The role of β_2 integrins on dendritic cells in tolerogenic and autoimmune environments

Leonie Schittenhelm

BSc

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Translational and Clinial Research Institute,

Faculty of Medical Sciences,

Newcastle University

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Abstract

The human immune system reacts swiftly to pathogens while remaining oblivious to self, an important concept termed self-tolerance. Dendritic cells (DCs) are pivotal in this process, as they mediate both initiation of adaptive immune responses as well as induction of tolerance. Recently, evidence has been accumulating that β_2 integrins, heterodimeric adhesion receptors that can assume an active conformation in response to stimulation through various receptors, may have both pro-inflammatory and immunoregulatory roles on DCs. In this thesis, I investigated the hypothesis that dysregulation of β_2 integrin signalling on DCs can contribute to aberrant inflammatory responses, such as in the autoimmune disease rheumatoid arthritis (RA).

To test this hypothesis, I first developed and optimised a multi-colour flow cytometry panel to measure both expression ('total' β_2 integrin) and expression of the active conformation ('active' β_2 integrin) of three β_2 integrin subunits: CD11a, CD11b and the pairing subunit CD18. As conformational state of integrins is highly important for the functionality, this provided important proof of concept that simultaneous staining for both 'total' and activation-specific epitopes is possible.

Then I utilised mature and tolerogenic monocyte-derived DCs (Mo-DCs) as a model system to explore the role of β_2 integrins in circumstances of immune activation and tolerance. I found that tolerogenic Mo-DCs expressed significantly more active CD11a, while expressing significantly less active CD11b compared to mature Mo-DCs. Furthermore, treating mature Mo-DCs with a CD11b-blocking antibody significantly decreased their T cell stimulatory ability, suggesting that CD11b might have pro-inflammatory roles on DCs.

Lastly, I compared total and active β_2 integrin expression on DCs and monocyte populations in RA patients and healthy controls. While no differences in β_2 integrin expression were observed in peripheral blood (PB), comparing synovial fluid (SF) to PB of active RA patients showed that expression of total and active CD11a was significantly reduced in SF, while CD11b was significantly increased.

To conclude, I found that CD11a and CD11b might play opposing roles on DCs in both tolerogenic and autoimmune environments. From my findings, it could be concluded that CD11a has immunoregulatory roles on DCs, while CD11b is pro-inflammatory.

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Definitions/Abbreviations

ACPA – anti-citrullinated peptide antibody

APC – antigen-presenting cell

BMDCs – bone marrow-derived dendritic cells

BSA – bovine serum albumin

Ca^{2+} - Calcium

CCP – anti-cyclic citrullinated peptide

cDC1 – conventional dendritic cell type 1

cDC2 – conventional dendritic cells type 2

CDP – common DC progenitor

CIA – collagen induced arthritis

CLR – C-type lectin receptors

CR3/4 – complement receptor 3/4

CRP – C-reactive protein

CTV – celltrace violet proliferatio dye

DAG – diaglycerol

DAMPS – damage associated molecular patterns

DAS28 – disease activity score 28

DC – dendritic cell

DMARD – disease-modifying anti-rheumatic drug

EAE - experimental autoimmune encephalomyelitis

ECM – extra-cellular matrix

EDTA – ethylenediamine tetraacetate acid

ELISA – enzyme-linked immunosorbent assay

ESR – eryhthrocyte sedimentation rate

FCS – fetal calf serum

FRC – fibroblast reticular cells

FRET – fluorescent resonance energy transfer

GWAS – gene wide associated study

H_2SO_4 – Sulfuric Acid

HBSS – Hanks' balanced salt solution

HCQ – hydroxychloriquine

HLA – human leukocyte antigen

HSP – heat shock protein

IBD – irritable bowel syndrome

ICAM-1 to -5 – intracellular adhesion molecule 1 to 5

IFN – interferon

IPEX - immune dysregulation polyendocrinopathy enteropathy X-linked

JAM – junction adhesion molecule

JC virus – human polyomavirus 2

KO – knock-out

LAD – leukocyte adhesion deficiency

LAP – latency-associated protein

LFA-1 – lymphocyte function associated antigen

LN – lymph node

LPS – lipopolysaccharide

mAb – monoclonal antibody

Mac-1 – macrophage-1 antigen

MFI – mean fluorescence intensity

$MgCl_2$ – Magnesium Chloride

MHC – major histocompatibility complex

MIP1α – macrophage inflammatory protein-1α

MLR – mixed lymphocyte reaction

Mn^{2+} – Manganese

Mo-DC – monocyte-derived DC

MS – multiple sclerosis

MS residual – mean squared residual

MTX – methotrexate

NaOH – sodium hydroxide

NK cells – Natural killer cells

OA – osteoarthritis

PAMPs- pathogen associated molecular patterns

PB – peripheral blood

PBMCs – Peripheral blood mononuclear cell

PBS – phosphate buffered saline

pDC – plasmacytoid DC

PFA – Paraformaldehyde

PGN – peptidoglycan

PKC – protein kinase C

PMA - phorbol 12-myristate 13-acetate

PML – progressive multifocal leukoencephalopathy

PMT voltages – photomultiplier voltages

PNPP - p-nitrophenyl phosphate

Pre-CDCs –pre-conventional DCs

PRR – pattern recognition receptor

P-SMAC – peripheral supramolecular activation cluster

RA – Rheumatoid Arthritis

RAGE - receptor for advanced glycation end products

Rap-GEF – Rap-guanine nucleotide exchanger

RF – rheumatoid factor

RIAM - Rap1-GTP interacting adaptor molecule

RT – room temperature

SD – standard deviation

SF – synovial fluid

SI – stain index

SLE – systemic lupus erythematosus

SSChi – SSC-high

SSClo- SSC-low

SSZ – sulfasalazine

ST – synovial tissue

TCR – T cell receptor

TGFβ – Transforming growth factor β

TLR – Toll like receptor

TNF – Tumour necrosis factor

Treg – regulatory T cell

VCAM – vascular cell adhesion protein

WT – wild type

βTD – β tail domain

Chapter 1 **Introduction**

1.1 Immune tolerance

The immune system consists of a variety of specialised cells, which work concordantly to protect an organism from danger, either in the form of foreign pathogens (non-self) or aberrant self, such as cancerous cells. To effectively protect an organism from these threats, the immune system needs to correctly distinguish between foreign or abnormal non-self, which needs to be eliminated, and the body's own cells (self), which need to remain protected from an immune response to remain healthy and functional.

The state of remaining non-responsive in the presence of self is called immune tolerance. If tolerance to self is lost, this can lead to emergence of autoimmunity, where the immune system attacks and progressively damages the body's own tissues. Common examples of autoimmune disorders include diabetes, inflammatory bowel disease (IBD) and rheumatoid arthritis (RA), where the pancreas, intestines and joint tissues, respectively, are targeted by the immune system causing pathology. Correctly distinguishing between self and non-self is therefore a highly important immunological process (Parijs and Abbas, 1998).

Recognising pathogens can occur non-specifically, by the innate immune system, and specifically, by the adaptive immune system. Briefly, phagocytic cells (neutrophils, monocytes, macrophages), cells releasing pro-inflammatory mediators (basophils, mast cells, eosinophils) and natural killer (NK) cells, as well as the complement system and interferons (IFNs) are all players of innate immunity. They recognise highly conserved properties of foreign molecules using so-called pattern recognition receptors (PRRs) and act quickly to eliminate them. While the innate immune system is therefore a highly important line of first defence against infection, it is less effective at reacting to pathogens that do not share conserved pattern recognition motifs. Another important role of the innate immune system is therefore that it can provide danger signals to activate the antigen-specific adaptive immune system. The adaptive immune system consists of T cells and B cells, which, through genetic rearrangement of their surface receptors, are able to recognise any novel antigens. This is largely due to the huge number of different antigen-specific T and B cell clones, which then in turn can be expanded to counteract the pathogen in question specifically. Importantly, some of the members of the expanded clone(s) can persist after the end of infection as memory cells, thereby providing more swift and effective responses to subsequent infections with the same pathogen. The

adaptive immune system is therefore the basis of vaccination being an effective mechanism to combat otherwise life-threatening diseases (Delves and Roitt, 2000).

1.2 Role of antigen-presenting cells in the immune system

Antigen-presenting cells (APCs) provide the important link between innate and adaptive immune responses. Importantly, they are also involved in maintaining tolerance to self. This is because APCs continuously sample, process and finally present self and non-self antigens in so-called major histocompatibility complexes (MHC) on their surfaces, which are termed human leukocyte antigens (HLAs) in humans. MHCs exist in two classes, MHC class I (MHC I), which present antigen to $CD8^+$ cytotoxic T cells and MHC class II (MHC II), which present antigen to $CD4^+$ helper T cells. This is where pathogenic and non-pathogenic antigens are presented and potentially recognised by both naïve and memory antigen-specific T cells, which are the drivers of the adaptive immune response. In contrast, self-antigen presented on cell surfaces is the basis of both central and peripheral tolerance (Miller and Basten, 1996; Miller and Morahan, 1992), which will be discussed in more detail in section 1.3. Although virtually all cells can act as APCs to present their own molecules to cytotoxic CD8 T cells via MHC I, only some APCs can present both foreign and self-antigen to helper CD4 T cells via MHC II. While macrophages, B cells and more recently γδ T cells have all been described to express MHC II (Lanzavecchia, 1985; Moser and Brandes, 2006; Unanue, 1984), dendritic cells (DCs) have been described as the dominant APC population that have the ability to stimulate naïve CD4 T cells, therefore being termed the professional APCs of the immune system (Lassila et al., 1988). Even though expression of MHC II was also found to be inducible under inflammatory conditions in a variety of cells from basophils to neutrophils, there is little evidence that they could replace DCs in any meaningful way (Kambayashi and Laufer, 2014). DCs are therefore key players in initiating either immune activation or tolerance and will be described in detail in section 1.2.1. Additionally, monocytes, which can develop into so-called monocyte-derived DCs (Mo-DCs) under inflammatory conditions, will also be discussed.

1.2.1 Dendritic cells

DCs are large cells with cytoplasmic protrusions of varying lengths, which were recognised in their own right by Steinmann and Cohn in murine lymphoid organs (Steinman and Cohn, 1973). In their role as ‘professional’ APCs, DCs are highly important in both initiating an adaptive immune response in the presence of

pathogens and conversely limiting aberrant immune activation in response to self. The majority of DCs reside in tissues and lymphoid organs, where they are surveying the area for antigens. However, DCs make up only 1-2% of peripheral tissues and circulating DCs only comprise 1% of PB, making them a comparatively small cell population. In addition to patrolling PB and tissues, they can also be found in lymph and lymphoid organs. In this section, development of different DC populations will be briefly described before exploring their roles in both immune activation and tolerance induction.

Development of DC subsets

DCs develop from common DC progenitors (CDP) in the bone marrow (Naik et al., 2007; Rhodes et al., 2019), which give rise to plasmacytoid DCs (pDCs) and pre-conventional DCs (pre-cDCs). Pre-cDCs then further differentiate into two types of conventional DCs: cDC1 and cDC2 DCs. While pDCs exit the bone marrow fully differentiated, pre-cDCs circulate in peripheral blood (PB) and enter lymphoid organs to differentiate into cDC1 or cDC2 DCs. More recent studies using single cell RNAseq have however identified further DC subsets (Villani et al., 2017), yielding a total of six DC subsets in PB. To specify, two different subtypes of cDC2 DCs have been described, with the newly discovered one (termed DC3 by Villani and colleagues) expressing high levels of CD163 and CD36 alongside other cDC2 markers. Further, one DC subset that lacks both cDC1 and cDC2 markers but expresses the monocyte marker CD16 have been described as DC4. Lastly, a pDC marker expressing DC subset, which can be delineated by its expression of Axl and Siglec6 was termed DC5 (Rhodes et al., 2019; Villani et al., 2017). However, these distinctions are still relatively recent, with the ontogeny of some of these previously undefined subsets still being unknown and available surface antibodies for flow cytometry being limited. This thesis will therefore focus on cDC1, cDC2 and pDCs, which will be discuss in more detail in respect to their expression of surface molecules, requirements for restriction factors and function.

cDCs are the major DC population responsible for detecting foreign antigen and presenting it to naïve T cells to initiate an adaptive immune response. cDC1 DCs are the rarest DC subset with only 0.1% of leukocytes belonging to this group. They are characterised by high expression of the thrombomodulin (CD141) as well as CLEC9A and XCR1. Their development is dependent on the transcription factor BATF3 (Poulin et al., 2012) and their murine equivalent are characterised by CD8α and

CD103 expression. In contrast, cDC2 DCs, which are of myeloid origin, have been shown to be dependent on the presence of IRF4 (Guilliams et al., 2016) and express CD1c, a member of the CD1 family responsible for presenting lipids, as well as CLEC6A and CLEC7A. In mice, cDC2 DCs are mainly characterised by their high expression of the β_2 integrin CD11b. Lastly, pDCs have a specialised role in response to viruses, where they produce large amounts of Type I IFNs (IFNα, IFNβ) to block viral replication and activate other immune cells (Asselin-Paturel and Trinchieri, 2005; Barchet et al., 2002). pDCs are characterised by expression of CD123 in human and Siglec H, BST-2 and B220 in mouse, as well as the absence of CD11c from their surface, which is a common marker for cDCs. The development of pDCs depends on the transcription factor E2-2 (Cisse et al., 2008).

Role of DCs in immune activation during infection

To appreciate the importance of DCs in the process of immune activation, a more detailed overview of antigen presentation and activation of adaptive immune responses is necessary. I will focus here on the presentation of a pathogenic antigen to illustrate how immune activation occurs when presented with a threat, but it has to be kept in mind that several steps in this process will occur similarly when a self-antigen is presented to induce tolerance. This aspect will be explored in detail in the following section, ‘Role of DCs in tolerance’.

Tissue-patrolling DCs, which make up the largest proportion of these APCs, as well as circulating DCs are largely in an immature state, meaning they can efficiently capture, process and present antigen on their surface, but have low T cell stimulatory abilities (Bell et al., 1999). Immature DCs are therefore characterised by high endocytic activity and expression of Fc receptors to mediate highly efficient antigen capture. While they have been reported to have high intracellular levels of MHC, surface expression is low, as is expression of costimulatory molecules such as CD80, CD83 and CD40. Lastly, immature DCs express little to no pro- or anti-inflammatory cytokines, explaining their low T cell priming abilities (Dudek et al., 2013). Upon encountering so-called danger signals, DCs rapidly increase surface expression of costimulatory molecules and MHC II while simultaneously downregulating processes of antigen capture. Furthermore, they undergo extensive cytoskeleton rearrangement and start expressing adhesion receptors, such as the integrin CD49 (Puig-Kröger et al., 2000), on their surface. However, a study comparing injection of wild type (WT) DCs into a murine footpad to DCs where all integrin subunits had been ablated,

showed that they arrived in the lymph node (LN) in similar numbers, suggesting that integrins are not required for migration of mature DCs to LN (Lämmermann et al., 2008). Much more importantly for migration, mature DCs also up-regulate the chemokine receptor CCR7, which mediates DC migration towards CCL19 and CCL20, chemokines produced by fibroblastic reticular cells (FRCs). These stromal cells are located in the T cell zone of secondary lymphoid organs and thereby guide DCs to their appropriate location within the LN (Geissmann et al., 2002). This cluster of processes enabling DCs to migrate from peripheral tissues to LNs and efficiently stimulate T cells is known as DC maturation (Théry and Amigorena, 2001). The danger signals eliciting this process can be exogenous by pathogen-associated molecular patterns (PAMPs), for example through presence of the bacterial cell wall component lipopolysaccharide (LPS) (Smedt et al., 1996), unmethylated CpG motifs in bacterial DNA (Sparwasser et al., 1998) or double-stranded viral DNA (Cella et al., 1999). To detect these PAMPs, DCs utilise so called pattern recognition receptors (PRRs), including Toll like receptors (TLRs), such as TLR2 and TLR4, and C-type lectin receptors (CLRs), such as CLEC9A and Dectin-2 (Lundberg et al., 2014). DC maturation can however also be induced by endogenous signals or damage-associated molecular patterns (DAMPs), such as the presence of heat-shock protein (HSPs) (Singh- Jasuja et al., 2000), breakdown products of extracellular matrix (ECM) such as heparin sulfates (Kodaira et al., 2000) and cytokines released by other activated leukocytes, such as interleukin 1β (IL-1β) and Tumour necrosis factor-α (TNF-α) (Zepter et al., 1997). Recognition of DAMPs can occur via DAMP receptors, such as TLR 4 and TLR9, but also via the receptor for advanced glycation end products (RAGE) (Nace et al., 2012).

After having acquired non-self antigen and undergone maturation, DCs migrate to the draining LN to present it to the resident naïve T cells. Once arrived in the LN, DCs present the antigen to a range of T cell clones, until a clone that specifically binds to the presented antigen is found (Ingulli et al., 1997). This antigen-specific interaction between DC and T cell is known as the immunological synapse. Three signals have been established to be necessary to initiate T cell activation and clonal expansion, which initiates the adaptive immune response (Figure 1.1A) (Gutcher and Becher, 2007). Signal 1 is the interaction between MHC II-bound peptide expressed by the DC and the T cell receptor (TCR) on the T cell side, ensuring that the response to the pathogenic origin of the presented peptide is antigen-specific. Signal 2, is mediated by

costimulatory factors CD80 and CD86 on DCs binding to CD28 expressed on CD4 T cells, thereby stimulating the T cell. Lastly, signal 3, mediated by cytokine released by DCs into the immunological synapse polarises T cell differentiation thereby targeting the immune response to the type of infective agent. For example, T cell stimulation occurring in the presence of IL-12 produced by APCs stimulates Th1 T cell responses targeted at bacteria and intracellular pathogens, while PGE_2 stimulates Th2 T cell responses, specifically targeting extracellular parasites such as helminths (Kaliński et al., 1999). Signal 3 can, therefore, provide valuable additional information collected by DCs in the periphery during maturation to naïve T cells in the LN. Furthermore, DCs play an important role in stimulating CD8 T cells in a process termed cross-presentation. This is a process whereby particularly cDC1 DCs present exogenous antigens via MHC I, which is usually reserved for cell-endogenous peptides, to stimulate CD8 T cells and enable them to mature into antigen-specific cytotoxic T cells (Bevan, 1976).

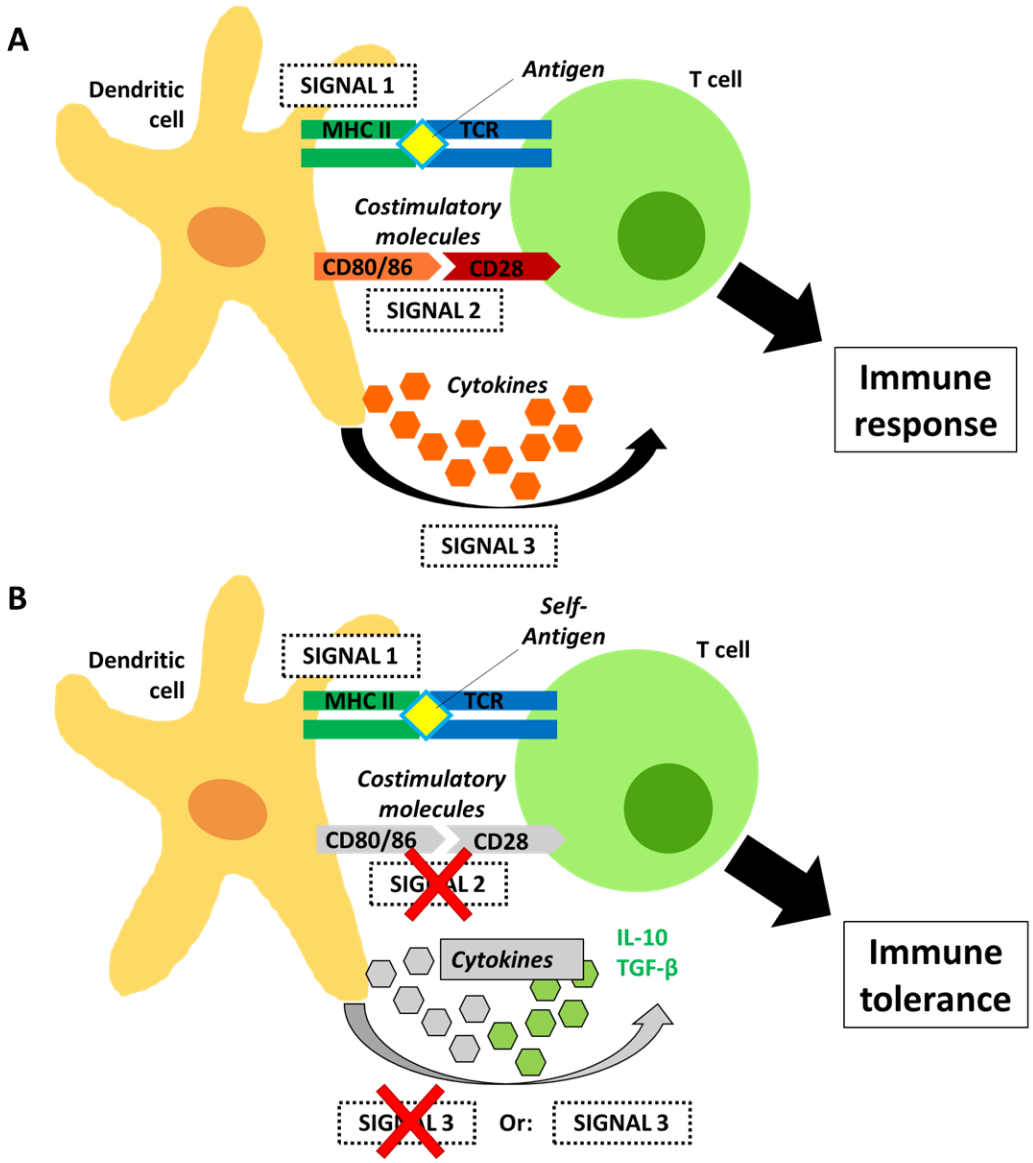

Figure 1.1 Antigen-specific T cell activation and tolerance induction

An APC, such as DC, and a T cell form an immunological synapse where the APC presents antigen in its MHC II, which is recognised by a TCR on the T cell (Signal 1). A. Initiation of immune activation occurs when DCs also provide co-stimulatory signals in the form of CD80/86 binding to CD28 on T cells (Signal 2) and produce cytokines that stimulate T cell responses (Signal 3). B. Non-pathogenic self-antigen is presented in the absence of Signal 2, Signal 3 or both, or in the presence of a immunoregulatory Signal 3 signalling involving IL-10 or TGF-β, thereby causing T cells specific for this antigen to become unresponsive, thereby maintaining peripheral tolerance to self. Adapted from Gutcher & Becher, 2007.

To summarise, DCs do not merely present antigens to T cells, they also actively stimulate T cell activation via costimulatory signals and shape the type of T cells responses elicited by polarising them towards pathogen-appropriate T cell phenotypes.

However, they also have important roles in maintaining tolerance, which I will explore in the following section.

Role of DCs in tolerance

As previously mentioned, DCs are not only important for the initiation of an immune response in the event of infection, they are also indispensable in preventing immune response to self-antigens, as well as commensals and harmless antigens ingested with food. The immense importance of DCs for tolerance is highlighted by the fact that complete ablation of DCs in a mouse model led to severe fatal multi-organ autoimmunity, suggesting that DCs play a highly important role in induction of tolerance to self (Ohnmacht et al., 2009).

There are a variety of ways in which DCs induce tolerance. To induce central tolerance, DCs induce cell death of T cells showing high affinity for self-antigen in the thymus, a process termed negative selection (Brocker et al., 1997). However up to 25-40% of self-reactive T cells were found to escape negative selection, necessitating further mechanisms to maintain tolerance to self (Bouneaud et al., 2000). Further contributing to central tolerance, DCs have also been found to contribute to the induction and expansion of $FoxP3^+$ regulatory T cells (Tregs) in the thymus (Proietto et al., 2008). Tregs are potent regulators of aberrant immune responses and depletion or reduction in numbers of Tregs has been associated with the emergence of autoimmune disease (Ochs et al., 2005; Singh et al., 2001). In fact, mutations in FoxP3 cause the fatal IPEX syndrome (immune dysregulation polyendocrinopathy enteropathy X-linked), an X-linked genetic disorder being characterised by excessive autoimmunity due to the absence of functional Tregs (Bennett et al., 2001). Furthermore, DCs are also involved in peripheral tolerance, by presenting self-antigen to T cells in the absence of costimulatory signals (signal 2) or pro-inflammatory cytokines (signal 3) in an immunological synapse (Figure 1.1B). In *in vitro* studies, absence of costimulation via CD28 rendered T cells anergic to stimulation with lectins, with low production of IL-2 and a decrease in IL-2 receptor expression, which was not possible to be reversed by external provision of IL-2 (Shahinian et al., 1993). The same study also reported that effective T cell responses were reduced *in vivo* studies when costimulation was absent, suggesting that absence of costimulation negatively impacts the ability of T cells to mount an immune response. Another possibility for DCs to mediate peripheral tolerance is by altering the cytokine profiles of stimulated T cells, thereby steering the direction of the

immune response. For example, DCs matured in the presence of the anti-inflammatory cytokine IL-10 were found to induce anergic T cells expressing low levels of IL-2, thereby being unlikely to elicit sustained T cell proliferation (Steinbrink et al., 1997). Furthermore, T cells activated by these so-called tolerogenic DCs did not only show reduced abilities raise an immune response themselves, they were also shown to actively suppress syngeneic T cells not exposed to IL10 in an antigen-specific manner (Steinbrink et al., 2002), thereby suggesting that these tolerised T cells can themselves tolerise other T cells. In addition to their roles in Treg induction in the thymus, DCs have also been shown to be able to induce Treg differentiation from CD4 T cells in the periphery (Coombes et al., 2007; Sun et al., 2007), further underlining their importance for tolerance induction.

In addition to routes to tolerance, it is also important to consider which types of DCs are able to induce tolerance. For a long time, it was believed that only immature or semi-mature DCs were able to induce tolerance, as they provided antigen presentation (signal 1) in the absence of costimulation and cytokines (signals 2 and 3). Immature DCs were repeatedly described to induce T cell anergy, which resulted in production of anti-inflammatory cytokine IL-10 by T cells (Jonuleit et al., 2000; Lutz et al., 2000b, 2000a). More recently, however, mature DCs have also been shown to have tolerogenic functions in certain immune environments. For example, introducing DCs matured with TNF-α , which was shown to produce an incomplete maturation of DCs in contrast with LPS+CD40 in respect to DC cytokine production, were shown to protect against the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), in an antigen-specific manner (Menges et al., 2002). In fact, in the complete absence of TNF-α, mice were shown to be more susceptible to EAE, further supporting that the cytokine had protective functions. In *in vitro* studies of Mo-DCs, a range of other compounds was found to drive a tolerogenic phenotype in DCs. For example, DCs matured in the presence of the anti-inflammatory cytokine IL-10 were shown to have reduced abilities to stimulate T cells (Steinbrink et al., 1997), as in the presence of transforming growth factor β (TGF-β) (Fogel-Petrovic et al., 2007), IL-6 (Hegde et al., 2004) or a combination thereof (Torres-Aguilar et al., 2010). Furthermore, non-immune compounds such as Vitamin D_3, or the dietary compounds sulforaphane or curcumin were shown to have similarly suppressive effects on DC maturation and enhance tolerogenic functions (Geisel et al., 2014; Penna and Adorini, 2000; Rogers et al.,

2010). Overall, this suggested that tolerogenic functions of DCs are not necessarily dependant only on immaturity, but also on surrounding factors.

Furthermore, different DC populations might have differential capacity to induce tolerance. For example, both thymus resident and migratory cDCs seem to be highly important for negative selection in the thymus, while pDCs were not shown to contribute to deletion of self-reactive T cells to the same degree (Guerri et al., 2013). In contrast, while ablation of all DCs caused fatal autoimmunity in mice, ablation of only cDCs specifically caused problems with raising an immune response but did not result in failure of either negative selection or Treg induction (Birnberg et al., 2008). Although these results have to be scrutinised due to the potential redundancy between different DC types, leading to pDCs potentially compensating functionally in the absence of cDCs, this suggests that pDCs might also contribute to self-tolerance. A role for pDCs in tolerance is further supported by studies reporting their tolerogenic functions in inflammatory models in the lung (Heer et al., 2004), the joint (Jongbloed et al., 2009) and models of organ transplantation (Ochando et al., 2006). While this suggests that both cDCs and pDCs contribute to induction of tolerance, they have also both been shown to be able to contribute to a breach of tolerance causing autoimmunity, which I will explore in more detail in the following section.

Role of DCs in breach of tolerance

While the previous section expanded on the important roles DCs play in immune tolerance, they can also be important contributors to development and progression of autoimmunity.

A range of autoimmune conditions show increased numbers of DCs within the affected tissue, suggesting that DCs play an important role in breach of tolerance and maintenance of autoimmunity. In the autoimmune disease RA, proportion and total number of DCs were found to be increased in synovial fluid and synovial membranes (Harding and Knight, 1986; Jongbloed et al., 2009; Lebre et al., 2008; Poulter et al., 1983). Similarly, in Sjögren's syndrome, an autoimmune condition targeting glands that produce moisture, increased numbers of DCs were observed in salivary glands (Blokland et al., 2000; Wildenberg et al., 2009). In MS, large numbers of circulating DCs expressing pro-inflammatory cytokines such as TNF-α and IL-6 (Huang et al., 1999). The study also reported increased levels of IFN-γ, which is likely to be due to increased T cell activation in MS.

Furthermore, animal studies have shown that DCs can effectively drive autoreactive T cell responses to cause autoimmunity. Introduction of DCs specific to a transgenic protein exclusively expressed in pancreatic β cells caused onset of diabetes in mice (Ludewig et al., 1998) and a similar study showed that adoptive transfer of DCs exposed to thyroglobulin were able to induce thyroiditis (Knight et al., 1988). Interestingly, high IFN levels due to persistent activation of pDCs has been described to contribute to systemic lupus erythematosus (SLE) and potentially other autoimmune disorders (Banchereau and Pascual, 2006), suggesting that pDCs can also play important roles in breach of tolerance.

There are different ways in which DCs could contribute to the initiation of autoimmunity. It is possible that DCs simply fail to provide tolerogenic signals to self-reactive T cells, either in the thymus, letting self-reactive T cells escape negative selection, or in the periphery, where presentation of innocuous self-antigen could falsely occur in the presence of costimulatory signals (signal 2) and pro-inflammatory cytokine release (signal 3). A third option is that T cells fail to react to self-antigen, but have strong affinity for self-antigen altered through post-translational modification. As post-translationally altered peptides would not be presented in the thymus, this means that T cells specific for altered peptides would not undergo deletion and could therefore become activated in the presence of post-transcriptionally altered peptide in the periphery.

To summarise, DCs are integral to the decision between immune activation and tolerance. If this careful balance is disturbed however, DCs can equally be important contributors to a breach of tolerance and thereby lead to autoimmune disease. In the next section I will explore the role monocytes play in these contexts before discussing the role these APCs play in the autoimmune condition RA.

1.2.2 Monocytes

Similar to DCs, monocytes arise from common monocyte progenitors (cMop) in the bone marrow before exiting to circulate in PB (Hettinger et al., 2013). Monocytes were originally characterised by their expression of the LPS co-receptor CD14 and occur in three distinct subsets: classical, intermediate and non-classical monocytes (Passlick et al., 1989; Ziegler-Heitbrock et al., 2010).

Classical monocytes show high expression of CD14 and are negative for CD16, a Fcγ receptor type III. Transcriptomic analysis of $CD14^+$ monocytes, thereby including both classical and intermediate monocyte populations, suggested roles in anti-bacterial and pro-inflammatory responses (Anbazhagan et al., 2014). While it was shown that $CD14^+$ monocytes do not undergo differentiation into DCs in the steady state, they contribute to antigen surveillance (Jakubzick et al., 2013). Under inflammatory conditions, such as in the presence of TNF-α and LPS, classical monocytes were shown to effectively differentiate into either so-called Mo-DCs or assumed a macrophage-like phenotype (Mo-macrophages) (Iwamoto et al., 2007) and $CD14^+$ monocytes could even differentiate into osteoclasts, when exposed to macrophage-colony stimulating factor (M-CSF) and RANK ligand (Sørensen et al., 2007). The question which cell type $CD14^+$ monocytes differentiate into is strongly reliant on the signalling factor present in the peripheral tissues where differentiation occurs. *In vitro* studies have shown that presence of IL-4 and GM-CSF produces differentiation of classical monocytes into DCs, while presence of M-CSF induces differentiation into either M1-like macrophages (+LPS) or M2-like macrophages (+IL-4) (Geissmann et al., 2010). Non-classical monocytes on the other hand express high levels of CD16 and low levels of CD14. They were shown to express higher levels of MHCII compared to classical monocytes and produce increased levels of TNF in response to TLR stimulation. Furthermore, non-classical monocytes were shown to expand in inflammatory conditions (Fingerle et al., 1993; Nockher and Scherberich, 1998), suggesting that they play a more important role in inflammation. This is further supported by a study reporting that $CD16^+$ monocytes expressed increased levels of TLRs and costimulatory molecules CD80 and CD86, as well as the previously mentioned HLA-DR, compared to $CD14^+$ monocytes, produced higher levels of pro-inflammatory cytokines TNF-α and IL-1β and showed low phagocytic activity (Mukherjee et al., 2015). The authors of the study therefore suggested that non-classical monocytes might contribute to antigen presentation. Indeed, others have suggested that studies detailing antigen-presentation to splenic marginal zone B cells by CD11c-low cells is not mediated by DCs but actually by $CD16^+$ monocytes (Balázs et al., 2002; Randolph et al., 2008), thereby mediating inflammatory immune responses directly. Additionally they are major producers of TNF in response to inflammatory signals (Belge et al., 2002). However, in the murine model of MS, EAE, immature non-classical monocytes were found to occur in high numbers in the CNS and suppressed T cell proliferation and induced apoptosis in T cells via Nitric Oxide

(Zhu et al., 2007), suggesting that CD16$^+$ monocytes also have anti-inflammatory roles. Intermediate monocytes, expressing high levels of CD14 as well as CD16, are found in much smaller numbers compared to the other two subsets in PB. While they were long thought to be merely a developmental intermediate, gene expression studies have confirmed that they are a transcriptomically distinct subset (Wong et al., 2011; Zawada et al., 2011). Although their distinct roles are still unclear, specifically intermediate monocytes have been described to be increased in RA, sarcoidosis and severe asthma (Hijdra et al., 2012; Moniuszko et al., 2009; Rossol et al., 2012). In contrast to the more pro-inflammatory roles of non-classical monocytes, intermediate monocytes were shown to express high amounts of IL-10 in response to LPS stimulation with decreased antigen-presentation but increased phagocytic capabilities (Skrzeczyńska- Moncznik et al., 2008). This suggested potential anti-inflammatory roles for intermediate monocytes.

In addition to their roles in inflammation and tolerance, it is also important to consider how monocytes can contribute to either a breach of tolerance or subsequent maintenance of autoimmunity. For example, activated CD14$^+$ monocytes isolated from inflamed RA joints were shown to induce exclusively Th17 T cells via cell-contact, thereby suggesting that classical monocytes actively contribute to autoimmunity and might be attractive targets to limit harmful Th17 responses (H. G. Evans et al., 2009). Furthermore, CD16$^+$ monocytes were found to be increased in both PB and SF of RA patients (Kawanaka et al., 2002). The same study suggested that increased CD16 expression on monocytes was likely due to increased cytokines such as IL-10, TGF-β1 and M-CSF, which recruited large numbers of CD16$^+$ monocytes to the joint, thereby sustaining joint inflammation. In EAE, non-classical monocytes expressing CCR2 were furthermore shown to be required for disease initiation, suggesting again that monocytes have important roles in contributing to autoimmunity.

As previously mentioned, an important characteristic of monocytes is their ability to exit the PB, enter tissues and differentiate into both macrophages and DCs (Randolph et al., 1999). While monocytes were originally believed to be the main precursor for both cell types, it is now known that macrophages are largely tissue-resident, where they arise from embryonic cells, while conventional DCs arise from a separate precursor, the CDP, in the bone marrow. However, the ability of monocytes

to differentiate into Mo-DCs at the infection site was found to be important in eliciting an effective Th1 response in *Leishmania* infection (León et al., 2007).

To summarise, both DCs in their capacity as professional APCs and monocytes in their ability to differentiate into potent APCs play a deciding role in initiating adaptive immune responses but also maintaining immune tolerance to avoid immune-mediated damage to self. Dysregulation of DC or Mo-DC function, especially in their communication with T cells, could therefore be a powerful contributor to the development of autoimmune diseases such as RA.

1.3 Breach of tolerance: the autoimmune disease RA

Especially as APCs mediate the decision between immune activation and tolerance, they also have an important role in breach of tolerance, which can contribute to emergence of autoimmune diseases. One example of such a disease is RA, where an aberrant immune response is raised targeting joint tissues thereby resulting in pain and progressive disability if unmedicated. In the following section, I will discuss the pathogenesis of RA in detail, including the role of specifically DCs in disease initiation and maintenance, before exploring DCs as potential therapeutics for RA.

1.3.1 Pathology of RA

The autoimmune disease RA is characterised by painful inflammation and swelling of joints, which can lead to progressive loss of function and disability due to destruction of cartilage and bone tissue. In addition to joint-specific symptoms, patients with RA are also more likely to suffer from systemic problems, such as a high vascular risk, metabolic syndrome and depression, which overall results in increased mortality (Mitchell et al., 1986; Pincus et al., 1984; Wolfe et al., 1994). Around 0.5-1% of people worldwide suffer from RA (Alamanos and Drosos, 2005), with women being twice as likely to be affected as men. While the reason for this discrepancy is not fully resolved, it is likely that hormonal differences, for example overproduction of prolactin in women who breastfed extensively or suffered from infertility or lower levels of the anti-inflammatory hormone testosterone, contribute to disease onset (Brennan and Silman, 1995).

RA is diagnosed using several indicators, including number of inflamed joints, serologic tests for the presence of auto-antibodies (Rheumatoid factor, RF, Anti-citrullinated peptide antibody, ACPA), increased inflammatory markers (e.g. erythrocyte sedimentation rate, ESR, C-reactive protein, CRP) and duration of

symptoms (Aletaha et al., 2010). Disease severity and response to treatment is most commonly measured using the disease activity score 28 (DAS28). This is a composite score calculated by the number of swollen and tender joints out of 28 (shoulders, elbows, wrists, knees and finger joints on each side), measurement of inflammation using either ESR or CRP and a global assessment of health of the patient using a health assessment questionnaire (HAQ) (Prevoo et al., 1995). Currently, a 'treat to target' therapy strategy is employed in the UK, meaning that treatment is adjusted regularly until improvement of symptoms as measured in a low disease activity DAS28 score is reached (National Institute for Health and Care Excellence, 2018). As a first line treatment a conventional disease-modifying anti-rheumatic drug (DMARD) is recommended as a monotherapy, with further DMARDs being added if neither remission (DAS28<2.6) or low disease activity (DAS28<3.2) can be achieved. DMARDs usually decrease immune system activity and include drugs such as methotrexate (MTX), hydroxychloriquine (HCQ) or sulfasalazine (SSZ). If multiple DMARDs do not achieve either low disease activity or remission or if side effects elicited by DMARDs are not acceptable, treatment can be further escalated to include biologics. These are drugs targeting specific molecules important for the immune system, such as abatacept, a CTLA4 fusion protein blocking T cell activation, or etanercept or infliximab, which both block TNF-α (Curtis and Singh, 2011). While this strategy has been proven efficient to decrease the level of disability that accumulates over time (Pincus et al., 2005), medicinal side effects (Bernatsky et al., 2007), persistence of pain in the absence of joint inflammation (Boyden et al., 2016) and difficulties in achieving stable long-term remission for treatment refractory patients (Gallego- Galisteo et al., 2012) remain to be solved to ensure patient well-being.

There is strong evidence that in RA tolerance to self is lost through a combination of genetic predisposition (susceptibility genes) and environmental factors, such as smoking, air pollution or periodontitis, which cause epigenetic changes (McInnes and Schett, 2011). This is believed to alter post-translational protein modification processes, which in turn leads to an increase in citrullination of self-proteins, where the amino acid arginine is replaced by citrulline. These alterations in self-proteins lead to the immune system falsely identifying them as non-self, causing immune activation and in turn eliciting the infiltration of the synovium with leukocytes (Hill et al., 2003). Autoantibodies, such as ACPA, tested for as anti-cyclic citrullinated

peptide (CCP), or RF are therefore a common hallmark of disease and contribute to diagnosis. The presence of autoantibodies can contribute to more aggressive disease progression and might therefore alter the clinical treatment strategy (Rycke et al., 2004), although they are currently used in the UK to expedite referral or patients with suspected RA from their primary care physician to specialist care. It is common that people with RA display autoantibodies years before the onset of symptoms, suggesting that dysregulation of the immune system occurs long before overt joint inflammation (Aho et al., 1991; Nielen et al., 2004; Rantapää- Dahlqvist et al., 2003). Especially considering that disease outcomes improve with early intervention (Emery, 2002; Quinn et al., 2001), it has been hypothesised that intervening before symptom onset might have merit. However, this idea is faced by two major challenges: first, the difficulty to correctly distinguish between the 1-2% of healthy people who are ACPA positive or the 4% who are RF positive and people who will definitely develop symptoms (Prüßmann et al., 2014; Ursum et al., 2009), and second, the question of how to induce the immune system to remain in a state of tolerance to self. This is especially important as current RA treatment strategies can only ameliorate symptoms of the disease not cure it. To achieve a cure for RA, the ultimate goal is therefore to re-establish tolerance that has been lost and is causing pathology.

To conclude, RA is an autoimmune disease where immune dysregulation leads to joint inflammation. If untreated using immunosuppressive drugs, joint tissue is destroyed leading to progressive accumulation of disability and significant morbidity.

1.3.2 Role of dendritic cells in Rheumatoid Arthritis

Auto-reactive Th1 and Th17 T cells have been implicated in driving the autoimmune response in RA (Chabaud et al., 1998; Yamada et al., 2008). Therefore, it is likely that DCs play a pivotal role in the initiation of RA due to their unique role as APCs in initiating T cell responses. Furthermore, recent evidence suggests that DCs also play a role in maintaining joint-specific immune responses in RA, making them a potential therapeutic target. For example, cDC2 DCs isolated from RA synovial fluid (SF) were shown to spontaneously activate autologous PB CD4 T cells and elicit increased production of pro-inflammatory cytokines such as IL-17, IFN-γ and IL-4 by co-cultured T cells (Moret et al., 2013).

In a mouse line expressing the RA susceptibility gene HLA-DRB1*0401, which was shown to contribute to altered citrullination levels (Snir et al., 2011), citrullination was shown to increase affinity between MHC II and peptide and thereby increase CD4 T cell activation by DCs (Hill et al., 2003). Further support for an important role of DCs in the initiation of RA was shown in a murine model of RA, where injection of collagen-pulsed mature DCs caused antigen-specific joint inflammation, mediated by both activation of autoreactive T cells and production of pro-inflammatory cytokines by DCs (Leung et al., 2002). It has been furthermore shown that induction of an RA murine model is critically dependant on cDCs (Benson et al., 2010), while pDCs were described to have immunoregulatory roles in this context and reduced autoimmune responses by adaptive immune cells (Jongbloed et al., 2009). In human, the strongest evidence for an important role for DCs in RA pathogenesis is that disease is strongly associated with specific alleles encoding the antigen-binding groove of MHC II (Winchester and Gregersen, 1988). This so-called shared epitope theory suggests that a number of alleles, specifically HLA-DRB1, all code for either the same or highly similar amino acid sequences, thereby conferring a higher risk of RA. This is interesting as patients with different HLA-DRB1 alleles were found to show distinct clinical phenotypes (Weyand et al., 1992), and presence of some alleles was found to be a reliable biomarker for efficacy of abatacept (Oryoji et al., 2018), an RA therapeutic blocking costimulation of T cells by DCs. Together, this provides strong evidence that DCs contribute to pathogenesis of RA by mediating the breach of tolerance via immunogenic presentation of altered self-antigen to T cells.

In addition to DCs contributing to onset of RA, there is also evidence that DCs contribute to the maintenance of RA pathology once it has been triggered. For example, DCs are found in much higher numbers in RA SF compared to synovium derived from patients with osteoarthritis (OA) (Harding and Knight, 1986; Zvaifler et al., 1985). Furthermore, while DCs have been described to be absent from healthy synovial tissue (ST), both cDC2s and pDCs occur in high numbers in RA ST, where they were shown to express cytokine profiles concurrent with maintaining RA pathology (Lebre et al., 2008). For example, cDC2 DCs were shown to produce large amounts of IL-12, which enhances Th1 differentiation and effector functions via increasing IFN-γ production by CD4 T cells and NK cells, and IL-23, a major driver of the Th17 response. pDCs produced high levels of both IL-18, which induces IFN-γ thereby also driving the Th1 phenotype, and IFNα/β, which similarly contribute to Th1

and autoreactive T cell responses, although IFNβ has been recently implied to also have some immunoregulatory roles in RA (van Holten et al., 2002). Lastly Lebre and colleagues reported that, cDC2 and pDCs from RA ST were shown to produce IL-15, which drives both sustained T cell and B cell responses. Therefore, it was suggested that DCs are an important contributor to on-going RA pathology. An on-going role of DCs is also likely due to the clinical success of the previously mentioned fusion protein of CTLA-4 and an IgG, abatacept, which competitively binds to CD80/CD86 on DC surfaces and thereby reduces their ability to stimulate potent T cell activation via CD28. In clinical trials, treating MTX-resistant RA patients with high disease activity with abatacept caused increased rates of remission and significantly lower disease activity scores (Kremer et al., 2005), strongly suggesting that on-going activation of auto-reactive T cells was partially responsible for RA disease severity.

To conclude, evidence suggests that DCs play not only a role in initiating an autoimmune response but also in maintaining active disease. DCs are therefore considered a valuable target for therapeutic intervention, which I will expand on in the next section.

1.3.3 Dendritic cell therapy for Rheumatoid Arthritis

Given the vast evidence indicating a dominant role for DCs in the initiation and progression of RA, DCs might hold the key to re-establish tolerance to self to achieve full remission. Tolerogenic Mo-DCs are promising cell therapeutics to re-educate the immune system by promoting T cell tolerance instead of restricting T cell activation using DMARDs or biologics. In this section I will introduce tolerogenic cell therapy approaches already underway in other autoimmune conditions, before discussing the properties of therapeutic tolerogenic Mo-DCs and exploring their potential significance in the treatment of RA.

As mentioned previously, DCs play a wide range of tolerogenic roles both in the thymus, where they delete self-reactive T cells, and in the periphery, where T cells that have escaped thymic selection are rendered unresponsive (Brocker et al., 1997; Proietto et al., 2008; Shahinian et al., 1993). These functions can be exploited for therapeutic causes, either by targeting endogenous DCs *in vivo* or by extracting monocytes, which are the precursors of Mo-DCs, culturing them to become tolerogenic Mo-DCs and reintroducing them into the patient. There is the potential to introduce an RA-relevant antigen to the tolerogenic Mo-DCs to make them disease-

specific and thereby more effective, which is however hindered by the absence of a singular antigen causing RA. A ‘disease-relevant’ antigen would therefore have to be highly specific to the patient or patient subset, in which pathology is caused by autoimmune reaction to this antigen, or a variety of RA-associated antigens, such as citrullinated peptides and collagen, would have to be used. The question of an RA relevant antigen thereby has implications for both availability of therapy to a wide range of patients, as therapeutic intervention might only be helpful if specific for the antigen causing disease in a patient, and cost of therapy, as the more personalised the tolerogenic Mo-DCs are, the more expensive the treatment would be. A further potential challenge in the use of tolerogenic Mo-DCs is the correct timing of cell therapy administration. Especially as breach of tolerance has been implied to occur years before disease onset, it is possible that only patients with very early disease would benefit from the treatment. However, when considering evidence of therapeutic use of tolerogenic Mo-DCs in other autoimmune diseases, a positive role for tolerogenic Mo-DCs even in established disease is likely.

Tolerogenic cell therapy has been successfully explored in a range of different autoimmune conditions. In Crohn’s disease, an autoimmune disorder targeting the intestine, intraperitoneal injection of tolerogenic Mo-DCs in Phase I clinical safety trial into patients refractory to treatment showed that tolerogenic Mo-DCs were safe with clinical responses in 2 of 9 study participants, with one further participant reaching full remission (Jauregui-Amezaga et al., 2015). A similar phase I clinical safety trial assessing intradermal injection of autologous tolerogenic Mo-DCs for Type 1 Diabetes showed no safety concerns either (Giannoukakis et al., 2011). Interestingly, injection with Mo-DCs caused increased presence of a $B220^{+}CD11c^{neg}$ B cell population, which was hypothesised to be regulatory in nature and might therefore positively contribute to disease outcomes. While tolerogenic cell therapy is still in its infancy, clinical safety trials in the last ten years show promise that this approach if feasible, safe and potentially beneficial to patients even at low doses. Although targeting DCs *in vivo* has shown promising results in other diseases, and is potentially more cost-effective because of the high cost of personalised cell therapy, the failure to identify a singular specific antigen in RA pointed to tolerogenic Mo-DCs as the more viable option in RA.

Tolerogenic Mo-DCs can be generated in culture in a variety of ways, but commonly require the presence of IL-4 and GM-CSF to initiate a DC-like phenotype in

monocytes (Sallusto and Lanzavecchia, 1994). To achieve a tolerogenic phenotype, a stimulating signal, such as LPS (Anderson et al., 2009), which would usually induce maturation of Mo-DCs, is combined with immunosuppressive drugs, such as blocking NF-κB , Dexamethasone and/or Vitamin D3 (Piemonti et al., 2000; Xia et al., 2005). The resulting tolerogenic Mo-DCs are described to exhibit a semi-mature state, marked by reduced expression of costimulatory molecules, decreased production of pro-inflammatory cytokines, such as IL-12, IL-6, IL-23 and TNF-α, and increased production of anti-inflammatory cytokines, such as IL-10, compared to mature DCs (Anderson et al., 2009; Harry et al., 2010). Therefore, tolerogenic Mo-DCs show reduced ability to stimulate T cells. Specifically, they have been described to cause skewing of naïve CD4 T cells towards a immunoregulatory cytokine profile low in IFN-γ and high in IL-10, while memory T cells were found to be rendered hyporesponsive to reactivation when co-cultured with tolerogenic DCs (Anderson et al., 2008). If injected, tolerogenic Mo-DCs could therefore render autoreactive T cells unresponsive either in the periphery or in the nearest LN, thereby returning the body to a state of self-tolerance. More specifically, Mo-DCs tolerised with Dexamethasone as well as Vitamin D3 are thought to induce their tolerogenic effects by their complete lack of IL-12 production, their high expression of LAP/TGF-β and lastly their ability to induce Type I Tregs, thereby indirectly promoting tolerance (Anderson et al., 2017, 2008; Spiering et al., 2019).

As immature DCs share many tolerising features of tolerogenic DCs, but can easily undergo maturation and become immunogenic in the presence of pro-inflammatory cytokines, it was important to ensure stability of tolerogenic DCs in the presence of inflammation to avoid further exacerbating disease when considering this as a cell therapy. However, re-stimulation of tolerogenic Mo-DCs with various pro-inflammatory mediators such as cytokines (IL-1β, IL-6, TNFα and IFNγ), LPS or peptidoglycan (PGN) did not alter their cytokine expression or semi-mature phenotype, strongly suggesting that tolerogenic Mo-DCs were stable and therefore unlikely to become reactivated in the presence of inflammation (Harry et al., 2010).

Another challenge in optimising tolerogenic Mo-DC therapy is considering their migratory ability and localisation *in vivo*, to ensure that tolerogenic cell therapy would not only suppress self-reactive T cells once they arrive in the periphery but also migrate to the nearest LN and present self-antigen in the absence of activating signals to induce tolerance. This would be more effective as it would potentially

provide lasting protection from autoreactive T cells that is not dependent on continuous presence of large numbers of therapeutic tolerogenic Mo-DCs. Indeed, tolerogenic Mo-DCs were found to migrate less effectively in a transwell system towards CCL19, which was proposed to be due to their reduced expression of CCR7 (Anderson et al., 2009). However, tolerogenic Mo-DCs maintained CCR7-mediated migration towards CCL19, albeit at lower levels, suggesting that tolerogenic Mo-DCs have the ability to migrate towards the nearest LN. Lastly, it is important to consider the antigenic stimulation that will be provided to tolerogenic Mo-DCs. The specific antigen eliciting RA is unknown and hypothesised to vary between patients, therefore making it difficult to achieve antigen-specific protection using tolerogenic Mo-DCs.

To date, the use of tolerogenic Mo-DC therapy for RA has been successfully tested in a range of animal models. For example, in mice, Mo-DCs tolerised using NF-κB inhibition and pulsed with collagen, were able to reduce the antigen-specific T cell response to collagen in the CIA model, thereby improving clinical scores when given 12 days after first injection with collagen but before onset of disease at 28 days (Popov et al., 2006). A different group found that intravenous injection of tolerogenic BMDCs pulsed with collagen but not without were able to migrate to draining LNs, decrease Th17 T cell responses and simultaneously increase the level of circulating IL-10 producing CD4 T cells (Stoop et al., 2010). A more recent study showed that murine tolerogenic BMDCs induced using vasoactive intestinal peptide could similarly improve clinical outcome in the CIA model when injected after onset of disease at day 40, suggesting that tolerogenic DC therapy could not only work preventatively but also in established disease (Wu et al., 2019).

In recent years, a range of early clinical trials has explored the viability of tolerogenic DC therapy in humans. One strategy involved tolerogenic Mo-DCs established through blocking NF-κB and exposed to four different citrullinated peptides, which were injected intradermally into patients positive for anti-CCP and carriers of the HLA-DRB1 shared epitope (Benham et al., 2015). Benham and colleagues showed a reduction in peripheral effector T cells with a concurrent increase in regulatory T cell numbers one month after injection. In addition, serum levels of pro-inflammatory cytokines and chemokines such as IL-15, IL-29 and CX3CL1 were reduced compared to untreated controls. These mediators have all be associated with worsened outcomes in RA, with IL-15 being described to sensitise TCRs to RA autoantigens (Deshpande et al., 2013), IL-29 being associated with increased

expression of TLR4 in both ST and PB (Xu et al., 2015) and expression of CX3CL1 being reduced inversely to clinical improvements in patients treated with infliximab (Odai et al., 2009). Furthermore, a phase I clinical trial was undertaken in 2016 to test the safety of injecting tolerogenic Mo-DCs directly into an inflamed joint (Bell et al., 2016). In the absence of a known common RA antigen, tolerogenic Mo-DCs were exposed to autologous SF, thereby providing a range of antigens that were likely to be relevant for the patient in question. Bell and colleagues established that treatment with tolerogenic Mo-DCs was feasible and safe, with minor symptom improvement observed in the small group of patients receiving the highest dose of tolerogenic Mo-DCs. While this paves the way for future clinical trials involving tolerogenic Mo-DCs to treat RA, determining a suitable antigen to benefit the largest amount of RA patients and finding the most suitable route of administration remain challenging. However, work on solving these problems is currently underway, with a Phase II clinical trial of tolerogenic Mo-DCs for RA currently in preparation.

To conclude, tolerogenic Mo-DCs represent a viable therapeutic opportunity for RA. Instead of current treatment strategies that aim to dampen unwanted immune activation, tolerogenic DC-based cell therapy aims to exploit the immune system's own mechanism to maintain tolerance to re-establish tolerance to self. However, while animal studies using tolerogenic Mo-DCs have been promising and clinical safety trials have been successful, certain challenges remain to be solved before this therapy can become available to patients. This includes the correct time and location to administer cell therapy as well as defining viable read-outs for therapeutic success (Hilkens and Isaacs, 2013).

1.4 β_2 integrins: immune regulation versus activation

As discussed in the previous section, APCs can both initiate and modulate T cell responses by direct contact with T cells. This can occur either at the site of inflammation or in the LN. One way that contact between DCs and T cells is established and maintained is by adhesion receptors, one group of which are integrins. β_2 integrins in particular, well known for their pro-inflammatory roles in lymphocytes, have recently been suggested to have immunoregulatory roles in DCs. In this section, I will give a brief outline of the family of integrin receptors before focusing on the roles of β_2 integrins, specifically in DCs. Lastly, I will explore the feasibility of targeting β_2 integrins therapeutically to influence the balance between immune activation and tolerance.

1.4.1 Integrins

Integrins are heterodimeric transmembrane receptors, which consist of two non-covalently associated proteins, more specifically one α- and one β-subunit (Hynes, 1987). To date, 18 α- and 8 β-subunits have been identified, which occur in various combinations yielding the 24 currently known members of the integrin family (Figure 1.2). While their large N-terminal extracellular domain can bind and interact with a number of different ligands, their short C-terminal cytoplasmic domain interacts directly and specifically with cytoskeletal proteins such as talin and filamin and induces downstream signalling pathways (Pfaff et al., 1998). This means that information between extracellular and intracellular space can be relayed and vice versa (Hynes, 1987). These properties make integrins important for a wide variety of biological functions including adhesion, cell migration and extravasation into tissues and cell communication.

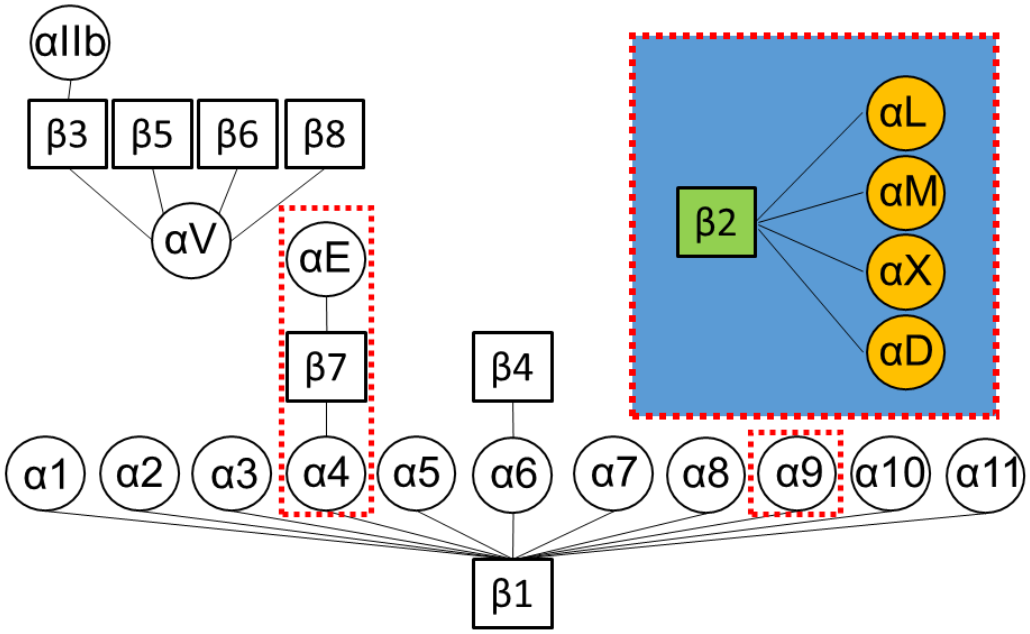

Figure 1.2 Integrin α and β subunit pairings

Adapted from Hynes & Naba, 2012. Integrin receptors consist of an α subunit and a β subunit. 18 α subunits (circles) and 8 β integrin subunits (squares) can pair to form a total of 24 recognised integrin receptors. Integrin receptors circled in red are expressed on leukocytes, but this thesis focuses on leukocyte-specific β_2 integrins and their role for the immune system specifically (blue background).

Integrin subunits consist of highly conserved domains, a visual representation of which can be seen in Figure 1.3 (Hynes and Naba, 2012). Briefly, integrin subunits consist of a head region, which forms part the ligand binding site, and a leg region,

important in positioning the head regions of each integrin subunit. An integrin α-subunit consists of one 7-bladed β propeller domain, followed by three β sandwich domains termed thigh and two calf domains (calf1, calf 2), forming the leg region of the integrin. Furthermore, 9 of the 18 α-subunits also have an I domain, which is located over the β propeller domain and thereby makes it an important factor in the integrin binding site. Interestingly, all four α-subunits (CD11a, CD11b, CD11c, CD11d) pairing with the β_2 integrin subunit (CD18), which is the focus of this thesis, have an I-domain as well as the β propeller and the thigh domain in their head region. The β_2 subunit CD18 on the other hand is made up of an I-like domain and a β sandwich hybrid domain forming the head of the integrin protein. The leg part of the protein consists of a cysteine rich plexine-semaphorin-integrin (PSI) domain, four EGF-like repeats and lastly a β tail domain (βTD) (R. Evans et al., 2009), which is located immediately above the transmembrane domains traversing the membrane and the short cytoplasmic tail.

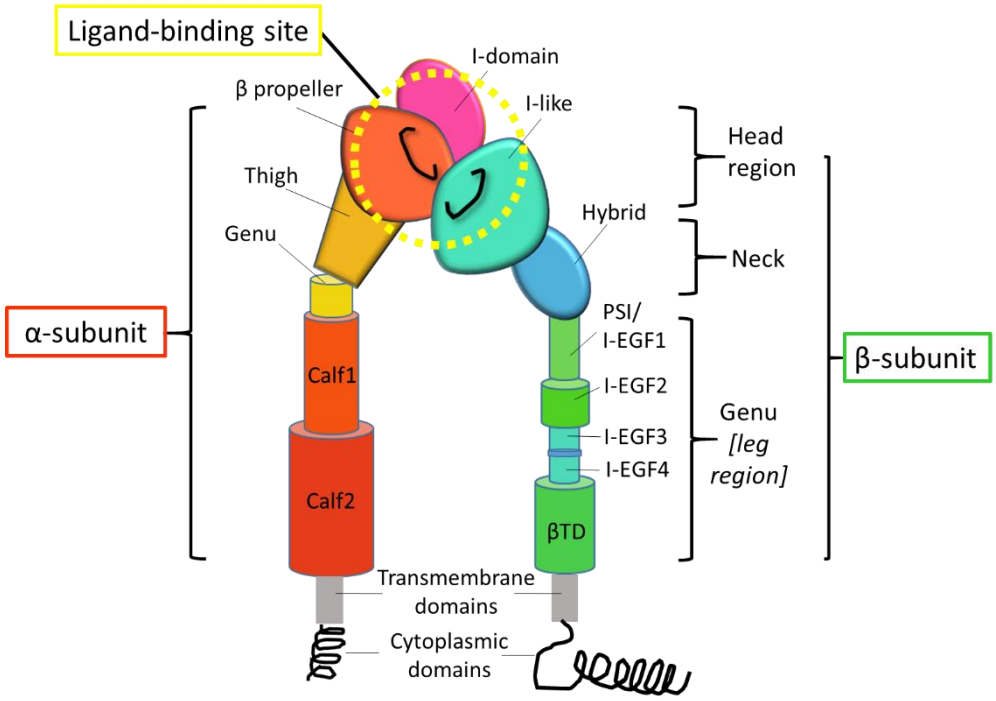

Figure 1.3 Protein domains of integrins

Adapted from Byron et al., 2009 and Evans et al., 2009. Both α and β subunits consist of distinct domains making up a leg region anchored in the cell membrane, a neck region and a head region, where the ligand binding site is located (circled in yellow).

Due to the wide variety of roles integrins can play on cell surfaces, adhesion to ligands has to be tightly controlled. Integrins have therefore been described to exist in three distinct of conformational states, which can limit or expand the access of

ligand binding sites and thereby control the level of binding and signalling through the transmembrane receptor. The three integrin conformational states are: inactive, intermediate and active. Movement from an inactive over an intermediate to an active integrin conformational state occurs via the so-called switchblade model (Jin et al., 2004). In an inactive state, the head regions of both integrin subunits are bent down towards the cell surface and the leg regions are clustering closely together, yielding the ligand binding site closed and the integrin receptor with low affinity for ligand (Figure 1.4) (Byron et al., 2009; R. Evans et al., 2009). Through chemokine or cytokine receptor activation, the leg regions move further apart from each other and extend upwards from their bent position, thereby forming an intermediate state. Even though the leg regions are extended, the head regions forming the ligand binding sites are still closed in this state, and were presumed to show intermediate affinity for ligand. To achieve full activation, the head regions have to open, exposing the ligand-binding sites to the extracellular environment. Due to the free availability of the binding sites, the active conformation of an integrin has the highest affinity for ligands, with up to 4,000 times higher affinity compared to the bent-closed inactive conformation (Li et al., 2017). A visual overview of this process can be seen in Figure 1.4.

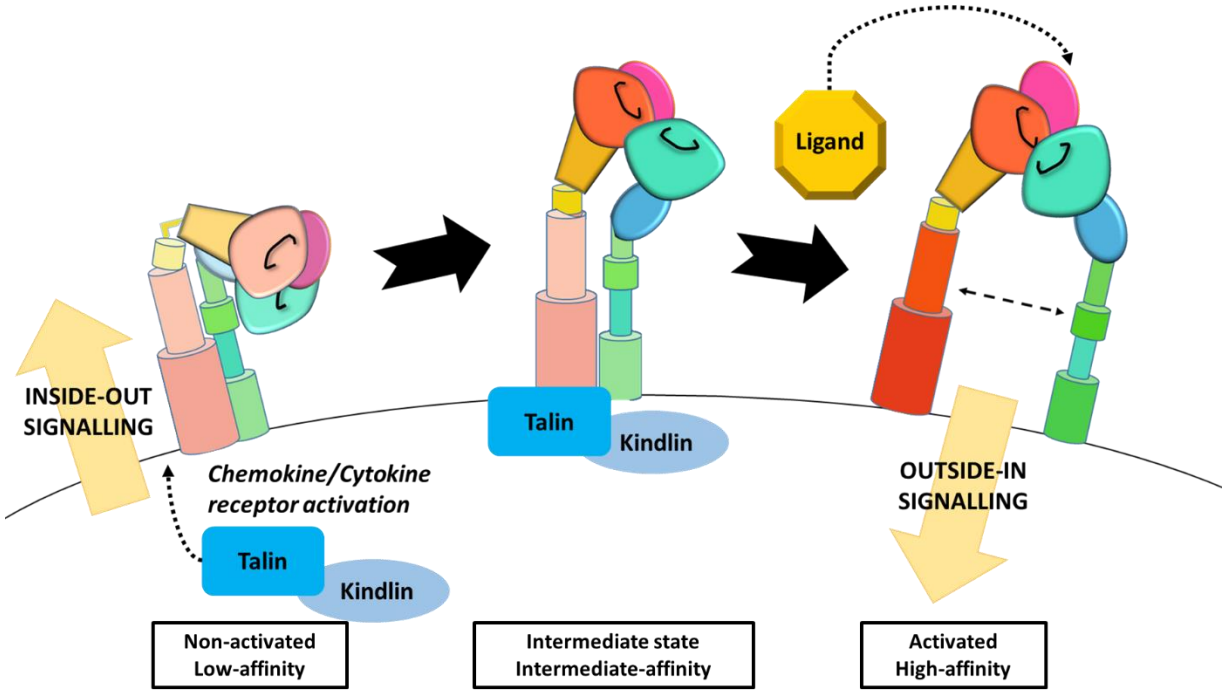

Figure 1.4 Integrin signalling and conformational states

Adapted from Evans et al., 2009. Integrins exist in three distinct conformational states: inactive with low affinity for ligand, intermediate with intermediate affinity for ligand and active with high affinity for ligand. Transition between inactive and active conformation is regulated by inside-out signalling, by which chemokine or cytokine receptor activation initiates the recruitment of talin and kindlin to the intracellular portion of the integrin subunits. This causes extension of neck regions and leg regions move apart from each other, thereby exposing the active ligand binding site to the extracellular space. When ligand binds to the ligand binding site, downstream signalling cascades transmit extracellular information towards the intracellular space via outside-in signalling.

Due to the need for tight regulation of integrin signalling, integrins have been described to be largely inactive on cell surfaces (Li and Springer, 2017), with 99.75% of $\alpha_5\beta_1$ integrins being in the bent-closed formation on a human leukaemia cell line. This poses the question how integrins can assume the extended-open (active) conformation efficiently and rapidly to mediate adhesion. The answer lies in integrin signalling, which consists of both inside-out and outside-in signalling (Figure 1.4). Fundamentally, inside-out signalling translates cell-internal signal into receptor affinity and clustering, thereby altering how the cell interacts with its immediate environment. Inside-out signalling is triggered by cell stimulation via chemokine or cytokine receptors (Shamri et al., 2005). This causes an increased formation of diaglycerol (DAG) and increase Ca^{2+} levels, which either activates protein kinase C (PKC) or a Rap-guanine nucleotide exchanger (Rap-GEF). Both of these signalling

proteins function in activating Rap1 by exchanging GDP for GTP (Han et al., 2006). This in turn causes relocation of Rap1-GTP to the cell membrane, where it was shown to interact with Rap1-GTP interacting adaptor molecule (RIAM) (Lee et al., 2009), which in turn causes the recruitment of talin and kindlin to the NPxY motifs of the integrin β subunit (Calderwood et al., 2002; Moser et al., 2008). By connecting the short cytoplasmic tail to the cytoskeleton, talin and kindlin function to separate the closely associated leg regions of the integrin alpha and beta subunits and stabilise them in the extended high-affinity conformation. Outside-in signalling then occurs once a ligand has bound to the integrin receptor. This is characterised by a downstream signalling cascade altering cell function in response to its environment (Shen et al., 2012), for example by activation and interaction with the g-protein coupled receptor $G\alpha_{13}$ (Gong et al., 2010). Outside-in signalling can mediate a range of possible outcomes for cells, including migration and motility, survival, cytoskeletal rearrangement and differentiation.

For the work done in this thesis, I focused specifically on β_2 integrins, due to their reported importance in immune function. In the following section, I will describe specific properties of β_2 integrins, particularly in our cell types of interest, namely monocytes and DCs.

1.4.2 A special role in the immune system: β_2 integrins

As mentioned previously, the β_2 integrin subunit (CD18) can pair with one of four α subunits (α_L-CD11a, α_M-CD11b, α_X-CD11c, α_D-CD11d) and are exclusively expressed by leukocytes. Due to their importance in immune function including adhesion, cell migration and cell-cell communication, they are known under a variety of names including lymphocyte function associated antigen I (LFA-1) (CD11a/CD18), macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3) (CD11b/CD18), p150/95 or complement receptor 4 (CR4) (CD11c/CD18) and CD11d/CD18. For clarity, I have used CD nomenclature for all β_2 integrins throughout this thesis.

The β_2 integrin receptor family can bind a variety of ligands, detailed in Figure 1.5. Briefly, CD11a/CD18 binds intracellular adhesion molecule-1 to 5 (ICAM-1 to ICAM-5) preferentially (Arnaout, 2016), as well as to junction adhesion molecule 1 (JAM-1) (Ostermann et al., 2002). CD11b/CD18 is the most promiscuous of all β_2 integrins with over 40 reported ligands (Podolnikova et al., 2015). CD11b binds a variety of complement proteins, including iC3b and C4b (Graham et al., 1989), as well as the adhesion molecules ICAM-1-4 and JAM-3, and ECM proteins fibrinogen and fibronectin. CD11c is highly homologous to CD11b, which makes it unsurprising that it shares many of its ligands, including iC3b, ICAM-1 as well as ICAM-4. Additionally, CD11c binds vascular cell adhesion protein 1 (VCAM-1). Lastly, CD11d was reported to bind specifically ICAM-3 and VCAM-1, as well as having many overlapping ECM-derived ligands with CD11b, such as fibronectin and fibrinogen as well as vitronectin (Van der Vieren et al., 1995; Yakubenko et al., 2006), which has also been described to be bound by CD11b (Kanse et al., 2004). Expression of CD11a, CD11b, CD11c and CD11d on human and mouse DCs and monocytes can found in Table 1.1. A more thorough review of the expression of beta2 integrin subunits in different DC and monocyte subsets can be found in my published review (Schittenhelm et al., 2017).

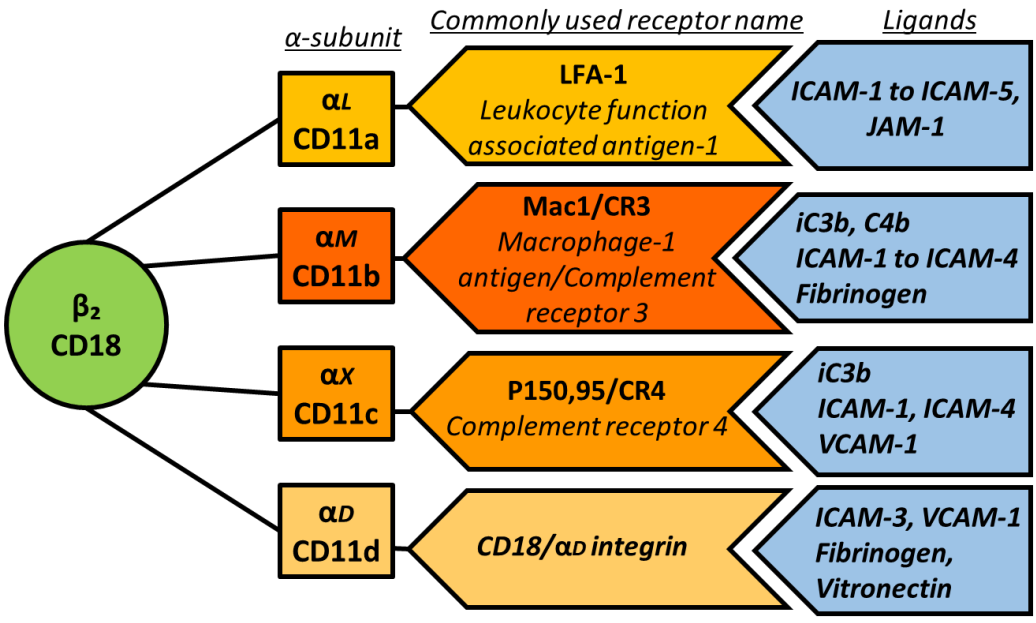

Figure 1.5 β_2 integrins and their ligands

Adapted from Schittenhelm et al., 2017. Shown are the four members of the β_2 integrin receptor subgroup with alternatively used names and respective most commonly described ligands.

Cell Type		Dendritic cells	Monocytes
CD11a (αL, *ITGAL*)	***Human***	**High on Mo-DCs, pDCs, reduced upon activation** (Ammon et al., 2000; de la Rosa et al., 2003; Freudenthal and Steinman, 1990; Geijtenbeek et al., 2000; Rieckmann et al., 2017; Sallusto and Lanzavecchia, 1994)	**Circulating monocytes** (Ammon et al., 2000; Bohuslav et al., 1995; Prieto et al., 1994)
	Mouse	**cDCs (especially CD8$^+$), pDCs** (Segura et al., 2007)	**Circulating monocytes** (Auffray et al., 2007)
CD11b (αM, *ITGAM*)	***Human***	**Mo-DCs, cDCs, but not on pDCs, reduced upon activation** (Freudenthal and Steinman, 1990; Geijtenbeek et al., 2000; Haniffa et al., 2012; Rieckmann et al., 2017; Robbins et al., 2008; Sallusto and Lanzavecchia, 1994)	**Circulating monocytes, differentially expressed on osteoclast precursors** (Bohuslav et al., 1995; Prieto et al., 1994; Robbins et al., 2008; Sprangers et al., 2017)
	Mouse	**BMDCs, CD8- DCs > CD8$^+$ splenic DCs, subpopulations of gut DCs, not on pDCs** (Cerovic et al., 2014; Nakano et al., 2001; Segura et al., 2007; Shortman and Liu, 2002)	**High on monocytes** (Sunderkötter et al., 2004)
CD11c (αX, *ITGAX*)	***Human***	**Mo-DCs, higher on cDC2 than cDC1 DCs, absent from pDCs, reduced levels upon activation** (Haniffa et al., 2012; Li et al., 2011; Rieckmann et al., 2017; Robbins et al., 2008)	**Circulating monocytes, classical, non-classical and intermediate monocytes** (Prieto et al., 1994; Rieckmann et al., 2017; Robbins et al., 2008)
	Mouse	**BMDCs, cDCs (used as marker, pDCs** (Helft et al., 2015; Miller et al., 2012; Segura et al., 2007)	**Thought to be absent from most monocytes, upregulated upon activation** (Geissmann et al., 2003a; Sunderkötter et al., 2004)
CD11d (αD, *ITGAD*)	***Human***	**Mo-DCs, single cell mRNA suggests low expression on DCs** (EMBL-EBI expression atlas, 2017; Miyazaki et al., 2014)	**Circulating monocytes, CD16$^+$ < CD16- monocytes** (Miyazaki et al., 2014)
	Mouse	**RNAseq data suggests medium *ITGAD* expression in murine DCs, CD8$^+$ > CD8^{neg} DCs** (EMBL-EBI expression atlas, 2017; Segura et al., 2007)	**Low *ITGAD* mRNA expression, low on circulating monocytes** (EMBL-EBI expression atlas, 2017; Noti, 2002)

Table 1.1 Expression of β_2 integrins on human and murine DCs and monocytes

The importance of β_2 integrin function for the immune system is highlighted by a group of congenital autosomal-recessive disorders termed leukocyte adhesion deficiencies (LAD), where a defective immune system arises from deficiencies in immune cell adhesion and migration. There are three different types of LAD, two of which show loss of expression or functionality of β_2 integrins. This is the case for LAD-I, where *ITGB2*, the gene coding for CD18 is mutated (Fischer et al., 1988), or the more recently identified LAD-III, where a mutation in kindlin-3 (*FERMT3*), a major cytoplasmic regulator of β_2 integrin activation, causes a failure of kindlin-3 binding to the β_2 integrin subunit, which directly results in the integrins inability to assume it active conformation and bind ligand (Jurk et al., 2010). Symptoms of LAD are recurrent bacterial and fungal infection and leukocytosis due to the inability of immune cells, especially neutrophils, to extravasate into tissue sites of infection in absence of β_2 integrins. Extravasation and homing into tissues is a major function of β_2 integrins (Grabbe et al., 2002), which will be discussed in more detail in the next section on immunoregulatory and pro-inflammatory roles of β_2 integrins. Many different mutations have been described to cause LAD of varying severity due to either a reduction of CD18 function or its complete absence. Furthermore, it has been shown that disease severity of LAD-I is directly correlated with the level of CD18 function preserved (Novoa et al., 2018). Together with the knowledge that effective β_2 integrin signalling is diminished in LAD-III, this strongly suggests the immense importance of β_2 integrins for raising effective immune responses. Interestingly, it was also shown that some symptoms of LAD are not due to the failure of raising an immune response, but rather due to an unregulated increase in inflammation. For example, bone destruction in periodontitis, another condition associated with LAD, was found to be driven by an increase in IL-17 production (Moutsopoulos et al., 2014), and was found to be mitigated by blocking IL-12 and IL-23, thereby reducing IL-17 signalling (Moutsopoulos et al., 2017). Furthermore, some patients with LAD have been described to suffer from ulcerative colitis (D'Agata et al., 1996; Uzel et al., 2001). While the exact causes for emergence of this autoimmune condition in LAD are not clear, it again suggests that impairment or complete absence of β_2 integrins might not only contribute to the failure to raise an appropriate immune response, but also the ability to regulate and dampen inappropriate immune activation.

To conclude, β_2 integrins are leukocyte-specific adhesion molecules with a wide range of ligands which are highly important for an effective response. In the following section, I will explore their roles in immune activation as well as more recent discoveries that suggest they play a distinct immunoregulatory role especially in DCs and other APCs.

1.4.3 Roles of β_2 integrins in immune activation and regulation

For a long time, β_2 integrins were primarily known for their various roles in initiating and maintaining immune responses. However, more recently, β_2 integrins have also been reported to be involved in a wide variety of functions involving immune regulation. In this section I will briefly summarise the roles of β_2 integrins in immune activation before exploring their important roles in immune regulation that form the basis of my thesis.

β_2 integrins in immune activation

β_2 integrins are involved in many stages of an effective immune response. I will highlight their immunostimulatory roles in the areas of antigen presentation/APC-T cell contact, cell migration and cell signalling.

Firstly, especially CD11b and CD11c are important mediators of phagocytosis by recognising iC3b- or C3b-opsonised particles or bacteria. The importance of these integrins for phagocytic activity can be supported by their high expression in immature DCs, which becomes downregulated upon DC activation, when phagocytosis is similarly downregulated in mature DCs (Rieckmann et al., 2017). Furthermore, macrophages, known primarily for their ability to phagocytose opsonised compounds, express both CD11b and CD11c at high levels on their surface (Beller et al., 1982; Drevets et al., 1993; Gautier et al., 2012). Phagocytosis is an important factor for effective downstream processing of antigen and final presentation on the cell surface, thereby contributing to immune activation. However, an allele of the gene coding for CD11b and being marked by reduced phagocytic ability is one the most common risk factors for SLE, suggesting that CD11b-mediated uptake of apoptotic cells and cellular debris also has important roles in maintaining immune tolerance (Fossati-Jimack et al., 2013; Rhodes et al., 2012). Furthermore, β_2 integrins play an important role in immunological synapse, where CD11a on T cells binding to ICAM-1 on APCs was found to cluster in the peripheral supramolecular activation cluster (P-SMAC), thereby stabilising the central SMAC and with it the

connection between MHC:peptide and TCR (Grakoui et al., 1999; Monks et al., 1998). By this process, CD11a was found to be an effective costimulatory signal, enhancing TCR signal transduction (Seventer et al., 1990). Furthermore, blocking either CD11a, CD18 or the integrin ligand ICAM-1 on human Langerhans cells from the skin significantly blocked their ability to stimulate T cells *in vitro* by over 70% (Simon et al., 1991), suggesting an important role of β_2 integrins on these DC-like cells in immune activation. However, examples of activating roles of β_2 integrins in 'true' DCs are difficult to find, as current research primarily focuses on the role of β_2 integrins on the T cell side.

In addition, β_2 integrins are important for cell adhesion and migration. While migration of leukocytes within tissues was found to occur independently of β_2 integrins (Lämmermann et al., 2008), the adhesion receptors are absolutely required for migration of leukocytes to inflamed tissues, as this requires adhesion to ICAM-1 on endothelial cells under shear-flow conditions (Alon and Dustin, 2007; Lämmermann et al., 2008). However, while blocking either CD11a specifically or all β_2 integrins by blocking CD18 caused a reduction in adherence of monocytes to vessel walls and migration (Chuluyan and Issekutz, 1993; Geissmann et al., 2003b), migration of monocytes to an inflammatory site was found to occur independently of either CD11a or CD11b (Henderson et al., 2003). Similarly, DCs deficient for all integrins migrated to the LNs to the same degree as WT DCs when injected into the footpad of a mouse, suggesting that DC migration towards the LN also occurred independently of integrins (Lämmermann et al., 2008). This suggests that in monocytes and DCs, β_2 integrin-mediated migration does not majorly contribute to inflammation, although this is not the case for all cell types. For example, increased expression of CD11d on macrophages was found to retain macrophages at the site of inflammation, while monocytes expressing lower levels could still migrate (Yakubenko et al., 2008).

Lastly, β_2 integrins play a pro-inflammatory role in signalling. As mentioned above, CD11a signalling in T cells acts a co-stimulatory signal, directly contributing to T cell activation. Additionally, ligation of both CD11b and CD11c on human monocytes elicited production of pro-inflammatory mediators such as macrophage-inflammatory protein 1-α (MIP1α), MIP1β and IL-8 (Rezzonico et al., 2001). Further supporting this, both CD11b in macrophages and CD11c in monocytes were shown to mediate activation in the presence of LPS (Fan and Edgington, 1991; Ingalls and Golenbock, 1995). Several studies also suggest that β_2 integrins positively regulate or mediate

TLR4 signalling. For example, CD14, CD11b and TLR4 on murine macrophages were reported to react in synergy to LPS stimulation (Perera et al., 2001). In DCs, CD11b was found to positively regulate TLR4, with loss of CD11b-mediated TLR4 signalling resulting in reduced ability to stimulate T cells (Ling et al., 2014).

β_2 integrins in immune regulation

While β_2 integrins, especially CD11a, were described to have pro-inflammatory functions in T cells, there is evidence suggesting that the opposite might be true in APCs. In murine models, increasing expression of both active CD11a (Balkow et al., 2010) and active CD11b (Varga et al., 2007) in DCs was found to decrease T cell activation, suggesting that β_2 integrin activation status on DCs is immunoregulatory. In humans, a similar mechanism has been described, whereby CD11b on APCs binding to ICAM-1 on inflamed lymphatic endothelial cells in the absence of pathogen-associated danger signals reduced expression of costimulatory molecule CD86 on APCs and suppressed their ability to stimulate T cells (Podgrabinska et al., 2009). Furthermore, expression of CD11b has been described to play an active role in Th17 T cell suppression, potentially by limiting IL-6 production by DCs (Ehirchiou et al., 2007), which was further supported by the finding that ligating CD11b on Mo-DCs resulted in a reduced ability to induce Th17 T cells, thereby conferring tolerogenic qualities on the Mo-DCs (Nowatzky et al., 2018a). Ligation of CD11b on murine bone marrow-derived DCs (BMDCs) was further shown to increase expression of MHC and costimulatory molecules, while production of inflammatory cytokine was blocked (Behrens et al., 2007), thereby suppressing T cell stimulatory capability.

While β_2 integrins on DCs were not found to be an important factor in mediating migration to the LN, they were reported to be indispensable for DCs strongly adhering to endothelium and extravasating into tissues (Alon and Dustin, 2007; Lämmermann et al., 2008). Interestingly, injecting murine BMDCs deficient in integrin signalling into the footpad of a mouse without LPS stimulation, BMDCs migrated to the LN in higher numbers, suggesting that β_2 integrins actually retain DCs in the periphery in the steady state. Furthermore, the study showed that BMDCs with deficient β_2 integrin function stimulated more IFN-γ production by T cells, indicative of driving a Th1 phenotye, thereby suggesting that β_2 integrins function in keeping DCs both non-migratory and non-stimulatory in the steady state (Morrison et al., 2014).

This strongly suggests an active role for β_2 integrins on DCs in immune regulation. In a murine model of skin inflammation, a larger number of signalling-deficient DCs were found both in the LN and at the site of inflammation, potentially suggesting that failure of β_2 integrin signalling further enhanced disease severity (Savinko et al., 2015). As mentioned above, some studies report that β_2 integrins have pro-inflammatory roles in immune cell signalling, especially in the context of TLR4. However, in the absence of β_2 integrins (CD18 KO mice), TLR signalling is increased, suggesting a regulatory role of β_2 integrins on TLR signalling (Yee and Hamerman, 2013). This however contradicts another study mentioned above, that specifically CD11b actually positively mediates TLR4 signalling (Ling et al., 2014). The discrepancy in these findings can potentially be explained by Yee and Hamerman utilising a complete CD18 knockout (KO), thereby not distinguishing between the potentially different roles of β_2 integrin α subunits, while Ling and colleagues focused specifically on the effects of CD11b deficiency. While this provides some insight into the distinct β_2 integrin subunit functions, other studies support the finding that β_2 integrins restrict TLR signalling. For example, TLR signalling was found to be restricted by β_2 integrins on both macrophages and DCs (Savinko et al., 2015). However, the role of β_2 integrins in immune cell signalling is complex. For example, a lack of CD11b in murine BMDCs was found to decrease signalling via TLR4, but simultaneously upregulated microRNA-146a, which functioned to increase DC-mediated cross-priming of cytotoxic T cells (Bai et al., 2012).Considering this evidence it becomes apparent that the literature exploring the role of β_2 integrins in immune cell signalling is complex, which makes it likely that the effect of β_2 integrins on TLR signalling might be dependent on cell type and environment.

To conclude, β_2 integrins play a wide variety of pro-inflammatory roles in the immune system. However, evidence specifically in DCs and monocytes suggests that they also play a potent role in regulation of immune responses (Figure 1.6).

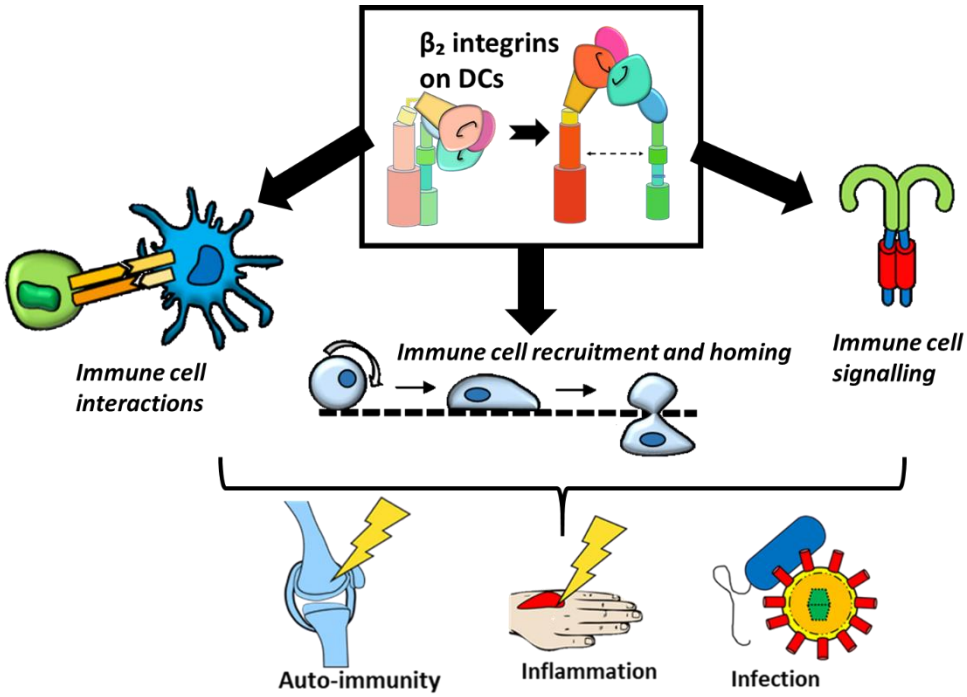

Figure 1.6 Roles of β_2 integrins in DCs

Adapted from Schittenhelm et al., 2017. Involvement of β_2 integrins in the immune system can be broadly categorised into interactions with immune cells, homing to LNs and recruitment to tissues as well as immune cell signalling. Evidence is accumulating that β_2 integrins have both pro-inflammatory and immunoregulatory roles in these areas. Dysregulation of β_2 integrins could therefore contribute to the aberrant immune response in autoimmune disease.

1.4.4 Evidence suggesting a role of β_2 integrins in RA

While β_2 integrins have not been identified as genetic risk factors for RA in gene wide association (GWAS) studies to my knowledge, there is still a wide range of evidence that suggests β_2 integrins might play a role in RA pathogenesis or progression. However, having explored the opposing functions of β_2 integrins, it is also possible that β_2 integrins might regulate or dampen aberrant immune processes. In the following section, I will give an overview of the published literature in this field and highlight where the limitations of current knowledge lie.

A range of autoimmune diseases and inflammatory disorders share β_2 integrins as genetic risk factors, which seem to be β_2 integrin α-subunit-specific. For example, the autoimmune condition IBD showed increased expression of alleles for CD11a (ITGAL), as well as its main ligand ICAM-1 (ICAM1) (de Lange et al., 2017). On the other hand, a genetic polymorphism of the gene coding for CD11b (ITGAM) was found to show increased risk of the autoimmune disease SLE (Nath et al., 2008),

which shares genetic risk factors with RA (Remmers et al., 2007). Lastly, in obesity, a condition marked by increased systemic inflammation, expression of CD11d was found to be increased in white adipose tissue of female study participants (Thomas et al., 2011). Furthermore, an increase in CD11d activation was found to elicit production of IL-1β (Miyazaki et al., 2014), a pro-inflammatory cytokine associated with autoinflammatory conditions (Dinarello, 2011). This suggests involvement of β_2 integrins in dysregulated immune responses including autoimmune conditions.

There is a large body of evidence in both animal models and human studies that suggest that beta2 integrins contribute to RA. For example, blocking all β_2 integrins in a rabbit model of arthritis using antigen directly injected into the joint reduced inflammation (Jasin et al., 1992). A different study showed that absence of CD11a specifically in a KB x N transfer mouse model caused animals to remain completely resistant to disease induction (Watts et al., 2005). Additionally, mice deficient for the β_2 integrin ligand ICAM-1 similarly showed reduced susceptibility to a collagen induced arthiritis (CIA) model (Bullard et al., 1996), suggesting that CD11a and ICAM-1 especially might have a role in RA initiation. It was furthermore shown that in the CIA model, treatment with either a mAb or a small-molecule antagonist targeting CD11a was protective and markedly reduced both inflammation-mediated joint destruction and synovial cytokine mRNA levels (Kakimoto et al., 1992; Suchard et al., 2010). However, these positive effects of blocking CD11a function in RA animal models are likely mediated by reduced cell recruitment to the joint, thereby improving symptoms.

In humans, surface expression of CD11a was found to be increased in cells isolated from RA ST but not from PB, where it was hypothesised to contribute to cell activation and maintenance of inflammation (Cush and Lipsky, 1988; Takahashi et al., 1992). However, considering that CD11a has a role in cell migration as well as immune cell activation and this study did not distinguish different cell types, it is possible that expression of CD11a is high due to the increased influx of immune cells into the joint cavity during synovitis. Another study did find CD11a, CD11b and CD18 to be elevated on PB monocytes from RA patients compared to healthy controls, an effect which was normalised after 4-6 weeks of low-dose steroid treatment (Torsteinsdóttir et al., 1999), suggesting that expression of β_2 integrins was associated with disease activity. Furthermore, while a wide range of self-peptides can be citrullinated thereby contributing to RA pathogenesis, citrullinated self-peptides

commonly identified in RA include several β_2 integrin ligands, such as fibrinogen, fibronectin and vimentin (McInnes and Schett, 2011). One could therefore hypothesise that altered self-protein might also alter β_2 integrin downstream signalling, for example by altering binding affinity of ligand to β_2 integrin. This is supported by a recent study showing that citrullination of fibronectin, a β_2 integrin ligand, altered clustering behaviour and adhesive capabilities of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ on stromal cells (Stefanelli et al., 2019). Similarly, RA synovial fibroblasts were found to show reduced adhesion and cell spreading on citrullinated fibonectin but not uncitrullinated fibronectin (Shelef et al., 2012). This suggests that citrullination of self-peptides might alter β_2 integrin function in RA.

To conclude there is a range of evidence suggesting β_2 integrins contribute to the development and/or progression of autoimmune disorders. Interestingly, roles of β_2 integrins are often α-subunit specific, highlighting that different β_2 integrins, while highly homologous, have distinct and potentially opposing functions. This is supported by the fact that different β_2 integrin subunits show differential expression in leukocyte subsets. Lastly, while extensive research in the role of β_2 integrins in RA exists, it is mostly focused on T cells and neutrophils, with no discernible reports on β_2 integrins specifically on APCs in RA. Considering especially the importance of DCs for initiation and maintenance of RA discussed at length in section 1.4.2, understanding how β_2 integrins on DCs might contribute to RA could reveal novel therapeutic pathways for the disease.

1.4.5 Targeting β_2 integrin receptors therapeutically

As discussed, β_2 integrins have a variety of important roles in the immune system from immune cell adhesion and migration to immune cell communication. This makes them a promising therapeutic target in conditions that are marked by dysregulation or aberrant activation of the immune system, such as autoimmune conditions. While the possibility of targeting β_2 integrins to control such conditions is encouraging, the wide range of roles played by β_2 integrins also make off-target effects a likely risk that will have to be circumvented. In this section, I will provide an overview of therapeutics that target integrins and the β_2 family specifically, and comment on their respective successes and failures. Lastly, I will identify the gaps in our understanding of the roles of β_2 integrins, the understanding of which will help to direct future strategies for therapeutic targeting of the beta2 integrin family.

To date, few examples of drugs targeting β_2 integrins specifically exist in the clinic. One exception is efalizumab, a monoclonal antibody (mAb) targeted against CD11a, which was originally licenced to treat psoriasis (Leonardi et al., 2005) and resulted in significant improvement in psoriatic plaque size and severity in 30-40% of patient after 12 weeks and up to 50-60% of patients after 24 weeks of efalizumab treatment. However, around 1 in 500 of patients several treated with the drug developed progressive multifocal leukoencephalopathy (PML), a rare, severe and potentially life-threatening condition where demyelination of brain white matter of the brain occurs due to reactivation of the human polyomavirus 2 (JC virus) (Khalili et al., 2019; Major, 2010). Due to the associated safety concerns, efalizumab was therefore withdrawn from European and American markets in 2009. Although the specific mechanism of action causing the increased incidence of PML in patients treated with efalizumab was not investigated, it was hypothesised to be either due to insufficient immune cell recruitment to the brain or the mAb itself entering the brain (Pavlovic et al., 2015; Schwab et al., 2012). However, in an animal model of joint inflammation, both a CD11a-targeting small-molecule antagonist (Suchard et al., 2010) and a mAB targeted against CD11a (Kakimoto et al., 1992) were successful in reducing inflammatory joint destruction and synovial cytokine levels. While efalizumab succeeded in reducing recruitment of leukocytes to inflamed sites, the severe side effects associated with its administration were not acceptable. However, lifitegrast, a competitive antagonist of CD11a, was found to be successful in treatment of dry eye disease by restricting recruitment of T cells to the ocular surface and activation of T cells, thereby reducing local inflammation and symptoms (Perez et al., 2016).

Similarly to CD11a, blocking of CD11b showed great promise in animal models. For example, in mice, a CD11b blocking antibody reduced liver injury in response to ischaemia due to reduced recruitment of neutrophils (Jaeschke et al., 1993) and in a rabbit model of arterial injury, blocking CD11b caused reduced intimal thickening due to reduced recruitment of cells to the site of injury (Rogers et al., 1998). This provided important proof of concept that blocking CD11b might be a viable therapeutic strategy to reduce the amount of immune cell influx into injury sites and the resulting tissue damage. However, results of blocking CD11b in human have largely been disappointing, with small or no visible improvement compared to placebo was found in most cases (Dove, 2000; Harlan and Winn, 2002). More recently, a different approach of using small-molecule agonists of especially CD11b, called leukadherins

(Faridi et al., 2009; Park et al., 2007), has shown great promise in reducing inflammation. The rationale for this counterintuitive approach was that increasing adhesion of immune cells to the vasculature due to the increase in CD11b activation led to a reduction in cell recruitment to the site of inflammation. As cells were less likely to transmigrate into tissues due to their strong adhesion to endothelium, inflammatory responses in a variety of animal inflammation models, including peritonitis, vascular injury and nephritis, were reduced (Maiguel et al., 2011). While this poses the risks of side effects in the form or endothelial damage or leakage due to increased adhesion of leukocytes to endothelium, this was not observed by the same group, suggesting that this approach is safe, although there is the possibility that recruitment to LNs of cells who are dependent on CD11b might be impaired. Another study on vascular injury confirmed that while both an CD11b activating antibody and the small-molecule agonist significantly increased adhesion to Fibrinogen, a reduction in inflammation was only observed in the animals treated with the leukadherin and not the activating antibody (Faridi et al., 2013). This was suggested to be due to the specificity of leukadherin, which only affected adhesive ability of cells, while the activating antibody furthermore induced both clustering of integrins and mediated outside-in signalling, suggesting that either or both of these properties made the activating antibody less effective. Clinically, the use of small-molecule agonists targeted at CD11b might be important for treatment of cancer: ADH-503, a different CD11b small molecule agonist, was shown to reduce recruitment of immunosuppressive myeloid cells to pancreatic tumours while simultaneously increasing DC-mediated immunogenic T cell responses (Panni et al., 2019). The positive effect on anti-tumour T cell response was proposed to be due to increased recruitment of cross-presenting cDC1 DCs into the tumour in the presence of ADH-503, which was in turn mediated by their naturally low expression of CD11b and increased expression of both MHCI and MHCII in the presence of CD11b agonism. Another option to avoid off-target negative side effects of drugs targeting β_2 integrins is by blocking β_2 integrin function in a ligand-specific way. For example, a mAb specifically blocking interaction between CD11b and one of its ligand CD40L was found to be protective in an animal model of sepsis, probably due to leukocytes not being able to adhere to CD40L on inflamed endothelium to extravasate into tissues, while broad blockade of CD11b worsened symptoms (Wolf et al., 2018).

In summary, there are a variety of drugs targeting β_2 integrins available. A big challenge is the wide variety of roles β_2 integrins play in the immune system, meaning that blocking these leukocyte-specific adhesion receptors has many unwanted off-target effects. Recent advances in β_2 integrin therapeutics highlight that activating rather than blocking β_2 integrins might be a viable option to prevent excessive immune activation by harnessing the immunoregulatory roles of these integrins. However, even though β_2 integrins were reported to have an immunoregulatory role specifically in DCs and macrophages, positive effects of CD11b activating drugs seem to be largely mediated by neutrophils (Celik et al., 2013; Dickinson et al., 2018). Understanding how these drugs that interfere with β_2 integrins affect DCs more specifically might therefore be important for the successful translation of these drugs into the clinic.

1.5 Hypothesis and Aims

Published literature suggests that β_2 integrins play an important role in DCs at the interface between immune activation and tolerance. While immunoregulatory roles of β_2 integrins especially on DCs have been reported in murine studies (Morrison et al., 2014; Savinko et al., 2015) as well as in human (Balkow et al., 2010; Podgrabinska et al., 2009; Varga et al., 2007), published knowledge in human DCs is still limited due to the complexity of β_2 integrin function. However, a range of evidence suggests that imbalance between β_2 integrins is able to potently contribute to aberrant immune activation (Torsteinsdóttir et al., 1999; Watts et al., 2005). Therefore, it was hypothesised that dysregulation of integrin signalling in APCs could alter the balance between activation and tolerance, thus contributing to the aberrant inflammation present in autoimmune diseases such as RA.

The hypothesis this thesis is based on can therefore be summarised as such: β_2 integrins on DCs play important pro-inflammatory and immunoregulatory roles. Dysregulation of β_2 integrins on DCs might therefore contribute to autoimmune conditions such as RA which would make β_2 integrins a viable therapeutic target. To investigate this hypothesis, I focused on three main questions.

First, integrin signalling is strongly reliant on conformational status of its subunits in response to cell-internal (inside-out signalling) and external signals (outside-in signalling) (Carman and Springer, 2003; Li et al., 2017). Whilst some studies suggest that differential expression of β_2 integrins, in this case CD11c, confers different

abilities and functions (Sándor et al., 2016a), activity status of β_2 integrins is not commonly measured. Due to published evidence suggesting that integrin conformation is highly important for their function, I hypothesised that alterations in conformational states of β_2 integrins might have functional effects on cells they are expressed on. Dysregulation in β_2 integrin signalling that could interfere with appropriate immune activation could therefore occur at the level of surface expression, as well as conformational state of β_2 integrin subunits. I therefore aimed to quantify both total β_2 integrin expression as well as the level of active conformation present on APCs, specifically DCs and monocytes. To this end, optimisation of staining for both 'total' β_2 integrin subunit expression as well as 'active' β_2 integrin expression, which used antibodies specific to an epitope exposed only when the β_2 integrin subunit was in its extended conformation is detailed in Chapter 3. This will tell us if either surface expression or regulation of conformational state of β_2 integrins differs between conditions or patient samples and healthy controls.

Second, tolerogenic Mo-DCs are not only a potential therapeutic option for DC-mediated therapy of RA, they also present a unique modelling system of DCs between immune activation and tolerance. Chapter 4 therefore aimed to characterise 'total' and 'active' β_2 integrin expression on tolerogenic compared to mature Mo-DCs to identify any potential differences in the respective immunoregulatory and pro-inflammatory model settings. In addition, this also allowed me to generate DC-like cells in large enough numbers for functional experiments, which is a challenge when using circulating human DCs, due to their low frequency in PB. Tolerogenic Mo-DCs were furthermore chosen as an optimal model system to investigate the pro-inflammatory and immunoregulatory function of β_2 integrins in DCs. This was because they show altered migration and adhesion abilities (Anderson et al., 2009) as well as altered T cell stimulatory activity (Anderson et al., 2008), which suggested differences in β_2 integrin expression or functionality. Investigating tolerogenic Mo-DCs was therefore also suited to explore the possibility of interfering with β_2 integrin function to potentially improve therapeutic efficacy of this cell-based therapy for RA.

Lastly, I aimed to investigate if β_2 integrins were indeed dysregulated in the autoimmune setting of RA, thereby potentially contributing to disease pathology. To do so, I first collected a comprehensive data set detailing 'total' and 'active' β_2 integrin expression on different human PB DC and monocyte subsets in health to act as a baseline. To investigate if dysregulation of β_2 integrins could indeed contribute

to RA pathology, I aimed to compare this healthy data set to β_2 integrin expression and conformation status in patients with RA. My findings comparing active RA, to remission and to healthy controls, as well as comparing RA PB to SF, are detailed in Chapter 5.

To conclude, the aims for my thesis were three-fold. First, I aimed to develop and optimise usage of flow cytometry to detect both expression and conformational status of β_2 integrins on APCs simultaneously. Second, I utilised mature and tolerogenic Mo-DCs as a model system to investigate both pro-inflammatory and immunoregulatory functions of β_2 integrins in DCs. Third and last, I collected a cohort of healthy, active RA and RA in remission PB and SF samples to investigate if either expression or conformational status of β_2 integrins was altered in the dysregulated autoimmune environment of RA.

Chapter 2 **Materials and Methods**

2.1 Cell isolation

2.1.1 Ethical approval

All healthy human samples were acquired under MVLS ethical approval 2012073, while patient samples collected in the clinic were acquired under REC approval 14/WS/1035 (additional tissue) and 16/WS/0207 (surplus tissue) for PB and SF/ST, respectively. Appropriate consent was given by all participants prior to sample collection. Additionally, every sample received a unique identifying number to anonymise the participant before cell isolation occurred.

2.1.2 Peripheral blood

Fresh whole blood was collected into 9ml VACUETTE® EDTA tubes (Greiner Bio-One). To isolate peripheral blood mononuclear cells (PBMCs), fresh human peripheral blood (PB) was mixed 1:1 with room temperature (RT) Hanks Balanced Salt Solution (HBSS, Gibco) + 2mM Ethylenediamine Tetraacetate Acid (EDTA, Fisher Scientific) before slowly layering onto 15ml RT lymphoprep (Axis-Shield PoC AS). Blood was centrifuged at 895g for 30min at RT and PBMCs were recovered from the surface of the high density medium with a sterile Pasteur pipette. Cell suspension was then washed in 50ml cold HBSS+1% Fetal Calf Serum (FCS, Gibco) at 600g, 4°C for 7min. Supernatant was discarded and pellet resuspended in HBSS+1%FCS for a further washing step at 250g, 4°C for 7min. Cells were filtered using a 70µm cell strainer and resuspended in appropriate volume for counting, with dead cells excluded by Trypan blue (Sigma), before being washed a third time at 400g, 4°C for 7min.

2.1.3 Synovial fluid

SF was collected into uncoated 20ml universal tubes as part of joint aspiration procedure and processed on the same day. To isolate cells from synovial fluid, the viscous sample was mixed 1:2 with RT HBSS + 2mM EDTA and filtered through a 100µm cell strainer before being carefully layered onto 15ml lymphoprep. After this cell isolation proceeded exactly as described for peripheral blood.

2.1.4 Synovial membrane

Synovial membrane or ST was collected as part of joint replacement operations and kept at 4°C suspended in RF10, consisting of RPMI-1640 (Gibco), 10% FCS, 2mM L-Glutamine (200mM, Invitrogen) and 100 units/ml of Penicillin and 0.1mg/ml Streptomycin solution (10,000 units of Penicillin and 10mg of Streptomycin per ml in 0.9% NaCl, Sigma-Aldrich) until processing the next day. ST samples were freed of

any visible fat and bone fragments before being cut into 1mm fragments using a scalpel. Care was taken to keep tissue immersed in RF10 to avoid drying out. Mechanically digested tissue in medium was resuspended in warm RF10, Then Liberase TH (high Thermolysin concentration, Roche) at a final concentration of 15µg/ml and DNase (Roche) at a final concentration of 30µg/ml were added. Samples were then transferred to a shaking incubator set at 260rpm at 37°C for 45min, with additional manual vigorous shaking every 15min. The solution was then filtered through a 70µm nylon filter. Whilst the filtrate was placed on ice immediately to prevent further enzymatic digestion, the tissue remaining in the filter was subjected to a second digestion of 45min, 260rpm at 37°C. Afterwards, filtrates were pooled, washed and cells counted.

2.2 Generating Mo-DCs

2.2.1 CD14^{+} monocyte isolation

PBMCs were isolated from human peripheral blood as described above. After the last washing step at 400g, 4°C for 7min, cells were resuspended in 80µl of ice-cold sterile-filtered MACS cell separation buffer per 10x10^{6} PBMCs, consisting out of Phospate Buffered Saline (PBS, Ca^{2+} free, Mg^{2+} free, Gibco), 5% FCS and 2mM EDTA. Then 10µl of human CD14 microbeads (MACS Miltenyi Biotec) per 10x10^{6} PBMCs were added and cells were incubated on ice for 20min, throughout which cells were gently shaken every 5min. Then, cells were washed in 25ml ice-cold MACS buffer as before. During this time, a cooled positive selection column (LS, MACS Miltenyi Biotec) was placed onto MACS separator magnet and pre-rinsed with 3ml ice-cold MACS buffer. Cell supernatant was discarded and cells were resuspended in 2ml ice-cold MACS buffer before being added to the column. CD14^{neg} cells were allowed to pass through the column, and it was washed a further three times using 3ml MACS buffer to ensure all negative cells had been flushed through. The MACS column was then removed from magnet and 5ml MACS buffer were added before releasing cells from column using a plunger. Cells were counted, washed in cold MACS buffer as described above and resuspended at 0.5x10^{6} cells/ml in cold RF10 medium.

2.2.2 Culture of Mo-DCs

Cells were seeded into an uncoated 24 well plate at 0.5x10^{6} cells in 1ml of cold RF10 per well. Human IL-4 (Immunotools) and GM-CSF (Immunotools) were added to CD14^{+} monocytes at a final concentration of 50ng/ml. Plates were incubated for 7 days

at 37°C in 5% CO_2. Cells were fed by removing 450µl of old culture medium and replacing with 500µl warm fresh RF10 supplemented with IL-4 and GM-CSF on day 3 of culture.

2.2.3 Generation of immature, mature and tolerogenic Mo-DCs

To culture immature Mo-DCs no further reagents were added. Mature Mo-DCs were stimulated with 0.1µg/ml LPS (Sigma-Aldrich) on day 6. To yield tolerogenic MO-DCs, cells received Dexamethasone (Sigma-Aldrich) at a final concentration of 10^{-6}M per well on day 3. The same amount of Dexamethasone was added on day 6 of the culture, together with Vitamin D_3 (Tocris, Biotechne) at a final concentration of 10^{-10}M and 0.1µg/ml LPS per well. All cells were harvested on day 7 for experimental use. To harvest, 24 well plates were placed on ice for 1hr to loosen cells. Cells were then gently scraped and removed from the wells. Cell suspension in RF10 was washed at least three times in ice-cold HBSS+1%FCS at 400g, 7min at 4°C, after which they were counted for further use.

2.3 Flow cytometry

All flow cytometry staining and washing steps were executed using cold FACS buffer, consisting out of PBS +3%FCS, 0.01% Sodium Azide (NaN_3, Sigma-Aldrich) and 1mM EDTA. Unless otherwise noted Sytox™ 7-AADvanced dead cell stain (Invitrogen) was added to cell suspension after washes at least 15min before analysis and was not washed off. Ultracomp beads (Invitrogen, Thermofisher Scientific) were used for compensation. 200µg/ml of human IgG was added to all staining mixes to act as a blocking agent to reduce non-specific binding to Fc receptors on human cell surfaces. In addition to controls mentioned specifically. Fluorescence Minus One (FMO) controls, containing all antibodies except for the one of interest, were used to ensure correct gating of populations, especially where data spread introduced by the use of multiple fluorochromes was likely. An overview of all antibodies used for flow cytometry with corresponding dilution used can be found in Table 2.1. Flow cytometry samples were acquired using a BD Fortessa and data was analysed using Flowjo software (Treestar).

2.3.1 Mo-DC phenotyping

After Mo-DC-harvest, cells were divided into two 96 well round bottom plates, to stain for β_2 integrin expression (at 37°C, as above) and phenotyping the different Mo-DC populations (at 4°C), respectively, for 30min in FACS buffer. For phenotyping Mo-DCs, cells were stained with antibodies to CD14 and CD1c, and MO-DCs identified as

$CD14^{neg}CD1c^{+}$. Furthermore, respective expression of HLA-DR, costimulatory molecules CD80 and CD86, as well as TLR2 and the latency-associated protein (LAP) portion of TGFβ1 were stained for. Cells were washed three times in cold FACS Buffer and resuspended in 200µl FACS buffer for acquisition.

2.3.2 Integrin activation staining

PBMCs were prepared as described. After the last wash, cells were rested for 60min in a 96 well plate in 200µl HBSS+2%FCS per staining condition in an incubator (37°C, 5% CO_2). This resting period was introduced to avoid DC death due to strenuous cell isolation process. In addition to 200µg/ml human IgG (Sigma), 1mM Magnesium Chloride ($MgCl_2$, Sigma) was added to the FACS buffer to ensure conditions in which β_2 integrins could fully extend. When staining for active β_2 integrins, an unstained control, a negative control containing no β_2 integrin antibodies, an isotype control and a positive control with the addition of 100ng/ml phorbol 12-myristate 13-acetate (PMA) were always included. After the resting period, cells were spun down in the plate for 5min, 400g at 4°C and stained for 30min at 37°C, 5% CO_2. Cells were then washed three times in 200µl cold FACS buffer as before and kept on ice between washes before being filtered and resuspended in 300µl for flow cytometry analysis.

2.3.3 Fluorescence activated cell sorting (FACS)

After PBMC isolation, cells were stained for 30min at 37°C with panel including HLA-DR, CD11c, CD14, CD16, CD1c and CD141 to yield cDC2s. CD3, CD19, CD20 and CD56 were also included to exclude T cells, B cells and NK cells. A representative gating strategy can be seen in Figure 2.1. Cells were washed three times in ice-cold FACS buffer and resuspended at $2x10^7$ cells/ml in FACS buffer, with further dilution of cells as needed to ensure optimal sorting conditions of ca. 7000 events/second. Cells were sorted into 5ml FACS tubes containing FACS buffer with 50%FCS to ensure cell survival. All sorting was done on a FACS Aria IIU or a FACS Aria II (BD Biosciences) using a 85µm nozzle. After sorting, cDC2s were rested overnight in RF10 in the presence of GM-CSF at 10ng/ml before use in further experiments.

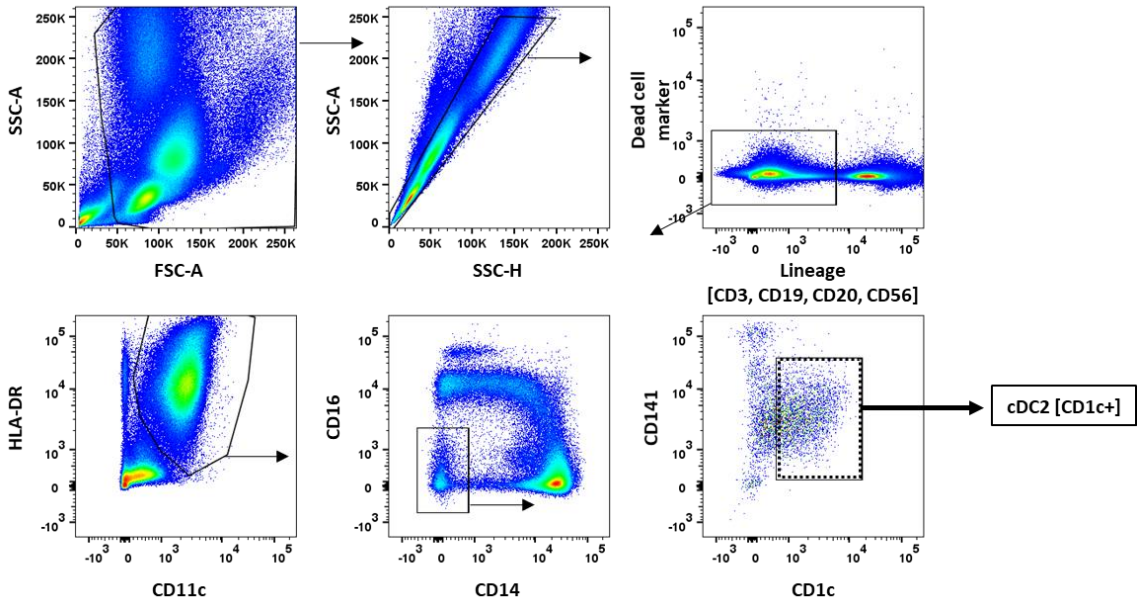

Figure 2.1 Gating strategy for sorting cDC2 DCs from human PB

cDC2s were sorted from healthy PBMCs under exclusion of doublets, T cells, B cells and NK cells. HLA-DR+CD11c+, bur CD14-CD16- cells were then shown in respect to their CD1c and CD141 staining and CD1c+ population was delineated using an unstained control. CD1c+ population was then sorted for. Representative plot of 3 different donors during 3 separate experimental repeats.

Antigen	Clone	Fluorochrome	Supplier	Optimal Titration	Final concentration
CD11a active	*MEM-83*	AF 700	*Novus bio*	1:100	9.5µg/ml
CD11a total	*HI111*	PE	*BioLegend*	1:50	2µg/ml
CD11b active	*CBRM1/5*	APC	*BioLegend*	1:50	4µg/ml
CD11b total	*ICRF44*	BV785	*BioLegend*	1:100	1.5µg/ml
CD11c	*BU15*	APC-Cy7	*BioLegend*	1:100	4µg/ml
CD14	*M5E2*	BUV737	*BD Biosciences*	1:50	1µg/ml
CD141	*M80*	PE-Cy7	*BioLegend*	1:50	4µg/ml
CD16	*3G8*	PE-Dazzle594	*BioLegend*	1:200	0.25µg/ml
CD18 active	*m24*	AF 488	*BioLegend*	1:10	10µg/ml
CD18 total	*6.7*	BV421	*BD Biosciences*	1:20	10µg/ml
CD19	*SJ25C1*	PE-Cy5.5	*eBioscience*	1:50	0.1µg/ml
CD1c	*L161*	PerCP-Cy5.5	*BioLegend*	1:100	2µg/ml
CD20	*2H7*	PE-Cy5.5	*eBioscience*	1:50	0.3µg/ml
CD3	*SK7*	PE-Cy5.5	*eBioscience*	1:100	5µg/ml
CD4	*OKT4*	PerCP-Cy5.5	*Biolegend*	1:50	2µg/ml
CD45	*HI30*	BUV395	*BD Biosciences*	1:50	2µg/ml
CD45RO	*UCHL1*	APC-Cy7	*Biolegend*	1:50	8µg/ml
CD56	*CMSSB*	PE-Cy5.5	*eBioscience*	1:100	0.06µg/ml
CD83	*HB15e*	PE	*BD Biosciences*	1:10	25µg/ml
CD86	*2331 (FUN-1)*	V450	*BD Biosciences*	1:50	4µg/ml
HLA-DR	*G46-6*	BV480	*BD Biosciences*	1:50	2µg/ml
HLA-DR	*L243*	APC	*BD Biosciences*	1:100	Unknown
h-LAP (TGFβ1)	*27232*	PE	*R&D systems*	1:10	Unknown
Isotype control Mouse IgG1, κ	*11711*	AF700	*R&D systems*	1:25	9.5µg/ml

Isotype control Mouse IgG1, κ	*MOPC-21*	APC	*BD Biosciences*	1:50	4µg/ml
Isotype control Mouse IgG1, κ	*MOPC-21*	AF488	*Biolegend*	1:5	10µg/ml
TLR2	*TL2.1*	AF647	*Biolegend*	1:10	40µg/ml

Table 2.1 Antibodies used for flow cytometry

2.4 Static adhesion assay

Static adhesion assays were used to quantify Mo-DC adherence to various β_2 integrin ligands. ICAM-1 (R&D systems), iC3b (Merck Millipore), C4b (Merck Millipore), Fibrinogen (Merck Millipore) and Fibronectin (R&D systems) diluted in PBS were coated overnight at 4°C onto COSTAR 96 well high-binding assay plates (Corning) at low, medium and high concentrations, which can be found in Table 2.2. Fibronectin was included as a positive control, while wells treated with PBS without any addition of ligand were included as a negative control. After 24h, wells were washed twice with PBS, before blocking with 1% Milk in PBS for 1h15min at 37°C. Plates were washed twice with cold PBS, left with 200µl PBS per well and placed on ice. Harvested Mo-DCs were resuspended in adhesion medium consisting of RPMI 1640, 0.1% Bovine Serum Albumin (BSA, Sigma), 20mM HEPES (pH 7.25) and 2mM $MgCl_2$ (Sigma), before adding $25x10^3$ Mo-DCs per well in duplicate or triplicate (400µl at a concentration of $0.0625x10^6$cells/ml).

Ligand	**Concentration (in µg/ml)**		
	Low	*Medium*	*High*
ICAM-1	0.1	1	10
iC3b	1	3.3	10
C4b	1	3.3	10
Fibrinogen	1	10	100
Fibronection	*Positive control:* 10		

Table 2.2 Ligand concentrations for static adhesion assay

After the addition of cells, plates were incubated for 8min on ice to allow cells to settle to the bottom of the well before being transferred to 37°C for 15min to allow cell adherence. Plates were then carefully placed upside down in 2.5 litre PBS+2mM $MgCl_2$ for 50min at RT to allow non-adhered cells to exit the well. Then, plates were inverted

and 350µl buffer was removed to leave 50µl per well, to which 100µl of lysis buffer, consisting out of p-nitrophenyl phosphate (PNPP, Calbiochem) dissolved in 1%Tx-100/50mM acetate buffer (pH 5) at a final concentration of 3mg/ml, was added. Adhered cells were lysed for 60min at 37°C protected from light and reaction was stopped by adding 50µl Sodium Hydroxide (NaOH) per well. Absorbance was measured using a Sunrise Absorbance Reader (Tecan) at 405nm. In order to quantify cell adhesion as a percentage of total cells, a 'total' plate was prepared by pelleting $25x10^3$ cells per Mo-DC subtype (400µl of $0.0625x10^6$ cells/ml cell suspension) and resuspending cells in 200µl PBS. 50µl of each cell suspension (i.e. x number of cells) was then transferred to a fresh uncoated COSTAR 96 well high-binding assay plate in triplicate, together with PBS in triplicate as a negative control. This 'total' plate was lysed and measured as described with the experimental plate.

2.5 18h PBMC stimulation

To understand the effects of extracellular factors on β_2 integrin expression and activation, PBMCs were stimulated with various factors for 18h. PBMCs were resuspended in RF10 and plated into 48 well plates (Corning) at $1.5x10^6$ cells per well. Then, stimuli diluted in RF10 were added to yield five conditions (untreated, LPS, IL-1β+TNFα, IL-10+TGF-β, SF), each in duplicate. Details of concentrations used can be found in Table 2.3. Cells were then returned to 37°C, 5% CO_2 for 18h before being harvested and stained for flow cytometry as above.

Condition	*Final concentration*	*Supplier*
Untreated	-	-
LPS	100ng/ml	*Sigma-Aldrich*
IL-1β + **TNF**	10ng/ml 10ng/ml	*Immunotools* *Immunotools*
IL-10 + **TGF-β**	10ng/ml 10ng/ml	*Immunotools* *Immunotools*
Synovial fluid	25% synovial fluid in RF10	*Biobanked samples under REC 16/WS/0207*

Table 2.3 Treatment conditions for 18h PBMC stimulation

2.6 Mixed Lymphocyte Reactions

To investigate the role of β_2 integrins on DCs in T cell activation, mixed lymphocyte reactions (MLRs) were set up with mature or tolerogenic Mo-DCs, or sorted cDC2s, together with CD4 T cells isolated from a different donor.

2.6.1 Naïve CD4 T cell isolation

5-10ml of buffy coat was taken to yield approximately 20-40x10^6 naïve CD4$^+$ T cells. Rosettesep Human CD4$^+$ enrichment cocktail (Stemcell Technologies) was added at 75µl/ml and incubated at RT for 20min. Sample was then diluted 1:2 using PBS+2%FCS at RT and gently layered onto 15ml lymphoprep. Density centrifugation occurred at 895g, 21°C for 30min after which enriched T cells were removed from the high density medium interface using a sterile Pasteur pipette. Cells were washed in warm PBS+2%FCS for 7min at 600G, RT, and again at 400G. Then, cells were counted and washed a third time in cold HBSS+1%FCS at 400G, 4°C for 7min. The pellet was then resuspended in 80µl ice-cold MACS buffer per 10x10^{6}cells. 20µl of human CD45RO Microbeads (MACS Miltenyi) per 10x10^{6}cells were added and gently mixed before cells were placed in the fridge for 15min. After incubation, non-adhered beads were washed off using ice-cold MACS buffer for 7min at 400g, 4°C, and resuspended in 500µl MACS buffer. A MACS separator column was pre-washed with 3ml MACS Buffer, before cells were added. CD45RO^{neg} cells passed through the column, while unwanted CD45RO$^+$ T cells remained bound to the column. The column was washed a further three times with 3ml MACS buffer each. Cells present in flow-through were counted for further analysis.

2.6.2 CellTrace Violet Proliferation Staining

After counting, naïve CD4 T cells were washed twice in PBS for 7min at 400g, 4°C. They were then stained with 5µM CellTrace Violet Proliferation Dye (CTV, Invitrogen, Thermofisher Scientific) in PBS for 15min at RT protected from light. After the incubation period, 1/5 of the staining volume of FCS was added for 5min to quench the reaction. Cells were then washed twice more in cold PBS+1%FCS for 7min at 400g, 4°C to remove excess dye. Cells were counted again and resuspended at 2x10^{6}cells/ml in cold RF10.

2.6.3 Setting up Mixed Lymphocyte Reaction

Mature and tolerogenic Mo-DCs, as well as overnight rested sorted cDC2s, were resuspended at 2x10^{5}cells/ml in cold RF10. MLR was set up in 48 well plates (Corning)

with 250µl of CTV-stained naïve CD4 T cells at $2x10^6$ cells per ml ($0.5x10^6$ T cells per well) and 250µl of the respective DCs ($0.05x10^6$ Mo-DCs or cDC2s per well) per well. This gives a DC:T cell ratio of 1:10. Where indicated, the CD11b agonist, Leukadherin-1 (R&D systems, Biotechne) in concentrations from 1µM to 10µM, with a corresponding DMSO control (0.1-1%) or a CD11b blocking antibody (Biolegend) at 20µg/ml with a corresponding isotype control (Biolegend) also at 20µg/ml were added to the culture. MLRs were cultured at 37°C, 5% CO_2 for 4 or 6 days. CD11b antagonists were replenished on Day 4, with a further 10µl of 1mg/ml stock solution of either CD11b blocking antibody or isotype control being added to respective culture dishes. CD11b agonist and DMSO control were not replenished on Day 4, but only added to the culture on Day 0 due to the inhibitory effect of even low DMSO concentrations on cell proliferation.

2.7 ELISA

Supernatants of MLRs were collected on Day 6 of culture and frozen at -80°C for cytokine measurements using enzyme-linked immunosorbent assay (ELISA). IL-10 and IFN-γ were measured using Human IL-10 ELISA MAX™ Deluxe (Biolegend) and human IFN-γ ELISA MAX™ Deluxe (Biolegend) kits, respectively, according to the manufacturer's protocol.

Briefly, capture antibodies against IL-10 and IFN-γ were coated overnight at 4°C onto COSTAR 96 well high-binding assay plates (Corning) at 50µl per well. Unbound capture antibody was washed off the following morning using PBS-T (PBS, 0.05% Tween-20) three times before blocking for 1h with the manufacturer's blocking solution. Plates were washed 3 times and 50µl of prepared stock solutions and sample dilutions were added to respective wells in duplicate. Plates were incubated for 2h on a shaker at RT for cytokine binding. After washing plates three times, 50µl of detection antibody was added to the plates and incubated a further 1h at RT on a shaker. Plates were washed three more times and 50µl of Avidin-HRP solution was added for 30min. For final 4 washes, plates were soaked in washing buffer for 30-60sec for each wash to reduce background signal. After the last washing step, 50µl of freshly mixed TMB substrate solution (components provided by the manufacturer) were added to each well and incubated in the dark. Once the characteristic blue colour appeared, after ca. 10-20min, the reaction was stopped by the addition of 50µl 10% Sulfuric Acid (H_2SO_4).

A Sunrise absorbance reader (Tecan) was used to measure absorbance at 450nm and 570nm, where the reading for A_{570} was subtracted from the reading for A_{450}.

Furthermore, background signal was removed by subtracting values measured in assay diluent-only negative control wells. Unknown cytokine concentrations were interpolated from a standard curve constructed using Graphpad Prism software and a defined series of 10 serial dilutions performed in duplicate, beginning with the top standard at 1000pg/ml and ending with a well without any top standard added to assay diluent (12 standards in total).

2.8 Microscopy

Microscopic imaging was used to validate cell blasting in MLRs and overall cell morphology as well as quantify cell clustering of different Mo-DC subtypes.

2.8.1 Quantification of Mo-DC clustering on glass bottom plates

To measure Mo-DC clustering a Leica DMi8 (Leica Microsystems) was used with a live cell chamber attachment at 37°C and 5% CO_2. Immature, mature and tolerogenic Mo-DCs were plated onto glass-bottom 24 well plates at a concentration of 200,000 cells per well in 1ml RF10 and kept at 37°C, 5% CO_2. Wells were then imaged 6h after plating to observe respective clustering behaviour. Up to 40 Bright field images of each well were taken at 10x magnification and stitched together to yield an overview of the whole well. Imaging acquisition software provided by Leica was used for all images acquired on this instrument.

To quantify clustering of Mo-DCs, a cellprofiler pipeline was designed with the help of Dr. Leandro Lemgruber (Imaging Technologist, Glasgow Imaging Facility), to identify both clusters of cells and single non-clustered cells respectively for measurement. An example of an image analysed by the pipeline can be found in Figure 2.2. Briefly, the origininal images were pre-processed to yield a high threshold black and white image showing cells as bright objects on a dark background (Step 2, using CellProfiler modules ColourToGrey, RescaleImage, ImageMath, EnhanceOrSuppressFeatures and Threshold). Each individual cell was then identified as an object, using parameters ranging between 30-40 pixels (ca. 18-24µm), which was based off repeated measurements of cells visualised (Step 3, IdentifyObjects). Then, to identify clusters of cells, cells which had one or more ‘neighbours’, which were defined as separate objects that were immediately adjacent to a cell, were identified (Step 4, MeasureObjectNeighbours). Clusters of cells were defined as cells that had neighbouring cells with touching borders. To enable measurement of these clusters, a threshold was applied to images containing just clusters and each cluster was

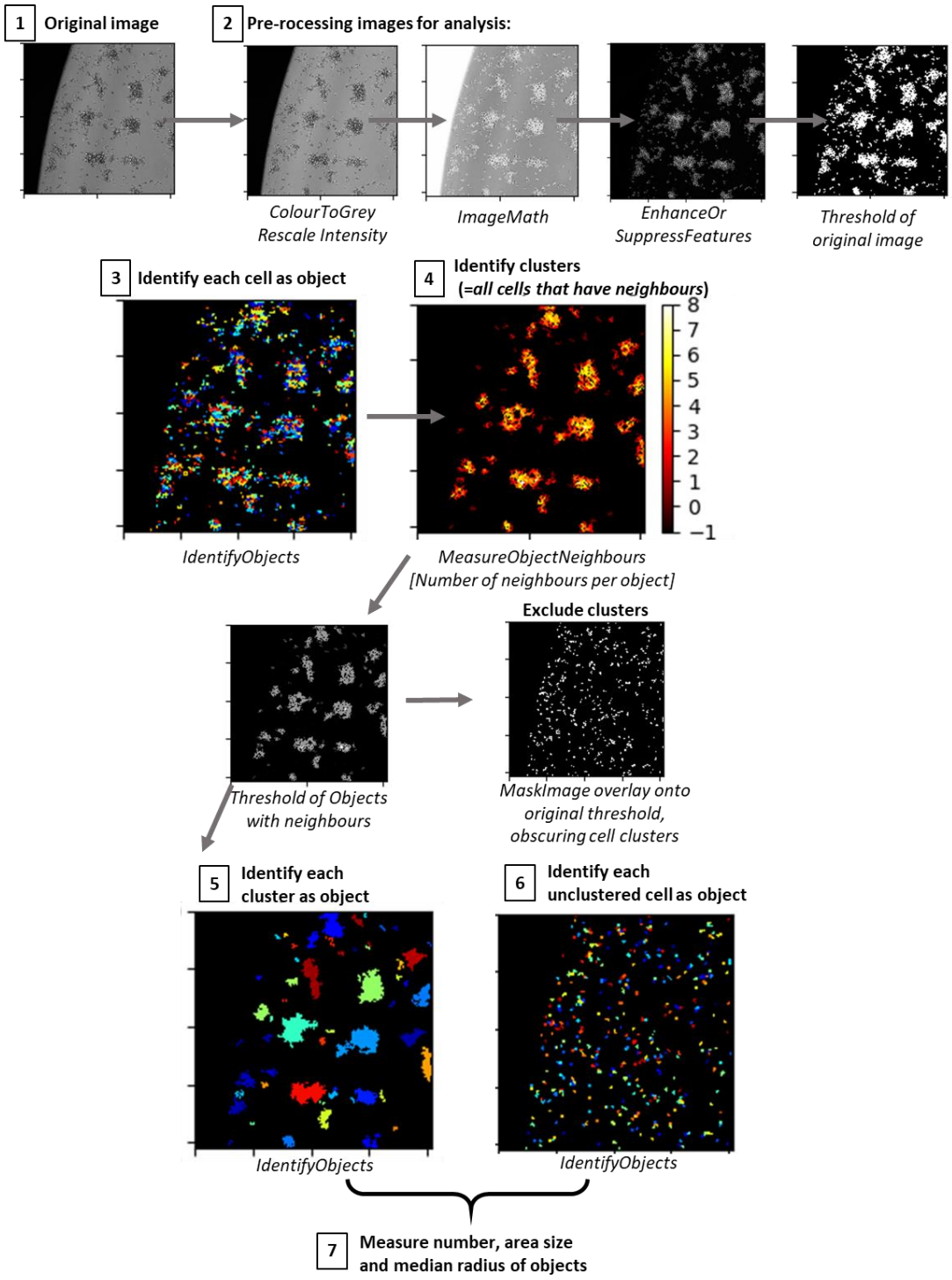

Figure 2.2 Visual representation of CellProfiler pipeline for cell clustering analysis

Analysis of sample image (mature Mo-DCs) with CellProfiler pipeline for cell clustering analysis, with different relevant steps shown to identify and quantify both clusters of cells as well as individual non-clustered cells. CellProfiler image processing modules utilised to yield respective stages can be found in cursive below each image.

identified as an object (Step 5, Threshold, IdentifyObjects). To detect single cells, that did not cluster together, the threshold image of clusters (Threshold ObjectsWithNeighbours) was overlaid as a mask onto the original threshold image (Threshold of original image), leaving only non-clustered cells. Then each non-clustered cell was identified as an object (Step 6, MaskImage, IdentifyObjects). After both cell clusters and single cells were identified as objects, measurements of object number, size and radius could be obtained (Step 7, MeasureImageAreaOccupied, MeasureObjectSizeShape) and compared between immature, mature and tolerogenic Mo-DCs.

2.9 Sample size estimation and power calculations

As very little is known about the expression levels of β_2 integrins, particularly in the active conformation, on human APCs, it was not possible to conduct a formal power calculation before beginning sample acquisition. It was therefore decided to utilise both a priori and compromise power calculations with Cohen's standard effect sizes (Cohen's *d*) (Cohen, 1992) initially and adapting these after 10 samples per group (healthy, active RA, remission) were collected to provide the preliminary data needed to estimate an appropriate effect size (Jones et al., 2003).

The null hypothesis for the clinical part of this thesis states that there is no difference in β_2 integrin expression or activation between RA patients (active or in remission) and healthy controls. The alternative hypothesis therefore states that there is a difference between these groups of people, with power calculations serving to avoid Type I (falsely accepting the null hypothesis) or Type II (incorrectly rejecting the null hypothesis) statistical errors. For all power calculations and sample size estimation Gpower 3.1 was used.

For a priori (Test family: F tests), a one-way ANOVA, fixed effects was used with varying levels of effect sizes (large – 0.4, medium – 0.25, small – 0.1, Cohen's *d*) and the lowest acceptable power (0.8) was compared to 0.95, which should be aimed for. Results of these calculations can be found in Table 2.4. For example, when acquiring 31 samples per group, medium effects would be detected correctly in 80% of cases.

For power calculations using compromise (Test family: F tests), the same statistical test was used with the difference that power was calculated from a respective sample size of 20 per group (total sample size 60), 25 per group (total sample size 75) and

32 per group (total sample size 96). Results of these calculations can be found in Table 2.5. For example, with a sample size of 25 samples per group, large effects could be correctly identified with 91% probability, neither medium nor small effects could be detected at high enough power to be reliable. Overall both a priori and compromise power calculations suggest that it would be impossible to detect small effects in the context of this thesis.

A priori	**POWER 0.95**		**POWER 0.8**	
Effect size	*Total samples*	*Samples per group*	*Total samples*	*Samples per group*
LARGE: 0.4	102	34	39	13
MEDIUM: 0.25	252	84	93	31
SMALL: 0.1	1548	516	570	190

Table 2.4 A priori sample size calculation

Compromise	*Power if total sample size=*		
Effect size	*60*	*75*	*96*
LARGE: 0.4	0.88	0.91	0.95
MEDIUM: 0.25	0.73	0.76	0.81
SMALL: 0.1	0.55	0.56	0.58

Table 2.5 Compromise power calculation

However, there is some criticism of using Cohen's small, medium and large effect sizes, as they are based on a normally distributed population and therefore might not fit all experiments (Rice and Harris, 2005). It is therefore recommended to calculate experiment-specific effect sizes using Cohen's *d* from means and standard deviation (SD) of relevant preliminary data.

The general formula states that (Ferguson, 2016):

$$Effect\ size = \frac{Mean\ of\ group\ A - Mean\ of\ group\ B}{SD\ of\ both\ data\ sets}$$

$$SD\ of both\ data\ sets = \sqrt{\frac{SD_A A^2 + SD_B A^2}{2}}$$

However, as three groups are compared to each other instead of two, effect sizes have to be calculated taking SDs and means of all three groups into account. Therefore, g*power was utilised to calculate respective effect sizes from the means of 10 samples per group. SD pooled from all three groups was calculated by the programme by providing the square root of the mean squared residual value (MS residual), which describes the mean difference between estimated sample mean and sample (Dekking, 2005).

2.10 Statistical Analysis

Data is shown as mean +SD if not specified otherwise. For all other statistical analysis Graphpad Prism software was used. Student's *t*-tests were used to compare two groups, while one-way ANOVA was utilised to compare single variables between 2 or more groups. In this case, Tukey's multiple comparison test was used to determine where differences between groups specifically occurred. P-values shown are as follows *$p<0.05$, **$p<0.01$, ***$p<0.005$, ****$p<0.001$.

Chapter 3 **Development and optimisation of a flow cytometry panel to quantify active β_2 integrins**

3.1 Introduction

The heterodimeric β_2 integrin receptors are exclusively found on leukocytes, where they play important role in cell adhesion and migration. However, evidence is growing that β_2 integrins can also regulate immune functions specifically in DCs (Schittenhelm et al., 2017) It was, therefore, hypothesised that dysregulation of β_2 integrin immune cell signalling is associated with the pro-inflammatory autoimmune environment prevalent in inflammatory diseases such as RA. To this end, the aim was to establish if total and active forms of β_2 integrins can be assessed in the same sample, and to develop a flow cytometry panel that will enable us to measure these β_2 integrin conformations on a range of APCs.

3.1.1 The β_2 integrins of interest

Receptors of the β_2 integrin family consist of a shared β_2 subunit (CD18) that non-covalently pairs with one of four α-subunits (CD11a, CD11b, CD11c or CD11d). These integrins are expressed on the cell surface and possess two main signalling modes. In inside-out signalling, cell internal signals drive activation or avidity of integrins on the cell surface to interact with the cells' immediate environment. On the other hand, outside-in signalling is triggered when an integrin encounters its ligand, which causes integrin activation and downstream signalling processes within the cell (Hogg et al., 2011). Importantly, both inside-out and outside-in β_2 integrin signalling is therefore modulated by conformational changes, which can increase ligand affinity between 4000 to 6000-fold between the inactive low affinity and active high-affinity state (Li et al., 2017). Considering the importance of these conformational changes for integrin signalling and function in immune cells, it is key to not only assess expression of a subunit but also consider its activation state. While published literature extensively explores differential expression of total β_2 integrins on various cell types (see Chapter 1, section 1.4.2, Table 1.1), a comprehensive overview of β_2 integrin activation status on different immune cell types has not been attained to date.

To address this knowledge gap, the aim was to quantify not only expression of the respective subunit (*total* β_2 integrin expression) but also the proportion of receptors present in the active high-affinity conformation (*active* β_2 integrin expression). This could provide important insights into the relationship between surface expression and activation, which are not well understood so far. CD11a, CD11b and the pairing subunit CD18 all have commercially available antibodies against binding sites that are exposed regardless of β_2 integrin conformational state (*total* β_2 integrin antibodies) and binding

sites that only become exposed when the β_2 integrin is extended into its high affinity conformation (*active* β_2 integrin antibodies) (Diamond and Springer, 1993). Comparing the α-subunits CD11a and CD11b is especially interesting, as they share ligands and are highly homologous, but have been described to have different functions (Ding et al., 1999). Measuring CD18 on the other hand yields slightly different information. As it pairs with both CD11a and CD11b, CD18 provides valuable information on how the different subunits can potentially compensate for each other if changes in one should occur. While an activation-specific antibody is commercially available for CD11c (Sadhu et al., 2007), this marker was not included as total CD11c was used to delineate APCs in the gating strategy (see Figure 2.1). CD11d, which is the most recent β_2 integrin subunit identified (Van der Vieren et al., 1995) and the least well-described, does not have flow cytometry antibodies targeted at either total CD11d or an activation-specific epitope, so it was not included. While CD11c and CD11d could not be assessed directly, CD18 might give us some information about these subunits indirectly.

3.1.2 Cells of interest

As my main focus was to investigate the roles of β_2 integrins in immune regulation, specifically in DCs, different subtypes of these professional APCs present in peripheral blood were of interest. This included both conventional DC subtypes, differing in the presence of $CD141^+$ staining (cDC1) and $CD1c^+$ staining (cDC2). Additionally, pDCs were considered. However, as there is some evidence that respective integrin expression or activation might contribute to the differing antigen-presenting capabilities between cell types (Sándor et al., 2016b), non-professional APCs were also included for comparison, such as classical and non-classical monocytes. Lastly, as the scope of my work was to investigate the role of β_2 integrins on myeloid APCs, lymphocytes such as T cells, B Cells and NK cells were not considered.

To conclude, expression of total and active forms of the β_2 integrin α-subunits CD11a and CD11b, as well as their common paired β-subunit CD18, were assessed in MO-DCs (*in vitro* work) and APCs present in peripheral blood (*ex vivo* work).

3.1.3 Application of the β_2 integrin flow cytometry panel

As I was interested in exploring β_2 integrin expression and conformation not only in peripheral blood of healthy controls, but in the context of autoimmune disease, further considerations had to be taken into account when designing the active β_2 integrin

panel. While RA is a systemic disease, many symptoms are joint-specific so it was important to test if synovial tissue could also be assessed. Developing a panel that can be dynamically used between different tissues is also important for potential future use in different disease contexts. To this end, I wanted to make sure this panel could also be used to measure total and active β_2 integrin expression in cells isolated not only from peripheral blood, but also from synovial fluid and synovial tissue samples. CD45 was therefore included as a marker into the panel to be able to distinguish between $CD45^{neg}$ stromal cells present in high numbers in these tissues and the $CD45^{+}$ APCs of interest. Furthermore, application of the panel to fresh and frozen samples alike would be tested, to make future use of the panel on samples stored in biobanks or other repositories possible.

3.2 Aims

To explore the potential roles β_2 integrins play in immune regulation on DCs, expression of both total and active forms of specific β_2 integrin subunits needed to be quantified in the different types of DCs. In this chapter, I aimed to develop and optimise a flow cytometry panel that will enable us to do this.

The specific aims of this chapter were:

1. To optimise staining conditions for total and active β_2 integrins in terms of staining temperature and time.
2. To validate an appropriate positive control for β_2 integrin staining.
3. To ensure concurrent staining of total and active β_2 integrins was not hindered by signal loss due to steric hindrance or FRET.
4. To titrate antibodies for optimal signal and develop a gating strategy to distinguish a range of APCs present in PB.
5. To assess potential future applications of the panel on both biobanked samples and synovial tissue.

3.3 Results

3.3.1 Temperature sensitivity of active integrin staining

Published literature suggests that integrin-mediated adhesion is highly temperature-sensitive (Cai and Wright, 1995; Rico et al., 2010), with little to no adhesion to ligand being observed at 4°C or even at RT. As integrin activation confers functionality, including adhesive capability of cells, it was hypothesised that integrins do not expose their activation-specific epitope under cold conditions, which hinders the binding of antibodies to active integrins. Differences between staining for an activation-specific epitope cold or at 37°C have been shown previously with the CD11b/CD18 activating clone KIM127 (Andrew et al., 1993). Different approaches concerning this limitation can be found in the literature, including bringing cells to 37°C for 30min before staining at 4°C (Kooyk et al., 1999) or staining separately for activation-specific epitopes at 37°C for a reduced time period of 10min (Shamri et al., 2005).

To test whether temperature influences the staining of active integrins, binding of antibodies to active and total CD11a, CD11b and CD18 in the fridge at 5.6°C and in the incubator at 37°C was compared (Figure 3.1). Staining of active CD11a was slightly higher at 37°C than at 5.6°C (Figure 3.1A), a difference which was significant (Figure 3.1B). Staining of active CD11b was also consistently higher at 37°C, which was especially true for the SSC-high (SSChi) population. However, this result was not significant due to the comparably small sample size with higher variation. Lastly, active CD18 staining could not be detected in any of the three donors when stained at 5.6°C, while staining at 37°C yielded a clear staining with distinct SSChi and SSC-low (SSClo) populations. This result was also not significant. Staining of total CD11a is also higher at 37°C compared to 5.6°C, but the result was not significant. Both total CD11b and total CD18 showed significantly higher staining at 37°C than at 5.6°C. Overall, SSC-hi and SCC-lo populations were the same between samples stained at different temperatures, with staining being shifted to the right in the cells stained at 37°C.

The aim was to develop a flow cytometry panel that detects both total and active expression to investigate the physiological relevance of β_2 integrins for disease. Being able to detect and measure active CD18 is a critical part of this aim, which is why staining of all samples at 37°C was deemed necessary. This is however associated with several caveats, such as increased cell death and an increase in non-specific

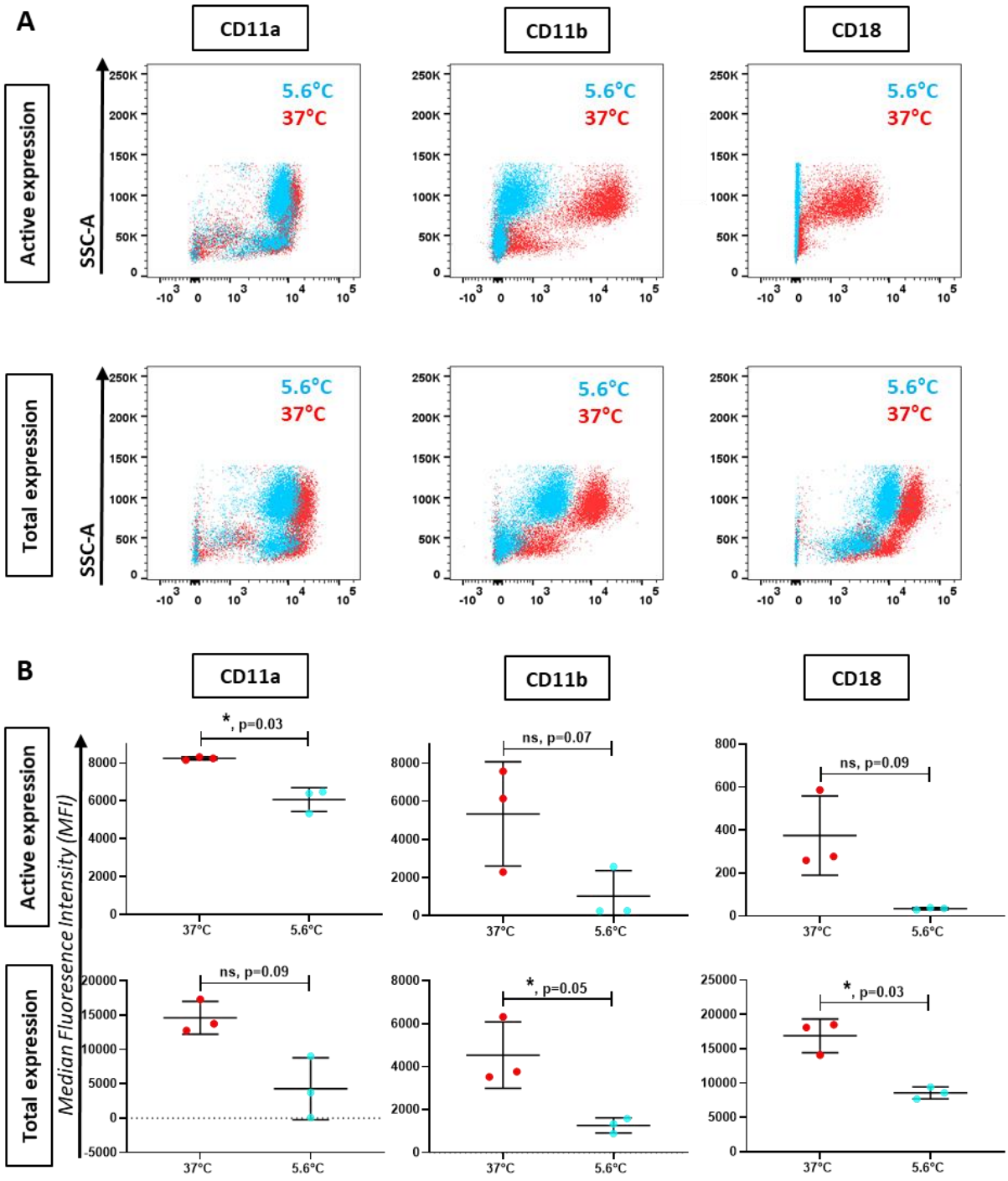

Figure 3.1: Staining of active β_2 integrin subunits is temperature sensitive.

Positive staining for the respective antibodies against active and total CD11a, CD11b and CD18 was assessed. Healthy unstimulated PBMCs were stained for 30min at 5.6°C or 37°C. A. Representative plots show positive staining for active and total β_2 integrins stained at 37°C (shown in red) overlaid onto cells stained at 5.6°C (shown in blue). Only live single cells negative for CD3, CD19, CD20 and CD56 are shown. B. Pooled data of three independent donors, stained on three separate days. Paired t-test, two-tailed.

staining, making the antibody staining less specific and reliable. Comparing the higher staining of total β_2 integrins at 37°C, it is possible that this is due to non-specific antibody binding, even though SSChi and SSClo populations have not changed in shape but merely in the brightness of signal recorded. Alternative methods were briefly explored, such as consecutive staining of total and active integrins at different temperatures. However, continuous integrin trafficking between cytoplasm and cell surface is a phenomenon widely described in the literature (Bretscher, 1992; Fabbri et al., 1999). To understand the relationship between β_2 integrin expression and activation, it is therefore essential that total and active forms are measured on the same cells in the same sample at the same time.

To summarise, staining of active β_2 integrins is highly temperature-sensitive. Therefore, staining samples at 37°C is necessary to detect the active integrin conformation, especially for active CD18. However, higher staining of total β_2 integrins at 37°C means that an increase in non-specific antibody binding could occur. A reduction in staining time was therefore tested to potentially recoup this effect.

3.3.2 Time sensitivity of active integrin staining

After it became apparent that β_2 integrin staining would have to be performed at 37°C, it is likely that molecular processes involved in the staining process, such as an antibody binding to its specific target, would occur faster than in cold temperatures. To minimise the risk of non-specific staining, it was hypothesised that staining time could be reduced from 30min.

Testing this hypothesis on CD11a showed promising results, as the positive population remained stable when comparing only 5min of staining to 30min of staining. The isotype control showed reduced non-specific specific binding (A). Approximately 10% of cells gained CD11a active staining in the 25min between experimental conditions. On the other hand, signal in isotype control was almost absent after 5min of staining time (1.48%), but rose to 8.39% after 30min, although the non-specific staining did not seem to overlap with cell populations stained with the CD11a active antibody. It was therefore considered reducing the staining time to 5min, with the signal lost being the caveat to reduce risks of non-specific staining. However, when staining for active CD11b for 5min or for 30min, clear separation of positive and negative population only became visible at 30min of staining (**Figure 3.2**B, top).

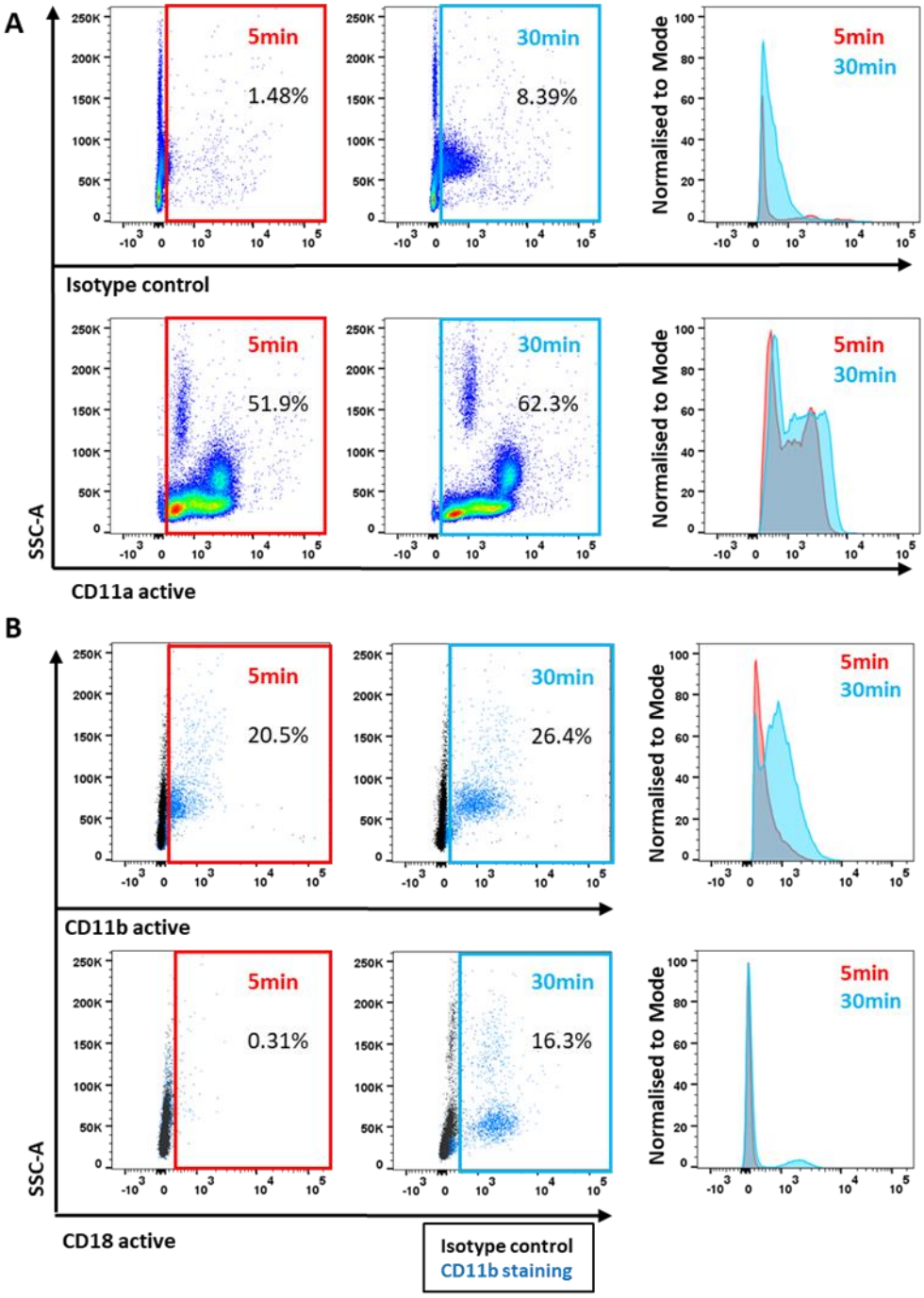

Figure 3.2 The effect of staining time on active β_2 integrin staining.

Healthy unstimulated PBMCs were stained for either 5min or 30min at 37°C. Cells were either stained with active CD11a (A), active CD11b (B, top) or active CD18 (B, bottom) or matched isotype controls. In the case of active CD11a and CD11b the same sample was stained for the respective times, while active CD18 was stained on two different samples. Experiment was done once for each β_2 integrin subunit. In B, Isotype control staining (shown in black) is overlaid over β_2 integrin staining (shown in blue).

Interestingly, while proportion of positive cells only increased by 5.9% when comparing 5min to 30min of staining time, cells gained signal brightness as staining time

increased. Furthermore, isotype control staining (shown in black) did not become more positive with increased staining time. A similar trend can be observed in active CD18 (**Figure 3.2**B, bottom), where only 0.31% of cells are positive for active CD18. After 30min of staining, not only signal brightness has increased but the percentage of active $CD18^+$ cells has risen to 16.3%. Similar to CD11b, non-specific binding in isotype control (shown in black) does not seem to increase with a longer staining time of 30min. As no clear positive populations could be distinguished in either active CD11b or active CD18 stain, the staining time experiments were not repeated further.

Clearly, β_2 integrin subunits differ in their optimal staining time. Even though β_2 integrin staining at 37°C risks increased levels of non-specific binding, this is not the case for every sample. From the different samples considered in **Figure 3.2**, this was only a minor problem in active CD11a, where non-specific binding was very low compared to active CD11a specific staining. For active CD11b and active CD18, non-specific staining could not be observed at all. Based on these results, all staining was performed for 30min so all active β_2 integrins could be detected. To ensure staining was not due to temperature-mediated non-specific binding isotype controls for the three active integrin antibodies were included in all future experiments.

3.3.3 Validating a suitable positive control

Before establishing if both total and active β_2 integrin antibodies can be used in the same panel, it first had to be ensured active β_2 integrins could be detected reliably and a suitable positive control could be validated. The positive control therefore requires high levels of active integrins. The three main ways to increase integrin activation are through monoclonal antibodies, which stabilise or induce the active conformation of the integrin subunit targeted (Byron et al., 2009), provision of divalent cations such as Manganese (Mn^{2+}) (Dransfield et al., 1992), and stimulation with phorbol esters (Kucik et al., 1996). Adding further antibodies, even if not fluorescently-labelled, was immediately eliminated due to likely binding site overlap. While the addition of Mn^{2+} has been described to increase activation specifically of CD11b, the positive effect of the divalent cation on β_2 integrins can be diminished by the presence of Calcium (Ca^{2+}) (Dransfield et al., 1992). While care was taken to only use Ca^{2+}-free media, the potential variability of Mn^{2+} as a positive control made it a less viable option. Therefore, the addition of phorbol esters in the form of PMA to stimulate β_2 integrin activation on cell surfaces was explored as a positive control.

Phorbol esters, specifically PMA, have been described to increase integrin-mediated adhesion via protein kinase C activation (Danilov and Juliano, 1989). When considering β_2 integrins specifically, total expression levels of CD11a were unaffected by PMA stimulation, but adhesion to ICAM-1 and mobility of the subunit on the cell surface were increased (Kucik et al., 1996). This suggests that an increase in active CD11a, rather than total, contributed to this effect, making PMA a feasible possibility as a positive control. 100ng/ml PMA was added to staining buffer with active β_2 integrin antibodies, stained for 30min at 37°C and compared to -PMA (Figure 3.3). In contrast to evidence in the literature, the effect of PMA on CD11a was the least pronounced, with PMA only inducing a small shift in active CD11a signal (Figure 3.3A and B). Both CD11b and CD18, on the other hand, show pronounced increase in activation after 30min of PMA stimulation, but result was not significant for any of the β_2 integrin subunits of interest (Figure 3.3B). However, increase in activation upon PMA stimulation is especially apparent in CD18, which in this donor shows little active staining in the absence of stimulation with PMA, an observation that fits with the literature describing that most β_2 integrins are inactive on the cell surface in the steady state (Li et al., 2017).

To conclude, PMA stimulation occurring concurrently with antibodies staining induced a visible shift in active CD11b and CD18 in the dot plot and histogram overlays. This result was not significant due to the variability of the response induced, but the marked shift visible in the histogram suggests that PMA stimulation is a viable positive control for activation-specific integrin staining. Although the shift observed in CD11a was very small, this could be due to the high levels of activated CD11a already detected on cell surfaces without stimulation, possibly leaving little room for further increase. This possibility could be assessed further when staining both active and total CD11a on the same cells, as the % of CD11a total expressing cells expressing CD11a active could be determined.

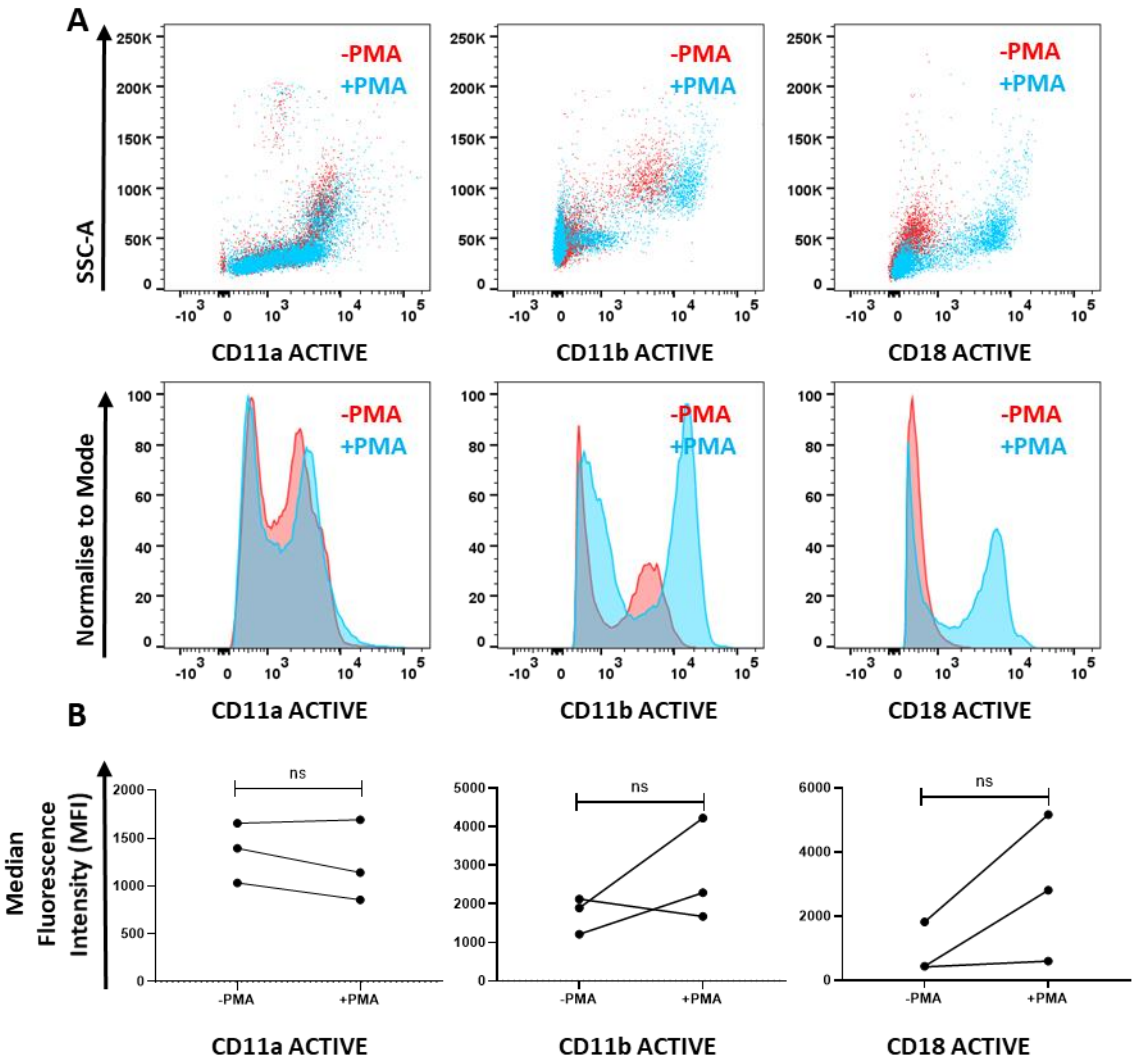

Figure 3.3 Effect of PMA stimulation on β_2 integrin activation

A healthy PBMC sample was stained for active CD11a, CD11b and CD18 respectively without or in the presence of 100ng/ml of PMA. A. Overlays of PMA-stimulated and unstimulated samples. Unstimulated cells are shown in red, PMA-stimulated cells are shown in blue, representative plots of both SSC-A (top) and histogram overlays (bottom) are shown for each active β_2 integrin subunit. Plots are representative of 3 donor samples in 3 independent experiments for each β_2 integrin subunit. B. Difference in MFI between 3 donor samples per β_2 integrin subunit, paired students t test.

3.3.4 Assessing steric hindrance and FRET

After ensuring activation-specific β_2 integrin staining can be detected and controlled for, it was then assessed if total and active integrin expression could be measured in the same sample. The challenge with this set-up is that an active subunit is the target of two antibodies simultaneously binding in close proximity. Furthermore, as α (CD11a or CD11b) and β (CD18) subunits are non-covalently associated with each other, up to four mAbs (two for determining total expression of the α and β subunits, and two for determining the active forms of these subunits) are vying for their epitope on the same adhesion protein complex. This could lead to loss or gain of signal either due to steric hindrance or fluoresecent resonance energy transfer (FRET), respectively, so both had to be kept in mind when designing and optimising this panel.

Steric hindrance describes the physical inaccessibility of antibody-binding sites due to another space-filling molecule, in this case the corresponding antibody, obstructing access to the binding site (Rubenstein and Leute, 1976). This would potentially lead to loss especially of the total signal, as activation-specific antibodies primarily bind in the β_2 integrin binding site only accessible when the respective integrin is in its extended form. This extension process rapidly increases binding affinity to its ligand, potentially making it a much easier target for activation-specific antibodies compared to total antibodies binding in an area which does not increase in binding affinity (Hughes and Pfaff, 1998) To assess if the respective total and active antibodies were susceptible to steric hindrance, double staining of both total and active integrin subunits was performed and compared to the respective single stained sample (for examples of CD11a, CD11b and CD18 see Figure 3.4A, B and C respectively). Signal loss as a percentage of the single stain was recorded in three separate healthy control samples, comparing both -PMA and +PMA samples to ensure that increased activation did not increase signal loss (see Figure 3.4D). A significant change in percentage of cells expressing either total or active β_2 integrin marker cannot be observed in any of the markers when stained simultaneously for active and total markers of CD11a, CD11b or CD18. Overall any differences in % seem to be between ±0.1-4.5%, with the highest recorded increase of signal in CD18 active at +11%. However, as this was only observed in one sample out of three and steric hindrance would not cause an increase in signal, this was not further followed up. In addition to % of cells expressing a marker after double staining, the staining brightness in the form of MFI was also considered (Figure 3.5). This is especially important as steric hindrance might occur on a % of cell

surface receptors rather than a % of cells. Figure 3.5A shows the single stains of total and active CD11a, CD11b and CD18 compared to their respective signal brightness in the double stained sample. While there is no visible signal loss in any of the active β_2 integrins, nor CD11b and CD18 total, CD11a total is higher in the single stain when comparing it to its respective double stain. This loss of signal brightness when staining total and active CD11a together is statistically significant in non-stimulated cells (Figure 3.5B), with cells stained with both antibodies having a 50-60% lower MFI than cells stained with the CD11a total antibody alone. Interestingly, this effect, while visible, is not statistically significant in the PMA-stimulated cells. This is puzzling as one could expect that higher activation due to PMA would increase steric hindrance and therefore CD11a total signal loss. However, when keeping in mind that PMA was less able to stimulate CD11a active compared to active CD11b and CD18, this could also point towards maximum activation being achieved even without PMA. In this case, one could speculate that PMA increased cycling rates of CD11a, which might in turn result in less CD11a total signal loss because inactive CD11a subunits are returned to the cell surface at a higher rate. It is also surprising that the loss of total CD11a signal does not affect active CD11a signal, as one might suspect. However, despite loss of signal, total CD11a can still be detected at high levels compared to an unstained control, suggesting detection of total CD11a on cells is still possible.

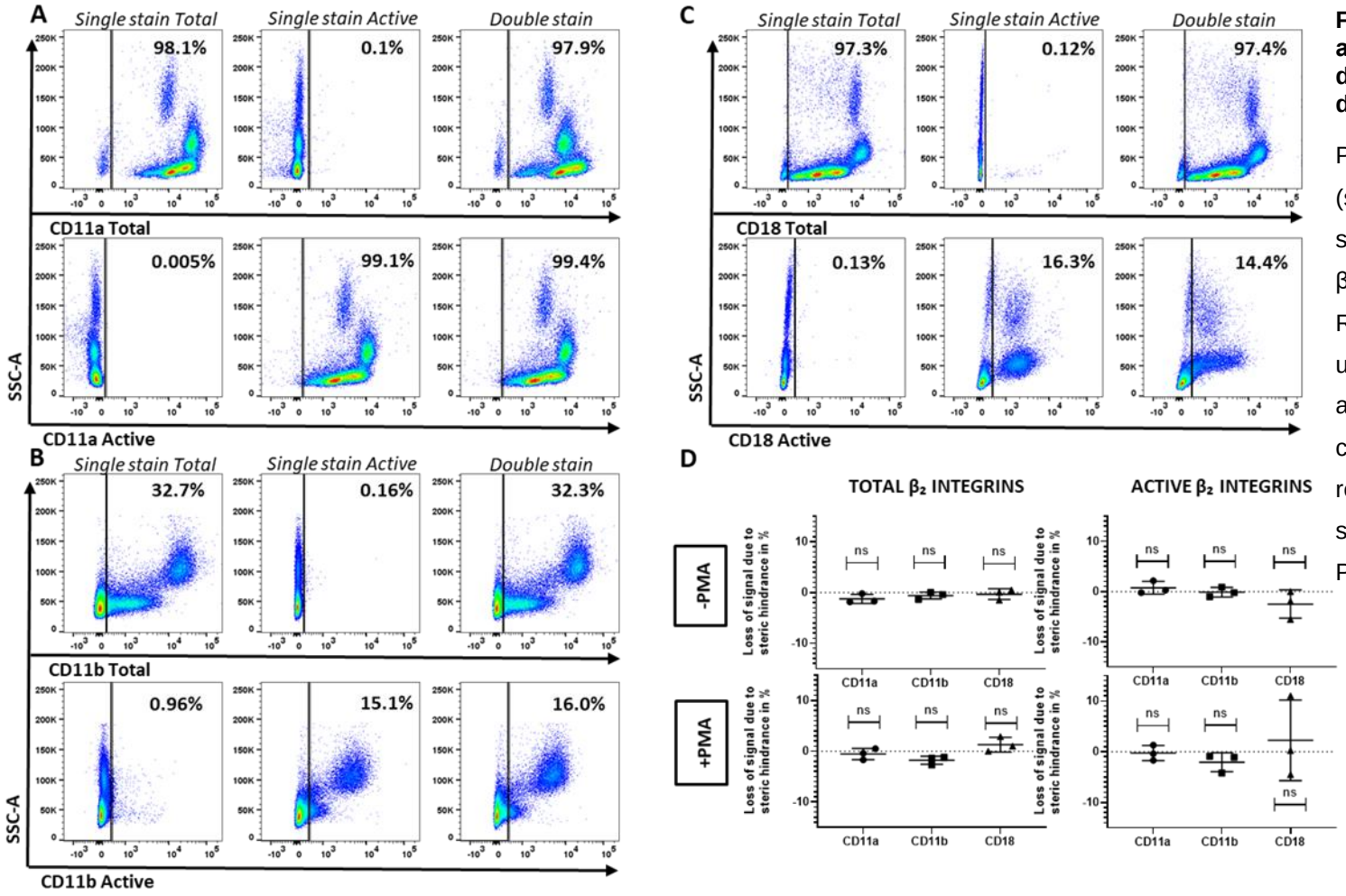

Figure 3.4 Staining for total and active β_2 integrins simultaneously does not lead to signal loss in % due to steric hindrance

PBMCs were stained with either total (single stain total), active (single stain active) or both total and active β_2 integrin antibodies (double stain). Representative plots show unstimulated CD11a (A), CD11b (B) and CD18 (C). D. Loss of signal was calculated by subtracting % of respective double stain from % of single stain with (+PMA) or without (-PMA) PMA stimulation. N=3.

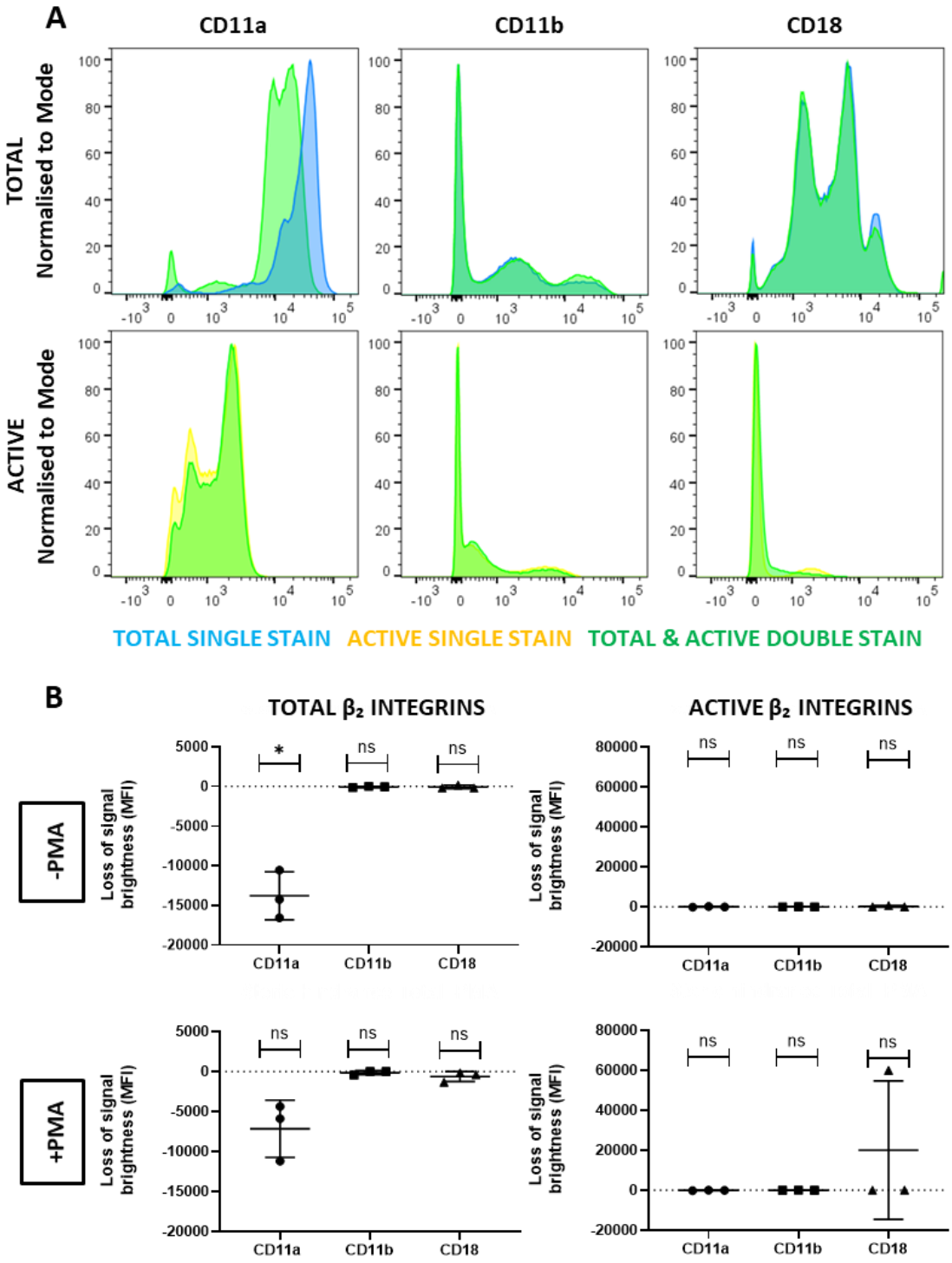

Figure 3.5 Loss of signal brightness due to steric hindrance

Data set shown is the same as in Figure 3.4, but in regards to signal brightness measured in MFI rather than %positive expression. A. Representative histograms show single total stain (shown in blue, top) and single active stain (shown in yellow, bottom) in comparison to respective double stain (shown in green). Only unstimulated samples are shown. B. Loss of signal brightness was calculated by subtracting MFI of respective double stain from MFI of single stain with (+PMA) or without (-PMA) PMA stimulation. Paired student's t test comparing single and double stain, N=3.

FRET describes the non-radioactive energy transfer between two fluorochromes where the emission energy of one corresponds with the activation energy of the other, resulting in an increase of signal (Horváth et al., 2005). While this can be exploited for flow cytometry and microscopy purposes, in this case FRET pairs were deliberately avoided when choosing which colours to use for the respective active and total antibody fluorochromes. As published literature has shown that the distance between associated CD11b and CD18 subunit is only 8.2nm (Fan et al., 2019) and FRET can occur over short distances from 1-10nm (or 10-100Å), care was also taken that α-subunit and β-subunit antibodies were not FRET pairs.

Overall, no significant signal loss occurs in percentage of cells expressing one of the β_2 integrin subunits due to steric hindrance. However, when considering loss in signal brightness, CD11a total showed considerable significant signal loss, which was not shared by any of the other total or active subunits. This is a valid concern, as loosing over half of the signal brightness of CD11a total suggest that signal loss due to steric hindrance occurs frequently. On the other hand, the high consistency in % of MFI signal lost between repeats using different healthy donors suggests that comparing a double-stained (i.e. total/active CD11a) sample to another double-stained sample would still give accurate results. Especially considering the absence of signal loss in the other markers considered, the ability to still record total CD11a expression levels although at lower levels and the importance of CD11a for this project, it was decided to proceed with simultaneous staining for active and total subunits of CD11b, CD18 and indeed CD11a. However, loss of CD11a total brightness due to steric hindrance has to be kept in mind when designing experimental controls.

3.3.5 Active integrin panel: Titration of antibodies

After having established that total and active β_2 integrins can indeed be stained together, although with some signal loss of CD11a total, I then turned to the development of an active integrin panel. Before the panel could be used to stain a healthy human sample, all antibodies had to be titrated for optimal performance on freshly isolated PBMCs (Hulspas et al., 2009).

Multicolour dot plots of each titration were compared to a histogram overlay of all four titrations. In addition, a titration curve was attained by calculating a stain index (SI) for each titration and plotting them against the increasing dilutions of antibodies. All information was taken into account when selecting the appropriate titration. Some

cases warranted choosing the titration with a clearly delineated positive population over the highest SI value. As an example, the dot plot, histogram overlay and titration curve of CD11b total are presented in Figure 3.6. Figure 3.6A shows a representative multi-colour flow plot and histogram of positive CD11b total staining (1:100 dilution), on healthy PBMCs where debris has been gated out. Histograms of different dilutions, 1:10, 1:50, 1:100, 1:200, were overlaid and signal brightness as well as cell number stained positive were considered (Figure 3.6B). 1:10 dilution showed the lowest signal both in brightness and cell numbers stained, followed by 1:50. 1:200 shows highest staining brightness, but 1:100 shows the largest number of cells stained positive for total CD11b. Then the respective SI values were calculated using the mean of the positive and negative populations as well as the SD of the negative population (Figure 3.6). SI values remained stable before falling off at 1:200, signalling that staining efficiency is compromised at this titration. Manufacturer's recommendation suggested 1:10 dilution, however 1:100 dilution shows most stained cells in overlay and highest stain index before signal intensity falls off. The 1:100 dilution was chosen for the CD11b total antibody. Optimal titrations for all antibodies can be found in Table 3.1.

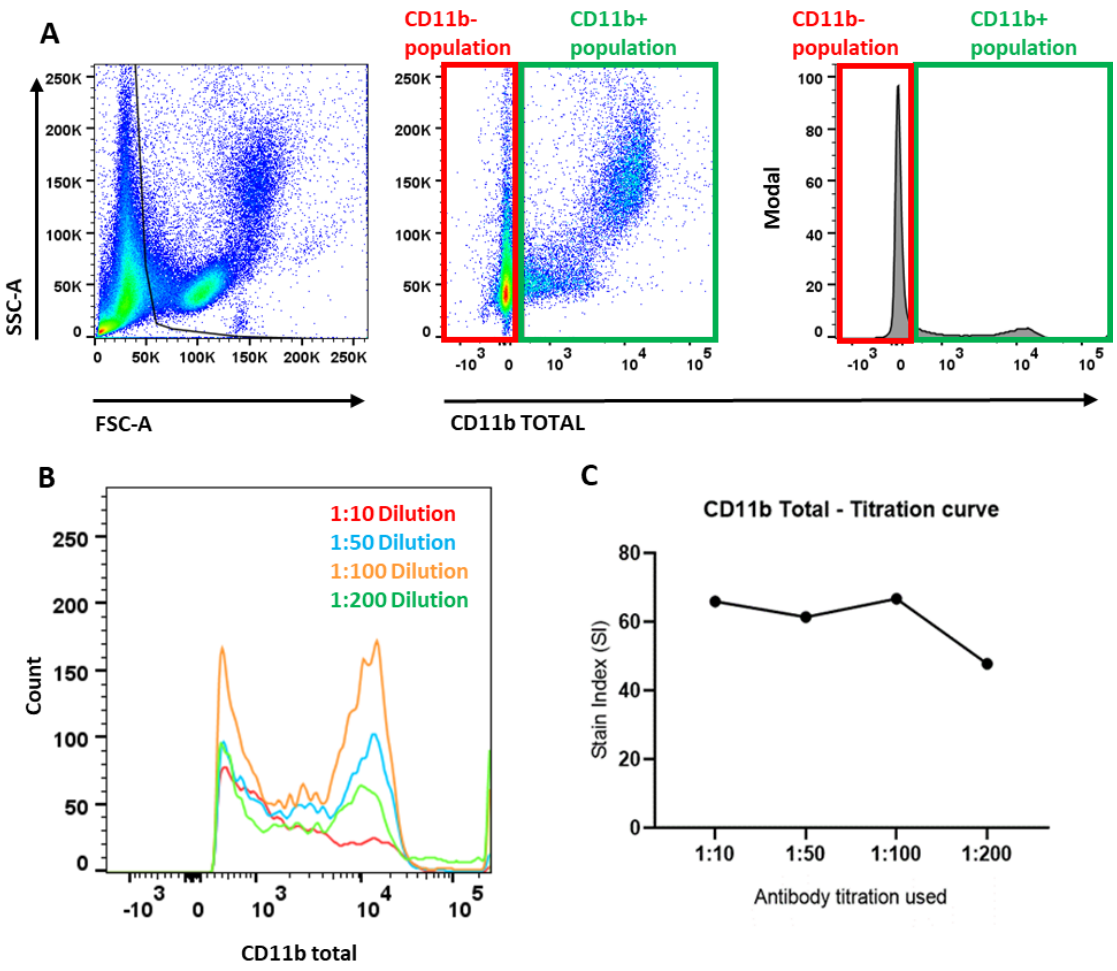

Figure 3.6: Example titration procedure using CD11b total antibody.

Healthy unstimulated donor PBMCs were stained with different dilutions of CD11b total antibody for 30min. **A.** Example of multi-colour flow plot for CD11b at 1:100 titration, clearly identifying positive (CD11b+) and negative (-ve) population. **B**. Histogram overlay of four titrations: 1:10, 1:50, 1:100 and 1:200

C. Titration curve of CD11b total antibody. The stain index (SI) was calculated using this formula:

$$SI = \frac{\mu_{pos} - \mu_{neg}}{2 \times \sigma_{neg}}$$

Where μ_{pos} and μ_{neg} are the mean of the +/-ve population respectively and σ_{neg} is the standard deviation of the –ve population based data spread in flowjo. N=1, although further repeats were necessary in some cases to settle on a final suitable concentration.

Antigen	Clone	Fluorochrome	Company	Optimal Titration	Final concentration
CD18 total	*6.7*	BV421	*BD Biosciences*	1:20	10µg/ml
CD18 active	*m24*	AF 488	*BioLegend*	1:10	10µg/ml
CD11a total	*HI111*	PE	*BioLegend*	1:50	2µg/ml
CD11a active	*MEM-83*	AF 700	*Novus bio*	1:100	9.5µg/ml
CD11b total	*ICRF44*	BV785	*BioLegend*	1:100	1.5µg/ml
CD11b active	*CBRM1/5*	APC	*BioLegend*	1:50	4µg/ml
CD11c	*BU15*	APC-Cy7	*BioLegend*	1:100	4µg/ml
CD141	*M80*	PE-Cy7	*BioLegend*	1:50	4µg/ml
HLA-DR	*G46-6*	BV480	*BD Biosciences*	1:50	2µg/ml
CD1c	*L161*	PerCP-Cy5.5	*BioLegend*	1:100	2µg/ml
CD14	*M5E2*	BUV737	*BD Biosciences*	1:50	1µg/ml
CD16	*3G8*	PE-Dazzle594	*BioLegend*	1:200	0.25µg/ml
CD45	*HI30*	BUV395	*BD Biosciences*	1:50	2µg/ml
Lineage:		PE-Cy5.5			
CD19	*SJ25C1*		*eBioscience*	1:50	0.1µg/ml
CD20	*2H7*		*eBioscience*	1:50	0.3µg/ml
CD56	*CMSSB*		*eBioscience*	1:100	0.06µg/ml
CD3	*SK7*		*eBioscience*	1:100	5µg/ml
AADvanced dead cell stain			*Thermo. Scientific*	1:500	1µM

Table 3.1 Antibodies used in active β_2 integrin flow cytometry panel

3.3.6 Gating strategy

After determining optimal antibody titration for this application, a gating strategy was devised to distinguish the different cells of interest (cDC1, cDC2 and pDCs, $CD14^+$ and $CD16^+$ monocytes) and measure β_2 integrin expression and activation on their respective surfaces.

The full gating strategy is presented in Figure 3.7, after a panel designed by Dr. David McDonald, Experimental Scientific Offier, Flow Cytometry Core Facility, Newcastle University. Markers chosen were based on a panel that had been successfully used to distinguish DCs in the lab before (Wood et al., 2019). First, cell debris and doublet cells were excluded, as well as all dead cells and $lineage^+$ cells. Lineage markers included CD3, to exclude both $CD4^+$ and $CD8^+$ T cells, as well as CD19 and CD20, to exclude B cells, and lastly CD56, to exclude NK cells. Then all $CD45^+$ cells are considered in respect to their HLA-DR and CD11c expression, identifying the HLA-DR high but $CD11c^{neg}$ pDCs and a HLA-DR^+CD11c^+ APC population. These APCs were split up into classical and non-classical monocytes, using high CD14 and high CD16 expression respectively. Lastly $CD14^{neg}CD16^{neg}$ cells were assessed according to their CD1c and CD141 expression, with cDC1 cells expressing high CD141 and cDC2 cells expressing high levels of CD1c.

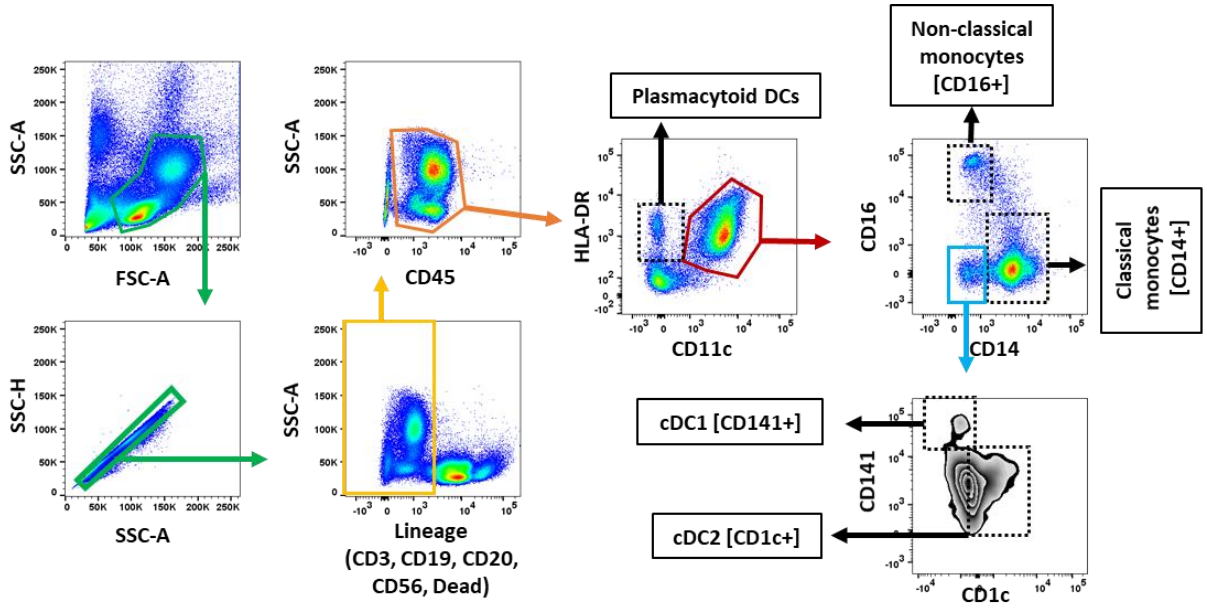

Figure 3.7 Gating strategy for APCs in active β_2 integrin panel

Demonstration of gating strategy on healthy control, to distinguish between different cell types capable of presenting antigen, including CD14+ monocytes, CD16+ monocytes, cDC1 DCs, cDC2 DCs and pDCs. Representative plots are from a healthy control PBMC sample. This gating strategy was used throughout Chapter 5 for PBMC, SF and ST analysis.

3.3.7 Experimental controls

Experimental controls were included to assess validity of each experiment. In addition to a completely unstained control, a 'negative' control where no β_2 integrin subunits were stained for was included to yield a baseline for integrin expression on the background of the lineage markers. As detailed above (Figure 3.3), a +PMA positive control was included. Lastly, an isotype control was included, where total β_2 integrin antibodies were stained as normal and individual active integrin antibodies were replaced by matched isotype controls with the fluorochromes and concentrations corresponding to the active β_2 integrin antibodies. This control also doubles a single stain reference for CD11a total, enabling us to validate any future results with the total stain unaffected by steric hindrance. While this does not satisfy our aim to detect total and active β_2 integrin subunits in the same sample at the same time, it can still provide a frame of reference how much signal was lost to steric hindrance and if this affects any results observed in the samples stained with both active and total CD11a simultaneously.

An example of all experimental controls in a healthy control sample can be seen in Figure 3.8. The negative control is void of all total and active integrin staining (2nd row),

the isotype control is void of active integrin staining only (3rd row), and the +PMA control (4th row) shows increased staining compared to staining for all β_2 integrin antibodies in the absence of stimulants (experimental condition, 1st row). Gates shown are based on the experimental condition (1st row) to demonstrate respective differences in β_2 integrin staining visible between different controls and experimental condition.

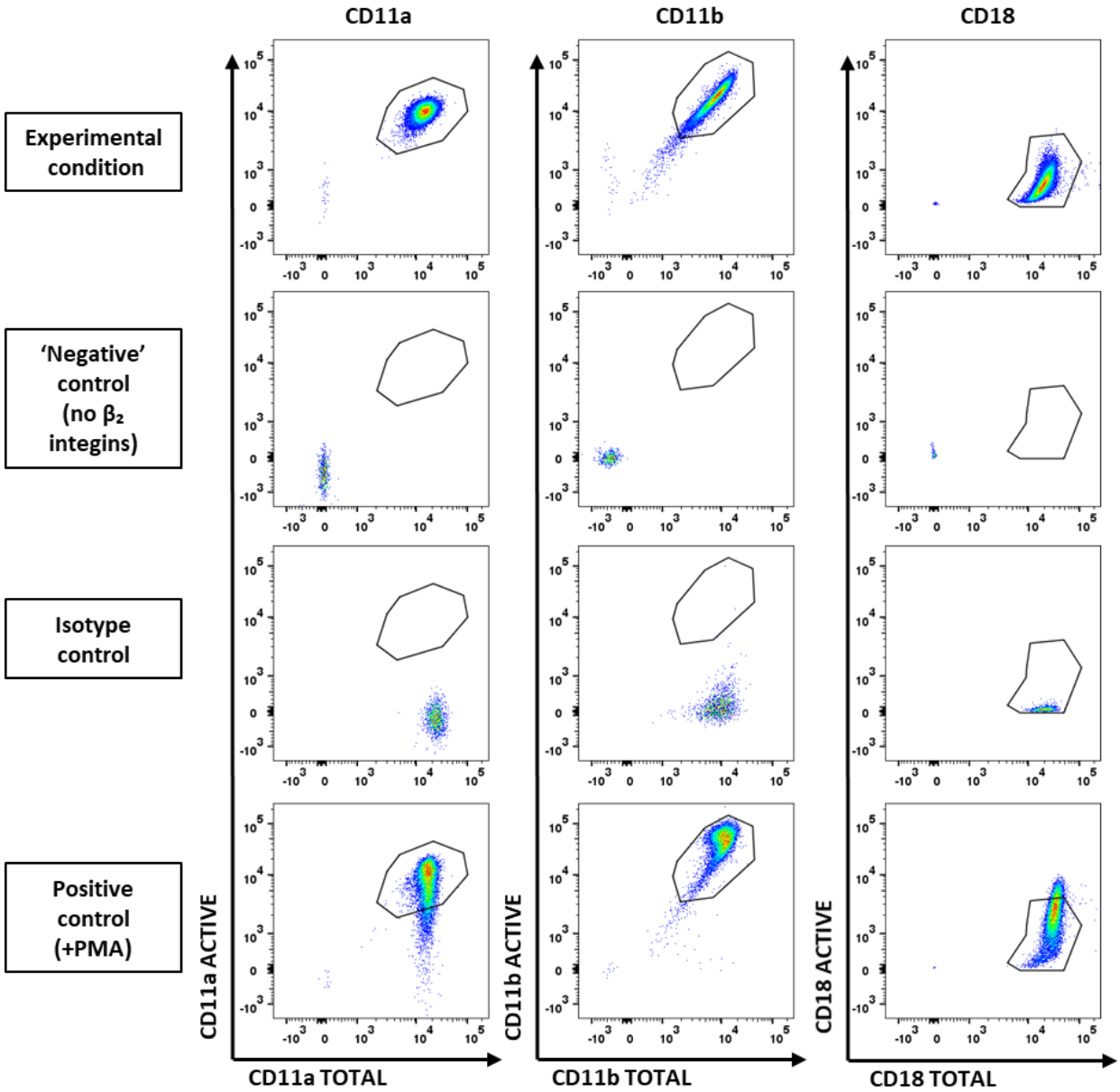

Figure 3.8: Experimental controls used in active β_2 integrin staining panel

Total and active CD11a, CD11b and CD18 are shown in the experimental condition (1st row), as well was three appropriate experimental controls below it. The negative control (2nd row) was stained with the lineage markers but without any total or active β_2 integrin antibodies. The isotype control (3rd row) was stained with the lineage markers and total β_2 integrin antibodies, but active β_2 integrin antibodies were replaced with matched isotype controls. The positive control (4th row) was stained exactly as the experimental condition but in the presence of 100ng/ml PMA. Representative plots from a single healthy control sample are shown, gates shown were drawn around experimental condition and copied to other control to demonstrate their differences.

3.3.8 Potential of using active integrin panel on human tissue

As this panel was developed for the investigation of β_2 integrin expression and activation states on APCs in the context of the autoimmune disorder RA, the joint-specific nature of the disease had to be taken into account. To this end, it was tested if the panel could not only assess β_2 integrins on cells isolated from PB and SF, but also on cells that were present in ST. As ST requires both mechanical and enzymatic digestion to yield a single cell suspension viable for flow cytometry analysis, it was therefore important to test if β_2 integrins would be cleaved or otherwise altered during the lengthy tissue digestion protocol.

To test for this eventuality without utilising precious synovial tissue samples, PBMCs were isolated and treated with the enzymatic digestion protocol optimised for ST (Wood et al., 2019). While ST is subjected to the enzymatic digestion for up to three cycles a 45min, PBMCs were only subjected to one round of digestion with the same concentrations used for ST of 15µg/ml Liberase and 30µg/ml DNAse for 45min at 37°C, as they already were in the single cell suspension the digestion is supposed to achieve. PB and ST cell exposure to the digestion enzymes is, therefore, assumed to be similar. Figure 3.9 shows the result of this investigation, with an example overlay of β_2 integrin staining in undigested and digested PB samples (Figure 3.9A). No significant signal loss occurs in any of the markers but CD18 total (Figure 3.9B), which is significantly lower in digested sample compared to undigested sample. On the other hand, CD18 active signal is not affected by the digestion process. CD11a total surprisingly showed an increase in the marker in one experiment, but this appears to be an outlier.

Overall, enzymatically digesting PBMCs does not cleave the majority of our β_2 integrins of interest off cell surfaces and staining of all subunits considered stays viable. The exception to this is CD18 total which is significantly reduced in digested samples. However, this is not mirrored in signal loss of total CD11a or CD11b, suggesting that this is a direct effect on CD18. It is however also possible that digestion enzymes preferentially cleave CD11c or CD11d, which would in turn cause the loss of CD18 total staining. Lastly the increase in sample processing time due the digestion step might have a different effect on CD18, or indeed CD11c and CD11d, than on CD11a and CD11b. If using this flow cytometry panel on ST samples, direct comparison to blood or other tissues would have to be exercised with caution due to the potential effect of the digestion process on CD18 total. As SF samples do not undergo a

digestion protocol, it can therefore be compared directly to PB in a more straightforward way.

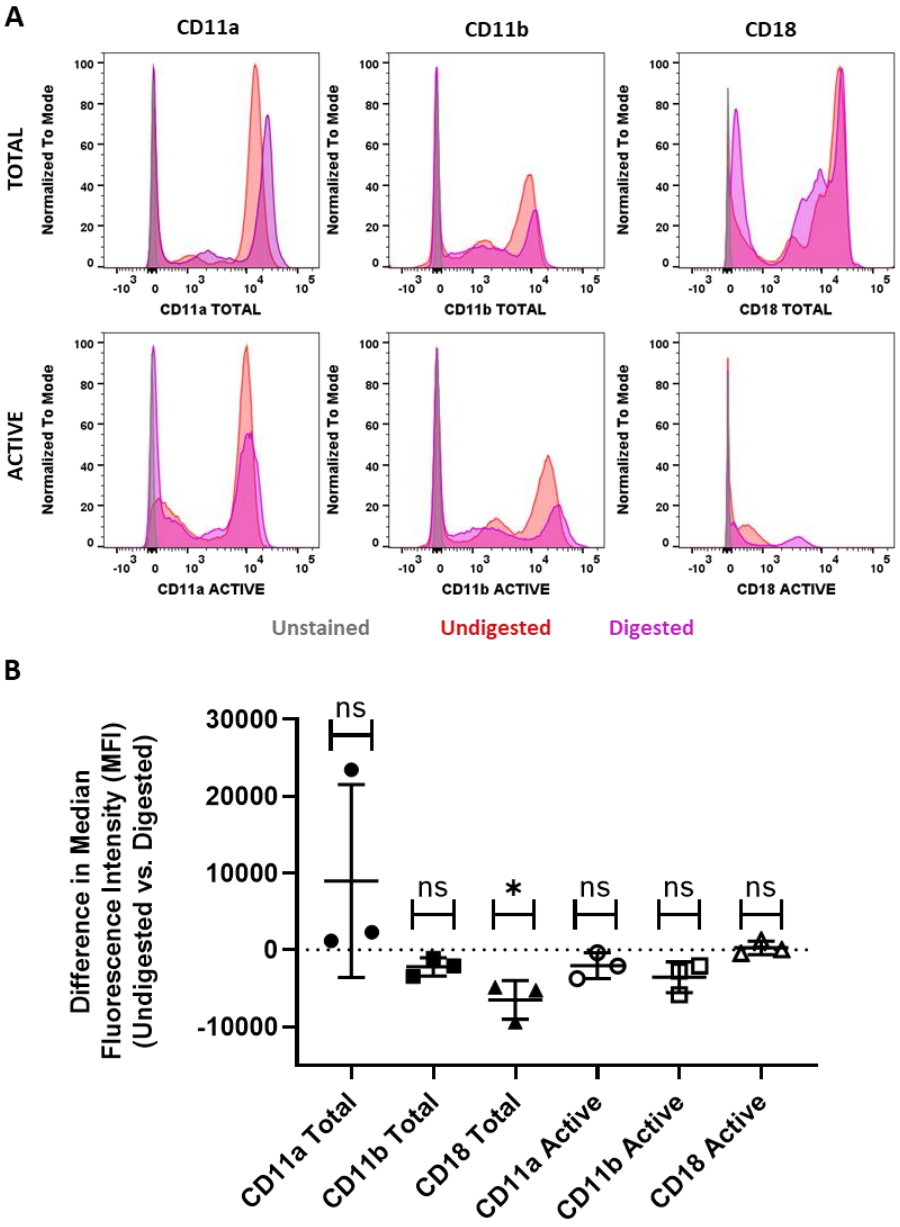

Figure 3.9: Effect of ST digestion protocol on total and active β_2 integrin staining

Healthy PBMCs were either kept on ice or subjected to a 45min digestion protocol in a shaking incubator at 37°C in the presence of 15µg/ml Liberase and 30µg/ml DNAse. After washing digestion enzymes off, undigested and digested samples were stained with the full active β_2 integrin panel. Cells shown are LIN-CD45+HLA-DR+. **A.** A representative overlay of undigested (shown in red), digested (shown in purple) and unstained control cells (shown in grey) in respect to their total and active β_2 integrin expression. Statistics shown were determined between undigested and digested sample MFI signals using a paired student's t test. N=3. **B.** Difference between MFI values of undigested and digested samples. A negative value signifies a loss of signal; a positive value indicates that signal has been increased due to digestion process.

3.3.9 The effect of sample preservation on active integrin staining

Circumstances of clinical research, including use of biobank samples and short-term unavailability of flow cytometers due to technical problems, sometimes make the use of sample preservation for later analysis of samples necessary. As the β_2 integrin activation panel was meant to be as widely usable as possible for potential future applications, the effect of common sample preservation methods on integrin expression and activation was assessed. These were (i) paraformaldehyde (PFA) fixation of stained samples; and (ii) freezing of PBMCs. This was of importance as available literature suggested that integrin expression and especially activation, which can occur on very short timeframes, can be heavily influenced by the medium surrounding it, such as in the case of cation availability (Zhang and Chen, 2012).

First, the effect of PFA-fixation, which is a widely accepted method for preserving flow cytometry samples for up to a week after staining (Lanier and Warner, 1981), on active β_2 integrin expression was assessed. For this, PB samples were stained with total and active β_2 integrin antibodies (CD11a, CD11b and CD18) and split in two, one to be analysed immediately and one to be fixed using 1% PFA, which was not washed off before flow cytometry analysis 6 days later. As visible in Figure 3.10, very little signal brightness was lost after 6 days of PFA-fixation. Neither total nor active CD11a, CD11b or CD18 showed any significant loss in signal brightness. Interestingly, CD18 total increased in signal brightness in two out of three repeats, although the increase was not significant. It is not likely that this increase is due to an increase in autofluorescence due to PFA fixation (Stewart et al., 2007), as such autofluorescence would affect colours in the green/yellow laser range, such as FITC, and not BV421, which is the fluorochrome used for CD18 total. Interestingly, active CD18 shows no significant difference in signal brightness between freshly stained and fixed samples, but all three repeats show variation in the percentage of cells positive at a specific brightness, as visible in the larger proportion of fixed cells high in active CD18 expression (Figure 3.10A). This change in proportions is however not mirrored in active CD18 MFI values, which could suggest some cell populations might be more prone to being gated out as debris after the fixing process.

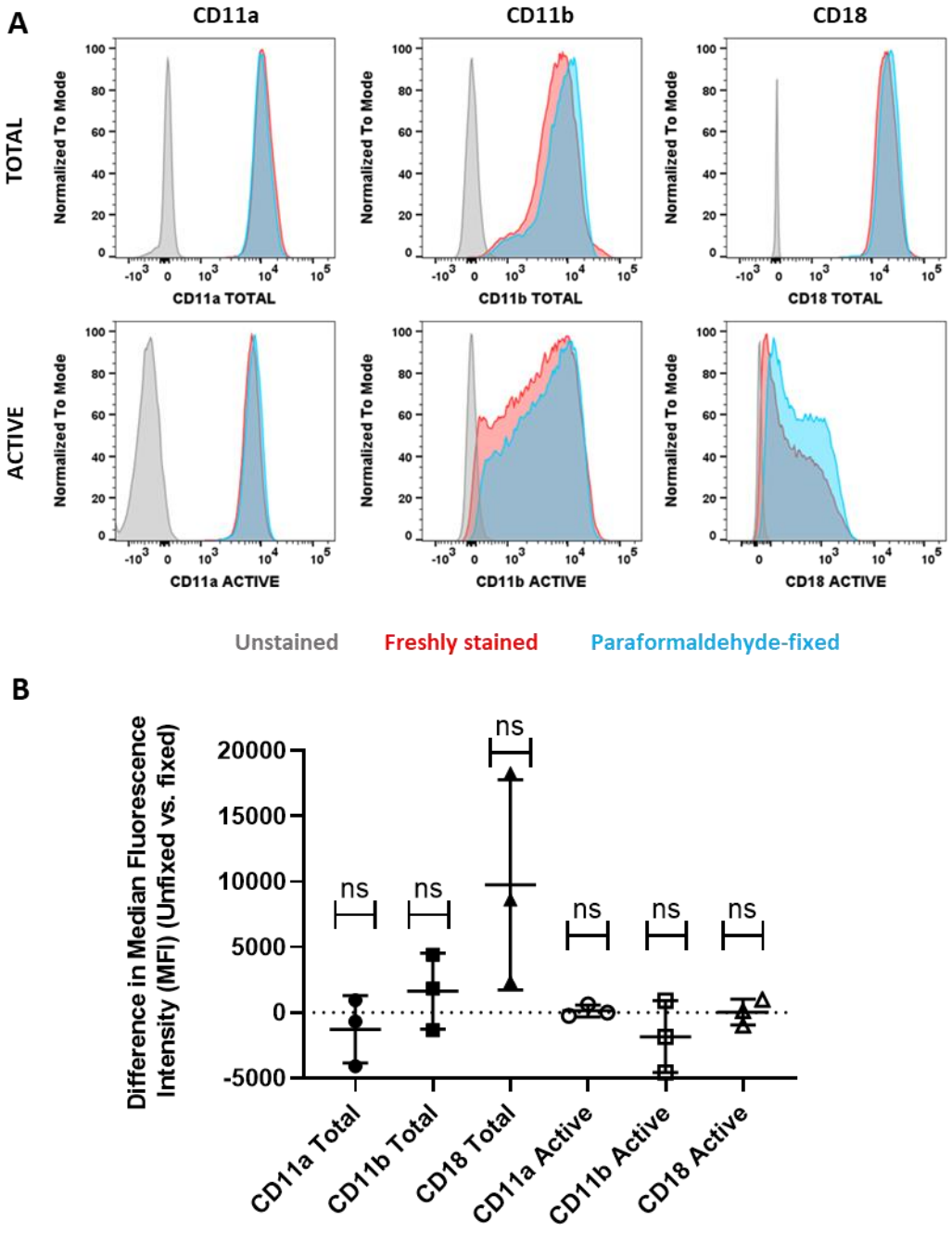

Figure 3.10: Effect of paraformaldehyde-fixation on total and active β_2 integrin staining

A healthy control PBMC sample was stained with the full active β_2 integrin panel and split into two, one to be acquired immediately, while the other was fixed using 1% PFA and acquired 6 days later. Cells shown are LIN-CD45+HLA-DR+. **A.** A representative overlay of freshly stained (shown in red), paraformaldehyde-fixed (shown in blue) and unstained control cells (shown in grey) in respect to their total and active β_2 integrin expression. **B.** Difference between MFI values of fixed and unfixed samples. A negative value signifies a loss of signal; a positive value indicates that signal has been increased due to the fixation process. Statistics shown were determined between freshly stained and PFA-fixed MFI signals using a paired student's t test. N=3.

In conclusion, fixation does not alter β_2 integrin staining in any of the active or total β_2 integrins, CD11a, CD11b or CD18. However, reasons for the high CD18 total staining in two out of three samples could not be completely elucidated. It was therefore decided to acquire samples immediately after staining and only rely on fixing samples in the event of an unusable flow cytometer. In this eventuality, measurements for total CD18 staining would not be included in the analysis to avoid skewing of the data.

While I had access to fresh samples from Rheumatology clinics and healthy donors alike, samples collected for disease-specific biobanks are frequently stored in frozen form. To explore the feasibility of using such biobank samples for active integrin staining, PBMCs were isolated and stained a proportion of cells immediately, whereas the rest was frozen for 2-4 weeks and stained after thawing.

Results of this enquiry can be seen in Figure 3.11, with an overlay of a healthy control sample that was either freshly stained or freeze-thawed before staining. Overall, there is no significant increase or decrease in any of the β_2 integrin subunit signals when comparing freshly stained to thawed samples (Figure 3.11B). However, when looking at the histogram overlays (Figure 3.11A), freshly stained and freeze-thawed samples do not overlap neatly, even though the differences are not significant. One possible explanation for this could be the freezing protocol utilised, which suspends cells in 10%DMSO 90%FCS. Both the addition of a high concentration of DMSO and the increased availability of FCS might cause subtler variations in β_2 integrins expressed and activated on the cell surface. Furthermore, the thawing process, which involves 3 washing steps to ensure DMSO is removed fully, could equally increase cell activation and increase cell death, changing the composition of the live cells gated on.

To conclude, neither fixing nor subjecting cells to a freeze-thawing cycle caused significant cleavage of β_2 integrins off the cell surface or deactivation of subunits, making both the use of fixed or biobanked samples possible with this panel. However, as small changes in both total expression and activation of β_2 integrins do seem to occur, it would be best practice to keep conditions between samples as similar as possible. As my project did not involve using biobanked samples, it was therefore decided to stain all samples fresh on the day of collection to avoid introducing further variability to data.

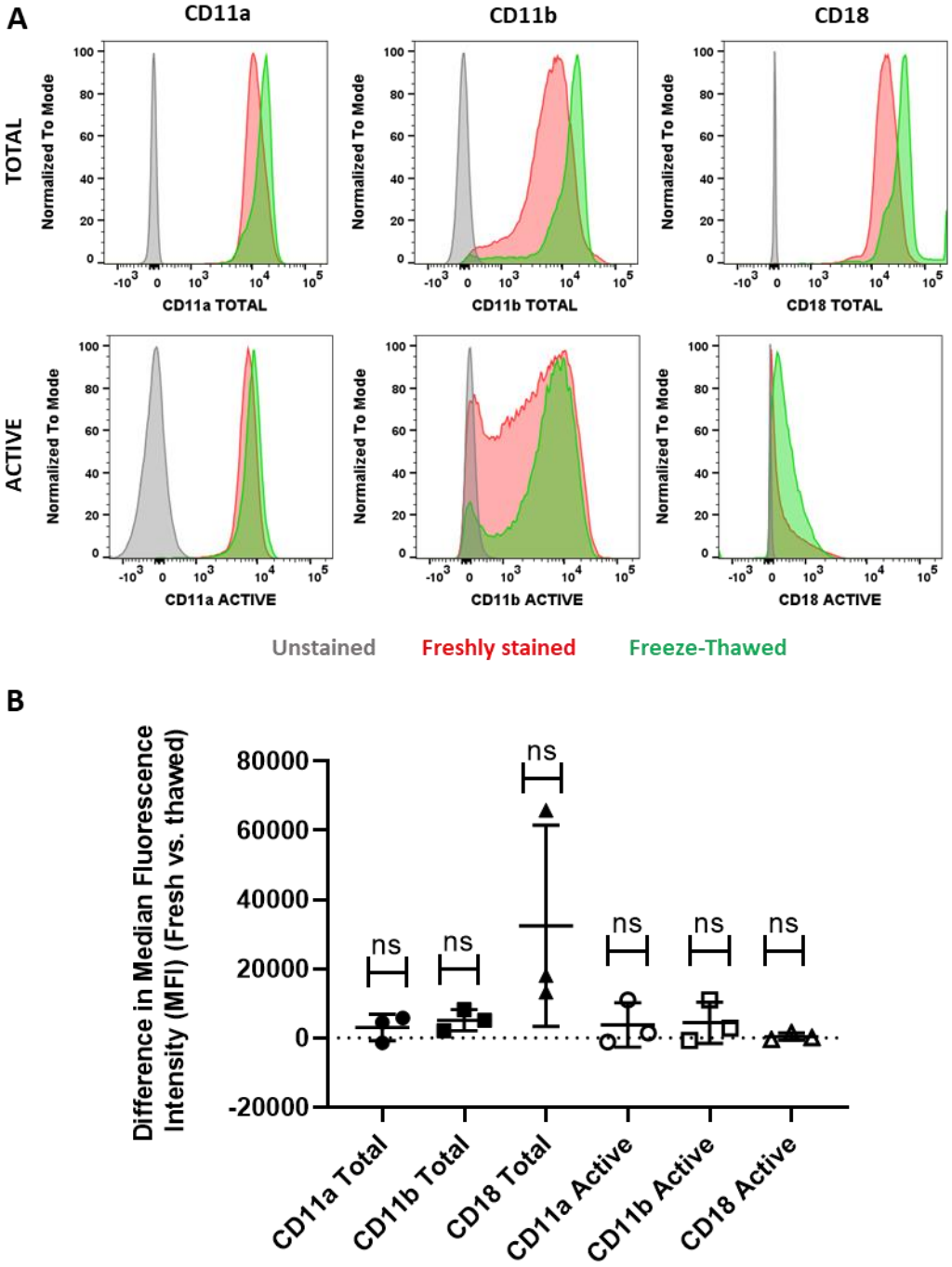

Figure 3.11 Effect of freeze-thawing on total and active β_2 integrin staining

Healthy control PB samples were stained with β_2 integrin antibodies (active and total) and split in two parts, one to be analysed on the BD LSR Fortessa immediately, while the other was fixed using 1%PFA and analysed a week later. Cells shown are LIN-CD45+HLA-DR+. **A.** A representative overlay of freshly stained (shown in red), freeze-thawed (shown in green) and unstained control cells (shown in grey) in respect to their total and active β_2 integrin expression. **B.** Difference between MFI values of freshly stained and freeze-thawed samples. A negative value signifies a loss of signal; a positive value indicates that signal has been increased due to the cryopreservation. Statistics shown were determined between freshly stained and freeze-thawed MFI signals using a paired student's t test. N=3.

3.4 Discussion

The aims of this chapter were the optimisation of detecting both total and active β_2 integrins on the surface of APCs. In this chapter, I have developed a flow cytometry panel that can measure expression of not only total but also active β_2 integrin subunits CD11a, CD11b and CD18 in a defined group of APC populations of interest. The staining protocol was optimised in respect to staining time and temperature and signal loss due to steric hindrance or FRET was assessed to be kept to a minimum. Positive and negative controls for the panel were developed. Furthermore, durability of this panel across cells from different sources or from different sample preservation methods was revealed, to ensure that the panel can be utilised as broadly as possible. This attempt to assess expression and activation on a variety of different APC populations present in blood holds both caveats and strengths, which are important to consider when evaluating any novel knowledge gained from this approach.

3.4.1 Limitations of the active β_2 integrin staining protocol and panel

There are several limitations of both the β_2 integrin staining procedure and the active integrin panel that need to be considered.

Macrophages

While the active integrin panel distinguishes a range of different APCs, one cell type is markedly absent: the macrophage. Although these cells could contribute to the initiation of RA, they are better known for their roles in pathology and disease activity (Janossy et al., 1981; Kinne et al., 2000). As an example, Haringman and colleagues (2005) showed that the number of macrophages present in the synovial sub-lining is a potent biomarker for disease activity and correlates well with successful therapeutic intervention. The option of including macrophages in our analysis was explored by trying to free up a flow cytometry channel to detect autofluorescence. In addition to being high in CD14 and HLA-DR, high autofluorescence of macrophages is used in the literature to distinguish them from monocytes (Wood et al., 2019). However, CD18 active, which was not available in a range of colours, already occupied the FITC channel and adding further parameters to a 14-colour flow cytometry panel proved difficult due to laser and fluorochrome limitations. The addition of further markers to allow the identification of macrophages would have had a negative impact on other, more important, aspects of the panel. Additionally, as the primary aim is to use this

panel in PB and SF where macrophage numbers are absent or low, respectively, exclusion of macrophage markers was thought not to impede the analysis significantly.

Integrin subunits

Other markers that had to be left out due to panel size restrictions, CD11c and CD11d, could similarly be considered a caveat. However, published literature points especially to the similarities as well as the differences between CD11a and CD11b. Research shows that adhesion to endothelial cells is 50% due to CD11a and 50% due to CD11b, while blocking CD11c had no effect on adhesive capabilities of cells (Lo et al., 1989). Similarly, increasing the amount of active CD11a (Balkow et al., 2010) and active CD11b (Varga et al., 2007) on DCs was found to inhibit their ability to activate T cells. While this highlights the overlapping functions of CD11a and CD11b, published literature also shows functions that are unique to either α-subunit respectively. Especially mouse KO studies have been helpful in elucidating β_2 integrin α-subunit specific functions. For example, CD11b is important for neutrophil adhesion and degranulation, but insignificant for cell emigration into tissues, which is mediated by CD11a (Lu et al., 1997). However, elucidating β_2 integrin α-subunit specific functions is very reliant on mouse KO models of the individual subunits. This means that there is very little knowledge about how CD11a and CD11b interact in a healthy person compared to someone who has a chronic inflammatory condition. Therefore, comparing CD11a and CD11b specifically is of great value to contribute to our knowledge of their similarities and differences.

Integrin signalling

Another aspect to keep in mind when interpreting results is that there are several aspects of integrin signalling that cannot be assessed using the active integrin flow cytometry panel. For example, as mentioned above, integrin clustering rather than its affinity (conformational state) can be as important for function as activation. Any results regarding β_2 integrin activation status will therefore have to be carefully examined to not draw conclusions prematurely. Additionally, measuring β_2 integrins using flow cytometry is quantitative on the population level and not on a cell-by-cell basis. This might potentially lead to misunderstandings or misinterpretation of results. Rather than measuring total number of active or total integrins per cell, this method can only state signal brightness or the proportion of cells that are positive for a marker. To give an example, published literature states that only ca. 1% of the $\alpha_1\beta_5$ integrins is in its active

conformation on cell surfaces under steady conditions (Li et al., 2017), while my results record levels of active integrins between 15-99% (Figure 3.4A, B and C). While this disparity could be due to differences between β_2 and β_1 integrin subfamilies, it is also due to the fact that this method cannot measure the exact number of integrin receptors per cell and, instead, measure the percentage of cells that have at least one active integrin on their surface. It is possible they have more, due to the potential removal of Ca^{2+} by EDTA (present in FACS buffer), which plays an important role to restrict integrin activation in blood (Dransfield et al., 1992), but this can only be measured non-quantitatively using the respective signal brightness. Therefore, all results gained from the active integrin flow cytometry panel will be compared to the same marker on different cell types or clinical samples.

These limitations discussed here should be kept in mind when assessing future results generated using the active flow cytometry panel. However, the strengths of this panel far outweigh its minor limitations.

3.4.2 Strengths of the active β_2 integrin staining protocol and panel

While there are some accepted limitations associated with this newly developed flow cytometry panel, it is also important to point out the strengths and scientific potential of this method.

Total and active integrins

Quantification of expression levels of both the total and active forms of the same β_2 integrin subunit in the same sample is of immense value to the scientific community. To my knowledge, this has not been done before. This is especially important as our knowledge of how integrin activation might differ between cell types or in disease is very limited. For example, differences in β_2 integrin expression or activation between cell types might contribute to our understanding of their distinct and overlapping functions. Furthermore, balance of β_2 integrin expression or activation might be altered in disease and either contribute to or further exacerbate symptoms. Assessing total and active β_2 integrins in the same sample will therefore give us a breadth of information not accessible before. It also provides valuable proof of concept, that will allow the practice to become more widespread, providing valuable further information on how β_2 integrin activation is regulated in different cell types.

It also important to mention here the use of several controls that strengthen any results generated using the active integrin panel. An appropriate positive control was validated in the form of PMA activation, and the negative control was expanded to include an isotype control that is necessary due to the temperature-sensitive nature of detecting activated β_2 integrin subunits, especially CD11b and CD18. Although some signal loss of CD11a total due to steric hindrance occurs when staining for total and active CD11a simultaneously, the isotype control includes a single stain of all total antibodies. Therefore, any results involving CD11a total can be confirmed with the single-stained CD11a total signal, further strengthening any findings.

APC populations of interest

Another strength of the active integrin panel is the wide range of APC populations it will assess. This is especially important as, due to the quantification of populations rather than single cells described above, comparing different specific cell subtypes (such as cDC1 vs. cDC2) will be valuable in understanding how these cells differ from each other in regards to their β_2 integrins. pDCs are similarly interesting, as they primarily respond to viral infections rather than activating naïve T cells. Comparing how conventional DCs differ from pDCs therefore might give insights into how β_2 integrins might contribute to these different ways of linking the innate to the adaptive immune system. While this project is focused on DCs in particular, monocytes also present antigen, although to a lesser level. Including them in our analysis will therefore give us valuable insight into how β_2 integrin surface expression and activation relates to a cells ability to adhere to β_2 integrin ligands, migrate and contribute to T cell activation.

Broad applications of the active integrin panel

Samples that were previously frozen or fixed after staining using PFA show no significant signal gain or loss in any of the β_2 integrin subunits assessed (Figure 3.10, Figure 3.11). This means that the panel could potentially be used on any biobanked disease samples, making it possible to validate if effects are disease-specific or in the broader context of inflammation. The possibility of fixing samples without significant signal loss is similarly important, as it ensures that staining remains viable if the flow cytometer usually used has technical problems. While not feasible in the context of this project, it would also allow staining to be done freshly on samples remotely, before analysis of the fixed sample after transport back to appropriate facilities. This might be especially important in diseases where sample collection involves long-distance travel.

Furthermore, it could be demonstrated that no signal loss occurred in any of the subunits except CD18 total when using a tissue digestion protocol (Figure 3.9). This means that the panel can not only be used in PB, SF or cells isolated from other bodily fluids, but also on cells isolated from tissues. This could be especially interesting when investigating the effect of β_2 integrins on highly tissue-specific conditions, such as RA or celiac disease. As β_2 integrins might also have an important role in cancer (Bednarska et al., 2016), this also suggests that the panel could be used on tumour biopsies to compare to healthy tissue samples and assess β_2 integrin contribution to tumour maintenance.

3.4.3 Differences between β_2 integrin subunits identified

Several structural differences between β_2 integrin subunits became apparent during the staining optimisation process. Mostly these differences exist between CD11a and both CD11b and CD18, which seem to behave more similarly to each other. While CD11a was the only subunit where the active conformation was significantly decreased in low temperatures, this was due to a very small amount of signal lost, as visible in the overlay (Figure 3.1). On the other hand, both active CD11b and CD18 were not significantly lower at cold temperatures, but a much larger shift occurred between warm and cold staining temperature, with active CD18 being virtually undetectable at 5.6°C in all three experiments. While this effect might simply be due to individual protein structure generally being more or less vulnerable to changes in temperature, the difference between CD11a and the other β_2 integrin subunits could also signify a functional difference. Although this has not been demonstrated for β_2 integrins, a link between fever and integrin-mediated homing of immune cells has been recently identified (Lin et al., 2019), potentially suggesting that differential temperature control between integrin subunits has to be in place to control for this.

While the effect of staining time on CD18 could not be assessed due to time constraints, differences occurred between CD11a and CD11b in regards to their staining time (**Figure 3.2**). Active CD11a was detected at high levels on the cell surface after only 5min, while active CD11b increased in signal brightness over time. One might argue that is merely due to more β_2 integrins becoming active, but this is unlikely as this occurs within very short time frames, so could easily be achieved in 5min of staining time. It is therefore more likely that this this effect is due to differences in β_2 integrin trafficking between cell surface and cytoplasm. Only 1% of CD11a molecules were found to cycle in a span of 20min, while CD11b cycles much faster, with 2.2% of

all cell surface molecules being endocytosed and returning to the cell surface every minute (Bretscher, 1992). This makes it likely that most CD11a subunits to be stained would be on the surface already when only stained for 5min, while the increased cycling speed of CD11b might lead to new receptors appearing on the cell surface throughout the 30min staining process. However, this also raises the question if the CD11b antibody would be internalised at higher rates due to the higher cycling speed of CD11b. While this is certainly a possibility, it seems unlikely that signal is lost due to internalisation as fast as signal is gained from more CD11b appearing on the cell surface, as we see an increase in signal over time. Another potential reason for this difference would be that CD11a and CD11b have different rates of cycling between active and inactive conformation on the cell surface, rather than cycling between cytoplasm and cell surface. However, by my knowledge this has not been shown in the published literature so far.

The difference between CD11a on one hand and CD11b/CD18 on the other is revealed again in the effects of PMA activation. Clear shifts in subunit activation occurred in CD11b and CD18, but only a subtle shift occurred in CD11a (Figure 3.3) although none of these were significant when looking at only 3 samples, with some experiments showing loss of CD11a activation upon PMA stimulation (Figure 3.8). This is especially puzzling because CD11a was one of the first β_2 integrins identified to increase adhesive capabilities of cells upon PMA-stimulation (Patarroyo et al., 1985), posing the question how adhesion can be increased without visible activation. While the specific mechanism by which the protein kinase C activator, PMA, increases integrin-mediated adhesion is not fully elucidated, there is some evidence that it is due to downstream-signalling events rather than a direct phosphorylation of the integrin subunit (Danilov and Juliano, 1989). One possibility is therefore that the increase in adhesion in response to PMA is due to receptor avidity rather than affinity. Supporting this, Kucik and colleagues showed that PMA increases non-directed CD11a mobility in lymphocytes, further suggesting that PMA releases cytoskeletal restraints on CD11a rather than acting on the integrin directly (Kucik et al., 1996). As active CD11a was detected on over 50% of cells, it is possible that levels of activation contribute less to adhesion than clustering behaviour of CD11a on the cell surface. Another possibility is that the mAb used to detect CD11a behaves differently compared to CD11b and CD18. While CBRM1/5, the antibody used to detect CD11b has been described to be a activation-specific antibody (Diamond and Springer, 1993), the antibody used to detect

active CD11a is more complex. As well as binding to active CD11a, clone MEM-83 has also been shown to induce adhesion of T cells to ICAM-1, which led to the assumption that MEM-83 also actively contributes to inactive CD11a assuming its high affinity state (Binnerts et al., 1994; Hogg et al., 1993; Landis et al., 1993). It is therefore likely that instead of CD11a reacting differently to PMA compared to CD11b, that the antibody used to detect already increased levels of active CD11a to its maximum, leaving very little room for further increases. Indeed, if gating on all cells positive for total CD11a, close to 100% of them are positive for active CD11a, suggesting that MEM-83 indeed actively contributes to activation of CD11a.

To conclude, several differences between CD11a and CD11b were detected during the panel optimisation process, which fit with published literature regarding differences in trafficking and avidity of these subunits. This is promising, as it further validates that we can effectively measure expression of total and active β_2 integrins, and that this measurement actually relates to their function.

3.5 Conclusions

I successfully developed a flow cytometry panel that allows me to quantify the total expression and activation-status of β_2 integrin subunits CD11a, CD11b and CD18. Furthermore, this panel can differentiate between a variety of APCs of interest, including cDC1 DCs, cDC2 DCs and pDCs, as well as $CD14^+$ and $CD16^+$ monocytes.

After having established that total and active forms of an integrin can be stained in the same sample, the next step was to quantify the expression of total and active integrins in Mo-DCs and explore how their expression changes under conditions of activation and tolerance.

Chapter 4 **Characterisation and function of β_2 integrins on tolerogenic monocyte-derived dendritic cells**

4.1 Introduction

After having established that total and active β_2 integrins can be assessed on the same cells, I investigated expression and function of β_2 integrins in *in vitro* generated Mo-DCs. I hypothesised that β_2 integrins play an important role in mediating immune tolerance on DCs, which is however challenging to investigate in vitro. Therefore, I focused specifically on two Mo-DC culturing conditions that represent two extremes: mature Mo-DCs, which were found to elicit immune cell activation (Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996), and tolerogenic Mo-DCs, which have tolerising and immunoregulatory roles (Anderson et al., 2008). While this does not mirror tolerance induction in DCs *in vivo,* mature and tolerogenic Mo-DCs still provided an excellent model system to investigate the roles of β_2 integrin expression and activation on DC-like cells between immune activation and tolerance.

4.1.1 Mature and tolerogenic Mo-DCs

Briefly, Mo-DCs were derived from human $CD14^+$ monocytes isolated from healthy PB, that became DC-like after 7 days of culture in the presence of IL-4 and GM-CSF. Tolerogenic Mo-DCs received tolerising agents Dexamethasone as well as Vitamin D_3, but both tolerogenic and mature Mo-DCs received stimulation with LPS on Day 6 of culture. There are several reasons for choosing Mo-DCs to investigate potential immunoregulatory roles of β_2 integrins in DCs:

First, they are an established *in vitro* model of DCs. While current literature suggests that most DCs present in PB in the steady state are myeloid- and not monocyte-derived (Guilliams et al., 2014), studies also suggest that *in vitro* generated Mo-DCs and PB DCs share many common features. For example, Mo-DCs and CD11c+ PB DCs express similar levels of the antigen-presenting complex HLA-DR as well as costimulatory factors such as CD40, CD83 and CD86 (Osugi et al., 2002). Furthermore, Mo-DCs are able to present antigen and elicit T cell proliferation in an MLR (Sallusto and Lanzavecchia, 1994). With the knowledge that DCs only constitute 1% of PBMCs, culturing Mo-DCs from healthy blood monocytes therefore provides a viable option to generate stable DCs in sufficiently high numbers to test expression as well as functionality of β_2 integrins.

Second, mature and tolerogenic Mo-DCs were selected because of their ability to activate and tolerise T cells, respectively, similar to cDCs. Tolerogenic Mo-DCs, cultured using the same protocol used for my studies, were characterised to have an

anti-inflammatory cytokine profile, with higher expression of IL-10 and concurrent lower expression of IL-12p70, IL-6 and TNF-α when compared to mature Mo-DCs (Anderson et al., 2008). The same study also observed that mature Mo-DCs induced activation and proliferation of both naïve and memory T cells, while tolerogenic Mo-DCs were significantly less capable to stimulate T cells. Lastly, naïve T cells that were primed by tolerogenic Mo-DCs produced more IL-10 upon restimulation with either mature Mo-DCs or anti-CD3/anti-CD28 while memory T cells were shown to be hyporesponsive to restimulation. Furthermore, tolerogenic Mo-DCs were found to control T cell activation by mature Mo-DCs in a co-culturing system where both mature and tolerogenic Mo-DCs were present (Harry et al., 2010). Comparing β_2 integrin expression and function between mature and tolerogenic Mo-DCs therefore provides the opportunity to investigate possible differences in a well-defined model system of immune stimulation (mature Mo-DCs) and regulation (tolerogenic Mo-DCs).

To conclude, respective phenotypes of both mature and tolerogenic Mo-DCs are well-described and large numbers of Mo-DCs can be generated *in vitro* relatively easily. Using mature and tolerogenic Mo-DCs therefore represent a useful tool to understand the role of β_2 integrins in immune activation and regulation. In addition, some properties of tolerogenic Mo-DCs described in the literature suggest a likely involvement of β_2 integrins in their function, which I will explore in the next section.

4.1.2 The potential role of β_2 integrins on Mo-DCs

After establishing that mature and tolerogenic Mo-DCs are a model system well-suited to investigate the roles of β_2 integrins between immune activation and tolerance, it is important to discuss published literature that suggest differences in β_2 integrins between the two Mo-DC subtypes.

Several studies have shown that genes encoding β_2 integrins are differentially expressed between CD14+ monocytes and Mo-DCs. For example, CD18 expression is increased in unstimulated (immature) Mo-DCs compared to monocytes (Angénieux et al., 2001). However, CD11a gene expression was downregulated in TNF-α stimulated Mo-DCs compared to monocytes, but did not differ between immature and mature Mo-DCs, alongside an upregulation of genes important for cell migration, such as macrophage capping protein and vimentin (Ivaska et al., 2007; Naour et al., 2001; Sun et al., 1995). This gene expression data is backed up by another study

measuring surface expression of β_2 integrins on mature Mo-DCs compared to CD14+ monocytes, which found higher expression of CD11c, CD11b and CD18, but lower expression of CD11a on Mo-DCs compared to monocytes (Ammon et al., 2000). Furthermore, previous experience in the Hilkens laboratory has shown that tolerogenic Mo-DCs adhere more tightly to plastic compared to mature Mo-DCs (unpublished observation). Keeping Mo-DCs for 60min on ice was therefore introduced into the protocol to enable sufficient harvesting of tolerogenic Mo-DCs. While plastic is obviously not a biological ligand for β_2 integrins, this suggests that adhesive capabilities are altered in tolerogenic Mo-DCs. This is further supported by the finding that mouse BMDCs with deficient β_2 integrin signalling show reduced adhesion to both ligands and plastic (Morrison et al., 2014). As adhesion is one function of β_2 integrins, it therefore seems likely that either expression or conformational state of the adhesion receptors differs between mature and tolerogenic Mo-DCs, thereby affecting Mo-DC adhesive capabilities.

Another important function of β_2 integrins is cell migration. Migration of immune cells towards the LN and correct positioning in T cell zones is primarily mediated by the chemokine receptor CCR7 binding to CCL19 and CCL21 located in the LN. While tolerogenic Mo-DCs can migrate towards CCL19, they are reported to migrate with 25-40% of the efficiency of mature Mo-DCs (Anderson et al., 2009, 2008), which was proposed to be due to the lower expression of CCR7 on tolerogenic compared to mature Mo-DCs. However, other studies suggest that β_2 integrins might also be involved. For example, in mouse, mature migratory DCs are shown to upregulate CCR7 while simultaneously downregulating β_2 integrins such as CD18 and CD11c (Morrison et al., 2014). Interestingly, the same study also showed that CCR7 is also upregulated in BMDCs when the kindlin-3 binding site of the β_2 integrin is mutated, reducing the integrins ability to form stable adhesions and making cells assume a mature migratory phenotype. Furthermore, both ligands of CCR7, namely CCL19 and CCL21, were shown to induce CD11a/CD18 activation on human Mo-DCs via inside-out signalling (Eich et al., 2011; Quast et al., 2009). These seemingly conflicting reports suggest that β_2 integrins might be important to mediate migration of cells into the LN once they receive CCR7 stimulation. This is supported by the finding that TNF-α stimulated Mo-DCs from two patients with LAD-1, where CD18 expression is impaired, show decreased trans-endothelial migratory ability (Fiorini et al., 2002). Previously described differences in both migration and CCR7 expression in

tolerogenic compared to mature Mo-DCs might therefore go alongside as of yet undescribed differences in β_2 integrins, which are potentially regulated by CCR7 signalling. When comparing different immunosuppressive agents (Vitamin D_3, IL-10, Dexamethasone, TGF-β, rapamycin) meant to induce tolerogenicity in Mo-DCs, it was furthermore shown that agents eliciting little immunosuppression also show no reduction in migratory ability towards CCL19 and vice versa (Boks et al., 2012). In fact, the same study did not pursue Vitamin D_3-treated tolerogenic Mo-DCs after finding that they showed lowest migratory ability of all agents tested. One could therefore hypothesise that gaining immunosuppressive and regulatory functions occurs simultaneously with loss of migratory capability, making a role for β_2 integrins in this context likely. This however also holds a caveat for future use of tolerogenic Mo-DCs as cell therapy, as low migratory ability might also impede their ability to travel to the draining LN and render T cells unresponsive.

Lastly, β_2 integrins have also been described to play a role in DCs forming contacts with T cells through the immunological synapse. For example, ligation of CD11b on human Mo-DCs reduced both their expression of CD86 and their ability to stimulate T cells (Podgrabinska et al., 2009), which has also been found in murine BMDCs (Behrens et al., 2007). CD11b was also found to be involved in controlling peripheral tolerance by restricting Th17 cell differentiation (Ehirchiou et al., 2007). Furthermore, both the active conformation of CD11a and CD11b were found to restrict T cell activation (Balkow et al., 2010; Varga et al., 2007), suggesting an active role of β_2 integrins in mediating T cell contacts from the DC side. While tolerogenic Mo-DCs were shown to have reduced T cell stimulatory abilities (Anderson et al., 2008), β_2 integrin expression on their surface has not been assessed so far.

To conclude, differences in adhesive and migratory capabilities between mature and tolerogenic Mo-DCs, as well as their respective ability to stimulate T cells, suggest that they differ in expression of total or active β_2 integrins. Identifying these differences and assessing their role for Mo-DC function could yield important insights into the immunoregulatory function of β_2 integrins on DCs.

4.1.3 Aims

The aims of this chapter are as follows:

1) Assess expression of total and active β_2 integrins on mature and tolerogenic Mo-DCs.

2) Define the adhesive and clustering capabilities of mature and tolerogenic Mo-DCs.
3) Investigate the functional impact of CD11b manipulation on Mo-DC phenotype and T cell priming ability.

4.2 Results

4.2.1 Generation and phenotypic analysis of mature and tolerogenic Mo-DC populations

First, populations of mature and tolerogenic Mo-DCs were generated by *in vitro* culture and characterised phenotypically and functionally. This was to ensure that culturing of mature and tolerogenic Mo-DCs was successful and could be compared to published literature. Additionally, phenotyping of Mo-DCs ensured consistency between Mo-DCs cultured from different healthy donors or on different days. To this end, immature Mo-DCs, mature Mo-DCs (LPS) and tolerogenic Mo-DCs (Dexamethasone+Vitamin D_3+LPS) were compared for expression of key cell surface markers. While immature Mo-DCs were included in all cultures as an internal control for phenotyping purposes, they were not of special interest to the understanding of the role of β_2 integrins in Mo-DC between activation and tolerance, so this group is included in figures only if they are of interest to the question at hand.

First, expression of CD14 and CD1c were assessed. A hallmark of successful *in vitro* DC induction is the loss of the monocyte marker CD14, while expressing CD1c (Sallusto and Lanzavecchia, 1994). As the monocytes utilised for this culture are selected for their CD14-positivity, the loss of this marker signifies that monocytes have indeed been transformed due to the presence of IL-4 and GM-CSF. An example of CD14 and CD1c expression in immature, mature and tolerogenic Mo-DCs generated from the monocytes is shown in Figure 4.1A.

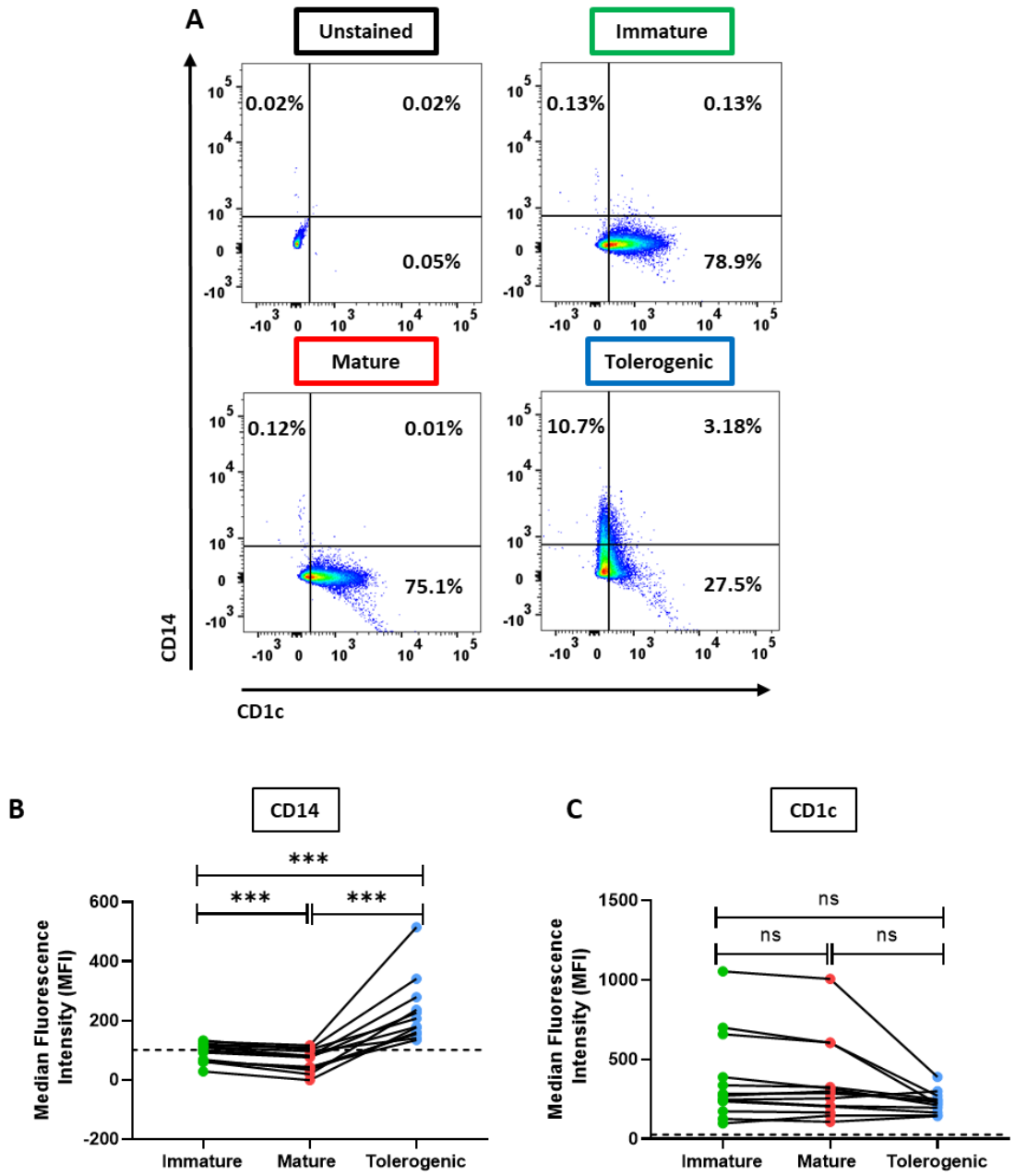

Figure 4.1 Expression of CD1c and CD14 on Mo-DCs

Expression of CD1c and CD14 on immature, mature and tolerogenic Mo-DCs. A. Representative expression of CD1c and CD14 on immature, mature and tolerogenic Mo-DCs cultured from CD14+ monocytes of a healthy donor compared to an unstained sample. B. MFI of CD14 expression, comparing immature, mature and tolerogenic Mo-DCs. Dotted line represents median of unstained control. One-way ANOVA, n=13. C. MFI of CD1c expression, comparing immature, mature and tolerogenic Mo-DCs to an unstained sample. Dotted line represents median of unstained control. One-way ANOVA, n=13.

CD14+ staining was absent from both immature and mature Mo-DCs, with signal brightness being highly similar to an unstained control. However, tolerogenic Mo-DCs showed some CD14+staining, some of which was CD1c positive (3.18%), while the largest part was CD1c negative (10.8%). CD1c expression was not significantly different between immature, mature or tolerogenic Mo-DCs, but all three Mo-DC subtypes expressed levels of CD1c above a median unstained signal (Figure 4.1C). As visible from the representative sample, immature and mature Mo-DCs showed very low CD14 expression which was not significantly different from unstained controls in immature Mo-DCs and actually significantly lower on mature Mo-DCs. However, the mean difference was very small, underlining that CD14 expression was comprehensively absent from mature Mo-DCs at the end of the 7-day culture. Interestingly, CD14 expression on tolerogenic Mo-DCs was significantly higher compared to an unstained control, suggesting that addition of immunosuppressive drugs inhibited the complete loss of CD14 from the cell surface. This could suggest that some cells did not complete the monocyte to DC differentiation process under tolerogenic cell culture conditions. Other studies have however also shown that Vitamin D_3 might induce expression of CD14 (Oberg et al., 1993), suggesting that the presence of this tolerogenic factor stimulates CD14 expression. Overall, CD14 was only expressed on around 14% of tolerogenic Mo-DCs, suggesting that only a small proportion of cells was affected by this and that the large majority of cells in tolerogenic cultures resembled Mo-DCs. To conclude, it can be assumed that immature and mature Mo-DCs harvested were Mo-DCs and not monocytes, and that at least 85% of tolerogenic Mo-DCs assumed a DC-like phenotype. CD1c expression compared to an unstained control is shown in Figure 4.1C, revealing that all Mo-DC subtypes express CD1c. Again, tolerogenic Mo-DCs differed from immature and mature Mo-DCs in that they expressed lower levels of CD1c, while this was not found to be significant, it suggests that addition of Dexamethasone and Vitamin D3 inhibited full Mo-DC development.

Immature Mo-DCs are known to increase in costimulatory markers such as CD83, CD86 and the antigen-presenting MHC HLA-DR upon LPS stimulation to yield mature Mo-DCs (Harry et al., 2010; Osugi et al., 2002). Tolerogenic Mo-DCs on the other hand were described to exhibit a semi-mature state, with significantly reduced expression levels of CD83 and CD86 and reduced expression of HLA-DR compared to mature Mo-DCs (Anderson et al., 2008; Harry et al., 2010). This observation was

confirmed in Figure 4.2A, which shows a representative overlay of expression of CD83, CD86 and HLA-DR on immature (shown in green), mature (shown in red) and tolerogenic (shown in blue) viable Mo-DCs. Immature Mo-DCs were lowest in all markers, while mature Mo-DCs showed the highest expression. Tolerogenic Mo-DCs showed medium to low expression of CD83, medium expression of CD86 (both intermediate between immature and mature) but expressed HLA-DR in similarly high levels to mature Mo-DCs. Expression of these three costimulatory molecules was quantified (Figure 4.2B), which confirmed that mature Mo-DCs express significantly higher levels of CD83 and CD86 compared to both immature and tolerogenic Mo-DCs, which were not shown to be significantly different from each other. However, while HLA-DR expression was significantly increased between immature and mature Mo-DCs, tolerogenic Mo-DCs were not shown to be significantly different from mature Mo-DCs. While representative histogram overlays showed that tolerogenic Mo-DCs were slightly higher in CD83 and CD86 when compared to immature Mo-DCs, this effect was not found to be significant in the pooled data.

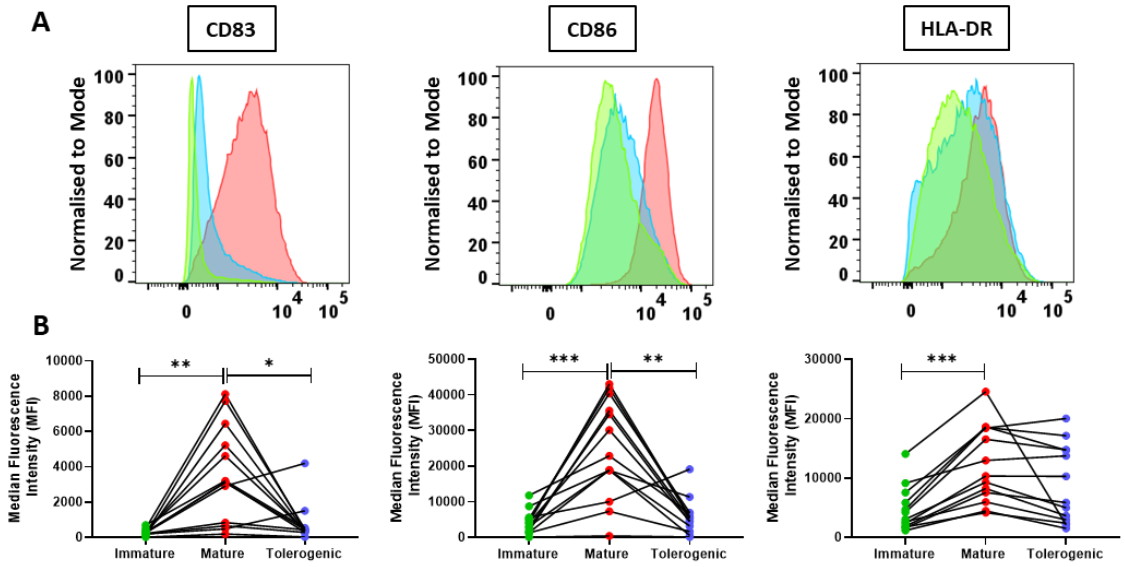

Figure 4.2 Expression of CD83, CD86 and HLA-DR on Mo-DCs

Expression of costimulatory receptors CD83 and CD86, as well as MHC class II HLA-DR on immature, mature and tolerogenic Mo-DCs. A. Representative histogram overlay of expression of CD83, CD86 and HLA-DR by immature (green), mature (red) and tolerogenic (blue) Mo-DCs. B. MFI expression of CD83, CD86 and HLA-DR comparing immature (green), mature (red) and tolerogenic Mo-DCs (blue). One-way ANOVA, n=13.

Overall, this suggests that the Mo-DC culturing system yielded three distinct Mo-DCs subtypes, which show differential expression of CD83, CD86 and HLA-DR. Here,

immature and mature Mo-DCs represented the low and high ends of costimulatory molecule expression, with tolerogenic Mo-DCs being more similar to immature Mo-DCs in respect to expression of CD83 and CD86, while HLA-DR expression was closer to that of mature Mo-DCs.

However, expression of MHC and costimulatory molecules alone are not sufficient to define DCs as either mature or tolerogenic. Distinct other markers are needed to distinguish tolerogenic from mature Mo-DCs. TLR2, a cell surface receptor that recognises bacterial lipoproteins and whose expression is induced by exposure to Dexamethasone , and the TGF-β receptor LAP have previously been described as markers of tolerogenic Mo-DCs (Harry et al., 2010; Shibata et al., 2009). Expression of TLR2 and LAP is shown using a multi-colour dot plot on immature, mature and tolerogenic Mo-DCs (Figure 4.3A). While immature and mature Mo-DCs showed some TLR2 expression, between 20-35% of Mo-DCs did not express TLR2. In contrast, tolerogenic Mo-DCs expressed much higher levels of TLR2 with only 2.2% of cells not expressing the marker. TLR2 signal was significantly brighter on tolerogenic Mo-DCs compared to both immature and mature Mo-DCs, and was also expressed on a larger percentage of tolerogenic Mo-DCs (Figure 4.3B). Interestingly, a significantly smaller percentage of mature Mo-DCs expressed TLR2 compared to the percentage of immature Mo-DCs. Furthermore, only 1-3% of immature and mature Mo-DCs showed expression of LAP, while up to 12% of tolerogenic Mo-DCs expressed LAP. Quantifying expression of LAP, no differences in MFI between the different Mo-DC subsets can be observed, although a significantly larger proportion of tolerogenic Mo-DCs expressed LAP when compared to mature Mo-DCs (Figure 4.3).

Overall, phenotyping of Mo-DC subtypes showed that culturing of the three different subtypes generated distinct populations that show significant differences in expression of key markers that matched their description in the literature. Mo-DCs lost CD14 expression but retained or in some cases gained CD1c expression as they differentiated into Mo-DCs. Addition of LPS caused a rapid increase in surface expression of CD83, CD86 and HLA-DR in mature DCs, but this was only the case for HLA-DR in tolerogenic Mo-DCs. Furthermore, tolerogenic Mo-DCs showed significantly increased surface expression of TLR2 compared to both immature and mature Mo-DCs, as well as an increased proportion of tolerogenic Mo-DCs expressing LAP. This shows that I was able to generate phenotypically distinct

mature and tolerogenic Mo-DCs reliably, which could therefore be used to study β_2 integrin expression and function in the context of immune activation and tolerance.

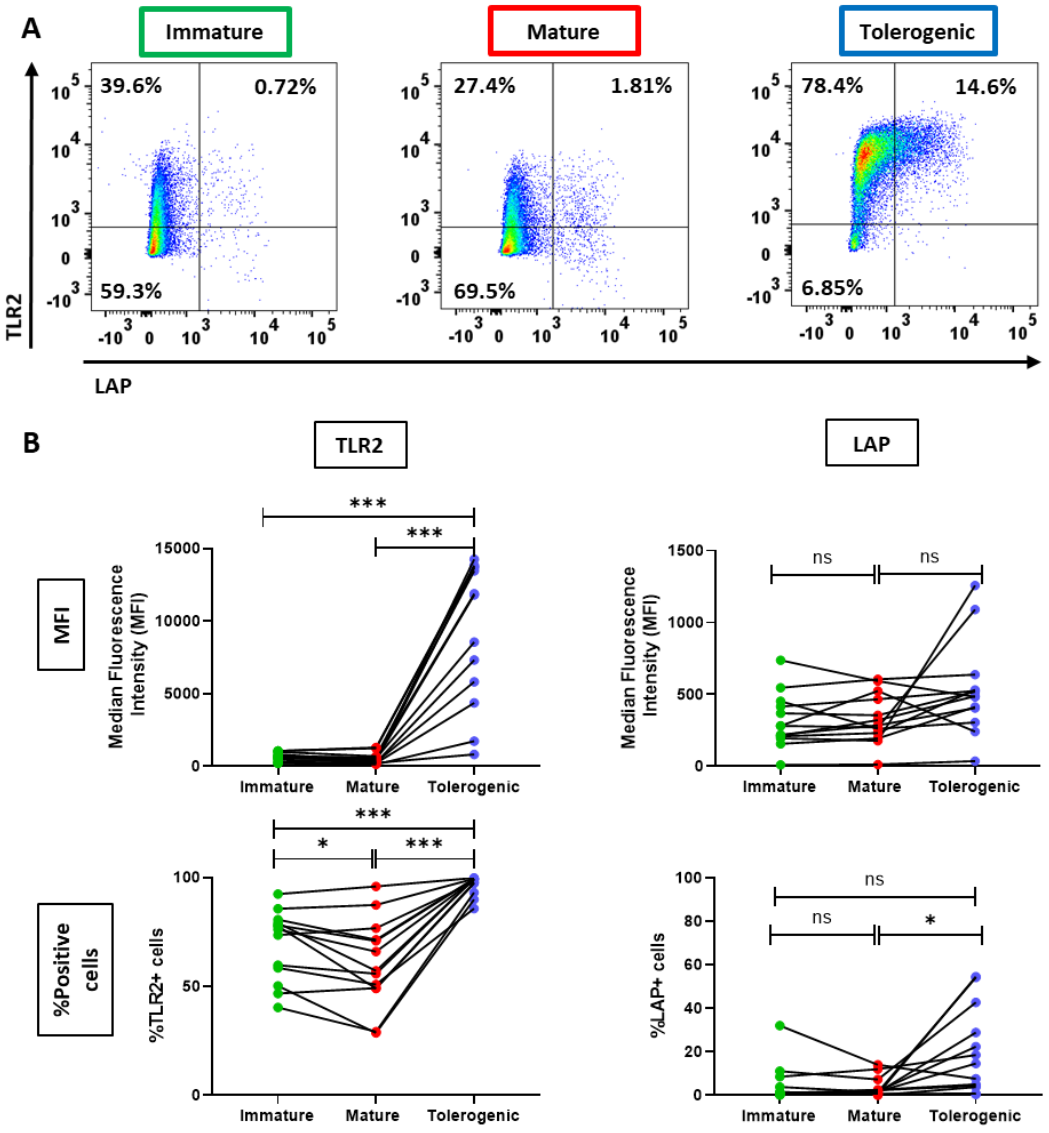

Figure 4.3 Expression of LAP and TLR2 on Mo-DCs

Expression of LAP and TLR2 on immature, mature and tolerogenic Mo-DCs. A. Representative multi-colour dot plot of expression of LAP and TLR2 on immature, mature and tolerogenic Mo-DCs. B. MFI expression of TLR2 (left) and LAP (right) on immature (green), mature (red) and tolerogenic Mo-DCs (blue). Both signal brightness (MFI, top) and %positive population (bottom) for both TLR2 and LAP are shown. One-way ANOVA, n=13.

T cell stimulatory abilities of mature and tolerogenic Mo-DCs

Mature Mo-DCs are described to induce higher levels of T cell proliferation and IFN-γ production compared to tolerogenic Mo-DCs in an MLR (Anderson et al., 2008). To test this, T cell proliferation in an MLR was tested using a cell proliferation dye (CTV, Figure 4.4A-B) and supernatants were tested for levels of IFN-γ (Figure 4.4C) and IL-10 (Figure 4.4D) using an ELISA. However, no significant difference in T cell proliferation was observed, neither in the total amount of proliferation nor when comparing different levels of division (Figure 4.4B). In fact, around 90% of T cells co-cultured with both mature and tolerogenic Mo-DCs were shown to have undergone 5-7 cell divisions, with only a small percentage of cells in either cell type having undergone 1-2 divisions. This suggested that at Day 6, that mature and tolerogenic Mo-DCs drive the same level of T cell division. This could potentially be due to the fact that it is difficult to detect further divisions after seven, as the CTV gets too diluted for detection. Therefore, potentially an earlier time point would show differences between mature and tolerogenic Mo-DCs ability to stimulate T cell proliferation, as the maximal amount of proliferation has not been reached yet. Another option however is that mature and tolerogenic Mo-DCs drive different T cell phenotypes. This is especially possible as IFN-γ levels in mature Mo-DC supernatants were significantly higher compared to tolerogenic Mo-DC-co-cultures (Figure 4.4C), confirming that the mature and tolerogenic Mo-DC states result in altered T cell priming. ln addition to IFN-γ, I also measured levels of the anti-inflammatory cytokine IL-10 in Mo-DC MLR supernatants to test if there was an increase in anti-inflammatory signalling in tolerogenic Mo-DC MLRs. No significant differences in IL-10 levels were found between MLRs using tolerogenic or mature Mo-DCs (Figure 4.4D). However, measuring IL-10 in MLRs had the inherent caveat that both T cells and Mo-DCs can produce it. This could mean that IL-10 production by either Mo-DC subset masked differences in IL-10 production by T cells that were present in the culture. Interestingly, the only donor that showed increased IFN-γ production in tolerogenic MLRs simultaneously also showed the only decrease in IL-10 production in tolerogenic compared to mature MLRs. This could suggest that this specific culture was unusual, possibly due to donor to donor variation of both Mo-DCs and naïve T cells or accidental switching of samples.

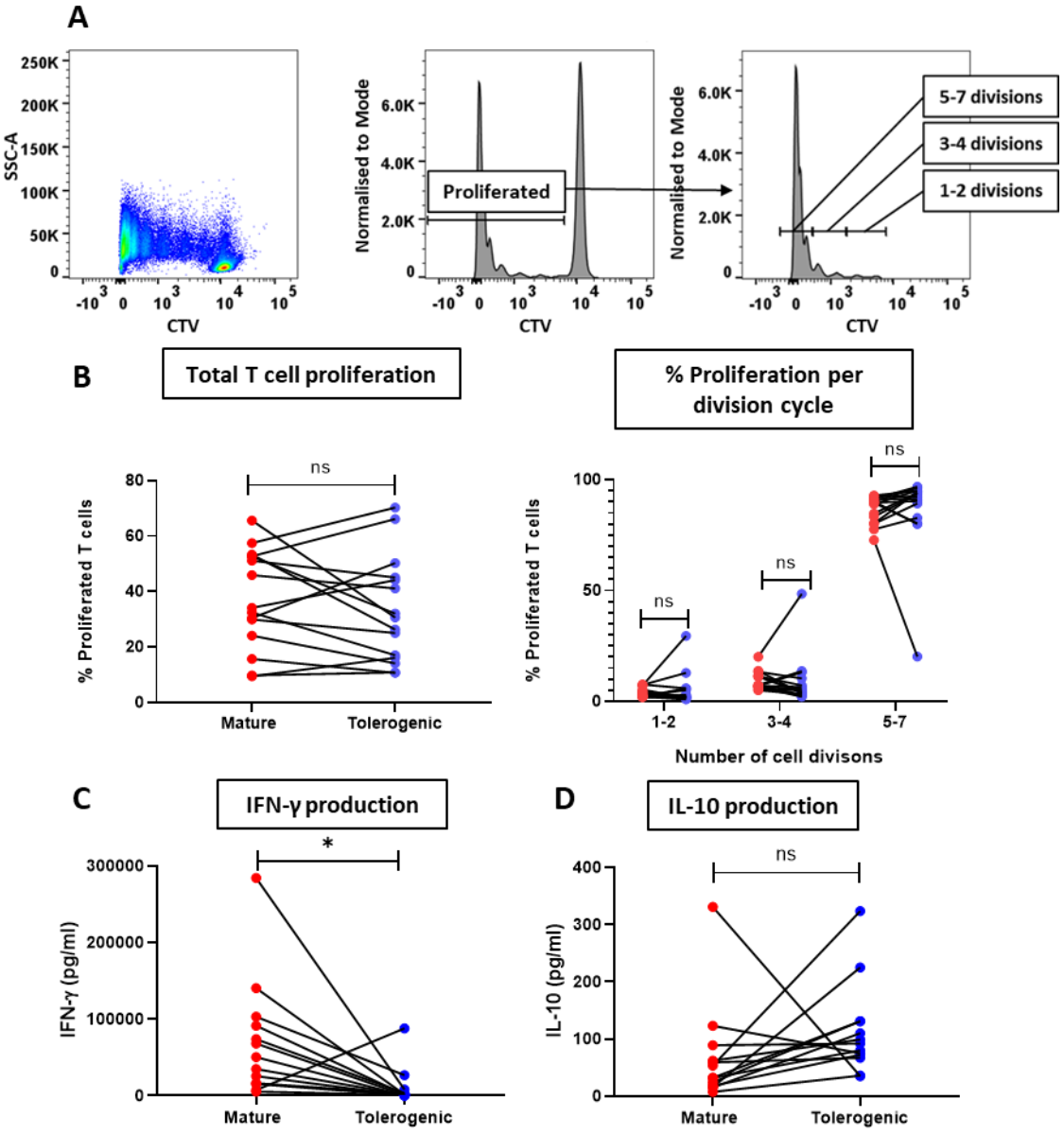

Figure 4.4 Effect of mature and tolerogenic Mo-DCs in an MLR

Effect of mature and tolerogenic Mo-DCs on T cell proliferation, IFN-γ and IL-10 production in an MLR. A. Gating strategy to yield proliferated cells in percent (Proliferated) and cells that had undergone 1-2, 3-4 and 5-7 divisions respectively. Single live cells, CD11c-CD3+CD4+. B. Total T cell proliferation in percent of T cells that have diluted CTV staining after 6 days of co-culture (left) and percent that had undergone 1-2, 3-4 and 5-7 cell divisions, comparing mature and tolerogenic Mo-DCs. C. IFN-γ concentration detected in MLR supernatants by ELISA. D. IL-10 concentration detected in MLR supernatants by ELISA. Student's *t* test, except C (right) two way ANOVA, n=15.

To conclude, I established that cultured mature and tolerogenic Mo-DCs are stable and

comparable to Mo-DCs discussed in previously published literature in respect to both their phenotype and their ability to stimulate T cells. Shortly, mature Mo-DCs had not only the ability to induce T cell proliferation but also showed to induce high levels of IFN-γ production by T cells. In contrast, tolerogenic Mo-DCs could induce T cell proliferation similar to mature Mo-DCs, the T cells produced significantly lower amounts of IFN-γ. Phenotypic differences, as well as immunostimulatory versus immunoregulatory abilities, in the form of T cell stimulatory abilities, were shown to be different between mature and tolerogenic Mo-DCs. This underlined that mature and tolerogenic Mo-DCs cultured by me are indeed two distinct phenotypes to investigate further.

4.2.2 Clustering abilities of Mo-DCs

In addition to differences in cell surface marker expression in Mo-DC subtypes, it was also important to assess if Mo-DC subtypes differ in their functionality in respect to cell adhesive behaviour. This is because differences in adhesive capabilities have been observed but so far not quantified. To this end, clustering phenotype of mature and tolerogenic Mo-DCs was assessed.

Clustering phenotypes of monocyte-derived dendritic cells

Mature Mo-DCs have been described previously to show DC morphology, including dendrite-like extensions, as well as to form tight clusters with each other (Zhou and Tedder, 1996). Given reports of altered adhesion of Mo-DC subtypes (Hilkens et al, unpublished observations), I then looked at Mo-DC morphology and clustering by imaging. Therefore, immature, mature and tolerogenic Mo-DCs were incubated for 6h at 37°C on glass after all stimulants were washed off, imaged using a bright field microscope and cell clustering was quantified using a CellProfiler pipeline, whereby both clusters of cells and individual unclustered cells could be detected as objects, enabling measurements of various parameters, including object area.

Immature (green), mature (red) and tolerogenic Mo-DCs (blue) were cultured on glass slides (representative images shown in Figure 4.5A). Differences in Mo-DC clustering was observed between conditions: immature Mo-DCs showed the least level of clustering, mature Mo-DCs showed few large clusters of cells and tolerogenic Mo-DCs showed a large number of smaller clusters. When quantifying the images using Cellprofiler, it was observed that clusters were significantly larger in mature Mo-DCs compared to both immature and tolerogenic Mo-DCs, and that tolerogenic Mo-

DCs in turn formed significantly larger clusters compared to immature Mo-DCs (Figure 4.5B, left). This result was furthermore supported when considering the median radius of clusters, which describes the median length of distance between any pixel within a cluster to the closest pixel outside of the cluster. Mature Mo-DC clusters are shown to have the largest median radius, with both tolerogenic and immature Mo-DC clusters having significantly lower median radius values (Figure 4.5B, right). Furthermore, the number of both clusters (left) and single unclustered cells (right) was determined for each condition to determine clustering behaviours of different Mo-DC subtypes (Figure 4.5). Mature Mo-DCs showed a significantly lower number of clusters compared to both immature and tolerogenic Mo-DCs, which did not differ in their number of clusters. Furthermore, mature Mo-DCs showed significantly less single unclustered cells compared to immature Mo-DCs, underlining again that mature Mo-DCs show higher rates of clustering compared to especially immature Mo-DCs. Overall, this confirmed that mature Mo-DCs show the highest level of cell clustering of all three Mo-DC types as the form a smaller number of larger clusters, with significantly less cells remaining unclustered. Immature Mo-DCs on the other hand show the least amount of clustering, with the smallest area and median radius of clusters observed. Lastly tolerogenic Mo-DCs possess an intermediate phenotype, with clusters that are significantly larger than immature Mo-DC clusters, but significantly smaller compared to mature Mo-DCs. Clustering ability of mature and tolerogenic Mo-DCs on glass slides suggests that mature Mo-DCs are less adhesive and therefore able to move faster together to form large clusters, while tolerogenic Mo-DCs could be supposed to be more adhesive, thereby only being able to transverse shorter distances to form smaller clusters. Although this is adhesion to glass, one could speculate that increased adhesive capabilities of tolerogenic Mo-DCs might alter their ability to migrate and affect how tightly they adhere to T cells, thereby influencing T cell activation.

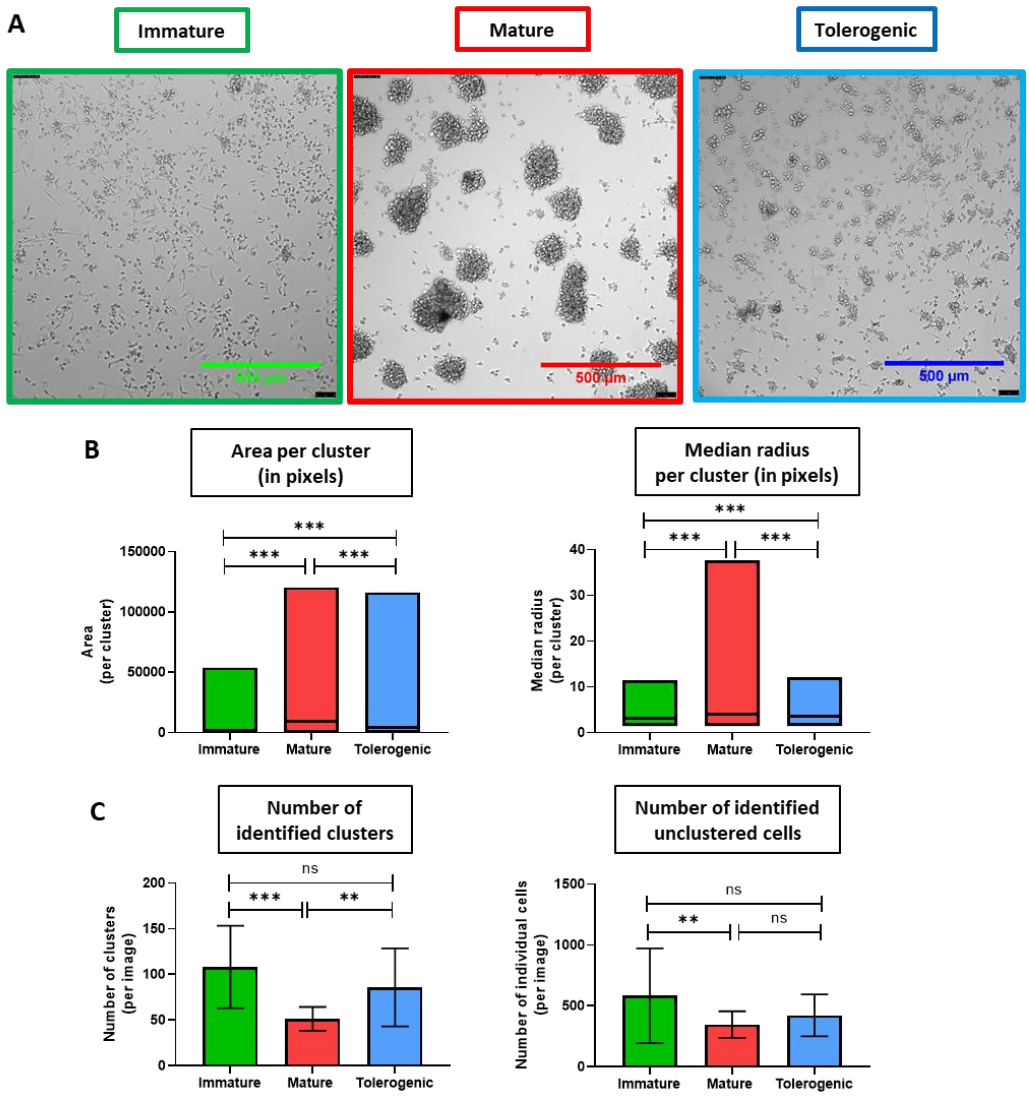

Figure 4.5 Cell clustering phenotype of Mo-DCs

Cell clustering of immature, mature and tolerogenic Mo-DCs incubated for 6h at 37°C on glass slides. A. Representative bright field microscopy images of immature (green), mature (red) and tolerogenic Mo-DCs (blue) on glass slides. 10x magnification. B. Area in pixels of clusters identified by cellprofiler (left) as well as median radius in pixels per cluster (right). Area is defined as the number of pixels in the region of the cluster, while median radius is defined as the median distance of any pixel in a cluster to the closest pixel outside of the object. One-way ANOVA, immature n=2490, mature=1493, tolerogenic=2405 individual clusters. C. Number of clusters identified per image by Cellprofiler pipeline (left) and number of unclustered individual cells identified per image by Cellprofiler pipeline (right). One-way ANOVA, immature n=23, mature n=29, tolerogenic n=28 healthy control donors. Experiment was done on three separate occasions using three different healthy Mo-DC donors.

4.2.3 The role of CD11a in Mo-DCs

As previously discussed in the introduction to this chapter, evidence supports an important role for β_2 integrins in mediating the functional differences between mature

and tolerogenic Mo-DCs. Indeed, the clustering data (Figure 4.5) indicate clear differences in adhesive properties between mature and tolerogenic Mo-DCs. However, very little is known about the expression and conformation of β_2 integrin subunits in Mo-DCs, and how integrin expression might influence Mo-DC function.

To assess a potential role of CD11a in Mo-DCs, total and active CD11a expression was assessed. As double staining for total and active CD11a led to a significant signal loss of total CD11a, due to steric hindrance (Chapter 3, section 3.3.4, Figure 3.4), total CD11a staining was assessed using both double stained (total and active CD11a mAbs) and single stained (total CD11a mAbs only) cells. Double stained total CD11a (left) was found to be significantly lower on tolerogenic DCs compared to both immature and mature Mo-DCs (Figure 4.6A). However, when considering the staining of the same samples not affected by steric hindrance (single stained, right), no significant differences in total CD11a expression between Mo-DC subtypes were observed. This suggests that the level of steric hindrance differs between cell types. Investigating this further, the level of steric hindrance between Mo-DC subtypes, both as the difference between single stained and double stained MFI (left) and as percentage of signal lost (difference single-double stained as percentage of single stained, right) was plotted (Figure 4.6B). This showed that tolerogenic Mo-DC indeed lost significantly more total CD11a signal compared to immature and mature Mo-DCs. This was the case both for total signal plotted as MFI, as well as percentage of signal loss, with immature Mo-DCs losing a mean of 13.9% of signal, mature Mo-DCs losing only a mean 0.7% of signal and tolerogenic Mo-DCs losing a mean of 34.7% of their total CD11a signal. Surprisingly, two out of 13 mature Mo-DCs actually gained signal when double stained. Furthermore, when excluding the three samples with the highest levels of steric hindrance shown from the double-stained data, no significant difference between expression of total CD11a was observed (Figure 4.6C), confirming that steric hindrance was the cause for the significantly different expression of total CD11a in tolerogenic Mo-DCs. As total CD11a expression was not significantly different between mature and tolerogenic Mo-DCs, but steric hindrance was increased in tolerogenic Mo-DCs, one could hypothesise that tolerogenic Mo-DCs have increased levels of active CD11a. This would explain why more total CD11a signal was lost, as more binding of active CD11a might reduce total CD11a binding to its target.

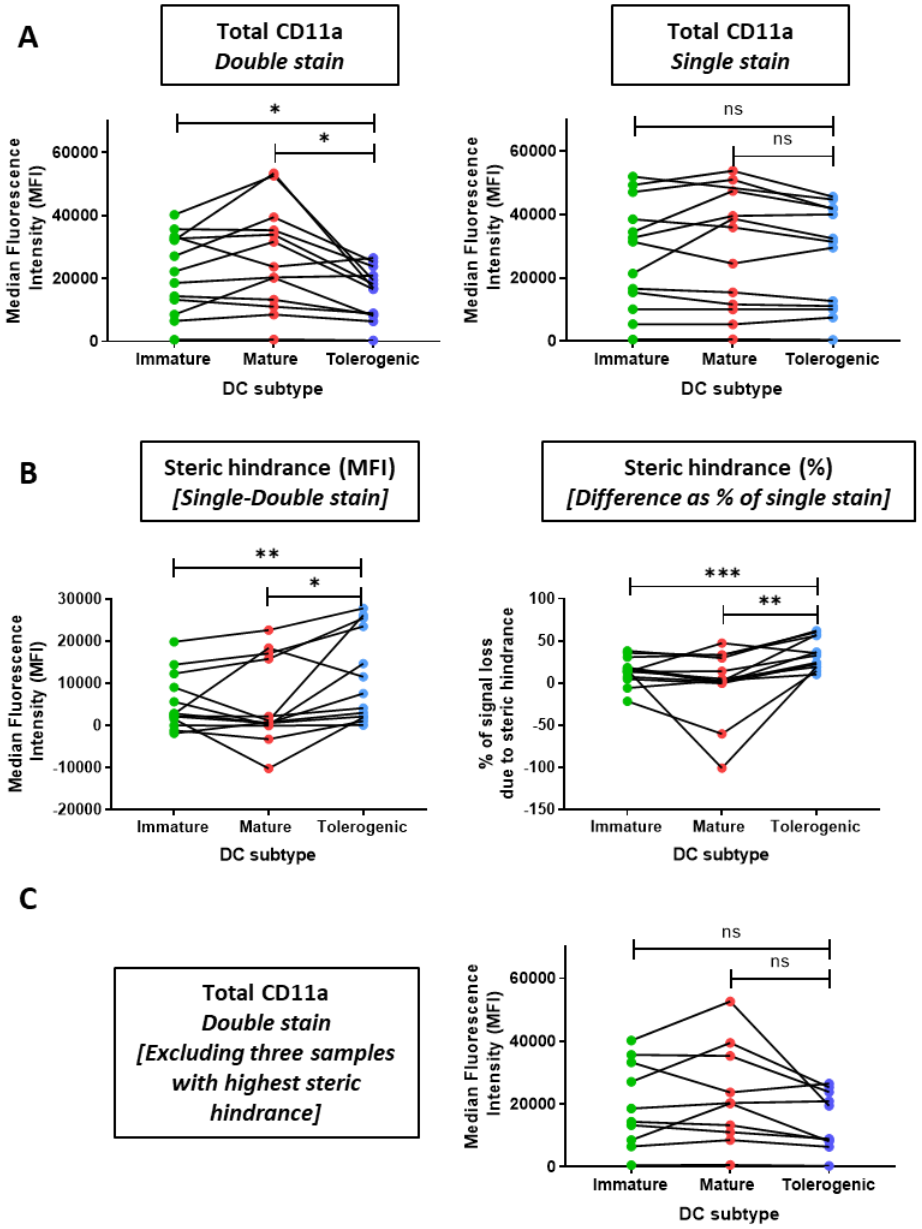

Figure 4.6 Expression of total CD11a in Mo-DCs

Expression of total CD11a in immature, mature and tolerogenic Mo-DCs. A. MFI of total CD11a, comparing double stain (left, total and active CD11a stained) and single stain (right, only total CD11a stained). B. Level of steric hindrance in immature, mature and tolerogenic Mo-DCs. Left shows the difference between single and double stain in MFI values (MFI lost to steric hindrance), right shows differences in steric hindrance as a percentage of single stain (% of signal lost to steric hindrance). C. MFI of total CD11a, double stain with three samples with highest levels of steric hindrance recoreded removed. Results shown are from single, live cultured cells. Matched one-way ANOVA, n=13, except C. n=10.

When considering active CD11a expression on tolerogenic Mo-DCs, this hypothesis was confirmed, as tolerogenic Mo-DCs expressed significantly more active CD11a compared to mature Mo-DCs (Figure 4.7). Expression of active CD11a was clearly higher in both a dot plot and a histogram overlay (Figure 4.7A). Interestingly, tolerogenic Mo-DCs did not differ significantly from immature Mo-DCs, which were also significantly higher in active CD11a expression compared to mature DCs. This was surprising, as one could therefore expect levels of steric hindrance due to higher active CD11a expression in immature Mo-DCs to be similar to tolerogenic Mo-DCs (Figure 4.7). However, this was not the case as there was significantly less steric hindrance in immature Mo-DCs compared to tolerogenic Mo-DCs (Figure 4.6B). This suggested that factors affecting steric hindrance of CD11a antibodies are potentially different in immature and tolerogenic Mo-DCs.

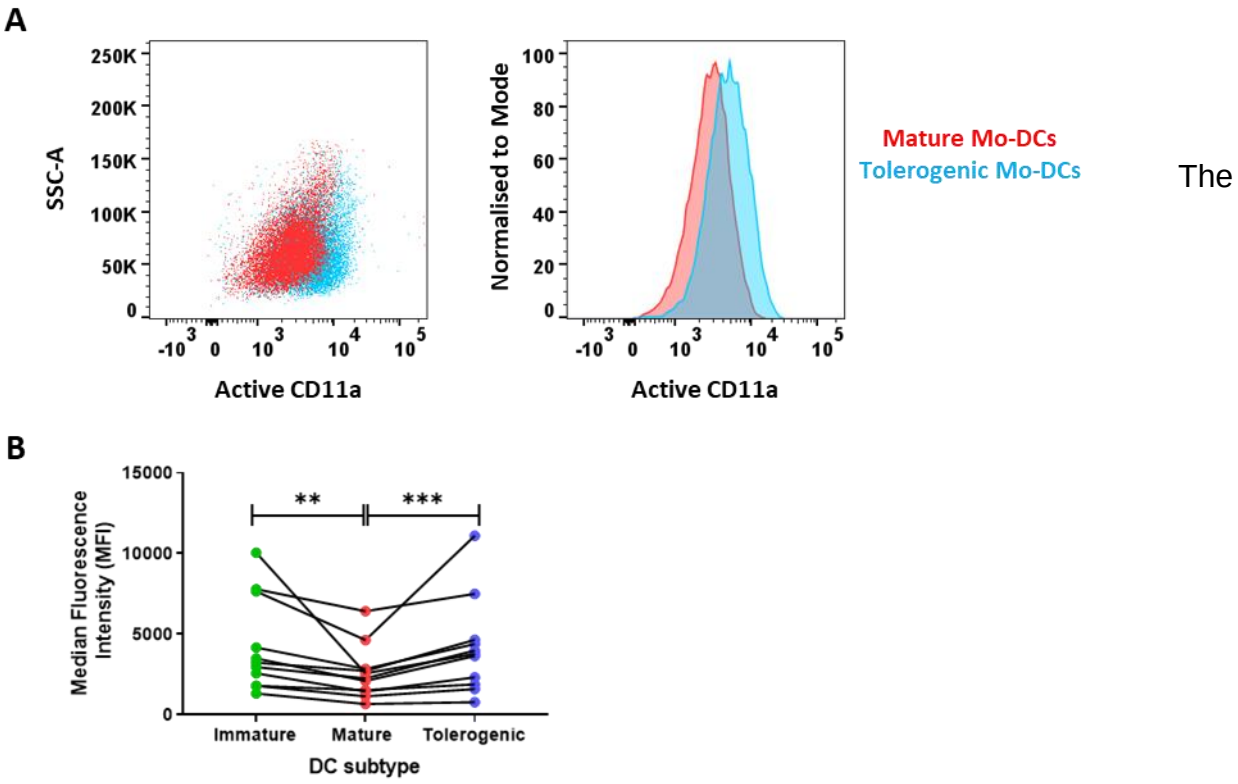

Figure 4.7 Expression of active CD11a in Mo-DCs

Expression of active CD11a in immature, mature and tolerogenic Mo-DCs. A. Representative dot plot and histogram overlay of active CD11a expression in mature (red) and tolerogenic Mo-DCs (blue). B. MFI expression of active CD11a in immature, mature and tolerogenic Mo-DCs (left, matched one-way ANOVA, n=13), or only comparing mature and tolerogenic Mo-DCs (right, paired Student's *t* test, n=13)

The significantly increased expression of active CD11a in tolerogenic compared to mature

Mo-DCs could suggest that β_2 integrins are altered on DCs under different circumstances. However, it is difficult to make assumptions about the potential functional impact of these differences on tolerogenic function of Mo-DCs. First, therefore, it was important to test if the observed difference in active CD11a expression affected cell adhesion. To this end, adhesion of mature and tolerogenic Mo-DCs to varying concentrations of the β_2 integrin ligand ICAM-1, which is a known ligand of CD11a (Landis et al., 1994), was compared. Given the increase in active CD11a expression seen in tolerogenic compared to mature Mo-DCs, we would predict increased adhesion of tolerogenic Mo-DCs to ICAM-1. Adhesion of mature and tolerogenic Mo-DCs to 0.1µg/ml (Figure 4.8A), 1µg/ml (Figure 4.8B) and 10µg/ml ICAM-1 (Figure 4.8C) is presented in Figure 4.8. Interestingly, no significant increase in adhesion was observed to any of the three concentrations of ICAM-1. Adhesion to the highest concentration of ICAM-1 (Figure 4.8C) showed a consistent upwards trend in all but one sample, which almost reaches statistical significance at p=0.051. This would suggest that the increase in active CD11a expression on tolerogenic Mo-DCs was not sufficient to increase adhesion to low and medium concentrations of ICAM-1, but that there might be a small but potentially important biological effect at high concentrations of ICAM-1. However, while ICAM-1 is described to be one of the main ligands for CD11a, both CD11b and CD11c can also bind ICAM-1 (Blackford et al., 1996; Diamond et al., 1990). This means that either one of the other β_2 integrin alpha subunits could potentially have compensated for the lower expression of active CD11a on mature Mo-DCs, making them equal to tolerogenic Mo-DCs. If given the ability to undertake further studies, β_2 integrin subunit specific blockade could be utilised to assess CD11a-mediated adhesion to ICAM-1 specifically.

To conclude, while total CD11a expression of CD11a did not differ between Mo-DCs, there was significantly higher expression of active CD11a on tolerogenic Mo-DCs compared to mature Mo-DCs. This explains the significantly increased level of total CD11a signal loss due to steric hindrance in tolerogenic Mo-DCs, as total CD11a binding sites may be obstructed by active CD11a binding. While increased expression of active CD11a on tolerogenic Mo-DCs did not translate to increased adhesion to one of the CD11a ligands ICAM-1, this could be explained by both CD11b and CD11c also being able to utilise ICAM-1 as a ligand, which could potentially mask effects of active CD11a expression. However, the almost significant increased adhesion of tolerogenic Mo-DCs to high levels of ICAM-1 suggests that

adhesion to especially T cells, which express ICAM-1 as part of stabilising the immunological synapse, might be altered as an effect of altered CD11a conformational state. It is also important to note that different β_2 integrin subunits might have distinct downstream-signalling pathways depending on ligand, thereby suggesting that even though CD11b or CD11c can maintain adhesion to ICAM-1 on mature Mo-DCs, downstream signalling might be altered due to the lower levels of CD11a binding ICAM-1. Because of the consistent and significant increase in active CD11a on tolerogenic Mo-DCs, one could hypothesise that active CD11a plays an immunoregulatory role in this *in vitro* system.

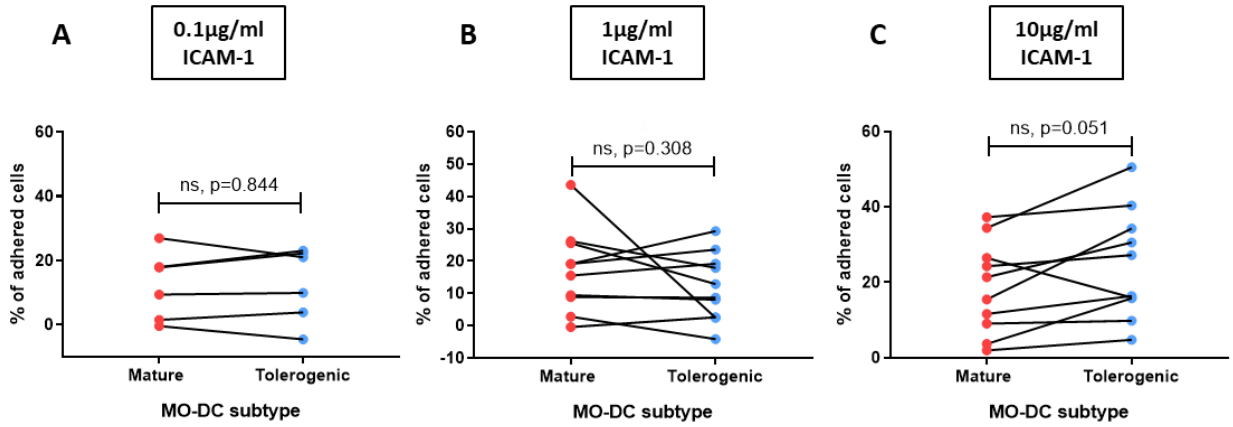

Figure 4.8 Adhesion of mature and tolerogenic Mo-DCs to ICAM-1

Adhesion assays comparing adhesion of mature to tolerogenic Mo-DCs to three concentrations of ICAM-1. A. Adhesion to ICAM-1 at 0.1µg/ml. B. Adhesion to ICAM-1 at 1µg/ml. C. Adhesion to ICAM-1 at 10µg/ml. Paired Student's *t* test, n=13.

4.2.4 The role of CD11b in Mo-DCs

Having explored total and active CD11a expression on Mo-DCs, I found that active CD11a expression was significantly higher on tolerogenic compared to mature Mo-DCs, but that the two Mo-DC subtypes did not differ significantly in their adhesion to ICAM-1. As CD11b, a different β_2 integrin alpha subunit, can also bind ICAM-1 and was hypothesised to be partially responsible for the similar adhesive capabilities of tolerogenic and mature Mo-DCs despite their differences in active CD11a, CD11b was also investigated. As no significant signal loss due to steric hindrance was observed between active and total CD11b (Chapter 3, section 3.3.4, Figure 3.4), only

samples where both total and active CD11b antibodies were used simultaneously were considered.

There were no significant differences in total CD11b expression between immature, mature and tolerogenic Mo-DCs, nor when comparing mature and tolerogenic Mo-DCs specifically to each other (Figure 4.9A-B). Neither expression of total CD11a nor total CD11b therefore differed between Mo-DC subtypes.

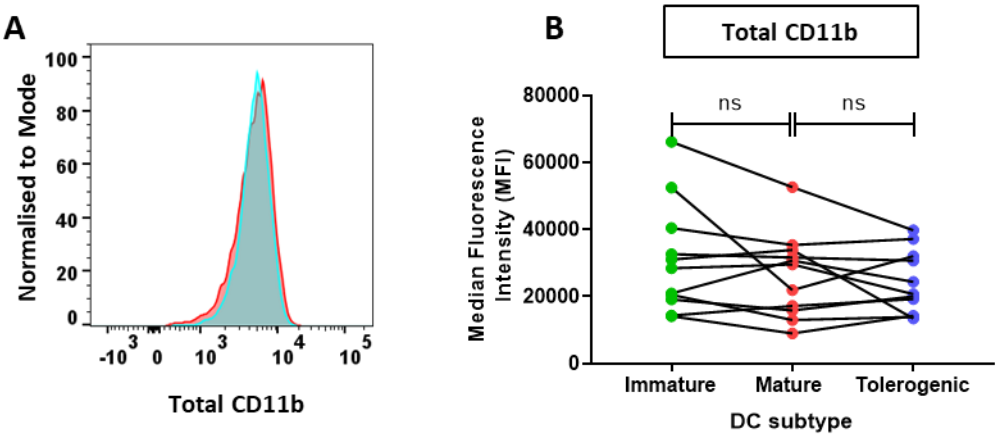

Figure 4.9 Expression of total CD11b on Mo-DCs

Expression of total CD11b on immature, mature and tolerogenic Mo-DCs. A. Representative overlay of expression of total CD11b in mature (red) and tolerogenic Mo-DCs (blue). B. MFI of total CD11b comparing immature (green), mature (red) and tolerogenic Mo-DCs (blue). Matched one-way ANOVA, n=13.

Levels of active CD11b were found to be lower on tolerogenic Mo-DCs when compared to mature Mo-DCs (Figure 4.10A-B), but only when comparing mature and tolerogenic Mo-DCs directly. When comparing immature, mature and tolerogenic Mo-DCs, tolerogenic Mo-DCs are not shown to be significantly different from mature Mo-DCs, but are significantly lower than immature Mo-DCs (Figure 4.10B, left). However, when only comparing mature and tolerogenic Mo-DCs, tolerogenic Mo-DCs were shown to have significantly lower expression of active CD11b. Interestingly, expression of active CD18, the shared β_2 integrin subunit of both CD11a and CD11b, was also significantly lower in tolerogenic Mo-DCs when compared only to mature Mo-DCs (Figure 4.11A-B). As active CD11a was found to be significantly increased in tolerogenic Mo-DCs, this could suggest that the lower active CD11b levels dominate over higher active CD11a levels on tolerogenic Mo-DCs, as active CD18 was also

significantly affected. However, when comparing active CD18 expression of all three Mo-DC subtypes to each other, no significant differences were observed. This could suggest that this effect is potentially smaller and therefore more relevant when comparing mature and tolerogenic Mo-DCs directly.

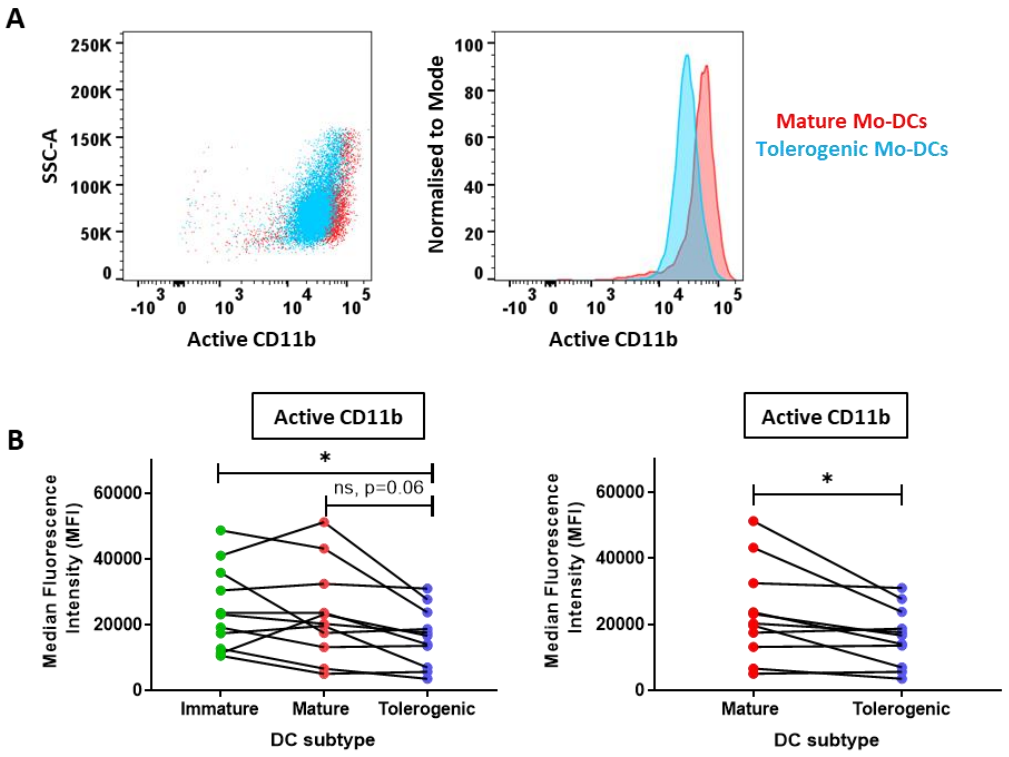

Figure 4.10 Expression of active CD11b in Mo-DCs

Expression of active CD11b in immature, mature and tolerogenic Mo-DCs. A. Representative dot plot and histogram overlay of active CD11b expression in mature (red) and tolerogenic Mo-DCs (blue). B. MFI of active CD11b expression in immature, mature and tolerogenic Mo-DCs (left, matched one-way ANOVA, n=13), or only comparing mature and tolerogenic Mo-DCs (right, paired Student's *t* test, n=13).

As active CD11b expression was significantly lower on tolerogenic compared to mature Mo-DCs, but active CD18 expression was similar, it was important to assess if different levels of active CD11b expression had functional consequences on Mo-DC adhesion. Similar to assessing adhesion of Mo-DCs to ICAM-1, adhesion was assessed to a range of CD11b ligands, including complement proteins iC3b and C4b as well as the blood protein fibrinogen. Due to the reduced expression of active CD11b on tolerogenic Mo-DCs, it was hypothesised that tolerogenic Mo-DCs would adhere less to CD11b ligands compared to mature Mo-DCs. Adhesion of mature and tolerogenic Mo-DCs to a range of concentrations of these CD11b ligands is shown in Figure 4.12.

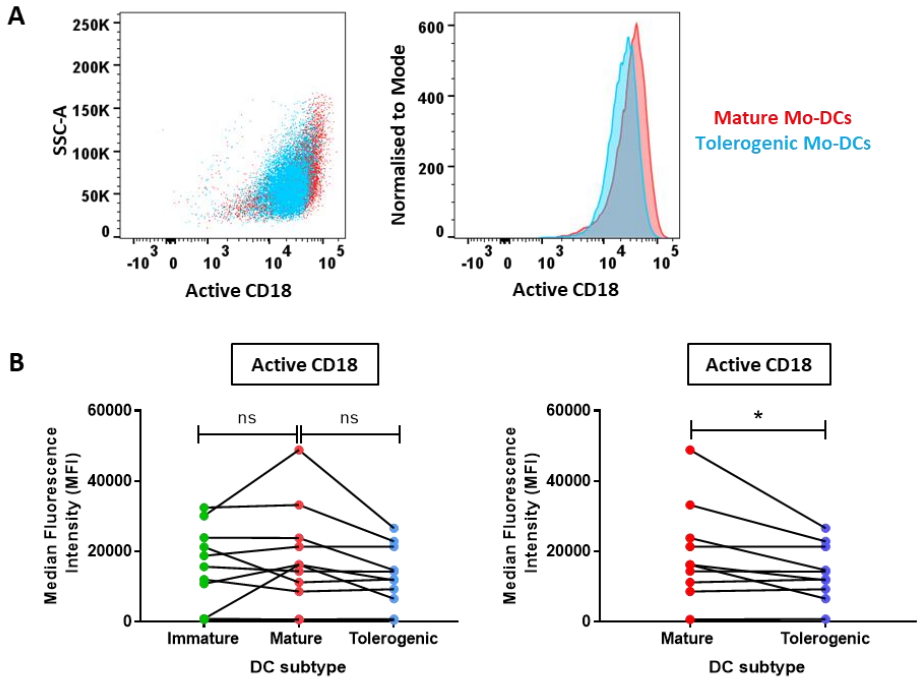

Figure 4.11 Expression of active CD18 in Mo-DCs

Expression active CD18 in immature, mature and tolerogenic Mo-DCs. A. Representative dot plot and histogram overlay of active CD18 expression in mature (red) and tolerogenic Mo-DCs (blue). B. MFI of active CD18 expression in immature, mature and tolerogenic Mo-DCs (left, matched one-way ANOVA, n=13), or only comparing mature and tolerogenic Mo-DCs (right, paired Student's *t* test, n=13).

As previously mentioned, iC3b (Figure 4.12A) was described to play an important role in the complement cascade and is produced when Complement Factor I cleaves C3b. Once it is cleaved, iC3b can act as an opsonin, enabling leukocytes to bind via CD11b/CD18 (Ross et al., 1985). Adhesion to iC3b was not found to be significantly different between mature and tolerogenic Mo-DCs in the lowest and highest ligand concentration, but adhesion to iC3b was significantly higher in tolerogenic Mo-DCs at 3.33mg/ml. This is surprising, as lower levels of active CD11b expression on tolerogenic compared to mature Mo-DCs would suggest a reduced ability to adhere to iC3b. C4b, a different member of the complement system, is located upstream of iC3b acting in concert with C2a to cleave C3 into C3a and C3b. It has also been described to act as opsonin mediating phagocytosis of opsonised cells mediated via outside-in signalling after CD11b binds to it (Gresham et al., 1991). No differences in adhesion between mature and tolerogenic Mo-DCs were found in any of the three concentrations of C4b (Figure 4.12B). Lastly, fibrinogen, a member of the coagulation cascade also termed Factor I, is an important factor for blood clotting and a

commonly used ligand to test adhesive capabilities of CD11b (Sándor et al., 2016b). Here, the breadth of ligand concentration used was larger with an additional concentrations added due to the wide variation in adhesion to fibrinogen between Mo-DCs derived from different donors. No significant differences in adhesion to fibrinogen were found in the lower concentrations, but at the highest ligand concentration of 100µg/ml tolerogenic Mo-DCs were found to be significantly more adhesive compared to mature Mo-DCs. These data fit with the increased adhesion to iC3b but contradict the hypothesis that reduced active CD11b expression would result in reduced adhesion to CD11b ligands.

The result that lower expression of active β_2 integrins resulted in increased adhesion to its ligand is puzzling and requires further discussion. There are several ways to interpret these results. First, while CD11b is lower on tolerogenic Mo-DCs, it could be more potent and therefore more likely to establish stable adhesion to a ligand that withstands washing steps of the adhesion assay protocol to a higher degree. This is however highly unlikely, as β_2 integrins in their active form have the highest affinity for ligand and integrin activation correlates well with adhesive capability (Coller, 1985; Shattil et al., 1985).

A more likely explanation is the ligand promiscuity of β_2 integrins, which was already discussed in the context of different β_2 integrin alpha-subunits binding to ICAM-1. Not only CD11b but also CD11c can bind both iC3b and fibrinogen (Loike et al., 1991; Malhotra et al., 1986; Myones et al., 1988), making it possible that CD11c compensates for reduced expression of active CD11b on tolerogenic Mo-DCs. While no difference in total CD11c expression on Mo-DCs was found (Figure 4.13), active CD11c expression was not measured. One could therefore hypothesise that active CD11c expression on tolerogenic Mo-DCs is increased, compensating for the reduction in active CD11b expression and leading to an overall increase in adhesion to iC3b and fibrinogen.

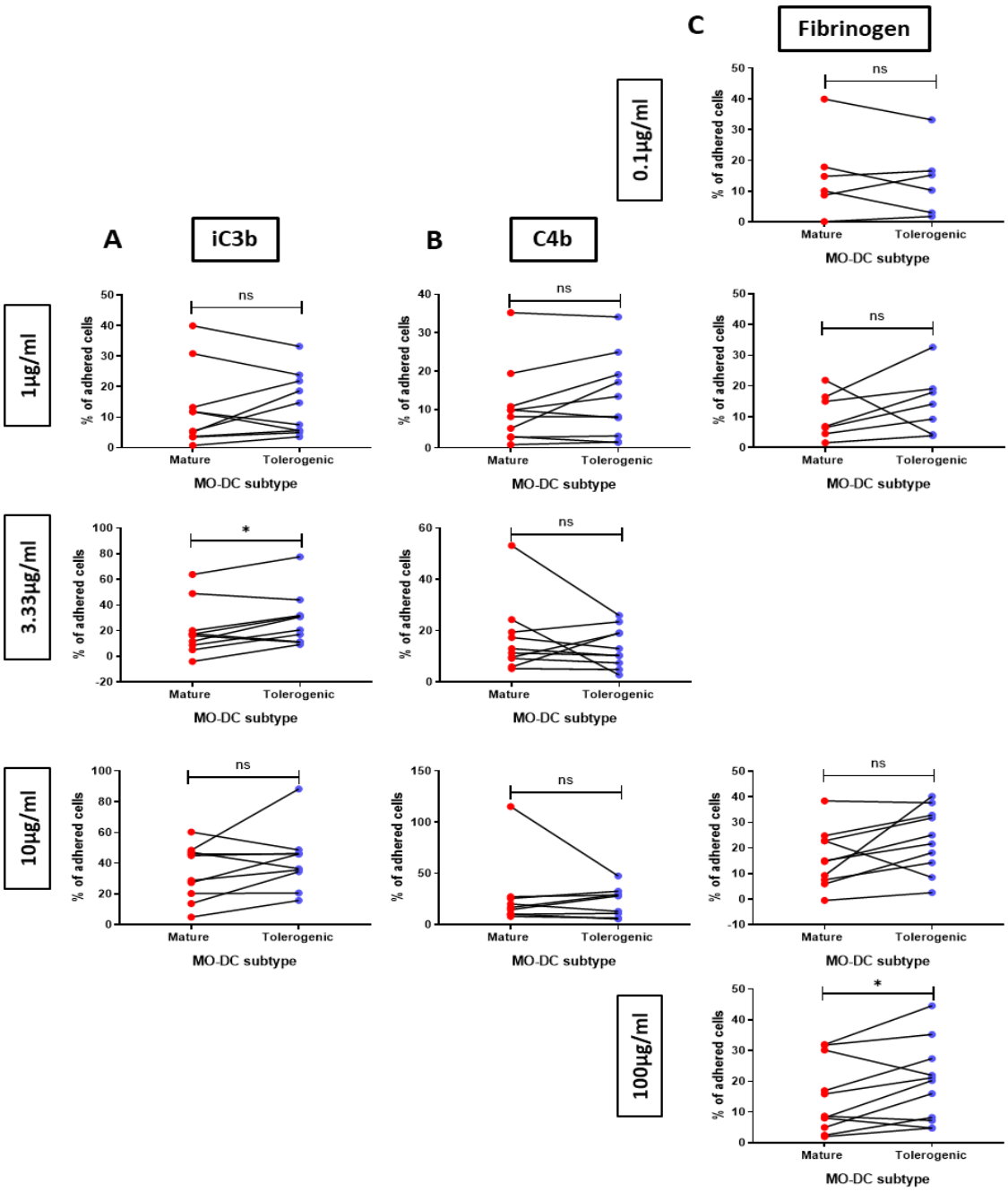

Figure 4.12 Adhesion of mature and tolerogenic Mo-DCs to iC3b, C4b and Fibrinogen

Adhesion assays comparing adhesion of mature (red) to tolerogenic Mo-DCs (blue) to three concentrations of iC3b and C4b, and four concentrations of fibrinogen. A. Adhesion to iC3b at 1µg/ml, 3.33µg/ml and 10µg/ml. Paired student's *t* test, n=10. B. Adhesion to C4b at 1µg/ml, 3.33µg/ml and 10µg/ml. Paired student's *t* test, n=10. C. Adhesion to Fibrinogen at 0.1µg/ml (n=6), 1µg/ml (n=7), 10µg/ml (n=10) and 100µg/ml (n=11). Paired student's *t* test.

This explanation is supported further by the fact that C4b is not described to be a ligand for CD11c, and adhesion to this protein did not show a similar pattern to iC3b and fibrinogen, and adhesion did not differ between mature and tolerogenic Mo-DCs. This could suggest that respective binding of different β_2 integrin subunits to ligand might be regulated either by subunit conformational state or interplay between different subunits, such as CD11b and CD11c.

In conclusion, active but not total CD11b expression was significantly lower on tolerogenic Mo-DCs when compared to mature Mo-DCs. Additionally, active CD18, the pairing β_2 integrin of both CD11a and CD11b, was also significantly lower in tolerogenic Mo-DCs. This suggests that the lower expression of active CD11b dominates over the higher expression of CD11a on tolerogenic Mo-DCs. The down-regulation of active CD11b on tolerogenic Mo-DCs, compared to both mature and immature Mo-DCs could suggest that lowering CD11b activation plays a role in the distinct phenotype tolerogenic Mo-DCs show compared to mature Mo-DCs. However, adhesion to β_2 integrin ligands iC3b, C4b and fibrinogen was not negatively affected by the decrease in active CD11b expression, suggesting that surface expression and functionality are potentially not directly linked. Indeed, adhesion to iC3b and fibrinogen was actually higher in tolerogenic compared to mature Mo-DCs, an effect presumed to be mediated by an increase in active CD11c. On the other hand, adhesion to C4b was not different between mature and tolerogenic Mo-DCs.

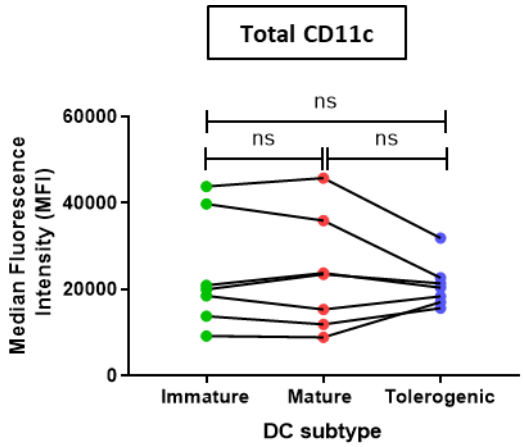

Figure 4.13 Expression of total CD11c on Mo-DCs

Expression of total CD11b on immature, mature and tolerogenic Mo-DCs. MFI of total CD11c comparing immature (green), mature (red) and tolerogenic Mo-DCs (blue). Matched one-way ANOVA, n=7.

To summarise my work up to this point, I found that mature and tolerogenic Mo-DCs differ in their active β_2 integrin expression, with tolerogenic Mo-DCs expressing more active CD11a and less active CD11b than mature Mo-DCs. I also found that adhesion to iC3b and fibrinogen, which have been reported to be ligands for both CD11b and CD11c, was significantly increased in tolerogenic Mo-DCs, while adhesion ICAM-1, bound by both CD11a and CD11b, was not significantly different between groups with the available sample size. Furthermore, tolerogenic Mo-DCs showed less clustering when adherent to glass. Together these data suggest that altered β_2 integrin conformational state on tolerogenic Mo-DCs contributes to altered adhesive and clustering behaviour. However, while tolerogenic Mo-DCs showed reduced T cell stimulatory abilities, it was important to investigate if this could also be due to altered β_2 integrins on tolerogenic Mo-DCs. To this end, I tested if altering availability of the active CD11b β_2 integrin subunit affected the T cell stimulatory abilities of mature and tolerogenic Mo-DCs.

4.2.5 Functional significance of β_2 integrins on Mo-DCs

To investigate the roles of β_2 integrins on Mo-DCs in mediating T cell proliferation and activation, both increasing β_2 integrin activation and blocking β_2 integrin adhesion were explored in MLRs. T cell proliferation was measured by CTV signal loss, and IFN-γ and IL-10 levels were quantified in MLR supernatants by ELISA.

I decided to focus on CD11b for the manipulation studies. This decision had several reasons. First, while active CD11a expression was significantly increased in tolerogenic compared to mature Mo-DCs, the significantly lower active CD18 expression on tolerogenic Mo-DCs suggested that CD11b might be a more dominant integrin. Second, manipulating either CD11a or CD18 in the context of an MLR held additional caveats: β_2 integrins not only play a role on DCs but are also important adhesion receptors on T cells. Indeed, investigation of total CD11a, CD11b, CD11c and CD18 expression on naïve CD45RO- T cells showed high levels of CD11a and CD18, and a low level of CD11c (Figure 4.14). On the other hand, naïve T cells showed no positive staining for CD11b, which is congruent with the published literature on β_2 integrins on T cells: In mice, absence of CD11b expression was shown to be one of the hallmarks of naïve CD4+ and CD8+ T cells (Christensen et al., 2001; Hataye et al., 2006; McFarland et al., 1992). This made manipulation of CD11b the best option, as any effects on T cell proliferation and differentiation in MLRs would be due to the DC-specific function of the β_2 integrin and not any T cell-mediated effects.

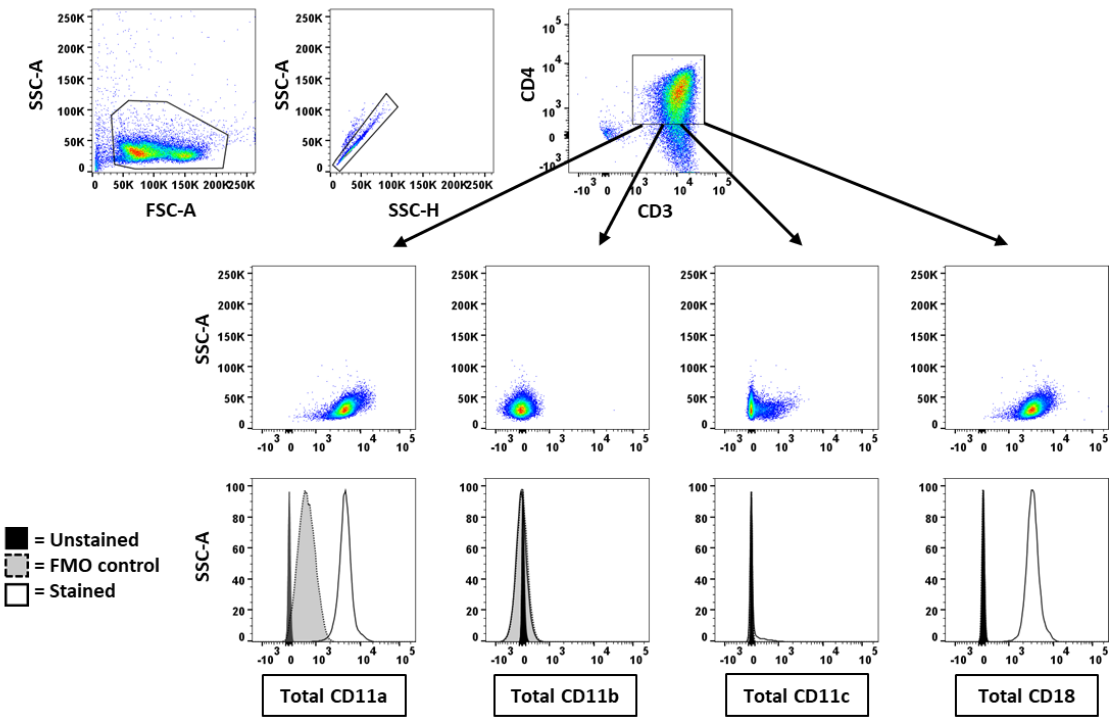

Figure 4.14 CD45RO-CD4+ T cells express CD11a and CD18, but not CD11b

CD45RO-CD4+ T cells isolated from healthy buffy coat for use in MLRs were stained for expression of total β_2 integrins CD11a, CD11b, CD11c and CD18. Multi-colour dot plots of stained single CD3+CD4+ T cells are shown above histogram overlays of unstained t cells (black), an FMO control (grey) and the stained sample (white). N=1.

Effect of activating the Mo-DC integrin subunit CD11b on T cell priming

Considering the significant decrease in active CD11b expression between mature and tolerogenic Mo-DCs, one could hypothesise that increasing active CD11b expression could enhance T cell proliferation and/or production of IFN-γ. This could be paired with a lower production of IL-10 in MLRs where CD11b activation was increased. Overall, I predict that increasing active CD11b on tolerogenic Mo-DCs might make them more similar to mature Mo-DCs in their ability to stimulate T cells. To investigate the role of CD11b on Mo-DCs in T cell proliferation, Mo-DCs were cultured as previously described, harvested and co-cultured with freshly isolated naïve T cells from healthy buffy coats. Proliferation and cytokine analysis from supernatants took place on Day 6 of culture.

To this end, a small-molecule agonist of CD11b, Leukadherin-1 (LA-1) was utilised to induce CD11b activation in MLRs. Leukadherins were identified to increase CD11b-

mediated adhesion to fibrinogen using a high-throughput screening system (Park et al., 2007), and have since been found to be a promising therapeutic agent (Dickinson et al., 2018). LA-1 specifically has been used in a wide range of studies and is commercially available. However, preliminary results testing the effect of LA-1 on MLRs showed that 15µM of LA-1, a concentration previously used in the literature (Maiguel et al., 2011), was not feasible due to the DMSO content: T cell proliferation was almost completely inhibited in both mature and tolerogenic Mo-DC cultures when treated with either LA-1 or the DMSO control (Figure 4.15). These data indicate that it is the DMSO, rather than the LA-1, that is inhibiting T cell proliferation.

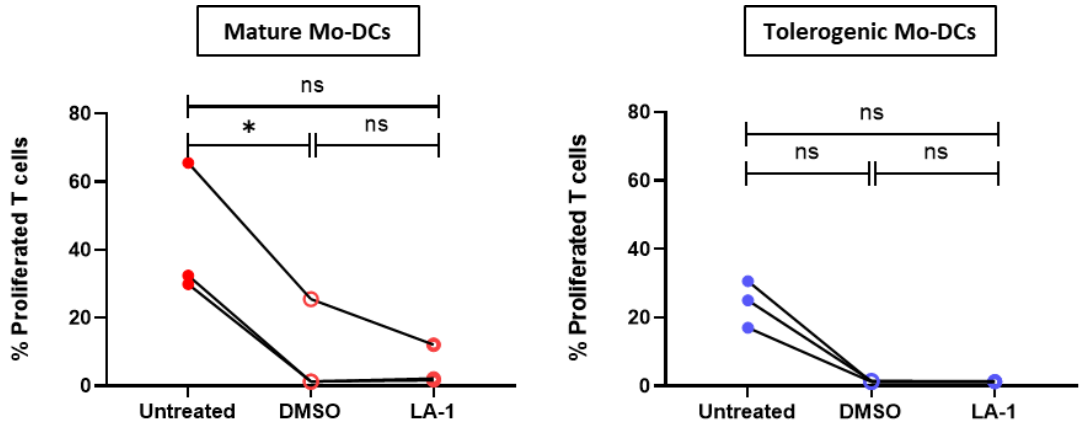

Figure 4.15 High concentrations of LA-1 dissolved in DMSO inhibits T cell proliferation in MLRs

Effect of 15µM LA-1 or equivalent volume of DMSO (1.5%) as a control in MLRs with either mature (left) or tolerogenic Mo-DCs (right), compared to an untreated culture. Matched one-way ANOVA, Mo-DCs n=3, with naïve CD4+ T cells from a single donor.

As per the recommendation of the supplier, LA-1 was dissolved in pure DMSO before being added to cells, which meant cells in Figure 4.15 were exposed to 1.5% of DMSO. As concentrations over 1% of DMSO have been described to be toxic in some cell lines (Galvao et al., 2013), it was decided to reduce the amount of DMSO added to MLRs. To this end, LA-1 concentration was titrated using concentrations between 0.5% and 0.1% DMSO per well, which is tolerated by T cells (Kloverpris et al., 2010).

It was found that adding LA-1 at a concentration of 5µM, resulting in a final concentration of 0.5% of DMSO, caused no negative effects on T cell proliferation in mature Mo-DCs, with T cell proliferation being equivalent between untreated and

DMSO-treated cultures in a representative experiment, at 57.4 and 53.0%, respectively (Figure 4.16A), which was confirmed in DMSO treated cultures not being significantly different from untreated cultures (Figure 4.16B). LA-1 treatment of mature Mo-DC MLRs resulted in a broadly similar level of T cell proliferation (63.6%), again confirmed by LA-1 treated cultures being not significantly different from untreated MLRs (Figure 4.16A-B). Conversely, the tolerogenic Mo-DC MLR was visibly affected by even the reduced amount of DMSO, with T cell proliferation in the DMSO control being reduced to 4.03% compared to 70.2% in the untreated culture (Figure 4.16C). However, it was interesting to observe that treatment with 5µM LA-1 partially rescued T cell proliferation, with approximately a third (33.5%) of T cells in the LA-1-treated tolerogenic Mo-DC cultures undergoing cell division (Figure 4.16C). However, quantification of tolerogenic MLR data showed that while both DMSO and LA-1 treated MLRs showed significantly lower T cell proliferation, no significant increase between DMSO and LA-1 could be observed. As active CD11b was higher in mature Mo-DCs (Figure 4.10), one could hypothesise that CD11b activation cannot be further increased as mature Mo-DCs already expressed the maximal amount possible. One the other hand, tolerogenic Mo-DCs did show significant loss of T cell proliferation in response to 0.5% DMSO, as well as 5µM of LA-1. This differential response to DMSO between mature and tolerogenic Mo-DCs was surprising, as it suggested either that T cells did not proliferate as effectively in the presence of DMSO or that tolerogenic Mo-DCs in turn show reduced ability to activate T cells due to DMSO exposure. However, LA-1-treated tolerogenic Mo-DC co-cultures had a trend for higher T cell proliferation than their DMSO control counterparts. When comparing DMSO control and LA-1 treatment directly to each other, however, the result was not significant. This would indicate that LA-1 cannot rescue the negative effect of DMSO on T cell proliferation and instead shows that CD11b activation in MLRs has no effect on T cell proliferation.

This was an unexpected result, as I had hypothesised that increasing activation of CD11b on tolerogenic Mo-DCs especially, due to their low expression of active CD11b (Figure 4.10), would increase Mo-DC-mediated T cell proliferation. To test the efficacy of the CD11b activating drug, effect of LA-1 treatment on active CD11b expression was investigated on Mo-DCs exposed to either 5µM LA-1 or equivalent DMSO and compared to untreated cells (Figure 4.17). Interestingly, no consistent increase in active CD11b expression was found in either mature or tolerogenic Mo-

DCs from four donors. In fact, results seemed to differ widely between donors: While donor 1 (Figure 4.17A) and donor 4 (Figure 4.17D) showed no effect of LA-1 treatment in either mature or tolerogenic Mo-DCs, donor 2 (Figure 4.17B) showed lower active CD11b expression on mature Mo-DCs exposed to DMSO, and with the LA-1 treated cells actually being lower in active CD11b in both mature and tolerogenic Mo-DCs, although this is likely to be due to the reduction in total CD11b expression observed in both Mo-DC subtypes. The only Mo-DC data-set where LA-1 treatment caused a visible increase in active CD11b expression was donor 3 (Figure 4.17C), but treatment with the equivalent percentage of DMSO increased active CD11b to a similar degree as LA-1 in both mature and tolerogenic Mo-DCs.

Overall, these data showed that treatment with 5µM LA-1 did not reliably induce an increase of active CD11b expression. LA-1 was reported to allosterically activate CD11b and thereby cause increased adhesion to fibrinogen and increased binding of an activation-specific antibody targeted at its pairing subunit CD18 (Maiguel et al., 2011). However, the same study also suggested that this was due to binding of LA-1 to the αI domain, which thereby stabilised the active conformation of CD11b. Therefore, while LA-1 was found to locally stabilise CD11b in its extended conformation, a global increase in expression of active CD11b was not observed in some studies (Faridi et al., 2013). Furthermore, using atomic force microscopy to test strength of ligand-integrin interaction, it was shown that LA-1 increased adhesion to ICAM-1 by increasing formation of membrane tethers with CD11b subunits showing weaker cytoskeletal linkage bonds (Celik et al., 2013). In light of this, it is possible that LA-1 is functional but that I cannot observe its functionality either with activation-specific antibodies, as it has not been reported to directly affect conformational state of CD11b, or in the more long-term culture conditions of an MLR, as it has been mostly reported to show effects in short-term cultures.

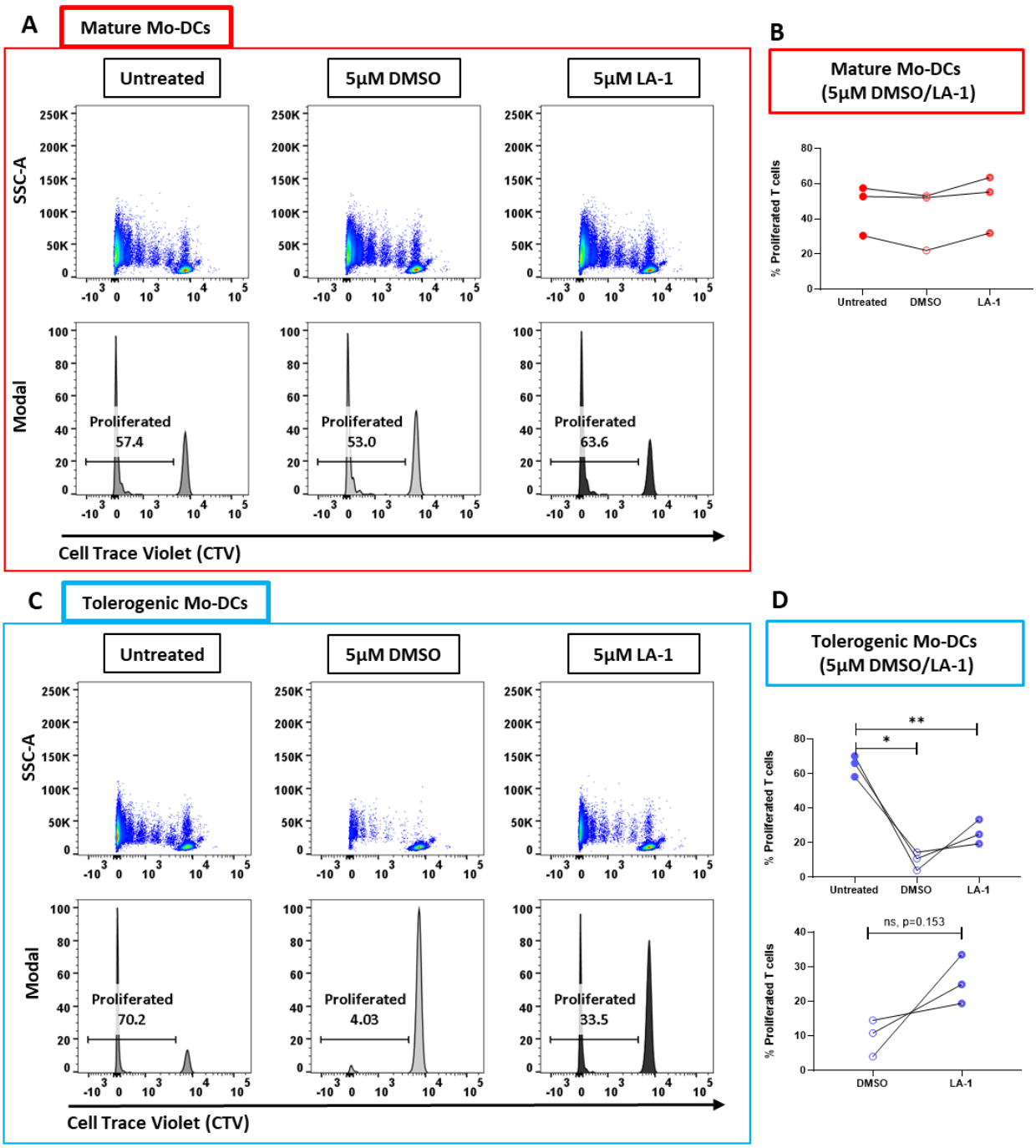

Figure 4.16 Effect of LA-1/DMSO on mature and tolerogenic Mo-DCs

Representative effect of 5µM LA-1 or 0.5% DMSO equivalent control on T cell proliferation in mature (A) and tolerogenic (B) Mo-DC MLRs, compared to untreated co-cultures. Effect of 5µM LA-1 or 0.5% DMSO equivalent control on mature and tolerogenic Mo-DC MLRs, compared to untreated co-cultures. A. Representative effect of LA-1/DMSO on mature Mo-DC MLRs. B. Quantification of effect of LA-1/DMSO on mature Mo-DC MLRs. One-way ANOVA, Mo-DCs n=3, naïve T cells n=3.C. Representative effect of LA-1/DMSO on tolerogenic Mo-DC MLRs. D. Quantification of effect of LA-1/DMSO on tolerogenic Mo-DC MLRs. One-way ANOVA, Mo-DCs n=3, naïve T cells n=3.

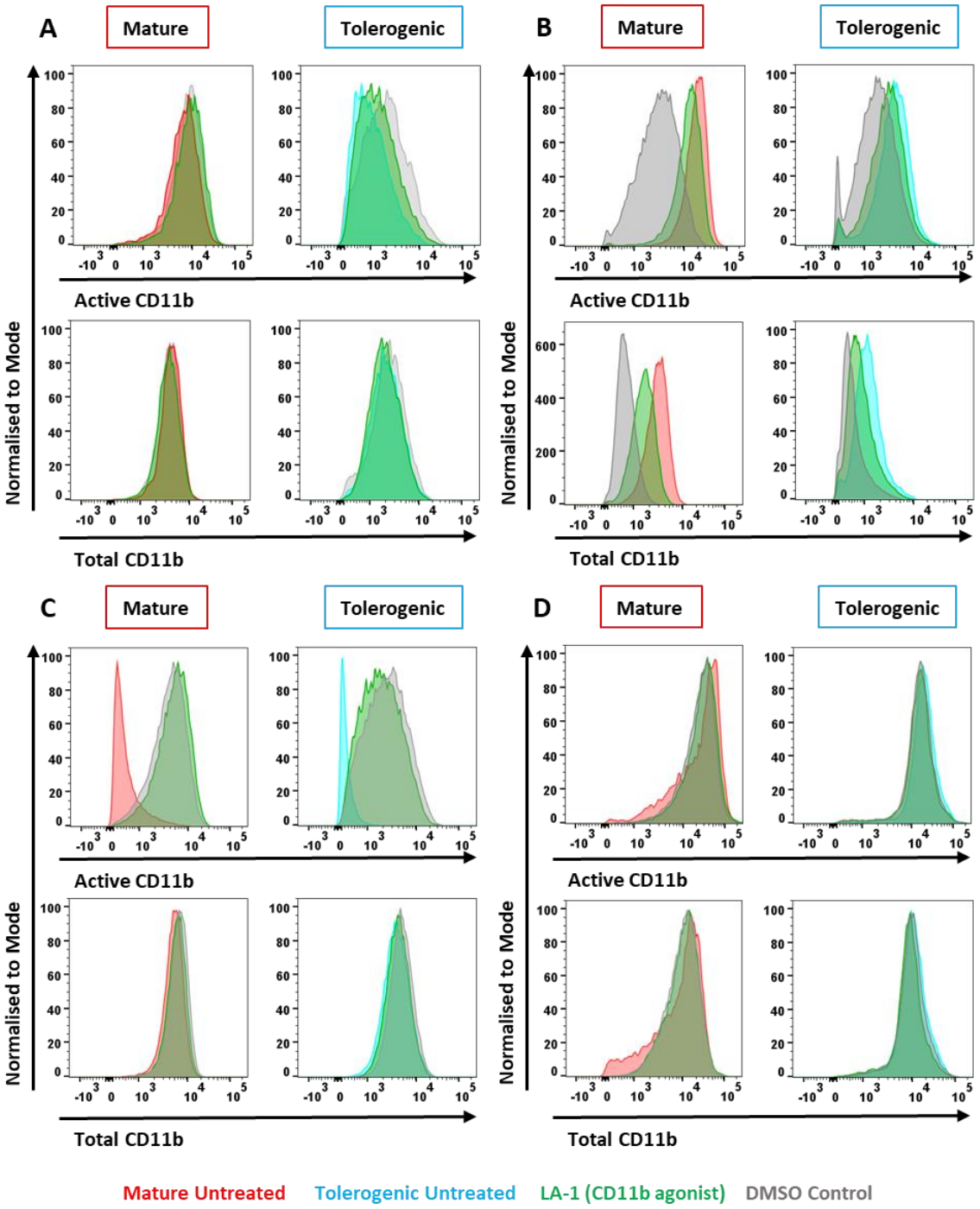

Figure 4.17 Effect of CD11b agonist LA-1 on expression of active and total CD11b

Effect of 5µM LA-1 or 0.5% DMSO control on expression of active and total CD11b in mature and tolerogenic Mo-DCs after 18h of incubation at 37°C. A-D represent four individual Mo-DC donors. N=4.

Another option is that CD11b is already in its active conformation in donors A, B and D (Figure 4.17). This argument can be supported by Figure 4.18, which shows mature and tolerogenic Mo-DCs with the respective lowest and highest MFI value for active CD11b. Comparing the highest recorded expression of active CD11b in both mature and tolerogenic Mo-DCs to the untreated conditions in Figure 4.17, this strongly suggests that CD11b is already in a highly active conformation and can therefore potentially not be activated further by LA-1.

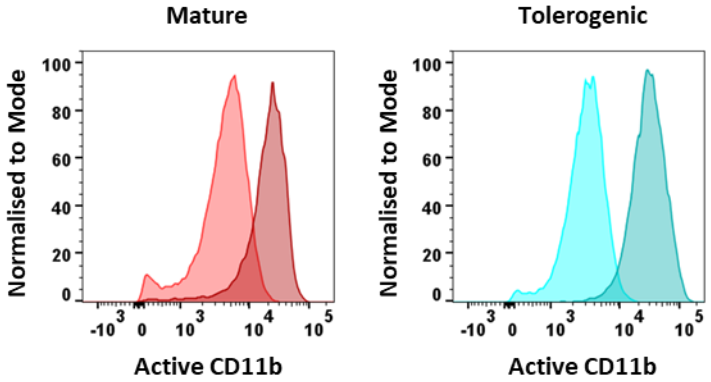

Figure 4.18 Range of expression of active CD11b on mature and tolerogenic Mo-DCs

Histogram overlay of mature (left) and tolerogenic Mo-DCs (right) with lowest (light coloured) and highest MFI (dark coloured) of active CD11b expression. Live, single untreated cells.

Lastly, it was important to consider if the negative effect of the DMSO vehicle was due to Mo-DCs or T cells being affected. To this end, percentage of dead cells, both CD11c+ to gain information on any Mo-DCs remaining in culture, and CD11c-, to assess if DMSO was directly toxic to T cells was being compared between untreated and the DMSO control (Figure 4.19). However, cell death was not significantly different between untreated and MLRs exposed to DMSO, and it was furthermore very low. This suggests that the negative effect of DMSO on T cell proliferation was not mediated by toxicity mediating increased cell death. However, considering that DMSO was shown to increase expression of active CD11b in Donor C (Figure 4.17C), which was the only donor shown to not have high expression of active CD11b regardless of treatment, it could suggested that DMSO might have a range of effects on both Mo-DCs and T cells that hinder effective proliferation. However, as three out of four donors showed high expression of active CD11b regardless of DMSO, it is unlikely that its effect is mediated via CD11b.

To conclude, treatment of MLRs with LA-1 had no significant effect on T cell proliferation as active CD11b expression on Mo-DCs was not increased. There was

an unexpected negative influence of DMSO on tolerogenic Mo-DCs that was not observed to affect mature Mo-DCs. This suggests that the DMSO vehicle, that LA-1 was dissolved in, inhibited either Mo-DCs, T cells or both in their ability to produce T cell proliferation to a similar level of the untreated control. As active CD11b was not consistently increased by LA-1 treatment independently of DMSO, it was decided to not pursue measurement of IFN-γ and IL-10 concentrations in culture supernatants or further LA-1 treatment studies. Further investigation, for example of other CD11b agonists, is required to fully define the impact of CD11b activation in MLRs.

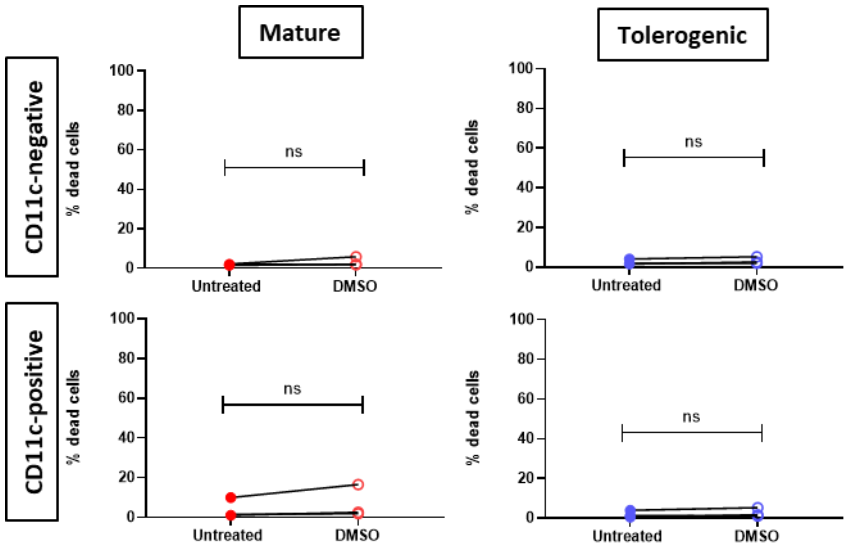

Figure 4.19 Effect of DMSO treatment on cell death

Assessing the percentage of cell death occurring in mature and tolerogenic Mo-DC MLRs in response to DMSO. Cells shown are single, positive for cell death marker and either CD11c- (T cells) or CD11c+ (Mo-DCs). Paired student's *t* test. N=3

Effect of blocking the β_2 integrin subunit CD11b

Due to the low expression of active CD11b on tolerogenic Mo-DCs and considering tolerogenic Mo-DCs reduced ability to stimulate IFN-γ production by T cells in an MLR, I hypothesised that downregulating active CD11b is an immunoregulatory process. Investigating this hypothesis, I predicted that blocking CD11b on Mo-DCs in an MLR would decrease T cell proliferation and IFN-γ production, while simultaneously increasing IL-10 production. To investigate the effect of blocking CD11b function, a CD11b-specific blocking antibody (clone M1/70) was compared to an isotype control which were added to the MLRs.

The effect of blocking CD11b on T cell proliferation is shown in Figure 4.20A. While tolerogenic Mo-DCs were not significantly affected in their ability to induce T cell proliferation with CD11b blocking, mature Mo-DCs induced significantly less proliferation of T cells when CD11b was blocked. In mature Mo-DC MLRs treated with a CD11b blocking antibody, significantly less T cells showed a high number of divisions (5-7), suggesting that blocking of CD11b caused T cells to either divide less quickly or stop dividing after less division cycles. As expression of active CD11b was already lower on tolerogenic compared to mature Mo-DCs, one could hypothesise that availability of active binding sites for ligand was already reduced. This would suggest that, even though tolerogenic Mo-DCs still expressed high levels of active CD11b, reduction of active CD11b might only be able to reduce T cell stimulatory capability to a certain degree, which was already reached in tolerogenic Mo-DCs. This would explain the absence of an effect in tolerogenic Mo-DC MLRs. One could therefore suggest that reducing CD11b activity on mature Mo-DC made them more tolerogenic Mo-DC-like. In support of this, there was a small but significant decrease in IFN-γ concentration measured in MLR supernatants in mature but not tolerogenic Mo-DC MLRs when CD11b was blocked (Figure 4.20B). This suggested that blocking CD11b did not only have an effect on T cell proliferation but also reduced the amount of pro-inflammatory cytokines produced by the T cells. On the other hand, no significant difference in IL-10 production was found with CD11b blocking of MLRs of either mature or tolerogenic Mo-DCs (Figure 4.20C). The absence of an effect of blocking CD11b on IL-10 production by T cells could be due to different reasons. As I am hypothesising CD11b to be more pro-inflammatory, it is possible that production of IL-10 was simply not affected by CD11b. As previously mentioned, T cells can also produce IL-10, which might render small differences due to treatment with the CD11b blocking antibody invisible.

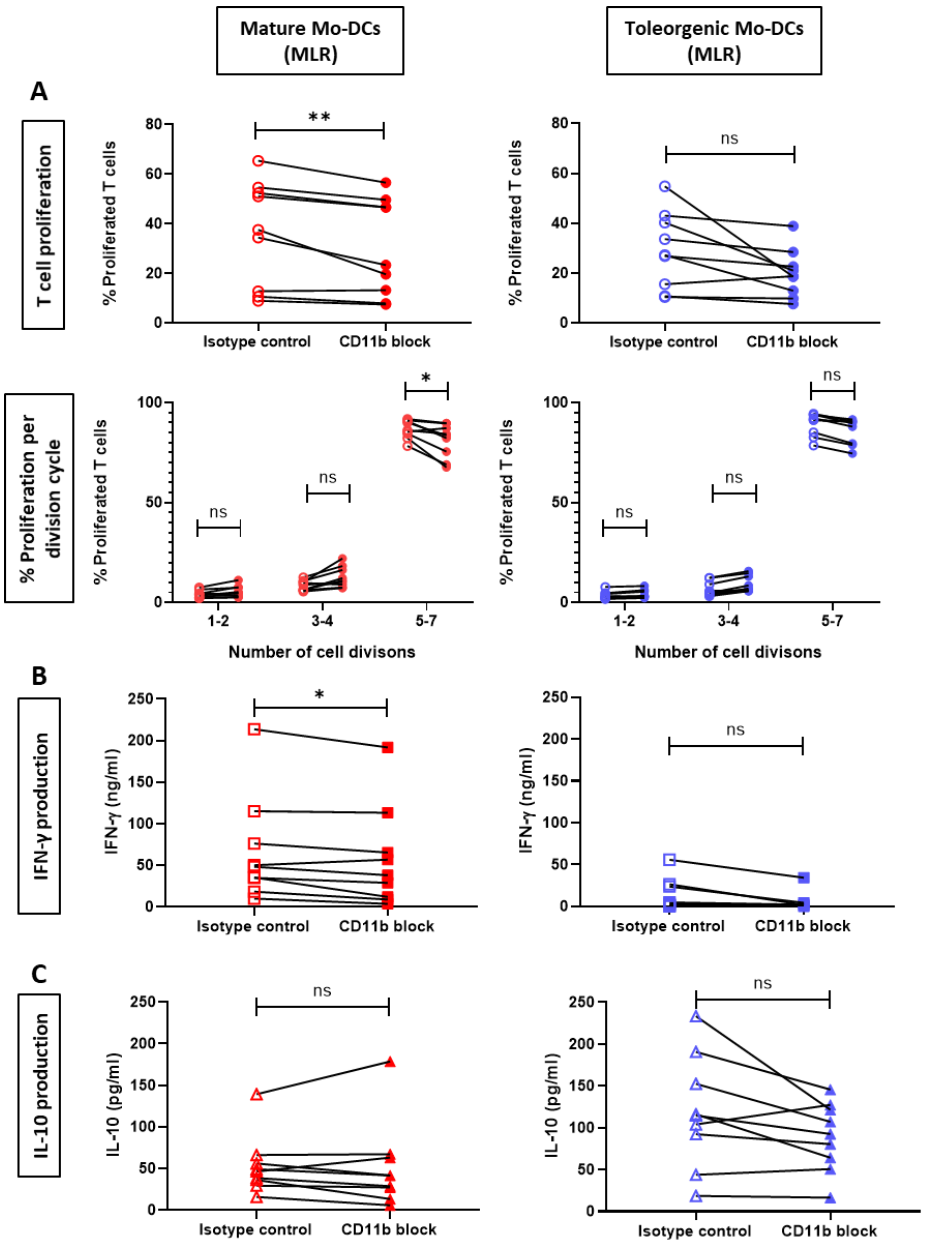

Figure 4.20 Effect of blocking CD11b on mature and tolerogenic Mo-DCs in MLRs

Effect of blocking of CD11b using a mAb (clone M1/70) compared to an isotype control (Rat, IgG2b, κ) in mature (red) and tolerogenic Mo-DC (blue) MLRs. A. Effect on T cell proliferation in percentage of T cells that showed diluted CTV signal due to cell division, percentage of T cells that have undergone proliferation (top) and percentage of cells per division category (1-2, 3-4, 5-7) (bottom) are compared between isotype control (unfilled circle) and CD11b blocking antibody (filled circle). B. Concentration of IFN-γ in MLR supernatants on day 6 measured by ELISA. C. Concentration of IL-10 in MLR supernatants on day 6 measured by ELISA. Paired Student's *t* test, Mo-DCs n=9, naïve T cells n=3.

To conclude, blocking CD11b significantly reduced the level of T cell proliferation and IFN-γ production elicited by mature Mo-DCs, but not tolerogenic Mo-DCs. One could therefore hypothesise that blocking CD11b on mature Mo-DCs made them more similar to tolerogenic Mo-DCs in terms of their T cell priming capacity.

4.3 Discussion

In this chapter, I investigated expression of both total and active CD11a and CD11b on mature and tolerogenic Mo-DCs, as well as their respective ability to adhere to a range of β_2 integrin ligands. There, I found that tolerogenic Mo-DCs express higher levels of active CD11a, but lower levels of active CD11b compared to mature Mo-DCs, which suggested opposing roles between the two subunits. While tolerogenic Mo-DCs actually adhered significantly better to CD11b ligands iC3b and fibrinogen, only a trend towards increased adhesion to ICAM-1 could be found at its highest concentration. Lastly, findings of differences in β_2 integrin expression were taken forward to test the functional role of the β_2 integrin CD11b in MLRs, using activating and blocking agents. This showed that blocking CD11b on mature Mo-DCs reduced their ability to induce T cell proliferation and downregulated IFN-γ production by T cells, suggesting that CD11b has pro-inflammatory roles in Mo-DCs. To summarise, *in vitro* study of mature and tolerogenic Mo-DCs has highlighted potential opposing roles for β_2 integrin subunits: high expression of active CD11a in tolerogenic Mo-DCs suggests an immunoregulatory role; whilst expression and intervention data point to a pro-inflammatory role for CD11b.

4.3.1 Opposing roles of CD11a and CD11b

While expression of the β_2 integrin CD11a in its active conformation was increased, in tolerogenic Mo-DCs compared to mature Mo-DCs, expression of active CD11b was significantly decreased in tolerogenic Mo-DCs. One can therefore conclude that they have opposing functions. While total CD11a or CD11b expression was not altered between Mo-DC subtypes, tolerogenic Mo-DCs expressed significantly higher levels of active CD11a and significantly lower levels of active CD11b compared to mature Mo-DCs. Tolerogenic Mo-DCs show decreased ability to stimulate IFN-γ production by T cells in an MLR, which may suggest that altered β_2 integrin conformational states could contribute to their tolerogenic function. Therefore, I hypothesised that CD11a has more immunoregulatory functions, as it was increased, while CD11b has more pro-inflammatory functions, as it was downregulated on tolerogenic Mo-DCs.

Evidence for immunoregulatory roles of CD11a

In the case of CD11a, an immunoregulatory role is supported by data in published literature. For example, constitutive activation of CD11a on BMDCs through a gene targeting approach actually reduced the amount of T cell proliferation DCs were ability to induce both *in vitro* and *in vivo* (Balkow et al., 2010). This was especially surprising, as constitutively active CD11a on DCs also increase their adhesion to ICAM-1 and prolonged contact time with T cells, both of which would have been expected to increase BMDC-mediated T cell stimulation. Furthermore, this effect could be rescued by blocking CD11a function using blocking antibodies, again suggesting that inactive CD11a is needed for effective T cell stimulation. Controlling activation of CD11a could therefore be understood as a highly effective immunoregulatory tool. However, this finding is surprising when considering what is known about timing DC-T cell interactions correctly. A large number of studies have shown that prolonged contact between T cells and DCs is needed for CD4+ T cell priming and proliferation (Celli et al., 2007; Hugues et al., 2004; Obst et al., 2005; Stoll et al., 2002). Additionally, Hugues and colleagues also reported that continuous short-term DC-T cell interactions are required for tolerance induction, and another study showed that Tregs limited development of diabetes by limiting contact duration between DCs and CD4+ T cells (Tang et al., 2006). However, another study showed that within the first 8h of antigen presentation, T cells only interacted briefly with DCs, after which they showed reduced motility and increased expression of activation markers (Mempel et al., 2004). After this time period, T cells were reported to associate with DCs for over 1h, finally resulting in T cell activation. This could suggest that early tight adhesion to T cells because of increased active CD11a might result in failure of T cells to upregulate activation markers CD44 and CD69 as well as increase cell motility. Seeing this in the context of my findings, it is therefore possible that the increase in active CD11a expression in tolerogenic Mo-DCs controlled contact duration with T cells. However, there is very little available information on CD11a in DCs, with no other studies finding a similar immunoregulatory role of CD11a in DCs.

It is also interesting that CCR7, which was found to be lower on tolerogenic Mo-DCs in previous studies (Anderson et al., 2009, 2008), usually mediates activation of CD11a (Kliche et al., 2012). One could have therefore expected expression of active CD11a to be lower on tolerogenic Mo-DCs due to their lower expression of CCR7.

However, as active CD11a expression was significantly increased in tolerogenic Mo-DCs this could have two reasons. Although CCR7 expression was not measured directly here, having utilised exactly the same protocol for generation of Mo-DCs and characterised their phenotype as identical to previous studies (Anderson et al., 2009, 2008; Harry et al., 2010), it is likely that CCR7 expression is similar to reported values in my cultures. It is therefore unlikely that expression of CCR7 on tolerogenic Mo-DCs was responsible for the increase in active CD11a. Another, more likely, possibility is that activation of CD11a in tolerogenic Mo-DCs is not mediated via inside-out signalling of CCR7. For example, Anderson and colleagues also found that expression of CCR1, CCR2 and CCR5 remained high in tolerogenic Mo-DCs even after stimulation with LPS (Anderson et al., 2009). This would suggest that higher expression of these cytokine receptors might mediate the higher expression of active CD11a instead. This is supported by studies finding that macrophages expressing CD11a were recruited at much lower levels to a site of nerve injury in a KO mouse model of CCR2 compared to CD11b positive macrophages (Siebert et al., 2000). Furthermore, CCL5, a ligand of CCR1, was shown to specifically mediate adhesion of T cells to ICAM-3 via CD11a, suggesting a role for especially CCR1 in mediating the increased expression of active CD11a (Szabo et al., 1997).

As discussed previously, targeting CD11a in an MLR holds caveats, as T cells also express the β_2 integrin subunit (Figure 4.14). This poses a technical challenge, as interfering with CD11a function in any way would always affect T cells as well as DCs, thereby making results less conclusive. However, there could be other ways to investigate the potential immunoregulatory role of CD11a on DCs. For example, an RNA silencing approach could be used to silence CD11a specifically on DCs before putting them in co-culture with T cells. One could expect that absence of CD11a from DCs would increase DC-mediated T cell stimulation. However, as transfection with a silencing RNA takes up to a week in culture, this approach would be difficult to do with mature and tolerogenic Mo-DCs, as tolerogenic Mo-DCs might alter their phenotype during this time and transfection rates might not be consistent between mature and tolerogenic Mo-DCs, making comparisons difficult. Possibly, a murine DC-specific KO of CD11a would give yield more information on the potential immunoregulatory role of CD11a.

Evidence for pro-inflammatory roles of CD11b

In contrast, the hypothesis that CD11b is a pro-inflammatory mediator of DC function due to my finding that it is significantly lower in tolerogenic Mo-DCs cannot be aligned with the published literature in the same way. Interestingly, active CD11b on DCs, similarly to active CD11a, was found to decrease the cells ability to stimulate T cells (Varga et al., 2007), which would suggest the significant reduction in active CD11b expression seen in Figure 4.10 actually represents a pro-inflammatory stimulus able to enhance T cell proliferation. However, this is not congruent with findings from my functional work, where I demonstrated that blocking CD11b on mature but not tolerogenic Mo-DCs significantly reduced the level of T cell proliferation and IFN-γ production elicited by Mo-DCs (Figure 4.20). Other studies have furthermore shown immunoregulatory roles of CD11b, especially in preventing Th17 immune responses. CD11b KO mice were shown to be unable to induce oral tolerance in response to a repeated antigen challenge, which was suggested to be due to increased IL-6 production of CD11b deficient APCs which in turn swayed T cell differentiation towards a Th17 phenotype (Ehirchiou et al., 2007). In addition, ligating CD11b on human Mo-DCs was found to efficiently restrict Th17 T cell expansion from memory T cells by reducing the production of cytokines such as IL-1β, IL-6 and IL-23 (Nowatzky et al., 2018a). However, as this study did not demonstrate a similar effect in naïve T cells, one could argue that this described immunoregulatory role of CD11b is not relevant in the experimental system utilised in this chapter whereby naïve T cell priming is quantified. Furthermore, ligation of CD11b was found to increase expression of MHC and costimulatory molecules on DCs, but restrict release of pro-inflammatory cytokines, thereby suppressing their stimulatory ability (Behrens et al., 2007), and CD11b was found to make DCs more tolerogenic (Škoberne et al., 2006). CD11b on DCs binding to ICAM-1 on inflamed endothelial cells similarly suppressed stimulatory function (Podgrabinska et al., 2009). Lastly, CD11b was found to be highly important in restricting TLR signalling on DCs (Han et al., 2010, p. 88; Yee and Hamerman, 2013), and was found to induce production of the anti-inflammatory cytokine IL-10 (Wang et al., 2010).

However, while several studies mentioned above suggest that CD11b negatively regulates TLR signalling, one study reported that CD11b deficiency caused reduction in T cell activation due to DCs inability to endocytose TLR4 in response to LPS stimulation, thereby suggesting CD11b as a positive regulator of TLR4 (Ling et al.,

2014). Still, considering the overwhelming breadth of evidence of CD11b playing immunoregulatory roles, the results of this chapter suggesting the exact opposite of CD11b being more pro-inflammatory have to be scrutinised.

One possibility is that the CD11b blocking antibody causes the downstream signalling associated with CD11b ligation, resulting in an immuno-regulatory effect in line with previous reports. While several studies have shown that blocking CD11b reduced CD11b-mediated adhesion (Altieri, 1991; Kusunoki et al., 1994), it is not well-described if binding of the blocking antibody could induce intracellular signalling despite blocking adhesion. Published evidence on this is conflicting. In monocytes for example, CD11b-dependent enhancement of TNF-α production in response to monocytes was reduced when confronted with a CD11b blocking antibody compared to ligation of CD11b (Fan and Edgington, 1993). This would suggest that blocking CD11b does not have the same effect as ligating it in terms of downstream cell signalling. However, another study reported that macrophages could produce Nitric oxide (NO) in a dose-dependent manner, when treated with different levels of CD11b blocking antibody in the presence of IFN-γ (Goodrum et al., 1994). Furthermore, ligation of CD11b with a different ligand did not have the same effect, suggesting that specifically M1/70, the CD11b blocking antibody used in this study, might have functional roles when binding to CD11b in addition to merely blocking access to other ligands. Thinking about this potential dual role of blocking CD11b and the wealth of literature describing anti-inflammatory roles of CD11b, this could imply that by blocking CD11b two distinct roles of the integrin subunits could be affected. First, its contribution to DC-T cell physical contacts via CD11b binding to its ligand ICAM-1 on T cells, which is a function that would be blocked. Second however, is the signalling role of ligated CD11b, which might have downstream effects on expression of co-stimulatory receptors and cytokine production and might potentially be increased by providing the CD11b blocking antibody in culture. CD11b blocking antibody acting in a similar way to ligation of CD11b, which was reported to be immunoregulatory, would explain the reduction in IFN-γ production and T cell proliferation in mature Mo-DCs treated with it (Figure 4.20). However, it does not explain why tolerogenic Mo-DCs, which showed to induce significantly lower IFN-γ production by T cells compared to mature Mo-DCs, have comparatively lower expression of active CD11b on their surface (Figure 4.10).

Balance between β_2 integrin subunits

Another possibility is that β_2 integrin subunits, such as CD11a and CD11b (and indeed CD11c and CD11d), act in careful balance with each other, which is an aspect that none of the CD11b-specific studies mentioned above took into consideration. For example, mutating CD11b could potentially alter the balance of other β_2 integrin subunits, such as CD11a and CD11c, in an attempt to compensate for the loss of CD11b. Rather than attributing results directly to CD11b, it is therefore possible that the balance between different β_2 integrin subunits contributes to their respective function. This is especially likely considering the inconclusive results gained from investigating adhesive ability of Mo-DCs to a variety of β_2 integrin ligands, where it was found that tolerogenic Mo-DCs were not significantly increased in their adhesion to ICAM-1, although close at the highest concentration of ligand, but surprisingly showed significantly higher adhesion to ligands iC3b and fibrinogen, but not C4b, which had been hypothesised to be reduced due to the significantly lower expression of active CD11b on tolerogenic Mo-DCs (Figure 4.8 and Figure 4.12, respectively). As discussed, even if other β_2 integrin subunits such as CD11c compensated for reduction in active CD11b to mediate increased adhesion, this does still not explain why adhesion to C4b was not altered. This could suggest that balance of different β_2 integrin subunits is important to mediate adhesion to ligand.

Role of β_2 integrins on tolerogenic Mo-DCs

An important point that has not been discussed yet is the question if differences in expression of CD11a and CD11b on tolerogenic compared to mature Mo-DCs are downstream or upstream of cells assuming a tolerogenic phenotype. So far, I have assumed that alteration in expression of active CD11a and CD11b part of the changes that infer tolerogenic Mo-DCs with immunoregulatory functions. However, it is also possible that tolerogenic phenotype in turn drives altered β_2 integrin conformation on tolerogenic Mo-DC surfaces, thereby implicating it as a potential biomarker for tolerogenicity of Mo-DCs. Considering that targeting CD11b using a CD11b blocking antibody caused a reduction in T cell proliferation and IFN-γ production elicited by mature Mo-DCs (Figure 4.20), it is likely that CD11b has a functional role, as well as the potential opportunity to use it as a biomarker for tolerogenic Mo-DCs. However, as manipulating CD11a was technically difficult, a functionally significant role for the increased expression of its active conformation

could not be confirmed in this study. Keeping in mind that another study did find that increases presence of active CD11a reduced T stimulatory capability of DCs (Balkow et al., 2010), it is however likely that both CD11a and CD11b might play distinct functional roles as well as being downstream of other tolerogenic mediators important for tolerogenic Mo-DC function.

While the proposed immunoregulatory role of active CD11a in Mo-DCs could not be proven functionally, it has been previously shown in the published literature. In contrast, the hypothesised pro-inflammatory role of active CD11b finds no relevant support in the very limited literature, and is actually found to be similarly immunosuppressive to active CD11a. However, when blocking CD11b on mature Mo-DCs I found T cell proliferation and IFN-γ production to be significantly lower, again supporting a pro-inflammatory role for CD11b. To conclude, CD11b might have pro-inflammatory roles on DCs that have not been extensively described so far and remit further investigation. Especially as CD11b seems to have both pro-inflammatory and immunoregulatory roles depending on context, involvement of other β_2 integrin subunits will have to be considered.

4.3.2 Possibility of modulating β_2 integrins to improve tolerogenic cell therapies for RA

As mentioned in Chapter 1, tolerogenic Mo-DCs tolerised with a patient-specific antigen contributing to RA disease in this individual is currently being explored as a promising cellular therapy for RA. In theory, a cell-based tolerogenic therapy would not only decrease immune activation short-term but also educate the immune system to re-establish tolerance to self that is lost in RA. While the phase I clinical trial established that tolerogenic Mo-DCs injected into the joint are safe and the highest dose was reported to have positive effects in some study participants (Bell et al., 2016), there are still some issues to resolve before this treatment can become available. Some of these issues, such as identifying a suitable antigen that is relevant for the immune response causing joint inflammation in a specific patient (Jansen et al., 2018), do not fall within β_2 integrin function. However, other aspects, such as the low migratory ability of tolerogenic Mo-DCs, could be partially explained and even potentially rectified by altered β_2 integrin function on tolerogenic Mo-DCs.

While earlier studies found that tolerogenic Mo-DCs express low levels of CCR7 and can migrate towards CCL19 in a transwell system, although less effectively

compared to mature Mo-DCs (Anderson et al., 2009, 2008), it remains unclear if tolerogenic Mo-DCs can migrate out of the joint to the LN and whether such migration to the LN is required for the induction of tolerance (Bell et al., 2016). As ability to present auto-antigen as well as migratory ability have been described as the hallmarks of yielding systemic tolerising effects (Hilkens et al., 2010), it is likely that tolerogenic Mo-DCs will need to reach the LN to interact with T cells and induce systemic tolerance. As the migratory ability of tolerogenic Mo-DCs using currently established protocols is poor, culturing methods may need to be adapted to ensure migratory ability of tolerogenic Mo-DCs is sufficient. In the following paragraphs, I will therefore briefly discuss the potential effects of β_2 integrin expression on the tolerogenic Mo-DCs ability to migrate and the possibility to alter their β_2 integrin functionality to improve migratory abilities.

Considering merely β_2 integrin expression on tolerogenic Mo-DCs, one cannot make assumptions about their ability to migrate. In mice, migration of cells from tissue to LN as found to occur independently of all β_2 integrins when in an inflammatory (Lämmermann et al., 2008; Morrison et al., 2014), which suggests that migration of Mo-DCs might occur independently of β_2 integrins in the inflammatory context of RA. However, the increased adhesion of tolerogenic Mo-DCs to plastic (observation in both Hilkens and Morrison labs), glass (Figure 4.5), as well as to iC3b and fibrinogen (Figure 4.12), suggests that altered composition of active β_2 integrin expression overall increases adhesion to various ligands, although adhesion to both plastic and glass could also be mediated by different adhesion receptors not considered in this study. This, in turn, might retain tolerogenic cells at the injection site, instead of being able to migrate to the draining LN.

Overall, testing migratory ability of tolerogenic Mo-DCs could not be attained due to limited time. However, migration towards LNs was shown to be independent of β_2 integrins in an inflammatory context (Lämmermann et al., 2008; Morrison et al., 2014) and blocking CD11b on tolerogenic Mo-DCs had no significant effects on T cell proliferation (Figure 4.20). In murine studies however, integrin-deficient DCs were shown to migrate to LN in larger numbers in steady state, which was hypothesised to be due to decreased tissue retention (Morrison et al., 2014). Keeping in mind that reduced expression of active CD11b also yielded more tolerogenic cells, blocking CD11b on tolerogenic Mo-DCs might potentially make them more tolerogenic and more migratory, although it has to be kept in mind that I could not observe any

changes in tolerogenic Mo-DC stimulatory capability when blocking CD11b (Figure 4.20). Furthermore, as tolerogenic Mo-DCs have been described to only express low levels of CCR7, blocking β_2 integrins might not improve exit from tissues and migration to LNs to a similar level as shown by Morrison and colleagues. Further work on this topic should therefore assess migratory ability in response to blocking β_2 integrin subunits alongside T cell stimulatory ability. However, instead of blocking CD11b, which was found to decrease T cell proliferation in mature Mo-DCs (Figure 4.20), it might be more interesting to block CD11a or CD11c to distinguish which subunit mediates the increase in adhesion to β_2 integrin ligands, which might impede migratory abilities.

When considering manipulation of β_2 integrins to improve tolerogenic Mo-DC therapy, any strategies will likely have spatially or temporally separate or multi-modal in nature. This is because of the range of functions β_2 integrins can be involved in, and they possibility that different subunits are playing potentially opposing roles in tolerogenic function. For example, if we assume that expression of integrins retains Mo-DCs in the joint after injection, it would be most feasible to block integrins to enable Mo-DCs to exit the joint and migrate towards LNs more effectively. However, considering β_2 integrins potential role in DC-T cell interactions, it is possible that balance of subunit conformation could contribute to tolerising T cells. Therefore, targeting β_2 integrins to enable migratory abilities of tolerogenic Mo-DCs might impair their ability to induce tolerance and vice versa. To add a further layer of complexity to this, β_2 integrins also have a potential role in inducing downstream signalling that alters expression of costimulatory molecules and production of pro- or anti-inflammatory cytokines. For example, a blocking antibody meant to increase migration out of the injection site could simultaneously increase pro-inflammatory signals produced by tolerogenic Mo-DCs by acting as a ligand for CD11b. Targeting β_2 integrins will therefore have to be carefully tested to reduce unwanted off-target effects, due to the distinct roles of β_2 integrins in tolerogenic Mo-DC function.

To conclude, there are still severe gaps in current knowledge on the effect of β_2 integrins on Mo-DCs migratory ability. However, as these gaps are filled and available β_2 integrin targeting therapeutics advance, targeting β_2 integrins on tolerogenic Mo-DCs might be a real opportunity to improve cell therapy for RA.

4.4 Conclusions

In this chapter, I identified potentially opposing roles of the β_2 integrin α-subunits CD11a and CD11b utilising mature and tolerogenic Mo-DCs as a model system for immune activation and tolerance in DCs. My findings suggest that active CD11a is more immunoregulatory, due to the increased expression of its active form on tolerogenic Mo-DCs. On the other hand, active CD11b was downregulated on tolerogenic compared to mature Mo-DCs and blocking CD11b on mature Mo-DCs in an MLR reduced their ability to stimulate proliferation of and IFN-γ production by T cells. Despite adhesion assays showing that tolerogenic Mo-DCs showed higher adhesion to iC3b and C4b and a range of literature suggesting the opposite, this is strong evidence that CD11b could also play a pro-inflammatory role on DCs.

Chapter 5 **Opposing roles of β_2 integrins on antigen-presenting cells in patients with Rheumatoid Arthritis**

5.1 Introduction

In an *in vitro* setting, I hypothesised that CD11b has more pro-inflammatory roles, while CD11a is potentially more immunoregulatory. This was based on my findings that active CD11b expression was reduced on tolerogenic Mo-DCs, while active CD11a expression was increased compared to mature Mo-DCs. This was further backed by the result that blocking CD11b on mature Mo-DCs reduced the amount of T cell proliferation they were able to induce in an MLR, as well as the amount of IFN-γ present in supernatants. Continuing this work, it was important to test if this finding could find support in an *ex vivo* setting. Specifically, in PB of healthy controls compared to patients with RA, a condition marked by a dysregulated immune system. The autoimmune condition RA was chosen as it represents a state of the immune system where tolerance to self is lost, which causes joint-specific as well as systemic pathology. Comparing healthy controls to patients who have active disease or are in remission therefore allows insights into how expression of β_2 integrins might potentially be altered in the context of loss of tolerance to self. As discussed previously, understanding both stimulatory and regulatory effects of β_2 integrins in DCs on immune function could make them more viable therapeutic targets for conditions such as RA, where immune regulation is lost, in the future.

5.1.1 Role of antigen-presenting cells in Rheumatoid Arthritis

While presentation of an auto-antigen to aberrant T and B cells is hypothesised to be the initiating event of RA pathogenesis, the role of APCs in RA pathogenesis as well as progression is not fully elucidated.

In mouse models of articular initiation of RA, conventional DCs were reported to have a critical role in T cell activation and concurrent joint inflammation (Benson et al., 2010), while pDCs were found to have more immunoregulatory roles in the murine joint (Jongbloed et al., 2009). However, the significance of DCs for RA progression is not well understood. Certain HLA-DR alleles, notably DR1 and DR4, are associated with more severe or more progressive disease, suggesting an important role for continuous T cell activation by DCs in RA (Weyand et al., 1992; Zeben et al., 1991). DCs were found to be present in higher numbers in synovial fluid of patients with established RA when compared to the less overtly inflammatory joint disease osteoarthritis (Harding and Knight, 1986), suggesting that there is an on-going role for DCs in maintaining joint inflammation. Circulating numbers of DCs in RA are a contentious issue, with several studies stating that circulating DCs are decreased in

RA (Cooles et al., 2018; Jongbloed et al., 2005), while others suggest DC numbers were actually higher (Estrada-Capetillo et al., 2013). This conflict in findings could be due to DCs becoming sequestrated in inflamed joint tissues at certain disease stages, removing cells from the circulating pool. However, it is established that Mo-DCs derived from RA patient blood monocytes are more pro-inflammatory than their healthy counterparts, characterised by increased IL-6 and IL-23 production and ability to induce Th17 cells (Estrada-Capetillo et al., 2013). Similarly, high numbers of DCs can be isolated from synovial fluid and were found to stimulate T cells better than cells isolated from peripheral blood (Zvaifler et al., 1985). Generally, as self-reactive T cells should be deleted in the thymus, it is hypothesised that DCs present self-protein that has been altered due to citrullination or other factors to T cells and provide sufficient pro-inflammatory cytokines and costimulatory factors to activate T cells, which are unable to recognise the altered self-protein (McInnes and Schett, 2011; Yu and Langridge, 2017). DCs therefore play important roles both in disease initiation as well as progression.

Monocytes are widely described to be more active in the context of RA, marked by increased expression of CD14 (Shinohara et al., 1992), a receptor for complexes of LPS and LPS binding protein (LBP) which can mediate immune sensitivity in response to infection (Wright et al., 1990). Interestingly, a range of β_2 integrins, including CD11a, CD11b and CD18 were also shown to be increased (Lioté et al., 1996; Torsteinsdóttir et al., 1999). While these changes are potentially due to the fact that monocytes are exposed to the inflammatory cytokine environment present in the systemic disease RA, evidence is accumulating that monocytes can also play an active role in creating the afore-mentioned pro-inflammatory environment. *In vivo* stimulated $CD14^+$ monocytes from inflamed joints of RA patients were found to promote specifically Th17 responses when compared to resting $CD14^+$ monocytes from PB (H. G. Evans et al., 2009), suggesting that monocytes have distinct pro-inflammatory roles in the joint that are independent of systemic cytokines. This is especially important, as Th17 T cells are considered a potent contributor to joint destruction in RA (van den Berg and Miossec, 2009). Another study found that CXCL16 expression, which was found to be a driver of immune cell recruitment to the joint as well as disease pathogenesis (Nanki et al., 2005; Voort et al., 2005), was not altered between monocytes from healthy donors and patients with RA. However, culturing monocytes together caused a significant increase in CXCL16, suggesting

than an increased influx of monocytes to the joint could in turn recruit other cells to the joint (Lieshout et al., 2009).

To conclude, it can therefore be said that both DCs and monocytes are thought to contribute to both initiation of RA as well as disease progression. Exploring the role of β_2 integrins on DCs and monocytes specifically could therefore be of special interest to understand how β_2 integrins might potentially contribute to these different functions.

5.1.2 Potential role of β_2 integrins in Rheumatoid Arthritis

There is a limited amount of published literature on the role of β_2 integrins in RA, but it is striking that β_2 integrin subunits CD11a and CD11b seem to have different reported functions in the context of RA (Schittenhelm et al., 2017).

In animal studies, CD11a was found to be important for the induction of RA disease models. Absence of CD11a in a KB x N serum transfer model of arthritis yielded mice resistant to disease induction (Watts et al., 2005). Supporting this, blocking all β_2 integrins in a rabbit model of RA caused a reduction of inflammation, suggesting that leukocytes could not be recruited to the joint in sufficient numbers to give rise to the full extent of the disease model (Jasin et al., 1992). Blocking CD11a, either using an anti-CD11a antibody (Kakimoto et al., 1992) or a small-molecule CD11a antagonist (Suchard et al., 2010), caused an improvement in clinical scores and reduced the amount of pro-inflammatory cytokine mRNA levels within the murine joint. Lastly, mice carrying a mutation in β_2 integrin ligand ICAM-1 similarly showed a reduction in susceptibility to the collagen-induced arthritis (CIA) model (Bullard et al., 1996). All of this suggests a pro-inflammatory role of CD11a in mice, specifically in leukocyte recruitment to the joint. In humans, however, the emerging role of CD11a seems to be more complex. CD11a expression was found to be increased in inflamed synovial tissue lymphocytes (Cush and Lipsky, 1988), but as the β_2 integrin subunit also plays an important role in lymphocyte migration, cause and effect are difficult to tease apart. On the other hand, blocking CD11a *in vitro* on healthy PB non-classical $CD16^+$ monocytes was found to increase induction of Th17 T cells in response to treatment with a superantigen and a TLR2/NOD receptor agonist, suggesting an immunoregulatory role of CD11a on $CD16^+$ monocytes but not $CD14^+$ monocytes (Traunecker et al., 2015).

When considering the β_2 integrin subunit CD11b on the other hand, animal studies have shown that knocking out CD11b causes exacerbated joint pathology in a KB x N serum transfer model of arthritis (Watts et al., 2005). This result was repeated in a study utilising the CIA model, which also found that the observed increase in IL-6 production and Th17 priming could be reversed when introducing a DC cell line expressing CD11b (Stevanin et al., 2017), suggesting that CD11b plays immunoregulatory roles on DCs in RA. Surprisingly, a different study using both the CIA model and serum transfer model, found that blocking CD11b immediately before onset of symptoms could ameliorate symptoms and render serum unable to induce arthritis in SCID mice (Taylor et al., 1996), potentially suggesting that CD11b might also contribute to cell recruitment to the joint. When FcγRIIB-deficient mice, which spontaneously develop arthritis, were treated with a CD11b monoclonal antibody, symptoms were improved. This occurred in tandem with a reduction in migration of inflammatory cells to the synovium (Ohtsuji et al., 2018). Despite these promising animal studies, a functional polymorphism of *ITGAM* (CD11b), was found to be significantly associated with SLE, but not with RA (Lee and Bae, 2015). While the polymorphism, coding for a single amino acid substitution at position 77 of the extracellular domain of CD11b, remains able to undergo conformational changes, it shows impaired ability to adhere to CD11b ligands and mediate phagocytosis (MacPherson et al., 2011; Rhodes et al., 2012). However, CD11b was also found to be increased in peripheral $CD14^+$ monocytes of RA patients (Lioté et al., 1996).

Consulting the published literature on a potential role of β_2 integrins in RA, it becomes apparent that several different aspects influencing β_2 integrin expression might occur simultaneously and might also differ between different cell types. First of all, there are systemic effects apparent in circulating cells as well as more local changes present in an inflamed joint. It is furthermore clear that β_2 integrins play a role in recruiting APCs to the joint, but other potentially more immunoregulatory roles in T cell activation are less well understood. Overall it is therefore possible that any additional roles carried out by β_2 integrins may be either subunit-specific, as suggested in the opposing roles of CD11a and CD11b observed in Chapter 4, or cell type specific, as suggested in studies finding differences in CD11b immunoregulatory function between classical and non-classical monocytes.

5.2 Aims

This chapter aims to assess expression and conformation of β_2 integrin subunits in APCs from RA patients and compare them to healthy controls. The aims of this chapter therefore were to:

1. Devise data analysis strategy that yields information on expression of both total and active β_2 integrins as well as how one relates to the other.
2. Quantify β_2 integrin expression and conformation on APC populations in PB from healthy controls
3. Compare expression and conformation of β_2 integrin α-subunits CD11a and CD11b specifically between PB of RA patients with active disease and in remission to healthy controls. Furthermore, compare expression and conformation of β_2 integrins in PB to SF of RA patients
4. Investigate if *in vitro* stimulation of PBMCs with various pro- and anti-inflammatory stimuli or RA SF can mimic the joint environment in respect to β_2 integrin expression and conformation
5. Test the ability of PB cDC2 DCs to stimulate T cell proliferation in an MLR in the presence of CD11b antagonism or agonism.

5.3 Results

5.3.1 Analysis and quantification of clinical data

β_2 integrin expression was to be assessed on clinical samples acquired over a period of 1-2 years. As expression level (MFI or brightness of signal) was one of the major read-outs anticipated, standardising flow cytometry data over time was necessary. This was because flow cytometer lasers undergo shifts over time, which could render samples collected at the beginning of the recruitment period more different from samples recruited towards the end than from a sample from a different group (Schwartz et al., 1998; Watson, 1987). For this, BD application settings were utilised, which were applied to Fortessa X20 baseline throughout the recruitment period. Application settings rely on recording standardised reference beads upon which photomultiplier (PMT) voltages can be adjusted to (Gratama et al., 1998).

Quantifying expression levels of both total and active β_2 integrins simultaneously had the potential to yield a wide breadth of novel information. This was particularly the case because there is no available literature on the relationship between total expression and active conformation. Considering both total and active expression at

the same time therefore provides unique insights into the role of β_2 integrin expression as well as conformational changes in different cell functions. To maximise information gained from this approach, a variety of analysis values were obtained for each sample. A detailed graphic representation of the different values obtained can be found in Figure 5.1.

For total expression of β_2 integrins, three values were obtained, including the MFI of the total population (1), the % of cells positive for the total marker in question (2) and the MFI of the cells that were positive for that specific marker (3) (Figure 5.1A). This is because different cell populations might have different proportions of cells positive for a marker. If, for example, the total β_2 integrin in question is only expressed on a small proportion of cells, the large negative population present will dominate the total MFI (1). In this case, %MFI (3) gives more accurate information on how much of that specific marker is present on the cells that express it. This also leads immediately into why it is important to consider both MFI (1 and 3) and %Positive (2). While MFI yields information on how bright the marker is in this cell population, suggesting more or less surface expression, percentage of cell positive for a specific marker gives important information on how widely the marker is expressed in a specific cell type.

For expression of active β_2 integrins, a total of five values were obtained. The first three values mirror what was obtained for the total expression of the subunit, with (4), (5) and (6) corresponding to MFI, %positive and %MFI respectively (Figure 5.1B). However, additionally to these values, two further measurements were taken into account to yield information on how total and active expression of a specific β_2 integrin subunit relate to one another (Figure 5.1C). To gather information on how many cells expressing the total β_2 integrin subunit also express the corresponding active subunit, the % of total-expressing cells positive for the active marker were recorded (T%) (7).

As this analysis was done for total and active β_2 integrin subunits CD11a, CD11b and CD18 on five APC subtypes, not all values acquired for each sample were possible to show in the following chapter. Instead, all data was considered and the value best suited to answer a specific question was selected.

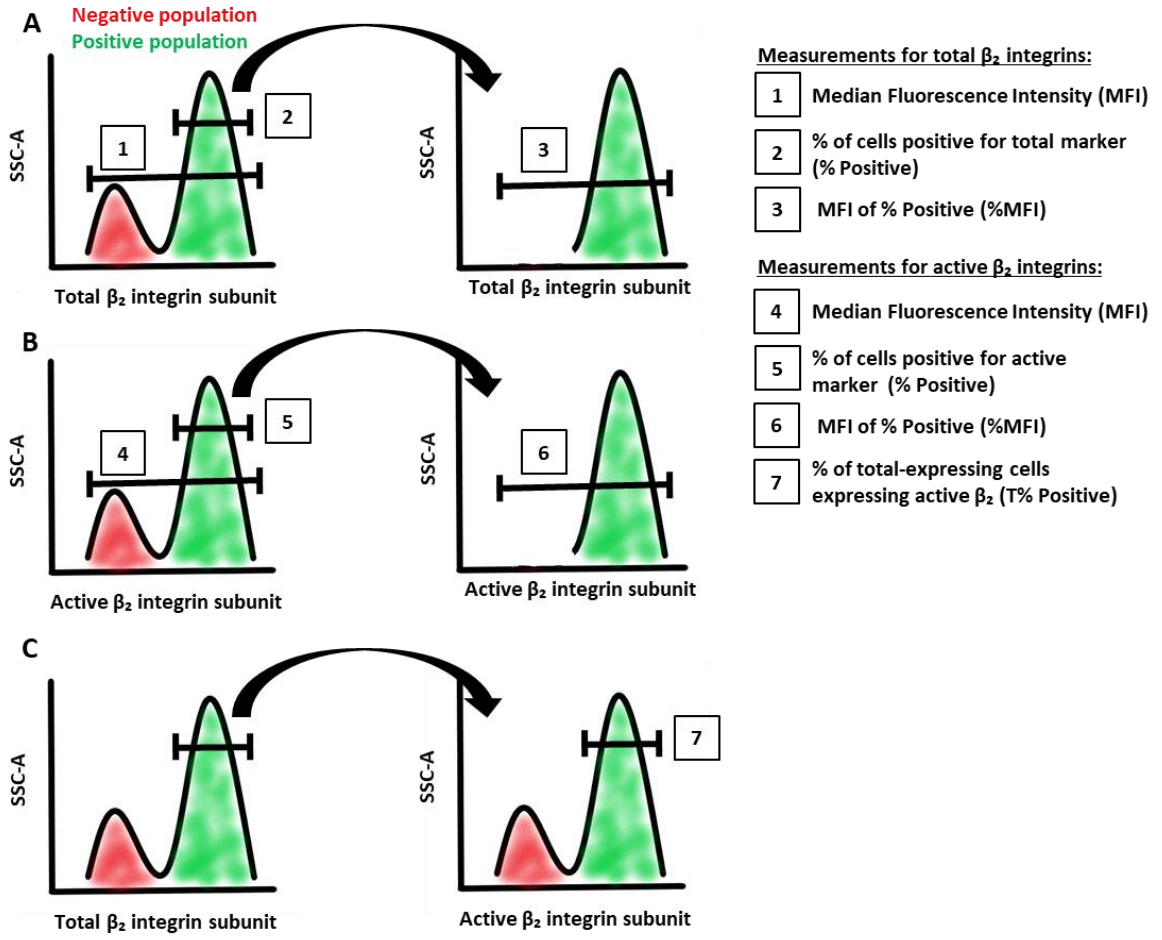

Figure 5.1 β_2 Quantification of β_2 integrin subunit expression

Graphical description of data analysis strategy used for each cell type and β_2 integrin subunit considered (CD11a, CD11b or CD18). A. Data analysis strategy for total β_2 integrins, yielding measurements 1, 2 and 3. B. Data analysis strategy for active β_2 integrins, yielding measurements 4, 5 and 6. C. Data analysis strategy to connect total β_2 integrin expression to active β_2 integrin expression, yielding measurements 7.

Effect of PMA activation on β_2 integrins

While the use of PMA as a positive control was validated in Chapter 3, the desired effect of increasing active β_2 integrins was not significant, as it was only tested on three healthy samples. Additionally, the effect of PMA on CD11b and CD18 seemed to differ from the effect on CD11a, with the latter seemingly less responsive to stimulation. Therefore, it was decided to investigate the effect of PMA activation on CD11a with a larger sample size of healthy controls, as well as any differences between APC types reacting to stimulation with PMA. Additionally, the effect of stimulation on total β_2 integrins was assessed. For this, only double stained CD11a

total (when co-stained with active CD11a, affected by steric hindrance) was analysed due to limited cell number.

PBMCs were gated in accordance with the gating strategy presented in Chapter 3, section 3.3.6, Figure 3.7. This yielded classical ($CD14^+$) as well as non-classical monocytes ($CD16^+$), as well as three DC subtypes, cDC1 DCs ($CD141^+$), cDC2 DCs ($CD1c^+$) and pDCs ($CD11c^{neg}$). Expression of active CD11a, CD11b and CD18 on unstimulated (-PMA) and stimulated (+PMA) APC populations is compared in Figure 5.2. Active CD11a was not significantly altered by PMA stimulation in $CD14^+$ monocytes or cDC2 DCs. Interestingly, however, active CD11a was significantly different in all other APC types, but instead of increasing the amount of active CD11a on the cell surface, PMA stimulation resulted in lower expression of active CD11a. Active CD11b was significantly increased upon PMA-stimulation in all APC types except pDCs. Lastly, active CD18 was significantly increased upon PMA stimulation in $CD14^+$ monocytes and $CD16^+$ monocytes, but none of the DC populations.

This reduction in active CD11a upon PMA stimulation could be due to increased endocytosis from the cell surface upon activation. However, this would suggest that active CD11a is endocytosed from the cell surface much quicker than active CD11b or CD18, where an increase in active β_2 integrin upon PMA stimulation can be observed. While this is possible considering my findings in Chapter 3 showing that sufficient CD11a staining can be achieved after 5min, while both CD11b and CD18 need to be stained for 30min to achieve sufficient signal (Chapter 3, 3.3.2, Figure **3.2**), it still seems unlikely. Another possibility is that PMA acts differently on CD11a than it acts on CD11b and CD18. The fact that CD18 reacted to PMA differently on different cell types again suggests that there is APC-type specific regulation of β_2 integrin activation. This is especially the case for CD18, as an increase in active CD18 likely also caused associated α-subunits to become activated. The fact that this was not the case for CD11a, and furthermore not shown in DC populations suggests that different cell types regulate β_2 integrin subunits differentially to yield migration or adhesion results necessary in the biological context of the cell. This was supported by findings in the literature, showing that if CD11a is transfected into a non-hematopoietic cell line remains constitutively active and is not affected by a protein kinase C inhibitor reported to reduce expression of active CD11a (Dustin and Springer, 1989; Larson et al., 1990). This suggests that different cell types might

have different pathways to modulate β_2 integrin expression via intracellular domains (Ginsberg et al., 1992).

Next, the effect of PMA stimulation on total β_2 integrin expression was analysed (Figure 5.3). There was no significant effect of PMA stimulation on total CD11a expression except in pDCs, where total CD11a expression was significantly lower in the stimulated sample. Interestingly, total CD11b was significantly higher in all APC types except pDCs upon PMA stimulation. This suggests that PMA not only increased the amount of CD11b subunits assuming their high-affinity form, but also the amount of total CD11b on the cell surface.

This could mean that CD11b trafficking, which occurs at a faster rate than CD11a (Bretscher, 1992), is further increased when cells are stimulated with PMA. Total CD18 was also significantly increased in PMA-stimulated $CD14^+$ monocytes, cDC2 DCs and cDC1 DCs, but not $CD16^+$ monocytes or pDCs. Considering the results of both CD11a and CD11b, it is possible that CD11a is more dominant on $CD16^+$ monocytes and pDCs, resulting in no significant difference in CD18, while the significant effect on total CD11b expression is more dominant on $CD14^+$ monocytes, cDC2 DCs and cDC1 DCs. Furthermore, it is important to keep in mind that CD11c, which is highly expressed on all APC types but pDCs due to the utilised gating strategy, can also pair with CD18 and might thereby affect expression of total or active CD18 in response to PMA stimulation.

In summary, PMA stimulation caused an increase in expression of active CD11b in all cell types but pDCs and CD18 in both classical and non-classical monocytes. Surprisingly, expression of active CD11a was significantly lower after stimulation in $CD16^+$ monocytes, cDC1 and pDCs, suggesting that the different β_2 integrin subunits react differently to PMA exposure. Furthermore, when testing the effect of PMA on total β_2 integrin expression, it was found that expression of total CD11b and CD18 is also significantly increased in several APC types after stimulation, potentially suggesting a role of PMA in β_2 integrin cycling. Expression of total CD11a was significantly reduced in pDCs but otherwise remained unaffected by PMA stimulation, again underlining that CD11a reacts to PMA in a fundamentally different way from both CD11b and CD18.

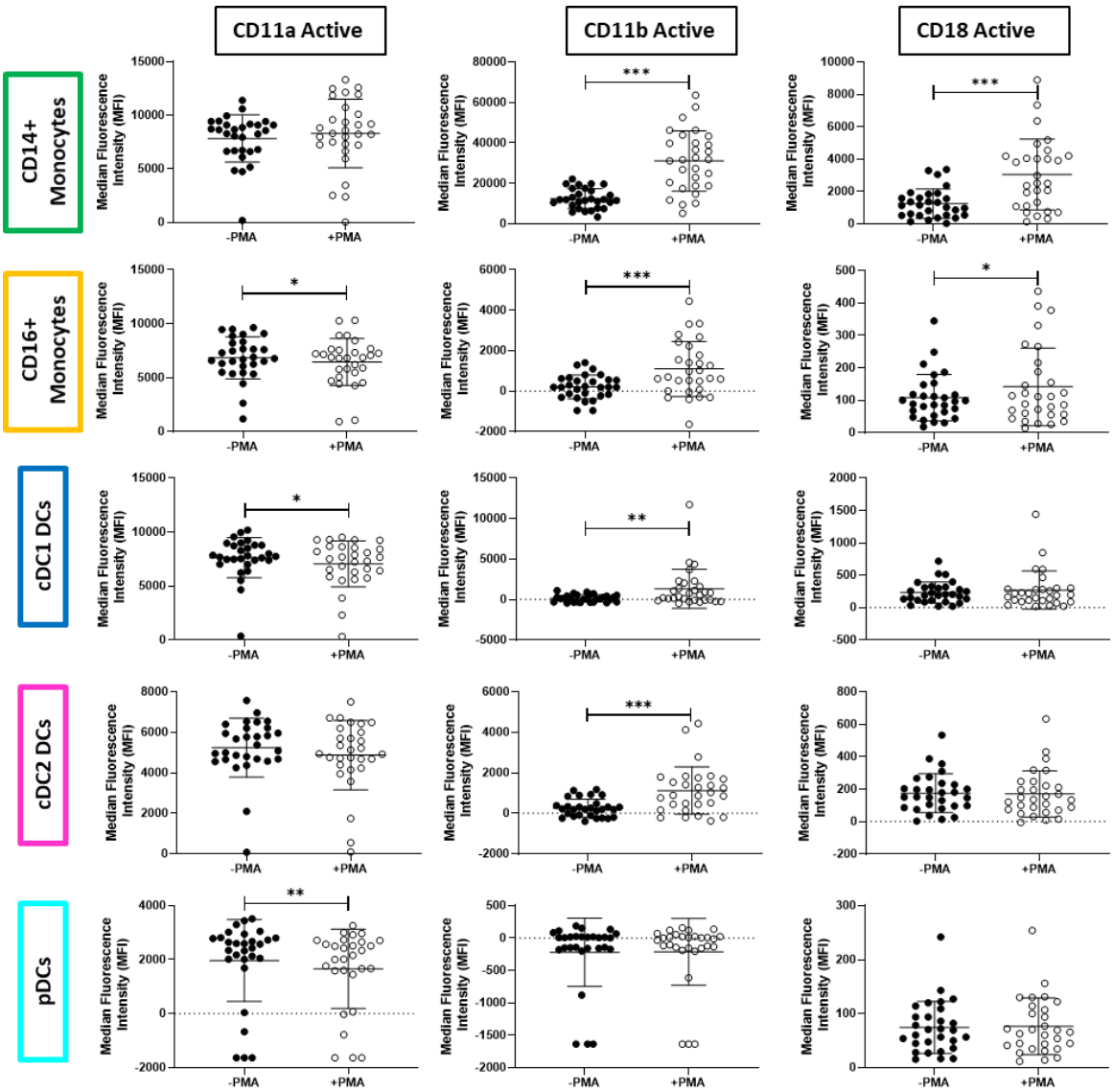

Figure 5.2 Effect of PMA on active β_2 integrin expression

Expression (MFI) of active CD11a, CD11b and CD18 was compared between unstimulated (black circles) and PMA-stimulated (open circles) healthy PB cells. Paired student's t test, n=29.

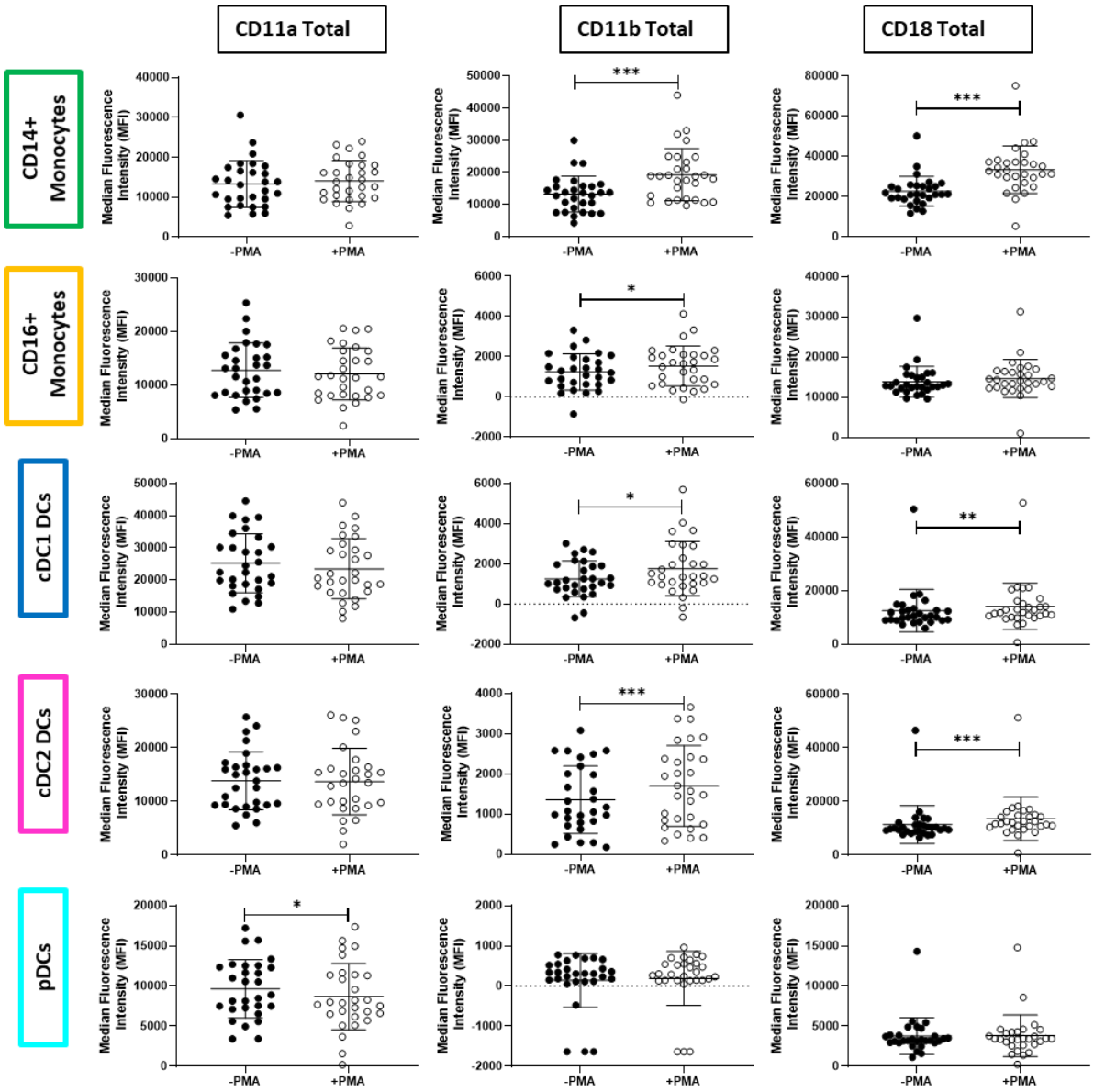

Figure 5.3 Effect of PMA on total β_2 integrin expression

Total expression (MFI) of CD11a, CD11b and CD18 was compared between unstimulated (black circles) and PMA-stimulated (open circles) healthy PB cells. Paired student's t test, n=29.

5.3.2 β_2 integrin expression on APCs in health

While there is evidence of different APC types expressing different levels of β_2 integrins, there is, to my knowledge, no information in the published literature on how this relates to β_2 integrin activation status (conformation). It was therefore of importance to assess a healthy control data set in respect to their total and active β_2 integrins on monocytes and DCs isolated from peripheral blood. To this end, 29 healthy controls were assessed using the active integrin panel described in Chapter 3 to quantify the expression of total and active integrin subunits on APCs.

Total β_2 integrins in healthy control APCs

Signal brightness of cells expressing the marker (MFI) for total CD11a, CD11b, CD18 and CD11c (MFI) for each of the APC types of interest is shown in Figure 5.4 (left). Additionally, the % of cells expressing the marker in question for CD11a, CD11b and CD18 was shown, as well as the MFI exclusively of cells expressing the marker (%MFI). Lastly, an overlay histogram of respective expression of total β_2 integrins on the five different APC subtypes was presented. As CD11c was used as a marker to delineate all APCs except for pDCs, % of cells expressing the marker is not shown as it would be 100% for all cell populations considered, except for pDCs. As discussed in Chapter 3, there is significant loss of CD11a total signal due to steric hindrance when staining for both active and total antibodies together. Total and active CD11a stained together ("double stain"), will therefore not only be shown in Figure 5.4A but will also be compared to the total CD11a single stain in Figure 5.5, allowing for validation of the double stained result. According to the double stain samples, CD11a expression is highest on cDC1 DCs, while expression on classical ($CD14^+$) and non-classical ($CD16^+$) monocytes is similar to CD11a expression on cDC2 DCs. Lastly, pDCs are shown to have the lowest amount of CD11a expression on their surface, significantly lower even than cDC2 DCs. The differences in CD11a expression are shown to be indeed due to differing amounts of CD11a total on the cell surface of the APCs, as the % of cells positive for the CD11a total marker is close to 100% for all samples in $CD14^+$ and $CD16^+$ monocytes, as well as cDC1 and cDC2 DCs. pDCs are the only cell types that do not express CD11a on all the cells in the population in some healthy donors, but this difference is not significant. Importantly, when comparing these results to the single stained CD11a (Figure 5.5), results are largely the same. The only exception is that both $CD14^+$ and $CD16^+$ monocytes show significantly higher CD11a expression compared to cDC2 DCs, a result which is not visible in the double stained samples. This strongly suggests that steric hindrance occurring largely does not differ between cell types but is mostly consistent, enabling direct comparison of double stained samples. It can therefore be concluded that assessment of total expression of CD11a is not hindered by the presence of steric hindrance in the used experimental systems, as relative differences between cells persist regardless of its presence.

CD11b expression shows a markedly different expression profile from CD11a in healthy control APCs. It is highest expressed in $CD14^+$ monocytes, and shows very

low expression on $CD16^+$ monocytes, cDC2, cDC1 and pDCs, which were all significantly lower than $CD14^+$ monocytes. Interestingly, CD11b expression showed more variation in % expression between cell populations. While almost 100% of $CD14^+$ monocytes expressed CD11b on their surface, this was not the case for the other cell types. CD11b was only expressed on around 60% of $CD16^+$ monocytes and cDC2 DCs, and 30% of cDC1. pDCs had the lowest %positive value, with only 20% of cells in the population expressing CD11b total (Figure 5.4B).

CD18 expression was highest on $CD14^+$ monocytes and lowest on pDCs, with expression being significantly lower on cDC2s compared to $CD16^+$ monocytes (Figure 5.4C). Similarly, to CD11a total, CD18 was expressed on almost 100% of the cell populations considered, meaning that graphs plotting MFI and %MFI look virtually the same, with the difference in signal brightness being due to the amount of CD18 expressed on the cell surface and not to the amount of cells expressing it.

Lastly CD11c was highest in $CD14^+$ monocytes and cDC2 DCs, and significantly lower $CD16^+$ monocytes and cDC1 DCs Figure 5.4D). As the absence of CD11c in the presence of HLA-DR was used to delineate pDCs it is not surprising that the marker was absent from this population. However, it is important to note that none of the β_2 integrins considered were highly expressed on pDCs, which always showed lowest expression compared to other APCs.

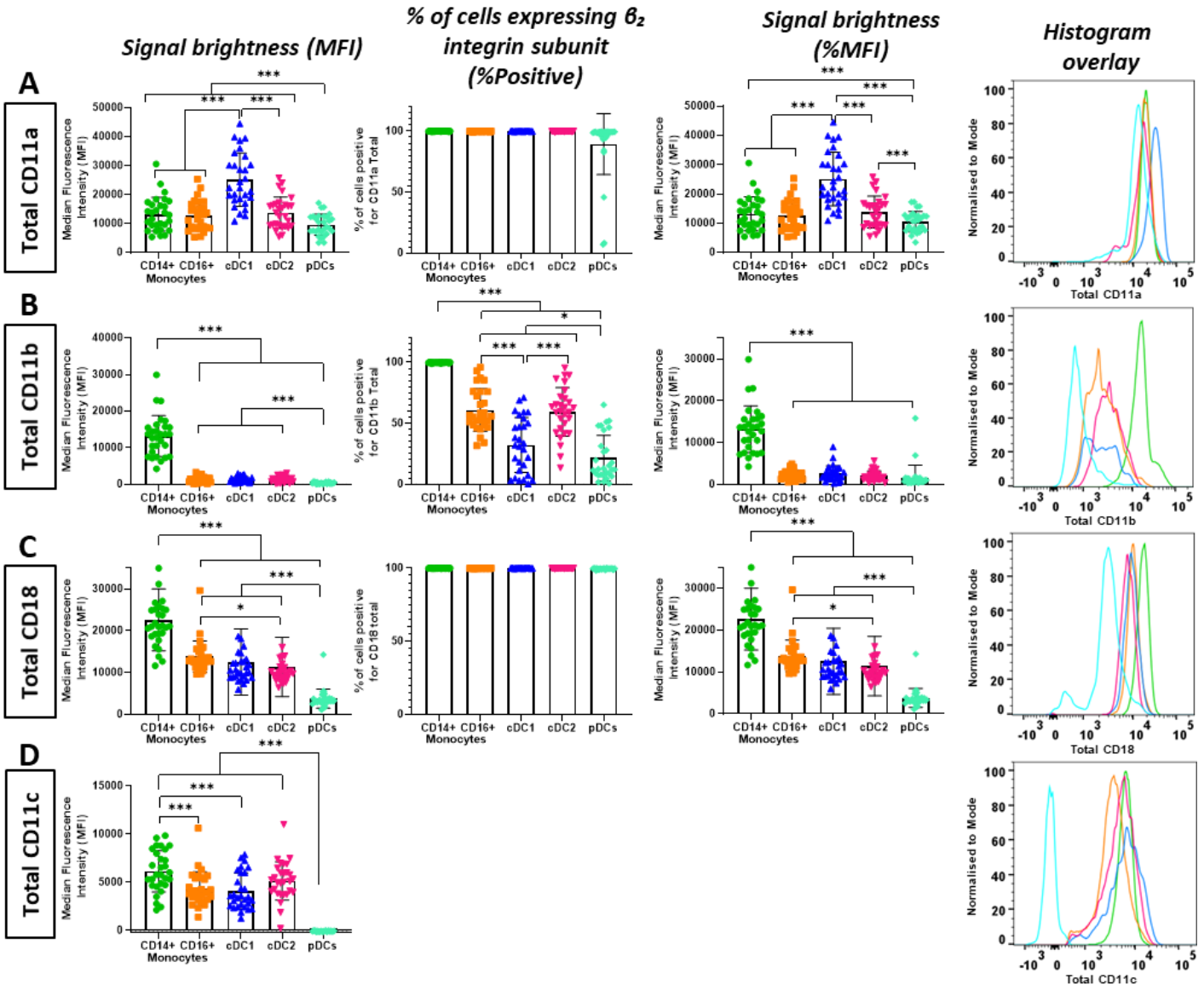

Figure 5.4 Total β_2 integrins in healthy controls

Total expression of CD11a, CD11b, CD18 and CD11c in five APC subtypes of interest, CD14+ monocytes+, CD16+ Monocytes, cDC2 and cDC1 DCs, as well as pDCs. Measurements shown are %MFI and %Positive cells, except for CD11c where only MFI was available. Repeated measures one-way ANOVA, n=29.

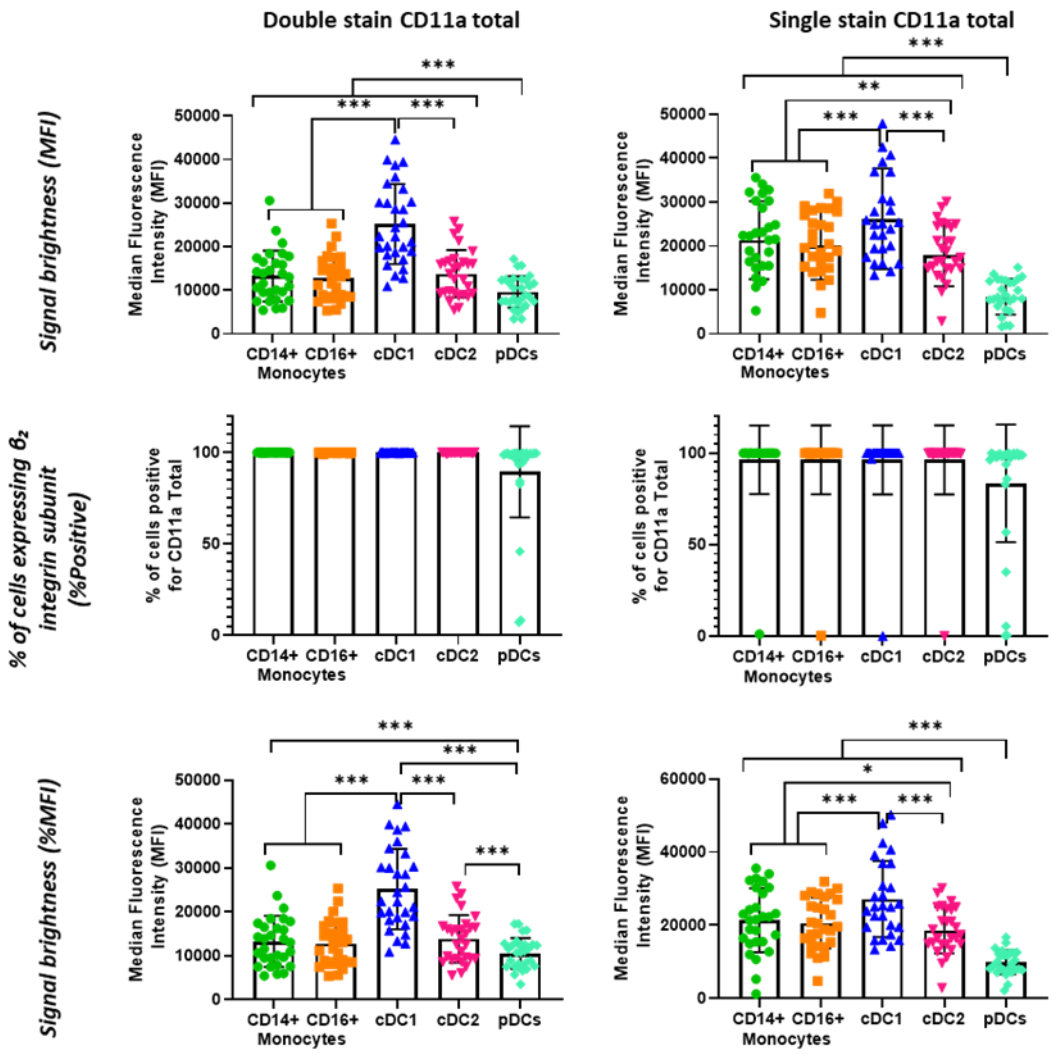

Figure 5.5 Effect of steric hindrance on total CD11a in healthy controls

Double stain of total and active CD11a affected by signal loss due to steric hindrance (left) compared to single stain of total CD11a unaffected by signal loss due to steric hindrance (right). %MFI and %Positive for total CD11a expression are shown. Repeated measures one-way ANOVA, n=29.

To summarise, total CD11a and CD18 were expressed on virtually all cells of any given population. This is maybe not surprising, as both are needed for homeostatic monocyte migration (Henderson et al., 2003), a role which could also be true for circulating DCs. CD11b on the other hand was present on nearly all $CD14^+$ monocytes, but percentage of cells stained positive for the marker was significantly lower in $CD16^+$ monocytes and cDC2 DCs, significantly lower again in cDC1 DCs and lowest values were recorded in pDCs. Considering brightness of signal only in cells positive for the total integrin, CD11a had a markedly different pattern from all other total β_2 integrin subunits considered. Total CD18, CD11b and CD11c were

highest expressed on $CD14^+$ monocytes, with significantly lower levels in $CD16^+$ monocytes, cDC2 and cDC1 DCs, and significantly lower levels again in pDCs. Total CD11a on the other hand was highest expressed on cDC1 DCs, with medium expression in $CD14^+$ and $CD16^+$ monocytes, as well as cDC2 DCs. What all β_2 integrins shared however, is their low expression on pDCs, which were significantly lower in all integrin subunits.

Considering how this compares to previously published date on β_2 integrin expression in human DCs and monocytes (Chapter 1, section 1.4.2, Table 1.1), this data set confirmed several previously published results, including that cDC2 DCs were significantly higher in CD11c compared to cDC1 DCs (Haniffa et al., 2012; Robbins et al., 2008), that pDCs expressed CD11a (Rosa et al., 2003) but almost no CD11b (Haniffa et al., 2012; Li et al., 2011; Rieckmann et al., 2017) and that circulating monocytes expressed CD11a, CD11b and CD11c. However, this data set did not confirm the findings that CD11b expression is higher in cDC2 DCs than in cDC1 DCs (Haniffa et al., 2012; Robbins et al., 2008), but rather shows that less cDC1 DCs are positive for the marker. Instead, the data in Figure Figure 5.4 indicate that CD11b expression was equivalent in these DC populations.

To summarise, I have found that different APC types differ not only in their respective expression of β_2 integrins, but also in the amount of cells expressing the β_2 integrin subunit. While all APC types were found to express CD11a and CD18 on their surface, expression of CD11b was more varied between cell types. Furthermore, I showed that the amount of signal lost due to steric hindrance when staining both total and active CD11a together is consistent in all APC types. This importantly means that total CD11a expression can be compared between APC types using double-stained samples affected by steric hindrance.

Active β_2 integrins in healthy control APCs

Having found high expression of β_2 integrin subunits in cDCs and monocytes, but not pDCs, I went on to assess the expression of the active β_2 integrins forms in these APCs. When investigating active β_2 integrin subunits, only CD11a, CD11b and C18 could be considered, as active CD11c was not included due to limitations of the panel design. Total MFI (left, all cells expressing the active β_2 integrin in question), as well as the percentage of cells expressing both total and active β_2 integrins (T %Positive, middle left, percentage of cells expressing total β_2 integrin that also

express the active β_2 integrin in question), the MFI of cells positive for the active conformation of the respective β_2 integrin (%MFI, middle right, MFI of active β_2 integrin on cells that also express its total form) and a histogram overlay of expression in all five APC types is shown in Figure 5.6. This allowed for both comparing the amount of positive staining between populations (MFI, %MFI, histogram overlay), as well as showing how many cells of a population express the respective active integrin subunit (T %Positive).

Similar to total CD11a (Figure 5.4,Figure 5.5), active CD11a was expressed on almost 100% of cells, that also expressed total CD11a, of all APC populations analysed. Again, pDCs had a lower percentage of cells positive for active CD11a in some samples, but this was not significant. Highest levels of active CD11a were measured on $CD14^+$ monocytes and cDC1 DCs, and significantly lower on $CD16^+$ monocytes. Expression of active CD11a was further reduced in cDC2 DCs, and significantly lower again in pDCs. It is interesting to note that expression of active CD11a on the cell surface was similar between $CD14^+$ monocytes and cDC1 DCs, even though cDC1 DCs express much higher levels of total CD11a (Figure 5.4). This suggests that CD11a activation on cDC1 DC surfaces is tightly controlled, as it did not directly correlate with the amount of total CD11a expression. The fact that different cell types showed significantly different amounts of active CD11a staining, with highest signal recorded in $CD14^+$ monocytes and cDC1 DCs, thereby implies that conformation of CD11a is cell type specific. This suggests two things: the first is that different cell types have different ‘optimum’ levels of active CD11a at steady-state. As an example, different APC populations might require different levels of active CD11a to adhere to endothelium and extravasate into tissue depending on cell size or density. However, the data did not fit this hypothesis, as active CD11a was found in lower levels on cDC2s compared to cDC1 DCs, even though cDC2 DCs are closer in cell size and granularity to cDC1 DCs than to $CD14^+$ monocytes. The other, more likely option, is that there are differences in CD11a expression and conformation between different APC subtypes, which might impact on the respective biological function of these cells.

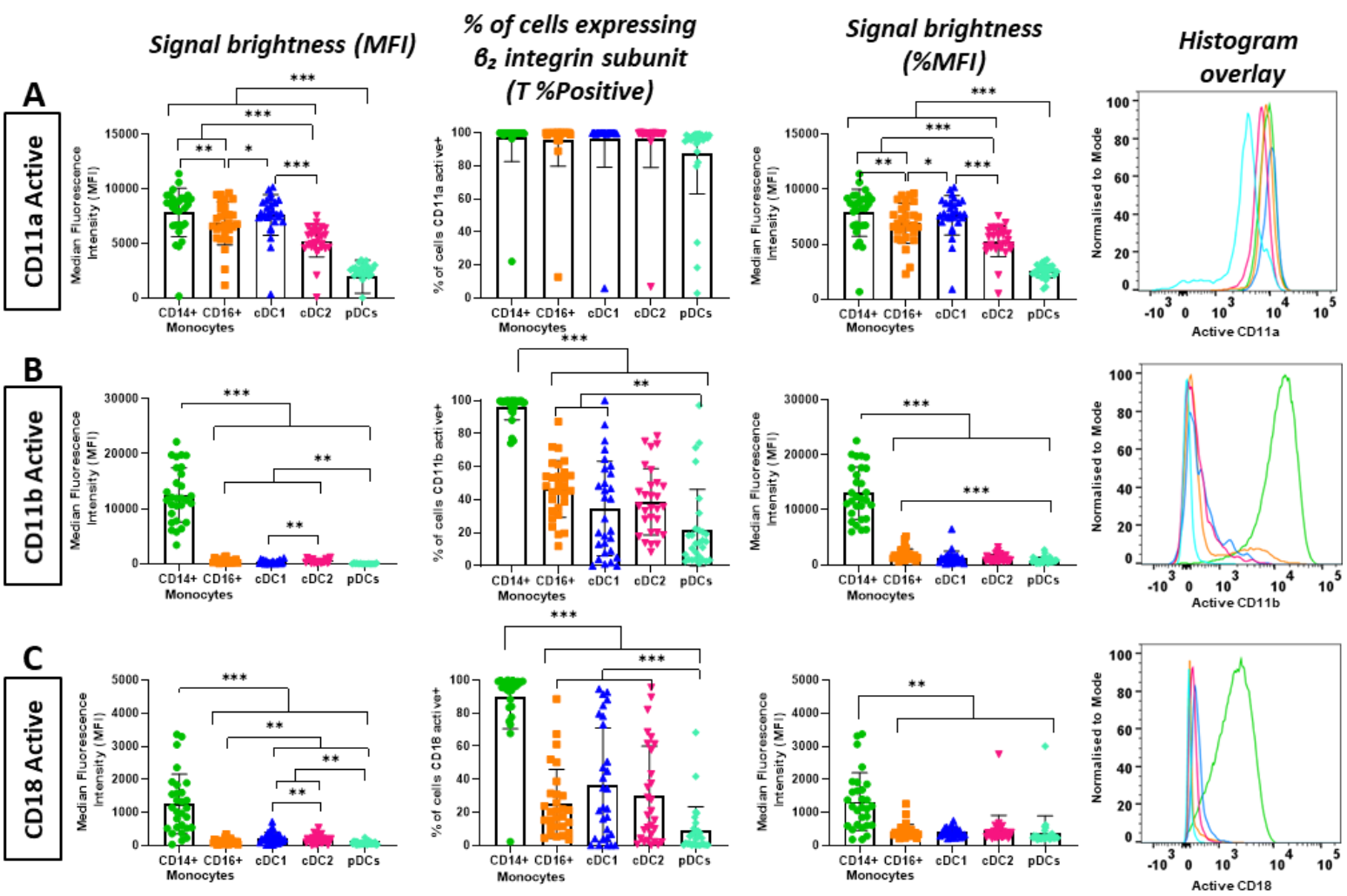

Figure 5.6 Active β_2 integrins in healthy control APCs

Active expression of CD11a, CD11b, CD18 in five APC subtypes of interest, CD14+ monocytes, CD16+ Monocytes, cDC2 and cDC1 DCs, as well as pDCs. Measurements shown are MFI, T %Positive cells and %MFI. Repeated measures one-way ANOVA, n=29.

The highest expression of active CD11b was found in $CD14^+$ monocytes, with close to 100% of this population expressing the total marker also expressing the active marker (Figure 5.6B). This was significantly different from only 40-50% of total $CD11b^+$ $CD16^+$ monocytes and cDC2 DCs expressing active CD11b. A mean of 30% of total $CD11b^+$ cDC1 DCs expressed active CD11b, but the spread between donor samples was very wide, with some donors having up to 90% of total $CD11b^+$ cDC1 DCs expressing active CD11b. Thus, active CD11b expression was not significantly different from $CD16^+$ monocytes and cDC2 DCs. Lastly, active CD11b was only expressed on around 20% of total $CD11b^+$ pDCs, which was significantly lower compared to all other APC populations except cDC1 DCs. MFI of the cells that did express active CD11b is much more uniform. $CD14^+$ monocytes expressed by far the greatest levels of active CD11b, while all other APC populations were quite similar to each other. However, the 20% of total $CD11b^+$ pDCs that expressed active CD11b on their cell surface did express significantly less than $CD16^+$ monocytes, but not any of the other APC types. It is striking that $CD14^+$ monocytes were found to have such high expression of active CD11b. Especially as published literature suggested β_2 integrins are largely inactive on cells in circulation (Li et al., 2017), this could again suggest that $CD14^+$ monocytes have a lower threshold for CD11b activation compared to other APC types.

Lastly, active CD18 showed a similar expression pattern to active CD11b. $CD14^+$ monocytes expressed significantly more active CD18 than all other APC types considered (Figure 5.6C). They were also close to 100% positive for active CD18, which was significantly different from only 25-35% of total $CD18^+$ $CD16^+$ monocytes, cDC1 DCs and cDC2 DCs. pDCs showed the lowest expression of active $CD18^+$ cells, with only 10% of total $CD18^+$ cells positive.

To summarise, CD11a has different expression and activation patterns across APC populations compared to the other β_2 integrin subunits. Importantly, cDC1 DCs expressed higher levels of both the total and the active forms of CD11a compared to cDC2 DCs. Total CD11b expression was lower in cDC1 compared to cDC2 DCs, although expression of active CD11b was equivalent. These differences between CD11a and CD11b in cDCs were especially interesting as it mirrored observations made during the active integrin panel optimisation process in Chapter 3, where CD11a showed different properties in regard to steric hindrance, staining duration and staining temperature, compared to CD11b and CD18. This suggests that while

CD11a and CD11b have overlapping functions, they are not interchangeable and show differences in which cell types they have which roles on.

The effect of age on β_2 integrin expression and conformation

After providing a thorough analysis of β_2 integrin subunit expression and conformation in APC populations in healthy control individuals, I now go on to assess the effect of an individual's age on expression and conformation of β_2 integrins. Assessing the effect of age on β_2 integrins is especially interesting in respect to a potential immunoregulatory role β_2 integrins might play, as senescent DCs were shown to be less able to stimulate T cells (Gardner et al., 2017) and that monocytes from individuals over the age of 65 are functionally and transcriptionally distinct upon activation from monocytes derived from younger donors (Metcalf et al., 2017). Testing the effect of age on β_2 integrin expression is therefore valuable not only to ensure age differences between healthy controls do not affect the results, but also to reveal potential differences in their function with age. As there is no published information on how β_2 integrins might differ with age of the individual, correlation between age of the donor and the signal of total and active β_2 integrins is shown in Figure 5.7.

Neither total nor active CD11a showed any significant correlation with age in any of the cell types. A similar picture emerges for total and active CD11b, suggesting that neither CD11a nor CD11b are in any way affected by age. Active CD18 is significantly correlated with age in $CD14^+$ monocytes and cDC1 DCs, while total CD18 is significantly correlated with age in cDC1 DCs and pDCs. However, when considering the R^2 values for the linear regression calculated for each correlation, they are very low, ranging from 0.139 to 0.258. This suggests that the correlation is not very strong and that the statistically significant result could arise due to a Type II statistical error. However, as the possibility remains that CD18 is correlated with the age of the donor, this needs to be considered later when comparing healthy controls to RA patients: the analysis will include age as a covariate to ensure that any effects seen are not due to a difference in age.

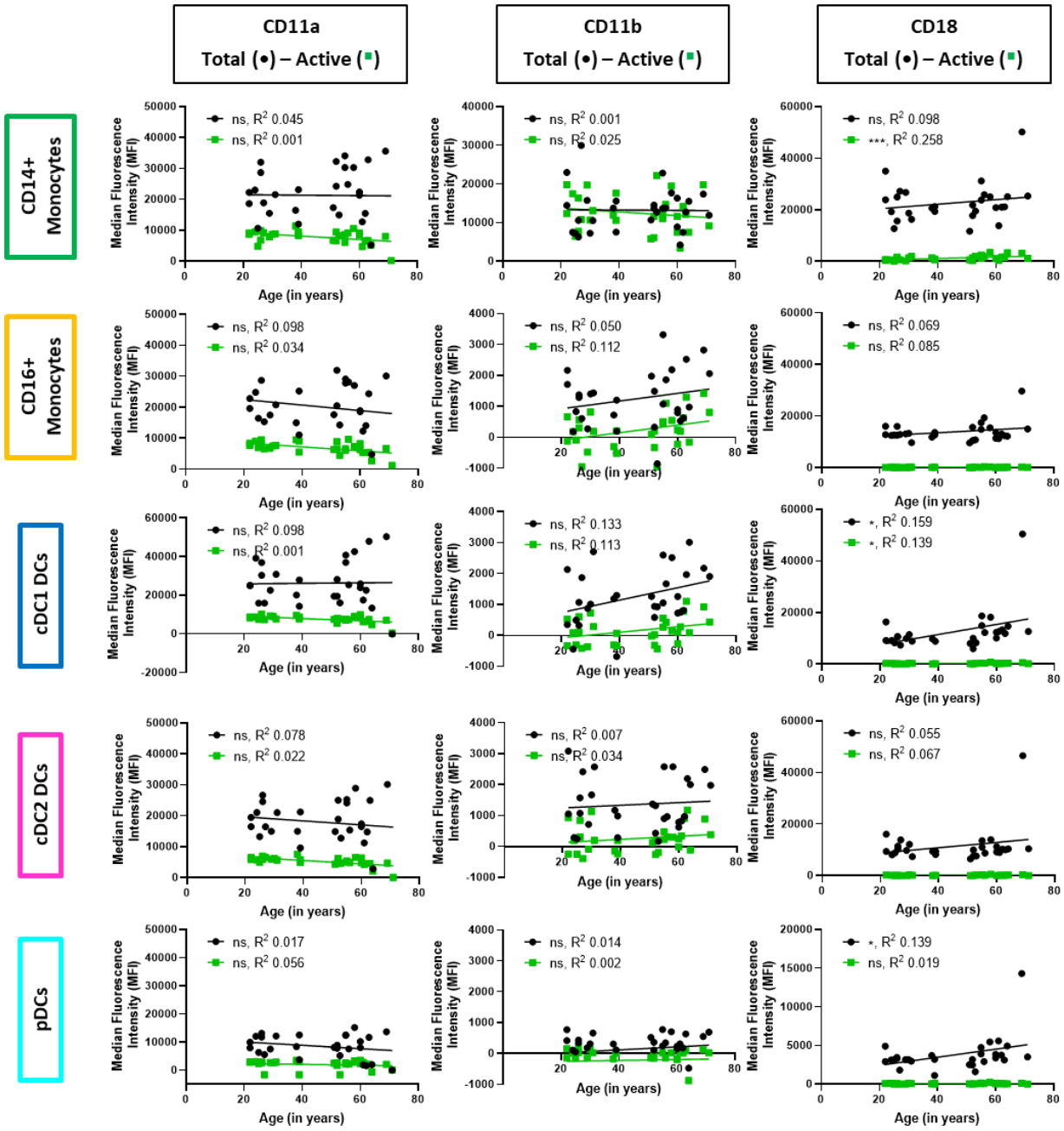

Figure 5.7 Correlation of total and active β_2 integrin expression with age in healthy controls.

Age in years was correlated to total and active CD11a (left), CD11b (middle) and CD18 (right) for APC subtypes of interest. Total β_2 integrins are shown in black, active β_2 integrins are shown in green, with respective R^2 values and significance noted above each plot. Linear regression with fitted line, n=29.

Effect of gender on total β_2 integrin expression

Similarly to age, it is not known if gender has any effect on β_2 integrin expression. However, several studies have highlighted differences in immune system function

depending on gender (Klein and Flanagan, 2016; Whitacre, 2001), making it important to investigate β_2 integrins in this context. Figure 5.8 shows total CD11a, CD11b and CD18 expression for healthy APCs from females and males, which shows that total CD11a is significantly higher in female than in male healthy controls. RA occurs in women at twice the rate than it occurs in men (Alamanos and Drosos, 2005). Due to the higher number of females, healthy controls could not be age-matched exactly, although 9 of 10 male healthy controls could have been matched to 9 female healthy controls. However, as the mean age of female healthy controls was 46.30 and the mean age of male participants was 43.78, the difference in ages was not considered to have significant impact. As this healthy control data set was collected to eventually be compared to RA patient samples, healthy female and male samples were collected in proportion to reflect this observed 2:1 ratio. CD11a expression is significantly lower in males, which is true for all APC types analysed. Note that this difference was apparent when plotting either single or double stained CD11a total (data not shown). On the other hand, total CD11b and CD18 expression show no significant increase or decrease associated with gender. As the effect of higher CD11a expression on APCs from female participants was so striking, it was next considered if this was due to total CD11a being expressed on a lower percentage of cells in male participants. However, when looking at the percentage of cells positive for total CD11a in different APC populations (Figure 5.9), both male and female study participants showed close to 100% of cells expressing total CD11a in all APC types.

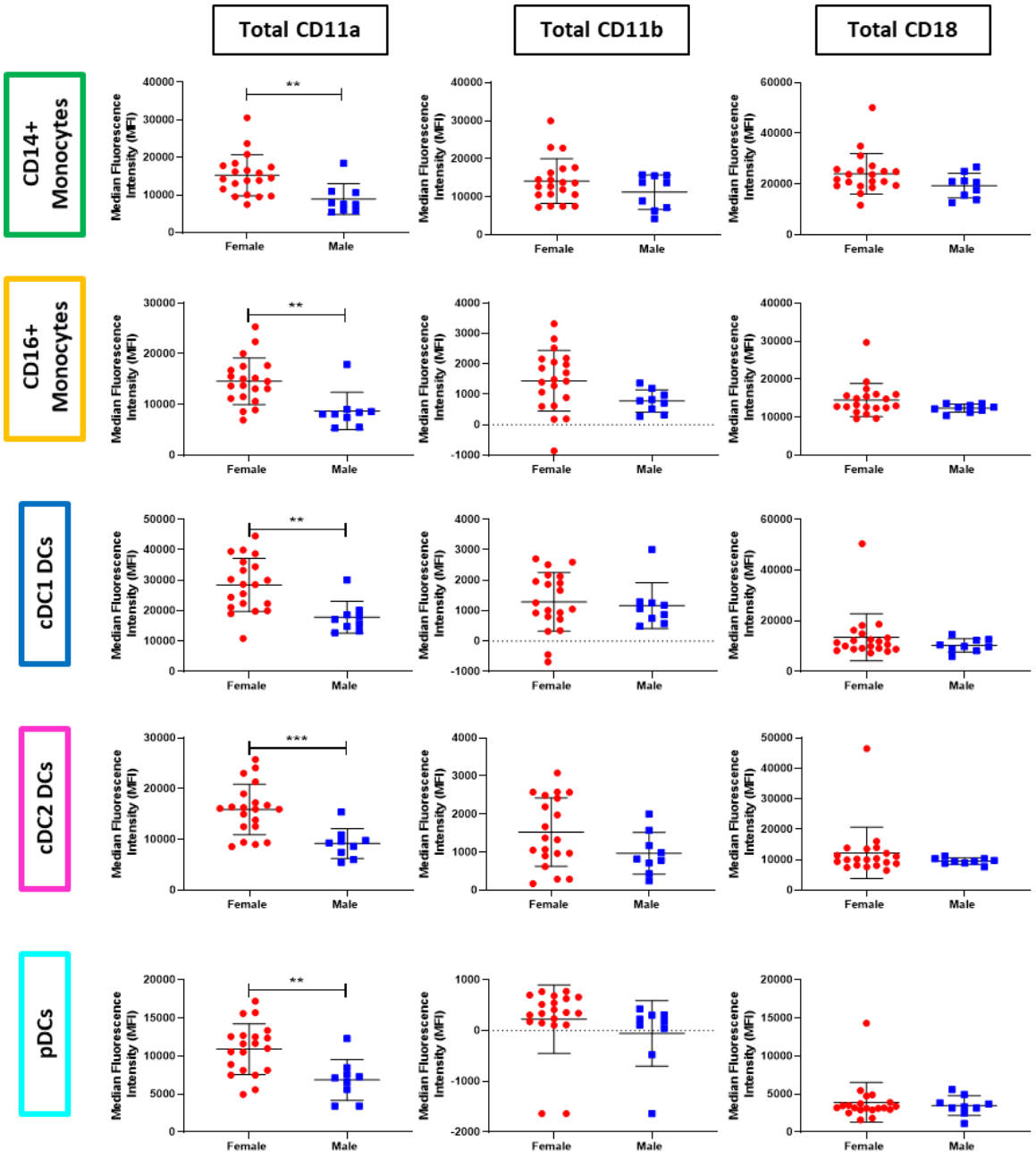

Figure 5.8 Effect of gender on total β_2 integrin expression in healthy controls

Total CD11a, CD11b and CD18 expression was compared between female (n=20, red circles) and male (n=9, blue squares) healthy controls in five APC types of interest. Unpaired t test, total n=29.

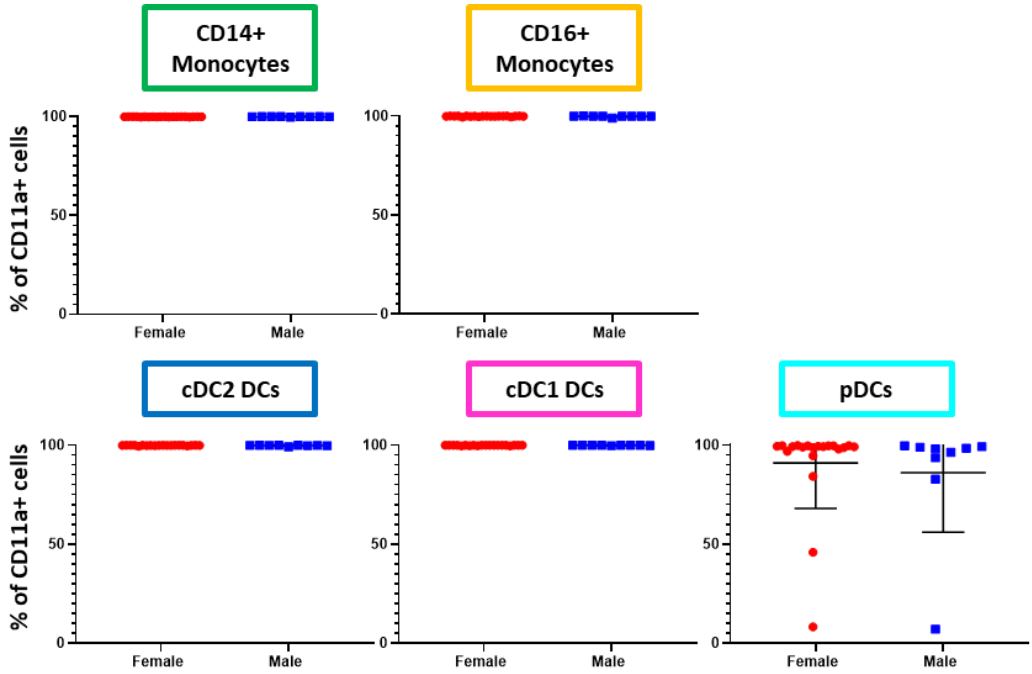

Figure 5.9 Percentage of male and female APCs expressing total CD11a

Percentage of APC population expressing total CD11a (% Positive) was compared between female (n=20, red circles) and male (n=9, blue squares) healthy controls. Unpaired t test, total n=29.

This suggests that women have higher total CD11a expression on APCs rather than increased proportion of APCs expressing total CD11a. However, when analysing active β_2 integrins on APCs from female and male participants (Figure 5.10), active CD11a does not show the same difference between genders, suggesting again that active β_2 integrins do not directly correlate to the level of total integrin expression. Active CD11b and CD18 are also not significantly different between genders.

In summary, gender of the study participant does not have any effect on conformation of β_2 integrins, but females express significantly more CD11a across APC types compared to males. This is an interesting finding that will be discussed in more detail in the discussion of this chapter.

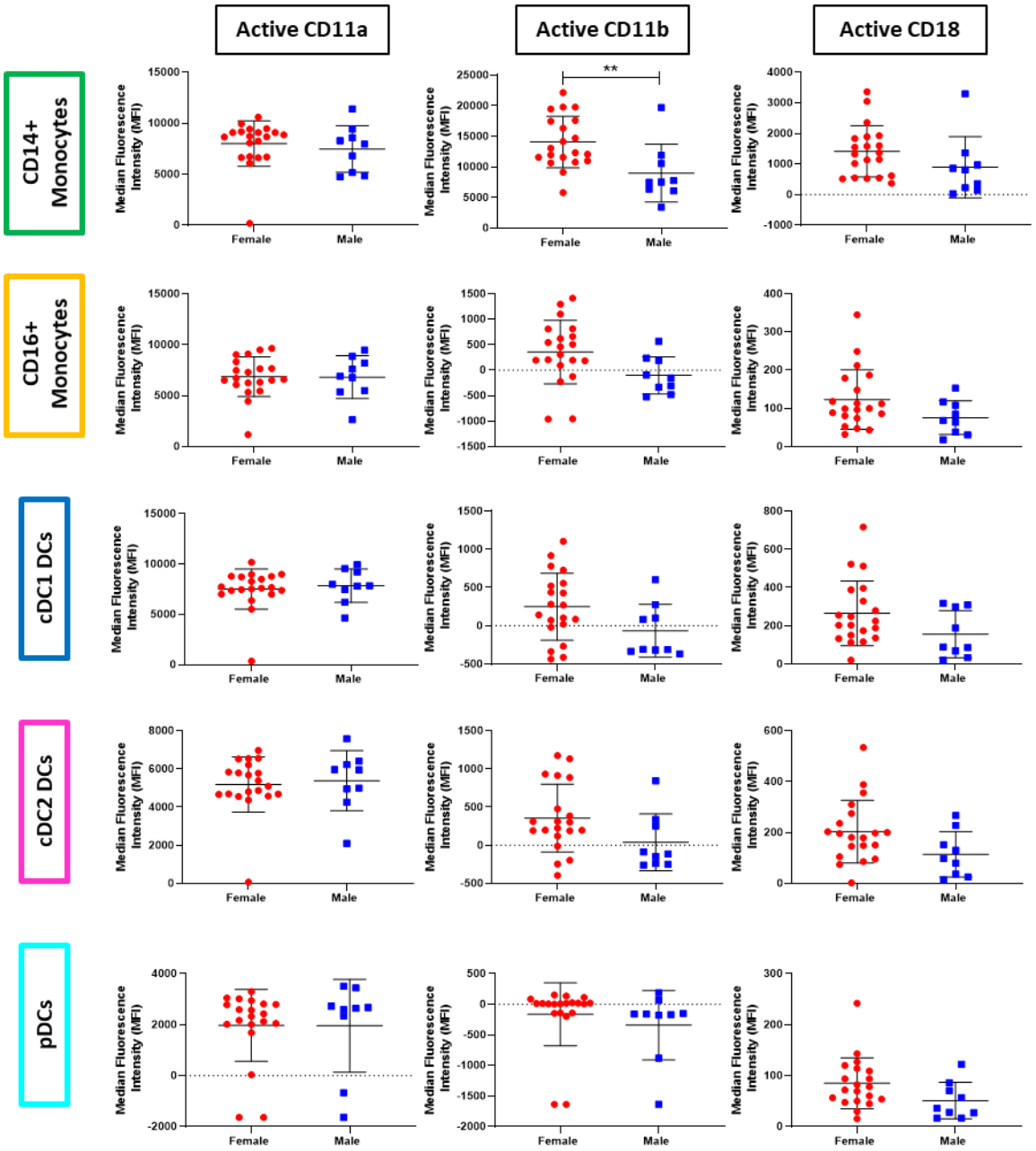

Figure 5.10 Effect of gender on active β_2 integrin expression

Expression of active CD11a, CD11b and CD18 was compared between female (n=20, red circles) and male (n=9, blue squares) healthy controls in five APC types from PB. Unpaired t test, total n=29.

5.3.3 β_2 integrin expression in APCs in Rheumatoid Arthritis

As discussed in the introduction to this chapter, there is a potential role for β_2 integrins in RA initiation or progression. However, due to range of β_2 integrin functions between migration and adhesion and the complexity of RA, it is difficult to draw clear conclusions from previously published literature. To gain more knowledge of the potential immunoregulatory roles of β_2 integrins, I aimed to identify any changes in expression and conformation of β_2 integrins in the inflammatory setting of the autoimmune disease RA. To investigate β_2 integrin expression on APCs in patients with RA, RA patients were recruited in a local clinic and processed immediately. Samples were categorised into active (DAS28>3.2) and remission (DAS28<2.6) groups according to 2010 EULAR RA classification criteria (Aletaha et al., 2010). Healthy controls showed no signs of joint pain and had not been diagnosed with a disease or disorder affecting the joints.

Power calculations for RA patient sample size

As there was no published information to the effect size that could be expected, power calculations were expected to be adjusted during the sample acquisition process (Jones et al., 2003). As discussed in the methods (Chapter 2, section 2.9, Table 2.4 and Table 2.5), *a priori* power calculations assuming a power of 0.95 showed that 34 samples per group would be sufficient to detect a large effect size after Cohen's *d* (Cohen, 1992). Assuming only 80% (0.8) probability that significant results were indeed correct lowered the amount of samples per group to 13 to effect large effect sizes, or to 31 samples per group to detect medium effect sizes. It was therefore decided to collect 10 samples in each group and repeat power calculations once effect sizes specific to total and active β_2 integrin expression in APCs could be attained (Lakens, 2013). Effect sizes were calculated using the mean squared residual (MSresidual), a value that describes the difference between the sample mean and the individual observed values, which derived from a one-way ANOVA and g*power. Effect sizes varied widely between β_2 integrin cell type combinations, with active CD18 expression needing 1344 samples per group to correctly detect a significant difference at 95% probability. Considering that small effect sizes between 10 samples per group in certain β_2 integrin APC types combinations (6 total and active β_2 integrin subunits x 5 APC types) signified that there was no significant difference to be detected, it was decided to only consider combinations where sample number per group could be reasonably recruited in the available time frame.

Calculating this, it was found that 32 samples per group yielded 12 of 30 β_2 integrin APC combinations to be correctly powered at 0.8 or above. These 12 correctly powered comparisons consisted out of five total CD11a comparisons (all APC types), four total CD11b comparisons (all APC types except pDCs), two active CD11a comparisons ($CD14^+$ and $CD16^+$ monocytes) and one active CD11b comparison ($CD14^+$ monocytes). The sample number needed to achieve the next tier of correctly powered comparisons per group, was not considered to be achievable. It was therefore decided to aim to recruit at least 32 samples per group.

Recruited RA patients

	Healthy Controls	**Active RA**	**Remission**
Recruitment criteria	No known joint disease	DAS28 (ESR) >3.2	DAS28 (ESR) <2.6
Final n number *(patients recruited)*	**29** (29)	**31** (29)	**24** (35)
Median age *(age range)*	52 years *(22-71)*	63 years *(35-87)*	65 years *(37-81)*
M:F ratio *(ratio)*	9:20 M:F *(1:2.1)*	5:26 M:F *(1:5)*	10:14 M:F *(1:1.4)*
Mean DAS28 *(±SD)*	N/A	4.48 *(±1.03)*	1.73 *(±0.67)*

Table 5.1 Recruitment details of healthy controls and patients with RA

The final number recruited for each group, as well as their median age, gender ratio and mean DAS28 is shown in Table 5.1. A total of 29 healthy controls, 31 active RA and 24 RA patients in remission were recruited for this study. The largest number of samples had to be excluded in remission patients, where more than 10 patients were diagnosed by their healthcare professional to be likely in remission but whose DAS28 was equal or higher than 2.6. Interestingly, 2 patients that were identified to be likely in remission in the clinic turned out to have a DAS28 over 3.2, which is why they were included in the active RA cohort. Differences in 'clinical remission' (no tender or swollen joints) and remission defined by DAS28 cut-off points have been observed in the past, with the criticism being raised that DAS28 might be an imperfect tool to assess remission (Mäkinen et al., 2005). However, while CRP-DAS28 was found to underestimate remission, this was not observed in ESR-DAS28 (Sheehy et al.,

2014), which was used throughout this study, as CRP-DAS28 could not be obtained for all patients. It was, however, also found that patients' perception of disease activity, which is a composite part of the DAS28 value, is dependent on the difference between disease activity at the beginning of treatment compared to a later time point (ALETAHA et al., 2009; Felson et al., 2011). This could be one reason why clinical perception of disease activity and DAS28 did not match.

Healthy controls were recruited to match age and gender ratio of RA patients as closely as possible. However, age-matching was found to be difficult. This was due to the fact that healthy donors were still largely in the work force, while recruitment of established RA patients included many who were of retirement age. While the oldest healthy donor was 71 years old, oldest donors in both active and remission RA were 10-15 years older, being 87 and 81 respectively. However, age-spread between healthy controls and RA patient samples was similar. Considering this, the age-gap of 10 years between the median healthy control and the median RA patient was thought to be acceptable. As studies have shown that RA occurs in twice as many women as men, healthy controls were recruited accordingly. However, this data set shows a much higher number of women in active RA with a ratio of 1:5, while the ratio in remission is closer to 1:1.4. Considering the differences in total CD11a between female and male healthy controls (Figure 5.8), the effect of gender on total CD11a expression will have to be considered in all further analysis. The mean DAS28 confirms that samples collected show high and low disease activity according to their group.

Disease status and medication in RA patient samples

Disease status as well as the drugs study participants were prescribed are shown in Table 5.2. Both active and remission groups had disease for 10-13 years. Participants with active RA were more likely to be RF+, while CCP+ status was similar between groups. In terms of medication, more patients in remission were on MTX (70.8%) compared to people with active disease (54.8%). This could potentially be explained by adverse reactions to MTX, which in turn could negatively affect disease activity. Alternatively, there is a higher likelihood that the first-line treatment MTX had not controlled disease in the past so had been discontinued in these patients with active disease. Approximately half of both groups were on various DMARDs, with HCQ being the most commonly prescribed drug in both groups. It is apparent that there was less DMARD variety in the remission group, potentially due

to the successful treatment with one of the first-line DMARDs. Lastly, approximately 10% less patients in remission were on biologics (20.8%), while 32.3% of patients with high disease activity were on biologics.

RA disease status		
	Active	**Remission**
Mean Disease duration *(±SD)*	12.73 (±8.68)	10.83 (±7.11)
Rheumatoid Factor (RF) status	*RF+ve:* 24 *RF-ve:* 4 *Unknown*: 3	*RF+ve:* 14 *RF-ve:* 6 *Unknown*: 4
Anti-cyclic citrullinated peptide (anti-CCP) status	*CCP+ve:* 21 *CCP-ve:* 4 *Unknown*: 6	*CCP+ve:* 15 *CCP-ve:* 2 *Unknown*: 7
RA Therapeutics		
No medication	**3** patients (2 <6months after diagnosis)	**1** patient
Methotrexate (MTX)	**51.51%** (**17/31**) (6 take no other drugs)	**70.8%** (**17/24**) (7 take no other drugs)
Disease-modifying anti-rheumatic drugs (DMARDs) Hydroxychloroquine(HCQ) Sulfasalazine (SSZ) Prednisolene (PRED) Leflunomide (LEF) Azathriopine (AZA)	**58.1%** (**18/31**) *HCQ:* 11 *SSZ:* 10 *PRED:* 3 *LEF:* 2 *AZA:* 1	**50%** (**12/24**) *HCQ:* 7 *SSZ:* 4 *PRED:* 1 *LEF:* 0 *AZA:* 0
Biologics Tocilizumab (TOC) Rituximab (RTX) Baricitinib (BAR) Certolizumab (CERT) Etanercept (ETA)	**32.3%** (**10/31**) *TOC:* 2 *RTX:* 1 *BAR:* 3 *CERT:* 1 *ETA:* 1 *Unknown*: 2	**20.8%** (**5/24**) *TOC:* 2 *RTX:* 1 *BAR:* 0 *CERT:* 2 *ETA:* 0 *Unknown*: 0

Table 5.2 Information on disease pattern and prescribed therapeutics in the recruited RA patients

The comparably smaller number of people prescribed Biologics is easily explained by the high cost of the medication, which usually makes it a last effort to reduce disease activity if other drugs have failed.

Even though sufficient numbers of samples could only be attained in the active RA cohort with 31 samples, patient recruitment can still be considered a tentative success. Comparisons of expression are still highly powered in 8 of the assumed 12 comparisons, including both total CD11a and CD11b in cDC1 and cDC2 DCs, which are the main cells of interest to understand the role of β_2 integrins in DCs. Furthermore, three comparisons are still correctly powered to compare active RA to healthy controls (CD11a active and CD11b active in $CD14^+$ monocytes, CD11a total in $CD16^+$ monocytes), with the only comparison that is not correctly powered due to the lower amount of healthy controls and remission samples recruited being CD11b total in $CD14^+$ monocytes. While having to exclude a large part of remission samples was unfortunate, this seems to be common in clinical research pertaining remission, but did not largely affect the comparisons made. The differences in age (Figure 5.7) and gender ratio (Figure 5.8) between different groups have to be considered. To further ensure age was not a significant factor, it was used as a covariate when differences between different patient groups were assessed. Furthermore, gender ratios between healthy controls and RA patients' groups were different, an aspect which might influence expression of especially total CD11a. This will be discussed in more detail in the discussion at the end of this chapter. Considering the RA patient groups, they compare well to published literature in terms of medication and disease status ("Guidelines for the management of rheumatoid arthritis," 2002; Song and Kang, 2010). It can therefore be concluded that the group of RA patients recruited into the study are representative of the wider patient community and can be compared to published studies.

5.3.4 The β_2 integrin CD11a in APCs from RA patients

First, it was assessed if expression of CD11a is altered in RA. For this, the data set discussed above was interrogated in terms of total and active CD11a expression.

Expression of total CD11a on different APC types between patients who have high disease activity or who are in remission is shown in Figure 5.11. No significant differences between RA patient groups were found, suggesting that CD11a expression on APCs is not a significant factor in either contributing to active disease

or controlling remission. When comparing healthy controls to both RA patient groups (Figure 5.12), representative histogram overlays show that expression of total CD11a is slightly lower on CD14^{+} monocytes from patients in remission and also on cDC2 DCs from RA patients in general (Figure 5.12A). However, Figure 5.12B shows that total CD11a is significantly lower in cDC2s from patients with both active disease and remission, when compared to healthy controls. However, the fold difference between active RA and healthy controls is quite small at 1.42, and the fold difference between remission and healthy controls is 1.63 in cDC2 DCs, which is similarly low. Interestingly, this trend is mirrored in CD14^{+} monocytes, cDC1 and pDCs, where only patients with low disease activity show significantly lower CD11a expression than healthy controls. This suggests that expression of total CD11a is altered, particularly on cDC2 DCs, in the inflammatory context of RA, but regardless of disease activity. Considering the higher expression of active CD11a on tolerogenic DCs compared to mature DCs shown in Chapter 4, one could hypothesise that the immunoregulatory function of CD11a is lost in RA, contributing to an altered immune phenotype that does not return to 'normal' despite disease remission.

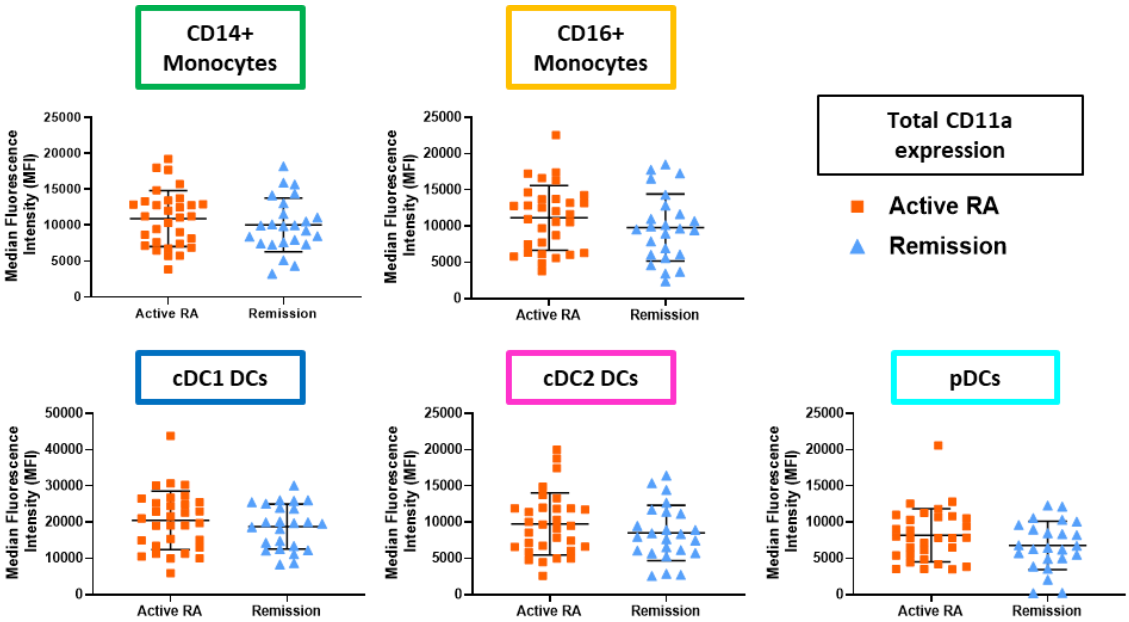

Figure 5.11 Total CD11a expression in RA

Total expression of CD11a (MFI) compared between active RA (orange squares, n=31) and disease in remission (blue triangles, n=24) on five APC types of interest isolated from PB. Unpaired student's t test.

As mentioned previously, total CD11a shows significant signal loss due to steric hindrance when stained together with active CD11a. To confirm the result of reduced

total CD11a in cDC2s isolated from RA patients compared to healthy controls, single stained total CD11a was plotted (Figure 5.13A, right). Surprisingly, no significant difference between RA patient samples and healthy controls was found in the single stained total CD11a samples. Looking further into this, it can be observed that rather than CD11a being lower in cDC2s from RA patients, it is the amount of steric hindrance that is increased in both active and remission RA groups. This was confirmed by considering both the difference in MFI (Figure 5.13B, left), showing significantly increased MFI between double and single stain, as well as the % of steric hindrance (Figure 5.13B, right), showing that a significantly greater percentage of total CD11a signal is lost in both RA patient groups.

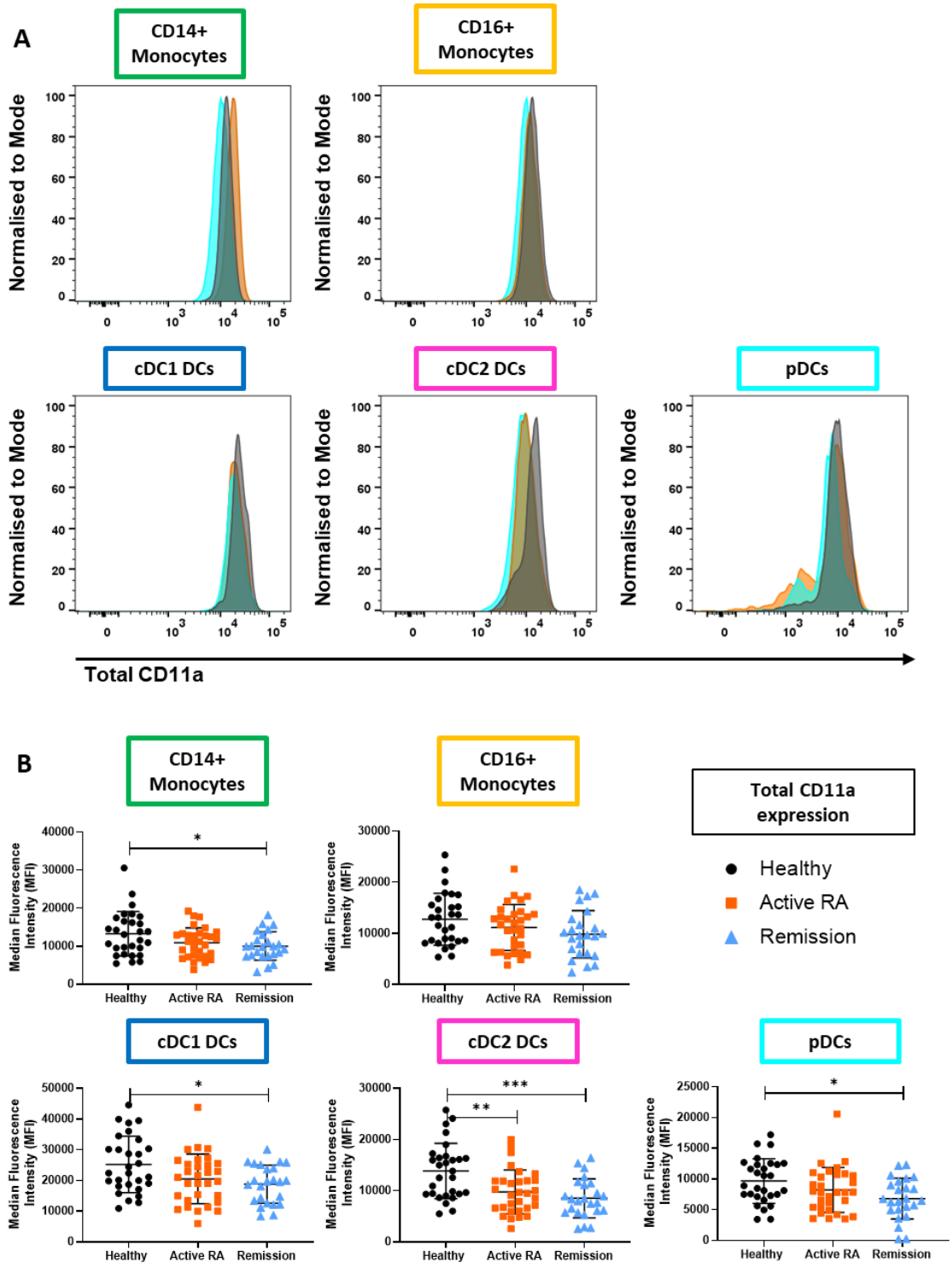

Figure 5.12 Total CD11a expression in healthy controls compared to RA patients

Expression of total CD11a comparing RA patient groups (active and remission) to healthy controls. A. Histogram overlays of representative total CD11a expression of each of the groups in each of the APC types. Representative samples were chosen as close to median of patient group as possible. B. Total CD11a expression (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

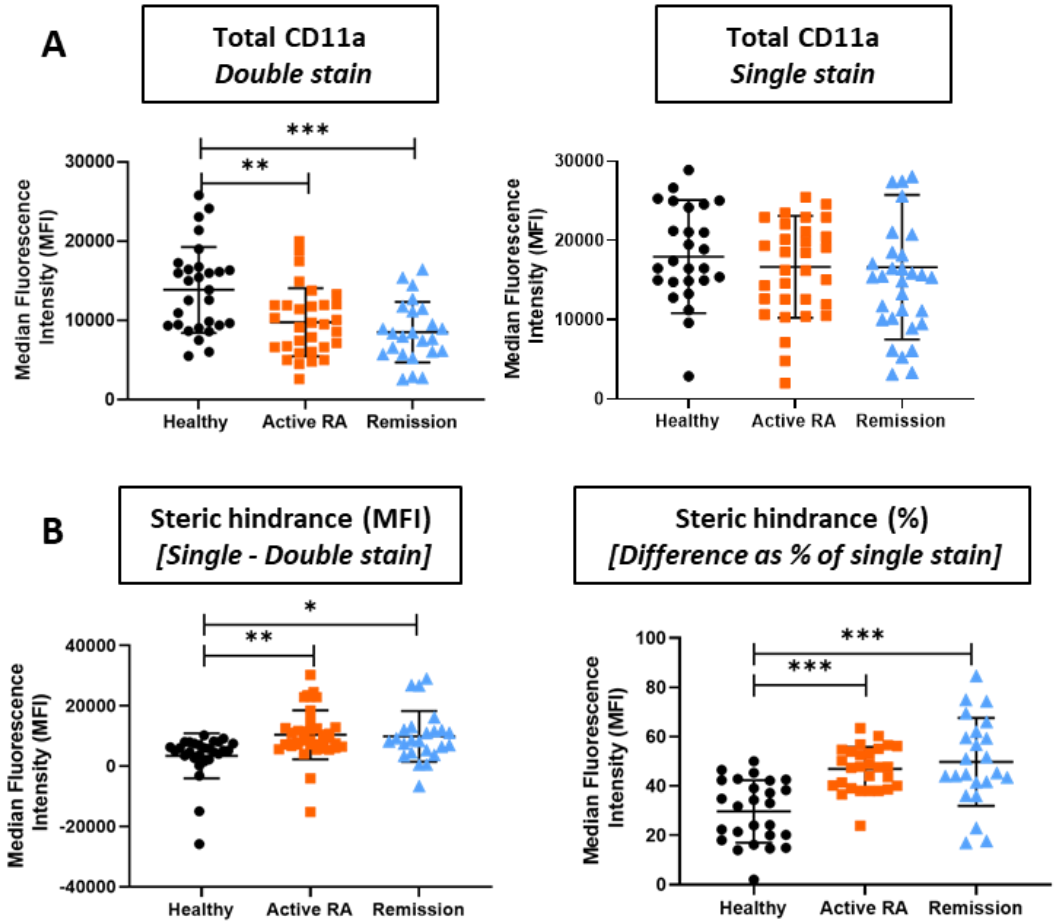

Figure 5.13 Effect of steric hindrance on total CD11a in RA

Total CD11a expression on cDC2 DCs compared between healthy controls (black circles, n=29) and patients with either active RA (orange squares, n=31) or in RA remission (blue triangles, n=24). A. Total CD11a expression on cDC2s in Double stain (shown before in Figure 5.12, and here left) and total CD11a single stain (right). B. Amount of steric hindrance on cDC2 DCs between healthy controls and RA patients. Left shows the difference between single and double stain in MFI values (MFI amount lost to steric hindrance), right shows differences in steric hindrance as a percentage of single stain (% of signal lost to steric hindrance). Unmatched one way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

Healthy control cDC2s lost a mean of 30% of total CD11a signal by staining alongside active CD11a, while a mean of 45% and 50% were lost respectively by patients with high and low disease activity. The fact that more total CD11a signal is lost in RA samples suggests that there is more active CD11a on RA patient cDC2s, which in turn cause an increase in steric hindrance. However, when looking at active CD11a expression, this was not the case. While there is a small but significant decrease in active CD11a expression between active RA and remission in cDC1 and

cDC2 DCs (Figure 5.14), there are no significant differences between healthy controls and RA patients (Figure 5.15). This result is supported by the representative overlays of the three groups considered, which show no increase in active CD11a expression in RA samples (Figure 5.15). This result is particularly puzzling, as it suggests that the increase in steric hindrance is not caused by an increase in active

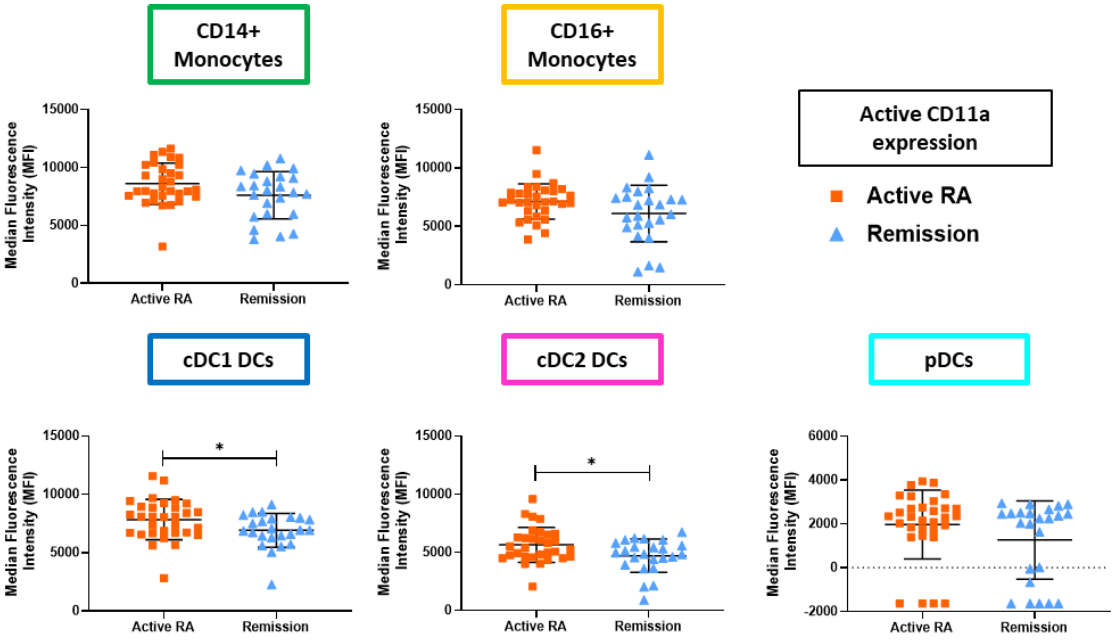

Figure 5.14 Active CD11a expression in RA

Expression of active CD11a (MFI) compared between active RA (orange squares, n=31) and remission (blue triangles, n=24) on five APC types from PB. Unpaired student's t test.

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Exploring if the increase in steric hindrance could be due to the prescribed therapeutics, total CD11a was assessed between patient groups taking different classes of anti-rheumatic drugs (Figure 5.16). For this, total CD11a expression determined by double staining (total and active CD11a together) was plotted. This is to visualise any differential effect between treatment groups.

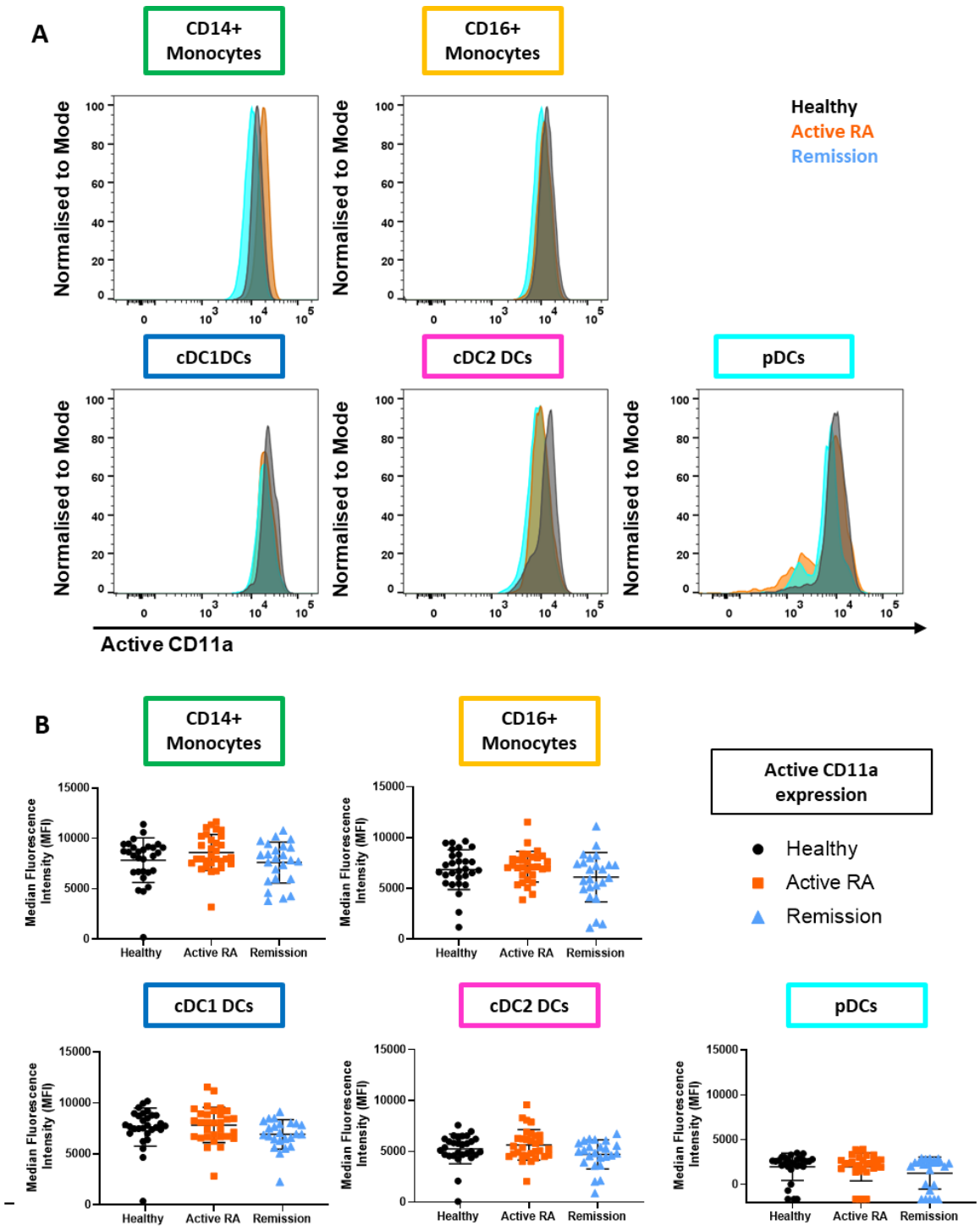


Figure 5.15 Active CD11a expression in healthy controls compared to RA patients

Expression of active CD11a comparing RA patient groups (active RA, remission) to healthy controls. A. Histogram overlays of representative total CD11a expression of each group in each APC type. Representative samples were chosen as close to median of patient group as possible. B. Total CD11a expression (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

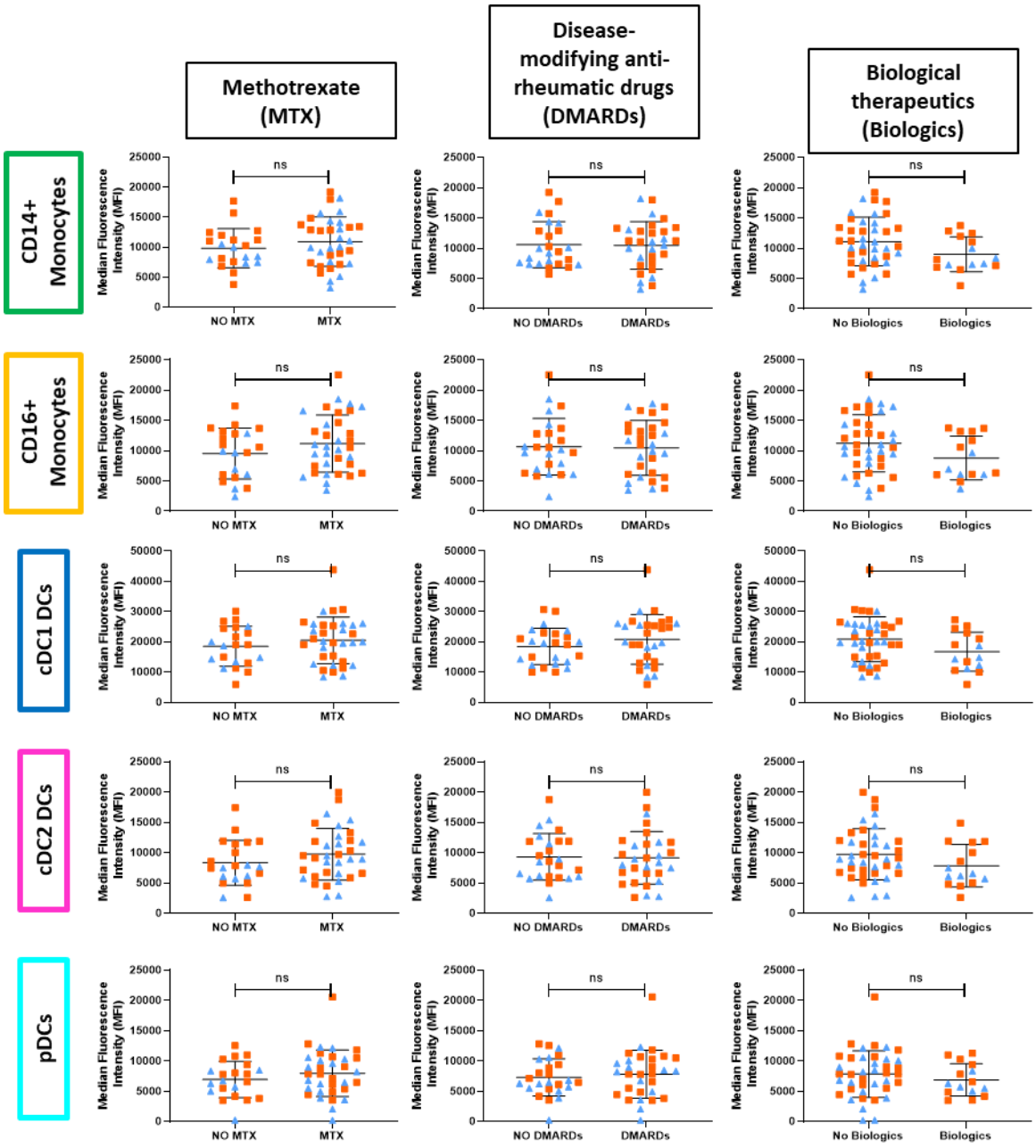


Figure 5.16 Effect of RA therapeutics on total CD11a expression in RA samples

Expression of total CD11a determined by double staining (affected by steric hindrance, MFI) on five APC subsets of interest, comparing between drug-naïve and drug exposed individuals both with active disease (orange) and in remission (blue). Specific therapeutics considered include Methotrexate (left, No MTX, n=21, MTX, n=34) and broader groups of therapeutics considered include other DMARDs (middle, No DMARDs, n=25, DMARDs, n=30) and Biologics (right, No Biologics, n=40, Biologics, n=15). Unpaired Student's t test.

Discussing patients in such broad treatment groups (e.g. no biologics vs. biologics) has its caveats, as different drugs of the same group might have differing effects on the amount of steric hindrance. Furthermore, many study participants are on combinational therapy, which is highly individualised. Both issues make it difficult to collate groups of patients on the exact same therapy that are larger than 2-4 samples. This makes it impossible to make statistically sound observations. Keeping the comparably small size of this data set in mind, broad grouping of patient samples by therapeutics is, while not perfect, the best available option.

It was found that neither taking MTX, DMARDs nor Biologics had any significant effect on total CD11a expression on any type of APC (Figure 5.16). As seen in Figure 5.11, there was no significant difference in total CD11a expression between active RA and patients in remission, with no clustering of either disease group occurring in any of the cell types or therapeutics taken. While there was no significant difference in total CD11a expression due to Biologics, this result could be complicated by the much lower number of patients on Biologics, making the group size uneven. Despite this, there was no significant effect of drugs on total CD11a that would explain the increase in steric hindrance observed in cDC2 DCs (Figure 5.16).

Having found no effect of drugs on CD11a steric hindrance, the influence of other disease factors was analysed. To this end, the effect of DAS28, disease duration and Rheumatoid factor (RF) positive or Anti-citrullinated protein (CCP) positive status of RA patients was considered in Figure 5.17. However, no significant relationship between total CD11a expression and any of the clinical information available was found. The reason for the increase in steric hindrance in RA samples can therefore not be fully elucidated using the information available from this current data set.

To summarise, RA patients with active disease or in remission show increased signal loss due to steric hindrance, which caused the observed reduction in total CD11a expression (Figure 5.12). Surprisingly, there is no equivalent increase in active CD11a expression in RA samples that would account for this observed increase. Further analysis did not associate any disease parameters available to me (DAS28, disease duration, RF or CCP status) with the increased steric hindrance. Therefore, it is likely that another factor, common to active and remission groups but not healthy controls, is responsible. Potential causes for this difference will be discussed in more detail at the end of this chapter (Paragraph 5.4.2, Discussion).Further hypotheses as

to why this increase in steric hindrance could occur will therefore be discussed in detail in the final part of this chapter.

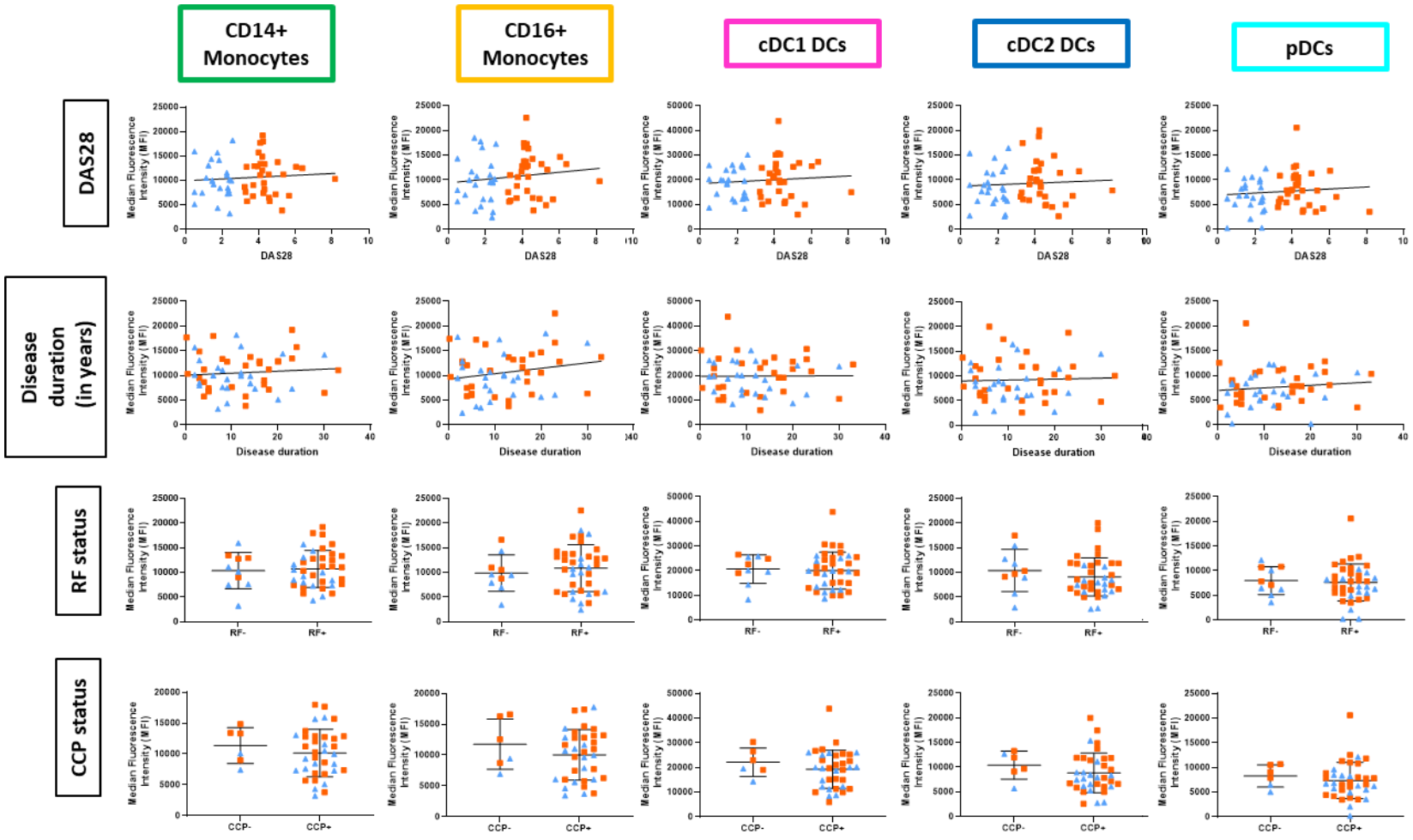


Figure 5.17 Effect of DAS28, disease duration and disease status on total CD11a expression.

Expression of total CD11a affected by steric hindrance (MFI, double stained) was investigated in terms of the effect of various RA patient variables. Both active RA (n=31, orange squares) and remission (n=24, blue triangles) were considered. A. Correlation between DAS28 and expression of total CD11a. Linear regression with fitted line. B. Correlation of disease duration in years with expression of total CD11a. Linear regression with fitted line. C. Comparing RF-negative with RF-positive patient samples, unpaired student's t test. D. Comparing CCP-negative with CCP-positive patient samples, unpaired Student's t test.

CD11a in RA synovial fluid

While considering total and active CD11a in PB is important, RA is an autoimmune disease primarily affecting the joints. To test if expression of the β_2 integrin subunit CD11a is different between the periphery and the site of inflammation, total and active CD11a expression was compared on different APCs between SF and PB from the same patient (Figure 5.18). Interestingly, both total and active CD11a expression were significantly decreased in SF in almost all cell types. The only exception were cDC1 DCs, which show no significant increase or decrease in total or active CD11a in SF compared to PB. However, as cDC1 DCs only make up around 0.1% of all PBMCs, their low number in PB might contribute to this finding. Considering the overlays of cells from SF (shown in blue) and PB (shown in red) (Figure 5.18, left), it is clear that the decrease in MFI was due to the larger spread of the population, and not due to the complete loss of total or active CD11a-high cells.

The result that total and active CD11a were lower on APCs found in SF compared to PB has several implications. First, considering the hypothesised immunoregulatory role of CD11a, this finding implies that immune regulation is either lost or actively downregulated in the inflammatory joint environment. To explain the difference, immunoregulatory and pro-inflammatory mechanisms have to be finely balanced to achieve a sufficient protective immune response, while simultaneously preventing immune-mediated tissue destruction. As the microenvironment of a swollen joint in RA is starkly pro-inflammatory, with the presence of high levels of cytokines such as IL-1β, IL-6, TGF-β and IFN-γ (Schlaak et al., 1996), CD11a could actively be downregulated to remove the influence of CD11a-mediate immune regulation and achieve a sufficient immune response in response to the loss of tolerance to self-antigen. On the other hand, it is also possible that the loss of total or active CD11a on all APCs except cDC1 DCs, is part of the immune dysregulation occurring as a result of, and potentially contributing to, the pathogenesis and maintenance of RA.

To summarise, while neither expression nor conformation of CD11a were found to be different between healthy controls and RA patients, expression of both total and active CD11a was significantly lower in almost all APC types found in SF when compared to PB of RA patients. This suggests that CD11a might play a different role in the inflammatory context of the joint that in PB.

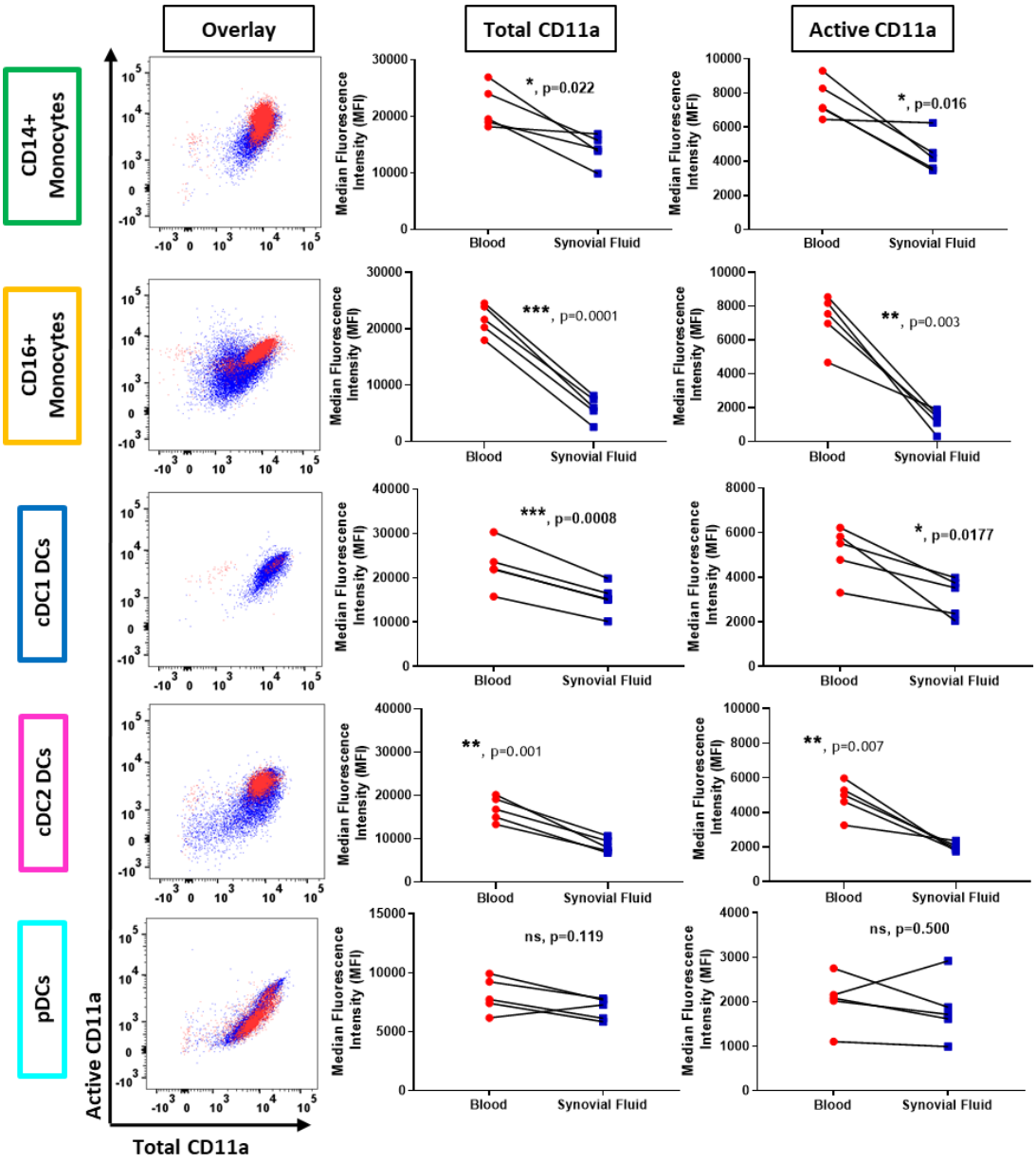


Figure 5.18 Total and active CD11a expression in RA PB and SF

Comparing expression of total and active CD11a in PB (red, circles, n=5) and matched SF (blue, squares, n=5) in five APC types of interest. Dot plot overlay of total and active CD11a expression in PB and SF (left), as well as total CD11a expression (middle, paired Student's t test, MFI of single-stained total CD11a) and active CD11a expression (right, paired Student's t test, MFI).

5.3.5 *The β_2 integrin CD11b in Rheumatoid Arthritis*

Having investigated the β_2 integrin subunit CD11a in the context of RA, I next analysed the role of CD11b. As previously mentioned, comparing CD11a and CD11b is of special interest, as the two subunits seem to have distinct functions in DCs. Furthermore, I hypothesised that CD11b has pro-inflammatory roles in DCs based on findings in Chapter 4 that blocking it on DCs was able to decrease T cell stimulatory capacity. Therefore, investigating CD11b in PB of RA could provide insight into whether this subunit is dysregulated in RA, due to either contributing to the disease or being affected by the presence of inflammation.

Total expression of CD11b in active RA and patients in remission is shown in Figure 5.19. No significant differences between RA patient groups were observed in any of the APC types, suggesting that total CD11b does not have a role in contributing to active disease or maintaining remission in PB and is not altered by the inflammatory environment in RA PB. Similarly, when comparing the two RA patient groups to healthy controls, there are no significant differences in total CD11b expression in any APC type (Figure 5.20). This finding is confirmed when looking at the representative histogram overlays (Figure 5.20A), which show high overlap.

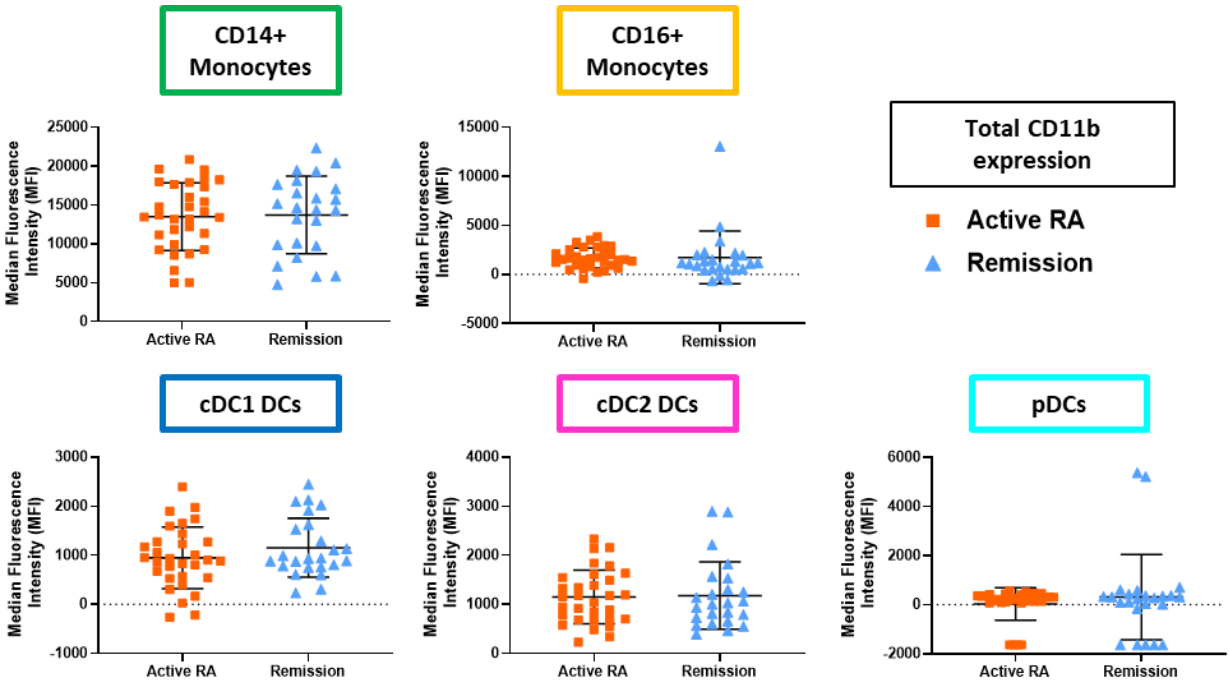


Figure 5.19 Total CD11b expression in RA

Expression of total CD11a (MFI) compared between active RA (orange squares, n=31) and remission (blue triangles, n=24) on five APC types. Unpaired Student's t test.

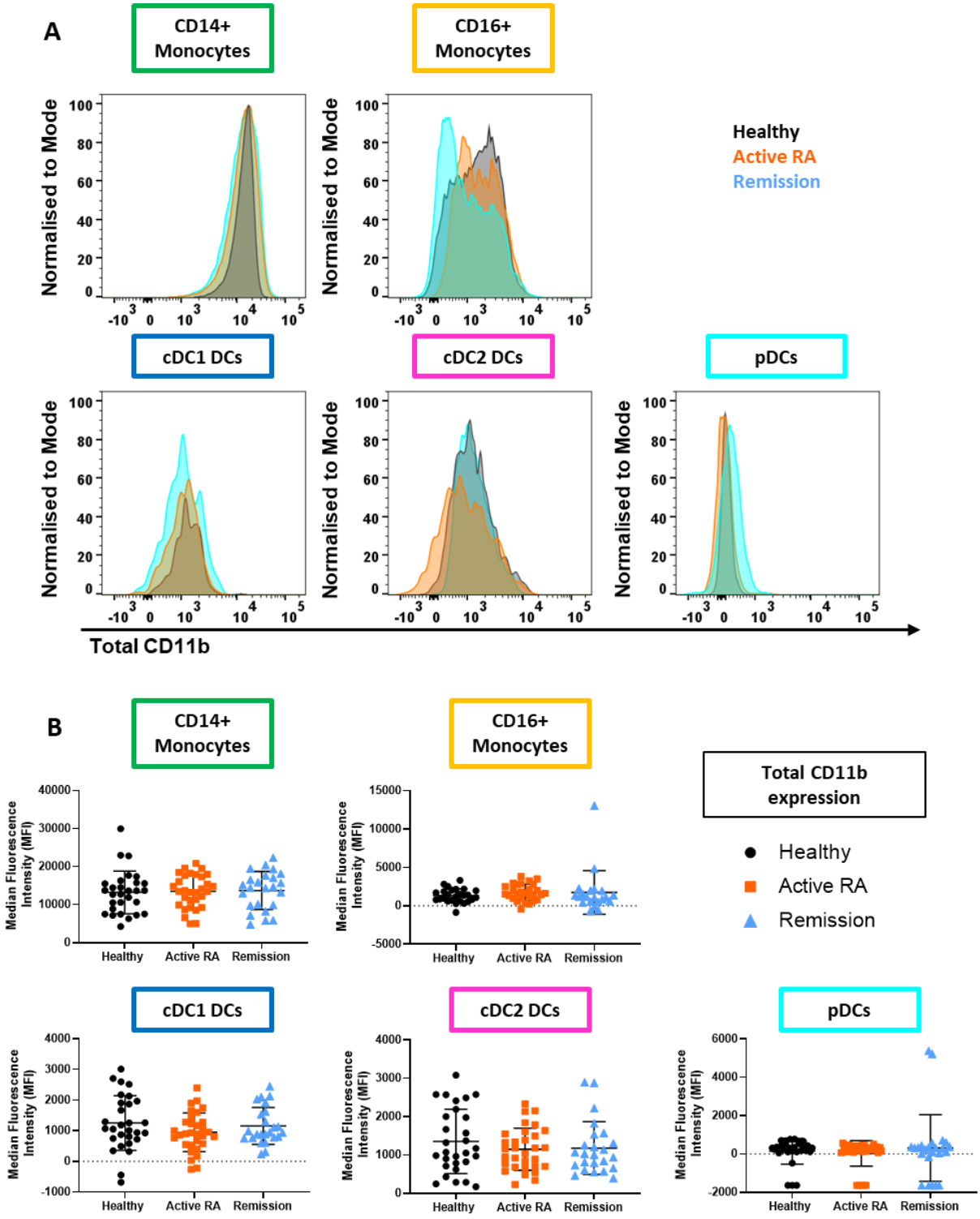


Figure 5.20 Total CD11b expression in healthy controls compared to RA patients

Expression of total CD11b comparing RA patient groups (active RA, remission) to healthy controls. A. Histogram overlays of representative expression of total CD11b of each group in each of the APC types. Representative samples were chosen as close to median of patient group as possible. B. Expression of total CD11b (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

While active RA and remission had indeed slightly higher means compared to healthy controls, this difference was not found to be significant.

Figure 5.21 shows that there are no significant differences in active CD11b expression between patients with active disease and in remission. Furthermore, when comparing healthy controls to RA samples (Figure 5.22), active CD11b expression is equivalent. Of note, active CD11b expression in remission sample cDC2 DCs had a slightly lower mean MFI compared to active disease and healthy controls (Figure 5.22A), but this was not significant.

In summary, total and active CD11b expression is not significantly different in PB APCs in RA compared to healthy controls, nor in active versus remission RA samples.

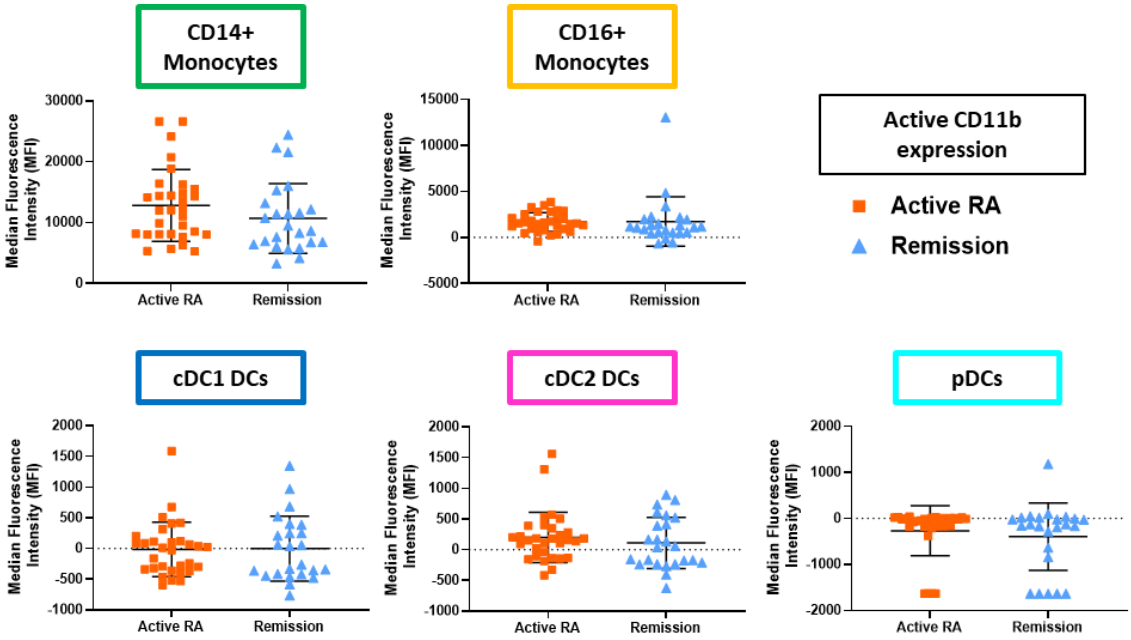


Figure 5.21 Active CD11b expression in RA

Expression of active CD11b (MFI) compared between active RA (orange squares, n=31) and remission (blue triangles, n=24) on five APC types. Unpaired Student's t test.

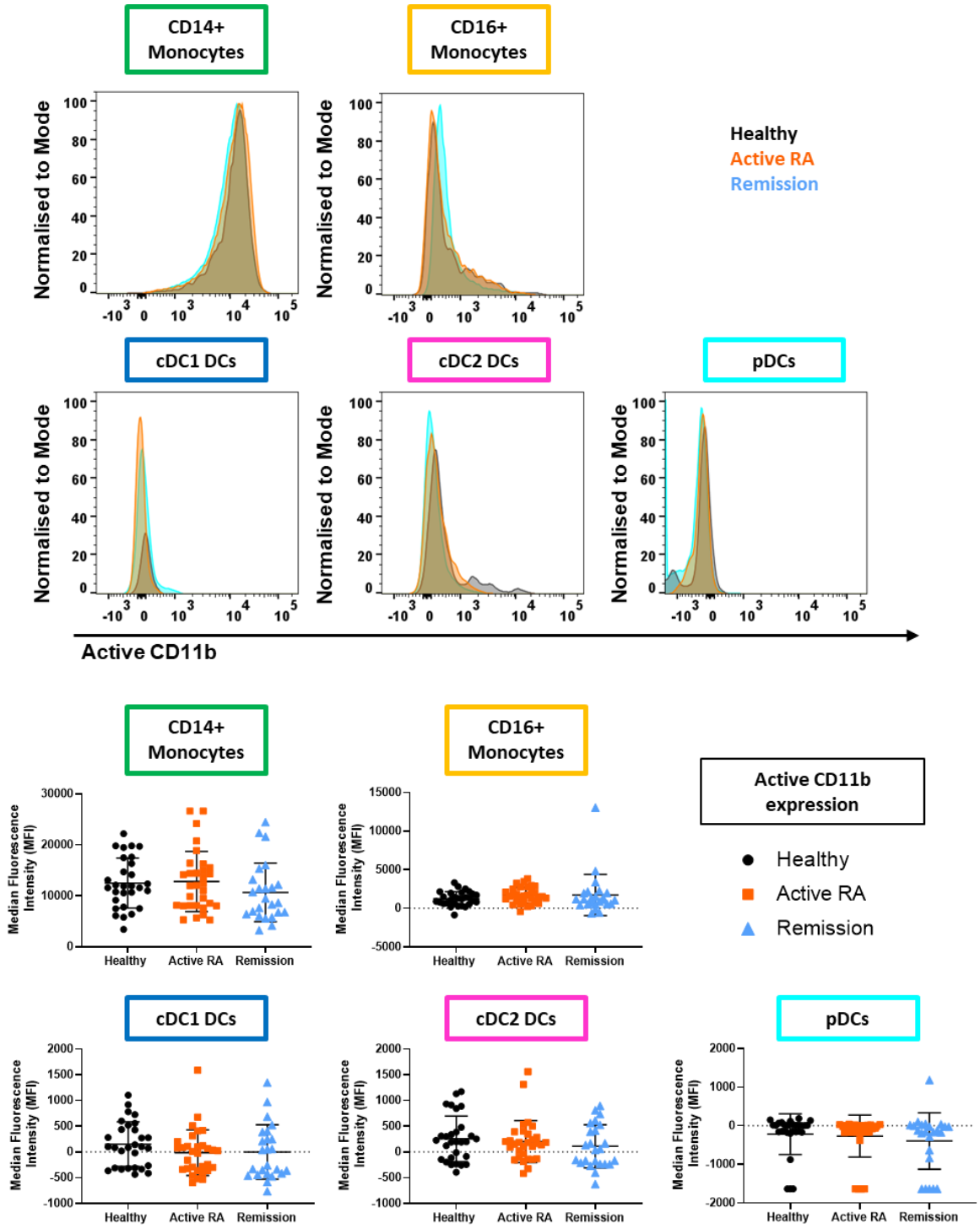


Figure 5.22 Active CD11b expression in healthy controls compared to RA patients

Expression of active CD11b comparing RA patient groups (active RA, remission) to healthy controls. A. Histogram overlays of representative active CD11b expression of each group in each APC type. Representative samples were chosen as close to median of patient group as possible. B. Expression of active CD11b (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

CD11b and CD18 in synovial fluid

While neither expression nor conformation of CD11b was altered in RA when compared to healthy controls, next CD11b expression was assessed on APCs in SF. This was done to gain further insights into the differences between the site of active inflammation and circulating cells.

Expression of total and active CD11b in RA PB compared to SF is shown in Figure 5.23. Total CD11b was found to be significantly higher in SF compared to PB in $CD16^+$ monocytes, cDC2 DCs and pDCs, but not $CD14^+$ monocytes and cDC1 DCs. On the other hand, active CD11b expression was significantly elevated only in cDC2 DCs and pDCs in SF compared to PB. This was reiterated by the overlays of cells from PB and SF (Figure 5.23, left), where SF cells showed a much larger spread of CD11b expression in $CD16^+$ monocytes, cDC2 and pDCs, while $CD14^+$ monocytes largely overlapped between SF and PB. While CD11b expression in cDC1 DCs was not significantly different between PB and SF, this could partially be due to the low numbers of cDC1 DCs present in PB.

As investigating β_2 integrins on mo-DCs *in vitro* suggested that CD11b is a pro-inflammatory molecule (Chapter 4), the increased expression of both total and active CD11b on cDC2 DCs and pDCs in the joint environment could suggest that CD11b is part of an increase in pro-inflammatory mechanisms being employed. Its increased expression in the joint might contribute to APC recruitment to and retention in the joint due to increased adhesive forces as well as increase interaction between T cells and DCs. The question of cause and effect is again difficult to tease apart. Is CD11b upregulated in response to pro-inflammatory stimuli in the joint, which then in turn contributes to maintaining the immune response? Or is it in fact part of the dysregulation that contributes to RA pathology? Overall, it is interesting that CD11b expression was shown to be increased in the same cells where CD11a expression was decreased, when comparing SF to PB. Both total and active CD11b therefore showed a completely reverse trend to CD11a in many APCs, with CD11b being significantly higher in synovial fluid than in PB, while CD11a was significantly lower. Together with the findings that CD11a was upregulated while CD11b was downregulated in tolerogenic Mo-DCs (Chapter 4), this strongly suggests that CD11a and CD11b might have opposing roles in APCs. This interesting finding will be discussed in more detail at the end of this chapter.

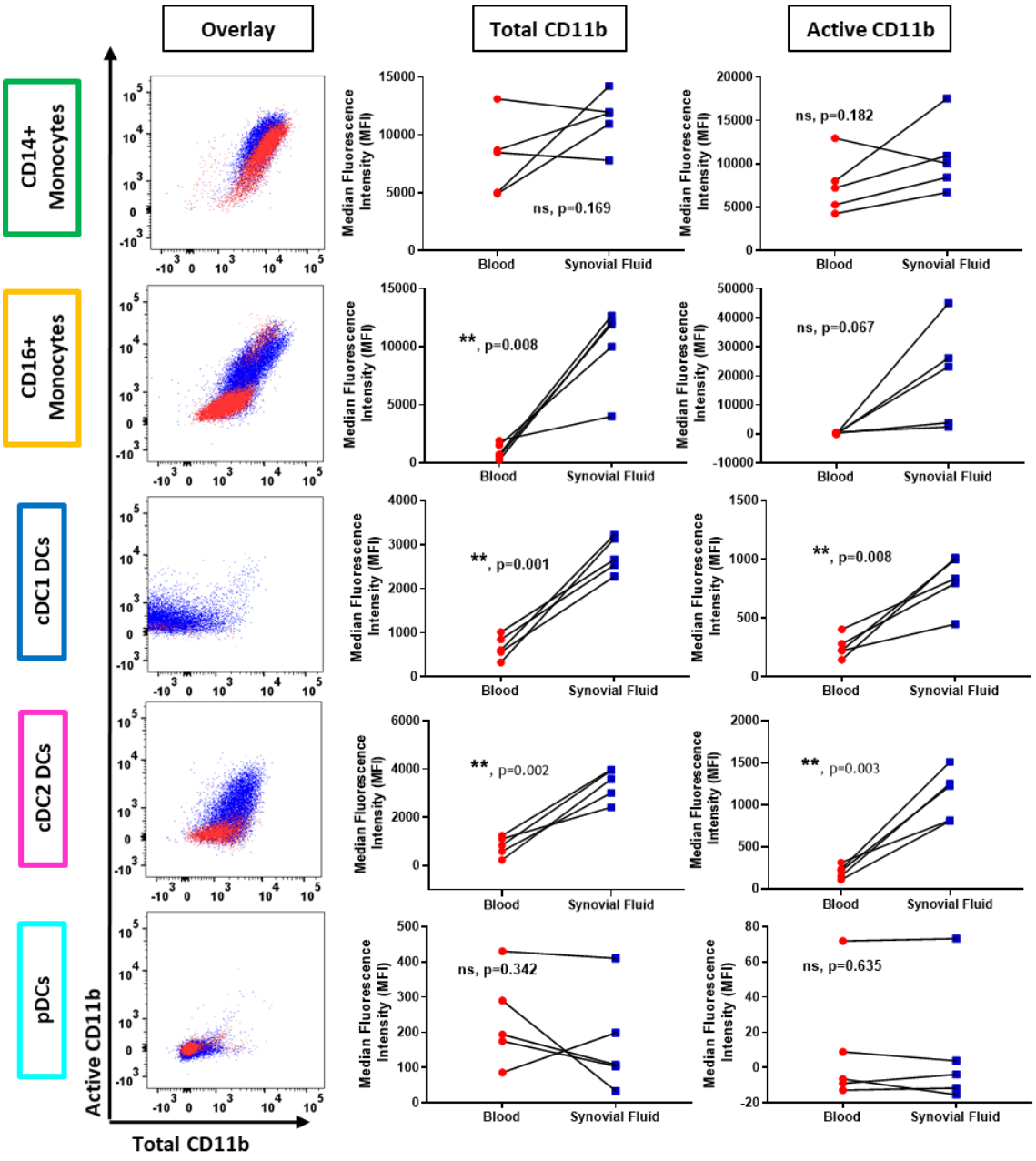


Figure 5.23 Total and active CD11b expression in RA PB and SF

Comparing expression of total and active CD11b in PB (red, circles, n=5) and matched SF (blue, squares, n=5) from the same RA patient in five APC types. Dot plot overlay of total and active CD11b expression in PB and SF (left), as well as total CD11b expression (middle, paired Student's t test, MFI) and active CD11b expression (right, paired Student's t test, MFI).

However, CD11a expression was lower in cells isolated from SF more globally, affecting all cell types except cDC1 DCs. On the other hand, total CD11b was significantly increased only in SF $CD16^+$ monocytes, cDC2 and pDCs, and active CD11b was only significantly increased in cDC2 and pDCs. This suggests that downregulation of CD11a has a more global role in the joint, while CD11b regulation is more cell-type specific.

It is surprising that the effect was so stark in pDCs, as they were shown to express the lowest levels of both total and active CD11b of all cell types considered (Figure 5.4,Figure 5.6). Consulting the literature on this topic, synovial pDCs from RA patients were found to be less mature compared to pDCs from PB, expressing lower amounts of CD40, CD83 and CD86 (Lande et al., 2004; Van Krinks et al., 2004). This could suggest that the increase in CD11b expression contributes to their immature state, meaning that the increase in CD11b is associated with less pDC activation. This has not been confirmed directly as our study did not segregate between immature and mature DCs. However, considering the inflammatory nature of the RA joint, it seems unlikely that the SF DCs are immature. Other studies have shown that SF pDCs are able to induce T cell proliferation and production of levels of IFN-γ, IL-10 and TNF-α that were comparable to SF myeloid DCs (Cavanagh et al., 2005). The role of synovial pDCs in RA and how β_2 integrin expression relates to maturity therefore has to be explored in more depth.

Expression of CD18, the pairing β-subunit of both CD11a and CD11b, was not shown in PB of RA patients, as no significant differences in either CD11a or CD11b were found. Here, however, it was important to include CD18 as it can give valuable information about the importance of either the significant decrease in CD11a or the significant increase in CD11b. Total and active CD18 staining in the five APC types is presented in Figure 5.24, showing that total CD18 was significantly higher in SF compared to PB in $CD14^+$ and $CD16^+$ monocytes, as well as cDC2s and pDCs. This suggests that the increase in CD11b dominated over the decrease of CD11a in SF, as CD18 expression was shown to increase. However, this effect was not replicated in active CD18, which was not significantly different in any of the cell types considered when comparing expression in SF to PB. All APC types except cDC1 DCs were significantly lower in active CD11a in SF, but only cDC2 and pDCs were significantly higher in active CD11b in SF.

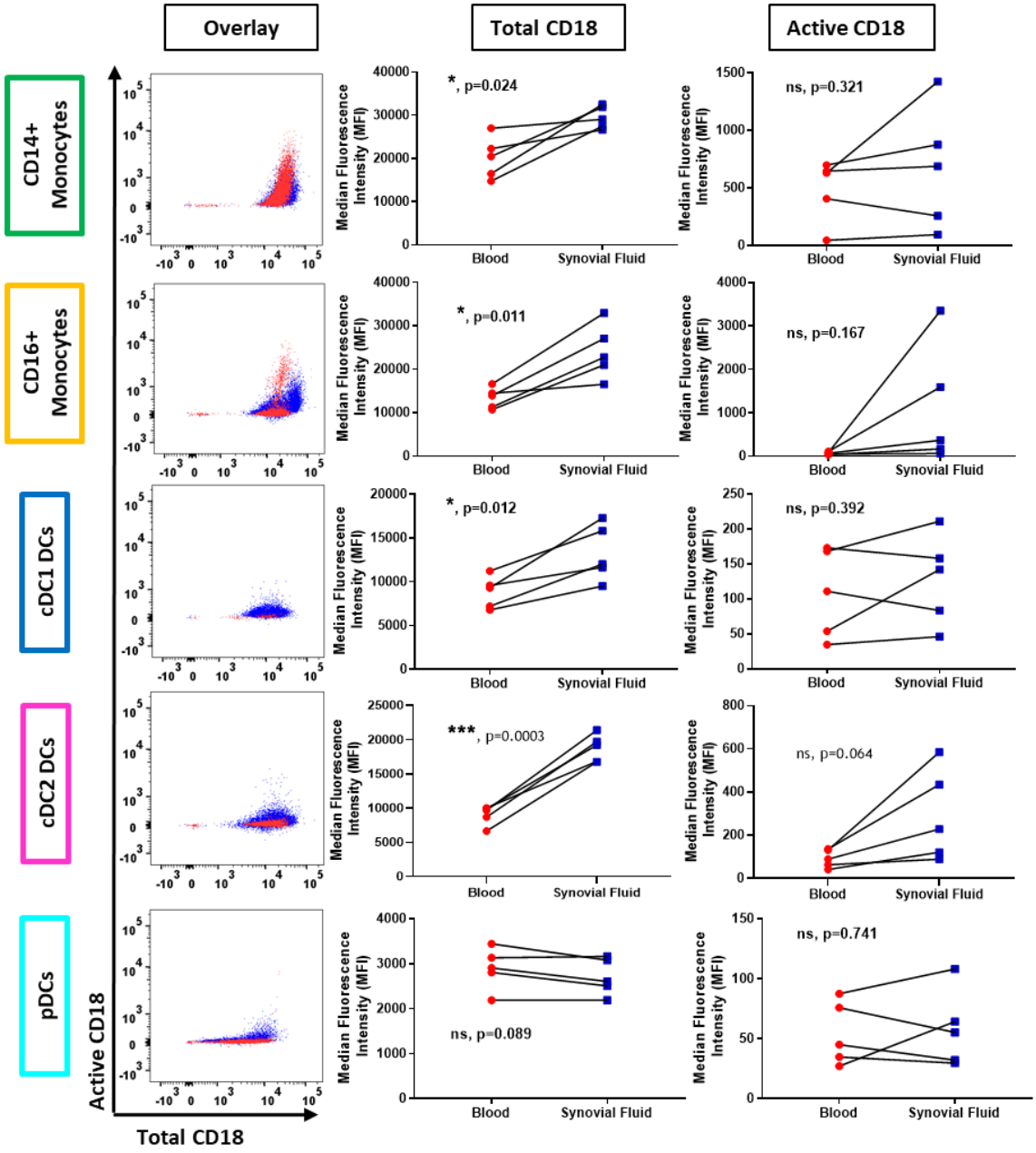


Figure 5.24 Total and active CD18 expression in RA PB and SF

Comparing expression of total and active CD18 in PB (red, circles, n=5) and matched SF (blue, squares, n=5) from the same RA patient in five APC types. Dot plot overlay of total and active CD18 expression in PB and SF (left), as well as total CD18 expression (middle, paired Student's t test, MFI) and active CD18 expression (right, paired Student's t test, MFI).

This, therefore, suggests that while total CD11b could determine total CD18 expression, this was not the case for active CD18, where the increase in active CD11b and decrease in active CD11a seem to balance each other out. However, as neither active CD11c nor active CD11d were considered in this panel, they would have influenced the expression of active CD18.

In summary, both expression of total CD11b and CD18 were found to be significantly increased in $CD16^+$ monocytes, cDC2 DCs and pDCs. But while expression of active CD11b was also significantly increased in cDC2 DCs and pDCs, no significant changes in expression of active CD18 were observed in any of the APC types when comparing PB to SF. Together with the finding that expression of total and active CD11a were increased on various APC types, this suggests that CD11b is playing an opposing role to CD11a.

5.3.6 Exploring functional roles of CD11a and CD11b ex vivo

Having investigated expression of total and active CD11a and CD11b in the context of RA, the data strengthens my result from Chapter 4, which suggested that the two β_2 integrin α-subunits have opposing functions, with CD11a being more regulatory while CD11b has more inflammatory roles. This role seems to be negligible in PB, where no significant differences between RA patients and healthy controls were found in either CD11a or CD11b expression. Comparing SF of RA patients to matched PB revealed total and active CD11a to be significantly reduced in SF (Figure 5.18), while total and active CD11b was increased on APCs in SF (Figure 5.23).

To investigate this result further, two questions were considered to explore the functional roles of CD11a and CD11b *ex vivo*. First, the effect of SF and various pro- and anti-inflammatory cytokines on total and active β_2 integrin expression was investigated. This was to test the hypothesis that β_2 integrins on synovial APCs respond to their pro-inflammatory environment, which would explain the stark differences seen between SF and PB. Second, following on from suppression of T cell proliferation after blockage of CD11b in Chapter 4, the effect of CD11b blocking on cDC2s sorted from peripheral blood was investigated.

5.3.7 Mimicking the synovial environment in vitro

Due to the wide-spread significant decrease in total and active CD11a expression and increase in total CD11b expression observed in SF compared to PB, it was

concluded that β_2 integrins have opposing function. It was considered that a more immunoregulatory role of CD11a was lost in RA, while the pro-inflammatory roles of CD11b found in Chapter 4 were further enhanced by the high concentration of pro-inflammatory cytokines present in an acutely inflamed joint. To test this hypothesis, healthy PBMCs were exposed to pro-inflammatory (+IL-1/TNFα, +LPS) or anti-inflammatory stimuli (+IL-10/TGFβ) for 18h and total and active β_2 integrins were measured on APCs. Importantly, IL-1 and TNFα, as well as IL-10 and TGFβ have been reported to be present in SF (Lettesjö et al., 1998; Schlaak et al., 1996). Additionally, PBMCs were exposed to thawed cell-free SF samples from three donors to determine if SF components could elicit a decrease in CD11a expression with a concomitant increase in CD11b. It was hypothesised that pro-inflammatory IL-1/TNFα and LPS stimulation would increase total CD11b expression and reduce total and active CD11a expression, while IL-10/TGFβ would increase CD11a expression and reduce total expression of CD11b by APCs.

CD11a expression in stimulated APCs

Total and active CD11a expression on APCs in the different conditions described above is shown in Figure 5.25. In $CD14^+$ monocytes, a significant decrease in total CD11a was seen in two conditions compared to untreated; however, it was lower in both LPS and IL-10/TGFβ treatment groups. This is surprising, as total CD11a expression in IL-1/TNFα treated cells was not significantly different from untreated. Counterintuitively, expression of total CD11a on $CD14^+$ monocytes was not affected by addition of any of the SF samples. Expression of active CD11a was globally significantly reduced when comparing any treatment to untreated cells. However, there are subtle differences between different treatment groups worth exploring. Considering the SF treatment conditions, more heterogeneity between the different donors is revealed. SF1 showed the lowest active CD11a expression of all conditions, while both other SF samples, SF2 and SF3 treatments both caused more expression of active CD11a. These differences were however not significant. This suggests that our hypothesis is not correct in the case of $CD14^+$ monocytes. While pro-inflammatory LPS treatment reduced the expression of total CD11a, it is not likely that this is what happens in the joint as the effect was not seen using IL-1/TNFα. Interestingly, SF did not affect expression of total CD11a. This could be explained by the fact that there was not sufficient biobanked synovial fluid available to test levels of any of the cytokines used as well as treat 8 PBMC samples with it. Therefore, it is

not clear how much of each cytokine is present. Simply put, cytokine concentrations could have been too low to elicit significant effects on total CD11a expression in $CD14^+$ monocytes. Another potential reason is that β_2 integrin expression and conformation are regulated by cell-cell contacts within the joint, which cannot be mimicked by adding cell-free SF. The global downregulation of active CD11a in all conditions is puzzling, as all cytokines were highly diluted in culture medium before being added to the well and compared to untreated, which only received medium.

$CD16^+$ Monocytes showed a different pattern of total CD11a expression to $CD14^+$ monocytes upon stimulation (Figure 5.25, top right). All pro- and anti-inflammatory stimuli significantly reduce the amount of total CD11a expression. The only exception is treatment with SF2, which caused no significant increase or decrease in total expression of CD11a total. The data. show again that while pro-inflammatory cytokines reduce total CD11a as hypothesised, the same can be said for treatment with the anti-inflammatory combination IL-10/TGFβ. Considering the absence of an effect of SF on total CD11a expression in $CD14^+$ monocytes, it could be hypothesised that SF samples from different donors have varying contents. Similarly, to $CD14^+$ monocytes, the $CD16^+$ monocytes data does not support my hypothesis. However, differences in expression of total and active CD11a suggest that β_2 integrins are differently controlled on the closely related monocyte types.

Looking at total CD11a expression on cDC2 DCs, total CD11a expression is significantly decreased in IL-1/TNFα and LPS treatment groups, while IL-10/TGFβ treatment does not significantly alter total CD11a expression compared to untreated cells. SF treated cells again show high heterogeneity between donors, with SF3 causing significant decrease in expression of total CD11a, while neither treatment with SF1 nor SF2 show any effect. There is no significant difference in active CD11a expression following any treatment condition. Similarly, to the results in $CD14^+$ and $CD16^+$ monocytes, cDC2 DCs reduce their total CD11a expression upon encountering pro-inflammatory stimuli. In contrast to both monocyte populations however, anti-inflammatory IL-10/TGFβ does not cause a decrease in total CD11a, instead maintaining it at a normal level.

Similar to cDC2 DCs, expression of total CD11a is significantly lower in cDC1 DCs when treated with IL-1/TNFα or LPS, but not with IL-10/TGFβ. Total CD11a is also lower on cDC1 DCs treated with SF3, but neither of the other SF samples. There are no significant differences in expression of active CD11a in cDC1 DCs in any of the

conditions. Both expression of total and active CD11a is significantly lower on pDCs treated with SF3, but none of the other treatments affect expression of CD11a.

Due to limited knowledge of the extent of steric hindrance affecting CD11a double staining at the time of experiment design, a single stained control for total CD11a was not included for all treatment groups. However, considering that difference in total CD11a between PB and SF became more and not less significant when plotting total CD11a not affected by steric hindrance, it was assumed that any effects seen would be due to a change in expression and not in steric hindrance. However, it cannot be assessed if pro- or anti-inflammatory cytokines could indeed affect steric hindrance, which is a caveat of this experiment.

In summary, pro-inflammatory stimuli (IL-1/TNFα, LPS), as well as treatment with SF3, caused a reduction in total CD11a expression on both cDC2 and cDC1 DCs, while conformation status was not affected. This supports the hypothesis of a immunoregulatory role of CD11a on DCs, as it was downregulated in response to pro-inflammatory stimuli. Considering differences in CD11a expression described between monocyte and DC types, one could furthermore hypothesise that expression is differentially regulated between monocytes on one side and DCs on the other side.

CD11b expression in stimulated APCs

The effect of different stimuli on total and active CD11b expression in different APC types is presented in Figure 5.26. In $CD14^+$ monocytes, total CD11b expression was significantly lower in all conditions when compared to untreated, including all SF samples. This was similar to the active CD11a results in $CD14^+$ monocytes (Figure 5.25). Despite reduced total CD11b, active CD11b was only significantly reduced when treated with LPS. As the PB-matched SF data (Figure 5.23) showed no significant increase in either total or active CD11b in synovial fluid, this result would fit the hypothesis that while CD11b might be pro-inflammatory on DCs, it might have opposite effects on monocytes.

In $CD16^+$ monocytes, neither expression of total nor active CD11b was significantly affected by any treatment condition. Also, the global downregulation of CD11b observed in $CD14^+$ monocytes upon addition of any kind of stimulus was not seen in $CD16^+$ monocytes. Considering that total CD11b was significantly increased in SF $CD16^+$ monocytes compared to PB (Figure 5.23), this result is surprising and

suggests that we cannot mimic the effects seen in SF cells in this *in vitro* stimulation system.

Expression of CD11b in cDC2 DCs was similarly unaffected by stimuli. The untreated samples showed a large data spread in both total and active CD11b, which did not appear to be typical for other APCs observed. No other significant differences in either total or active CD11b expression were observed.

cDC1 DCs showed a significant reduction in total CD11b in LPS, IL-10/TGFβ and SF2 conditions compared to untreated, but no significant differences in active CD11b. Similarly to $CD14^+$ monocytes, lower CD11b expression was apparent in all treatment conditions, but is not as strong as in the monocytes.

Lastly, pDCs showed no significant differences in stimulated compared to untreated cells in either total or active CD11b expression, but heterogeneity of SF samples was shown again.

Overall, data showing expression of total and active CD11b in response to various stimuli did not support the hypothesis that increased CD11b expression in SF is due to the pro-inflammatory cytokines present in the joint environment. However, several considerations have to be kept in mind when putting this result into context. First, it was not possible to use cells isolated from PB or SF of RA patients for these experiments. While no differences in total or active CD11b expression between healthy controls and RA patients could be found, it could still be possible that β_2 integrins of RA patients react differently to stimuli that those of healthy controls. This is likely as myeloid DCs from RA SF were found to express greater levels of costimulatory factors compared to PB DCs and were far more proficient at inducing T cell proliferation (Moret et al., 2013). This suggests that DCs are likely to be functionally altered in the inflammatory joint environment of RA. Another consideration is that the increased total and active CD11b expression in $CD16^+$ monocytes, cDC2 and pDCs may be due to chronic rather than short-term exposure to cytokines. As healthy PBMCs were only stimulated for 18h, it is possible that either chronic joint inflammation or the event of a flare on the background of a dysregulated immune system could majorly influence how expression of CD11b reacts to stimuli.

CD18 expression by stimulated APCs

Levels of total and active CD18 expression in APCs in response to overnight exposure to various stimuli are shown in Figure 5.27.

As both total CD11a and total CD11b were reduced on $CD14^+$ monocytes when exposed to any of the stimuli, it is unsurprising that a similar pattern emerges with total CD18 expression. Expression of total CD18 in the untreated condition was significantly higher than with IL-1/TNFα, LPS or SF2 stimulation, but not than any of the other conditions. Considering the different SF samples, it appears that total CD18 expression following SF1 and SF3 treatment was similar, in contrast to the significantly lower expression of total CD18 in SF2 treatment. Expression of active CD18 was significantly reduced in LPS treatment, but not in any of the other treatment conditions, suggesting again that active CD18 expression was not immediately dependent on total expression.

Considering CD18 on $CD16^+$ monocytes, no significant differences in active CD18 were observed with stimulation. However, total CD18 expression was significantly lower in cells treated with SF1 compared to untreated cells, suggesting that SF1 might contain either higher concentrations or different cytokines that cause total CD18 reduction.

Total CD18 expression in cDC2 DCs was more similar in pattern to total CD11b expression, although here no significant differences were observed between treatment groups. Similarly, active CD18 expression was not significantly different in cDC2 DCs in any treatment group.

In cDC1 DCs, untreated cells had significantly greater levels of total CD18 compared to LPS, IL-10/TGFβ and SF2, while no significant differences in active CD18 were observed.

Lastly, total CD18 expression in pDCs was reduced with SF3 treatment but with no other stimuli. No significant differences in active CD18 expression in pDCs between different conditions were found.

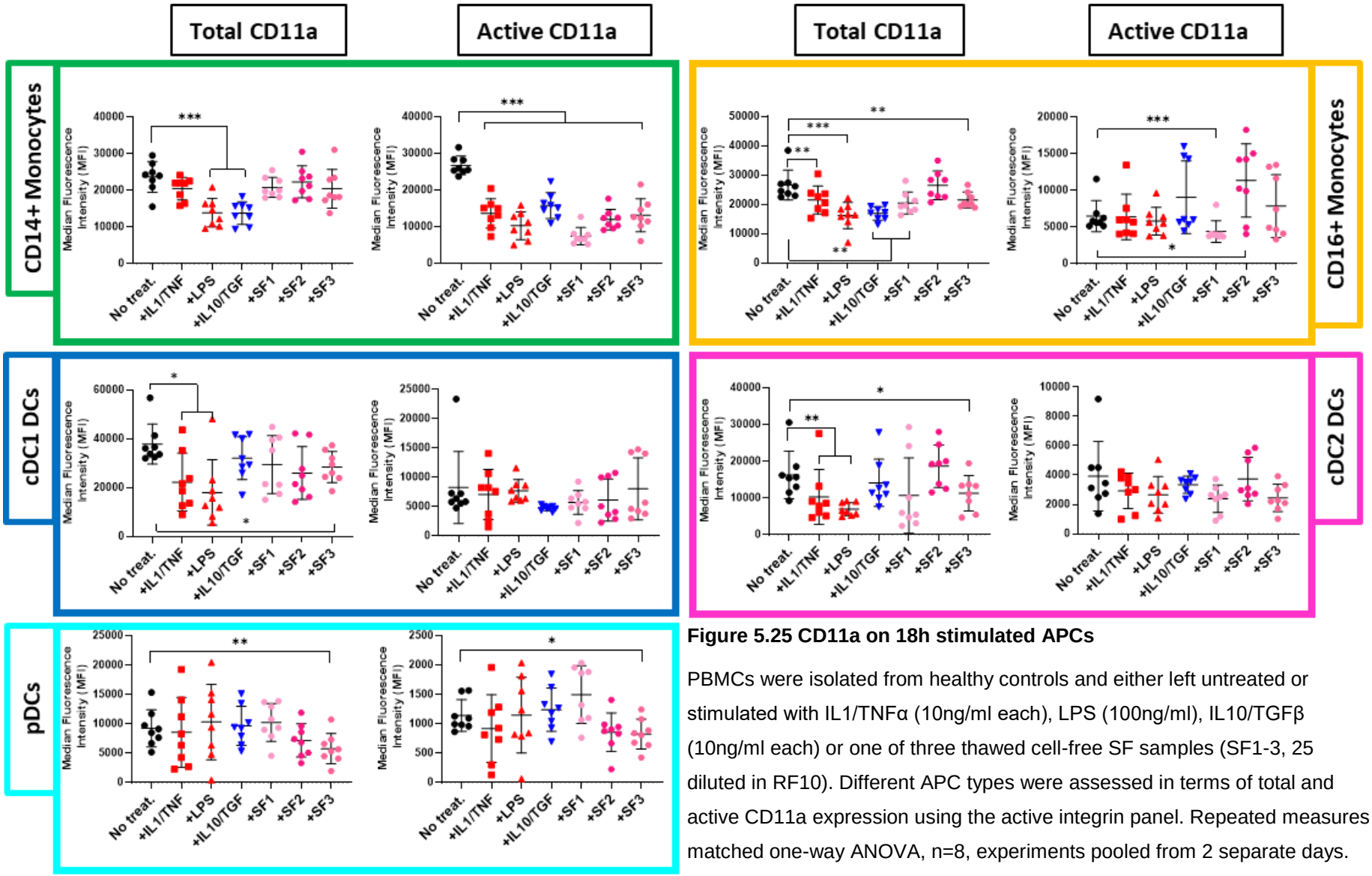


Figure 5.25 CD11a on 18h stimulated APCs

PBMCs were isolated from healthy controls and either left untreated or stimulated with IL1/TNFα (10ng/ml each), LPS (100ng/ml), IL10/TGFβ (10ng/ml each) or one of three thawed cell-free SF samples (SF1-3, 25 diluted in RF10). Different APC types were assessed in terms of total and active CD11a expression using the active integrin panel. Repeated measures matched one-way ANOVA, n=8, experiments pooled from 2 separate days.

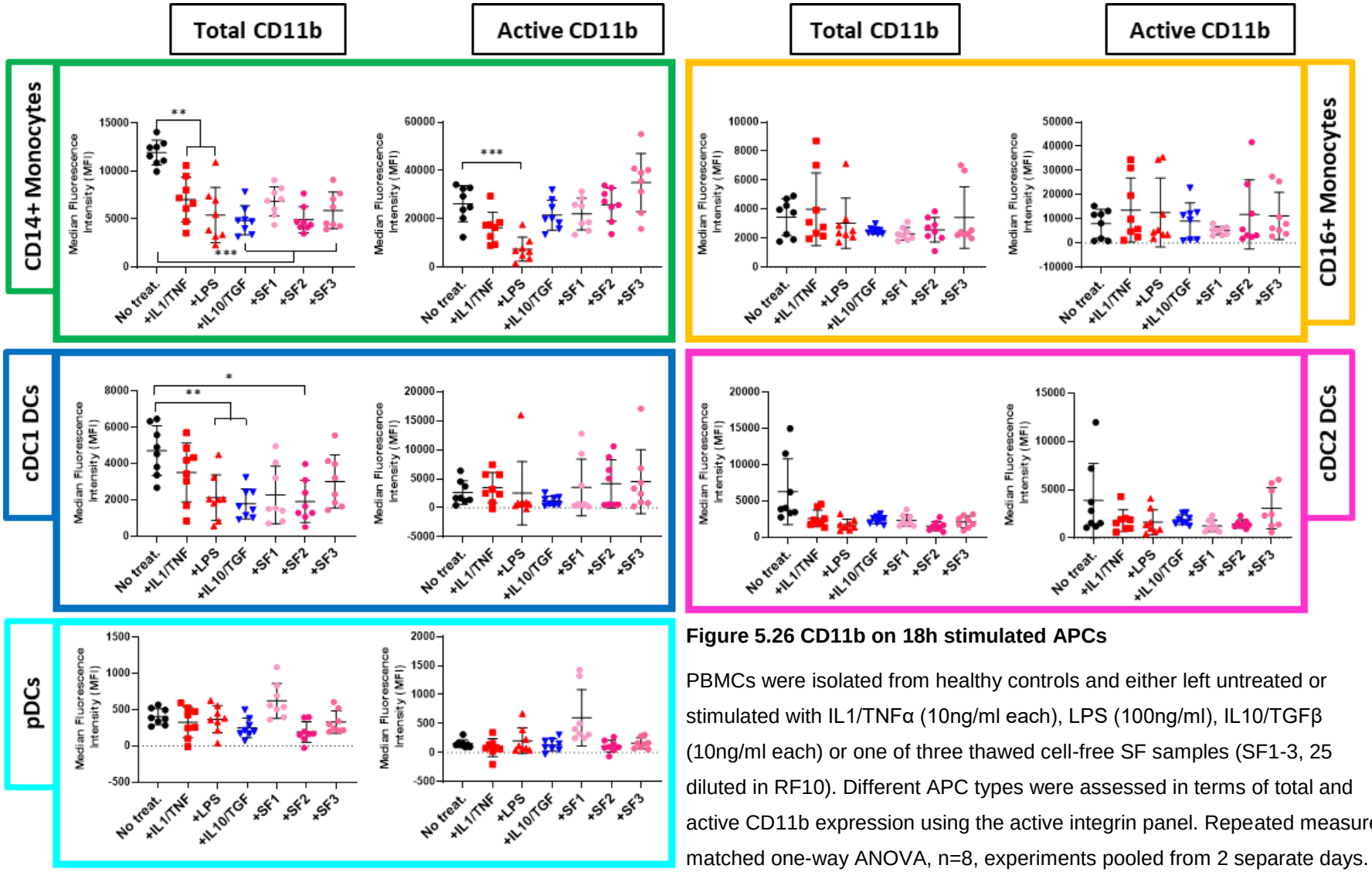


Figure 5.26 CD11b on 18h stimulated APCs

PBMCs were isolated from healthy controls and either left untreated or stimulated with IL1/TNFα (10ng/ml each), LPS (100ng/ml), IL10/TGFβ (10ng/ml each) or one of three thawed cell-free SF samples (SF1-3, 25 diluted in RF10). Different APC types were assessed in terms of total and active CD11b expression using the active integrin panel. Repeated measures matched one-way ANOVA, n=8, experiments pooled from 2 separate days.

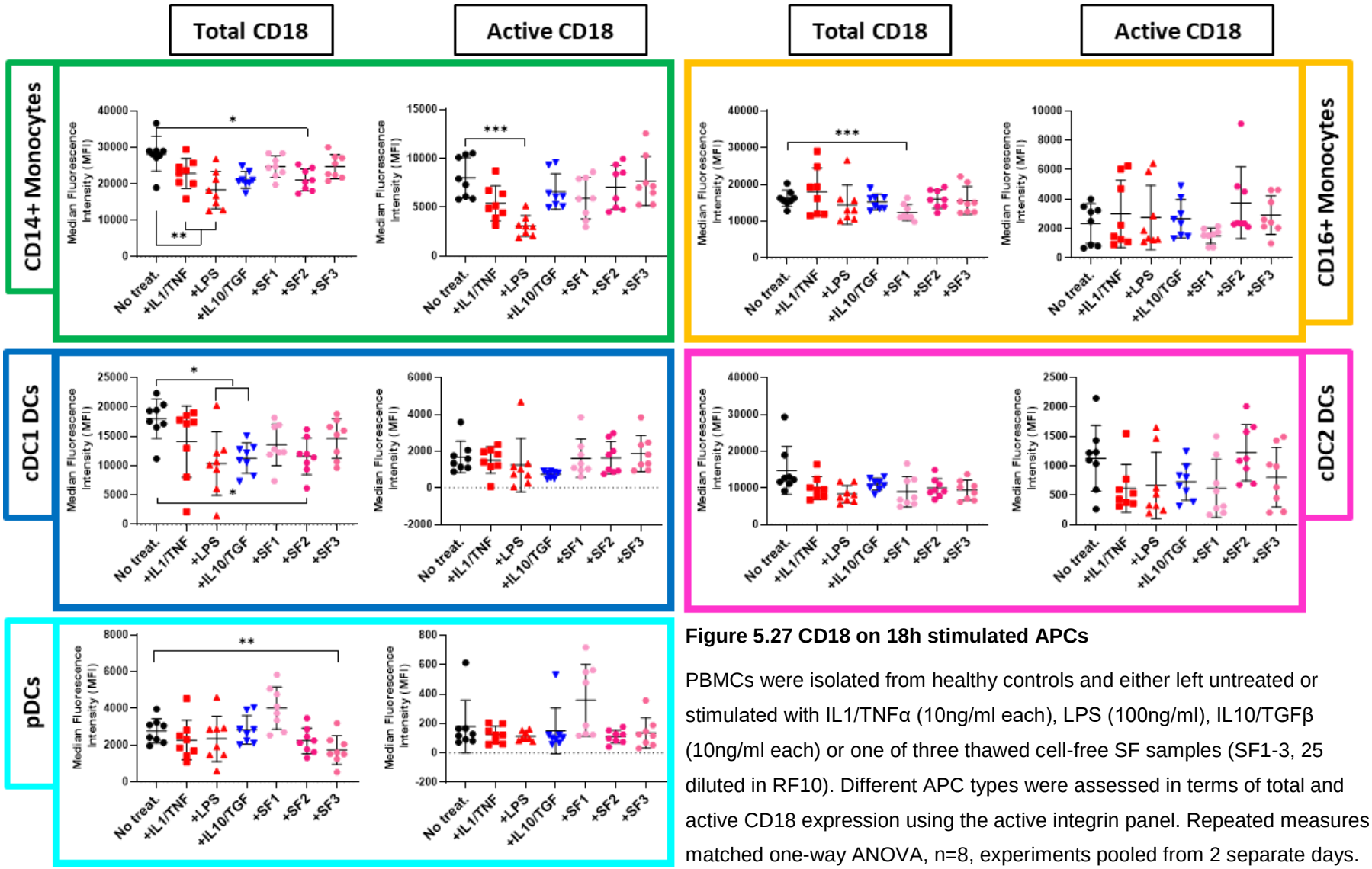


Figure 5.27 CD18 on 18h stimulated APCs

PBMCs were isolated from healthy controls and either left untreated or stimulated with IL1/TNFα (10ng/ml each), LPS (100ng/ml), IL10/TGFβ (10ng/ml each) or one of three thawed cell-free SF samples (SF1-3, 25 diluted in RF10). Different APC types were assessed in terms of total and active CD18 expression using the active integrin panel. Repeated measures matched one-way ANOVA, n=8, experiments pooled from 2 separate days.

In summary, expression or conformation of CD18 is not altered in cDC2s, but total expression is reduced in cDC1 and pDCs in response to both pro- and anti-inflammatory stimuli or SF. Total and active CD18 expression in different APC types therefore does not clearly follow either CD11a or CD11b expression patterns, suggesting that the other pairing subunits CD11c and CD11d may react to the various stimuli and influence these results.

5.3.8 Effect of blocking CD11b on cDC2 stimulatory ability

First, it was assessed if cDC2s sorted from peripheral blood were impeded in their ability to stimulate T cell proliferation when the CD11a/CD11b ratio was shifted by blocking CD11b. This question was based on a series of observations from both *in vitro* and *ex vivo* experiments utilising Mo-DCs (Chapter 4). There I found that expression of active CD11a is increased, while active CD11b was decreased, on tolerogenic compared to mature Mo-DCs, and that blocking CD11b on mature Mo-DCs made them less efficient at stimulating T cells. From this I hypothesised that CD11b is a pro-inflammatory mediator in DCs. Therefore, I wanted to test if blocking CD11b on PB cDC2 DCs had the same effects. However, one caveat of this experiment was that cDC2s in PB are likely to be immature. Furthermore, considering the hypothesis that the increase in synovial CD11b is linked to its increased pro-inflammatory roles in the joint environment of RA, this is also an opportunity to question if expression links up with function. It was therefore hypothesised that blocking CD11b would result in reduced T cell proliferation and IFN-γ production, while potentially increasing IL-10 production by both DCs and T cells. Conversely, it is predicted that activating CD11b will enhance its inflammatory ability, thus increasing T cell proliferation and IFNγ production.

To test these hypotheses, cDC2 DCs were sorted from PB of three healthy donors and co-cultured with naïve CD4 T cells for 6 days, either untreated or in the presence of CD11b antagonism (M1/70 or isotype control mAbs, Figure 5.28A) or CD11b agonism (LA-1 or control DMSO, Figure 5.28B). As naïve CD4 T cells express CD11a as well as CD18, only CD11b could be targeted specifically on DCs in an MLR (Chapter 4, section 4.2.5, Figure 4.14). No significant difference in percentage of proliferated T cells between untreated MLRs and CD11b blocking by M1/70 treatment was found (Figure 5.28B). There was also no significant difference in either IFN-γ or IL-10 levels measured in MLR supernatants. However, it is important to note that M1/70 treatment led to a small decrease in IFN-γ production in two out of three

donors with a simultaneous increase in IL-10 levels visible in all three donors, though not significant. This could suggest that while there was little effect of CD11b blocking on T cell proliferation, production of pro- and anti-inflammatory cytokines might be impacted by the blockage of CD11b. Ideally, additional samples would be analysed to increase N, as only three donors were assessed due to time constraints.

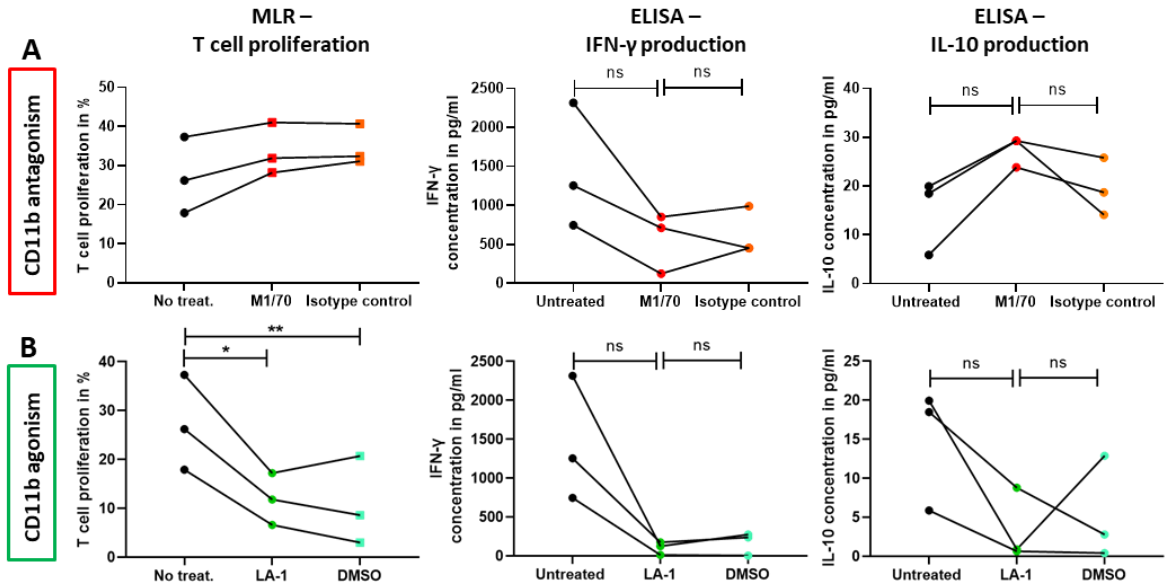


Figure 5.28 Effect of CD11b in FACS -sorted cDC2 MLRs

cDC2s were sorted from three healthy donors and co-cultured with naïve T cells for 6 days, in the presence of either CD11b blocking antibody (M1/70, control: Isotype control) or a small-molecule CD11b agonist (LA-1, control: DMSO). Shown are T cell proliferation in % of single live CD11c-CD3+CD4+ cells which had lost a proportion of their CTV staining (left), as well as the concentration of IFNγ (middle) and IL-10 (right) in the co-culture supernatants. Matched one-way ANOVA, n= 3 healthy cDC2 donors co-cultured with 3 separate buffy coat donors, from which naïve T cells were isolated.

On the other hand, LA-1 treated MLRs showed a significant decrease in T cell proliferation compared to untreated. However, the same was true for DMSO treated cells, suggesting that the negative effect of DMSO on cell proliferation is to blame for the significant decrease in T cell proliferation observed in the LA-1 culture. As upregulation of active (or indeed total) CD11b using LA-1 could not reliably be achieved *in vitro* (Chapter 4, section 4.2.5, Figure 4.17), it is likely that the DMSO rather than LA-1 is responsible for the loss of T cell proliferation. Indeed, DMSO has previously been reported to block T cell proliferation (Vanherwegen et al., 2019). Neither IFN-γ nor IL-10 production was significantly affected by treatment with LA-1,

but it seems as if IFN-γ production followed the pattern of T cell proliferation, with almost no IFN-γ being produced in LA-1 or DMSO controls (Figure 5.28).

To conclude, blocking CD11b on sorted PB cDC2s from healthy donors did not reduce T cell proliferation. However, one could hypothesise that no effect can be seen because of the immaturity of cDC2s circulating in PB. It could therefore be possible that investigating this further using a larger sample number or a longer co-culture might reveal differences that cannot be observed in this culturing system. Furthermore, it might be necessary to induce DC maturation before any effects of CD11b blocking can be observed.

5.4 Discussion

The aims of this chapter were to investigate the roles of total and active β_2 integrins in RA patient PB and SF samples compared to healthy controls in order to identify potential differences. In this chapter, I have devised a way to ensure data acquired over time is consistent and comparable, and developed a data analysis strategy that fully utilises the breadth of information captured by the integrin panel. Furthermore, I have characterised active and total β_2 integrin expression levels on APCs in detail in healthy samples, creating a valuable baseline of expression and activation status in a healthy context. There, I found that total CD11a expression is significantly higher in females than in males. I then compared the properties of β_2 integrin α-subunits CD11a and CD11b in the autoimmune context of RA, comparing both PB and SF to this healthy baseline. While no differences between healthy controls and RA patients were found in PB, comparing PB with matched SF of active RA patients again suggested opposing roles of β_2 integrins. While both total and active CD11a were downregulated in SF cells, total and active CD11b were upregulated particularly on DC populations. Lastly, I investigated how expression of active and total β_2 integrin subunits related to function, utilising different methods of *in vitro* manipulation of β_2 integrins (agonism and antagonism). There, I found that total expression of CD11a is downregulated in response to pro-inflammatory but not immunoregulatory stimuli in cDC1 and cDC2 DCs, further supporting the hypothesis that it has immunoregulatory roles. In contrast, neither total nor active CD11b were affected by either pro-inflammatory or immunoregulatory stimuli in cDC2 or pDCs, but were decreased in cDC1 DCs, not supporting my hypothesis of CD11b as a pro-inflammatory modulator. This chapter poses several interesting questions on the significance of these findings, which I will discuss in the following paragraphs.

5.4.1 Gender differences in β_2 integrins

Considering the gender differences observed in total CD11a expression (Figure 5.8), with female study participants showing higher CD11a expression in all APC types analysed, it is important to interrogate this effect in more detail.

There is no prior evidence in the literature for this phenomenon, which is potentially unsurprising considering the previously acknowledged lack of cell-specific information on β_2 integrins. Furthermore, the field of immunology is potentially prone to under-reporting gender differences as less than 10% of published articles in the field analyse at least part of their data by gender (Beery and Zucker, 2011). This is surprising, as there are several established differences in immune responses between male and female study participants, including larger T cell numbers, antibody responses to infection and Type I interferon responses in human females (Klein and Flanagan, 2016).

In mice, it was observed that APCs from female mice are more efficient at presenting antigen either to female or male T cells, suggesting that the difference between genders lies on the APC rather than the T cell side (Weinstein et al., 1984). Putting this into context with the increased CD11a expression in female study participants (Figure 5.8), it is possible that the increased surface expression of total CD11a also improves formation of the immune synapse, in turn increasing the efficiency of T cell activation. This hypothesis would also fit with the absence of significant differences in active CD11a between genders (Figure 5.10), as shifting the balance towards more active CD11a was found to actually decrease the amount of T cell activation (Balkow et al., 2010).

Another potential reason for the increase in total CD11a in females is the role the hormone oestrogen plays in the female immune system. For example, pDCs from both female humanised mice and human female adults were found to become more activated and increase TLR7 and TLR9 signalling in response to oestrogen (Seillet et al., 2012). On the other hand, absence of all β_2 integrins caused an increase in TLR-mediated signalling (Yee and Hamerman, 2013), suggesting that β_2 integrins play an important role in dampening down TLR-signalling. It could therefore be suggested that CD11a is upregulated in female study participants to counteract the comparably larger TLR-mediated signalling caused by the presence of oestrogen in the system. However, oestrogen levels decrease after menopause (Brzezinski, 2019) yet we

found no significant negative correlation of total CD11a expression with age, refuting this theory.

Interestingly, no differences in total CD11a expression between male and female study participants who had RA were found (data shown in appendix). This suggests that comparably lower total CD11a expression in APCs of female study participants may contribute to RA pathology. This is interesting as women are more susceptible to many autoimmune disorders (Whitacre, 2001). Therefore, the role of gender in β_2 integrin expression, particularly in CD11a expression, should be investigated in more detail.

To conclude, gender differences in total CD11a expression in APCs have not been described in the published literature to my knowledge. While it could not be identified why female healthy controls express significantly higher levels of total CD11a compared to males, one could hypothesise that the higher expression of total CD11a on female APCs is connected to the immune functions in females, including their increased ability to present antigen and the potential dampening of increased TLR-responses caused by oestrogen. Future studies on β_2 integrins should therefore ensure that any gender differences are sufficiently reported on and studied in more detail. With differing gender ratios, 1:2.1 in healthy controls and 1:5 in active RA patients, one could have assumed that patients with active RA express higher levels of total CD11a due to the larger numbers of females. This is however not the case, as there were no differences in total CD11a when steric hindrance did not influence the results. Furthermore, differences in expression of total CD11a between female and male participants with RA could not be found. The increased expression of total CD11a in female healthy controls therefore does not affect any conclusions drawn from comparing the RA data set to healthy controls.

5.4.2 Increase of steric hindrance in RA samples

As mentioned previously, I observed significantly reduced expression of total CD11a in cDC2 DCs in RA patients compared to healthy controls (Figure 5.12). Further analysis revealed that expression was not reduced in the single stained condition and that the effect was caused by an increase in steric hindrance (Figure 5.13). This result is puzzling as the increase in steric hindrance was not accompanied by an increase in active CD11a expression, which was previously observed to be the

source of the steric hindrance (Chapter 4, section 4.2.3, Figure 4.6). Considering the highly significant differences, this effect has to be explored in more depth.

First, one explanation is that total CD11a staining is lost due to its close proximity to the non-covalently associated β subunit, CD18. As steric hindrance was only tested between total and active antibodies of each β_2 integrin subunit in isolation, it would be possible that total or active CD18 might interfere with binding of the antibody raised against total CD11a. However, neither total nor active CD18 were shown to be significantly increased in any of the APC types in RA samples to explain this increase in steric hindrance (data shown in appendix). The only exception was pDCs, where a significantly smaller percentage of pDCs expressed total CD18 in remission samples. However, the percentage of pDCs expressing active CD18 was lower and not higher than in healthy controls, and this effect could not be observed in any of the other APC types. It is, therefore, considered unlikely that the increase in steric hindrance in RA samples is due to increased binding of active or total CD18 antibodies.

A second option to consider is that the increase in steric hindrance is due to an increase in avidity of CD11a/CD18. As β_2 integrin avidity is potentially more important than affinity in the context of inside-out signalling (Bazzoni and Hemler, 1998), this could potentially point to a difference in CD11a signalling in RA. To explain, increased clustering of CD11a might result in an increased likelihood of close proximity of total CD11a binding sites *to each other* and therefore a loss of total CD11a antibody access. This would explain the increase in steric hindrance, not only would signal be lost due to steric hindrance between active and total CD11a mAbs, but also between total mAbs binding to epitopes that have brought closer together due to increase β_2 integrin clustering. While this is possible, it does however not explain why there is no significant signal loss of total CD11a in the RA samples where no steric hindrance to active CD11a occurred. RA single stained total CD11a should at least show 15-20% less signal compared to the healthy controls, if this hypothesis was valid, as this is the increased amount of signal lost in active RA samples. Furthermore, it seems unlikely that steric hindrance would occur *between different* CD11a subunits, but not *within the same* CD11a protein. It is therefore unlikely that increased avidity of CD11a in RA samples is the cause for the significant difference in steric hindrance.

A third possibility is that the active CD11a antibody binds to an intermediate state of the β_2 integrin, where total and active antibody binding sites are positioned in closer proximity to another and steric hindrance increases. Inactive and active forms of β_2 integrins are extreme situations and a variety of intermediate states have been described in the literature (Carman and Springer, 2003). However, this would also imply that either intermediate CD11a states occur more frequently or intermediate CD11a activation states are recognised at significantly higher rates in the context of either active RA or remission. Additionally, affinity of the active CD11a antibody for its target was proposed to be quite promiscuous, demonstrated by its ability to bind its target even in cold temperatures (Chapter 3, section 3.3.1, Figure 3.1). The group first identifying MEM-83, the clone used to detect active CD11a, reported that it binds in I domain of the alpha-subunit (Landis et al., 1994), which is located immediately in the active binding site between the β-propeller of CD11a and the I-like domain of CD18. While this would suggest that MEM-83 binds exclusively when CD11a is in its fully extended conformation, Landis and colleagues actually found that the antibody increased adhesion of T cells to ICAM-1 and bound regardless of cation availability. This would not only explain why no further increase in active CD11a expression could be elicited by PMA, it also strongly suggests that MEM-83 binds to the intermediate affinity states of CD11a and activates them. It is therefore a real possibility that significantly increased levels of steric hindrance in RA signal increased levels of β_2 integrins in their intermediate state. This increased readiness for activation might contribute or be elicited by the pro-inflammatory environment in RA.

While it is likely that binding to intermediate affinity β_2 integrin states contribute to the increase in steric hindrance, the idea that binding sites of total and active CD11a are somehow closer together in RA patients could reveal a further possibility. X-ray crystallography studies on the structure of CD11a revealed a 5-stranded parallel β-sheet surrounded by 7 α-helices on both sides (Binnerts and van Kooyk, 1999; Qu and Leahy, 1995), which was hypothesised to be the active conformation of CD11a as it was shown to be bound to Mn^{2+}, a potent cation activator if β_2 integrins. Keeping in mind that β_2 integrin conformation can be altered by cation concentrations in the surroundings (Zhang and Chen, 2012), it is possible that an altered cation balance in PB of RA patients might contribute to the increase in intermediate CD11a that in turn causes the increase in steric hindrance. Indeed, Ca^{2+} levels were found to be

reduced in the PB of RA patients (Scott et al., 1981). Considering that Ca^{2+} was found to be one of the main cations inhibiting β_2 integrin activation in PB (Dransfield et al., 1992), it is somewhat surprising that the potentially lower Ca^{2+} levels of the RA data set would not in turn increase the levels of active CD11a expressed by APCs. One could therefore tentatively hypothesise that CD11a is structurally altered in response to the state of chronic inflammation present in RA, which is accompanied by low Ca^{2+} expression. This would explain both the increase in steric hindrance, as well as the absence of increased active CD11a in response to low Ca^{2+}. However, there are still caveats to this hypothesis, as it does not sufficiently explain why neither active CD11b nor CD18 are similarly altered in response to low Ca^{2+} levels. However, keeping previous observations of the different properties of CD11a compared to CD11b/CD18 in mind (Chapter 3), one could propose that CD11b and CD18 might react to the pro-inflammatory environment with different modifications that would not affect steric hindrance.

To conclude, the specific reason why CD11a mAb steric hindrance is significantly increased in APCs from RA patient samples could not be elucidated and further tests on the subject were not within the time limits of this project. However, considering all possible options, one could propose that CD11a structure or avidity is consistently altered in RA patients in a way that total and activation-specific binding sites move closer together, causing an increase in β_2 integrins in their intermediate state which in turn causes a loss of signal due to steric hindrance. Future studies should explore this link in more detail.

5.4.3 Opposing functions of CD11a and CD11b

My findings from Chapter 4 suggest that β_2 integrin pairing subunits CD11a and CD11b have opposing functions in Mo-DCs, with CD11a being more immunoregulatory, while CD11b has more pro-inflammatory roles. I will now explore this conclusion in the context of the knowledge on β_2 integrins in APCs in a human *ex vivo* setting gained in this chapter.

There were no differences in expression of total or active CD11a and CD11b in APCs isolated from PB of RA patients compared to healthy controls, suggesting that function of β_2 integrins in PB is not altered in RA. However, the striking increase in steric hindrance affecting total CD11a staining when comparing RA patients to

healthy controls (Figure 5.13) suggests that the two β_2 integrin subunits might react differently to the changed environment in RA PB.

However, when considering CD11a and CD11b expression in RA SF, evidence of opposing functions clearly emerges. While total and active CD11a are significantly lower in SF APCs compared to PB (Figure 5.18), total and active CD11b are significantly higher in SF (Figure 5.23). These results are partially supported by other studies reporting large amounts of 'shed' CD11a in SF effusions (Evans et al., 2006), suggesting that CD11a is actively lost from cell surfaces rather than internalised. This finding would support my conclusion from Chapter 4, that CD11a is immunoregulatory in DCs and is actively downregulated to maintain joint inflammation. To achieve the inflammation present in an RA joint during flare, it would make sense that immunoregulatory mediators, such as CD11a, are downregulated. Shedding of CD11a is one way to achieve this. On the other hand, pro-inflammatory mediators, as I suggested CD11b to be, would increase to enable and perpetuate this pro-inflammatory local microenvironment, as I have shown to be the case in SF APCs.

Following this finding further, I exposed healthy PBMCs to various pro- and anti-inflammatory stimuli to investigate if both CD11a and CD11b could be manipulated by mimicking the potential factors present in SF. However, the data from short-term *in vitro* stimulation of APCs did not show the same effects of integrin subunit expression as we see in SF. cDC1 and cDC2 DCs indeed decrease total CD11a expression upon stimulation with pro-inflammatory mediators IL-1/TNFα or LPS and are not affected by immunoregulatory stimuli, supporting my conclusion. In contrast, neither expression of total nor active CD11b was affected by stimulation, and cDC1 DCs confusingly expressed significantly less total CD11b after both stimulation with LPS (pro-inflammatory stimuli) and IL-10/TGFβ (anti-inflammatory stimuli).

To summarise, while the *ex vivo* PB versus SF data is striking, I have not been able to completely replicate this result with short-term stimulation *in vitro*. It is therefore likely that this effect is dependent on either a state of chronic inflammation or on other environmental aspects, that cannot be replicated *in vitro*. One example of such an environmental factor would be the presence of multiple other cell types in the inflamed joint. However, as the failure of β_2 integrin-targeted therapeutics is largely credited to the failure of drugs to target different subunits specifically, increasing our

understanding of the specific properties and functions could improve future therapeutic interventions.

5.5 Conclusion

In this chapter, I successfully characterised, in detail, total and active β_2 integrin expression in healthy study participants, finding that different APC types have distinct expression of both total and active β_2 integrins. In addition, total expression of CD11a is significantly higher in females compared to males. Furthermore, I investigated if β_2 integrins were in any way altered in the context of the autoimmune disorder RA PB and SF. While no differences were found in PB between healthy controls and RA patients, comparing RA PB to SF yielded striking results, with expression of total and active CD11a being decreased and expression of total and active CD11b being increased in SF. Lastly, the reaction of β_2 integrins to outside stimuli as well as their functional role was tested to gain further insight into β_2 integrin functionality. Here, I found that neither blocking nor stimulating CD11b had any significant effect on T cell stimulatory capacities of PB cDC2 DCs, although this is likely due to their immature state. Furthermore, I found that pro-inflammatory stimuli decrease expression of total CD11a in DCs, but not monocytes. In contrast, expression of conformation of CD11b was not altered in DCs by stimuli, except in cDC1 DCs, where pro-inflammatory stimuli caused a decrease in total CD11b.

The most striking result of this chapter is the opposite relationship between CD11a and CD11b demonstrated in the RA PB to SF comparison, finding that CD11a is downregulated in the joint, while CD11b is upregulated. This mirrors my findings in Chapter 4, which leads me to conclude that CD11a and CD11b have opposing roles in DCs. In my final discussion, I will therefore explore the respective significance of CD11a and CD11b on DCs.

Chapter 6 **Final Discussion**

The role of β_2 integrins in the immune systems is multifactorial. While the heterodimeric adhesion receptors were primarily associated with playing pro-inflammatory roles due to their roles in migration, cell signalling and maintaining the immunological synapse, more recent work has also highlighted their potential immunoregulatory roles on macrophages and, of special interest in this thesis, on DCs. This thesis therefore investigated the hypothesis that dysregulation of β_2 integrins on DC surfaces might contribute to aberrant inflammatory responses and autoimmunity. To this end, expression and conformation of β_2 integrin subunits CD11a, CD11b and CD18 were assessed simultaneously in human Mo-DCs, as a model of immune tolerance versus activation (mature and tolerogenic Mo-DCs) and a dysregulated immune system where tolerance to self has been lost and is causing pathology (RA patient samples). Furthermore, the impact of activating or blocking specific integrin subunits in DCs on subsequent T cell priming was defined. In the following discussion, I will discuss the challenges in detecting and targeting β_2 integrins encountered during this thesis, before delving into my findings that β_2 integrins are dysregulated in RA. Furthermore, I will discuss my findings that CD11a and CD11b play opposing roles on DCs and comment on how this would impact targeting β_2 integrins therapeutically. Lastly, I will explore further work that arises from the findings presented in this thesis.

6.1 Challenges in targeting β_2 integrins for research and therapeutic reasons

The question if a β_2 integrin-targeted antibody is blocking adhesion, mediating outside-in signalling or both arose predominantly when targeting CD11b on DCs in MLRs. As discussed in Chapter 4 (section 4.3.1), there is a wide range of published evidence for an immunoregulatory role of CD11b on DCs (Nowatzky et al., 2018b; Varga et al., 2007; Yee and Hamerman, 2013). This made my finding that T cell proliferation and production of IFN-γ was reduced in a mature Mo-DC MLR when adding a CD11b-specific antibody surprising, as it suggested pro-inflammatory roles for CD11b.

The antibody clone used in this thesis to block CD11b, M1/70, has been well established to block CD11b-mediated functions in multiple species. For example, M1/70 strongly inhibited resetting of erythrocytes in response to stimulation with complement (Springer et al., 1982) and reduced adhesive ability to fibrinogen in a dose-dependent manner (Rogers et al., 1998). The same study also reported that administrating M1/70 to a rabbit model of angioplasty reduced leukocyte recruitment

to the site of artery injury. In a rabbit model of muscle injury, M1/70 blocked neutrophil-mediated oxidative burst and limited the severity of injury (Brickson et al., 2003). Interestingly, this study also observed that treatment with M1/70 actually reduced CD11b receptor density on neutrophils, suggesting that the blocking effect of M1/70 was not exclusively mediated by reducing CD11b binding to its endogenous ligands. Potentially, binding of M1/70 to CD11b caused increased internalisation of the receptor, therefore reducing its presence on the cell surface.

However, in murine DCs, M1/70 was found to reduce CpG-mediated production of IL-6 (Stevanin et al., 2017), which led the authors to propose that the relative resistance of $CD11b^+$ C57BL/6 to CIA compared to CD11b KO mice is mediated via tight control of IL-6 production by CD11b. Furthermore, both murine and human macrophages were shown to increase production of tissue factor (TF), an important initiating factor in the coagulation cascade, two to eight-fold when treated with M1/70 (Fan and Edgington, 1991). This suggested that M1/70 is not merely functioning as a blocking antibody, it is also able to induce functional outside-in signalling when binding to CD11b and therefore act as ligand in its own right. However, it is puzzling how M1/70 could induce downstream signalling without also inducing the active conformation of CD11b, as the essential signalling molecules talin and kindlin are only recruited after integrin activation. One would therefore have to assume that M1/70 binding activates CD11b but simultaneously blocks integrin-mediated adhesion and binding by other ligands to explain its effects on cell function.

Considering the evidence suggesting that M1/70 can both act as a blocking agent and a functional ligand, one could assume that this is an isolated phenomenon, potentially only true for the binding site of M1/70. However, clone ICRF44 (or clone 44), which binds total CD11b and was used in this study for flow cytometry purposes, has been used to block or bind CD11b in a similar fashion to M1/70, suggesting that clear boundaries between ‘blocking’ and ‘ligating’ are difficult to assess when targeting β_2 integrins. Similar to M1/70, clone ICRF44 has been described to block a variety of CD11b-mediated functions. For example, it was shown to block adhesion to iC3b, as well as chemotactic abilities and cell spreading when seeded on plastic coverslips (Dana et al., 1986). Another study showed that clone ICRF44 treatment of COS cells, exclusively expressing CD11b, CD11c and CD18, but not CD11a, reduced IL-23 mediated release of pro-inflammatory cytokines (Lecoanet-Henchoz et al., 1995). Furthermore, treatment of neutrophils with clone ICRF44 reduced

adhesion in the presence of hydrogen peroxide (H_2O_2) (Blouin et al., 1999), as well as CD86-mediated adhesion of monocytes to fibrinogen (Sitrin et al., 1996).

While this would suggest that usage of clone ICRF44 is suitable for blocking CD11b-mediated adhesion, several studies have also reported that it has functional effects on cells. For example, *in vitro* treatment of neutrophils with clone ICRF44 caused rapid release of neutrophil gelatinase B (MMP-9), which was proposed to contribute to their transmigration into sites of inflammation (Wize et al., 1998). Furthermore, the antibody was found to induce translocation of NF-κB from the cytoplasm to the nucleus and subsequently mediate production of macrophage inflammatory protein 1α (MIP-1α) and MIP-1β by primary human monocytes (Rezzonico et al., 2001). This, again, suggested that clone ICRF44 actively mediated cell function via outside-in signalling. Lastly, treatment of human Mo-DCs with clone ICRF44 was previously shown to reduce Mo-DC-mediated T cell proliferation and IFN-γ production, although CD11b-mediated inhibition could be overturned when Mo-DCs were simultaneously infected with *Listeria monocytogenes* (Škoberne et al., 2006). Indeed, a follow-up study reported that ligating CD11b on human Mo-DCs using clone ICRF44 efficiently restricted memory T cell development into Th17 T cells, by skewing cytokine production by Mo-DCs (Nowatzky et al., 2018b). However, while both studies observed that DC function was more tolerogenic after treatment with clone ICRF44, neither assessed signalling occurring after binding of the CD11b-specific antibody directly. Together, this suggests that while clone ICRF44 might block CD11b-mediated adhesion, there is a wide range of evidence supporting that it induces functional differences in cells by ligating CD11b.

So how does the potential dual role of CD11b targeting antibodies in blocking and ligation affect interpretation of my results? I used M1/70 to block CD11b in MLRs and found it to reduce T cell priming by mature Mo-DCs (Chapter 4, section 4.2.5, Figure 4.20). Considering that treatment of human Mo-DCs with CD11b-specific antibody clone ICRF44 caused similar reduction in their ability to induce proliferation and IFN-γ production by T cells (Škoberne et al., 2006), it is possible that this result could be similarly explained by ligating effects of M1/70 on CD11b. To clearly distinguish between ligation and blocking action of M1/70, further tests on mature Mo-DCs in the presence of antibody would have to be made to detect if outside-in signalling molecules are phosphorylated after exposure to M1/70. While treatment with the CD11b agonist LA-1 did not have any significant effect on T cell proliferation, this

was potentially due to its solving agent DMSO, which had unforeseen negative effects. Ligating CD11b would therefore be testing if a functioning agonist of CD11b has the same effect on T cell proliferation and IFN-γ production. However, the suggestion that immunoregulatory functions are induced by ligation rather than blocking of CD11b does not make sense when considering my finding that active CD11b is significantly lower on tolerogenic Mo-DCs. It is therefore likely that M1/70 did act as a blocking antibody in the context of an MLR, as mature Mo-DCs became more tolerogenic Mo-DC-like, which expressed lower levels of active CD11b. Furthermore, the ICRF44 CD11b antibody was used when staining for 30min at 37°C for flow cytometry to detect total CD11b expression. Despite the evidence that this antibody can also elicit downstream signalling, it is unlikely that this aspect altered staining results, as CD11b could still be activated further by PMA, thereby suggesting that ICRF44 did not cause activation of all CD11b subunits it bound to.

In summary, both M1/70 and clone ICRF44 have been classically described as ‘blocking’ antibodies, largely due to their ability to reduce CD11b-mediated adhesion. However, taking all evidence into account, this is not factually correct, as both antibodies mediate a range of cellular functions, including production of both pro- and anti-inflammatory mediators (Nowatzky et al., 2018b; Rezzonico et al., 2001). It is, therefore, likely that interpretation of the effects of CD11b-targeting antibodies in the literature has so far primarily been based on testing a narrow range of functions in each paper, resulting in the term ‘blocking’ if a functional aspect was reduced or ‘ligating’ if a functional aspect was induced. While this is reasonable due to limited space for publication and the need for a coherent story, it makes interpretation of published data and its potential therapeutic impact highly complex, as exact functions of mAbs currently used in basic research could be multifactorial and potentially dependent on cell-type and environment. Future studies should acknowledge the potential dichotomy of mAbs or other therapeutic targets blocking some functions but activating others, thereby potentially impacting interpretation of results. Furthermore, it would be important to test if downstream outside-in signalling actually occurs as a direct result of individual antibodies binding, as this is not known so far.

An important question that arises from the difficulty to correctly distinguish between effects mediated by ‘blocking’ and ‘ligation’ of a β_2 integrin subunit is what this means for the targeting β_2 integrins therapeutically in the future. Currently, the most promising avenue to target β_2 integrins are small-molecule CD11b agonists termed

leukadherins (Faridi et al., 2013; Panni et al., 2019). One possibility is that these therapeutic agents are not only promising because they reduced inflammation in animal models (see Chapter 1, Section 1.4.5) (Faridi et al., 2013; Park et al., 2007), but because of their defined function on integrins: they increase adherence of the β_2 integrin to its ligand. It is therefore possible that failure of other CD11b-targeting therapies to outperform placebo in human (see Chapter 1, Section 1.4.5) (Dove, 2000; Harlan and Winn, 2002) was partially due to not fully understanding their functionality and thereby affecting β_2 integrins and in turn cell properties in unforeseen ways.

6.2 Evidence of dysregulation of β_2 integrins in RA

As the main aim of my thesis was to define the roles of β_2 integrins on DC in tolerogenic and autoimmune environments, I will first explore which of my findings support my original hypothesis that integrin expression and/or conformation may be dysregulated in RA.

First, no differences in the expression of total or active β_2 integrin subunits were observed in any of the DC subtypes or monocytes isolated from PB between healthy controls and RA patients (Chapter 5, sections 5.3.4 & 5.3.5, Figure 5.12, Figure 5.15, Figure 5.20, Figure 5.22). This suggested that, in PB, β_2 integrin expression or conformational regulation were neither actively contributing to pathology nor altered in response to the presence of inflammation due to established autoimmunity.

However, while this result does not support a role of β_2 integrins in systemic immune dysregulation, several other results presented in this thesis do support the hypothesis that β_2 integrins are dysregulated in autoimmunity. CD11a, in particular, was found to have different properties when comparing healthy controls and RA patients. For example, total CD11a expression was significantly lower in healthy male controls compared to healthy females. However, when comparing total CD11a expression between male and female RA patients, no significant differences between genders was observed (Appendix, Figure 7.1). Considering the fact that women are twice as likely to suffer from RA and other autoimmune disorders compared to men (Whitacre, 2001), this could suggest that dysregulation of CD11a expression leading to a change in immune cell communication could contribute to RA pathology. This is further supported by evidence that APCs derived from healthy female mice showed higher abilities to elicit a T cell response in an MLR compared to APCs derived from

male (Weinstein et al., 1984). The authors suggested that the higher reactivity of APCs from female mice might contribute to an increased likelihood to breach self-tolerance. Finally, the female hormone oestrogen has been found to increase TLR-signalling (Seillet et al., 2012), while β_2 integrins have been described to dampen and control TLR signalling (Yee and Hamerman, 2013). As no difference in CD11a expression between male and female RA patients was found, one could therefore suppose that decreased CD11a on female RA patients fails to regulate the TLR signalling mediated by oestrogen. This would further support the theory that the loss of difference in total CD11a expression between male and female RA patients could contribute to disease pathology in women. To conclude, this data therefore suggests that β_2 integrins, especially loss of CD11a, might play a role in mediating autoimmune diseases such as RA in women. It is here also important to state that, to my knowledge, no other differences in integrin expression or function have been reported to be different between genders.

Another difference between healthy controls and RA patients concerning CD11a was observed in the significantly increased steric hindrance between total and activation-specific antibodies in both active and remission RA samples compared to healthy control samples (Chapter 5, section 5.3.4, Figure 5.13). By elimination of other possibilities, I concluded that this was likely to be due to a consistent alteration in either molecular structure, such as an increase in the intermediate activation state, or avidity (clustering) of CD11a, that resulted in total and active binding sites moving closer together, therefore increasing loss of total CD11a signal due to steric hindrance. While exploring this altered state was not within the remit of this thesis, this finding suggested that the β_2 integrin subunit CD11a is consistently altered in RA. These alterations in CD11a expression and molecular properties may directly contribute to disease or, alternatively, may reflect a downstream consequence of the dysregulated immune environment in RA.

Considering that I did find β_2 integrins to be dysregulated in RA, it is furthermore interesting to speculate if these changes are specific to RA and autoimmune disease more generally, or if this change in CD11a is likely to occur in any inflammatory environment. It is interesting that the difference in steric hindrance was seen in both active RA and remission patients, suggesting that this dysregulation exists even in the absence of inflamed joints. One could therefore speculate that CD11a would not be similarly altered in acute infection. However, further work on understanding if and

how the change in CD11a affects DC function in RA and potentially other immune diseases would be necessary to understand the aspect of chronic versus acute inflammation and infection versus autoimmunity better.

My data showing that tolerogenic and mature Mo-DCs differ in their expression of active CD11a and CD11b, and that treating mature Mo-DCs with a blocking antibody against CD11b reduced their ability to effectively stimulate T cells, further supports the possibility that alteration of β_2 integrins might affect immune functions such as antigen presentation and cytokine production. In the following section, I will therefore explore the potential opposing roles of the β_2 integrin α subunits CD11a and CD11b evidence of support from throughout my thesis.

6.3 Summary of opposing roles of CD11a and CD11b

A theme that was persistent throughout my thesis were the differences observed specifically between β_2 integrin subunits CD11a and CD11b, which led me to conclude that they likely play opposing roles on DCs. In the following paragraphs, I will summarise the findings presented in my three results chapters, put them into context with the published literature and explore what they can tell us about the respective roles of CD11a and CD11b on DCs.

Opposing roles of CD11a and CD11b on Mo-DCs

I quantified β_2 integrin expression and conformation in mature and tolerogenic Mo-DCs to investigate if they are involved in respective immunogenic and tolerogenic roles of mature and tolerogenic Mo-DCs. While I detected no differences in total CD11a or total CD11b expression, active CD11a was significantly increased in tolerogenic compared to mature Mo-DCs, while active CD11b was significantly decreased (Chapter 4, sections 4.2.3 & 4.2.4, Figure 4.7 and Figure 4.10). Especially as the active CD11a antibody (clone MEM-83) likely also contributes to activation of CD11a, one could speculate that tolerogenic Mo-DCs were able to activate CD11a to a higher degree compared to mature Mo-DCs. Furthermore, surprising adhesion assay data suggested a potential role for CD11c, which would further support that a fine balance between different b2 integrin alpha subunits has to be maintained to mediate tolerogenic Mo-DC function. Lastly, blocking CD11b on mature Mo-DCs in the context of an MLR reduced the level of T cell proliferation and cytokine production. Although this result was not repeated in tolerogenic Mo-DCs, which showed no differences in T cell stimulatory ability in response to blocking CD11b, this

fits well with the data showing that active CD11b is significantly reduced on tolerogenic Mo-DC surfaces (Chapter 4, section 4.2.4, Figure 4.10). Therefore, it suggests that active CD11b is somehow maintained in its inactive state or downregulated on tolerogenic Mo-DCs to mediate immunoregulatory functions, potentially via an increase in active CD11a and that blocking CD11b on mature Mo-DC surfaces makes them assume a more tolerogenic phenotype. The mechanism of how integrins could be maintained in an inactive state have not been reported, but one could speculate that as inside-out signalling is largely mediated via talin and kindlin binding to the β_2 integrin β subunit CD18, that specific inactivation of CD11b could be mediated by increasing non-covalent interactions between CD11b and CD18. Possibly, phosphorylation of CD11b intracellular domains might thereby keep leg regions of CD11b and CD18 close together despite talin and kinlin binding to CD18 to induce activation.

So how could differences in active CD11a and active CD11b relate to the function of mature and tolerogenic Mo-DCs respectively? One could speculate that increasing active CD11a on tolerogenic DCs would make them adhere tighter to T cells via binding to ICAM-1, thereby prolonging contact time, which was previously shown to reduce T cell activation (Balkow et al., 2010). However, contact with T cells is not the only aspect that could be affected: changing the balance of active CD11a and active CD11b on tolerogenic Mo-DCs could alter ligands the cells primarily interact with and thereby affect cell signalling. For example, it is possible that tolerogenic Mo-DCs resistance to restimulation with LPS is partially mediated by their low active CD11b expression, as LPS-mediated gene changes were shown to be dependent on CD11b signalling in macrophages (Perera et al., 2001). Increasing signalling mediated via CD11a and decreasing signalling mediated via CD11b might also alter type and levels of cytokines released by tolerogenic Mo-DCs by shifting downstream signalling processes, potentially explaining why tolerogenic Mo-DCs do not induce as much IFN-γ production by T cells.

To summarise, active CD11a and CD11b were found to be differentially expressed between mature and tolerogenic Mo-DCs. In addition to other mature and tolerogenic Mo-DC properties that were hypothesised to be due to altered β_2 integrin expression, such as cell adhesion, clustering and ability to stimulate T cell responses, targeting CD11b specifically altered functionality of mature Mo-DCs by reducing their ability

stimulate T cells. This provided first concrete evidence that β_2 integrin α subunits CD11a and CD11b play opposing roles in immune activation versus tolerance.

Opposing roles for CD11a and CD11b in RA

As I found evidence of opposing functions of CD11a and CD11b in a model of immune versus tolerogenic environments (mature vs. tolerogenic Mo-DCs), I went on to validate these findings in real human DCs from relevant clinical samples (healthy controls vs. RA patients). As previously mentioned, no opposing functions for β_2 integrin α subunits were observed in PB, with only CD11a being implicated to be dysregulated between RA patients and healthy controls. Importantly, the finding that CD11a and CD11b might play opposing roles on DCs was further supported when comparing PB monocytes and DCs to their counterparts located in SF. Compared to PB APCs, SF APCs showed significantly increased expression of total and active CD11b, while expression of total and active CD11a was significantly decreased compared to PB (Chapter 5, sections 5.3.4 and 5.3.5, Figure 5.18, Figure 5.23 and Figure 5.24). Therefore, in the joint, where DCs have been shown to be in a mature state, I found CD11b expression to be increased and CD11a expression decreased, compared to PB. This mirrors my finding that mature Mo-DCs expressed more active CD11b and less active CD11a compared to tolerogenic Mo-DCs. Especially as CD11a, the proposed immunoregulatory mediator, was found to be shed from cell surfaces in large quantities in the synovium (Evans et al., 2006), one could speculate that to maintain joint inflammation, the immunoregulatory roles of CD11a have to be actively down modulated, while the pro-inflammatory functions of CD11b are increased.

In line with my findings in tolerogenic Mo-DCs, I proposed that increased presence of pro-inflammatory cytokines within the inflamed joint cavity likely cause downregulation of immunoregulatory mediators, such as CD11a, while simultaneously stimulating pro-inflammatory mediators, such as CD11b. Cytokines that could mediate driving DCs to a mature state could be IFN-γ, IL-1β and IL-6, which have all been found at increased levels in RA synovial fluid (Lettesjö et al., 1998; Schlaak et al., 1996). To test if this was true, I therefore quantified expression of total and active β_2 integrins on monocytes and DCs when exposed to pro- and anti-inflammatory stimuli, as well as RA synovial fluid. Expression of CD11b in response to pro- and anti-inflammatory stimuli did not support my hypothesis, with CD11b either not being altered by stimuli or, confusingly, being significantly

decreased by both the pro-inflammatory stimulus LPS and the anti-inflammatory stimulus IL-10/TGFβ in cDC1 DCs. However, total CD11a expression was significantly decreased specifically in cDC1 and cDC2 DCs when treated with the pro-inflammatory stimuli LPS or IL-1/TNFα, further supporting my hypothesis that CD11a holds immunoregulatory roles on DCs that are downregulated in the presence of inflammation.

Overview of opposing roles of CD11a and CD11b

To summarise, evidence that roles of CD11a and CD11b might be different and potentially even in direct opposition of each other was presented in both Mo-DCs and in RA clinical samples. Due to CD11a being upregulated on tolerogenic Mo-DCs (chapter 4) while being downregulated in a setting of inflammation (chapter 5), I propose that CD11a plays an immunoregulatory role on DCs. While very little research on the role of CD11a on DCs exists, this conclusion finds support in the group reporting that constitutively active CD11a on DCs decreased their ability to induce T cell proliferation (Balkow et al., 2010; Semmrich et al., 2005). This previous publication fits well with data presented in this thesis on both Mo-DCs and in RA SF DCs. Increased levels of active CD11a on tolerogenic Mo-DCs, could thereby contribute to their variety of tolerogenic functions, such as low expression of costimulatory molecules, reduced expression of pro-inflammatory cytokines and increased expression of immunoregulatory cytokines (Chapter 4, section 4.1.1), alongside their reduced abilities to induce IFN-γ production by T cells (Chapter 4, section 4.2.1, Figure 4.4). Conversely, in an inflamed RA joint, it is likely that local pro-inflammatory factors, such as IFN-γ, IL-1β IL-6 (Lettesjö et al., 1998; Schlaak et al., 1996), would activate DCs present in the joint and increase their ability to stimulate T cells (Grohmann et al., 2001), therefore explaining the shedding of CD11a from DC surfaces. On the other hand, I suggested CD11b to be pro-inflammatory due to its significantly decreased expression on tolerogenic Mo-DCs, while being upregulated on APCs found in RA SF. Evidence from the literature on this topic is more conflicting, as there are a number of studies to suggest immunoregulatory roles for CD11b in DCs (Chapter 1, section 1.4.3) (Behrens et al., 2007; Ehirchiou et al., 2007; Nowatzky et al., 2018b; Podgrabinska et al., 2009; Škoberne et al., 2006; Varga et al., 2007; Yee and Hamerman, 2013). However, one study suggested that absence of CD11b from DCs restricted their ability to stimulate

T cells by restricting endocytosis of TLR4 (Ling et al., 2014), suggesting that CD11b can mediate pro-inflammatory functions under some circumstances.

How can this discrepancy between findings presented in this thesis and the wider published literature be explained? First, it is possible that CD11b has both pro-inflammatory and immunoregulatory functions that are mediated depending on context. It is therefore possible that both inducing mature Mo-DCs from $CD14^+$ monocytes and APCs present in an inflamed joint represent an environment where pro-inflammatory functions of CD11b are more prevalent in comparison to its immunoregulatory roles. This is further supported by the fact that the study reporting a pro-inflammatory role for CD11b in TLR4 signalling (Ling et al., 2014) found this in murine BMDCs that were matured with LPS. Another possibility would be that CD11a and CD11b are reciprocally regulated, potentially together with CD11c, with a change in expression of CD11a automatically altering the balance of β_2 integrins on the cell surface and therefore affecting expression and conformation of CD11b. One can speculate that such a reciprocal regulation of β_2 integrin α subunits could be mediated by downstream signalling processes causing increased internalisation of a certain β_2 integrin subunit or by altering their ability to assume their active conformation in response to either outside-in or inside-out signalling.

6.4 Targeting β_2 integrins therapeutically

Data presented in this thesis highlight opposing roles of CD11a and CD11b, with CD11a potentially being more immunoregulatory and CD11b potentially playing pro-inflammatory roles on DCs. Therefore, therapeutic manipulation of this β_2 integrin axis, for example by blocking CD11b and/or promoting CD11a, might have benefit in RA and other autoimmune conditions by inducing DCs with a more tolerogenic phenotype.

A major challenge in interpreting scientific results as well as developing therapeutic strategies involving β_2 integrins is the difficulty in distinguishing how mAbs or therapeutic agents mediate their effects. To explain, an agent binding specifically to a β_2 integrin subunit could be 'blocking' β_2 integrin function, by obstructing their access to ligands. However, a mAb or therapeutic agent could equally well mediate outside-in signalling by 'ligating' the β_2 integrins, which might elicit phenotypic changes to cells via downstream outside-in signalling pathways. Interestingly, both approaches might be useful intervention strategies in disease. For example, blocking adhesion of

DCs to T cells might reduce the amount of T cells becoming activated and blocking adhesion to endothelium would restrict access of cells to sites of tissue inflammation, thereby potentially contributing to resolving inflammation. On the other hand, downstream β_2 integrin signalling in DCs has been described to be immunoregulatory (Chapter 1, section 1.4.3), suggesting that ligating β_2 integrins might increase control of TLR signalling (Yee and Hamerman, 2013), thereby contributing to limiting immune responses. Therapeutically, it might therefore even be desirable to block access of immune cells into tissues, while simultaneously inducing immunoregulatory signalling in DCs already present in either inflamed tissues or LNs.

6.5 Future Directions

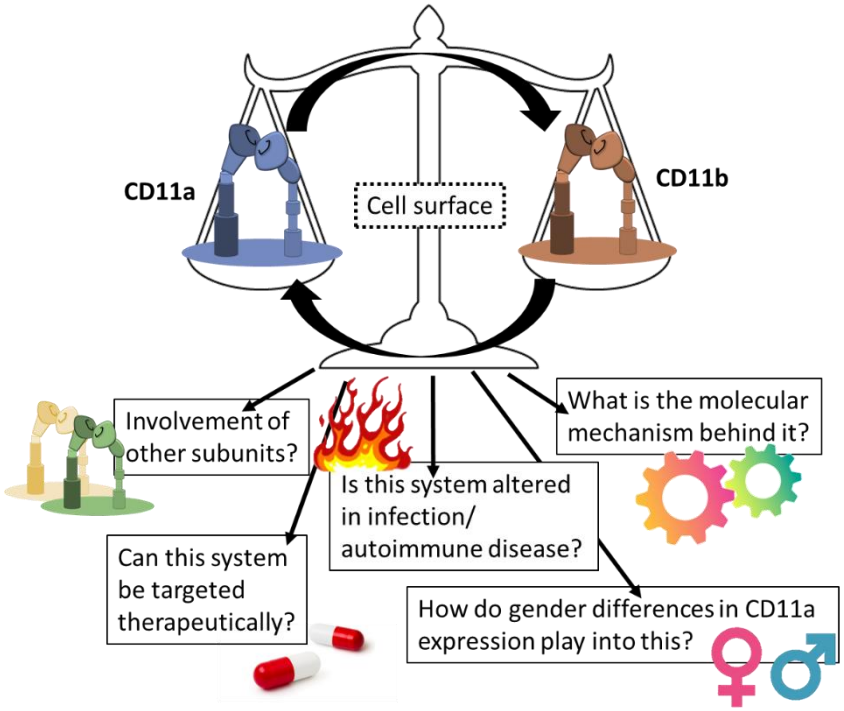


Figure 6.1 Graphical summary

The work presented in this thesis suggests that β_2 integrin subunits CD11a and CD11b are reciprocally controlled on immune cell surfaces. This raises a variety of novel questions for future research, including involvement of other subunits, underlying molecular mechanism and role of found gender differences as well as potential alterations in autoimmune disease or infection, making this system a potential therapeutic target.

The finding that β_2 integrin subunits CD11a and CD11b appear to be reciprocally regulated, with one increasing when the other decreases in surface expression under several circumstances, is one of the main findings of this thesis (Figure 6.1). It generates a variety of novel questions and directions for future research. The most

obvious questions would be how such a system of integrin regulation works on a molecular basis and how other subunits, such as CD11c and CD11d are involved. Another question that was touched on by my finding that β_2 integrins, especially CD11a, do seem to be dysregulated in RA, is if this system is altered in autoimmune disease or infection and if it is therefore a viable therapeutic target. Lastly, gender differences in CD11a total expression suggest that this mechanism might operate differently in males and females, adding a further dimension. In the following paragraphs, I will explore how these questions could be answered in the future.

While the evidence presented in the previous section supports a role for dysregulation of the β_2 integrin subunit, CD11a, in RA, the question of cause and effect is especially difficult to answer in a human setting. Continuing this work, I would focus on the functional aspects of β_2 integrins on DCs, including adhesive, migratory and T cell stimulatory functions, between male and female healthy controls and healthy controls and RA patients. One could here hypothesise that such CD11a-mediated functions would be altered in RA patients, and might even be shown to contribute to on-going inflammation. Here, it would be especially important to compare RA patient samples functionally to healthy controls, as the specific nature of CD11a dysregulation is not yet clear and would therefore be difficult to recreate *in vitro*. This furthermore suggests that while measuring both total and active β_2 integrin expression yields novel information, this is not exhaustive of integrin function. Even though expression of the active integrin conformation likely reflects functional ability, further measurements, such as receptor avidity, might affect function as well and might therefore be important to take into account.

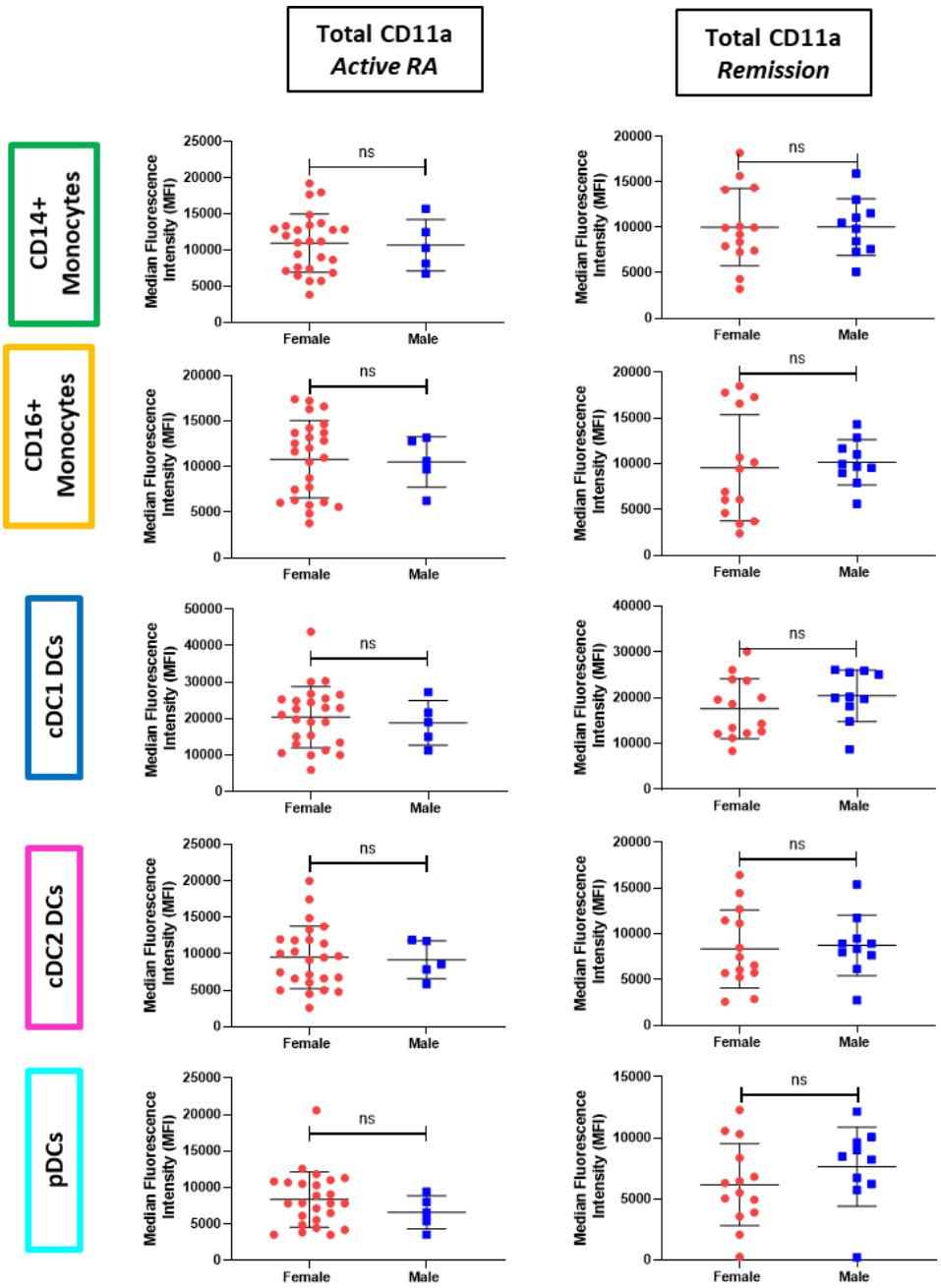
For further exploring the potential opposing functions of CD11a and CD11b in the future, targeting CD11a and CD11b specifically on human DCs would be a top priority to investigate the respective functional importance of CD11a and CD11b on DCs. RNAsilencing of individual β_2 integrin subunits, which would allow for quantification of expression and conformation of other β_2 integrins to test if any reciprocal regulation between β_2 integrin subunits exists, could potentially do this. This approach would also allow to pinpoint the functional significance of different β_2 integrin subunits, as it would allow testing of DC abilities to migrate, stimulate T cells and produce cytokines. Furthermore, as the read-outs used to detect differences in specifically tolerogenic function of Mo-DCs were limited, further read-outs could be added once specific integrin subunits can be blocked or activated. Examples of this

could include measuring cytokine signalling, such as the presence of IL-12 or TGF-β after blocking or activating integrin subunits to test if tolerogenic functions are altered. Similarly, surface marker expression of CD83, CD86, HLA-DR, LAP or TLR2 might give insight in how far integrins are actually involved in these differences between mature and tolerogenic Mo-DCs. Lastly migratory and clustering properties could be similarly assessed to test how functionally important the different subunits are.

6.6 Concluding remarks

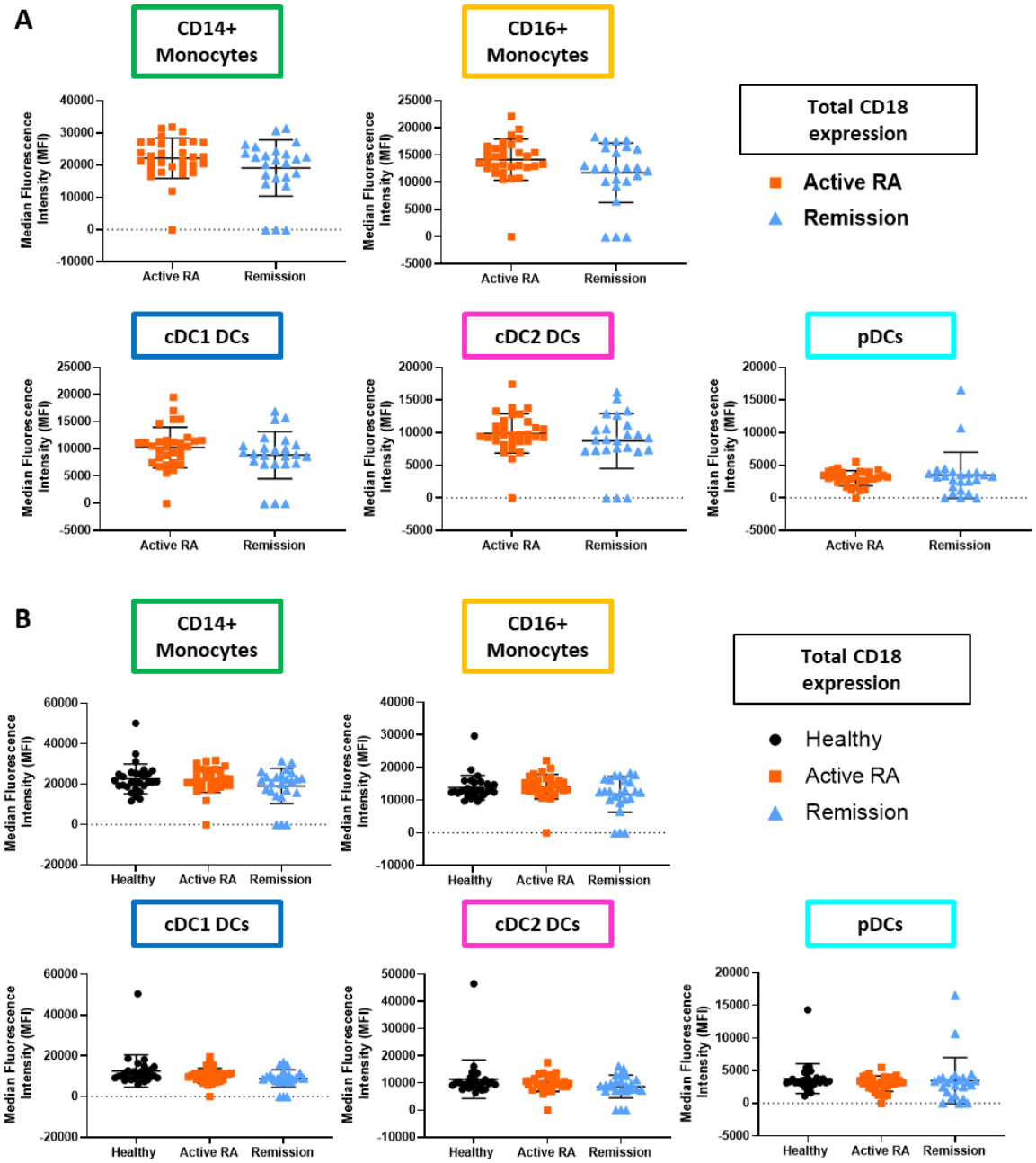
In this thesis I present evidence that the β_2 integrin CD11a is dysregulated in RA. These alterations of CD11a may indicate a downstream consequence of inflammation or actively contribute to disease pathology, or potentially even do both. Especially the finding that expression of CD11a differed between healthy men and women, but was found to be the same between men and women with RA, could provide valuable insights into how gender might contribute to the development of autoimmune disease. Furthermore, my work uncovered potentially opposing roles of β_2 integrin subunits CD11a and CD11b on DCs, with CD11a being likely to mediate immunoregulatory roles, while CD11b was suggested to play more pro-inflammatory roles. Although technical problems with β_2 integrin targeting therapies persist and would need to become more targeted to specific cell types or cell locations, together, this suggests that β_2 integrins represent a viable therapeutic target on DCs specifically to treat RA and potentially other autoimmune disorders by driving a more tolerogenic phenotype in DCs.

Appendix



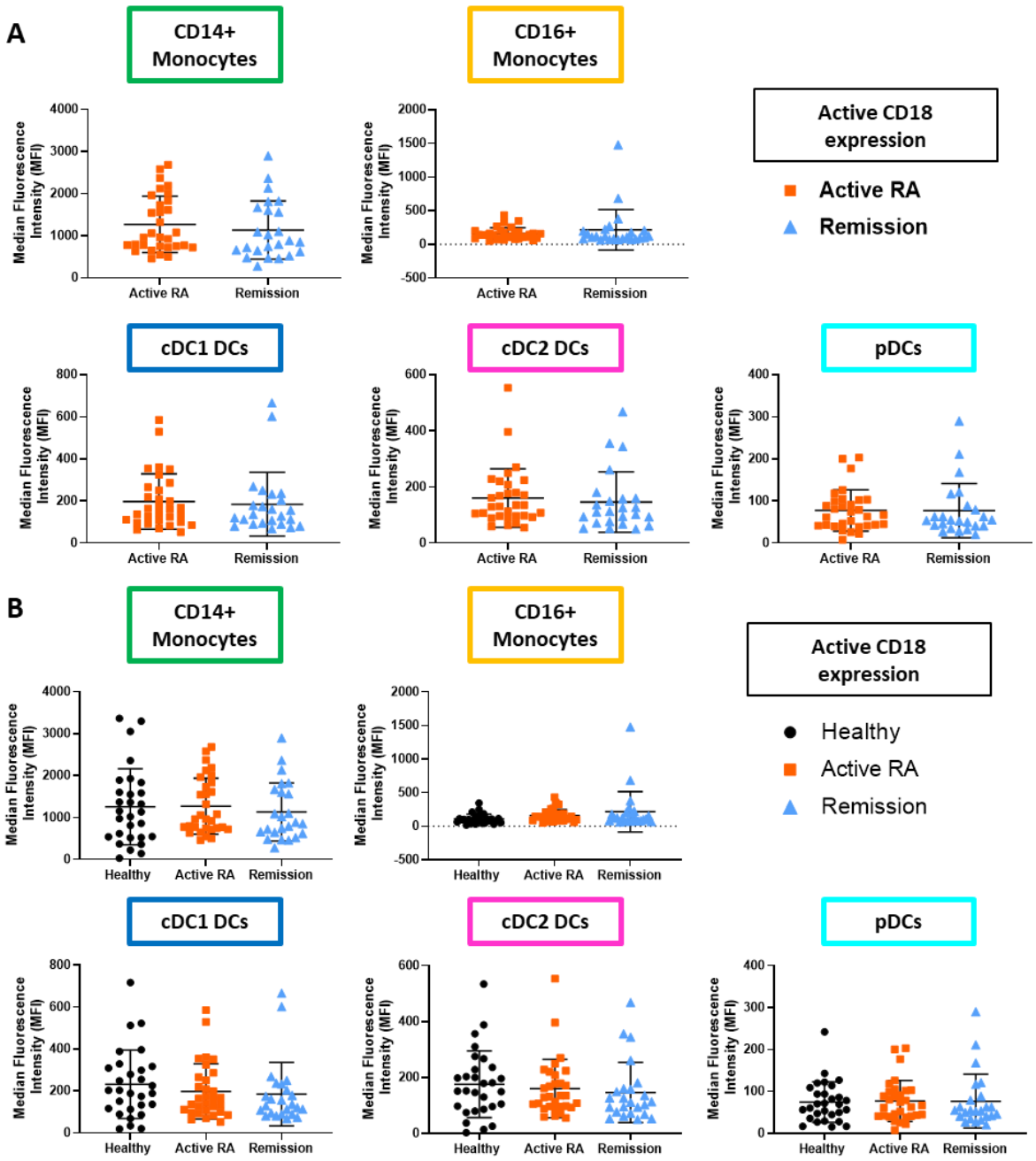
Appendix figure 1 Expression of total CD11a does not differ between genders in RA

Total CD11a expression was compared between female (red circles) and male (blue squares) RA patients in five APC types of interest. Both active RA (left, female n=26, male n=5) and remission samples (right, female n=14, male n=10) are shown. Unpaired t test, total n=29.



Appendix figure 2 Total CD18 expression in healthy controls compared to RA patients

Total CD18 expression in RA samples and RA samples compared to healthy controls A. Total expression of CD18 (MFI) compared between active RA (orange squares, n=31) and disease in remission (blue triangles, n=24) on five APC types of interest isolated from PB. Unpaired student's t test. B. Total CD18 expression (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.



Appendix figure 3 Active CD18 expression in healthy controls compared to RA patients

Active CD18 expression in RA samples and RA samples compared to healthy controls A. Expression of active CD18 (MFI) compared between active RA (orange squares, n=31) and disease in remission (blue triangles, n=24) on five APC types of interest isolated from PB. Unpaired student's t test. B. Active CD18 expression (MFI) between healthy controls and RA patients. One way ANOVA, n=29 for healthy control, n=31 in active RA, n=24 in remission.

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