Immunophenotyping of tissue immune responses in Chronic Lung Allograft Dysfunction after lung transplantation

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Abstract

Background

Long-term survival after lung transplantation remains limited, mainly due to the development of chronic lung allograft dysfunction (CLAD), with its main phenotypes bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS). The pathophysiology of CLAD is multifactorial, yet the exact immunological drivers are poorly understood. To investigate the immunological processes in CLAD, a systematic review was performed, followed by detailed immunophenotyping of human lung transplant tissue using imaging mass cytometry with laser ablation of regions of interest (ROIs).

Methods

Explanted lung tissue from 23 recipients, 20 with and 3 without CLAD, was sectioned and stained with a 40-plex antibody panel before 81 ROIs from airways, blood vessels and lung parenchyma were ablated. 190,851 single cells across 41 mm^2 tissue were captured before 26 distinct immune and structural cell populations were identified and interrogated across CLAD phenotypes.

Results

The systematic review confirmed that alloreactive T and B cells, neutrophils and eosinophils are key drivers of CLAD. Our findings support this, with evidence of classical cellular (cytotoxic T cells) and humoral (B cells, especially plasma cells) immune responses, alongside infiltration of eosinophils. Within CLAD, BOS was characterised by increased γδ T cells and non-classical M2 macrophages, but RAS by an increase in Th1 cells and intermediate M2 macrophages. Fibrotic remodelling of airways and parenchyma was associated with common cell profiles; however, different profiles in RAS (M2 macrophages, Th1 cells) and in BOS (γδ T cells) were identified.

Conclusion

In-depth immunophenotyping of cells in their local tissue microenvironment identified major differences in CLAD versus non-CLAD and between BOS and RAS. The innate immune system appeared to be more activated in BOS, while more pronounced alloimmune and repair responses were observed in RAS. Our findings in the fibrotic progression of CLAD suggest γδ T cells and M2 macrophages in particular merit further investigation.

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Abbreviations

αSMA	alpha smooth muscle actin
ACR	acute cellular rejection
BAL	bronchoalveolar lavage
BOS	bronchiolitis obliterans syndrome
cGvHD	chronic graft-versus-host disease
CLAD	chronic lung allograft dysfunction
CT	computed tomography
CyTOF	cytometry by time-of-flight
EMT	epithelial-to-mesenchymal transition
EnMT	endothelial-to-mesenchymal transition
FEV_1	forced expiratory volume in one second
FFPE	formalin-fixed paraffin-embedded
ROI	region of interest
FVC	forced vital capacity
HLA	human leukocyte antigen
IFN-γ	interferon gamma
IL	interleukin
ILD	interstitial lung disease
IMC	imaging mass cytometry
ISHLT	International Society for Heart and Lung Transplantation
MHC	major histocompatibility complex
NK	natural killer
OB	obliterative bronchiolitis
RAS	restrictive allograft syndrome
ROI	region of interest
TBB	transbronchial biopsies
TLC	total lung capacity
TGF-β	transforming growth factor beta
TNF	tumour necrosis factor
Tregs	regulatory T cells

"Without the organ donor, there is no story, no hope, no transplant.
But when there is an organ donor, life springs from death, sorrow turns to hope
and a terrible loss becomes a gift."[1]

[1] United Network for Organ Sharing (UNOS)

Chapter 1 Introduction

Lung transplantation is a valuable, life-saving treatment for a small number of carefully selected patients with chronic end-stage lung diseases with the primary goal of extending life expectancy, reducing breathlessness and improving quality of life. The number of lung transplantations performed each year has risen almost constantly since the 1990s and more than 70,000 procedures in adults have been reported worldwide to date. (Chambers et al., 2019) The field of lung transplantation has made significant progress over the past decades with improvements of donor lung utilisation and allocation, surgical techniques and patient care. (Bos et al., 2020) However, despite these favourable trends, lung transplantation still carries a high risk of short- and long-term complications and related morbidity and mortality remain high. (Chambers et al., 2019) Understanding factors associated with the natural course of lung transplantation, from waiting list to short- and long-term survival is important for predicting and possibly improving outcomes. The main focus of this PhD is on the most common long-term complication, chronic lung rejection or so-called chronic lung allograft dysfunction (CLAD).

1.1 Lung transplantation

Lung transplantation has evolved from an experimental surgical technique, first developed in dogs by Vladimir Demikhov in the 1940s and later performed in humans by James Hardy in 1963, to an established treatment option for well-selected patients with various end-stage lung diseases. (Panchabhai et al., 2018) Nowadays, over 4,500 adult lung transplant procedures are reported to the International Society for Heart and Lung Transplantation (ISHLT) Registry every year, which now contains data from nearly 70,000 recipients through June 2018. (Chambers et al., 2019) The actual number is assumed to be higher, since the ISHLT Registry is voluntary and does not include data from all lung transplant centres worldwide.

According to recent data, the main indications for lung transplantation are chronic obstructive pulmonary disease/alpha-1 antitrypsin deficiency (29.6%) and idiopathic pulmonary fibrosis (29.0%), followed by cystic fibrosis (14.2%) (ISHLT Registry data Jan 2010 – Jun 2018). (Perch et al., 2022) (Figure 1.1)

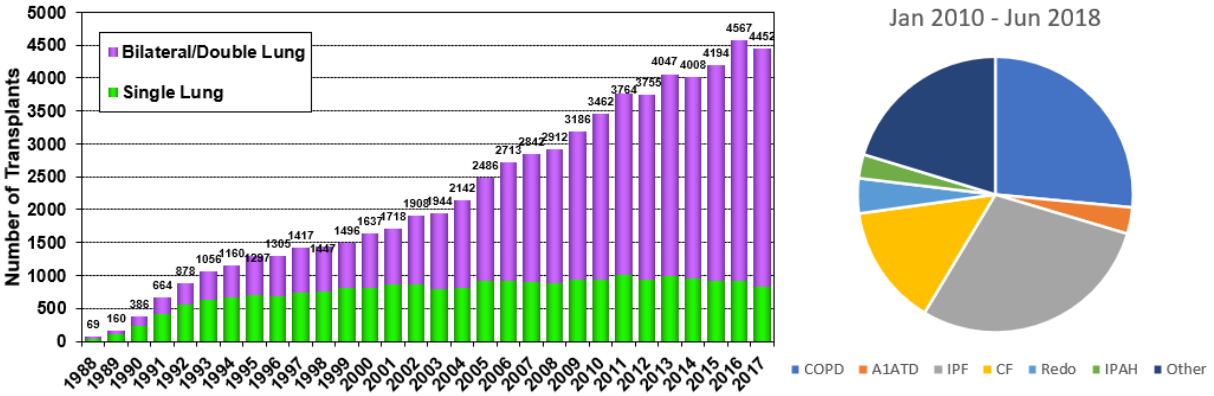


Figure 1.1. Number of lung transplants and main indications
Left: number of adult lung transplants by year and procedure type. Data from ISHLT Registry. (Chambers et al., 2019) Right: overview of main indications for lung transplantation between January 2010 and June 2018. Adapted from ISHLT registry data. (Perch et al., 2022)
A1ATD: alpha-1 antitrypsin deficiency, COPD: chronic obstructive pulmonary disease, CF: cystic fibrosis, IPAH: idiopathic pulmonary arterial hypertension, IPF: idiopathic pulmonary fibrosis, ISHLT: International Society for Heart and Lung Transplantation.

Over the past decades, significant improvements in patient outcomes have been achieved as a result of careful donor and recipient selection, improved organ retrieval, preservation and surgical techniques, development of immunosuppressive drugs, and better perioperative management and treatment of postoperative complications. (Bos et al., 2020) However, long-

term graft and patient outcomes still lag well behind that of other solid organ transplants. The ISHLT Registry reports a 1- and 5-year overall survival of 85% and 59%, respectively, and a median survival of 6.7 years for adult lung transplant recipients across all indications in the latest era (2010-2017). (Chambers et al., 2019) (Figure 1.2) According to recent UK data, which included data from adult and paediatric patients transplanted between 1995 and 2017, the mean survival estimate for lung transplantation is 9.3 years. This is much lower than the mean survival estimate of 26.6 years for kidney transplants, followed by 20.4 years for liver and 15.9 years for heart transplants. (Graham et al., 2022)

CLAD and side effects of chronic immunosuppressive treatment, including infections and an increased risk of solid organ malignancies, remain the most important challenges impairing long-term survival. (Bos et al., 2020)

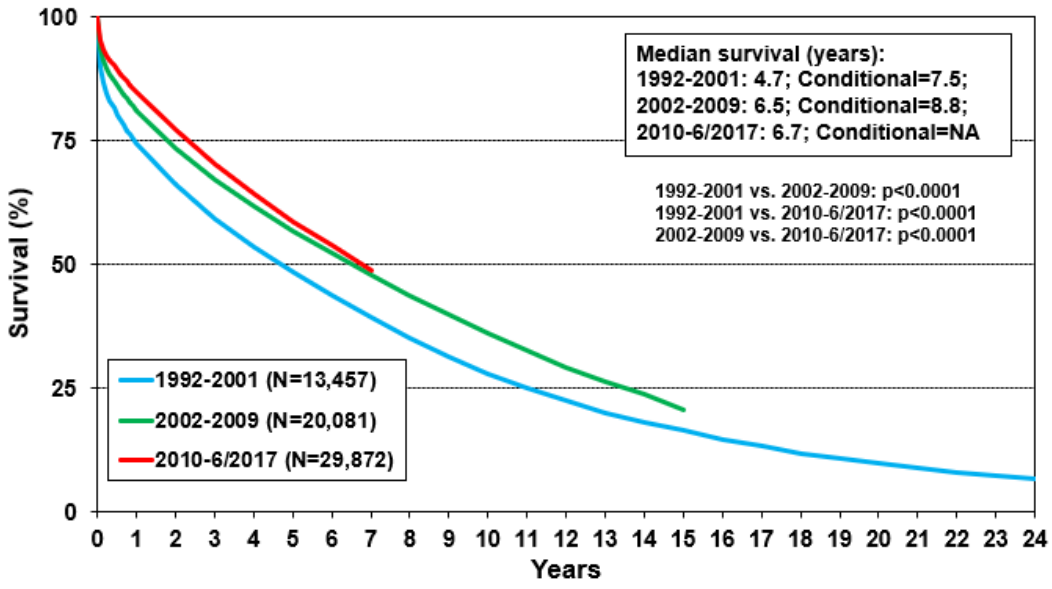


Figure 1.2. Median survival after lung transplantation
International Society for Heart and Lung Transplantation Registry data showing improved post-transplant overall survival over the past decades, mainly due to increased early post-transplant survival (red line: most recent decade January 2010 - June 2017). (Chambers et al., 2019)

1.2 Chronic lung allograft dysfunction

The long-term success of lung transplantation continues to be challenged by the development of chronic lung rejection, so-called CLAD, occurring in up to 50% of recipients within five years post-transplant. (Chambers et al., 2019) Allograft failure is the leading cause of post-transplant morbidity and mortality, and accounts for > 40% of deaths beyond the first year of transplantation. (Verleden et al., 2019a) Other common causes of death include infection, malignancy and cardiovascular diseases. (Figure 1.3)

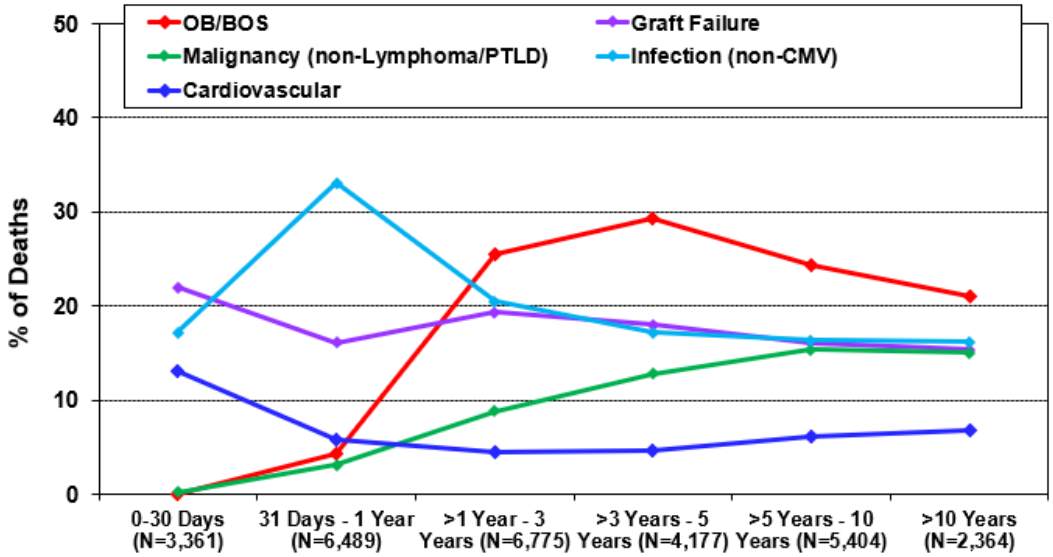


Figure 1.3. Leading causes of death after lung transplantation
International Society for Heart and Lung Transplantation Registry data showing that CLAD (in this graph annotated as OB/BOS) is the leading cause of death beyond the first year post-transplant, followed by infection, malignancy and cardiovascular diseases. (Chambers et al., 2019)
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, CMV: cytomegalovirus, OB: obliterative bronchiolitis, PTLD: post-transplant lymphoproliferative disease.

Among solid organ transplants, lung transplant recipients continue to have the highest rates of acute and chronic rejection. (Angaswamy et al., 2013) (Table 1.1)

Organ	Acute rejection rate at 1 year (%)	Chronic rejection rate at 5 years (%)
Lung	30-60	40-70
Heart	10-25	25-60
Kidney	10-20	40-50
Liver	7-22	4-12

Table 1.1. Acute and chronic rejection rates in solid organ transplants
Rates of acute rejection 1 year post-transplant and chronic rejection 5 years post-transplant among solid organ transplant recipients, with the highest rates seen in lung transplant recipients. Adapted from Angaswamy et al. (Angaswamy et al., 2013)

For a long time, obliterative bronchiolitis (OB), and its clinical surrogate bronchiolitis obliterans syndrome (BOS), was the sole recognised manifestation of and synonymous with chronic lung rejection. The term CLAD was first introduced in 2010 and has since been more specifically defined and redefined. (Verleden et al., 2014a, Verleden et al., 2019a) CLAD is an overarching clinical diagnosis based on objective physiological parameters. In 2019, a standardised definition of CLAD was adopted by the ISHLT, a crucial step in the standardisation of descriptions of post-transplant graft dysfunction, allowing the international community to compare standardised subpopulations across a vastly heterogenous post-transplant cohort. (Verleden et al., 2019a)

Nowadays, CLAD is an umbrella term which includes two main clinical phenotypes, BOS and restrictive allograft syndrome (RAS). (Verleden et al., 2019a) At least partly different pathophysiological mechanisms are believed to be involved in these phenotypes, as is reflected by differences in disease course, radiographic imaging, histology, and different cytokine, chemokine and growth factor expression. (Vos et al., 2015) In addition to these two phenotypes, a mixed phenotype exists with both obstructive and restrictive features. (Verleden et al., 2019a)
Since CLAD is a major barrier to long-term survival, advances in prevention, earlier detection and treatment are critical to further improve outcomes after lung transplantation, as will be discussed in more depth later.

1.2.1 Bronchiolitis obliterans syndrome

BOS is the most prevalent phenotype in approximately 70% of CLAD patients and has a median survival of 3-5 years after onset. (Verleden et al., 2019a) It has been described in lung transplant recipients since 1984 and is characterised by progressive airway obliteration

leading to airflow obstruction. (Burke et al., 1984) BOS is associated with a gradual loss of allograft function, which is clinically marked by a persistent and progressive decline in forced expiratory volume in one second (FEV_1). (Verleden et al., 2019a) The histological hallmark of BOS is OB (see also subsection 1.2.4); however, histopathological confirmation of OB by transbronchial biopsies (TBB) is difficult due to the patchy nature of the disease and small sample size of the biopsies. Therefore, the clinical correlate 'bronchiolitis obliterans syndrome' was introduced in 1993 by Cooper et al. as a non-invasive clinical surrogate solely based on pulmonary function testing. (Cooper et al., 1994)

1.2.2 Restrictive allograft syndrome

Although findings of interstitial lung disease have repeatedly been described in lung transplant recipients since the 1980s, it was only more recently that RAS was acknowledged as a separate clinical phenotype of CLAD. (Sato et al., 2011b, Glanville et al., 2019) RAS occurs in up to 30% of CLAD patients, with a significantly worse prognosis compared with BOS of only 6-18 months after diagnosis. It is characterised by parenchymal fibrosis and distortion of lung architecture with a restrictive pulmonary function decline and persistent pleuroparenchymal abnormalities on computed tomography (CT). (Verleden et al., 2019a, Glanville et al., 2019) Patients with RAS often progress stepwise with episodes of exacerbation followed by intervals of relative clinical stability. Less common patterns of progression are acute hypoxaemic respiratory failure with rapid worsening, or a gradually progressive decline with a slow but steady drop in total lung capacity (TLC). (Glanville et al., 2019) The latter is more commonly seen in patients with predominant upper lobe fibrosis and has a better prognosis than the other two progression patterns. (Verleden et al., 2016a)

1.2.3 Diagnosis

The diagnosis of CLAD is primarily based on pulmonary physiological changes measured via pulmonary function test, and in the case of RAS and mixed phenotype in combination with the presence of specific radiological features. All phenotypes require a patient to have an irreversible loss of more than 20% of their post-transplant baseline to meet criteria for diagnosis. Post-transplant baseline is defined as the average of the two best post-transplant FEV_1 values obtained at least three weeks apart. (Verleden et al., 2019a) Inherent in this definition is the exclusion or treatment of other potential causes of allograft dysfunction that can be either allograft-related (e.g., anastomotic bronchial stenosis, acute rejection,

respiratory infection) or non-allograft-related (e.g., diaphragmatic dysfunction, pleural disorders, obesity), or a combination of both. (Verleden et al., 2014a)

Evaluation of the FEV_1 trajectory over time is important to help detect any deterioration in allograft function and distinguish CLAD from acute or subacute complications after lung transplantation. A critical decline in $FEV_1 \geq 20\%$ from baseline corresponds with possible (< 3 weeks), probable (persistent > 3 weeks) or definite (persistent > 3 months and after exclusion or treatment of other causes of lung function decline) CLAD diagnosis. (Verleden et al., 2019a) This approach allows for close follow-up and to assess the effects of any therapeutic intervention. Moreover, a decline of 10% or more ("potential CLAD") should prompt close monitoring and investigation for possible causes for the observed lung function decline that may respond to treatment. (Verleden et al., 2019a) Radiographic imaging, preferably by high-resolution chest CT with inspiratory and expiratory phase imaging, is important in the work-up. Chest CT can detect diagnostically useful changes, such as air trapping or pleuroparenchymal abnormalities, to which routine chest X-rays are not sensitive enough. (Meyer et al., 2014) If a specific cause is identified, it must be treated appropriately. If this is not the case and the patient is not already treated with azithromycin, a prolonged course of at least eight weeks should be prescribed, which may result in some patients responding with a substantial FEV_1 increase, so-called azithromycin-responsive allograft dysfunction (or ARAD). (Verleden et al., 2019a)

A lung transplant recipient meets the criteria for BOS if there is a persistent obstructive pulmonary function decline, with FEV1 ≤ 80% compared with baseline, exclusion or adequate treatment of possible other causes, and no opacities on chest CT. (Verleden et al., 2019a) Chest CT can be normal, but often signs of mosaic attenuation and air trapping, due to small airways disease and hypoxic vasoconstriction, or bronchiolitis (e.g., tree-in-bud nodules, bronchial wall thickening, bronchiectasis) are seen. (Byrne et al., 2021) (Figure 1.4A-B)
The diagnosis of RAS can be made in case of a persistent decline in FEV1 ≤ 80% from baseline in combination with a decline in TLC ≤ 90% from baseline, with persistent pleuroparenchymal abnormalities on chest CT, and after ruling out or treating other possible causes. TLC monitoring is recommended after lung transplantation, but since this is not routinely performed and/or feasible in most transplant centres, a decrease in forced vital capacity (FVC) of ≥ 20% can be used as a surrogate marker. However, it should be borne in mind that FVC can

also decrease due to air trapping, and therefore TLC should preferably be used as confirmation at the time of RAS diagnosis. (Glanville et al., 2019, Verleden et al., 2019a) Examples of CT abnormalities in RAS are ground-glass opacities, consolidation, interstitial reticular and septal thickening, (sub)pleural reticulation and thickening, (traction) bronchiectasis, architectural distortion, and volume loss. (Byrne et al., 2021) (Figure 1.4C-F)

Furthermore, patients may present de novo with a mixed phenotype, or shift from one phenotype (often BOS) to another (RAS/mixed phenotype) over time. (Glanville et al., 2019) Lastly, the term undefined is used if there is a persistent decline in $FEV_1 \leq 80\%$ from baseline with CT opacities, or a persistent combined pulmonary function decline without CT opacities, and no other causes can be identified. (Verleden et al., 2019a)

The severity of CLAD (both BOS and RAS) is determined based on the grade of lung function decline (in FEV_1) from baseline and ranges from stage 1 to 4. (Verleden et al., 2019a) (Table 1.2)

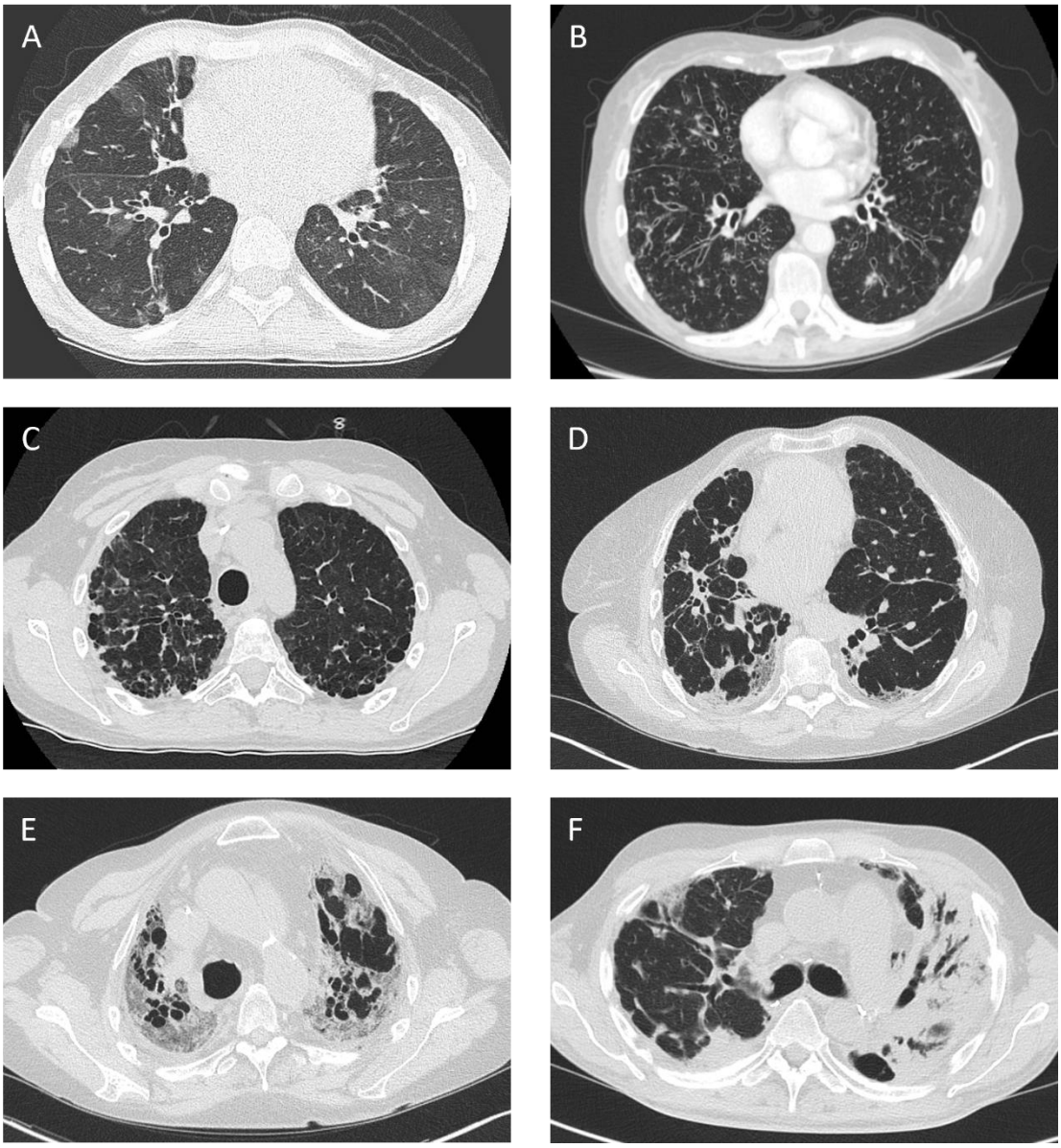


Figure 1.4. CT images of BOS and RAS
Examples of CT findings in BOS (A-B) and RAS (C-F) patients. **A.** Air trapping. **B.** Airway thickening and bronchiectasis. **C-D.** Fibrotic changes in RAS with interstitial reticular and septal thickening, ground glass opacities, consolidation, traction bronchiectasis, and/or volume loss.

CLAD definition	Potential	≥ 10% decline in FEV_1 ± FVC/TLC from baseline*: perform investigations
	Possible	< 3 weeks ≥ 20% decline in FEV_1 ± FVC/TLC from baseline
	Probable	3 weeks - 3 months ≥ 20% decline in FEV_1 ± FVC/TLC from baseline
	Definite	> 3 months ≥ 20% decline in FEV_1 ± FVC/TLC from baseline
CLAD grading	CLAD 0	FEV_1 > 80% FEV_1 baseline
	CLAD 1	FEV_1 65-80% FEV_1 baseline
	CLAD 2	FEV_1 50-65% FEV_1 baseline
	CLAD 3	FEV_1 35-50% FEV_1 baseline
	CLAD 4	FEV_1 ≤ 35% FEV_1 baseline

CLAD phenotypes

	Obstruction (FEV1/FVC < 0.7)	Restriction (TLC ≤ 90% from baseline)	CT opacities
BOS	Yes	No	No
RAS	No	Yes	Yes
Mixed	Yes	Yes	Yes
Undefined	Yes	No	Yes
	Yes	Yes	No

	BOS	**RAS**
Prevalence	Up to 70% of CLAD patients	20-30% of CLAD patients
CT findings	Air trapping Bronchiolitis (e.g., tree-in-bud) Bronchiectasis	Ground-glass opacities Consolidation Pleural/septal thickening Bronchiectasis Volume loss
Histology	OB	Most common: DAD, AFE, PPFE Concurrent OB
Diagnosis	FEV_1 ≤ 80% compared with baseline	FEV_1 ≤ 80% compared with baseline + TLC ≤ 90% or FVC ≤ 80% compared with baseline** + persistent pleuroparenchymal CT abnormalities
Prognosis	Median survival 3-5 years	Median survival 1-2 years

Table 1.2. CLAD diagnosis and phenotypes
CLAD definition, grading and phenotypes and characteristics, based on the latest 2019 International Society for Heart and Lung Transplantation consensus document. (Verleden et al., 2019a)
AFE: alveolar fibroelastosis, BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, CT: computed tomography, DAD: diffuse alveolar damage, FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, OB: obliterative bronchiolitis, PPFE: pleuroparenchymal fibroelastosis, RAS: restrictive allograft syndrome, TLC: total lung capacity.
* Baseline: mean of the two best post-transplant FEV_1 values (in L), taken at least three weeks apart, without administration of a bronchodilator.
** Baseline: mean of the two measurements obtained at the same time as or very near to the two best post-operative FEV_1 values.

1.2.4 CLAD histopathology

BOS is histologically typified by small airway obstruction, OB, or constrictive bronchiolitis, the histological hallmarks of BOS. (Figure 1.5) Obliterative and constrictive bronchiolitis are thought to arise as lymphocytic bronchiolitis of the respiratory and terminal bronchioles, followed by recruitment and proliferation of (myo)fibroblasts and submucosal and peribronchiolar fibrosis, ultimately leading to a partial or complete obstruction (OB) or constriction ('constrictive bronchiolitis') of the small airway. (Belperio et al., 2009, Verleden et al., 2016b) Verleden et al. demonstrated that about 50% of the airways in BOS are obstructed at the 9th generation and up to 70-100% at generation 14-17. (Verleden et al., 2019b) The airways can either be collapsed by peribronchiolar fibrosis or obstructed by intraluminal accumulation of granulation tissue and organising extracellular matrix. Interestingly, OB lesions very often only cause a segmental occlusion of the airway lumen, with a preserved luminal diameter more distally. This may be due to collateral ventilation through interalveolar pores, broncho-alveolar or interbronchiolar connections. (Verleden et al., 2019b, Terry and Traystman, 2016)

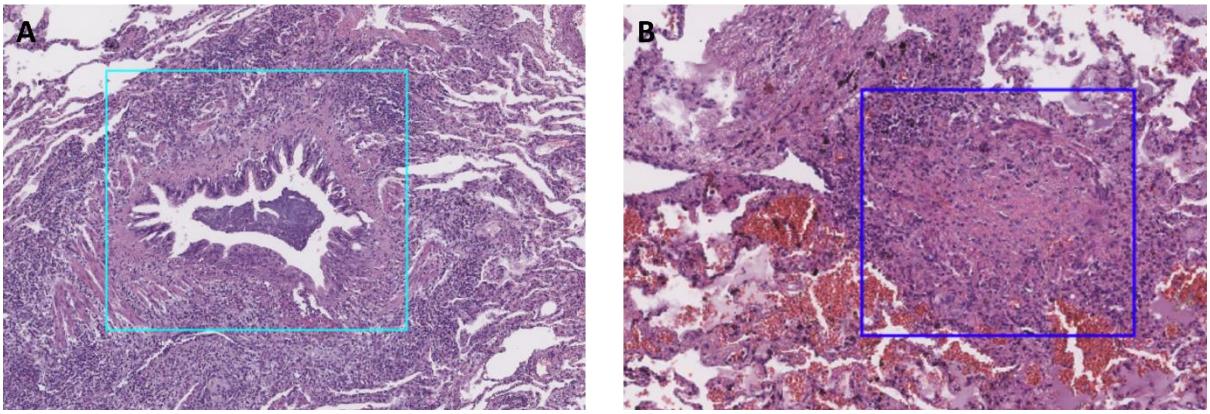


Figure 1.5. Inflammatory and fibrotic OB lesion
A. H&E staining of inflammatory OB lesion in a BOS patient (denoted in turquoise). **B.** H&E staining of fibrotic OB lesion with complete obliteration of the airway lumen in a BOS patient (denoted in blue).
BOS: bronchiolitis obliterans syndrome, H&E: haematoxylin and eosin, OB: obliterative bronchiolitis.

In RAS, various findings can be seen on histological examination, including intra-alveolar and pleuroparenchymal fibroelastosis, diffuse alveolar damage, organising pneumonia, non-specific interstitial pneumonia, and acute fibrinous and organising pneumonia. (Verleden et al., 2019a, Ofek et al., 2013) (Figure 1.6) Concurrent OB lesions are frequently seen in up to 62-100% of RAS patients and are often found in areas of ongoing fibrosis. (Verleden et al., 2019b)

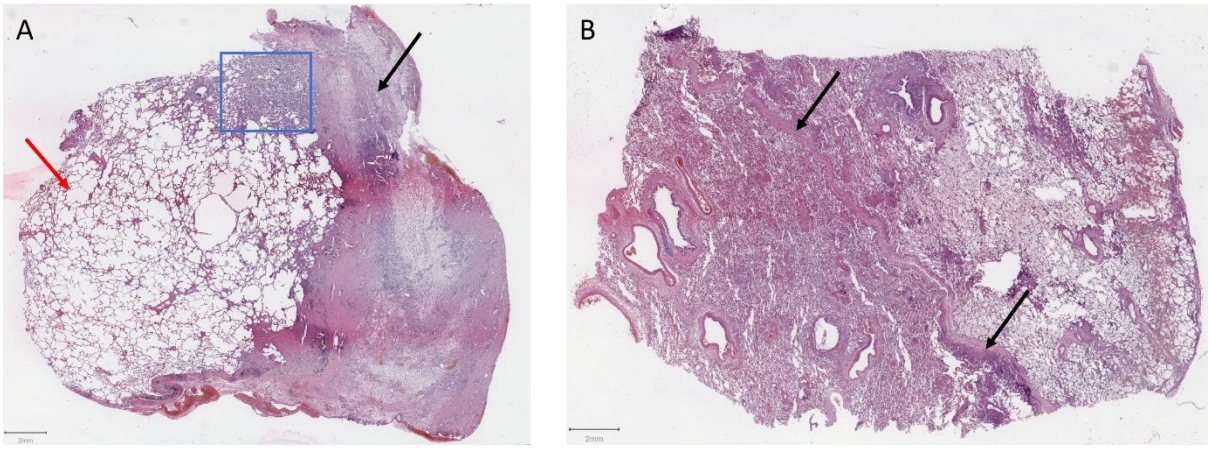


Figure 1.6. Pleuroparenchymal fibrosis in RAS
A. RAS patient with pleural fibrosis (black arrow), parenchymal fibrosis (indicated by blue rectangle) and areas of preserved alveoli (red arrow). H&E staining. **B.** RAS patient with varying degrees of parenchymal fibrosis as well as septal fibrosis (black arrow). H&E staining. Scale bar 2 mm.
H&E: haematoxylin and eosin, RAS: restrictive allograft syndrome.

However, histology is rarely used in clinical practice to support the diagnosis of BOS or RAS. This is because, on the one hand, the yield of TBB is low due to the small sample size and patchy nature of the disease. On the other hand, surgical biopsies are more invasive and do not always contribute to the diagnosis. In general, bronchoscopy with bronchoalveolar lavage (BAL) and TBB is primarily used to rule out other causes of allograft decline, such as infection and acute rejection. (Meyer et al., 2014)

1.2.5 Risk factors and multiple hits theory

Several risk factors for the development of CLAD have been identified that can broadly be grouped into two categories. Firstly, alloimmune triggers, including T cell-mediated rejection, antibody-mediated rejection, and antibodies to human leukocyte antigens (HLA), non-HLA and self-antigens. Secondly, non-alloimmune triggers such as ischaemia-reperfusion injury, respiratory infections, gastro-oesophageal reflux, and pollution. (Vos et al., 2015) (Table 1.3)
Noteworthy, in many – often older – studies, RAS was not recognised as a separate clinical phenotype and all CLAD phenotypes were pooled together, making it difficult to distinguish individual risk factors for each phenotype. However, several risk factors appear to be similar for BOS and RAS. (Bos et al., 2022c)

Alloimmune-dependent risk factors	Alloimmune-independent risk factors
Acute cellular rejection Lymphocytic bronchiolitis HLA mismatching Preformed or de novo antibodies to HLA, non-HLA and/or self-antigens Antibody-mediated rejection Donor and recipient genetic variants Non-adherence to immunosuppressive therapy	Ischaemia-reperfusion injury Respiratory infection (bacterial, viral, fungal) Gastro-oesophageal reflux Air pollution Inhaled toxins

Table 1.3: Risk factors for the development of CLAD
Risk factors for CLAD can be divided in alloimmune-dependent and alloimmune-independent risk factors. CLAD: chronic lung allograft dysfunction, HLA: human leukocyte antigen.

The driving factor of CLAD is innate and adaptive immune activation, as will be explained in more detailed below, which is elicited by these alloimmune and non-alloimmune triggers. Since neither ongoing alloimmune recognition nor non-alloimmune stimulation can be completely avoided, CLAD could be considered an inevitable consequence of lung transplantation. Depending on the effectiveness of the immunosuppressants used and the number and severity of post-transplant 'hits' (injuries), CLAD may develop sooner or later after transplantation. Thus, from this perspective, most patients will develop structural and/or functional abnormalities of their graft at some point. The concept of multiple hits that may contribute to the onset of CLAD is also nicely illustrated in a review paper by Beeckmans et al. (Beeckmans et al., 2023)

1.2.6 BOS and RAS: common pathway with a dissimilar endpoint?

The similarity in risk factors for both BOS and RAS may imply that the underlying disease mechanisms also bear similarities. This can also be supported from a histological perspective, where similar findings can be found in both phenotypes. As mentioned, OB lesions are also commonly seen in RAS patients. But also vice versa, and very interestingly, parenchymal fibrosis can be observed in advanced BOS patients too. As such, a (partial) overlap in underlying immunological and fibrotic mechanisms between BOS and RAS is suspected. In fact, OB lesions are not specific to CLAD and can be found in other diseases such as pulmonary chronic graft-versus-host disease (cGvHD) after allogeneic haematopoietic stem cell transplantation or after radio-chemotherapy. This could suggest a comparable process of tissue remodelling despite different clinical backgrounds and preceding triggers. (Verleden et al., 2020a, Jonigk et al., 2017) Similarly, alveolar fibroelastosis is not only seen in RAS, but can

also be found in pulmonary cGvHD or be idiopathic in nature. (Verleden et al., 2020a) Even on a molecular level, analogous gene expression patterns of extracellular matrix remodelling were observed in CLAD and pulmonary cGvHD, both in OB lesions and in parenchymal fibrotic lesions, albeit with minor differences regarding fibrin upregulation and fibroblast recruitment. (Jonigk et al., 2015)

Although there are clear differences in clinical presentation, radiological findings and disease course between BOS and RAS, there is thus some overlap especially on a histological and molecular level. This suggests, at least partly, similar underlying disease mechanisms and tissue remodelling processes. (Bos et al., 2022a) This is also supported by the fact that patients may present clinically with a mixed phenotype with features of both obstruction and restriction, or evolve from one phenotype to another over time. (Verleden et al., 2019a)
This overlap in disease findings may be explained by the involvement of the most distal, respiratory bronchioles extending into the adjacent alveolar spaces (previously referred to as bronchiolitis obliterans organising pneumonia), as well as involvement of the alveolar rather than the bronchiolar compartment in the ongoing disease process. (Beeckmans et al., 2023) This in turn is supported histologically by the fact that areas of diffuse alveolar damage and pleuroparenchymal fibrosis or non-specific interstitial pneumonia and interstitial fibrosis are often found next to each other, suggesting a temporal sequence of the disease process. (Ofek et al., 2013)

Finally, since many risk factors for BOS and RAS are similar, it seems plausible that chronic or repeated lung injury, caused by different factors, serves as a common denominator leading to inflammation and subsequent fibrosis in both BOS and RAS. Depending on the primary site of injury (bronchiolar, alveolar and/or vascular compartment), different clinical manifestations can occur. For example, some external stimuli could affect several compartments of the secondary pulmonary lobule (e.g., infection affecting the alveolar spaces), while other causes are more airway-centred (e.g., gastro-oesophageal reflux). (Bos et al., 2022a, Beeckmans et al., 2023)
Histologically, this presents mainly as an involvement of the bronchovascular axis in BOS, although other parts of the lung (alveoli, pleura) can be affected to a greater or lesser extent as well. While in RAS, mainly other regions of the secondary pulmonary lobule are involved, specifically the alveoli, interlobular septa, interstitial space, and pleura. The wide range of

anatomical structures that can be affected also explains the variety of histological findings in RAS. (Beeckmans et al., 2023) (Figure 1.7)

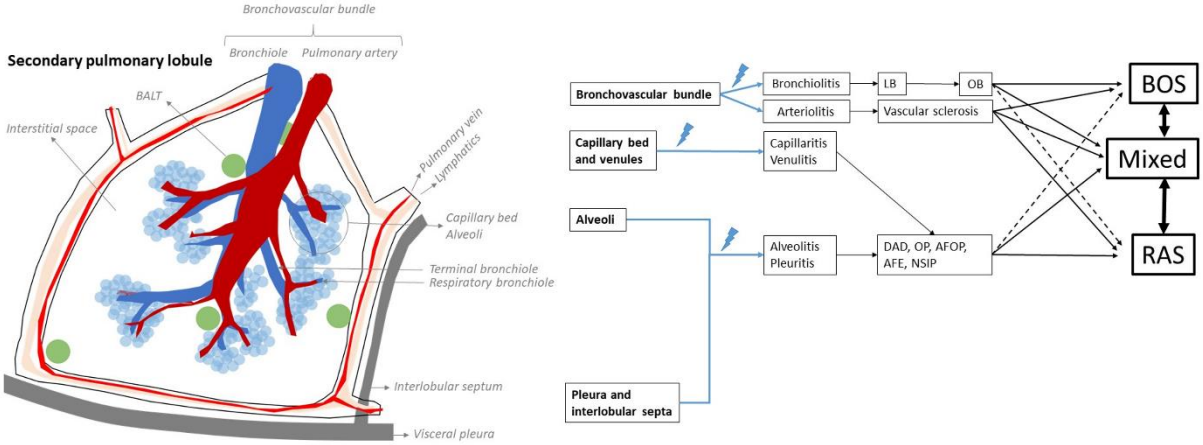


Figure 1.7: CLAD as a disease of the secondary pulmonary lobule
Schematic representation of CLAD as a disease of the secondary pulmonary lobule in which the bronchovascular bundle, alveoli, pleura, and interlobular septa, and/or capillary bed and venules can be involved. Adapted from Beeckmans et al. (Beeckmans et al., 2023) Reprinted with permission.
AFE: alveolar fibroelastosis, AFOP: acute fibrinous and organising pneumonia, BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, DAD: diffuse alveolar damage, LB: lymphocytic bronchiolitis, NSIP: non-specific interstitial pneumonia, OB: obliterative bronchiolitis, OP: organising pneumonia, RAS: restrictive allograft syndrome.

CLAD could thus be regarded as a common endpoint due to multifactorial injuries, which helps to explain the different phenotypes, with the predominant anatomical location of the injury determining the dominant phenotype, and transition from one CLAD phenotype to another. This theory would shift our understanding of CLAD as an umbrella term, with distinct entities, towards CLAD as a common endpoint of several mechanisms of tissue damage affecting the secondary pulmonary lobule as a whole. (Beeckmans et al., 2023)

1.2.7 CLAD pathophysiology

Parts of this section have been the subject of the following publication: **Bos S**, Milross L, Filby AJ, Vos R, Fisher AJ. Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle. Eur Respir Rev. 2022;31(165). See Appendix A p. 210. Reprinted with permission.

For a long time, lung allograft rejection was primarily considered a manifestation of cellular immune responses. Today, however, we are aware of the array of complex, interacting and multifactorial mechanisms that contribute to the pathogenesis of CLAD, with an impact of alloimmune, non-alloimmune, autoimmune, and fibroproliferative processes. The pathophysiology is thought to be mediated via activation of both innate and adaptive arms of the immune system in response to a myriad of immunological, infectious and mechanical insults. (Bos et al., 2022c) There is constant crosstalk between every aspect of the immune system and in particular the innate system communicates with the adaptive, and the adaptive communicates with the innate. We have thus gained a better understanding of how the immune system contributes to inflammatory responses, airway and parenchymal remodelling, and fibrosis after lung transplantation. However, in order to make therapeutic advances in the prevention and treatment of CLAD, it is critical to develop a full picture of how exactly all immune processes at play in the lung allograft interact in the pathogenesis of CLAD. As part of this doctoral project, we published an update on immune processes in CLAD pathogenesis, including advanced insights into the role of innate immune pathways and crosstalk between innate and adaptive immunity. The aim of this review was also to identify gaps in current knowledge. (Bos et al., 2022c)

As highlighted in the previous subsection, partly similar underlying disease mechanisms and tissue remodelling processes are expected to be involved in the disease onset of BOS and RAS. However, due to the only relatively recent acknowledgement of RAS as a clinical phenotype of CLAD and the smaller amount of pooled evidence for RAS at present, it is currently difficult to clearly categorise the different pathophysiological mechanisms. As a result, most of the data on CLAD pathophysiology to date stem from the BOS population. (Bos et al., 2022c) The main findings are summarised in the following subsections.

Lung as an immunogenic organ

Lung allografts are particularly immunogenic as is evidenced by higher rates of rejection and decreased survival compared with other solid organ transplants. (Angaswamy et al., 2013) Unlike other solid organ transplants, the lung allograft is uniquely susceptible to injury from exogenous agents due to its direct exposure to the external environment. (Gauthier et al., 2018) The lungs therefore harbour a robust innate immune presence primed to respond to environmental and microbiological challenges and contain more tissue-resident and

interstitial immune cells. It is therefore not surprising that, in addition to alloimmune injuries, non-alloimmune injuries have been described as important risk factors for later development of CLAD. Non-immune risk factors include ischaemia-reperfusion injury, respiratory infections, air pollution, and inhaled toxins. (Belperio et al., 2009, Vos et al., 2015, Martinu et al., 2009) Furthermore, the lung is at risk of exposure to gastric contents through gastro-oesophageal reflux and (micro)aspiration since the oesophagus and trachea are anatomically connected. (Kawashima and Juvet, 2020, Hathorn et al., 2017) These 'non-immune' factors most likely promote tissue damage and local inflammation that in turn can initiate and intensify an alloimmune response. (Meyer et al., 2014) This ultimately predisposes to CLAD through subsequent recruitment of fibroproliferative growth factors, excessive airway and/or tissue remodelling, and eventually fibrosis and allograft dysfunction. (Bos et al., 2022c) Some of the main mechanisms involved in these 'non-alloimmune' factors are displayed in Figure 1.8.

T cell-mediated immunity

Cell-mediated immunity is probably the best understood alloimmune pathway. It is predominantly driven by T cells following presentation of alloantigens by antigen-presenting cells via major histocompatibility complex (MHC) molecules, also called HLA. Two main modes play a role in this allorecognition: the direct pathway, where allogeneic MHC is presented directly to recipient T cells by donor antigen presenting cells, and the indirect pathway, in which recipient antigen presenting cells phagocytise and present alloantigens to recipient T cells. MHC classes I and II are, respectively, recognised by CD8+ and CD4+ T cells. Following allorecognition, T cells require secondary costimulatory signals, resulting in proliferation and differentiation. (Pishesha et al., 2022) Besides cytotoxic CD8+ T cells, immunological responses are regulated by CD4+ helper T cells, which include the main subtypes Th1 cells, Th2 cells, regulatory T cells (Tregs), and Th17 cells. (Gracon and Wilkes, 2014) (Figure 1.9) These subtypes have different characteristics, ranging from cytolytic activity, activation of innate and other adaptive immune cells, to propagating or dampening inflammation and are assumed to all be involved in CLAD pathogenesis. (Yamada et al., 2019, Nakagiri et al., 2012) Little is currently known about the exact role of other T-cell subsets, such as γδ T cells, T follicular helper cells, Th9 cells, Th22 cells, and natural killer (NK) T cells in the onset of CLAD. Specific roles of these different T-cell subsets will be discussed in detail in the Results Chapter (Chapter 3: Effector immune cells in CLAD: a systematic review).

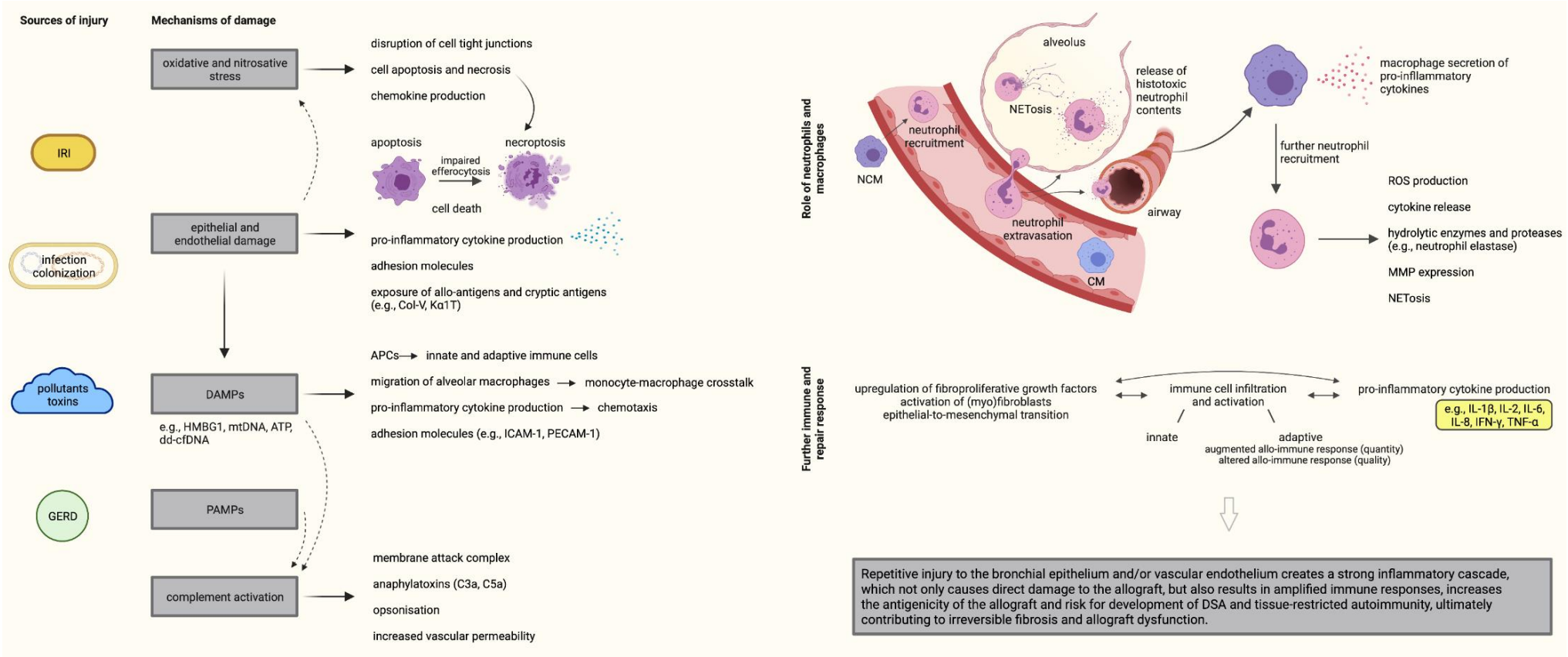


Figure 1.8: Non-alloimmune factors contributing to CLAD
Simplified representation of pathways involved in non-alloimmune mechanisms which may contribute to CLAD onset.
APC: antigen-presenting cell, ATP: adenosine triphosphate, CLAD: chronic lung allograft dysfunction, CM: classical monocyte, Col-V: collagen V, DAMP: damage-associated molecular pattern, dd-cfDNA: donor-derived cell-free DNA, DSA: donor-specific antibody, GERD: gastro-oesophageal reflux disease, HMGB1: high-mobility group box 1, ICAM-1: intercellular adhesion molecule 1, IFN: interferon; IL: interleukin, IRI: ischaemia-reperfusion injury, Kα1T: K-alpha 1 tubulin, MMP: matrix metalloproteinase, mtDNA: mitochondrial DNA, NCM: non-classical monocyte, NET: neutrophil extracellular trap, PAMP: pathogen-associated molecular pattern, PECAM-1: platelet endothelial cell adhesion molecule 1, ROS: reactive oxygen species, TNF-α: tumour necrosis factor alpha. Figure partially created with BioRender.com. Reprinted with permission. (Bos et al., 2022c)

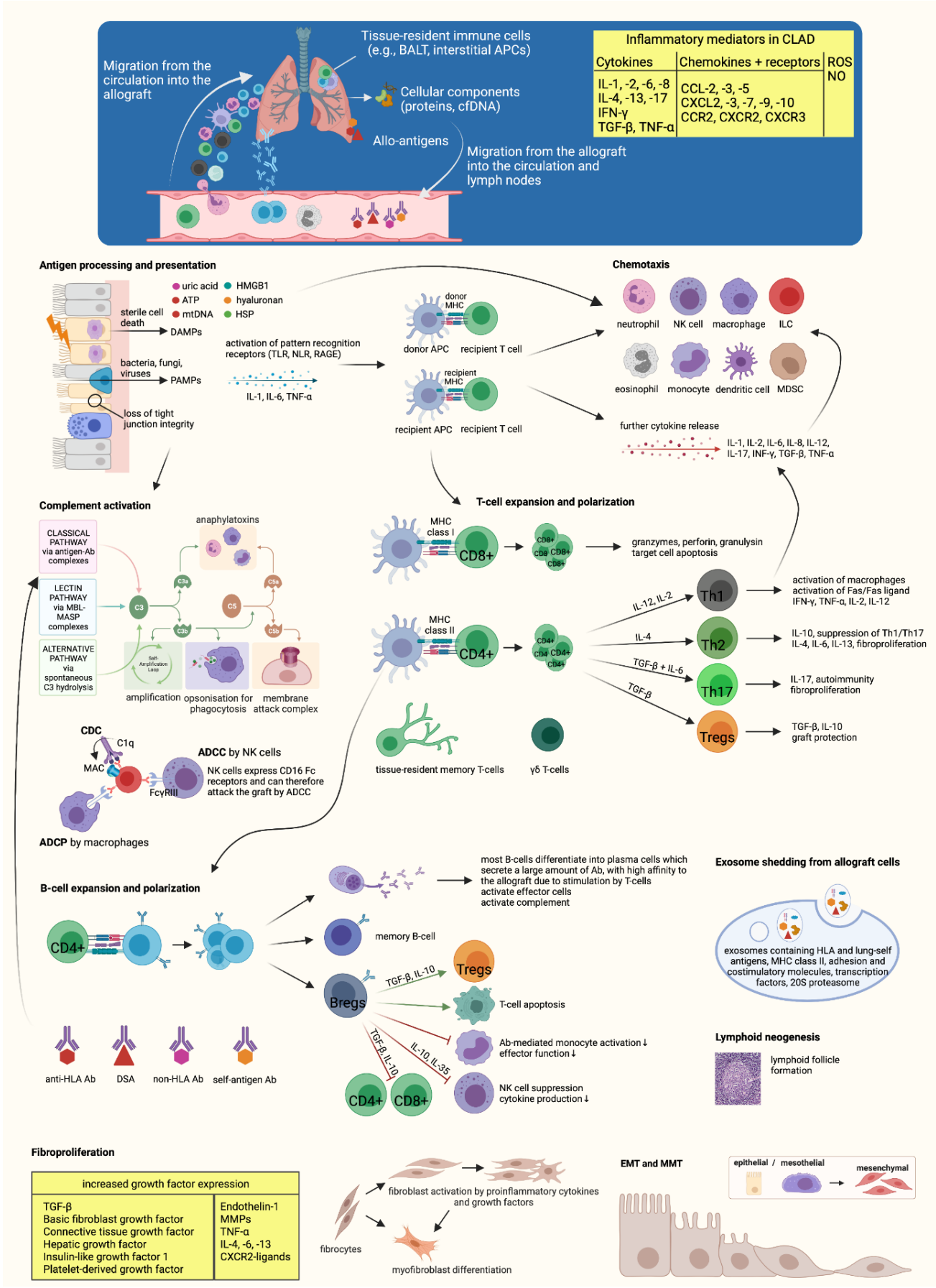

Migration from the circulation into the allograft
Tissue-resident immune cells (e.g., BALT, interstitial APCs)
Cellular components (proteins, cfDNA)
Allo-antigens
Migration from the allograft into the circulation and lymph nodes
Inflammatory mediators in CLAD
Cytokines
IL-1, -2, -6, -8
IL-4, -13, -17
IFN-γ
TGF-β, TNF-α
Chemokines + receptors
CCL-2, -3, -5
CXCL2, -3, -7, -9, -10
CCR2, CXCR3
ROS
NO
Antigen processing and presentation
uric acid
ATP
mtDNA
HMGB1
hyaluronan
HSP
sterile cell death
DAMPs
bacteria, fungi, viruses
PAMPs
loss of tight junction integrity
activation of pattern recognition receptors (TLR, NLR, RAGE)
IL-1, IL-6, TNF-α
donor MHC
donor APC
recipient T cell
recipient MHC
recipient APC
recipient T cell
Chemotaxis
neutrophil
NK cell
macrophage
ILC
eosinophil
monocyte
dendritic cell
MDSC
further cytokine release
IL-1, IL-2, IL-6, IL-8, IL-12, IL-17, INF-γ, TGF-β, TNF-α
Complement activation
CLASSICAL PATHWAY via antigen-Ab complexes
LECTIN PATHWAY via MBL-MASP complexes
ALTERNATIVE PATHWAY via spontaneous C3 hydrolysis
anaphylatoxins
C3
C3a
C3b
C5
C5a
C5b
amplification
opsonisation for phagocytosis
membrane attack complex
CDC
C1q
MAC
FcγRIII
ADCC by NK cells
NK cells express CD16 Fc receptors and can therefore attack the graft by ADCC
ADCP by macrophages
T-cell expansion and polarization
MHC class I
CD8+
granzymes, perforin, granulysin
target cell apoptosis
MHC class II
CD4+
IL-12, IL-2
Th1
activation of macrophages
activation of Fas/Fas ligand
IFN-γ, TNF-α, IL-2, IL-12
IL-4
Th2
IL-10, suppression of Th1/Th17
IL-4, IL-6, IL-13, fibroproliferation
TGF-β + IL-6
Th17
IL-17, autoimmunity
fibroproliferation
TGF-β
Tregs
TGF-β, IL-10
graft protection
tissue-resident memory T-cells
γδ T-cells
B-cell expansion and polarization
CD4+
most B-cells differentiate into plasma cells which secrete a large amount of Ab, with high affinity to the allograft due to stimulation by T-cells
activate effector cells
activate complement
memory B-cell
Tregs
TGF-β, IL-10
Bregs
T-cell apoptosis
Ab-mediated monocyte activation↓
effector function↓
TGF-β, IL-10
IL-10, IL-35
NK cell suppression
cytokine production↓
CD4+
CD8+
anti-HLA Ab
DSA
non-HLA Ab
self-antigen Ab
Exosome shedding from allograft cells
exosomes containing HLA and lung-self antigens, MHC class II, adhesion and costimulatory molecules, transcription factors, 20S proteasome
Lymphoid neogenesis
lymphoid follicle formation
Fibroproliferation
increased growth factor expression
TGF-β
Basic fibroblast growth factor
Connective tissue growth factor
Hepatic growth factor
Insulin-like growth factor 1
Platelet-derived growth factor
Endothelin-1
MMPs
TNF-α
IL-4, -6, -13
CXCR2-ligands
fibroblast activation by proinflammatory cytokines and growth factors
fibrocytes
myofibroblast differentiation
EMT and MMT
epithelial / mesothelial
mesenchymal

Figure 1.9. Key elements in the pathogenesis of CLAD
Overview of the pathogenesis of CLAD with some of the main immune mechanisms and cytokines involved. Tissue injury by alloimmune-dependent and -independent mechanisms induces the release of tissue damage-associated molecular patterns, pathogen-associated molecular patterns and inflammatory cytokines, followed by antigen presentation to donor and host antigen-presenting cells by pattern-recognition receptors. This is followed by an advanced interplay between innate and adaptive immune responses, with infiltration of innate and adaptive immune cells into the allograft. Activation of alloreactive T and/or B cells and suppression of regulatory T cells further perpetuate an inflammatory milieu. Finally, fibrotic growth factors are upregulated and (myo)fibroblasts are activated, leading to deposition of extracellular matrix and, ultimately, fibrosis and allograft dysfunction.
Ab: antibodies, ADCC: antibody-dependent cellular cytotoxicity, ADCP: antibody-dependent cellular phagocytosis, APC: antigen-presenting cells, ATP: adenosine triphosphate, BALT: bronchus-associated lymphoid tissue, Breg: regulatory B cell, CCL: C-C motif ligand, CCR: C-C motif receptor, CDC: complement-dependent cytotoxicity, cfDNA: cell-free DNA, CLAD: chronic lung allograft dysfunction, CXCL: C-X-C motif ligand, CXCR: C-X-C motif receptor, DAMPs: damage-associated molecular patterns, DSA: donor-specific antibody, EMT: epithelial-to-mesenchymal transition, HLA: human leukocyte antigen, HMGB1: high-mobility group box 1, HSP: heat-shock protein, IFN-γ: interferon gamma, IL: interleukin, ILC: innate lymphoid cell, MAC: membrane attack complex, MASP: MBL-associated serine protease, MBL: mannan-binding lectin, MDSC: myeloid-derived suppressor cell, MHC: major histocompatibility complex, MMP: matrix metalloproteinase, MMT: mesothelial-to-mesenchymal transition, mtDNA: mitochondrial DNA, NK: natural killer, NLR: nucleotide-binding oligomerisation domain-like receptor, NO: nitric oxide, PAMPs: pathogen-associated molecular patterns, RAGE: receptor for advanced glycation end products, ROS: reactive oxygen species, TGF-β: transforming growth factor beta, Th: T-helper, TLR: Toll-like receptor, TNF-α: tumour necrosis factor alpha, Treg: regulatory T cell. Figure partially created with BioRender.com. Reprinted with permission. (Bos et al., 2022c)

Humoral immunity

Today, we know that chronic organ rejection is caused by more than just traditional T cell-mediated immune responses. Antibodies to MHC/HLA and minor histocompatibility antigens play an important role, as well as alloreactive B cells. The presence of donor-specific antibodies is strongly associated with CLAD, through alloimmune responses and complement activation, but also via complement-independent mechanisms. (Yoshiyasu and Sato, 2020) Moreover, anti-HLA antibodies can induce the release of fibrotic growth factors, including platelet-derived growth factor, insulin-like growth factor-1 and transforming growth factor beta (TGF-β). These events culminate in the activation of myofibroblasts and extracellular matrix regeneration, hereby contributing to the development of CLAD. (Yoshiyasu and Sato, 2020, Gracon and Wilkes, 2014)

Importantly, T cells are required for the growth and maturation of antigen-specific B cells for them to go into a terminally differentiated state to produce antibodies. Beyond their role in antibody production, B cells can either contribute to or limit the development of CLAD through regulation of T-cell immunity via improved antigen presentation and co-stimulation, enhanced cytokine production, and influence on accommodation or tolerance via, for example, B-

regulatory cells. (Dijke et al., 2016, Thaunat, 2012, Schmitz et al., 2020) However, the specifics of B-cell regulation in CLAD remain to be established, and the manifold and complex interactions between B and T cells are not yet fully understood. (Dijke et al., 2016)

Autoimmunity

Mounting evidence has emerged that alloimmunity is not only directed against HLA and non-HLA, but also against lung-associated self-antigens, hereby suggesting a role for autoimmunity in CLAD pathogenesis. Both pre-existing and de novo lung self-antigens appear to contribute to acute and chronic lung rejection through an interplay between allo- and autoimmunity. Two prominent self-antigens that most likely play a role in the onset of CLAD are collagen V and K-alpha 1 tubulin. These are both components of small airways that are normally not expressed to the host immune system. (Gauthier et al., 2018, Sureshbabu et al., 2020)

Collagen V can be found in the lung epithelium and perivascular and peribronchial tissues, but also in the skin and placenta. It normally effectively masks its epitopes from the immune system because it is assembled in the same fibril as collagen I. (Gracon and Wilkes, 2014) However, allograft injury (e.g., due to ischaemia-reperfusion injury, infection) enhances exposure of these antigenic proteins and results in the release of lung-derived autoantigens as soluble antigens, exosomes or apoptotic bodies. These are detected and subsequently presented by antigen-presenting cells, leading to the induction of autoimmune responses via the Th17 / interleukin (IL)-17 axis. (Gracon and Wilkes, 2014, Sureshbabu et al., 2020) This is possibly initiated by increased cleavage of collagen V due to upregulation of matrix metalloproteinases 2 and 9 (Sureshbabu et al., 2020, Tiriveedhi et al., 2013), alongside loss of peripheral tolerance due to downregulation of Tregs and loss of IL-10 response to self-antigens (Tiriveedhi et al., 2013, Bharat et al., 2006).

K-alpha 1 tubulin is a gap junction protein, essential for cytoskeletal structure and normal cellular function, that gets exposed in cases of injury of the airway epithelium, leading to expression of transcription and growth factors involved in fibroproliferation. (Sureshbabu et al., 2020, Goers et al., 2008)

In other solid organ transplant recipients, several other autoantibodies have been identified that may contribute to chronic rejection (e.g., antibodies to MHC class I-related chain A, angiotensin type 1 receptor or endothelin type A receptor). These antibodies may also play a

role in CLAD. (Sumitran-Holgersson, 2008, Lyu et al., 2012, Angaswamy et al., 2013, Reinsmoen et al., 2017)

Innate immunity

It is increasingly recognised that an advanced interplay between innate and adaptive immunity drives graft injury. Several innate immune pathways facilitate recruitment of inflammatory cells into the allograft and are key elements in the pathogenesis of primary graft dysfunction, acute rejection and CLAD. (Kawashima and Juvet, 2020)

Innate immunity encompasses a broad spectrum of immune responses mediated by elements that do not rely on gene rearrangement, including neutrophils, macrophages, NK cells, and eosinophils. In addition, the complement system plays an important role in innate immunity via the alternative and lectin pathways. Innate immunity is usually activated through pathogen- and damage-associated molecular patterns. The latter are endogenous molecules that are released from injured cells, such as adenosine triphosphate, donor-derived cell-free DNA, mitochondrial DNA, high-mobility group box 1, heat-shock protein, and hyaluronan. (Kawashima and Juvet, 2020) These molecular patterns are recognised by pattern recognition receptors such as Toll-like receptors, the receptor for advanced glycosylation endproducts and nucleotide-binding oligomerisation domain-like receptors. (Belperio et al., 2009) Recognition induces (sterile) inflammation, characterised by recruitment of mainly neutrophils and macrophages, upregulation of MHC expression and antigen presentation, followed by activation of the adaptive immune system. (Yoshiyasu and Sato, 2020) (Figure 1.9) More details on the role of innate immune cells in CLAD are provided in Chapter 3: Effector immune cells in CLAD: a systematic review.

Genetic variants associated with CLAD

Several genetic variants appear to contribute to the development of CLAD and as such may increase an individual's risk to develop CLAD. Not only recipient-related genetic variants play a role, but also donor-related genetic variants. Overall, these variants affect the innate defence system, hereby altering immune responses to injury. They may increase susceptibility to airway inflammation or allograft infection and may therefore contribute to CLAD pathogenesis. (Bos et al., 2022c) Examples are single nucleotide polymorphisms in Toll-like receptors (TLR2, TLR4, TLR9) and gene polymorphisms in HLA-E, CD14, dectin-1, interferon gamma (IFN-γ), IL-6, IL-17A, killer immunoglobulin-like receptors, mannose-binding lectin,

matrix metalloproteinase 7, and TGF-β. (Kastelijn et al., 2010b, Palmer et al., 2005, Luijk et al., 2019, Calabrese et al., 2019c, Kastelijn et al., 2010a, Ruttens et al., 2013) Regarding donor-related genetic variants, gene polymorphisms in surfactant proteins, donor Clara cell secretory proteins, mannose-binding lectin, and CD59 correlated with increased CLAD risk. (Ali et al., 2018, Aramini et al., 2013, D'Ovidio et al., 2020, Budding et al., 2016, Bourdin et al., 2012)

Repair and regeneration processes

Dysregulated epithelial repair and airway and/or tissue remodelling are cornerstones in the pathogenesis of CLAD. Severe, repetitive or persistent alloreactive, autoreactive, infective, or non-specific epithelial injury leads to the loss of epithelial integrity and dysregulated repair. (Belperio et al., 2009) Inflammation can induce an excessive fibroblastic response with excessive extracellular matrix remodelling, resulting in small airways and/or parenchymal fibrosis. Multiple growth factors are involved in this process and are secreted by epithelial cells, fibroblasts and inflammatory cells. (Borthwick et al., 2009) (Figure 1.9)

Epithelial-to-mesenchymal transition (EMT) is a key factor in the onset of fibrosis in which the normal epithelium is replaced by fibroblastic scar tissue. In this process, epithelial cells lose their epithelial properties and acquire a mesenchymal cell phenotype, including deposition of extracellular matrix and production of metalloproteinases. TGF-β1 plays a key role, by inducing fibroblast proliferation and differentiation into myofibroblasts, but also as a main driver of EMT. (Borthwick et al., 2009) Metalloproteinases can in turn facilitate uncontrolled extracellular matrix turnover, epithelial damage, fibrosis, and tissue remodelling. (Banerjee et al., 2011, Heijink et al., 2015) A similar process of mesothelial-to-mesenchymal transition has been described in RAS. (Sacreas et al., 2019)

Next to epithelial injury, increased angiogenic activity and vascular remodelling, initiated by airway inflammation and damage to the airway microvasculature, are also believed to be important. (Belperio et al., 2009, Walters et al., 2008) A recent study showed that nearly 50% of BOS patients had chronic vascular abnormalities, such as pulmonary arteriopathy, pulmonary venopathy and bronchial arterial vasculopathy. (Vanstapel et al., 2022)

1.2.8 CLAD treatment options anno 2023

Parts of this section have been the subject of the following publication: **Bos S**, Pradère P, Beeckmans H, Zajacova A, Vanaudenaerde BM, Fisher AJ, Vos R. Lymphocyte depleting and modulating therapies for chronic lung allograft dysfunction. Pharmacol Rev. 2023;75(6):1200-17. See Appendix B p. 228. Reprinted with permission.

Standard immunosuppressive maintenance treatment after lung transplantation usually consists of triple therapy, including a calcineurin inhibitor (i.e., tacrolimus or cyclosporine), a cell cycle inhibitor (i.e., mycophenolate mofetil or azathioprine) and corticosteroids. (Nelson et al., 2022) Anno 2023, there are few effective treatment options to slow the progressive decline in lung function in CLAD and an effective medical treatment, targeting specific immune cells or pathways, is one of the greatest unmet needs. (Bos et al., 2023) First-line treatment often includes intensification and optimisation of maintenance immunosuppression, such as augmentation of corticosteroids and switching to more potent immunosuppressive drugs, such as from cyclosporine to tacrolimus and azathioprine to mycophenolate mofetil. (Nelson et al., 2022) This, often in combination with the addition of azithromycin (if not already initiated as preventive treatment post-transplant), is usually instituted as an early measure to aim to halt CLAD progression. (Verleden et al., 2019a) Beyond this first line of treatment, several options to attenuate the progression of CLAD have been examined and are directed against both innate and adaptive arms of the immune response in CLAD. Commonly used second-line treatment options include extracorporeal photopheresis, total lymphoid irradiation and anti-thymocyte globulin. (Bos et al., 2023) Some of the main mechanisms of these and other drugs examined in CLAD are shown in Figure 1.10. However, most of these therapies have only been studied in small retrospective single-centre studies and only have limited efficacy, with further disease progression over time in the majority of patients. Treatment options in RAS are even more limited and experimental. (Bos et al., 2023)

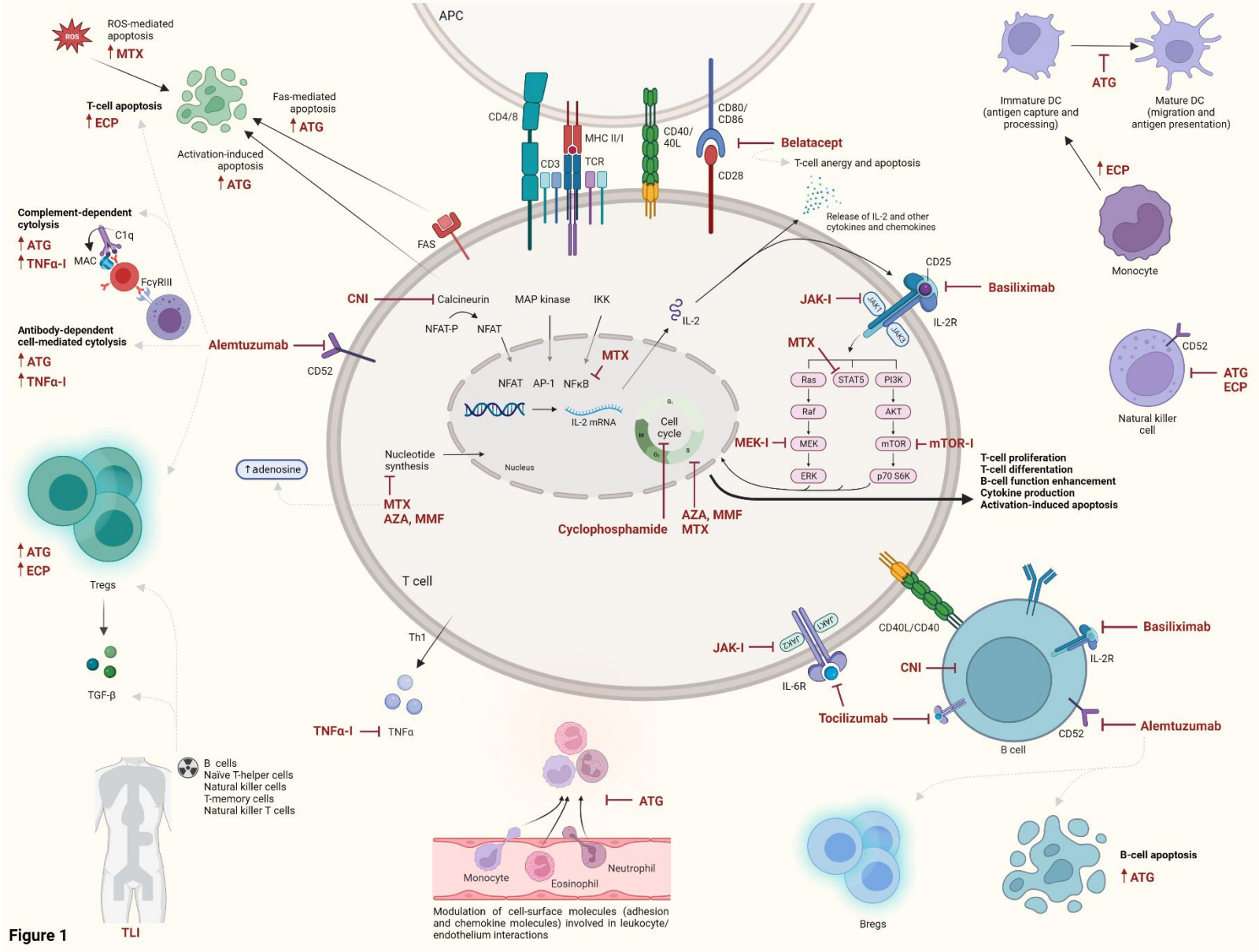

APC
ROS-mediated apoptosis
↑MTX
T-cell apoptosis
↑ECP
Activation-induced apoptosis
↑ATG
Fas-mediated apoptosis
↑ATG
FAS
CD4/8
MHC II/I
CD3
TCR
CD40/ 40L
CD80/ CD86
CD28
Belatacept
T-cell anergy and apoptosis
Release of IL-2 and other cytokines and chemokines
Immature DC (antigen capture and processing)
ATG
Mature DC (migration and antigen presentation)
↑ECP
Monocyte
Complement-dependent cytolysis
↑ATG
↑TNFα-I
C1q
MAC
FcγRIII
Antibody-dependent cell-mediated cytolysis
↑ATG
↑TNFα-I
Alemtuzumab
CD52
CNI
Calcineurin
NFAT-P
NFAT
MAP kinase
IKK
NFAT
AP-1
NFκB
MTX
IL-2 mRNA
IL-2
CD25
Basiliximab
IL-2R
JAK-I
JAK1
JAK3
MTX
Ras
STAT5
PI3K
Raf
AKT
MEK-I
MEK
mTOR
mTOR-I
ERK
p70 S6K
CD52
ATG
ECP
Natural killer cell
Cell cycle
Nucleotide synthesis
Nucleus
↑adenosine
MTX
AZA, MMF
Cyclophosphamide
AZA, MMF
MTX
T-cell proliferation
T-cell differentation
B-cell function enhancement
Cytokine production
Activation-induced apoptosis
↑ATG
↑ECP
Tregs
TGF-β
T cell
Th1
TNFα-I
TNFα
JAK-I
JAK1
JAK2
IL-6R
Tocilizumab
CD40L/CD40
Basiliximab
IL-2R
CNI
CD52
Alemtuzumab
B cell
B cells
Naïve T-helper cells
Natural killer cells
T-memory cells
Natural killer T cells
TLI
ATG
Monocyte
Eosinophil
Neutrophil
Modulation of cell-surface molecules (adhesion and chemokine molecules) involved in leukocyte/ endothelium interactions
Bregs
B-cell apoptosis
↑ATG

Figure 1

Figure 1.10. Main mechanisms of different drugs studied in CLAD
Overview of main mechanisms of several lymphocyte depleting and/or modulating therapies for CLAD. APC: antigen-presenting cell (e.g., dendritic cell, macrophage, B cell), AZA: azathioprine, Bregs: regulatory B cells, CLAD: chronic lung allograft dysfunction, CNI: calcineurin inhibitor, DC: dendritic cell, JAK-I: Janus kinase inhibitor; MEK-I: mitogen-activated protein kinase kinase inhibitor, MMF: mycophenolate mofetil, MTX: methotrexate, TNFα-I: tumour necrosis factor alpha inhibitor, Tregs: regulatory T cells. Created with BioRender.com. Reprinted with permission. (Bos et al., 2023)

There are no real prevention strategies, other than adequate immunosuppressive treatment and compliance, and prevention and treatment of risk factors, including infection prophylaxis and adequate treatment of gastro-oesophageal reflux. Only prophylactic treatment with azithromycin appeared to significantly reduce the occurrence of CLAD (especially BOS) and to improve long-term survival. (Ruttens et al., 2016, Li et al., 2020) In established BOS, azithromycin improved long-term survival in a significant proportion of patients and correlated with BAL neutrophilia. (Corris et al., 2015) The actions of azithromycin are mediated by anti-inflammatory and immunomodulatory effects. (Vos et al., 2012)

There is some evidence that montelukast, a selective leukotriene receptor antagonist, may reduce the rate of FEV_1 decline in CLAD patients. Rapid decliners and patients with RAS were less likely to respond, and those who responded appeared to have higher blood eosinophils. (Vos et al., 2019, Ruttens et al., 2018)

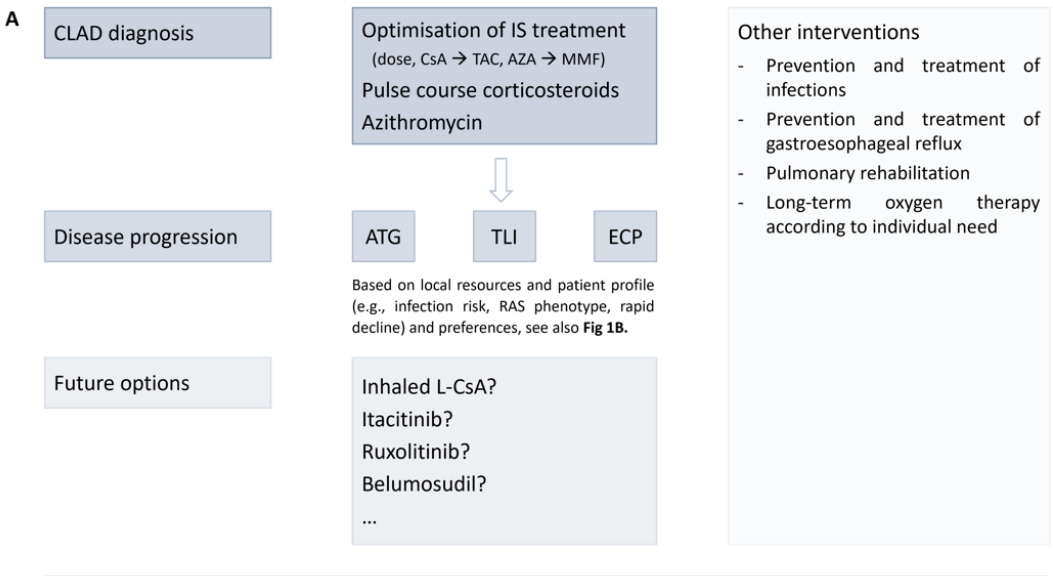
There is a larger body of evidence from multiple retrospective and prospective, single- and multicentre studies that extracorporeal photopheresis may reduce the rate of lung function decline in CLAD, without an increased risk of infection or significant adverse events. (Bos et al., 2023) Efficacy appeared to be more pronounced in BOS patients with a slowly progressive FEV_1 decline and increased blood or BAL neutrophilia. (Del Fante et al., 2015, Greer et al., 2013, Hage et al., 2021, Vazirani et al., 2021) Disease progression was less likely to be attenuated in rapid decliners or in patients with RAS. (Del Fante et al., 2015, Greer et al., 2013) The actions of extracorporeal photopheresis are not completely understood, but are believed to be primarily based on immunomodulatory effects. (Bos et al., 2023) (Figure 1.10) Given that this response appeared to be independent of CLAD duration as well as disease stage at treatment initiation, extracorporeal photopheresis should be considered a viable second-line treatment option. However, larger prospective clinical trials are needed to help predict treatment response and ultimately guide the placement of extracorporeal photopheresis in the treatment algorithm for CLAD. (Bos et al., 2023) As such, the results of a currently ongoing

multicentre randomised controlled trial comparing extracorporeal photopheresis plus standard of care versus standard of care alone in patients with progressive CLAD in the UK (NIHR130612) are eagerly awaited.

In rapid decliners, total lymphoid irradiation may be more effective, especially in BOS patients, but possibly also in RAS patients. (Fisher et al., 2005, Lebeer et al., 2020, Geng-Cahuayme et al., 2022) Data remain currently relatively scarce, although findings were consistent across most studies in which total lymphoid irradiation appeared to attenuate the decline in lung function. (Bos et al., 2023) However, due to the intense depletion of immune cells, there is a higher risk of side effects, including pancytopenia and secondary infections. This suggests that total lymphoid irradiation should be used with caution, although reassuringly the incidence of serious adverse events was low. However, thrombocytopenia, neutropenia and infections often led to treatment being delayed or terminated prematurely. (Fisher et al., 2005, Geng-Cahuayme et al., 2022, Lebeer et al., 2020, O'Hare et al., 2011)

Anti-thymocyte globulin is most commonly used as an induction immunosuppressant in recipients of lung and other solid organ transplants, but can also be used in the treatment of CLAD. Anti-thymocyte globulin appeared to be effective in stabilising or attenuating lung function decline in a subgroup of CLAD patients, including RAS patients. However, the body of evidence is small and multicentre, randomised controlled trials are needed to better determine predictors of response. (Bos et al., 2023)

An overview of these second-line treatment options is given in Figure 1.11, along with some associated characteristics.



B

	ATG	TLI	ECP
Time to start	Days	Weeks	Weeks
Treatment duration	Days	Weeks (usually 5)	Months
Inpatient stay	Yes	No	Yes/no
Start of treatment effect	Days-weeks	Weeks	Weeks-months
Estimated efficacy (stabilisation or improvement)	≥ 50%	≥ 50%	≥ 50%
Duration of treatment effect	Weeks-months	Weeks-months	Weeks-months
Side effects	+	++	+/-
Costs	+	+/++	+++

Figure 1.11. Second-line treatment options in CLAD
A: Suggested treatment algorithm for CLAD based on existing data taking into account the efficacy and risk of side effects as well as some potential safer future options that require more investigation. **B:** Overview of features associated with ATG, TLI and ECP treatment. Which therapeutic option is chosen mainly depends on local resources and patient profile (e.g., risk of infection, CLAD phenotype, rapid versus slow lung function decline) and preferences.
ATG: anti-thymocyte globulin, AZA: azathioprine, CLAD: chronic lung allograft dysfunction, CsA: cyclosporine A, ECP: extracorporeal photopheresis, IS: immunosuppressive, L-CsA: liposomal cyclosporine A, MMF: mycophenolate mofetil, TAC: tacrolimus, TLI: total lymphoid irradiation. Reprinted with permission. (Bos et al., 2023)

Next to these lymphocyte depleting and/or modulating therapies, several other treatment options have been tested, including antifibrotic agents. Some small studies showed beneficial results of antifibrotic agents in CLAD. (Bos et al., 2021) However, a recent multicentre, randomised trial of pirfenidone in new-onset progressive BOS patients could not demonstrate a significant effect on pulmonary function decline. (Perch et al., 2020) So far, no data are available on the effects of nintedanib in BOS. Antifibrotic treatment may have a greater effect in RAS patients, and some case reports and one small case series showed a decrease in the rate of pulmonary function decline. (Vos et al., 2013, Vos et al., 2018, Suhling et al., 2016) However, larger randomised trials are pivotal and pending.

Lastly, redo lung transplantation might be a feasible option in well-selected CLAD (mainly BOS) patients with acceptable long-term outcomes. (Verleden et al., 2019a, Verleden et al., 2015b)

In conclusion, several therapeutic options have been used in attempts to prevent, reverse or slow CLAD progression. However, there are only limited effective therapeutic options and there is currently no consensus on the most effective option. Furthermore, interpretation of these results is overshadowed by the fact that randomised controlled trials are almost universally lacking, which may make it unclear whether the attenuated rate of FEV_1 decline represents true treatment response or merely the natural course of the disease. In advanced CLAD stages, a less pronounced decline in lung function may also be due to limited residual lung function. However, some studies showed sustained lung function stabilisation or improvement even in advanced CLAD stages. (Bos et al., 2023)

A better understanding of the underlying immunopathology and more research into prevention and treatment of CLAD, with development of individualised therapies specific to each phenotype and individual patient profiles, are imperative to further improve long-term outcomes after lung transplantation. Multicentre randomised controlled trials, preferably also including RAS patients, with longer follow-up as well as platform trials moving rapidly between investigational agents are urgently needed to define the most appropriate treatment algorithm for CLAD. (Bos et al., 2023) Given that increased risk of infection is a common and important side effect that can contribute significantly to morbidity and mortality, new treatment options should also be explored and focus should be on potentially safer immunosuppressants. (Figure 1.12)

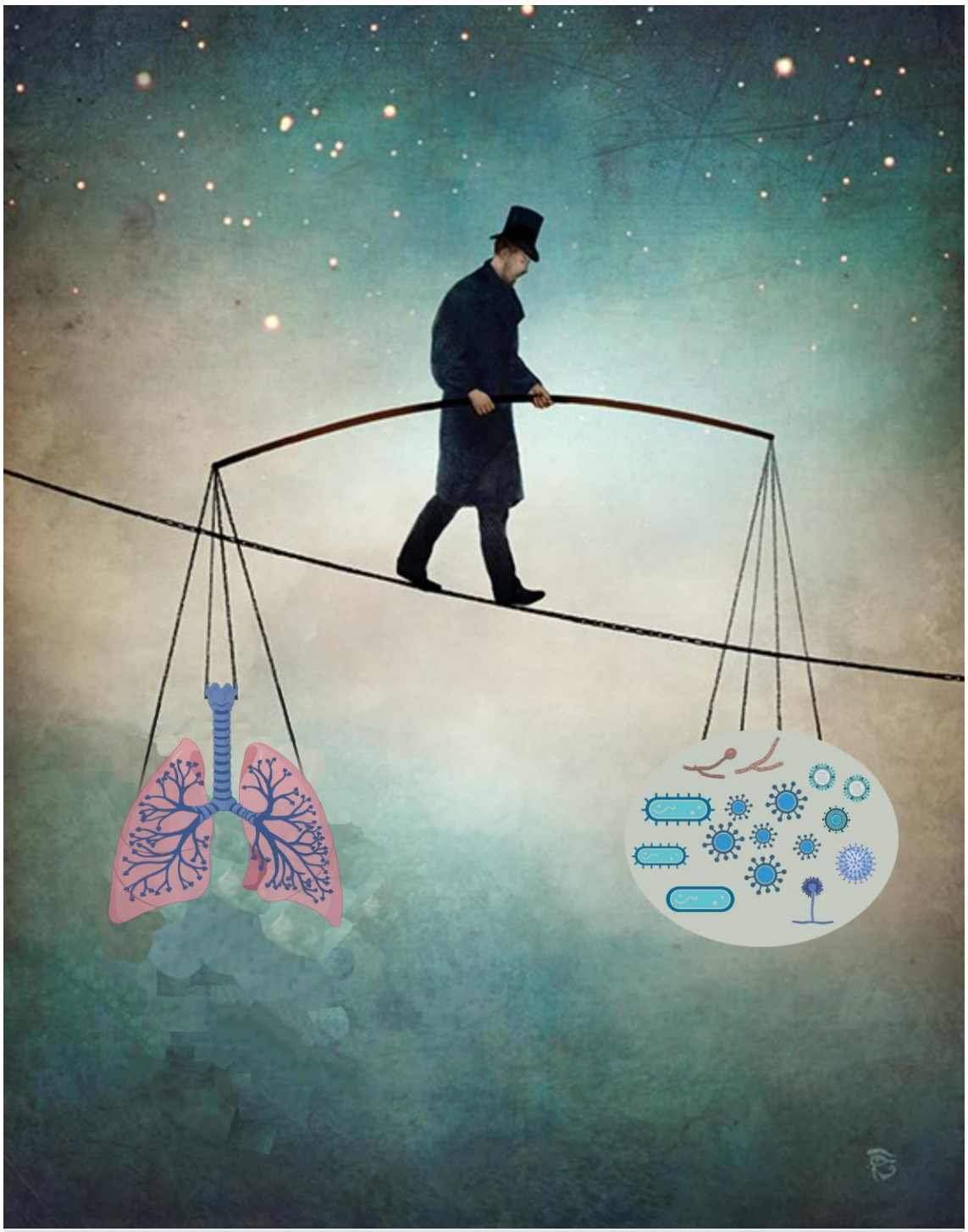


Figure 1.12: The balance between graft protection and risk of infection
Adapted from Christian Schloe, partially created with BioRender.com.

1.2.9 Challenges related to CLAD

A better understanding of the exact underlying immunopathology of CLAD is needed to promote earlier detection, the development of biomarkers with high accuracy, and new preventive and therapeutic drugs.

Currently, one of the biggest challenges is that at the time of CLAD diagnosis, immune activation and subsequent repeated/persistent inflammation have already caused significant lung damage and fibrosis. Although the definition of CLAD as a clinical entity represents a step forward in describing and standardising post-transplant graft loss, there are inherent limitations. It is purely a descriptive term referring to a progressive, irreversible decline in lung function. More specifically, the diagnosis of CLAD requires that a patient has already irreversibly lost more than 20% of their baseline lung function. (Verleden et al., 2019a) In addition, there are many risk factors that can contribute to the development of CLAD, but the term itself does not provide insight into the cause, nor the underlying pathophysiology in a specific individual. A fundamentally important question facing clinicians remains the ability to predict which patients are likely to develop CLAD at a time when graft loss may be reversible.

Vast heterogeneity in clinical features of lung transplant recipients combined with small cohort sizes internationally has hitherto hampered efforts to identify robust biomarkers for early disease identification. To date, none of the potential biomarkers tested, such as circulating interleukins, profibrotic factors or donor-derived cell-free DNA, have been specific enough to predict the onset of CLAD and establish a diagnosis before loss of graft function manifests. (Pradère et al., 2023) There is a vast area of unmet clinical need to develop biomarkers of graft dysfunction prior to CLAD, to stratify patients according to their personal individualised prospect of CLAD development, and to facilitate personalised intervention based on their own specific combination of risk factors. In other words, there is an urgent need to develop clinically relevant biomarkers to identify those patients that are at risk of developing CLAD at a time when intervention may prevent the development of fibrosis and preserve valuable lung function. Furthermore, biomarkers are likely to provide valuable insights into complex pathophysiological pathways leading to CLAD with hope for subsequent development of novel therapeutics. (Pradère et al., 2023)

The other major challenge is that, as explained in the previous subsection, therapeutic interventions are of limited benefit with best outcomes restricted to cessation of ongoing decline and stabilisation of lung function. There is a compelling need for more effective treatments to prevent the onset and progression of CLAD. Additionally, biomarkers are needed to better understand an individual's balance between over- and underimmunosuppression. After all, if there is insufficient immunosuppression, the allograft is at risk of acute and chronic rejection. However, in the case of overimmunosuppression, there is increased risk of infection and associated morbidity and mortality. This balance is very precarious, varies from patient to patient and may vary within an individual over time. (Figure 1.12) Thus, since each patient's post-transplant journey is different, it is important to understand an individual's (immune) profile and risk of infection/rejection in order to implement precision medicine in the hope that each individual can be treated in the best possible way. The intention is to move away from a one-size-fits-all model that is still widely used today.

Nevertheless, to make progress in any of these areas (e.g., detection, prevention, treatment, biomarkers), a better understanding of the underlying immunopathophysiology is imperative.

1.3 From conventional flow cytometry to imaging mass cytometry

Deciphering the immunological mechanisms that underlie cellular function and disease pathogenesis is a central goal in biology and translational research. Technological advances have provided scientific insights across many areas of research. Given the complexity of the human immune system, immunology has benefited from innovations in various single-cell technologies. And research has been driven by the need to perform as many measurements on as many cells as possible towards the goal of identifying every single-cell type, transition state and functional state. (Filby and Houston, 2017, Hartmann and Bendall, 2020) Recent technologies based on high-dimensional methodologies have facilitated in-depth study of the composition and activation of immune cell populations and their relation to disease. (Hartmann and Bendall, 2020)

1.3.1 Cytometry

Cytometry stems from two Greek words, 'cyto' comes from the word kytos and means cell, and 'metry' from metria, meaning the process of measuring. Accordingly, cytometry can be defined as the process of cell measurement. More specifically, cytometry is the measurement of a cell's phenotype, morphology and function at the single-cell level, always performed on a population of cells in order to understand and decipher the cellular heterogeneity inherent in all biological systems. (Filby and Houston, 2017)

There are three tenets of cytometry. The first pillar is that measurements should be performed in a controlled manner, where measurements are performed simultaneously or in a way that successive measurements can be correlated back to single-cell data. Secondly, cytometry should be (semi-)quantitative. This controlled and quantitative approach allows fair measurement comparisons between individual cells and across samples. These principles are based on the premise that cytometry is multiparameter (i.e., multiple measurements of single cells) and that measurements are made in a relatively high-throughput manner (i.e., able to analyse many cells as quickly as possible). The latter also promotes the identification and analysis of rare cell types and short-lived cellular transition states. (Filby and Houston, 2017)

With data obtained from cytometry, one can try to answer the following questions:

- What types of cells are present in our sample?

- What are the relative frequencies of the different types of cells and phenotypes in our sample?
- What do they do functionally?
- What role do they have in the context of disease?

1.3.2 Cytometers

There are many different types of cytometers that can be broadly divided into two classes. The first class are zero-resolution systems where no image information is derived from the measured cells. This includes flow cytometry and mass-based cytometry. The second class is image-based cytometry, such as imaging flow and mass cytometry. There are differences in the number of parameters that these cytometers can measure on each individual cell, ranging from low for conventional fluorescence-based cytometry to very high for imaging mass cytometry (IMC). The trade-off, however, is that as the number of parameters increases, the throughput tends to decrease. (Vembadi et al., 2019)

1.3.3 Conventional cytometry: lack of spatial information

For many years, immune monitoring and cell phenotyping have relied on conventional flow cytometry and immunohistochemistry and immunofluorescence to capture and quantify heterogeneity and its relationship to disease. (Hartmann and Bendall, 2020) Conventional flow cytometry provides rapid analysis of a limited number of parameters of single cells in a liquid suspension. As such, this is very useful for samples derived from liquid biopsies (e.g., blood, BAL). However, when using tissue samples, they must first be disaggregated into single-cell suspension. The development of new fluorophores and laser systems has made it possible to investigate a wider range of parameters, with the discovery of new immune cell subtypes and functional cell states. Nevertheless, the number of parameters than can be analysed simultaneously is still low and restricts analyses in terms of cellular properties and composition of cell populations. (Hartmann and Bendall, 2020)

Flow cytometers work by aligning single cells using flow techniques. In conventional fluorescence-based flow cytometry, targeted fluorescence is added to the cells after which the cells move in a fluid stream through laser beams, producing scattered and fluorescent light signals that are read by detectors. (Figure 1.13) Cell populations can be analysed based on their fluorescent and light-scattering properties. This allows simultaneous quantitative

measurements of several parameters at the single-cell level, within a potentially highly heterogeneous cell population. (Cossarizza et al., 2017)

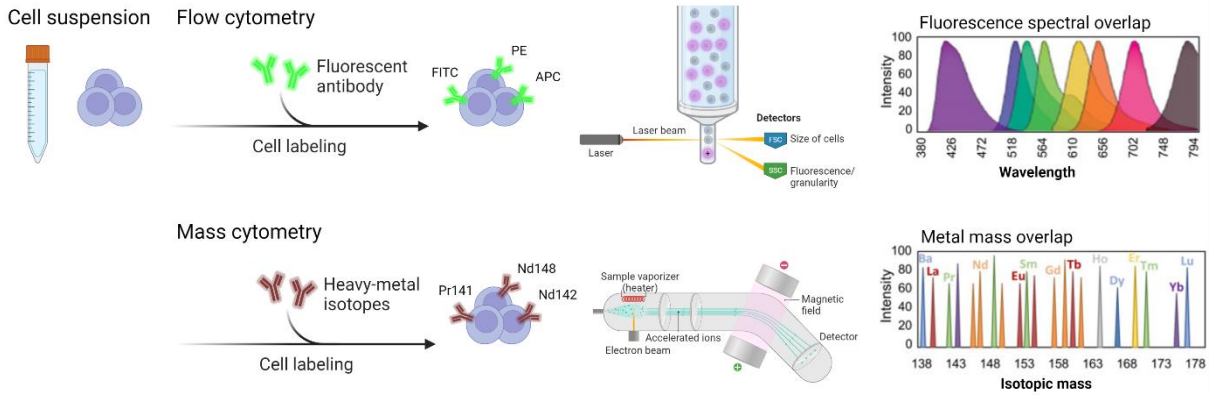


Figure 1.13. Flow and mass cytometry
Schematic overview of mechanisms behind flow and mass cytometry. Partially created with BioRender.com.

Fluorophore-conjugated antibodies, however, are of limited use for high-parameter studies due to signal overlap or fluorescence spillover from one channel to the next. Fluorophore emission spectra are usually broad, leading to overlap into adjacent analysis channels, which results in a non-specific signal in that channel. (Figure 1.13) An additional source of background signal comes from cellular autofluorescence. Many cells contain intrinsically fluorescent molecules across the entire spectrum, leading to autofluorescence and background signal. These limitations add considerable complexity to experimental design and data interpretation. (Filby and Houston, 2017, Hartmann and Bendall, 2020) As a result, addition of further parameters is hindered and simultaneous measurement of the entire immune state is impeded. (Hartmann and Bendall, 2020)

A solution to the physical limitations of fluorescence-based cytometry was the substitution of these fluorescence-based reporters with elemental isotopes and a mass cytometry-based approach. (Hartmann and Bendall, 2020) Cytometry by time-of-flight (also called CyTOF) is a combination of both flow cytometry and elemental mass cytometry. Using antibodies tagged to rare heavy-metal isotopes, CyTOF overcomes the limitations of fluorescence-based detection modalities by separating signals based on differences in mass instead of wavelength. (Filby and Houston, 2017, Hartmann and Bendall, 2020) (Figure 1.13) Indeed, problems related to spectral overlap between different analysis channels are limited because heavy-metal

isotopes have non-overlapping atomic masses that can be accurately resolved and quantified. Therefore, there is minimal spillover between different channels. In addition, the availability of many different heavy-metal isotopes has increased the multiplexing capacity compared with fluorescent-based flow cytometry. Realistically, however, the availability of isotopes of sufficient purity and antibody conjugation chemistries limit applications to a maximum of 40-60 parameters per mass cytometry panel. (Hartmann and Bendall, 2020)

Another advantage over conventional flow cytometry is that there is little variation in signal intensity across channels. With CyTOF technology, they are all ionised to a similar extent and detected with similar efficiency by the time-of-flight instrument; whilst with fluorophores, some fluorophores are very bright whilst others are dim. Finally, since these heavy metals are not naturally present in cells, there is a very low background signal. (Hartmann and Bendall, 2020)

Table 1.4 summarises the main differences between conventional flow cytometry and mass cytometry.

	Conventional flow cytometry	Mass cytometry	Imaging mass cytometry
Antibody labelling	Fluorophores	Heavy metals	Heavy metals
Detector	Fluorescence detector	Mass cytometer	Mass cytometer
Multiplex	Up to 20	Up to 60	Up to 40
Status of cells	Both live and fixed cells can be analysed	Cells are required to be fixed	Cells are required to be fixed
Cell throughput	Thousands of cells per second	Hundreds of cells per second	Hundreds of cells per second
Cell sorting	Cells can be further sorted for functional studies	Cells are destroyed during ionisation	Cells are destroyed during ionisation
Sources of non-specific signal	Spectral overlap Autofluorescence Differences in signal intensity Fluorophore degradation	Almost no spectral overlap No autofluorescence Uniform staining intensity Isotopic impurity Metal oxidation	Almost no spectral overlap No autofluorescence Uniform staining intensity Isotopic impurity Metal oxidation
Spatial resolution	No	No	Yes
Data analysis	Simple	Complex	Complex
Cost	Low	Moderate	High

Table 1.4. Flow cytometry, mass cytometry and imaging mass cytometry
Overview of key differences in flow versus mass cytometry and imaging mass cytometry.(Palit et al., 2019, Hartmann and Bendall, 2020)

1.3.4 Moving towards high-dimensional imaging technologies

A significant limitation of conventional flow and mass cytometry is that cells are converted to a number but without any spatial resolution. That is, the set of parameters per channel for each measured cell contain no multipixel or multivoxel information about the morphology of the cell and location-specific features, such as cellular shape, volume, molecular migration, concentration gradients, etc. However, in many situations in cell biology, location and spatial context are essential to the underlying biological question. (Filby and Houston, 2017) Tissue-based imaging methods can offer a unique opportunity to examine cells in their native or pathological context, and spatial analysis provides insights into the cellular microenvironment and cell-to-cell interactions. (Hartmann and Bendall, 2020)

One option is to use many cycles of imaging, usually done with fluorophore-conjugated antibodies. These are then analysed in multiple cycles of staining, imaging and quenching. In addition to the standard limitations associated with fluorescent-based techniques, this cyclic

process can lead to changes in epitope accessibility and altered tissue quality and morphology. (Hartmann and Bendall, 2020) Another option is novel imaging-based technologies. These have now evolved to sophisticated, high-content, automated imaging cytometers. Imaging cytometry is still based on the basic principle of cytometry, which is to measure a cell phenotype at a single-cell level on a population of cells. However, in imaging cytometry, the single-cell level data are derived mathematically from an image. Consequently, information about signal intensity, texture, shape, and location can be derived. As such, imaging cytometry provides important information on cell morphology and spatial context of a signal within the cell in the form of two- and three-dimensional data. (Filby and Houston, 2017)

Imaging cytometry is a much more diverse field compared with conventional cytometry. A single-channel digital image is far more parameter-rich than single pulses from flow cytometry detectors or CyTOF peak values. The future lies in single-cell analyses with imaging data that provide full per-cell transcriptome or proteome information across a spatial dimension. (Filby and Houston, 2017)

1.3.5 Imaging mass cytometry

IMC is one such imaging-based technology, developed to meet the need for analysing the cellular complexity, cell-to-cell interactions and functional diversity of the immune system. (Hartmann and Bendall, 2020) The Hyperion Imaging System consists of two functional units, the Hyperion Tissue Imager that performs laser ablation of selected tissue regions and a mass cytometer with CyTOF technology. High-resolution scanning laser ablation followed by mass cytometry facilitates simultaneous high-multiplex interrogation of up to 40 different protein markers. This allows deep immunophenotyping of infiltrating cells in the tissue and can be performed on formalin-fixed paraffin-embedded (FFPE) tissue sections. Thus, there is no need to disaggregate to single-cell suspension, thereby preserving the structural context in tissue architecture, cellular morphology and spatial relationships. (Chang et al., 2017, Hartmann and Bendall, 2020) The process of IMC is shown schematically in Figure 1.14.

Images reconstructed from tissue sections scanned by IMC have a resolution comparable to light microscopy, with the high content of mass cytometry, providing detailed single-cell information, highly multiplexed (simultaneous analysis of 4-40 specific protein markers), combined with spatial relationships (cellular location, proximity to other cells, sub- and extracellular structures). (Chang et al., 2017, Hartmann and Bendall, 2020) As a result, IMC

offers unique advantages for the analysis of tissue samples in unprecedented detail. It allows for a comprehensive understanding of complex cellular phenotypes, insight into cell-cell interactions and their relationship to the cellular microenvironment, and offers a unique opportunity to investigate cells in their native and pathological context. (Hartmann and Bendall, 2020)

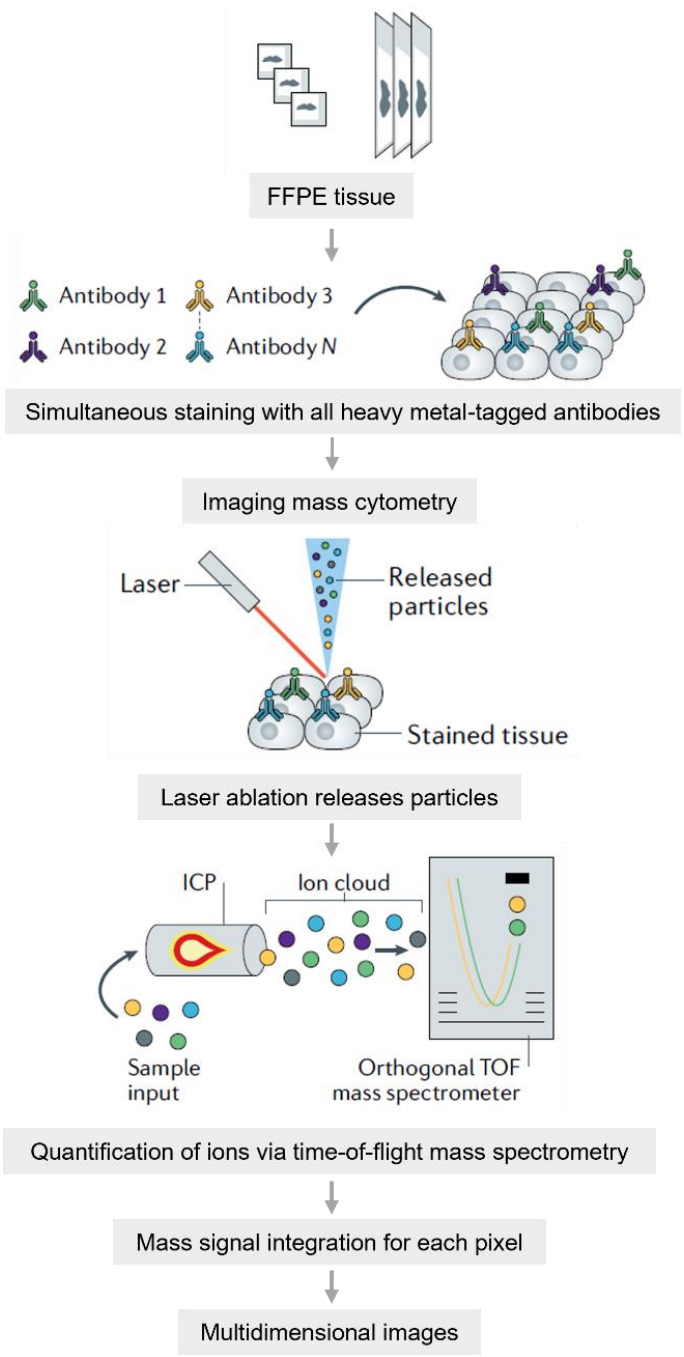


Figure 1.14. Imaging mass cytometry
Overview of basic principles of high-dimensional imaging analysis of tissue sections by imaging mass cytometry. Adapted from Hartmann et al. (Hartmann and Bendall, 2020)
FFPE: formalin-fixed paraffin-embedded

1.4 Conclusions

Lung transplantation is a viable treatment option for well-selected patients with chronic end-stage lung diseases. CLAD is the most important long-term complication, affecting an average of 50% of lung transplant recipients five years after transplantation. The pathophysiology of CLAD is multifactorial, although a better understanding of all immune cells at play is needed to improve early detection, prevention and treatment. In addition, increasing insights into the complex pathophysiological pathways leading to CLAD may support the development of clinical biomarkers, which will not only be useful in diagnostics, but also enable progress towards personalised medicine.

To gain a better understanding of the complex underlying immunopathology of CLAD, new high-dimensional imaging technologies such as IMC could be very valuable. More specifically because IMC allows multiparametric analysis of immune cells and their functional states at the lung tissue level, while preserving spatial information.

Key points

- Over 4,500 adult lung transplant procedures are performed worldwide annually.
- Median overall survival after lung transplantation is approximately seven years in the most recent era, and is limited primarily due to the development of CLAD.
- CLAD develops in up to 50% of recipients within five years of transplantation.
- The most common phenotype of CLAD is the obstructive phenotype BOS in about 70% of CLAD patients. BOS is characterised by an obstructive lung function decline with FEV1 ≤ 80% from baseline due to airway inflammation and fibrosis.
- The restrictive phenotype RAS is a less common phenotype in 20-30% of CLAD patients, characterised by pleuroparenchymal fibrosis with a restrictive decline in lung function with FEV1 ≤ 80% and TLC ≤ 10% from baseline, together with persistent pleuroparenchymal opacities on chest imaging.
- The pathophysiology of CLAD is multifactorial and is thought to be mediated via activation of both innate and adaptive arms of the immune system in response to a myriad of immunological, infectious and mechanical insults. CLAD has alloimmune-dependent and alloimmune-independent risk factors.

- There are only a few treatment options for CLAD that provide limited benefit, with best outcomes restricted to cessation of ongoing decline and stabilisation of lung function. As such, there is a compelling need for more effective treatments to prevent the onset and progression of CLAD.
- To make progress in better and earlier detection of CLAD, as well as in prevention and treatment, a better understanding of the underlying immunopathophysiology is of primary importance.
- Advanced single-cell proteomic approaches might be of value for the study of a wide variety of clinical samples.
- The combination of mass cytometry with high-dimensional imaging techniques in IMC enables in-depth characterisation of the phenotype and functional state of immune cells at the single-cell level and adds unique information about spatial relationships and cell-to-cell interactions.
- Immune cell phenotyping with such high-dimensional single-cell approaches has the potential to uncover new insights into pathological processes, diagnostics and therapeutic options for CLAD.

1.5 Aims and objectives

1.5.1 Aims

To improve patient outcomes and prolong survival after lung transplantation, it is paramount to better understand the specific immunopathological mechanisms that contribute to the onset of CLAD. Due to the extensiveness of immune pathways active in CLAD pathogenesis, we believe that a broader global assessment is needed to uncover key immune cells involved in the trajectory from a stable lung allograft to inflammation and ultimately fibrosis in CLAD. Therefore, the two overriding aims of this PhD were firstly to summarise our current knowledge of CLAD pathogenesis, and secondly to investigate the immunological processes involved in CLAD development through immunophenotyping of human lung transplant tissue in a translational research project.

The specific aims of this PhD were as follows:

- Summarise current data on key immune processes in CLAD pathogenesis.
- Gain a better understanding of what immune cell phenotypes contribute to the difference in immune profile between CLAD patients and stable lung transplant recipients.
- Gain a better understanding of the differences at the immunological level between BOS and RAS.
- Examine differences in immune cell types between regions of interest (ROIs) with different degrees of inflammation and fibrosis.
- On the trajectory from stable graft function to CLAD via episodes of acute rejection and infection, what differences in immune cell phenotypes emerge.
- Identify future research priorities.

1.5.2 Objectives

How we intended to achieve these aims is explained in the following work packages. More details are given in the Methods Chapter.

Work package 1: Perform a narrative literature review to summarise available data on key processes in CLAD pathogenesis.

The first objective was to perform a narrative literature review to summarise the current knowledge on key processes in CLAD pathogenesis.

Work package 2: Perform a systematic literature review to summarise available data describing the phenotype of immune cells in CLAD tissue and BAL.

The second objective was to perform a systematic review to provide an overview of data available to date on the phenotype of immune cells in CLAD tissue and BAL. This then helped us design a panel of structural and immune cells of interest to be examined in CLAD tissue using IMC in our translational research project.

Work package 3: High-dimensional tissue profiling of immune cell responses in CLAD using explanted lungs and IMC.

The objective of this translational research project was to investigate the immunological processes in CLAD, both BOS and RAS, through detailed immunological profiling (40 markers) of explanted lung tissue using high-resolution IMC. By doing so, we aimed to obtain detailed single-cell information, as well as insights into cell-cell interactions and their relationship to the cellular microenvironment. At the start of my PhD, there was almost no information about IMC in CLAD. Only one small pilot study by Renaud-Picard et al. was available in abstract form, demonstrating the feasibility of IMC and confirming interest in further research with IMC in CLAD. (Renaud-Picard et al., 2020, Renaud-Picard et al., 2022) Also at the end of my PhD, data from single-cell resolution spatial studies using lung tissue from patients with CLAD remained scarce.

Work package 4: Set up a prospective study to collect research samples (blood, BAL, TBB, and endobronchial biopsies) during bronchoscopy.

The objective of this prospective study was to restart systematic collection of extra samples for research purposes (blood, BAL, TBB, and endobronchial biopsies) at times when patients are already scheduled for bronchoscopy, according to their routine follow-up schedule or when clinically indicated (IRAS 296641). Ethical approval by the Research Ethics Committee (REC 21/PR/0981), Health Research Authority and hospital sponsor (The Newcastle upon Tyne Hospitals NHS Foundation Trust, R&D 09899) was obtained, with final approval in January 2022. Samples have been collected and used for other projects outside the scope of this PhD Thesis, and will as such not be discussed further:

- Collaboration with Therasure, Oncocyte: prospective donor-derived cell-free DNA study.

- Collaboration with Prof. Dr. Anna Reed, Harefield Hospital, Royal Brompton & Harefield hospitals, London and Prof. Dr. Darius Armstrong, Imperial College London: immune cell landscape in blood and BAL in CLAD, Aspergillus study.

Work package 5: Identify possible new pathways, functional biomarkers and targets for the detection, prevention and treatment of CLAD, and indicate future (research) opportunities.
We hoped that new insights into the immunological processes in CLAD, obtained from work packages 2-4, would help us discover structural and immune proteins of interest for specific further research, development of potential functional biomarkers or therapeutic targets for the prevention and treatment of CLAD.

Chapter 2 Materials and methods

2.1 Narrative literature review: Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle

2.1.1 Summary

The purpose of this narrative literature review was to introduce and critically assess key literature on the pathogenesis of CLAD and to highlight missing pieces of the puzzle. Results of the literature review are included in the Introduction Chapter.

2.1.2 Methods: narrative review

Because the pathogenesis of CLAD is much broader than alloimmune responses alone, we felt it was important to provide a state-of-the-art overview on what we currently know about key processes involved in CLAD pathogenesis. The methodology used for this literature review was that of a narrative review to summarise the existing literature and to provide an overview of the topic. Narrative reviews are ideal for presenting a broad perspective (Green et al., 2006), in this case providing specific context and background information on CLAD pathogenesis. No systematic search and evaluation were carried out. The electronic databases of PubMed (MEDLINE) and EMBASE were consulted for relevant articles. Results were described using a narrative approach.

2.1.3 Topics of interest

We specifically wanted to synthesise available data on the following (sub)topics:

- Immune processes in CLAD
    - T cell-mediated immunity
    - Humoral immunity
    - Autoimmunity
    - Innate immunity
- Repair and regeneration processes
    - Aberrant epithelial repair
    - Angiogenesis and vascular changes
- Alloimmune-dependent risk factors

    - Acute cellular rejection and lymphocytic bronchiolitis
    - Antibody-mediated rejection
- Alloimmune-independent risk factors

In addition, we wanted to identify the missing pieces of the puzzle, namely what is missing from our current understanding of this puzzle and the areas we believe future research should focus on.

Results of this literature review are incorporated in the Introduction Chapter. The full published paper can be found in Appendix A.

2.2 Narrative literature review: Lymphocyte depleting and modulating therapies in chronic lung allograft dysfunction

2.2.1 Summary

The purpose of this narrative literature review was to assess key literature on the role of lymphocyte depleting and modulating therapies in CLAD management. Results of the literature review are included in the Introduction Chapter.

2.2.2 Methods: narrative review

Since immune responses in CLAD, in which lymphocytes play a crucial role, are the main topic of this PhD Thesis, we felt it was important to update where we stand regarding the use of lymphocyte depleting and modulating therapies in CLAD. The methodology used for this literature review was also that of a narrative review to summarise the existing literature. No systematic search and evaluation were conducted. The electronic databases of PubMed (MEDLINE) and EMBASE were consulted for relevant articles. Results were described using a narrative approach.

2.2.3 Topics of interest

We specifically wanted to describe available data on the following (sub)topics:

- Immunodepleting therapies
    - Alemtuzumab
    - Anti-thymocyte globulin
    - Total lymphoid irradiation
- Immunomodulating therapies
    - Methotrexate
    - Cyclophosphamide
    - mTOR (mammalian target of rapamycin) inhibitors
    - Belatacept and basiliximab
    - Tumour necrosis factor (TNF) alpha inhibitors
    - Extracorporeal photopheresis
- B cell-directed treatment
- Future directions

- Tyrosine kinase inhibitors
- Janus kinase inhibitors
- Rho kinase inhibitors
- MEK (mitogen-activated protein kinase kinase) inhibitors
- IL-6 inhibitors
- Inhaled liposomal cyclosporine A

Results of this literature review are incorporated in the Introduction Chapter. The full published paper can be found in Appendix B.

2.3 Systematic review: Effector immune cells in chronic lung allograft dysfunction

2.3.1 Summary

The aim of this systematic review was to comprehensively assess the phenotype of immune cells in allograft tissue or BAL from patients with CLAD. The following subsections explore each of the stages of the methodology in more detail.

2.3.2 Research question

The PICO question was as follows: "In lung transplant patients with CLAD, what immune cell phenotypes are found in the allograft tissue or BAL?". Ideally compared with lung transplant patients without CLAD, but the comparator was not strict and could also be other controls. Other controls were typically healthy controls or unused donor lung tissue in the case of studies involving tissue samples.

P: lung transplant patients with CLAD, both BOS and RAS.

I: allograft tissue or BAL.

C: lung transplant patients without CLAD, other controls (e.g., healthy controls, non-used donor lung tissue) or no controls.

O: immune cells including lymphocytes, NK cells, neutrophils, eosinophils, monocytes, macrophages, dendritic cells, basophils, and mast cells. But also other relevant factors, namely: complement, matrix metalloproteinases, cytokines, and chemokines. A full list of outcomes captured by these MeSH and non-MeSH terms that were of interest to us is displayed in Table 2.1.

2.3.3 Inclusion and exclusion criteria

The following inclusion criteria were used: prospective and retrospective original research studies, human data, data in lung transplant recipients only, analyses on CLAD tissue or bronchoalveolar lavage fluid. Articles were further restricted to publications from January 2000 onwards, English-language articles and articles with full-text access. Results from blood analysis, in vitro analysis, studies in animals, case reports, and conference abstracts were excluded.

Immune cells	Cytokines and chemokines	Other
Granulocytes	Cytokines	Complement
Basophils	Chemokines	Mannose-binding lectin
Eosinophils	Chemokines, C	MMPs
Neutrophils	Chemokines, CC+	MMP 1
Leukocytes, Mononuclear	Chemokines, CX3C+	MMP 2
Cytokine-Induced Killer Cells	Chemokines, CXC+	MMP 3
Lymphocytes+	Macrophage Inflammatory Proteins+	MMP 7
Monocytes	Interferons	MMP 8
Macrophages	Interferon gamma	MMP 9
	Interferon Type I+	MMP 10
	IL-1 Receptor Antagonist Protein	MMP 11
	Interleukins	MMP 12
	IL-1+	MMP 13
	IL-10	MMP 14
	IL-11	MMP 15
	IL-12+	MMP 16
	IL-13	MMP 17
	IL-15	MMP 20
	IL-16	
	IL-17	
	IL-18	
	IL-2	
	IL-23+	
	IL-27	
	IL-3	
	IL-33	
	IL-4	
	IL-5	
	IL-6	
	IL-7	
	IL-8	
	IL-9	
	Monokines	
	Transforming Growth Factor beta	
	Transforming Growth Factor beta 1	
	Transforming Growth Factor beta 2	
	Transforming Growth Factor beta 3	
	Tumour Necrosis Factors	
	Tumour Necrosis Factor alpha	

Table 2.1. Outcomes of interest
Overview of outcomes of interest captured with the MeSH and non-MeSH search terms used. + means there were more subgroups for this search term.
IL: interleukin, MMP: matrix metalloproteinase.

2.3.4 Literature search and screening

The systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. (Page et al., 2021) The literature search for the PICO question was based on a priori defined inclusion and exclusion criteria as outlined above. A systematic search on the electronic databases of PubMed (MEDLINE) and EMBASE was performed using keywords related to immune cells and CLAD. The search strings and filters used were as follows:

PubMed

("Leukocytes"[Mesh] OR "Macrophages"[Mesh] OR "complement" OR "mannose-binding lectin" OR "Matrix Metalloproteinases"[Mesh] OR "Cytokines"[Mesh] OR "neutrophilia" OR "eosinophilia" OR "Bronchoalveolar Lavage"[Mesh] OR "flow cytometry" OR "Histology"[Mesh] OR "lung biopsy" OR "transbronchial biopsy" OR "lung tissue") AND ("chronic lung allograft" OR "CLAD" OR "chronic lung rejection" OR "restrictive allograft" OR "rCLAD" OR "bronchiolitis obliterans syndrome" OR "obliterative bronchiolitis")

Filter: Publication start date: 01 Jan 2000, language: English, text availability: full text.

EMBASE

('matrix metalloproteinase'/exp OR 'mannose binding lectin' OR 'leukocyte'/exp OR 'macrophage'/exp OR 'cytokine'/exp OR 'complement'/exp OR 'neutrophilia' OR 'eosinophilia' OR 'bronchoalveolar lavage fluid'/exp OR 'histology'/exp OR 'flow cytometry'/exp OR 'lung biopsy'/exp OR 'lung tissue') AND ('chronic lung allograft dysfunction'/exp OR 'bronchiolitis obliterans syndrome'/exp OR 'restrictive allograft syndrome'/exp OR 'rclad' OR 'obliterative bronchiolitis' OR 'chronic lung rejection') AND [english]/lim AND [2000-2021]/py

2.3.5 Screening

Results of the systematic searches were uploaded on Rayyan (https://rayyan.ai). A preliminary screening of titles and abstracts was performed, followed by full screening of all potentially eligible articles. Screening was performed by one reviewer (Saskia Bos), with discrepancies discussed and resolved by consensus with a second reviewer (Andrew Fisher).

2.3.6 Data extraction

One reviewer (Saskia Bos) extracted the data from relevant publications. If needed, data collection was discussed within the author team (Andrew Fisher) until consensus was reached. Relevant study characteristics including study design, sample size, CLAD phenotype, type of analysis, and results were collected.

2.3.7 Data analysis

Due to heterogeneity of the study populations, laboratory techniques used and ways of reporting, meta-analyses could not be performed and a narrative approach was used to synthesise the findings.

Results of this systematic review are presented in Chapter 3.

2.4 High-dimensional tissue profiling of immune cell responses in chronic lung allograft dysfunction using explanted lungs and imaging mass cytometry

2.4.1 Summary

The aim of this study was to perform a detailed immunophenotyping of explanted lung tissue from patients with and without CLAD, obtained at the time of redo lung transplantation or post-mortem, using a bespoke 40-plex antibody panel and IMC.

2.4.2 Acquisition of human bio-samples

To conduct this research, a close collaboration was established with the University Hospitals of Leuven, Belgium to obtain sufficient tissue samples. This happened in collaboration with Prof. Dr. Robin Vos and Prof. Bart M. Vanaudenaerde, Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), Leuven, Belgium. Leuven has a biobank with human explanted lungs, obtained during redo lung transplantation or autopsy (local ethical approval: S51577, P2011/243). Ethical approval (S65670) was obtained to use research samples from Leuven for this study. (Appendix C p. 246) Exchange of tissue samples took place under a Material Transfer Agreement, which was approved and signed on 22nd December 2021.
In addition, explanted lung tissue samples from Newcastle were used, which were obtained during redo lung transplantation and stored in the biobank (reference number 04/Q0906/88).

Initially, readily available FFPE tissue slides provided by Leuven were assessed. These tissue slides had been used in a previous project and were immediately available, along with all clinical metadata. However, these tissue sections were cut several years ago, raising concerns about tissue quality for use in IMC. Some of these slides were therefore evaluated with a mini-panel of common antibodies (CD45, CD3, CD4, CD8, CD79a, CD68, and alpha smooth muscle actin (αSMA)) and two intercalators (Iridium 191 and 193) to assess tissue quality. Unfortunately, antibody uptake was significantly less compared with what we normally see. (Figure 2.1) Therefore, the decision was made to collect FFPE tissue blocks and prepare new tissue slides instead.

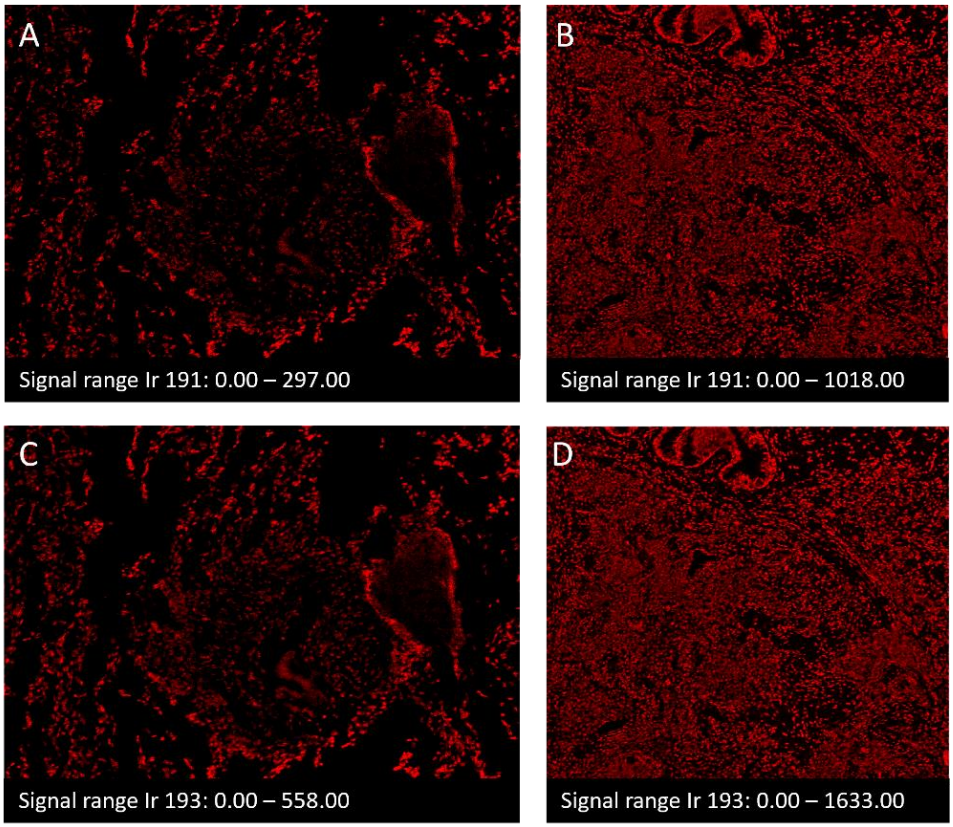


Figure 2.1. Quality check tissue slides
Quality of the tissue slides was checked using a mini-panel of common antibodies and two intercalators to stain the nuclei (red), Ir 191 **(A-B)** and Ir 193 **(C-D)**. The ROIs shown are an OB lesion in a BOS sample **(A, C)**, which was compared to a ROI from a patient with idiopathic pulmonary fibrosis analysed in a previous project **(B, D)**. The Iridium staining showed reduced maximum signal in the CLAD sample.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, Ir: iridium, OB: obliterative bronchiolitis, ROI: region of interest.

Eight FFPE tissue blocks were provided by the Newcastle Biobank after being reviewed by clinical pathologists (Joaquim Majo, Julian Pulle), confirming that the selected blocks were representative of CLAD pathology (in this case BOS). In addition, 17 CLAD FFPE tissue blocks were shipped from Leuven to Newcastle, including six tissue blocks from BOS patients and 11 from RAS patients. With respect to control tissue, the intention was to use tissue blocks from explanted lungs from lung transplant patients who had died of non-respiratory causes. However, these tissue blocks were all stored as frozen cores (-80° C), which are more difficult to handle for IMC and, most importantly, antibody uptake is different compared with FFPE samples. Prior research conducted by the Leuven group has shown that there is no difference in sample quality between immediate embedding and later embedding after freezing at -80° C. (Unpublished data) Therefore, at the time it was decided to use these samples for our study, tissue samples from three non-CLAD patients were thawed and embedded in paraffin by the

local research group according to their local protocol. Examples of what the cores looked like and which ones were chosen are shown in Figure 2.2.

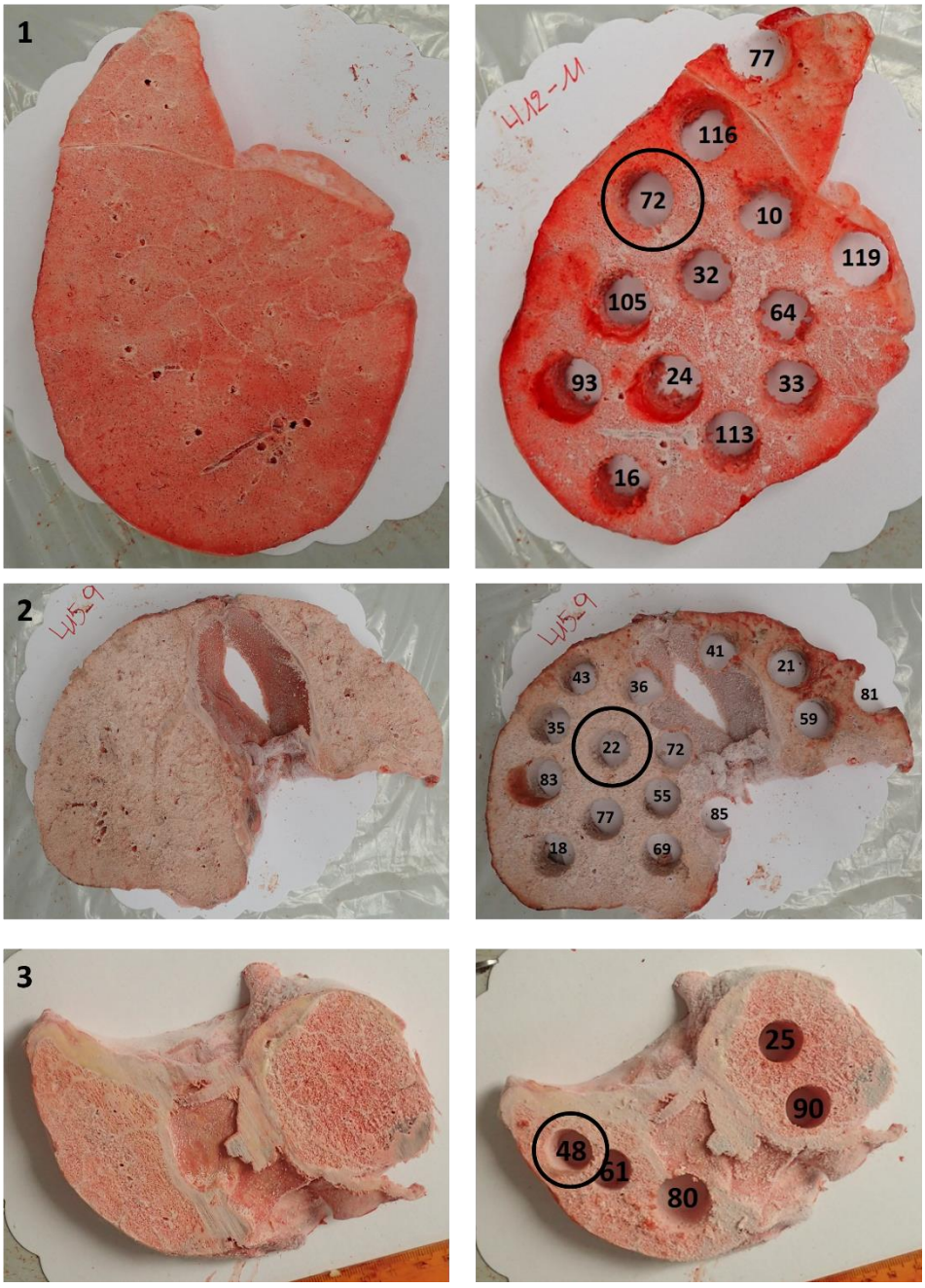


Figure 2.2. Lung slices and cores
Examples of lung slices and cores. Explanted lungs were inflated with air, frozen, cut into lung slices (left column) and sampled with core biopsies of 1.4 cm in diameter and 2 cm in height (right column). The three cores selected for inclusion in this study are circled.

Lastly, tonsil tissue was collected for use as a positive control. FFPE tissue blocks of human tonsil tissue (cores of 2 mm) were obtained from Novopath Tissue Biobank, Newcastle.

2.4.3 Tissue preparation

Selected FFPE lung and tonsil tissue blocks were cut at 8 µm (HM 325 Rotary Microtome, Fisher Scientific, USA) and mounted onto frosted microscope slides (SuperFrost Plus Adhesion Slides, Epredia). Some of the CLAD tissue was quite fragile, especially in the RAS cases, and repeatedly floated off the tissue slides during the antigen retrieval process. In an effort to optimise adhesion, some additional tissue sections were cut and mounted onto gelatine-coated microscope slides that we had coated ourselves. This coating solution was made of gelatine and chromium potassium sulphate dodecahydrate. The coating protocol was as follows (R&D Systems):

- Prepare the gelatine-coating solution by dissolving 5 g of gelatine in 1 L of heated, deionised water (temperature should not exceed 45°C).
- After the gelatine has dissolved, add 0.5 g of chromium potassium sulphate dodecahydrate. Chromium potassium sulphate dodecahydrate will positively charge the slides allowing them to attract negatively charged tissue sections.
- Filter this solution and store at 2-8°C until use. It is recommended that this solution be filtered again immediately before use (adjust to room temperature before filtration).
- Place the microscope slides into a metal rack. The slides should first be cleaned by washing them in soapy water and rinsing them thoroughly, first in tap water and finally in deionised water.
- Dip the rack containing the slides 3 to 5 times (about 5 seconds each) into the gelatine-coating solution.
- Remove the rack containing the slides and let them drain. Blot excess solution from the racks onto filter paper.
- Place the rack containing the slides on a lab bench and cover them with paper towels to protect them from dust and dry at room temperature for 48 hours.

2.4.4 Haematoxylin and eosin staining

Successive FFPE sections were used, with the first section stained with haematoxylin and eosin to guide selection of ROIs. The following staining protocol was used:

- Dewax the slides for 2x5 mins in Histo-clear.
- Rehydrate the slides in graded series of alcohol, 2 minutes each in 100%, 90% and 70%.

- Wash the slides in running tap water for 2 minutes.
- Place the slides in Mayer's haematoxylin for approximately 1 minute and 30 seconds.
- Wash the slides in running tap water for 2 minutes.
- Blue the nuclei by placing in Scott's tap water substitute for 30 seconds.
- Wash the slides in running tap water for 2 minutes.
- Stain the slides with eosin by using a Pasteur pipette and covering the tissue thoroughly for 45 seconds.
- Wash the slides in running tap water briefly for 15 seconds (eosin will start to leech out of the tissue the longer it is washed in water and low-grade alcohols).
- Dehydrate briefly through graded series of alcohols of 70%-100%.
- Clear the slides in Histo-clear.
- Mount the tissue with DPX Mountant and coverslip.

2.4.5 Selection of regions of interest

The slides stained with haematoxylin and eosin were used for the selection of ROIs up to 1x1 mm. ROIs were selected in different lung compartments to include airways, blood vessels, lung parenchyma, septa, and pleura using QuPath (v0.3.0, University of Edinburgh, UK (Bankhead et al., 2017)). The proposed ROIs were discussed with clinical pathologists (Joaquim Majo and Julian Pulle for the Newcastle samples, Arno Vanstapel for the Leuven samples) for their expert input. (Figure 2.3)

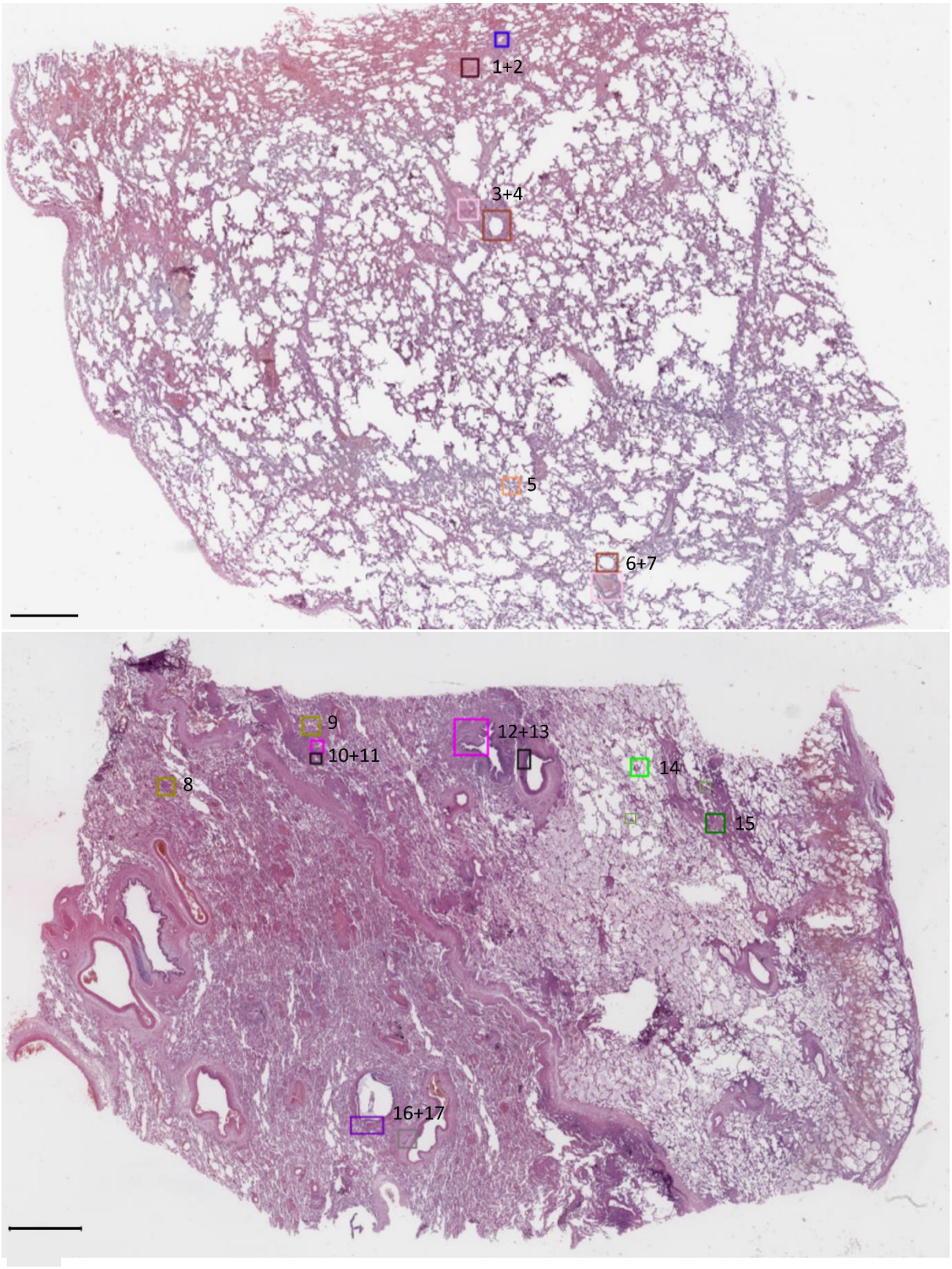


Figure 2.3. ROI selection
Examples of selected ROIs in a BOS (top) and RAS (bottom) sample.
ROI 1-2: fibrotic OB lesion and adjacent blood vessel, 3-4 and 6-7: normal airway and adjacent blood vessel, 5: relatively preserved alveoli, 8-9: less fibrotic parenchymal area, 10-11 and 12-13: inflammatory OB lesion and adjacent blood vessel, 14: relatively preserved alveoli, 15: more fibrotic parenchymal area, 16-17: normal airway and adjacent blood vessel. H&E staining (40x magnification). Scale bar 2 mm.
BOS: bronchiolitis obliterans syndrome, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

2.4.6 ROI tissue area correction

Often the metric used to quantify immune cells in lung tissue is cells per unit area of tissue section (cells/mm²). However, this does not take into account areas without tissue (e.g., after floating off) or the presence of air in airways, alveoli or blood vessels. To adjust for these airspaces, total tissue area was corrected using ImageJ 1.54d (National Institutes of Health, USA) to include only the cellular area for analysis. (Figure 2.4)

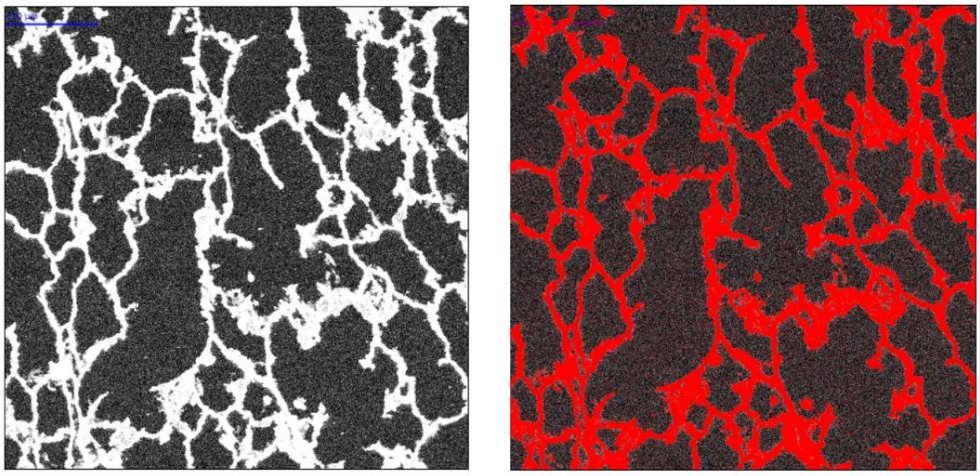


Figure 2.4. Tissue area correction
Tissue area was corrected to account for airspace contributions to section area across alveolar, airway and vascular compartments. Example based on a ROI of relatively preserved alveoli. The size of the ROI is 1x1 mm. If the total tissue section were used to measure the tissue area, this would correspond to a total area of 1 mm² (left). If you adjust for airspaces, the actual cellular area is only 0.44 mm² (right, highlighted in red). Scale bar 200 µm.
ROI: region of interest.

2.4.7 Antibody panel design

The antibody panel was designed based on findings from our systematic review to detect immune cells considered potentially important in the pathogenesis of CLAD. Antibodies were chosen based on cell surface markers of these immune cells. In addition, markers were included to identify activation or differentiation states of these immune cells, if applicable (e.g., T cells). Finally, structural markers were included to indicate lung structures.

Candidate antibodies had to be free of BSA (bovine serum albumin), as this can interfere with heavy-metal conjugation.

2.4.8 Antibody conjugation

Several antibodies had already been used and optimised by members of the team for previous projects. Antibodies to be tested and validated were: CCR10, CD161, CD141, CD16, CD194,

CD196, CD1a, CD203c, CD25, CD38, CD63, CD86, E-Cadherin, and Siglec 8. Unfortunately, for some of the desired cell markers, no suitable clone for use in IMC could be found. This was the case for CD161 and CD203c. CD161 was omitted because a suitable clone for CCR10 was available, which is also a marker to distinguish between Th17 and Th22 cells. Because there were no alternatives, markers for mast cells/basophils (CD203c and CD63) could not be included.

All antibodies were conjugated to rare heavy-metal isotopes. In general, stronger metals were conjugated to weaker antibodies. MaxPar X8 Antibody Labelling Kit (Standard BioTools, USA) was used to conjugate antibodies to lanthanide metals. The conjugation protocol used is displayed in Table 2.2. Antibody conjugations to cisplatin 194Pt and 198Pt (Standard BioTools, USA) were performed as described previously by Mei et al. (Mei et al., 2016)

Metal-tagged antibody conjugation
1. Antibody concentration check
- Add 1 µL of the antibody to be conjugated to the nanodrop. - Measure the absorbance of the antibody at 280 nm. This will give the antibody concentration in mg/mL. - Repeat until you get 3 similar readings and take an average of this. The trace displayed by the nanodrop must be smooth.
2. Pre-load the polymer with lanthanide
- Spin the polymer tube for 10 seconds in a microfuge to ensure the reagent is at the bottom of the tube. - Resuspend the polymer with 95 µL of L-buffer. Mix thoroughly by pipetting. - Add 5 µL of lanthanide metal solution to the tube. Mix thoroughly by pipetting. - Incubate at 37°C for 30 minutes.
3. Buffer exchange and partially reduce the antibody
- Add 300 µL of R-buffer to a 30 kDa filter. - Add up to 100 µg in up to 200 µL of stock antibody to the R-buffer in the filter. - Centrifuge at 12,000 xg for 10 minutes at room temperature. - During centrifugation, dilute 0.5 M TCEP stock to 4 mM in R-buffer by mixing 8 µL of 0.5 M TCEP stock with 992 µL of R-buffer. For each antibody being labelled, 100 µL of 4 mM TCEP-R-buffer is required. - Discard column flow-through. - Add 100 µL of the 4 mM TCEP-R-buffer to each antibody and mix by pipetting. - Incubate at 37°C for 30 minutes.
4. Purify lanthanide-loaded polymer
- Add 200 µL of L-buffer to a 3 kDa filter. - Add the metal-loaded polymer mixture to the filter containing the L-buffer. - Centrifuge at 12,000 xg for 25 minutes at room temperature.

- Discard column flow-through. - Add 300 µL of C-buffer to the filter and centrifuge at 12,000 xg for 30 minutes at room temperature.
5. Purify the partially reduced antibody
- Retrieve the partially reduced antibody from the incubator. - Add 300 µL of C-buffer to each 30 kDa filter. - Centrifuge at 12,000 xg for 10 minutes at room temperature. - Discard flow through. - Add 400 µL of C-buffer to the filter. - Centrifuge at 12,000 xg for 12 minutes at room temperature.
6. Retrieve the partially reduced antibody and lanthanide-loaded polymer
- Retrieve 3 kDa filter containing the lanthanide-loaded polymer from the centrifuge and discard column flow through. - Retrieve 30 kDa filter containing the partially reduced antibody from the centrifuge and discard column flow through.
7. Conjugate antibody with lanthanide-loaded polymer
- Using a pipette, re-suspend the lanthanide-loaded polymer in 100 µL of C-buffer. - Transfer the re-suspended contents to the corresponding partially reduced antibody in the 30 kDa filter. Mix briefly by pipetting. - Incubate at 37°C for at least 60 minutes (up to 2 hours).
8. Wash metal-conjugated antibody
- Add 300 µL of W-buffer to the antibody conjugation mixture. - Centrifuge at 12,000 xg for 5 minutes. - Discard flow through. - Repeat 3 more times with 400 µL of W-buffer (for a total of 4 washes with W-buffer).
9. Recover metal-conjugated antibody
- Add 50 µL of W-buffer to the 30 kDa filter, pipette to mix and rinse the walls of the filter. - Invert the 30 kDa filter over to a new collection tube. - Centrifuge the inverted filter/collection tube assembly at 1000 xg for 2 minutes. - Remove the inverted filter from the collection tube, rinse walls of the filter with an additional 50 µL of W-buffer and replace it, inverted, back to the collection tube. - Centrifuge the inverted filter/collection tube assembly at 1000 xg for 2 minutes.
10. Yield determination and storage of metal-conjugated antibody
- Quantify the conjugated antibody by measuring the absorbance at 280 nm against a W-buffer blank. Repeat until you get 3 similar readings and take an average of this. The trace displayed by the nanodrop must be smooth. - For storage, dilute the antibody to a final concentration of 0.1 mg/mL in a commercially available antibody stabilisation buffer supplemented with 0.05% sodium azide and store at 4°C until ready to titrate.
11. Validation of antibody
- Stain some antibody capture beads with the conjugated antibody and run on the CyTOF. Signal in the specific lanthanide metal channel will confirm the metal is present.

Table 2.2. Antibody conjugation protocol
Protocol for antibody conjugation to lanthanide metals.

New antibodies, which had not been used before, were checked for signal detection by binding the antibody to iridium-labelled antibody capture beads (Thermo Fisher, USA) and then tested and optimised for performance using tonsil and lung tissue. After an initial immunofluorescence check, we moved relatively quickly to testing the performance of all antibodies on the Hyperion system, as IMC does not suffer from autofluorescence. Based on these findings, antibody concentrations were adjusted. Unfortunately, despite attempts at optimisation, antibody staining with CD194, CD196 and Siglec 8 did not work. We therefore choose to include CD183 as a Th1-cell marker instead of CD194 and CD196. For eosinophils, RNAse3 (eosinophil cationic protein) was included in place of Siglec 8.

The antibodies ultimately included and used in our study, as well as the heavy metals they were conjugated to, are listed in Table 2.3.

Metal	Antibody	Clone	Vendor	Cat No.
113In	ECAD	24E10	CST	3195BF
115In	CD45RO	4CHL1	Invitrogen	14-0457-82
141Pr	CD45	D9M8I	CST	13917BF
142Nd	CD68	KP1	Biolegend	916104
143Nd	CD8	C8/144B	Biolegend	372902
144Nd	C3	Polyclonal	Invitrogen	PA5-114921
145Nd	Collagen 1	3D5E8	Proteintech	66761-1-Ig
146Nd	CD138	4F3A8	Proteintech	67155-1-Ig
147Sm	FoxP3	236A/E7	Abcam	ab96048
148Nd	IL-1R	Polyclonal	Invitrogen	PA5-28834
149Sm	CD45RA	HI100	Invitrogen	14-0458-82
150Nd	TGF-β1	Polyclonal	Proteintech	21898-1-AP
151Eu	CD141	5A4C5	Proteintech	67831-1-Ig
152Sm	CD169	SP213	Abcam	ab245735
153Eu	CD56	E7X9M	CST	99746BF
154Sm	HELIOS	E4L5U	CST	89270BF
155Gd	CD69	15B5G2	Novus	NBP2-25236
156Gd	EPCAM	Polyclonal	Abcam	ab71916
157Gd	CD206	2A6A10	Proteintech	60143-1-Ig
158Gd	CD79	EP3618	Abcam	ab239891
159Tb	CD16	EPR16784	Abcam	ab256582
160Gd	CD127	EPR2955	Abcam	ab240225
161Dy	γδ-TCR	H-41	SCB	Sc-100289
162Dy	CD1c	2A7C11	Novus	NBP2-61726
163Dy	CD15	W6D3	Biolegend	323002
164Dy	CD25	SP176	Abcam	ab238272
165Ho	CD57	HNK-1	Biolegend	359602
166Er	αSMA	1A4/asm-1	Novus	NBP2-33006
167Er	CD11b	1C7C2	Proteintech	66519-1-Ig
168Er	CD38	EPR4106	Abcam	ab226034
169Tm	CCR10	Polyclonal	Proteintech	22071-1-AP
170Er	CD3	Polyclonal	Fluidigm	3170019D
171Yb	CD183	EPR25373-32	Abcam	ab288446
172Yb	CD31	EPR3094	Abcam	ab207090
173Yb	CD1a	Polyclonal	Proteintech	17325-1-AP
174Yb	CD4	EPR6855	Abcam	ab181724
175Lu	CD86	E2G8P	CST	76755SF
176Yb	CD14	EPR3653	Abcam	ab226121
194Pt	C4d	Polyclonal	Proteintech	22233-1-AP
198Pt	RNASE3	Polyclonal	Proteintech	55338-1-AP

Table 2.3. Antibodies, clones and conjugates
Overview of antibodies used in the panel with vendor, category number, clone and metal conjugates. CD: cluster of differentiation, CCR: CC chemokine receptor, CST: cell signalling technology, ECAD: E-Cadherin, ECP: eosinophil cationic protein, EPCAM: epithelial cellular adhesion molecule, FoxP3: forkhead box P3, IL-1R: interleukin 1 receptor, RNAse: ribonuclease, SCB: Santa Cruz Biotechnology, αSMA: alpha smooth muscle actin, TCR: T-cell receptor, TGF-β: transforming growth factor beta.

2.4.9 IMC immunostaining protocol

Tissue samples were stained with all antibodies simultaneously. The immunostaining protocol is explained in Table 2.4. Heat-induced epitope retrieval was applied. Initially, this was done using a pressure cooker. However, some tissue samples were quite fragile and tissue pieces repeatedly floated off the tissue slides during the immunostaining process. Since the epitope retrieval step was particularly concerning and contributed to this, a different epitope retrieval method was implemented in the hope of preserving tissue adherence. So we changed to the PreTreatment (PT) module (Epedria, UK) to enable consistent antigen retrieval and reduce deleterious effects of temperature variations and pressure. Both methods are described in Table 2.4. There were no differences in antibody signal detection between both methods. (Figure 2.5)

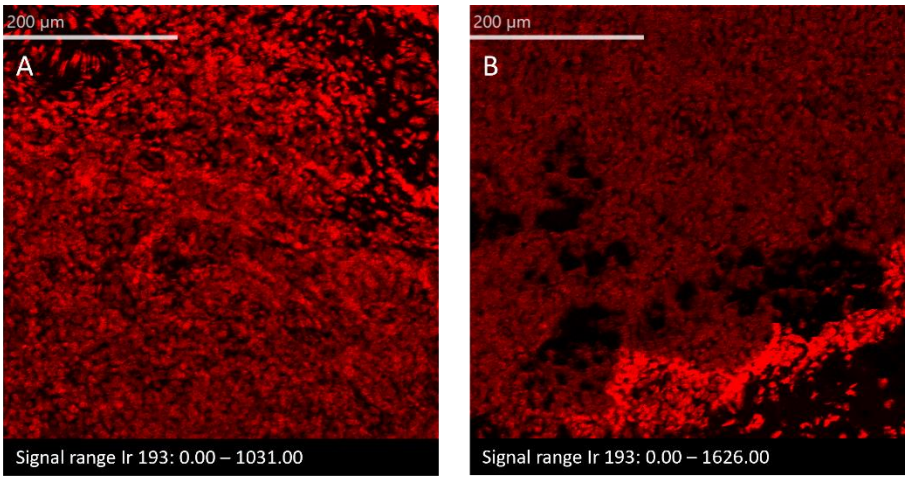


Figure 2.5. Quality check pressure cooker versus PT module
Quality of the PT module as antigen retrieval method was checked and compared with the pressure cooker method. Tonsil tissue stained for Ir 193, nuclei (red). **A.** Pressure cooker. **B.** PT module. The Iridium staining showed a good signal range in both methods. Scale bar 200 µm.
Ir: iridium, PT module: PreTreatment module.

Immunostaining protocol	
1. Tris EDTA buffer solution	
- Make a stock solution of 1 L ultra-pure water, 12.1 g Tris and 3.7 g EDTA. - At the time of staining, make 1 L (pressure cooker) or 1.5 L (PT module) solution of 100 mL Tris EDTA buffer in 900 mL ultra-pure water. Adjust pH using NaOH to reach a pH of 9. Add 500 µL Tween per liter buffer solution.	
2. Hyperion staining Day 1	
- Bake slides on 60°C for 2 hours. - Dewax sections in two changes of fresh xylene. - Rehydrate sections through graded series of alcohols prepared with ultra-pure water: 100%, 90%, 70% and 50%, 5 minutes each. - Wash in ultra-pure water in plastic Coplin jar with gentle agitation twice for 5 minutes.	
3. Heat-induced epitope retrieval (HIER) using Tris EDTA pH9 buffer 0.5% Tween	
Pressure cooker	**PT module (Epedria, UK)**
- Fill the microwave safe pressure cooker with 1 L of retrieval solution. - Put the slides in the pressure cooker and microwave for 20 minutes. - Remove cooker from microwave and allow sections to cool to a minimum of 70°C before proceeding.	- Fill the PT module tank with 1.5 L of retrieval solution. - Preheat the PT module to 85°C. - Mount the slides into the autostainer rack and place the slide rack into the PT module tank. - Heat to 98°C for 20 minutes and cool down to 85°C before taking the slides out.
4. Hyperion staining Day 1 continued	
- Wash sections in two washes of ultra-pure water with gentle agitation for 5 minutes each. - Wash sections in two washes of PBS with gentle agitation for 5 minutes each. - Use PAP pen to circle tissue sections. - Block each section with 180 µL of 3% BSA in PBS for 45 minutes. - Add 180 µL of antibody cocktail to each section in a final concentration of 0.5% BSA solution in PBS. - Incubate overnight in humidified staining tray at 4°C.	
5. Hyperion staining Day 2	
- Wash slides in 0.2% Triton X-100 in PBS with gentle agitation for 8 minutes. - Wash slides in two washes of BPS with gentle agitation for 8 minutes each. - Incubate sections with the intercalator in PBS 1:400 dilution for 30 minutes. - Wash slides in ultra-pure water with gentle agitation for 5 minutes. - Air-dry slides at room temperature for at least 20 minutes.	

Table 2.4. Immunostaining protocol for IMC
Protocol showing the steps for staining the tissue slides with the antibody cocktail, with details of epitope retrieval, including both methods used (pressure cooker and PT module).
BSA: bovine serum albumin, EDTA: ethylenediaminetetraacetic acid, IMC: imaging mass cytometry, PAP pen: hydrophobic barrier PAP pen, PBS: phosphate buffered saline, PT module: PreTreatment module.

2.4.10 Image acquisition using IMC

Tissue acquisition was performed on a Helios time-of-flight mass cytometer coupled to a Hyperion Imaging System (Standard BioTools, USA). After system tuning, a tissue slide was loaded onto the Hyperion Tissue Imager. A panorama image was created, providing a low-resolution overview of the tissue surface (1 µm per pixel, comparable with 10x magnification on an optical imaging system) to select as closely as possible the same ROIs that were selected based on the haematoxylin and eosin staining. Then, after flushing the ablation chamber with helium, ROIs were ablated by a UV laser spot-by-spot at a resolution of 1 µm² and frequency of 200Hz. Each laser shot produced a plume of ablated tissue particles that were transported to the mass cytometer by a stream of inert gas. The ablated tissue is ionised in a high-energy argon plasma and the resulting ions, and thus antibodies associated with each spot, are simultaneously analysed by CyTOF. For each tissue spot, specific isotope abundance can be mapped back to the original coordinates, generating an intensity map of all target proteins. So each spot of ablated tissue corresponded to an image pixel associated with its content in different metal ions.

The result consisted of a multichannel multiparametric image in the form of a MCD file that gathered data for the different pixel coordinates and metals and was used for further analysis.

2.4.11 Assessment of staining

The MCD files from the Hyperion system were evaluated for staining intensity using MCD Viewer (v1.0.560.6, Standard BioTools, USA). (Figure 2.6) All images were then converted to 16-bit single multi-level TIFF files for further analysis.

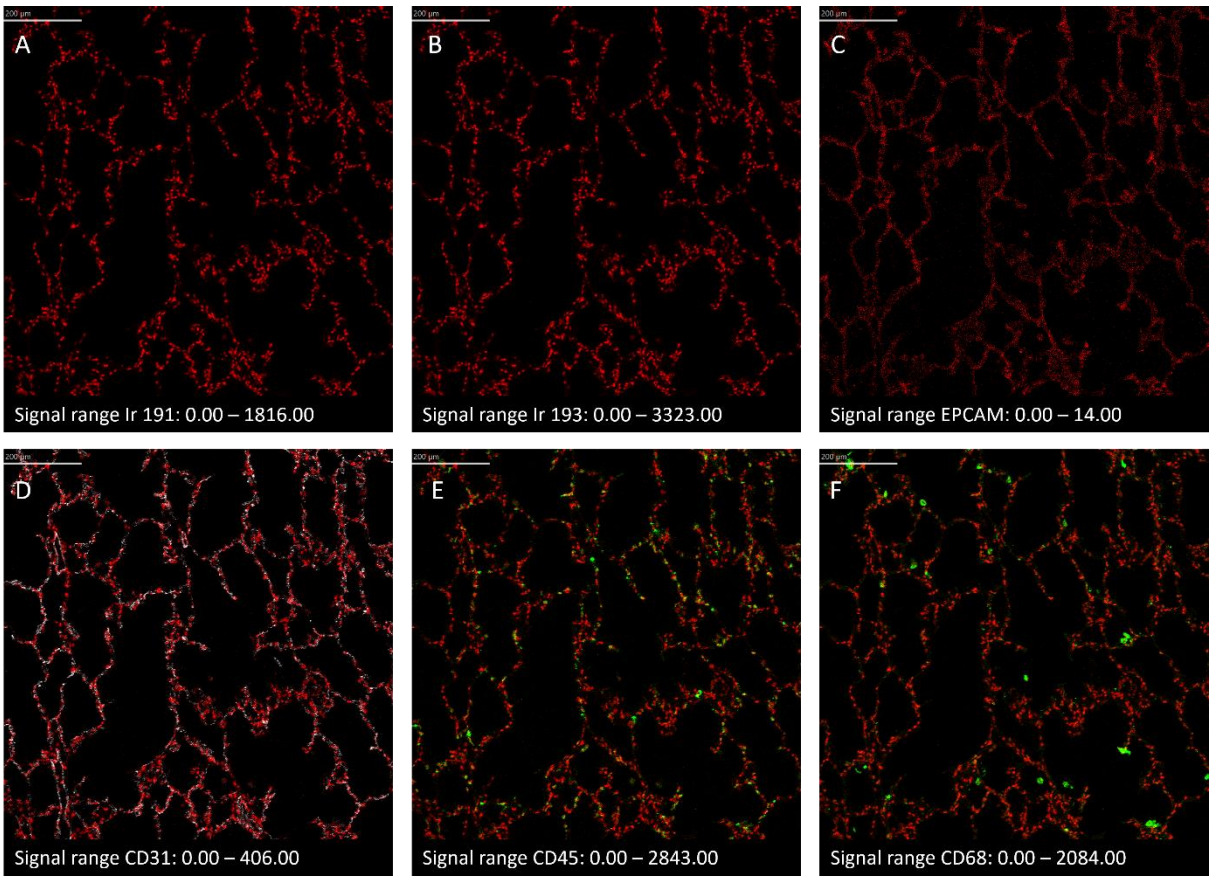


Figure 2.6. Assessment of signal intensity
Assessment of MCD files for evaluation of staining intensity. **A.** Ir 191, nuclei (red). **B.** Ir 193, nuclei (red). **C.** EPCAM, epithelium (red). **D.** CD31, capillaries (white) and Ir 191, nuclei (red). **E.** CD45, leukocytes (green) and Ir 191, nuclei (red). **F.** CD68, macrophages (green) and Ir 191, nuclei (red). Scale bar 200 µm. CD: cluster of differentiation, EPCAM: epithelial cellular adhesion molecule, Ir: iridium.

2.4.12 OPTIMAL framework

The OPTIMAL framework, designed by members of the research group, was used as an optimised approach for cell segmentation, parameter transformation, batch effect correction, dimensionality reduction, clustering, and spatial neighbourhood analysis. (Hunter et al., 2023) This pipeline was run on our data by someone from the Image Analysis Unit (George Merces).

Single-cell segmentation

Accurate segmentation of individual cells is crucial for correct identification of cell phenotypes and interpretation of spatial relationships. Four cell segmentation models had been tested in the OPTIMAL framework and Ilastik-derived probability maps were found to be the most successful method. (Hunter et al., 2023) However, given the tissue thickness used for Hyperion (8 µm), one should always be cautious as segmentation might remain flawed to some extent as information from different z-planes might mix.

Without explaining in detail, Ilastik models (Ilastik v1.3.2) were created to perform cell segmentation based on distinguishing nuclear versus non-nuclear pixels. Subsequently, nuclear probability maps were uploaded onto CellProfiler (v4) to create cell masks, which were used to extract single-cell information. Cell boundaries were determined based on EPCAM (epithelial cellular adhesion molecule) signal.

The initial pipeline run for single-cell segmentation was suboptimal, failing to detect all single cells (see Figure 2.7A and 2.7C). This was much better after optimisation of the pipeline. (Figure 2.7B and 2.7D)

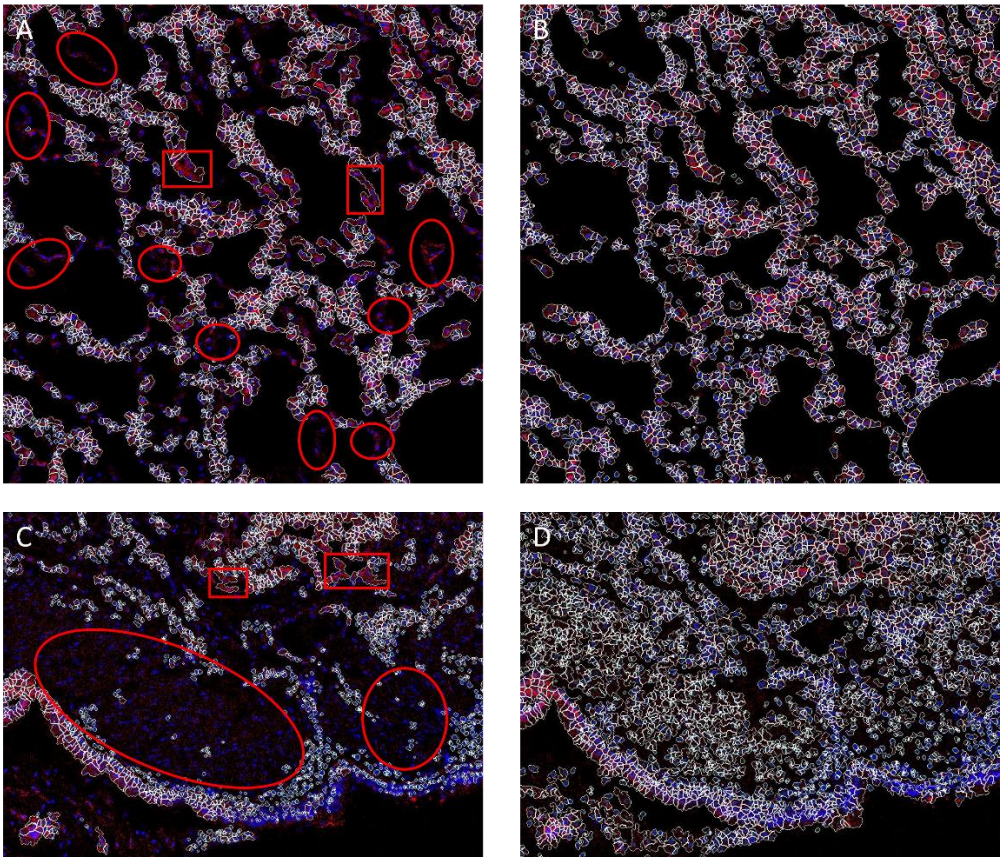


Figure 2.7. Assessment of single-cell segmentation
A, C. Initial single-cell segmentation was suboptimal due to under-segmentation, with areas of undetected cells (circles) or large cells containing more than one nuclei (rectangles). **B, D.** Much improved identification of single cells after optimisation.

Spillover correction

Although the amount of spillover in (imaging) mass cytometry is generally low, spillover can considerably complicate data interpretation. Correction of spillover between isotopes was done by applying spillover correction to all mean pixel values for all metal ion channels.

Parameter transformation

To maximise the statistical separation between negative and positive signal distributions, arcsinh transformation was applied. In other words, this maximised signal resolution. Based on a range of different arcsinh cofactor parameter transformation values that had been tested for the OPTIMAL framework, a cofactor of 1 was used in our project.

Batch effect correction

Although efforts should and have been made to minimise any batch effects (staining by the same individuals, reduction of number of conjugations and batches, etc.), full elimination of batch effects is virtually inevitable. Therefore, in addition to arcsinh transformation, Z-score normalisation was used to eliminate batch effects and to normalise marker intensities relative to each other for subsequent heatmap creation. (Figure 2.8)

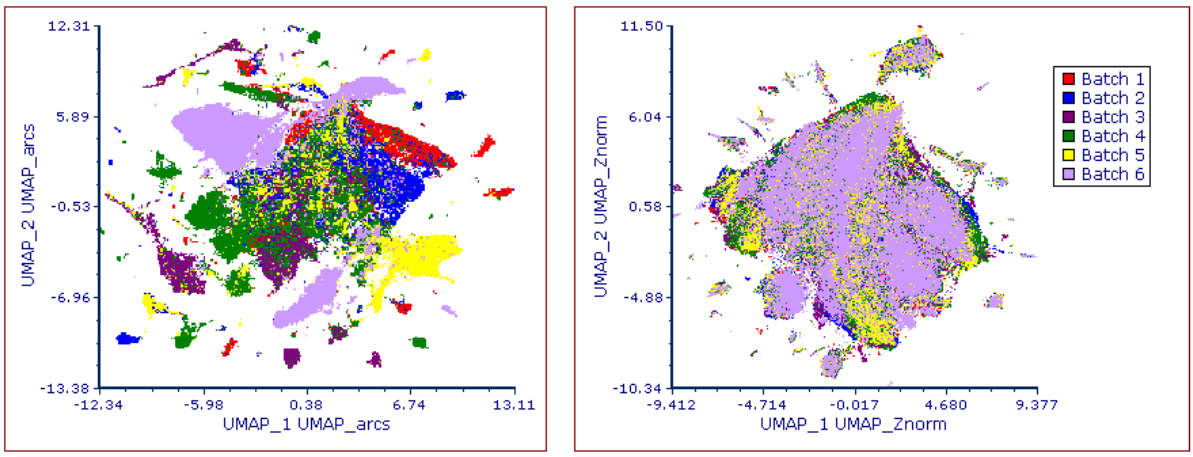


Figure 2.8. Batch effect correction
Dimensionality reduction plots using UMAP (Uniform Manifold Approximation and Projection). Application of arcsinh transformation only on the left, demonstrating the presence of batch effects. Correction of batch effects on the right by applying Z-norm correction (UMAP arcsinh and Z-norm correction).

These transformations and corrections helped achieve the best resolution from our IMC dataset. Final matrix data were then converted to .FCS files for visualisation, clustering and single-cell data analysis in FCS express (v7.18.0015, De Novo software by Dotmatics, USA).

2.4.13 Dimensionality reduction

Dimensionality reduction plots were used to visualise and understand high-dimensional data sets. Herein, high-dimensional data are visualised in a low-dimensional space of two (or three)

dimensions by assigning each datapoint a location on a two-dimensional map. This is done in such a way that similar objects are modelled by nearby points and dissimilar objects are modelled by distant points with high probability. In other words, it groups similar categories together.

Using the Python interface in the FCS Express pipeline module, PaCMAP (Pairwise Controlled Manifold Approximation) dimensionality reduction plots were created, as they were found to be the best among five different dimensionality reduction approached tested in the OPTIMAL framework. It outperformed commonly used approaches such as UMAP (Uniform Manifold Approximation and Projection) and tSNE (t-distributed Stochastic Neighbour Embedding). (Hunter et al., 2023) PaCMAP separated groups of similar categories much more clearly.

2.4.14 Clustering

Next we applied FlowSOM clustering, which proved to be beneficial over PhenoGraph in terms of identifying cell types. (Hunter et al., 2023) Different numbers of consensus clusters were created, ranging from 30 to 65, to detect which was most accurate in identifying enough specific clusters (representing structural and immune cell types), but with the least noise (e.g., unidentifiable, nonsense clusters). A heatmap was created showing the transformed and normalised antibody-derived signals in the rows, and the consensus clusters were displayed in the columns. Median values were normalised by column (i.e., cluster) to aid interpretation of the heatmap. Once the most accurate heatmap (and therefore the number of consensus clusters) was chosen, all data were extracted for further data analysis in GraphPad Prism (v10.0.2, San Diego, USA).

2.4.15 Spatial neighbourhood analysis

After merging consensus clusters into meaningful biological phenotypes at different levels (e.g., level 1: T cells, level 2: Th1 cells), spatial neighbourhood analysis was performed by the Image Analysis Unit (George Merces). Each cell was assessed for the number of unique cell identities within a pixel-defined threshold distance (i.e., 5 pixels) from the cell edge. Partial or fragmented cells around the edge of the image were not included, and a size-based filter was also used to exclude possibly over- and under-segmented objects. These findings were compared to a 90% confidence interval of random iterations. Positive, neutral and negative interactions were then averaged to create an overall heatmap for a given condition (i.e., clinical phenotype). (Hunter et al., 2023) While interesting, data from a heatmap that

represents the average of all ROIs is almost uninterpretable due to structural and clinical heterogeneity. This is why it is best to look at heatmaps for specific conditions (i.e., clinical phenotype).

Results of this study are presented in Chapter 4 and 5.

Chapter 3 Effector immune cells in chronic lung allograft dysfunction: a systemic review

Parts of this Chapter have been the subject of the following publications: **Bos S**, Filby AJ, Vos R, Fisher AJ. Effector immune cells in Chronic Lung Allograft Dysfunction: a Systematic Review. Immunology 2022;166(1):17-37. See Appendix D p. 249.

Bos S, Milross L, Filby AJ, Vos R, Fisher AJ. Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle. Eur Respir Rev. 2022;31(165). See Appendix A p. 210.

3.1 Introduction

Long-term success of lung transplantation continues to be challenged by the development of CLAD, occurring in up to 50% of recipients within five years of transplantation. (Chambers et al., 2019) The acknowledgement that there are distinct phenotypes of CLAD, as explained in the main Introduction, suggests – at least partly – different underlying immunological mechanisms. However, the exact immunopathological mechanisms leading to BOS and RAS remain unclear. Although it is generally postulated that multiple (immune) mechanisms contribute, with interactions between external factors and innate and adaptive immune responses, ongoing crosstalk and communication within the immune system, and the pathogenic properties of certain subsets of immune cells. (Belperio et al., 2009, Bos et al., 2022c) A better understanding of the mechanistic differences between CLAD phenotypes and involved pathways in the inflammatory and remodelling processes is crucial to identify disease-specific biomarkers that enable early diagnosis, better patient stratification and could lead to more personalised and efficacious therapies.

The primary objective of this systematic review was to comprehensively assess the phenotype of effector immune cells present in allograft tissue or BAL from lung transplant recipients with CLAD. We hypothesised that most of the findings would come from BOS patients, as the RAS/mixed phenotypes were only recognised more recently. Because changes in effector immune cells at the peripheral blood level may differ from what is detected at the allograft level, studies with peripheral blood analyses were not included in this systematic review.

3.2 Methods

The systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. (Page et al., 2021) Details of the PICO question, outcomes of interest, inclusion and exclusion criteria, search strategy, and data extraction are explained in the main Methods (Section 2.3 p. 49-52).

3.2.1 Search strategy and eligibility criteria

In brief, a systematic search was conducted on the electronic databases of PubMed and EMBASE using keywords related to immune cells and CLAD. The last search was performed on 22nd April 2021. The search was limited to publications from January 2000 onwards, English-language articles, and articles with full-text access. All titles and abstracts were reviewed thoroughly, followed by full-text review if deemed eligible for inclusion. Further eligibility criteria were limited to original research articles, human data, analyses on lung tissue or BAL from patients with CLAD. Studies that did not match the topic of interest and conference abstracts were excluded.

3.2.2 Screening and data extraction

All titles and abstracts were reviewed by one reviewer (Saskia Bos) followed by full-text screening and data collection if the inclusion criteria were met. In case of unclarity, inclusion was discussed until consensus was reached. Relevant study characteristics including study design, sample size, CLAD phenotype, and type of analysis and its results were collected.

3.3 Results

3.3.1 Literature search

The systematic search revealed 1,351 potentially relevant articles. After deleting duplicate records and primary screening of titles and abstracts, 101 articles were included for full-text screening. (See PRISMA flow diagram Figure 3.1) Of these, 25 were excluded because they did not match the topic or study design. Detailed characteristics of the 76 included studies are presented in Appendix E, p. 270. Fifty-one studies investigated BAL samples, 15 tissue samples, and nine examined both tissue and BAL samples.

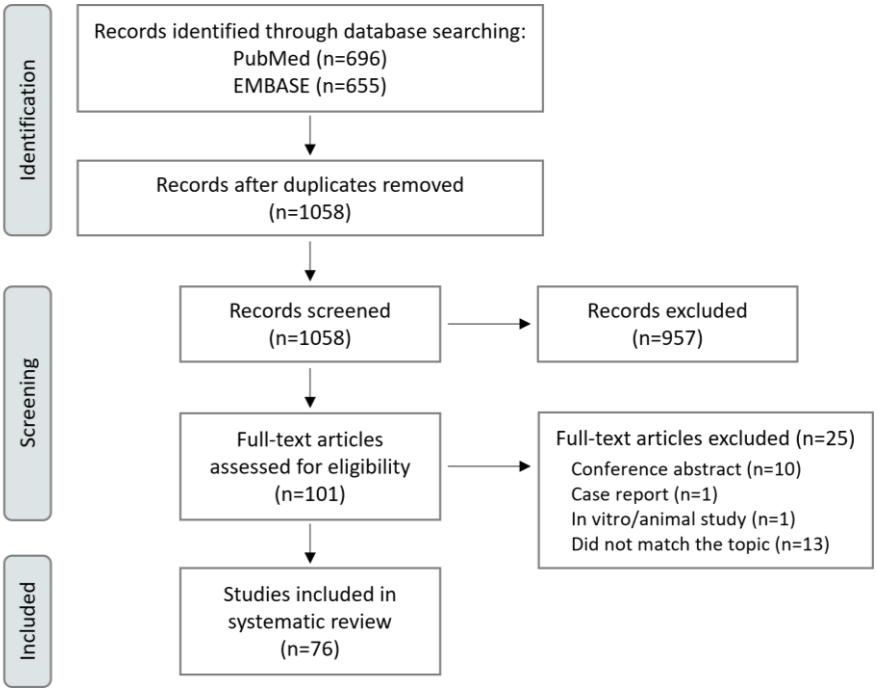


Figure 3.1. PRISMA flow diagram
PRISMA flow diagram showing number of studies identified, screened and included. Reprinted with permission. (Bos et al., 2022b)

A more detailed description of the results can be found in the publication, see Appendix D, p. 249. A summary of the results with a focus on innate and adaptive immune cells relevant to our Hyperion project, as well as an outline of how these cells may play a potential role in the onset of CLAD, is presented below. To facilitate interpretation, an overview of immune cells according to their cell lineage is displayed in Figure 3.2.

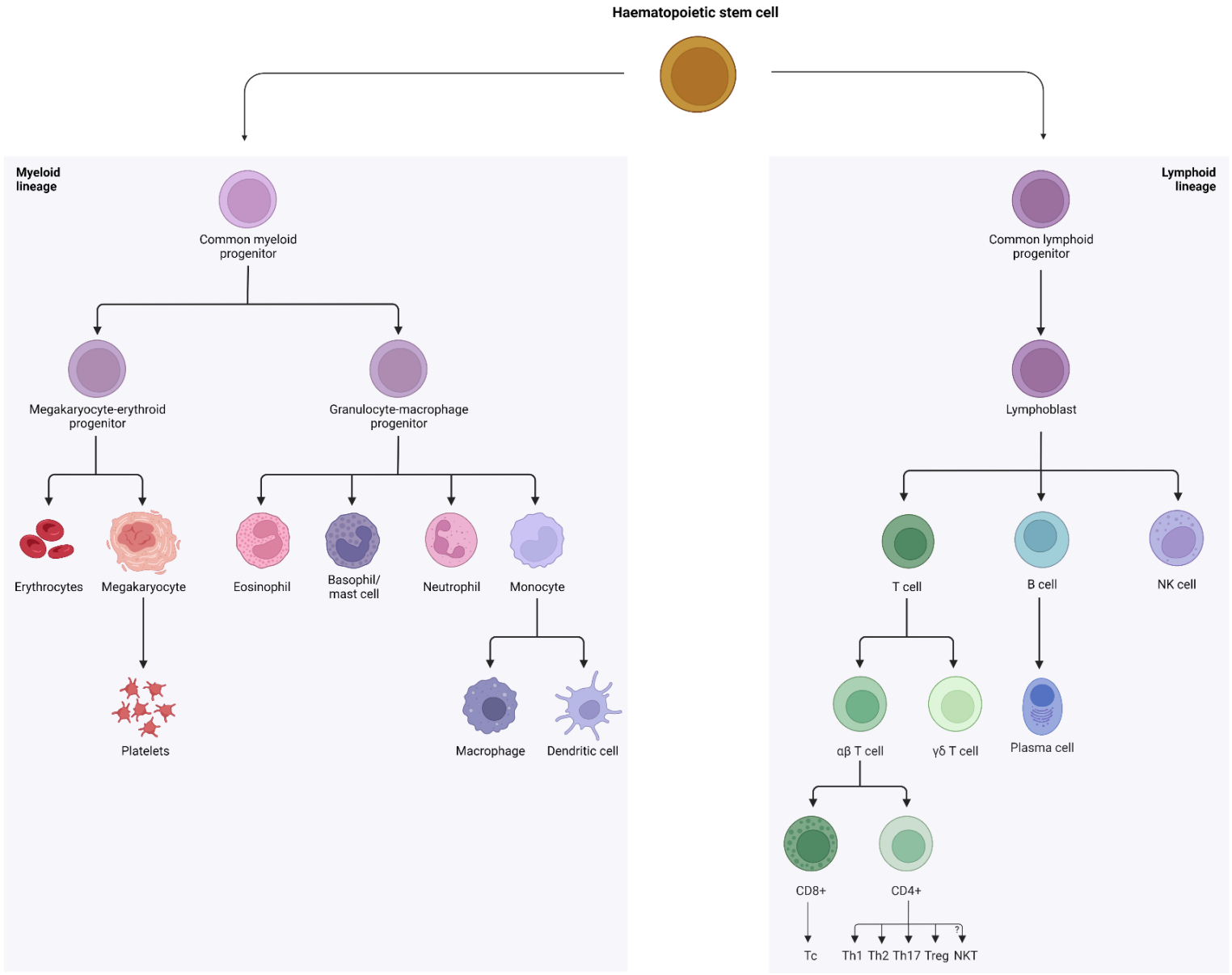

Haematopoietic stem cell
Myeloid lineage
Common myeloid progenitor
Megakaryocyte-erythroid progenitor
Granulocyte-macrophage progenitor
Erythrocytes
Megakaryocyte
Eosinophil
Basophil/ mast cell
Neutrophil
Monocyte
Platelets
Macrophage
Dendritic cell
Lymphoid lineage
Common lymphoid progenitor
Lymphoblast
T cell
B cell
NK cell
αβ T cell
γδ T cell
Plasma cell
CD8+
CD4+
Tc
Th1 Th2 Th17 Treg NKT

Figure 3.2. Myeloid and lymphoid cell lineages
Overview of myeloid and lymphoid immune cells according to their cell lineage. Created with BioRender.com.
NK: natural killer, Tc: cytotoxic T cell.

3.3.2 Neutrophils

Overall, neutrophils seem to play a clear role in CLAD. Based on differential cell count, most studies found a significantly higher percentage of neutrophils in BAL in BOS compared with stable lung transplant recipients (Zheng et al., 2000, Devouassoux et al., 2002, Fisichella et al., 2013, Meloni et al., 2004b, Vos et al., 2009, Reynaud-Gaubert et al., 2002c, Elssner et al., 2000, Verleden et al., 2011, Laan et al., 2003, Heijink et al., 2015, Hübner et al., 2005, Vanaudenaerde et al., 2008a, Reynaud-Gaubert et al., 2002b), with also an increase in absolute numbers (Zheng et al., 2000, Vos et al., 2009, Elssner et al., 2000, Vandermeulen et al., 2016, Belperio et al., 2002, Vanaudenaerde et al., 2008a, Borthwick et al., 2013, Reynaud-Gaubert et al., 2002b). Similar findings were found in studies that included RAS patients, with increased neutrophils in both BOS and RAS patients. (Berastegui et al., 2017, Yang et al., 2019, Verleden et al., 2015a, Vandermeulen et al., 2016, Suwara et al., 2014) Some studies compared with healthy controls and also noted increased neutrophils in stable lung transplant recipients compared with them. (Zheng et al., 2000, Ward et al., 2001, Zheng et al., 2006)
With respect to tissue analyses, more neutrophils were seen in RAS lungs and airways of both BOS and RAS patients compared with controls. (Vandermeulen et al., 2017) Zheng et al. found not only more neutrophils in the airways of BOS patients, but also in stable patients compared with healthy controls. (Zheng et al., 2000) The same group noted that airway wall neutrophilia, assessed by endobronchial biopsies, was similar to healthy controls at baseline, but increased over time in BOS patients. (Zheng et al., 2006)

Several studies with longitudinal analyses documented increased BAL and/or endobronchial neutrophils at the time of BOS diagnosis compared with pre-BOS samples. (Hübner et al., 2005, Reynaud-Gaubert et al., 2002b, Zheng et al., 2006, Borthwick et al., 2013) Others already found increased neutrophils in patients who would go on to develop BOS compared with those who would remain stable. (Reynaud-Gaubert et al., 2002b, Scholma et al., 2000, Neurohr et al., 2009) Increased neutrophils correlated with increased BOS risk (Neurohr et al., 2009, Scholma et al., 2000), and Neurohr et al. showed that a neutrophil percentage of ≥ 20% in BAL was a significant predictor for subsequent BOS stage ≥ 1. (Neurohr et al., 2009). In a group of stable lung transplant recipients with high (≥ 15%) versus low BAL neutrophil counts, an

increased incidence of CLAD and lower CLAD-free and overall survival was seen in the high-neutrophil group. (Vandermeulen et al., 2015) The same group demonstrated that increased neutrophils (> 10%) in RAS patients also correlated with worse graft survival. (Verleden et al., 2016a) On the other hand, other studies could not demonstrate a difference in BAL neutrophils in future BOS or RAS patients compared with those who would remain stable. (Belperio et al., 2002, Zheng et al., 2006, Suwara et al., 2014)

Possible role of neutrophils in CLAD

Neutrophils perform a very important first line of innate defence by patrolling the circulatory system and lung allograft, as well as other tissues, and have strong phagocytic capabilities. However, they not only play an important role in innate immunity, but can also enhance antigen presentation and Th1-driven alloimmune responses. IL-8, secreted by alveolar type II epithelial cells, bronchial epithelial cells and macrophages upon release of proinflammatory cytokines, is a major neutrophil chemo-attractant. (Elssner and Vogelmeier, 2001) Several studies have shown higher levels of BAL IL-8 in BOS patients with a correlation between neutrophils and IL-8 levels. (Neurohr et al., 2009, Vanaudenaerde et al., 2008b) A key cytokine in the induction of IL-8 is IL-17, resulting in IL-17-mediated tissue/airway neutrophilia, which is also the driver in azithromycin-responsive allograft dysfunction (formerly called neutrophilic reversible allograft dysfunction). (Vandermeulen et al., 2015) IL-17 is produced by Th17 cells, but also by invariate NKT cells and γδ T cells, three cell types that are all involved in so-called border control. The IL-17 receptor is found on many cell types, not only on immune cells, but also on vascular endothelial cells and lung endothelium. In addition, neutrophil induction can also be mediated through a non-IL-17-dependent pathway via IL-1 (especially agonists IL-1α and IL-1β, and receptor antagonist IL-1RA). (Vandermeulen et al., 2015, Suwara et al., 2014)

Activated neutrophils have remarkable potential to cause lung allograft damage through a variety of mechanisms, including: 1) respiratory or oxidative burst releasing large amounts of reactive oxygen and nitrogen species, 2) activation of hydrolytic enzymes and proteases, 3) disruption of cellular function, barrier integrity and local cell death, 4) further release of cytokines, and 5) expression of matrix metalloproteinases that can lead to degradation of collagen matrix. An additional mechanism of neutrophil-induced injury is the formation of neutrophil extracellular traps, a process known as NETosis. Neutrophil extracellular traps are extracellular networks of DNA clad with granular proteins that were cast out from neutrophils

and are thought to exert an effector function of neutrophils. (Frye et al., 2021, Bos et al., 2022c) (Table 3.1)

Cell type	Characteristics	Location
Neutrophils	First line of defence Strong phagocytic activity Respiratory burst with reactive oxygen and nitrogen species Activation of hydrolytic enzymes and proteases Release of cytokines Expression of matrix metalloproteinases Generation of neutrophil extracellular traps (NETosis) Epithelial-to-mesenchymal transition	Migration from circulation into tissue
Eosinophils	Release of cytokines, chemokines, reactive oxygen species, cytotoxic cationic granule proteins, and other mediators Recruitment of fibroblasts Release of TGF-β Epithelial-to-mesenchymal transition Weak phagocytic activity	Migration from circulation into tissue
Macrophages	Phagocytosis Antigen presentation Respiratory burst with reactive oxygen and nitrogen species Antibody-dependent cell-mediated cytotoxicity Release of cytokines and chemokines	Migration from circulation into tissue Tissue-resident macrophages
NK cells	First line of defence against infected or transformed cells Cytolytic granule-mediated cell apoptosis Antibody-dependent cell-mediated cytotoxicity Release of cytokines and chemokines Activating and inhibitory receptors Downregulating MHC I regulator Clearance of senescent cells	Migration from circulation into tissue
Mast cells	Barrier control Defence against environmental pollutants Allergic reactions together with IgE Release of histamine, heparin, serine proteases (e.g., tryptase, chymase), cytokines, and other mediators	Mucosal and epithelial tissues Migration of mast cell progenitors upon antigen-induced inflammation
Dendritic cells	Antigen presentation Only cell type that can directly alert naive T-helper cells Release of cytokines and chemokines	Present in lymphoid organs, blood, epithelial tissue Migration to lymph nodes upon activation

Table 3.1. Characteristics of innate immune cells
Overview of main characteristics of innate immune cells. Adapted from Bos et al. Reprinted with permission. (Bos et al., 2022b)
MHC: major histocompatibility complex, IgE: immunoglobulin E, NK: natural killer, TGF-β: transforming growth factor beta.

3.3.3 Eosinophils

With respect to eosinophils in CLAD, data vary. Most studies reported no increase in BAL levels in BOS patients compared with stable lung transplant recipients (Zheng et al., 2000, Fisichella et al., 2013, Ward et al., 2001, Berastegui et al., 2017, Yang et al., 2019, Elssner et al., 2000, Belperio et al., 2002, Laan et al., 2003, Hübner et al., 2005, Vanaudenaerde et al., 2008a, Borthwick et al., 2013), while some found increased levels (Devouassoux et al., 2002, Verleden et al., 2011). Scholma et al. described elevated numbers in the bronchial, but not alveolar, BAL fraction of future BOS patients, and elevated levels correlated with BOS risk. (Scholma et al., 2000) In RAS patients, eosinophil percentages were higher than in stable patients (Verleden et al., 2015a, Vandermeulen et al., 2016, Suwara et al., 2014) or BOS patients (Verleden et al., 2015a). Also in explanted RAS lungs, more eosinophils were found than in controls, and these were mainly located in the lung parenchyma and around blood vessels. (Vandermeulen et al., 2017)

Notably, BAL eosinophilia ≥ 2% correlated with CLAD and CLAD-free survival, and the worst outcome was seen in patients with high BAL and high blood (> 8%) eosinophils. (Kaes et al., 2020) Episodes of BAL eosinophilia (≥ 2%) predisposed to CLAD, mainly RAS but also BOS, and the risk of CLAD and mortality was higher in case of multiple episodes of increased BAL eosinophilia. (Verleden et al., 2014b) A strong association between increased BAL eosinophils (≥ 2%) and survival after RAS diagnosis has also been described. (Verleden et al., 2016a)

Possible role of eosinophils in CLAD

Based on these findings, a role for eosinophils in the pathological process of CLAD, mainly RAS but possibly also BOS, has been implicated. Eosinophils are terminally differentiated granulocytic leukocytes that reside primarily in mucosal tissues and function in host defence. They respond to IgE antibodies and are involved in Th2 responses, playing a key role in allergic reactions and targeting parasites. In many cases, the effects of eosinophils are due to the release of toxins, and eosinophils have only weak phagocytic activity. (Stone et al., 2010) The mechanisms of action of eosinophils in CLAD have not been clearly elucidated, but are thought to be secondary to profibrotic features, by recruiting fibroblasts and stimulating TGF-β release, as well as through direct toxic effects on airway epithelial cells (e.g., increased membrane permeability, ciliary damage). (Darley et al., 2021, Verleden et al., 2014b) Interestingly, translational data from animal models have recently illustrated that eosinophils, however, may also be involved in the downregulation of alloimmunity, possibly through the release of

suppressive molecules or interactions with dendritic cells and lymphocytes. (Onyema et al., 2020) These immunosuppressive effects are though to originate from other subtypes of eosinophils, such as tissue-resident eosinophils, although this needs further study. (Bos et al., 2022c)

3.3.4 Macrophages

In general, BAL macrophage percentages were often lower in BOS patients compared with stable lung transplant recipients, most likely secondary to an increase of other leukocytes, mainly neutrophils. (Zheng et al., 2000, Devouassoux et al., 2002, Fisichella et al., 2013, Meloni et al., 2004b, Berastegui et al., 2017, Yang et al., 2019, Reynaud-Gaubert et al., 2002c, Elssner et al., 2000, Verleden et al., 2011, Vandermeulen et al., 2016, Laan et al., 2003, Hübner et al., 2005, Vanaudenaerde et al., 2008a) The same was reported in RAS patients. (Berastegui et al., 2017, Yang et al., 2019, Verleden et al., 2015a, Vandermeulen et al., 2016, Suwara et al., 2014) Most studies found no difference in absolute macrophage numbers, although Vandermeulen et al. reported an increase in BOS versus RAS and stable patients. (Vandermeulen et al., 2016) Macrophages have not been well studied in CLAD tissue, but one study noted more CD68+ macrophages in explanted RAS lungs compared with BOS and non-transplanted controls. (Vandermeulen et al., 2017)

Possible role of macrophages in CLAD

The main functions of macrophages are phagocytosis and antigen presentation, and they can set off an inflammatory response with pro-inflammatory cytokines (e.g., TNF, IL-1, IL-4, IL-6, IL-8, IL-12, IL-13, IFN-γ) in particular to alert cytotoxic T cells and T-helper cells. (Santa, 2023) To facilitate recognition of possible pathogens, toll-like receptors are found on the surface of macrophages. Upon phagocytosis, a respiratory (also called oxidative) burst can be seen, as in neutrophils, with release of reactive oxygen and nitrogen species. Furthermore, macrophages are involved in antibody-dependent cell-mediated cytotoxicity, along with NK cells and neutrophils. These mechanisms also play a role in allograft rejection. (Bos et al., 2022c)

Worth mentioning, it is important to consider the different subtypes of macrophages, including M1 and M2 macrophages depending on their pro- or anti-inflammatory properties, as well as alveolar and interstitial macrophages based on their anatomical position. All these macrophage subtypes can exert different functions. M1 macrophages, formerly also called

classically activated macrophages, are the dominant phenotype observed in early stages of inflammation and are activated by TNF, IFN-γ and damage-associated molecular patterns. They secrete high levels of IL-12, TNF and low levels of IL-10. Unlike M1 macrophages, M2 macrophages, previously referred to as alternatively activated macrophages, are activated by exposure to certain cytokines such as IL-4, IL-10 or IL-13, and produce high levels of anti-inflammatory cytokines like IL-10 and TGF-β. (Byrne et al., 2015, Hu and Christman, 2019) M2 macrophages are the main phenotype of tissue-resident macrophages, involved in wound healing and repair.

Macrophages are highly plastic and their polarisation states are not mutually exclusive; cells can exhibit elements of both M1 and M2 macrophages. In addition, these polarised macrophages can depolarise to M0 macrophages or exhibit the opposite phenotype by repolarising, depending on the types of cytokines present in the specific microenvironment. Both alveolar macrophages and monocyte-derived macrophages can be polarised to the M2 phenotype. (Cheng et al., 2021) Importantly, data defining the M1/M2 paradigm are largely based on in vitro studies using monocytes isolated from peripheral blood. Caution should be exercised in extrapolating these results to activation states during disease and to the level of the lung parenchyma or airways. (Byrne et al., 2015, Hu and Christman, 2019)

3.3.5 Natural killer cells

Less data is available on the role of NK cells in CLAD. Some studies found increased CD16+/CD56+ NK cells in BOS (Ward et al., 2001) and stable patients (Ward et al., 2001, Hodge et al., 2017, Hodge et al., 2019) compared with healthy controls. Interestingly, more NK cells were seen in small airway brushings in BOS patients compared with stable lung transplant recipients and controls, with no changes in large airway brushings. (Hodge et al., 2017, Hodge et al., 2019) Fildes et al. also found more NK cells in TBB from BOS patients than from stable patients. (Fildes et al., 2008b) Interestingly, Calabrese et al. showed that a certain subtype of NK cells, NKG2C+ NK cells, correlated with CLAD incidence. (Calabrese et al., 2019a)

Possible role of NK cells in CLAD

NK cells are innate lymphoid cells and form an important link between the innate and adaptive immune system. They act as a first-line defence against infected or transformed cells, have a downregulating MHC I regulator, and can induce apoptosis via granzymes and perforin, FAS ligand and antibody-dependent cell-mediated cytotoxicity. This allows them to respond

directly to alloantigens and non-self cells without prior activation. In addition, NK cells can release cytokines, such as IFN-γ and TNF-α, and chemokines via which they can summon T cells, skew immune responses to Th1, increase MHC class I and II expression, and induce graft infiltration of dendritic cells, macrophages and neutrophils. (Fildes et al., 2008a)

Increased numbers of activated NK cells were found in the lungs of CLAD patients, with corresponding peripheral blood depletion, suggesting systemic activation and subsequent migration into the allograft. (Fildes et al., 2008b) There is growing evidence that NK cells have crucial and sometimes opposing roles in lung allograft rejection, due to either activating or inhibitory actions of different NK receptors. In addition to the cytotoxic and inflammatory effects described above, it has been postulated that NK cells might promote graft tolerance through depletion of donor antigen-presenting cells and alloreactive T cells via killer immunoglobulin-like receptors or possibly via IL-15/IL-15Ra complex expansion. (Fildes et al., 2008a, Calabrese et al., 2019b) Nonetheless, the exact mechanisms by which NK cells contribute to CLAD remain to be explored.

3.3.6 Mast cells

Only few studies investigated the presence of mast cells in lung transplant recipients. One study showed an increase of mast cells in RAS lungs compared with non-transplant controls. These mast cells were primarily located in the lung parenchyma and around blood vessels. (Vandermeulen et al., 2017) Another study differentiated between subtypes of mast cells and found an increase in total number of mast cells and subtype mast cell tryptase-chymase over time after transplantation. Moreover, they noted an increase in mast cell tryptase-chymase in CLAD patients compared with non-CLAD patients. (Banga et al., 2016)

Possible role of mast cells in CLAD

Currently, not much is known about any possible role of mast cells in CLAD. Mast cells are innate immune cells located at many barrier sites in the body, where they respond to injury and promote local inflammation. Furthermore, they work together with basophils to respond to worms and environmental pollutants, and are important in allergic responses via interaction with IgE antibodies through their Fc receptor. They act via the release of histamine and heparin, leading to an upregulation of leukotrienes and prostaglandins. Cross-linking of their Fc receptors also initiates a kinase cascade that leads to activation of nuclear factor kappa B, which will attach to the promoters of inflammatory genes and upregulate their

transcription, in turn leading to more leukotrienes. Their response, which is also mediated via IL-4 and IL-5, is restrained to a Th2 response. Finally, type IV cellular or delayed hypersensitivity plays a role in allograft rejection and is mediated by T_{DTH} (delayed type hypersensitivity) cells, a subset of Th1 cells. Interestingly, this response can be downregulated by mast cells, upon signalling from other mast cells or through their IgG receptors, via secretion of IL-10. (Gilfillan et al., 2011, Kalesnikoff and Galli, 2008, Nagata and Nishiyama, 2021) As such, it appears that mast cells may be involved in local allograft inflammation, but are also capable of downregulating immune responses through IL-10.

3.3.7 Dendritic cells

In their function as antigen-presenting cells, dendritic cells form an important link between innate and adaptive immunity. Not many studies looked at differences in the number of dendritic cells in CLAD versus non-CLAD patients. One study reported more dendritic cells, characterised by CD1a, MHC class II or RFD1, in BOS patients compared with stable lung transplant recipients on both trans- and endobronchial biopsies. (Leonard et al., 2000) A more recent study including RAS patients identified more CD1a+ dendritic cells in the lung parenchyma in RAS lungs than in BOS lungs or non-transplant biopsies. More resident mucosal, langerin-positive dendritic cells were present in the lung parenchyma in RAS compared with controls, but these were decreased around the airways. (Vandermeulen et al., 2017)

Possible role of dendritic cells in CLAD

Sentinel dendritic cells, along with macrophages and B cells, are professional antigen-presenting cells. Dendritic cells are particularly effective at alerting naive T-helper cells, as they constitutively express class II MHC and costimulatory (B7) molecules and are the only ones that can directly activate naive CD4+ T cells. (Stockwin et al., 2000) Besides their role as antigen-presenting cells, not much is known of dendritic cells in the context of CLAD.

In addition to antigen-presenting sentinel dendritic cells, there are other types of dendritic cells with other functions, such as follicular dendritic cells. Unlike sentinel dendritic cells, follicular dendritic cells are not derived from haematopoiesis, but are of mesenchymal origin. The latter are important for improving immune function; they are found in lymphoid tissues, present antigen-antibody complexes (iccosomes) to B cells and have the ability to enhance the affinity of immunoglobulins. (Tew et al., 1997)

3.3.8 T cells

Total lymphocyte counts in BAL based on differential cell count often did not differ between patients with and without CLAD. (Zheng et al., 2000, Meloni et al., 2004b, Ward et al., 2001, Berastegui et al., 2017, Yang et al., 2019, Reynaud-Gaubert et al., 2002c, Verleden et al., 2015a, Elssner et al., 2000, Verleden et al., 2011, Belperio et al., 2002, Laan et al., 2003, Suwara et al., 2014, Hübner et al., 2005, Zheng et al., 2006, Borthwick et al., 2013, Reynaud-Gaubert et al., 2002a) Similar for CD3+ lymphocytes, some studies found no difference between groups (Reynaud-Gaubert et al., 2002c, Bhorade et al., 2010, Hodge et al., 2009), while others reported an increase in lung transplant recipients, including BOS patients, compared with healthy controls (Ward et al., 2001), or even a decrease in BOS versus stable patients (Hodge et al., 2021) or healthy controls (Hodge et al., 2021, Hodge et al., 2017, Hodge et al., 2019). Various studies described increased CD8+ T cells, often with proportionally decreased CD4+ T cells, in BOS versus stable lung transplant recipients (Hayes et al., 2020, Hodge et al., 2017, Hodge et al., 2019, Hodge et al., 2021) or controls (Hodge et al., 2021), or BOS and stable patients versus healthy controls (Ward et al., 2001, Hodge et al., 2009).

With respect to CD4+ T-helper cells, increased Th1 and reduced Th2 activity was seen in evolving BOS versus stable BOS, with both being elevated compared with non-BOS patients. (Mamessier et al., 2007) Regarding Tregs, Bhorade et al. noted less FoxP3+ Tregs in BAL in BOS patients versus stable lung transplant recipients. Furthermore, they identified more Tregs at one year post-transplant in patients who would remain stable than in those who would eventually develop BOS. More specifically, a threshold of 3.2% Tregs distinguished stable patients from those who would go on to develop BOS within the first two years post-transplant. (Bhorade et al., 2010) Gregson et al. also demonstrated a protective effect of increased CCR7+ Tregs against subsequent development of BOS. (Gregson et al., 2010) Finally, another study found more CD25highCD69- Tregs in stable and evolving BOS patients compared with stable lung transplant recipients, with higher levels in stable versus evolving BOS patients. (Mamessier et al., 2007)

With regard to tissue analyses, no difference was seen in CD4+ or CD8+ T cells in TBB taken during the first year post-transplant between patients who would remain stable and those who would develop BOS. However, there were more activated (CD25+ and CD69+) T cells in future BOS patients. (Devouassoux et al., 2001) Vandermeulen et al. identified more cytotoxic T cells in explanted BOS and RAS lungs compared with non-transplant controls.

(Vandermeulen et al., 2017) Sato et al. also found more T cells in explanted BOS lungs, especially in areas of active lymphocytic and obliterative bronchiolitis. These T cells were mainly effector memory T cells and were clustered into aggregates. (Sato et al., 2009)

Possible role of T cells in CLAD

T cells are activated by presentation of antigen on MHC I or II molecules. CD8+ T cells recognise antigens on MHC class I, which is present on nearly all nucleated cells, after which they interact with T-helper cells before differentiating into cytotoxic T cells. They subsequently attack cells and induce apoptosis via granzymes, perforins and FAS ligand. They are especially active against intracellular pathogens. In addition, there are several types of CD4+ T-helper cells that respond to antigens presented on MHC II molecules on antigen-presenting cells, and coordinate the immune response in a variety of ways. (Pishesha et al., 2022)

Firstly, the Th1 response, which is considered cellular given the response is quite similar to a cytotoxic T-cell response. That is, Th1 cells will mount a very strong, highly inflammatory immune response against intracellular pathogens at the risk of damaging normal tissue. The Th1 pathway is mostly promoted by dendritic cells and macrophages via pathogen-associated molecular patterns in combination with granulocyte-macrophage colony-stimulating factor, IL-3, IL-12, IL-18, and IFN-γ. (Figure 3.3) Th1 cells will then upregulate IL-2, TNF-α, TNF-β, and IFN-γ, which in turn will upregulate cytotoxic T cells and macrophages. With respect to antibodies, IgG1 and IgG3 can be generated by Th1 cells, which activate complement and phagocytic cells. Abundant evidence demonstrates the ability of Th1 cells to mediate acute and chronic rejection. (Iasella et al., 2021, Yamada et al., 2019, Bergantini et al., 2021)

Secondly, the Th2 response. This is a more restrained, containment response that is moderately inflammatory and is induced by mast cells, basophils and NK cells in combination with granulocyte-macrophage colony-stimulating factor, IL-3 and IL-4. Th2 cells can induce upregulation of antibody production, IgM, IgE and the non-complement activating IgG4. In addition, they can summon eosinophils and secrete a variety of effector cytokines (e.g., IL-4, IL-5, IL-6, IL-10, IL-13), some of which downregulate further cytokine production, while others promote humoral immunity. IL-4 together with IL-5 will also promote antibody class switching to IgE. (Yamada et al., 2019, Nakagiri et al., 2012)

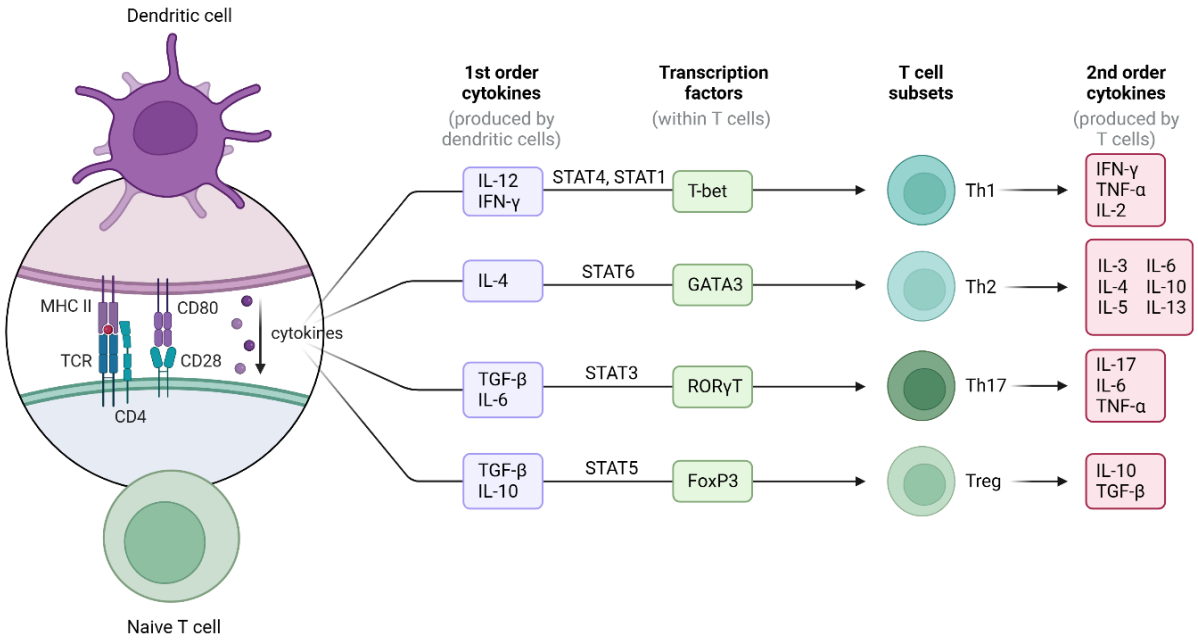


Figure 3.3. T-cell activation and differentiation
Some of the key factors that determine the differentiation state of naive T cells, together with key factors produced by differentiated T-cell subtypes. Created with BioRender.com
FoxP3: forkhead box P3, GATA3: GATA binding protein 3, IFN-γ: interferon gamma, IL: interleukin, RORγT: retinoic acid-related orphan receptor gamma T, STAT: signal transducer and activator of transcription, T-bet: T-box protein expressed in T cells, TGF-β: transforming growth factor beta, TNF-α: tumour necrosis factor alpha, Treg: regulatory T cell.

Thirdly, Th17 cells, which are involved in border defence, produce IgA and trigger an inflammatory response against extracellular pathogens. Th17 cells are induced by TGF-β in combination with IL-6, whilst TGF-β in combination with retinoic acid (which enhances IL-10) leads to the development of Tregs. (Nakagiri et al., 2012, Kimura and Kishimoto, 2010) Th17 cells are mainly activated by dendritic cells with bacterial and especially fungal pathogen-associated molecular patterns, and produce IL-17, IL-22, IL-6, and TNF-α. Both Th17/IL-17 and IL-6 are thought to be involved in the pathogenesis of CLAD, partly through endothelial cell activation and fibroblast activation and proliferation. IL-17 has also been shown to trigger a positive-feedback loop of IL-6 expression. (Kimura and Kishimoto, 2010, Nakagiri et al., 2012, Gupta et al., 2017)

Fourthly, the T-regulatory response, which is an anti-inflammatory response and an essential component of the normal immune system, responsible for maintaining homeostasis and balancing activated immune responses. Like other T-helper cells, Tregs have an αβ T-cell receptor, display the co-receptor CD4, and respond to IL-2 by clonal expansion and activation. (Sakaguchi et al., 2020) However, other Treg subtypes have also been reported, including

CD8+ Tregs. (Kimura and Kishimoto, 2010) Unlike other T cells, Tregs can respond immediately to new antigens and decide whether or not to induce an immune response because it is pre-equipped with CD25, the α-subunit of the IL-2 receptor. Cytotoxic T cells and other T-helper cells must first synthesise the α-subunit before they can be upregulated by IL-2. In addition, Tregs have high levels of CTLA-4 (cytotoxic T-lymphocyte associated protein 4), something that is usually induced later in most T-helper cells, which interacts with B7 that could otherwise stimulate other T cells. Tregs are therefore able to downregulate the adaptive immune system with antigenic specificity and are crucial for active peripheral tolerance. They actively suppress other lymphoid cells, rendering them anergic. (Sakaguchi et al., 2020) The function of Tregs is mediated by TGF-β; TGF-β with IL-21 and IL-6 directs the immune system to an adaptive attack, while TGF-β plus retinoic acid/IL-10 supports the expansion of Tregs. Other pro-inflammatory cytokine signals, such as IFN-γ, also inhibit Tregs. Tregs in turn also produce TGF-β. In other words, it responds to TGF-β and produces more of it. They also produce IL-10, which tends to dampen any immune responses. (Kimura and Kishimoto, 2010)

There are two categories of Tregs, natural Tregs and induced Tregs. Natural Tregs are produced in the thymus and are released into the circulatory system as functioning Tregs. Induced Tregs are, as the word suggests, induced most likely from naive T-helper cells in secondary lymphoid organs, and are activated when an antigen is presented in the absence of a danger signal. (Nakagiri et al., 2012, Kimura and Kishimoto, 2010)

Tregs have been shown to reduce the onset of CLAD and to establish immune tolerance in animal models. (Gracon and Wilkes, 2014, Yamada et al., 2019, Snyder et al., 2019) Increased proportions of Tregs, especially in the lung allograft, appeared to stabilise allograft function, while a decline of this cell population has been described in progressive CLAD. (Gracon and Wilkes, 2014, Bhorade et al., 2010, Ius et al., 2020, Mamessier et al., 2007, Meloni et al., 2004a, Salman et al., 2017)

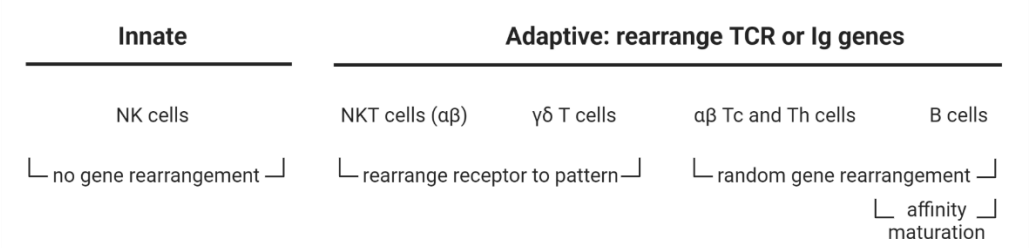


Figure 3.4. Lymphoid cells in order of innate to most adapted
Overview of innate and adaptive lymphoid cells, ranked from innate to most adapted.
Ig: immunoglobulin, NK: natural killer, Tc: cytotoxic T cell, TCR: T-cell receptor, Th cells: T-helper cell.

Next, the NKT cell. This is a heterogeneous group of T cells that share characteristics of both NK cells and quasi-innate T cells. NKT cells have an αβ receptor and typically have CD4, but are sometimes double negative. They mainly recognise innate triggers and do not form memory cells. NKT cells can respond directly to lipid antigens that bind with CD1 molecules; they can also be activated indirectly in response to Toll-like receptor signals and cytokines. (Godfrey et al., 2004) They produce inflammatory cytokines similar to T-helper and γδ T cells and have a CD16 Fc antibody receptor. Activated NKT cells promote Th1, Th2 and Th17 differentiation and NK cell functions. (Mak, 2014)

There are two types. Type one, also known as invariant NKT cell, is highly specific and produces a receptor that always uses the same alpha option and a limited number of beta options. So, it is quasi-innate but still has a form of adaptive development. (Figure 3.4) Type two has a bit more variety in its receptor and in the types of lipids it can recognise. (Godfrey et al., 2004) Not much is known about the role of NKT cells in CLAD.

Lastly, γδ T cells. Instead of an αβ receptor, these cells have a γδ receptor. Compared with αβ genes that produce the αβ receptor, there are a limited number of gene regions, and they rearrange the receptor to a pattern using a limited subset of options. Because they do not have a completely random variety of receptors, they are considered quasi-innate, and they can interact with antigen directly. (Latha et al., 2014) (Figure 3.4) In general, these cells have receptors that recognise lipid antigens presented on CD1, they patrol mucous membranes and epithelia, and cooperate with Th17 cells in barrier defence. They have a plethora of slightly modified specific functions and act through phagocytosis, apoptosis and upregulation of immune responses similar to T-helper cells via cytokines. (Zhao et al., 2018) For instance, they can secrete IFN-γ, which is a Th1 promoter, and they can secrete IL-17, which is part of the fundamental switch away from Tregs and towards the upregulation of Th17 cells. But they

also have cytokines that function like Tregs. As such, there are several subpopulations of γδ T cells, some with proinflammatory features and some with inhibitory properties. In addition, they have Fc receptors, Toll-like receptors, and they can even present antigen. (Zhao et al., 2018, Dar et al., 2014) Therefore, γδ T cells have a wide spectrum of possible functions and it seems likely that they might play a role in the onset of CLAD.

An overview of main characteristics of these different T-cell subtypes is displayed in Figure 3.5. Little is known about the precise role of other T-cell subsets, including T follicular helper cells, Th9 and Th22 cells, in the lung transplant setting. The exact role of memory T cells and γδ T cells in the onset of CLAD also remains unclear. (Snyder et al., 2019, Sullivan et al., 2019) Memory T lymphocytes are commonly viewed as an important barrier to long-term survival of organ allografts; however, Krupnick et al. demonstrated an unsuspected role in lung allograft tolerance of central memory CD8+ T cells in a murine model. (Krupnick et al., 2014) Further research on T-cell subsets is warranted.

Responding cell	Cytotoxic T cell	T-helper cell	NKT cell	γδ T cell
Response	attacks and kills cell	coordinates immune response	regulates immune response	barrier defence
Binds antigen with	αβ T-cell receptor	αβ T-cell receptor	αβ T-cell receptor	γδ T-cell receptor
Diversity	high	high	limited, quasi-innate	limited, quasi-innate
Co-receptor	CD8	CD4	CD4 but can be double negative	usually not, but may have Fc and Toll-like receptors
Antigen presented on	class I MHC	class II MHC	MHC class I-like molecules (e.g., CD1)	MHC class I-like molecules (e.g., CD1)
Cells presenting	all nucleated cells except sperm	sentinel dendritic cells, macrophages and B cells (APCs)	APCs, epithelial and vascular smooth muscle cells	APCs, epithelial and vascular smooth muscle cells
Phagocytosis and antigen presentation	no	no	no	yes
Location	secondary lymphoid organs and systemic circulation	secondary lymphoid organs and systemic circulation	secondary lymphoid organs and peripheral tissues (e.g., liver)	mucosa, including lungs

Response	Th1-mediated (cellular)	Th2-mediated (humoral)	Treg-mediated (inhibitory)	Th17-mediated (epithelial)
Setting	acute infection (intracellular pathogens) and inflammation	chronic or parasitic infection, allergy	modulation of inflammatory responses	acute infection (extracellular pathogens), opposes Tregs
Activated by	macrophages and dendritic cells	mast cells, basophils and NK cells	resting thymus epithelial cells and dendritic cells, dampened by inflammatory milieu	dendritic cells
Promoted by	GM-CSF, IL-3, IL-12, IL-18, IFN-γ	GM-CSF, IL-3, IL-4	TGF-β with retinoic acid (IL-10) blocked by IL-6	TGF-β with IL-6, IL-21, IL-23 blocked by IL-10
Effector cytokines	IFN-γ, TNF-α, TNF-β, IL-2	IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13	IL-10, TGF-β	IL-6, IL-17, IL-21, IL-22, TNF-α, GM-CSF
Effector cells	stimulates development and activity of cytotoxic T cells and macrophages	stimulates development and activity of mast cells, basophils and eosinophils	activates transcription factor FoxP3, production Tregs, suppression dendritic cells and effecto	stimulates neutrophil production and recruitment
Antibody response	moderately strong antibody response, especially IgG1 and IgG3, which activates complement and phagocytes	very strong antibody response, especially IgE, also IgM and non-complement activating IgG4	can attack B cells, reducing antibody production	IgA
Overall response	highly inflammatory, damage to normal tissue, autoimmunity	moderately inflammatory, allergic reactions	anti-inflammatory	inflammatory, autoimmunity

Figure 3.5. T-cell subtypes
Overview of the different T-cell subtypes and their characteristics.
APC: antigen-presenting cell, GM-CSF: granulocyte-macrophage colony-stimulating factor, IFN-γ: interferon gamma, IL: interleukin, MHC: major histocompatibility complex, NK: natural killer, TGF-β: transforming growth factor beta, TNF-α: tumour necrosis factor alpha, Treg: regulatory T cell.

3.3.9 B cells, lymphoid follicles and immunoglobulins

Surprisingly, only a few studies to date have looked at the presence of B cells in CLAD. One study noted more B cells in areas of lymphocytic bronchiolitis and active OB than in areas with inactive OB or healthy tissue. (Sato et al., 2009) Also, more lymphoid aggregates were seen in explanted CLAD lungs versus non-transplant controls. (Sato et al., 2011a) Finally, a recent study using explanted BOS and RAS lungs found more CD20+ B cells in both phenotypes in comparison with non-transplant controls. In addition, they found that RAS lungs contained more lymphoid follicles ("tertiary lymphoid organs") than BOS lungs and non-transplant biopsies. These lymphoid follicles were predominantly located around blood vessels and in the lung parenchyma. (Vandermeulen et al., 2017)

Immunoglobulin deposition has been described in the bronchial epithelium, basement membrane zone, bronchial wall microvasculature, and chondrocytes in TBB from BOS patients compared with stable patients and non-transplant controls. (Magro et al., 2003b, Magro et al., 2003a) When differentiating between BOS and RAS phenotypes, higher levels of IgG (total IgG and IgG1-4) and IgM were seen in BAL of RAS patients versus BOS and stable patients. IgA and IgE levels were also elevated in RAS patients compared with stable lung transplant recipients, and higher total IgG and IgE levels were found in BOS versus stable lung transplant recipients. Finally, increased IgG (total IgG, IgG1, IgG3, and IgG4) and IgM levels correlated with poorer survival. (Vandermeulen et al., 2016)

Possible role of B cells in CLAD

Upon signalling by T cells, B cells will multiply and mature into antibody-producing plasma cells and some will form memory cells. Antibodies are highly specific for a particular epitope, and B cells have the ability to improve their antibody binding through affinity maturation and class switching. Those antibodies, depending on their functions, can in turn signal macrophages, neutrophils and NK cells. (Schroeder and Cavacini, 2010)

Secondary follicles develop after antigen exposure and have active germinal centres where B cells develop in response to signals from follicular dendritic cells, T-helper cells and macrophages. B cells that have spent time in a secondary follicle are able to produce more effective antibodies. In addition, lymphoid neogenesis with the transformation of intragraft inflammatory infiltrates into tertiary lymphoid tissue probably also plays a role in CLAD, as has been reported in several other allograft types. (Yoshiyasu and Sato, 2020, Thaunat, 2012)

The functions of the different classes of immunoglobulins differ greatly and are determined by the Fc stem of the antibody. IgM and IgD receptors are present on naive B cells and memory B cells, while the other immunoglobulins, IgG, IgE and IgA, only develop after class switching and their receptors are only present on memory B cells. (Schroeder and Cavacini, 2010)

IgM is a so-called first responder because it is the first antibody class expressed on naive B cells. It is a strong complement-activating antibody and can summon neutrophils, macrophages and NK cells through antibody-dependent cell-mediated cytotoxicity, together with IgG. There are four types of IgG. IgG1 and IgG3 are strong activators of complement, neutrophils and macrophages, and produce a highly inflammatory Th1 response. IgG2 is a weak complement activator, produces a mildly inflammatory response, and can interact with IgA and IgE antibodies during Th2 responses. Finally, IgG4 does not activate complement, but is a strong phagocyte activator. (Schroeder and Cavacini, 2010)

IgE antibodies attach to the Fc receptors of mast cells, basophils and eosinophils and are involved in allergic reactions and Th2 responses against worms, parasites and environmental pollutants. Finally, IgA is a protective antibody that crosses epithelia and protects boundaries. IgA antibodies are in general considered to be non-inflammatory, but there are two subclasses, one that is moderately inflammatory and one that is not inflammatory at all. (Schroeder and Cavacini, 2010)

Although it seems likely that mainly IgG and IgM antibodies participate in the onset of CLAD via complement activation and antibody-dependent cell-mediated cytotoxicity, elevated levels of all classes of antibodies have been described, although data are scarce. (Vandermeulen et al., 2016)

3.3.10 Complement

Various studies noted increased complement levels in BAL and tissue deposition in CLAD patients, and higher levels of complement deposition (e.g., C1q, C3d, C4d) predisposed to CLAD development. Complement deposition was seen in the bronchial epithelium, basement membrane zone, bronchial wall microvasculature, chondrocytes, and septae. (Bos et al., 2022b)

Possible role of complement in CLAD

The complement system is a complex immune surveillance system consisting of a cascade of multiple proteins that are crucial in innate defence. But the complement system also plays a

role in adaptive immunity through cell-mediated and humoral processes. (Ali et al., 2018) It basically works by poking holes in a cell membrane (membrane lysis), which facilitates phagocytosis by macrophages and neutrophils. In addition to the formation of a membrane attack complex, the complement system exerts its function via opsonisation, which again facilitates phagocytosis, and through immune complex clearing and release of anaphylatoxins (C3a, C5a). The latter serve as chemo-attractants to trigger chemotaxis of mast cells, neutrophils and macrophages. (Ali et al., 2018)

There are three different ways to activate the complement system. (Figure 1.9) The classical pathway is typically activated by antigen-antibody complexes, consisting of either IgM or IgG antibodies, that bind with C1q. Activation of C1q by antibody binding is also referred to as complement fixation. (Nesargikar et al., 2012, Schroeder and Cavacini, 2010) The alternative pathway is based on innate pattern recognition and relies on spontaneous cleavage of C3. It is initiated by a variety of compounds characteristic of pathogenic surfaces, such as lipopolysaccharide (gram-negative bacteria), teichoic acid (gram-positive bacteria) and zymosan (fungi). Lasty, the lectin pathway is very similar to the classical pathway, but is activated by mannose-binding lectin which has a recognition region for carbohydrates. (Nesargikar et al., 2012)

3.3.11 Cytokines

Numerous studies have examined cytokines in CLAD patients. (Bos et al., 2022b) In summary, the clearest correlation was seen between BAL IL-8 and neutrophils, with elevated IL-8 levels in BOS patients, especially neutrophilic BOS patients. (Bos et al., 2022b) Similarly, there was also a correlation between BAL IL-8 and endobronchial neutrophil counts. (Zheng et al., 2006) Interestingly, no difference in IL-8 levels was seen between RAS patients and stable lung transplant recipients. (Suwara et al., 2014, Yang et al., 2019)

Even though Th17/IL-17 appears to play a role in the pathogenesis of CLAD, IL-17 levels in BAL often did not differ between BOS and/or RAS patients and stable lung transplant recipients. (Fisichella et al., 2013, Neujahr et al., 2012, Verleden et al., 2015a) However, one study examined both protein and mRNA levels, and IL-17 mRNA levels were increased in BOS patients compared with stable lung transplant recipients, while protein levels remained below the detection level. (Vanaudenaerde et al., 2008a)

Some studies reported increased TGF-β levels in BOS patients, although several other studies failed to support this finding. (Bos et al., 2022b) Interestingly, a recent study that included both BOS and RAS patients documented elevated levels in RAS patients, which correlated with worse graft survival, perhaps suggesting a more prominent role for TGF-β in this phenotype. (Sacreas et al., 2019)

With respect to other cytokines, levels were often not consistently different across groups, except some studies showed elevated IL-1β and/or IL-1RA levels in BOS patients, and some noted increased IL-6 levels in BOS and/or RAS patients. (Bos et al., 2022b)

Since mRNA and protein levels can differ, it is important to consider both methods of analysis.

Possible role of cytokines in CLAD

As signalling molecules, cytokines play a key role in the management of immune responses by coordinating the immune response via upregulation and downregulation of innate and adaptive immune cells. A complex network of cytokines and chemokines and their receptors are involved in the inflammatory processes leading to CLAD. (Bos et al., 2022b) There are several types of cytokine receptors with which cytokines interact.

Firstly, IL-1 is one of the most fundamental upregulatory signals of all immune responses and acts via an immunoglobulin IL-1 receptor. Its signalling function appears to be similar to that of Toll-like receptors, ultimately leading to the upregulation of nuclear factor kappa B, one of the most fundamental transcription factors. (Dinarello, 2018)

Secondly, type I receptors are a group of related receptors called the haematopoietin superfamily, with which cytokine ligands IL-2, IL-4, IL-5, IL-6, and granulocyte-macrophage colony-stimulating factor interact. (Uings and Farrow, 2000) IL-2 is also one of the most fundamental upregulators of the adaptive immune system, while IL-4 and IL-5 are important in Th2 responses. Binding of IL-2 to the IL-2 receptor will phosphorylate Janus kinases, which in turn will phosphorylate cytoplasmic regions of the receptor. This leads to the formation of a STAT (signal transducer and activator of transcription) dimer, which will enter the nucleus and upregulate gene transcription. However, their action will differ depending on which of several Janus kinases and STATs are activated, leading to upregulation of different genes. (Ross and Cantrell, 2018) Several commonly used immunosuppressants act on the IL-2 receptor or downstream signalling molecules (e.g., basiliximab, Janus kinase inhibitors). (Bos et al., 2023) (Figure 1.10)

Cytotoxic T cells and Th1 cell are key sources of IFN-γ production, a major inflammatory signal considered important in both acute and chronic rejection. IFN-γ, along with IL-12, amongst others, act via type II receptors that are part of the IFN family. The mechanisms of phosphorylation of Janus kinases and cytoplasmic extensions of the receptor, followed by STAT dimerisation and upregulation of gene transcription are very similar to those of the IL-2 receptor. (Uings and Farrow, 2000)

Next, the IL-17 receptor, which is unique in comparison with all other known receptor families. As previously mentioned, IL-17 and the IL-17 receptor are involved in border defence and IL-17 is produced by Th17 cells, γδ T cells and invariant NKT cells. When the cytokine binds, it sets off a series of signals leading to activation of downstream pathways that include nuclear factor kappa B and mitogen-activated protein kinases to induce expression of anti-microbial peptides, cytokines (e.g., IL-6, TNF-α) and chemokines. (Gu et al., 2013) IL-17 plays an important role in enhancing chemotaxis as related to the innate immune response. Chemokines upregulated by IL-17 include CXCL1, CXCL2, CXCL5, CXCL8 (IL-8) (3), CXCL9, CXCL10, CCL2, and CCL20. In addition, IL-17 is especially potent in expansion and recruitment of neutrophils. (Xie et al., 2010)

Finally, one of the other important cytokines and its receptor are TNF-α and the TNF receptor. Binding of TNF-α to its receptor triggers a complex series of events through nuclear factor kappa B, caspase and c-Jun N-terminal kinase pathways, which mediate inflammation and apoptosis. (Uings and Farrow, 2000)

3.3.12 Chemokines

With respect to chemokines in BAL in CLAD, elevated levels of CCL2/MCP-1 (C-C motif chemokine ligand/monocyte chemoattractant protein), CCL3/MIP-1α (macrophage inflammatory protein-1 alpha), CCL4/MIP-1β (macrophage inflammatory protein-1 beta), CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted), and CXCL10/IP-10 (C-X-C-L motif chemokine ligand/interferon gamma-induced protein 10) have been described in BOS and/or RAS patients, whilst other studies could not find differences between patient groups. (Bos et al., 2022b)

Possible role of chemokines in CLAD

Chemokine literally means “chemical motion” and is a small type of cytokine or regulatory chemical involved in redirecting (i.e., recruiting and activating) leukocytes, especially neutrophils, macrophages and lymphocytes, to the site where they are needed. Chemokine receptors can crosstalk, that is, one chemokine receptor will often respond to several different chemokines, and one chemokine can often activate several different receptors. (Hughes and Nibbs, 2018) Several subtypes of chemokines and their receptors are thought to be important in the pathogenesis of CLAD as part of the overall signalling and crosstalk that mediates immune responses. (Bos et al., 2022b)

3.4 Discussion

Post-transplant airway and/or interstitial fibrosis results from a chronic immunological insult that ultimately leads to fibroproliferation and obliteration of distal airways and/or fibrosis of the lung parenchyma. (Bos et al., 2022b) As presented here, multiple innate and adaptive immune cells, including neutrophils, eosinophils, NK cells, and alloreactive T and B cells, are involved in the pathogenesis of CLAD, along with upregulation of various cytokines and chemokines.

Today we are aware of this multifactorial aetiology of CLAD, although currently used immunosuppressive regimens still primarily intervene in T-cell immune responses, long thought to be the sole driver of rejection. (Bos et al., 2022c) However, the fact that not one specific innate or adaptive immune cell is involved, but almost all types of immune cells, makes targeted immunosuppressive treatment difficult. Likewise, this also complicates the development of a diagnostic biomarker for CLAD that has high sensitivity and specificity. (Bos et al., 2022b)

Effector T cells, along with B cells, remain cornerstones in the pathogenesis of CLAD, with immune responses regulated by different subsets of T cells. The actions of these different subtypes range from cytolytic activity (CD8+ T cells, Th1 cells), activation of innate and adaptive immune cells, to propagating (pro-inflammatory/profibrotic cytokine release from Th1 and some Th2 cells) or dampening inflammation (Tregs, anti-inflammatory cytokine release from Th2 cells). (Bhorade et al., 2010, Mamessier et al., 2007, Yamada et al., 2019) Overall, more cytotoxic T cells were identified in CLAD patients, especially in areas of ongoing fibrosis. (Hayes et al., 2020, Hodge et al., 2017, Hodge et al., 2019, Hodge et al., 2021) However, surprisingly few BAL and tissue studies focused on the effects of different subtypes of T and B cells in CLAD. Therefore, it is of utmost importance in future CLAD studies to examine in more detail the precise role of different subtypes, such as effector memory T cells, tissue resident cells, γδ T cells, and regulatory B cells, as well as their activation states.

In addition to alloreactive immune responses from T and B cells, neutrophilic inflammation has been identified as a driving force behind CLAD in numerous studies. (Zheng et al., 2000, Devouassoux et al., 2002, Neurohr et al., 2009, Vandermeulen et al., 2015) Whether neutrophils were attracted to the lungs due to infection and innate immune reaction, or as

part of an alloreactive immune response against foreign antigens, they are potent effector cells. (Bos et al., 2022b) Similarly, eosinophils appear to be involved in the pathogenesis of CLAD, especially RAS, as well. (Scholma et al., 2000, Kaes et al., 2020, Verleden et al., 2014b, Verleden et al., 2016a) Both neutrophils and eosinophils can cause significant tissue damage, as highlighted above, via the release of potent cytotoxic granule products, cytokines and chemokines to further propagate immune responses and attract fibroblasts. (Frye et al., 2021, Stone et al., 2010)

The exact role of other innate cells, such as dendritic cells, macrophages and NK cells, other than their normal function (e.g., antigen presentation, phagocytosis), in the pathogenesis of CLAD needs to be further studied. It is currently unclear whether these cells are actively involved in CLAD pathogenesis, or merely present because of a more pronounced activation of and attraction by other cells. (Bos et al., 2022b) For instance, increased dendritic cells in CLAD patients presumably reflect upregulation of expression of foreign allograft antigens. (Leonard et al., 2000) Interestingly, in CLAD patients, peripheral blood NK cells were decreased but activated, while there was an increase in the lung allograft, suggesting systemic activation and migration to the lung during CLAD. (Fildes et al., 2008b) Importantly, most of the included studies did not differentiate between different subtypes of these innate cells. For example, several subtypes of NK cells exist with either activating or inhibitory actions depending on the type of receptor. (Calabrese et al., 2019b) Likewise, the role of different subtypes of macrophages (e.g., different polarisation states, tissue-resident macrophages) and possibly eosinophils in the onset of CLAD needs to be further clarified. (Kopecky et al., 2020, Onyema et al., 2020)
Altogether, this again underlines the importance of looking not only at the presence of certain types of immune cells, but also at the differentiation and activation states and subtypes.

Currently, there is not much data about the differences at an immunological level between BOS and RAS, as BOS was first considered as a unique manifestation of chronic lung rejection. However, it is highly likely that many studies predating the official recognition of the RAS phenotype also contained RAS patients. (Bos et al., 2022b) After all, interstitial lung abnormalities have been described in lung transplant patients since the 1980s. (Glanville et al., 2019) Recent studies that distinguished between both clinical phenotypes identified that there are both similarities as well as differences in the immunological findings, which seems

very plausible analogous to the clinical presentation. For example, neutrophils were found to be higher in BAL in both BOS and RAS patients, and post-transplant BAL neutrophilia correlated with an increased incidence of CLAD and mortality. (Yang et al., 2019, Vandermeulen et al., 2016, Berastegui et al., 2017, Verleden et al., 2015a, Suwara et al., 2014) Also in RAS patients, BAL neutrophilia correlated with worse graft survival. (Yang et al., 2019) In contrast, the presence of eosinophils appeared more pronounced in RAS patients, although BAL eosinophilia predisposed to both CLAD phenotypes, but RAS in particular. (Vandermeulen et al., 2017, Verleden et al., 2015a) In addition, BAL eosinophilia also correlated strongly with survival after RAS diagnosis. (Verleden et al., 2014b, Verleden et al., 2016a) Elevated eosinophil levels after transplantation may indicate a subtherapeutic dose of corticosteroids for that individual or (relative) steroid resistance.

Another difference between the BOS and RAS phenotype is a more prominent humoral response in RAS with more pronounced presence of B cells, immunoglobulins, complement deposition, and lymphoid follicles. (Vandermeulen et al., 2016, Vandermeulen et al., 2017) This has raised the question of whether a continuum exists between antibody-mediated rejection, which is also primarily mediated by antibodies, and RAS, and whether RAS arises from a chronic form of antibody-mediated rejection. Nonetheless, evidence supporting this paradigm is currently lacking. (Vandermeulen et al., 2016)

Besides the more pronounced presence of eosinophils and humoral immunity, not much is known about the differences at the immunopathological level between BOS and RAS. The same counts for the mixed phenotype. The reason why some patients transition from one phenotype to another remains poorly understood, although some patients had an episode of infection or antibody-mediated rejection between CLAD and mixed diagnosis. (Verleden et al., 2020b) In addition, as in RAS ab initio patients, higher numbers of circulating donor-specific antibodies were seen in patients with a mixed phenotype, again suggesting a role for humoral immunity. (Verleden et al., 2020b)

Several limitations of the studies included in this systematic review need to be addressed, in addition to the fact that most of them focused on the BOS phenotype. Most studies had a cross-sectional study design and a small study population. Different types of analyses and techniques were used, making adequate comparison difficult, and findings were often inconsistent. This heterogeneity precluded the performance of a meta-analysis, making it more difficult to draw general conclusions.

The impact of other factors, such as respiratory infection or colonisation, was not discussed in this review, although many studies took this into account or excluded these patients. Finally, this systematic review focused on immune cells and cytokines and chemokines involved in CLAD pathogenesis, but CLAD is a much more complex pathology, as illustrated in the main Introduction.

3.5 Conclusions

Multiple innate and adaptive immune cells, including neutrophils, eosinophils, NK cells, and alloreactive T and B cells, have been implicated in the pathogenesis of CLAD, along with upregulation of various cytokines and chemokines. Currently, there is not much data on the differences at an immunological level between BOS and RAS. Although it seems that there is a more pronounced presence of eosinophils and humoral immunity in RAS.

The fact that CLAD is not driven by one specific innate or adaptive immune cell, but by many, makes both targeted immunosuppressive treatment and the development of an accurate diagnostic biomarker difficult. Surprisingly few studies focused on the effects of different immune cell subtypes in CLAD. Therefore, it is of utmost importance to explore in more detail the precise role of such subtypes in future CLAD studies, including effector memory T and B cells, macrophage subtypes, tissue resident cells, γδ T cells, as well as their differentiation and/or activation states.

Key points

- Post-transplant airway and/or interstitial fibrosis results from chronic immunological injury in which many innate and adaptive immune cells are involved.
- Alloreactive T and B cells, along with neutrophils and eosinophils, are key drivers in the onset of BOS and/or RAS.
- Post-transplant BAL neutrophilia (≥ 15-20%) correlated with an increased incidence of CLAD and lower CLAD-free and overall survival.
- Episodes of post-transplant BAL eosinophilia (≥ 2%) predisposed to CLAD, mainly RAS but also BOS, and the risk of CLAD and mortality was higher in case of multiple episodes.
- Future studies should focus on specific mechanistic differences between CLAD phenotypes, especially BOS versus RAS.
- The precise role of immune cell subtypes should be examined in future CLAD studies, as well as their differentiation and activation states.
- Disease-specific biomarkers for timely diagnosis and endo/phenotyping of CLAD should be explored, as well as to stratify patients at risk for CLAD.
- Future studies should aim at identifying specific immune cells or (profibrotic) pathways in the pathogenesis of CLAD that are targetable for treatment.

Chapter 4 High-dimensional tissue profiling of immune responses in chronic lung allograft dysfunction

Parts of this Chapter are the subject of the following paper that is in submission: **Bos S**[*], Hunter B[*], McDonald D, Merces G, Sheldon G, Pradère P, Majo J, Pulle J, Vanstapel A, Vanaudenaerde BM, Vos R, Filby AJ, Fisher AJ. High-dimensional tissue profiling of immune responses in chronic lung allograft dysfunction.

4.1 Introduction

Over the past decades, we have gained a better understanding of how the immune system contributes to inflammatory responses, airway and parenchymal remodelling, and fibrosis after lung transplantation. (Bos et al., 2022c) However, a clearer picture of how all immune processes at play in the lung allograft interact in the pathogenesis of CLAD is needed in order to improve early diagnostic tools, such as diagnostic biomarkers, make therapeutic progress and optimise prevention. Until recently, tools for assessing lung allograft cells were limited to histological examination with immunohistochemistry and immunofluorescence imaging, and fluorescent flow and mass cytometry. However, the cellular complexity, cell-to-cell interactions and functional diversity of the immune system necessitate the use of high-dimensional single-cell tools to uncover its role. (Hartmann and Bendall, 2020) To obtain such integrative picture, a multiparametric approach is essential. Several high-dimensional technologies have recently emerged based on RNA sequencing and cytometry that enable exploration of cell heterogeneity at the single-cell level, but these often lack tissue contextual information. (Efremova et al., 2020) Importantly, data from single-cell resolution spatial studies in lung tissue from patients with CLAD are scarce. (Bos et al., 2022b)

IMC is a novel technology that couples laser ablation of tissue ROIs to a mass cytometer and can be applied to FFPE tissue sections. Using antibodies conjugated to rare heavy-metal isotopes, simultaneous high-multiplex interrogation of up to 40 different protein markers on the same tissue section is possible without the need to disaggregate to single-cell suspension. Thereby, the structural context in tissue architecture, cellular morphology and spatial relationship is preserved. (Hartmann and Bendall, 2020, Chang et al., 2017) IMC has thus led to a major advancement in the ability of multiplex immunodetection of protein markers in

tissue samples, on each cell simultaneously within the tissue, greatly surpassing the multiplex possibilities of immunohistochemistry and immunofluorescence.

The first aim of this study was to perform a detailed assessment of immune cell profiles and structural cell composition in lung tissue from lung transplant recipients with and without CLAD. More specifically, we aimed to investigate what immune cell phenotypes contribute to the difference in immune profile between patients with and without CLAD and between BOS and RAS. The second aim was to assess the temporal evolution of fibrosis in CLAD, which will be described in the next Chapter. Lastly, we aimed to describe whether there is a common immunological rejection pathway in both CLAD phenotypes.

4.2 Methods

A detailed description of the methods can be found in the main Methods, Chapter 2. An overview of the experiment set-up is displayed in Figure 4.1.

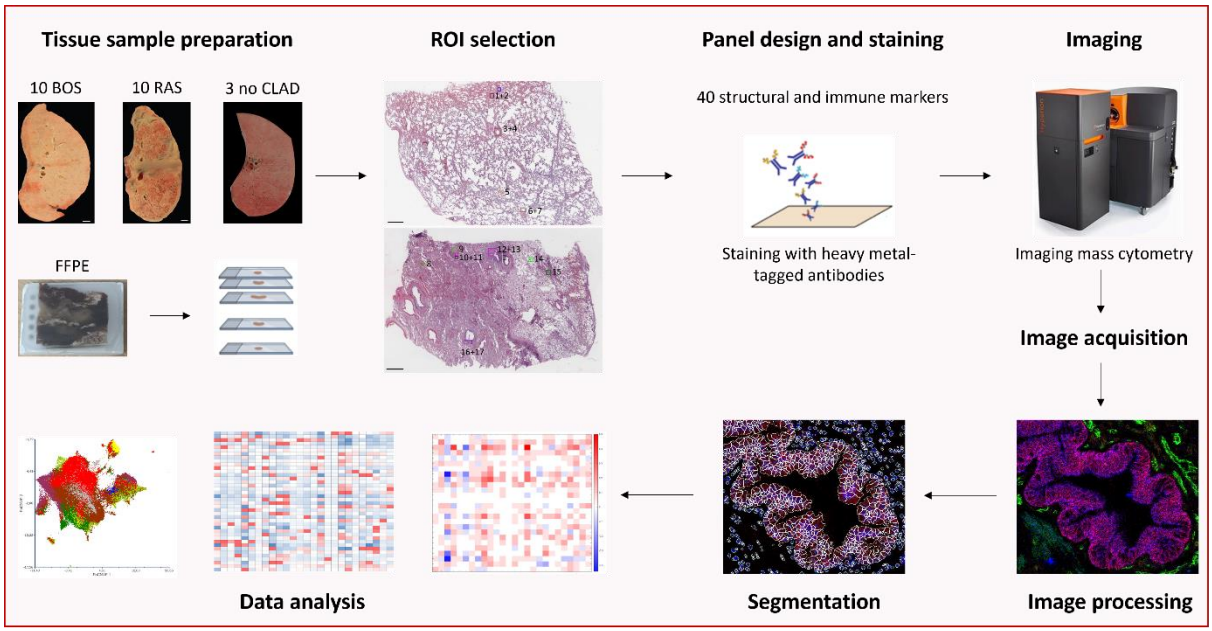


Figure 4.1. Experiment set-up
Overview of the experiment set-up highlighting the most important steps, based on the OPTIMAL approach. (Hunter et al., 2023)
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, FFPE: formalin-fixed paraffin-embedded, RAS: restrictive allograft syndrome, ROI: region of interest.

4.2.1 Tissue samples

Explanted lung tissue samples from patients who developed CLAD after primary lung transplantation were used, with either a BOS (n=10) or RAS (n=10) phenotype, along with control samples from lung transplant patients who did not have CLAD and died of non-respiratory causes (n=3). One tissue sample per patient was used. Because of the heterogeneity of CLAD, only patients with a clear BOS or RAS phenotype were included in this study, excluding patients with a mixed phenotype, as results from the latter would complicate interpretation given the limited number of patients and samples included.

Tissue samples were obtained from two lung transplant centres, Newcastle Hospitals (n=6) and University Hospitals Leuven (n=17), according to local ethics (Newcastle 04/Q0906/88, Leuven S51577 and S65670). All patients had provided written informed consent to use their clinical and biobanked data for research purposes, and relevant clinical metadata were retrieved from the participants' (electronic) medical files.

4.2.2 Tissue section preparation and ROI selection

Selected FFPE lung tissue blocks were cut at 8 µm and mounted onto frosted microscope slides (SuperFrost Plus Adhesion Slides, Epredia) or gelatin-coated microscope slides (RAS samples that floated off repeatedly, coating solution made of gelatin and chromium potassium sulphate dodecahydrate). Successive FFPE sections were used, with the first section stained with haematoxylin and eosin to select ROIs up to 1x1 mm in different lung compartments (bronchiolar/vascular/interstitial).

4.2.3 Panel design

The antibodies selected in the bespoke 40-plex antibody panel were designed to allow identification of i) key immune cell subsets considered potentially important in CLAD pathogenesis, ii) their activation or differentiation states, and iii) structural markers to indicate lung structures and reveal tissue organisation. Antibodies were chosen based on characteristic cell surface markers and their availability for use in IMC (Table 4.1 and Figure 4.2). This selection was based on findings from our systematic review (see Chapter 3) (Bos et al., 2022b).

Myeloid immune cell markers	Lymphoid immune cell markers		Structural cell markers and other
CD1a	CCR10	CD57	αSMA
CD1c	CD127	CD69	CD31
CD11b	CD138	CD79a	Collagen I
CD14	CD183	CD8	Complement C3
CD141	CD25	FoxP3	Complement C4d
CD15	CD3	Helios	E-Cadherin
CD16	CD38	γδ-TCR	EPCAM
CD169	CD4		IL-1R
CD206	CD45		TGF-β1
CD68	CD45RA		
CD86	CD45RO		
ECP	CD56		

Table 4.1. Antibody panel
40-plex antibody panel consisting of markers for immune cell subsets considered important in CLAD pathogenesis, their activation or differentiation states, and structural markers to mark lung structures.
αSMA: alpha smooth muscle actin, CD: cluster of differentiation, CCR: C-C chemokine receptor, ECP: eosinophil cationic protein, EPCAM: epithelial cellular adhesion molecule, FoxP3: forkhead box P3, IL-1R: interleukin 1 receptor, TCR: T-cell receptor, TGF-β: transforming growth factor beta.

In our selection, we found it important to focus on T-cell subtypes (e.g., γδ T cells) and their differentiation states (e.g., effector memory T cell) and macrophage subtypes (e.g., polarisation state). We were more limited in doing this for other immune cells, such as B cells

and other innate immune cells (e.g., NK cell subtypes), due to the limited number of markers that can be included and the difficulty in identifying some subtypes (e.g., many markers for regulatory B cells). Although cytokines and chemokines are important in CLAD pathogenesis, as illustrated in our systematic review, we were limited in their inclusion in our Hyperion panel also due to the fact that cytokines are soluble and soluble and intracellular markers are more difficult to assess via IMC. IMC relies mainly on cell surface markers to phenotype immune cells. In addition, it is important to consider not only protein but also mRNA levels when analysing cytokines, as demonstrated in our systematic review. Therefore, we limited ourselves to the inclusion of IL-1R, which can exist in both a transmembrane form and a soluble form, and TGF-β1. Finally, two important complement factors, C3 and C4d, were included.

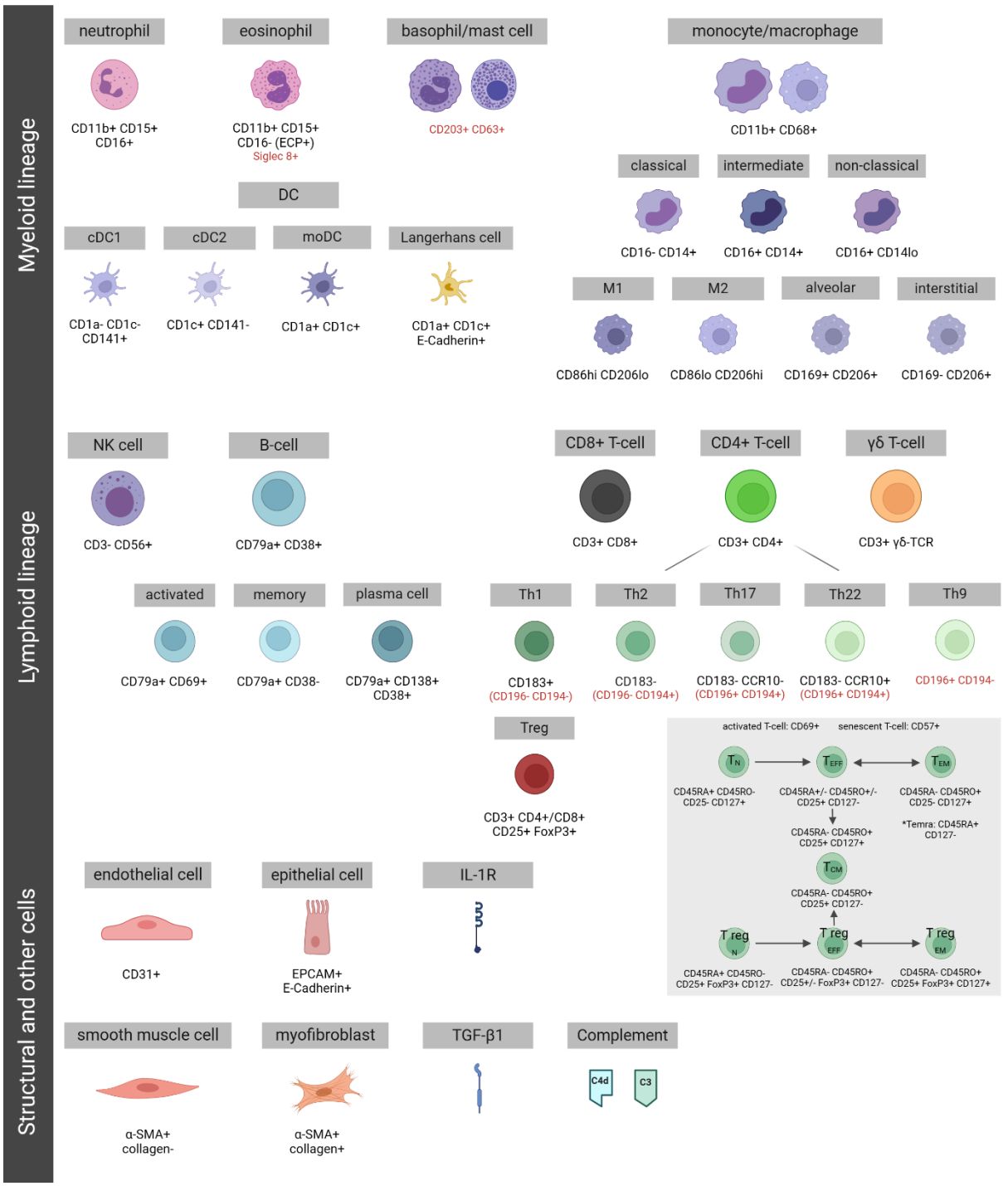


Figure 4.2. Identification of immune and structural cells

Overview of key cell markers for identification of immune and structural cells targeted by our antibody panel. Markers in red could unfortunately not be used because either no suitable clone was available or validation in tonsil and lung tissue proved unsuccessful for use in imaging mass cytometry.

αSMA: alpha smooth muscle actin, CD: cluster of differentiation, CCR: C-C chemokine receptor, CM: central memory, ECP: eosinophil cationic protein, EFF: effector, EM: effector memory, EPCAM: epithelial cellular adhesion molecule, FoxP3: forkhead box P3, IL-1R: interleukin 1 receptor, N: naive, TCR: T-cell receptor, TGF-β: transforming growth factor beta.

4.2.4 Antibody validation and conjugation

All antibodies were conjugated to rare heavy-metal isotopes and were optimised and initially tested for performance on tonsil tissue. Details regarding antibody conjugation and validation can be found in the main Methods, Chapter 2. Briefly, MaxPar X8 Antibody Labelling Kit (Standard BioTools, USA) following manufacturer's instructions was used to conjugate antibodies to lanthanide metals. Antibody conjugations to cisplatin 194Pt and 198Pt (Standard BioTools, USA) were performed as described previously by Mei et al. (Mei et al., 2016) Post-conjugation, all coupled antibodies were eluted in 50 µL W-buffer (Standard BioTools, USA) and 100 µL antibody stabiliser buffer (supplemented with 0.05% sodium azide). They were then stored at 4°C.

4.2.5 IMC immunostaining protocol

Slides were baked at 60°C for 2 hours, after which they were deparaffinised in xylene (2x5 min), rehydrated in descending series of ethanol (100%, 90%, 70% and 50%, 5 min each) and washed in Milli-Q® Type 1 ultra-pure water (2x5 min). Heat-induced antigen retrieval was performed using Tris-EDTA (pH9) buffer 0.5% Tween in a PT module (Epedria, UK) to enable consistent antigen retrieval and reduce damaging effects of temperature variations or pressure. After washing in ultra-pure water (2x5 min) and PBS (2x5 min), tissue sections were blocked with 3% BSA in PBS for 45 min at room temperature, after which they were stained with the metal-conjugated antibody cocktail and left at 4°C overnight. After washing with 0.2% Triton X-100 in PBS (8 min) and PBS (2x8 min), tissue sections were incubated with a DNA intercalating agent (191Ir and 193Ir) for 30 min at room temperature and washed with ultra-pure water before airdrying.

4.2.6 Image acquisition

Tissue acquisition was performed on a Helios time-of-flight mass cytometer coupled to a Hyperion Imaging System (Standard BioTools, USA) over six batches, with a tonsil tissue control slide processed and stained alongside. Briefly, after flushing the ablation chamber with helium, ROIs were ablated by a UV laser spot-by-spot at a resolution of 1 µm² and frequency of 200Hz. For each tissue spot, specific isotope abundance can be mapped back to the original coordinates. So each spot of ablated tissue corresponds to an image pixel associated with its content in different metal ions. The result consists of a multichannel multiparametric image in the form of a MCD file that gathers the data for the different pixel coordinates and metals,

which was evaluated for staining intensity (MCD Viewer v1.0.560.6, Standard BioTools, USA) and then converted to 16-bit single multi-level TIFF files for further analysis. ROI tissue area was corrected using ImageJ (v. 1.54d, NIH, USA) to adjust for airspaces.

4.2.7 Segmentation, clustering and spatial analysis

The previously described OPTIMAL framework was used as an optimised approach for cell segmentation, parameter transformation, batch effect correction, dimensionality reduction, clustering, and spatial neighbourhood analysis. (Hunter et al., 2023) (See main Methods, Chapter 2) Briefly, cell segmentation was performed using Ilastik (v1.3.2). Subsequently, the probability maps were uploaded onto CellProfiler (v4) to create cell masks which were used to extract single-cell information. An arcsinh transformation cofactor of 1 was applied to all metal signal parameters for optimal separation between negative and positive signal distributions. Z-score normalisation was used for batch effect correction. Final matrix data were converted to .FCS files and visualised using FCS Express (v7.18.0015, De Novo software by Dotmatics, USA). PaCMAP dimensionality reduction with FLOWSOM clustering was performed, creating 35 consensus clusters. These clusters were presented on a heatmap, all representing immune and structural cell types. The 35 clusters were further merged to eight Tier 1 clusters, representing key immune and structural cells (e.g., T cells), and 26 distinct Tier 2 clusters, consisting of subtypes of these Tier 1 clusters (e.g., CD8+ cytotoxic T cells), based on expert annotation. (Figure 4.3-4.5)

4.2.8 Neutrophil elastase staining

Since we were unable to identify neutrophils, an additional neutrophil elastase staining was performed on a serial tissue section of two BOS cases. Sections were deparaffinised in xylene (2x5 min), rehydrated in descending series of ethanol (100%, 90%, 70% and 50%, 5 min each) and washed in distilled water (2x5 min) and PBS (2x5 min). Tissue sections were blocked with 3% BSA in PBS for 45 min at room temperature, after which they were stained with the primary antibody (neutrophil elastase, 1:100 dilution) and left at 4°C overnight. After washing with 0.2% Triton X-100 in PBS (8 min) and PBS (2x8 min), tissue sections were incubated with the secondary antibody (568, 5 µg/mL in PBS) for 60 min at room temperature. After two washes in PBS (2x5 min), the slides were incubated with DAPI for 15 min, followed by two washes in PBS (2x5 min). The tissue sections were mounted using Prolong Glass Antifade Mountant seal with coverslip and stored at room temperature.

4.2.9 Definitions

- Endothelial-to-mesenchymal transition (EnMT): EnMT is characterised by endothelial cells losing their endothelial properties and acquiring a mesenchymal cell phenotype. We should note that EnMT should be interpreted with caution in our study, as we noted more EnMT than expected in ROIs other than blood vessels, most likely due to the close proximity of capillaries and respiratory epithelium that can express collagen and αSMA.
- Epithelial-to-mesenchymal transition (EMT): EMT is characterised by epithelial cells losing their epithelial properties and acquiring a mesenchymal cell phenotype. EMT1 are epithelial cells positive for collagen I, TGF-β and/or αSMA. EMT2 are epithelial cells positive for TGF-β.
- Intermediate M2 monocytes/macrophages: monocytes can generally be divided into three subsets: classical, intermediate and non-classical. Once monocytes reach the tissue, they typically undergo polarisation towards M1 or M2 macrophages. In our clusters we could recognise the polarisation state but also still the original subset, possibly because these macrophages had recently transitioned from monocytes. For convenience, they were called intermediate M2 macrophages.
- Unclassified lymphoid cells: the cluster unclassified lymphoid cells consisted of T-cell, B-cell and NK-cell markers that could not be further divided.

4.2.10 Statistical analysis

All analyses were performed using GraphPad Prism 10.0.2 (San Diego, USA). Results from continuous data are expressed as median [interquartile range, IQR] and compared across groups using Mann-Whitney U test and Kruskal-Wallis tests with Dunn's multiple comparisons test if significant. Categorical data were compared using Fisher's exact test for small groups and Chi-square tests for groups with more than two categories with post-hoc Bonferroni correction for multiple significance tests if applicable. P-values are two-tailed, and $p<0.05$ is considered statistically significant.

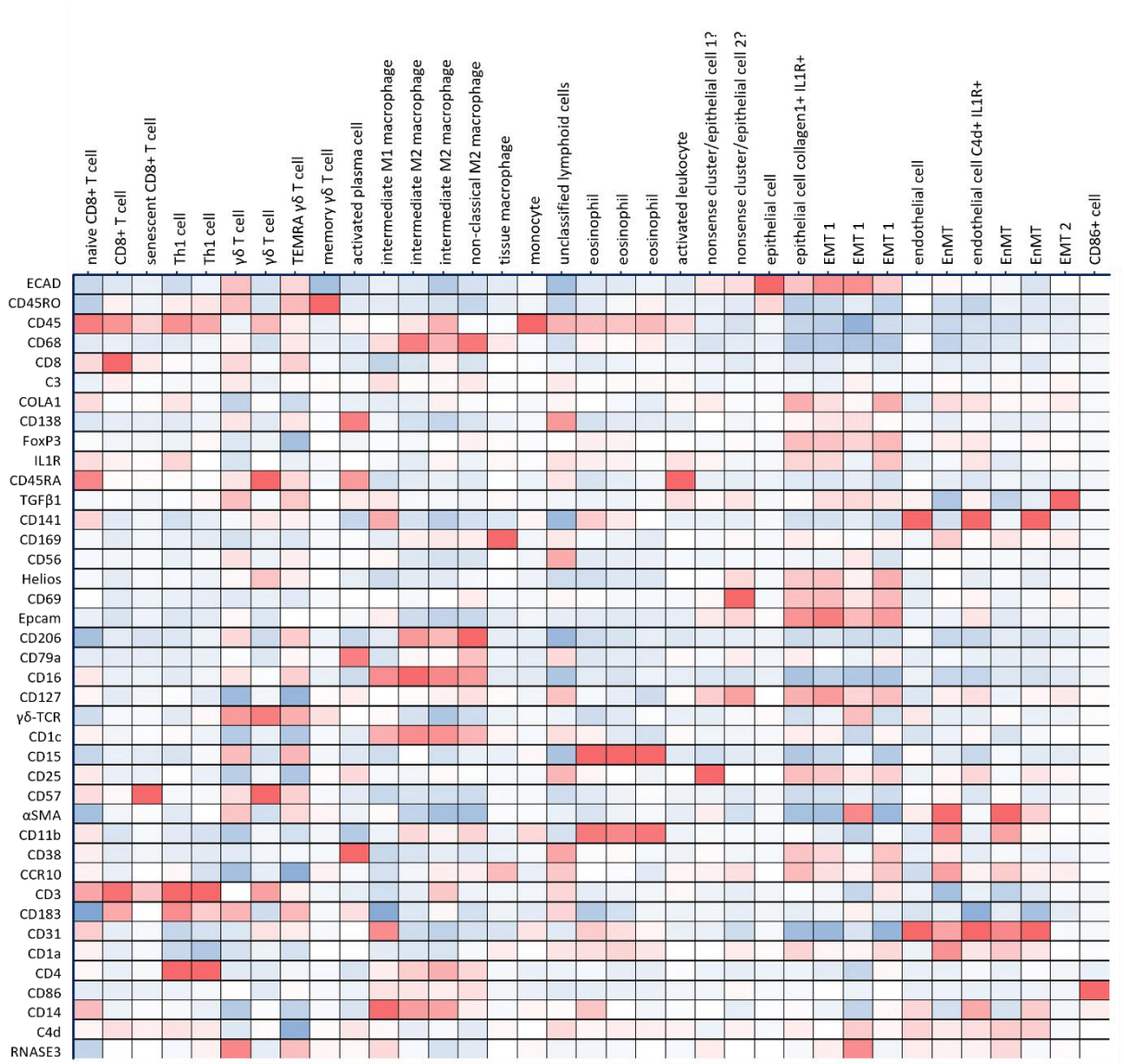


Figure 4.3. Consensus clusters

Heatmap of all 35 consensus clusters showing the median Z-score normalised values for all 40 phenotypic and functional markers. Red represents upregulated marker expression, blue downregulated marker expression and white insignificant marker expression.

αSMA: alpha smooth muscle actin, C3: complement 3, C4d: complement 4d, CCR: C-C chemokine receptor, CD: cluster of differentiation, ECAD: E-Cadherin, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, EPCAM: epithelial cellular adhesion molecule, Foxp3: forkhead box P3, IL-1R: interleukin 1 receptor, TCR: T-cell receptor, TEMRA: terminally differentiated effector memory T cells, TGF-β: transforming growth factor beta.

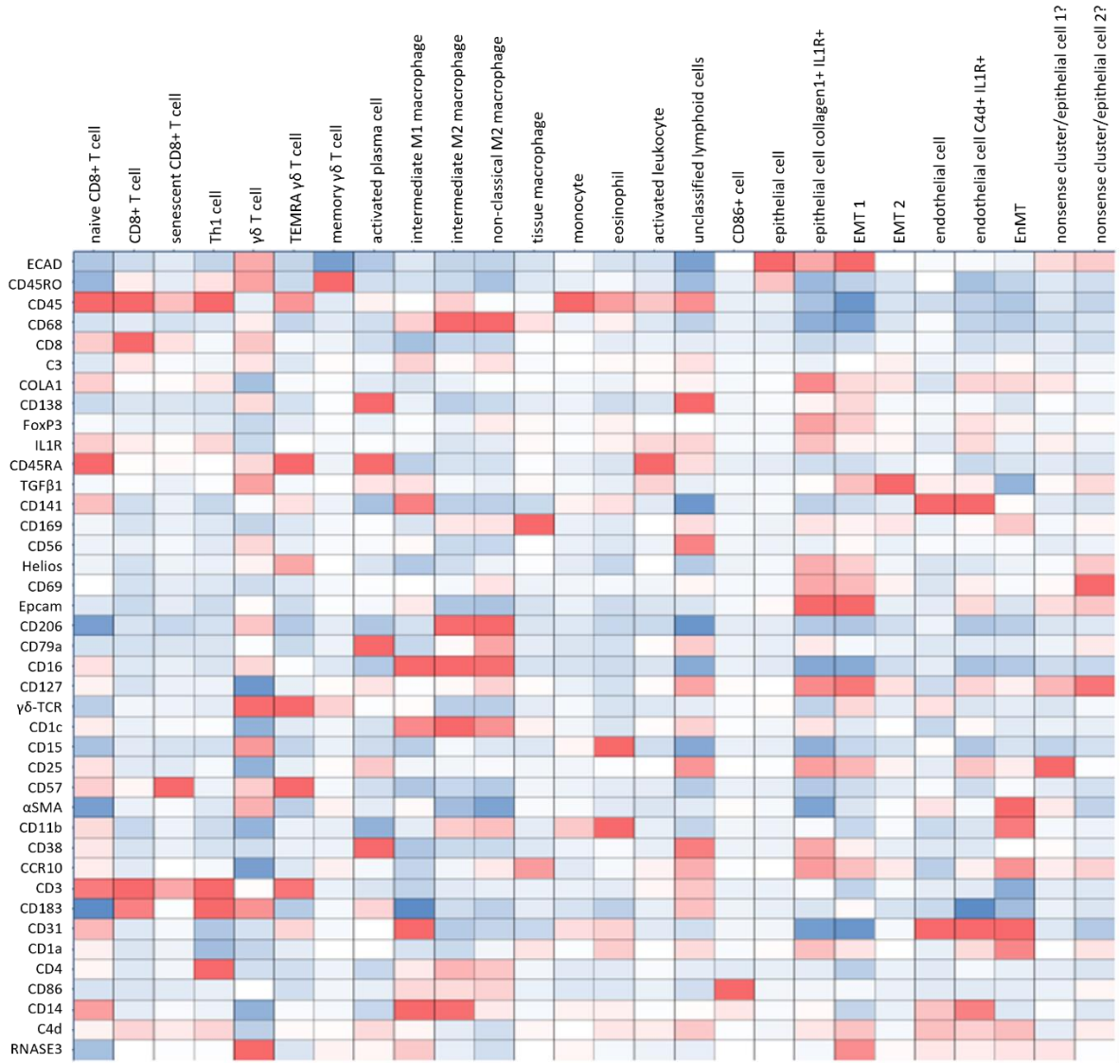


Figure 4.4. Tier 2 consensus clusters

Heatmap of 26 distinct Tier 2 consensus clusters, which were merged from the initial 35 consensus clusters, showing the median Z-score normalised values for all 40 phenotypic and functional markers. Red represents upregulated marker expression, blue downregulated marker expression and white insignificant marker expression.

αSMA: smooth muscle actin, C3: complement 3, C4d: complement 4d, CCR: C-C chemokine receptor, CD: cluster of differentiation, ECAD: E-Cadherin, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, EPCAM: epithelial cellular adhesion molecule, Foxp3: forkhead box P3, IL-1R: interleukin 1 receptor, TCR: T-cell receptor, TEMRA: terminally differentiated effector memory T cells, TGF-β: transforming growth factor beta.

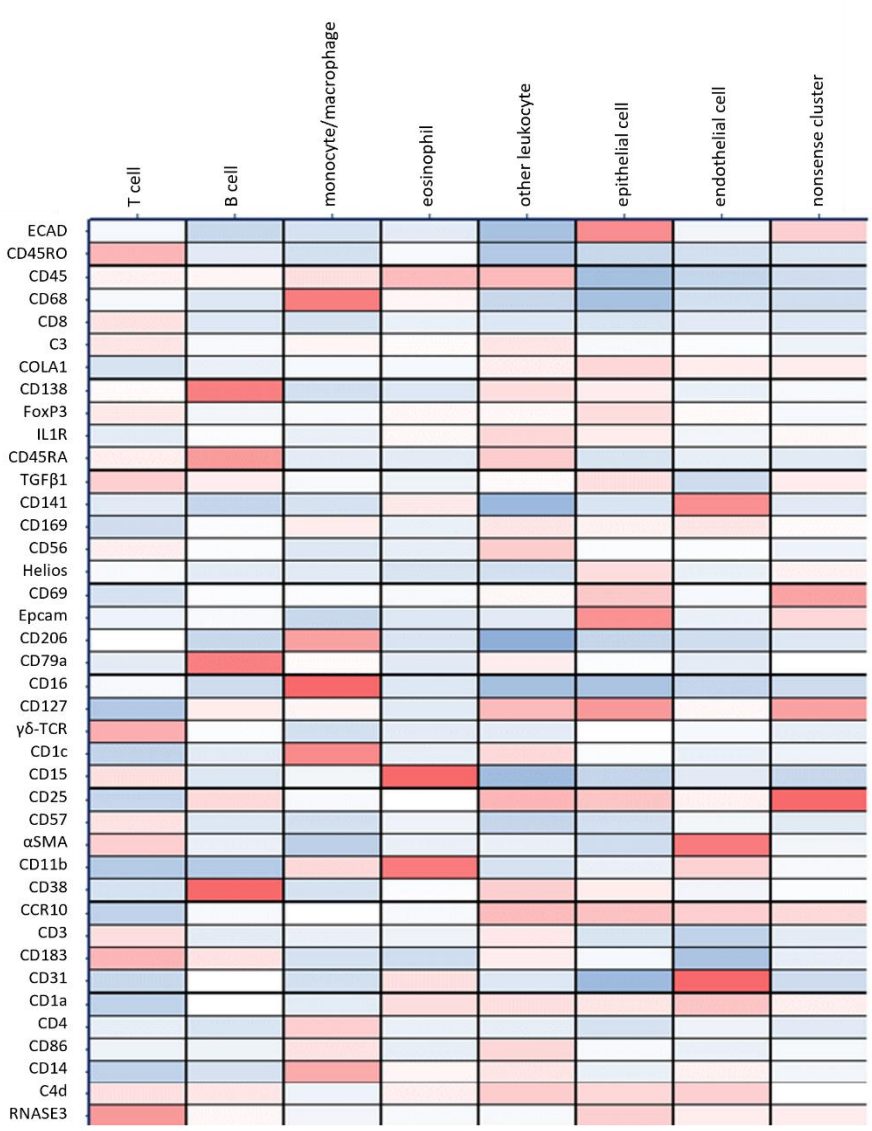


Figure 4.5. Tier 1 consensus clusters

Heatmap of eight distinct Tier 1 consensus clusters, which were merged further from the 26 Tier 2 consensus clusters, showing the median Z-score normalised values for all 40 phenotypic and functional markers. Red represents upregulated marker expression, blue downregulated marker expression and white insignificant marker expression.

αSMA: smooth muscle actin, C3: complement 3, C4d: complement 4d, CCR: C-C chemokine receptor, CD: cluster of differentiation, ECAD: E-Cadherin, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, EPCAM: epithelial cellular adhesion molecule, Foxp3: forkhead box P3, IL-1R: interleukin 1 receptor, TCR: T-cell receptor, TEMRA: terminally differentiated effector memory T cells, TGF-β: transforming growth factor beta.

4.3 Results

4.3.1 Patient cohort

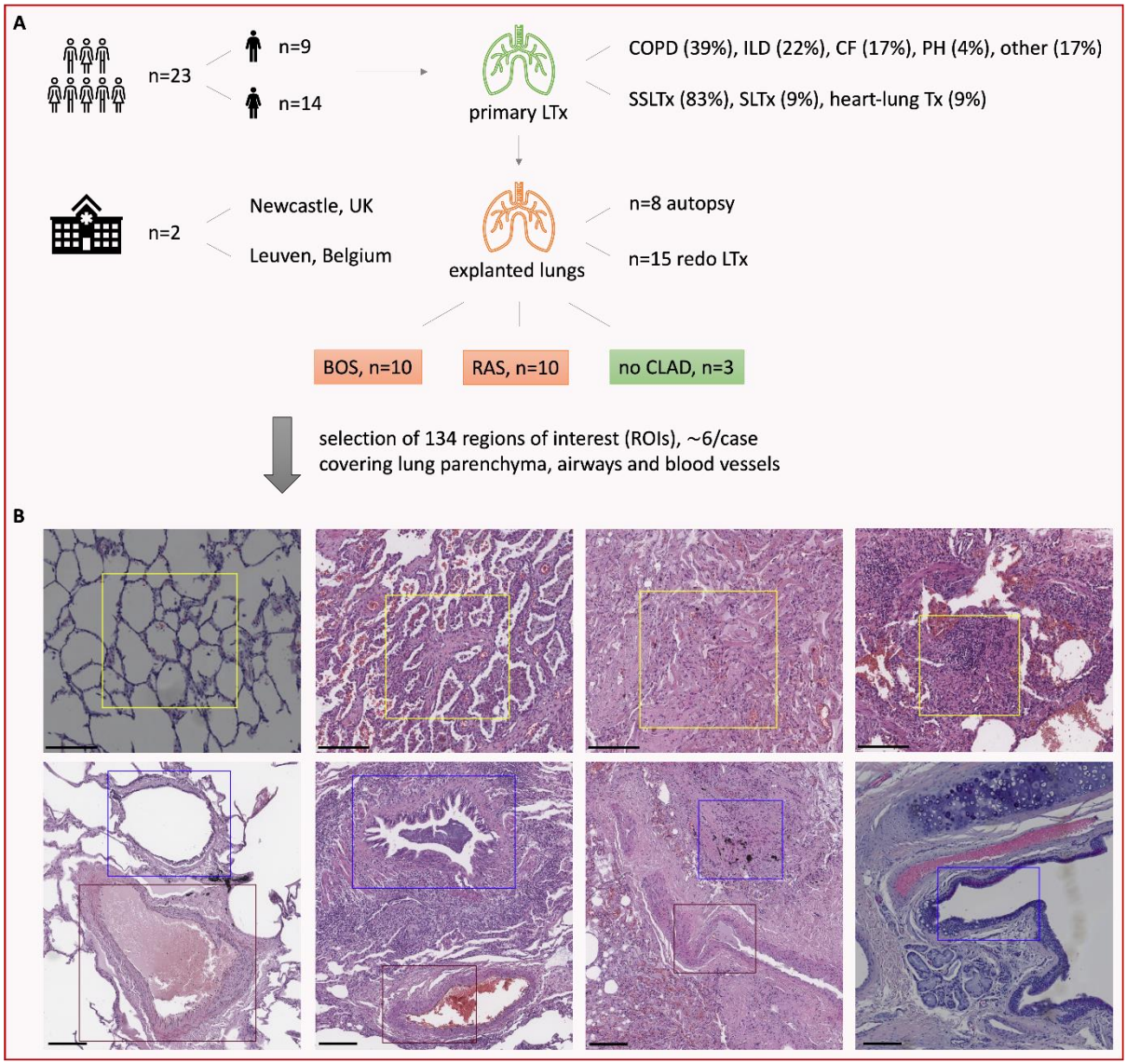


Figure 4.6. Patient demographics and histological model
A. Graphical overview of cohort composition and key clinical metadata. Detailed patient characteristics can be found in Table 4.2. **B.** Examples of selection of regions of interest (ROIs) that were selected based on H&E images (x40, scale bar 200 µm) and expert pathologist input. Top from left to right: preserved alveoli, less fibrotic lung parenchyma, more fibrotic lung parenchyma, lymphocytic parenchymal area. Bottom from left to right: non-proliferative airway and adjacent blood vessel, inflammatory obliterative bronchiolitis lesion with adjacent blood vessel, fibrotic obliterative bronchiolitis lesion with adjacent blood vessel, large airway.
BOS: bronchiolitis obliterans syndrome, CF: cystic fibrosis, CLAD: chronic lung allograft syndrome, COPD: chronic obstructive pulmonary disease, H&E: haematoxylin and eosin, ILD: interstitial lung disease, LTx: lung transplantation, PH: pulmonary hypertension, RAS: restrictive allograft syndrome, SLTx: single lung transplantation, SSLTx: sequential single lung transplantation, Tx: transplantation.

Figure 4.6 displays the patient cohort. The explanted lung tissue samples consisted of six BOS samples from Newcastle and four BOS samples from Leuven, along with ten RAS and three non-CLAD samples from Leuven. Sixty-one percent of patients were female. Main indications for lung transplantation were chronic obstructive pulmonary disease (39%) and interstitial lung disease (22%). Most patients (83%) underwent sequential single lung transplantation and were on triple immunosuppressive therapy (71%). Sixty-five percent of lung samples were obtained at the time of redo lung transplantation. (Table 4.2A)

There were no significant differences in patient demographics among the three groups, except that the median age at time of transplantation was lower in BOS patients than in non-CLAD controls (median 26 [21-45] vs 61 [60-66] years, p=0.0115). However, patient characteristics did not differ between BOS patients from Newcastle and Leuven. (Table 4.2B) In addition, no obvious differences in data spread were seen on PaCMAP dimensionality reduction plots coloured for demographic characteristics, including indication and type of lung transplantation, and autopsy versus redo lung transplantation. (Figure 4.7)

A. Patient characteristics

Characteristics	All patients (n=23)	BOS (n=10)	RAS (n=10)	Non-CLAD controls (n=3)	p-value
Male sex (n, %)	9 (39)	3 (30)	4 (40)	2 (67)	0.5199
Age at primary LTx (years)	43 [23-56]	26 [21-45] *	50 [32-57]	61 [60-66]	**0.0115**
Indication primary LTx (n, %)					0.2614
- COPD	9 (39)	2 (20)	5 (50)	2 (67)	
- Cystic fibrosis	4 (17)	2 (20)	2 (20)	0 (0)	
- ILD	5 (22)	1 (10)	3 (30)	1 (33)	
- Pulmonary hypertension	1 (4)	1 (10)	0 (0)	0 (0)	
- Other	4 (17)	4 (40)	0 (0)	0 (0)	
Type of primary LTx (n, %)					0.1782
- SSLTx	19 (83)	6 (60)	10 (100)	3 (100)	
- SLTx	2 (9)	2 (20)	0 (0)	0 (0)	
- Heart-lung	2 (9)	2 (20)	0 (0)	0 (0)	
Time to CLAD after primary LTx (days)#	837 [316-1413]	445 [315-660]#	942 [568-1760]	N/A	0.0992
Time to redo-LTx or autopsy after primary LTx (days)	1627 [681-2620]	1636 [761-2400]	1649 [1298-3050]	374 [218-3354]	0.5301
- Redo-LTx		1525 [735-2138]	1762 [1314-3247]	N/A	
- Autopsy		2620 [2620-2620]	1567 [881-2686]	374 [218-3354]	
Immunosuppression##					0.0984
- CNI/CCI/CS	12 (71)	2 (50)##	9 (90)	1 (33)	
- CNI/CS	5 (29)	2 (50)##	1 (10)	2 (67)	

B. BOS patients Newcastle versus Leuven

Characteristics	BOS Newcastle (n=6)	BOS Leuven (n=4)	p-value
Male sex (n, %)	3 (50)	0 (0)	0.2000
Age at primary LTx (years)	23 [17-36]	38 [24-54]	0.1714
Indication primary LTx (n, %)			0.3093
- COPD	0 (0)	2 (50)	
- Cystic fibrosis	1 (17)	1 (25)	
- ILD	1 (17)	0 (0)	
- Pulmonary hypertension	1 (17)	0 (0)	
- Other	3 (50)	1 (25)	
Type of primary LTx (n, %)			0.1084
- SSLTx	2 (33)	4 (100)	
- SLTx	2 (33)	0 (0)	
- Heart-lung	2 (33)	0 (0)	
Time redo-LTx or autopsy after primary LTx (days)	1848 [1347-2641]	735 [630-2162]	0.1714

Table 4.2. Patient characteristics
A. Overview of patient characteristics with key clinical metadata. **B.** Comparison of clinical metadata in BOS patients from Newcastle versus Leuven.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, COPD: chronic obstructive pulmonary disease, ILD: interstitial lung disease, LTx: lung transplantation, RAS: restrictive allograft syndrome, SLTx: single lung transplantation, SSLTx: sequential single lung transplantation.
* Kruskal-Wallis with Dunn's multiple comparison test. Significant compared with controls (p=0.0101).
# Data only available for 5/10 BOS patients.
Data only available for 4/10 BOS patients.

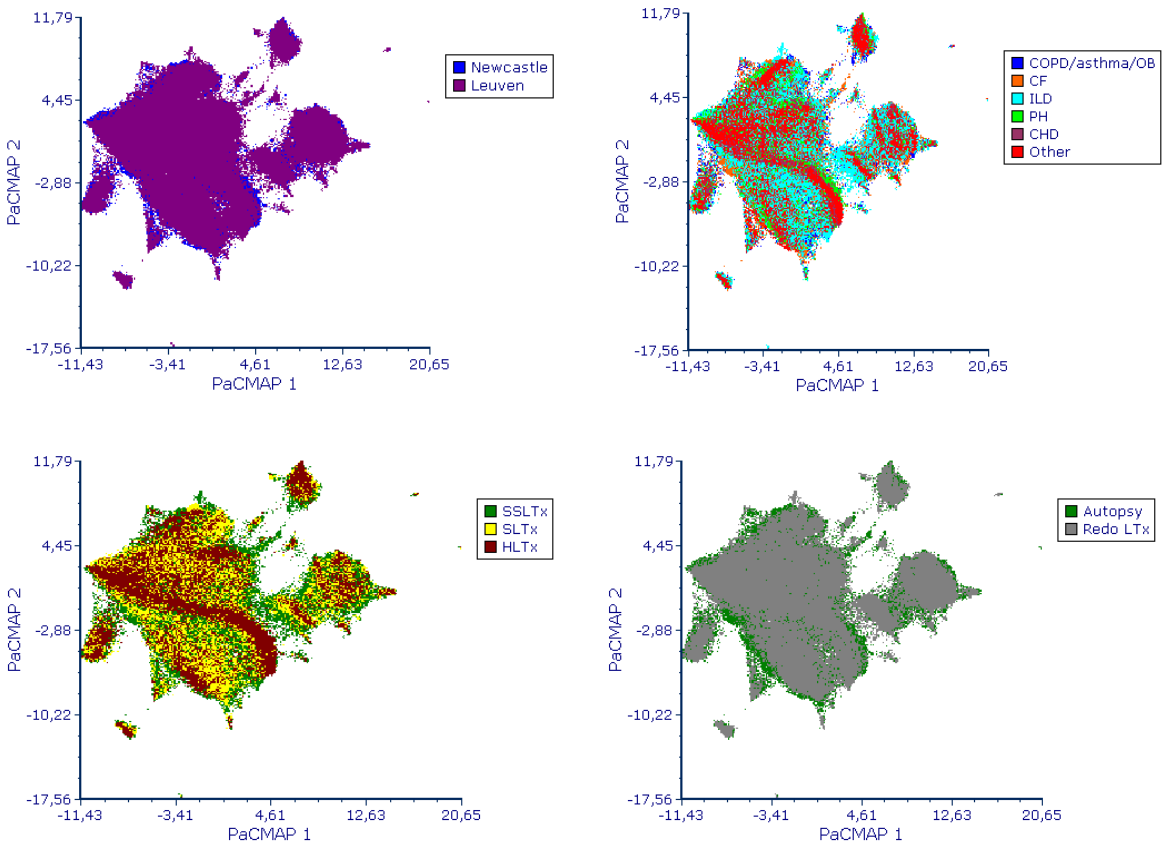


Figure 4.7. Cohort characteristics

PaCMAP dimensionality reduction plots coloured by lung transplant centre, indication for lung transplantation, type of lung transplantation and retrieval of explant lungs (autopsy versus at time of redo lung transplantation). The plots show no obvious differences in data spread for these variables, as evidenced by the distribution of datapoints across the entire plots.

CF: cystic fibrosis, CHD: congenital heart disease, COPD: chronic obstructive pulmonary disease, HLTx: heart-lung transplantation, ILD: interstitial lung disease, LTx: lung transplantation, OB: obliterative bronchiolitis, PH: pulmonary hypertension, SLTx: single lung transplantation, SSLTx: sequential single lung transplantation.

4.3.2 Evaluation of staining success

Unfortunately, not all selected ROIs could be included for data analysis. Due to the fragile status of some of the (mainly RAS) tissue samples, some tissue repeatedly floated off the tissue slides during the antigen retrieval process, despite various attempts to improve tissue adherence (e.g., special-coated slides, changes in antigen retrieval method). Additionally, some of the ROIs were not in the laser ablation field that can be ablated by IMC. Eventually, 40 out of 52 (77%) identified BOS ROIs could be included, 28/69 RAS (41%) and 13 (100%) non-CLAD ROIs, covering airways, (adjacent) blood vessels and lung parenchyma with varying degrees of inflammation and fibrosis. (Table 4.3)

A total ROI area of 56.30 mm^2 was ablated and ROI tissue area was corrected to adjust for airspaces, corresponding to a total cellular area of 41.13 mm^2.

	Included ROIs		**Non-evaluable ROIs**
Non-CLAD samples	3 alveoli 4 airways 3 blood vessels 1 septum	2 adjacent blood vessels	
	Total: 13 ROIs		
BOS samples	9 non-proliferative small airways 3 inflammatory OB lesions 4 fibrotic OB lesions 3 large airways 9 relatively preserved alveoli 3 fibrotic parenchymal areas	5 adjacent blood vessels 2 adjacent blood vessels 2 adjacent blood vessels	 1 inflammatory OB lesion, 2 adjacent blood vessels 4 fibrotic OB lesions, 2 adjacent blood vessels 1 large airway 1 relatively preserved alveoli 2 fibrotic parenchymal areas
	Total: 40/52 ROIs		
RAS samples	4 less fibrotic parenchymal areas 3 more fibrotic parenchymal areas 0 lymphocytic areas 1 fibrotic pleural areas 0 lymphocytic pleural areas 1 fibrotic septum 6 relatively preserved alveoli 2 non-proliferative small airways 3 inflammatory OB lesions 1 fibrotic airway 1 large airways	 2 adjacent blood vessels 3 adjacent blood vessels 1 adjacent blood vessels	2 less fibrotic parenchymal areas 3 more fibrotic parenchymal areas 3 lymphocytic areas 4 fibrotic pleural areas 5 lymphocytic pleural areas 2 fibrotic septa 4 relatively preserved alveoli 2 non-proliferative small airways, 1 adjacent blood vessel 2 inflammatory OB lesions, 2 adjacent blood vessels 6 fibrotic airways, 5 adjacent blood vessels
	Total: 28/69 ROIs		

Table 4.3. Overview of included and non-evaluable ROIs
Overview of ROIs that were included for data analysis as well as ROIs that could unfortunately not be included. Due to the fragile status of some of the (mainly RAS) tissue samples, not all initially selected ROIs could be included.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

4.3.3 High-dimensional cellular profiling

Single-cell segmentation resulted in a total output of 190,851 single cells, with a median of 4536 [3444-5701] cells per ROI. High-level analysis of immune and structural markers identified eight main consensus clusters (so-called Tier 1), which were substantially discrete when mapped back to a PaCMAP dimensionality reduction plot. (Figure 4.9A-B) Subsequently, these clusters were further divided into 26 different cellular clusters with distinct expression profiles (Tier 2). An overview of immune cell phenotypes included in each Tier (initial unmerged consensus clusters, Tier 2 and Tier 1) is provided in Table 4.4. All heatmaps, illustrating the average marker expression in each Tier cluster, are shown in Figures 4.3 - 4.5.

Initial 35 consensus clusters	Merged Tier 2 consensus clusters	Merged Tier 1 consensus clusters
Naive CD8+ T cells	Naive CD8+ T cells	T cells
CD8+ T cells	CD8+ T cells	
Senescent CD8+ T cells	Senescent CD8+ T cells	
Th1 cells	Th1 cells	
Th1 cells	γδ T cells	
γδ T cells	TEMRA γδ T cells	
γδ T cells	Memory γδ T cells	
TEMRA γδ T cells	Activated plasma cells	B cells
Memory γδ T cells	Intermediate M1 macrophages	
Activated plasma cells	Intermediate M2 macrophages	
Intermediate M1 macrophages	Non-classical M2 macrophages	Monocytes/macrophages
Intermediate M2 macrophages	Tissue macrophages	
Intermediate M2 macrophages	Monocytes	
Non-classical M2 macrophages	Eosinophils	Eosinophils
Tissue macrophages	Activated leukocytes	
Monocytes	Unclassified lymphoid cells	Other leukocytes
Eosinophils	CD86+ cells	
Eosinophils	Epithelial cells	Epithelial cells
Eosinophils	Epithelial cells collagen 1+, IL1R+	
Activated leukocytes	EMT 1	Endothelial cells
Unclassified lymphoid cells	EMT 2	
CD86+ cells	Endothelial cells	Nonsense clusters
Epithelial cells	Endothelial cells C4d+	
Epithelial cells collagen 1+, IL1R+	EnMT	
EMT 1	Nonsense cluster 1	
EMT 1	Nonsense cluster 2	
EMT 1		
EMT 2		
Endothelial cells		
Endothelial cells C4d+		
EnMT		
EnMT		
EnMT		
Nonsense cluster 1		
Nonsense cluster 2		

Table 4.4. Overview of consensus clusters
Overview of immune cell phenotypes in initial 35 consensus clusters, which were afterwards merged to 26 Tier 2 consensus clusters and eight Tier 1 consensus clusters based on expert annotation.
C4d: complement 4d, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, IL-1R: interleukin 1 receptor, TEMRA: terminally differentiated effector memory T cells.

4.3.4 Difference in immune cell profile between CLAD and non-CLAD

The immune cell profile of CLAD differed from that of non-CLAD and was characterised on the one hand by classical cellular and humoral alloimmune responses with increased T cells, including cytotoxic CD8+ T cells, and B cells, which were mainly plasma cells. On the other hand, we also observed involvement of the innate immune system, with an increase in γδ T cells, eosinophils, monocytes/macrophages, and other leukocytes. The macrophages were predominantly intermediate M2 macrophages. Increases in these immune cells also corresponded to an increase in total cellularity (cells/mm²). Finally, more epithelial cells showed markers of EMT (i.e., EMT1) in CLAD than in non-CLAD. (Figure 4.8)

A summary of key findings in CLAD and BOS versus RAS is displayed in Table 4.5.

CLAD	
↑ cellularity ↑ T cells: CD8+ T cells, γδ T cells ↑ B cells: plasma cells ↑ macrophages: intermediate M2 macrophages ↑ eosinophils ↑ EMT	
BOS	**RAS**
↑ γδ T cells ↑ intermediate M2 macrophages ↑ non-classical M2 macrophages ↑ B cells ↑ eosinophils ↑ EMT	↑ Th1 cells ↑ intermediate M2 macrophages ↑ B cells ↑ eosinophils ↑ EMT

Table 4.5. Summary of findings
Summary of main findings of Tier 1 and 2 analyses across clinical phenotypes.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, RAS: restrictive allograft syndrome.

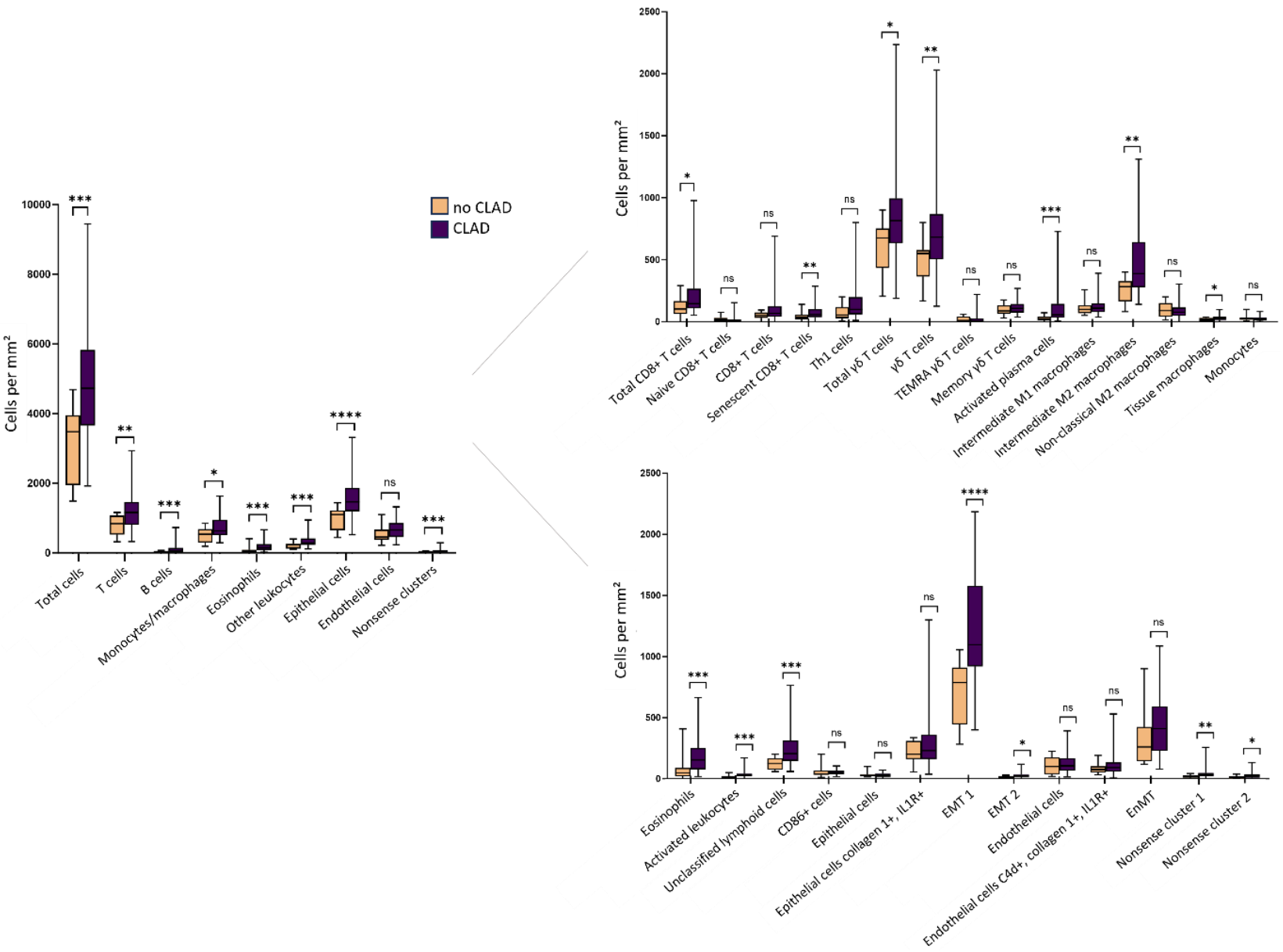

no CLAD
CLAD
Cells per mm²
Total cells
T cells
B cells
Monocytes/macrophages
Eosinophils
Other leukocytes
Epithelial cells
Endothelial cells
Nonsense clusters
Total CD8+ T cells
Naive CD8+ T cells
CD8+ T cells
Senescent CD8+ T cells
Th1 cells
Total γδ T cells
γδ T cells
TEMRA γδ T cells
Memory γδ T cells
Activated plasma cells
Intermediate M1 macrophages
Intermediate M2 macrophages
Non-classical M2 macrophages
Tissue macrophages
Monocytes
Eosinophils
Activated leukocytes
Unclassified lymphoid cells
CD86+ cells
Epithelial cells
Epithelial cells collagen 1+, IL1R+
EMT 1
EMT 2
Endothelial cells
Endothelial cells C4d+, collagen 1+, IL1R+
EnMT
Nonsense cluster 1
Nonsense cluster 2

Figure 4.8. Tier 1 and 2 analyses of CLAD versus non-CLAD
Graphs displaying Tier 1 (left) and Tier 2 (right) analysis of CLAD versus non-CLAD based on median cell counts (corrected per mm² of tissue). Mann-Whitney U test. *: < 0.05, **: <0.01, ***: < 0.001, ****: < 0.0001. C4d: complement 4d, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, IL-1R: interleukin 1 receptor, TEMRA: terminally differentiated effector memory T cells.

4.3.5 Immunological differences between BOS and RAS: Tier 1

Tier 1 consisted of seven main immune and structural clusters: T and B cells, monocytes/macrophages, eosinophils, other leukocytes, epithelial and endothelial cells. (Figure 4.9) In addition, there was a small group (1%) of unidentifiable (nonsense) clusters that could not be further classified. Nonetheless, 99% of the cells could be successfully allocated a Tier 1 and Tier 2 phenotype.

Cellularity was higher in both BOS and RAS compared with non-CLAD controls, with significantly more T cells in BOS versus controls, and B cells and eosinophils in both BOS and RAS versus controls. (Table 4.5)

Taken together, there were no significant differences between BOS and RAS in absolute numbers, but the proportions of B cells, macrophages and other leukocytes were higher in RAS than in BOS, and a similar trend was observed in absolute cell counts.

Regarding structural cells, CLAD samples contained fewer endothelial cells than controls, while the percentage of epithelial cells was higher in BOS than in RAS and controls. The former could correspond to the larger proportion of blood vessels analysed in the control samples; similarly, more airways were included in BOS samples, which could explain the latter. However, the median number of epithelial cells per mm² was significantly higher in BOS and RAS compared with non-CLAD controls, with a similar pattern in endothelial cells, although not significant (p=0.0549).

All significance levels are displayed in Figure 4.9 and Supplementary Table F.1, Appendix F, p. 293. Figure 4.10 shows a visual representation of Tier 1 clusters in common ROI types.

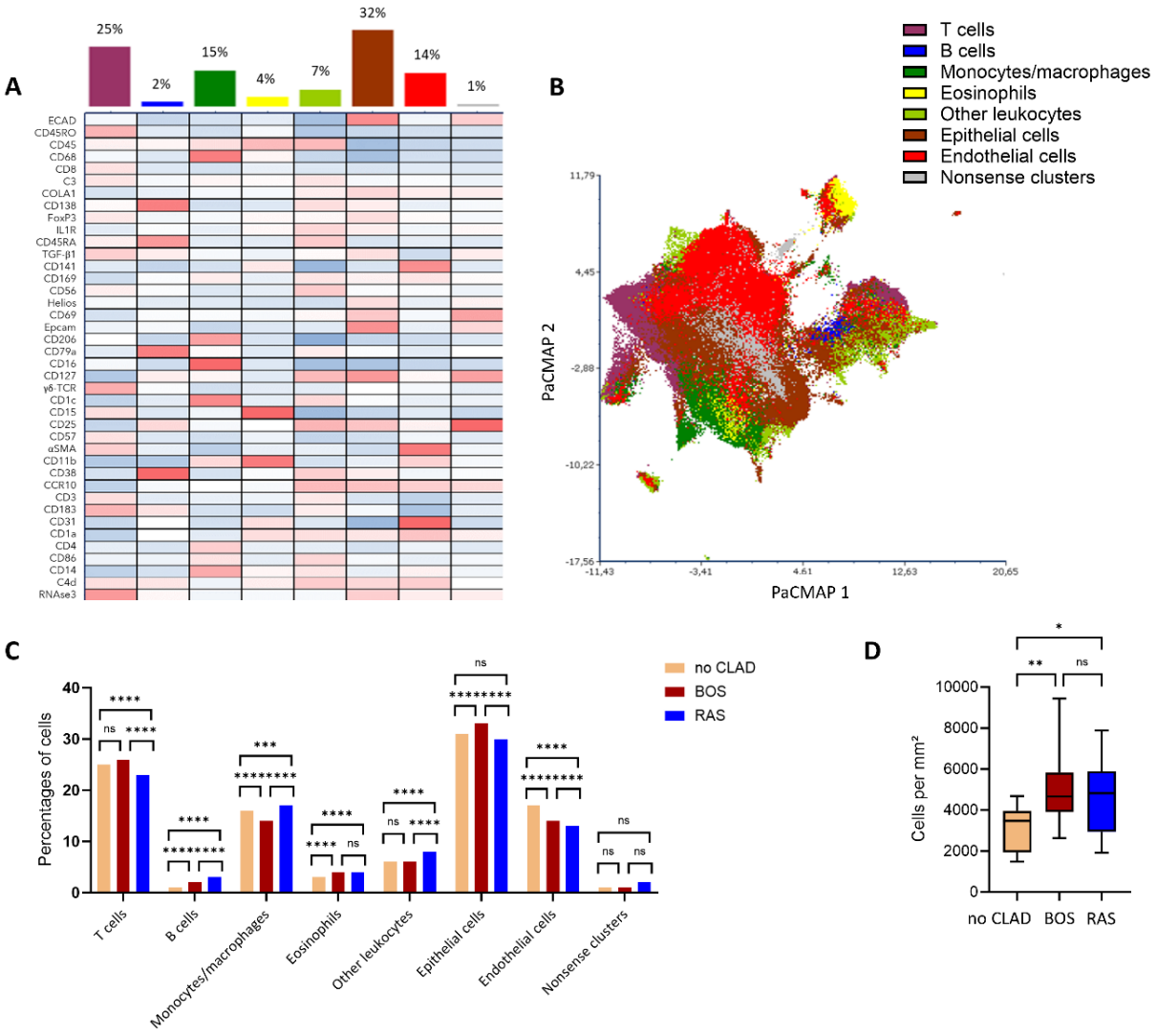

A
25%
2%
15%
4%
7%
32%
14%
1%
ECAD
CD45RO
CD45
CD68
CD8
C3
COLA1
CD138
FoxP3
IL1R
CD45RA
TGF-β1
CD141
CD169
CD56
Helios
CD69
Epcam
CD206
CD79a
CD16
CD127
γδ-TCR
CD1c
CD15
CD25
CD57
αSMA
CD11b
CD38
CCR10
CD3
CD183
CD31
CD1a
CD4
CD86
CD14
C4d
RNAse3
B
T cells
B cells
Monocytes/macrophages
Eosinophils
Other leukocytes
Epithelial cells
Endothelial cells
Nonsense clusters
PaCMAP 2
PaCMAP 1
11,79
4,45
-2,88
-10,22
-17,56
-11,43
-3,41
4,61
12,63
20,65
C
no CLAD
BOS
RAS
Percentages of cells
T cells
B cells
Monocytes/macrophages
Eosinophils
Other leukocytes
Epithelial cells
Endothelial cells
Nonsense clusters
D
Cells per mm²
no CLAD
BOS
RAS
E
T cells
B cells
Monocytes/macrophages
Eosinophils
Other leukocytes
Epithelial cells
Endothelial cells
Nonsense clusters
Cells per mm²
no CLAD
BOS
RAS

Figure 4.9. Analysis of Tier 1 clusters

A. Heatmap of Tier 1 consensus clusters showing the median Z-score normalised values for all 40 phenotypic and functional markers. Coloured bars denote the percentages of each cluster across the entire single-cell data set. A larger version of the heatmap can be found in Figure 4.5. **B.** PaCMAP dimensionality reduction plot of single-cell data coloured by Tier 1 clusters. **C.** Overview of proportions of Tier 1 clusters corrected per mm² of tissue and significance levels between clinical phenotypes. Non-CLAD samples contained more endothelial cells, corresponding to the larger proportion of blood vessels analysed in non-CLAD samples. Similarly, the percentage of epithelial cells was higher in BOS than in RAS and controls, corresponding to the higher number of airways included in BOS samples. Chi-square test with Bonferroni correction for multiple significance tests. ***: < 0.001, ****: < 0.0001. **D.** Median total number of cells per clinical phenotype. **E.** Overview of median cell counts (corrected per mm² of tissue) of Tier 1 clusters and significance levels between clinical phenotypes. Kruskal-Wallis tests with Dunn's multiple comparisons test. *: < 0.05, **: < 0.01, ***: < 0.001, ****: < 0.0001. Full dataset can be found in Supplementary Table F.1, Appendix F.

BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, RAS: restrictive allograft syndrome.

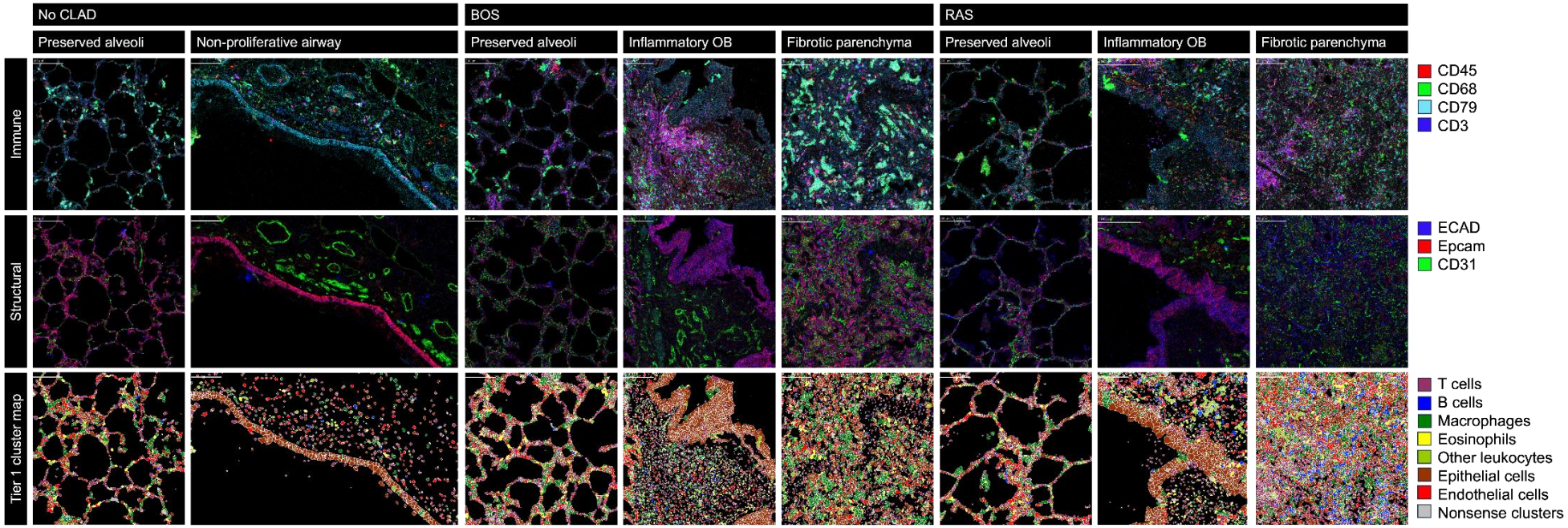


Figure 4.10. Examples of Tier 1 expression in key ROIs

Figures showing raw IMC images and cluster maps for Tier 1 populations in some key pathology classes, visually displaying variations in immune and structural populations. Immune cells highlighted using CD45 (leukocytes), CD68 (macrophages), CD79 (B cells) and CD3 (T cells.) Structural features highlighted are epithelial cells (ECAD and Epcam) and endothelial cells (CD31). Scale bar 200 µm.

BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, ECAD: E-Cadherin, Epcam: epithelial cellular adhesion molecule, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome.

4.3.6 Immunological differences between BOS and RAS: Tier 2

Figure 4.11 provides details of the 26 Tier 2 clusters. Interestingly, regarding T-cell subtypes, we noticed mainly CD8+ T cells and Th1 cells, with no other types of T-helper cells that could be classified further.

BOS and RAS were both associated with cellular and humoral immune responses. Cytotoxic CD8+ T cells were elevated in BOS and RAS samples, although this no longer reached significance at a Tier 2 level (p=0.0628). All B cells that could be further differentiated were activated plasma cells, with greater numbers in both BOS and RAS than in non-CLAD controls. Compared with RAS, we observed a more pronounced innate immune response in BOS, with an increase in γδ T cells and non-classical M2 macrophages. By contrast, Th1 cells and intermediate M2 macrophages were proportionally higher in RAS, with a similar trend in absolute numbers. Furthermore, there were significantly more unclassified lymphoid cells, a group consisting of T-cell, B-cell and NK-cell markers, in BOS and RAS compared with non-CLAD, as well as activated leukocytes. Finally, both CLAD phenotypes were associated with a higher number of cells showing EMT (i.e., EMT1). (Table 4.5)

In summary, there was an expansion of γδ T cells in BOS, Th1 cells in RAS and plasma cells in both. Intermediate M2 macrophages were numerically increased in both BOS and RAS, but proportionally higher in RAS, whereas non-classical M2 macrophages were elevated in BOS. Eosinophils and EMT1 were higher in both CLAD phenotypes compared with non-CLAD. All significance levels are displayed in Figure 4.11 and Supplementary Table F.1 Appendix F, p. 293.

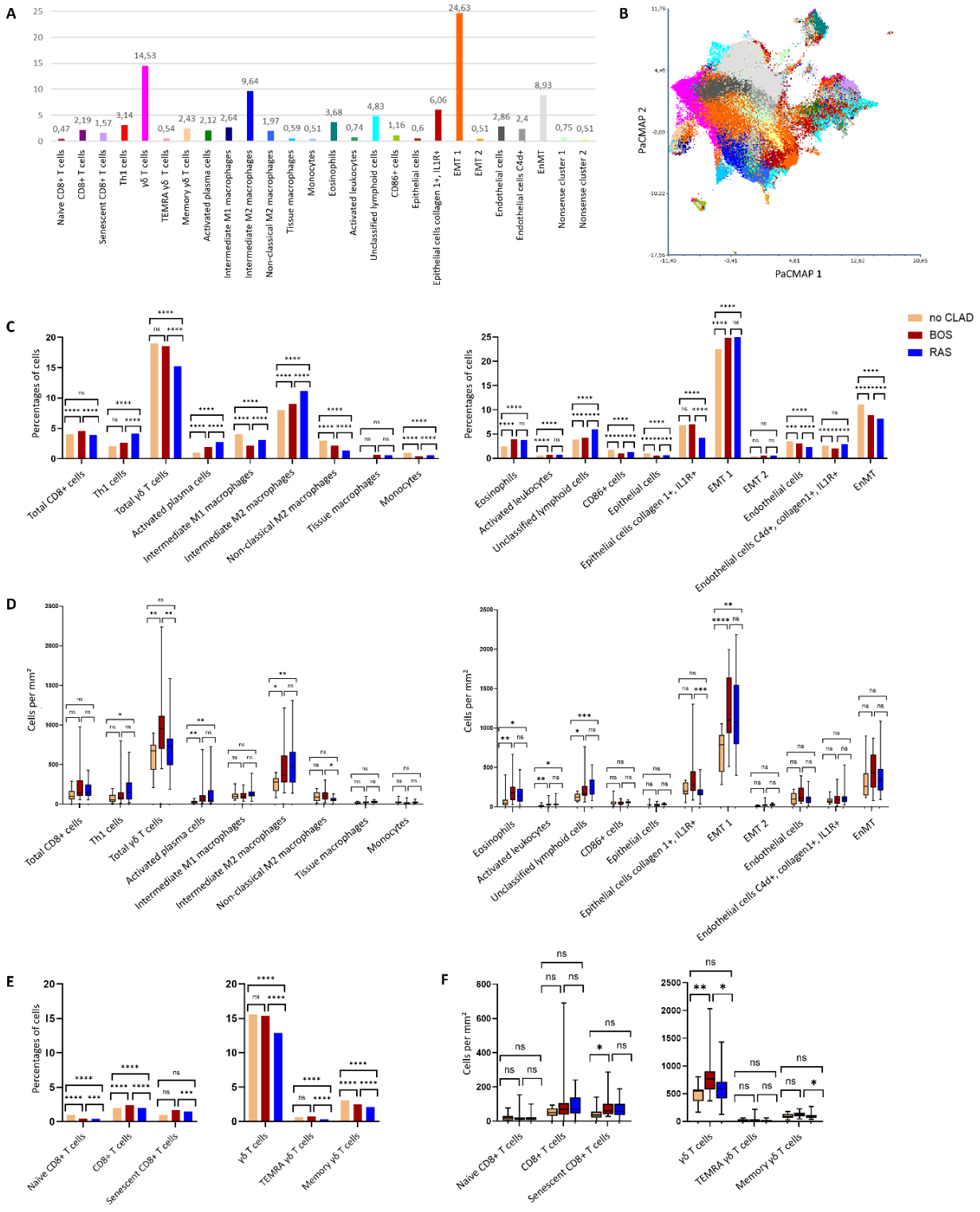


Figure 4.11. Analysis of Tier 2 clusters

A. Overview of Tier 2 consensus clusters. Coloured bars denote the percentages of each cluster across the entire single-cell data set. **B.** PaCMAP dimensionality reduction plot of single-cell data coloured by Tier 2 clusters. **C.** Detailed overview of proportions of Tier 2 clusters corrected per mm² of tissue and significance levels between clinical phenotypes. Chi-square test with Bonferroni correction for multiple significance tests. ***: < 0.001, ****: < 0.0001. **D.** Median cell counts per mm² for Tier 2 clusters. Kruskal-Wallis tests with Dunn's multiple comparisons test. *: < 0.05, **: < 0.01, ***: < 0.001, ****: < 0.0001. **E-F.** Subgroup of CD8+ T cells and γδ T cells according to percentages **(E)** and median cell counts **(F)**. Full dataset can be found in Supplementary Table F.1, Appendix F.

BOS: bronchiolitis obliterans syndrome, C4d: complement 4d, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, IL-1R: interleukin 1 receptor, RAS: restrictive allograft syndrome, TEMRA: terminally differentiated effector memory T cells.

4.3.7 Immune cell interactions

Cellular neighbourhoods of different clinical phenotypes using interaction/avoidance heatmaps are presented in Figure 4.12. Immune cell interactions showed many similarities between BOS and RAS and were more widespread than in patients without CLAD. For example, eosinophil counts were increased in both BOS and RAS and had a wide interaction profile, including increased interaction with plasma cells and T-cell subsets. Across all clinical phenotypes, T cells interacted with B cells, macrophages and epithelial cells.

In BOS, γδ T cells were spatially co-located with cytotoxic T cells, macrophage subsets, plasma cells, eosinophils, and epithelial cells. In RAS, both Th1 cells and intermediate M2 macrophages had a wide range of interactions, including with cytotoxic T cells, plasma cells, macrophages, and epithelial cells.

Overall, spatial analysis demonstrates the complexity and scale of the immune system, as many cells interacted with other cells to some extent.

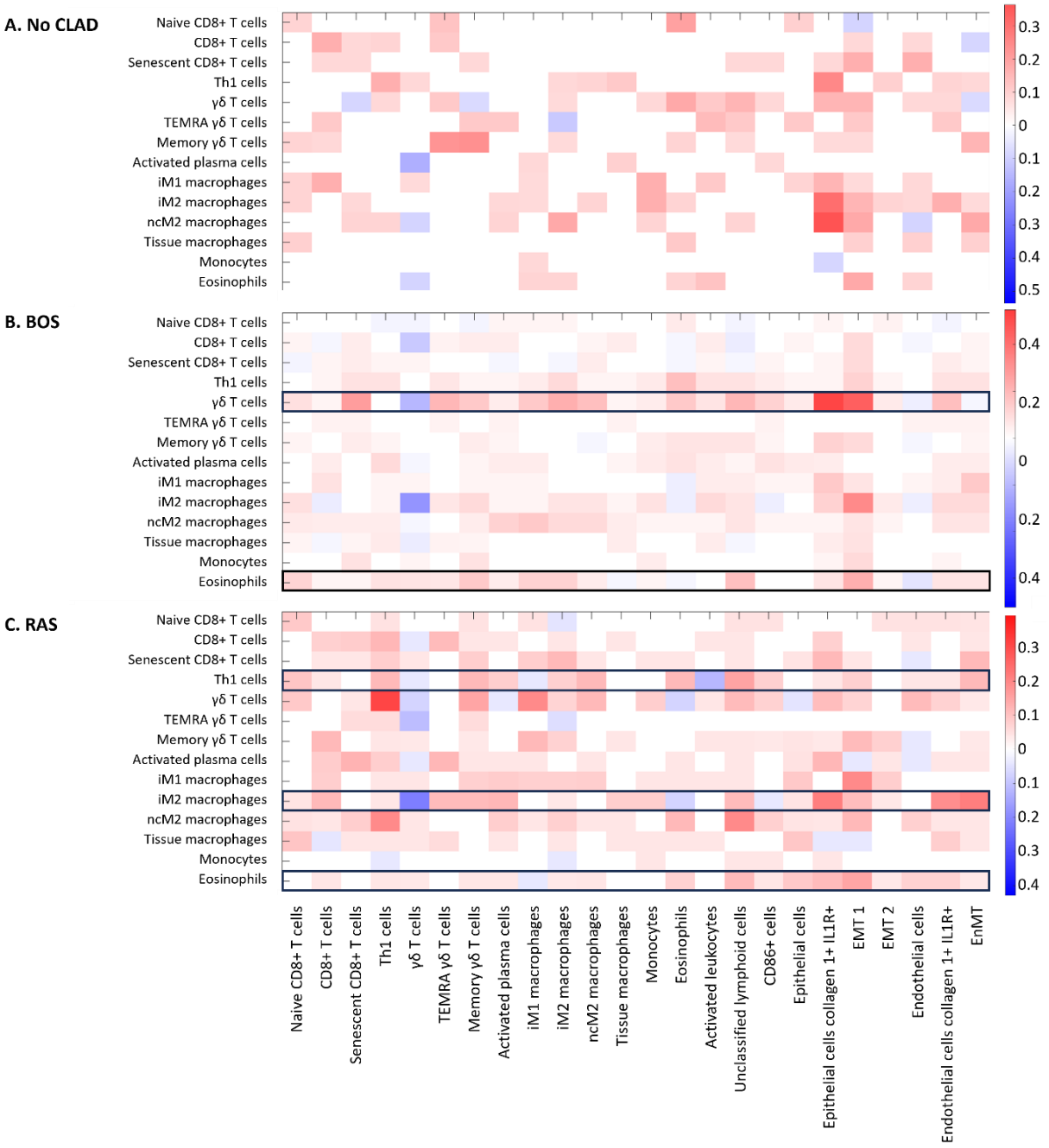


Figure 4.12. Neighbourhood analyses

Neighbourhood analyses showing immune cell interactions in no CLAD **(A)**, BOS **(B)** and RAS **(C)**. Cell-to-cell interactions displayed as a heatmap with red representing a positive (neighbourhood) association, white as an insignificant association and blue as a negative (avoided) association. Rows signify cells surrounding a cell type of interest. Columns signify the cell type of interest. The main immune cells that were differentially increased in BOS (γδ T cells) and RAS (Th1 cells, intermediate M2 macrophages) are marked with black bars, as well as eosinophils, which were increased in both BOS and RAS compared with non-CLAD samples.

BOS: bronchiolitis obliterans syndrome, C4d: complement 4d, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, i: intermediate, IL-1R: interleukin 1 receptor, nc: non-classical, RAS: restrictive allograft syndrome, TEMRA: terminally differentiated effector memory T cells.

4.4 Discussion

Characterisation of recipient immune responses to the transplanted lung at the lung tissue level using multimodal approaches is critical to improving our understanding of CLAD pathophysiology. Studies with blood and BAL have shown that the systemic immune response in CLAD shows major changes in lymphoid and myeloid compartments. (Bos et al., 2022b) Since these studies provide mere inferences about the cellular responses and architectural injury hidden at the tissue level where end-organ dysfunction occurs, detailed immunophenotyping of affected tissue is needed to complete the picture.

In this study, we present a detailed assessment of the immune cell profile and structural cell composition in lung tissue from lung transplant recipients with and without CLAD. In summary, we have evidence of cellular and antibody-mediated responses against the allograft in CLAD, mediated by cytotoxic T cells and B cells. In addition, we have novel observations of involvement of γδ T cells and M2 macrophage polarisation. Both suggest that specific innate immune responses occur alongside classical alloimmune responses, as does the infiltration of eosinophils. Furthermore, we noted mesenchymal and fibrotic proliferation, supporting previous observations made in CLAD. (Borthwick et al., 2009, Zhang et al., 2017) With regard to CLAD phenotypes, we observed increased γδ T cells and non-classical M2 macrophages in BOS, whereas increased intermediate M2 macrophages and Th1 cells in RAS. Interestingly, our findings show that the same type of immune (over)activation, albeit to a lesser extent, is present in patients without CLAD, which itself may be a consequence of transplantation.

4.4.1 Immune profile of CLAD

Major immune cell shifts detected in CLAD compared with non-CLAD included significant expansion of T and B lymphocytes (especially cytotoxic T cells, γδ T cells and plasma cells), M2 macrophage polarisation and eosinophil infiltration. These findings confirm the presence of both classical cellular and humoral alloimmune responses in CLAD, as have been described before, as well as specific innate immune responses. (Bos et al., 2022b) Regarding similarities and differences in immune profiles in BOS and RAS, we noted that infiltration of eosinophils and fibrotic remodelling occurred in both phenotypes. However, strikingly, RAS appeared to be characterised by an increase in Th1 cells and BOS by an increase in γδ T cells. Furthermore, our findings confirmed increased proportions of plasma cells and unclassified lymphoid markers (which also included B-cell markers) in RAS compared with BOS. A similar trend was

observed in absolute cell numbers, but did not reach significance, probably because of the low sample size. Increased B cells in RAS have been described before. (Vandermeulen et al., 2017)

The increase in γδ T cells and non-classical M2 macrophages in BOS may indicate an exaggerated innate immune response, as they both play a role in patrolling. The role of these cells in CLAD is still unclear. As explained in the previous Chapter, γδ T cells are considered quasi-innate and can interact with antigen directly. They patrol mucous membranes and epithelium and cooperate with Th17 cells in barrier defence. (Sullivan et al., 2019) As such, this could explain the higher number in BOS where they may be involved in airway inflammation and remodelling. There were indeed more γδ T cells in the airways than in the vascular compartment (post-hoc analysis, p=0.0003). Airway-centred insults, such as respiratory infections or gastro-oesophageal reflux, and the related epithelial injury may explain the higher number of γδ T cells found in BOS. Indeed, regarding the contribution of infectious and/or alloimmune events, all BOS patients from whom data were available had had one or more respiratory infections requiring hospitalisation or episode of acute cellular rejection before CLAD onset. (Table 4.6)
γδ T cells have a plethora of slightly modified specific functions and act through phagocytosis, apoptosis and upregulation of immune responses via cytokines, such as IFN-γ and IL-17. (Sullivan et al., 2019) According to our interaction profiles, they interacted with a variety of other cells, including cytotoxic T cells, Th1 cells, plasma cells, macrophages, eosinophils, and epithelial cells, including cells showing EMT. (Figure 4.12)

Interestingly, all T-helper cells were polarised towards a more inflammatory Th1 subtype. Our knowledge of the role of Th1 cells in RAS is still small, but their role in chronic rejection has been described in experimental (Yamada et al., 2019) and human (Mamessier et al., 2007, Iasella et al., 2021) studies. The Th1 response is very similar to a cytotoxic T-cell response, and the Th1 pathway is mostly promoted by dendritic cells and macrophages and upregulates IL-2, IFN-γ and TNF-α. These in turn upregulate cytotoxic T cells and macrophages. Indeed, we observed that Th1 cells interacted with cytotoxic T cells and macrophage subsets. In addition, Th1 cells can upregulate IgG1 and IgG3, which activate complement and phagocytic cells. Increased proportions of macrophages in RAS versus BOS may play a role in this upregulation of Th1 in RAS secondary to antigen presentation. This is also supported by interactions between not only M1 but also M2 macrophages and Th1 cells. (Figure 4.12) Higher numbers

of macrophages and dendritic cells have previously been identified in RAS lungs compared with BOS lungs. (Vandermeulen et al., 2017)

Thirdly, we observed polarisation towards M2 macrophages. M2 macrophages play a central role in antigen presentation but are also involved in fibrogenesis, as will be explained in more depth in the next Chapter. Furthermore, most monocytes/macrophages were from an intermediate subset. It is well known that monocyte subsets exhibit remarkable heterogeneity in the expression and function of their surface markers. While classical monocytes are primed for phagocytosis, migration and inflammation and non-classical monocytes mainly patrol, intermediate forms are well suited for antigen presentation and T-cell activation. (Kapellos et al., 2019) They are the only subset that express CCR5, which regulates trafficking and effector functions of T lymphocytes and macrophages via CCL5/RANTES and CCL3/MIP-1α. (Yang et al., 2014) This suggests these monocytes are primed from a first-line defence against pathogens and other insults as part of the innate immune system towards driving alloimmune responses. Under normal conditions, the majority of human monocytes (85%-90%) is represented by classical monocytes (CD14+CD16-). The remaining population is divided between the intermediate subset (CD14+CD16+) and non-classical monocytes (CD14-CD16+). However, intermediate monocytes/macrophages were the most common subset in our patients, followed by a small percentage of non-classical monocytes/macrophages, and we could no longer identify any classical monocytes/macrophages. This distribution was also similar in patients without CLAD and might be a consequence of transplantation and/or immunosuppressive treatment.

Taken together, the increase in both Th1 cells and intermediate M2 macrophages in RAS may allude to pro-inflammatory activation and attempted repair of alloimmune-mediated injury. We observed that in CLAD, and especially in RAS, intermediate M2 macrophages interacted with Th1 cells. Overall, intermediate M2 macrophages had a wide range of interactions with other immune cells, including cytotoxic T cells, B cells, other macrophages, eosinophils, as well as epithelial and endothelial cells. (Figure 4.12)

4.4.2 Other immune cells

With respect to other immune cells, we observed a higher number of cytotoxic T cells in CLAD patients compared with non-CLAD patients, which has been described before. (Bos et al., 2022b) Although this no longer reached significance when comparing across the three clinical

phenotypes (p=0.0628). As can be expected due to immune activation, we noted a shift from naive CD8+ T cells towards more non-naive and senescent CD8+ T cells in BOS and RAS compared with non-CLAD controls.

With respect to other T-helper cells, we were unfortunately unable to further phenotype these. We could therefore unfortunately not identify Th2 cells, Th17 cells or Tregs, although these cells were most likely included in the group of unclassified lymphoid cells (a group with markers of T cells, B cells and NK cells). Additionally, a decline in Tregs has been described in progressive CLAD. (Bhorade et al., 2010)

All B cells that could be differentiated further were plasma cells, although the cluster of unclassified lymphoid cells was also positive for B-cell markers and most likely contained other types of B cells as well. It is known that lymphoid follicles can develop in CLAD, especially RAS. (Vandermeulen et al., 2017) Unfortunately, the tissue sections/ROIs that contained lymphoid follicles did not survive the antigen retrieval process and could therefore not be analysed. It would have been interesting to see what type of cells these were made of, especially with regard to the subsets of T cells (cytotoxic T cells? Th1 cells? γδ T cells? all of the above?) in addition to B cells/plasma cells.

Previous literature showed that the presence of eosinophils was more prominent in RAS patients, although BAL eosinophilia predisposed to both CLAD phenotypes, but RAS in particular. (Vandermeulen et al., 2017, Verleden et al., 2015a) In our study, eosinophil counts were higher in CLAD compared with non-CLAD, but were similarly elevated in both BOS and RAS phenotypes. Therefore, they indeed appear to play a role in both CLAD phenotypes. The mechanisms of action of eosinophils in CLAD have not been clearly elucidated, but eosinophils can cause significant tissue damage as explained in the previous Chapter. The effects are most likely caused by the release of cytotoxic granule products, which can lead to direct epithelial damage, and cytokines and chemokines. The latter further propagate immune responses, but also fibrotic remodelling and EMT by recruiting fibroblasts and stimulating TGF-β release. (Darley et al., 2021, Verleden et al., 2014b)

4.4.3 Epithelial and endothelial mesenchymal transition

In addition to characterising the immune profile in CLAD, our findings also confirmed the presence of more cells with markers of EMT in CLAD, both BOS and RAS, supporting previous

studies. (Borthwick et al., 2009, Zhang et al., 2017) EMT is a typical feature of fibrotic remodelling in CLAD in which epithelial cells lose their epithelial properties and acquire a mesenchymal cell phenotype. However, since many epithelial cells had markers of EMT, we have to be cautious as we might have overinterpreted it. On the other hand, EMT is a continuum and certain markers suggestive of EMT were present (i.e., collagen 1, TGF-β and/or αSMA), although E-Cadherin was also still positive. This suggests that these cells were still transitioning, but that the EMT process had been activated.

Besides epithelial injury and EMT, damage to the microvasculature and EnMT can occur as well. (Walters et al., 2008, Vanstapel et al., 2022) With respect to the interpretation of EnMT, we also have to be careful as we observed more EnMT than expected in ROIs other than blood vessels. This can most likely be explained by the close proximity of capillaries and respiratory epithelium, the latter being able to express collagen and αSMA, also under normal conditions. In addition to vascular remodelling, increased angiogenic activity in BOS has been described in literature before. This angiogenesis could be caused by airway inflammation directly or via vascular endothelial growth factor and would be interesting to investigate further in the future. (Walters et al., 2008)
Likewise, there is still a paucity of evidence as to whether or not lymphangiogenesis occurs in CLAD, which might also be of interest for further research. (Traxler et al., 2017)

4.4.4 BOS and RAS: similar immunological rejection pathways?

The above findings demonstrate both similarities and differences in immune reactions in BOS and RAS. Overall, relatively similar adaptive and innate immune responses occurred, except for a more exaggerated innate immune response in BOS, with activation of γδ T cells and non-classical M2 macrophages, and more pronounced alloimmune response in RAS via intermediate M2 macrophages and Th1 cells. These differences in immune reactions might be mediated by differences in the types of injuries contributing to the onset of CLAD. Indeed, regarding the contribution of infectious and alloimmune responses in BOS, we found that these patients had had more respiratory infections requiring hospitalisation or episodes of acute rejection prior to CLAD. (Table 4.6) On the other hand, we had evidence of more antibody-mediated rejection events before the onset of CLAD in RAS patients. (Table 4.7) We will discuss these rejection pathways in BOS and RAS further in the next Chapter, after reviewing differences in fibrotic remodelling in BOS and RAS.

Number, sex	Age at LTx (years)	Indication for LTx	Time CLAD after LTx (days)	Maintenance immunosuppression/ immunomodulation before redo LTx/ autopsy	Respiratory infection/ colonisation 6 months before redo LTx or autopsy	Respiratory infection (requiring hospitalisation) or alloimmune events before CLAD onset	DSA	Other
1, F	56	COPD	870	TAC, CS, AZI	Frequent infections No colonisation	2 episodes of ACR (1x A1, 1x B1) 1 infection with hMPV	-	
2, F	49	COPD	449	TAC, MMF, CS, AZI	Colonisation with P. aeruginosa Infection with A. fumigatus	3 episodes of ACR (1x A1, 1x A2, 1x A3) No respiratory infections	-	No ROIs included from this patient as tissue floated off repeatedly
3, F	27	asthma	313	TAC, CS, AZI	1 respiratory infection No colonisation	2 episodes of ACR (1x A1B1, 1x A2) 1 infection with S. aureus, 1 with P. aeruginosa and 1 with S. pneumoniae	-	
4, F	23	CF	445	TAC, MMF, CS, AZI, MLK	Colonisation with P. aeruginosa and A. xylosoxidans	2 episodes of ACR (1x A2, 1x A3) AMR (treated with PLEX, IVIG and rituximab) 3 infections with P. aeruginosa	DQ DR	PTLD > 1 year before CLAD onset B-cell counts were still relatively low (7-40/mm²) after rituximab treatment compared with other BOS patients
5, F	21	OB	NA	NA	NA	NA	NA	
6, M	33	ILD	NA	NA	NA	NA	NA	
7, F	19	ILD	NA	NA	NA	NA	NA	Treatment with infliximab but no information if close to redo LTx
8, F	43	PH	316	NA	NA	NA	NA	
9, M	24	CHD	NA	NA	NA	NA	NA	
10, M	10	CF	NA	NA	History of Aspergillus cavity, no other information	NA	NA	PTLD > 1 year before CLAD onset

Table 4.6. Characteristics BOS patients

Characteristics of BOS patients, including any episodes of acute rejection, antibody-mediated rejection or infection before CLAD onset, infectious events and/or colonisation in the six months before the lungs were obtained, and maintenance immunosuppression and immunomodulation therapy at the time of redo lung transplantation or autopsy.

ACR: acute cellular rejection, graded based on the International Society for Heart and Lung Transplantation Lung Rejection Working Group (Stewart et al., 2007), AMR: antibody-mediated rejection, AZI: azithromycin, BOS: bronchiolitis obliterans syndrome, CF: cystic fibrosis, CHD: congenital heart disease, CLAD: chronic lung allograft dysfunction, COPD: chronic obstructive pulmonary disease, CS: corticosteroids, DSA: donor-specific antibodies, hMPV: human metapneumovirus, ILD: interstitial lung disease, IVIG: intravenous immunoglobulins, LTx: lung transplantation, MLK: montelukast, MMF: mycophenolate mofetil, NA: not available, OB: obliterative bronchiolitis, PLEX: plasmapheresis, PTLD: post-transplant lymphoproliferative disease, ROI: region of interest, TAC: tacrolimus.

Number, sex	Age at LTx (years)	Indication for LTx	Time CLAD after LTx (days)	Maintenance immunosuppression/ immunomodulation before redo LTx/ autopsy	Respiratory infection/ colonisation 6 months before redo LTx or autopsy	Respiratory infection (requiring hospitalisation) or alloimmune events before CLAD onset	DSA	Other
11, M	56	COPD	652	TAC, MMF, CS, AZI, MLK	1 respiratory infection No colonisation	No ACR 1 infection with Serratia	DQ	No ROIs included from this patient as tissue floated off repeatedly
12, F	54	COPD	884	TAC, MMF, CS, AZI, MLK Pirfenidone	No colonisation	No ACR No respiratory infections AMR treated post-CLAD onset (rituximab + PLEX + IVIG)	DQ	1 week pre-autopsy very small dose ATG (stopped hypotension) B cells were still low (21-38/mm² ROI tissue area)
13, M	39	COPD	1678	TAC, AZA, CS, AZI, MLK	No colonisation	2 episodes of ACR (2x B1) 2 infections with P. aeruginosa related to bronchial stricture issues	DQ DR	No ROIs included from this patient as tissue floated off repeatedly
14, F	20	CF	3521	TAC, MMF, CS, AZI, MLK	Colonisation with P. aeruginosa	No ACR 2 infections with P. aeruginosa (1 empyema)	-	
15, F	58	ILD	2004	TAC, MMF, CS, AZI, MLK Pirfenidone	No colonisation	No ACR 1 respiratory infection	-	
16, F	36	ILD	1413	TAC, MMF, CS, AZI, MLK Pirfenidone	2 respiratory infections Colonisation with S. maltophilia	2 episodes of ACR (1x A2, 1x B1) Several respiratory infections (2x influenza A, hMPV, P. aeruginosa and 2 with negative cultures) AMR (1x treated with PLEX + IVIG, 1x with rituximab + PLEX + IVIG)	A	B cells still relatively low compared with other RAS patients (34-56/mm² ROI tissue area)
17, F	65	COPD	291	TAC, CS, AZI, MLK Pirfenidone	Infection with A. fumigatus No colonisation	No ACR No respiratory infections AMR (1x treated with rituximab + IVIG + PLEX, 2x with PLEX + IVIG)	DQ DR	No ROIs included from this patient as tissue floated off repeatedly
18, M	45	ILD	837	TAC, AZA, CS, AZI, MLK Pirfenidone	Donor-derived m. tuberculosis infection No colonisation	No ACR No respiratory infections AMR (2x treated with rituximab + IVIG + PLEX)	DQ DR	No ROIs included from this patient as tissue floated off repeatedly

19, M	55	COPD	1000	TAC, MMF, CS, AZI, MLK Pirfenidone	Infection with A. fumigatus and CMV No colonisation	1 episode of ACR (A1) No respiratory infections AMR (treated with PLEX + IVIG + rituximab)	-	
20, F	15	CF	315	TAC, MMF, CS, AZI	2 respiratory infections Colonisation P. aeruginosa and S. marcescens	4 episodes of ACR (2x A2, A4, B1) No respiratory infections	-	Treatment with ATG 7 months before redo LTx No ROIs included from this patient as tissue floated off repeatedly

Table 4.7. Characteristics RAS patients

Characteristics of RAS patients, including any episodes of acute rejection, antibody-mediated rejection or infection before CLAD onset, infectious events and/or colonisation in the six months before the lungs were obtained, and maintenance immunosuppression and immunomodulation therapy at the time of redo lung transplantation or autopsy.

ACR: acute cellular rejection, graded based on the International Society for Heart and Lung Transplantation Lung Rejection Working Group (Stewart et al., 2007), AMR: antibody-mediated rejection, ATG: anti-thymocyte globulin, AZA: azathioprine, AZI: azithromycin, CF: cystic fibrosis, CLAD: chronic lung allograft dysfunction, COPD: chronic obstructive pulmonary disease, CS: corticosteroids, DSA: donor-specific antibodies, hMPV: human metapneumovirus, ILD: interstitial lung disease, IVIG: intravenous immunoglobulins, LTx: lung transplantation, MLK: montelukast, MMF: mycophenolate mofetil, NA: not available, PLEX: plasmapheresis, RAS: restrictive allograft syndrome, ROI: region of interest, TAC: tacrolimus.

4.4.5 Strengths and limitations

The strength of this study comes from the relatively high number of single cells captured for analysis, covering different lung compartments, interrogated at the tissue level using a multiparametric spatial approach. Furthermore, we used a correction that removed variability in airspaces as a confounder. After all, the metric commonly used to quantify immune cells in lung tissue, namely cells per unit area of tissue section, can be confounded by changes in airspace contributions to section area across alveolar, airway and vascular compartments. (Milross et al., 2023) An additional strength is that the control group consisted of lung transplant patients without CLAD who had been exposed to standard immunosuppressive treatment.

Several limitations need to be addressed, such as the fact that not all identified ROIs could be analysed, contributing to a more limited number of ROIs for some clinical phenotypes and ROI types. However, given the rarity of explanted CLAD tissue available, this is still a unique study examining the immune profile in such depth across different lung compartments. Furthermore, only one tissue sample per patient was included, not capturing intra-patient variability, although all patients had end-stage disease and the blocks were selected by pathologists as representative of the pathology. We were unable to identify some other leukocytes (e.g., dendritic cells, neutrophils) and T-cell subsets (e.g., Th2 cells, Th17 cells, Tregs), although these cells might have been included in the clusters activated leukocytes and unclassified lymphoid cells, respectively. Because neutrophils play an important role in inflammation and tissue injury after lung transplantation, an additional neutrophil elastase immunofluorescence staining was performed on a serial section to ensure no large number of neutrophils were missed. This staining confirmed the presence of a small number of neutrophils. (Figure 4.13) We therefore assume these cells were captured in the activated leukocytes cluster. Furthermore, it would have been interesting to include other immune cells markers, such as for mast cells/basophils and innate lymphoid cells. Unfortunately, there were no clones available for use in IMC for the former. And the latter are difficult to identify because they lack the expression of typical cell surface identification molecules (cell lineage marker negative, Lin-). Finally, it is important to take into account that B cells were still suppressed in some RAS patients who had received prior treatment with rituximab.

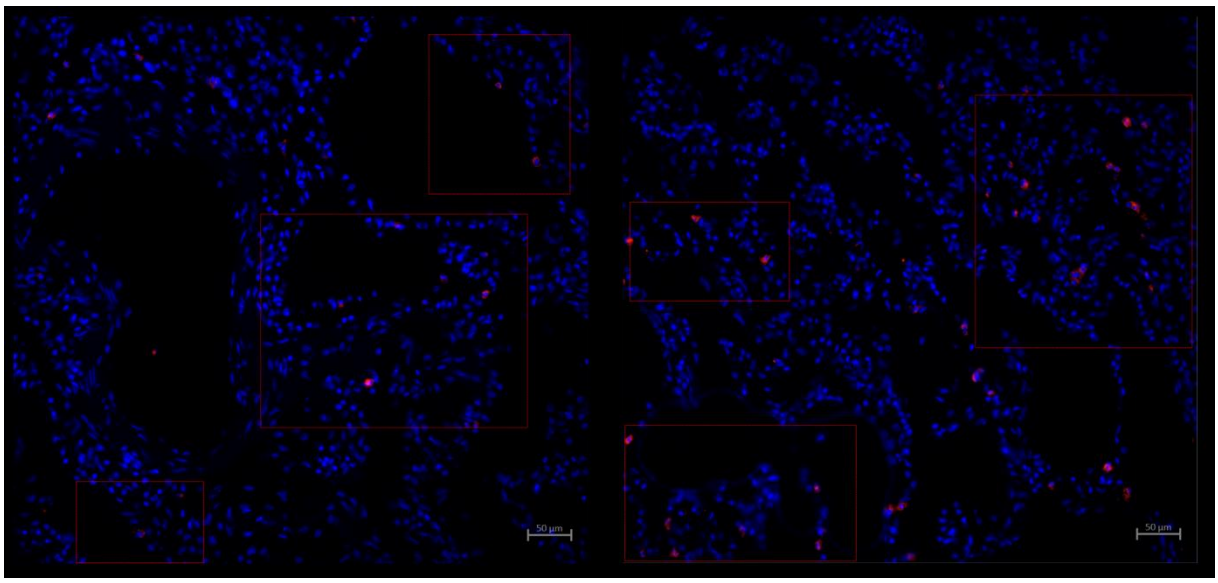


Figure 4.13. Neutrophil elastase staining
Fluorescence microscopy images of two BOS cases stained with DAPI (blue) and neutrophil elastase (red), showing the presence of a small number of neutrophils in the lung tissue. Images were taken at 20x magnification (scale bar 50 µm).
BOS: bronchiolitis obliterans syndrome.

4.5 Conclusions

Highly multiplexed imaging of lung tissue at single-cell resolution revealed major differences in cellularity and cell populations in CLAD versus non-CLAD. Differences were also observed between BOS and RAS. BOS was characterised by more γδ T cells and non-classical M2 macrophages, suggesting an exaggerated innate immune response compared with RAS. In contrast, Th1 cells and intermediate M2 macrophages were more common in RAS, pointing to upregulated alloimmune and repair responses in RAS. Lastly, we confirmed the occurrence of EMT in both CLAD phenotypes.

Key points

- The immune cell profile of CLAD differed from that of non-CLAD.
- CLAD was characterised by classical cellular (cytotoxic CD8+ T cells) and humoral (B cells, especially plasma cells) immune responses.
- In addition to adaptive immune responses, innate immune cells were involved in CLAD, including infiltration of eosinophils and polarisation of macrophages towards M2 macrophages.
- Non-classical M2 macrophages were increased in BOS, whereas intermediate M2 macrophages were proportionally higher in RAS, with a same trend in absolute numbers.
- BOS appeared to be associated with a more pronounced innate immune response of γδ T cells and non-classical M2 macrophages.
- RAS was characterised by alloimmune responses through intermediate M2 macrophages and Th1 cells.
- More epithelial cells in CLAD showed markers of EMT than in non-CLAD samples.

Chapter 5 Temporal evolution of fibrotic remodelling in chronic lung allograft dysfunction

Parts of this Chapter are the subject of the following paper that is in submission: **Bos S**[*], Hunter B[*], McDonald D, Merces G, Sheldon G, Pradère P, Majo J, Pulle J, Vanstapel A, Vanaudenaerde BM, Vos R, Filby AJ, Fisher AJ. High-dimensional tissue profiling of immune responses in chronic lung allograft dysfunction.

5.1 Introduction

Fibrosis is a hallmark of a number of chronic diseases. Following an injurious insult, tissue repair is an essential homeostatic mechanism and the development of fibrosis has been associated with a dysregulated or excessive wound healing response. In the lungs, fibrosis can affect both the parenchyma and airways. Fibrosis is a characteristic pathological change in the parenchyma in patients with idiopathic pulmonary fibrosis and other fibrosing interstitial lung diseases (ILDs), while in asthma and chronic obstructive pulmonary disease fibrosis occurs in the airways. (Boorsma et al., 2014) Dysregulated epithelial repair and airway and/or tissue remodelling are also cornerstones in the pathogenesis of CLAD. (Bos et al., 2022c)

The current paradigm suggests that repeated or persistent clinical or subclinical injury causes epithelial damage and promotes fibrosis. Epithelial-mesenchymal crosstalk appears to be a key feature in this process, resulting in the release of fibrogenic growth factors and activation of mesenchymal cells, leading to the infiltration of myofibroblasts, key effector cells in fibrosis. (Boorsma et al., 2014) The process by which the normal epithelium is replaced by fibroblastic scar tissue is termed EMT, in which epithelial cells lose their epithelial properties and acquire a mesenchymal cell phenotype, including deposition of extracellular matrix and production of matrix metalloproteinases. (Borthwick et al., 2009) A similar mechanism of mesothelial-to-mesenchymal transition has been postulated in RAS. (Sacreas et al., 2019)

The pathogenesis of fibrosis is not fully understood and there is no single unifying mechanism that explains the entire process of pulmonary fibrosis. (Boorsma et al., 2014) The mechanisms are complex and involve a range of different mediators and signalling pathways. (Bos et al., 2022c) Although there may be distinct differences in the patterns and pathways involved

between parenchymal and airway fibrosis, it seems likely that the general mechanisms regulating the fibrogenic response may be broadly similar. (Boorsma et al., 2014)

In addition to detailed immune profiling of lung tissue from lung transplant recipients with and without CLAD, the second aim of our study was to place particular emphasis on spatial and temporal differences in the evolution of fibrosis in CLAD. Rather than analysing typical profibrotic growth factors in the pathogenesis of fibrosis, our aim was to look at differences in the immune cell profile in the temporal evolution of fibrosis and how these immune cells could potentially contribute to the onset and progression of fibrosis.

5.2 Methods

5.2.1 Study set-up

With regard to the patient cohort, tissue processing, image acquisition, and analysis, the methods are similar to those described in Chapter 4, the first part of this study.

5.2.2 Definitions

To evaluate fibrotic parenchymal remodelling, relatively preserved alveoli and less and more fibrotic parenchymal areas were compared. In non-CLAD and BOS cases, areas of preserved alveoli were easy to include. In RAS, the degree of parenchymal fibrosis varied across cases and areas with the best-preserved alveoli were selected.

To evaluate fibrotic remodelling of airways, we compared non-proliferative small airways, inflammatory OB lesions and fibrotic OB lesions/fibrotic airways. In BOS cases, we distinguished between inflammatory and fibrotic OB lesions. In RAS, it was difficult to differentiate between fibrotic OB lesions and fibrotic airways due to RAS itself; we therefore choose to use only the term fibrotic airway in RAS.

5.2.3 Statistical analysis

Because the number of ROIs per type of ROI and per clinical phenotype was relatively low, a descriptive approach was used to compare ROI types.

5.3 Results

5.3.1 Cellularity across main ROI types

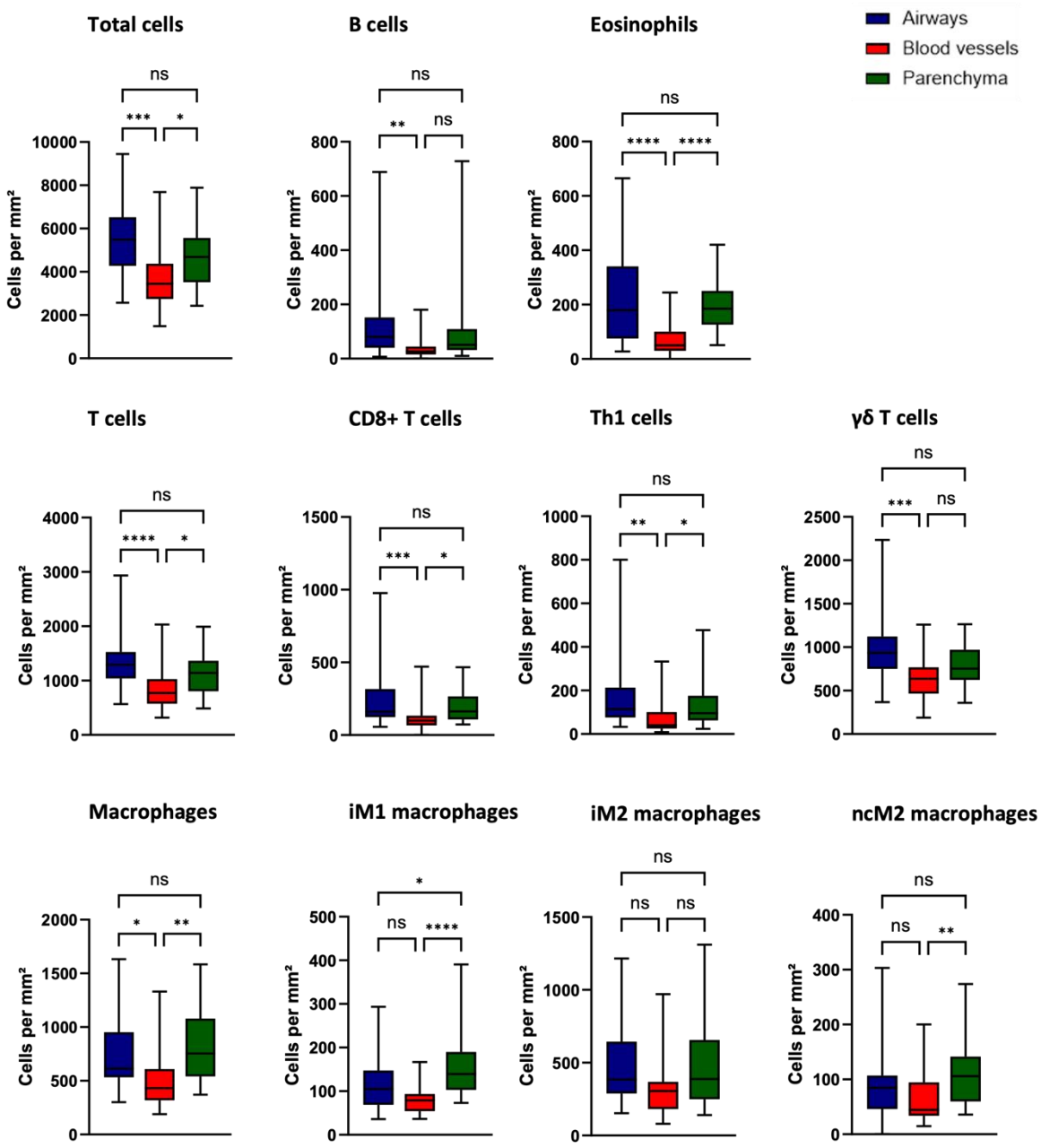


Figure 5.1. Cellularity across main ROI types
Graphs showing median cell counts per mm² tissue for total cell counts and key immune cells across the main types of ROIs, airways, blood vessels and lung parenchyma.
Kruskal-Wallis with Dunn's multiple comparison test. *: < 0.05, **: < 0.01, ***: < 0.001, ****: < 0.0001.
There was a significant difference for intermediate M2 macrophages (Kruskal-Wallis, p=0.0454), although comparison of the different subgroups using Dunn's multiple comparison test no longer reached significance between the subgroups.
i: intermediate, nc: non-classical, ROI: region of interest.

Total cellularity as well as cellularity of key immune cells across the main ROI types, namely airways, blood vessels and lung parenchyma, are displayed in Figure 5.1. Total cell counts were highest in the airways, followed by the lung parenchyma, and both were significantly higher compared with blood vessels. The same was true for T cells, macrophages, eosinophils, and the T-cell subsets CD8+ T cells and Th1 cells. B cells were only significantly higher in the airways compared with blood vessels, which was also the case for γδ T cells. The highest numbers of intermediate M1 macrophages and non-classical M2 macrophages were found in the lung parenchyma, being significantly higher compared with blood vessels and the former also compared with airways.

Comparison of these main ROI types across different clinical phenotypes is difficult as it is confounded by the subsets of ROIs included. For instance, airways in non-CLAD patients consisted only of normal, non-proliferative airways, whereas in CLAD patients, non-proliferative airways, inflammatory OB lesions and fibrotic OB lesions/fibrotic airways were included. Comparison between these subsets of ROIs will be explored in the next section.
With respect to total cellularity in these clinical phenotypes, the highest number of cells was observed in the airways in BOS patients, although only significantly higher compared with non-CLAD patients and not compared with RAS. Regarding blood vessels, we noticed an increasing trend from non-CLAD to BOS to RAS, although without significant differences. A similar trend was observed with regard to the lung parenchyma, with the highest cellularity in RAS patients, although again not significantly different. (Figure 5.2)

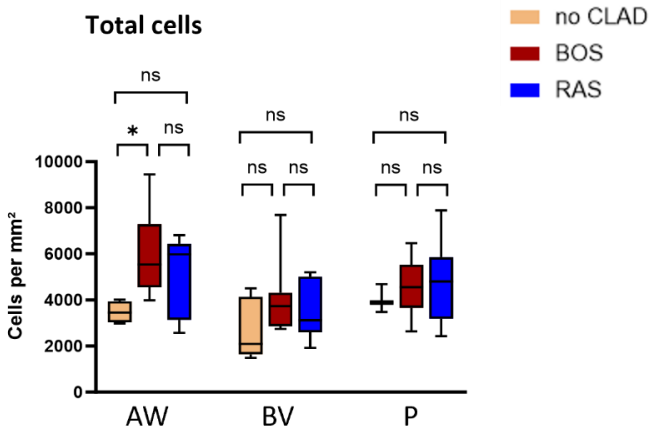


Figure 5.2. Total cellularity of main ROI types across clinical phenotypes
Graphs showing total median cell counts per mm² tissue for airways, blood vessels and lung parenchyma across clinical phenotypes.
Kruskal-Wallis with Dunn's multiple comparison test. *: < 0.05.
AW: airways, BOS: bronchiolitis obliterans syndrome, BV: blood vessels, CLAD: chronic lung allograft dysfunction, P: parenchyma, RAS: restrictive allograft syndrome, ROI: region of interest.

5.3.2 Temporal evolution of fibrosis in CLAD

We studied adapted Tier 1 clusters (using EMT1 and EnMT instead of total epithelial and endothelial cells) across different ROI types to assess the temporal evolution of parenchymal and airway fibrosis. (Figure 5.3-5.5 and Supplementary Table F1.2, Appendix F, p. 293)

Comparing relatively preserved alveoli, less and more fibrotic parenchymal areas, cellularity increased with severity of fibrosis and this was mainly accounted for by an increase in T and B cells, macrophages and EMT. Differences between clinical phenotypes were not very pronounced in preserved alveoli. However, in more fibrotic areas, there were more cells, including B cells and macrophages, in RAS than in BOS, while eosinophils were more abundant in BOS. (Figure 5.3)

With regard to airways, the highest cellularity was observed in inflammatory OB lesions, which contained the most B cells. Contrarily, macrophages in particular were more abundant in fibrotic OB lesions/fibrotic airways, while T cells, eosinophils and EMT were increased in both inflammatory OB lesions and fibrotic OB lesions/fibrotic airways. Cellularity was higher in non-proliferative small airways in BOS than in RAS, corresponding to an increase in γδ T cells and EMT. However, findings were quite similar when comparing inflammatory OB lesions between BOS and RAS, except for a higher number of B cells in RAS. On the other hand, in fibrotic OB lesions/fibrotic airways, macrophages, eosinophils and T cells were higher in RAS. (Figure 5.4)

Interestingly, fibrotic remodelling of both parenchyma and airways was associated with an increase in γδ T cells in BOS, but Th1 cells and intermediate M2 macrophages in RAS. (Figure 5.6 and 5.7) A summary of findings is displayed in Table 5.1.

Relatively preserved alveoli → fibrotic parenchyma		**Non-proliferative small airways → fibrotic airways**	
↑ cellularity ↑ T cells: CD8+ T cells, Th1 cells, γδ T cells ↑ B cells: plasma cells ↑ macrophages: intermediate M2 macrophages ↑ EMT		↑ cellularity ↑ T cells: CD8+ T cells, γδ T cells ↑ macrophages: intermediate M2 macrophages ↑ EMT	
BOS	**RAS**	**BOS**	**RAS**
↑ CD8+ T cells ↑ γδ T cells ↑ eosinophils ↑ EMT	↑ CD8+ T cells ↑ Th1 cells ↑ B cells: plasma cells ↑ intermediate M2 macrophages ↑ EMT	↑ CD8+ T cells ↑ γδ T cells ↑ B cells: plasma cells ↑ non-classical M2 macrophages ↑ EMT	↑ CD8+ T cells ↑ Th1 cells ↑ intermediate M2 macrophages ↑ eosinophils ↑ EMT

Table 5.1. Summary of findings
Summary of main findings of Tier 1 and Tier 2 analyses across clinical phenotypes and areas of fibrotic remodelling.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, RAS: restrictive allograft syndrome.

Lastly, we also looked at the blood vessels and noticed more cells overall in CLAD than in non-CLAD samples. There was a greater number of especially eosinophils but also intermediate M2 macrophages in RAS and γδ T cells in BOS. B cells, γδ T cells and Th1 cells were increased in blood vessels next to fibrotic airways. (Figure 5.5 and Figure 5.8)

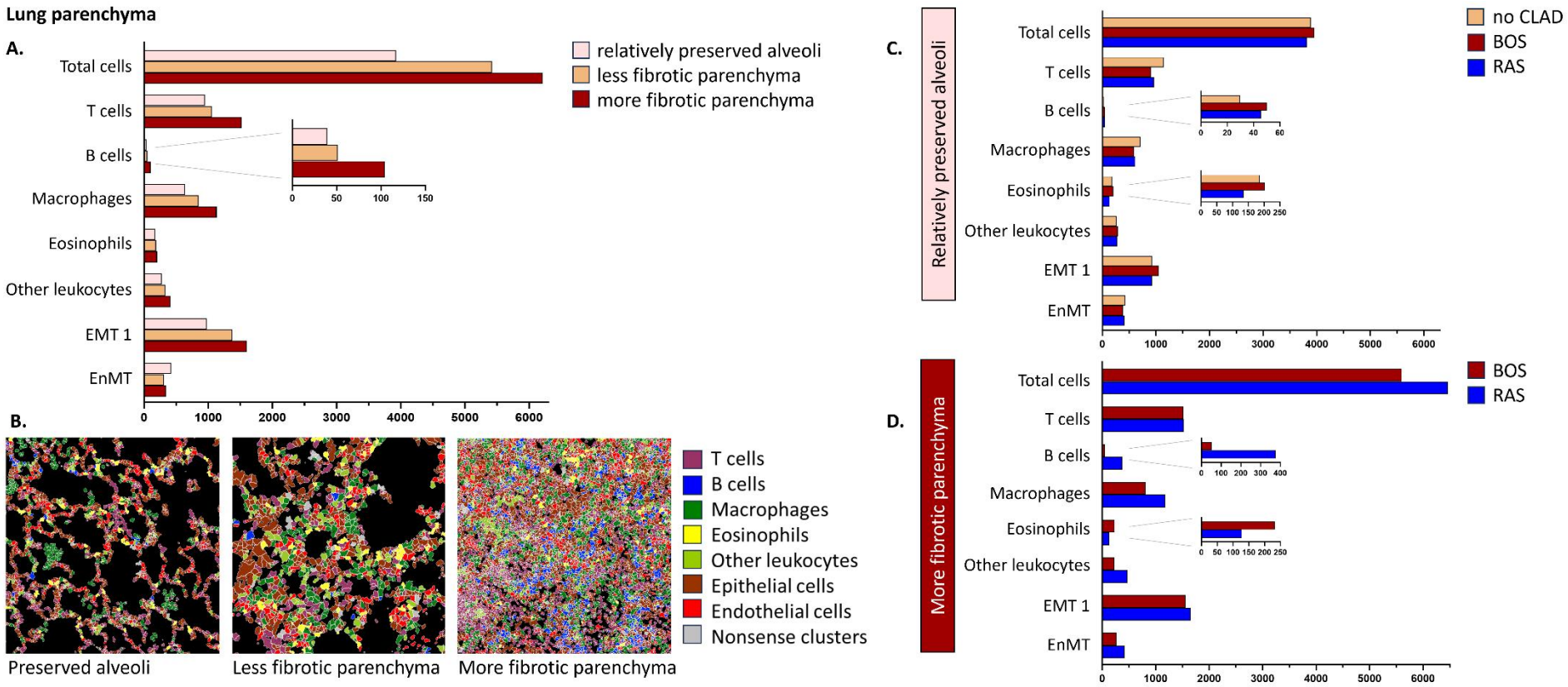


Figure 5.3. Adapted Tier 1 clusters across different ROI types: lung parenchyma

Graphs showing median cell counts per mm² tissue for adapted Tier 1 clusters (using EMT and EnMT instead of total epithelial and endothelial cells). **A.** Across different types of ROIs, relatively preserved alveoli, less fibrotic and more fibrotic parenchyma. **B.** Representative examples of these ROI types. Sizes of ROIs in the images are 1000x1000 µm, 794x758 µm and 1000x1000 µm (left to right). All ROI images are from RAS patients. **C.** Differences in adapted Tier 1 clusters between clinical phenotypes (no CLAD, BOS and RAS) in relatively preserved alveoli. **D.** Differences in adapted Tier 1 clusters between clinical phenotypes (BOS and RAS) in more fibrotic parenchyma.

BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, RAS: restrictive allograft syndrome, ROI: region of interest.

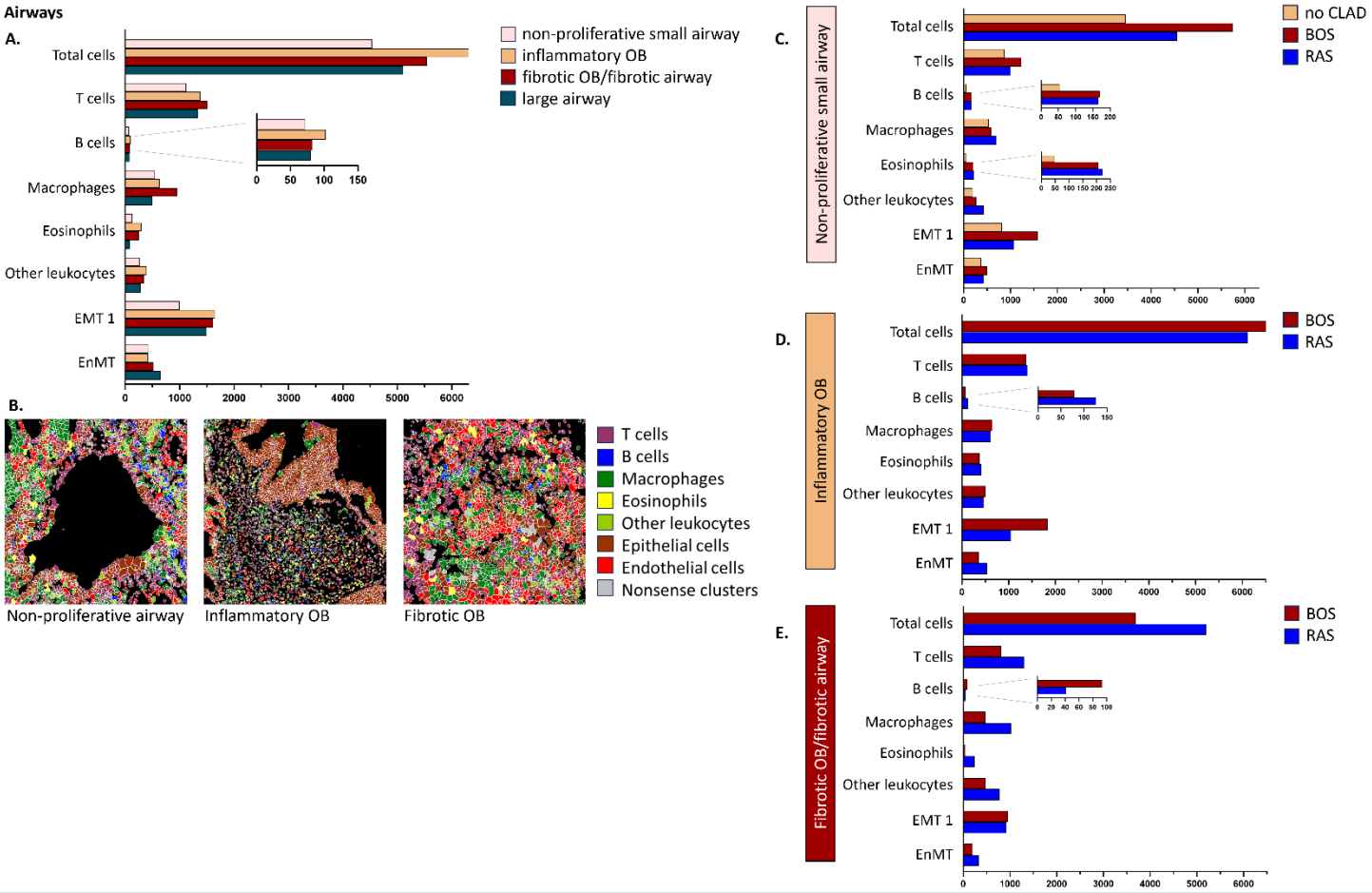


Figure 5.4. Adapted Tier 1 clusters across different ROI types: airways

Graphs showing median cell counts per mm² tissue for adapted Tier 1 clusters (using EMT and EnMT instead of total epithelial and endothelial cells). **A.** Across different types of ROIs, non-proliferative small airways, inflammatory OB, fibrotic OB/fibrotic airways, and large airways. **B.** Representative examples of these ROI types. Sizes of ROIs in the images are 663x604 µm, 1000x1000 µm and 750x680 µm (left to right). All ROI images are from BOS patients. **C-E.** Differences in adapted Tier 1 clusters between clinical phenotypes (no CLAD, BOS and/or RAS) in **C.** non-proliferative small airways, **D.** inflammatory OB, **E.** fibrotic OB/fibrotic airways. Unfortunately, no inflammatory OB lesions were identified in the patients without CLAD.

BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, OB: obliterative bronchiolitis RAS: restrictive allograft syndrome, ROI: region of interest.

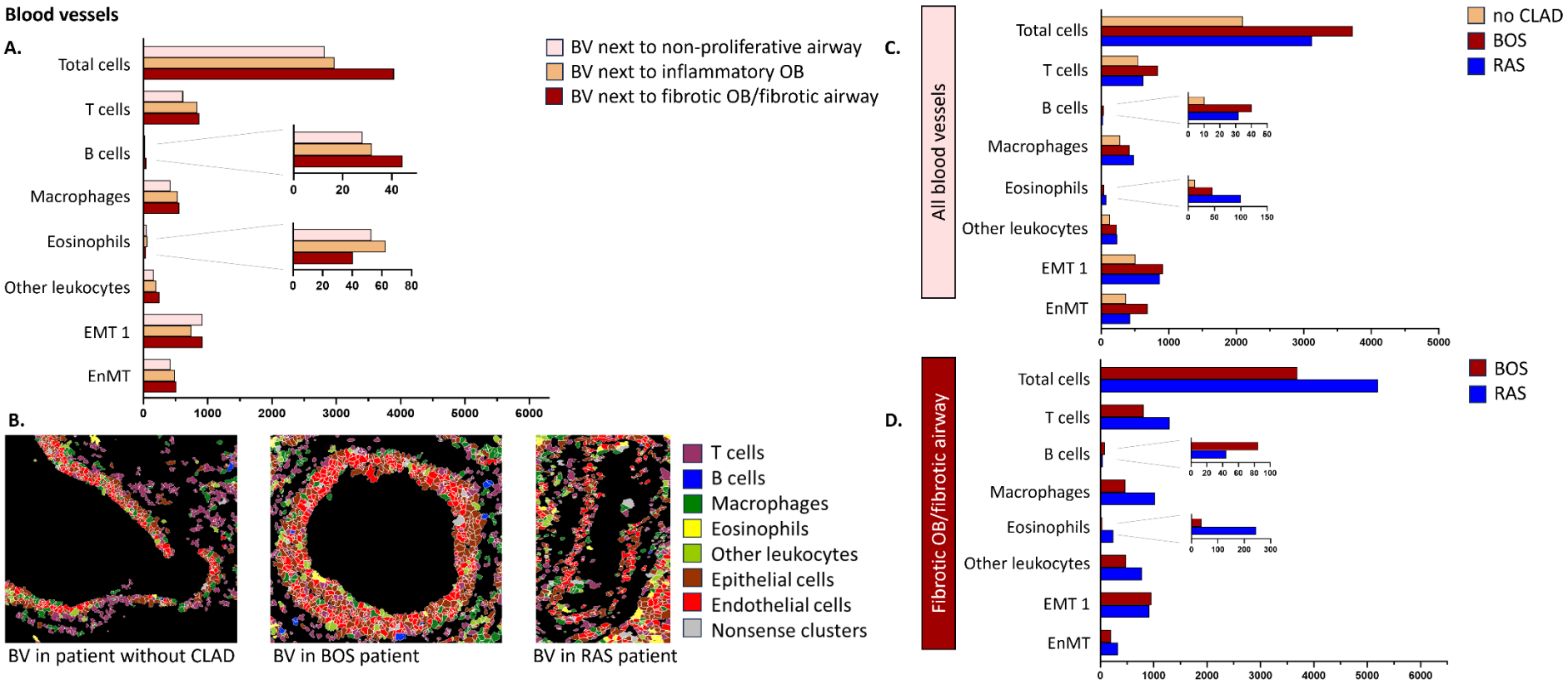


Figure 5.5. Adapted Tier 1 clusters across different ROI types: blood vessels

Graphs showing median cell counts per mm² tissue for adapted Tier 1 clusters (using EMT and EnMT instead of total epithelial and endothelial cells). **A.** Across different types of ROIs, blood vessels next to non-proliferative airways, inflammatory OB and fibrotic OB/fibrotic airways. **B.** Example of blood vessel in patient without CLAD, a BOS patient and a RAS patient. Sizes of ROIs in the images are 1000x1000 µm, 786x656 µm and 366x844 µm (left to right). **C.** Differences in adapted Tier 1 clusters between clinical phenotypes (no CLAD, BOS and RAS) across all blood vessels. **D.** Differences in adapted Tier 1 clusters between clinical phenotypes (BOS and RAS) in blood vessels next to fibrotic OB/fibrotic airways.

BOS: bronchiolitis obliterans syndrome, BV: blood vessels, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

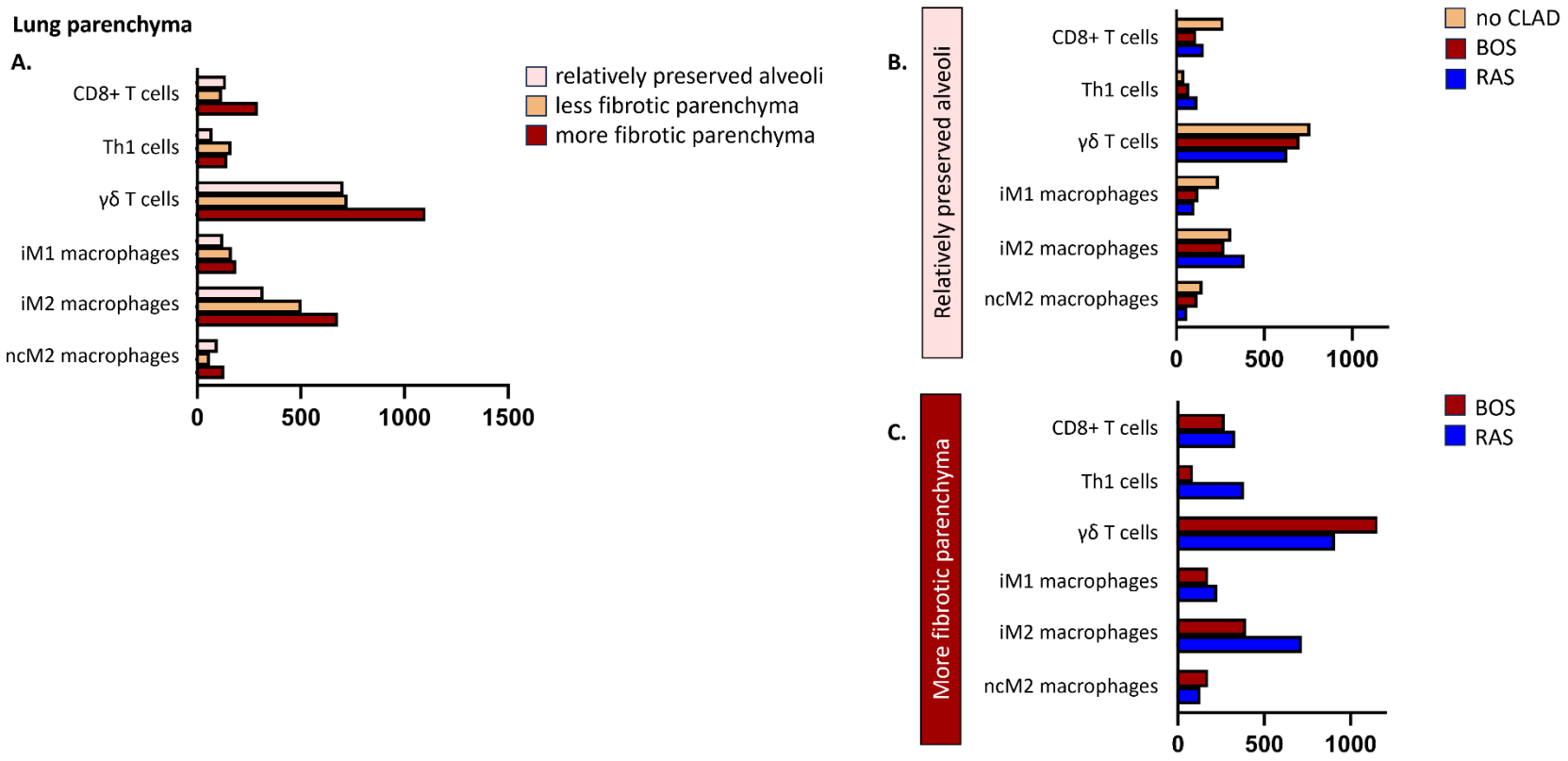


Figure 5.6. Adapted Tier 2 clusters across different ROI types: lung parenchyma
Graphs showing median cell counts per mm² tissue for adapted Tier 2 clusters (subsets of T cells and macrophages). **A.** Across different types of ROIs, relatively preserved alveoli, less and more fibrotic parenchyma. **B.** Differences in adapted Tier 2 clusters between clinical phenotypes (no CLAD, BOS and RAS) in relatively preserved alveoli. **C.** Differences in adapted Tier 2 clusters between clinical phenotypes (BOS and RAS) in more fibrotic parenchyma.
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, i: intermediate, nc: non-classical, RAS: restrictive allograft syndrome, ROI: region of interest.

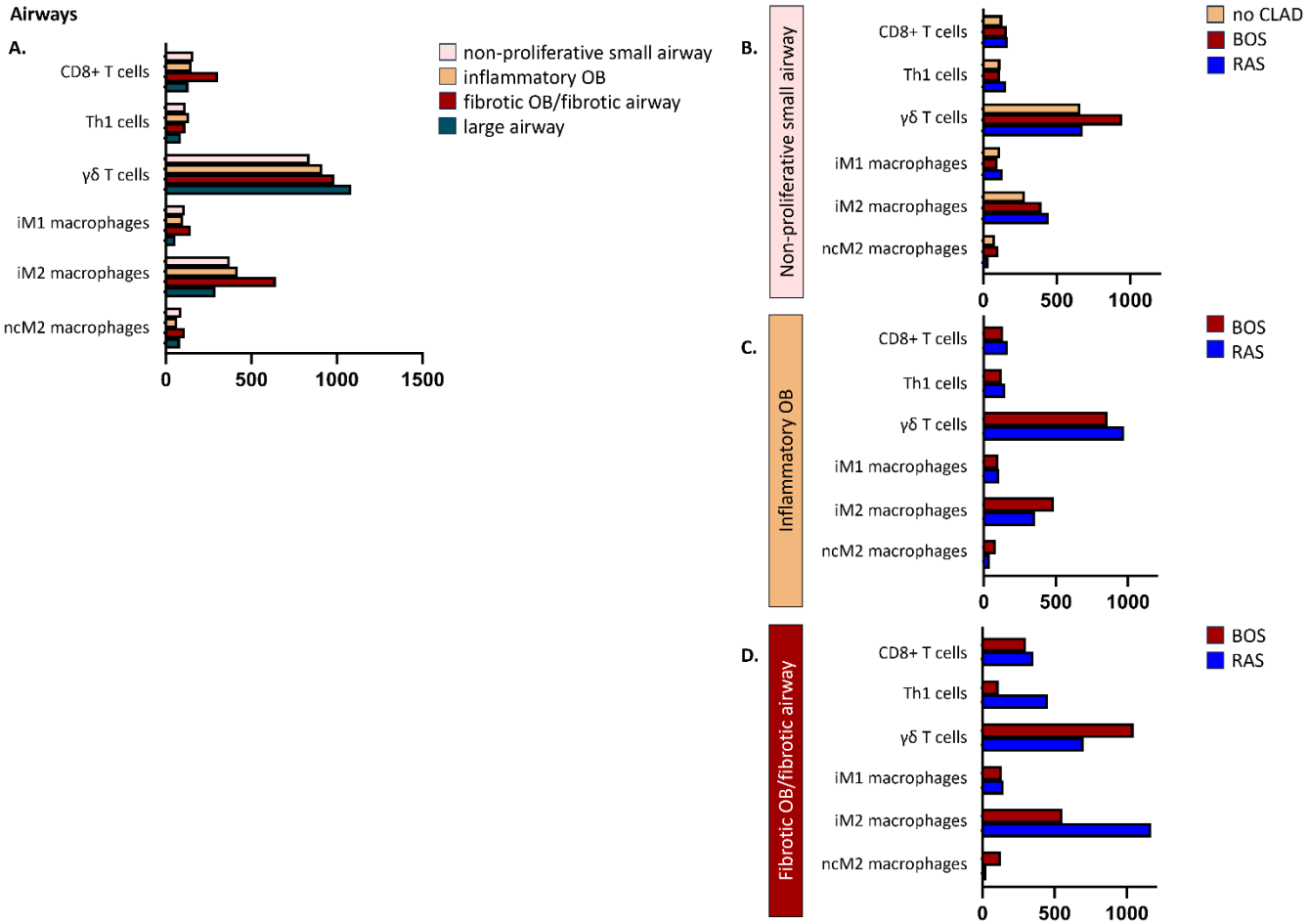


Figure 5.7. Adapted Tier 2 clusters across different ROI types: airways

Graphs showing median cell counts per mm² tissue for adapted Tier 2 clusters (subsets of T cells and macrophages). **A.** Across different types of ROIs, non-proliferative small airways, inflammatory OB, fibrotic OB/fibrotic airways, and large airways. **B-D.** Differences in adapted Tier 2 clusters between clinical phenotypes (no CLAD, BOS and/or RAS) in **B.** non-proliferative small airways, **C.** inflammatory OB, **D.** fibrotic OB/fibrotic airways. Unfortunately, no inflammatory OB lesions were identified in the patients without CLAD.

BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, i: intermediate, nc: non-classical, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

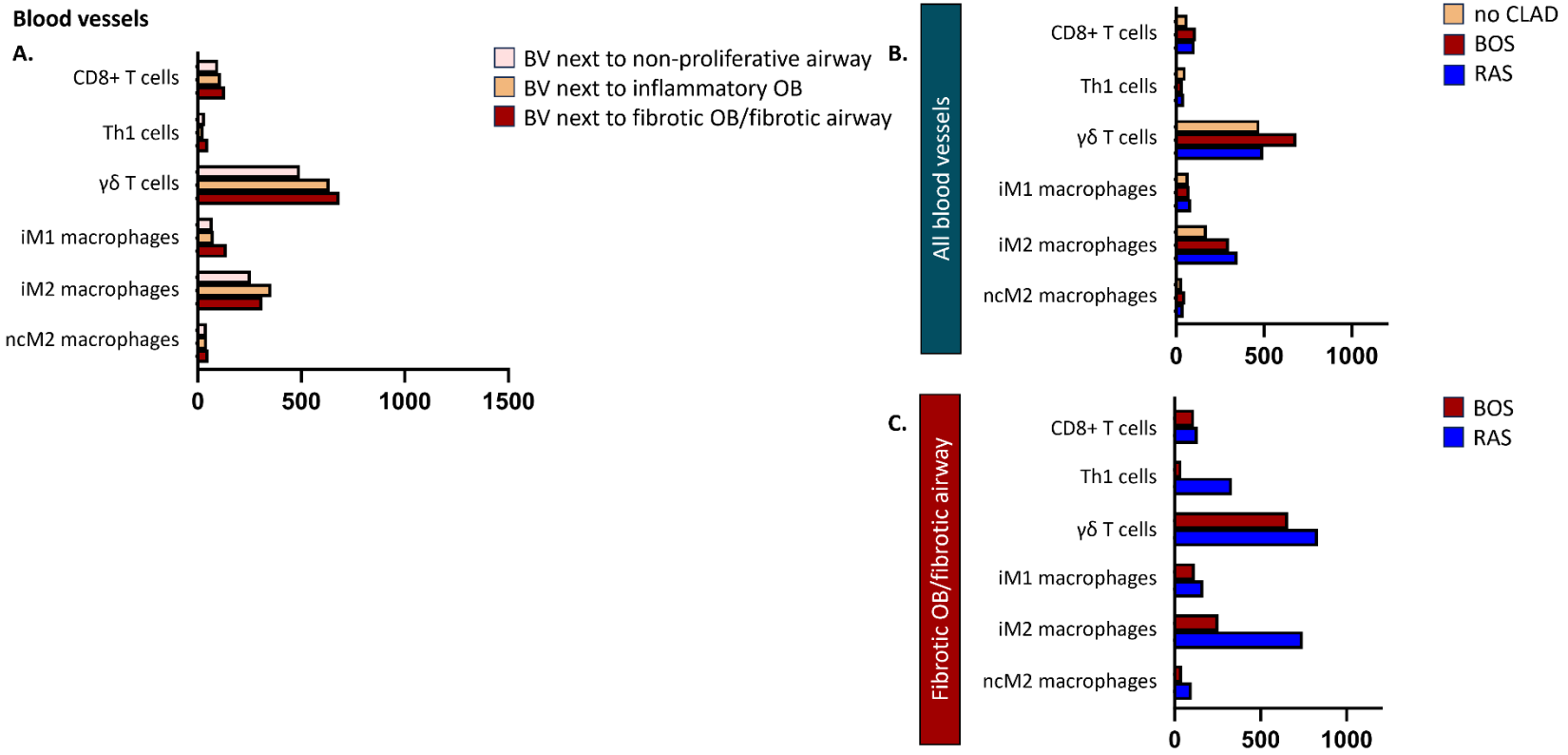


Figure 5.8. Adapted Tier 2 clusters across different ROI types: blood vessels
Graphs showing median cell counts per mm² tissue for adapted Tier 2 clusters (subsets of T cells and macrophages). **A.** Across different types of ROIs, blood vessels next to non-proliferative airways, inflammatory OB and fibrotic OB/fibrotic airways. **B.** Differences in adapted Tier 2 clusters between clinical phenotypes (no CLAD, BOS and RAS) across all blood vessels. **C.** Differences in adapted Tier 2 clusters between clinical phenotypes (BOS and RAS) in blood vessels next to fibrotic OB/fibrotic airways.
BOS: bronchiolitis obliterans syndrome, BV: blood vessels, CLAD: chronic lung allograft dysfunction, i: intermediate, nc: non-classical, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

5.4 Discussion

Fibrotic remodelling is an important feature of many chronic diseases and one of the key features of CLAD. With this study, we identified potential novel immunological insights into the pathogenesis and fibrotic progression of CLAD. Progression from preserved lung architecture to advanced fibrosis with complete destruction of the lung architecture was associated with cellular (cytotoxic T cells, Th1 cells) and humoral (B cells, especially plasma cells) immune responses. In addition, several innate immune cell types increased, with infiltration of γδ T cells and intermediate M2 macrophages. But most importantly, fibrosis was partially associated with different factors in RAS (Th1 cells, intermediate M2 macrophages) and in BOS (γδ T cells).

5.4.1 M2 macrophages in fibrotic remodelling in CLAD

Interestingly, although perhaps not very surprisingly, the same immune cell types that were found in higher numbers in CLAD versus non-CLAD were also found to be key factors in fibrotic remodelling. Intermediate M2 macrophages in particular seemed to play a role in RAS.

Macrophages are among the most abundant non-structural cells in the respiratory tract and are important for lung homeostasis. Although the traditional function of macrophages is a first-line defence against pathogens as part of the innate immune system, several studies have also documented a role in fibrogenesis. (Boorsma et al., 2014) M2 macrophages in particular may have a direct fibrotic effect because they aid in wound healing, post-inflammatory tissue repair and remodelling via tissue regeneration and cell proliferation. (Alexander et al., 2019, Italiani and Boraschi, 2014) After all, tissue repair is an essential homeostatic mechanism after (lung) injury and macrophages are also able to degrade extracellular matrix proteins again and thereby promote resolution of fibrosis after completion of the initial repair response. As such, the development of fibrosis has been associated with a dysregulated or excessive wound healing response that results in excessive accumulation of extracellular matrix components, such as collagen and fibronectin. (Boorsma et al., 2014) Failure to control macrophage plasticity could thus lead to such abnormal repair responses and the development of pathological fibrosis. (Cheng et al., 2021) Evidence from studies in idiopathic pulmonary fibrosis shows that a predominant M2 profile is detrimental to pulmonary fibrosis. (Desai et al., 2018, Lis-López et al., 2021) We now also demonstrate the importance of M2 macrophages in CLAD pathogenesis and fibrotic remodelling.

The repair response induced by M2 macrophages is mediated by production of growth factors (e.g., platelet-derived growth factor, vascular endothelial growth factor, connective tissue growth factor), arginase 1, matrix metalloproteinase 13, and polyamine and collagen synthesis. Secretion of profibrotic cytokines (e.g., IL-4, IL-13) can further contribute to fibrosis. Additionally, macrophages express profibrotic chemokines, including CCL2 and CCL24, which recruit fibrocytes and M2 macrophages also produce TGF-β1, which induces differentiation of fibroblasts into myofibroblasts and extracellular matrix deposition. (Cheng et al., 2021)
TGF-β1 was one of the markers used to identify EMT, but the staining was otherwise unfortunately too diffuse for accurate assessment and direct comparison between clinical phenotypes and ROI types. Given that increased levels of TGF-β1 have been described particularly in RAS patients, this would have been interesting to assess. (Sacreas et al., 2019)

In RAS, many cells interacted with intermediate M2 macrophages and could therefore have played a role in the upregulation of M2 macrophages, including cytotoxic T cells, Th1 cells and macrophage subsets. In turn, spatial analysis confirmed direct interaction between intermediate M2 macrophages and epithelial cells with markers of EMT. (Figure 4.12)

Although several studies have linked M2 polarisation of macrophages to lung repair and fibrosis, the role of M2 macrophage subtypes remains insufficiently understood. M2 macrophages consist of four subtypes, M2a, M2b, M2c, and M2d, which are activated by different cytokines. (Cheng et al., 2021) We can currently only hypothesise which main subset (or subsets) is involved in CLAD fibrosis.
In general, M2a macrophages are mainly induced by IL-4 and IL-13, leading to upregulated expression of arginase 1, mannose receptor MRc1 (CD206), CCL17, CCL18, CCL22, antigen presentation by MHC II, and production of IL-13 but also IL-10 and TGF-β. The M2b phenotype is usually stimulated by IL-1 receptor ligands, produces IL-1β, IL-6 and TNF-α and can activate Tregs through IL-10. IL-10, TGF-β and glucocorticoids promote the synthesis of M2c macrophages, which in turn secrete IL-10 and TGF-β. Lastly, M2d macrophages are mainly induced by adenosine A2A receptor agonists and IL-6, and express high levels of IL-10, TGF-β and vascular endothelial growth factors, promote angiogenesis and tumour progression and are often referred to as tumour-associated macrophages. (Cheng et al., 2021, Italiani and Boraschi, 2014, Yao et al., 2019)

So it is important to realise that macrophages can adopt a variety of functional phenotypes with specific biological characteristics and functions depending on subtle and continuous changes in the tissue microenvironment. (Italiani and Boraschi, 2014) It is observed that macrophage states change over the time course of inflammation and disease, and macrophages can depolarise to M0 macrophages or repolarise to the opposite phenotype, depending on the types of cytokines present in the specific microenvironment. The same counts for M2 macrophage subsets. (Cheng et al., 2021) Polarisation and phenotype switching are associated with global changes in cell transcriptome and proteome that are strictly regulated by exogenous and endogenous stimuli. (Orekhov et al., 2019, Li et al., 2022)

Thus, polarised macrophages exhibit enormous plasticity and dysregulation of the balance of M1/M2 macrophages most likely plays a critical role in uncontrolled inflammation and repair. However, M1 and M2 activation states most likely represent extremes with a continuum of diverse functional states in M1 - M2 polarisation of macrophage functions. Although M1/M2 polarisation might thus be viewed as a simplified dichotomous stratification describing a continuum of diverse functional states, the M1/M2 distinction is still useful in describing functional features in broad terms. (Italiani and Boraschi, 2014)

5.4.2 γδ T cells in fibrotic remodelling in CLAD

γδ T cells appeared to be involved in fibrotic remodelling of airways but also lung parenchyma in BOS. Not surprisingly, γδ T cells were found in the highest concentrations in the airways, as they play an important role in patrolling, followed by the lung parenchyma. (Sullivan et al., 2019) (Figure 5.1) When we looked at the blood vessels, we also noticed a higher number of γδ T cells in BOS than in RAS, and an increase in γδ T cells in blood vessels next to fibrotic airways.

Limited data are available on the possible mechanisms of fibrosis via γδ T cells. Chen et al. showed that explanted lungs from a patient with COVID-19-induced pulmonary fibrosis contained abundant γδ T cells. (Chen et al., 2021) As in the liver, the functional effect of γδ T cells on lung disease progression likely depends on the subsets involved in a cytokine-specific manner. (Hammerich and Tacke, 2014) It was found that IFN-γ-producing γδ T cells had a protective effect, while IL-17-secreting γδ T cells by contrast promoted fibrosis via extracellular matrix production, collagen deposition and TGF-β signalling. (Bank, 2016) Similarly, in a bleomycin-induced pulmonary fibrosis mouse model, fibrosis was attenuated by IFN-γ-producing γδ T cells through suppression of IL-17. (Segawa et al., 2016) γδ T cells and IL-

17 have also been linked to airway inflammation and remodelling in asthma. (Yao et al., 2022) It is also possible that γδ T cells induce fibrosis via different mechanisms than cytokine production. In systemic sclerosis, the prototypical human fibrotic disease, γδ T cells promoted fibrosis via upregulation of pro-alpha2(I) collagen (COL1A2) expression in fibroblasts. (Ueda-Hayakawa et al., 2013)

Unfortunately, we were unable to identify Th17 cells nor could we quantify whether the γδ T cells we detected secreted IL-17 or not. As such, it is unclear whether the presumed IL-17-mediated effects were exerted directly by γδ T cells or also via stimulation of IL-17 production by Th17 cells.

5.4.3 Th1 cells in fibrotic remodelling in CLAD

In contrast, Th1 cells seemed to be mainly involved in fibrotic remodelling in RAS. This is intriguing because Th1 cells are generally considered anti-fibrotic. Similar to the protective effect of IFN-γ-producing γδ T cells, Th1 cells are generally thought to attenuate fibrosis via their IFN-γ production, which suppresses fibroblast-induced collagen synthesis. (Zhang and Zhang, 2020) However, although not much evidence is available, it is possible that Th1 cells mediate fibrosis through different mechanisms. Indeed, in cardiac fibrosis, it has been described that Th1 cells were able to induce TGF-β expression in myofibroblasts. (Nevers et al., 2017) Further research on the possible role and mechanisms of Th1 cells in fibrogenesis is needed.

Figure 5.9 illustrates the presence of key immune cells in BOS and RAS (Th1 cells, γδ T cells, plasma cells, intermediate M2 macrophages) as well as EMT and EnMT.

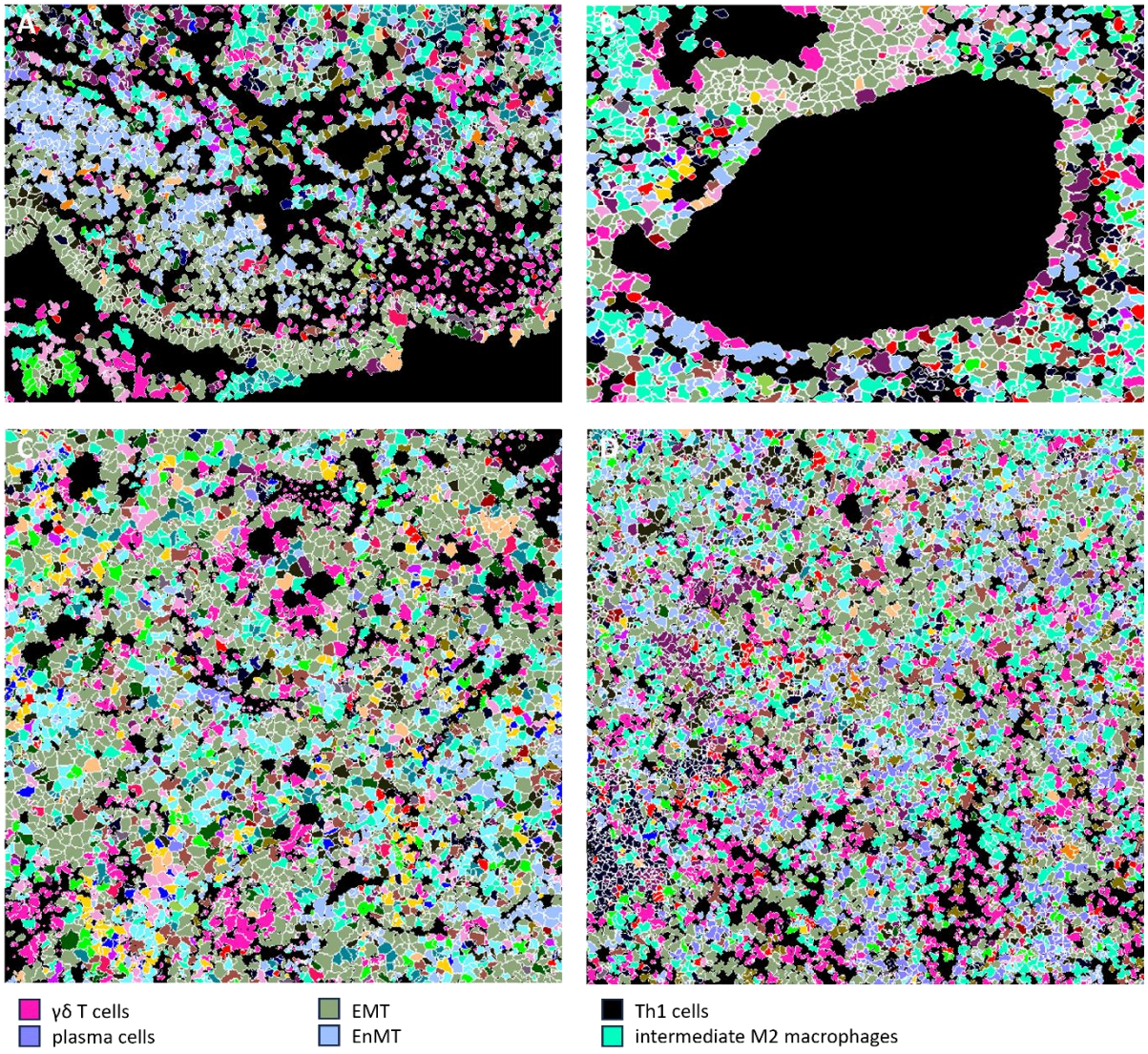


Figure 5.9. Tier 2 cluster maps

Images of fibrotic airways and parenchyma in BOS and RAS patients showing the distribution of Tier 2 clusters. **A.** Fibrotic OB lesion in BOS patient showing fibrotic remodelling of the respiratory epithelium (EMT), the presence of a high number of γδ T cells, as well as some plasma cells, and fibrotic remodelling of the adjacent capillaries (EnMT). Image size 1000x636 µm. **B.** Fibrotic airway in RAS patient showing EMT of the respiratory epithelium, alongside a high number of surrounding intermediate M2 macrophages and some Th1 cells. Image size 598x500 µm. **C.** More fibrotic parenchymal area in BOS patient showing fibrotic remodelling of the parenchyma (EMT) and the presence of many γδ T cells. Image size 1000x1000 µm. **D.** More fibrotic parenchymal area in RAS patient showing fibrotic remodelling of the parenchyma (EMT), numerous intermediate M2 macrophages, as well as many Th1 cells and plasma cells. Image size 1000x1000 µm.

BOS: bronchiolitis obliterans syndrome, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome.

5.4.4 B cells and fibrotic remodelling in CLAD

Only a limited number of studies have analysed B cells in lung tissue in CLAD patients. One study reported more B cells in areas of lymphocytic bronchiolitis and active OB than in areas of inactive OB or healthy tissue. (Sato et al., 2009) A recent study using explanted BOS and RAS lungs found more B cells in both phenotypes in comparison with non-transplant controls. (Vandermeulen et al., 2017)

Similarly, we found an increase in B cells in both CLAD phenotypes, albeit with small differences across the different types of ROIs. More specifically, fibrotic parenchymal remodelling was associated with an increase in plasma cells, which were more abundant in RAS. On the other hand, there were more B cells in fibrotic OB lesions in BOS than in fibrotic airways in RAS, with also more B cells in adjacent blood vessels. Regardless, the number of B cells in non-proliferative airways was very similar in BOS and RAS.

In addition to their antibody production capacity, more and more evidence has accrued suggesting that plasma cells can directly contribute to fibrosis through promotion of recruitment, proliferation and differentiation of fibroblasts and myofibroblasts. (Goodwin et al., 2022, Della-Torre et al., 2020) Administration of bortezomib, a proteasome inhibitor that induces plasma cell depletion, has been shown to reduce pulmonary fibrosis in mouse models. (Prêle et al., 2022, Penke et al., 2022)

5.4.5 Eosinophils and fibrotic remodelling in CLAD

Lastly, the role of eosinophils in fibrotic remodelling in CLAD remains uncertain. Differences regarding eosinophils were less consistent and pronounced, with only a slight increase in more fibrotic parenchymal areas compared with less fibrotic areas and relatively preserved alveoli. Contrary to expectations, their number was higher in BOS than in RAS in more fibrotic areas. On the other hand, higher numbers of eosinophils were found in blood vessels and fibrotic airways in RAS patients compared with BOS.

Eosinophils normally migrate quickly from the bloodstream to the tissue and have a limited half-life of approximately 18 hours. During their transit from the bloodstream to the tissue, eosinophils interact with endothelial cells using selectins and integrins. M2 macrophages have a pivotal role in recruiting eosinophils to tissues, but similarly, eosinophils are also capable of recruiting M2 macrophages. (Rosenberg et al., 2013) In all clinical phenotypes, we noted interaction of eosinophils with M2 macrophages and endothelial and epithelial cells. Furthermore, in CLAD there was a broader range of interactions of eosinophils with other

immune cells, including T and B lymphocytes. Eosinophils can indeed directly interact with and stimulate T cells in an antigen-specific manner, via upregulation of MHC II molecules as well as T-cell costimulatory CD80 and CD86 molecules, resulting in T-cell proliferation and cytokine release. (Puga et al., 2013) Furthermore, they can also regulate the production of Th2 chemoattractants (including CCL17 and CCL22) and thereby promote recruitment of Th2 cells. Additionally, they can indirectly stimulate T cells via the release of preformed cytokines (e.g., IL-4, IL-13 and IFN-γ) that promote either Th2 or Th1 responses. (Rosenberg et al., 2013) The exact contribution of eosinophils in CLAD and fibrotic remodelling in CLAD remains currently unclear. However, we noted that eosinophils interacted directly with cytotoxic T cells, Th1 cells and γδ T cells to a greater extent in CLAD relative to non-CLAD according to our cellular neighbourhood analyses. (Figure 4.12)

5.4.6 BOS and RAS: common pathway with a dissimilar endpoint?

An important finding of this study is that both parenchymal fibrosis and airway fibrosis appear to be mediated by the same mechanisms, confirming our hypothesis that the general mechanisms regulating the fibrogenic response may be broadly similar. Indeed, there seems to be a common pathway of fibrotic remodelling independent of location. This is partly associated with classical cellular and humoral immune responses. However, this is partly also differentially mediated across clinical phenotypes. More specifically, fibrotic remodelling was characterised by increased Th1 cells and intermediate M2 macrophages in RAS, while by γδ T cells in BOS. We can only hypothesise what causes the partial difference in immune cells involved in fibrotic remodelling in BOS and RAS. One likely cause is the difference in lung compartments involved. It has been postulated that the predominant anatomical location of the injury (bronchiolar, alveolar and/or vascular compartment) determines the dominant clinical phenotype. (Beeckmans et al., 2023) (See also Figure 1.7 p. 15)

In our study, we noted that more cells were present in non-proliferative airways in BOS than in RAS, reaffirming that BOS is – at least partly – an airway-centred disease with impact of external stimuli (e.g., respiratory infection, gastro-oesophageal reflux, inhaled toxins). We could hypothesise that airway injury induces upregulation of γδ T cells in BOS, which may induce airway remodelling, but could afterwards also contribute to parenchymal remodelling. In RAS, endogenous stimuli are thought to play a greater role and may be responsible for the increase in M2 macrophages and Th1 cells, and may contribute to fibrosis especially via the

former. Hence, it is possible that the primary site of injury determines the difference in immune cells involved. This is something that should be further explored in future studies, as well as the correlation between our observations and other fibrotic diseases, such as parenchymal fibrosis in idiopathic pulmonary fibrosis and other fibrosing ILDs and airway remodelling in chronic obstructive pulmonary disease and asthma.

5.4.7 Strengths and limitations

The strengths and limitations of this study are similar to those described in Chapter 4. With regard to the second aim of this study, special attention should be paid to the added value provided by the different lung compartments included, the ability to evaluate the temporal evolution of fibrosis and the spatial information. On the other hand, the fact that not all identified ROIs could be analysed is an important limitation that contributed to a more limited number of ROIs for some clinical phenotypes and ROI types. For example, we could not evaluate fibrotic remodelling of the pleura and septa, and the number of fibrotic airways in RAS was unfortunately low.

5.5 Conclusions

IMC is a powerful tool that enables highly multiplexed imaging of lung tissue at single-cell resolution, hereby providing new insights into the temporal evolution of fibrosis in CLAD. Despite several commonalities, fibrotic remodelling appeared to be differentially mediated in BOS and RAS, with parenchymal and airway fibrosis associated with γδ T cells in BOS, whereas with intermediate M2 macrophages and Th1 cells in RAS. Importantly, as hypothesised, the mechanisms of fibrosis appeared to be independent of location (parenchyma versus airway). These findings need to be confirmed in a larger cohort and future research should focus on subsets of these immune cells and their activation states to assess the potential for targeted treatment and diagnostic tools.

Key points

- Total cellularity as well as cellularity of several immune cells (e.g., T cells, macrophages, eosinophils) was highest in the airways.
- Fibrotic remodelling of the parenchyma and airways in CLAD occurred in a similar manner.
- Fibrotic remodelling in CLAD was characterised by cellular (cytotoxic T cells, Th1 cells) and humoral (B cells, especially plasma cells) immune responses.
- In addition to adaptive immune responses, innate immune cells appeared to be involved in the onset and progression of fibrosis in CLAD, especially γδ T cells and intermediate M2 macrophages.
- Although fibrosis occurred similarly at different sites (parenchyma and airways), it was differentially mediated in BOS (γδ T cells) and RAS (intermediate M2 macrophages, Th1 cells).
- We hypothesise that the predominant anatomical location of the injury (bronchiolar, alveolar and/or vascular compartment) might be responsible for the immunological and clinical differences between BOS and RAS.

Chapter 6 Discussion

6.1 Summary of key findings

CLAD, with its main phenotypes BOS and RAS, is the major hurdle to long-term survival after lung transplantation. (Chambers et al., 2019) The pathophysiology of CLAD is multifactorial and many risk factors for CLAD have been recognised. These risk factors can be broadly divided into alloimmune and non-alloimmune risk factors, including acute cellular rejection and antibody-mediated rejection and ischaemia-reperfusion injury, respiratory infections and gastro-oesophageal reflux, respectively. (Figure 6.1) As illustrated in our literature review, many immune cells play a role in the onset and progression of CLAD, in addition to various cytokines, chemokines and fibrotic factors. (Bos et al., 2022c) Based on our systematic review, we know that alloreactive T and B cells are key drivers of CLAD. Furthermore, the innate arm of the immune system is activated, most likely in response to a myriad of immunological, infectious and mechanical insults, mainly involving neutrophils and eosinophils. (Bos et al., 2022b) However, we still do not know how exactly all immune cells at play contribute and to what extent. As was clear from the systematic review, more research is needed on the subtypes of immune cells, including T cells and macrophages. And the systematic review also highlighted the lack of specific data on the immunopathological mechanisms in RAS, although it seemed that involvement of eosinophils and humoral immunity was more pronounced in RAS. Both these points have been taken into account in our Hyperion work.

Through a single-cell, multiparametric, proteomic approach using IMC and lung tissue specimens from CLAD and non-CLAD patients, we performed detailed immunophenotyping of non-CLAD, BOS and RAS samples. Studies involving spatial analysis of CLAD tissue are scarce to date, as is the use of IMC on CLAD tissue. (Renaud-Picard et al., 2020, Renaud-Picard et al., 2022) Our study revealed major differences in cellularity and immune cell populations in CLAD versus non-CLAD, and also between BOS and RAS. The innate immune system appeared to be more activated in BOS, as evidenced by an increase in γδ T cells and non-classical M2 macrophages. On the other hand, Th1 cells and intermediate M2 macrophages were increased in RAS, suggesting more pronounced alloimmune and repair responses in RAS. In addition to these differences, we also found commonalities between BOS and RAS with evidence of classical cellular (cytotoxic CD8+ T cells) and humoral (B cells, especially plasma cells) immune

responses, as well as infiltration of eosinophils and fibrotic remodelling (EMT). Fibrotic remodelling of the airways and/or lung tissue is a cornerstone in the pathogenesis of CLAD. By examining ROIs with varying degrees of inflammation and fibrosis, we found that the same immune cells that were differentially increased in BOS and RAS also appeared to be involved in this fibrotic remodelling. More specifically, parenchymal and airway fibrosis were associated with γδ T cells in BOS, while with intermediate M2 macrophages and Th1 cells in RAS. Notably, as hypothesised, the mechanisms of fibrosis appeared to be independent of the location. In other words, fibrotic remodelling of the lung parenchyma and airways occurred in a similar manner, mediated by the same immune cells. (Figure 6.1)

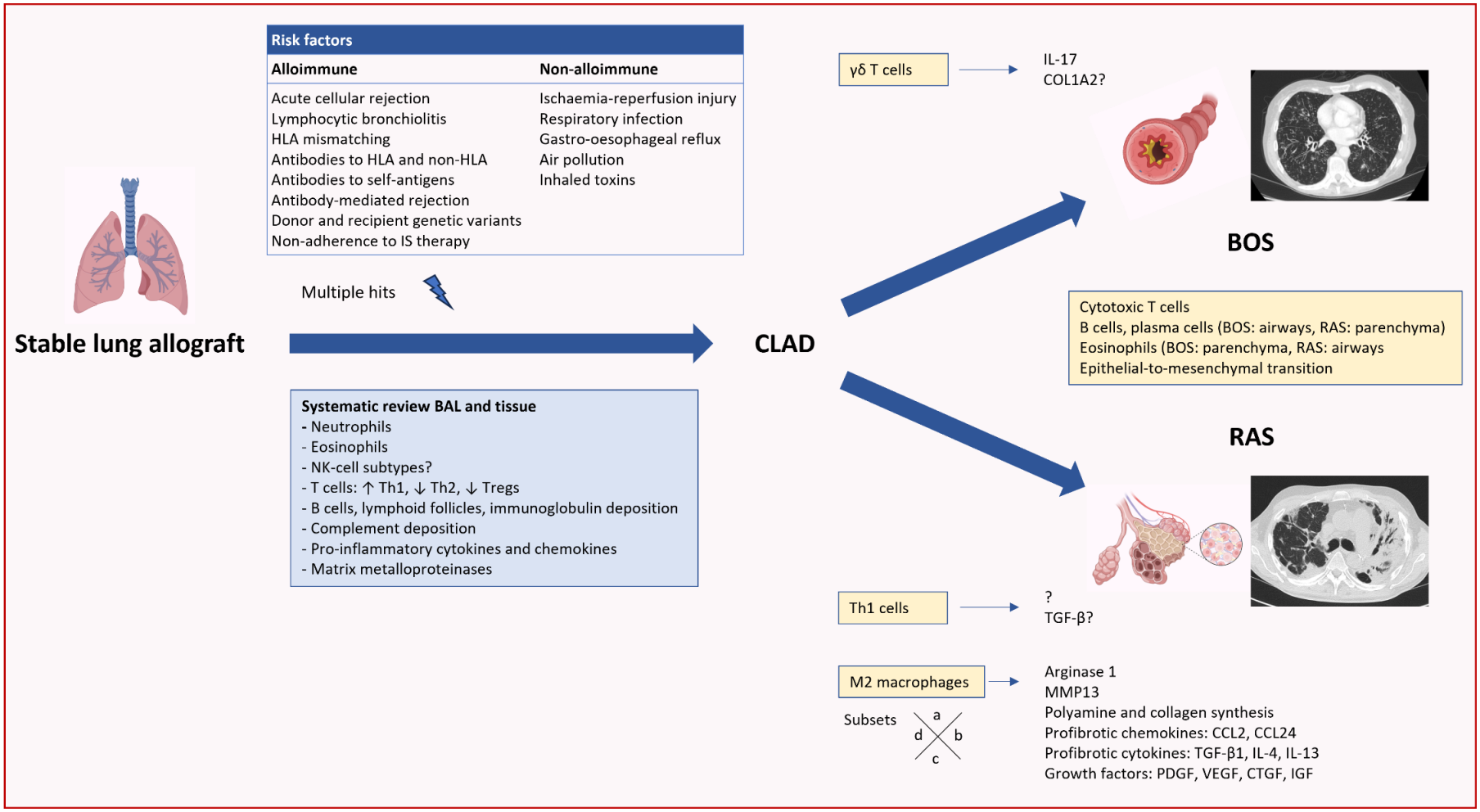


Figure 6.1. Summary of findings
Schematic overview of findings from a stable, healthy lung allograft to the development of CLAD to which various risk factors can contribute (see box). The blue box denotes findings from our systematic review. Yellow boxes highlight key findings from our Hyperion work.
BOS: bronchiolitis obliterans syndrome, CCL: C-C chemokine ligands, CLAD: chronic lung allograft dysfunction, COL1A2: collagen type I alpha 2 chain, CTGF: connective tissue growth factor, HLA: human leukocyte antigen, IGF: insulin-like growth factor, IL: interleukin, IS: immunosuppressive therapy, MMP: matrix metalloproteinase, NK: natural killer, PDGF: platelet-derived growth factor, RAS: restrictive allograft syndrome, TGF-β: transforming growth factor beta, Tregs: regulatory T cells, VEGF: vascular endothelial growth factor.

6.2 BOS and RAS: a common immunological rejection pathway?

One of our objectives was to see whether BOS and RAS share a similar immunological rejection pathway. We hypothesised that there would be both similarities and differences between BOS and RAS at the immunological level. This hypothesis was based on the following points. As emphasised in the main introduction, similar histological findings can be found in both CLAD entities, such as OB lesions in RAS and areas of alveolar fibrosis in BOS. Moreover, these lesions are often not disease specific. Indeed, there is considerable overlap between OB after lung transplantation, after allogeneic haematopoietic stem cell transplantation and in some non-transplant settings (e.g., post-infectious). (Verleden et al., 2019b, Bos et al., 2022a) Similarly, findings of alveolar and pleuroparenchymal fibroelastosis are not limited to RAS, but can also occur after allogeneic haematopoietic stem cell transplantation, radiation, or drug exposure, and can sometimes be idiopathic. (Bos et al., 2022a, Verleden et al., 2019b) This suggests a comparable immunological reaction to lung injury. Secondly, the fact that BOS and RAS share many risk factors (e.g., acute rejection, infection, non-specific triggers of lung injury), and that patients can transition from one phenotype to another, also supports the hypothesis that BOS and RAS may form a continuum of the same disease. (Bos et al., 2022b) On the other hand, differences in clinical presentation, disease course, histology, and cytokine, chemokine and growth factor expression suggest at least partly different underlying mechanisms. (Vos et al., 2015)

Findings of our Hyperion work support this paradigm. Both innate and adaptive arms of the immune system were involved in CLAD pathogenesis, but some immune cells were differentially mediated between BOS and RAS. As explained before, it seems plausible that this difference is caused by the primary site of injury. (Beeckmans et al., 2023) For example, γδ T cells are primarily found in the airways, where their normal function is to patrol the airway epithelium as part of border defence. We can speculate that damage to airway epithelial cells, either by immunological (e.g., lymphocytic bronchiolitis), infectious (e.g., bacterial infection/colonisation) or mechanical insults (e.g., gastro-oesophageal reflux or aspiration), leads to activation of these γδ T cells. A subsequent uncontrolled inflammatory reaction may lead to fibrotic remodelling, ultimately leading to organ dysfunction. (Figure 6.1) Similarly, it seems very plausible that intermediate M2 macrophages are important drivers of fibrosis in RAS. Overall, macrophages are among the most abundant immune cells in the lung, and the

intermediate monocyte/macrophage subtype in particular plays a key role in antigen presentation. (Kapellos et al., 2019) Given that humoral immunity is upregulated in RAS, it seems very likely that there is increased activation of macrophages. This appears to be followed by polarisation towards M2 macrophages, possibly initiated as a normal repair mechanism after an alloimmune reaction. M2 macrophages are considered a double-edged sword because of their protective and pathogenic roles. Most likely, in this setting, as a result of severe, chronic or repetitive stimulation, a dysregulated repair response occurs, leading to excessive accumulation of extracellular matrix components and fibrosis.

Thus, it seems plausible that different causes of severe, repetitive or chronic lung injury may serve as a common denominator, leading to inflammation, activation of immune cells and ultimately to pulmonary fibrosis, with partly different immunological and clinical manifestations depending on the principal site of injury (bronchiolar/alveolar/vascular compartment). (Beeckmans et al., 2023)

6.3 Findings in a broader context

6.3.1 From a healthy lung allograft to CLAD via episodes of acute rejection and/or infection

Using explanted lungs from patients with end-stage CLAD, we established what occurs at the immunological level in advanced CLAD. However, there are multiple steps on the trajectory from a healthy lung allograft to advanced CLAD that we could not to take into account in our research. Most likely, multiple 'hits' (injury) to the lung allograft lead to the development of CLAD. (Beeckmans et al., 2023) How exactly these hits contribute is currently unclear and detailed immunophenotyping of effector immune cells in acute rejection and infection as part of the evolution from allograft injury to CLAD would be necessary.

We hypothesise that following immunological, infectious or mechanical injury, certain immune cell phenotypes reside or remain upregulated in the lung tissue and may provide a mechanistic link between acute rejection/infection and the development of CLAD. For example, it is possible that tissue-resident memory T cells persist and thereby contribute to the onset of CLAD.

The ideal aim was to also look at TBB taken in our CLAD patients before the onset of CLAD, to assess what type of immune cells were present during and after episodes of acute rejection and infection. Unfortunately, however, mainly due to logistical challenges (e.g., most TBB were completely sectioned for clinical purposes, longitudinal samples were often not available), we were unable to do this. Still, it would be very valuable to pursue this in the future and relevant research objectives and questions could be:

- Longitudinal assessment of TBB to determine when the first evidence of immunological responses consistent with CLAD are present.
- Identification of immunological responses at times of acute rejection and/or infection and their relationship to the onset of CLAD (e.g., reversible versus irreversible changes, persistence of certain immune cell phenotypes).
- Is the immune response in the perivascular area and in lymphocytic bronchiolitis in acute rejection and the immune response in infection similar or very different from what is seen in CLAD.
- Can we identify a difference between "true" rejection and a non-specific lymphocytic response to infection or other allograft injury.

- Are there immunological differences between clinical and subclinical acute rejection and according to the severity of acute rejection (e.g., A1 versus A2), and in function of their contribution to the development of CLAD.

Tissue material is still the ideal method to answer questions like these and to uncover what exactly is happening at the level of end-organ dysfunction. Though there are significant limitations, specifically that tissue samples cannot be obtained easily and frequently, which complicates longitudinal assessment. In addition, the limited number and size of samples and their primary use for clinical purposes often means that not much remains for research purposes. BAL and especially blood samples can be easily obtained, yet are considered less ideal because they provide mere inferences about the cellular responses occurring at the tissue level. Nonetheless, one of the objectives of this doctoral project was to restart systematic collection of these samples (blood, BAL, trans- and endobronchial biopsies at the time of bronchoscopy to have simultaneous information on infection and acute rejection).
We have contributed blood and BAL samples to a research project being performed at Harefield Hospital and Imperial College, where they are examining blood and BAL samples to define the immune cell landscape in CLAD. These findings are still confidential and will not be discussed here, but are very promising. In brief, using spectral flow cytometry, we found clear differences in immune cell types in peripheral blood in patients with and without CLAD and also before the onset of CLAD. As such, this is a promising tool that will enable simpler, yet in-depth assessment of immune cells before, during and after episodes of acute rejection and infection. It may be a more convenient approach to assess how and when certain effector immune cell types arise and contribute to the trajectory of a stable, healthy lung allograft, through episodes of acute rejection and/or infection to the onset and progression of CLAD.

6.3.2 Diagnostic markers and therapeutic targets

Based on the findings of this doctoral project, we hoped to identify potential new pathways, functional biomarkers or targets for the detection, prevention and/or treatment of CLAD. Our findings are too preliminary in doing so, but they are promising for determining future research opportunities. We believe the subtypes of γδ T cells and M2 macrophages in particular merit further investigation. Indeed, they seem to play an important role in fibrotic remodelling in CLAD. If we can identify which subtype(s) is primarily responsible (e.g., IL-17-producing γδ T cells in BOS?), these cells could be targets for diagnostics and therapeutic

interventions. However, given that many innate and adaptive immune cells are involved in the pathogenesis of CLAD, we must be realistic that it is likely that a drug targeting a single immune cell (sub)type or pathway will most likely be insufficient. In my opinion, it seems likely that we will need a drug that targets multiple sites, or a combination of drugs that each target a specific immune cell (sub)type or pathway.

6.3.3 Pulmonary cGvHD

Recent studies suggest that similar injury mechanisms are in place across different solid organ transplants. (Sacreas et al., 2018) Aside from that, there are even more similarities between chronic lung rejection after lung transplantation and after allogeneic haematopoietic stem cell transplantation, also called pulmonary cGvHD in this setting. Although the aetiology is different, namely host-versus-graft disease after lung transplantation and graft-versus-host disease after stem cell transplantation, many of the subsequent inflammatory and fibrotic processes and clinical presentations are similar. Two clinical phenotypes have also been observed in pulmonary cGvHD: an obstructive phenotype, also called BOS, and a restrictive phenotype, which closely resembles RAS after lung transplantation. (Bos et al., 2022a)

Given these resemblances in clinical presentation and pathophysiology, both communities could benefit from joint research efforts. This would also help increase the size of study populations, which is currently often a limitation. Furthermore, patients might also benefit from similar therapeutic options. (Bos et al., 2022a)

It is therefore quite likely that our current findings will be applicable to the pulmonary cGvHD population. And it will be of interest to investigate whether γδ T cells and M2 macrophages play a similar role, in addition to classical cellular, humoral and innate immune responses. We aim to include pulmonary cGvHD samples in future research endeavours.

6.3.4 Fibrosing ILDs

Besides similarities between CLAD and pulmonary cGvHD, similarities have also been identified between CLAD, especially RAS, and fibrosing ILDs. (Bos et al., 2021) ILDs comprise a heterogeneous group of > 200 parenchymal lung diseases, characterised by varying degrees of inflammation and fibrosis. (Cottin et al., 2018) Idiopathic pulmonary fibrosis is the most common form of idiopathic interstitial pneumonia and is associated with a poor prognosis. (Raghu et al., 2018) In addition, some patients with other forms of ILD (e.g., chronic hypersensitivity pneumonitis, non-specific interstitial pneumonia, related to rheumatoid

arthritis or systemic sclerosis) may develop a progressive fibrosing phenotype. (Cottin et al., 2018)

Similar to our hypothesis for CLAD, fibrosing ILDs are also thought to be caused by chronic or repeated micro-injury to the alveolar epithelium, leading to an altered wound healing process with defective attempts at regeneration, aberrant epithelial-mesenchymal crosstalk, and an imbalance between pro- and antifibrotic mediators. (Glass et al., 2020, Sgalla et al., 2018) In response to epithelial injury, bronchiolar and alveolar epithelial cells migrate to damaged areas for repair and stimulate production of profibrotic chemokines, matrix metallo-proteinases and migration, proliferation and differentiation of lung fibroblasts. Ultimately, fibroblasts differentiate into myofibroblasts, with synthesis of excessive amounts of extracellular matrix and abundant collagen deposition. TGF-β1 and, to a lesser extent, platelet-derived growth factors drive this myofibroblast formation. (Glass et al., 2020)

Given that there are clinical and anatomopathological similarities between RAS and fibrosing ILDs, it seems highly likely that there are also similarities at the immunological level. Indeed, M2 macrophages are thought to play an important role in the dysregulated repair response in fibrosing ILDs, and higher levels of M2 markers have been found in blood and BAL in patients with idiopathic pulmonary fibrosis. Furthermore, depletion of M2 macrophages during the fibrotic phase in a murine model of lung fibrosis resulted in a reduction of extracellular matrix production. (Boorsma et al., 2014) Next to this, Th2 and Th17 cells have been linked to the pathogenesis of idiopathic pulmonary fibrosis, while reduced levels of Tregs were found, similar to what has been described in CLAD. Via secretion of IL-4 and IL-13, Th2 cells can directly induce M2 macrophage activation, thereby promoting fibrogenesis. (Sgalla et al., 2018) IL-4 is also known to induce macrophage M2 polarisation via activation of PI3K/AKT and JAK1/STAT6. (Cheng et al., 2021) In addition, IL-4 and IL-13 are directly implied in fibroblast activation. Furthermore, Th17 cells can promote fibrosis by increasing TGF-β1 levels and via production of IL-17. (Sgalla et al., 2018)

As such, there seem to be similarities between CLAD, especially RAS, and fibrosing ILDs at the clinical, pathological and immunological levels. To further explore commonalities in these inflammatory and fibrotic processes, it would be worthwhile to include samples from patients with fibrosing ILDs in future research efforts. Also with respect to treatment, patients might

benefit from similar treatment options. (Bos et al., 2021) Blocking recruitment of mononuclear-derived macrophages, promoting apoptosis of M2 macrophages or inhibiting M2 macrophage polarisation may be beneficial for the treatment of pulmonary fibrosis in both fibrosing ILDs and RAS, worthy of investigation. Although many studies have linked M2 polarisation of macrophages to lung repair and fibrosis, there is currently still relatively little information on the role of M2 macrophage subsets in lung repair and fibrosis. (Cheng et al., 2021)

6.4 Limitations

To date, most data on CLAD, also those included in our systematic review, regarding the underlying immunopathophysiology come from small, retrospective studies. Most data focused on the BOS phenotype, as the RAS and mixed phenotype were recognised more recently. However, study populations were often heterogeneous and most likely also included patients with interstitial lung abnormalities. In addition, different types of analyses and techniques used made it difficult to compare findings from different studies. Moreover, findings were often inconsistent, making it difficult to draw general conclusions.

With respect to our Hyperion work, our results should be interpreted in light of several limitations. Despite the multicentre approach, the number of samples was relatively low and the main limitation is that not all identified ROIs could be analysed due to the fragility of some tissue samples. This led to a more limited number of ROIs for some clinical phenotypes and ROI types. Furthermore, we did not capture intra-patient variability as only one tissue sample was included per patient. However, it is important to mention that the fact that we used non-CLAD control specimens from lung transplant recipients is a great added value. Many available studies used non-transplant control samples, which does not allow for accurate comparison because these patients have not been exposed to immunosuppressive therapy routinely used following transplantation. Similarly, most centres do not correct for airspaces present in tissue sections (e.g., air in airway lumens), meaning they quantify cells per unit area of tissue section. However, this can be significantly confounded by changes in airspace contribution to section area, as recently demonstrated by the Newcastle group. (Milross et al., 2023)

Another limitation is that it was impossible to include all factors possibly relevant to CLAD in this study. On the one hand, this is due to the maximum number of markers that can be included and on the other hand, some factors require different techniques to analyse them (e.g., not all markers are available for IMC, some non-immune cells might be better analysed with different technologies). Consequently, the antibody panel was limited to key adaptive and innate immune cells. Although we aimed to identify differentiation and activation states, this proved not to be simple. Furthermore, we were unable to further phenotype some clusters (e.g., activated leukocytes, unclassified lymphoid cells) and were unable to identify some leukocytes (e.g., neutrophils) and T-cell subsets (e.g., Th17 cells).

Another important limitation is that we looked at end-stage CLAD disease and compared areas of no, limited and more severe fibrosis within those samples. These results cannot be fully extrapolated to different stages along the trajectory from a stable lung allograft to end-stage CLAD. And while it would be interesting to investigate the actual different stages of CLAD disease (according to time of onset and/or severity), this is limited by the need for good tissue samples.

Lastly, we strived for as homogeneous a population as possible (e.g., only inclusion of clear BOS and RAS phenotypes, no mixed phenotype). Nevertheless, it remains very difficult to eliminate all heterogeneity in a study population. In our study, there may have been an effect of colonisation or respiratory infection in the period before the lungs were retrieved, or of certain effects of immunosuppressive treatment that can last for a long time (e.g., B-cell depletion after treatment with rituximab in RAS patients). (See also Table 4.6 and 4.7)

6.5 Future directions

Based on the systematic review and our Hyperion work, we believe that future research opportunities should focus on the following:

Confirmation of findings in a larger cohort (i.e., validation of results) and comparison of findings with other fibrotic diseases. We are currently conducting a multicentre (n=4) study using MICS (MACSima Imaging Cyclic Staining) technology (Miltenyi Biotec). This is a novel imaging system for fully automated cyclic immunofluorescence analysis that enables immunofluorescence imaging of hundreds of protein targets in a single sample at subcellular resolution. MICS is based on cycles of immunofluorescence staining, multi-field imaging and signal erasure, using up to three fluorochrome-conjugated antibodies per cycle. Our aim is to increase the sample size of CLAD specimens (n>40) and to include samples from patients with idiopathic pulmonary fibrosis, other fibrosing ILDs and pulmonary cGvHD. This technology will allow inclusion of more markers, including more cytokine and chemokine markers. The aim of this project is to focus more on fibrotic remodelling and pathways involved. Therefore, we designed an immune and a fibrotic panel of approximately 100 markers in total for 1) identification of immune cells and 2) assessment of fibrotic markers and pathways.

Further studies are also necessary to identify subtypes of γδ T cells (especially IL-17- versus IFN-γ-producing γδ T cells) and M2 macrophages (Ma, Mb, Mc, Md) that are associated with fibrotic progression. Because different subtypes of immune cells exist that exert different functions, it is important to characterise the metabolic state that regulate their function and to analyse the dynamic interaction of those immune cells in response to insults. It will be important to identify the exact subtype responsible for the inflammatory and fibrotic responses in CLAD. Identification of the responsible subtype(s) will hopefully also aid in the development of diagnostic tools and therapeutic agents. Multi-omics techniques can be used for this, although IMC might not be the ideal method due to the limited number of markers that can be included and the fact that soluble markers are more difficult to analyse. MACSima could be a step forward as it allows for a larger antibody panel and better identification of cytokines. Therefore, we aim to include both IFN-γ and IL-17 so that we can hopefully determine whether the γδ T cells we identified produce IL-17 as we expect. It will be more difficult to identify macrophage subsets using MACSima and it may be better to assess this

using single-cell RNA sequencing. Single-cell RNA sequencing provides whole transcriptome expression profiles of individual cells and is considered the gold standard for defining cell states and phenotypes.

Lastly, future studies should also focus on other adaptive and innate immune cells and their subsets, as some of these subsets may have opposing effects:

- Further investigation of memory T cells and less common and/or less studied T-cell subsets, such as NKT cells, Th9 cells, Th22 cells, and T follicular helper cells, in CLAD.
- The function of regulatory B cells in general and in CLAD.
- Exact role and mechanisms of action of eosinophils in CLAD. Further research on the existence of eosinophil subtypes with immunosuppressive effects, as observed in animal studies.
- Similar for NK cells, due to either activating or inhibitory actions of different NK-cell receptors, the functions of different NK-cell subtypes need to be explored.
- Whether mast cells or mast cell subtypes contribute to the onset and progression of CLAD.
- Whether dendritic cells play a role in CLAD in addition to their function as antigen-presenting cells. Research should also focus on follicular dendritic cells, as they can present iccosomes (antigen-antibody complexes) to B cells and enhance the affinity of immunoglobulins and could thus play a role in CLAD.
- Whether innate lymphoid cells play a part in the pathogenesis of CLAD.

6.6 Conclusions

In order to gain a better understanding of the complex immunopathology of CLAD, we used IMC to enable highly multiplexed imaging of lung tissue at single-cell resolution. Using this approach, we identified potential novel immunological insights into the pathogenesis and fibrotic progression of CLAD. In-depth immunophenotyping of cells in their local tissue microenvironment identified major differences in CLAD versus non-CLAD and between BOS and RAS. CLAD was characterised by classical cellular and humoral immune responses, including cytotoxic T cells and plasma cells, but additionally eosinophil infiltration. Novel observations included M2 macrophage polarisation and expansion of Th1 cells in RAS and γδ T cells in BOS, possibly pointing to more pronounced alloimmune responses in RAS and more active innate involvement in BOS. Common immune cell profiles were observed in evolving fibrosis in both lung parenchyma and airways, involving both adaptive and innate immune cells as well as EMT. However, different profiles in RAS (intermediate M2 macrophages, Th1 cells) and in BOS (γδ T cells) were also identified. Our findings in fibrotic progression of CLAD suggest γδ T cells and M2 macrophages in particular merit further investigation.

Chapter 7 References

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properties of azithromycin involved in treatment and prevention of chronic lung allograft rejection. *Transplantation,* 94**,** 101-9.

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YAMADA, Y., BRÜSTLE, K. & JUNGRAITHMAYR, W. 2019. T Helper Cell Subsets in Experimental Lung Allograft Rejection. *J Surg Res,* 233**,** 74-81.

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YANG, J. Y. C., VERLEDEN, S. E., ZARINSEFAT, A., VANAUDENAERDE, B. M., VOS, R., VERLEDEN, G. M., SARWAL, R. D., SIGDEL, T. K., LIBERTO, J. M., DAMM, I., WATSON, D. & SARWAL, M. M. 2019. Cell-Free DNA and CXCL10 Derived from Bronchoalveolar Lavage Predict Lung Transplant Survival. *J Clin Med,* 8.

YAO, Y., XU, X. H. & JIN, L. 2019. Macrophage Polarization in Physiological and Pathological Pregnancy. *Front Immunol,* 10**,** 792.

YAO, Y. E., QIN, C. C., YANG, C. M. & HUANG, T. X. 2022. γδT17/γδTreg cell subsets: a new paradigm for asthma treatment. *J Asthma,* 59**,** 2028-2038.

YOSHIYASU, N. & SATO, M. 2020. Chronic lung allograft dysfunction post-lung transplantation: The era of bronchiolitis obliterans syndrome and restrictive allograft syndrome. *World J Transplant,* 10**,** 104-116.

ZHANG, C., NIU, Y., YU, L., LV, W., XU, H., ABUDUWUFUER, A., CAO, J. & HU, J. 2017. The role of epithelial-mesenchymal transition in the post-lung transplantation bronchiolitis obliterans. *J Cardiothorac Surg,* 12**,** 119.

ZHANG, M. & ZHANG, S. 2020. T Cells in Fibrosis and Fibrotic Diseases. *Front Immunol,* 11**,** 1142.

ZHAO, Y., NIU, C. & CUI, J. 2018. Gamma-delta (γδ) T cells: friend or foe in cancer development? *J Transl Med,* 16**,** 3.

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ZHENG, L., WHITFORD, H. M., ORSIDA, B., LEVVEY, B. J., BAILEY, M., WALTERS, E. H., WILLIAMS, T. J., KOTSIMBOS, T. & SNELL, G. I. 2006. The dynamics and associations of airway neutrophilia post lung transplantation. *Am J Transplant,* 6**,** 599-608.

Chapter 8 PhD-related publications and abstracts

8.1 Publications

Bos S, Milross L, Filby AJ, Vos R, Fisher AJ. Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle. Eur Respir Rev. 2022;31(165). See Appendix A.

Bos S, Filby AJ, Vos R, Fisher AJ. Effector immune cells in Chronic Lung Allograft Dysfunction: a Systematic Review. Immunology 2022;166(1):17-37. See Appendix E.

Bos S, Pradère P, Beeckmans H, Zajacova A, Vanaudenaerde BM, Fisher AJ, Vos R. Lymphocyte depleting and modulating therapies for chronic lung allograft dysfunction. Pharmacol Rev. 2023;75(6):1200-17. See Appendix B.

Bos S[*], Hunter B[*], McDonald D, Merces G, Sheldon G, Pradère P, Majo J, Pulle J, Vanstapel A, Vanaudenaerde BM, Vos R, Filby AJ, Fisher AJ. High-dimensional tissue profiling of immune responses in chronic lung allograft dysfunction. In submission.

8.2 Abstracts

Bos S, Hunter B, McDonald D, Merces G, Sheldon G, Pradère P, Majo J, Pulle J, Vanstapel A, Vanaudenaerde BM, Vos R, Filby AJ, Fisher AJ. Profiling immune cell responses in chronic rejection after lung transplantation using imaging mass cytometry. See Appendix G.
British Transplant Society, Annual Congress 2024, 6th – 8th March 2024, Harrogate, UK.
Finalist Medawar Medal Award session.

Bos S, Hunter B, McDonald D, Merces G, Sheldon G, Pradère P, Majo J, Pulle J, Vanstapel A, Vanaudenaerde BM, Vos R, Filby AJ, Fisher AJ. High-dimensional lung tissue imaging reveals temporal changes in immune cell populations and cell interactions during progression of Chronic Lung Allograft Dysfunction (CLAD). See Appendix H.
International Society for Heart and Lung Transplantation, 44th Annual Meeting and Scientific Sessions, 10th – 13th April 2024, Prague, Czech Republic.
Finalist Philip K. Caves Award session.

Chapter 9 Curriculum vitae

Personalia

Name	Saskia Bos
Date and place of birth	30th October 1989, Hasselt, Belgium
Residence	Newcastle upon Tyne, United Kingdom
LinkedIn	https://www.linkedin.com/in/saskia-bos-5396a3204
Web of science Researcher ID	AAC-4899-2021
Orcid ID	https://orcid.org/0000-0002-5336-5914
E-mail	saskia.bos@newcastle.ac.uk
	saskia.bos@uzleuven.be

Education and training

High school	2001 – 2007	Sint-Jan Berchmans college, Genk, Belgium
	Major: Latin and mathematics, graduated *Magna Cum Laude*	
Academic education	2007 – 2010	Universiteit Hasselt, Diepenbeek, Belgium
	2010 – 2014	Katholieke Universiteit Leuven, Leuven, Belgium
	Faculty of Medicine, graduated *Magna Cum Laude*	
Residency Internal Medicine	2014 – 2017	University Hospitals Leuven, Leuven, Belgium
		Ziekenhuis Oost-Limburg, Genk, Belgium
		Jessa ziekenhuis, Hasselt, Belgium
Residency Respiratory Med.	2017 – 2020	Algemeen Ziekenhuis Groeninge, Kortrijk, Belgium
		University Hospitals Leuven, Leuven, Belgium

Work (pulmonologist)

01-08-2020 – 31-12-2020	Senior resident, Dept. of Respiratory Medicine/Lung Transplantation
	University Hospitals Leuven, Leuven, Belgium
18-01-2021 – present	Clinical Research Associate and PhD Candidate Lung Transplantation
	Honorary lung transplant physician, Freeman Hospital
	Paul Corris Training Scholarship
	Newcastle University, Newcastle upon Tyne, United Kingdom

Past work experiences

2005 – 2007	Nursing home Prinsenpark, Genk, Belgium. Logistics function.
2007 – 2014	Mise en Place, Hasselt, Belgium. Hospitality industry.
2011 – 2012	Nursing home De Olijfboom, Genk, Belgium. Nursing assistant.

Additional training and certificates

Aug. 2010	Volunteering work in Rwanda: building a maternity clinic, millennium goals 4 and 5.
2010 – 2011	Course in Tropical Diseases, KULeuven, Belgium.
2011 – 2012	Course in Biomedical Cooperation, KULeuven, Belgium.
2013 – 2014	Course in Electrocardiography, KULeuven, Belgium.
2017 – 2020	Pulmonary Function Course, Belgian Respiratory Society, Belgium.
March 2020	Development cooperation project in Congo, cooperation between Lumos, University Hospitals Leuven, Belgium and Memisa, Hôpital St. Luc, Kisantu, DR Congo.
01-05-2020	Good Clinical Practice e-learning course, The Global Health Network.
02-09-2020	IELTS academic (International English Language Testing System) Band score 8/9. Corresponding to Common European Framework of References for Languages C1.
11-09-2020	Good Clinical Practice interactive training, European forum for good clinical practice.
13-04-2021	Interactive course on lung transplant rejection and extracorporeal photopheresis. Provided by Therakos. Center of Excellence Vienna. Virtual due to COVID-19.
June 2021	ERS virtual school courses on lung transplantation basic and advanced. Virtual due to COVID-19.
27-04-2021	Human Tissue Act (research sector) e-learning course.
2021 – 2023	Online course Fundamentals of Immunology Specialization, Rice University, USA.
Feb. 2022	ISHLT academies, BSTR core competencies course, basic and translational science.
May 2022	**Cochrane Interactive Learning: Conducting an Intervention Review**
09-08-2023	Good Clinical Practice e-learning course, The Global Health Network.
Nov. 2023	ESCMID Postgraduate course on Infections in Immunocompromised Host

Prizes, grants and scholarships

2019	Forum Vlaamse Longartsen – Colloquium, 1st prize
2021 – 2023	**Paul Corris International Clinical Research Training Scholarship**
2022	ESOT educational scholarship
2022	ISHLT Basic Science and Translational Research core competencies course, 1st prize
17-06-2022	Prize of Best Speaker at RMSCT Theme Away Day 3-min PhD Talk: "Immunophenotyping of tissue immune responses in chronic lung allograft dysfunction"
2023 – 2024	**Transplant International Editorial Fellowship**

Memberships and leadership board positions

Forum Vlaamse Longartsen (FVL)	2017 – 2020	member
Belgian Respiratory Society (BeRS)	2018 – present	member
European Respiratory Society (ERS)	2018 – present	member

International Society for Heart and Lung Transplantation (ISHLT)	2020 – present	member
European Society for Organ Transplantation (ESOT)	2020 – present	member
North of England Thoracic Society (NETS)	2021 – 2023	member
British Transplant Society (BTS)	2023 – 2024	member
ERS Clinical Practice Guidelines Methodology Network	2022 – 2025	board
ERS Long-Range Planning Committee	2022 – present	board
ESOT Infectious Disease Task Force	2022 – present	board
ESOT Scientific Programme Committee biennial congress London 2025	2023 – 2025	board
ERN-LUNG Core Network Chronic Lung Allograft Dysfunction	2023 – present	board
ESOT Guidelines Task Force (including organisation TCJ 4.0, 2025)	2023 – 2025	Deputy Chair
ERS Guidelines Working Group	2024 – 2027	board

Guideline development

ERS/EBMT Clinical Practice Guideline on treatment of pulmonary chronic graft versus host disease, 2021-2023, junior ERS chair and methodology.

ESOT Consensus Statement on the use of non-invasive biomarkers for cardiothoracic transplant rejection surveillance. TLJ 3.0, 2022, Prague, Czech Republic. Jury member and methodology.

ERS/ESTI/ESR/ESTRO/ESTS Clinical Practice Guideline on the management of positive findings from low-dose CT screening for lung cancer, 2023-2025, methodology.

ESCMID/ESOT Clinical Practice Guideline on vaccines in solid organ transplants, 2023-2024, ESOT representative and panel member.

Clinical Trials

Sub-investigator of NCT03978637, Safety and Efficacy of Itacitinib in Participants With Bronchiolitis Obliterans Syndrome Following Lung Transplantation, Phase 1 multicentre study, University Hospitals Leuven, Belgium, 2020.

Sub-investigator of NIHR130612, Extracorporeal Photophoresis in the treatment of Chronic Lung Allograft Dysfunction: a randomised controlled trial (E-CLAD), multicentre study, Freeman Hospital, Newcastle upon Tyne, UK, 2023-2024.

Co-PI of Outcomes and risk factors for survival of lung transplantation after prior allogeneic haematopoietic stem cell transplantation: a study from the Transplant Complications Working Party of the EBMT, multicentre, retrospective study, 2021 – present.

Eurofins – Transplant Genomics Inc., Assessment of allograft health using donor-derived cell-free DNA and gene expression profiling after lung transplantation (Allo-GEN), prospective, multicentre study, set-up of study and PI Newcastle site, Newcastle upon 2021 – present.

(Co-)supervisorships

David van Eijndhoven, MD dissertation Medical School, topic: "Monoclonal antibodies in prevention and early treatment of COVID-19 in lung transplant recipients: a systematic review", Katholieke Universiteit Leuven, Leuven, Belgium. (co-supervisor 2022-2024)

Georgia Sheldon, MRes Biomedical Sciences, topic: "Characterising the immune response of T cells in Chronic Lung Allograft Dysfunction after lung transplantation". Newcastle University, Newcastle upon Tyne, UK. (co-supervisor 2023)

Arisha Tahir, Undergraduate Student Biomedical Sciences, topic: "Profiling cellular immune responses in chronic rejection after lung transplantation". Newcastle University, Newcastle upon Tyne, UK. (co-supervisor 2024)

Presentations and lectures

Poster presentation, Belgian Pneumology Days, Brussels, Belgium. Cardinaels N, Bos S, Santy L, et al. "Respiratory failure due to fibrothorax". (30-11-2018)

Oral abstract presentation, Belgian Pneumology Days, Brussels, Belgium. Cardinaels N, Van Rompaey W, Bos S (presenter), et al. "EBV-related pulmonary lymphomatoid granulomatosis". (30-11-2018)

Workshop "Chronic cough", severe asthma congress, Davos, Switzerland. (14-03-2019, 15-03-2019)

Workshop "Chronic cough", Leuven, Belgium. (07-11-2019)

Oral abstract presentation, European Respiratory Society International Congress, virtual congress due to COVID-19. Bos S, De Sadeleer LJ, Vanaudenaerde BM, et al. "Real life experience with mTOR-inhibitors after lung transplantation". (09-09-2020)

Lecture "Pulmonary GvHD", annual meeting of EBMT (European Society for Blood and Marrow Transplantation), Nurses group, virtual congress due to COVID-19. (16-03-2021)

Poster presentation, International Society for Heart and Lung Transplantation, virtual congress due to COVID-19. Bos S, Daniëls L, Michaux L, et al. "An unusual course of donor-transmitted angiosarcoma after lung transplantation". (27-04-2021)

Lecture "When the tissue is the issue: pathophysiology of lung GvHD versus CLAD", Therakos Lung Day UK Meeting, London. (24-09-2021)

ERN-LUNG Academy, session "CLAD after Lung Transplantation: Diagnosis and differential diagnosis". (2022)

RMSCT (Regenerative Medicine Stem Cell and Transplant) Theme Away Day, 3-min PhD Talk: "Immunophenotyping of tissue immune responses in chronic lung allograft dysfunction". (17-06-2022)

Lecture "Lung GvHD", EBMT-BHS Nurses Group 14th International Study Day, Brussels, Belgium. (6-10-2022)

Lecture "Lung GvHD", 2nd North BMT Training Day, Manchester, UK. (7-10-2022)

Lecture "Lung GvHD", 5th South-West BMT Training Day, Bristol, UK. (13-10-2022)

Presentation "Immunophenotyping of tissue immune responses in Chronic Lung Allograft Dysfunction after lung transplantation", Association of Lung Transplant Physicians, UK. (12-05-2023)

Poster presentation, European Society for Organ Transplantation, annual congress 2023, Athens, Greece. Bos S, Majo J, Funston W, et al. Silicone depositions, an unusual finding in the explanted and newly transplanted lungs. (19-09-2023)

TV studio panel, European Society for Organ Transplantation, annual congress 2023, Athens, Greece. Berney T, Gilbo N, Bos S, Selzner N. Diversity, collaboration and equity – the TI way. (19-09-2023)

Lecture "Pulmonary graft-versus-host disease and chronic lung allograft dysfunction: two sides of the same coin?", Therakos Institute Webinar. (26-09-2023)

Lecture "When the issue is the tissue: pathophysiology of lung GvHD versus CLAD", Round table lung GvHD, Paris, France. (04-10-2023)

Lecture "Lung GvHD, the importance of early detection", UK EBMT Nurses and AHP Group, virtual. (13-10-2013)

Presentation "Imaging mass cytometry for high-dimensional tissue profiling of chronic lung allograft dysfunction", NECS (Newcastle, Edinburgh, Cambridge, Sheffield) research meeting, Edinburgh, UK. (7-12-2023)

Conference chair

European Respiratory Society, annual congress 2022, Barcelona, Spain. Thematic poster session "Risk stratification in lung transplant patients". (04-09-2023)

European Society for Organ Transplantation, annual congress 2023, Athens, Greece. Full oral session "Molecular monitoring of lung allograft rejection". (17-09-2023)

Editorial functions

Editorial Fellow Transplant International 2023-2024

Guest Editor Special issue Organoids, Transplant International

Reviewer for medical journals

American Journal of Transplantation
BMC Pulmonary Medicine
Chest Disease Reports
Chest Pulmonary
Current Challenges in Thoracic Surgery
European Respiratory Society Monograph
International Immunopharmacology
Journal of Clinical Medicine
Journal of Lung Health and Diseases
Journal of Thoracic Disease
Lung
PLOS One
The Lancet Respiratory Medicine
Therapeutic Advances in Respiratory Disease
Thorax
Transplantation
Transplantation Direct
Transplant International
World Journal of Clinical Cases
World Journal of Clinical Infectious Diseases
World Journal of Gastroenterology
World Journal of Transplantation

Cardinaels N, Van Rompaey W, Bos S, Bode H, Tousseyn T, Van Bleyenbergh P. An atypical case of a pulmonary mass in an immunocompromised patient. Acta Clin Belg 2020;75(5):370-74.

Bos S, Vos R, Van Raemdonck DE, Verleden GM. Survival in adult lung transplantation: where are we in 2020? Curr Opin Organ Transplant 2020;25(3):268-73.

Bos S, De Vleeschouwer S, Van Raemdonck DE, Verleden GM, Vos R. Intracerebral abscess due to Cutibacterium acnes after lung transplantation. Transpl Infect Dis 2021 Feb;23(1):e13398.

Ceulemans LJ, Van Slambrouck J, De Leyn P, Decaluwé H, Van Veer H, Depypere L, Ceuterick V, Verleden SE, Vanstapel A, Desmet S, Maes P, Van Ranst M, Lormans P, Meyfroidt G, Neyrinck AP, Vanaudenaerde BM, Van Wijngaerden E, Bos S, Godinas L, Carmeliet P, Verleden GM, Van Raemdonck DE, Vos R. Successful double-lung transplantation from a donor previously infected with SARS-CoV-2. Lancet Respir Med 2021;9(3):315-18.

De Mol W*, Bos S*, Beeckmans H, Lagrou K, Spriet I, Verleden GM, Vos R. Antifungal prophylaxis after lung transplantation: where are we now? *Contributed equally. Transplantation 2021;105(12):2538-2545.

Bos S, De Sadeleer LJ, Vanaudenaerde BM, Yseryt J, Dupont LJ, Godinas L, Verleden GM, Vos R. Real life experience with mTOR-inhibitors after lung transplantation. Int Immunopharmacol 2021;94:107501.

Beeckmans H, Bos S, Vos R. Selection Criteria for Lung Transplantation: Controversies and New Developments. Semin Respir Crit Care Med 2021;42:329–45.

Bos S, De Sadeleer LJ, Vanstapel A, Beeckmans H, Sacreas A, Yserbyt J, Wuyts W, Vos R. Antifibrotic drugs in lung transplantation and chronic lung allograft dysfunction: a review. Eur Respir Rev 2021; 30:210050.

Decaesteker T*, Bos S*, Lorent N, Everaerts S, Bullens D, Dupont LJ. Elevated serum calprotectin (S100A8/A9) in patients with asthma. *Contributed equally. J asthma 2022;59(6):1110-1115.

Bos S, Michaux L, Daniels L, Vanden Bempt I, Vermeer S, Woej-A-Jin S, Schöffski P, Weynand B, Sciot R, Declerq S, Van Raemdonck DE, Dupont LJ, Verleden GM, Vos R. Case report: An unusual course of angiosarcoma after lung transplantation. Front Immunol 2021;12:789851.

Bos S, Fisher AJ. Is downregulation of liver kinase B1 the major factor driving epithelial-to-mesenchymal transition? Am J Transplant 2022;22(3):689-690.

Bos S, Filby AJ, Vos R, Fisher AJ. Effector immune cells in Chronic Lung Allograft Dysfunction: a Systematic Review. Immunology 2022;166(1):17-37.

Bos S, Ricciardi S, Caruana EJ, Öztürk NAC, Magouliotis D, Pompili C, Migliore M, Vos R, Meloni F, Elia S, Hellemons M. ERS International Congress 2021: highlights from Assembly 8 Thoracic Surgery and Lung Transplantation. ERJ Open Res 2022;23;8(2):00649-2021.

Bos S, Beeckmans H, Vanstapel A, Sacreas A, Geudens V, Willems L, Schreurs I, Vanaudenaerde BM, Schoemans H*, Vos R*. Pulmonary Graft-*versus*-Host Disease and Chronic Lung Allograft Dysfunction: Two Sides of the Same Coin? Lancet Resp Med 2022;10(8):796-810.

Bos S, Milross L, Filby AJ, Vos R, Fisher AJ. Immune processes in the pathogenesis of Chronic Lung Allograft Dysfunction: identifying the missing pieces of the puzzle. Eur Respir Rev 2022;31(165): 220060.

Beeckmans H*, Ambrocio GPL*, Bos S, Geudens V, Vanstapel A, Vanaudenaerde BM, De Baets F, Malfait T, Emonds MP, Van Raemdonck DE, Schoemans H**, Vos R** and for the Leuven Lung Transplant Group. Allogeneic Hematopoietic Stem Cell Transplantation After Prior Lung Transplantation for Hereditary Pulmonary Alveolar Proteinosis: A Case Report. Front Immunol 2022; 13:931153.

Beeckmans H*, Bos S*, Vos R**, Glanville AR**. Acute Rejection and Chronic Allograft Dysfunction: Obstructive and Restrictive allograft dysfunction. Clin Chest Med 2023;44:137–157.

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Appendix A: Publication "Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle"

Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle

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Shareable abstract (@ERSpublications)
CLAD is the end-stage of a disease continuum marked by complex, interacting, innate and adaptive, cellular and humoral, allo- and autoimmune mechanisms, repeated lung injury, tissue remodelling and repair, ultimately leading to allograft dysfunction. https://bit.ly/3Ny3LZz

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Abstract

Lung transplantation is the optimal treatment for selected patients with end-stage chronic lung diseases. However, chronic lung allograft dysfunction remains the leading obstacle to improved long-term outcomes. Traditionally, lung allograft rejection has been considered primarily as a manifestation of cellular immune responses. However, in reality, an array of complex, interacting and multifactorial mechanisms contribute to its emergence. Alloimmune-dependent mechanisms, including T-cell-mediated rejection and antibody-mediated rejection, as well as non-alloimmune injuries, have been implicated. Moreover, a role has emerged for autoimmune responses to lung self-antigens in the development of chronic graft injury. The aim of this review is to summarise the immune processes involved in the pathogenesis of chronic lung allograft dysfunction, with advanced insights into the role of innate immune pathways and crosstalk between innate and adaptive immunity, and to identify gaps in current knowledge.

Introduction

Chronic lung allograft dysfunction (CLAD) remains the major limitation to the long-term success of lung transplantation, occurring in up to 50% of recipients within 5 years post-transplant [1]. CLAD encompasses two distinct but overlapping phenotypes, of which bronchiolitis obliterans syndrome (BOS) is the most prevalent, featuring in ~70% of CLAD patients. The histological hallmark of BOS is small airways fibrosis, known as "obliterative bronchiolitis", which is clinically characterised by persistent and progressive airflow limitation [2]. Restrictive allograft syndrome (RAS) occurs in 20–30% of CLAD patients and is defined by a restrictive pulmonary function decline and persistent pleuroparenchymal abnormalities on computed tomography [2, 3]. In addition, patients might present with a mixed phenotype or shift from one phenotype to another over time [3]. Different pathophysiological mechanisms have been suggested to be involved in these phenotypes, given the differences in disease course, radiographic imaging, histology, and cytokine, chemokine and growth factor expression. However, it is difficult to clearly categorise the different pathophysiological mechanisms because of the relatively smaller amount of pooled evidence for RAS at present [2, 4].

Unlike other solid organ transplants, the lung allograft is continuously exposed to the external environment and therefore harbours a robust innate immune presence primed to respond to environmental or microbiological challenges and contains more tissue-resident and interstitial immune cells [5]. It is therefore not surprising that various insults to the lung allograft have been identified as important contributing factors to CLAD, and can broadly be described as alloimmune-dependent and -independent (table 1) [4, 6]. Over

TABLE 1 Risk factors contributing to chronic lung allograft dysfunction onset

Alloimmune dependent
Acute cellular rejection
Lymphocytic bronchiolitis
Human leukocyte antigen (HLA) mismatching
Preformed or *de novo* anti-HLA antibodies, non-HLA antibodies and antibodies to self-antigens
Antibody-mediated rejection
Donor and recipient genetic variants
Non-adherence to immunosuppressive therapy
Alloimmune independent
Ischaemia-reperfusion injury
Allograft infection (bacterial, fungal, viral)
Gastro-oesophageal reflux
Air pollution
Inhaled toxins

the past decades, we have gained a better understanding of how the immune system contributes to inflammatory responses, airway and parenchymal remodelling, and fibrosis after lung transplantation. However, in order to make therapeutic advances in the prevention and treatment of CLAD, it is critical to develop a full picture of how all the immune processes at play in the lung allograft interact in the pathogenesis of CLAD. Here, we review evidence established to date, with advanced insights into the role of innate immune pathways and crosstalk between innate and adaptive immunity before identifying what is missing from our current understanding of this puzzle.

Immune processes in CLAD

T-cell-mediated immunity

Cell-mediated immunity is perhaps the best understood alloimmune pathway. It is predominantly driven by T-cells following the presentation of alloantigens by antigen-presenting cells (APCs) *via* major histocompatibility complex (MHC) molecules, also called human leukocyte antigen (HLA) [7, 8]. HLA genes are highly polymorphic and large interindividual differences in allelic variants are the major immunological barrier to transplantation [9]. Two main modes play a role in this allorecognition. In the direct pathway, allogeneic MHC is presented directly to recipient T-cells by donor APCs. In the indirect pathway, recipient APCs phagocytise and present alloantigens to recipient T-cells as MHC–peptide complexes [8, 10]. MHC classes I and II are, respectively, recognised by $CD8^+$ and $CD4^+$ T-cells [7]. Following allorecognition, T-cells require secondary costimulatory signals, resulting in proliferation and differentiation [11].

Besides cytotoxic $CD8^+$ T-cells, immunological responses are regulated by $CD4^+$ helper T-cells, whose subtypes have different characteristics, ranging from cytolytic activity, activation of innate and other adaptive immune cells, to propagating or dampening inflammation [12, 13]. T-helper 1 (Th1) cells are a key source of interleukin (IL)-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, which drive a cytotoxic immune response. They are highly effective in activating macrophages, but can also cause direct allograft damage through Fas/Fas ligand-mediated cytotoxicity [6, 12, 14]. Abundant evidence demonstrates the ability of Th1 cells to mediate acute rejection and CLAD [6, 12, 15, 16]. Th2 cells can produce a variety of cytokines (IL-4, -5, -6, -10, -13), some of which downregulate further cytokine production, while others promote humoral immunity [12, 13]. For example, a Th1/Th2 balance in favour of Th2 and IL-10 can reduce rejection rates, and on the other hand, Th2 cells can accelerate rejection by releasing proinflammatory and potent profibrotic mediators such as IL-6 and IL-13 [12, 17]. Next to cytokines, a complex network of chemokines and their receptors, which function to recruit and activate various leukocyte subsets, are involved in the inflammatory processes leading to the development of BOS or RAS (*e.g.*, CCR2/CCL2, CXCR2/ligand, CXCR3/ligand, and CCL5/RANTES interactions) [6, 18, 19] (figure 1).

Two other T-cell subtypes play important roles in the onset of CLAD. Firstly, Th17 cells, which secrete IL-6, IL-17, IL-22 and TNF-α, help to clear pathogens through recruitment and activation of neutrophils and macrophages, but are also associated with autoimmunity in cases of dysregulation or overproduction of IL-6 [13, 14]. Secondly, a unique subset of lymphocytes called regulatory T-cells (Tregs) have an important role in immune homeostasis [13]. Th17 and Tregs both develop from naive T-cells on stimulation by transforming growth factor (TGF)-β. IL-6, a proinflammatory cytokine, has a pivotal role in regulating the Th17/Treg balance, inducing the generation of IL-17-producing Th17 cells in concert with

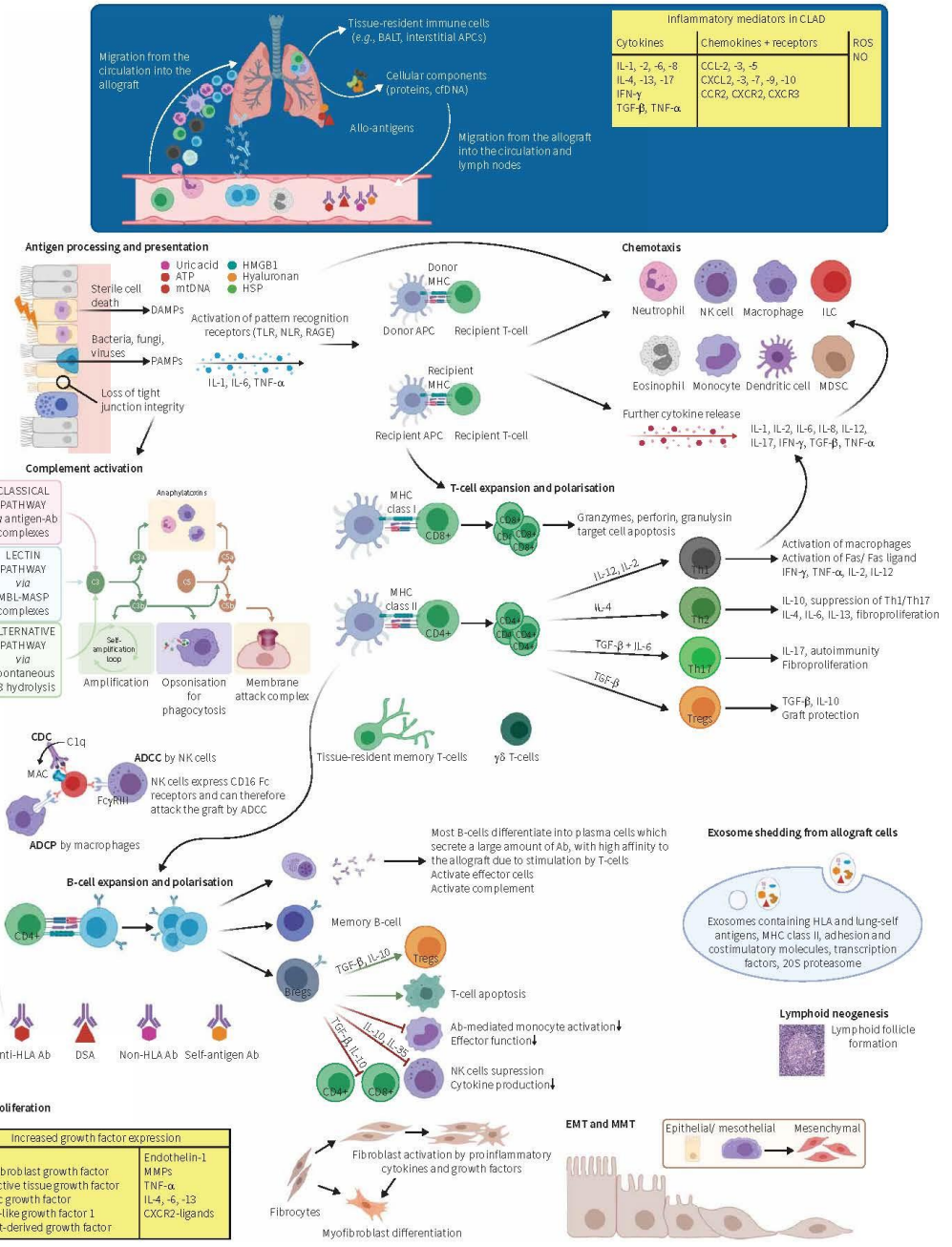

FIGURE 1 Key elements in the pathogenesis of chronic lung allograft dysfunction (CLAD). Overview of the pathogenesis of CLAD with some of the main immune mechanisms and cytokines involved. Tissue injury by alloimmune-dependent and -independent mechanisms induces the release of

tissue damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and inflammatory cytokines, followed by antigen presentation to donor and host antigen-presenting cells (APCs) by pattern-recognition receptors. This is followed by an advanced interplay between innate and adaptive immune responses, with infiltration of innate and adaptive immune cells into the allograft. Activation of alloreactive T- and/or B-cells and suppression of regulatory T-cells further perpetuate an inflammatory milieu. Finally, fibrotic growth factors are upregulated and (myo)fibroblasts are activated, leading to deposition of extracellular matrix and, ultimately, fibrosis and allograft dysfunction. Ab: antibodies; ADCC: antibody-dependent cellular cytotoxicity; ADCP: antibody-dependent cellular phagocytosis; ATP: adenosine triphosphate; BALT: bronchus-associated lymphoid tissue; Breg: regulatory B-cell; CCL: C-C motif ligand; CCR: C-C motif receptor; CDC: complement-dependent cytotoxicity; cfDNA: cell-free DNA; CXCL: C-X-C motif ligand; CXCR: C-X-C motif receptor; DSA: donor-specific antibody; EMT: epithelial–mesenchymal transition; HLA: human leukocyte antigen; HMGB1: high-mobility group box 1; HSP: heat-shock protein; IFN: interferon; IL: interleukin; ILC: innate lymphoid cell; MAC: membrane attack complex; MASP: MBL-associated serine protease; MBL: mannan-binding lectin; MDSC: myeloid-derived suppressor cell; MHC: major histocompatibility complex; MMP: matrix metalloproteinase; MMT: mesothelial–mesenchymal transition; mtDNA: mitochondrial DNA; NK: natural killer; NLR: nucleotide-binding oligomerisation domain-like receptor; NO: nitric oxide; RAGE: receptor for advanced glycation end products; ROS: reactive oxygen species; TGF: transforming growth factor; Th: T-helper; TLR: Toll-like receptor; TNF: tumour necrosis factor; Treg: regulatory T-cell. Figure partially created with BioRender.com

TGF-β, whilst inhibiting TGF-β-induced Treg differentiation [13, 14]. Both Th17/IL-17 and IL-6 are thought to be involved in the pathogenesis of CLAD, partly through endothelial cell activation and fibroblast activation and proliferation. IL-17 has also been shown to trigger a positive-feedback loop of IL-6 expression [13, 14, 20].

Tregs are essential components of the normal immune system and are responsible for maintaining homeostasis and balance activated immune responses. This is accomplished by the release of immunosuppressive cytokines (TGF-β, IL-10) as well as direct cell–cell interactions (*e.g.*, regulation of dendritic cell maturation and function) [11]. These actions prevent excessive effector T-cell responses [14]. By promoting the differentiation and/or activity of IL-10-secreting T-cells, Tregs also protect against autoimmunity [21]. Tregs have been shown to reduce the onset of CLAD and to establish immune tolerance in animal models [11, 12, 22]. Increased proportions of Tregs, especially in the lung allograft, seemed to stabilise allograft function, while a decline of this cell population has been described in progressive CLAD [11, 23–27].

Apart from classical $CD8^+$ cytotoxic cells, it has recently been shown that other cytotoxic cells with a senescent pattern are thought to be involved in CLAD development and associated with uncontrolled regulation by Tregs or immune checkpoints. For example, increased senescent T- and natural killer T-like lymphocytes with loss of CD28 expression were identified in BOS patients and correlated with increased expression of granzyme B, IFN-γ and TNF-α [28]. Brugière *et al.* [29] investigated whether the immune checkpoint HLA-G/immunoglobulin-like transcript (ILT)2 expressed by peripheral T-cell subpopulations could predict CLAD and found that an early increase after lung transplantation of cytotoxic $CD4^+CD57^+ILT2^+$ T-cells, selectively inhibited by HLA-G, may be associated with CLAD onset. The importance of the role of these cells remains to be confirmed in large cohorts, but could open new avenues for targeted therapies.

Little is known about the precise role of other T-cell subsets including T follicular helper cells, Th9 cells and Th22 cells in the lung transplant setting yet, and the exact role of memory T-cells and γδ T-cells in the onset of CLAD remains also unclear [22, 30]. Memory T-lymphocytes are commonly viewed as an important barrier to long-term survival of organ allografts; however, Krupnick *et al.* [31] demonstrated an unsuspected role in lung allograft tolerance of central memory $CD8^+$ T-cells, characterised by high surface expression of CD62 ligand and CD44, in a murine model. Further research on these T-cell subsets is warranted.

Humoral immunity

Traditionally, CLAD was thought to be primarily a manifestation of T-cell-mediated immune responses; however, antibodies and pathological alloreactive B-cells play a significant role in CLAD [7, 32]. HLAs have a crucial role in immune surveillance by presenting peptides to T-cell receptors [8]. T-cells are required for the growth and maturation of antigen-specific B lymphocytes, which produce alloantibodies against mismatched MHC and minor histocompatibility antigens [11, 21]. The presence of donor-specific antibodies (DSAs) is strongly associated with CLAD, through alloimmune responses, complement activation, and complement-independent mechanisms [10]. Moreover, anti-HLA antibodies can induce the release of fibrotic growth factors, including platelet-derived growth factor, insulin-like growth factor-1 and

TGF-β. These events culminate in the activation of myofibroblasts and extracellular matrix regeneration, thereby contributing to the development of CLAD [10, 11]. The onset of anti-HLA antibodies and subsequent complement activation are discussed in more detail in the section on antibody-mediated rejection (AMR).

Beyond their role in antibody production, B-cells can either contribute to or limit the development of CLAD *via* their regulation of T-cell immunity. B-cells influence T-cell responses through improved antigen presentation, co-stimulation, enhanced cytokine production, or induction of accommodation or tolerance [33–35]. However, the specifics of B-cell regulation in lung transplantation remain to be established, and the manifold and complex interactions between B- and T-cells are not yet fully understood [33]. A recent study demonstrated an increase in absolute peripheral B-cell count in patients with BOS, with a significant increase in specific subtypes of memory B-cells and a decrease in naive and transitional B-cells shown [36]. Similarly, Vandermeulen *et al.* [32] found higher levels of B-cells in BOS and RAS explant lungs and more lymphoid follicles in RAS tissue. The transformation of intragraft inflammatory infiltrates into tertiary lymphoid tissue, also called lymphoid neogenesis, probably also plays a role in lung allograft dysfunction, as has been reported in several other allograft types [10, 34].

Under some circumstances, humoral immune responses seem to cause little or no damage to the allograft. Accommodation describes a biological state in which the graft function remains stable despite alloantibodies or alloimmune responses, and is probably achieved by graft exposure to low concentrations of DSA or an altered affinity and/or specificity of the immune response [35]. Growing evidence demonstrates that B-cells also play a pivotal role in transplant tolerance [35]. Regulatory B-cells (Bregs) are thought to represent a stage of B-cell development before their differentiation into plasma cells and are potent inhibitors of the immune system, able to suppress allo- and autoimmune responses [33]. Bregs function, at least partly, through the production of IL-10, IL-35 and TGF-β, to suppress antigen presentation and cytokine secretion by APCs, T-cell proliferation, and actions from natural killer (NK) cells, neutrophils and other effector cells. Moreover, Bregs promote T-cell apoptosis and generation of Tregs by directly interacting with T-cell differentiation [35]. In addition to Bregs, other specific B-cell populations may be associated with long-term graft acceptance, such as IL-10 secreting $CD9^+$ transitional B-cells as described by Brosseau *et al.* [37].

As a result, B-cells are increasingly acknowledged as crucial mediators at the centre of immune regulation with the power to enhance or inhibit allograft immunity.

High incidences of CLAD have been described in patients with previous episodes of AMR, and DSAs are a strong risk factor for acute cellular rejection (ACR), AMR and CLAD [38–42]. Numerous studies have attempted to identify DSA characteristics that correlate with worse outcome. Patients with anti-HLA antibodies prior to transplantation had increased risk post-transplant of developing antibodies to HLA and non-HLA molecules, AMR, CLAD and mortality, although some reports failed to substantiate this. Moreover, there is currently no consensus on the use of peri-operative desensitisation protocols in these patients [43–48]. Post-transplant *de novo* DSAs are also strongly linked to acute and chronic rejection and graft failure [40, 45–47, 49–54].

Detailed examination of DSA characteristics identified a greater risk for AMR, BOS and allograft loss in patients with DSAs against class II MHC molecules, especially DQ, compared to class I [40, 44, 55, 56]. A link between the number of total HLA mismatches and incidence of BOS has also been described [57]. Furthermore, the impact of circulating DSA depends on its ability to bind complement. Generally, complement-binding DSA was associated with worse CLAD-free and graft survival compared to non-complement-binding DSA [43, 49, 55]. Patients who cleared DSA after therapy had greater freedom from BOS and better survival rates than those who did not, which suggests that ongoing lung injury in the setting of persistent DSA results in accelerated graft dysfunction [46, 55, 58]. Based on these findings, many centres frequently monitor for DSA using highly sensitive immunoassays [10]. However, antibodies detected in the blood do not necessarily represent antibodies acting on the graft [34]. Importantly, it has been recognised that DSA might be absent in serum yet persist in allograft tissue [59].

Few studies have distinguished the effects of DSA or AMR on the development of BOS *versus* RAS. Until recently, AMR was believed to mainly occur early after transplantation as (hyper)acute rejection. However, AMR is increasingly seen beyond the first year post-transplant, which is likely partly due to increased awareness and implementation of sensitive detection methods. This raises the possibility of chronic AMR as cause for CLAD [60]. Moreover, patients with chronic AMR or persistent DSA seemed to be more prone to develop RAS than BOS [10, 46]. In patients with RAS, the level of tissue-bound DSA in the

allograft seemed higher than in BOS, which might indicate a strong relationship with fibrosis [59]. It is appealing to consider whether RAS is an end-stage of chronic AMR, but definitive data are lacking to date and elevated B-cells, DSA and immunoglobulin G (IgG) were also seen in BOS, implying that chronic (less severe) AMR might also be a driving factor for CLAD phenotype BOS [32, 61].

Autoimmunity

A critical feature of the immune system is to establish effective cell-mediated and humoral responses to foreign antigens while remaining unresponsive to self-antigens. This is checked centrally by negative selection of immature $CD4^+$ T-cells recognising self-antigen and peripherally by anergy, apoptosis, and/or production of Tregs [11]. Mounting evidence has emerged that alloimmunity is not only directed against HLA, but also non-HLA and lung-associated self-antigens, suggesting a role for autoimmunity in the pathogenesis of CLAD [7, 21].

Collagen V (Col-V) and K-alpha 1 tubulin (Kα1T), two prominent self-antigens, are both components of small airways and are normally not exposed to the host immune system [7]. Col-V is found in the skin, lung epithelium and perivascular and peribronchial tissues, and placenta. It is an immunogenic self-protein that normally effectively masks its epitopes from the immune system because it is assembled in the same fibril as collagen I [11]. However, allograft injury (*e.g.*, due to ischaemia-reperfusion injury, infection, DSA) enhances exposure of these antigenic proteins and results in the release of lung-derived autoantigens as soluble antigens, exosomes or apoptotic bodies. These are detected and then presented by APCs leading to the propagation of autoimmune responses through the Th17–IL-17 axis [11, 21]. This is possibly initiated by increased cleavage of Col-V due to upregulation of matrix metalloproteinases (MMP) 2 and 9 [21, 62], alongside loss of peripheral tolerance due to downregulation of Tregs and loss of IL-10 response to self-antigens [10, 62, 63].

Kα1T is a gap junction protein, essential for cytoskeletal structure and normal cellular function [11]. Similar to Col-V, repeated injury of the airway epithelium exposes Kα1T, resulting in expression of transcription and growth factors involved in fibroproliferation, suggesting that antibodies to Kα1T are directly pathogenic [21, 64].

A strong correlation between these antibodies and CLAD has been reported, in some instances in the absence of classic HLA antibodies. Conversely, autoantibody-mediated graft damage can trigger *de novo* DSA generation [64–66]. Although DSA can be transient, antibodies to self-antigens are often persistent. In patients with antibodies to both DSA and self-antigens, those who cleared DSA but had persistent autoantibodies were significantly more likely to develop BOS [67]. Moreover, patients with pre-existing autoantibodies had increased risk of developing *de novo* antibodies to DSA and non-HLA, AMR, primary graft dysfunction and CLAD [21, 65, 66]. Large cohort studies revealed that up to 30% of patients undergoing lung transplantation had pre-existing antibodies to lung self-antigens, primarily in patients with idiopathic pulmonary fibrosis and cystic fibrosis [65].

Taken together, both pre-existing and *de novo* lung self-antigens contribute to acute and chronic lung rejection through an interplay between allo- and autoimmunity, in which allograft immune responses may trigger autoimmune responses, which in turn further activate alloimmune responses. While alloimmunity may have initiated allograft injury, autoimmunity may ultimately contribute to the progression of CLAD [10, 21].

Several other autoantibodies have been described in other solid organ transplant recipients, and data on these autoantibodies are gradually becoming available in lung transplant recipients [68]. Firstly, antibodies to MHC class I-related chain A, expressed on endothelial cells and monocytes, have been associated with increased graft failure after kidney transplantation [68]. Likewise, Lyu *et al.* [69] and Angaswamy *et al.* [70] described a correlation between these antibodies and BOS. Secondly, the presence of angiotensin type 1 receptor or endothelin type A receptor antibodies correlated with allograft rejection in kidney and heart transplants [71]. Reinsmoen *et al.* [71] investigated the impact of these antibodies on graft outcome in lung transplantation and reported a trend toward higher ACR rates and an increased risk of *de novo* DSA. Follow-up time was not sufficient to observe CLAD outcome.

Innate immunity

It has been increasingly recognised that an advanced interplay between innate and adaptive immunity drives graft injury. Several innate immune pathways facilitate recruitment of inflammatory cells into the allograft and are key elements in the pathogenesis of primary graft dysfunction, acute rejection, and CLAD [72]. Innate immunity encompasses a broad spectrum of immune responses mediated by elements that are

not reliant on gene rearrangement, including polymorphonuclear leukocytes, macrophages, NK cells and the complement system [72]. Innate recognition depends on pathogen- and damage-associated molecular patterns (PAMPs/DAMPs), recognised by pattern recognition receptors such as Toll-like receptors (TLRs), the receptor for advanced glycosylation endproducts and nucleotide-binding oligomerisation domain-like receptors [6]. DAMPs are endogenous molecules released from injured cells, such as high-mobility group box 1, heat-shock protein, hyaluronan, adenosine triphosphate, donor-derived cell-free DNA and mitochondrial DNA [72]. Recognition leads to immediate (sterile) inflammation, characterised by recruitment of mainly neutrophils and macrophages, upregulation of MHC expression and antigen presentation, followed by activation of the adaptive immune system (figure 1) [10]. The exact immune mechanisms in RAS have yet to be elucidated, but DAMPs appeared to be upregulated to a greater extent compared to BOS [73].

TLRs are transmembrane receptors mainly expressed by macrophages and dendritic cells, serving as a bridge between innate and adaptive immunity because of their ability to induce T-cell responses [10]. On the other hand, TLRs might contribute to CLAD directly [10]. For example, TLR4 signalling can induce fibroblast activation together with TGF-β, and in the case of sustained innate immune activation, the process of fibroblast activation might persist, leading to excess repair and fibrotic tissue remodelling [72].

Neutrophils play an important role not only in innate immunity, but also by enhancing antigen presentation and Th1-driven alloimmune responses [7]. Elevated bronchoalveolar lavage (BAL) and allograft neutrophilia have been repeatedly observed in patients with BOS and RAS, and early or persistent BAL neutrophilia correlated with subsequent CLAD occurrence [32, 74–79]. The relevance of neutrophils was further supported by the emergence of neutrophilic reversible allograft dysfunction, characterised by IL-17-mediated airway neutrophilia, in which azithromycin was able to attenuate pulmonary function decline [80]. IL-17 can induce IL-8, a major neutrophil chemo-attractant. Multiple studies demonstrated higher levels of BAL IL-8 in BOS patients with a correlation between neutrophils and IL-8 levels [77, 79]. IL-8 is secreted by alveolar type II epithelial cells, bronchial epithelial cells and macrophages after the release of proinflammatory cytokines [81]. In some patients, neutrophilia was not suppressed or redeveloped despite azithromycin, suggesting a non-IL-17-dependent pathway. Indeed, Vandermeulen *et al.* [80] found worse CLAD-free and overall survival in those patients, possibly driven by increased levels of IL-1β and IL-1β-induced proinflammatory cyto-/chemokines (*e.g.*, IL-6, IL-8, macrophage inflammatory proteins, eosinophil attractants). Suwara *et al.* [78] also demonstrated an increase in IL-1α and IL-1β in patients with persistent airway neutrophilia. Activated neutrophils have remarkable potential to cause tissue damage through a variety of mechanisms: 1) release of large quantities of reactive oxygen species, 2) release of cytokines, 3) activation of hydrolytic enzymes and proteases, 4) expression of MMP that leads to degradation of collagen matrix [82]. An additional mechanism of neutrophil-mediated injury is the formation of neutrophil extracellular traps (NETs), a process known as NETosis. NETs are extracellular networks of DNA clad with granular proteins that were cast out from neutrophils and are thought to be an effector function of neutrophils [82] (figure 2).

Eosinophils have also been implicated in the pathological process of CLAD. Two decades ago, Scholma *et al.* [83] had already noted that BAL eosinophilia correlated with increased BOS risk (RAS was not yet identified then). Likewise, a more recent study demonstrated a significant correlation between BAL eosinophilia and the development of CLAD, in particular RAS, and mortality [84]. The same group also found higher eosinophil levels in allograft tissue from RAS patients, and worse CLAD-free survival in patients with high blood eosinophils [32, 85]. Additionally, Darley *et al.* [86] demonstrated that detection of eosinophils on transbronchial biopsies was independently associated with an increased risk of CLAD and mortality. The actions of eosinophils are thought to be secondary to profibrotic features, by attracting fibroblasts and stimulating TGF-β release, as well as through toxic effects on airway epithelial cells (*e.g.*, increased membrane permeability, ciliary damage) [84, 86]. Conversely, translational data from animal models recently illustrated a role for eosinophils in the downregulation of alloimmunity, potentially by the release of suppressive molecules or interactions with dendritic cells and lymphocytes [87]. These immunosuppressive effects are presumably exerted by a different subtype of eosinophils, such as tissue-resident eosinophils, although this needs to be further elucidated [87].

NK cells act as the first line of defence against infected or transformed cells and can directly respond to alloantigens and non-self cells through an arsenal of effector functions that are vital in innate–adaptive bridging [88, 89]. Increased numbers of activated NK cells were found in the lungs of CLAD patients, with corresponding peripheral blood depletion, suggesting systemic activation and subsequent migration into the allograft tissue [89]. Once activated, NK cells release a wide range of cytolytic proteins, such as granzymes and perforin, and chemotactic cytokines such as IFN-γ and TNF-α, which were found to be

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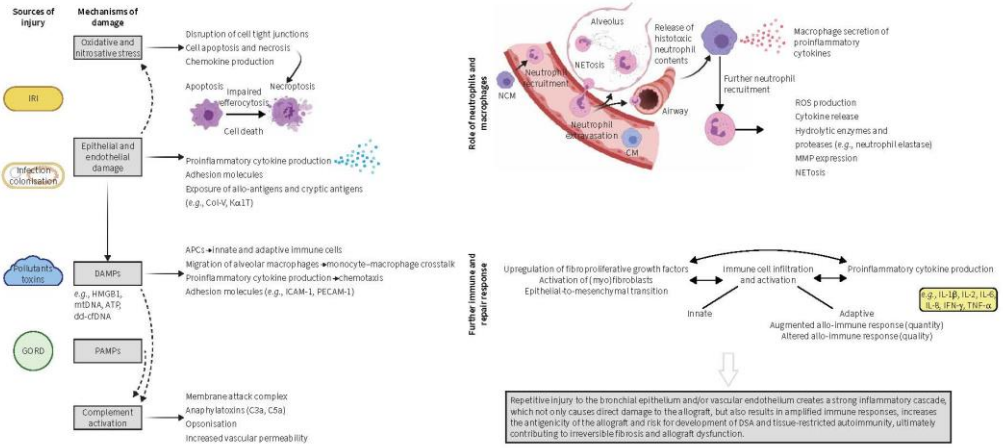

FIGURE 2 Non-alloimmune factors contributing to chronic lung allograft dysfunction (CLAD). Simplified representation of pathways involved in non-alloimmune mechanisms which may contribute to CLAD onset. APC: antigen-presenting cell; ATP: adenosine triphosphate; CM: classical monocyte; Col-V: collagen V; DAMP: damage-associated molecular pattern; dd-cfDNA: donor-derived cell-free DNA; DSA: donor-specific antibody; GORD: gastro-oesophageal reflux disease; HMGB1: high-mobility group box 1; ICAM-1: intercellular adhesion molecule 1; IFN: interferon; IL: interleukin; IRI: ischaemia-reperfusion injury; Kα1T: K-alpha 1 tubulin; MMP: matrix metalloproteinase; mtDNA: mitochondrial DNA; NCM: non-classical monocyte; NET: neutrophil extracellular trap; PAMP: pathogen-associated molecular pattern; PECAM-1: platelet endothelial cell adhesion molecule 1; ROS: reactive oxygen species; TNF: tumour necrosis factor. Figure partially created with BioRender.com

upregulated in CLAD [90]. Through the release of these cytokines, NK cells commit T-cells, skew immune responses to Th1, increase MHC class I and II expression, and induce graft infiltration by macrophages, dendritic cells and neutrophils [90]. Moreover, NK cells' upregulation of Fc-receptors plays an important role in antibody-dependent T-cell-mediated cytotoxicity [88].

There is mounting evidence that NK cells have crucial and sometimes opposing roles in lung allograft rejection, due to either activating or inhibitory actions through different NK receptors [88]. NK cells might enhance CLAD through the above-described cytotoxic and inflammatory effects. On the other hand, it has been postulated that they may promote graft tolerance through depletion of donor APC and alloreactive T-cells *via* killer immunoglobulin-like receptors or possibly *via* IL-15–IL-15Ra complex expansion [88, 91]. Nonetheless, the exact mechanisms by which NK cells contribute to CLAD remain to be investigated.

Other innate lymphoid cells (ILC1, ILC2, ILC3) are a recently recognised and understudied group of immune cells, which are difficult to analyse because of their tissue-resident and lineage negative features. However, they can exert different actions such as type 1 immunity with macrophage activation and cytotoxicity, type 2 immunity and the formation of tertiary lymphoid structures by their different subtypes *via* the release of IFN-γ, granzymes, perforin, TNF-α, IL-13, IL-17, *etc*. [92, 93]. As such, it does not seem unlikely that they contribute to the pathogenesis of CLAD, and further investigation is warranted [94–96].

The complement system is a complex immune surveillance system made up of a cascade of multiple proteins, crucial in innate defence, and it plays a role in adaptive immunity *via* cell-mediated and humoral processes [97]. There are three different activation pathways (classical, lectin and alternative), all leading to the formation of a membrane attack complex which induces cell lysis. Furthermore, it stimulates immune complex clearing by opsonisation and, during activation of the complement cascade, signalling components known as anaphylatoxins are released and are capable of summoning various other innate and adaptive immune cells by stimulation of proinflammatory cytokines and chemotaxis [97]. Complement activation plays a role in the pathogenesis of primary graft dysfunction and AMR, which are risk factors for CLAD, but a direct contribution in the pathogenesis of CLAD is also assumed. Deposition of complement factors C1q, C3d and C4d in lung allografts was found to be independently associated with CLAD [97]. Higher levels of complement and IgG deposition were found in RAS compared to BOS patients, pointing to the role of humoral immunity and activation of B-cells in RAS, and the possible overlap between AMR and RAS [98, 99]. Several studies yielded some evidence that mannose-binding lectin, part of the lectin pathway, is involved in CLAD development. Higher levels were found in BOS *versus* stable patients, and presence of mannose-binding lectin at 3 and 6 months post-transplant correlated with later onset of BOS [97].

In conclusion, innate immune responses provide an early, robust trigger that augment adaptive alloimmunity, ultimately promoting CLAD development.

Exosomes

Recently, exosomes have begun to attract attention as a trigger in CLAD development through activation of cellular and humoral immunity. Exosomes are dual-layer membrane vesicles which can contain HLA and lung self-antigens, adhesion and costimulatory molecules, MHC class II molecules, transcription factors, and 20S-proteasome. They are shed from allograft cells after lung injury and are highly efficient in presenting antigens to the immune system [100–102]. Exosomes have been shown to induce T-cell-mediated immune responses, and the induction and continuous release of exosomes from the allograft may stimulate the process of CLAD [21]. Furthermore, a recent animal study demonstrated the ability of exosomes, derived from lung transplant recipients with respiratory viral infections, to induce epithelial-to-mesenchymal transition (EMT) [103]. The role of exosomes in promoting EMT has been highlighted in cancer research and could be another way by which exosomes might initiate the process leading to CLAD [103]. Several studies demonstrated higher levels of exosomes, which also contained more of the aforementioned factors, in BOS patients [101, 104]. Furthermore, Sharma *et al.* [102] found that increased levels of circulating exosomes preceded the onset of BOS and could be detected 6–12 months before diagnosis.

Genetic variants associated with CLAD

Several donor- and recipient-related genetic variants may contribute to the development of CLAD [4]. Specific single nucleotide polymorphisms in TLR2, -4 and -9, were associated with a higher incidence of BOS [105]. Other types of polymorphisms in TLR4 correlated with a reduced risk of acute rejection and a trend toward reduced onset of BOS [106]. These findings again reinforce the importance of the link between innate immune responses and alloimmune response in the development of CLAD.

A polymorphism in HLA-G seemed to have a protective role by modulating cytotoxic T-cells and NK cells, while a specific HLA-E allele negatively influenced CLAD onset [107]. There is some evidence that functional polymorphisms in the genes of CD14, dectin-1, IFN-γ, IL-6, IL-17A, killer immunoglobulin-like receptors, mannose-binding lectin, MMP-7 and TGF-β1 are linked to CLAD development [108–110]. Regarding donor-related polymorphisms, gene polymorphisms in surfactant proteins, donor Clara cell secretory proteins, mannose-binding lectin and CD59 correlated with increased CLAD risk [97, 111–114].

In general, these genetic variants affect the innate defence system, altering immune responses to injury, possibly increasing susceptibility for airway inflammation or allograft infection, thereby contributing to the pathogenesis of CLAD [4].

Repair and regeneration processes

Aberrant epithelial repair

Dysregulated epithelial repair and airway and/or tissue remodelling are cornerstones in the pathogenesis of CLAD [115]. Repetitive or persistent alloreactive, autoreactive, infective or non-specific epithelial injury leads to the loss of epithelial integrity and dysregulated repair [6]. A disbalance between pro- and anti-inflammatory cytokines can induce an excessive fibroblastic response with excessive extracellular matrix remodelling, leading to small airways and/or parenchymal fibrosis [115]. Multiple growth factors are involved in this process and are secreted by epithelial cells, fibroblasts and inflammatory cells [6]. TGF-β1 plays a key role, by inducing fibroblast proliferation and differentiation into myofibroblasts [116]. The process by which the normal epithelium is replaced by fibroblastic scar tissue is believed to be based on TGF-β1-driven EMT, as illustrated in animal models and *in vitro* [115–117]. This can be further stimulated by activated macrophages *via* TNF-α [116]. During EMT, epithelial cells lose their epithelial properties and acquire a mesenchymal cell phenotype, including deposition of extracellular matrix and production of MMPs [115]. A similar mechanism has been postulated in RAS. Indeed, *in vitro* treatment of human pleural mesothelial cells with TGF-β1 led to mesothelial-to-mesenchymal transition [118].

MMPs, derived from bronchial/bronchiolar airway epithelium and parenchymal cells, are capable of degrading extracellular matrix proteins, cleaving collagen, and are involved in cell proliferation, migration and apoptosis [119]. Significantly increased MMP levels (MMP-2, -3, -7, -8, -9) were found in BAL and airway epithelial cells from BOS patients, and excess MMP activity may facilitate uncontrolled extracellular matrix turnover, epithelial damage, fibrosis and tissue remodelling [119–121]. In addition to epithelial cells, neutrophils may be another source of MMPs, able to store and release MMPs from their granules [119]. Several studies showed that MMP-8 and -9 levels correlated with BAL neutrophilia in patients with BOS, and along with their role in tissue remodelling, these MMPs may also perpetuate neutrophilic inflammation *via* a self-sustaining loop [119, 121].

In addition to TGF-β, liver kinase B1 (also known as serine-threonine kinase 11) might also have a role in the process of EMT in CLAD. Liver kinase B1 is a protein kinase that activates adenosine monophosphate-activated protein kinase and many related kinases, and regulates cell growth, cell polarity, cell metabolism and autophagy [122, 123]. Hereby, liver kinase B1 inhibits EMT and tissue fibrosis and Rahman *et al.* [122] recently demonstrated that liver kinase B1 was significantly downregulated in patients with BOS.

Most studies analysing profibrotic mediators in CLAD focus on (myo)fibroblasts, TGF-β, TNF-α, MMPs and tissue inhibitors of metalloproteinases. With respect to other common fibrotic factors, little is known about the role of connective tissue growth factor (CTGF) in CLAD, though it is an important mediator in several fibrotic diseases, such as idiopathic pulmonary fibrosis. A recent study demonstrated higher levels of tissue CTGF expression in BOS and RAS compared to controls. Interestingly, BAL levels of CTGF were higher in RAS compared to BOS and stable patients, and also elevated at 3 months post-transplant in future RAS patients, perhaps suggesting a more specific role for CTGF in the pathogenesis of RAS [124].

Angiogenesis and vascular changes

Besides epithelial injury, damage to the airway microvasculature also seems important. Lung tissue analyses showed that obliterative bronchiolitis was associated with increased angiogenic activity, and vascular remodelling was an important feature of tissue remodelling [6, 125]. Airway inflammation itself appeared to be the main determinant of this angiogenic remodelling, through proinflammatory cyto-/chemokines, with a smaller role for vascular endothelial growth factor [125]. Regardless, the role of angiogenesis in CLAD remains incompletely understood with opposing findings in the literature [126]. Further research on vascular changes in CLAD is needed, especially given a recent study showed that

nearly half of BOS patients had chronic vascular abnormalities (*e.g.*, pulmonary arteriopathy and venopathy, bronchial arterial vasculopathy) [61].

Alloimmune-dependent risk factors

Acute cellular rejection and lymphocytic bronchiolitis

Alloimmune-dependent factors such as ACR and lymphocytic bronchiolitis (LB) are strongly linked to CLAD [6]. ACR has been studied in more detail, but independent of acute vascular rejection, the onset and severity of LB is also associated with long-term outcomes after lung transplantation and an increased risk of BOS and death [127]. ACR and LB are driven by T-lymphocytes and many actions are similar as described in CLAD such as a predominance of Th1 cells with increased production of IFN-γ, IL-2 and TNF-α, activation of macrophages, direct allograft damage through Fas/Fas ligand-mediated cytotoxicity, and reduced Tregs [16, 25, 122]. There is currently too little evidence, but donor tissue-resident memory T-cells may play a protective role in ACR [22].

Although T-lymphocytes are regarded as the main culprit in ACR, other mechanisms contribute as well. It is well known that increased leukocytes, including lymphocytes and neutrophils, are found in BAL and tissue of patients with ACR, and increasingly more awareness is given to eosinophils and NK cells [84, 128–131]. Not much is currently known about the role of B-cells in acute rejection, but a recent study reported a decrease in the number of Bregs in peripheral blood and BAL during acute rejection and the role of B-cells in local lymphoid follicle formation could also be of importance in LB [16, 132]. It is worth noting that HLA antibodies do not only appear to be involved in the onset of AMR and CLAD, but also in ACR and LB [133, 134]. On the other hand, ACR and LB may predispose to *de novo* DSA [135].

In LB, there is convincing evidence of an IL-17-mediated pathway, which triggers IL-8-driven neutrophilic airway inflammation [130, 136]. Verleden *et al.* [136] found that patients with LB had significantly more IL-17$^+$ cells on transbronchial biopsies compared to patients with ACR, and the number of IL-17$^+$ cells correlated with BAL neutrophilia. Not Th17 cells, but CD8$^+$ T-cells were the major source of this IL-17 production, which could be attenuated by azithromycin [137].

Ultimately, the alloreactive T-cell response and IL-17-mediated inflammation generate a profibrotic environment which can contribute to CLAD [130].

Antibody-mediated rejection

AMR results from the recipient's immune system recognising pre-existing or *de novo* antibodies to HLA, non-HLA or self-antigens [60]. Pre-existing anti-HLA antibodies may arise after prior sensitizing events such as pregnancy, blood transfusion or organ transplantation [41]. Risk factors for *de novo* DSA are only beginning to be identified. It is postulated that immunising events (*e.g.*, transfusion, ACR) and lung injury (*e.g.*, ischaemia-reperfusion injury, allograft infection) upregulate the expression of HLA molecules, thereby increasing the graft's immunogenicity [60]. Antibodies may develop to MHC class I antigens (HLA-A, -B, -C) which are expressed on nearly all nucleated cells, or MHC class II antigens (HLA-DQ, -DR, -DP) on professional APCs [41].

The binding of antibodies to directly accessible allogenic targets expressed by endothelial cells activates the classical complement pathway. This begins with binding of C1q, and eventually leads to membrane attack complex formation and cytotoxicity [34, 41]. Deposition of complement and IgG in lung allograft tissue have both been demonstrated [41, 138]. Activation of endothelial cells leads to the release of adhesion molecules and cytokines that, together with anaphylatoxins C3a and C5a, attract neutrophils, monocytes and NK cells to the allograft, propagating inflammation and graft injury [34, 41]. Complement-mediated allograft injury is a defining pathophysiological characteristic of AMR. However, graft injury can also occur independently of complement pathways through antibody-dependent cell-mediated cytotoxicity [88]. The latter is likely mediated by NK cells, recognising antibodies through their Fc-receptor, CD16, although antibodies can also bind the Fc-receptor of myeloid cells such as macrophages and neutrophils. In this way, antibodies bridge the innate and adaptive arms of the immune system [88].

Both complement-dependent and -independent mechanisms lead to the production of IFN-γ and other proinflammatory cyto-/chemokines, increased MHC expression, recruitment of leukocytes and platelets, amplification of innate and adaptive immunity, and upregulation of adhesion molecules and fibroblast growth factor receptor on endothelial cells. All these mediators contribute to microangiopathy, tissue injury and graft dysfunction [9, 41, 88].

Not all patients with DSA develop AMR, the clinical relevance of DSA may depend on the variable pathogenicity of IgG subclasses. Complement-binding IgG (IgG1, IgG3) seemed more damaging than non-complement-binding IgG (IgG2, IgG4) [43, 55]. Higher rates of early BOS were found in cases of increased C3d and C4d deposition early after transplantation [139]. Similarly, DSA-positive patients with increased C3d deposition had lower graft survival than those without C3d activation [138].

Although AMR might be a reversible cause of acute graft dysfunction, it generally portends a poor prognosis with a high incidence of CLAD amongst survivors and worse long-term survival compared to ACR [60].

Alloimmune-independent risk factors

In addition to immune-mediated lung injury, various other factors have been linked to the onset of CLAD, including ischaemia-reperfusion injury, respiratory infections, gastro-oesophageal reflux, air pollution and (inhaled) toxins [4]. Lung allografts are uniquely susceptible to injury from exogeneous agents due to their constant exposure to the external environment and, since the oesophagus and trachea are anatomically connected, the lung is at risk of exposure to gastric contents through gastro-oesophageal reflux and (micro) aspiration [7, 72].

In general, it is postulated that these "alloantigen-independent" lung injuries contribute to CLAD by direct damage to the allograft epithelium and/or endothelium as well as upregulating the tissue inflammatory milieu. The induction of a strong inflammatory cascade by epithelial injury directs an alloimmune response *via* downstream effects, promoting clonal expansion of alloreactive T- and B-cells, upregulation of HLA class II molecules and enhanced antigen presentation. This facilitates allorecognition, thereby increasing the antigenicity of the allograft and risk for development of DSA as well as tissue-restricted autoimmunity. This ultimately predisposes to CLAD through subsequent recruitment of fibroproliferative growth factors, excessive airway/tissue remodelling, and eventually airway/tissue fibrosis and allograft dysfunction [6, 7, 140].

Some of the main mechanisms involved in these non-alloimmune factors are displayed in figure 2.

Missing pieces of the puzzle

Based on these findings, we believe future research in the lung transplant setting should focus on:

- The role of Tregs in preventing or slowing down CLAD onset and progression.
- The role of Th9 and Th22 T-cell subsets, memory T-cells, and γδ T-cells in the pathogenesis of CLAD.
- The specifics of B-cell regulation and interactions between B- and T-cells in CLAD pathogenesis, and the possible role of Bregs in immunomodulation and suppression of immune responses in CLAD.
- Subtypes of innate immune cells (*e.g.*, eosinophils, NK cells, macrophages) and their potential to promote or inhibit alloimmune responses in CLAD.
- Description of (chronic) vascular changes in BOS and RAS, and the role of lymphoid neogenesis and angiogenesis in the onset of CLAD.
- How to deal with anti-HLA, non-HLA and autoantibodies prior to and after transplantation.
- Identification of specific immune cells or profibrotic pathways (*e.g.*, EMT) which are targetable for treatment.
- Ways to establish immune tolerance after lung transplantation.

Conclusion

Over the last decades, we have gained a better understanding of how the immune system contributes to the development of CLAD, although the exact pathophysiological mechanisms are still not completely understood. Complex and overlapping immune-mediated mechanisms, including cellular, humoral, innate, adaptive and autoimmune processes, have been implicated as the leading causes of CLAD. It is increasingly recognised that non-alloimmune mechanisms have a crucial role due to (repetitive) epithelial injury, creating a privileged immune microenvironment, resulting in amplified immune responses. The central belief is that CLAD is the end-stage of a disease continuum marked by continuous/repeated lung injury, immune activation, tissue remodelling and repair, ultimately leading to irreversible fibrosis and allograft failure.

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Appendix B: Publication "Lymphocyte depleting and modulating therapies in chronic lung allograft dysfunction"

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PHARMACOLOGICAL REVIEWS

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Lymphocyte Depleting and Modulating Therapies for Chronic Lung Allograft Dysfunction

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***Abstract*——Chronic lung rejection, also called chronic lung allograft dysfunction (CLAD), remains the major hurdle limiting long-term survival after lung transplantation, and limited therapeutic options are available to slow the progressive decline in lung function. Most interventions are only temporarily effective in stabilizing the loss of or modestly improving lung function, with disease progression resuming over time in the majority of patients. Therefore, identification of effective treatments that prevent the onset or halt progression of CLAD is urgently needed. As a key effector cell in its pathophysiology, lymphocytes have been considered a therapeutic target in CLAD. The aim of this review is to evaluate the use and efficacy of lymphocyte depleting and immunomodulating therapies in progressive CLAD beyond usual maintenance immunosuppressive strategies. Modalities used include anti-thymocyte globulin, alemtuzumab, methotrexate, cyclophosphamide, total lymphoid irradiation, and extracorporeal photopheresis, and to explore possible future strategies. When considering both efficacy and risk of side effects, extracorporeal photopheresis, anti-thymocyte globulin and total lymphoid irradiation appear to offer the best treatment options currently available for progressive CLAD patients.**

***Significance Statement*——Effective treatments to prevent the onset and progression of chronic lung rejection after lung transplantation are still a major shortcoming. Based on existing data to date, considering both efficacy and risk of side effects, extracorporeal photopheresis, anti-thymocyte globulin, and total lymphoid irradiation are currently the most viable second-line treatment options. However, it is important to note that interpretation of most results is hampered by the lack of randomized controlled trials.**

I. Introduction

Lung transplantation is a life-saving therapeutic option in well-selected patients with end-stage chronic lung diseases. Advancements in surgical techniques and early post-transplant care, such as maintenance immunosuppressive therapy and management of infections, have improved post-transplant outcomes in the past decades (Bos et al., 2020). Nevertheless, survival after lung transplantation still lags behind that of recipients of other solid organ transplants, with a median post-transplant survival of only 6.7 years (Chambers et al., 2019). To a larger extent, this poor long-term survival is related to the high incidence of and difficulty managing chronic lung rejection, so-called chronic lung allograft dysfunction (CLAD), a progressive life-threatening condition affecting 50% of patients within five years post-transplant, leading to lung allograft failure, respiratory insufficiency and death (Chambers et al., 2019).

CLAD encompasses two main phenotypes, bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS), along with a mixed phenotype with features of both. BOS is the commonest phenotype in approximately 70% of CLAD patients and is characterized by progressive airway obliteration leading to airflow obstruction. RAS occurs in up to 20%–30% of CLAD patients and is characterized by parenchymal and/or pleural fibrosis with a restrictive pulmonary function decline. RAS has a very poor prognosis, with a median survival of only 1-2 years after diagnosis compared with 3–5 years for BOS. The diagnosis of CLAD is made based on a decline in forced expiratory volume in one second (FEV_1) of $\geq$20% from post-transplant baseline, defined as the mean of the two best post-operative FEV_1 measurements taken >3 weeks apart, in combination with a concurrent decline in forced vital capacity of $\geq$20% and persistent opacities on chest imaging for the RAS phenotype (Verleden et al., 2019). CLAD leads to a progressive decline in FEV_1; this decline is often stepwise, in which after an initial decrease a plateau phase is reached. However, some patients have a steep and rapidly progressive decline, while others have a slower decline over years (Belperio et al., 2009; Sato et al., 2013). CLAD severity is graded from 1–4 based on the severity of FEV_1 decline (stage 1: 66–80%, stage 2: 51–65%, stage 3: 36–50%, stage 4: $\leq$35% of baseline) (Verleden et al., 2019).

It is postulated that CLAD occurs as a result of the host's adaptive and innate immune responses directed to the lung allograft, in which a complex array of immune cells and mechanisms is involved (Bos et al., 2022b,c). Next to medical non-compliance with immunosuppressive treatment, various risk factors for CLAD have been identified, both alloimmune and non-alloimmune factors, including ischemia-reperfusion injury, acute cellular rejection, antibody-mediated rejection, respiratory infections, gastroesophageal reflux, and air pollution (Verleden et al., 2019).

The type of standard immunosuppressive maintenance treatment after lung transplantation varies between centers, but usually consists of triple therapy with a calcineurin inhibitor (tacrolimus/cyclosporine), a cell cycle inhibitor (mycophenolate mofetil/azathioprine) and corticosteroids (Nelson et al., 2022). Currently, therapeutic options to slow the progressive

ABBREVIATIONS: ATG, anti-thymocyte globulin; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; ECP, extracorporeal photopheresis; FEV_1, forced expiratory volume in one second; GvHD, graft-*versus*-host disease; JAK, Janus kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; RAS, restrictive allograft syndrome; TLI, total lymphoid irradiation; TNFα, tumor necrosis factor alpha.

decline in lung function in CLAD are very limited. These include intensification and optimization of maintenance immunosuppression, such as augmentation of corticosteroids and switching to more potent maintenance immunosuppressive drugs, such as from cyclosporine to tacrolimus and azathioprine to mycophenolate mofetil (Nelson et al., 2022). This, often in combination with the addition of azithromycin (if not already initiated as preventive treatment post-transplant), is usually instituted as an early measure to aim to halt CLAD progression (Verleden et al., 2019). The immunomodulatory properties of azithromycin in CLAD are summarized in a review by Vos et al. (Vos et al., 2012).

Beyond this first line of treatments, several lymphocyte depleting and/or modulating therapies have been studied in patients with progressive CLAD, including methotrexate, cyclophosphamide, alemtuzumab, anti-thymocyte globulin (ATG), total lymphoid irradiation (TLI), and extracorporeal photopheresis (ECP). Most of these therapies have only been evaluated in small retrospective single-center studies, and the effect reported is often temporary with further disease progression over time in the majority of patients. Therefore, there is a compelling need for more effective treatments to prevent the onset and progression of CLAD (Verleden et al., 2019).

This review summarizes the data available to date on the efficacy of lymphocyte depleting and modulating therapies in CLAD beyond optimized maintenance immunosuppressive strategies and explores possible future directions in this area. For this, the electronic databases of PubMed and EMBASE were searched in July 2022 and publications related to our predefined topic were included. There is little data available on use of these modalities for the treatment of RAS, as such, most of the data presented in this review focuses on experience from the treatment of BOS.

II. Immunodepleting Therapies

A. Alemtuzumab

Alemtuzumab is a recombinant humanized IgG1 monoclonal antibody directed against CD52, which is expressed on the cell surface of mainly T and B lymphocytes, and to a lesser extent on natural killer cells, macrophages, and monocytes, and is believed to play a role in cell signaling and homeostasis (Bhowmick et al., 2016; Syed, 2021). Alemtuzumab induces a rapid, profound and prolonged (i.e., several months) lymphocyte depletion through antibody-dependent cell-mediated cytolysis, complement-dependent cytolysis, and induction of apoptosis, but also leads to an expansion of regulatory T and B cells during repopulation (Bhowmick et al., 2016) (Fig. 1). Because of prolonged lymphodepletion, the potential for sustained bone marrow suppression is of concern, especially given the susceptibility of lung transplant recipients to infections and malignancies (Trindade et al., 2020).

Alemtuzumab has been used primarily for the treatment of chronic lymphocytic leukemia (Hallek, 2017) and relapsing-remitting multiple sclerosis (Syed, 2021), but also off-label for induction immunosuppression in solid organ transplantation (Small et al., 2022).

1. Evidence in CLAD. In an effort to more effectively deplete T cells and other immune cells that may contribute to CLAD, Reams et al. investigated the effect of alemtuzumab (30 mg i.v.) in ten BOS patients after failure of prior therapy with methylprednisolone and ATG (Reams et al., 2007). They found a stabilization or improvement of BOS stage in 70% of patients. Alemtuzumab caused a long-lasting decrease in CD4 count and only 27% of patients remained free of infectious complications in the entire cohort, which also included patients with acute cellular rejection (Reams et al., 2007). Another study involving 17 BOS patients mainly demonstrated efficacy of alemtuzumab (30 mg i.v.) in early BOS. BOS-free progression was seen in 53% of patients at 6 months with freedom from FEV_1 decline >10% in 70% of early BOS (stage 1) *versus* only 14% in advanced BOS (stage 2–3). Also, in this study, the infection rate was high (77%) (Ensor et al., 2017).

Moniodis et al. compared the efficacy of alemtuzumab (30 mg i.v. or s.c.) ($n = 13$) to ECP ($n = 17$) for the treatment of CLAD (Moniodis et al., 2018). The rate of FEV_1 decline improved significantly at 3 and 6 months in both groups, compared with pre-treatment, with a benefit also at 1 month in the alemtuzumab group. Subgroup analyses for alemtuzumab in RAS only showed a slowing in slope at 3 months, while the BOS subgroup resembled the overall CLAD cohort. Interestingly, alemtuzumab reduced the number of rapid decliners (>25% drop FEV_1) more markedly than ECP at 1, 3, and 6 months following treatment. There were no differences between alemtuzumab and ECP with regard to infections, with 29% of alemtuzumab-treated patients having a clinically significant infection in the year after treatment. There was no difference in survival at 6 months and 1 year between the alemtuzumab, ECP, and untreated (i.e., slowly progressive CLAD) group (Moniodis et al., 2018).

Trindade et al. examined the safety of alemtuzumab in a specific group of lung transplant recipients with short telomeres who are at increased risk of clinically significant leukopenia (Trindade et al., 2020). In this small study (14 CLAD patients of whom three with short telomeres), alemtuzumab treatment appeared safe, with no significant difference in infections necessitating hospitalization, although it was associated with an increased incidence of neutropenia, thrombocytopenia, and anemia in the short telomere group (Trindade et al., 2020).

Lastly, a conference abstract, looking at 1-year overall survival after alemtuzumab administration in 14

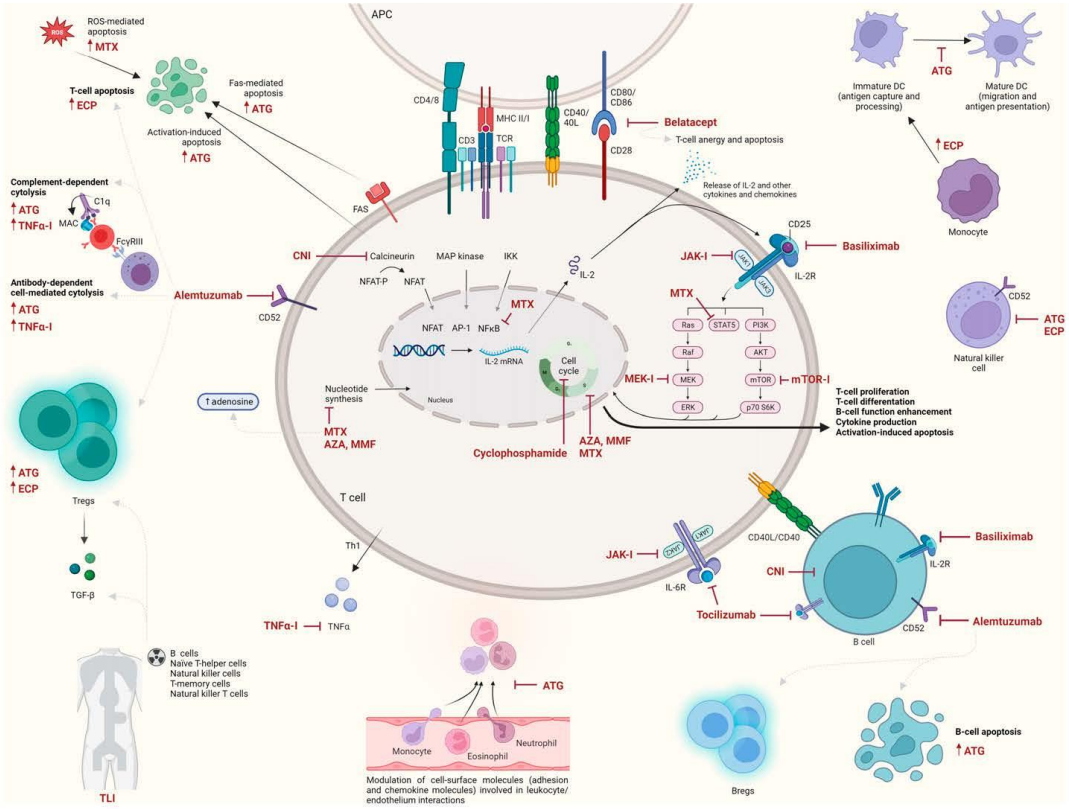


Fig. 1. Overview of main mechanisms of several lymphocyte depleting and/or modulating therapies for CLAD. APC, antigen-presenting cell (e.g., dendritic cell, macrophage, B cell); AZA, azathioprine; Bregs, regulatory B cells; CNI, calcineurin inhibitor; DC, dendritic cell; JAK-I, janus kinase inhibitor; MEK-I, mitogen-activated protein kinase kinase inhibitor; MMF, mycophenolate mofetil; MTX, methotrexate; TNFα-I, tumor necrosis factor alpha inhibitor; Tregs, regulatory T cells. Created with BioRender.com.

patients with severe CLAD, reported that 64% were alive with a stable FEV_1 in 67% of survivors (Thachuthara-George et al., 2015). Another conference abstract documented that the rate of lung function decline during the 3 months post-treatment (30 mg s.c.) was significantly lower than the 3 months prior to treatment in eight BOS patients with rapid loss of lung function (75% stage 3–4). Clinically symptomatic infections occurred in 50% of patients (Girgis et al., 2020).

Treatment with alemtuzumab appears to attenuate lung function decline, especially in BOS patients. It is, however, difficult to determine whether this change simply represents the natural course of BOS or is a direct treatment effect, although some studies (Moniodis et al., 2018; Thachuthara-George et al., 2015) have documented sustained results. Ensor et al. mainly observed efficacy in BOS stage 1 *versus* higher stages. Reduced efficacy in more advanced CLAD may be due to a significant delay in therapy to a point beyond where allograft function can be stabilized, because of too severe structural injury to the allograft (Ensor et al., 2017). On the other hand, beneficial results were seen by Girgis et al. where 75% of patients were in CLAD stage 3–4 (Girgis et al., 2020). While alemtuzumab may have a potential benefit in BOS, it carries a high risk of infectious complications. Randomized controlled trials are required to better establish efficacy and safety.

B. Anti-Thymocyte Globulin

ATG is a polyclonal antibody preparation, derived from rabbits or horses immunized with thymocytes or T-cell lines (Mohty, 2007). The polyclonal nature of ATG is reflected in its diverse immune effects, including prolonged (i.e., several weeks) depletion of cytotoxic T cells by complement-mediated lysis, antibody-dependent cell-mediated cytotoxicity, apoptosis (activation-associated and Fas-dependent apoptosis), and opsonization. Alongside T-cell-depleting properties, other potential mechanisms

of action involve B-cell apoptosis, depletion of natural killer cells, interference with dendritic cells, modulation of cell surface adhesion proteins and chemokine receptors, and induction of regulatory T cells (Mohty, 2007). One should keep in mind that, despite sharing some common traits, equine and rabbit ATG are strictly different drugs (Mohty, 2007). Rabbit ATG is thought to have a better efficacy and side effect profile than equine ATG, and is more easily accessible than alemtuzumab in some countries.

ATG has been used in conditioning regimens for hematopoietic stem cell transplantation and as induction immunosuppression in solid organ transplants, including lung transplant recipients (Mohty et al., 2014; Small et al., 2022).

Common adverse events related to ATG include transfusion-related reactions, cytokine release syndrome, leukopenia, thrombocytopenia, and infections (Mohty et al., 2014).

1. Evidence in CLAD. In addition to some older studies(Date et al., 1998; Kesten et al., 1996; Snell et al., 1996) published in the early era of lung transplantation that showed some efficacy, there are several larger, recent, retrospective studies that have examined the potency of ATG in slowing CLAD progression. In a study of 25 CLAD patients, 32% had stabilization of FEV_1 for at least 6 months after ATG (1.5 mg/kg/d for 7 days i.v.), with an improved survival rate (Izhakian et al., 2016). However, these patients appeared to have a slower decline in FEV_1 pre-treatment, suggesting an already slower disease progression (Izhakian et al., 2016). January et al. found an increase in FEV_1 (defined by a shift from a negative to a positive slope) in the 6 months after ATG (5–7.5 mg/kg over 3-6 days) compared with before in 40% of a total of 108 patients (93% BOS) (January et al., 2019). Additionally, 44% of the non-responders had a less negative FEV_1 slope. It is worth noting that this study included 20% BOS stage 0p (10%–20% FEV_1 decline and/or $\geq$25% decline in $FEF_{25-75\%}$) patients, and that no predictors of response were identified, neither disease severity at time of treatment, nor steepness of FEV_1 decline or RAS phenotype (January et al., 2019). Kotecha et al. reported 71 patients receiving mostly equine ATG (500 mg on day 1, subsequent dosing days 2–5 based on CD2/CD3 lymphocyte counts) for CLAD (83% BOS). Twenty-three percent were complete responders who had stabilization or improvement in FEV_1, while 40% were partial responders with a $\geq$20% improved rate of FEV_1 decline (Kotecha et al., 2021). Risk of death or retransplantation was significantly lower in these groups, with a 70% and 65% reduction, respectively. CLAD stage 2–3 and younger age were predictors of partial, but not complete, response. CLAD phenotype did not correlate with response. Interestingly, as many centers only try ATG treatment once, 30% of patients had received ATG twice with a median interval of 3 months (Kotecha et al., 2021). Finally, another small study of 13 CLAD patients (77% BOS; ATG 1.5 mg/kg/d, total target dose 10–20 mg/kg) reported stabilization or improvement (>5%) of FEV_1 in half of the patients (Margallo Iribarnegaray et al., 2021). Most patients who responded were in CLAD stage 1–2 (71%). Worse survival was observed in rapid decliners (monthly FEV_1 drop >100 ml) (Margallo Iribarnegaray et al., 2021).

Most important side effects reported in these studies were mild infusion-related reactions (January et al., 2019; Margallo Iribarnegaray et al., 2021), infections (up to 19%) (January et al., 2019), severe leukopenia (4%) (Izhakian et al., 2016), and neutropenia (14%) (Margallo Iribarnegaray et al., 2021).

ATG appears to be effective in stabilizing or attenuating lung function decline in a subgroup of CLAD patients, including RAS, and may lead to prolonged survival. Although certain predictors of response have been identified, such as early disease stages (Kotecha et al., 2021; Margallo Iribarnegaray et al., 2021), these were not consistent across all studies (January et al., 2019). Multicenter, randomized controlled trials are needed to better determine predictors of response to ATG in CLAD.

C. Total Lymphoid Irradiation

Radiation therapy is undoubtedly best known for its role in cancer treatment, but its use extends beyond this (McKay et al., 2014). TLI targets the main structures of the lymphatic system as most lymphocytes are highly radiation sensitive (Schaue and McBride, 2012). TLI therefore has a strong immunosuppressive nature; it produces a selective and long-lasting (i.e., several weeks) reduction of certain subsets of T-cell and B-cell populations. In general, there is a spectrum of radiosensitivity from B cells through naive T-helper cells, natural killer cells, toward more radioresistant T-memory cells and natural killer T cells. As a result, irradiation shifts the balance of the immune system. Regulatory T cells and natural killer T cells are relatively radioresistant and their proportion within the lymphoid tissues increases rapidly following irradiation. Further induction and activation of regulatory T cells can occur via TGF-β, which is induced by TLI (Schaue and McBride, 2012). TLI is often administered in ten fractions of 0.8 Gy twice weekly, via mantle, paraaortic and inverted-Y fields (McKay et al., 2014).

1. Evidence in CLAD. A first study in 1998 described poor efficacy of TLI in 11 BOS patients; most patients died within eight weeks of cessation due to further disease progression or infection, and only 36% had sustained stabilization of FEV_1 with a mean follow up of 24–72 weeks (Diamond et al., 1998). Later, Verleden et al. documented a significant attenuation in the rate of FEV_1 decline in a small group ($n = 6$) compared with historical controls ($n = 5$), although half of them failed within the first year after TLI (Verleden et al., 2009). The Newcastle Group also reported that TLI significantly decreased the rate of FEV_1 decline in 12

BOS patients (Chacon et al., 2000) and in a further, larger study of 37 BOS patients (Fisher et al., 2005), the majority of whom had BOS stage 2–3. Interestingly, the latter study found that the most pronounced effect appeared to occur in patients with the fastest progression prior to TLI (Fisher et al., 2005). Lastly, in a recent study, the Leuven Group reported the outcome of 20 BOS patients (65% BOS 3) treated with TLI, including the six previously reported (Verleden et al., 2009) patients (Lebeer et al., 2020). Four patients (20%) died during or shortly after TLI due to progressive respiratory insufficiency, while the decline in FEV_1 slowed significantly in 94% of the remaining patients, again especially in those with a rapid decline pre-TLI ($\geq$ 100 ml/mo) (Lebeer et al., 2020). An absolute increase in FEV_1 was seen in 13% 6 months post-treatment, even though these patients were already in BOS 3. Freedom from graft loss was 27% 2 years after TLI (Lebeer et al., 2020). Lastly, a recently published study (Geng-Cahuayme et al., 2022) included 23% RAS patients and showed significant attenuation of FEV_1 slope in both BOS and RAS phenotypes and both rapid and slow decliners. They found that a Karnofsky Performance Status of >70 was a prognostic marker for survival (Geng-Cahuayme et al., 2022).

In addition to these studies, several conference abstract reports were available. Most of these had similar findings with a decrease in FEV_1 decline post-treatment compared with before (Afolabi et al., 1996; Arbeláez et al., 2014; Hunt et al., 2019; Low et al., 2017; Miller et al., 2016; Soresi et al., 2015; Sáez et al., 2014). Hunt et al. reported a mean survival of 4.2 (range 0.75–7.5) years post-TLI, and Soresi et al. a 2-year overall survival of 59% after initiation of treatment (Hunt et al., 2019; Soresi et al., 2015). Schmack et al. attempted to correlate specific lymphocyte phenotypes with response to TLI in a prospective study of 26 patients with progressive BOS (Schmack et al., 2017). They found an inverse correlation between the total number of peripheral B cells, naive B cells, memory B cells, plasmablasts, and naive CD8+ T cells pre-treatment and patient survival (Schmack et al., 2017).

Frequently reported side effects were neutropenia, thrombocytopenia, infections, gastrointestinal symptoms, and fatigue (McKay et al., 2014). The first three often led to treatment being delayed or terminated prematurely (Fisher et al., 2005; Geng-Cahuayme et al., 2022; Lebeer et al., 2020; O'Hare et al., 2011).

Although data on TLI in CLAD remain relatively scarce, the findings are consistent across most studies in which TLI appeared to attenuate the decline in lung function in BOS and RAS. Importantly, it also seemed to be effective in CLAD patients with a rapid decline in lung function at the time of treatment initiation. Early initiation after CLAD onset may be warranted, although good results have also been documented in patients with advanced BOS (Fisher et al., 2005; Lebeer et al., 2020). Reported complications, such as neutropenia, thrombocytopenia, and risk of infection, suggest that TLI should be used with caution, although the incidence of serious side effects was low.

III. Immunomodulating Therapies

A. Methotrexate

Methotrexate is a folic acid analog and acts via several suggested mechanisms, including inhibition of purine and pyrimidine synthesis, suppression of transmethylation reactions with accumulation of polyamines, prolonged (i.e., several weeks) reduction of antigen-dependent T-cell proliferation, apoptosis of T cells through the generation of reactive oxygen species, as well as selective downregulation of B cells, interference with cytokines and matrix metalloproteinases, and promotion of extracellular release of adenosine (Alqarni and Zeidler, 2020; Amrouche and Jamin, 2017; Bedoui et al., 2019). Adenosine is a potent anti-inflammatory mediator that acts through interactions with a variety of immune cell subtypes, such as neutrophils, macrophages, and T cells (Bedoui et al., 2019). In addition, recent insights suggest that methotrexate may also exert its anti-inflammatory effects via inhibition of nuclear factor-κB and the JAK/STAT pathway (Alqarni and Zeidler, 2020; Bedoui et al., 2019).

As a drawback, methotrexate has a high toxicity profile and can cause considerable side effects, including cytopenia, stomatitis, subcutaneous nodulosis, hepatic and renal toxicity, fatigue and lethargy (Bedoui et al., 2019). Although most of these mainly occur when higher doses (usually >30 mg/m^2) are used as part of a chemotherapy regimen. Importantly, methotrexate can also cause pulmonary toxicity, such as drug-induced pneumonitis (Pivovarov and Zipursky, 2019).

Methotrexate has been used extensively in the treatment of neoplasms as a chemotherapeutic agent, autoimmune and connective tissue diseases such as rheumatic arthritis (Alqarni and Zeidler, 2020), interstitial lung diseases, including sarcoidosis (van den Bosch et al., 2022), and is commonly used after hematopoietic stem cell transplantation to prevent graft-*versus*-host disease (GvHD) (Martinez-Cibrian et al., 2021).

1. Evidence in CLAD. Evidence for methotrexate in CLAD is sparse and limited to BOS. A small study of ten patients showed that methotrexate could reduce the rate of lung function decline in BOS (Dusmet et al., 1996). Boettcher et al. also reported some benefit of methotrexate (single dose of 5 mg/kg or 7.5 mg/week) in three BOS patients (Boettcher et al., 2002). The same was found in a larger, retrospective study of 30 BOS patients, the majority of whom had BOS stage 3 at the time of treatment initiation (5–10 mg/week) (Sithamparanathan et al., 2016).

A decrease in the rate of lung function decline was seen in 95% of patients treated for at least 6 months (70% of the cohort), with a significant median increase in FEV_1 at 3 and 6 months. The reduced rate of lung function decline remained significant in those treated for at least 12 months. However, methotrexate had to be discontinued in 30% of patients due to nausea, fatigue or leukopenia. This number was higher than that seen in, for example auto-immune diseases, but may be explained by the combination of other immunosuppressants and transplant-related drugs (Sithamparanathan et al., 2016).

Although based on a limited number of small uncontrolled retrospective studies, methotrexate might slow the rate of lung function decline in BOS patients, even in patients with severe BOS. With the recent insights on the involvement of methotrexate in the JAK/STAT pathway, one could reconsider further prospective studies as it is a less expensive alternative to more specific Janus kinase (JAK) inhibitors (discussed later in this review) (Alqarni and Zeidler, 2020). However, toxicity is still a concern and lack of tolerability and side effects were the main cause of drug withdrawal (Sithamparanathan et al., 2016).

B. Cyclophosphamide

Cyclophosphamide is an alkylating agent belonging to the group of oxazaphosporines (Ahlmann and Hempel, 2016). It is an inactive prodrug, requiring bioactivation by P450 enzymes to exhibit cytotoxic activity. Since cyclophosphamide has been used for over 40 years, there is plenty of experience in its use for the treatment of cancer and as a highly potent immunosuppressant for the treatment of autoimmune and immune-mediated diseases including vasculitis, systemic sclerosis, connective tissue disease-related interstitial lung disease (Ahlmann and Hempel, 2016; Barnes et al., 2018; Emadi et al., 2009; van den Bosch et al., 2022).

Cyclophosphamide halts cell division by cross-linking DNA strands (Ahlmann and Hempel, 2016). Therefore, it is a non-specific cell-cycle inhibitor affecting most cell lines, although it has some selectivity toward T and B lymphocytes, causing prolonged (i.e., several weeks) immunosuppressive effects. It is therefore now widely adopted in tumor vaccination protocols and to control alloreactivity after hematopoietic stem cell transplantation (Ahlmann and Hempel, 2016; Nunes and Kanakry, 2019). Interestingly, cyclophosphamide can also increase the number of myeloid-derived suppressor cells (Ahlmann and Hempel, 2016).

Important side effects are cytopenia, nausea, hemorrhagic cystitis, cardio-, liver- and nephrotoxicity, and carcinogenicity with an increased risk of hematological and solid organ malignancies (e.g., secondary acute leukemia, bladder cancer, skin cancer) (Ahlmann and Hempel, 2016; Barnes et al., 2018).

1. Evidence in CLAD. In 1999, Verleden et al. reported the outcome of oral cyclophosphamide (0.5–1 mg/kg daily) in seven BOS patients. In 86% of patients, FEV_1 stabilized or increased 3 and 6 months after initiation and remained stable for at least 24 ± 7 months in five patients who were able to continue treatment (Verleden et al., 1999). Cyclophosphamide was well tolerated and had to be discontinued in only one patient because of persistent leukopenia (Verleden et al., 1999). Other than this study, however, no further data in CLAD are available. As such, the role of cyclophosphamide in the treatment of CLAD remains unclear.

C. mTOR Inhibitors

Mammalian target of rapamycin (mTOR) inhibitors have been used after lung transplantation for several indications, such as a cell cycle inhibitor alternative, as part of a calcineurin inhibitor-sparing regimen or adjunctive immunosuppressive agent in the setting of rejection, cytomegalovirus infection or in patients with malignancies (Fine and Kushwaha, 2016). mTOR inhibitors block mammalian target of rapamycin, a serine/threonine kinase, and thereby inhibit growth factor-stimulated proliferation of lymphocytes and mesenchymal cells. In addition, mTOR inhibitors also interfere with B and dendritic cell maturation and function (Thomson et al., 2009) and possibly NK cell-mediated endotheliitis (Koenig et al., 2019). Common adverse events are gastrointestinal intolerance, leukopenia, edema, thromboembolic events, and drug-induced pneumonitis.

1. Evidence in CLAD. Most recent studies on mTOR inhibitors have focused on their use in maintenance immunosuppression as part of a calcineurin inhibitor-sparing regimen with the aim of preserving kidney function. The combination of low-dose everolimus and low-dose tacrolimus appeared safe, with no difference in incidence of acute rejection or CLAD compared with high-dose calcineurin inhibitor therapy (Gottlieb et al., 2019; Ivulich et al., 2023; Kneidinger et al., 2022). Few studies looked at the use of everolimus or sirolimus as a treatment of CLAD. Cahill et al. found that in patients with rapidly declining pulmonary function, sirolimus resulted in stabilization or improvement of FEV_1 slope (Cahill et al., 2003). Everolimus also improved the FEV_1 slope 3 and 6 months after *versus* before treatment in a study by Fernandez et al. (David Iturbe et al., 2019). Patrucco et al. also found stabilization in FEV_1 in CLAD patients, however, subgroup analysis showed progressive functional loss in RAS patients (Patrucco et al., 2021). In another small study, three CLAD patients (60%) remained stable after introduction of everolimus, whereas 40% progressed (Turkkan et al., 2022). Nonetheless, side effects often necessitated discontinuation of mTOR inhibitors (Bos et al., 2021; Cahill et al., 2003; Kneidinger et al., 2022).

D. Belatacept and Basiliximab

Little is known about the use of these two agents for CLAD. Belatacept is a selective CD80/86-CD28 T-cell

costimulation blocker widely used in kidney transplantation for induction and maintenance immunosuppression (Masson et al., 2014). The role of belatacept in the setting of lung transplantation remains uncertain with only a few small studies reporting its use in maintenance immunosuppression as part of a calcineurin inhibitor-sparing regimen (Huang et al., 2022; Iasella et al., 2018; Timofte et al., 2016), and a conference abstract on antibody-mediated rejection (Zaffiri et al., 2022), while data in CLAD is lacking. Importantly, one randomized controlled trial with 27 lung transplant patients had to be discontinued prematurely due to increased rates of death in the belatacept arm (Huang et al., 2022).

Basiliximab, a chimeric monoclonal antibody that selectively binds to the α–subunit (CD25) of interleukin-2 receptors, is used for induction therapy in lung transplantation (Small et al., 2022). In addition, there are some case series describing its use in maintenance immunosuppression to avoid calcineurin inhibitor-related nephrotoxicity (Högerle et al., 2016; Kim et al., 2021; Ross et al., 2020). Again, there is no data on any potential benefit in CLAD.

E. TNF-Alpha Inhibitors

Tumor necrosis factor alpha (TNFα) is a cytokine that acts as a major regulator of inflammatory reactions via the initiation of signal transduction pathways leading to cytotoxicity and upregulation of various cytokines, chemokines and growth factors (Jang et al., 2021). TNFα is also a key factor in the pathogenesis of CLAD (Bos et al., 2022b).

Several TNFα inhibitors are used for the treatment of inflammatory and autoimmune conditions, such as the monoclonal antibodies infliximab, adalimumab, certolizumab, and golimumab, and the recombinant fusion protein etanercept (Jang et al., 2021). Some of these have also been tested in solid organ transplants to mediate inflammatory responses in ischemia-reperfusion injury and rejection (Pascher and Klupp, 2005).

Anti-TNF agents are generally well tolerated, with common adverse effects being minor. General side effects include infusion-related reactions, injection site reactions, anemia, transaminitis, and mild infections; although there is a risk of severe infections and possibly an increased risk of malignancies, especially lymphomas and non-melanoma skin cancers (Jang et al., 2021).

1. Evidence in CLAD. Next to a few preclinical animal studies (Alho et al., 2003; Aris et al., 2002; Smith et al., 2001), there is one proof-of-concept study that reported the use of infliximab (3 mg/kg i.v. at 0-2-6 weeks minimally) in five patients with progressive BOS (Borthwick et al., 2013). FEV_1 and 6-minute walk distance improved in four patients and stabilized in a fifth patient with rapid lung function decline. All patients remained stable for at least 18 months. Infliximab was generally well tolerated; one patient developed a fungal infection (Borthwick et al., 2013).

F. Extracorporeal Photopheresis

ECP is a leukapheresis-based immunomodulatory procedure, currently approved for the management of cutaneous T-cell lymphoma, GvHD and rejection after solid organ transplantation (Hage et al., 2021). ECP is a procedure in which whole blood is collected from the patient and circulating leukocytes are removed by density centrifugation. The collected buffy coat is then treated with a photosensitizing agent (i.e., 8-methoxypsoralen) and exposed to UV A light before reinfusion into the patient (Cho et al., 2018). The exact mechanisms of therapeutic action are elusive, but ECP is thought to induce apoptosis of lymphoid cells, largely natural killer cells and T cells, and differentiation of activated monocytes into immature dendritic cells which in turn stimulate phagocytosis of lymphoid cells, and maturation and presentation of antigenic peptides (so-called transimmunization). Furthermore, ECP might modify the cytokine profile with induction of anti-inflammatory cytokines (IL-4, IL-10, transforming growth factor beta) and reduction of pro-inflammatory cytokines (e.g., tumor necrosis factor alpha), and stimulate upregulation of regulatory T cells (Cho et al., 2018). Different schedules are being used, often with a more intensive induction phase, followed by a maintenance schedule. However, the treatment effects after ECP initiation take time to come into effect. Next to this, there is no consensus on how long this therapy should be continued and there is uncertainty as to whether a sustained response can be observed and for how long after cessation. Furthermore, ECP is not reimbursed by health systems or insurance providers in many countries.

1. Evidence in CLAD. There are numerous publications describing the effects of ECP in CLAD, including several studies and various conference abstracts. Two prospective studies are available (Table 1). Firstly, a prospective multicenter study with 31 BOS patients (58% stage 2–3) from ten lung transplant centers (Hage et al., 2021). Rate of FEV_1 decline was reduced by 93% at 6 months, with a reduction ≥50% in 95% of patients. Multivariate analysis identified that pre-enrolment FEV_1 rate of decline was associated with both 6- and 12-month mortality. Notably, study enrollment was terminated prematurely due to a higher-than-expected mortality rate within the first year after enrollment of 32% and 41% at 6 and 12 months, respectively. There was no difference in mortality between the ECP group and an observational cohort; worth noting, the slope of FEV_1 decline pre-enrollment was much steeper in the former group (Hage et al., 2021). Another prospective single-center study by Jaksch et al. included 51 BOS patients and reported FEV_1 stabilization (variation <5%) in 61% of patients with an improvement in survival in these patients compared with both non-responders and non-treated BOS patients (Jaksch et al., 2012). Factors associated with inferior treatment response

were cystic fibrosis as underlying lung disease and a longer time between transplant and BOS onset (Jaksch et al., 2012).

Furthermore, several recent retrospective single-center studies included both BOS and RAS patients, varying from 12 to 65 CLAD patients per study, of whom the majority had CLAD stage 2–3 (Table 2). Del Fante, Greer, and Vazirani all reported a significant reduction in rate of lung function decline (Del Fante et al., 2015; Greer et al., 2013; Vazirani et al., 2021) with a stabilization or improvement (≥10%) in lung function around 54%–60% (Del Fante et al., 2015; Greer et al., 2013). Notably, patients who did not complete the initial 3-month induction treatment or at least eight procedures were excluded in Greer's (Greer et al., 2013) and Del Fante's (Del Fante et al., 2015) studies, respectively. Robinson et al. looked at the lung function trajectory after forced cessation of ECP due to loss of reimbursement in 12 CLAD patients who had undergone long-term ECP treatment (median 1001 days) (Robinson et al., 2017). FEV_1 significantly and rapidly declined within 6 months of cessation, while lung function was stable in all patients before. Moreover, 58% died within 12 months mostly due to CLAD progression (Robinson et al., 2017).

Survival seemed to correlate with response to ECP (Greer et al., 2013; Vazirani et al., 2021) though predictors of response varied across the studies. Some studies documented that female sex (Vazirani et al., 2021), a rapid decline in FEV_1 pre-ECP (Del Fante et al., 2015; Greer et al., 2013), RAS phenotype (Greer et al., 2013), a low baseline neutrophil count in blood ($< 1.9 \times 10^9$/L) (Vazirani et al., 2021) or bronchoalveolar lavage (≤15%) (Greer et al., 2013), prior exposure to ATG (Vazirani et al., 2021), and time from transplant to CLAD onset (Del Fante et al., 2015) adversely affected response to ECP. Although others could not find an impact of sex (Del Fante et al., 2015), CLAD phenotype (Del Fante et al., 2015; Vazirani et al., 2021), timing of CLAD onset (Greer et al., 2013), CLAD stage (Del Fante et al., 2015; Greer et al., 2013), or time from CLAD diagnosis to ECP initiation (Del Fante et al., 2015; Greer et al., 2013).

These findings corroborate the results from multiple previous studies where ECP was administered for BOS, as summarized in Table 2. Again, in these studies, that included between 5 and 88 patients, there was a significant reduction in FEV_1 decline with in most studies a stabilization in lung function in 60%–80% of patients (Baskaran et al., 2014; Benden et al., 2008; Isenring et al., 2017; Karnes et al., 2019; Leroux et al., 2022; Meloni et al., 2007; Moniodis et al., 2018; Morrell et al., 2010; Pecoraro et al., 2017; Salerno et al., 1999). Lastly, there are numerous conference abstracts reporting similar outcomes.

TABLE 1
Prospective studies of ECP in BOS

Reference	Study design and period	Number of patients	CLAD stages	Duration ECP	Median slope FEV_1 pre-ECP (ml/month)	Median slope FEV_1 post-ECP (ml/month)	Response rate to ECP	Mortality within study	Predictors of response
Hage et al., 2021	Prospective, multicenter, 04/2015–07/2016	31 BOS ECP 13 BOS controls (7 crossover)	BOS 1 42% BOS 2 29% BOS 3 29%	6 months	Mean -136 ± 117	Mean -10 ± 58†	- Reduction ≥50% in 95% of patients (data 16/30, 63%) - Higher rate FEV_1 decline in non-survivors at 6 and 12 months post-ECP start - FEV_1 improvement in 30% (12% 3–6 months, 18% >12 months) - Stabilization in 31%	39% and 48% at 6 and 12 months (87% CLAD, 13% infection)	FEV_1 rate of decline pre-ECP correlated with 6- and 12-month mortality.
Jaksch et al., 2012	Prospective, single-center, 01/2000-06/2010	51 BOS ECP 143 BOS controls At least 3 months treatment	BOS 1 12% BOS 2 20% BOS 3 68%	3 months (51 patients) 12 months (25 patients)	−123†	−14* and -18‡			Negative impact: BOS onset >3 years post-transplant, rapid FEV_1 decline pre-ECP, BOS stage, cystic fibrosis as primary lung disease. Overall survival: response to ECP.

Period of 3*, 6† or 12‡ months pre-/post-ECP initiation.

TABLE 2
Retrospective, single-center studies of ECP in CLAD

Reference	Study design and period	Number of patients	CLAD stages	Duration ECP	Median slope FEV_1 pre-ECP (ml/month)	Median slope FEV_1 post-ECP (ml/month)	Response rate to ECP	Mortality within study	Predictors of response
Baskaran et al., 2014	Retrospective, single-center, 01/2000–06/2011	88 BOS		6 months	−127[†]	−47[†]	−63% reduction in rate of FEV_1 decline, 23% stabilized or improved (data 69/88, 78%)	19% at 2 years	No impact: % reduction of DSA or lung-associated self-antigens, or level of cytokines (IL-1β, IL-2, IL-4, IL-10, IL-17, IFN-γ, IP-10, MCP-1).
Benden et al., 2008	Retrospective, single-center; 1997–2007	12 BOS	BOS 1 42% BOS 2 17% BOS 3 42%	12 cycles	112 From baseline until start ECP	12 After 12 cycles of ECP until last value		33% (100% CLAD) Median OS 4.9 years after ECP start (BOS + ACR cohort)	
Del Fante et al., 2015	Retrospective, single-center; 02/2003–12/2013	34 BOS 14 RAS 58 controls At least 8 procedures	CLAD 1 58% CLAD 2 21% CLAD 3 21%	Median 26 (IQR 17-42) procedures	−48 (95% CI -61; -36)[†]	−19 (95% CI -35; -3)[†] −4 (95% CI -15; +7) 12-24 months post ECP start	−60% stable graft function at 6 months	42% (85% CLAD, 5% cancer, 10% other), no difference ECP group and controls	Negative impact: rapid decliners (>100 ml FEV_1 /month), time from transplant to CLAD onset. No impact: CLAD phenotype, CLAD stage, time from CLAD onset to ECP. Overall survival: RAS (trend: $P = 0.06$), no influence CLAD stage, rapid decline, time CLAD diagnosis and start ECP.
Greer et al., 2013	Retrospective, single-center, 11/2007–09/2011	65 CLAD At least 3 months treatment	CLAD 0p 5% CLAD 1 9% CLAD 2 32% CLAD 3 54%	Median 15 (IQR 12–18) cycles			−12% improvement (≥10%) −42% stabilization in FEV_1	2-year OS 97% in responders	Negative impact: rapid decline (>100 ml FEV_1 /month), RAS phenotype, ≤15% BAL neutrophils. No impact: CLAD stage, time to CLAD diagnosis and initiation of ECP. Overall survival: response to ECP.
Isenring et al., 2017	Retrospective, single-center, 01/2008–12/2012 Update from 2008 study(Benden et al., 2008)	9 of initial 12 BOS, 2 continued ECP after re-transplant	BOS 1 44% BOS 2 11% BOS 3 44%	Range 48–119 months			- Progression in 17% still alive at end of follow up	33% (67% cancer, 33% CLAD)	Negative impact: CLAD stage 2-3.

(*continued*)

TABLE 2—*Continued*

Reference	Study design and period	Number of patients	CLAD stages	Duration ECP	Median slope FEV_1 pre-ECP (ml/month)	Median slope FEV1 post-ECP (ml/month)	Response rate to ECP	Mortality within study	Predictors of response
Karnes et al., 2019	Retrospective, single-center, 01/2000–12/2007 Update from 2010 study(Morrell et al., 2010)	60 BOS	See(Morrell et al., 2010)	6 months	See(Morrell et al., 2010)	See(Morrell et al., 2010)	See(Morrell et al., 2010)	17% <6 months, 50% <16 months of ECP initiation	12-fold higher chance of response if FEV_1 decline >40 ml/ months pre-ECP. FEV_1 at start ECP correlated linearly with time to mortality and mortality at 16 months after start ECP.
Leroux et al., 2022	Retrospective, single-center, 01/2012–07/2019	12 BOS ECP 13 BOS controls At least 6 months treatment	BOS 1 33% BOS 2 25% BOS 3 42%	Median 32 (IQR 12-58) months	−44 (IQR -112; -8)†	+11 (IQR -0.8; +41)†	−75% FEV_1 stabilization (±5%) within 12 months −63% improvement (>5%), 25% stabilization within 24 months of initiation - Lower risk of >20% drop in FEV_1 in ECP-treated group versus control decliners.	33% (100% sudden death)	No impact: rate of FEV_1 decline pre-ECP, time BOS diagnosis and ECP start.
Meloni et al., 2007	Retrospective, single-center	5 BOS At least 4 months treatment	BOS 2 60% BOS 3 40%	Range around 4–32 months			−60% FEV_1 stabilization	40% (100% infection)	Tregs stabilized or increased in patients who stabilized and declined in non-responders.
Moniodis et al., 2018	Retrospective, single-center, 01/2005–12/2014	13 BOS ECP 4 RAS ECP 9 BOS alemtuzumab 5 RAS alemtuzumab 78 controls	CLAD 1 88% CLAD 2 12%	6 months	−122 (IQR -164; -77)*	−27 (IQR -82; -36)* and -12 (-56–22)†	- Significant reduction in rate of FEV_1 decline at 3 and 6 months	OS at 6 months 0.82 (95% CI 0.55–0.94)	Negative impact: RAS phenotype.
Morrell et al., 2010	Retrospective, single-center, 01/2000–12/2007	60 BOS	BOS 1 8% BOS 2 33% BOS 3 58%	6 months	−116†	−29† and -21‡	- Reduction of FEV_1 rate of decline in 79% - FEV_1 improved in 25% at 6 and 12 months (data 56/60, 93%)	Median OS 2.6 years after ECP start	No impact: BOS stage, time of BOS onset, rate of FEV_1 decline pre-ECP.
Pecoraro et al., 2017	Retrospective, single-center, 11/2013–06/2016	15 BOS 39 controls	BOS 1 7% BOS 2 27% BOS 3 67%	13 cycles			−80% FEV_1 stabilization - FEV_1 significantly higher 12 months after start ECP compared with controls	13% (50% CLAD, 50% cancer), better OS in ECP versus controls	No impact: CLAD stage, time CLAD onset, time BOS diagnosis and ECP.

(*continued*)

TABLE 2—*Continued*

Reference	Study design and period	Number of patients	CLAD stages	Duration ECP	Median slope FEV_1 pre-ECP (ml/month)	Median slope FEV_1 post-ECP (ml/month)	Response rate to ECP	Mortality within study	Predictors of response
Robinson et al., 2017	Retrospective, single-center, Patients who had to stop ECP end 2014 due to stop reimbursement	10 BOS 2 RAS	BOS 2 30% BOS 3 70% RAS unknown	Median 44 (range 8–142) procedures	−13 (range -8–110)	−17 (range -6–163)	- FEV_1 rapidly declined within 6 months after ECP cessation	58% within 12 months of treatment cessation (43% CLAD, 43% infection + CLAD, 14% cancer)	
Salerno et al., 1999	Retrospective, single-center, 1992–1998	8 BOS 20 controls	BOS 3 88%	Median 6 (range 3–13) months	Range -366; +8 From baseline until start ECP	Range -30; +44	−71% FEV_1 stabilization or improvement −63% clinical stabilization −67% (<20% decrease in FEV_1 within 6 weeks of ECP start)	50% alive without retransplant after median 36 months	
Vazirani et al., 2021	Retrospective, single-center, 01/2013–06/2018	5 BOS 2 RAS 5 mixed	CLAD 2 17% CLAD 3 83%		Mean 9 (95% CI 5; 12) ml/day in responders Mean 7 (4; 10) m/day in non-responders	Mean 1.4 (95% CI 0; 4) ml/day In responders Mean 5 (3; 7) ml/day in non-responders		Graft-failure in all non-responders (33%) within 6 months of ECP start	Negative impact: female sex, low baseline neutrophil count (< 1.9×10^9/L), prior exposure to ATG. No impact: CLAD phenotype.

OS, overall survival; RAS, restrictive allograft syndrome. Period of 3*, 6† or 12‡ months pre-/post-ECP initiation.

In all published data to date, ECP has generally been found to be a safe treatment without significant adverse effects.

In summary, clinical evidence suggests that ECP is associated with improvement or stabilization in lung function and decreases the rate of lung function decline in BOS, without an increased risk of infections or significant adverse events, with some studies also showing improved survival. Given that this response appeared to be independent of CLAD duration as well as stage at treatment initiation in most studies, ECP should be considered a viable second-line treatment option.

Large prospective clinical trials are needed to help predict response to therapy, and ultimately guide the placement of ECP in the treatment algorithm for CLAD. The results of a multicentre randomized controlled trial comparing ECP plus standard of care *versus* standard of care alone in patients with progressive CLAD in the UK (NIHR130612) are therefore eagerly awaited.

IV. B-cell-Directed Treatment

The effects of immunomodulatory and lymphodepleting treatments primarily targeting B cells and anti-human leukocyte and donor-specific antibodies, such as rituximab (anti-CD20), bortezomib and carfilzomib (both proteasome inhibitors), are mainly described in the context of antibody-mediated rejection (Neuhaus et al., 2022; Pham et al., 2021; Razia et al., 2022; Roux et al., 2016; Vacha et al., 2017; Yamanashi et al., 2020). However, evidence for their relevance as part of CLAD treatment is lacking. We can speculate that these agents might have a beneficial effect when given in combination with other therapies, as antibodies and various subsets of B cells are involved in CLAD pathogenesis (Bos et al., 2022c). However, combination therapy may increase the complexity of treatment and risk of side effects.

V. Future Directions

Interestingly, there are many similarities between CLAD and pulmonary chronic GvHD after hematopoietic stem cell transplantation, as described elsewhere (Bos et al., 2022a). This could imply that therapies developed for (pulmonary) GvHD may also be effective in CLAD, and vice versa, which deserves further attention. Indeed, efforts are needed from both academia and industry for devoted development of novel (more efficacious and safer) immunosuppressive agents, or drug repurposing, along with innovative trial designs with relevant clinical endpoints focusing on these devastating conditions, which are an unmet need.

A. Tyrosine Kinase Inhibitors

Imatinib and ibrutinib are two tyrosine kinase inhibitors commonly used in chronic GvHD with some evidence

for their use in pulmonary GvHD. Imatinib (100–400 mg daily) seemed to stabilize FEV_1 in some BOS patients after allogeneic hematopoietic stem cell transplantation, and in subgroup analyses of some patients treated with imatinib for chronic GvHD (Magro et al., 2009; Olivieri et al., 2013, 2009; Parra Salinas et al., 2021; Stadler et al., 2009; Sánchez-Ortega et al., 2016; Watanabe et al., 2015). There is minimal data from preclinical animal studies regarding the use of imatinib in CLAD, showing that imatinib improved luminal airway obstruction in experimental bronchiolitis obliterans (Pandolfi et al., 2020; von Suesskind-Schwendi et al., 2013; Watanabe et al., 2017), possibly through reduction of migration and differentiation of fibrocytes in the allograft (Watanabe et al., 2017).

Currently, no data are available on ibrutinib (140–420 mg daily) in CLAD nor from pulmonary GvHD-specific studies, although some stabilization of lung function was observed in subgroup analyses of chronic GvHD studies (Doki et al., 2021; Kaloyannidis et al., 2021). More data on the use of tyrosine kinase inhibitors in pulmonary GvHD and CLAD are needed to decide whether there is sufficient efficacy in stabilizing lung function or not.

B. Janus Kinase Inhibitors

Ruxolitinib (5–10 mg b.d.) is a relatively new JAK-1/2 inhibitor used with good results in chronic GvHD and some promising results in pulmonary GvHD as well (Streiler et al., 2020; Zhao et al., 2021). There is currently no data in CLAD yet. Promising results of another JAK-1 inhibitor, itacitinib (400–600 mg daily), in a phase 1 study with 23 BOS patients were recently presented, demonstrating that treatment with itacitinib resulted in stabilization of FEV_1 in all participants who continued treatment with an absolute increase of $\geq$10% in 22% of patients (Diamond et al., 2022). Further results from phase 2 as well as results from a phase 2 trial in steroid-refractory chronic GvHD (NCT04200365) and phase 1 trial in pulmonary GvHD (NCT04239989) are awaited.

C. Rho Kinase Inhibitors

Belumosudil (200–400 mg daily) is a rho kinase inhibitor recently approved for the treatment of chronic GvHD after failure of at least two prior lines of systemic therapy in the USA, and will soon be available in the UK as well. Its efficacy merits further investigation in both pulmonary GvHD and CLAD, as in two recent phase 2 chronic GvHD studies, it resulted in a $\geq$ 10% increase in FEV_1 in 55% of 47 (Cutler et al., 2021) and 71% of 17 (Jagasia et al., 2021) subjects with pulmonary GvHD.

D. MEK Inhibitors

MAPK/ERK kinase (MEK) inhibitors inhibit the mitogen-activated protein kinase kinase enzymes MEK1 and/or MEK2. Trametinib, a MEK-1/2 inhibitor, ameliorated the onset of GvHD (Itamura et al., 2021, 2016) and chronic rejection after lung transplantation (Takahagi et al., 2019) in some animal studies, highlighting the need for further translational research.

E. IL-6 Inhibitors

The humanized IL-6 receptor antibody tocilizumab prevents binding of IL-6 to its receptor and signal transducer glycoprotein 130 complex, inhibiting downstream JAK/STAT signaling, and has been used in a limited number of studies after allogeneic hematopoietic stem cell transplantation for both acute and chronic GvHD prevention and treatment (Drobyski et al., 2011; Ganetsky et al., 2019; Kattner et al., 2020; Kennedy et al., 2021; Melgarejo-Ortuño et al., 2021; Roddy et al., 2016; Yucebay et al., 2019). In a study of chronic GvHD patients (8 mg/kg q4w), the response rate for pulmonary GvHD ranged between 17 and 33% within the first year of treatment initiation (Kattner et al., 2020). One case report is available in the setting of CLAD in a patient transplanted for COPA syndrome, a genetic disorder leading to upregulation of pro-inflammatory cytokines (primarily IL-1β and IL-6) and development of interstitial lung disease (Riddell et al., 2021). Involvement of IL-6 in the pathogenesis of CLAD has also been documented (Bos et al., 2022b), and tocilizumab (4 mg/kg monthly for 3 doses) effectively suppressed IL-6 upregulation though without clinical improvement in this patient (Riddell et al., 2021). Moreover, one conference abstract demonstrated stabilization of lung function in nine CLAD patients who received tocilizumab (4–8 mg/kg monthly) for at least 3 months, but in combination with other therapies such as ATG, rituximab and immunoglobulins (Ross et al., 2019). Another conference abstract reported reduced onset of rejection when tocilizumab was added in a preclinical animal model, possibly via transient expansion of regulatory T cells (Aoyama et al., 2016).

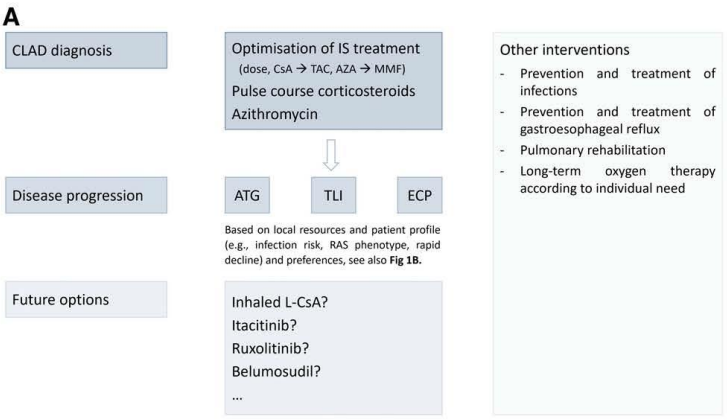
Further evaluation of a potential role for tocilizumab in the treatment of pulmonary GvHD and CLAD in larger trials is warranted.

F. Inhaled Liposomal Cyclosporine A

Local intrapulmonary lymphocyte suppression and immunomodulation via nebulized immunosuppressive drugs, such as liposomal cyclosporine, may be an elegant way to prevent systemic side effects and ensure high local efficacy in CLAD. Following prior studies demonstrating a possible beneficial effect for CLAD prevention (Groves et al., 2010; Iacono et al., 2019; Neurohr et al., 2022), currently two studies in CLAD are ongoing (BOSTON-1 and BOSTON-2) which results are eagerly awaited.

VI. Conclusion

CLAD is the leading cause of death beyond the first year after lung transplantation (Chambers et al., 2019). Some patients experience an accelerated loss of lung function, whereas others have a slower progression



B

	ATG	TLI	ECP
Time to start	Days	Weeks	Weeks
Treatment duration	Days	Weeks (usually 5)	Months
Inpatient stay	Yes	No	Yes/no
Start of treatment effect	Days-weeks	Weeks	Weeks-months
Estimated efficacy (stabilisation or improvement)	≥ 50%	≥ 50%	≥ 50%
Duration of treatment effect	Weeks-months	Weeks-months	Weeks-months
Side effects	+	++	+/-
Costs	+	+/++	+++

Fig. 2. Lymphocyte depleting and/or modulating therapies in CLAD. (A) Suggested treatment algorithm for CLAD based on existing data taking into account the efficacy and risk of side effects as well as some potential safer future options that require more investigation. (B) Overview of features associated with ATG, TLI, and ECP treatment. Which therapeutic option is chosen mainly depends on local resources and patient profile (e.g., risk of infection, CLAD phenotype, rapid *versus* slow lung function decline) and preferences. AZA, azathioprine; CsA, cyclosporine A; IS, immunosuppressive; L-CsA, liposomal cyclosporine A; MMF, mycophenolate mofetil; TAC, tacrolimus.

with intermittent loss of function (Belperio et al., 2009; Sato et al., 2013). Several therapeutic options have been used in attempts to prevent, reverse, or slow CLAD progression; however, there are only limited effective therapeutic options and there is currently no consensus on the most effective option (Verleden et al., 2019). Interpretation of these results is overshadowed by the fact that randomized controlled trials are almost universally lacking; thus, it is unclear whether the attenuated rate of FEV_1 decline represents true treatment response or merely the natural course of the disease. In advanced CLAD stages, a less pronounced decline in lung function may also be due to limited residual lung function (Kotecha et al., 2021). However, some studies showed sustained lung function stabilization (Jaksch et al., 2012; Kotecha et al., 2021; Moniodis et al., 2018; Robinson et al., 2017) or improvement even in advanced CLAD (Del Fante et al., 2015; Girgis et al., 2020; January et al., 2019; Lebeer et al., 2020; Thachuthara-George et al., 2015; Vazirani et al., 2021). Secondly, comparing studies is complicated because of different treatment dosages and regimens used, also with respect to other transplant-related drugs and center-specific policies. Furthermore, the comparison of results is hampered by the use of different definitions of CLAD prior to an international consensus and of treatment response, highlighting the need for standardization and harmonization.

Knowledge of the mechanisms-of-action of existing drugs is an essential prerequisite that allows us to understand how a treatment works, but also the expected side effects, and may allow identification of other treatment options, targeting similar immune cells or pathways. Taking into account the efficacy and risk of side effects, we believe that ECP, ATG, and TLI currently have the most promising data to suggest they could be considered second-line lymphocyte-targeted treatment options for CLAD patients (Fig. 2). As intercurrent infections may drive CLAD onset and progression, however, the need for safer lymphocyte-directed therapies has become clear.

To improve future treatments in lung transplantation, standardization of care, trial protocols, and relevant study endpoints, which include lung function, overall survival and preferably also quality of life and exercise capacity between different transplant centers, are key. Larger randomized controlled multi-center trials, preferably also including RAS patients, with longer follow up as well as platform trials moving rapidly between investigational agents and further investigation of novel treatment options are urgently needed to define the most appropriate treatment algorithm for CLAD. A list of currently ongoing clinical trials is provided in Table 3.

TABLE 3
Ongoing clinical trials in CLAD using lymphocyte depleting or modulating drugs (registered at clinicaltrials.gov or NIHR)

Study identifier, country	Title	Study design
NCT02181257, USA	Extracorporeal Photopheresis for the Management of Progressive Bronchiolitis Obliterans Syndrome in Medicare-Eligible Recipients of Lung Allografts	Randomized controlled open-label multicenter trial
NIHR130612, UK	Extracorporeal Photophoresis in the treatment of Chronic Lung Allograft Dysfunction: a randomized controlled trial (E-CLAD UK)	Randomized controlled open-label multicenter trial
NCT04792294, Austria	Multicenter Analysis of Efficacy and Outcomes of Extracorporeal Photopheresis as Treatment of Chronic Lung Allograft Dysfunction	Retrospective multicenter trial
NCT03978637, USA, Canada, Belgium	An Open-Label, Single-Arm, Phase 1/2 Study Evaluating the Safety and Efficacy of Itacitinib in Participants With Bronchiolitis Obliterans Syndrome Following Lung Transplantation	Phase 1–2 open-label multicenter trial
NCT04640025, USA, Canada, Europe	A Phase 2, Open-Label, Multicenter, Rollover Study to Provide Continued Treatment of Participants Previously Enrolled in Studies of Itacitinib (INCB039110)	Phase 2 open-label multicenter trial
NCT03657342, USA and Europe	A Phase III Clinical Trial to Demonstrate Efficacy / Safety of Liposomal Cyclosporine A + Standard of Care (SoC) versus SoC Alone in Treating Chronic Lung Allograft Dysfunction / Bronchiolitis Obliterans in Patients Post Single Lung Transplant (BOSTON-1)	Phase 3 randomized controlled multicenter trial
NCT03656926, USA and Europe	A Phase III Clinical Trial to Demonstrate Efficacy / Safety of Liposomal Cyclosporine A + Standard of Care (SoC) versus SoC Alone in Treating Chronic Lung Allograft Dysfunction / Bronchiolitis Obliterans in Patients Post Double Lung Transplant (BOSTON-2)	Phase 3 randomized controlled multicenter trial
NCT04039347, USA and Europe	A Phase III, Extension Clinical Trial to Demonstrate Efficacy and Safety of Liposomal Cyclosporine A Via the PARI Investigational eFlow Device and SoC in Treating Bronchiolitis Obliterans in Patients Post Single or Double Lung Transplant	Phase 3 open-label multicenter trial

Authorship Contributions

Participated in research design: Bos, Vos.

Performed data analysis: Bos.

Wrote or contributed to the writing of the manuscript: Bos, Pradère, Beeckmans, Zajacova, Vanaudenaerde, Fisher, Vos.

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Appendix C: Ethical approval Leuven

Leuven, 06 Dec 2021

Ethics Committee
Research UZ/KU Leuven
Herestraat 49
B 3000 Leuven (Belgium)

Email : ec@uzleuven.be

prof. dr. Robin Vos
IG PNEUMOLOGIE

Our reference:	EudraCT-nr:	Belg. Regnr:
S65670		

Understanding the interactions between the immune system and transplanted lungs.

Dear colleague

The Ethics Committee Research (EC Research) of University Hospitals Leuven (UZ Leuven) has examined and discussed the above mentioned dossier at its meeting of 11 Oct 2021.

EC Research has no objection to the project provided that the data is managed confidentially and in compliance with the Belgian legislation on privacy.

EC Research emphasizes the responsibility of the PI/promotor of this study concerning the privacy of the person/patient data in contact with patients, or when accessing patient data, including the correct implementation thereof by coworkers and students. The PI/promotor is responsible for the implementation of the project proposal in accordance with applicable laws and regulations including, but not limited to, the EU regulation 2016/679 (General Data Protection Regulation), the Belgian Law on patients' rights of 22/8/2002, the regulations of the hospital concerning tissue management and the regulations of the law of December 19, 2008.

This project does not fall within the scope of the Law of 7/5/2004.

We wish to remind the researcher of his responsibility not to use material from donors who have opposed the future use of residual human body material.

Although EC Research gives a positive advice for the study, EC would like to communicate to include in the protocol the analyses that will be performed and that this study is exploratory as mentioned in the resubmission form. Please update this in the protocol when submitting an amendment to this study.

For the assessment of this dossier, documents/answers submitted on 01 Oct 2021 and 25 Nov 2021 have been taken into account.

This letter concerns:

Protocol:
Version 2 dd 25Nov2021
GDPR questionnaire:
Submitted on 24Nov2021

EC Research confirms working in accordance with the ICH-GCP principles (International Conference on Harmonization Guidelines on Good Clinical Practice), the latest version of the Declaration of Helsinki, the Oviedo Convention on Human Rights and Biomedicine and applicable laws and regulations.

EC Research confirms that - in case of conflict of interest - involved members do not take part in the vote concerning the study.

List of members: see appendix.

Points of concern: (if applicable)

The conformity of translated documents compared to the Dutch documents, is the responsibility of the sponsor.

Provided that there is a Clinical Trial Agreement, the study can only be conducted if the Clinical Trial Agreement has been approved and signed by the managing director of UZ Leuven (and/or by an authorized representative of KU Leuven R&D).

This advice of EC Research does not imply that she will assume responsibility for the planned study. You will remain responsible for the study. In addition, you should ensure that your opinion as an involved researcher is reproduced in publications, reports for the government, etc. which are the result of this study.

We request you to inform us if the study will not be initiated, or when it will be closed or prematurely ended (stating the reason).

Yours sincerely,

Prof. Dr. Minne Casteels
Chair
Ethics Committee Research UZ/KU Leuven

Cc:
FAMHP (Federal Agency for Medicines and Health Products)

CTC (Clinical Trial Center UZ Leuven)

List of members EC Research UZ/KU Leuven on 11 Oct 2021:

Chair	prof. dr. Maria-Reinhilde Casteels	Clinical Pharmacology
Vice chair	prof. dr. Dominique Bullens	Paediatrics
	De heer Aernout De Raemaeker	Medical Legislation alternate
	De heer Jean-Jacques Derèze	Medical Legislation alternate
	De heer Mathijs Swaak	Healthy volunteer repres.
	Mevr. Angélique Rézer	Medical Legislation alternate
	Mevr. Annick Vanclooster	Nurse
	Mevr. Katelijne Van Overwalle	Pt representative (alternate)
	Mevr. Lia De Wilde	Pt representative (alternate)
	Mevr. Liliane Vandergeeten	Pt representative (alternate)
	Mevr. Marilien Vandeputte	Nurse
	Mevr. Michèle Dekervel	Medical Legislation alternate
	Mevr. Teresia De Fraye	Pt representative
	Mevr. Veerle Vanparys	Pharmacist (alternate)
	apr. Josse R. Thomas	Clinical Pharmacology
	dr. Kristel Van Landuyt	Reumatology
	dr. Lut De Groote	General Practitioner
	dr. Marleen Renard	Paediatrics
	prof. André Loeckx	Pt representative (alternate)
	prof. Ben Van Calster	Statistics
	prof. Guy Bosmans	Clinical Psycology (alternate)
	prof. Pascal Borry	Ethics
	prof. dr. Anne Smits	Paediatrics
	prof. dr. Anne Uyttebroeck	Paediatrics
	prof. dr. Ariel Alonso	Statistics (alternate)
	prof. dr. Benoit Nemery	Pneumology
	prof. dr. Gregor Verhoef	Haematology
	prof. dr. Jan Verhaegen	Laboratory Medicine
	prof. dr. Jan de Hoon	Clinical Pharmacology
	prof. dr. Karin Sipido	Experimental Cardiology
	prof. dr. Koen Luyckx	Clinical Psycology (alternate)
	prof. dr. Maria Schetz	Intensive care
	prof. dr. Simon Brumagne	Physiotherapy
	prof. dr. Xavier Bossuyt	Immunology
	prof. dr. apr. Erwin Dreesen	Pharmacist (alternate)

Appendix D: Publication "Effector immune cells in chronic lung allograft dysfunction: a systematic review"

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REVIEW

Effector immune cells in chronic lung allograft dysfunction: A systematic review

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Abstract

Chronic lung allograft dysfunction (CLAD) remains the major barrier to long-term survival after lung transplantation and improved insight into its underlying immunological mechanisms is critical to better understand the disease and to identify treatment targets. We systematically searched the electronic databases of PubMed and EMBASE for original research publications, published between January 2000 and April 2021, to comprehensively assess current evidence on effector immune cells in lung tissue and bronchoalveolar lavage fluid from lung transplant recipients with CLAD. Literature search revealed 1351 articles, 76 of which met the criteria for inclusion in our analysis. Our results illustrate significant complexity in both innate and adaptive immune cell responses in CLAD, along with presence of numerous immune cell products, including cytokines, chemokines and proteases associated with tissue remodelling. A clear link between neutrophils and eosinophils and CLAD incidence has been seen, in which eosinophils more specifically predisposed to restrictive allograft syndrome. The presence of cytotoxic and T-helper cells in CLAD pathogenesis is well-documented, although it is challenging to draw conclusions about their role in tissue processes from predominantly bronchoalveolar lavage data. In restrictive allograft syndrome, a more prominent humoral immune involvement with increased B cells, immunoglobulins and complement deposition is seen. Our evaluation of published studies over the last 20 years summarizes the complex multifactorial immunopathology of CLAD onset and progression. It highlights the

Abbreviations: AMR, antibody-mediated rejection; BALF, bronchoalveolar lavage fluid; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; Ig, immunoglobulins; LTR, lung transplant recipients; MMP, matrix metalloproteinases; NK, natural killer; RAS, restrictive allograft syndrome; Tregs, T-regulatory cells.

Take Home Message: The underlying immunopathological mechanisms in chronic lung allograft dysfunction are complex, involving many effector immune cells, both innate and adaptive, as well as cytokines, chemokines and matrix remodelling.

expressed are those of the author(s) and not necessarily those of the NIHR, the Department of Health and Social Care or NHSBT.

phenotype of several key effector immune cells involved in CLAD pathogenesis, as well as the paucity of single cell resolution spatial studies in lung tissue from patients with CLAD.

KEYWORDS

adaptive immunity, chemokines, chronic lung allograft dysfunction, cytokines, immune cells, innate immunity, lung transplantation

MULTIPLE FACES OF CHRONIC LUNG REJECTION

Lung transplantation is an established treatment option for patients with end-stage lung diseases. However, long-term success continues to be challenged by the development of chronic lung rejection, occurring in up to 50% of recipients within five years post-transplant [1]. For a long time, obliterative bronchiolitis, and its clinical surrogate bronchiolitis obliterans syndrome (BOS), was the sole recognized manifestation of chronic lung rejection. Nowadays, the term chronic lung allograft dysfunction (CLAD) is used as an umbrella, which includes two main phenotypes, BOS and restrictive allograft syndrome (RAS), and a mixed phenotype [2,3]. BOS is the best known and most common phenotype, in ~70% of CLAD patients, characterized by progressive airway obliteration leading to airflow obstruction [3]. RAS has more recently been acknowledged as another phenotype of CLAD, occurring in 20–30% of CLAD patients. It is characterized by interstitial fibrosis and distortion of lung architecture, a restrictive pulmonary function decline and persistent pleuroparenchymal abnormalities on computed tomography, and is associated with a poor median survival of only 1–2 years after diagnosis [3,4]. Moreover, patients can switch from one phenotype (often BOS) to another (RAS/mixed) over time or present *de novo* with a mixed phenotype, characterized by mixed obstructive-restrictive pulmonary function limitation and persistent parenchymal opacities [4]. The acknowledgement that there are different phenotypes suggests different underlying immunological mechanisms, although BOS and RAS also share commonalities such as the presence of obliterative bronchiolitis lesions in both entities, and areas of alveolar fibrosis in BOS. [5–7]

COMPLEXITY OF THE UNDERLYING IMMUNOPATHOLOGY: A CHALLENGE

The exact immunopathological mechanisms leading to CLAD remain unclear, although multiple (immune) mechanisms are thought to contribute. Complex interactions between innate immune responses, alloreactive T, B, natural killer (NK) and dendritic cells, and subsequent adaptive immune mechanisms are considered to be fundamental [8]. Over the last decades, we have gained better understanding of the interactions between innate immunity, adaptive immunity and autoimmunity [9]. A better insight into all these processes is of utmost importance because, of all solid organ transplants, lung transplantation has the worst overall median survival of approximately 7 years [1,10–12]. A better understanding of the mechanistic differences between CLAD phenotypes and involved pathways in the inflammatory and remodelling processes is crucial. On the one hand, this might help us to identify disease-specific biomarkers that allow for early diagnosis, differentiation, and ideally predict CLAD development. On the other hand, it could lead to a personalized medicine approach through development of individualized therapies specific to each condition [13].

The primary objective of this systematic review is to comprehensively assess the phenotype of effector immune cells present in allograft tissue or bronchoalveolar lavage fluid (BALF) from lung transplant recipients (LTR) with CLAD. We postulate that most findings will be described in BOS patients, as the RAS/mixed phenotypes have only been recognized more recently. Since changes in effector immune cells at the peripheral blood level may contradict with what is detected at the allograft level, studies focusing on peripheral blood analyses were not included in this systematic review.

METHODS

The systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines [14].

Search strategy and eligibility criteria

We conducted a systematic search on the electronic databases of PubMed and EMBASE using keywords related to immune cells and CLAD. Details on the search string can

be found in Supplement 1, the last search was performed on 22 April 2021. The search was limited to publications from January 2000 onwards, English-language articles, and articles with full-text access. All titles and abstracts were reviewed thoroughly, followed by full-text review if deemed eligible for inclusion. Further eligibility criteria were limited to original research articles, human data and analyses on lung tissue or BALF from patients with CLAD. We excluded studies that did not match the topic of interest and conference abstracts. In case of unclarity, inclusion was discussed until consensus was reached.

Data extraction and synthesis

One reviewer (SB) screened all titles and abstracts and reviewed full-text articles for study selection and collected data from the reports. If needed, data collection was discussed within the author team until consensus was reached. Relevant study characteristics including study design, sample size, CLAD phenotype, and type of analysis and its results were collected.

RESULTS

Literature search

The systematic search revealed 1351 potentially relevant articles. After deleting duplicate records and primary screening, 101 articles were included for full-text evaluation (Figure 1). Of these, 25 were excluded because they did not match the topic or study design. Characteristics of the included studies are presented in Supplement 1. Fifty-one studies investigated BALF, 15 tissue analyses and 9 both tissue and BALF. Abbreviations for the factors analysed in BALF and tissue can be found in Table 1.

Innate immune cells

Neutrophils

Numerous studies have described involvement of neutrophils in CLAD. Based on differential cell count, most studies found a significantly increased percentage in BALF in BOS compared to stable LTR [15–27], with also an increase in absolute numbers [15,19,21,26–30]. Similar findings were found in studies that included RAS patients, with increased neutrophils in both BOS and RAS patients compared to stable LTR [13,28,31–33]. Few studies made a comparison with healthy controls and also noted increased neutrophils in stable LTR compared to them [15,34,35]. Upregulation of neutrophils (by neutrophil elastase staining) was also seen in BALF from RAS patients compared to stable LTR and BOS patients [36], and BOS patients versus stable LTR [36,37].

Tissue analyses demonstrated increased neutrophils (by myeloperoxidase staining) in RAS explant lungs and airways of RAS and BOS patients compared to controls [38]. Zheng and colleagues demonstrated more neutrophils (by

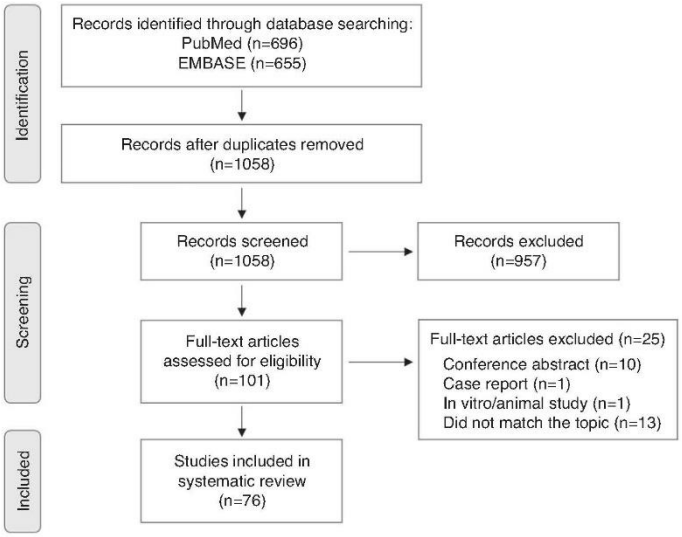


FIGURE 1 PRISMA 2020 flow diagram for systematic review

TABLE 1 Abbreviations for factors analysed in bronchoalveolar lavage fluid and tissue

C-C motif chemokine ligand	CCL
C-C motif chemokine receptor	CCR
Cluster of differentiation	CD
C-X-C-L motif chemokine ligand	CXCL
Epithelial-neutrophil activating peptide	ENA
Forkhead box P3	FoxP3
Granulocyte chemotactic protein	GCP
Human leucocyte antigen	HLA
Interferon gamma	IFN-γ
Interferon gamma-induced protein 10	IP-10
Interferon–inducible T-cell alpha chemo-attractant	ITAC
Interleukin	IL
Interleukin 1 receptor antagonist	IL-1RA
Macrophage inflammatory protein	MIP
Macrophage-derived chemokine	MDC
Major histocompatibility complex	MHC
Matrix metalloproteinases	MMP
Monocyte chemo-attractant protein	MCP
Monokine induced by interferon gamma	MIG
Pulmonary and activation-regulated chemokine	PARC
Regulated upon activation, normal T-cell expressed and secreted	RANTES
Thymus- and activation-regulated chemokine	TARC
Tissue inhibitor of metalloproteinases	TIMP
Transforming growth factor beta	TGF-β
Tumour necrosis factor alpha	TNF-α

neutrophil elastase staining) in the airways in BOS as well as stable LTR compared to healthy controls, with no difference in the lung parenchyma (RAS was not yet identified at that time) [15]. The same group noted that airway wall neutrophilia, assessed by endobronchial biopsies, was similar to healthy controls at baseline, but increased over time in BOS patients [35].

Longitudinal analyses demonstrated increased BALF and/or endobronchial neutrophils at time of BOS diagnosis compared to pre-BOS samples [25,27,30,35]. Others already showed increased neutrophils in LTR who would go on to develop BOS compared to those who would remain stable [27,39,40]. Moreover, increased neutrophils correlated with increased BOS risk [39,40]; more specifically, a BALF neutrophil percentage of ≥20% was a significant predictor for subsequent BOS ≥1 in a study by Neurohr et al. [40] Conversely, other studies could not demonstrate a difference in BALF neutrophils in future BOS or RAS patients compared to those who would remain stable [29,33,35].

Interestingly, Devouassoux et al. found no difference in neutrophil percentages in BOS stage 1 compared to stable LTR. In BOS stage 2, the increase of neutrophils occurred at BOS diagnosis, while in BOS stage 3, BALF neutrophilia preceded the diagnosis by 6 months [16]. Similarly, Heijink et al. found increased neutrophils in BALF from patients in BOS stage 1 who would progress to BOS stage 3 [24]. Finally, Vandermeulen et al. investigated a group of stable LTR with high (≥ 15%) versus low BALF neutrophil counts and found increased CLAD incidence and lower CLAD-free and overall survival in the high-neutrophil group [41]. The same group demonstrated that increased neutrophils (> 10%) in RAS patients correlated with worse graft survival [42].

Eosinophils

Data on eosinophils vary. In BOS patients, most studies found no elevated levels compared to stable LTR [13,15,17,21,23,25,26,29–31,34], while others noted an increase based on differential cell count [16,22]. Scholma et al. found elevated numbers in the bronchial, but not alveolar, BALF fraction of future BOS patients, and elevated levels correlated with BOS risk [39]. In a study comparing stable LTR with high and low neutrophil counts, increased eosinophils were seen in the high-neutrophil group [41]. In RAS patients, eosinophil percentages were higher than in stable LTR [28,32,33] or BOS patients [32]. More eosinophils (marked by EG2) were found in RAS explant lungs compared to controls and were primarily located in the lung parenchyma and around blood vessels [38].

BALF eosinophilia ≥2% correlated with CLAD and CLAD-free survival, and the worst outcome was seen in LTR with high BALF and high blood (>8%) eosinophils [43]. Verleden et al. investigated the effects of episodes of eosinophilia in LTR and demonstrated that an episode of BALF eosinophilia (≥2%) correlated with worse CLAD-free and overall survival, and predisposed to CLAD, mainly RAS but also BOS. The risk for CLAD and mortality was higher in case of multiple episodes of increased BALF eosinophilia [44]. The same group described a strong association between increased BALF eosinophils (≥2%) and survival after RAS diagnosis [42].

Macrophages

The percentage of BALF macrophages on differential cell count is often reported to be decreased in BOS patients compared to stable LTR, most likely secondary to an increase in other leucocytes, mainly neutrophils [13,15–18,20–23,25,26,28,31]. The same was true for

patients with RAS compared to stable LTR [13,28,31–33]. Ward et al. found decreased expression of alveolar macrophage surface markers (CD11a, CD11b, CD11c, CD14 and HLA-DR) in BOS and stable LTR compared to controls [34]. Most studies showed no difference in absolute macrophage numbers, although Vandermeulen et al. described an increase in BOS versus stable LTR and RAS patients [28]. On the other hand, on tissue analyses, more macrophages (CD68+) were found in RAS explant lungs compared to BOS and non-transplant controls [38]. Zheng et al. described an increase on endobronchial biopsies in BOS and stable LTR over time compared to healthy controls [35].

Natural killer cells

Ward et al. found increased NK cells (CD56/CD16+) in both BOS and stable patients compared to healthy controls [34]. Other studies also noted increased BALF NK cells (CD56+) in BOS patients versus healthy controls, but not versus stable LTR [45,46]. In addition, more NK cells were seen in small airway brushings in BOS patients compared to stable LTR and controls, with no changes in large airway brushings [45,46]. In a study by Fildes et al., more NK cells (CD16+) were found on transbronchial biopsies from BOS patients than from stable patients [47]. Notably, Calabrese et al. showed that a certain subtype of NK cells, NKG2C+ NK cells, correlated with CLAD incidence [48]. Noteworthy, this impact on CLAD incidence may have been mediated by an effect on cytomegalovirus, as higher levels of NKG2C+ NK cells were found prior to and during cytomegalovirus infection, although the elevated risk remained after adjusting for cytomegalovirus serostatus and viraemia [48].

Mast cells

Few studies provide information on the presence of mast cells after lung transplantation. One study demonstrated an increase (marked by tryptase) in RAS explant lungs compared to non-transplant controls. These mast cells were primarily located in the parenchyma and around blood vessels [38]. Another study differentiated between subtypes of mast cells and found an increase in total number of mast cells and subtype mast cell tryptase-chymase over time after transplantation, with more mast cell tryptase in stable LTR >6 months post-transplant compared to before. Moreover, they noted an increase in mast cell tryptase-chymase in CLAD patients versus stable LTR [49].

Summary for innate immune cells

In summary for innate immune cells, we can state that neutrophils were generally elevated in BALF and lung tissue from BOS and RAS patients, and increased levels after transplantation correlated with increased CLAD incidence and lower CLAD-free and overall survival. Higher levels of eosinophils were especially detected in RAS patients, while data varied in BOS studies. However, a clear correlation was again seen between elevated eosinophils and CLAD incidence (mainly RAS, but also BOS) and CLAD-free survival.

It is too early to draw conclusions about changes in macrophages, NK cells or mast cells in BALF or lung tissue from CLAD patients. Usually, a decrease in BALF macrophage percentages was seen, secondary to an increase in other leucocytes, without a difference in absolute numbers; while one study showed higher numbers in RAS explant lungs compared to BOS. For NK cells, looking at different subtypes is promising.

Adaptive immune cells

Dendritic cells

Dendritic cells form a link between innate and adaptive immunity. Leonard et al. found increased dendritic cells, marked by CD1a, MHC class II or RFD1, in BOS patients compared to stable LTR on both trans- and endobronchial biopsies. Markedly greater numbers were detected when using MHC class II expression and dendritic morphology than only CD1a as a marker [50]. A more recent study that included RAS patients, identified more dendritic cells (CD1a+) in the lung parenchyma in RAS explant lungs than in BOS or non-transplant biopsies. More resident mucosal, langerin-positive dendritic cells were present in the parenchyma in RAS compared to controls, but were decreased around the airways [38].

Lymphocytes

The majority of studies demonstrated no difference in BALF total lymphocytes based on differential cell count between CLAD patients and stable LTR [13,15,18,20–23,25,27,29–35]. A few found elevated lymphocyte percentages or numbers in BOS [17,24,26,28] or RAS [28] patients compared to stable, or in LTR with high versus low neutrophil counts [41]. Scholma et al. described increased lymphocyte numbers in the bronchial, but not alveolar, BALF fraction of future BOS patients compared to those who would remain stable, and

elevated levels correlated with increased BOS risk [39]. In contrast, Zheng et al. found an almost significantly decreased lymphocyte percentage after BOS onset versus before ($p = 0.057$) [51]. With respect to tissue analyses, the same group found that the number of endobronchial lymphocytes was similar to healthy controls at baseline but increased over time in all LTR [35].

T-lymphocytes

The proportion of BALF CD3+ lymphocytes was not significantly different between groups in some studies [20,52,53], while others showed an increase in BOS and stable LTR compared to healthy controls [34], or a decrease in BOS versus stable LTR [54] or healthy controls [45,46,54]. Various studies described increased CD8+ T cells with proportionally decreased CD4+ T cells in BOS versus stable LTR [55], or BOS and stable LTR versus healthy controls [34,53]. Others found increased CD8+ and decreased CD4+ T cells in BOS patients versus controls, with increased CD8+ T cells in BOS versus stable LTR [45,46,54] and controls [54]. One study described opposing findings with increased CD4+ and decreased CD8+ T cells in BOS patients compared to stable LTR [20], while another study could not demonstrate a difference between groups [52].

A longitudinal study of Zheng et al. noted decreased BALF CD3+ T cells over time in BOS patients, and after BOS diagnosis compared to pre-BOS samples. They could not demonstrate a longitudinal difference in CD4+ or CD8+ T cells [51]. Opposing findings were seen on endobronchial biopsies, with an increase in CD3+ and CD8+ T cells over time after transplantation, which was more pronounced in BOS patients. There was no significant difference after BOS diagnosis compared to before, but a trend was seen towards more CD8+ T-cell infiltration in BOS patients than in stable LTR [51]. Another longitudinal study also demonstrated increased BALF CD8+ and decreased CD4+ T cells after BOS onset versus before [55].

Based on the varying data found in BALF regarding lymphocyte differential cell count and CD4/CD8 subtypes (i.e. stable vs. decreased vs. increased, as described above), it is difficult to make conclusions about underlying tissue processes. Devouassoux et al. found no difference in CD4+ or CD8+ T-cells in transbronchial biopsies taken during the first year post-transplant between patients who would remain stable and those who would develop BOS. However, there were more activated (CD25+ and CD69+) T cells in future BOS patients [56]. Vandermeulen et al. identified more cytotoxic T cells in RAS and BOS explant lungs than in non-transplant controls [38]. Sato et al. also found more T cells in BOS explant lungs compared to non-transplant controls, especially in areas of active obliterative and lymphocytic bronchiolitis compared to inactive obliterative bronchiolitis. These T cells were mainly effector memory T cells and were clustered into aggregates [57].

CD4+ T-cell subsets

Several CD4+ helper T-cell subtypes, including Th1, Th2 and T-regulatory cells (Tregs), play a role in the pathogenesis of CLAD. Mamessier et al. demonstrated that there were more Th1 and Th2 cells in stable BOS than in non-BOS patients, and more Th1 cells in evolving BOS than in stable LTR. Th2 activation was increased and Th1 activation was reduced in stable versus evolving BOS [58]. Several studies focused on Tregs, which are believed to have a role in regulating or suppressing effector T-cell immune responses [52]. Bhorade et al. found less BALF FoxP3+ Tregs in BOS versus stable LTR. Furthermore, they identified more Tregs at one year post-transplant in patients who would remain stable than those who would eventually develop BOS. More specifically, a threshold of 3·2% Tregs distinguished stable LTR from those developing BOS within the first two years post-transplant. Additionally, CCL22, a chemokine involved in recruitment of Tregs, was also increased in the majority of stable patients, suggesting a potential mechanism by which these cells were attracted to the lung allograft [52]. Gregson et al. described no difference in total Tregs (CD25highFoxP3+) and CCR4 or CD103 subsets (essentially all Tregs were CCR4+ and CD103-) in BALF from future BOS patients. On the other hand, increased CCR7+ Tregs protected against subsequent development of BOS. The CCR7-ligand CCL21 correlated with CCR7+ Tregs and inversely with BOS, suggesting that this ligand might mediate recruitment of this Treg subset and downregulate alloimmunity [59]. Another study found more CD25highCD69- Tregs in stable and evolving BOS patients compared to stable LTR, with higher levels in stable versus evolving BOS patients [58]. Finally, Krustrup et al. noticed the highest number of FoxP3+ Tregs on transbronchial biopsies two weeks after transplantation. However, there was no effect of the number of FoxP3+ cells on BOS onset, nor did it predict time to BOS onset [60].

B-lymphocytes and lymphoid follicles

Few studies focused on the presence of B cells in LTR and CLAD patients. A study investigating transbronchial biopsies during the first year post-transplant noted increased CD20+ B cells in all LTR compared to non-transplant controls [56]. More B cells were seen in areas of lymphocytic and active obliterative bronchiolitis than in areas of

inactive obliterative bronchiolitis or healthy tissue [57]. Another study by Sato et al. demonstrated an increase in lymphoid aggregates in CLAD explant lungs versus non-transplant controls, no further differentiation into BOS or RAS was made at that time [61]. Finally, a recent study investigating BOS and RAS explant lungs found more CD20+ B cells in both phenotypes compared to non-transplant controls. Additionally, they found that RAS explant lungs contained more lymphoid follicles ('tertiary lymphoid organs') compared to BOS explant lungs and non-transplant biopsies. These lymphoid follicles were predominantly localized around blood vessels and in the lung parenchyma [38].

Immunoglobulins

Deposition of immunoglobulins (Ig) has been described in the bronchial epithelium, basement membrane zone, bronchial wall microvasculature and chondrocytes in transbronchial biopsies from BOS patients compared to stable LTR and non-transplant controls [62,63]. A more recent study differentiated between BOS and RAS phenotypes, and found increased levels of IgG (total IgG and IgG1-4) and IgM in BALF from RAS compared to BOS patients and stable LTR. IgA and IgE levels were also higher in RAS patients than in stable LTR, and higher total IgG and IgE levels were found in BOS versus stable LTR. Finally, increased IgG (total IgG, IgG1, IgG3 and IgG4) and IgM levels correlated with worse survival [28].

Summary for adaptive immune cells

With respect to adaptive immune cells, discordant data on BALF lymphocytes and CD4/CD8 subtypes have been reported, making it difficult to draw conclusions about underlying tissue processes. Most studies found no difference in total BALF lymphocytes, although a few found elevated levels in BOS and/or RAS patients. Data on lymphocyte subtypes varied: a majority found elevated CD8+ T cells with proportionally decreased CD4+ T cells in BOS patients, although others reported opposing findings or no differences. With regard to tissue analyses, findings were more consistent, with in general more cytotoxic T cells in CLAD patients (both RAS and BOS, especially in areas of active obliterative and lymphocytic bronchiolitis).

Surprisingly, few studies focused on the role of CD4+ T-cell subtypes in CLAD. Both Th1 and Th2 cells were elevated in BOS compared to non-BOS patients, with higher Th1 activity in evolving BOS and greater Th2 activation in stable BOS. Higher levels of Tregs were seen in stable LTR or stable compared to evolving BOS patients, and increased post-transplant levels might protect against subsequent CLAD development.

Currently, there is limited published data on the presence of B cells in CLAD patients, but they showed more B cells in areas of lymphocytic and active obliterative bronchiolitis, Ig deposition and lymphoid aggregates, especially in RAS.

Complement

Increased C3a was seen in BALF from BOS patients compared to non-transplant controls [64]. Looking at both CLAD phenotypes, C4d [28,65] and C1q [28] levels were elevated in RAS versus BOS and stable LTR, and correlated with mortality [28]. Two studies demonstrated lower levels of mannose-binding lectin in BOS patients compared to stable LTR or controls [66]; and detection of mannose-binding lectin at 3 and 6 months post-transplant correlated with later development of BOS [67]. Deposition of mannose-binding lectin was seen in the basement membrane and vasculature in BOS [68].

Magro et al. demonstrated increased C1q, C3, C4d, and C5b-9 deposition in the bronchial epithelium, basement membrane zone, bronchial wall microvasculature and chondrocytes in BOS patients compared to stable LTR and non-transplant controls [62]. Another study of the same group described bronchial wall deposition of C1q, C4d, and C5b-9 in BOS patients, in which C1q deposition was the strongest predictor of BOS [63].

Intermediate and high levels of C3d correlated with BOS and bronchial wall or septal fibrosis, and all LTR with higher values of C3d within the septae or bronchial wall eventually developed BOS [69]. Similarly, Ngo et al. described that all LTR with high, multifocal C4d deposition developed CLAD [70]. Westall et al. found no association between early (<3 months post-transplant) C3d or C4d deposition and BOS, but found significant intracapillary C3d/C4d deposition in all LTR with early BOS, along with light-microscopic features suggestive of antibody-mediated rejection (AMR) [71]. Ionescu et al. looked at C4d deposition in LTR with and without HLA antibodies and demonstrated that all patients with antibodies and subendothelial C4d deposition eventually developed BOS and/or graft loss [72]. Finally, downregulation of tissue complement-regulatory proteins (CD55, CD46) has been described in BOS patients compared to non-transplant controls [64].

In summary, various studies demonstrated increased complement levels and deposition in CLAD patients, and

TABLE 2 BALF analyses of MMP in CLAD patients

	MMP-1 concen-tration	MMP-1 activity	MMP-2 concen-tration	MMP-2 activity	MMP-3 concen-tration	MMP-3 activity	MMP-7 concen-tration	MMP-7 activity	MMP-8 concen-tration	MMP-8 activity	Pro-MMP-9	MMP-9 concen-tration	MMP-9 activity	MMP-12 concen-tration	MMP-12 activity	MMP-13 concen-tration	MMP-13 activity	MMP-8/TIMP-1	MMP-9/TIMP-1	TIMP-1	TIMP-2	TIMP-3	TIMP-4	Comments
BOS vs. stable LTR																								
Hübner et al. [25]												↑	↑						↑*	↓				* elevated post-BOS vs. pre-BOS
Vandermeulen et al. [28]											↑	↑	↑											
Banerjee et al. [74]				↑									↑											Increased expression on bronchial/bronchiolar airway epithelium in BOS vs. stable LTR and healthy controls
Riise et al. [37]				=								↑	↑											
Hardison et al. [75]									↑	↑		↑	↑											Increased in post-BOS vs. pre-BOS
Verleden et al. [22]									↑			↑						↑	↑					Caused by a difference in protein concentrations in BOS patients with high BALF neutrophil counts with no differences between BOS patients with low neutrophil counts and stable LTR
Saito et al. [36]									↑															
Heijink et al. [24]	=	=	↑	=	↑	=	↑	=	↑	=		↑	=	=	=	=	=			↑	↑	=	=	No active MMPs in BOS patients, only MMP-7 activity was detected in stable LTR. However, TIMP-1-bound MMP-7, -8, and -9 and TIMP-2-bound MMP-8 and -9 were increased in BOS, suggesting earlier activity of these MMPs
Future BOS vs. stable LTR																								
Ramirez et al. [73]													↑											
RAS vs. stable LTR																								
Vandermeulen et al. [28]											↑	↑	↑											
Saito et al. [36]									↑															

Note: Overview of studies showing BALF analyses of MMP in CLAD patients.

Abbreviations: ↑: increase; ↓: decrease; =: stable; BALF: bronchoalveolar lavage fluid; BOS: bronchiolitis obliterans syndrome; CLAD: chronic lung allograft dysfunction; LTR: lung transplant recipients; MMP: matrix metalloproteinases; RAS: restrictive allograft syndrome; TIMP: tissue inhibitor of metalloproteinases.

higher levels of complement deposition (e.g. C3d, C4d and C1q) predisposed to CLAD development.

Matrix metalloproteinases

A summary of studies investigating matrix metalloproteinases (MMP) is provided in Table 2. [22,24,25,28,36,37,73–75] In general, most studies found an upregulation of MMP-8 and/or MMP-9 concentration and/or activity in BALF from CLAD patients compared to stable LTR. Neutrophils were the main source of MMP-9 production [25], and MMP-3 [24], MMP-7 [24], MMP-8 [22,24], MMP-9 [22,24,25,37,74] and TIMP-1 [22] concentration and/or activity correlated with BALF neutrophils. Another study showed the airway epithelium itself as a direct source of MMP-2 and MMP-9 expression [74].

Cytokines

IL-8

With the exception of one study [76], increased BALF IL-8 levels were found in BOS patients compared to stable LTR [13,17,18,21,24,26,27,30,33], or compared to stable LTR and healthy controls, and stable LTR compared to healthy controls [15]. Increased IL-8 was also seen in stable LTR with high versus low neutrophil counts [41]. A correlation between IL-8 and BALF neutrophils has been demonstrated in numerous studies [15,22,27,35,40], and also between BALF IL-8 and endobronchial neutrophil numbers [35]. Interestingly, Verleden et al. found upregulation of IL-8 in CLAD patients due to an upregulation in neutrophilic BOS with no difference between non-neutrophilic BOS patients and stable LTR [22]. Longitudinal data showed increased levels after BOS diagnosis compared to pre-BOS samples in many [27,30,35,75], but not all [33], studies. Some studies demonstrated that IL-8 was elevated in future BOS patients compared to those who would never develop BOS [27,39,40], and correlated with increased BOS risk [39], while Zheng et al. found persistently elevated levels in both future BOS patients and those who would remain stable compared to healthy controls [35]. Two recent studies included RAS patients and found no difference in IL-8 levels between RAS and stable LTR [13,33].

Regarding tissue analyses, increased IL-8 expression was found on bronchial epithelial cells in a study by Elssner et al. [21] Finally, looking at donor lung biopsies, there was no difference in IL-8 expression in future BOS or RAS patients compared to patients who would remain stable [77].

IL-17

Several studies [17,32,78] demonstrated no differences in IL-17 BALF levels between BOS and/or RAS patients and stable LTR, although elevated levels at 6–12 months post-transplant were predictive of early BOS in a study by Fisichella et al. [17] Similarly, no difference was seen in stable LTR with high versus low neutrophils counts [41]. In a study looking at protein and mRNA levels, protein levels were under the detection level, but IL-17 mRNA levels were increased in BOS patients compared to stable LTR [26]. Snell et al. looked at endobronchial presence of IL-17, which was elevated early after transplant and subsequently decreased over time. There was a correlation with endobronchial CD8+ cells, but not with BALF IL-8 levels, neutrophil percentages or BOS [79].

TGF-β

Several studies described no differences in BALF TGF-β levels between BOS and stable LTR [17,29,80,81] or future BOS patients and those who would remain stable [73]. One study demonstrated that increased levels during the first 24h post-transplant were associated with increased BOS risk, also after adjusting for primary graft dysfunction [82]. TGF-β was expressed by bronchial epithelial cells, subepithelial mononuclear cells and alveolar macrophages, and TGF-β receptor I by airway epithelium, peri-airway and interstitial mononuclear cells, stromal cells and alveolar macrophages [82]. Elssner et al. found increased levels in BOS patients compared to stable LTR, but no increased TGF-β expression on BALF or bronchial epithelial cells [21]. Hodge et al. noticed a longitudinal increase in BOS compared to pre-BOS samples, but these data were only available in one patient [81]. Vanaudenaerde et al. differentiated between TGF-β protein levels and mRNA and demonstrated no difference in protein levels, but an increase in TGF-β mRNA in BOS patients compared to stable LTR [26]. On the other hand, Meloni et al. found a trend towards decreased TGF-β in BOS compared to stable patients [18].

One recent study investigated both BOS and RAS patients and found increased levels in RAS compared to stable LTR. RAS patients with high TGF-β levels had worse graft survival than those with low levels. On tissue analyses of RAS patients, TGF-β1 was located in the (sub)pleural areas and patients with high TGF-β1 expression had

TABLE 3 BALF analyses of cytokines in CLAD patients

	IL-1β	IL-1RA	IL-2	IL-4	IL-5	IL-6	IL-7	IL-9	IL-10	IL-12	IL-13	IL-15	IL-16	IL-23	TNF-α	IFN-γ	Comments
BOS vs. stable LTR																	
Fisichella et al. [17]	↑	=		=	=	=	=	↓		↓	=	=			=	=	Increased IL-15, IL-17, and TNF-α 6-12m post-transplant was predictive of early-onset BOS
Meloni et al. [18]									=	↓						=	Lower levels of IL-12 were predictive of BOS
Vos et al. [76]						=											
Elssner et al. [21]									=						=		
Belperio et al. [29]	=	↑							=						=		Increased IL-1RA preceded BOS onset
Laan et al. [23]													=				No difference at any time point
Vanaudenaerde et al. [26]	↑		↓			↑								↑			
Borthwick et al. [30]	↑														↑		Increased after BOS compared to before
Berastegui et al. [31]				=	=	=			=		=				=	↑	
Yang et al. [13]						=											
Keane et al. [84]																	
BOS vs. stable LTR				=							↑						
fibrotic BOS vs. stable LTR				=							↑						
treated BOS vs. stable LTR				=							↑						
Verleden et al. [22]																	IL-1β correlated with BALF neutrophils
neutrophilic BOS vs. stable LTR	↑														=		
neutrophilic vs. non-neutr. BOS	↑														=		
non-neutrophilic BOS vs. stable	=														=		
Verleden et al. [32]																	
neutrophilic BOS vs. stable LTR	↑	↑	=	↑			↑	=	=		=						
non-neutrophilic BOS vs. stable	=	=	=	=			=	=	=		=						
neutrophilic vs. non-neutr. BOS	↑	=	=	=			↑	=	=		=						
Suwara et al. [33]																	
ARAD vs. stable LTR	↑	↑				=									=		Increased IL-1α after BOS compared to pre-BOS
PAN vs. stable LTR	↑	↑				↑									↑		
Future BOS vs. stable LTR																	
Ramirez et al. [73]	=		=	=		=			=	=					=		
Scholma et al. [39]						↑											Increased IL-6 correlated with increased BOS risk

TABLE 3 (Continued)

	IL-1β	IL-1RA	IL-2	IL-4	IL-5	IL-6	IL-7	IL-9	IL-10	IL-12	IL-13	IL-15	IL-16	IL-23	TNF-α	IFN-γ	Comments
Stable LTR with high neutrophil count vs. low neutrophil count																	
Vandermeulen et al. [41]	↑	↑	=	↑	↑		=	↑	↑	=	=	=			↑	=	Correlation between IL-1β and IL-4, IL-8, CCL2, CCL3, CCL4, and CCL11. Correlating trend between IL-1β and CLAD-free survival ($p = 0.084$)
RAS vs. stable LTR																	
Suwara et al. [33]	=	=				↑									=		
Yang et al. [13]						=											
Berastegui et al. [31]																	
vs. stable LTR				=	↑	=			=		=				=	↑	
vs. BOS				=	↑	=			=		=				=	=	
Verleden et al. [32]																	IL-6 was associated with survival after RAS diagnosis
vs. stable LTR	↑	↑	=	=	=	↑		=	=		=						
vs. non-neutrophilic BOS	=	↑	=	=	=	↑		=	=		=						
vs. neutrophilic BOS	=	=	=	=	=	↑		=	=		=						
Other																	
Verleden et al. [85]	High IL-6 levels first 24h post-transplant correlated with better CLAD-free and graft survival IL-6 correlated with BALF neutrophils and IL-8																
Neujahr et al. [78]	No correlation IL-1RA, IL-13 or IL-17 during first year post-transplant and future BOS or graft failure																

Note: Overview of studies showing BALF analyses of cytokines in CLAD patients.

↑: increase; ↓: decrease; =: stable; ARAD: azithromycin-reversible allograft dysfunction; BALF: bronchoalveolar lavage fluid; BOS: bronchiolitis obliterans syndrome; CLAD: chronic lung allograft dysfunction; LTR: lung transplant recipients; PAN: persistent airway neutrophilia; RAS: restrictive allograft syndrome; other: see Table 1.

TABLE 4 BALF analyses of chemokines in CLAD patients

	CCL2/MCP-1	CCL3/MIP-1α	CCL4/MIP-1β	CCL5/RANTES	CCL7/MCP-3	CCL11/eotaxin-1	CCL17/TARC	CCL18/PARC	CCL19/MIP-3β	CCL20/MIP-3α	CCL22/MDC	CCL25/eotaxin-3	CXCL5/ENA-78	CXCL6/GCP-2	CXCL9/MIG	CXCL10/IP-10	CXCL11/ITAC	Comments
BOS vs. stable LTR																		
Fisichella et al. [17]	↓	=	=	↑		=										↑		
Meloni et al. [18]	↑			=														
Belperio et al. [87]															↑	↑	↑	Levels were not increased 4.5m before BOS onset
Belperio et al. [86]	↑																	Sources of CCL2 were airway epithelium and mononuclear cells
Reynaud et al. [27]	↑			↑														CCL2 correlated with BALF neutrophils and IL-8
Verleden et al. [22]																		
neutrophilic vs. non-neutr. BOS	↑			↑														CCL2 and CCL5 correlated with BALF neutrophils
Verleden et al. [32]																		
neutrophilic BOS vs. stable LTR		↑	↑	=	↑			↓					=	=	=		=	
neutrophilic vs. non-neutr. BOS		↑	=	=	=			↓					=	=	=		=	
non-neutr. BOS vs. stable LTR		=	=	=	=			=					=	=	=		=	
Sinclair et al. [80]																		
BOS and stable LTR vs. healthy controls	↑																	
Future BOS vs. future stable LTR																		
Meloni et al. [88]		=	=				=		↑	↑	↑	=						Increased CCL19, CCL20 and CCL22 levels at 6m post-transplant predicted BOS onset.
Scholma et al. [39]	=																	Increased CCL2 correlated with BOS risk.
Reynaud et al. [27]	↑			↑														
Stable LTR with neutrophil high vs. low counts																		
Vandermeulen et al. [41]	↑	↑	↑	↑		↑		=			=		=	=		↑		
RAS vs. stable LTR																		
Yang et al. [13]																=		Trend towards increased CXCL10 ($p = .08$).
Verleden et al. [32]																		CXCL10 and CXCL11 were associated with survival after RAS diagnosis.
vs. stable LTR	↑	↑	↑	=				=					=	=	=	↑	=	
vs. non-neutrophilic BOS	=	↑	↑	=				=					=	=	=	=	=	
vs. neutrophilic BOS	=	=	=	=				↑					=	=	=	=	=	

TABLE 4 (Continued)

	Comments	CXCL11/ITAC	CXCL10/IP-10	CXCL9/MIG	CXCL6/GCP-2	CXCL5/ENA-78	CCL25/eotaxin-3	CCL22/MDC	CCL20/MIP-3α	CCL19/MIP-3β	CCL18/PARC	CCL17/TARC	CCL11/eotaxin-1	CCL7/MCP-3	CCL5/RANTES	CCL4/MIP-1β	CCL3/MIP-1α	CCL2/MCP-1
Other																		
Meloni et al. [88]	No difference in CCR4, -6, or -7 expression but higher density of CCR6 in future BOS vs. stable LTR with increased CCR4 and -6 expression on CD68+ cells																	
Agostini et al. [89]	T-cells expressing CXCR3 were found in areas of active obliterative bronchiolitis on transbronchial biopsies and BALF in BOS patients																	
Belperio et al. [87]	Prolonged elevation of CXCR3 ligands correlated with increased CLAD risk																	
Neujahr et al. [78]	Cumulative increased CXCL9 and CXCL10 during first year post-transplant correlated with BOS and graft failure and preceded BOS onset by 3 and 9 months																	
Neujahr, Agostini, Shino et al. [78,89,90]	CXCL9, CXCL10 and CXCR3 were expressed by airway epithelial cells, mononuclear cells, and alveolar macrophages																	

Note: Overview of studies showing BALF analyses of chemokines in CLAD patients.
↑: increase; ↓: decrease; =: stable; BALF: bronchoalveolar lavage fluid; BOS: bronchiolitis obliterans syndrome; CLAD: chronic lung allograft dysfunction; LTR: lung transplant recipients; RAS: restrictive allograft syndrome; other: see Table 1.

more local CD20+ B cells, CD4+ and CD8+ T cells, and CD68+ cells [83].

Other cytokines

Table 3 displays the main analyses of other cytokines in BALF in CLAD patients [13,17,18,21–23,26,29–33,39,41,73,76,78,84,85]. Additionally, in a study of donor lung biopsies, increased IL-1β and IL-6 expression were seen in future CLAD patients, and increased IL-6 expression in pre-implanted lungs of future BOS patients compared to RAS and stable LTR. There was a significant association between high IL-6 expression and later BOS development [77].

Summary for cytokines

Overall, numerous studies have examined BALF cytokines in CLAD patients and we can conclude that a correlation between IL-8 and neutrophils is present, with elevated IL-8 levels in BOS patients, especially neutrophilic BOS patients, and no change in RAS patients. Some studies reported increased TGF-β levels in BOS patients, although several other studies failed to support this finding. Interestingly, a recent study documented increased levels in RAS patients that correlated with worse graft survival, perhaps suggesting a more prominent role for TGF-β in this phenotype. Regarding other cytokines, levels were often not consistently different across groups, except that several studies reported increased IL-1β and IL-1RA in BOS patients, and some showed elevated IL-6 levels in BOS and/or RAS patients. Finally, since mRNA and protein levels may differ, it is important to consider both methods of analysis.

Chemokines

Table 4 provides an overview of BALF chemokines investigated in CLAD patients [13,17,18,22,27,32,39,41,78,80,86–90]. To summarize, several studies found elevated levels of chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES or CXCL10/IP-10 in BOS and/or RAS patients, while others did not. With respect to tissue analysis, Sato et al. found increased CXCL12 in alveolar and airway epithelial cells and CCL21+ lymph vessels in CLAD explant lungs compared to non-transplant controls [61].

DISCUSSION

Post-transplant airway and/or interstitial fibrosis results from a chronic immunological, inflammatory insult that

leads to fibroproliferation and obliteration of distal airways and/or fibrosis of the lung parenchyma [27]. As presented here, multiple mechanisms are involved in CLAD (both BOS and RAS phenotypes), including allograft infiltration of innate immune cells, alloreactive T, B and NK cells, upregulation of numerous cytokines and chemokines, and matrix remodelling. Although BOS was first considered as a unique manifestation of chronic lung rejection, the identification of the RAS phenotype has changed our perception of this pathology [9]. As expected, less data are currently available on the specific mechanisms in RAS and the differences between RAS and BOS. After all, many studies predated the establishment of the RAS phenotype, although these chronic rejection groups probably also sometimes contained RAS patients.

Various findings overlap, such as the presence of neutrophils in BALF from patients with BOS and RAS, without differences between the two phenotypes [13,28,31–33]. On the other hand, the presence of eosinophils seemed more pronounced in RAS [32,38]. Episodes of BALF eosinophilia predisposed to both CLAD phenotypes, but particularly RAS, with a strong correlation between increased BALF eosinophils and survival after RAS diagnosis [42,44]. Theoretically, steroids inhibit eosinophil accumulation. However, increased eosinophilia in CLAD patients may indicate subtherapeutic steroid dosing or (relative) corticosteroid resistance as it was even present in patients with higher doses of corticosteroids, indicating that eosinophils might have an important role [41]. Eosinophilic granulocytes are able to release potent cytotoxic granule products, including proteins and cytokines, associated with cellular damage, and can regulate immune responses by attracting other immune cells via stored chemokines [39,43]. Additionally, the release of eosinophilic cationic protein attracts fibroblasts and stimulates TGF-β1 release, a known inducer of fibrosis [43,44]. (Table 5) This makes us speculate about a possible role for eosinophils in the mechanism of tissue fibrosis in RAS [43].

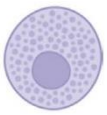
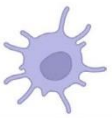
Secondly, RAS has a more prominent humoral immune involvement, and the increase in B cells, immunoglobulins, and the presence of organized lymphoid follicles and complement is more specific in RAS [28,38]. This raises the question whether there is a continuum between AMR and RAS [28]. AMR is usually caused by donor-specific antibodies directed against donor human leucocyte antigens, leading to complement dependent and independent recruitment of immune cells leading to tissue injury and allograft dysfunction. AMR can present itself in a hyperacute (though currently rare due to improved antibody detection assays), acute or chronic form [91]. This has raised the thought whether RAS arises from a chronic form of AMR, although evidence supporting this paradigm is lacking. However, studies in this systematic review confirmed the higher presence of B-cells, lymphoid follicles and immunoglobulins in RAS.

Besides the more pronounced presence of eosinophils and humoral immunity, not much is known about the differences at an immunopathological level between BOS and RAS. Reinvestigating old data in the light of our current knowledge would be useful, but presumably difficult to accomplish because not all details will be available, and we will therefore have to look for additional studies in the near future.

The same goes for the mixed phenotype. The reason why some patients transition from one phenotype to another remains poorly understood, although in some patients an episode of infection or AMR occurred between CLAD and mixed diagnosis [7]. Moreover, like in RAS *ab initio* patients, a higher number of circulating donor specific antibodies was seen in mixed phenotype patients, suggesting a role for humoral immunity. Additionally, similar histopathology findings were reported in patients that evolved from BOS to mixed and RAS *ab initio* patients, with survival rates comparable to RAS *ab initio* patients, suggesting a similar pathophysiology [7]. Regarding BALF analysis, Verleden et al. found no difference in total cell count, macrophages, neutrophils or lymphocytes between the mixed phenotype and RAS patients, but a higher percentage of eosinophils in the RAS group [7].

Given that a lot of risk factors (e.g. acute rejection, infection, non-specific triggers of lung injury) are shared between BOS and RAS, combined with some similar findings in both entities (e.g. obliterative bronchiolitis lesions in RAS, areas of alveolar fibrosis in BOS) and the fact that patients can transition from one phenotype to another supports the hypothesis that BOS and RAS may be a continuum of the same disease [5–7]. Interestingly, there is considerable overlap between obliterative bronchiolitis after lung transplantation, after allogeneic hematopoietic stem cell transplantation and in clinical settings other than post-transplant (e.g. post-infectious) [92,93]. Similarly, findings of alveolar and pleuroparenchymal fibroelastosis are not limited to RAS, but can also be found after allogeneic hematopoietic stem cell transplantation, drug exposure, radiation and occasionally idiopathic, suggesting a comparable immunological reaction to lung injury [92–94]. It therefore seems plausible that different causes of severe, repetitive or chronic lung injury can serve as a common denominator leading to inflammation and immune cell activation, and ultimately to pulmonary fibrosis, in which different clinical manifestations can be seen depending on the principal site of injury (bronchiolar/alveolar/vascular compartment) [5].

TABLE 5 Function of innate immune cells

Cell type	Characteristics	Location	
Neutrophils [109]	Chemotaxis Phagocytosis Release of pro-inflammatory cytokines, reactive oxygen species, hydrolytic enzymes and proteases,... Generation of neutrophil extracellular traps (NETosis) Epithelial-to-mesenchymal transition	Migration from circulation into tissue	
Eosinophils [110]	Release of cytokines, chemokines, reactive oxygen species, cytotoxic cationic granule proteins, enzymes,... Production of TGF-β Epithelial-to-mesenchymal transition	Circulation in blood and migration into tissue	
Macrophages [111]	Phagocytosis Antigen presentation Production of enzymes, complement proteins, and regulatory factors M1 (classically activated) macrophages: pro-inflammatory cytokine release, bactericidal and phagocytic function, promotion of a local Th1 environment M2 (alternatively activated) macrophages: participation in type 2 immune responses, anti-inflammatory cytokine release, tissue repair, production of TGF-β	Tissue resident macrophages: alveolar macrophages, interstitial macrophages Migration from circulation into tissue	
NK cells [112]	Activating and inhibitory receptors Cytolytic granule mediated cell apoptosis Antibody-dependent cell-mediated cytotoxicity Secretion of cytokines and chemokines Tumour cell surveillance Missing-self (MHC I) recognition Clearance of senescent cells	Circulation in blood and migration into tissue	
Mast cells [113]	Release of histamine, serine proteases (e.g. tryptase, chymase), cytokines, reactive oxygen species, and other mediators	Mucosal and epithelial tissues (including respiratory epithelium) Migration of mast cell progenitors upon antigen-induced inflammation	
Dendritic cells [114]	Antigen presentation Release of pro-inflammatory cytokines and chemokines	Present in lymphoid organs, blood, epithelial tissue (including lungs) Migration to lymph nodes upon activation	

Note: Overview of some of the main general actions of innate immune cells. Images from BioRender.com.

Traditionally, CLAD was thought to be primarily elicited by T-cell immune responses, on which our currently used immunosuppressive regimens are based. However, we are nowadays aware of the multifactorial aetiology and contribution of many other factors, including pathologic B cells, innate immune cells and growth factors [8]. BALF profiles have been looked at in many studies and demonstrate involvement of neutrophils, eosinophils, NK cells, and possibly dendritic cells and mast cells. However, these results have proven to be not sensitive or specific enough to be relied on for accurate CLAD diagnosis [27]. Furthermore, the fact that not one specific innate immune cell is involved, but almost all types of innate immune cells, makes targeted therapy difficult.

Numerous studies illustrated neutrophilic inflammation as a driving force in this process, and BALF neutrophilia correlated with CLAD onset and severity [15,16,39–41]. Whether neutrophils were attracted to the airways because of infection and innate immune reaction, or as part of an alloreactive immune response to 'non-self' antigens, they are potent effector cells [35]. Neutrophils contain strong pro-inflammatory mediators, such as reactive oxygen metabolites, hydrolytic enzymes and proteases, which potentially induce tissue

injury and extracellular matrix degradation [15]. An additional mechanism of neutrophil-mediated cell injury is the formation of neutrophil extracellular traps and induction of epithelial-to-mesenchymal transition of lung epithelial cells [95,96]. (Table 5) IL-8 has been identified to account for a large portion of neutrophil chemotactic activity, and significantly higher percentages of neutrophils and IL-8 levels were also detected in future BOS patients [15,40]. IL-17 might trigger IL-8 and subsequent neutrophil chemotaxis [17,97]. In contrast to this IL-17-driven neutrophilia, which is also the driver in azithromycin-reversible allograft dysfunction [26,98], IL-1 (especially agonists IL-1α and IL-1β, and receptor antagonist IL-1RA) can also be a source of persistent neutrophilia [33,41]. Neutrophils play a key role not only in the onset of CLAD, but also in primary graft dysfunction for example, but given their important role in fighting infections, neutrophil actions cannot be completely negated [99].

The role of other innate cells in CLAD, for example dendritic, NK and mast cells, needs to be further clarified and some general immune functions of these cells are listed in Table 5. It is currently unclear whether these cells are actively involved in CLAD pathogenesis, or merely present because of more pronounced activation of and attraction by other cells. For example, increased dendritic cells in CLAD patients presumably reflect upregulation of expression of foreign allograft antigens [50]. Interestingly, in CLAD patients, peripheral blood NK cells were decreased but activated, while there was an increase in lung tissue, suggesting systemic activation and migration to the lung during CLAD [47]. This also highlights the importance of looking at the activation status, and not just the amount of immune cells present.

Thirdly, the precise involvement of macrophages in CLAD remains understudied and most of the included studies did not differentiate between macrophage subtypes. Macrophages are an essential component of the innate immune system, able to contribute to CLAD through pro-inflammatory cytokine production, antigen processing and presentation, and tissue remodelling, but it is unclear whether they contribute solely by initiating immune responses or more specifically [100].

Finally, what has become less clear in these studies is the importance of different immune cell subtypes. Similar to T cells ranging from protective Tregs to cytotoxic T cells, more protective and more damaging NK cells exist, due to either activating or inhibitory actions through different receptors [101]. Calabrese and colleagues demonstrated that a specific subtype, NKG2C+ NK cells, correlated with CLAD incidence [48]. On the other hand, NK cells may promote graft tolerance through depletion of donor antigen-presenting cells and alloreactive T cells via killer immunoglobulin-like receptors [101]. The same probably also applies to eosinophils, where it has recently been illustrated in animal models that eosinophils can downregulate alloimmunity. These immunosuppressive effects are presumably exerted by a different subtype of eosinophils [102].

We deliberately excluded studies with peripheral blood analyses, as these findings do not always reflect what is happening at a tissue level in the allograft. For example, immune cells can be attracted from the systemic circulation into the allograft (and thus be normal or decreased in serum while elevated in the allograft). Furthermore, even lung tissue and BALF analyses can be contradictory, which we saw especially in the lymphocytes and their subsets, where the data were not always consistent with more consistent findings in tissue, highlighting the importance of tissue analyses.

The actions of effector T and B cells remain crucial in the pathogenesis of CLAD, and immunological reactions are regulated by different subsets of T cells, ranging from cytolytic activity (CD8+ T cells, Th1 cells), activation of innate and adaptive immune cells, to propagating (pro-inflammatory/profibrotic cytokine release from Th1 and some Th2 cells) or dampening inflammation (Tregs, anti-inflammatory cytokine release from Th2 cells) [52,58,103]. Overall, increased cytotoxic T cells were present in CLAD patients, especially in areas of ongoing fibrosis. It is surprising how few BALF and/or tissue studies were found that focused on the effects of these subtypes in CLAD. In future research, it will be important to look at more detail not only at the presence of these adaptive immune cells but also their activation status as well as the exact roles of different subtypes, including effector memory T and B cells, tissue resident cells, and γδ-T cells in the onset of CLAD.

The adaptive immune response relies on the ability of T and B cells to undergo extensive cell division and clonal expansion to generate an adequate immune response to antigen exposure. Therefore, in contrast to many other somatic cell lineages, T and B cells express high levels of telomerase activity at regulated stages of development and upon activation of mature cells. Telomeres and telomerase play a critical role in the regulation of the replicative lifespan of cells. Briefly, telomeres are repetitive nucleotide sequences located on the terminal region of chromosomes that protect the integrity of chromosomes during cell replication. Telomere length decreases with cellular ageing and biologic stressors, but excessive shortening triggers cellular senescence or apoptosis. Telomerase is an enzyme that synthesizes telomeres and compensates for telomere loss that occurs with cell division [104–107]. Consequently, individuals with short telomeres (whether or not caused by mutations in the telomerase maintenance mechanism) are more susceptible to a range of premature organ dysfunctions such

as pulmonary fibrosis. After lung transplantation, it has been shown that these patients had a higher incidence of clinically significant leukopenia and CLAD, with decreased CLAD-free survival [106,107].

Finally, the actions of many immune cells rely on the presence of cytokines and chemokines to activate and direct them into the allograft [31]. Of the chemokines found to be upregulated in CLAD, CCL3/MIP-1α, CCL5/RANTES, CCL7/MCP-3 and CCL11/eotaxin are known to attract eosinophils, while most chemokines are able to recruit macrophages and/or T cells [32,41].

The three IFN-γ-induced CXCR3 ligands, CXCL9/MIG, CXCL10/IP-10 and CXCL11/ITAC, have been shown to be important in CLAD [78,87–89]. Persistent expression leads to ongoing peribronchial/-bronchiolar leucocyte infiltration, which eventually promotes fibrotic remodelling, and blockade of CXCR3 was associated with a significant reduction in intra-graft mononuclear cell infiltration [87,88]. Similar results were seen with CCL2/MCP-1, a potent mononuclear phagocyte chemo-attractant. CCL2 also correlated with neutrophils and IL-8, demonstrating distinct mechanisms by which a specific receptor/chemokine biological axis may be involved in the pathogenesis of BOS and RAS [18,22,27,32,86,88].

The role of CCL19/MIP-3β has not been widely studied, but CCR7, the receptor for CCL19 and CCL21, is involved in migration of central memory T cells and mature dendritic cells, and maturation and differentiation of T cells [88]. In addition, a role in tissue repair mechanisms has been implicated as CCR7 is expressed on peripheral blood fibrocytes, airway smooth muscle, and fibroblasts. The CCR7/CCL19 axis seemed to play a role in airway smooth muscle hyperplasia in asthmatics and CCR7 was also expressed on fibroblasts in fibrotic areas of idiopathic pulmonary fibrosis patients [88]. Altogether, a possible involvement of CCL19/CCR7 interaction in the fibroproliferative process of CLAD has been suggested [88].

Several limitations of the studies included in this systematic review need to be addressed, in addition to the fact that most focused on the BOS phenotype. Most studies had a cross-sectional study design and a small study population. Different types of analyses and techniques have been used, making an adequate comparison difficult, and findings were often inconsistent. The impact of other factors, such as airway infection or colonization, is not discussed in this review, although many studies took this into account or excluded these patients.

Finally, this systematic review focused on immune cells and cytokines and chemokines involved in CLAD pathogenesis, but we know CLAD is a much more complex pathology involving many other factors, such as different types of antibodies and fibrotic growth factors. Also, emerging evidence underscored significant interactions between autoimmunity and alloimmunity after transplantation, with involvement of Th17 cells and IL-17, and lung-associated self-antigens (e.g. collagen V, K-alpha 1 tubulin) [108].

FUTURE RESEARCH DIRECTIONS

Based on these findings, future research should include studies to address the following:

- Specific mechanistical differences between CLAD phenotypes, especially BOS versus RAS;
- Use of single cell and spatial studies in lung tissue;
- Disease-specific BALF biomarkers for timely diagnosis and endo/phenotyping of CLAD;
- Identifying specific immune cells or (profibrotic) pathways in the pathogenesis of CLAD which are targetable for treatment;
- Use of BALF gene expression profiling to identify LTR at risk for acute rejection and/or CLAD;
- Developing immunosuppressive drugs specifically targeting certain subtypes of T and B cells, upregulating Tregs, and/or modulating other immune cells involved in CLAD pathogenesis.

CONFLICT OF INTEREST

None of the authors of this manuscript have any conflicts of interest to disclose in relation to this manuscript. The authors confirm that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form in English or in any other language, without the written consent of the copyright holder.

The data that support the findings of this study are available on request from the corresponding author. All authors contributed in an important manner to the study design, data collection and analysis, or writing of the paper according to the guidelines of the International Committee of Medical Journal Editors (ICMJE). All authors have read and approved the manuscript, all authors take responsibility for the manuscript, and the submitting author has permission from all authors to submit the manuscript on their behalf.

AUTHOR CONTRIBUTION

SB performed research, collected data, wrote the manuscript. AF and RV critically revised the manuscript. AJF co-ordinated and designed the research, critically revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Appendix E: Appendix of the systematic review

1. Overview of included articles

Abbreviations used in table			
Ab	antibodies	**IP-10**	interferon gamma-induced protein 10
ACR	acute cellular rejection	**ITAC**	interferon–inducible T-cell alpha chemoattractant
AMR	antibody-mediated rejection		
AR	acute rejection	**LB**	lymphocytic bronchiolitis
ARAD	azithromycin-reversible allograft dysfunction	**LTR**	lung transplant recipient
BALF	bronchoalveolar lavage fluid	**LTx**	lung transplantation
BB	bronchial brushing	**MBL**	mannose-binding lectin
BOS	bronchiolitis obliterans syndrome	**MCP**	monocyte chemoattractant protein
CCL	C-C motif chemokine ligand	**MDC**	macrophage-derived chemokine
CCR	C-C motif chemokine receptor	**MHC**	major histocompatibility complex
CD	cluster of differentiation	**MIG**	monokine induced by interferon gamma
CLAD	chronic lung allograft dysfunction	**MIP**	macrophage inflammatory protein
CMV	cytomegalovirus	**MMP**	matrix metalloproteinases
CRP	C-reactive protein	**MPO**	myeloperoxidase
CXCL	C-X-C motif chemokine ligand	**NK**	natural killer
DAD	diffuse alveolar damage	**NRAD**	neutrophilic reversible allograft dysfunction
DSA	donor-specific antibodies		
EBB	endobronchial biopsy	**OB**	obliterative bronchiolitis
fBOS	fibrotic bronchiolitis obliterans syndrome	**PGD**	primary graft dysfunction
FoxP3	forkhead box P3	**RANTES**	regulated upon activation, normal T-cell expressed and secreted
GCP	granulocyte chemotactic protein		
GM-CSF	granulocyte-macrophage colony-stimulating factor	**RAS**	restrictive allograft syndrome
		TARC	thymus- and activation-regulated chemokine
HLA	human leukocyte antigen		
IF	immunofluorescence	**TBB**	transbronchial biopsy
IFN-γ	interferon gamma	**TGF-β**	transforming growth factor beta
Ig	immunoglobulin	**TIMP**	tissue inhibitor of metalloproteinases
IHC	immunohistochemistry	**TNF-α**	tumour necrosis factor alpha
IL	interleukin	**Tregs**	regulatory T-cells
IL-1RA	interleukin 1 receptor antagonist	**VEGF**	vascular endothelial growth factor

Author	Study design	Population	BALF/tissue	Analysis	Results
Agostini et al., 2001[1]	Retrospective	24 LTR (3 BOS and 8 TBB with AR)	BALF TBB	CXCL10/IP-10, CXCR3 In vitro chemotaxis	BALF: T-cells expressed CXCR3 and IFN-γ during AR and BOS. TBB: areas of AR and active OB were infiltrated by T-cells expressing CXCR3. TTB and BALF: high expression of CXCL10 by macrophages and occasionally by epithelial cells in AR and BOS. Higher expression of CXCR3 and IFN-γ on BALF T-cells and CXCR3 on TBB T-cells in higher grade AR than lower grade (p<.01).
Banerjee et al., 2011[2]	Prospective	8 BOS 18 stable LTR 10 healthy controls	BALF BB	MMP-2, MMP-9, TIMP-1, TIMP-2	Increased BALF neutrophils (%) in BOS vs stable LTR (p=.08). Increased MMP-2 and -9 activity in BALF and bronchial and bronchiolar airway epithelium expression in BOS vs stable LTR and controls (all p≤0.01). Airway epithelium was a direct source of MMP-2 and -9 expression in BOS patients. Increased MMP-9/TIMP-1 and MMP-2/TIMP-2 ratio in BOS vs stable LTR (both p≤0.01). No difference in TIMP-1 or -2 expression. No difference in MMP-2 and MMP-9 expression between small and large airways. No correlation between BALF neutrophils and MMP-2 or -9 expression, correlation between BALF neutrophils and MMP-2 and -9 activity (p≤0.01).
Banga et al., 2016[3]	Retrospective Longitudinal	5 CLAD	TBB	Mast cell (MC), MC-tryptase (MC-t), MC-tryptase/chymase (MC-tc) determined during early stable post-LTx (< 6m), late stable post-LTx (> 6m), ACR, and CLAD	MC (#) and MC-tc (#) increased over time (both p<.01). Increased MC-tc (#) in CLAD vs others (all p<.05), increased MC-tc/MC-t ratio in CLAD vs others (all p<.001). Increased MC-t (#) in late stable vs early stable (p=.04).
Belperio et al., 2001[4]	Prospective Cross-sectional	20 BOS 27 AR 30 stable LTR	BALF	CCL2/MCP-1 In vitro chemotaxis, role of MCP-1 in murine model	Increased CCL2 in BOS vs stable LTR, and AR vs stable LTR, with more mononuclear cell chemotaxis (all p≤.01). Sources of CCL2 were airway epithelium and mononuclear cells.
Belperio et al., 2002[5]	Prospective Longitudinal	108 LTR	BALF	CXCL9/MIG, CXCL10/IP-10, CXCL11/ITAC Role of CXCR3 in murine model	Increased CXCL9, CXCL10, and CXCL11 in BOS and AR vs stable LTR (all p<.05). Elevated levels were predictive of acute or chronic rejection. No increase of CXCL9, CXCL10, and CXCL11 at a mean of 4.5 months before BOS onset.
Belperio et al., 2002[6]	Prospective Longitudinal	22 BOS 33 AR 30 stable LTR	BALF	IL-1RA, IL-1β, IL-10, TGF-β, TNF-α	Increased neutrophils (#) in BOS vs AR and stable LTR (both p<.05), no difference in future BOS. Increased IL-1RA in BOS vs AR and stable LTR (both p<.05), and this preceded BOS onset. No difference in IL-1β, IL-10, TGF-β, or TNF-α.
Berastegui et al., 2017[7]	Retrospective Cross-sectional	15 BOS 7 RAS 29 stable LTR	BALF	Differential cell count, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-γ GM-CSF	Increased neutrophils (%) and decreased macrophages (%) in BOS and RAS vs stable LTR (all p≤0.01). Increased IFN-γ in BOS and RAS vs stable LTR, increased IL-5 in RAS vs BOS and stable LTR (all p<.05).
Bhorade et al., 2010[8]	Prospective Longitudinal	20 LTR (6 developed BOS)	BALF	CD3, CD4, CD8, CD25, FoxP3, CCL17, CCL22/MDC Blood analyses	Decreased CD4+FoxP3+ cells (%) and CCL22 in BOS vs stable LTR (both p<.05), no difference in CCL17.

Author	Study design	Population	BALF/tissue	Analysis	Results
					Increased CD4+FoxP3+ cells (%) at 1y in LTR who would remain stable vs future BOS (p=.017). Threshold of 3.2% CD4+FoxP3+ distinguished stable LTR from those developing BOS within first 2y post-LTx. No difference between BOS and stable LTR CD3+, CD4+, CD8+ T-cells in BALF. During AR, more BALF CD4+FoxP3+ cells (%) in LTR who did not progress to BOS (p=.002). More CD3+ T-cells in blood than in BALF in BOS and stable LTR. No difference in blood CD4+FoxP3+ cells, CD3+, CD4+, CD8+ T-cells in BOS vs stable LTR. More CD4+FoxP3+ cells (%) in BALF vs blood in stable LTR at 1y.
Borthwick et al., 2013[9]	Retrospective Longitudinal	52 LTR (26 developed BOS)	BALF	Differential cell count, IL-1β, IL-8, TNF-α In vitro analyses	Increased total cell count and neutrophils (#) in < 3 mos. prior to BOS vs > 3 mos. prior to BOS and stable LTR (both p<.01). No difference in macrophages, eosinophils, lymphocytes (#). Increased IL-1β, IL-8, and TNF-α in < 3 mos. prior to BOS vs > 3 mos. prior to BOS (> 3 mos.) and stable LTR (all p<.001). Increased total cell count, neutrophils, TNF-α, IL-1β, and IL-8 in *P. aeruginosa* culture positive LTR vs culture negative LTR (all p<.05).
Budd et al., 2012[10]	Retrospective Cross-sectional	8 BOS explant lungs 6 at time of implantation 6 DAD in non-LTx controls	Biopsies	MBL Blood MBL, C3, C4	MBL localized to vasculature and basement membrane during cold ischemia and BOS. Increased plasma MBL in BOS vs stable LTR and in LTR < 5 years vs > 5 years post-LTx (all p<.05). Increased plasma C3 in BOS vs stable LTR, increased plasma C4 in > 5 years post-LTx BOS vs stable LTR (all p<.05).
Calabrese et al., 2019[11]	Prospective	130 LTR	BALF	NK cells (NKG2A, NKG2C, KIR2D, KIR3D, KIR3DL1, CD56, CD3, CD45) Blood analyses (n=40 LTR)	Increased NKG2C+ NK cells correlated with CLAD. 7.2% of the NK cells were NKG2C+. NKG2C+ NK cells were more mature and proliferative than NKG2C- NK cells (all p<.001). Increased NKG2C+ NK cells in LTR with high CMV viraemia (p=.0001). No difference or correlation between BALF and blood NK cells, no difference but positive correlation between BALF and blood NKG2C+ NK cells.
Carroll et al., 2011[12]	Retrospective Longitudinal	37 LTR	BALF	MBL Blood analyses	Detection of MBL in BALF at 3 and 6m post-LTx correlated with later development of BOS (both p<.05). Blood MBL correlated with MBL-mediated C4d deposition (p<.001). Increased blood MBL at 3, 6, and 12m post-LTx vs pre-LTx (all p<.05). LTR who developed BOS or died had higher blood MBL at 6 and 12m post-LTx vs stable LTR (p<.05). No correlation blood MBL and PGD, ACR, sepsis, or micro-organism isolation (p<.05). Low pre-LTx blood MBL correlated with CMV reactivation (p=.04).
DerHovanessian et al., 2016[13]	Retrospective Cross-sectional	75 LTR 5 surgical biopsy	BALF < 24h post-LTx Surgical biopsy	BALF: TGF-β, procollagen Biopsy: TGF-β and TGF-β receptor I expression (TGF-βRI)	Increased BALF TGF-β and procollagen < 24h post-LTx were associated with increased BOS risk (both p<.05), TGF-β remained significant after adjusting for PGD (p=.01). Correlation between TGF-β and procollagen (p<.001), no longer significant in multivariable models after adjustment for PGD severity.

Author	Study design	Population	BALF/tissue	Analysis	Results
					TGF-β expression by bronchial epithelial cells, subepithelial infiltrating mononuclear cells, alveolar macrophages; TGF-βRI expression by airway epithelium, peri-airway and interstitial infiltrating mononuclear cells, stromal cells, and alveolar macrophages. No correlation between TGF-β or procollagen and AR or LB. Increased BALF TGF-β in transient or severe PGD2-3 vs PGD0-1 (both p<.01). Increased BALF procollagen in transient or severe PGD2-3 vs PGD0-1 (both p<.01). Association between transient or severe PGD2-3 and BOS, increased with PGD severity.
Devouassoux et al., 2001[14]	Retrospective	8 LTR (4 developed BOS) 9 non-LTx controls	TBB (first year post-LTx)	CD45, CD20, CD5, CD4, CD8, CD25, CD69, CD1 HLA class I and II, Ki-67, ELAM, LECAM, VCAM, ICAM, PECAM, VLA-4, LFA-1, Mac-1	Increased leukocyte (CD45+) infiltration in grafts with future BOS (p=.003), CD20+ B-cells in LTR vs controls (p=.005), no difference in CD5+, CD4+, CD8+. More CD25+ and CD69+ cells in future BOS vs stable LTR (both p<.05). Increased HLA class I expression in future BOS and stable LTR vs controls, decreased expression on alveolar macrophages in stable LTR (all p<.05). Increased Ki-67+ cells on capillary endothelium, alveolar and bronchial epithelium in grafts, and bronchial epithelium in future BOS vs stable LTR (all p<.05). Increased ELAM-1, VCAM-1, ICAM-1, VLA-4, LFA-1, Mac-1 expression on grafts, and VLA-4 in future BOS vs stable LTR (all p<.05).
Devouassoux et al., 2002[15]	Retrospective Longitudinal	22 BOS 22 stable LTR	BALF	Differential cell count	Increased total cell count, neutrophils (%), eosinophils (%) and decreased macrophages (%) in BOS vs stable LTR (all p<.05). No difference in total cell count and neutrophils (%) in BOS 1 vs stable LTR, increased total cell count and neutrophils (%) in BOS 2 and 3 (all p<.05). BOS < 12 mos. post-LTx was associated with rapid increase of neutrophils (1-3 mos. p<.01, 3-6 mos. p<.05), delayed increase of neutrophils if BOS > 12 mos. post-LTx (6-9 mos. p<.05, 9-12 mos. p<.01), > 9 mos. no difference between early and late BOS. BOS 1: low neutrophilia, not influenced by BOS 1. BOS 2: low neutrophilia 3 mos. before diagnosis, increase at onset (p<.01), and peak 6 mos. later (p<.01). BOS 3: neutrophilia preceded diagnosis by 6 mos. (p<.01), peaked 9 mos. later (p<.01).
Elssner et al., 2000[16]	Prospective	10 BOS 9 stable LTR	BALF BB	BALF: differential cell count, IL-8, IL-10, TGF-β, TNF-α BB: IL-8, IL-10, TGB-β, TNF-α	Increased BALF neutrophils (#/%) and decreased macrophages (%) in BOS vs stable LTR (all p<.05). Increased BALF IL-8 and TGF-β in BOS vs stable LTR (p<.005). Higher IL-8 expression on bronchial epithelial cells (p<.05), not on BALF cells. No increased expression of TGF-β on BALF cells or bronchial epithelial cells in BOS vs stable LTR. No difference in IL-10 or TNF-α.
Fildes et al., 2008[17]	Prospective Cross-sectional	9 BOS 21 stable TLR	TBB	NK cells (CD16) Blood analyses (n=41 LTR)	Increased lung tissue NK cells in BOS vs stable LTR (p=.001). Decreased but activated blood NK cells in BOS vs stable LTR.
Fisichella et al., 2013[18]	Prospective Longitudinal	105 LTR (29 developed BOS)	BALF	Differential cell count, 34 cytokines, chemokines, and growth factors Analyses in AR and aspiration	Increased lymphocytes (%) and neutrophils (%) and reduced macrophages (%) in BOS vs stable LTR (all p<.05). Increased IL-1β, IL-8, CCL5, CXCL10, and decreased IL-9, IL-12 (p=.053), and CCL2 in BOS vs stable LTR (all p<.05). Neutrophils (%) strongly correlated with time after LTx in BOS (p<.05). Increased IL-15, IL-17, and TNF-α 6-12 mos. post-LTx predictive of early-onset BOS.

Author	Study design	Population	BALF/tissue	Analysis	Results
					No difference in IL-1RA, IL-4, IL-5, IL-6, IL-7, IL-13, IL-15, IL-17, IFN-γ, TGF-β, TNF-α, CCL3, CCL4, and CCL11.
Gregson et al., 2010[19]	Prospective	47 LTR (13 developed BOS)	BALF TBB	BALF: Tregs (CD3+CD4+CD25hi-FoxP3+), CCR4, CCR7, CD103, CD45RA subsets TBB: CC21 expression	Tregs were essentially all CD45RA-, CCR4+, and CD103-. No difference in total Treg frequency nor CCR4+ nor CD103- subsets in future BOS vs stable LTR. Increased CCR7+ Tregs correlated with reduced risk of future BOS (p=.04). CCR7 ligand CCL21 correlated with CCR7+ Treg frequency and inversely with BOS (both p<.05). CCL21 protein is predominately expressed on bronchiolar epithelial cells and alveolar macrophages.
Hardison et al., 2009[20]	Retrospective Longitudinal	7 BOS 8 AR 7 stable LTR	BALF	IL-8, MMP-8 and -9 activity and concentration MPO, proline-glycine-proline (PGP), prolyl endopeptidase (PE), in vitro analyses	Increased IL-8 in BOS vs pre-BOS (p<.05). Increased MMP-8 and MMP-9 concentration and activity in BOS vs stable LTR, pre-BOS, and AR (all p<.05). Increased MPO in BOS vs stable LTR and pre-BOS (both p<.05). Increased PGP in BOS vs stable LTR, pre-BOS and AR (all p<.05). Increased PE detection and activity in BOS vs stable LTR, pre-BOS, and AR (all p<.01). Correlation between MMP-9 activity and PGP levels (p<.05), PGP and PE (p<.01) and PGP and FVC (p<.05).
Hayes et al., 2020[21]	Retrospective Longitudinal	16 CF LTR (10 developed BOS)	BALF	CD3, CD4, CD8	Increased CD8+ and decreased CD4+ T-cells in BOS vs pre-BOS and vs stable LTR (all p<.05). No change in T-cell profile prior to BOS onset. No change over time in CD4+ or CD8+ in stable LTR.
Heigl et al., 2021[22]	Retrospective Cross-sectional	14 BOS 16 RAS 13 stable LTR	BALF	C4d (ELISA) AR and HLA-Ab analyses	Increased C4d in RAS vs stable LTR and BOS (both p<.01), not in BOS vs stable LTR. Increased C4d in ACR, LB, AMR, and infection vs stable LTR (all p<.01). Increased C4d in C4d (ICH)-/HLA-Ab-, C4d-/HLA-Ab+, C4d+/HLA-Ab+ vs stable LTR (all p<.05). Correlation between C4d and CRP (p<.0001).
Heijink et al., 2017[23]	Retrospective Cross-sectional	20 BOS stage III 20 stable LTR	BALF (taken in stage I)	Differential cell count, MMP-1, -2, -3, -7, -8, -9, -12, -13, and TIMP-1, -2, -3, -4 concentration and activity	Increased neutrophils (%), lymphocytes (%), and IL-8 in BOS vs stable LTR (all p<.05). Increased MMP-2, -3, -7, -8, and -9 levels in BOS vs stable LTR (all p<.05). Activity of MMP-7, but none of the other MMPs, was detected in stable LTR. No active MMPs in BOS. Increased TIMP-1 and -2 in BOS vs stable LTR (both p<.01). Increased TIMP-1-bound MMP-7, -8, and -9 and TIMP-2-bound MMP-8 and -9 levels in BOS vs stable LTR (all p<.0005). MMP-3, -7, -8, -9 levels correlated with BALF neutrophil numbers in BOS.
Hodge G et al., 2009[24]	Retrospective Longitudinal	12 BOS 35 stable LTR 18 healthy controls	BALF BB	BALF: differential cell count, CD3, CD4, CD8, CD45/CD14 BB: CD4, CD8, CD3, CD45/CD14 In vitro T-cell cytokine production IL-2, IL-4, IFN-γ, TNF-α, blood analyses	No difference in BALF leukocytes (#), macrophages (#), T-cells (#). Increased CD8+ and decreased CD4+ in BOS and stable LTR vs controls (all p<.05). No difference in BB T cell count (#/%), CD4+ or CD8+ cells. More BALF CD8+ T-cells producing IFN-γ, IL-2, TNF-α in BOS and stable LTR vs controls, more CD4+ T-cells producing IFN-γ in BOS vs controls (all p<.05). No difference in BB cytokine production.

Author	Study design	Population	BALF/tissue	Analysis	Results
					No difference in blood leukocytes (#), lymphocytes (#), T-cells (#). Increased blood CD8+ and decreased CD4+ in BOS and stable LTR vs controls. More blood CD4+ T-cells producing IL-2 in BOS and stable LTR vs controls, less blood CD4+ T-cells producing TGF-β in BOS and stable LTR vs controls. Less blood CD4+ and CD8+ T-cells producing TGF-β in BOS vs stable LTR. (all p<.05)
Hodge G et al., 2017[25]	Retrospective Cross-sectional	8 BOS 18 stable LTR 10 healthy controls	BALF BB	BALF/BB: CD3, CD4, CD8, NK T-like cells, NK cells (CD56) Expression of granzyme B, perforin, in CD8+, CD4+, NK T-like and NK cells, and TNF-α, TFN-γ in CD8+, CD4+ and NK T-like cells, glucocorticoid receptor (GCR) expression, blood analyses	Decreased BALF T-cells and CD4+ T-cells, increased CD8+ T-cells, NK T-like, and NK cells (%) in BOS vs controls. Increased CD8+ T-cells in BOS vs stable LTR. Increased large airway CD8+ T-cells, decreased CD4+ T-cells in BOS vs stable LTR. Increased small airway CD8+ T-cells, NK T-like, NK cells, and decreased CD3+, CD4+ T-cells in BOS vs stable LTR and controls. (all p<.05) More BALF CD8+ T-cells producing IFN-γ and TNF-α in BOS vs stable LTR and controls, and stable LTR vs controls. More CD8+ and CD4+ T-cells expressing granzyme B and perforin. More large airway CD8+ T-cells and NK-cells producing IFN-γ and TNF-α in BOS vs stable LTR and controls. No difference in expression of granzyme B or perforin in T-cells, NK T-like, or NK cells, or GCR in CD8+ T-cells and NK cells. More small airway CD8+ T-cells, NK T-like, and NK cells producing IFN-γ, TNF-α, granzyme B, perforin, and CD4+ T-cells granzyme B, perforin in BOS vs stable LTR and controls. And CD8+ IFN-γ, TNF-α, granzyme B in stable LTR vs controls. Less CD8+GCR+ T-cells in BOS vs stable LTR and controls and NKT-like GCR+ cells in BOS vs stable LTR. GCR expression by small airway CD8+ T-cells correlated with FEV1. Decreased blood T-cell count, CD4+ T-cells and increased CD8+ T-cells, NK T-cell like (%) in BOS vs stable LTR and controls. Increased NK cells (%) in BOS and controls vs stable LTR. More blood CD8+ T-cells expressing IFN-γ, TNF-α, granzyme B, perforin, and less CD8+GCR+ T-cells in BOS vs stable LTR. More NK-cells expressing granzyme B, perforin in BOS vs stable LTR. More NK T-like cells expressing granzyme B, perforin in BOS and stable LTR vs controls. Less NK T-like GCR+ and NK GCR+ cells in BOS vs stable LTR and controls.
Hodge G et al., 2018[26]	Retrospective Cross-sectional	12 BOS 18 stable LTR 13 healthy controls	BALF BB	BALF/BB: CD3, CD4, CD8, NK T-like cells, NK cells (CD56) Expression of TNF-α, IFN-γ, and HDA2c by T and NK T-like cells, blood analyses	Increased BALF CD8+ T-cells, NK T-like, NK cells, and decreased CD3+ and CD4+ (%) T-cells in BOS vs controls. Increased BALF CD8+ T-cells (%) in BOS vs stable LTR. Increased large airway CD8+ and decreased CD4+ T-cells in BOS vs stable LTR. No changes in NK T-like and NK cells. Increased small airway CD8+ T-cells, NK T-like, NK cells, and decreased CD3+, CD4+ T-cells in BOS vs stable LTR and controls. (all p<.05) More BALF CD8+ and CD4+ producing IFN-γ and TNF-α in BOS vs stable LTR and controls, and CD8+ producing IFN-γ and TNF-α in stable LTR vs controls. More large airway CD8+ T-cells and NK-cells producing IFN-γ and TNF-α in BOS vs stable LTR and controls. Less CD8+ T-cells and NK-cells expressing HDAC2 in BOS. More small airway CD8+ T-cells producing IFN-γ and TNF-α in BOS vs stable LTR and controls and stable LTR vs controls. More NK T-like and NK cells producing IFN-γ and TNF-

Author	Study design	Population	BALF/tissue	Analysis	Results
					α in BOS vs stable LTR and controls. Less CD8+ T-cells and NK T-like cells expressing HDAC2 in BOS. HDA2c expression by small airway CD8+ T cells correlated with FEV1. Increased blood CD8+ T-cells, NK T-like cells and decreased T-cells, CD4+ T-cells (%) in BOS vs stable LTR and controls. Increased NK cells (%) in BOS and controls vs stable LTR. More CD8+ T-cells expressing IFN-γ and TNF-α, loss of HDA2c expression by CD8+ T-cells, NK T-like and NK cells in BOS.
Hodge G et al., 2021[27]	Retrospective Cross-sectional	10 BOS 11 stable LTR 10 healthy controls	BALF BB	BALF/BB: CD3, CD4, CD8, NK T-like cells, NK cells (CD56) Granzyme B, IFN-γ, TNF-α expression, blood analyses.	Decreased T-cells, increased NK T-like cells, CD8+ T-cells and NK T-like cells, CD28null CD8+ T-cells and NK T-like cells in BOS vs stable LTR and controls in BALF, large and small airway brushings, and blood (all p<.05). Increased BALF granzyme B+ CD28null CD8+ T- and NKT-like cells in BOS vs stable LTR and controls, and stable LTR vs controls. Increased IFN-γ+ and TNF-α+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls. Increased large airway granzyme B+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls, and stable LTR vs controls. Increased IFN-γ+ and TNF-α+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls, and TNF-α+ CD28null CD8+ T- and NK T-like cells in stable LTR vs controls. Increased small airway granzyme B+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls, and stable LTR vs controls. Increased IFN-γ+ and TNF-α+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls, and stable LTR vs controls. Loss of CD28 expression by CD8+ T-cells was associated with FEV1. Increased blood granzyme B+ CD28null CD4+ and CD8+ T- and NK T-like cells in BOS vs stable LTR and controls, increased granzyme B+ CD28null CD8+ T- and NK T-like cells and CD28null CD4+ NK T-like cells in stable patients vs controls. Increased IFN-γ+ and TNF-α+ CD28null CD8+ T- and NK T-like cells in BOS vs stable LTR and controls.
Hodge S et al., 2009[28]	Prospective Longitudinal	6 BOS 16 stable LTR	BALF BB	BALF: TGF-β1 BALF hepatocyte growth factor (HGF) BB alpha smooth muscle actin (α-SMA), S100A4, extra-domain-A fibronectin (ED-A FN), HLA-DR	No difference in BALF TGF-β1 in BOS vs stable LTR. Longitudinal increase of TGF-β1 in BOS vs pre-BOS (n=1). Increased BALF HGF in BOS vs stable LTR (p<.05). Increased bronchial epithelial cell expression of α-SMA, S100A4, ED-A FN, and HLA-DR in BOS vs stable LTR (all p<.05). Longitudinal increase of HGF, α-SMA, S100A4, and ED-A FN in BOS vs pre-BOS (n=1 LTR).
Hodge S et al., 2011[29]	Retrospective Cross-sectional	25 BOS 34 infection 16 stable LTR 14 healthy controls	BALF	MBL, MBL-mediated C4d deposition Blood analyses, efferocytosis of apoptotic bronchial epithelial cells	Reduced MBL in BOS vs stable LTR and controls (both p<.05). Reduced efferocytosis by alveolar macrophages from BOS vs stable LTR and controls (both p<.05). Increased plasma MBL and MBL-mediated C4d deposition in infected LTR vs stable LTR (both p<.05). Significant correlation between MBL and MBL-mediated complement deposition (p<.001), no correlation between blood and BALF MBL.

Author	Study design	Population	BALF/tissue	Analysis	Results
Hubner et al., 2005[30]	Retrospective Longitudinal	8 BOS 12 stable LTR	BALF	Differential cell count, MMP-9, TIMP-1 concentration and activity	Increased neutrophils (%) and decreased macrophages (%) in BOS vs stable LTR and vs pre-BOS (both p<.05). Increased MMP-9 and decreased TIMP-1 concentrations, and increased MMP-9/TIMP-1 ratio in BOS vs stable LTR (all p<.05). Increased MMP-9/TIMP-1 ratio in BOS vs pre-BOS (p<.05). Increased MMP-9 activity in BOS vs stable LTR, neutrophils were the main source. MMP-9 correlated with neutrophil numbers and negatively with lymphocytes (both p<.02). MMP-9/TIMP-1 ratio correlated negatively with FEV1 (p=.003).
Ionescu et al., 2005[31]	Retrospective Longitudinal	32 LTR (16 HLA-Ab+, 16 HLA-Ab-) 18 LTR with PGD or CMV pneumonitis	TBB	C4d deposition (IHC)	All subendothelial C4d+ HLA-Ab+ LTR developed BOS and/or graft loss. More C4d deposition in HLA-Ab+ LTR vs HLA-Ab- (p<.05), all C4d+ cases were in LTR with DSA, no significant C4d deposition in PGD or CMV pneumonitis.
Kaes et al., 2020[32]	Retrospective	376 LTR	BALF	Differential cell count Blood analyses	High BALF eosinophilia (≥ 2%) correlated with CLAD (p=.001) and CLAD-free survival (p=.003). Decreased CLAD-free survival in LTR with high blood and high BALF eosinophils, high blood and low BALF eosinophils, and low blood and high BALF eosinophils vs low blood and low BALF eosinophils (all p<.05); worst outcomes in LTR with high blood and high BALF eosinophils (p<.0001). High blood eosinophils (≥ 8%) was associated with worse graft and CLAD-free survival (both p<.05). Within the high blood eosinophil group, 23.5% had RAS vs 3% in the low eosinophil group (p < 0.0001). More episodes of LB and more severe LB, and more DSA in high vs low blood eosinophil group (all p<.05).
Keane et al., 2007[33]	Prospective Cross-sectional	30 BOS 28 fBOS 10 treated BOS (tBOS) 47 stable LTR	BALF	IL-4, IL-13 Fibroblast proliferation, procollagen type I and III expression, in vitro and murine models	Increased IL-13 in BOS, fBOS, and tBOS vs stable LTR (all p<.05). No difference in IL-4. Increased fibroblast proliferative response and procollagen type I and III expression in BOS, fBOS, and tBOS vs stable LTR (all p<.05). Reduced fibroblast proliferation and procollagen type I and III expression in BOS, fBOS, and tBOS in the presence of anti-IL-13, no effect anti-IL-4 or anti-TGF-β.
Krustrup et al., 2015[34]	Retrospective Longitudinal	58 LTR (28 developed BOS)	TBB	FoxP3+ Tregs	Highest number of FoxP3+ cells/mm² 2w post-LTx. No effect of FoxP3+ cells/mm² on BOS (p = 0.84), significant effect of A-score. The number of FoxP3+ cells/mm² after 2w did not predict the time interval to BOS (p=.65), also not as a time-dependent covariate (p=.77).
Laan et al., 2003[35]	Prospective Longitudinal	7 BOS 7 stable LTR	BALF	Differential cell count, IL-16, IL-2R AR analyses	No difference in IL-16 in BOS vs stable LTR at any time point (p=.6), no correlation with lymphocytes or IL-2R (p=.7). Increased neutrophils (%) and decreased macrophages (%) in BOS vs stable LTR (both p<.05). Lower IL-16 in AR vs stable LTR (p=.03), correlated negatively with IL-2R (p=.03), no correlation with lymphocytes. Increased lymphocytes (%) and decreased macrophages (%) in AR vs stable LTR (both p<.05).

Author	Study design	Population	BALF/tissue	Analysis	Results
Leonard et al., 2000[36]	Prospective Longitudinal	8 BOS 14 stable LTR	TBB EBB	Dendritic cell (DC) staining (CD1a, RFD1, MHC class II), suppressor macrophages (RFD1 and RFD7)	Increased CD1a and MHC class II DC in BOS vs stable LTR (both p<.05), increased DC using dendritic morphology and class II MHC expression vs CD1a expression (p<.000001). DC declined over time in BOS and stable LTR, no longer reaching statistical difference. More MHC class II DC in EBB vs TBB (p<.003), no difference in CD1a DC. No difference in DC CD80 or CD86 expression in BOS vs stable LTR. No difference in RFD1 staining vs CD1a staining. No correlation between CD1a or MHC class II DC and AR or CMV pneumonitis.
Magro et al., 2003[37]	Retrospective Cross-sectional	13 BOS 7 stable LTR 7 non-Tx controls	TBB	C1q, C4d, C5b-9, IgG, IgM, IgA (IF) Pathology findings, serum anti-endothelial antibodies	Increased C1q, C3, C4d, C5b-9, and immunoglobulin deposition in the bronchial epithelium, chondrocytes, basement membrane zone of the bronchial epithelium, and bronchial wall microvasculature in BOS vs others.
Magro et al., 2003[38]	Retrospective	7 BOS	TBB	C4d, C3, C1q, C5b-9, IgG, IgM, IgA (IF) AR analyses	Bronchial wall deposition of C1q, C4d, C5b-9, IgM, and IgA in BOS. Bronchial wall C1q deposition was the strongest predictor of BOS (p=.0038), C4d (p=.04), IgA (p=.04), and C5b-9 (p=.03). C4d and c1q correlated with the degree of humoral rejection pathologically (both p<.01). No correlation with C3, C5b-9, and Ig. High and intermediate C4d levels correlated with a clinical diagnosis of AR (p<.0001). Absent or minimal C4d deposition correlated with a state of clinical wellbeing. No correlation between C4d deposition and presence of ACR.
Magro et al., 2006[39]	Retrospective Longitudinal	24 LTR	TBB	C3d deposition (IF and IHC)	Intermediate and high levels of C3d correlated with BOS (p<.0001) and bronchial wall or septal fibrosis (p<.0016). All LTR with higher values of C3d within septae or bronchial wall eventually developed BOS. Good correlation between C3d and C4d staining (p<.00001), no correlation between extent of C3d and ACR or AMR. IHC staining was superior to IIF.
Mamessier et al., 2007[40]	Prospective	13 BOS (7 stable and 13 evolving BOS samples) 7 AR 14 stable LTR	BALF	T-cell subtypes Sputum and blood analyses	Increased CD4+CD25highCD69-Tregs (BALF, sputum, blood), Th1 (BALF, sputum IFN-γ+CD3+T cells), and Th2 (BALF, sputum IL13+CD3+, blood IL-4+CD3+cells), IL-13+ CD8+ T-cells (BALF, sputum), and IFN-γ+ CD8+ T-cells (BALF) in stable BOS vs stable LTR (all p<.05). Increased Tregs (BALF, sputum, blood), Th1 (BALF, sputum), IFN-γ+ CD8+ T-cells (BALF) in evolving BOS vs stable LTR (all p<.05). Higher Treg and Th2 activation and a lower Th1 activation in stable BOS vs evolving BOS (all p<.05). Blood TGF-β was increased in AR and evolving BOS vs stable LTR (both p<.05). Increased blood IL-4 and TGF-β in evolving BOS vs stable LTR (both p<.01). Th1 activation was observed in AR. In AR, the proportion of Increased blood T cells expressing CD69 and CD103 in AR vs stable LTR, no difference in Tregs.
Meloni et al., 2004[41]	Prospective Longitudinal	44 LTR (8 developed BOS)	BALF	Differential cell count, IL-8, IL-10, IL-12, IFN-γ, TGF-β, CCL2/MCP-1, CCL5/RANTES	Increased neutrophils (%), IL-8, CCL2, and decreased macrophages (%) and IL-12 in BOS vs stable LTR (all p<.05). Trend towards decreased TGF-β in BOS vs stable LTR (p=.06). Lower levels of IL-12 were significantly predictive of BOS (p=.03).

Author	Study design	Population	BALF/tissue	Analysis	Results
Meloni et al., 2008[42]	Retrospective Longitudinal	8 future BOS 8 stable LTR	BALF (at month 6)	CCL3/MIP1-α, CCL4/MIP1-β, CCL17/TARC, CCL19/MIP3-β, CCL20/MIP3-α, CCL22/MDC, CCL26/eotaxin CCR4, CCR6, CCR7 on CD3+ and CD68+ cells	CCL19, CCL20, CCL22 levels at 6 mos. post-LTx predicted BOS onset (all p<.02), with a significantly different temporal trend in future BOS vs stable LTR. No difference in CCR4, CCR6, CCR7 expression on CD3+ lymphocytes. Higher density of CCR6 in future BOS vs stable TLR (p=.02), no difference in CCR4. Increased CCR6 and CCR4 expression on CD68+ cells in future BOS vs stable LTR (both p<0.02), trend CCR7 (p=.07). No difference in receptor density on CD68+ cells.
Neujahr et al., 2012[43]	Prospective Longitudinal	40 LTR (15 developed BOS)	BALF during first year	IL1-RA, IL-13, IL-17, CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG, CXCL10/IP-10	Cumulative increased CXCL9 and CXCL10 were associated with BOS and graft failure (both p<.01), and preceded BOS onset by 3 and 9 mos. Sources of CXCL9 and CXCL10 were airway epithelium and alveolar macrophages. No correlation between IL1RA, IL-13, IL-17, CCL2, CCL5 and BOS or graft failure.
Neurohr et al., 2009[44]	Retrospective	63 stable LTR (16 developed BOS)	BALF	Differential cell count, IL-8 Secretory leukocyte protease inhibitor (SLPI)	Increased total cell count and neutrophils (%) and decreased macrophages (%) in future BOS vs stable LTR (all p<.05). Increased IL-8 in future BOS vs never BOS, and correlated with number of neutrophils (both p=.01). BALF neutrophil percentage of ≥ 20% was a significant predictor for BOS ≥ 1 (p<.05). Trend towards increased risk of death in future BOS (p=.056). Reduced SLPI in future BOS vs stable LTR, correlated negatively with neutrophils (p=.01).
Ngo et al., 2019[45]	Retrospective	48 LTR	TBB	C4d deposition (IHC)	All C4d3+ LTR developed early persistent DSA, AMR, and CLAD (2 BOS, 1 RAS, 1 mixed). Microvascular inflammation and acute lung injury were rare but more frequent in C4d1-3+, C4d+ was more frequent in infection.
Ramirez et al., 2008[46]	Retrospective Cross-sectional	13 future BOS 21 stable LTR	BALF (last BALF before BOS onset)	TGF-β1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, MMP-9 gelatinase activity GM-CSF, in vitro fibronectin expression in murine fibroblasts	No difference in TGF-β1 level in future BOS vs stable LTR. Higher MMP-9 activity in future BOS vs stable LTR (p<.005). Trend towards higher IL-8 in future BOS vs stable LTR (p=.08). TGF-β1 correlated with fibronectin gene transcription (r=0.71). Higher fibronectin promoter activity in future BOS vs stable LTR (p=.026).
Reynaud-Gaubert et al., 2002[47]	Retrospective	26 LTR	BALF	Differential cell count, lymphocytes staining (CD3, CD4, CD8, CD37, CD57, HLA-DR)	Increased total cell count in BOS, AR, and infection vs stable LTR (p<.01). Increased neutrophils (%) in BOS and infection vs stable LTR (both p<.0001). Neutrophils outside the CI correlated with BOS (p<.01). Decreased macrophages (%) in BOS, AR, and infection vs stable LTR, and BOS and infection vs AR (all p<.01). Increased CD4+ and decreased CD8+ cells in BOS and infection vs stable LTR (all p<.05). Increased lymphocytes (%) in AR, decreased CD4+ T-cells in infection, increased HLA-DR+ lymphocytes (%) during infection and AR vs stable LTR, increased expression of NK-cell associated CD57 in infection vs others (all p<.05).
Reynaud-Gaubert et al., 2002[48]	Prospective Longitudinal	21 LTR (8 developed BOS)	BALF	Differential cell count, IL-8, CCL2/MCP-1, CCL5/RANTES	Increased total cell count, neutrophils (#/%), and decreased macrophages (%) in BOS vs stable LTR (all p<.01).

Author	Study design	Population	BALF/tissue	Analysis	Results
				Soluble intercellular adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule-1 (VCAM-1)	Increased IL-8, CCL2, and CCL5 in BOS vs stable LTR (all p<.05). Increased neutrophils (#/%), IL-8, CCL2, and CCL5, and decreased macrophages (%) in future BOS vs stable LTR (all p<.05). Increased neutrophils (%) and IL-8 in post-BOS vs pre-BOS (both p=.02). Neutrophils correlated with IL-8 and CCL2. Correlation between IL-8 and CCL2. Negative correlation between neutrophils and IL-8 and FEV1. (all p<.01) No difference in sICAM-1 and VCAM-1 between groups. Neutrophils correlated negatively with sICAM-1.
Riise et al., 2010[49]	Retrospective	12 BOS 12 stable LTR	BALF	MMP-2 and MMP-9 concentration and gelatinase activity Serine protease, neutrophil elastase, secretory leukocyte protease inhibitor	Increased net gelatinase activity in BOS vs stable LTR (p<.005). MMP-9 activity exceeded MMP-2 activity in BOS and stable LTR (both p<.01). Increased MMP-9 activity and concentration in BOS vs stable LTR (both p<.05), but not MMP-2. Gelatinase activity correlated with MMP-9 concentration and neutrophils (%) (both p<.01). Increased neutrophil elastase in BOS vs stable LTR (p<.01). Serine protease correlated with neutrophil elastase concentration and neutrophils (both p<.05).
Sacreas et al., 2019[50]	Retrospective Cross-sectional	1) BALF: 23 BOS, 26 RAS, 20 stable LTR 2) Explant lungs: 19 BOS, 19 RAS, 14 non-LTx controls	BALF Explant lungs	BALF: TGF-β1 Explant lungs: TGF-β1, CD4, CD8, CD20, CD68 Calretinin, serum mesothelin, explant lung CT and protein and mRNA analyses, in vitro analyses	Increased BALF TGF-β1 in RAS vs stable LTR (p = 0.02). Worse post-diagnosis graft survival in RAS LTRs with high TGF-β1 levels vs those with low TGF-β1 levels (p = 0.033). TGF-β1 was located in the (sub)pleural area in explant lungs. Low TGF-β1 expression: little and dispersed CD4+, CD20+, and CD68+ cells. Intermediate TGF-β1 expression: low number of CD4+, CD8+, CD20+, and CD68+ cells. High TGF-β1 expression: higher number of CD4+, CD8+, CD20+, and CD68+ cells. Increased volume fraction of pleura in RAS explant lungs vs BOS and non-LTx controls, higher proportion of calretinin+ staining in RAS vs BOS and controls, decreased E-cadherin mRNA expression in RAS pleura vs controls, increased α-smooth muscle actin mRNA and protein expression in RAS pleura vs controls, increased blood mesothelin in RAS vs stable LTR (all p<.05). TGF-β1 stimulation of pleural mesothelial cells led to a phenotypical switch to mesenchymal cells, accompanied with an increased migratory capacity. IL-1α was able to accentuate TGF-β1–induced mesothelial-to-mesenchymal transition.
Saito et al., 2013[51]	Retrospective Cross-sectional	50 BOS 21 RAS 38 stable LTR	Donor lung biopsies	IL-1β, IL-6, IL-8, IL-10, IFN-γ, TNF-α mRNA	Increased IL-6 expression in pre-implanted lungs from future BOS patients vs RAS and stable LTR (both p<.03). Increased IL-1β and IL-6 in future CLAD vs no-CLAD (both p<.05). Association between high IL-6 and CLAD, BOS, and early BOS development (all p<.01).
Saito et al., 2018[52]	Retrospective Cross-sectional	18 BOS 10 RAS 25 stable LTR	BALF	MMP-8 Neutrophil elastase, α-defensins, long pentraxin-3 (PTX3)	Upregulated MMP-8 in RAS (p<.001) and BOS (p=.002) vs stable LTR. Upregulated neutrophil elastase, α-defensins, and PTX3 in RAS vs stable LTR (all p<.001), neutrophil elastase, α-defensins and PTX3 vs BOS (all p<.01), and neutrophil elastase in BOS vs stable LTR (p=.024).
Sato et al., 2009[53]	Retrospective Cross-sectional	12 explant BOS lungs	Explant lungs	CD3, CD20, CD45RO, CCR7, lymphocyte aggregations	More T- and B-cells in LB and active OB vs inactive OB and controls. T-cells in LB and active OB lesions were mainly CD45RO+ CCR7- effector memory T-cells.

Author	Study design	Population	BALF/tissue	Analysis	Results
		1 surgical BOS biopsy 15 non-LTX controls		Peripheral lymph node addressin (PNAd), high endothelial venules (HEV), Ki-67, animal analyses	Effector memory T-cell aggregates did not completely meet the anatomical criteria of secondary or tertiary lymphoid tissue, because they did not include segregated T-cell and B-cell zones or B-cell follicles positive for CD21+ follicular dendritic cells. Large number of PNAd+ HEVs in the airways of BOS lungs vs controls (p<.01). HEVs existed in almost all of the LB and active OB lesions in the bronchiolar wall vs a small number of HEVs in inactive OB lesions.
Sato et al., 2011[54]	Retrospective Cross-sectional	20 CLAD explant lungs 20 non-LTx controls 20 LTR (7 developed CLAD)	CLAD explant lungs TBB	CXCL12, CXCL13, CCL21 Peripheral lymph node addressin (PNAd)	Increased lymphoid aggregates, CXCL12 in alveolar and airway epithelial cells, CCL21+ lymph vessels (all p<.01), and infiltration of DC-specific intercellular adhesion molecule-grabbing nonintegrin+ immature DCs (p=.056) in CLAD explant lungs vs controls. Increased PNAd+ high endothelial venule like vessels in CLAD explant lungs vs controls, and in TBB of future CLAD vs stable LTR (all p≤0.001).
Scholma et al., 2000[55]	Prospective Cross-sectional	60 LTR (19 developed BOS)	BALF on mean day 41	Differential cell count, IL-6, IL-8, CCL2	Total cell count (#), lymphocytes (#), eosinophils (#), IL-6, and IL-8 were higher in future BOS vs stable LTR in bronchial fraction (all p<.05). Increased neutrophils (#), IL-6, and IL-8 in alveolar fraction in future BOS vs stable LTR (p<.05). No difference in CCL2. Increased total cells, neutrophils, lymphocytes, eosinophils, IL-6, and IL-8 in bronchial fraction and total cells, neutrophils, IL-6, IL-8, and CCL2 in alveolar fraction correlated with increased BOS risk.
Shino et al., 2013[56]	Retrospective Longitudinal	224 LTR	BALF	CXCL9/MIG, CXCL10/IP-10, CXCL11/ITAC Pathologic findings of 441 LTR	Prolonged elevation of CXCR3 ligands (CXCL9, -10, -11) correlated with increased CLAD risk. CXCL9, CXCL10, and CXCR3 were expressed by epithelial cells, mononuclear cells, and alveolar macrophages. CXCL11 was mainly expressed by vascular endothelial cells. Increased CXCL9 and CXCL10 during DAD, AR, and LB, and CXCL11 during DAD. Episode of DAD correlated strongly with increased risk of CLAD, especially RAS, and allograft failure. AR and LB correlated with increased risk of CLAD, AR correlated with BOS and RAS. OP correlated with allograft failure. (all p<.05)
Sinclair et al., 2021[57]	Retrospective Cross-sectional	7 CLAD 7 early stable LTR (< 1y) 7 stable LTR (> 1y) 7 healthy controls	BALF	CCL2, TGF-β Hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet derived growth factor BB (PDGF-BB), lysophosphatidic acid (LPA), autotaxin, mesenchymal stromal cells (MSc) migration, in vitro analyses	Increased CCL2 in early post-LTx, CLAD, and stable LTR vs controls (all p<.05). No difference in TGF-β in CLAD vs stable LTR. CLAD BALF increased MSc migration (all p<.05), BALF from healthy controls and early post-LTx LTR (<1j) did not induce MSc migration. Increased HGF in CLAD vs early post-LTx and controls. Increased autotaxin in early post-LTx, CLAD, and stable LTR vs controls. Autotaxin mRNA was increased in LTR who developed CLAD early post-LTx, autotaxin expression was inversely correlated with time to CLAD. No difference in EGF and PDGF-BB. Increased LPA species 16:0 and 22:4 in LTR vs controls. LPA inhibition completely blocked the effect of CLAD BALF on chemotaxis.
Snell et al., 2007[58]	Prospective Longitudinal	34 stable LTR	BALF EBB	BALF: differential cell count, CD3, CD8, IL-8 EBB: CD3, CD4, CD8, CD45, IL-17	No correlation between EBB IL-17+ cells and BALF IL-8, neutrophils (%), acute rejection, or BOS. EBB IL-17 was elevated early and subsequently fell with time post-LTx.

Author	Study design	Population	BALF/tissue	Analysis	Results
					EBB IL-17 correlated with EBB CD8+ cells, increased BALF lymphocytes, and correlated negatively with time post-LTx (all p<.05). EBB IL-17 increased in CMV mismatch and clinical infection (all p<.05).
Suwara et al., 2014[59]	Retrospective Longitudinal	9 RAS 13 BOS 10 LB/ARAD 10 persistent airway neutrophilia (PAN) 13 stable LTR	BALF	Differential cell count, IL-1α, IL-1β, IL-6, IL-8, TNF-α Plasma CRP, in vitro viability of bronchial epithelial cells	Increased leukocytes (#) in ARAD, PAN, pre-BOS, and BOS vs controls. Increased neutrophils (%) in ARAD, PAN, BOS, and RAS. (all p<.01) No difference in neutrophils in pre-BOS or pre-RAS vs stable LTR. Increased eosinophils (%) in RAS vs control (p=.01). Decreased macrophages (%) in ARD, PAN, and RAS vs controls (all p<.05). Increased IL-1α and IL-1β in ARAD and PAN vs controls, increased IL-6 in PAN and RAS vs controls, increased IL-8 in ARAD, PAN, and BOS vs controls, increased TNF-α in PAN vs controls (all p<.05). Increased IL-1α in BOS vs pre-BOS (p=.02). Increased plasma CRP in PAN and RAS vs controls (both p<.01). Decreased epithelial cell viability after exposure to BALF in PAN (p<.01).
Suzuki et al,. 2013[60]	Retrospective Cross-sectional	BALF: 6 BOS, 10 non-LTx controls Tissue: 4 BOS, 4 non-LTx controls	BALF Explant lungs	BALF: C3a Tissue: CD55, CD46 In vitro, murine analyses	Upregulation of BALF C3a in BOS vs controls (p<.05). Downregulation of tissue complement-regulatory protein (CD55, CD46) in BOS vs controls (both p<.05).
Vanaudenaerde et al., 2008[61]	Retrospective Cross-sectional	36 BOS 11 infection 43 AR 42 stable LTR	BALF	IL-1β, IL-2, IL-6, IL-8, IL-17, IL-23, TGF-β	Increased neutrophils (#/%) in BOS, infection, and AR vs stable LTR (all p<.01). Increased lymphocytes (#) in BOS, infection, and AR vs stable LTR (all p<.05). Decreased macrophages (%) in BOS and infection vs stable LTR (both p<.001). Increased IL-1β, IL-6, IL-17 mRNA, IL-23 mRNA, TGF-β mRNA, and decreased IL-2 in BOS vs stable LTR (all p<.05). Increased IL-8 protein in BOS, infection, and AR vs stable LTR, increased IL-8 mRNA in BOS vs stable LTR (all p<.05). TGF-β protein levels did not significantly differ. Increased IL-1β, IL-6, IL-17, IL-23, TGF-β mRNA in infection vs stable LTR (all p<.05). Increased IL-6 and decreased IL-2 in AR vs stable LTR. Increased total cell count (#) in infection and AR vs stable LTR (both p<.001). Increased macrophages (#) in infection and AR vs stable LTR (both p<.01). Increased eosinophils (#/%) in infection vs stable LTR (p<.05).
Vandermeulen et al., 2015[62]	Retrospective Cross-sectional	72 stable LTR with high BALF neutrophilia (≥ 15%) 37 stable LTR with low BALF neutrophilia	BALF	Differential cell count, 33 cytokines, chemokines, and growth factors	Increased total cell count, neutrophils (#/%), eosinophils (#), lymphocytes (#), IL-1β, IL-1RA, IL-4, IL-6, IL-8, IL-9, IL-10, TNF-α, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL8, CXCL10, and decreased macrophages (%) in neutrophil-high vs neutrophil-low group (all p<.05). Correlation between IL-8 and BALF neutrophilia. Correlation between IL-1β and IL-8, markers of eosinophils (IL-4, CCL11) and markers of macrophages (CCL2, CCL3, CCL4) (all p<.05), correlating trend IL-1β and CLAD-free survival (p=.084). Increased CLAD incidence, lower CLAD-free, and overall survival in neutrophil-high vs neutrophil-low group (all p<.05).

Author	Study design	Population	BALF/tissue	Analysis	Results
					No difference in IL-2, IL-7, IL-12, IL-13, IL-15, IL-17, IFN-γ, CXCL5, CXCL6, CCL18, CCL22. Increased FGF-β, G-CSF, PDGF, VEGF (%) in neutrophil-high vs neutrophil-low group (all p<.05). No difference in GM-CSF.
Vandermeulen et al., 2016[63]	Retrospective Cross-sectional	15 BOS 16 RAS 14 stable LTR	BALF	Differential cell count, C4d, C1q, IgA, IgE, IgG1-4, IgG, IgM, proMMP-2, proMMP-9, MMP9	Increased total cell count in BOS vs stable LTR, increased neutrophils (#/%), lymphocytes (#), and decreased macrophages (%) in BOS and RAS vs stable LTR, increased macrophages (#) in BOS vs stable LTR and RAS, increased eosinophils (#/%) in RAS vs stable LTR (all p<.05). Increased IgG, IgG1-4, IgM in RAS vs stable LTR and BOS. Increased IgA and IgE in RAS vs stable LTR, and increased total IgG and IgE in BOS vs stable LTR (all p<.05). Increased IgG, IgG1, IgG3, IgG4, IgM correlated with worse survival (all p<.05). Increased C4d and C1q in RAS vs BOS and stable LTR, and correlated with mortality and IgG1-4, IgG, IgE, IgA (all p<.05). Increased proMMP-9 and MMP-9 levels and activated MMP-9 in RAS and BOS vs stable LTR, and increased MMP-9 induced gelatin degradation in BOS vs stable LTR. Increased proMMP-2 in RAS vs stable LTR. (all p<.01) Increased blood DSA in RAS vs BOS and stable LTR (p=.017).
Vandermeulen et al., 2017[64]	Retrospective Cross-sectional	19 BOS 18 RAS 21 non-Tx controls	Explant lungs (BOS/RAS) Biopsies (controls)	Neutrophils (MPO), eosinophils (EG-2), macrophages (CD68), mast cells (tryptase), dendritic cells (CD1a, CD207), B-cells (CD20), cytotoxic T-cells (CD8), T-helper cells (CD4), lymphoid follicles DSA	Increased number neutrophils, eosinophils, macrophages, mast cells, B-cells, and cytotoxic T-cells in RAS vs controls (all p<.05). Increased B-cells and cytotoxic T-cells in BOS vs controls (both p<.05). Lymphoid follicles in RAS vs BOS and controls, predominantly localized around the blood vessels and in the parenchyma (all p<.05). Myeloid cell types were more prevalent around the airways vs parenchyma or around blood vessels. Increased neutrophils in airway component in RAS and BOS vs controls. Eosinophils and mast cells in RAS were primarily located in the parenchyma and around blood vessels. Macrophages were more abundant in RAS vs controls and BOS in every compartment. More CD1a dendritic cells in the parenchyma in RAS vs BOS and controls. Increased resident mucosal (langerin positive) DC in the parenchyma in RAS vs controls and decreased around the airways in RAS vs controls. (all p<.05) DSA were more prevalent in RAS vs BOS (p=.04).
Verleden et al., 2011[65]	Retrospective Cross-sectional	9 fBOS 9 NRAD 10 stable LTR	BALF	Differential cell count, 32 cytokines, chemokines, growth factors	Increased neutrophils (%) and eosinophils (%) and decreased macrophages (%) in CLAD vs stable LTR. Increased total cell count, neutrophils (%) and decreased macrophages (%) in NRAD vs stable LTR (all p<.05). Upregulated IL-1β, IL-8, MMP-8, MMP-9, MMP-8/TIMP-1, MMP-9/TIMP-1 in CLAD/NRAD vs stable LTR (all p<.05). No difference in fBOS vs stable LTR. Upregulated IL-1β, IL-8, CCL2, CCL5, TIMP-1, MMP-8, MMP-9 in NRAD vs fBOS (all p<.05). IL-1β, IL-8, CCL2, CCL5, TIMP-1, MMP-8, and MMP-9 correlated with BALF neutrophils (%). No differences in TNF-α and TGF-β.

Author	Study design	Population	BALF/tissue	Analysis	Results
					Upregulated HGF, MPO, and downregulated RAGE, SP-C, and PDFG-AA in NRAD vs stable LTR. Upregulated HGF, MPO, bile acids, and downregulated PDGF-AA in NRAD vs fBOS. FGFb, PLGF, HGF MPO, RAGE, SP-C, and bile acids correlated with BALF neutrophils (%).
Verleden et al., 2014[66]	Retrospective Longitudinal	66 LTR with eosinophilia (≥ 2%) 253 LTR without eosinophilia	BALF	Differential cell count Blood analyses	Increased BALF eosinophilia (≥ 2%) correlated with worse CLAD-free and overall survival vs controls (both p<.01), and predisposed to BOS and especially RAS (p<.0001). Higher CLAD and mortality risk if multiple BALF eosinophilia vs once (both p<.01). Correlation between BALF (%) and blood eosinophilia (%) (p<.0001), higher blood eosinophils in CLAD LTR in eosinophil group vs those who did not develop CLAD (p=.07). Increased CRP in eosinophil group vs controls (p<.0001).
Verleden et al., 2015[67]	Retrospective Cross-sectional	20 BOS 17 neutrophilic BOS 20 RAS 20 stable LTR	BALF	Differential cell count, 34 cytokines, chemokines, growth factors	Increased total cell count and neutrophils (#/%) and decreased macrophages (%) in neutrophilic BOS and RAS vs non-neutrophilic BOS and stable LTR, and higher eosinophils (%) in RAS vs BOS and stable LTR (all p<.05). Upregulated IL-1β, IL-1Rα, IL-6, IL-8/CXCL8, CCL2, CCL3, CCL4, CXCL10 and decreased VEGF in RAS vs stable LTR (all p<.05). Increased IL-1Rα, IL-6, IL-8, CCL3, CCL4 in RAS vs non-neutrophilic BOS and IL-6, CCL18, and decreased VEGF vs neutrophilic BOS (all p<.05). Upregulated IL-1β, IL-1Rα, IL-4, IL-7, IL-8/CXCL8, CCL3, CCL4, CCL7, and decreased CCL18 in neutrophilic BOS vs stable LTR (all p<.05) and upregulated IL-1β, IL-7, IL-8, CCL3, CCL7 and decreased CCL18 vs non-neutrophilic BOS. No difference between non-neutrophilic BOS and stable LTR. IL-6, CXCL10, CXCL11 were associated with survival after diagnosis in RAS (all p<.05). No difference in IFN-γ, TNF-α, IL-2, IL-5, IL-9, IL-10, IL-13, IL-17, CCL5, CXCL5, CXCL6, CXCL9, and CXCL11.
Verleden et al., 2016[68]	Retrospective Cross-sectional	33 RAS	BALF	Differential cell count Radiology, pathology and pulmonary function test findings, blood eosinophilia (n=53)	Increased neutrophils and eosinophils (%) and decreased macrophages (%) correlated with worse graft survival (all p<.05). Strong association between increased eosinophils (≥ 2%) and survival after diagnosis (p=.0002), and neutrophils (> 10%) and survival (p=.019). BALF eosinophilia correlated with blood eosinophilia (#/%). Increased blood eosinophilia (#) and lower lobe or diffuse infiltrates correlated with worse graft survival. Blood eosinophil count > 240 x 10^6/L correlated with worse outcome (p=.0015).
Verleden et al., 2018[69]	Retrospective Cross-sectional	336 LTR	BALF < 24-48h post-LTx	Differential cell count, IL-6, IL-8	High IL-6 < 24h post-LTx was associated with better CLAD-free and graft survival (both p<.05). Weak correlation between neutrophilia (%) and IL-6 and inverse correlation with macrophages (both p<.05). IL-8 correlated with IL-6 (p<.0001). Increased IL-6 < 24h post-LTx was associated with longer ICU and hospital stay and increased PGD3 prevalence (all p<.01). Increased IL-8 < 24h post-LTx correlated with PGD3 and ECMO use, higher donor paO2, younger donor age, but not with other short-or long-term outcome (p<.01).

Author	Study design	Population	BALF/tissue	Analysis	Results
Vos et al., 2009[70]	Retrospective Cross-sectional	121 LTR (54 developed BOS)	BALF	Differential cell count, IL-6, IL-8 BALF and plasma CRP	Increased neutrophils (#/%) in BOS vs stable LTR (p<.03). Trend for increased total cell count in BOS vs stable LTR (p=.053). No difference in IL-6 or IL-8. BALF CRP at D90 was an independent predictor for graft failure (p=.004), trend for plasma CRP (p=.077). Increased BALF and plasma CRP in BOS vs stable LTR (p<.03 resp. p=.056). Increased plasma CRP, BALF CRP and neutrophils in AR vs without AR (all p≤.02). Increased BALF neutrophils in colonized vs non-colonized LTR (p=.047).
Ward et al., 2001[71]	Retrospective Cross-sectional	5 BOS 19 stable LTR 18 healthy controls	BALF	Differential cell count, lymphocyte, alveolar macrophages (AM), and NK surface markers (CD3, CD45, CD4, CD8, CD14, CD25, HLA-DR, CD56, CD16, CD11a, CD11b, CD11c, CD18)	Increased neutrophils (%) in stable LTR and BOS vs controls (p<.05) and trend in BOS vs stable LTR (p=.08). Increased NK cells (CD56/CD16+), CD11b+ and CD11a+ CD3+ lymphocytes, CD8+ lymphocytes, and decreased CD4+ cells (%) in stable LTR and BOS vs controls (all p<.05). Increased CD11a CD3+ lymphocytes in stable LTR vs controls (p<.05). Decreased expression of AM surface markers CD11a, CD11b, CD11c, HLA-DR and CD14 in stable LTR and BOS vs controls (all p<.05). Increased HLA-DR expression in CD8+ cells in stable LTR and BOS vs controls (all p<.05).
Westall et al., 2008[72]	Retrospective Cross-sectional	33 LTR (9 developed early BOS, mean 79 weeks)	TBB first 3 months post-LTx	C3d and C4d deposition (IHC) Pathologic findings, correlation AMR, PGD, respiratory infection, CMV pneumonitis	Early (< 3 mos. post-LTx) C3d/C4d deposition was not associated with ACR, lung function, BOS, or mortality. 9 LTR with early BOS all had significant intracapillary C3d/C4d deposition and features of AMR. Good correlation between C3d and C4d staining (p<.05), no correlation between degree and extent of C3d/C4d and morphologic features of AMR, increased C3d/C4d deposition in severe PGD3 (p=.07/.01) and respiratory infection (p=.01/.02). No association between C3d/C4d deposition and CMV pneumonitis.
Yang et al., 2019[73]	Retrospective Cross-sectional	20 BOS 20 RAS 20 stable LTR	BALF	Differential cell count, IL-6, IL-8, CXCL10/IP-10 Cell-free DNA (cfDNA)	Increased neutrophils (%) and decreased macrophages (%) in BOS and RAS vs stable LTR (all p=.01). No difference in eosinophils. Higher IL-8 in BOS vs stable LTR (p=.0163), no differences in IL-6. Trend towards higher CXCL10 in RAS vs stable LTR (p=.08). Higher cfDNA in BOS vs RAS and stable LTR (both p<.01). Association between overall survival and cfDNA, CXCL10, and cfDNA-CXCL10 interaction (all p<.05).
Zheng et al., 2000[74]	Retrospective Cross-sectional	10 BOS 19 stable LTR 20 healthy controls	BALF TBB, EBB	BALF: differential cell count, IL-8 TBB/EBB: neutrophil elastase staining	Increased BALF neutrophilia (#/%) in BOS vs stable LTR and controls, and in stable LTR vs controls (all p<.01). Decreased alveolar macrophages (%) in BOS vs controls and stable LTR (both p<.01). Increased BALF IL-8 in BOS vs stable LTR and controls, and stable LTR vs controls (all p<.01). BALF IL-8 strongly correlated with neutrophils (%) in BOS (p<.05), not in stable LTR. Increased airway wall neutrophilia in BOS and stable LTR vs controls (both p<.05). No difference in neutrophils in lung parenchyma in BOS vs stable LTR.

Author	Study design	Population	BALF/tissue	Analysis	Results
Zheng et al., 2005[75]	Prospective Longitudinal	29 LTR (23 developed BOS0p, 17 BOS ≥ 1) 6 healthy controls	BALF EBB	BALF/EBB: differential cell count, CD3, CD4, CD8, HLA-DR	Increased BALF total cell count in LTR vs controls, no difference in lymphocytes (%) in LTR vs controls, decreased lymphocytes (%) in BOS vs pre-BOS (p=.057). Decreased BALF CD3+ over time in BOS, and after BOS vs pre-BOS (all p<.05). Increased BALF CD8+ and decreased CD4+ early post-LTx vs controls (all p<.05). No difference in CD4 or CD8 in BOS vs pre-BOS. Increased EBB CD3+ and CD8+ lymphocytes over time vs early post-LTx, and more pronounced in BOS (all p<.05). No difference after BOS vs pre-BOS, trend towards higher CD8+ infiltration in BOS vs stable LTR.
Zheng et al., 2006[76]	Prospective Longitudinal	28 stable LTR (16 developed BOS) 15 healthy controls	BALF EBB	BALF: differential cell count, IL-8 EBB: lymphocytes (CD3, CD4, CD8), macrophages (CD68), neutrophils (neutrophil elastase)	Increased BALF baseline total cell counts, neutrophils (%), and IL-8 in never BOS and future BOS vs controls (all p<.05), and persisted over time. No difference in BALF lymphocytes and macrophages (%). EBB lymphocytes and macrophages (#) were similar to controls at baseline, but increased over time. EBB neutrophils were similar to controls at baseline, but increased over time in BOS (p=.0004). Increased EBB and BALF neutrophils and BALF IL-8 in post-BOS vs pre-BOS (all p<.01). BALF IL-8 correlated with BALF neutrophils (%) (p<.001) and EBB neutrophils (#) (p=.01). Increased BALF neutrophils (%), but not EBB neutrophils (#), in case of bronchopulmonary infection in LTx patients who developed BOS (p=.002). In the presence of concomitant infections, BALF neutrophilia was more marked post-BOS vs pre-BOS (p=.01).

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Appendix F: Supplementary files Hyperion

1. Tier 1 and 2 consensus clusters across clinical phenotypes

A. Cells and ROIs	All	No CLAD	BOS	RAS	p-value
Total number of cells	190,851	14,013	100,006	76,832	
Total number of ROIs	81	13	40	28	
Total area of ROIs (mm^2)[a]	41.13	4.78	20.45	15.90	
Median area of ROI (mm^2)[a]	0.47 [0.31-0.72]	0.42 [0.19-0.48]	0.51 [0.35-0.67]	0.53 [0.28-0.90]	0.1240
Median number of cells per mm^2	4536 [3444-5701]	3476 [1946-3954]	4670 [3912-5826]**	4825 [2952-5908]*	**0.0021**
B. Clusters	**All**	**No CLAD**	**BOS**	**RAS**	**p-value**
T cells	1118 [763-1370]	841 [527-1072]	1201 [878-1521] **	1133 [608-1304]	**0.0035**
- CD8+ T cells	134 [101-254]	103 [63-166]	146 [106-304]	149 [108-251]	0.0628
▪ Naive CD8+ T cells	13 [6-23]	18 [5-30]	11 [6-21]	13 [8-22]	0.6880
▪ CD8+ T cells	67 [38-103]	50 [30-75]	69 [38-105]	71 [45-138]	0.1401
▪ Senescent CD8+ T cells	57 [36-99]	36 [21-56]	62 [42-101] *	58 [34-101]	**0.0216**
- Th1 cells	94 [54-182]	55 [29-118]	83 [59-152]	151 [61-272] *	**0.0448**
- γδ T cells	769 [620-977]	675 [434-751]	951 [700-115] ** ##	725 [494-826]	**0.0003**
▪ γδ T cells	621 [480-828]	550 [366-578]	765 [578-901] ** #	593 [416-710]	**0.0003**
▪ TEMRA γδ T cells	9 [3-29]	9 [5-43]	7 [3-37]	9 [0-20]	0.5042
▪ Memory γδ T cells	106 [72-139]	88 [65-133]	127 [94-159] #	91 [62-110]	**0.0062**
B cells	50 [25-120]	25 [10-41]	61 [35-112] **	50 [29-178] **	**0.0038**
- Activated plasma cells	50 [25-120]	25 [10-41]	61 [35-112] **	50 [29-178] **	**0.0038**
Monocytes/macrophages	615 [476-859]	536 [292-685]	588 [518-932]	722 [456-1004]	0.0738
- Intermediate M1 macrophages	106 [75-146]	100 [68-134]	96 [73-139]	126 [100-159]	0.1217
- Intermediate M2 macrophages	357 [270-559]	283 [165-327]	372 [278-615] *	476 [273-662] **	**0.0063**
- Non-classical M2 macrophages	78 [47-117]	89 [41-151]	103 [60-148] ##	56 [43-79]	**0.0058**
- Tissue macrophages	23 [13-34]	13 [0.8-29]	23 [17-35]	26 [13-42]	0.0511
- Monocytes	19 [12-32]	23 [15-35]	16 [9-25]	22 [13-36]	0.1487
Eosinophils	130 [62-248]	48 [20-89]	177 [89-255] **	133 [69-232] *	**0.0017**
Other leukocytes	269 [200-395]	150 [119-259]	263 [201-355]	344 [260-465] ***	**0.0006**
- Activated leukocytes	27 [19-40]	13 [8-23]	30 [22-41] **	31 [20-42] *	**0.0021**
- Unclassified lymphoid cells (NK/T/B cells)	178 [136-275]	125 [73-168]	167 [143-263] *	260 [156-347] ***	**0.0003**
- CD86+ cells	47 [35-69]	39 [33-69]	39 [33-66]	60 [46-71]	0.0756
Epithelial cells	1379 [1109-1770]	1100 [652-1221]	1501 [1315-1998] ****	1321 [983-1771] *	**0.0001**
- Epithelial cell	25 [17-39]	28 [22-33]	19 [10-35]	28 [22-42]	0.0694
- Epithelial cell, collagen1+, IL-1R+	228 [162-336]	200 [160-310]	282 [201-450] ###	174 [148-223]	**0.0006**
- EMT1	1012 [835-1536]	788 [443-909]	1101 [936-1641] ****	1070 [796-1546] **	**0.0002**
- EMT2	21 [11-30]	12 [7-24]	20 [12-30]	22 [14-37]	0.0802
Endothelial cells	629 [441-849]	457 [375-667]	715 [551-875]	539 [389-802]	0.0549
- Endothelial cell	105 [64-169]	100 [35-176]	112 [70-243]	102 [52-127]	0.1164
- Endothelial cell, C4d+, IL-1R+, collagen1+	87 [56-133]	75 [52-102]	89 [41-135]	107 [63-134]	0.4050
- EnMT	383 [215-552]	261 [144-424]	435 [244-665]	332 [208-482]	0.1653
Nonsense clusters	51 [29-76]	29 [15-49]	47 [27-69] #	69 [47-86] ***	**0.0002**

Table F.1. Tier 1 and 2 consensus clusters according to clinical phenotypes
Overview of cell counts of Tier 1 and 2 clusters for all ROIs and per CLAD phenotype (no CLAD, BOS and RAS). Data expressed as median [IQR] number of cells per mm2 ROI.
[a] Corrected taking sections without tissue and airspaces into account.
Kruskal-Wallis with Dunn's multiple comparison test * Compared with controls. *: < 0.05, **: < 0.01, ***: < 0.001, ****: < 0.0001. # compared with RAS.
BOS: bronchiolitis obliterans syndrome, C4d: complement 4d, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, IL-1R: interleukin 1 receptor, NK: natural killer, RAS: restrictive allograft syndrome, ROI: region of interest, TEMRA: terminally differentiated effector memory T cells.

2. Adapted Tier 1 cell counts per type of ROI

A. Lung parenchyma

Type of ROI	All (n=81 ROIs)	No CLAD (n=13 ROIs)	BOS (n=40 ROIs)	RAS (n=28 ROIs)
Relatively preserved alveoli (n)	18	3	9	6
- Area of ROI (mm^2)	1 [1-1]	1 [1-1]	1 [1-1]	1 [1-1]
- Cells per mm^2	3925 [3443-4711]	3891 [3476-4684]	3959 [3491-4906]	3821 [2605-4722]
- T cells	949 [763-1189]	1149 [841-1163]	909 [763-1358]	965 [550-1187]
- Plasma cells	39 [28-59]	30 [24-43]	50 [32-61]	46 [23-195]
- Monocytes/macrophages	641 [526-856]	706 [664-853]	592 [520-995]	611 [379-876]
- Eosinophils	173 [118-248]	186 [51-408]	202 [129-287]	134 [92-171]
- Other leukocytes	278 [196-354]	269 [204-287]	286 [190-369]	277 [223-414]
- EMT 1	982 [892-1110]	926 [833-1055]	1053 [927-1211]	932 [691-1040]
- EnMT	419 [350-483]	428 [410-457]	385 [349-552]	414 [337-508]
Less fibrotic parenchymal area (n)	4	0	0	4
- Area of ROI (mm2)	0.68 [0.38-0.96]			0.68 [0.38-0.96]
- Cells per mm^2	5423 [3520-5674]			5423 [3520-5674]
- T cells	1057 [612-1262]			1057 [612-1262]
- Plasma cells	51 [24-121]			51 [24-121]
- Monocytes/macrophages	849 [552-1412]			849 [552-1412]
- Eosinophils	189 [162-363]			189 [162-363]
- Other leukocytes	329 [267-535]			329 [267-535]
- EMT 1	1379 [884-1546]			1379 [884-1546]
- EnMT	309 [163-522]			309 [163-522]
More fibrotic parenchymal area (n)	6	0	3	3
- Area of ROI (mm2)	0.98 [0.91-0.99]		0.93 [0.84-1.00]	0.98 [0.97-0.99]
- Cells per mm^2	6211 [5529-6820]		5602 [5311-6465]	6463 [5959-7884]
- T cells	1520 [1414-1707]		1517 [1483-1613]	1523 [1205-1991]
- Plasma cells	104 [49-465]		52 [41-95]	377 [113-728]
- Monocytes/macrophages	1131 [781-1346]		811 [691-1451]	1179 [1082-1311]
- Eosinophils	203 [121-274]		232 [174-256]	127 [102-327]
- Other leukocytes	411 [217-492]		224 [196-391]	471 [432-556]
- EMT 1	1603 [1421-1680]		1556 [1506-1651]	1652 [1166-1765]
- EnMT	344 [248-426]		265 [200-304]	421 [384-440]

B. Airways

Type of ROI	All (n=81 ROIs)	No CLAD (n=13 ROIs)	BOS (n=40 ROIs)	RAS (n=28 ROIs)
Non-proliferative airway (n)	15	4	9	2
- Number of ROI	15	4	9	2
- Area of ROI (mm^2)	0.38 [0.28-0.45]	0.33 [0.21-0.43]	0.34 [0.27-0.53]	0.44 [0.43-0.45]
- Cells per mm^2	4536 [3721-5976]	3452 [3032-3942]	5739 [4518-8258]	4558 [3140-5976]
- T cells	1118 [933-1370]	875 [714-1035]	1231 [1079-2608]	1002 [730-1273]
- Plasma cells	72 [25-197]	54 [29-71]	170 [41-284]	166 [12-320]
- Monocytes/macrophages	550 [533-951]	535 [358-547]	583 [544-1410]	701 [451-951]
- Eosinophils	130 [66-259]	47 [28-76]	207 [97-356]	222 [140-304]
- Other leukocytes	264 [203-587]	192 [145-245]	265 [225-694]	428 [270-587]
- EMT 1	1005 [837-1817]	819 [729-977]	1583 [979-1826]	1074 [691-1458]
- EnMT	430 [261-537]	376 [284-437]	503 [290-612]	421 [191-651]
Inflammatory OB lesion (n)	6	0	3	3
- Area of ROI (mm^2)	0.45 [0.06-0.64]		0.52 [0.38-0.86]	0.07 [0.03-0.57]
- Cells per mm^2	6463 [4152-7041]		6958 [4681-7288]	6112 [2567-6814]
- T cells	1385 [948-2171]		1368 [1075-2128]	1402 [567-2300]
- Plasma cells	103 [67-159]		79 [79-145]	126 [33-200]
- Monocytes/macrophages	633 [569-848]		650 [615-1019]	614 [433-791]
- Eosinophils	397 [174-455]		383 [210-450]	412 [67-471]
- Other leukocytes	395 [260-581]		358 [240-433]	537 [267-714]
- EMT 1	1385 [922-1877]		1838 [996-1995]	1043 [700-1728]
- EnMT	479 [317-749]		497 [356-637]	461 [200-1086]
Fibrotic OB lesion/fibrotic airway (n)	5	0	4	1
- Area of ROI (mm^2)	0.49 [0.20-0.58]		0.50 [0.26-0.61]	0.22
- Cells per mm^2	5539 [5397-6986]		5484 [5381-7043]	6427
- T cells	1509 [1358-1814]		1510 [1287-1960]	1509
- Plasma cells	82 [48-106]		94 [62-107]	41
- Monocytes/macrophages	955 [704-1264]		850 [684-1105]	1373
- Eosinophils	250 [132-339]		202 [121-326]	327
- Other leukocytes	345 [272-394]		314 [266-407]	359
- EMT 1	1614 [1303-1805]		1559 [1202-1866]	1659
- EnMT	522 [451-691]		508 [429-718]	600
Large airway	4	0	3	1
- Area of ROI (mm^2)	0.53 [0.37-0.76]		0.38 [0.36-0.67]	0.79
- Cells per mm^2	5105 [4413-5943]		4547 [4369-6037]	5662
- T cells	1341 [1217-1497]		1371 [1185-1539]	1311
- Plasma cells	80 [32-124]		69 [19-92]	134
- Monocytes/macrophages	498 [414-654]		482 [392-513]	701
- Eosinophils	82 [56-277]		98 [53-337]	66
- Other leukocytes	288 [236-418]		245 [233-331]	447
- EMT 1	1496 [912-2108]		1114 [845-1879]	2185
- EnMT	654 [290-775]		674 [633-809]	176

C. Blood vessels

Type of ROI	All (n=81 ROIs)	No CLAD (n=13 ROIs)	BOS (n=40 ROIs)	RAS (n=28 ROIs)
Blood vessels	20	5	9	6
- Area of ROI (mm²)	0.34 [0.09-0.65]	0.47 [0.05-0.68]	0.42 [0.18-0.71]	0.18 [0.08-0.36]
- Cells per mm²	3446 [2749-4367]	2095 [1642-4138]	3729 [2863-4313]	3122 [2595-5013]
- T cells	773 [574-1028]	549 [402-1006]	838 [687-1017]	625 [507-1125]
- Plasma cells	27 [16-44]	10 [3-22]	40 [22-97]	30 [22-71]
- Monocytes/macrophages	431 [320-609]	283 [213-713]	423 [342-558]	482 [421-768]
- Eosinophils	51 [30-100]	13 [2-74]	46 [37-104]	81 [45-142]
- Other leukocytes	181 [130-280]	129 [107-275]	231 [138-670]	235 [151-408]
- EMT 1	809 [568-957]	507 [324-794]	920 [770-1012]	870 [692-1114]
- EnMT	502 [365-819]	364 [239-869]	685 [430-842]	428 [285-694]
Adjacent blood vessel non-proliferative airway	12	5	5	2
- Area of ROI (mm2)	0.41 [0.14-0.68]	0.47 [0.05-0.68]	0.67 [0.23-0.73]	0.30 [0.27-0.32]
- Cells per mm²	3115 [2258-4319]	2095 [1642-4138]	3729 [2752-6175]	3115 [2819-3411]
- T cells	639 [559-1053]	549 [402-1006]	791 [617-1577]	625 [591-659]
- Plasma cells	24 [12-39]	10 [3-22]	40 [29-113]	21 [15-28]
- Monocytes/macrophages	423 [285-606]	283 [213-713]	417 [294-940]	431 [428-433]
- Eosinophils	51 [19-106]	13 [2-74]	53 [42-160]	80 [53-107]
- Other leukocytes	156 [119-280]	129 [107-275]	231 [125-377]	216 [163-270]
- EMT 1	797 [577-957]	507 [324-794]	941 [857-1421]	876 [789-963]
- EnMT	543 [318-847]	364 [239-869]	729 [418-860]	482 [297-667]
Adjacent blood vessel inflammatory OB lesion	5	0	2	3
- Area of ROI (mm²)	0.09 [0.07-0.61]		0.42 [0.09-0.74]	0.08 [0.06-0.47]
- Cells per mm²	2969 [2378-4458]		3468 [2969-3967]	2832 [1925-4950]
- T cells	838 [447-989]		875 [838-911]	568 [325-1067]
- Plasma cells	32 [19-97]		28 [12-44]	32 [25-150]
- Monocytes/macrophages	530 [412-619]		489 [423-556]	530 [400-683]
- Eosinophils	63 [24-83]		48 [28-67]	63 [19-100]
- Other leukocytes	200 [130-433]		190 [146-233]	200 [115-633]
- EMT 1	744 [455-1192]		627 [510-744]	817 [400-1567]
- EnMT	489 [308-759]		759 [685-833]	367 [250-489]
Adjacent blood vessel fibrotic OB lesion/fibrotic airway	3	0	2	1
- Area of ROI (mm2)	0.25 [0.09-0.42]		0.34 [0.25-0.42]	0.09
- Cells per mm²	3896 [3481-5200]		3688 [3481-3896]	5200
- T cells	868 [755-1300]		811 [755-868]	1300
- Plasma cells	44 [21-148]		85 [21-148]	44
- Monocytes/macrophages	560 [386-1022]		473 [386-560]	1022
- Eosinophils	40 [36-244]		38 [36-40]	244
- Other leukocytes	256 [143-333]		199 [143-256]	333
- EMT 1	922 [896-1012]		954 [896-1012]	1012
- EnMT	514 [440-778]		447 [440-514]	778

Table F.2 Adapted Tier 1 cell counts per type of ROI
Median number of cells per mm² tissue for adapted Tier 1 clusters across different types of ROI and clinical phenotypes (no CLAD, BOS and RAS).
BOS: bronchiolitis obliterans syndrome, CLAD: chronic lung allograft dysfunction, EMT: epithelial-to-mesenchymal transition, EnMT: endothelial-to-mesenchymal transition, OB: obliterative bronchiolitis, RAS: restrictive allograft syndrome, ROI: region of interest.

Appendix G: Abstract BTS conference

Profiling immune cell responses in chronic rejection after lung transplantation using imaging mass cytometry

Introduction: Chronic rejection or Chronic Lung Allograft Dysfunction (CLAD) severely limits long-term survival after lung transplantation. CLAD has two phenotypes, bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS), characterised by airway-centred or parenchymal fibrosis, respectively. The effector immune cell response driving CLAD phenotypes is poorly understood. Imaging mass cytometry (IMC) allows a large bespoke panel of immune and structural markers to be simultaneously localised at single-cell resolution in tissue.

Methods: Lung tissue from 20 recipients with CLAD, obtained during re-transplantation or post-mortem, and 3 recipients who died with healthy grafts was sectioned and stained with a 40-plex antibody panel. Eighty-one pathologist-guided regions of interest from airways, blood vessels and parenchyma were laser ablated using IMC. 190,851 cells across 41 mm^2 tissue were captured allowing 26 distinct immune and structural cells to be identified. Cell numbers and % were compared across BOS, RAS and non-CLAD groups.

Results: IMC revealed classical cellular and humoral immune responses in CLAD, including cytotoxic T cells and plasma cells, but additionally eosinophil infiltration. Novel findings showed more M2 macrophage polarisation and expansion of Th1 cells in RAS and increased γδ T cells in BOS. There were common cell profiles in evolving fibrosis in both parenchyma and airways, involving both adaptive and innate cells as well as epithelial-to-mesenchymal transition. (Fig.) However different profiles in RAS (M2 macrophages, Th1 cells) and in BOS (γδ T cells) were also identified.

Discussion: In-depth immunophenotyping of cells in their native tissue microenvironment identified major differences in CLAD versus non-CLAD and between BOS and RAS. Our findings in fibrotic progression of CLAD suggest γδ T cells and M2 macrophages merit further investigation. IMC provides powerful immunological insights that may be important across all organ transplants.

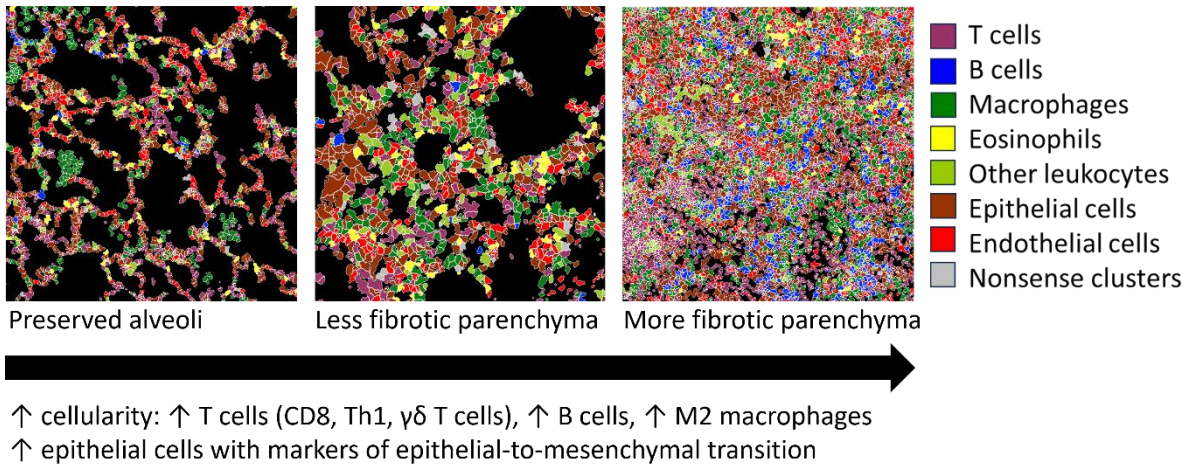

Fig. Temporal evolution of parenchymal fibrosis

Appendix H: Abstract ISHLT conference

High-dimensional lung tissue imaging reveals temporal changes in immune cell populations and cell interactions during progression of Chronic Lung Allograft Dysfunction (CLAD)

Purpose

The immunological drivers of progressive CLAD at a tissue level are poorly understood. Tissue imaging using mass spectrometry (IMC) and laser ablation of regions of interest (ROI) offers single-cell resolution of distinct immune cell populations and their spatial relationships in disease and may improve our understanding of CLAD pathophysiology.

Methods

Explant lung tissue from 23 recipients, 20 with and 3 without CLAD, was sectioned and stained with a bespoke 40-plex antibody panel before 81 pathologist-selected ROIs from airways, blood vessels and lung parenchyma were ablated using IMC. 190,851 individual segmented cells across 41 mm^2 tissue were captured before 26 distinct immune and structural cell populations were identified and interrogated across BOS, RAS and non-CLAD controls using the OPTIMAL analysis pathway.

Results

CLAD was associated with increased total cellularity and specifically expansion of cytotoxic T cells, Th1 cells, plasma cells, and γδ T cells compared to non-CLAD, even after correction for airspace differences. Regions with marked fibrotic remodelling showed M2 polarisation of macrophages and eosinophil infiltration. Within CLAD ROIs, RAS was characterised by more Th1 cells and fewer γδ T cells than BOS.

The temporal evolution of fibrotic remodelling appeared to be driven by γδ T cells in BOS and intermediate M2 macrophages in RAS, along with B cell expansion and infiltration of eosinophils. A strong spatial interaction between eosinophils and endothelial cells was observed in less fibrotic areas, while in more fibrotic areas, eosinophils interacted with B cells especially in RAS.

Conclusions

IMC is a powerful tool, enabling highly multiplexed imaging of lung tissue at single-cell resolution. The interaction between eosinophils and B cells raises the possibility that eosinophils, which can act as antigen-presenting cells, may be important in stimulating B cells to produce antibodies. In addition, increased proportions of M2 macrophages may play a role in lung tissue remodelling in RAS; whereas in BOS, γδ T cells, which are quasi-innate and patrol mucous membranes and epithelia, are more numerous than in RAS. Using this approach we identified potential novel immunological insights into the pathogenesis and fibrotic progression of CLAD.