Conventional dendritic cells for regeneration of immune tolerance

A thesis submitted for the requirements of the degree of Doctor of Philosophy

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Abstract

Tolerogenic dendritic cells (tolDC) are a promising cell therapy for the treatment of autoimmune disease due to their ability to regenerate immune tolerance. The most commonly used cells for their generation are monocyte-derived DC (moDC). However, this approach has some limitations, including the poor migratory ability of these cells. The work presented in this thesis aims to generate a conventional DC (cDC)-based tolDC therapy, because cDC have excellent migratory capacity towards T cell areas in lymphoid tissues, where they can educate naïve $CD4^+$ T cells.

The first objective of this thesis was to select the most appropriate cDC subset for tolDC generation. Therefore, the Nanostring nCounter platform was used to compare the gene expression profiles of cultured $CD34^+$ stem cell-derived type 1 and type 2 cDC (cDC1 and cDC2) and monocyte-derived tolerogenic DC (Mo-tolDC). This analysis revealed that cDC2 was more closely aligned than cDC1 with Mo-tolDC, with regard to the expression of tolerogenic genes, including *PDL-1*, *PRDM1* and *MRC1*. In addition, both cultured and peripheral blood-derived cDC2 expressed significantly higher level of *LILRB4* (ILT3 encoding gene) compared to cDC1, suggesting its contribution to the regulatory function of cDC2. Therefore, the cDC2 subset was chosen for the generation of cDC-derived tolDC.

Another objective of this project was to investigate the optimal tolerogenic treatment to generate migratory cDC-derived tolDC and to assess the impact of different culture media on the phenotype and migratory ability of cDC-derived tolDC. Hence, peripheral blood cDC2 were magnetically isolated from leukocyte reduction cones (LRS) and cultured *in vitro* in the presence of GM-CSF with or without tolerogenic agents and LPS. Cells were phenotypically characterized by flow cytometry, and their cytokine secretion profiles were determined by Meso-Scale-Discovery (MSD) multiplexing or ELISA. In addition, CCR7-dependent migration towards CCL19 and CCL21 was assessed. Treating freshly isolated cDC2 with the active form of vitamin D3 (VitD3) and LPS (VitD3-cDC2) significantly enhanced the expression of the tolerogenic marker PDL-1. Remarkably, IL-10 secretion was also high in VitD3-cDC2, suggesting its contribution to their regulatory capacity. However, while secretion of IL-12p70 was very low, VitD3-cDC2 secreted high levels of the pro-inflammatory cytokines IL-6 and TNF-α. Prominently, CCR7 expression remained high on VitD3-cDC2, and they exhibited good migratory ability, albeit slightly reduced compared to LPS-activated cDC2.

To further characterize the stimulatory capacity of VitD3-cDC2, co-culture assays with allogeneic naïve $CD4^+$ T cells were performed. VitD3-cDC2 demonstrated lower T cell stimulatory capacity with significant reduced levels of IFN-γ secretion than untreated and unstimulated cDC2. Finally, T cells that were primed with VitD3-cDC2 and rested before restimulation with LPS-treated cDC2 demonstrated an increase in their expression of CD25 and FOXP3, which could be indicative of Treg induction. In addition, I also observed higher IL-10 secretion and lower IFN-γ/IL-10 ratio in T cells primed with VitD3-cDC2 after re-stimulation assays, suggesting a potential regulatory capacity of these cells.

The findings in this thesis support the use of cDC2 as an alternative to moDC for the generation of therapeutic tolDC. Additionally, this thesis has provided the basis for future work on the generation of tol-cDC2 for treating autoimmune diseases. Further work is required to understand the mechanism(s) by which tol-cDC2 regulate T cell responses.

COVID-19 impact statement

The COVID-19 pandemic has significantly impacted the trajectory of this Ph.D. project. The unprecedented global situation has necessitated adaptations in project planning and implementation. Challenges related to limited access to laboratory facilities, disruptions in data collection, and altered experimental timelines have influenced the study's progression. The safety protocols and restrictions imposed during the pandemic have led to modifications in research methodologies, hindering the original project plan and timelines. Furthermore, upon my return to my home country with my children and husband during the pandemic, I encountered difficulties due to the enforced lockdown measures upon entry. However, when transportation options became accessible, I returned to Newcastle and continued my research activities. Despite these difficulties, the study has persevered through innovative remote work strategies, adjustments in experimental protocols, and utilizing available resources to maintain progress.

Declaration

The content within this thesis is entirely the author's original work, unless specified otherwise, and has not been previously presented for a degree at this university or any other academic institution.

Dedication

Dedicated to the loving memory of my dearest mother*, Abeer Madani*, may ALLAH rest her soul in an eternal piece and a place in the highest ranks of Paradise. Your unwavering love, guidance, and boundless support were the pillars of my life and education. Though you are no longer by my side, your spirit and strength continue to inspire me every day. This thesis stands as a tribute to your enduring belief in my dreams. You are deeply missed and will always reside in my heart.

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List of abbreviation

Ab	Antibody
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AIRE	Autoimmune regulator protein
ANOVA	Analysis of variance
APCs	Antigen presenting cells
bl-cDC	Peripheral blood derived conventional dendritic cells
BSA	Bovine serum albumin
BTLA	B- and T-lymphocyte attenuator
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDC1	Conventional dendritic cell type 1
cDC2	Conventional dendritic cell type 2
CDP	Common dendritic cell progenitor
CG	CellGenix culture medium
CLEC4A	C-type lectin receptor
CLEC10A	C-type lectin receptor
CLRs	C-type lectin receptors
cMoP	Common monocyte progenitors
CTFR	Cell Trace Far Red
CTLA-4	Cytotoxic T lymphocyte associated protein 4
Cul-cDC	Cultured $CD34^+$ derived dendritic cells
CXCL	C-X-C motif chemokine ligand
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DEGs	Differentially expressed genes
Dex	Dexamethasone
Dex-cDC2	Dexamethasone treated cDC2
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESAM	Endothelial cell-selective adhesion molecule
FACS	Fluorescence associated cell sorting
FCS	Foetal calf serum
FMO	Fluorescence minus one
FoxP3	Forkhead box P3
FSC A	Forward scatter area
FSC H	Forward scatter height
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GSEA	Gene set enrichment analysis
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT3	Immunoglobulin-like transcript 3
ILT4	Immunoglobulin-like transcript 4
IRF	Interferon regulatory factor
ImmDC	Immature DC
LAG-3	Lymphocyte activation gene 3
LC	Langerhans cells
LP	Lymphoid progenitor
LPS	Lipopolysaccharide
LRS	Leukocyte reduction cones
MACS	Magnetic-activated cell sorting
MatDC	Mature DC
MDP	Macrophage and dendritic cell progenitor
MERTK	Receptor tyrosine kinases of the TAM family
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MoDC	Monocyte-derived dendritic cell
MP	Myeloid progenitor
MPLA	Monophosphoryl Lipid A
MRC1	Mannose receptor C type 1
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSD	Meso Scale Discovery Immunoassay
mTOR	Mammalian target of rapamycin
NFkB	Nuclear factor kappa B
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cells
PDL-1	Programmed death-ligand 1
PDL-2	Programmed death-ligand 2
PGE2	Prostaglandin E2
PRR	Pattern recognition receptor
RA	Rheumatoid Arthritis
Rapa	Rapamycin
Rapa-cDC2	Rapamycin treated cDC2
RPMI	Royal Park Memorial Institute culture medium
RT	Room temperature
SD	Standard deviation

SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
SSC A	Side scatter area
SSC H	Side scatter height
STAT	Signal transducer and activator of transcription
Stim-cDC2	LPS stimulated cDC2
T1D	Type 1 diabetes mellitus
TCR	T-cell receptor
Tfh	T follicular helper
TGF	Tissue growth factor
Th	T helper CD4+ effector cell
TIM-3	T cell immunoglobulin and mucin domin-3
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TolDC	Tolerogenic dendritic cell
Treg	Regulatory T cell
$\mathbf{T_{Stim\text{-}cDC2}}$	CD4+ T cells primed with Stim-cDC2
$\mathbf{T_{Un\text{-}cDC2}}$	CD4+ T cells primed with Un-cDC2
$\mathbf{T_{VitD3\text{-}cDC2}}$	CD4+ T cells primed with VitD3-cDC2
Un-cDC2	Unstimulated and untreated cDC2
VISTA	V-domain Ig suppressor of T-cell activation
VitD3	Vitamin D3 or 1,25-dihydroxyvitamin D (calcitriol)
VitD3-cDC2	Vitamin D3 treated cDC

Chapter 1: Introduction

1.1 Immunity and Immune Tolerance

The immune system can distinguish between self and non-self-antigen through various mechanisms to protect our body (Huber et al., 2018). Immune tolerance is a central role of the immune system that prevents auto-reactive T cells from reacting against self-antigens to avoid the development of autoimmune diseases (Audiger et al., 2017). Over the last decades, a growing number of studies have shown the contribution of different immune and non-immune cells in maintaining the balance between tolerance and immunity (Waldmann, 2014). Among these cells, antigen-presenting cells (APCs) such as dendritic cells (DC) have notably been found to link the innate and adaptive immune systems in order to develop an accurate immune response (Huber et al., 2018; Waldmann, 2014).

Consequently, extensive research has found the importance of DC as critical immune system regulators. DC are professional APCs crucial in maintaining the balance between immunity and tolerance (Fucikova et al., 2019; Obregon et al., 2017). These occur through either promoting effector T cells to react against invading pathogens and provide protective immunity or through regulation of immune cells and protect the host from pathogenicity of auto-reactive T cells (Abbas et al., 2004; Fucikova et al., 2019). In line with this, it has been shown that many innate immune recognition receptors such as pattern recognition receptors (PRRs), toll-like receptors (TLRs), C-type lectin receptors (CLRs) and Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) are expressed on the surface of DC, which allow them to recognize and respond to microbial molecules or cytokines in the early stage of infection (Collin and Bigley, 2018a; Manh et al., 2013). Furthermore, besides their role in immunity, DC play a crucial role in the induction of both central and peripheral tolerance through different mechanisms (Audiger et al., 2017; Fucikova et al., 2019). All these mechanisms will be discussed in detail in the next section.

1.1.1 Self-Tolerance induction

Immunological tolerance is a state of unresponsiveness to self-antigen and the ability of the immune system to discriminate between self and non-self-antigen (Waldmann, 2014). This

vital property of the immune system is generated by a series of mechanisms established at the time of birth (Romagnani, 2006; Waldmann, 2014). Tolerance to self-antigen is mediated in the thymus or peripheral tissues, known as central and peripheral tolerance, respectively (Hilkens et al., 2010). DC are key players in regulating and maintaining both central and peripheral tolerance (Hilkens et al., 2010).

1.1.1.1 Central Tolerance

During lymphocyte development in the thymus, DC is essential in establishing self-tolerance either by negative selection of auto-reactive T-cells or positive selection of regulatory T cells (Tregs) (Domogalla et al., 2017). So, after the positive selection of $CD4^+$ and $CD8^+$ T cells in the thymic cortex, those T cells migrate into the thymic medulla and interact with tissue-specific antigens (TSA) that are presented on MHC molecules by medullary thymic epithelial cells (mTECs) and DC (Takaba and Takayanagi, 2017). Thus, these interactions usually lead to the elimination of almost all auto-reactive T-cells by apoptosis, which will prevent the release of self-reactive T-cells into the periphery, a process referred to as negative selection (Perry et al., 2014; Takaba and Takayanagi, 2017). In addition, DC and mTECs regulate the differentiation of some T cells into $Foxp3^+$ Tregs, which is required to avoid the development of spontaneous autoimmune disease (Takaba and Takayanagi, 2017). Moreover, deletion of self-reactive T cells (negative selection) is mainly dependent on medullary thymic epithelial cells (mTECs) under the control of AIRE (autoimmune regulator), a transcription factor required for TSA presentation to T cells (Osorio et al., 2015). However, induction of Treg differentiation (positive selection) is predominantly regulated by the thymic DC (Devi and Anandasabapathy, 2017; Osorio et al., 2015). Over the years, it has been found that there are two main subtypes of Tregs: natural Tregs (nTregs) that develop in the thymus and adaptive Tregs (also referred to as induced or peripheral Tregs) that are induced in the periphery (Hilkens et al., 2010). Interestingly, growing evidence indicates a crucial role of DC in promoting both types of Tregs (Osorio et al., 2015).

1.1.1.2 Peripheral Tolerance

Despite the deletion of the majority of self-reactive T-cells in the thymus, some of them will escape and migrate into the periphery, which may lead to the development of autoimmunity (Hilkens et al., 2010). However, these self-reactive T cells are regulated by different checkpoint mechanisms in the periphery, such as deletion by apoptosis, induction of T cell anergy, or

induction of adaptive Tregs (Horton et al., 2017; Waldmann, 2014). DC have been found to play a central role in the induction of peripheral tolerance by all these distinctive mechanisms (Domogalla et al., 2017; Hilkens et al., 2010; Horton et al., 2017) (see figure 1.1).

1.1.1.2.1 T cell anergy

Three important signals are required to achieve full T cell activation during the interaction between APCs (such as DC) and T cells. The first signal is dependent on the interaction between MHC/peptide complexes on the APCs and the T cell receptor (TCR) on the T cells, whereas the second signal occurs after the engagement of CD28 on T cells and the co-stimulatory molecules (CD80, CD86) on APCs (Renz and Herz, 1998; Tai et al., 2018). After that, a third signal occurs through APC-derived or microenvironmental factors, for example, the pro-inflammatory cytokines IL-12, IFNα/β, and IL-2, which lead to the proliferation of effector T cell (Goral, 2011; Kaliński et al., 1999; Macián et al., 2004). Hence, presenting the Ag on MHC molecules without having a second signal (i.e., loss of co-stimulatory molecules) leads to a state of T cell hypo-responsiveness that is called T cell anergy, resulting in inhibition of effector T cell proliferation and decreased IL-2 production (Horton et al., 2017; Xing and Hogquist, 2012). Another molecule crucial in inducing T cell anergy is cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Macián et al., 2004). Interestingly, immature DC (iDC) in steady-state conditions regulates peripheral tolerance by induction of T-cell anergy via IL-10 (Hilkens et al., 2010; Tuettenberg et al., 2009).

1.1.1.2.2 T cell apoptosis

Apoptosis of T cells, also referred to as clonal deletion, which includes elimination of T cells, is another mechanism of peripheral tolerance. T cell apoptosis mainly occurs as a result of binding between death receptors such as Fas (CD95) on the T cell surface, with its ligand FasL (CD95L), which is expressed by APCs including DC, resulting in activation of caspase 8-mediated apoptosis of T cells (Hasegawa and Matsumoto, 2018). Another mechanism that induces T cell apoptosis is through IDO tryptophan catabolism that is expressed by DC as well (Fallarino et al., 2002). (More detailed information about this catabolic enzyme will be demonstrated in section 5, markers of tolerance).

1.1.1.2.3 Regulatory T cell (Treg) expansion and induction

The process of FoxP3^{+} Treg differentiation and expansion is one of the prominent roles of DC in controlling the immune peripheral tolerance (Hilkens et al., 2010; Osorio et al., 2015). DC have the ability to induce Tregs in the periphery via several mechanisms, such as expression of the programmed cell death ligand-1 (PD-L1) molecule or indoleamine 2,3 deoxygenase (IDO) (Mok, 2015), which promotes the differentiation into Foxp3+ Tregs (Osorio et al., 2015). In addition to that, tolerogenic DC can also enhance Foxp3^{+} Treg differentiation through the secretion of transforming-growth-factor-beta (TGF-β) and/ or IL-10 (Raker et al., 2015).

Therefore, the breakdown of one of these mechanisms of immune tolerance could lead to the development of autoimmunity and autoimmune disease.

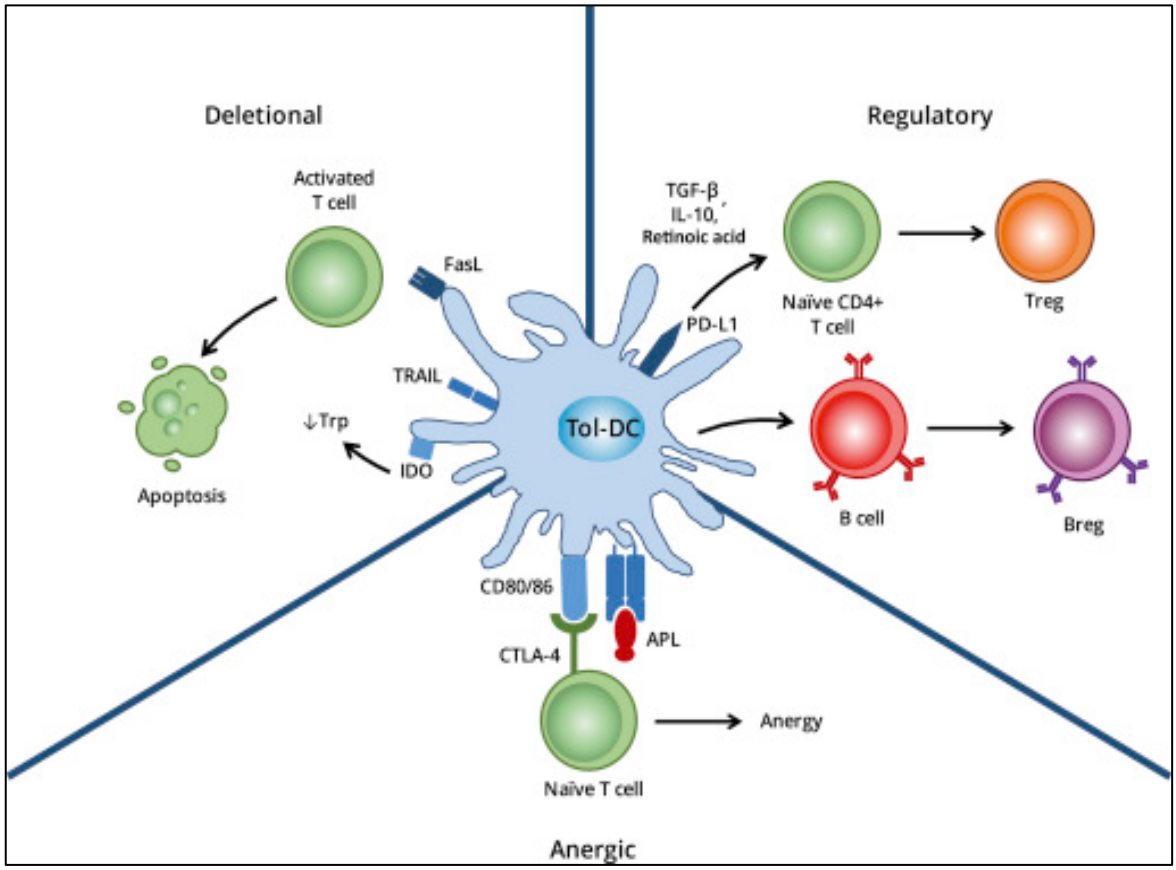


Figure 1.1: Peripheral tolerance mechanisms mediated by tolDC. These mechanisms include Clonal deletion (apoptosis), which is mediated through the involvement of FasL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and IDO enzyme on tolDC. Treg induction by tolDC is another mechanism of tolerance induction that is mediated by the

involvement of PDL-1 molecule, while T cell anergy is the third mechanism that is mediated through the interaction of costimulatory molecules CD86/CD80 on tolDC with CTLA-4 (immune inhibitory molecule) on the T cell. This figure is obtained from (Horton et al., 2017).

1.1.2 Breach of Tolerance

Failure of tolerance induction results in the inability of the immune system to discriminate between self and non-self-antigen, leading to failure of autoreactive T cell elimination, which consequently leads to the development of pathological autoimmune diseases (Abbas et al., 2004; Romagnani, 2006; Waldmann, 2014). As mentioned in the previous section, a breakdown of one or more mechanisms responsible for maintaining self-tolerance will lead to autoimmunity.

There are multiple interacting factors that could lead to a breach or breakdown of tolerance. Of note, genetic polymorphisms that lead to a mutation in some genes essential in either central or peripheral tolerance induction are important factors leading to autoimmunity (Romagnani, 2006). One of the vital genetic polymorphisms linked to autoimmune diseases include variations in the Human Leukocyte Antigen (HLA) genes, particularly HLA class I and II, in various autoimmune conditions (Dendrou et al., 2018). This aligns with the essential function of HLA class I and II molecules in presenting a wide range of antigens to T cells, allowing the immune system to distinguish between the self and non-self antigens. For example, in type 1 diabetes mellitus (T1D), a mutation in class II HLA has been reported with a strong association with *HLA-DQ and DR* genes (Sticht et al., 2021). Another example of an autoimmune disease associated with polymorphism in the *HLA-II* gene is multiple sclerosis (MS) (Hafler et al., 2005). However, mutations in other non-HLA genes were also reported with the development of many autoimmune diseases. For instance, variation in IL-7 receptor α-chain (IL-7Rα) that is crucial for the signaling and functioning of the IL-7 cytokine in supporting the development and homeostasis of T cells, was reported to be associated with susceptibility to MS (Thewissen et al., 2014). Additionally, polymorphisms in CTLA-4 and PD-1(immune checkpoint) have been reported to be associated with the development of systemic lupus erythematosus (SLE) (Chen et al., 2022).

Another important factor that could also lead to the failure of tolerance induction is environmental factors. For instance, microbial infection and ischemic injury are the most vital

environmental factors that may lead to the development of autoimmune diseases (Romagnani, 2006).

1.2 Autoimmune disease and therapeutic interventions

Over the last decade, there has been an increase in the prevalence of autoimmune and autoinflammatory diseases among the population of the developed world (Van Brussel et al., 2014). To date, there are no curative therapies for these diseases. Although until now, immunosuppressive and immune-modifying drugs are the most successful at slowing the progression of autoimmune diseases (Domogalla et al., 2017), various limitations and drawbacks are associated with the prolonged use of these drugs (Macedo et al., 2012). For instance, lifelong usage of immunosuppressive medications can make the patient susceptible to infection and failure to encourage tolerance induction (Macedo et al., 2012). Because autoimmune diseases result from a breakdown in immune tolerance against self-antigen (Mosanya and Isaacs, 2019; Van Brussel et al., 2014), investigations of various cell types to re-establish immune tolerance have been made. So, in this section, the main potential cellular therapeutic intervention for autoimmune diseases will be discussed.

1.2.2 Cellular therapy

Recently, researchers believed that the ideal treatment for autoimmune disease is therapy that can return the state of self-tolerance in the immune system (Bell et al., 2017). For this reason, several cellular therapies have been investigated for treating autoimmune diseases. CAR (Chimeric Antigen Receptor) T-cell therapy, an innovative immunotherapy, has primarily been utilized in the treatment of cancer. However, its potential application in autoimmune diseases is an area of ongoing research and exploration (Riet and Chmielewski, 2022). For example, a study by Fransson et al. (2012) in the murine experimental autoimmune encephalomyelitis (EAE) model of MS determined that CAR Treg that targeted the myelin oligodendrocyte glycoprotein (MOG) has a suppressive capacity in vitro. In addition, they also observed a reduction in the symptoms of ongoing encephalomyelitis, accompanied by decreased levels of IL-12 and IFN-gamma mRNA in the brain tissue (Fransson et al., 2012).

Another attractive cellular therapy is tolerogenic dendritic cells (tolDC; in vitro-generated monocyte-derived (moDC) that have been modulated to increase their tolerogenic properties),

which have been broadly studied in the last decades for their ability to regenerate immune tolerance in patients with autoimmune diseases.

Several clinical trials have proven the safety and feasibility of tolDC in the treatment of various diseases such as autoimmune diseases (e.g., rheumatoid arthritis (RA), type 1 diabetes (T1D), multiple sclerosis (MS)) and Crohn's inflammatory disease (Kim et al., 2018; Phillips et al., 2017; ten Brinke et al., 2019). For instance, the first clinical trial of tolDC was performed on patients with type 1 diabetes mellites autoimmunity (T1D) by Giannoukakis et al., 2011 showing the safety of using this cellular therapy at a time near the clinical onset of the disease (Giannoukakis et al., 2011). Another successful example of a completed trial of tolDC therapy is the AuToDeCRA trial (**Au**tologous **To**lerogenic **De**ndritic **C**ells for **RA**) (Bell et al., 2017). In this trial, tolDC was generated from monocytes plus dexamethasone and vitamin D3 as the tolerogenic agents (Bell et al., 2017; Harry et al., 2010a). However, several concerns have been mentioned, such as the route of administration (intra-articular), lack of migratory ability to the draining LN, and the possible secretion of proinflammatory cytokines by these monocyte-derived tolDC (Bell et al., 2017; Cooke et al., 2022). In addition, work co-led by Hilkens and Isaacs showed that ^{111}Indium-labelled tolDC remained in the joint (Cooke et al., 2022), which suggests that tolDC did not migrate to the draining lymph nodes and therefore did not induce any systemic regulatory effects on the immune system. Therefore, the AuToDeCRA 2 trial was initiated to inject tolDC through (intra-articular) i.a., (intra-dermal) i.d., and also into the lymph node, and this trial is due to commence early 2024.

Another interesting ongoing trial that investigates a more reliable route of tolDC administration in MS patients is performed by Willekens B et al. (2019). They compare two different ways of administration, intradermal and intranodal, to assess the feasibility and safety of this tolDC therapy (Willekens et al., 2019). Notably, in this trial, moDC is also used as a cellular approach for generating tolDC. However, unlike AuToDeCRA, they only used VitD3 treatment as a tolerising agent. Furthermore, a recent review by Mansilla et al., (2023) summarised the pros and cons of different tolDC administration in various autoimmune diseases, including MS, T1D, and RA (Mansilla et al., 2023).

In addition, another example of a completed phase I trial is the administration of autologous tolDC in patients with Crohn's disease (Jauregui-Amezaga et al., 2015). The protocol for tolDC generation in this trial was similar to the AuToDeCRA one in terms of the source of DC

(monocyte-derived DC) while they used different tolerogenic agents, which are dexamethasone and Vitamin A (Kim et al., 2018; Phillips et al., 2017). In this trial, Jaureguie-Amezaga et.al. showed that intraperitoneal administration of tolDC in patients with refractory Crohn's disease appears to be safe and feasible (Jauregui-Amezaga et al., 2015). Hence, a second tolDC clinical trial for Crohn's disease was initiated; however, no information about the results is available yet.

In addition to these diseases, other studies are ongoing to assess tolDC immunotherapy in other autoimmune diseases, such as systemic lupus erythematosus and organ transplantation. One of the significant challenges in using tolDC immunotherapy is the optimization protocol to generate the highest number of tolDC with stable and effective tolerogenic and migratory properties (Kim et al., 2018). Nevertheless, the completed and ongoing trials on tolDC immunotherapy provide hope in using these as a cellular therapy for autoimmune diseases in the future.

1.2.2.1 Alternative cellular approach

Recently, a considerable number of studies have identified the potential use of DC (both moDC and conventional DC (cDC) in cancer immunotherapy. The reason for that is the critical role of DC in promoting the protective anti-tumor effect by cytotoxic T lymphocyte (CTL) to induce the immunogenicity of cancer patients (Cancel et al., 2019). As such, Cancel and colleagues (2019) have reported that conventional DC1 (cDC1), a unique subtype of 'natural' DC (as opposed to moDC), has an essential role in promoting a spontaneous anti-tumor activity in a mouse model by educating CTL, natural killer cells (NK cells) and NKT cells (Cancel et al., 2019). Moreover, it has been confirmed that cDC1 has superior cross-presentation activity to $CD8^+$ T cells (CTL), which increases the support of their utility in anti-tumor immunity (Noubade et al., 2019).

Another subtype of natural DC that has recently been investigated in the context of cancer immunotherapy is cDC2. These cells are characterized by unique strong priming of naïve $CD4^+$ T cells leading to strong polarization towards various T cell subsets. For example, cDC2 has been demonstrated to promote both Th1 (Butcher and Zhu, 2021) and Th2 (Eisenbarth, 2019; Gao et al., 2013; Tussiwand et al., 2015) differentiation in humans leading to defense against both intracellular pathogen and helminth infection, respectively (Butcher and Zhu, 2021). In

addition, Schreibelt and colleagues' clinical study of metastatic melanoma patients found that vaccination with autologous cDC2 is feasible and safe leading to promising antitumor response (Schreibelt et al., 2016). Furthermore, another group identified that cDC2 vaccination in patients with metastatic prostate cancer is very effective and well tolerated (Prue et al., 2015).

Recently, some studies determined the tolerogenic and immunoregulatory activity in both cDC subsets that could be promising for use in autoimmune disease immunotherapy. For example, Gargaro and his group demonstrated crosstalk between cDC1 and cDC2 to promote tolerance through the IDO1 pathway. In this study, they identified that after LPS stimulation, IDO^{+} cDC1 educated mature cDC2 to express IDO1 tolerogenic activity that depended on the Aryl hydrocarbon receptor (AhR) mechanism in the cDC2 (Gargaro et al., 2022).

Overall, DC therapy has proved its safety and immunomodulatory/stimulatory capacity in many clinical trials regarding different types of autoimmune diseases and cancer.

1.3 Dendritic cell origin, subset, and maturation

DC are a vital orchestrator in regulating the balance between immunity against invading pathogens and establishing immunological tolerance (Domogalla et al., 2017; Rhodes et al., 2019). Their role in immunity and tolerance has been summarised in the previous section. However, the origin and subsets of these specialized cells will be discussed in this section.

1.3.1 Human DC origin and subsets

DC are derived from bone marrow precursors. They can pick up and process the invading organism and then present the antigen to naïve T cells that promote various immunological responses (Collin and Bigley, 2018; Thomas and Lipsky, 1996). Because of these broad varieties of mechanisms and responses, five major subtypes of DC were discovered in the peripheral blood, lymphoid organs, and tissues (Collin and Bigley, 2018). These subtypes are classified based on the origin, location, specific function, and gene expression profile into conventional or classical DC1 (cDC1), conventional or classical DC2 (cDC2), plasmacytoid DC (pDC), Langerhans cells (LC), and monocyte-derived DC (moDC) (Cancel et al., 2019; Collin and Bigley, 2018). A summary of the characteristic features, markers, and functions of DC subtypes is shown in table 1.1.

Distinct dendritic cell subsets have diverse origins and lineages in vivo. cDC are derived from hematopoietic stem cells in the bone marrow that give arise to myeloid progenitor (MP), which then originate the monocyte dendritic cell progenitor (MDP) that gives arise to common dendritic cell precursor (CDP) in the presence of FLT3. Then, both types of cDC will originate from the pre-cDC precursor. However, moDC derived from circulating monocytes originate from monocyte dendritic cell progenitor (MDP) (Collin and Bigley, 2018).

Furthermore, DC can also be generated *in vitro* from either monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 cytokines to produce moDC or derived from bone marrow mononuclear cells to generate the conventional or classical type of DC (cDC) and plasmacytoid DC (pDC) (Balan et al., 2018). An overview of the DC origin and subset are described in figure 1.2.

Recently, there have been several technologies that can help characterize and identify DC ontogeny and DC subpopulations. Of special interest is single-cell RNAseq, which has been used by Villani et al. (2017), allowing them to discover new subsets of DC and monocytes (Villani et al., 2017). For instance, two subdivisions of cDC2, namely CD1c A (DC2) and CD1c B (DC3), have been identified that share the characteristic surface marker CD1c (Villani et al., 2017). Despite that both are potent stimulators of naïve T cells, functional differences have been observed after activation; for example, CD1c A DC was found to secrete higher levels of immune mediators CCL19, IL-10, and IL-12B (Villani et al., 2017).

Furthermore, cDC can also be subclassified based on anatomical location into tissue-resident cDC that are localized in lymphoid tissues where they educate T cells, and migratory cDC (also known as non-lymphoid tissue cDC) that constitutively migrate from tissues to the lymph node (Boltjes and van Wijk, 2014).

DC subsets	Localization	Transcription factor	Main surface marker	Main function
cDC1	Various distribution in blood and lymphoid organs and peripheral tissues such as lung, intestine and skin.	IRF8 BATF3	CD141^{+}/ XCR1 CLEC9A CD11c^{+}	Superior cross presentation and priming to CD8^{+} T cytotoxic cell. Produce high level of type III IFN.
cDC2		IRF4 KLF4 NOTCH2	CD1c^{+}, CD172a^{+} CLEC10A CD11c^{+} CD5$^{+/-}$	Strong Priming of naïve CD4^{+} T cell that promote wide range of T cell response such as Th2 & Th17.
DC3	Peripheral blood	IRF8^{Low}	CD1c^{+} CD163^{+} CD14$^{+/-}$	Ability to Prime of both CD4+ and CD8+ T cells
pDC	Tonsils and resident in lymphoid tissue	E2-2 IRF8 &IRF4	CD123^{+}, CD303^{+} CD304^{+}	Type I interferon secretion and Antiviral immunity
moDC	Peripheral tissue in inflammation	MAFB IRF4?	CD14^{+}, CD1c^{+} HLA-DR^{+}	Direct priming of CD8^{+} T cell in tissues during inflammation.
LC	Skin	PU.1 ID-2 RUNX3	Langerin/CD207 DEC-205	Antigen presentation to CD4+ and CD8+ T cells to promote tolerance and immunity.

Table 1.1: Human dendritic cell subsets and characteristics (Ashour et al., 2020; Bourdely et al., 2020a; Canavan et al., 2021; Cancel et al., 2019; Chopin et al., 2013; Collin and

Bigley, 2018a; Dutertre et al., 2019; Eisenbarth, 2019; Patente et al., 2019; Romani et al., 2003; Stoitzner et al., 2022; Villani et al., 2017).

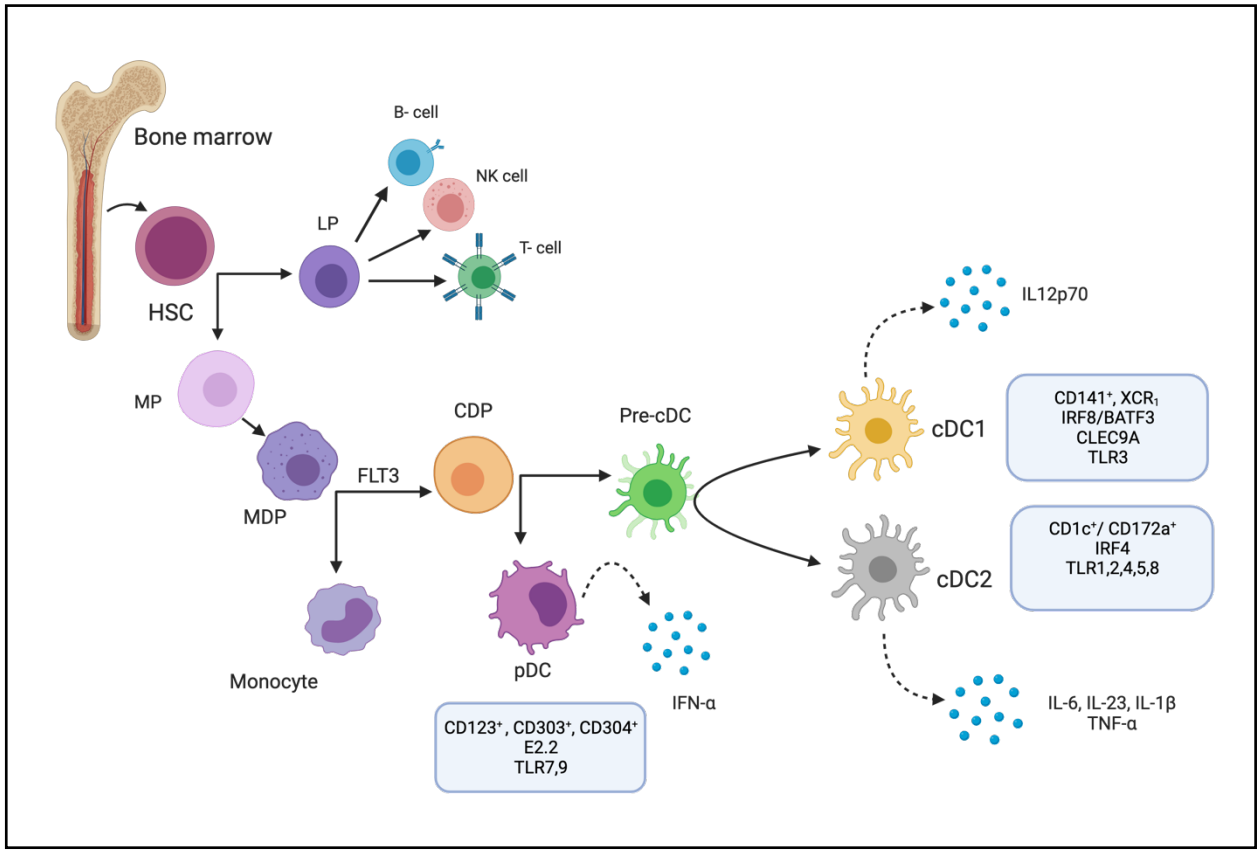


Figure 1.2: A simplified summary of DC ontogeny and main characteristic features and differences of cDC1, cDC2, and pDC. $CD34^+$ human stem cells (HSC) give rise to lymphoid progenitor (LP) and myeloid progenitor (MP), which then differentiate into monocytes, macrophages, and dendritic precursor (MDP). Monocytes and common DC precursor (CDP) will arise from MDP. Then, CDP can differentiate into pre-DC and pDC. Pre-DC will then give rise to conventional DC1 and 2 (cDC1 & cDC2). Each one of these DC subsets has a specific characteristic feature. The figure is based on information obtained from reference (Cancel et al., 2019; Collin and Bigley, 2018).

1.3.1.1 Conventional DC (cDC)

Across all tissues, two distinct types of cDC have been identified, which are cDC1 and cDC2 that are entirely dependent on fms-like tyrosine kinase three ligand (FLT3L) for differentiation from pre-cDC precursor (Eisenbarth, 2019; Kirkling et al., 2018). Each one of these subtypes

has distinctive phenotypic expression and function, but they both express CD11c and MHC-II in varying amounts (Guilliams et al., 2014). cDC1 has less migratory ability to lymphoid tissue than cDC2 (Granot et al., 2017; Segura, 2022).

1.3.1.1.1 Classical or conventional DC type 1 (cDC1)

cDC1 is a unique subtype of cDC that has a superior ability to cross-present and prime $CD8^+$ cytotoxic T cells due to high expression of $CD141^+$ ($BDCA3^+$), XCR1(which is believed to have a selective role in crosstalk between cDC1 and $CD8^+$ T cells) and CLEC9A (which is essential for cross-presentation and stimulation of $CD8^+$ T cells). cDC1 is a rare DC population in the peripheral blood that accounts for approximately 0.02-0.05 % of PBMC (Collin and Bigley, 2018; Villani et al., 2017). In addition to FLT3L, the development of cDC1 depends on the expression of IRF8 and BATF3 transcription factors (Schlitzer et al., 2015). cDC1 uniquely expresses toll-like receptor3 (TLR3), which enables them to sense viral infection and produce a large amount of type III interferon (Schlitzer et al., 2015; Shan Pang et al., 2020). Furthermore, cDC1 produced larger amounts of IL-12p70 than cDC2 and is thought to play a prominent role in the priming of Th1 cells (Ashour et al., 2020, p. 12; Eisenbarth, 2019).

1.3.1.1.2 Classical or conventional DC type 2 (cDC2)

Unlike cDC1, cDC2 is a heterogenous subtype of conventional DC that has been determined to express less specific markers, which makes them overlap with monocytes (Dutertre et al., 2019). They are the dominant DC population in the peripheral blood, tissues, and lymphoid organs, accounting for approximately 0.3-1% of the total PBMC (Collin and Bigley, 2018a; Dutertre et al., 2019). cDC2 differentiation mainly depends on the IRF4 transcription factor, and the cells typically express the surface marker $BDCA1^+$ ($CD1c^+$) in humans or $CD11b^+$ $CD4^+$ $CD8^-$ in mouse (Castell-Rodríguez et al., 2017). More recently, single-cell RNA sequencing experiments have revealed further phenotypic markers for cDC2, which include CLEC10A, FCGR2B, and FCER1A, thereby discriminating them from moDC and other types of cells (Shan Pang et al., 2020). CLEC10A is a C-type lectin receptor that was originally reported to be expressed on immature Mo-DC. More recently it has been discovered to be associated with enhancing the activation of antigen-specific $CD8^+$ T cells and it was also reported as a specific marker of human $CD1c^+$ DC (Heger et al., 2018; Tang et al., 2022).

Interestingly, this receptors has been also reported to be rapidly internalized by human $CD1c^+$ DC, which could contribute to antigen-targeting approaches (Heger et al., 2018).

Moreover, a recent study by Ashour et al. demonstrated that two subdivisions of cDC2 are present in vivo, which depend on different transcription factors for their development, including mainly Klf4 and Notch2 (Ashour et al., 2020). These subdivisions mainly depend on the expression of surface molecule Endothelial cell-selective adhesion molecule ($ESAM^{)}$. $ESAM^{low}$ $CD11b^+$ cDC2 and $ESAM^{hi}$ $CD11b^+$ cDC2 have been discovered that are dependent on Klf4 and Notch2 transcription factors, respectively (Ashour et al., 2020; Brown et al., 2019). In addition, it has been found that expression of this adhesion molecule contributes to the function or expression of other surface molecules by cDC2. For example, $ESAM^{hi}$ cDC2 have been identified to express higher levels of MHC-II than $ESAM^{low}$ cDC2, which therefore could lead to stronger stimulation of CD4+ T cells (Saito et al., 2022).

Interestingly, cDC2 express various type of pattern recognition receptors such as TLR2, 5, and 6 and also intracellular receptors such as Retinoic Acid-Inducible Gene (IRIG-I), which support recognition of a broad spectrum of pathogens to stimulate Th2 and Th17 response (Schlitzer et al., 2015). Moreover, cDC2 has been identified to secrete various ranges of cytokines, including IL-12p70, IL-1β, IL-6, and IL-23 in response to several stimuli (Leal Rojas et al., 2017). Due to all these heterogeneous subsets of cDC2, they exert various regulatory and stimulatory effects on the immune system. Until to date, the heterogeneity of cDC2 subsets is considered to be controversial, with many new subsets identified; see Table 1.2 for an overview of the most reported surface markers in all cDC2s and DC3.

There are at least two main subsets of cDC2 based on the expression of CD5 and CD163 molecules. $CD163^-$ $CD5^+$ cDC2 and $CD163^+$ $CD5^-$ cDC2, each with a distinct transcriptional regulation and gene expression profile (Collin and Bigley, 2018; Yin et al., 2017). However, the functional differences between each subset need to be better understood. One study on mice with CD5-deficient DC and wild-type DC determined that the presence of CD5 on DC suppresses the production of IL-12 and exerts a regulatory influence on their capacity to activate T cells (Li et al., 2019). Moreover, it has been determined that $CD5^+$ cDC2 has a higher ability to migrate to the T cell area than the $CD5^-$ subset with higher expression of the CCR7 migratory molecule in the human (Yin et al., 2017). On the other hand, CD163 is a scavenger receptor that has been found to be highly expressed on macrophages. This receptor is involved

in many biological functions, including cell adhesion and tolerance induction (Onofre et al., 2009).

1.3.1.1.3 CD163^{+} CD14^{+} CD1c^{+} (DC3)

Recently, single-cell RNA sequencing on human myeloid DC and monocytes identified a new subtype of DC with a characteristic feature between CD1c^{+} cDC2 and monocytes, but they are functionally and developmentally different from them (Villani et al., 2017). This DC subtype is called DC3 and is mainly characterized by CD1c^{+} CD163^{+} and CD14$^{+/-}$ expression, see table 1.2.

Moreover, DC3 arise by an individual pathway activated by GM-CSF from MDP, independent from IRF8^{high} CDP (that give rise to both cDC1 and cDC2) and cMoP (Bourdely et al., 2020; Cytlak et al., 2020). Notably, cDC2 stimulated with GM-CSF does not give rise to DC3 *in-vitro* (Bourdely et al., 2020). Activated DC3 express CCR7 migratory molecules and secrete high amounts of pro-inflammatory cytokines IL-1β, IL-23, IL-27, IL12p70, and TNF-α as well as anti-inflammatory cytokine IL-10 (Bourdely et al., 2020; Cytlak et al., 2020). DC3 transcriptionally and functionally possess a pro-inflammatory capacity with strong priming of naïve CD4^{+} T cells towards Th2 and Th17. They were also identified to have a specific ability to prime tissue-resident memory CD8^{+} T cells (Bourdely et al., 2020).

In addition, a study by Dutertre and colleagues identified the accumulation of this proinflammatory DC3 subset in the peripheral blood of systemic lupus erythematosus (SLE) patients, which correlated with the activity of the disease (Dutertre et al., 2019). Furthermore, DC3 was also found in the peripheral blood of patients with severe COVID-19 (Segura, 2022; Winheim et al., 2021). Thus, this DC population could contribute to the pathogenicity of these diseases.

Surface marker	cDC2 subset DC2		DC3
	CD5+	**CD5-**	
CD1c	++	+	+
CD163	-	-	+
CD14	-	-	- /+
BTLA	+	+	-
MHC-II	++	++	+
CD11c	++	++	++

Table 1.2: Overview of cell surface marker of cDC2 subtype and DC3 reported in the literature (Collin and Bigley, 2018; Cytlak et al., 2020; Dutertre et al., 2019; Heger et al., 2020).

1.3.1.2 Plasmacytoid DC (pDC)

Plasmacytoid DC are a unique DC type initially found in human LN. They were discovered to be the main producer of type I interferons, which mediate an antiviral innate immune reaction to many viral infections through the expression of TLR7 and TLR9 pattern recognition receptors (Collin and Bigley, 2018; Manh et al., 2013; Swiecki and Colonna, 2015). pDC can also contribute to the adaptive immune response by producing IDO and TGF-beta, which are involved in the induction of tolerance (Shan Pang et al., 2020). The development of pDC is mainly controlled by the expression of transcription factor E2-2 (Castell-Rodríguez et al., 2017). In contrast to cDC, pDC have been determined to exert limited interaction with naïve T cells (Fucikova et al., 2019), possibly due to the low expression of co-stimulatory molecules (Merad et al., 2013). Unlike other DC, pDC have low expression of CD11c (Merad et al., 2013). In addition, the distribution of pDC in blood and lymphoid organs is similar to cDC, although they are present in lower numbers in other tissues (Obregon et al., 2017).

1.3.1.3 Monocyte-derived DC (moDC)

Monocytes are essential precursors that give rise to moDC, the most abundant type of APCs in tissue during inflammation (Tang-Huau et al., 2018). In addition to that, over the last few years, moDC have been documented to be the most common *in vitro* model of DC (Noubade et al., 2019, p. 1; Obregon et al., 2017). moDC have a unique capacity to cross-present the Ag to cytotoxic T cells directly from peripheral tissues during infection and inflammation, which is considered to be a crucial feature that attracted researchers to design an anti-tumor-based immunotherapy (Tang-Huau et al., 2018).

Human moDC can be generated *in vitro* from monocytes upon treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Posch et al., 2016). Various phenotypic markers are expressed by moDC, such as $CD14^+$, $CD11c^+$, $CD1c^+$, and $HLA\text{-}DR^+$(Devi and Anandasabapathy, 2017; Patente et al., 2019). In terms of transcription factor usage, intriguingly, moDC is similar to cDC2, which depends on IRF4 for their differentiation (Patente et al., 2019).

1.3.2 DC maturation

The central role of DC in priming and directing T cells toward an immunogenic or tolerogenic response mainly depends on the maturation state of DC (see figure 1.3**)** (Devi and Anandasabapathy, 2017). It is also found that the microenvironments around DC (steady-state or inflammation) and the localization (blood, lymphoid organ, and peripheral tissues) are responsible for the functional difference between immunogenic and tolerogenic DC, as well as the maturation state (Kim et al., 2018; Oppenheim et al., 2002; Švajger and Rožman, 2018). Each maturation state endows DC with specific functional capabilities that lead to a particular immune response (Oppenheim et al., 2002). Moreover, during the maturation and activation of DC, several changes occur in the expression of surface molecules and cytokine production that contribute to their ability to interact with exogenous Ag and with other cells (Castell-Rodríguez et al., 2017).

DC in an immature state (immDC) are responsible for the uptake and processing of exogenous and endogenous Ag by endocytosis or pinocytosis (Schülke, 2018). Thus, they express a large number of PRRs to sense and capture a broad spectrum of Ag. In contrast, they have lower expression of MHC and co-stimulatory molecules that contribute to the priming of T cells,

making them inferior to mature DC (matDC) in producing an immune response (Yoo and Ha, 2016). Upon activation by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or damage-associated molecular pattern (DAMPs), immDC undergo a maturation process (Mbongue et al., 2017).

Throughout maturation, DC upregulates MHC and co-stimulatory molecules (CD83, CD86) as well as chemokine receptors such as CCR7, thus promoting migration from their place in peripheral tissues to the closest lymph node (Kim and Kim, 2019; Mbongue et al., 2017). Then, upon arrival in the T cell area, matDC present MHC/peptide complexes to naïve T cells to promote differentiation of effector T cells to produce the desired immune responses (Mbongue et al., 2017). As previously mentioned, three necessary output signals are required by matDC to induce the T cell immune response (Kaliński et al., 1999).

Furthermore, during maturation, DC undergo several morphological changes such as the formation of dendrites and phenotypical modifications that correspond to their function to induce immune response (Alloatti et al., 2016; Kim and Kim, 2019).

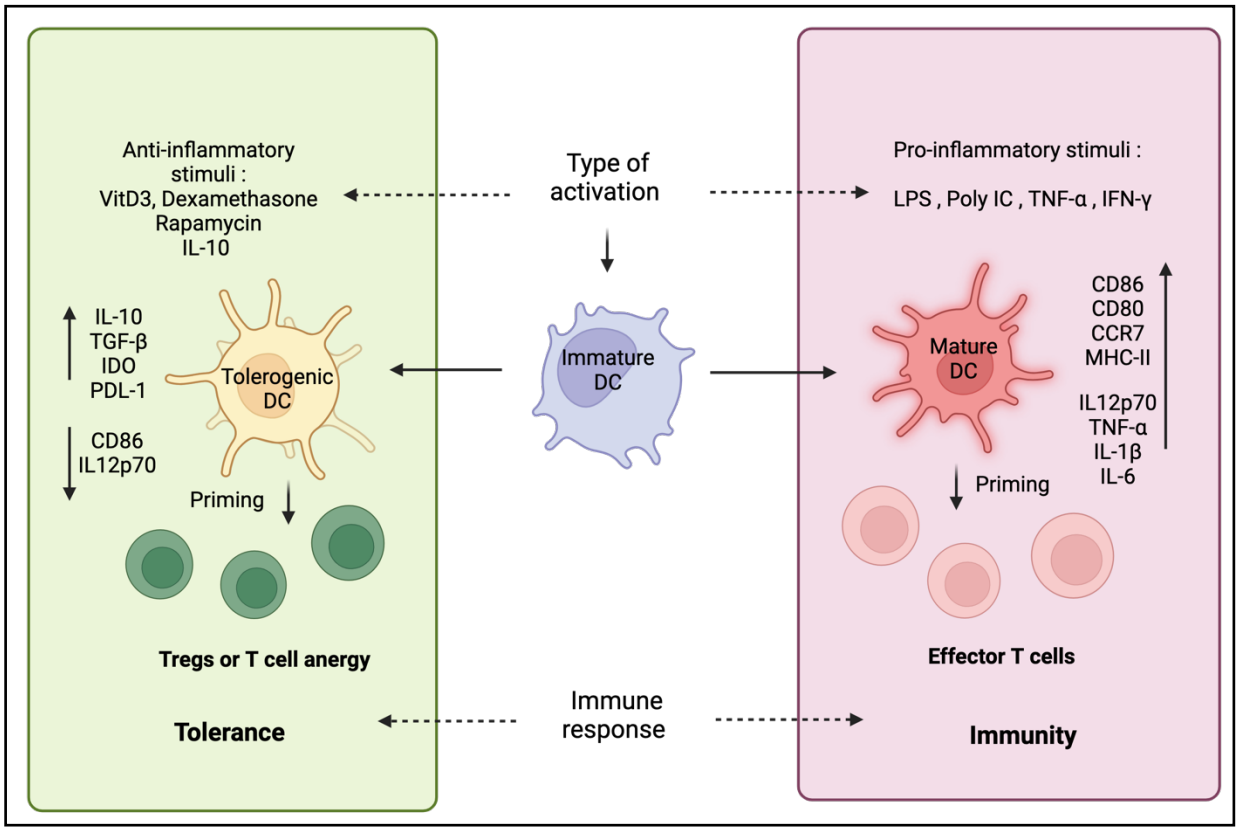


Figure 1.3: Role of DC in balancing tolerance and immunity. The differentiation of immature DC into tolerogenic DC or mature DC mainly depends on the cytokines

microenvironments and other molecules such as LPS and anti-inflammatory agents (Fucikova et al., 2019). Created with BioRender.com

1.4 Tolerogenic dendritic cells (tolDC)

1.4.1 Main characteristic and function

Monocyte-derived tolerogenic dendritic cells (tolDC) exhibit a semi-mature phenotype with high to intermediate expression of MHC-II, low expression of co-stimulatory molecules such as (CD80/CD86) and maturation marker CD83, and increased expression of co-inhibitory molecules such as programmed death ligand 1 and 2 (PDL-1, PDL-2) and Ig-like transcript 3 and 4 (ILT3, ILT4), and increased secretion of anti-inflammatory cytokines such as IL-10 and TGF-ß (Anderson et al., 2017; Macedo et al., 2012). Therefore, these features of tolDC contribute to promoting tolerance rather than immunity through various mechanisms, which include mainly induction of T cell anergy, T cell apoptosis, and increase in regulatory T cell expansion and induction (De Laere et al., 2018; Horton et al., 2017; ten Brinke et al., 2019; Yoo and Ha, 2016).

In addition, the suppressive properties of tolDC are mainly mediated by the interaction between co-inhibitory molecules such as PDL-1 on the surface of tolDC with their receptor PD-1 on the surface of T cell. Further to these inhibitory molecules, anti-inflammatory cytokines contribute to the suppressive microenvironments and induce the differentiation of Tregs (Yoo and Ha, 2016). Among these cytokines, IL-10 is a critical cytokine that prevents excessive immune response to exogenous pathogens, which helps prevent chronic inflammation and tissue damage (Schülke, 2018). Figure (1.4) summarizes the main tolDC phenotype and functions.

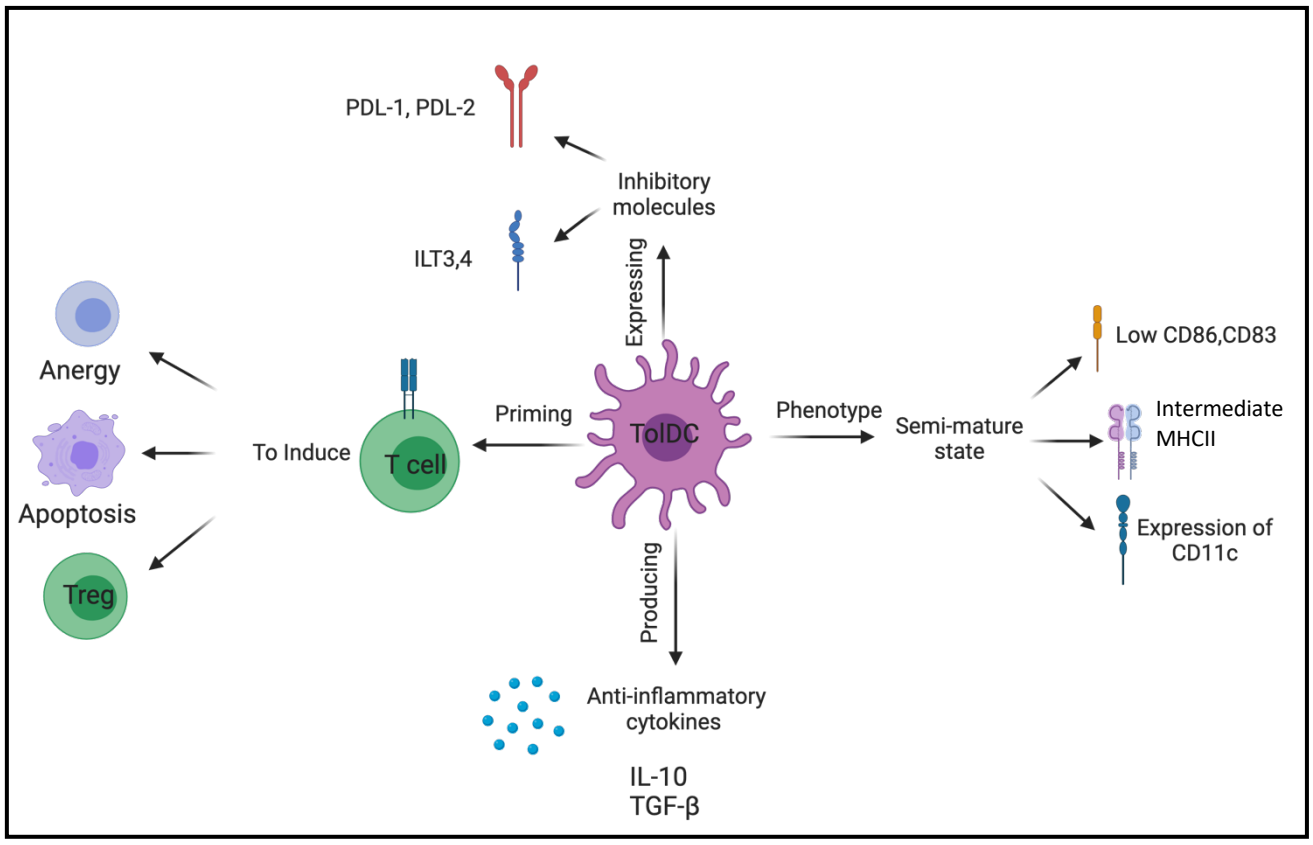


Figure 1.4: The main characteristics feature of tolDC. TolDC has a semi-mature phenotype with low expression of co-stimulatory molecules and intermediate to high expression of MHCII molecule . They also express a wide range of co-inhibitory molecules that help produce an effective priming to naïve $CD4^+$ T cells. Besides that, tolDC produces several immunomodulatory cytokines, such as IL-10 and TGF-β that support the induction of Tregs and tolerance. Created with BioRender.com

1.4.2 Generation of tolDC in vitro

Extensive research has shown that tolDC can be generated from either bone marrow or peripheral blood monocytes in specific media supplemented with GM-CSF and IL-4 and tolerogenic agents such as immunosuppressive drugs or cytokines. These agents help promote the tolerogenicity of DC by inhibiting maturation and/or regulating the cytokine secretion profile (Thomson and Ezzelarab, 2018). Besides that, these DC can also be stimulated with inflammatory stimuli such as LPS to acquire antigen-processing and enhance migratory ability, which is both essential for tolDC to migrate to the draining lymph node and present Ag to T cells (Anderson et al., 2009). However, it has been found that even after LPS stimulation, tolDC has limited migration to the T cell area in vivo (Cooke et al., 2022). Each one of the tolerogenic

agents has specific beneficial effects as well as drawbacks effect, which will be discussed in detail below.

1.4.2.1 Pharmacological agents

In recent years, various immunosuppressive drugs have been used to generate DC with tolerogenic properties in vitro, such as dexamethasone, rapamycin, and the active form of Vitamin D3 (VitD3) (Anderson et al., 2017; Kim et al., 2018). However, many studies found that distinctive immunosuppressive drugs vary in their immunomodulatory effects (Sordi et al., 2006). For example, previous studies have shown that dexamethasone and VitD3 inhibit the maturation and differentiation process of DC (Nikolic and Roep, 2013; Ritprajak et al., 2019), whereas rapamycin affects DC functional activity by controlling the metabolic activity of DC (Snyder and Amiel, 2019; Sordi et al., 2006). Therefore, combining therapy may produce clinically successful DC with tolerogenic properties.

Dexamethasone (Dex) is a synthetic glucocorticoid with potent immunoregulatory effects on immune cells, especially DC. It has a range of anti-inflammatory effects through binding to its receptors in the nucleus of DC that lead to inhibition of the NF-kB pathway, leading to inhibition of maturation and inducing a tolerogenic state (Kim et al., 2018; Phillips et al., 2017; Švajger and Rožman, 2018). It also strongly inhibits monocyte to DC differentiation through suppression of co-stimulatory molecules such as CD80, CD86, and the maturation marker CD83, as well as hinders the expression of essential transcription factors such as IRF4 (Švajger and Rožman, 2018). Therefore, to generate a tolDC, Dex is added on day three after the monocyte differentiates to DC (Hilkens et al., 2023). In addition, it suppresses the production of IL12p70 but enhances the production of IL-10 (Raker et al., 2015).

Rapamycin (sirolimus) is an immunosuppressive macrolide that was first used to prevent allograft rejection through inhibition of T cell proliferation by blocking the mammalian target of rapamycin (mTOR) signaling pathway (Hackstein and Thomson, 2004; Stallone et al., 2016). In addition, it has been shown that rapamycin has a unique immunoregulatory activity as well as the enhanced surface expression of CCR7 on DC, leading to increased migration of DC to lymphoid tissue, which is mainly dependent on mTOR signaling pathway (Sordi et al., 2006). mTOR serves as a crucial regulator of glycolytic metabolism and is pivotal in orchestrating metabolic activity in the DC (Snyder and Amiel, 2019).

VitD3 (calcitriol) is one of the most profoundly studied agents for induction of tolDC (Švajger and Rožman, 2018). It has immunoregulatory properties through activation of its receptor (VDR), which is expressed by many APCs, including monocytes and DC (Iberg and Hawiger, 2020). The effect of VitD3 treatment on DC is to inhibit the maturation process and increase IL-10 production, resulting in the generation of DC with favourable tolerogenic properties (Suuring and Moreau, 2021; Van Brussel et al., 2014). In addition, VitD3-treated DC show a high level of PD-L1 besides ILT3 and ILT4 expression, promoting T cell apoptosis and Treg differentiation (Horton et al., 2017; Unger et al., 2009).

All trans Retinoic acid (RA) is a vitamin A metabolite important in regulating mucosal immune response (Cassani et al., 2012). It has been found that treatment of DC with RA results in establishing tolDC that produces the same metabolite (i.e., RA), which in turn promotes the $Foxp3^+$ Tregs expansion (Di Caro et al., 2013).

1.4.2.2 Cytokines

Extensive research has shown that various immunomodulatory cytokines could be used to generate clinically successful DC with tolerogenic properties, which are either derived from bone marrow or monocytes (Švajger and Rožman, 2018; Yoo and Ha, 2016). Each of these cytokines has specific immune regulatory mechanisms that change the microenvironment around DC in favor of a tolerogenic response (Torres-Aguilar et al., 2010). The most critical cytokines that have been extensively shown to induce tolerance either by promoting T cell anergy or induction of Tregs are IL-10 and TGF-ß, respectively (Schülke, 2018).

Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that plays a significant role in generating tolerance through either stimulation of a tolerogenic state in DC or inducing Treg development (Horton et al., 2017; Švajger and Rožman, 2018). One of the preliminary studies of using IL-10 immunosuppressive agent to establish an *in vitro* tolDC showed that IL-10-treated DC displayed a low expression level of co-stimulatory molecules such as CD86 and notably a decrease in allo-stimulatory effects (Švajger and Rožman, 2018).

In addition, it has been found that treatment of DC with IL-10 promotes the secretion of high amounts of IL-10, TGF-β, and PGE_2 (prostaglandin E2), immune inhibitory receptors including ILT3 and ILT4, besides a decrease in the production of IL-6 and IL-12, which facilitates the induction of Tregs (Castell-Rodríguez et al., 2017; Comi et al., 2018; Horton et al., 2017). Furthermore, IL-10 can be produced by and have pleiotropic activity on various types of cells, such as tolDC and FOXP3 Tregs, which both lead to establishing a peripheral tolerance (Schülke, 2018).

Transforming growth factor-beta (TGF-ß)

TGF-β is a pleiotropic cytokine that can be produced by various cells, such as DC, which play a crucial role in regulating immunity (Worthington et al., 2012). Similar to IL-10, TGF-β can be both secreted and have a regulatory effect on tolDC. TGF-β exerts its tolerogenic effect on DC by downregulating MHC and co-stimulatory molecules beside upregulation of PDL-1 inhibitory molecule, resulting in decreased Ag presentation and T cell anergy, respectively (Domogalla et al., 2017).

Overall, these tolerogenic agents have specific effects on either DC maturation or function, including cytokine production, resulting in generating DC with stable tolerogenic activity. For example, it has been found that TGF-β and VitD3 both decrease the immunogenic functional capacity of DC through altering cytokines and chemokines production, such as IL-12 pro-inflammatory cytokine (Lyakh et al., 2005). In addition, it has been suggested that a combination of two tolerogenic agents may boost the effect of each other. For example, combining dexamethasone and VitD3 in the generation of tolDC boosts the tolerogenic potential of each other (Anderson et al., 2017; Švajger and Rožman, 2018). The combination of VitD3 with IFN-γ is another recent attractive combination therapy that has shown a synergistic effect in producing tolDC with low T cell stimulatory capacity and high IL-10 production, leading to Treg induction (Švajger and Rožman, 2019).

1.4.2.3 Genetic engineering

Another method for generating tolDC in vitro is the genetic engineering of DC to express immunosuppressive molecules. An example of this method is the engineering of DC with a retroviral vector to produce a high amount of IL-10, leading to T cell hyperresponsiveness and a reduced stimulation and proliferation of cytotoxic T cells (Kim et al., 2018; Takayama et al.,

1998). In addition, a recent study by Passeri et al. generated high IL-10-producing tolDC with a lentiviral vector co-encoding for immunodominant antigen-derived peptides and IL-10. This IL-10-engineered tolDC has been shown to produce high amounts of IL-10 that induce T regulatory type 1 (Tr1) in chimeric transplanted mice (Passeri et al., 2023).

1.5 Markers of tolerance

1.5.1 PDL-1 and PDL-2

PDL-1 and PDL-2 are the main co-inhibitory molecules that act as a ligand on the surface of DC that interact with the PD-1 receptor molecule on T cells to limit the responsiveness of triggered T cells (Zhang et al., 2019). It is believed that the PD-1/PD-L axis notably induces peripheral tolerance through limiting T cell responses, prevention of some autoimmune diseases (e.g., autoimmune diabetes), and promotion of feto-maternal tolerance (Versteven et al., 2018). The mechanism of tolerance induction by this axis is mainly due to two important mechanisms. First, induction of T cell anergy with lower IL-2 secretion after binding of PD-1 with its ligand leads to inhibition of T cell proliferation and differentiation (Bishop et al., 2009). Second, induction of Treg development and function, which might help sustain the immune hemostasis (Francisco et al., 2010).

1.5.2 ILT3 and ILT4

ILT3 (immunoglobulin-like transcript 3) and ILT4 (immunoglobulin-like transcript 4) belong to the Ig superfamily. They are receptors containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) expressed on the surface of professional APCs such as DC and macrophages (De Goeje et al., 2015). Also, it has been discovered that they are essential inhibitory receptors expressed on the surface of tolerogenic DC (Stallone et al., 2016). ILT3 exerts its inhibitory function on T cells through the cell-to-cell contact-dependent system leading to tolerance induction by inducing T cells anergy (De Goeje et al., 2015) as well as promotion of the Treg development (Penna et al., 2005; Vlad et al., 2010). Many substances can induce the expression of ILT3 on the surface of DC, including VitD3 (Penna et al., 2005), IL-10, and IFN-α (Manavalan et al., 2003).

1.5.3 MERTK

MERTK is a member of the tyrosine kinase receptor (TAM family) that has a crucial role in immune regulation. It is mainly expressed on the surface of M2 macrophages, NK, and tolerogenic dendritic cells (Cabezo´n et al., 2015). The primary ligands of the MERTK receptor are growth arrest-specific 6 (Gas6), which binds with low affinity, and protein S (PROS1), which binds MERTK with high affinity. Both receptors were expressed on the surface of activated T cells. MERTK was found to be highly expressed on the surface of dexamethasone-treated tolDC (Cabezo´n et al., 2015; Giroud et al., 2020). Cabezo´n and his group determined that MERTK has a role in inhibiting naïve $CD4^+$ T cell activation, leading to tolerance induction. (Cabezo´n et al., 2015). MERTK plays a crucial role in facilitating the induction of immune tolerance through two key mechanisms. First, post receptor signaling, after engagement to its ligand on the surface of activated T cells, leading to inhibition of NF-κB signalling thereby diminishing the pro-inflammatory cytokine response (Lahey et al., 2022). Second, through phagocytosis of apoptotic cells (a process known as efferocytosis), which prevents the release of pro-inflammatory signals and promotes an anti-inflammatory response, leading to tolerance induction (Lahey et al., 2022). Researchers discovered that mice deficient in MERTK (MERTK knockout) develop a condition resembling lupus-like autoimmunity. This phenomenon occurs due to the gradual accumulation of apoptotic cells in organs such as the spleen and thymus, thereby triggering an inflammatory response and the release of self-antigens, which can activate the immune system and lead to the production of autoantibodies (Shao et al., 2010).

1.5.4 Indolamine 2,3-dioxygenase (IDO)

IDO is a rate-limiting enzyme that is responsible for degrading the essential amino acid tryptophan, which leads to the production of immunosuppressive metabolites, such as kynurenine, that contribute to tolDC activity (Hwang et al., 2005; Mellor et al., 2017). Kynurenine binds and stimulates AhR that promotes Treg differentiation leading to a tolerance induction (Matteoli et al., 2010). Thus, IDO upregulation by DC is crucial for converting DC into tolDC with immunosuppressive phenotype through increasing expression of inhibitory receptors such as ILT3 and ILT4 promoting the differentiation of Tregs (Hasegawa and Matsumoto, 2018; Mellor et al., 2017). Moreover, another immune regulatory mechanism of IDO is inducing tolerance to apoptotic self-antigens, which inhibit the development of systemic

autoimmune diseases (Ravishankar et al., 2012). In addition, the IDO gene is known to contain elements that respond to IFN leading induction of IDO expression by DC. Hence, IFN-I and IFN-II (produced at the site of inflammation) are both known to be potent inducers of IDO expression (Mellor et al., 2017).

1.5.5 Forkhead box protein 3 (Foxp3)

Foxp3 is a nuclear transcription factor that is an essential regulator for the development and maintenance of Tregs that in turn are responsible for suppressive immunomodulatory effects (Dominguez-Villar and Hafler, 2018; Lu et al., 2017). Deficiency or mutation in the Foxp3 gene can lead to a lack of Treg differentiation and development, thereby causing a defect in tolerance induction and hence, the development of the autoimmunity (Lu et al., 2017). For example, Foxp3 mutation in humans can lead to fetal immune dysregulation poly-endocrinopathy enteropathy X-linked (IPEX) syndrome (Lu et al., 2017).

1.5.6 FAS/FAS ligand

Fas and Fas Ligand (FasL) belong to the tumor necrosis factor (TNF)-receptor and TNF family, respectively. Fas is a transmembrane protein of type 1, possessing a death domain within its cytoplasmic region (Fesik, 2000). When Fas (on the surface of T cell) binds with FasL (on the surface of DC), it triggers a series of caspase activations, initiating the process of T cell apoptosis leading to peripheral deletion of autoreactive T cell (Volpe et al., 2016). Recently, it has been found that the immunosuppressive function of tolDC can be enhanced after ligation of FAS with its ligand via the ERK/β-Catenin pathway, suggesting a negative feedback route of this axis in the immune response (Qian et al., 2013).

1.5.7 Cytotoxic T-lymphocyte antigen 4 (CTLA-4)

CTLA-4 is a central negative regulator of T-cell responses. It acts by binding to co-stimulatory receptors (CD80, CD86) on the surface of DC with higher affinity than CD28 (Laurent et al., 2010; Paterson et al., 2015). Hence, CTLA-4 inhibits T cell activation by disrupting signal 2 delivery, resulting in a state of T cell anergy (Xing and Hogquist, 2012). Furthermore, CTLA-4 is also identified to be constitutively expressed on the surface of Treg (Ovcinnikovs et al., 2019; Paterson et al., 2015), suggesting its contribution in their regulatory function through

transendocytosis of co-stimulatory molecules (CD86 and CD80) on the surface of DC (Ovcinnikovs et al., 2019). In addition, it has been determined that mutation in CTLA-4 will decrease this protein's expression by Treg in patients with autosomal-dominant immune dysregulation syndrome (Schubert et al., 2014).

1.5.8 Lymphocyte activation gene 3 (LAG-3)

LAG-3 is an inhibitory receptor that is expressed on the surface of activated T cells and binds to its ligand MHC-II on the surface of DC. Engagement of this receptor/ ligand will lead to downregulating T cell activity and regulating self-reactivity through decreasing cytokine production in effector T cells and promoting differentiation of regulatory T cells (Tregs) (Rodríguez-Guilarte et al., 2023). In addition, it has been identified that LAG-3 mediates immunometabolic programming of T cells through limiting oxygen consumption and preventing T cell proliferation, leading to tolerance induction (Previte et al., 2019).

1.5.9 B and T lymphocyte attenuator (BTLA)

It is a co-inhibitory receptor expressed on various immune cells including T cells, B cells and DC. BTLA plays a crucial role in regulating immune responses by transmitting inhibitory signals upon interaction with its ligand, HVEM (Herpes Virus Entry Mediator), also known as TNFRSF14. This ligand is expressed on various cell types, including T cells, B cells, dendritic cells, and NK cells. Upon receptor/ ligand interaction, an inhibitory signal will be induced in T cells leading to tolerance induction. Another mechanism of inhibitory signal by this receptor/ ligand is through inhibiting the secretion of cytokines including IL-2, IFN-γ, IL-4, and IL-10 leading to downregulation of immune response (Kamali et al., 2023).

1.5.10 T-cell immunoglobulin and mucin domain 3 (TIM-3)

TIM-3 is a cell surface protein expressed on various immune cells, including T cells, natural killer cells, DC, and macrophages. This immune checkpoint involves interaction with four distinct ligands: galectin-9, phosphatidylserine (PtdSer), high mobility group protein B1 (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) (Kamali et al., 2023). Upon interaction with its ligand, TIM-3 delivers inhibitory signals that dampen immune activation and contribute to the termination of immune responses through

inhibition of the production of many pro-inflammatory cytokines, including TNF-alpha and IFN-gamma. In addition, Increased levels of Tim-3 expression have been observed on $CD4^+$ regulatory T cells (Tregs) within human and murine tumor environments, correlating with the presence of Foxp3 expression (Kamali et al., 2023).

1.5.11 T-cell immunoreceptor with Ig and ITIM domains (TIGIT)

TIGIT is a protein found on the surface of T cells and NK cells. It belongs to the immunoglobulin superfamily and plays a role in regulating immune responses by transmitting inhibitory signals upon interaction with its ligands CD112, CD112 and CD155, which are expressed on antigen-presenting cells and tumor cells (Harjunpää and Guillerey, 2020). When TIGIT binds to these ligands, it can inhibit T cell activity through directly delivering inhibitory signals to the effector cell. In addition, Tregs have been reported to express TIGIT, which contribute to their suppressive function (Kamali et al., 2023).

1.5.12 V-domain Ig suppressor of T-cell activation (VISTA)

VISTA is a checkpoint protein that belongs to the B7 family of immune regulators. It is mainly expressed on myeloid cells including macrophages and DC. VSIG-3 is a main ligand that has been found on the surface of T cells and interacts with VISTA, which therefore promotes inhibitory or suppressive function to T cells (Kamali et al., 2023).

1.6 DC migration

Localization of DC is mainly controlled by chemotactic signals that are produced upon interaction of specific chemokine receptors on DC with chemokine ligands, which leads to either recruitment of immature DC from BM to peripheral tissues or migration of mature DC from the periphery to lymphoid tissues (Hackstein and Thomson, 2004; Tiberio et al., 2018). This complex mechanism of ideal DC localization helps maintain an appropriate immune response in pathological and steady-state environments. For this reason, understanding chemokines and their role in each state will help to discover a new target for therapeutic intervention in cancer and autoimmunity (Tiberio et al., 2018).

Chemokines are small proteins that have a crucial role in cell migration. A considerable amount of literature has been published on the importance of various chemokines and chemokine receptors responsible for DC migration. For instance, CCR5 is a crucial molecule that directs DC to the site of inflammatory lesions (De Laere et al., 2018). It has also been found that the CCR5 ligands (CCL3, CCL4, and CCL5) were significantly expressed on the surface of inflammatory lesions such as on the lesions of multiple sclerosis (MS) and central nervous system (CNS) inflammation (De Laere et al., 2018). On the other hand, CCR4 is an important chemokine receptor overexpressed in hematological malignancies such as adult T-cell leukaemia. Hence, inhibition of this overexpressed receptor by a human anti-CCR4 antibody (Mogamulizumab) can directly destroy tumor cells, but several immunological responses could result as a side effect of this process (Mollica Poeta et al., 2019).

Furthermore, the chemokine receptor profile exhibited by DC aids in monitoring their maturation status and assessing the migratory capacity of generated tolDC. Notably, in regards to tolDC, it has been found that expression of the crucial chemokine receptors that are mainly responsible for migration and localization (CCR7 and CXCR4) are downregulated on the surface of DC in tolerogenic state (Ritprajak et al., 2019). Therefore, stimulation of tolDC with inflammatory stimuli such as LPS is essential to upregulate these important receptors (Anderson et al., 2009; Ritprajak et al., 2019a). However, CCR7 expression may still not be as high as on matDC, leading to the limited migratory ability of tolDC that should be considered. So, in this section, the main chemokine receptors and ligands that regulate DC migration will be discussed in detail.

1.6.1 Chemokine receptors (CCR7)

CCR7 is a crucial chemokine receptor that is upregulated on the surface of DC during maturation in response to various environmental factors such as inflammatory cytokines, infectious agents, and some stimuli (e.g., pharmacological substances). It interacts with its ligands CCL19 and CCL21, which are mainly expressed on the surface of stromal cells in the T cell area of the LN (Riol-Blanco et al., 2005; Tiberio et al., 2018). This process will lead to migration of DC to the lymph node to prime T cells (Riol-Blanco et al., 2005). CCR7 upregulation is mainly required for two main purposes. The first one is inducing the chemotactic signals and the second one is controlling the speed of the DC migration (Riol-Blanco et al., 2005). These two functions of CCR7 are made through activating two

independent signaling pathways in human DC. The first signaling pathway controls the chemotaxis by activating MAPK and G protein. In contrast, the second signaling pathway regulates the speed of DC migration by activating Rho/Pyk2/cofilin (Riol-Blanco et al., 2005).

Recently, it has been found that each one of the CCR7 ligands (CCL19 and CCL21) has a different chemotactic effect and both are highly expressed on the surface of the secondary lymphoid organ (SLO), which includes LN and the spleen (Ricart et al., 2011). CCL21 is mainly required to direct DC to and along the lymphatic vessels, whereas both CCL19 and CCL21 are involved in the migration of DC in the LN (Tiberio et al., 2018).

Within recent years, it has been found that treatment of DC with rapamycin to generate tolDC is significantly associated with an up-regulation of CCR7 (Sordi et al., 2006; Stallone et al., 2016). Thus, this will likely lead to an increase in the migration of DC to LN and the priming of naïve T cells to generate an adaptive immune response. Moreover, it has been discovered that CCR7-dependent DC migration regulates the induction of organ-specific Tregs, which will result in promoting peripheral tolerance (Tiberio et al., 2018).

1.6.2 CXCR4

Despite the fact that CXCR4 chemokine receptors are mainly expressed on the surface of immDC with other chemokine receptors (CCR1, CCR2, CCR5, and CCR6), all these receptors will be downregulated upon maturation of DC in response to inflammatory agents, except for CXCR4 (Ricart et al., 2011). CXCL12 is the widely expressed ligand of CXCR4 that upon binding will allow the migration of DC to various peripheral tissues (DÃ¶ring et al., 2014).A summarized overview of important tolerogenic phenotype and chemokine receptor expression in differently treated moDC is illustrated in Table 1.3 below.

DC type	Inhibitory receptors	Chemokine receptors expression	Cytokine secretion profile	Main functional properties
Dex-tolDC	Low PDL-1 and ILT3 High MERTK	Intermediate CCR7 CXCR4	High IL-10 Low IL-12p70	Low IFN-γ secretion by T cell And induction of Tr1
Rapa-tolDC	Not reported	High CCR7	low IL-10 and high IL-12p70	Induce FOXP3+ Tregs and T cell apoptosis
VitD3-tolDC	High PDL-1 and ILT3	High/intrmediate CCR7	High IL-10 &TGF-β, low IL12p70 and Intermediate TNF-α	Induce T cell apoptosis, Low IFN-γ secretion by T cell and Ag specific Treg induction
IL-10-tolDC	High ILT3, ILT4 and HLA-G	Low CCR7	High IL-10	Induction of type 1 Tregs (Tr1) and Ag-specific T cell anergy
VitD3/Dex-tolDC	High PDL-1 and TLR2 Intermediate ILT3 and MERTK	Intermediate/low CCR7	High IL-10 &TGF-β Low IL12p70, IL-1β and IL6	Ag-specific Treg induction

Table 1.3: Overview of the main surface molecules expression and chemokine receptors in different treated Human moDC and its function (Anderson et al., 2017; Cabezo´n et al., 2015; Colvin et al., 2008; Nikolic and Roep, 2013; Sordi et al., 2006, 2006; Spiering et al., 2019; Suuring and Moreau, 2021; Unger et al., 2009).

1.7 Alternative Approach for Generation of tolDC *in vitro*

Interestingly**,** it has been found that the characteristic features of tolDC, as well as the capability to promote induction of Tregs (Amodio et al., 2015) could be different depending on the tolDC generation protocol (Ritprajak et al., 2019a). Therefore, both the cellular source (monocyte or $CD34^+$ stem cell) and the tolerogenic agent used in establishing DC tolerogenicity may contribute to the generation of competent, stable, and efficient tolDC applicable to use as immunotherapy. This section will discuss a new idea for the in vitro generation of tolDC.

1.7.1 Stem cell-derived tolerogenic dendritic cell (Sc-tolDC)

As mentioned before, DC can be cultured from $CD14^+$ monocytes in the presence of GM-CSF and IL-4 for seven days, giving rise to moDC (Shan Pang et al., 2020). They can also be generated from $CD34^+$ hematopoietic stem cells in the presence of GM-CSF and TNF-alpha for about 14 days (Paczesny et al., 2007). Unlike the generation of DC from monocytes, the generation of DC from $CD34^+$ cells can give rise to conventional dendritic cells (cDC), which have a unique ability to prime and educate naïve T cells (Paczesny et al., 2007). In addition, it has been reported that DC derived from $CD34^+$ hematopoietic precursors possess a greater expression of chemokine receptors than moDC, which could increase the migratory ability to T cell area (Syme et al., 2005). However, it is still unclear if one of these DC generation protocols is better than the other regarding regeneration of tolerance and reliability to use in cellular immunotherapy. Hence, further investigation and comparison between these cellular sources is needed to establish an effective and safe tolDC immunotherapy.

1.7.2 Peripheral blood-derived conventional dendritic cell-based tol-DC

Another interesting possible alternative way for a generation of tolDC is from cDC that are isolated from peripheral blood. Although the number of cDC that are circulating in the blood is limited, there are recent research studies that have highlighted the use of blood-derived cDC1 (Johnson et al., 2022; Noubade et al., 2019) and cDC2 (Schreibelt et al., 2016) in the context of cancer immunotherapy. These studies have proven the efficacy and feasibility of using cDC for cancer immunotherapy. For example, a study by (Schreibelt et al., 2016) identified that vaccination of IV melanoma patients with a small amount of autologous cDC2 that activated and cultured ex vivo and then loaded with tumor-associated antigens is safe and effective in

induction of anti-tumor immune response. In addition, (Canavan et al., 2021) determined the accumulation of mature stimulatory cDC2 in the synovium of RA patients.

Conversely, (Price et al., 2015) identified in mice that cDC2 can inhibit T cell proliferation by increasing the *ZBTB32* transcription factor, which inhibits T cell differentiation. Together, these findings and observations mean that cDC have heterogeneous functions in different circumstances, mainly depending on the microenvironment. Therefore, cDC could be one of the promising alternative sources of tolerogenic DC generation.

1.8 Hypothesis, Aim and Objectives

As mentioned above, many pharmacological drugs used to treat immunological diseases appear to have several unfavorable side effects, such as general immunosuppression, making the patient more susceptible to infections. Thus, extensive studies have shown the safety of using tolDC as cellular immunotherapy in different diseases, such as autoimmune diseases and GVHD.

In line with this, Hilkens and colleagues clearly showed that monocyte-derived tolDC have potent immunomodulatory capacity *in vitro* and are safe to use *in vivo*. However, several limitations have been discovered in relation to moDC. Firstly, they have shown a poor migratory ability that will prevent them from reaching secondary lymphoid organs and inducing a favourable immune response. Secondly, monocytes and monocyte-derived DC do not readily proliferate *in vitro*, limiting the number of tolDC that can be generated. Thirdly, the length of DC generation from monocytes in culture could affect the viability and sterility of the cells, as the longer the culture, the higher the chance that contamination may occur.

Therefore, we hypothesize that a solution to these problems may be switching the generation of tolDC from monocyte-derived DC products to natural, conventional DC products. The reasons for that are:

1. An essential feature of conventional DC (cDC) is the ability to migrate to the T cell area on the lymphoid tissue.
2. cDC have a unique capacity to prime and educate naïve T cell.
3. cDC can be either produced from a stem cell progenitor or easily isolated from peripheral blood.

4. cDC can be directly tolerized, reducing the length of tolDC generation in culture and, therefore will reduce the risk of microbial contamination.

Hence, this project aims to design and characterize a novel tolerogenic cDC-based therapy for the regeneration of immune tolerance.

Specific objectives are:

- To determine whether cDC1 or cDC2 is the most effective option for cDC-based tolDC therapy.
- To generate and characterize the phenotype of human cDC-based tolDC.
- To assess the migratory ability and functional capacity of cDC-based tolDC.

Chapter 2: Material and Methods

A class 2 biological hazard laminar flow air hood was used for all the *in vitro* cell culture work. Newcastle University Health and Safety guidelines for good laboratory practice were followed throughout this study.

2.1 Samples, general tissue culture reagents and buffers

2.1.1 Sample collection and ethical approval

Informed consent from healthy donors and ethical approval from the Animal Welfare, Ethical Review Body (AWERB no.633) and the Faculty of Medical Sciences Ethics Committee were obtained. Leukocyte Reduction System (LRS) Cones were procured from healthy donors at the NHS Blood and Transplant, specifically from the Newcastle Blood Donor Centre, to be utilized in the research project.

2.1.2 Lymphoprep solution

Lymphoprep (STEMCELL Technologies) is a density gradient solution used to isolate human peripheral blood mononuclear cells (PBMC). During centrifugation, granulocytes and erythrocytes sediment through the Lymphoprep medium due to their higher density, while the mononuclear cells form a layer on top of the solution.

2.1.3 Dulbecco's Phosphate-Buffered Saline (PBS)

PBS is a buffered salt solution used for the dilution and washing of mammalian cells. The Cytiva brand origination used in this thesis does not contain calcium and magnesium ions, which can cause cell clumping.

2.1.4 Fetal calf serum (FCS)

FCS is a medium supplement that provides nutrients and growth factors for cell culture. Heat inactivation at 56°C for 30 minutes is commonly performed to hinder the complement system and any potential cell growth inhibitors in culture.

2.1.5 MACS buffer

Magnetic activated cell sorting (MACS) buffer is a solution used for cell separation and isolation through a magnetic field. It is made up of PBS (without calcium and magnesium) (Cytiva), 2mM EDTA and 0.5% FCS. It is used in this project for all cell isolation protocols.

2.1.6 FACS buffer

Flow cytometry staining buffer is a saline solution based on PBS without calcium and magnesium (Cytiva) and contains 0.5% BSA, 1mM EDTA and 0.01% sodium azide. This buffer is used for all cell surface and intracellular staining of cells for flow cytometry analysis.

2.1.7 Thawing media

Hanks' Balanced Salt Solution (HBSS, Ca2+ and Mg2+ free, Sigma-Aldrich) plus 10% FCS was used to thaw the freezing cells.

2.1.8 Culture media

In this study, different culture media were used depending on the type of cultured cell.

RF10 is a cell culture medium that was used in this project for the generation of monocyte-derived DC (moDC). It is composed of RPMI 1640 medium (Sigma), with 10% FCS (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), and 2mM L- glutamine (Sigma-Aldrich). This medium contains biotin, vitamin B12, para-aminobenzoic acid, inositol and choline. It is considered suitable for various mammalian cells and is commonly used for the culture of primary immune cells.

CellGenix DC (CellGenix) is a serum-free and xeno-free Good Manufacturing Practice (GMP)-compatible medium formulated for the culture of clinical-grade DC. In this thesis, it was used for both cDC2 and moDC cultures.

X-VIVO15 (LONZA) is also a serum-free hematopoietic cell medium. In this thesis, it was used in combination with 2% human serum (HS; Sigma) for cDC2 culture. It was also used for

DC-T cell co-culture assay in combination with 2% HS and 100 U/ml penicillin + 100 μg/ml streptomycin.

2.1.8 Freezing solution

The freezing solution was prepared from 90% FCS (Gibco) and 10% dimethyl sulfoxide (NBS Biologicals), which acts as a cryoprotectant.

2.1.9 Coating buffer

This buffer was used in ELISA to assess cytokine secretion in moDC. To prepare the coating buffer, 300 ml of deionized water dH_2O was used in combination with 4.35g Na_2HPO_4 and 5.37g $NaH_2PO_4.H_2O$ (both from Sigma-Aldrich).

2.1.10 Freezing and thawing of cells

For cryopreservation, cells were frozen using a freezing medium (described before) at an appropriate cell number concentration containing DMSO as a cryoprotectant to prevent the formation of extra- and intra-cellular crystals in cells during freezing. Cell suspensions were frozen in 1.5ml cryopreservation vials (1ml/vial) and placed in a CoolCell® (BioCision, CA, USA), and stored at -80°C overnight before being transferred and stored in liquid nitrogen (LN).

For cell thawing, the vial of cells was removed from LN and placed on dry ice for five minutes to allow any LN that may have entered the vial to evaporate. Then, the vial was thawed in the water bath at 37 °C, after which the cells were transferred into a universal tube with the pre-warmed thawing medium. Finally, the cells were centrifuged at room temperature and resuspended in a wash buffer.

2.2 Methods for Chapter 3

2.2.1 Gene expression datasets

The two datasets used in this analysis (figure 2.1) were generated using the nCounter Human Immunology_V2 panel, quantifying the expression level of 594 genes plus 30 additional genes that were important for classical or conventional DC (see Table 2.1 for the additional genes).

The first dataset was generated by Dr. Rachel Spiering and includes 12 samples corresponding to three different conditions of moDC, namely tolDC, immature DC (immDC) and mature DC (matDC) that were obtained from four independent experiments (unpublished data). The tolDC generation protocol was identical to our 'gold standard' tolDC (see section 2.2.4). However, the mature DC was obtained by treatment with MPLA and IFN-γ. Full details about the DC generation method and its functional effects on $CD4^+$ T cells are presented in a previously published paper by the Hilkens group (Spiering et al., 2019).

The second dataset was generated by Dr. Ursula Cytlak and included cell subsets derived from bone marrow $CD34^+$ cells that had been cultured with GM-CSF, FLT3L and SCF on OP9 cells and then harvested and FACs-purified at Day 21. It is composed of four different cell subtypes (cDC1, cDC2, pDC and cultured monocytes), each from three donors. The data obtained were derived from 10,000 cells/sample and there were the same number of cells for all samples. Full details of the culture technique are available in ((Kirkling et al., 2018) and (Cytlak et al., 2020)). Gating strategy for the cultured and blood-derived DC subsets is represented in figure 2.1; full details of the gating strategy (including mAbs used) are available in (Cytlak et al., 2020).

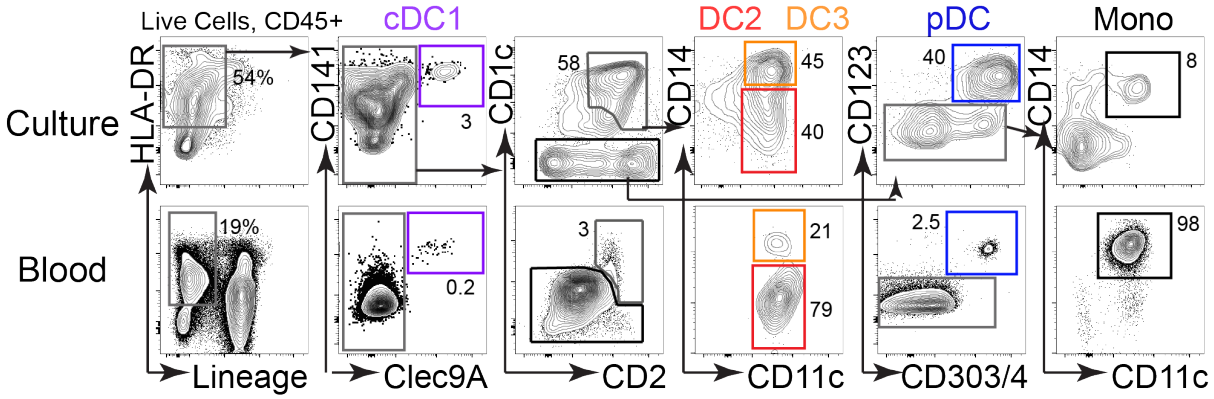


Figure 2.1: Cell sort gating strategy of cultured and blood-derived DC subsets. The numbers next to the gates represent percentage of parent. Purple boxes: gates for cDC1; red (lower) boxes: gates for DC2; orange (upper) boxes: gates for DC3; blue boxes: gates for pDC; black boxes: gates for monocytes (Mono). This gating strategy has been published by Cytlak et al 2020.

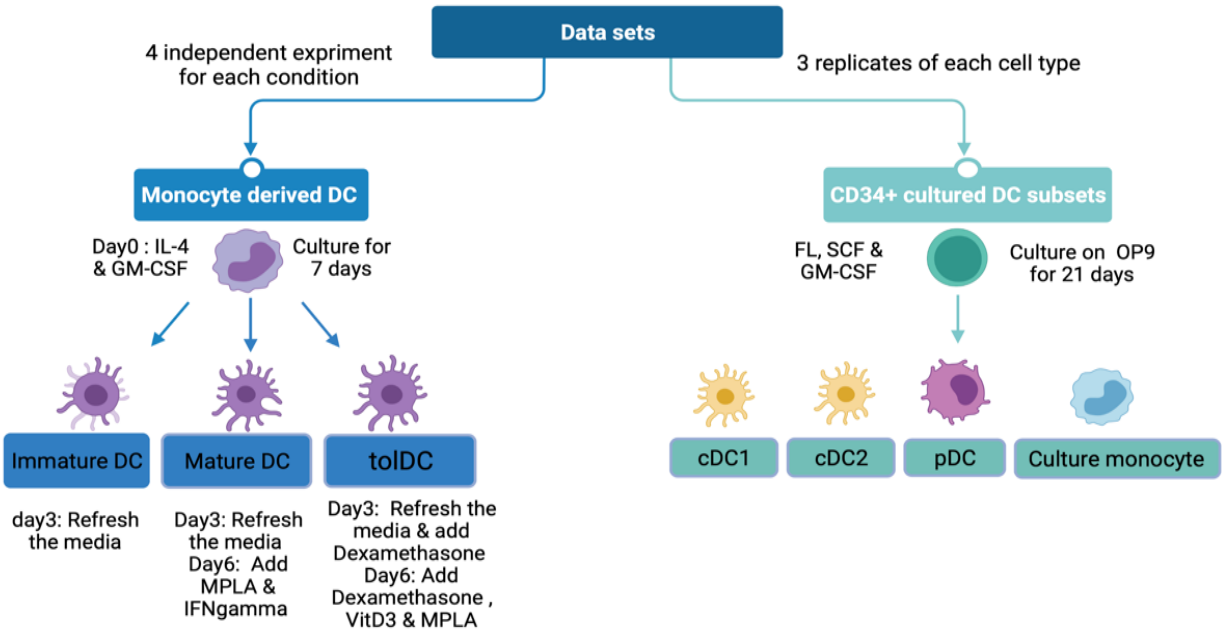


Figure 2.1: Schematic diagram showing the data sets used in the analysis. (Cytlak et al., 2020; Kirkling et al., 2018; Spiering et al., 2019) Created with BioRender.com

ASIP	DAXX	MERTK
C19orf59	DBN1	Ki67
CCL17	F13A1	NDRG2
CD1c	FGD6	PACSIN1
CD207	FLT3	PPM1N
CLEC10A	GCSAM	PRAM1
CLEC9A	GGT5	S100A12
CLNK	LPAR2	TMEM14A
COBLL1	LYVE1	UPK3A
CXCL5	MAFF	ZBTB46

Table 2.1: The 30 additional genes that were included in the Nanostring panel.

2.2.2 Bioinformatic techniques and statistical analysis pipeline

All parameters were analysed using nSolver software v4.0 (Nanostring nCounter analysis software) with the Advanced Analysis Module v2.0 and/or R software (version 3.2.3) for further analysis. Gene expression data analysis with R was performed by Najib Naamane, a bioinformatician in our group. Several CRAN and Bioconductor R packages were used to perform quality control (QC), normalization, differential expression (DE) analysis, functional enrichment analysis, as well as heatmap and venn diagram production. In addition, Advanced Analysis Module software was used as an open-source R program for DE analysis, pathway scoring, and gene-set enrichment analysis.

2.2.2.1 Data normalization and quality assessment

Raw data (RCC) files were imported into R software for QC and normalization using NanoStringQCPro R package. The raw counts from each dataset were first subject to the standard pre-processing steps recommended by the NanoString Gene Expression Data Analysis Guidelines, which produces different quality metrics and normalizes the data based on different sets of control genes. In brief, the background signal estimates were calculated as the geometric mean of the negative control probes for each sample using the getBackground function and then subtracted from the raw counts using the subtractBackground function. Subsequently, the background subtracted values were adjusted for platform-associated sources of variation using the posCtrlNorm function (i.e. Positive Control Normalization). This

function first calculates the geometric mean of the positive spike-in RNA hybridization controls in each sample then divides the arithmetic mean of all the resulting geometric means by the geometric mean of each sample to calculate a sample specific correction factor (i.e. positive control scaling factor). The gene counts of each sample are then multiplied by its scaling factor to obtain the normalised counts. Following this, a set of 13 optimal housekeeping genes were determined by the geNorm algorithm (Vandesompele et al., 2002) and used by the contentNorm function to adjust for total RNA sample input variability (i.e. Housekeeping Gene Normalization). Like in the Positive Control Normalization, this function multiplies the positive control-normalised counts of each sample by its scaling factor which is calculated using the housekeeping genes instead of the positive controls. The final log2 scale normalised counts obtained at this step were further adjusted for sample donor effect using the removeBatchEffect function from the Limma package (Ritchie et al., 2015) and finally used for clustering and plotting (e.g. in heatmaps and box plots).

Sample-level quality assessment (i.e., identification of sample outliers) was performed based on different quality metrics suggested by both the NanoString Gene Expression Data Analysis Guidelines and the arrayQualityMetrics R package (Kauffmann et al., 2009). The quality metrics defined by NanoString, such as the percentage of Fields Of View (FOV) with successful imaging, the Binding Density, the Positive Control Linearity, the Limit of Detection and the positive control and content normalization scaling factors, were computed using the NanoStringQCPro R package. The values of these metrics were within the NanoString-recommended ranges for all samples. Furthermore, three additional sample quality metrics were calculated using the arrayQualityMetrics package namely the sum of the distances to all other samples, the Kolmogorov-Smirnov statistic (compares the sample and the pooled data distributions) and the Hoeffding's D-statistic (test for the independence of M (log2(I1) - log2(I2)) and A (1/2 (log2(I1)+log2(I2))) values, where I1 is the intensity of the sample, and I2 is the intensity of a "pseudo"-sample that consists of the median across samples). Samples were flagged if their quality metric was higher than the third quartile plus 1.5 times the interquartile range. All samples were included in downstream analysis as none of them has more than one metric exceeding the threshold.

When cell subset comparisons were made between datasets, the three datasets were re-normalized together using the remove unwanted variation (RUV) approach (Risso et al., 2014) which has been shown to eliminate technical variation from multisite datasets more reliably

than the NanoString's normalization procedure (Bhattacharya et al. 2020). Initially, RCC files from the three datasets were combined into one RccSet object (NanoStringQCPro container for high-throughput assays) and only genes present in the three datasets were kept for further analysis. Raw counts were then preprocessed using the varianceStabilizingTransformation function which estimates sample-specific size factors and gene-specific dispersions, normalizes the count data with respect to library size and applies a variance stabilizing transformation (VST) to the normalized counts making them approximately homoscedastic (i.e. with constant variance across the range of mean values) (Anders and Huber, 2010). To adjust for unwanted technical variation including the dataset confounding effect, the previously selected housekeeping genes were first used by the RUVg function to estimate an unwanted technical variation factor from the original data which was subsequently regressed out from the VST-transformed data using the removeBatchEffect function. The resulting adjusted counts were used for clustering and plotting. When using nSolver, data were normalized using Advanced Analysis Module v2.0, which uses the geNorm algorithm to select the best housekeeping genes and internal positive controls.

2.2.2.2 Differential expression and pathway enrichment analysis

The DESeq2 R package (Love et al., 2014) was used to compare the average gene expression level between the moDC conditions (tolDC, matDC and immDC). In addition, it was also used for the $CD34^+$ cultured DC subsets dataset. In brief, sample-specific library size factors were first estimated based on the previously selected housekeeping genes using the estimateSizeFactors function which implements the median ratio method described in (Anders and Huber, 2010). Subsequetly, the estimateDispersions function was used to calculate the gene-specific dispersions estimates. In the last step of DESeq2, the pre-calculated size factors and dispersion estimates were used by the nbinomWaldTest function to fit a Generalized Linear Model (GLM) on the raw counts of each gene and test the significance of the model's coefficients (i.e. log2 fold changes) by Wald tests (Love et al., 2014). The donor ID was included as a covariate in the GLM to adjust for the donor effect when cell subsets comparisons were performed within each dataset, whilst the factor of unwanted variation estimated by RUVg was included in the models used for between-dataset comparisons. All possible pairwise comparisons between the cell subsets were performed. Differentially expressed genes (DEGs) were defined as those with a Benjamini-Hochberg corrected p-value <0.01 and an absolute fold-change >2. Resulting lists of DEGs were tested for GO biological processes and KEGG

pathways enrichment using the RDAVIDWebService R package (Fresno and Fernandez, 2013), which offers the main functionalities of DAVID (Database for Annotation, Visualization and Integrated Discovery). In addition, pathway enrichment analysis of moDC and cultured $CD34^+$ derived DC subsets was performed using the advanced analysis module in nSolver software. A p-value <0.05 was set as the cut-off criterion.

2.2.3 Cell isolation for tolerogenic marker validation in tolDC

2.2.3.1 Peripheral blood mononuclear cells (PBMC)

Healthy peripheral blood mononuclear cells (PBMC) were separated by density centrifugation using Lymphoprep at room temperature (RT). Briefly, samples from the LRS cone were flushed into 100 ml with HBSS at RT supplemented with two mM EDTA. Then, 20 ml of diluted blood was carefully placed on top of a 15 ml layer of lymphoprep at RT and subsequently centrifuged at 900g for 30 minutes in RT. PBMC were isolated from the buffy layer at the interface and washed using a cold wash buffer (Hanks supplemented with 1% FBS) by centrifugation at 600g for 8 minutes at 4 °C to remove any residual lymphoprep. Next, A second cold wash was conducted, centrifuging the cells at 300g for 8 minutes at 4°C to eliminate platelet contamination. The cells were filtered through a 70-micron nylon filter to eliminate any aggregations. Then, the cells were quantified using a Burker counting chamber along with trypan blue. After that, it was used for either monocyte or $CD1c^+$ isolation.

2.2.3.2 Monocyte isolation

Monocyte isolation was performed by positive bead selection using anti-CD14 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, monocytes were isolated by labeling PBMC with $CD14^+$ microbeads and then separated on a column in a permanent magnet, the MACS separator (Miltenyi Biotech, UK). In this procedure, the unlabelled cells are lost as they pass through the column and the $CD14^+$ cells can then be eluted after removal of the column from the magnetic field.

2.2.4 Generation of monocyte-derived dendritic cells (moDC)

Generation of moDC subsets, including tolDC, was carried out according to the protocol developed by the Hilkens group (Hilkens et al., 2023). Briefly, $CD14^+$ monocytes were seeded

in a 24-well plate ($0.5x10^6$ cells/ml) and cultured in either RF10 or CellGenix GMP DC medium supplemented with GM-CSF and IL-4 (both at 50 ng/ml; Immunotools). The cells were incubated under humidified conditions (37°C, 5% CO_2) for 7 days. Refreshment with pre-warmed media containing GM-CSF and IL-4 was done on day 3 of culture. TolDC was generated by adding dexamethasone (Dex; 10^{-8} M) on day 3, followed by adding Dex (10^{-8}M) and vitamin D3 (VitD3; 10^{-10} M) on day 6. For maturation of DC, LPS (Sigma; 0.1µg/ml) or MPLA (Sigma; 1µg/ml) plus or minus IFN-γ (Peprotech; 1000 U/ml; matDC only where indicated) was added on day 6 in both matDC and tolDC, while immDC were left untreated for another 24h. MPLA is a non-toxic analogue of the lipid A portion of LPS and is used to generate matDC and to stabilise the tolDC product making it resistant to further maturation (Harry et al., 2010). In addition, as previously shown by the Hilkens group, TLR4-mediated activation of tolDC is required for these cells to acquire antigen-presenting and migratory capacity (Anderson et al., 2009). On day 7, supernatants were harvested and frozen at -80 C°. Cells were then harvested (by pipetting and scraping the cells with a blue pipette tip after a 1-hour incubation on ice) to perform further analysis.

2.2.5 Cell count and viability

moDC from the different culture conditions were counted using a Burker counting chamber. Cell viability was measured using trypan blue (Sigma) in a 1:1 dilution with a suitably diluted cell suspension.

2.2.6 ELISA for cytokine secretion analysis

IL-12p70 and IL-10 levels in stored moDC samples were determined by sandwich ELISA, carried out in 96-well EIA/RIA flat bottom plates. Briefly, purified rat anti-human IL-10 and purified rat anti-human IL12p70 capture mAbs (both from BD Pharmingen Biosciences) were coated on the plates in coating buffer (as described before in the reagents 2.1.9) and incubated overnight. Then, the plates were washed and the capture mAbs were discarded. The plate was incubated for 1 hour with the block (1%BSA in PBS). Next, the plates were washed three times, and then diluent was added to the standard wells. The samples were added into the other wells in an appropriate ratio as needed and incubated overnight in moist at 4C. The next day, biotin anti-human IL-10 and biotin mouse anti-human IL-12p70 detection mAbs (all from BD Pharmingen Biosciences) were added to the plate and incubated at room temperature for 1

hour. Extravadin peroxidase and O-Phenylenediamine dihydrochloride (OPD) tablets were employed in the detection steps (both from Sigma Aldrich). The reaction was halted using 3M sulphuric acid and the plates were read at an optical density (OD) of 490nm.

2.2.7 Flow cytometry for cell surface marker analysis

To assess the low and high expression of markers by moDC types, a multicolour fluorescence antibody panel was used (Table 2.2). Harvested moDC were stained for surface molecules of interest. Briefly, after harvesting of moDC after gentle scraping with a blue pipette tip, the wells were washed once with cold HBSS+ 1% FCS to ensure that the majority of the cells were harvested, then centrifuged at 400g, 4ºC for 8 minutes, and washed four times to ensure removal of reagents (Dex, VitD3 and LPS/ MPLA). After that, a minimum of 1×10^5 DC of each sample (immDC, matDC and tolDC) was transferred into a 96-well v-bottom plate and stained with Zombie Aqua (ZA, Biolegend) to assess the viability of cells. Briefly, the cells were stained with ZA 1:100 in PBS and incubated at RT in the dark for 15 minutes. Then FACS buffer was added to quench the ZA dye and centrifuged at 400g for 3 minutes before staining with the mAb panels. The 4µg/ml of Human IgG (Sigma, UK) was added to the cells to block binding to FcR. After that, the cells were stained with a master mix of mAbs in a 50 µl volume, as shown in Table 2.2. Cells were stained with panels of a maximum of five fluorophore-compatible mAbs as described in Table 2.2 to reduce compensation issues and non-specific background signal and then incubated on ice for 30 minutes. Unstained cells were used as a negative control. The cells were then fixed in 1 % formaldehyde (TAAB Labs) and stored away from light at 4 °C for a maximum of 7 days. The data were acquired on the BD LSR Fortessa X20TM Cell Analyzer (BD Biosciences) (5000 cells were acquired for each sample) and then analysed by FlowJo version 10.8.1 (BD, OR, USA).

Panel	mAb name	Clone	Dilution	Company	Isotype control
Panel 1	CD83: PerCP CY5.5	HB15e	1:50	Biolegend	Mouse IgG1 PerCP CY5.5
	CD86: V450	FUN-1	1:20	BD Bioscience	Mouse IgG1 V450
	HLA-DR: FITC	L243	1:10	BD Bioscience	Mouse IgG2A FITC
	ILT3: APC	ZM4.1	1:50	Biolegend	Mouse IgG1 APC
	ILT4:PE	42D1	1:20	Biolegend	Rat IgG2A PE
Panel 2	PDL1:BB515	MIH-1	1:20	BD Bioscience	Mouse IgG1 BB515
	PDL2:BV421	24F.10C12	1:50	Biolegend	Mouse IgG2A BV421
	CXCR4:APC	REA649	1:100	Miltenyi	Human IgG1 APC
	MERTK:BV711	590H11G1E	1:10	Biolegend	Mouse IgG1 BV711
	TLR2:PE	TL2.1	1:10	Thermo Scientific	Mouse IgG2A PE
Panel 3	IL1R2:FITC	34141	1:10	Life Tech	Mouse IgG1 APC
	CLEC4A: APC	216110	1:10	R&D	Mouse IgG1 BV421
	CD32:PE	190723	1:10	R&D	Mouse IgG1 FITC
	MRC1: BV421	15-Feb	1:20	Biolegend	Mouse IgG2A PE
Panel 4	CCR7: PE	G043H7	1:20	Biolegend	Mouse IgG2A PE

Table 2.2: List of mAbs used for flow cytometry of moDC types. Isotype controls were also used in the staining of moDC.

2.2.8 Gating strategy for moDC

An example of the moDC gating strategy is shown in figure 2.2 below. In all experiments, the cells were first gated for cells of interest within the FCS/SSC plot and then single cells within the SSC-A/SSC-W plot to exclude any debris and doublet cells from the analysis, respectively. Median fluorescence intensity (MFI) and percentage of positive cells were then be calculated from this population after exclusion of dead cells.

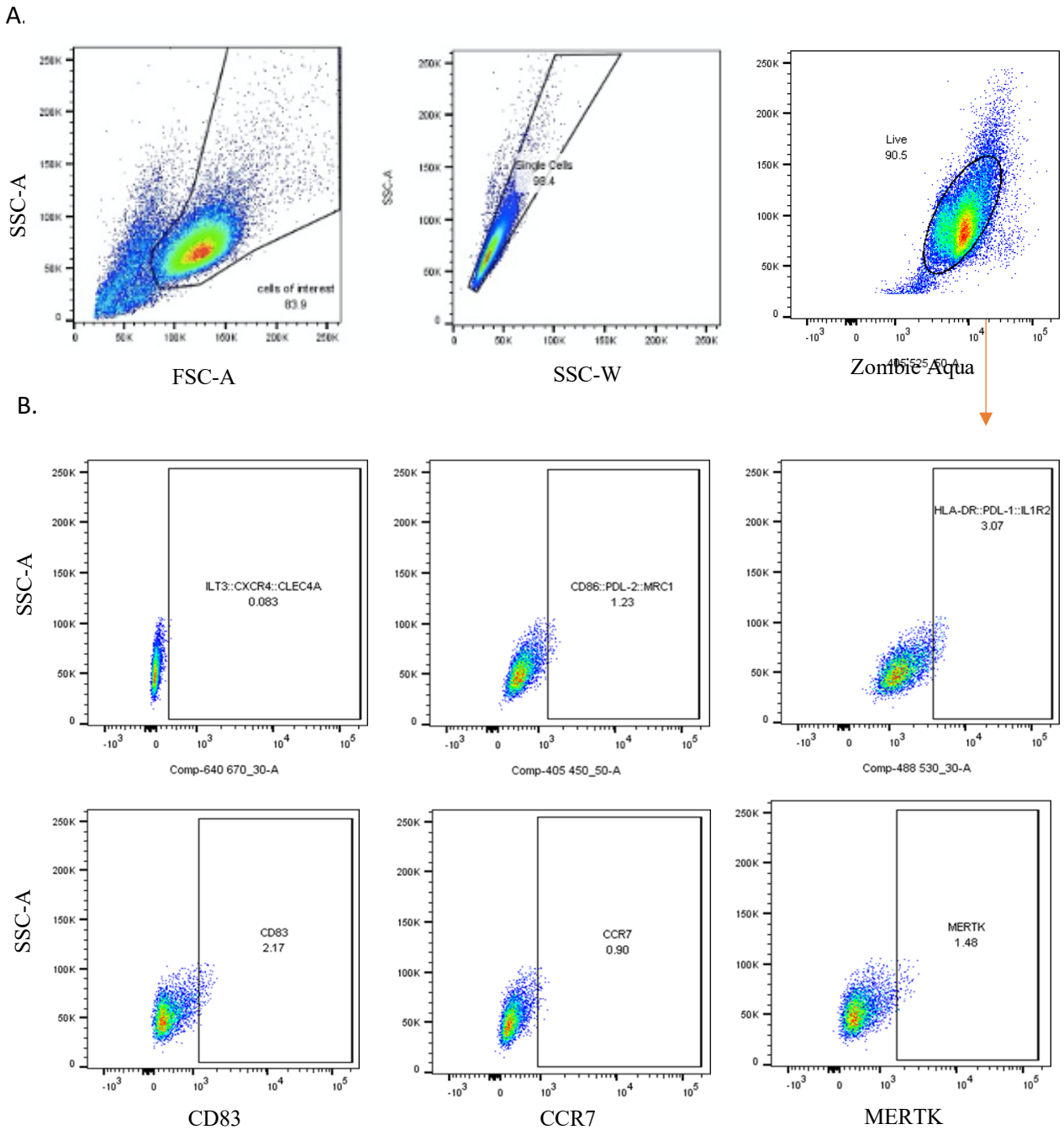


Figure 2.2: An example of moDC gating strategy in unstained cells of immDC condition. After the exclusion of debris, doublet and dead cells (A), markers of interest in different moDC conditions were gated (B).

2.3 Material and Methods for Chapters 4 and 5

2.3.1 Cell isolation

2.3.1.1 PBMC

PBMC were isolated from the peripheral blood of healthy donors as described in section 2.2.3.1. These cells were either used for further cDC2 isolation or cryopreserved in freezing medium and stored in liquid nitrogen.

2.3.1.2 cDC2 cell isolation

Conventional DC type 2 (cDC2) was separated from PBMC by using the CD1c (BDCA-1) $^{+}$ human DC isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, PBMC were labelled with CD19 and CD14 microbeads and separated on LD column in a permanent magnet, the MACS separator (to deplete them). The unlabelled cells (negative for CD19 and CD14) passing through the column were then collected for the next $CD1c^{+}$ positive selection step. If required, $CD14^{+}$ and $CD19^{+}$ cells can be eluted after removal from the magnetic field. $CD14^{-}$ and $CD19^{-}$ cells were passed through MS column 2 times to increase the purity of the $CD1c^{+}$ selected cells.

2.3.1.3 $CD4^{+}$ Naïve T cell isolation

Human $CD4^{+}$ T cells were isolated from the LRS cone by using the RosetteSep^{TM} Human $CD4^{+}$ T cell enrichment cocktail (StemCell Technologies). The cocktail facilitates the binding of undesired cells in human blood samples to multiple red blood cells (RBCs), leading to the formation of immunorosettes. This results in an elevated density of the unwanted (rosetted) cells, causing them to pellet along with the free RBCs upon centrifugation over Lymphoprep. Subsequent to a 20-minute incubation period with the T cell enrichment cocktail at room temperature (RT), the blood sample underwent a 1:2 dilution with PBS and 2% FCS. The diluted sample was then layered onto Lymphoprep and centrifuged at 900g for 30 minutes. The collection of CD4+ cells took place at the interface between the plasma and Lymphoprep.

After that, CD45RO microbeads and MACS magnetic cell separation were used to negatively isolate naïve $CD4^{+}$ T cell as per the manufacturer's instructions (MACS, Miltenyi Biotec, Germany). Naïve $CD4^{+}$ $CD45RO^{-}$ T cells were then frozen at -80 C° after resuspension in

freezing medium before transfer to LN. Purity of the naïve $CD4^+$ $CD45RO^-$ T cells were usually around 85-90 % after checking with flow cytometry (figure 2.3).

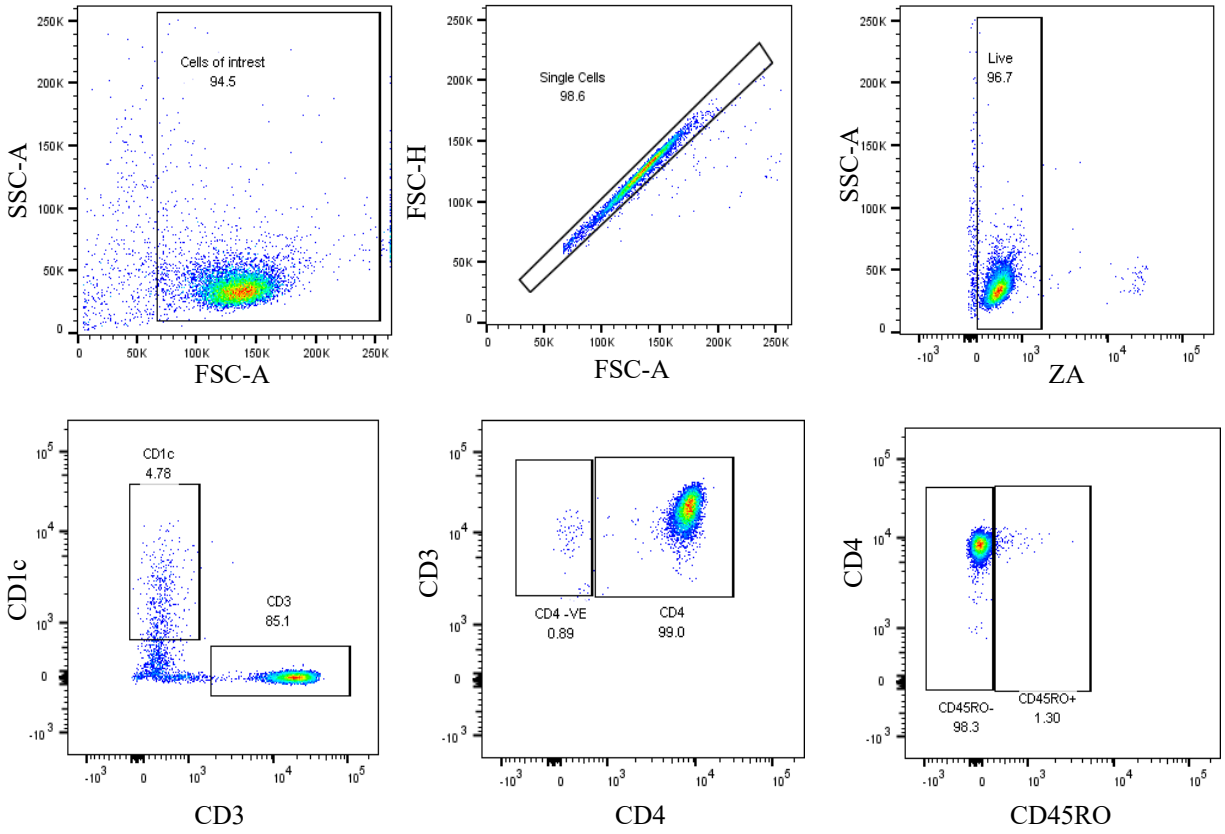


Figure 2.3: An example of $CD4^+$ $CD45RO^-$ (naive T cells gating) for purity checking. Briefly, after exclusion the debris, doublet and dead cells, the cells were gated on $CD3^+$ population first to exclude any other cells contamination. Then $CD3^+CD4^+$ cells were gated. After that naïve $CD45RO^-$ cells were gated.

2.3.2 Tol-cDC2 generation and culture

Isolated cDC2 were cultured in either CellGenix GMP DC medium (CellGenix) or X-VIVO15 medium + 2% HS; both supplemented with either 10 or 50 ng/ml GM-CSF in 96 well flat bottom plates in a concentration of 150,000 cells/well. Tolerogenic cells were generated by adding either Dexamethasone (Dex) 10^{-8}M, VitD3 10^{-8} M, rapamycin (Rapa) 10 ng/ml, or various combinations (Dex and VitD3 or Rapa and VitD3). The cells were first pre-primed with immunomodulatory agents for 1 hour to ensure activation of tolerogenic pathways before

the addition of the maturation factor LPS (0.1µg/ml). Untreated cDC2 cells were cultured as a negative control. The cells were then incubated under humidified conditions (37°C, 5% CO_2) for overnight around 16-20 hours. After that, the cells were harvested (by gentle pipetting up and down with yellow tip) for further analysis.

2.3.3 cDC2 and allogenic naïve $CD4^+$ T cell co-culture (Allo-MLR)

After harvesting, cDC2 was washed three times to ensure the removal of all treatments. cDC2 were resuspended in X-VIVO15 medium + 2% HS and 100 U/ml penicillin + 100µg/ml streptomycin. Previously frozen naïve $CD4^+$ T cells were thawed (see section 2.1.10) and resuspended in the same medium.

For the mixed lymphocyte reaction (MLR) assay, all cDC2 conditions (Un-cDC2, Stim-cDC2 and VitD3-cDC2) were co-cultured with naïve $CD4^+$ T cells in round bottom 96-well plates. Different cDC2/naïve $CD4^+$ T cell ratios were performed: 1:10 (10,000 cDC2 to 100,000 T cells), 1:20 (5000 cDC2 to 100,000 T cells) and 1:40 (2500 cDC2 to 100,000 T cells) in 200µl medium per well. The MLRs were incubated under humidified conditions (37°C, 5% CO_2) for 4 to 6 days. Supernatants were collected on days 3, 4 and 5 to assess cytokine secretion at different time points and frozen in -80 for MSD analysis. Untreated naïve CD4+ T cells were cultured independently, serving as a negative control. The cells were then harvested on day 4 or 6 for flow cytometry staining. For subsequent MLRs, 4-day cultures were selected as an optimal time for cDC2/ T cells co-culture.

2.3.4 T cell proliferation assay

Proliferation of naïve $CD4^+$ T cells was assessed by labeling T cells before the start of the MLR with 0.5µM CellTrace Far Red (CTFR) (ThermoFisher) following the manufacturer's instructions. Unlabeled T cells were cultured as a control. On Day 4 the cells were acquired by flow cytometer and the proliferation percentages were obtained as a negatively stained population of CTFR on the APC channel laser Bandpass (635-670/30). See figure 2.4 for a simplified diagram that summarizes the allo-MLR experiment. In addition, the proliferation index of the $CD4^+$ T cell was calculated with the following formula:

Proliferation Index: Total Number of Divisions / Cells that went into division

This information was obtained from the proliferation modeling tool from FlowJo.

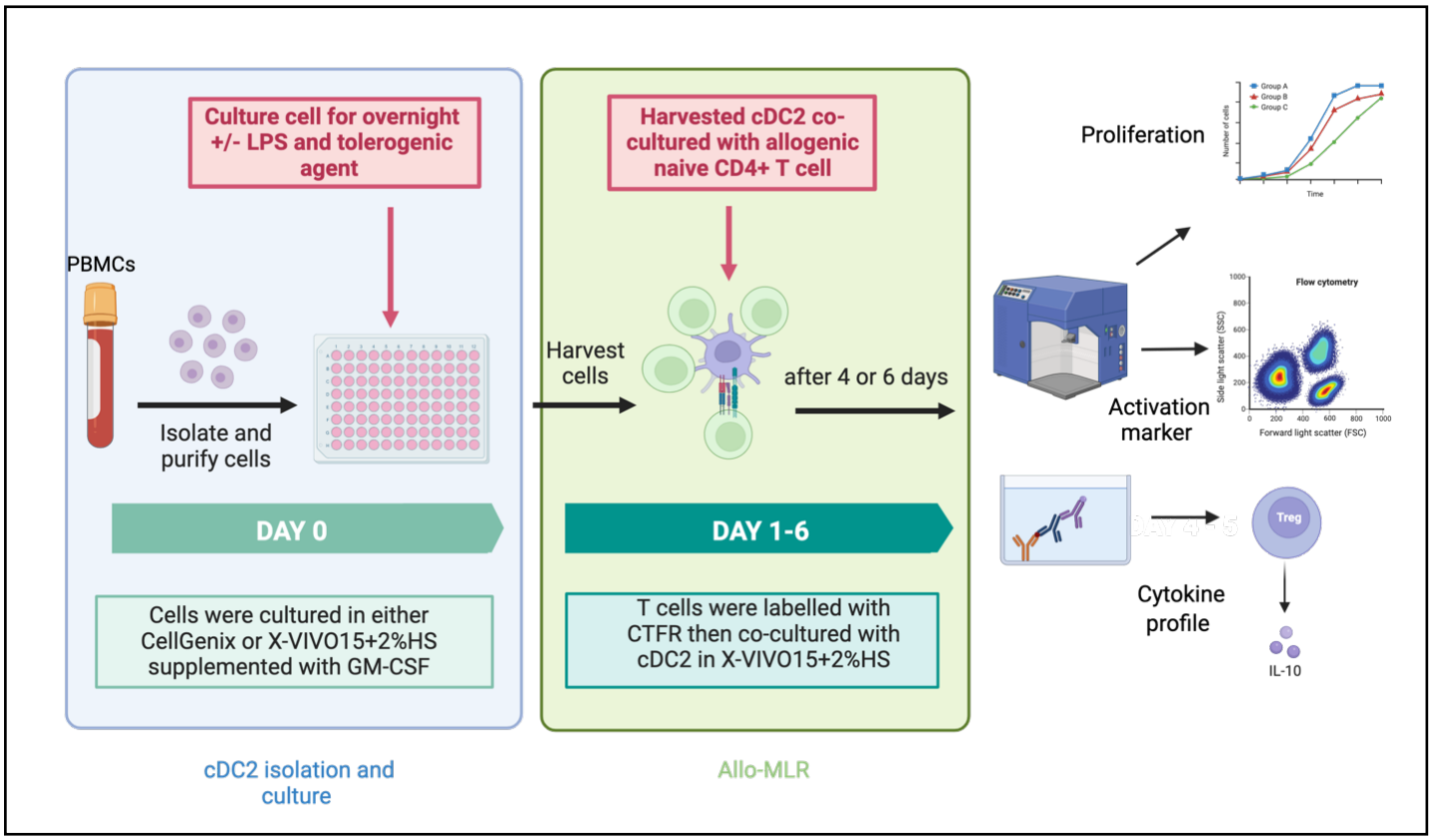


Figure 2.4. Summary of the allo-MLR and proliferation assay. First cDC2 was isolated and purified then cultured for overnight +/- LPS. After that harvested cDC2 were co-cultured with allogenic naïve CD4+ T cells for 1-6 days after labeling of T cells with CTFR. T cells were then acquired by flow cytometry to assess T cell proliferation and activation markers. Created with BioRender.com.

2.3.5 T cell re-stimulation assay

Allogenic naïve $CD4^+$ T cells were primed with Un-cDC2, Stim-cDC2 or VitD3-cDC2 at a 1:10 ratio (10,000 cDC2/ 100,000 T cells) in a total volume of 200 µl /well in 96 well round bottom plate. The cells were incubated for 5 days (at 37°C, 5% CO_2), after which IL-2 was added (10U/ml; Sigma) and cells were rested in these conditions for another 3-4 days. Cells were split into a 48-well plate once the media started to become slightly yellow, to avoid over-acidification of the medium; fresh media was added to the split wells. Then, primed T cells were harvested, washed, and stimulated with LPS-treated cDC2 from the original cDC2 donor at a 1:10 ratio (10,000 cDC2/ 100,000 T cells) or T cell CD3/CD28 expander and activator beads (Gibco by ThermoFisher Scientific) at either 1:1 or 1:5 ratio in 200µl /well in 96 well

flat bottom plate. On day 3, supernatants were harvested to assess the cytokine secretion profiles by MSD, and the cells were phenotyped by flow cytometry.

2.3.6 Migration assay

cDC2 migration was assessed by CCR7-dependent migration towards the chemokines CCL19 and CCL21 using a transwell plate system (pore size, 0.5M; Corning Life Sciences, UK). 100 µL that contain $2x10^5$ cDC2 were added in the upper chamber ('input DC'), and either cellGenix or X-VIVO15 medium, with or without a mixture of CCL19 and CCL21 (both at 250 ng/ml; R&D Systems), added to the lower chamber as shown in figure 2.5. The migration of cDC2 was evaluated following a 2-hour incubation at 37°C by collecting the cells from the lower chamber and quantifying them using trypan blue and a hemocytometer. The expression of cDC2 migration is represented as the percentage of input DC that underwent migration. See figure 2.5 which summarizes the migration assay.

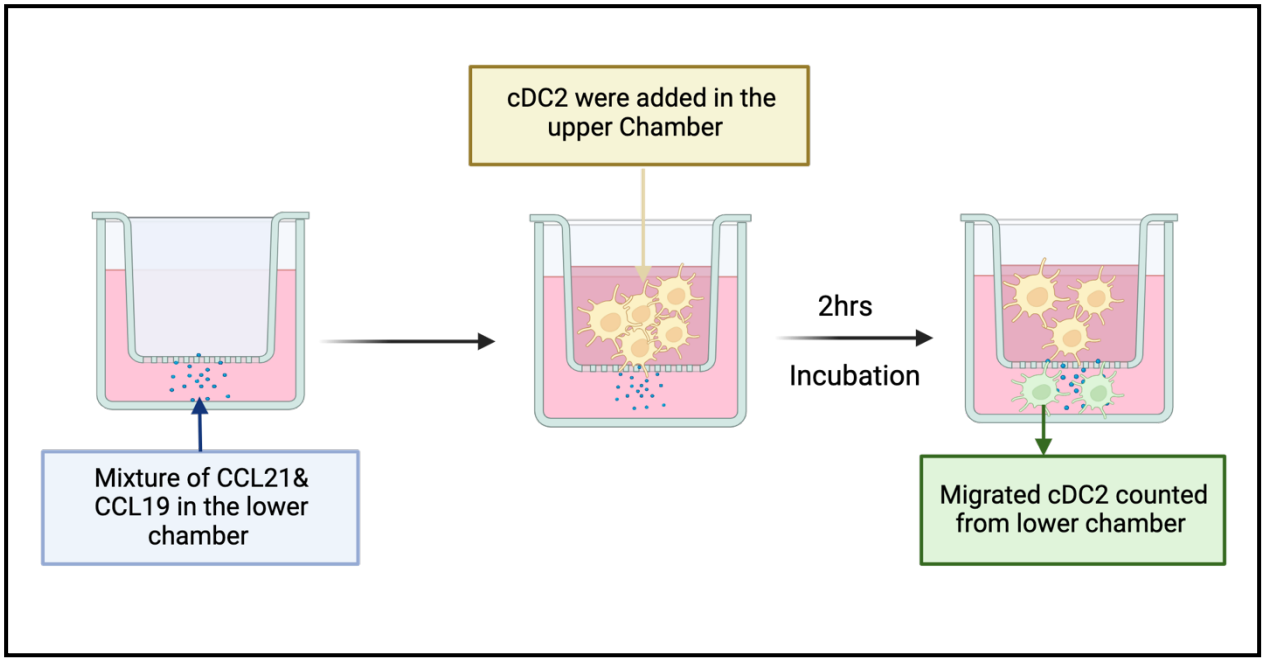


Figure 2.5: Simplified diagram displays the process of Migration assay. Created with BioRender.com

2.3.7 Flow cytometry staining

Both harvested cDC2 and T cells were first stained for viability by Zombie Aqua (ZA, Biolegend) in order to exclude dead cells. Briefly, as mentioned before in the context of moDC, cells were stained with 1:100 ZA in PBS for 15 minutes in the dark at RT. After that FACS buffer was added to quench the dye and then the cells were washed and centrifuged at 400g for 3 minutes at RT before adding surface stain mAb.

2.3.7.1 Cell surface staining

After staining for viability, both cDC2 and T cells were stained for surface molecules of interest using the same flow cytometry surface staining protocol described in section 2.2.7. Then, the cells were fixed by 1% formaldehyde and stored until acquired by LSRFortessa ™ X-20 Cell Analyzer (BD Biosciences). The antibodies panel design for cDC2 or T cells are illustrated in Table 2.3 or Table 2.4, respectively. Isotype control and/or Flow Minus one (FMO) control were performed to exclude background autofluorescence in cDC2 or T cell.

mAB name	Clone	Dilution	Company
CD3: FITC	UCHT1	1:20	BD Bioscience
CD19: FITC	UG7	1:50	BD Bioscience
CD16: FITC	NKP15	1:200	BD Bioscience
CD14: BV650	M5E2	1:100	Biolegend
HLA-DR: AF700	G46-6	1:10	BD Bioscience
ILT3:APC	ZM4.1	1:50	Biolegend
CD1c: PE-CY7	L161	1:50	Biolegend
CD163: PE-CF594	GHI161	1:20	BD Bioscience
CD5: APC-CY7	L17F12	1:100	Biolegend
CD86: PE	IT2.2	1:20	BD Bioscience
CD83: PerCp cy5.5	HB15e	1:50	Biolegend
MERTK: BV711	590H11G1E	1:10	Biolegend
PDL-1: BUV395	MIH1	1:10	BD Bioscience
CCR7:BV421	150503	1:20	BD Bioscience

Table 2.3: List of mAb used for flowcytometry surface staining in cDC2. FMO were also used in the staining of cDC2.

Antibody	Clone	Dilution	Company
CD4: AF700	SK3	1:100	Biolegend
CD3: BUV395	UCHT1	1:100	BD Bioscience
CD45RO: FITC	UCHL1	1:50	BD Bioscience
CD25: PE	M-A251	1:20	BD Bioscience
CD127: BV650	A019D5	1:100	Biolegend
CD1c: PE-CY7	L161	1:50	Biolegend

Table 2.4: List of Antibodies used for T cell surface staining for flow cytometry in MLRs. FMO and unstained control were both used to remove background autofluorescent and gating.

2.3.7.2 Intracellular staining

Briefly, after viability and surface staining the cells were washed by FACS buffer and centrifuged at 400g for 3 minutes. T cells were then resuspended in 1X (the solution after dilution) Fixation and Permeabilization (Fix/Perm) buffer (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen) and incubated at 4 °C for 30 minutes in the dark. After that, the cells were washed and resuspended in 1X Foxp3 Permeabilization (Perm) buffer and incubated for 15 minutes followed by blocking with 2% mouse serum for 15 min at 4 °C in the dark. Antibodies for intracellular staining were added and incubated for 30 minutes, followed by washing and resuspension in FACS buffer, after which the cells were immediately acquired by LSRFortessa ™ X-20 Cell Analyzer (BD Biosciences). Table 2.5 lists the antibodies used for T cell-surface and -intracellular staining after the re-stimulation assay.

Antibody	Clone	Dilution	Company
CD4: BV786	Sk3	1:100	BD Bioscience
CD3: BUV395	UCHT1	1:100	BD Bioscience
CD25: PE	M-A251	1:20	BD Bioscience
CD137: BV650	4B4-1	1:50	Biolegend
Foxp3:AF647(Intracellular)	206D	1:20	Biolegend

Table 2.5: List of mAb used for T cell staining after the re-stimulation assay.

2.3.7.3 Flow cytometry: data acquisition and analysis

Appropriate voltages were selected for the five-laser BD LSRFortessa ™ X-20 Cell Analyzer (BD Biosciences) in order to distinguish between positive and negative populations. Compensation was then calculated using data from negative and positive-stained beads, using FCSDiva^{TM} software. The experiment file was also saved as a template for subsequent analysis, while FCS files were exported from the analyser and data analysis was performed using FlowJo version 10.8.1 (BD, USA).

For cDC2 gating, cells of interest followed by single cell gating were used to exclude debris and doublet cells, respectively. Dead cells were also excluded from the analysis by high ZA staining. Lymphocytes were then excluded by gating out cells positive in the 'dump' channel i.e. $CD3^+$ $CD19^+$ and $CD16^+$ cells. The majority of the HLA-DR^+ population were $CD1c^+$ cells, which consisted of $CD5^+$ DC2 and $CD163^+$ DC3 subsets as shown in (figure 2.6). In order to evaluate the expression of functional markers of interest (this will be described in detail in Chapter 4 and 5), bulk $CD1c^+$ populations were used. For T cell proliferation and activation marker characterization, the cells were first gated on cells of interest and single cells to exclude debris and doublet, respectively. Then after the exclusion of dead cells, $CD1c^+$ cells were gated out and then cells were gated on $CD3^+CD4^+$ population to assess all markers of interest as well as proliferation percentage (Figure 2.6).

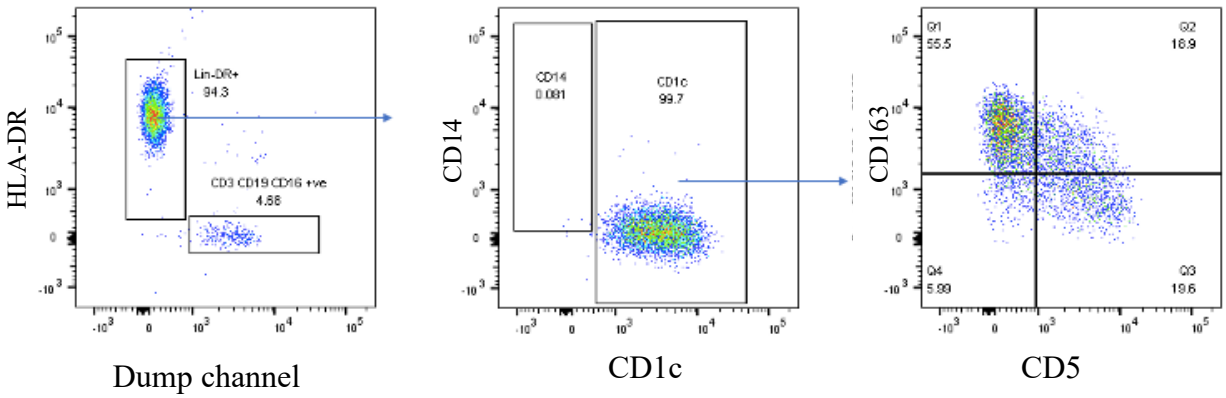


Figure 2.6: An example of cDC2 gating strategy after the exclusion of debris, doublet and dead cells.

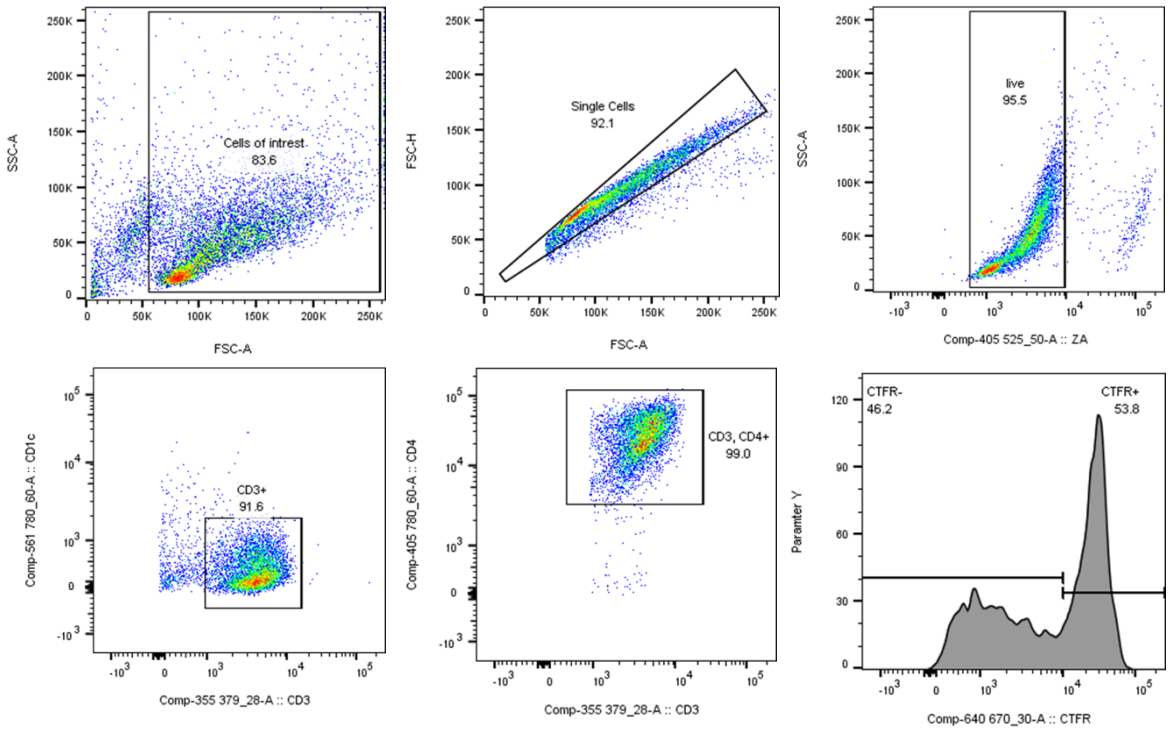


Figure 2.7: An example of T cell gating strategy to assess both proliferation and activation marker expression.

2.3.8 Meso Scale Discovery (MSD) for multiplexed analysis of secreted cytokines

MSD is a multiplex ELISA (U-PLEX assay kit) that was performed as per the manufacturer's instructions to measure the levels of the cytokines panel IL-10, IL-12p70, IL-1β, IL-6, IFN-γ and TNF-α. Briefly, samples (supernatant) were first diluted with 1:2 with the same media used for the cell culture. Then 25 µl of this diluted supernatant was added into pre-coated multiplex U-plate wells and allowed to incubate for 1 hour at room temperature with continuous shaking. Concurrently, eight-point calibrator standards were included in the plate to verify the establishment of the standard curve. The plate underwent three washes with PBS/Tween, following which 25 µl of the detection antibody was added and allowed to incubate for 1 hour at RT with continuous shaking. Subsequently, after three additional washes, 150 µl of Read buffer was applied, and the plate was examined using a Meso Sector S600 Imager. (MSD Company).

2.3.9 Statistical analysis and production of Figures

Graphs and statistical analyses were conducted using GraphPad Prism software (GraphPad Software, San Diego, California) or JMP Statistical Discovery LLC. Flow cytometry data were analyzed using FlowJo software. The figures presented in this thesis were created using Biorender.com, based in Canada.Nanostring data were analysed by using both nSolver and R software.

Chapter 3: Comparative transcriptomic profile analysis of monocyte-derived tolerogenic DC and conventional DC subsets

3.1 Introduction

Tolerogenic dendritic cells (tolDC) have been promoted as a promising cellular therapy for the treatment of autoimmune disease due to their ability to re-establish immune tolerance in an antigen-specific approach (Navarro-Barriuso et al., 2018). To date, the most used cellular source for generation of tolDC therapy is monocytes. However, this cellular approach has some limitations including poor migratory ability. So, we aim to generate a conventional (cDC)-based tolDC therapy as a potential solution. Hence as a first step, we aim to compare the transcriptomic profile of our 'gold standard' monocyte derived-tolDC (Mo-tolDC) (induced with active form of VitD3 and dexamethasone) with both the peripheral blood and cultured $CD34^{+}$-derived conventional DC subsets (cDC1 and cDC2) through a Nanostring gene expression analysis system.

3.1.1 Nanostring nCounter Technology

The NanoString nCounter gene expression analysis is a reliable technology designed for the identification of the expression of as many as 800 genes in a single reaction. The methodology involves the direct digital detection of mRNA molecules of interest through the utilization of target-specific, multiplexed color-coded probe pairs. The main advantages of the nCounter® technology are the ability of measuring total mRNA transcripts without requiring cDNA conversion or target amplification, requires minimal sample preparation and has been repeatedly demonstrated to correlate well with other microarray platforms (Reis et al., 2011).

N.B All Mo-tolDC mentioned in this section are generated by treatment with dexamethasone and the active form of vitamin D3 (the 'gold-standard' protocol used in the Hilkens Laboratory (Anderson et al., 2017; Harry et al., 2010a; Spiering et al., 2019). Mature DC is either stimulated with MPLA & IFN-γ (in Good Manufacture Practice (GMP)-compliant CellGenix DC medium) or LPS alone (in RF10 medium). See table 3.1 for the nomenclature of all cells used in this chapter.

Monocyte derived dendritic cells (moDC)	*Cultured CD34$^+$ derived dendritic cells (cul-cDC)*	*Peripheral blood derived dendritic cells (bl-cDC)*
Immature dendritic cells (immDC) Mature dendritic cells (matDC) Tolerogenic dendritic cells (tolDC)	Conventional dendritic cell 1 (cul-cDC1) Conventional dendritic cell 2 (cul-cDC2) Plasmacytoid dendritic cell (cul-pDC) Cultured monocyte (cul-Monocyte)	Conventional dendritic cell 1 (bl-cDC1) Conventional dendritic cell 2 (bl-cDC2) Plasmacytoid dendritic cell (bl-pDC) Blood monocyte (bl-Monocyte)

Table: 3.1 Nomenclature of all DC used in this chapter.

3.2 Chapter aims

1. To identify the tolerogenic signature genes in tolDC.
2. To identify the natural tolerogenic genes expressed in cDC subsets.
3. To select the most appropriate cDC subset to generate a cDC-based tolerogenic DC therapy.
4. To investigate the transcriptomic relationship between cDC and tolDC.

3.3 Experimental approach

To address these aims the NanoString nCounter gene profiling system was used. Briefly, moDC data were generated by Rachel Spiering in the Hilkens laboratory (Spiering et al., 2019),

while the cDC data were generated by Ursula Cytlak in the Bigley laboratory (Cytlak et al., 2020; Kirkling et al., 2018). An overview of the significantly up- and down-regulated genes in our tolDC were obtained. Then, these genes were compared with the differentially expressed genes (DEGs) in both cul-cDC and bl-cDC subsets in order to identify the similarity and differences between them. In addition, the most closely related conventional DC (cDC) subsets to our tolDC were identified in line with the main objective of my project. Moreover, I performed a literature study to create a list of DC-related tolerogenic genes (tolGenes) in order to do a heatmap overview of tolDC, both cul- and bl- cDC1 and cDC2 subsets, based on these tolGenes.

The transcriptomic analysis pipeline was performed in four steps as follows:

1. Comparative transcriptomic profile of the moDC conditions (immDC, matDC and tolDC).
2. Defining of tolerogenic signature genes in our tolDC through selecting the intersection or overlap between the differentially expressed genes (DEGs) in (tolDC vs matDC) and (tolDC vs immDC).
3. Comparative transcriptomic profile of moDC conditions with both cultured $CD34^{+}$ derived and blood DC subsets to detect any similarities or differences in gene expression profiles.
4. In order to investigate whether any of conventional DC (cDC) expressed any pro-tolerogenic genes, a heatmap based on DEGs were performed.

Next, tolerogenic genes and other molecules of interest that were significantly upregulated in tolDC were validated at the protein level by flow cytometry.

3.4 Results

3.4.1 Transcriptional tolerogenic signature of monocyte-derived tolDC

To identify the differential gene expression profile and the unique molecular mechanism in tolDC, I performed a comparative transcriptomic analysis between tolDC, matDC and immDC. Of the 578 genes in the Immunology V2 Nanostring panel plus the 30 custom DC-related genes, 416 genes remained in the analysis after normalization and pre-processing steps described in Chapter 2, section (2.2).

In order to find out the significantly up/down regulated genes in tolDC, the DEGs between tolDC vs matDC were compared in 4 healthy donors. The representation of the gene expression profile of the tolDC and matDC in a heatmap evidenced the separation into 3 clusters of gene sets that have opposing expression in tolDC and matDC (hierarchical clustering displayed as a dendrogram above and on the right side of the heatmap to differentiate between the cell type and genes set expression, respectively) (figure 3.1A). Next, in order to distinguish and find the relationship between different condition of moDC, principal component analysis (PCA) was performed. As shown in figure 3.1B each group of samples clustered well together indicating clear differences in the cell types. Each one of PC represent a pattern or variation in the gene expression data to distinguish between the three conditions. However, PC1 and PC2 captured the most significant pattern or variation in the gene expression profile between the group of cells showing a prominent difference between tolDC, matDC and immDC (figure3.1B)

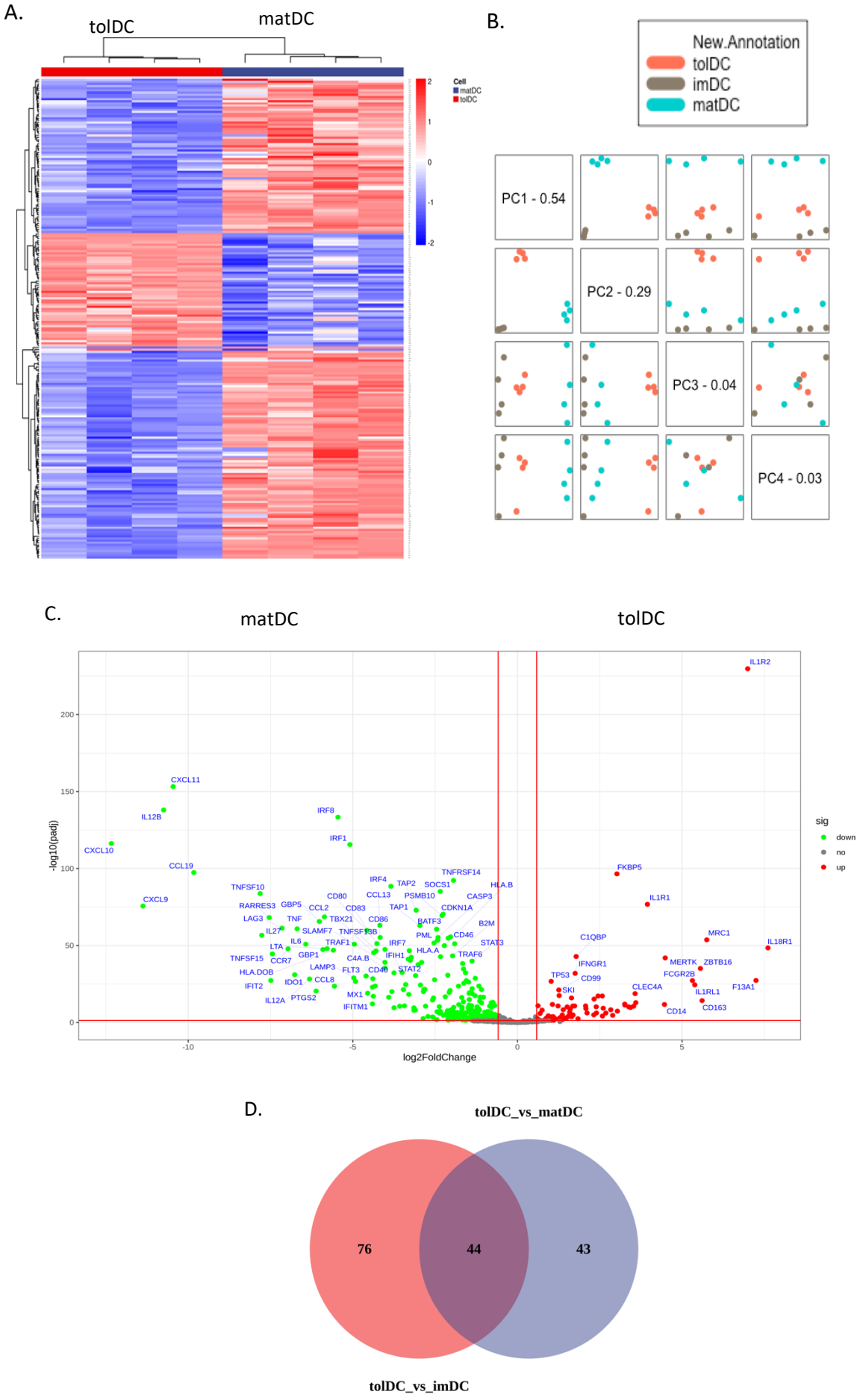
Then, using greater than 2-fold change and adjusted p-value < 0.01, I identified 87 statistically significant upregulated genes and 300 downregulated genes in tolDC in comparison with matDC (figure 3.1C). As demonstrated in Table 3.2 and figures 3.1C and 3.2, several of the overexpressed genes in tolDC were directly involved in immune tolerance such as *MERTK* (Receptor tyrosine kinases of the TAM family) and in other immune-related function such as complement activation (*C1QA and C1QBP*), innate immune system (*S100A8, S100A9* and *FCGR2B*), while others mainly related to metabolism and cell cycle function.

Interestingly, *IL1R2* (interleukin-1 receptor-2) was one of the most striking differentially expressed genes in tolDC (figure 3.1C and figure 3.2A). This gene encodes the IL1R2, which act as a decoy receptor that binds IL-1β with high affinity (Molgora et al., 2018). This receptor was found to be expressed at very high levels by a number of cells, including monocytes, M2 macrophages, neutrophils and B cells (Peters et al., 2013). Hence, it will be of interest to validate this gene at the protein level in our tolDC, which may contribute to our understanding of how these cells exert their regulatory actions.

Other interesting genes that were significantly up regulated were *FKBP5* and *F13A1*. Notably, *FKBP5* was one of the top 6 up regulated genes in tolDC in comparison with both matDC and immDC (table.3.2, figure 3.1C and 3.2B). See discussion section 3.5.2 for more information about this gene.

Surprisingly, Indoleamine 2,3-dioxygenase 1 (*IDO1*) and *LILRB4* (ILT3-encoding gene); both important tolerogenic regulators; were found to be expressed at lower levels in our tolDC as compared to matDC (figure 3.3). This could be because of using both MPLA and IFN-γ for the maturation of DC, of which IFN-γ is a known inducer of IDO (Mellor et al., 2017; Scheler et al., 2007), while using MPLA alone for maturation of tolDC. In contrast, *CLEC4A* (C-type lectin receptor), of which the protein contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and *MRC1* (Mannose receptor C type 1), of which the protein is mainly associated with phagocytosis (Hoober et al., 2019), were both found to be highly up regulated in tolDC compared to matDC (figure 3.2). In addition, *SOCS3* (suppressor of cytokine signaling-3) a major regulator of inflammation with immunosuppressive effects was expressed lower or higher in tolDC compared to matDC and immDC, respectively. This could contribute to the tolerogenic function of tolDC as reported in the literature. As expected, the expression of a number of genes with immunostimulatory function (*AHR, IL12A&B, CD83, CD86, PRDM1 and TNF)* was lower in tolDC than in matDC.

Furthermore, in order to identify the overlap in the upregulated genes between tolDC-*vs*-matDC and tolDC-*vs*-immDC, a venn diagram was created, showing that 44 upregulated genes overlap; thus, these genes appeared to be strongly related to the tolDC profile. See figure 3.1D and table 3.3). Significantly, of these 44 overlapping genes, *TLR2* (Toll like receptor 2) and *IL18R1* (interleukin-18 receptor -1) that are mainly involved in signaling function were highly up regulated in tolDC compared to both matDC and immDC. Thus, both genes could have a regulatory function that contribute to generation of tolerance (table 3.3 and figure 3.2).


A.
tolDC
matDC
Cell
matDC
tolDC
2
1
0
-1
-2
B.
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tolDC
imDC
matDC
PC1 - 0.54
PC2 - 0.29
PC3 - 0.04
PC4 - 0.03
C.
matDC
tolDC
-log10(padj)
log2FoldChange
sig
down
no
up
IL1R2
CXCL11
IL12B
CXCL10
IRF8
IRF1
CCL19
CXCL9
FKBP5
IL1R1
MRC1
IL18R1
C1QBP
IFNGR1
MERTK
ZBTB16
FCGR2B
TP53
CD99
SKI
CLEC4A
IL1RL1
F13A1
CD14
CD163
TNFRSF14
IRF4
TAP2
SOCS1
HLA.B
TNFSF10
CD80
CCL13
PSMB10
CASP3
RARRES3
GBP5
CCL2
CD83
TAP1
CDKN1A
LAG3
TNF
TBX21
CD86
BATF3
B2M
IL27
SLAMF7
TNFSF13B
PML
CD46
IL6
TRAF1
IRF7
STAT3
LTA
GBP1
HLA.A
TNFSF15
CCR7
C4A.B
IFIH1
TRAF6
HLA.DOB
LAMP3
FLT3
CD40
STAT2
IFIT2
IDO1
CCL8
IL12A
PTGS2
MX1
IFITM1
D.
tolDC_vs_matDC
76
44
43
tolDC_vs_imDC

Figure 3.1: Differential expressed genes in tolDC. A) Overview Heatmap of transcriptomic expression profile of tolDC and matDC: red samples are tolDC while blue is mature DC. B) Principal component analysis (PCA) of the 3 moDC conditions: (tolDC red dots, matDC blue dots and immDC grey dots). The first four principal components of the gene expression data are plotted against each other and coloured by the values of the selected covariate. C) Volcano plot of top differentially expressed genes in tolDC (the red dots) vs matDC (the green dots) based on log 2-fold change. D) Venn diagram of up regulated genes in (tolDC *vs* matDC) blue circle and (tolDC *vs* immDC) red circle. There are 44 genes in the overlap between (tolDC vs matDC) and (tolDC vs immDC) which defined as the mostly related to tolDC expression profile.

RANK	Gene symbol	Gene name	Fold change (FC)
	Up regulated in tolDC		
1	IL1R2	Interleukin 1 receptor 2	127.6
2	FKBP5	FK506 binding protein 5	8.1
3	IL1R1	Interleukin 1 receptor 1	15.4
4	MRC1	Mannose Receptor C-type 1	53.9
5	IL18R1	Interleukin 18 Receptor 1	195.5
6	C1QBP	Complement C1Q binding protein	3.4
7	MERTK	MER Proto-oncogene tyrosine kinase	22.5
8	ZBTB16	Zinc finger And BTB domain containing 16	47.1
9	IFNGR1	Interferon Gamma Receptor1	3.4
10	F13A1	This gene encodes the coagulation factor XIII A subunit	152
	Downregulated in tolDC		
1	CXCL11	C-X-C motif chemokine ligand 11	-1406.8
2	IL12B	Interleukin 12 B	-1718.5
3	IRF8	Transcription factor, interferon regulatory factor 8	-43.7
4	CXCL10	C-X-C motif chemokine ligand 10	-5166.1
5	IRF1	Transcription factor, interferon regulatory factor 1	-34
6	CCL19	C-C motif chemokine ligand 19	-910.4
7	TNFRSF14	TNF Receptor Superfamily Member 14	-3.8
8	IRF4	Transcriptional activator, interferon regulatory factor 4	-14.3
9	SOCS1	Suppressor of cytokine signaling 1	-5.1
10	TNFSF10	TNF ligand Superfamily Member 10	-225.3

Table 3.2: Top 10 Differentially expressed genes in tolDC *vs* matDC. P value is <0.001 in all top 10 up/down regulated genes.

Genes symbol
Gene related to innate immune function C1QBP, C3, C1QA, C1QB, CD163, CD14, S100A8, S100A9, FCGR2A.C, FCRG2B, FCGR2A, IRAK3, CLEC4E, CLEC4A, CLEC9A, CFH, NLRP3, MIF, DBN1, MASP1. ***Gene related to signaling and trafficking*** IL1R2, IL1R1, IL6ST, IL18R1, TLR2, IL1RL1, CCR6, CCL17, LAIR1/CD305, IL1A, CXCL5, CD3EAP, CD24, CD79B ***Gene related to immune metabolism and cell cycle function*** FKBP5, SLC2A1, ADA, KCNJ2, GGT5, CCND3 ***Gene related to transcriptional regulation*** Ki67, CEBPB, ZBTB16 ***Gene related to tolerance induction*** MERTK

Table 3.3: Summarized List of the 44 upregulated genes in tolDC after overlap with matDC and immDC according to Venn diagram. The genes were classified according to related function

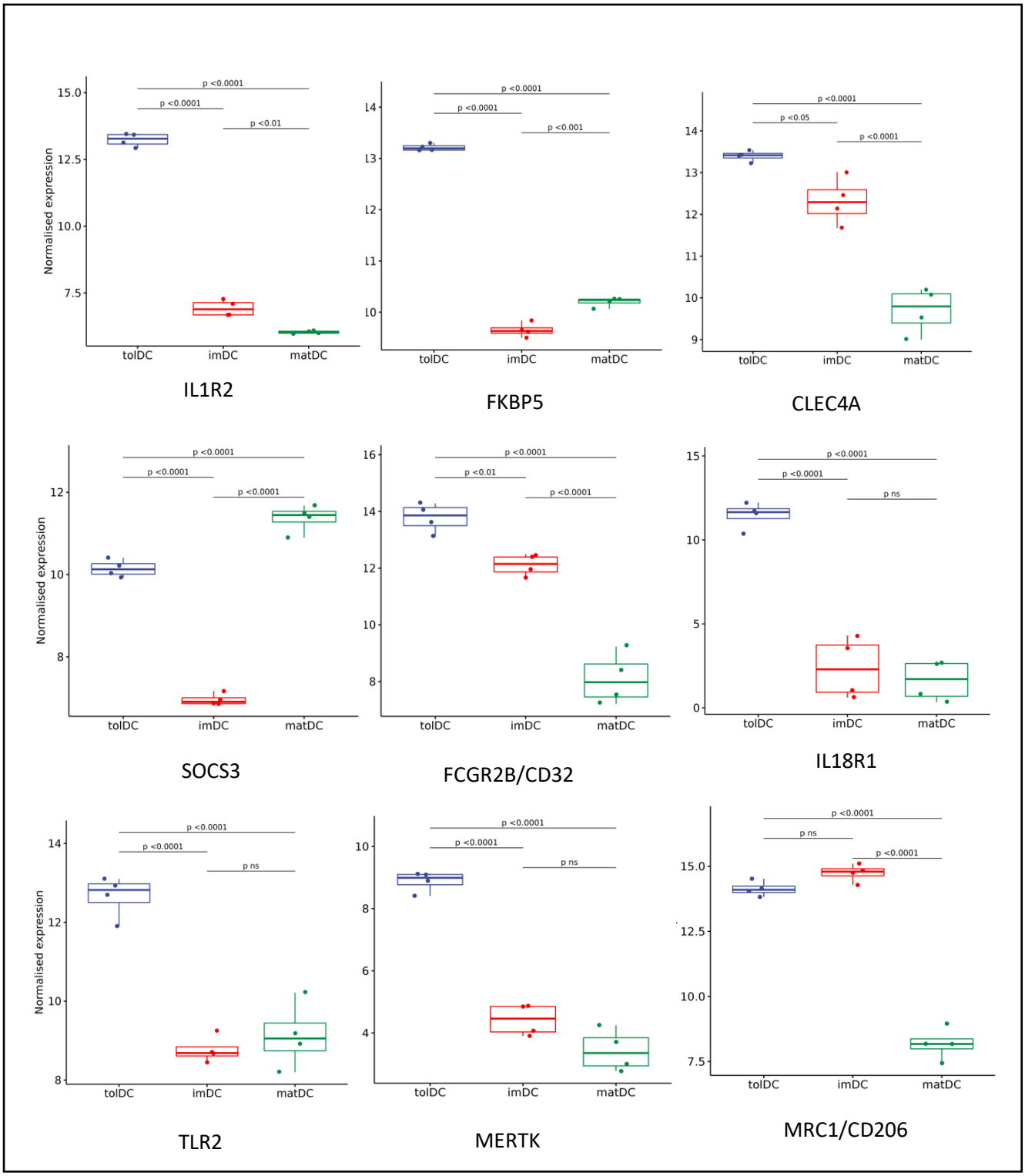


Figure 3.2: Box plot of some important significantly up-regulated genes in tolDC (blue) in comparison with matDC (green) and immDC (red). P-value represent the results of pairwise comparison with Wald test.

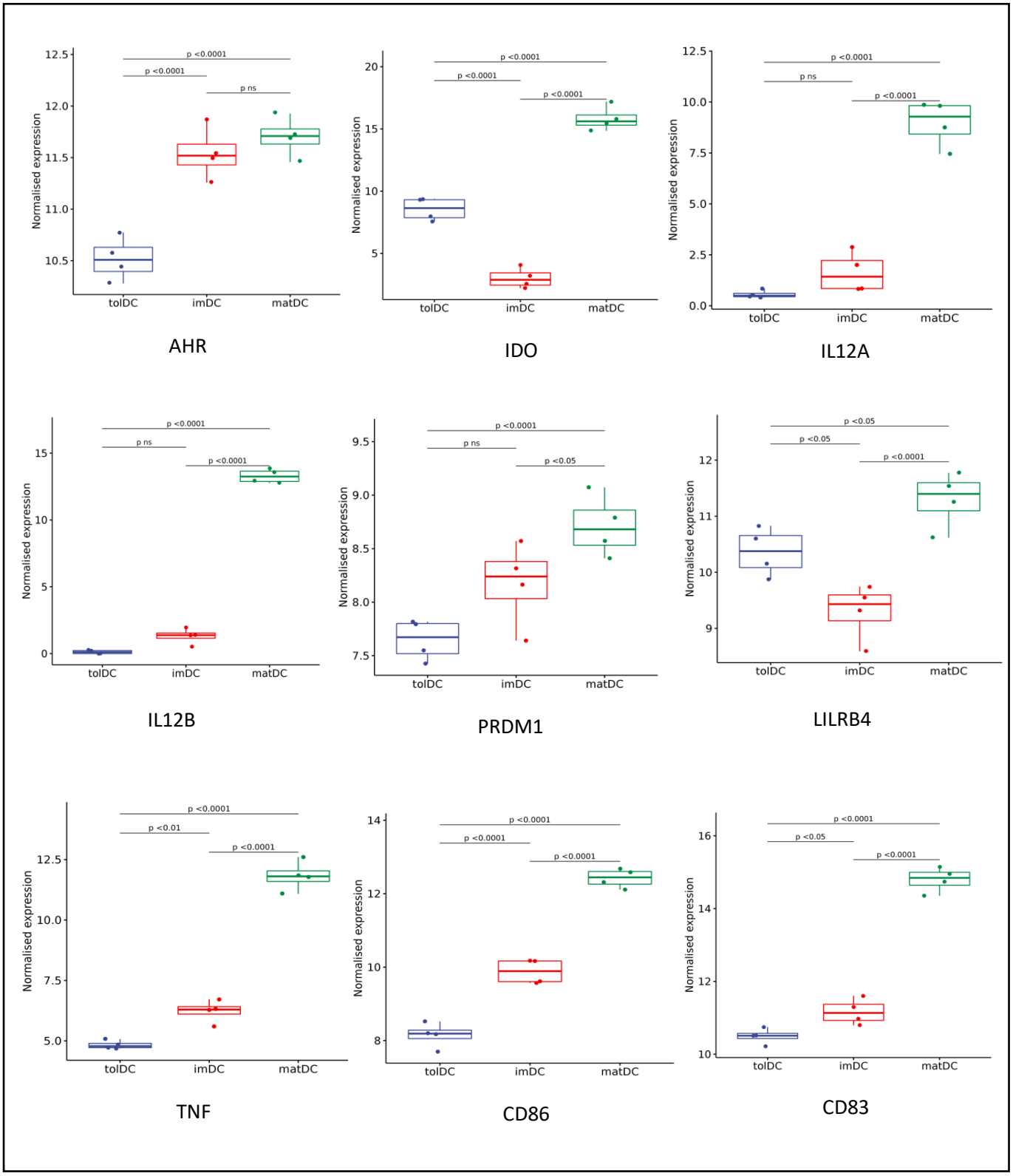


Figure 3.3: Box plot demonstrate significantly down regulated genes in tolDC vs matDC. Blue is tolDC, red immature DC and green is mature DC. P-value represent the results of pairwise comparison with Wald test. Full process of normalisation is explained in method section page 40 and 41.

3.4.2 Differential expression analysis of tolDC with other DC subsets

Hierarchical clustering and heatmap of DEGs was performed in order to investigate the gene expression profile similarities and differences between tolDC and both cul- and bl-cDC subsets. In addition, 42 genes that i) were reported in the literature as up- or down-regulators of tolerogenicity (tolGenes) in various tolDC types and ii) were also present in the Nanostring panel were identified (see tables 3.4, 3.5 and 3.6). These tolGenes were also included in the analysis, in addition to the tolDC-related signature genes that were identified in this thesis (see table 3.4). It is to be noted that there are other regulators of tolerance that are reported in literature but were, unfortunately, not present in the Nanostring panel used, such as *GILZ*, *ANXA1* and *THBS1*(Navarro-Barriuso et al., 2018).

3.4.2.1 Comparison of tolDC with cul-cDC subsets

In line with the PCA analysis (figure 3.4A), the unsupervised hierarchical clustering showed two main separate branches, where the DC differentiated from $CD14^+$ monocytes (tolDC, immDC and matDC) clustered together and the DC derived from $CD34^+$ cells (cDC1, cDC2 and pDC) clustered together (figure 3.4B). Interestingly, the cultured $CD34^+$ derived monocyte sample was clustering with the monocyte-derived DC types, which suggests that the clustering is not just driven by culture conditions, but that the cell linage is also influential. As expected, the closest match in transcriptional profiles was seen between immDC and tolDC, which clustered next to each other. However, matDC clustered in a separate branch, demonstrating a distinct transcriptional profile (figure 3.4B).

To have a closer view at similarities and differences between the cDC subsets and tolDC, tissue culture signature genes were removed from the analysis as described in Chapter 2, section 2.2.2.2. In addition, because the gene expression profile in tolDC is mainly dependent on the immunomodulation treatment that is used for generation of tolDC, tolDC signature genes as well as tolerogenic genes (tolGenes) were included in the analysis (see Table 3.4 for definitions). tolGenes are previously literature reported regulators of tolerogenicity in tolDC and present in the Nanostring panel.

Subsequently, to identify which cDC subset was more closely related to the gene expression profile of tolDC, a heatmap was generated based on comparative expression of tolGenes between tolDC, cDC1 and cDC2 (figure 3.5A). Notably, the results indicated that both cDC

subsets naturally express some of the important tolerogenic genes, such as *IDO1* (gene encoding the indoleamine 2,3-dioxygenase) and *BTLA* (B and T lymphocyte attenuator) with greater expression in cDC1. Interestingly, our tolDC showed highest expression of *MERTK* and *IL10* as compared to both cDC subsets (figure 3.5A). Moreover, I found a strongly contrasting gene expression profile between tolDC and both cDC subsets (figure 3.5 A and B). However, of the two cDC subsets, cDC2 showed greater similarity to tolDC in expression of tolerogenic genes, such as: *PD-L1*(CD274), *PRDM1* and *MRC1* (figure 3.5B). Notably there is general trend gene downregulation in tolDC in comparison with all other DC subsets, which may be due to the treatment with potent immunosuppressive drugs.

Tolerogenic signature genes	**Defining method**
tolGenes (literature)	Genes that were reported to be either up- or down-regulated in various tolDC types (generated by different methods) and described in papers including: (Navarro-Barriuso et al., 2018b, 2018a; Ritprajak et al., 2019b; Takenaka and Quintana, 2017) and that were present in the NanoString panel (42 genes; see Tables 3.5 and 3.6).
tolDC signature genes (this thesis)	There are 111 DEGs that results from the intersection or overlap between (tolDC vs matDC) and (tolDC vs immDC). These includes 44 upregulated genes and 67 downregulated genes (figure 3.6C).

Table 3.4: Method of defining tolerogenic signature gene.

Gene symbol	Function
NFKB1	Transcription factor that stimulates the expression of genes involved in in induction of inflammatory cytokines.
AHR	A ligand-activated transcription factor that regulates the activity and degradation of transcription factors important for the control of DC function.
IRF4	Transcription factor that in DC show to modulate TLR signal transduction and regulate expression of CCR7.
CD86 CD80 CD40	Costimulatory molecule that provides necessary signals for T cell activation and also a DC maturation marker
CD83	DC maturation marker
CD1c	DC marker
IL12A IL12B	Pro- inflammatory cytokine that considered to be inflammation associated markers.
IL6	Pro-inflammatory cytokine.
PRDM1/BLIMP1	This molecule regulates CIITA expression (master regulator of MHC II genes) in human moDC, and suppressess IL-6 and Ccl2 transcription
MAPK1/ERK	Important component of the MAP kinase signal transduction pathway which mediates diverse biological functions including decrease of NF-κB DNA binding.
TNFAIP3/A20	The ubiquitin editing enzyme that have a role in degradation of NF-κB molecules
CD74/HLA-DR	Antigen presenting molecule.
LAMP3	lysosomal-associated membrane protein 3 which linked with the maturation of dendritic cells

Table 3.5: List of tolGenes reported to be down regulated in tolDC types (Navarro-Barriuso et al., 2018b, 2018a; Ritprajak et al., 2019b; Takenaka and Quintana, 2017)

Gene symbol	Function
IDO	Indolamine-2,3-dioxygenase enzyme that metabolite tryptophan, the necessary component of T cell proliferation and differentiation.
C1QA &C1QBP	Both involve in vital tolerogenic activity including increased PD-L2 surface expression and decreased CD86. Also related to reduce induction of Th1 and Th17 proliferation (Navarro-Barriuso et al., 2018b).
MERTK	Inhibits T cell activation directly through competition of PROS1 on the surface of T cells (Suwandi et al., 2017).
CD274/PDL-1	Programmed death-ligand that enables tolDC to inhibit effector T cells and trigger Treg induction.
IL-10	An anti-inflammatory cytokine that inhibits the production of IL-12 and the expression of co-stimulatory molecules by DC.
LILRB4/ILT3 LILRB2/ILT4	Inhibitory receptors that promoted DC tolerogenicity and subsequent T-cell suppression.
CCL18	Treg chemotactic factor upregulated in tolDC induced by dexamethasone.
CCL22	Treg chemotactic factor upregulated in tolDC induced by vitD3.
MRC1/CD206	Mannose receptor C-type lectin that is both immature DC and M2 macrophages marker.
CD14	A monocyte marker which identified to be also associated with the immunosuppressive function of tolDC (van Wigcheren et al., 2021).
FCGR3A	Fc fragment of IgG receptor IIIa,
IL1RN	IL1Ra cytokine that are antagonist ligand for IL1 receptor and also inhibit DC maturation and subsequent T cell activation and polarization.
FCGR2B	An immunoregulatory receptor that reported to maintain tolerance in tolDC.
TLR2	Toll like receptor that have a role in maintain tolerance through increase production of IL-10 (Chamorro et al., 2009; Raker et al., 2015).
CD163	A marker of monocyte/macrophages cell lineage.
ZBTB16/PLZF	A transcriptional repressor that play a role in inhibition of DC maturation
F13A1	Coagulation factor XIII A that has critical roles in blood clot stabilization and may have a role in RA production and induce Foxp3+ Tregs.
CTSC	Cathepsin C is considered to be a pan-regulatory tolDC marker that expressd in all tolDC type.
CLEC4A	DC immunoreceptor 2 (DCIR2) is a C-type lectin receptor mainly expressed on $CD8\alpha^-$ conventional DC and involved in regulating immunity and impairing inflammation and T cell activation (Uto et al., 2016).
CD209	DC-SIGN is a C-type lectin receptor that mainly expressed on immDC and often associated with suppression of inflammation , inhibition of pro-inflammatory cytokine and induction of regulatory immune response (Castenmiller et al., 2021).
PPARG	Nuclear receptor that involve in immunometabolism such as Inhibition of NF-κB nuclear localization.
PTPN6/SHP-1	This gene encodes shp-1 enzyme that act as inhibitor for RAS–MAPK pathway.
SOCS1	A member of the SOCS family of intracellular proteins that has a critical inhibitory function in DC by controlling the cytokine response and antigen presentation.
STAT3	A cytoplasmic transcription factor that has a role as a negative regulator of inflammatory responses.
SOCS3	A member of the SOCS family of intracellular proteins that suppress cytokine signaling and Th2 induction
IL27	Member of IL-12 family and known to have antagonistic function on Th17 and capacity to generate IL-10 producing, type 1 regulatory T cells (Tr1). Also, it induced expression of PDL-1 in DC .

Table 3.6: List tolGenes reported to be up regulated in tolDC types (Navarro-Barriuso et al., 2018b, 2018a; Ritprajak et al., 2019a; Schinnerling et al., 2015; Takenaka and Quintana, 2017)

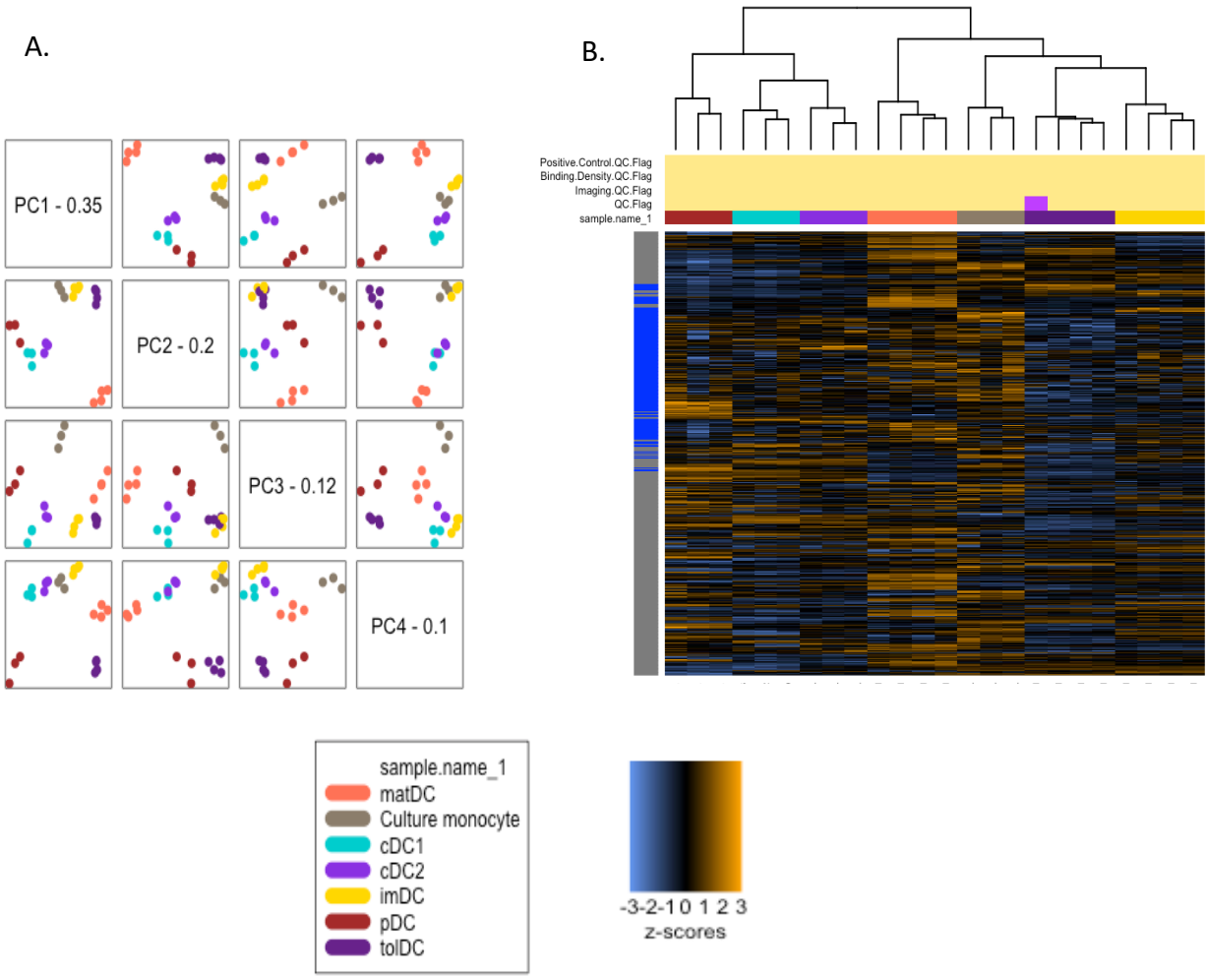
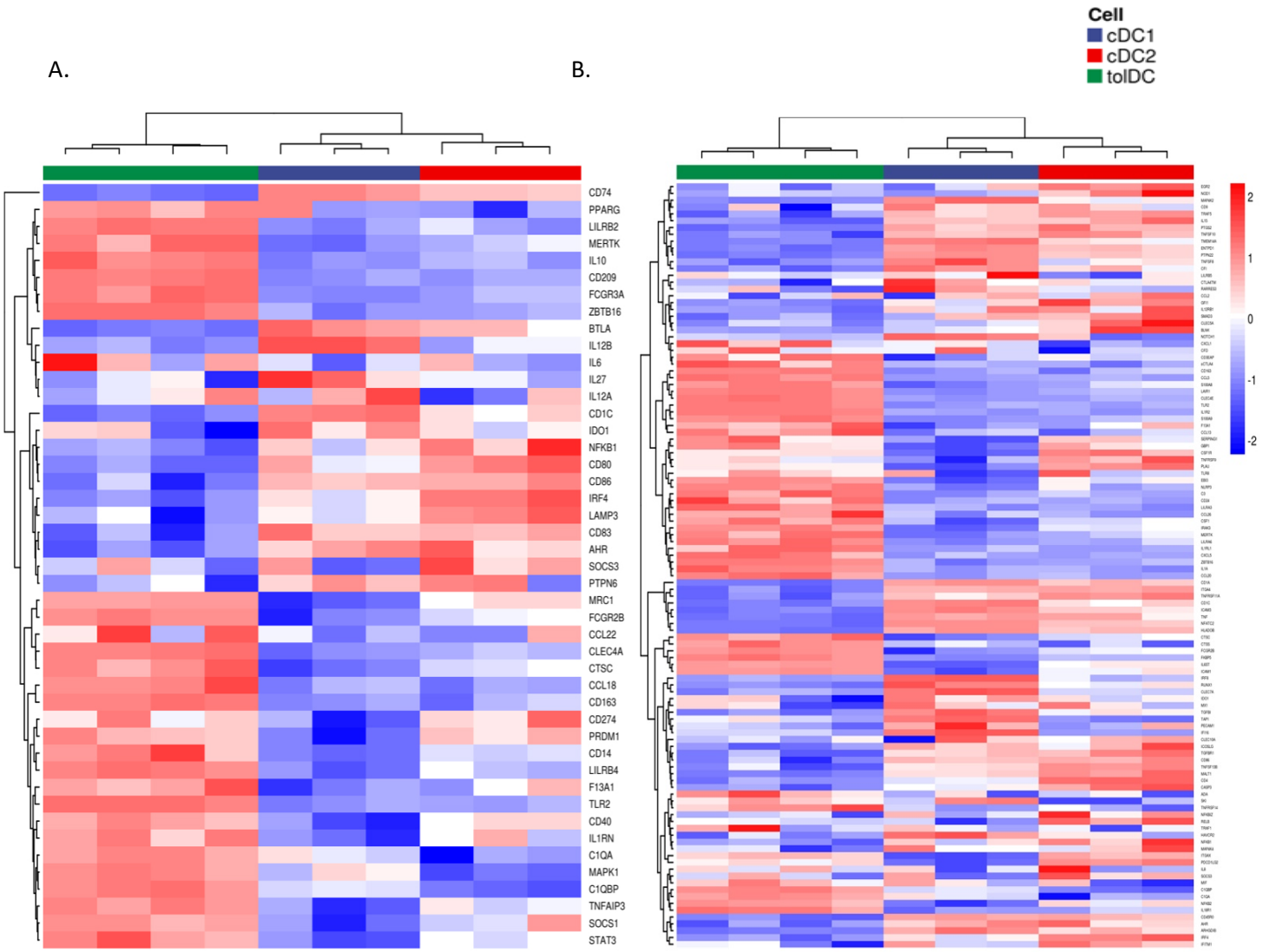


Figure 3.4: Relationship between moDC types, cul-DC subsets and cultured monocytes. A) PCA plot of the first four principal components of the gene expression data are plotted against each other and colored by the values of the selected covariate as light red (matDC), yellow (immDC), dark purple (tolDC), gray (cultured monocyte), blue (cul-cDC1), light purple (cul-cDC2) and dark red (cul-pDC). B) Heatmap and hierarchical clustering of moDC type and cul-DC based on DEGs. Orange indicates high expression; blue indicates low expression.



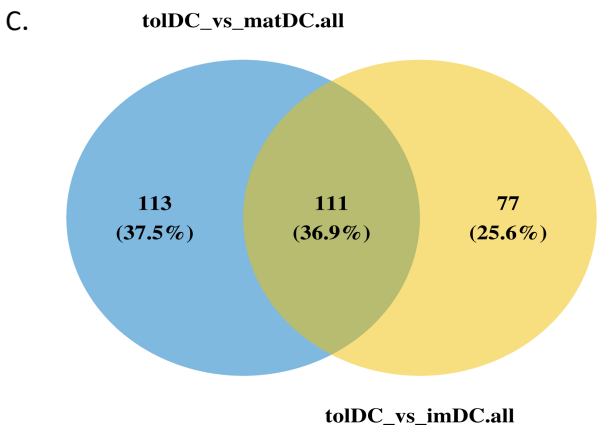


Figure 3.5: Identification of similarity and differences in differentially expressed genes between tolDC and cul-cDC subsets. A) Heatmap demonstrate the gene expression profile

of tolDC (green), cDC1 (blue) and cDC2 (red) based on tolGenes. B) Heatmap of tolDC, cDC1 and cDC2 based on tolDC markers. C) Venn diagram show the 111 DEGs overlap between (tolDC vs matDC) and (tolDC vs immDC).

3.4.2.2 Comparison of tolDC with bl-cDC subsets

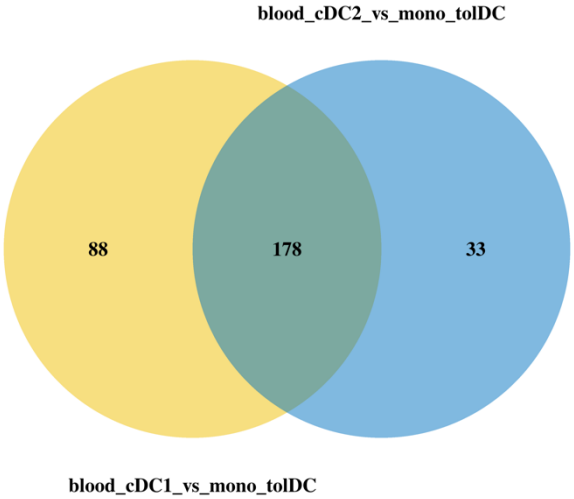
To identify the similarities and differences in gene expression profile between tolDC and peripheral blood cDC (cDC1 and cDC2), overlap in the differentially expressed genes between tolDC vs bl-cDC2 and tolDC vs cDC1 were performed. As shown in figure 3.6A, 178 tolDC signature related genes were found on the overlap between tolDC vs bl-cDC2 (blue circle) and tolDC vs bl-cDC1 (yellow circle). Next, in order to determine the most closely related bl-cDC to tolDC in gene expression profile, hierarchical clustering with heatmap based on the 178 tolDC signature genes as well as tolGenes that reported in the literature were performed. As shown in figure 3.6B, both bl-cDC clustered in one main branch whereas tolDC cluster in different branch according to the 178 toDC signature genes. In addition, generally there is contrasting gene expression between tolDC and both bl-cDC. However, bl-cDC2 demonstrated greater similarity than bl-cDC1 in the expression of some genes with tolDC. For example, both tolDC and bl-cDC2 demonstrated higher upregulation of *MRC1*, *PDL-1* and *FCGR2B* genes compared to bl-cDC1.

Interestingly, as demonstrated in figure 3.6C, bl-cDC2 and tolDC were clustered together in one branch, while cDC1 clustered in another separate branch suggesting a higher similarity between tolDC and bl-cDC2 than bl-cDC1 according to tolGenes expression profile. In addition, bl-cDC1 demonstrates contrasting expression of tolGenes compared to tolDC, where the genes that were highly expressed in tolDC showed lower expression in cDC1 and vers versa. However, bl-cDC2 was determined to have some similarity with tolDC in the expression of tolGenes. For example, both tolDC and bl-cDC2 were found to be highly expressed *SOCS3*, *MRC1*, *FCGR2B,IL1RN* and *F13A1* genes in contrast to cDC1. In addition, both tolDC and bl-cDC2 demonstrate downregulation of *IL12A* and *IDO1* genes compared to bl-cDC1. However, bl-cDC2 also demonstrates some differences with tolDC in the expression of tolGenes with upregulation, specifically in the genes that related to maturation and co-stimulation *CD83, CD86* and *CD74* genes. Conversely, *MERTK* and *IL-10* genes were found to be downregulated in both bl-cDC1 and bl-cDC2 compared to tolDC. Notably, I found upregulation in both

CLEC4A and *LILRB4* genes in tolDC and slightly higher upregulation in bl-cDC2 as well compared to bl-cDC1.

To this end, based in both tolDC signature and tolGenes, bl-cDC2 was found to be more closely aligned to tolDC in gene expression than bl-cDC1.

A.



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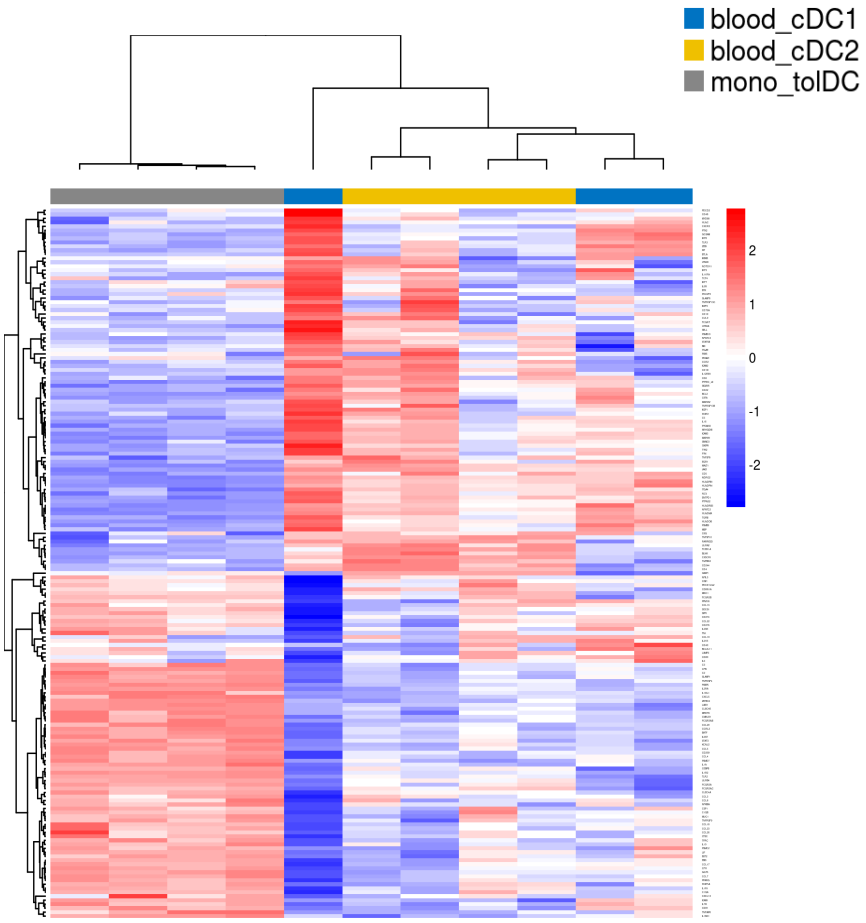


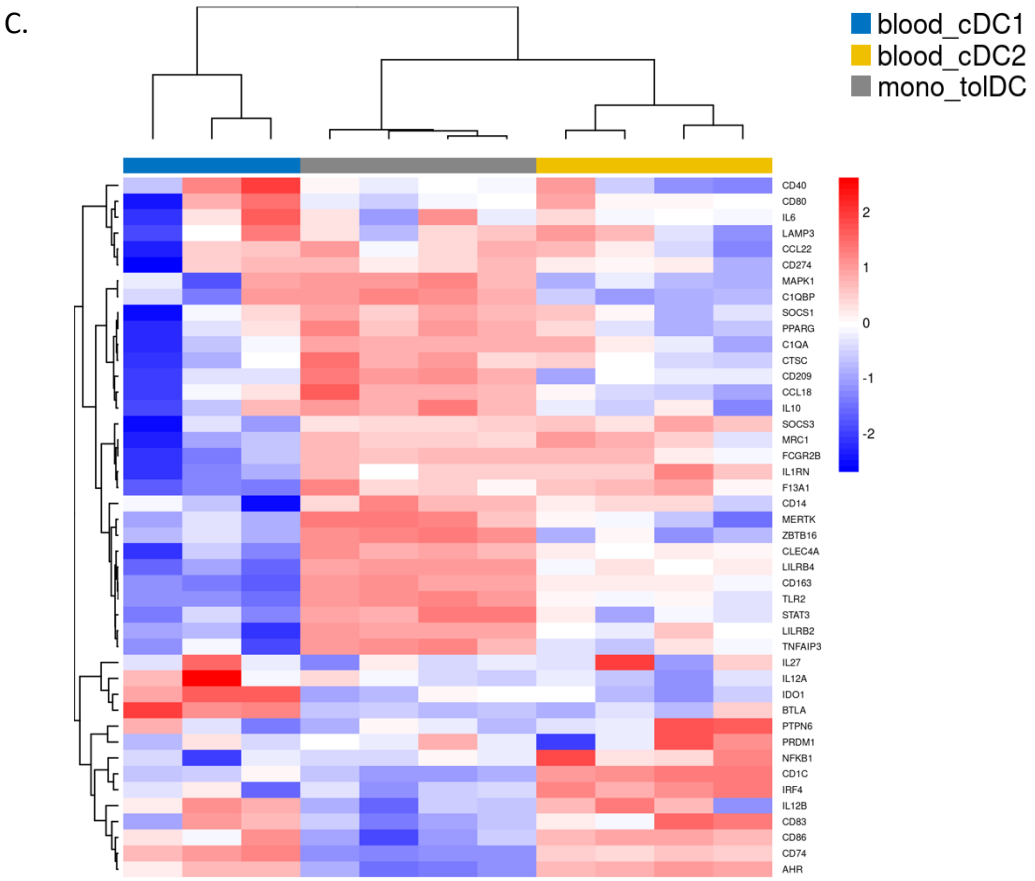


Figure 3.6: Transcriptomic expression profile comparison between tolDC and bl-cDC. A) Venn diagram showing the gene expression profile overlap between tolDC vs bl-cDC2 (blue circle) and tolDC vs bl-cDC1 (yellow circle). B) Heatmap and hierarchical clustering dendrogram demonstrate the up and down regulation of 178 tolDC signature genes in tolDC (gray), cDC1 (blue) and cDC2 (yellow). Red demonstrates high expression of gene while blue demonstrate the low expression of gene. C) Heatmap and hierarchical clustering dendrogram demonstrate the up and down regulation of 42 tolGenes in tolDC (gray), bl-cDC1 (blue) and bl-cDC2 (yellow).

3.4.3 Pathway enrichment analysis of tolDC

The transcriptomic profile of tolDC was mostly consisting of down-regulated gene sets, with evidence of 10 pathways that were repressed in comparison with matDC. Most of these down-regulated pathways were related to inflammation, infection and cancer (table 3.7).

Interestingly, TNF signaling pathway and NF-kappa B signaling pathway where in the list of pathways that are significantly downregulated in comparison with matDC (table 3.8 and figure 3.7B), which in turn may support the tolerogenic function.

3.4.3.1 Pathway Comparison with cultured CD34$^+$ derived DC subsets

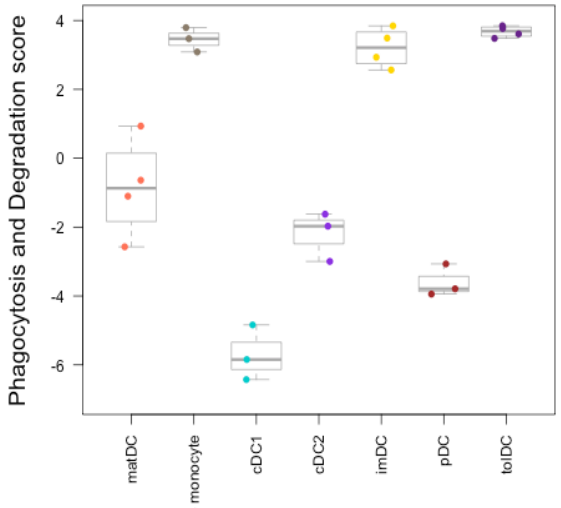
In order to explore and compare the possible functional properties of DEGs in tolDC and other CD34$^+$ derived DC subsets, pathway analysis was done by advanced analysis module through KEGG database. The results demonstrated that 'immunometabolism' and 'phagocytosis and degradation' are the most upregulated enriched pathways in our tolDC compared to matDC and CD34$^+$ derived DC subsets (figure 3.7A). However, cDC2 showed slightly higher 'phagocytosis and degradation' score than cDC1 and pDC. Notably, among CD34$^+$ cultured DC, pDC have the lowest TNF and NFκB signaling enriched pathway (figure 3.7B).

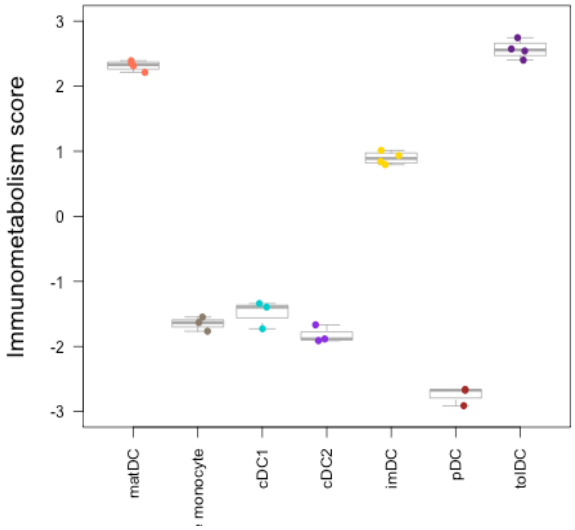
Furthermore, gene set enrichment analysis (GSEA) was performed in order to identify groups of genes in relation to different immune function. Interestingly, *FKBP5* was one of the genes that was significantly up regulated and directly related to regulation of immunometabolism activity in tolDC in comparison to matDC (figure 3.7C). On the other hand, *AHR* (Aryl Hydrocarbon Receptor), a gene also related to immunometabolism, was significantly down regulated in tolDC. However, IDO (Indolamine-2,3-dioxygenase enzyme), considered one of the important tolerogenic genes, was also down regulated in our tolDC (figure 3.7C). As mentioned previously, this could be due to stimulation of mature DC with IFN-γ, which is known as a master regulator of IDO.

Pathway name	Count	P-Value	Genes name
TNF signaling pathway	23	0.0009	SOCS3;CXCL1;RELA;PTGS2;FAS;NFKB1;CXCL10;TRAF5;BCL3; IL6;MAPK11;VCAM1;ICAM1;LTA;TRAF2;IL15;CCL5;CSF1;TNF; TRAF1;CCL20;CCL2;CASP3
Prolactin signaling pathway	13	0.0011	SOCS3;RELA;NFKB1;STAT5A;JAK2;IRF1;MAPK11;TNFRSF11A; SOCS1;STAT1;STAT3;STAT5B;SRC
Herps simplex infection	32	0.0019	SOCS3;TICAM1;CD40;RELA;FAS;NFKB1;TRAF5;PML;IRF7; HLA-A;IL12B;IL6;JAK2;HLA-B;IL12A;LTA;TRAF2;TRAF6;IL15; STAT1;HLA-C;CCL5;STAT2;TNFRSF14;TAP1;HLA-DOB;TNF,JAK1 & Others.
NF-kappa B signaling pathway	24	0.0041	TICAM1;CD40;RELA;MALT1;PTGS2;NFKB1;BCL2;TRAF5; NFKB2;TNFSF13B;CCL19;VCAM1;ICAM1;RELB;BLNK;TNFRSF1 1A;CCL13,LTA;TRAF2;TRAF6;TNF;TRAF1;IL8;PLAU
Hepatitis B	23	0.0097	TICAM1;TGFBR1;RELA;STAT4;FAS;NFKB1;BCL2;NFATC2;IRF7; STAT5A;EGR2;IL6;CDKN1A;STAT1;STAT2;TNF;JAK1;STAT3; IFIH1;IL8;STAT5B;SRC;CASP3
Viral carcinogenesis	18	0.0112	RELA;NFKB1;TRAF5;NFKB2;IRF7;HLA-A;STAT5A; EGR2;CDKN1A;HLA-B;TRAF2;HLA-C; JAK1;TRAF1;STAT3;STAT5B;SRC;CASP3
Pathways in cancer	27	0.0126	TGFBR1;CTNNB1;RELA;PTGS2;FLT3;FAS;NFKB1;BCL2;SMAD3; TRFF5;PML;NFKB2;STAT5A;IL6;CDKN1A;PTGER4;RUNX1;TRA F2;TRAF6;STAT1;JAK1;TRAF1;STAT3;IL8 & Others
Influenza A	26	0.0203	SOSC3;TICAM1;RELA;FAS;NFKB1;CXCL10;MX1;PML;IRF7;IL12 B;IL6;JAK2;MAPK11;ICAM1;IL12A;STAT1;CCL5;STAT2;TNFSF10 ;HLA-DOB;TNF;JAK1;IFIH1;IL8;CCL2;CASP1
Epstein-Barr virus infection	20	0.0276	CD40;RELA;NFKB1;BCL2;TRAF5;ITGAL;ENTPD1;NFKB2;HLA-A;CDKN1A;CD58;MAPK11;ICAM1;RELB;HLA-B;TRAF2;TRAF6; HLA-C;JAK1;STAT3
RIG-I-like receptor signaling pathway	14	0.0446	RELA;TMEM173,NFKB1;CXCL10;IRF7;IL12B;ATG12;MAPK11;IL 12A;TRAF2,TRAF6;TNF;IFH1;IL8

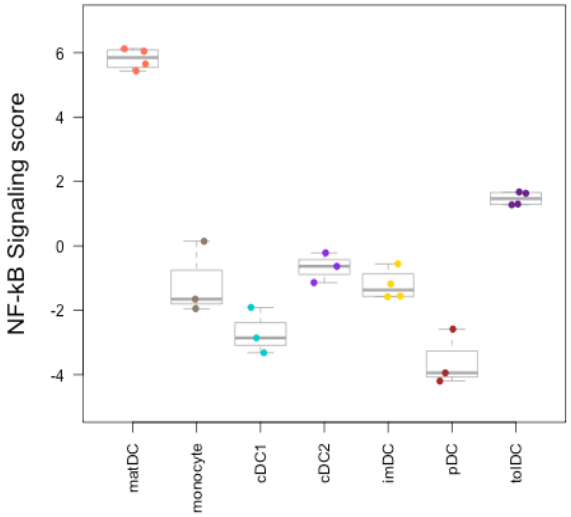
Table 3.7: List of 10 down regulated pathway in tolDC vs matDC determined by DAVID database and KEGG pathway.

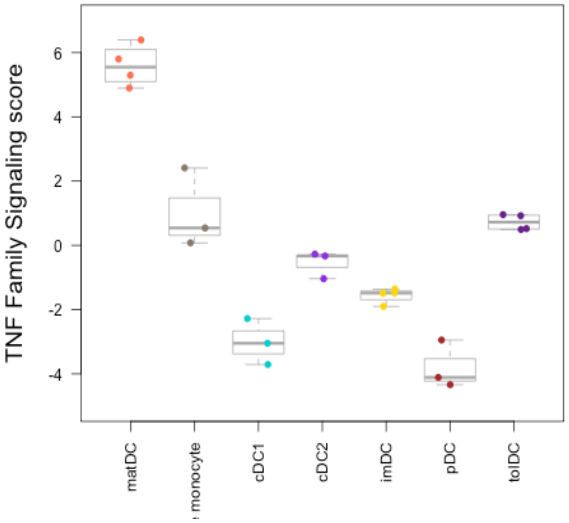
A.


Phagocytosis and Degradation score
4
2
0
-2
-4
-6
matDC
monocyte
cDC1
cDC2
imDC
pDC
tolDC


Immunometabolism score
3
2
1
0
-1
-2
-3
matDC
monocyte
cDC1
cDC2
imDC
pDC
tolDC

B.


NF-kB Signaling score
6
4
2
0
-2
-4
matDC
monocyte
cDC1
cDC2
imDC
pDC
tolDC


TNF Family Signaling score
6
4
2
0
-2
-4
matDC
monocyte
cDC1
cDC2
imDC
pDC
tolDC

C.

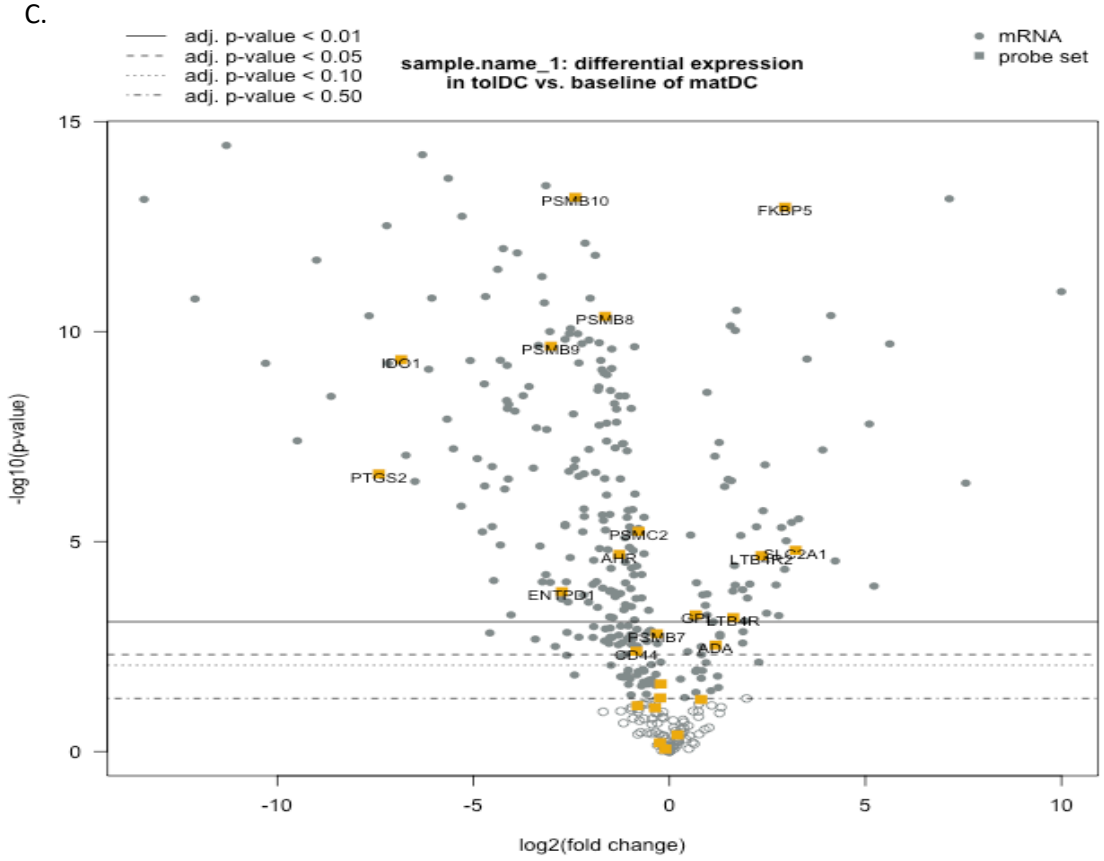


Figure 3.7: Pathway analysis in tolDC vs cultured CD34+ DC subset. A) Box plots of highly enriched pathways in tolDC vs all other moDC conditions and subtypes. B) Box plots show the lower pathway score of tolDC and across different DC subtypes. C) Volcano plot demonstrates significantly upregulated genes related to immunometabolism in tolDC vs matDC.

3.4.3.2 Pathway Comparison with other moDC conditions

Pathway enrichment analysis was performed between tolDC with matDC and immDC to investigate the pathways that are mainly related to tolDC function. There are only a few pathways that are upregulated in tolDC, which are mainly related to phagosome and metabolic activity. The rest of pathways were down regulated in tolDC in comparison with matDC (table 3.7).

GSEA determined that most of the NFκB signaling-related genes were significantly lower in tolDC than in matDC. *CD14*, *SYK* and *IL1R1* were the only exception genes that related to NFκB signaling pathway and were found to be higher in tolDC than matDC (figure 3.8 and

3.9). In addition, a completely contrasting expression of genes that related to 'antigen presentation', 'checkpoint and execution', 'cytokines and chemokines' pathways were identified in tolDC vs matDC (Figure 3.8). Remarkably, genes that are linked to the 'phagocytosis and degradation' pathway (one of the highly enriched pathways in tolDC vs matDC) include MRC1, TLR2, FCGR2B (CD32), CD14, and *CD163* (figure 3.9). These genes could be considered as a signature for tolDC. To this end, combined action of genes as a pathway or sets may control the tolerogenic function in tolDC more than relying on a single gene.

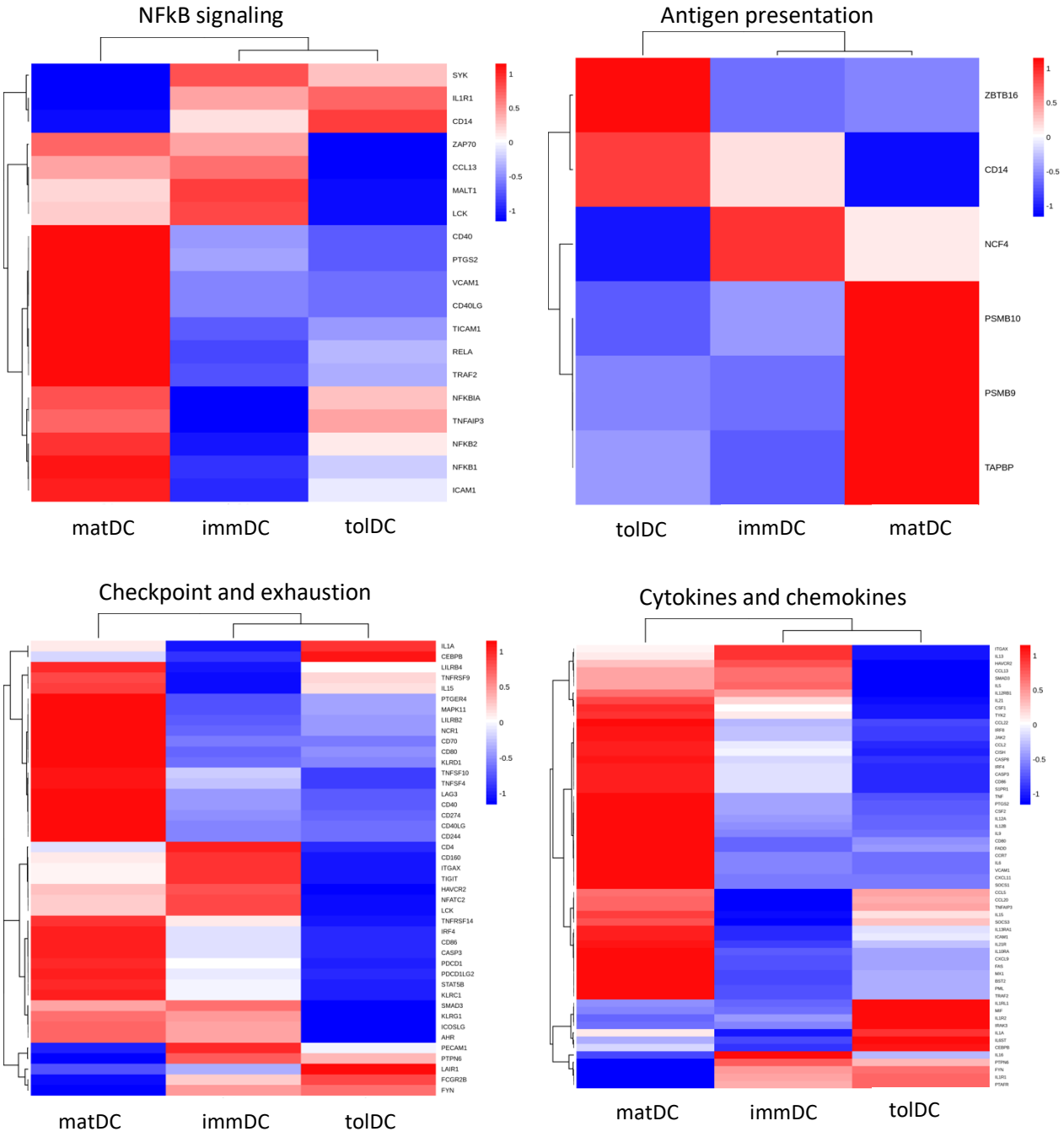


Figure 3.8: Heatmap demonstrate some important pathways expression in tolDC vs matDC and immDC.

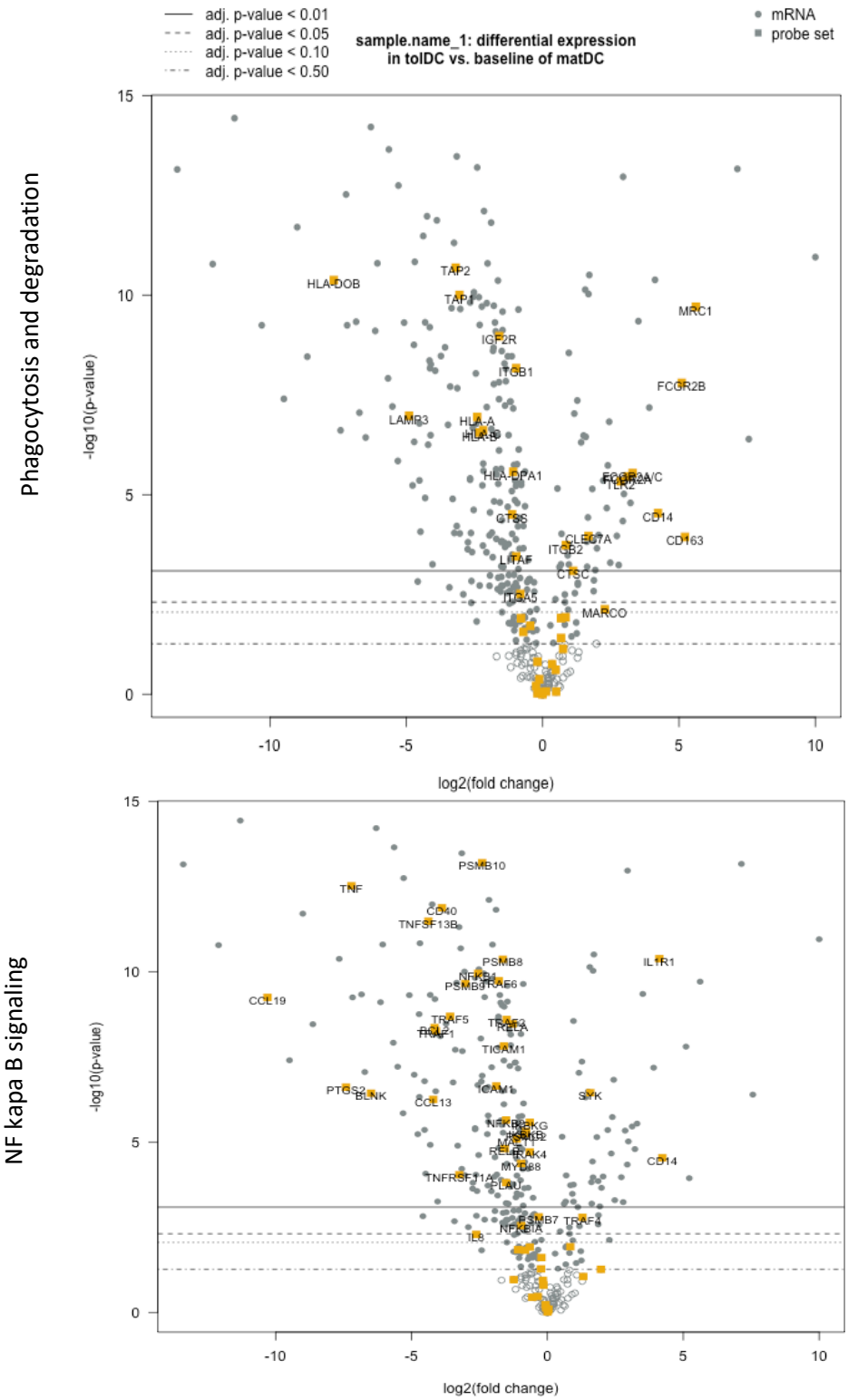


Figure 3.9: Volcano plot of differentially expressed genes in tolDC vs matDC according to pathway analysis shows the log2 fold change vs log10. Adjusted P-value is shown in the upper left of the image.

3.4.4 Identification of pro-tolerogenic genes in conventional DC subsets

In order to investigate the natural pro-tolerogenic genes in cDC, a volcano plot and heatmap that depend on unsupervised hierarchical clustering according to DEGs were performed between the cDC subsets.

3.4.4.1 Pro-tolerogenic genes in cultured CD34^{+} derived cDC

To evaluate the extent to which cul-cDC subsets have a pro-tolerogenic genes, the DEGs between cul-cDC (cDC1 and cDC2) were compared. As expected, there is a contrasting gene expression profile between cDC1 and cDC2 where the genes that are mainly reported in the literature as regulators of tolerance are higher in cDC1 than cDC2 (figure 3.10). For example, *BTLA* and *IDO1* both significantly have higher expression in cDC1 compared to cDC2. However, other important genes that also related to tolerance were significantly higher in cDC2 than cDC1, which include *PDL-1*, *MERTK* and *LILRB4* (ILT3) (figure 3.11).

In addition, there is significant up regulation of an *AIRE* (autoimmune regulator; an essential gene for central tolerance) in cDC2 compared to cDC1 (figure 3.11). Interestingly, cDC2 is also demonstrated to have higher expression of *SOCS3* (suppressor of cytokine signaling-3) (figure 3.11) that were considered to be a major regulators of infection and inflammation , although it have a contrasting influence in inflammation (Carow and Rottenberg, 2014). Notably, some of the highest DEGs in our tolDC (mentioned in section 3.4.1) were also significantly up regulated in cul-cDC2 vs cDC1 including: *FKBP5* and *MRC1*.

To summaries this section, both cul-cDC subsets expressed some of the important pro-tolerogenic genes, but to a different extent. However, cDC2 show more similarity to tolDC with regard to the expression of some important genes that could contribute to its tolerogenic function.

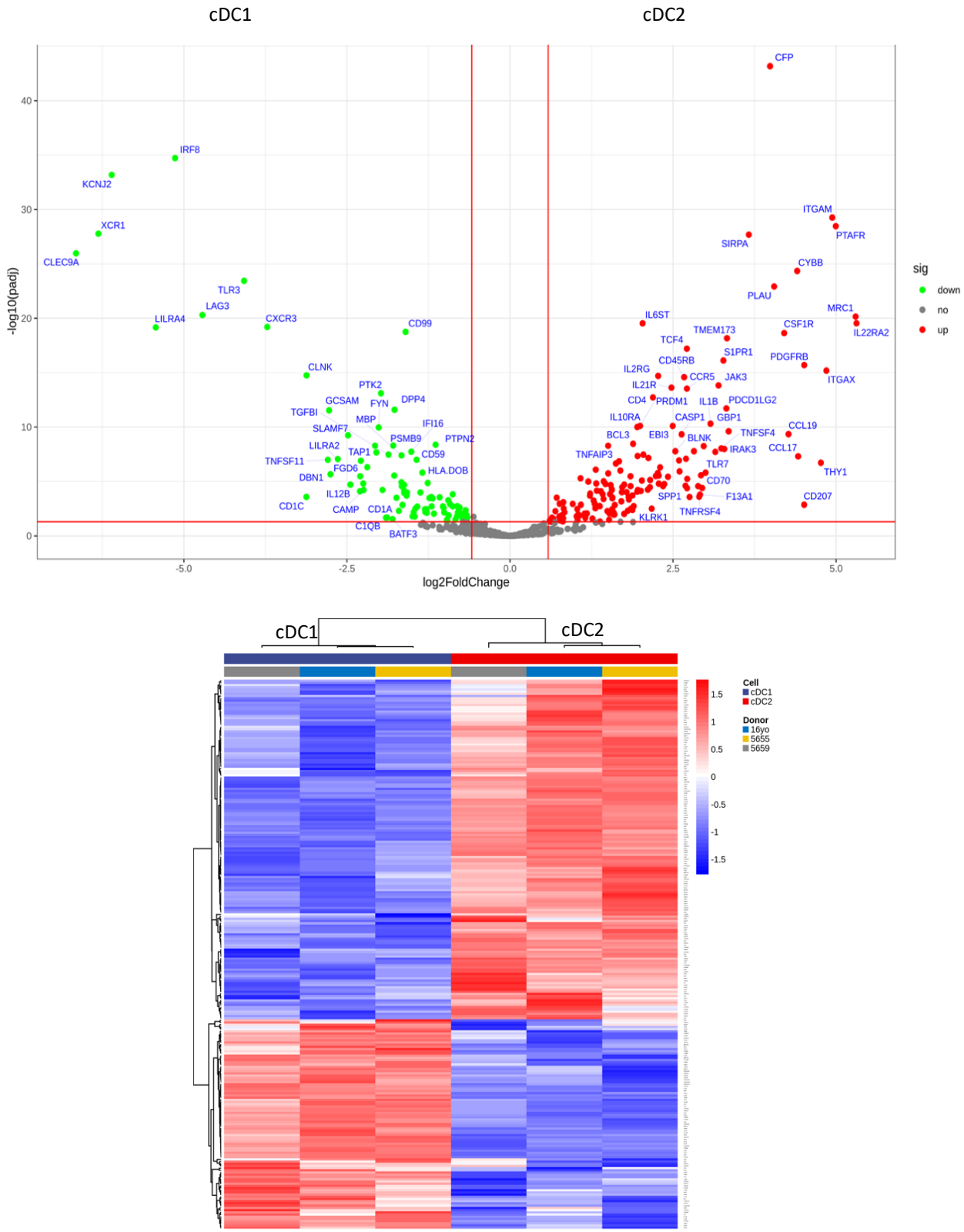


Figure 3.10: Differential expression genes in cul-cDC2 vs cDC1. A) Volcano plot of cDC2 vs cDC1 show a log2 Fold change against the log10. Red is the significantly up regulated genes in cDC2 vs cDC1 and green is significantly down regulated genes in cDC2 vs cDC1. B)

Heatmap of DEGs in cul-cDC2 (Red rectangle) vs cul-cDC1 (Blue rectangle) in 3 different doners. Red demonstrates high expression genes and blue demonstrate low expression genes.

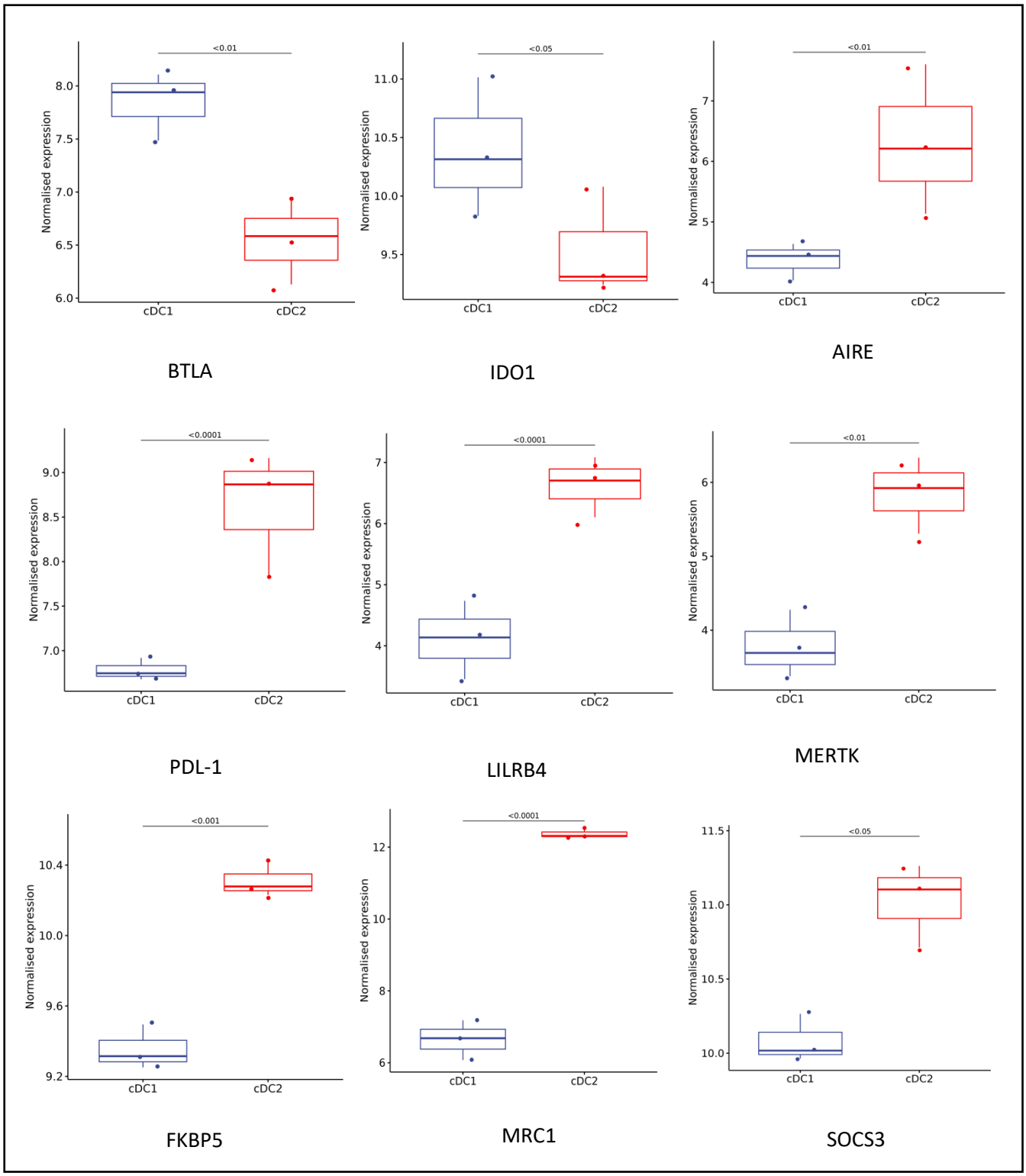


Figure 3.11: Box plot illustrate some of the significantly up/down regulated genes in cDC1 (blue) vs cDC2 (Red). Statistically significant was determined by using pairwise

comparison with Wald test to compare the fold change in gene expression between cDC1 and cDC2. * = $p<0.05$ ** = $p<0.01$ *** = $p<0.0001$.

3.4.4.2 Pro-tolerogenic genes in peripheral blood cDC

Peripheral blood cDC may show a different gene expression profile compared to cul-cDC which mainly depend on the cytokine environment during differentiation. Hence, to investigate the pro-tolerogenic genes in peripheral blood cDC, a DEG analysis and unsupervised hierarchical clustering with heatmap were performed between cDC1 vs cDC2 (figure 3.12A and 3.12B).

Likewise, cul-cDC, peripheral blood cDC demonstrate contrasting gene expression profile between cDC1 and cDC2 (figure 3.12 A and B). However, bl-cDC2 demonstrate striking expression of *CLEC10A* and *FCGR2B* compared to cDC1, while were no significant difference in these tow markers between cul- cDC2 vs cDC1. In addition, similar to cul-cDC1, peripheral bl-cDC1 exhibit high expression of both *BTLA* and *IDO1* tolerance related genes compared to cDC2 which demonstrate significant up regulation of *LILRB2* (ILT4), *LILRB4* (ILT3) genes that encode immunoinhibitory proteins (figure 3.13). However, unlike cul-cDC2, bl-cDC2 express non-significant level of *MERTK* and *PDL-1* genes compared to cDC1 (data not shown).

Interestingly, in alignment with cul-cDC2, bl-cDC2 significantly have higher expression of *MRC1* compared to cDC1. In addition, similar to tolDC, bl-cDC2 have higher up regulation of *CLEC4A* and *IL1R2* genes in contrast with cDC1. Furthermore, *SOCS3* gene were also significantly expressed in bl-cDC2 compared to cDC1. Thus, this gene could contribute to the regulatory function of cDC2 (figure 3.13).

To this end, although, bl-cDC exhibited a greater similarity in differentially expressed genes to cul-cDC, there are some differences in genes up regulation that could be due to linage origination. Finally, cDC2 showed closer gene upregulation profile to our tolDC than cDC1.

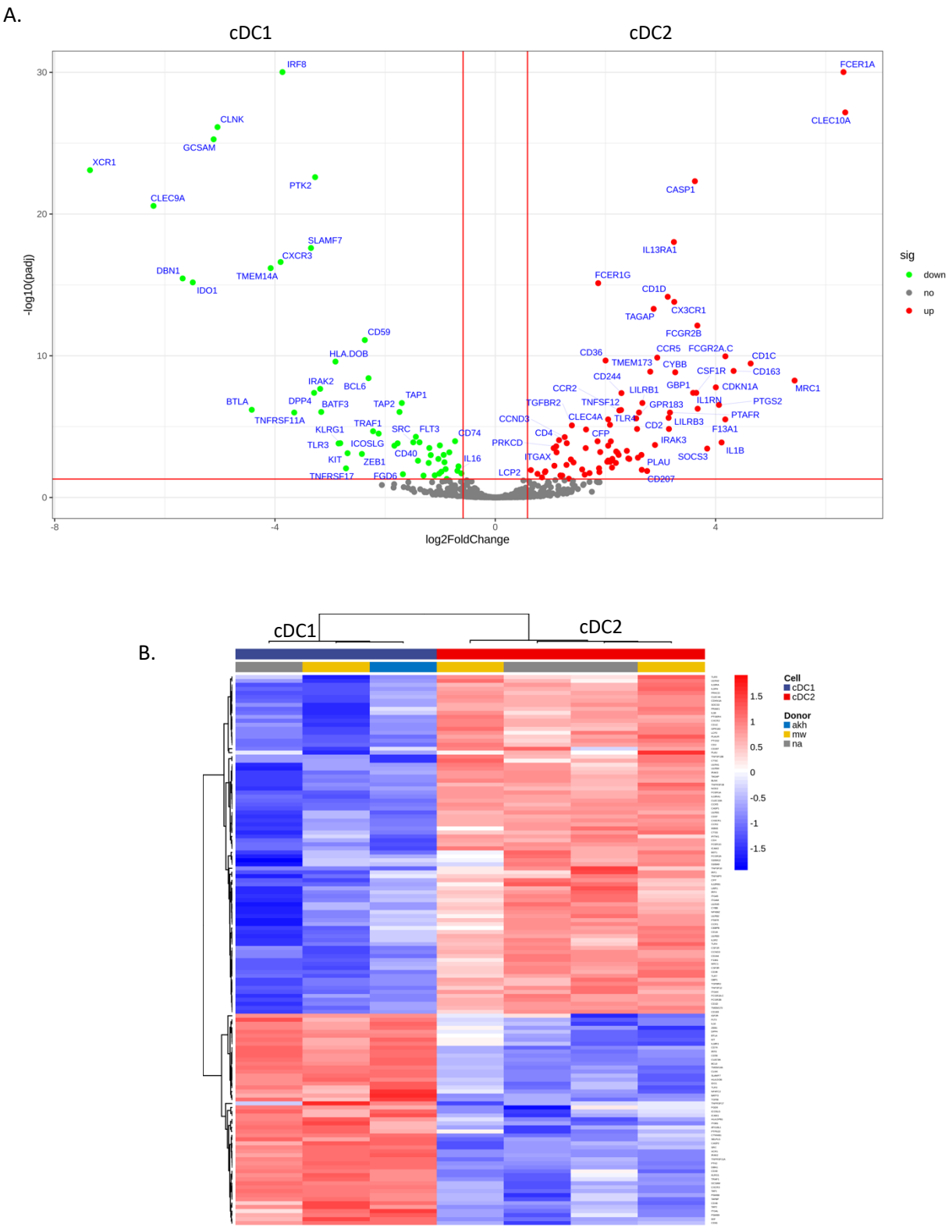
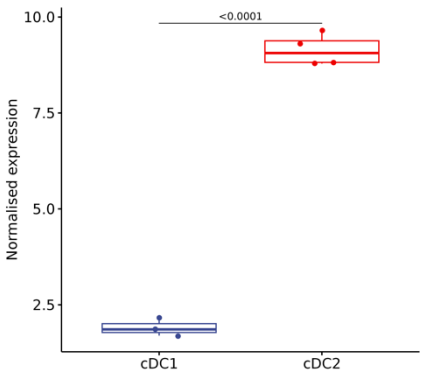
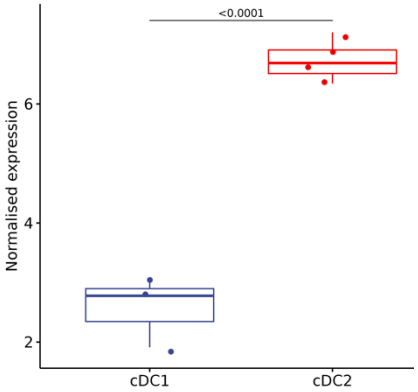


Figure 3.12: Differentially expressed genes in blood derived cDC. A) Volcano plot of DEGs between cDC1 vs cDC2. B) Heatmap of DEGs in bl-cDC2 (Red rectangle) vs bl-cDC1

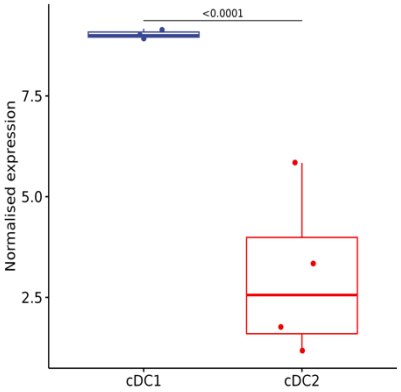
(Blue rectangle) in 3 different donors. Red demonstrates high expression genes and blue demonstrate low expression genes.



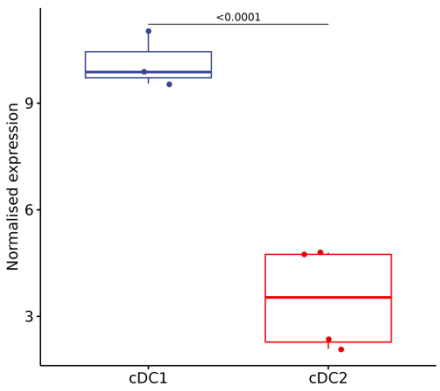
CLEC10A



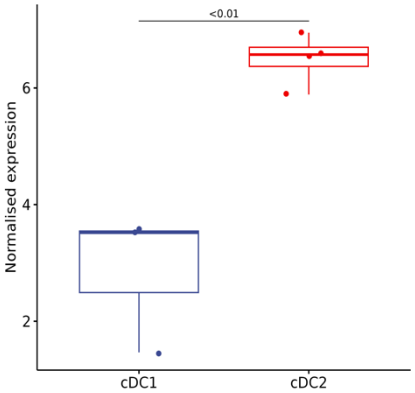
FCGR2B



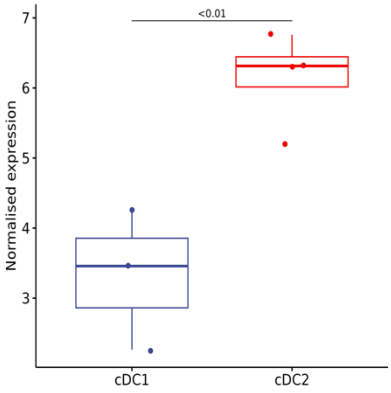
BTLA



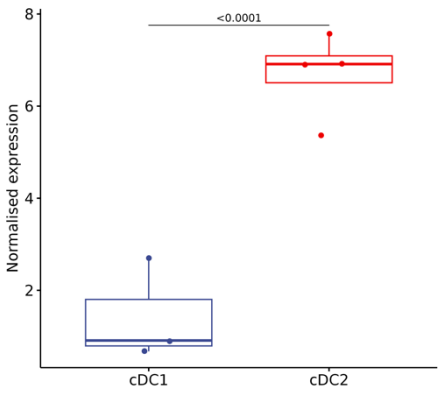
IDO1



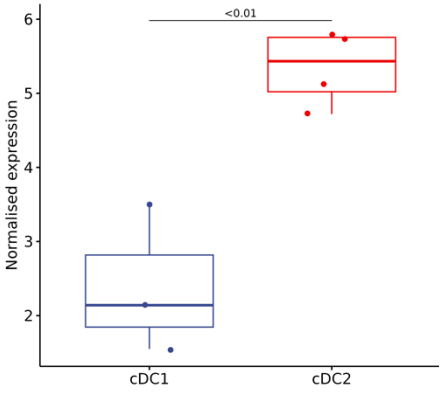
LILRB4 (ILT3)



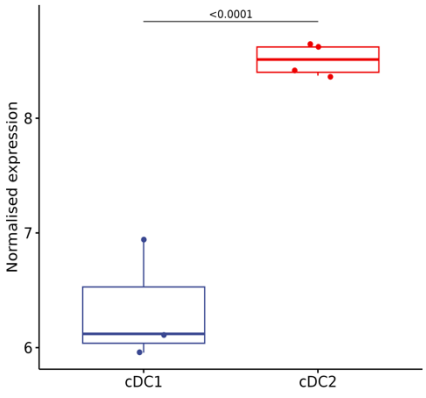
LILRB2 (ILT4)



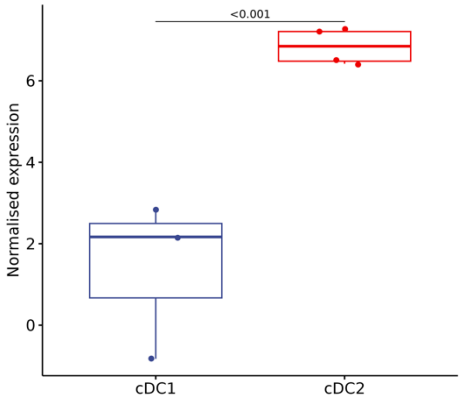
MRC1



IL1R2



CLEC4A



SOCS3

Figure. 3.13: Box plot demonstrates gene expression in blood-derived cDC1 (blue) and cDC2 (Red). P-value represents the results of pairwise comparison with the Wald test.

3.4.5 Validation of tolerogenic signature proteins in our tolDC

Having identified some of the significantly upregulated genes in our tolDC, which may contribute to its tolerogenic function, we thought to validate these genes and other molecules of interest at the protein level to confirm their expression and evaluate their function. To do so, different moDC conditions (immature, mature and tolDC) were cultured in CellGenix media as described in method section 2.2.3, as this was the media used to generate the Nanostring data in moDC. In addition, because in our immunotherapy group, we usually used RF10 culture media as the gold standard protocol for research grade tolDC generation, tolDC were generated in both RF10 and CellGenix DC to assess the effect of culture media on the expression of the tolDC markers as well.

The microscopic picture of the cells after seven days of culture clearly demonstrates a difference in the maturation state of the cells with a semi-mature feature in tolDC compared to mature and immature DC, irrespective of culture media (figure 3.14).

After gating the cells as described in method section 2.2.8, the marker of interest plus the phenotypic marker of different moDC conditions were assessed. As anticipated, tolDC shows the characteristic feature of a semi-mature state with low expression of co-stimulatory molecule

CD86 and maturation marker CD83 compared to matDC (positive control) with no difference between cultured media (figure 3.15). In addition, tolDC also demonstrates higher expression of important immunoregulatory molecules ILT-3, ILT4, PDL-1, PDL-2 and MERTK compared to matDC and immDC, both culture media (figure 3.16). However, both ILT-3 and ILT-4 were also high in matDC that cultured in CG, which is possibly due to stimulation of these cells with IFN-γ besides MPLA, which have a role in the expression of many immune regulatory molecules (Mimura et al., 2018; Švajger and Rožman, 2019). Interestingly, TLR2 (toll-like receptor 2) was highly expressed in tolDC in contrast to matDC, which may contribute to its tolerogenic function in both culture media (figure 3.16).

Furthermore, tolDC demonstrated higher expression of CXCR4 migratory molecule compared to matDC in both culture media while showing lower expression of CCR7 compared to matDC cultured in CG media (figure 3.17). Of note, I observed higher expression of CCR7 in tolDC that were cultured in RF10 media than the cells that were cultured in CG. This may mean that tolDC has a higher expression of the immature state's migration molecule than the mature state's migration molecule.

Attractively, the IL1R2 marker that were significantly upregulated in tolDC at gene level were also highly expressed at protein level in tolDC compared to matDC in both culture media with slightly higher expression in tolDC cultured in RF10 medium (figure 3.18). Thus, this marker could have a regulatory function that contributes to tolerance induction. In addition, the pan CD32 marker (encoded in FCGR2A&B genes) demonstrated high expression in tolDC compared to matDC irrespective of culture media. However, CLEC4A and MRC1 molecules were found to be expressed in tolDC as well but at a lower level compared to matDC and immDC in CG media, whereas they have approximately the same expression level in all moDC conditions cultured in RF10 medium (figure 3.18).

Besides this phenotypical characterization of tolDC, the cytokines secretion profile also showed a typical tolDC profile with the significantly higher secretion of IL-10 and lower IL12p70 production than matDC (figure 3.19).

To this end and in line with other studies, our tolDC has a phenotypical feature of tolerogenic state and tolerogenic cytokines profile with high IL-10 and low IL12p70. However, the

expression of CCR7 was lower in tolDC, while CXCR4 showed higher expression in tolDC compared to matDC. Finally, markers of interest IL1R2, CD32, MRC1 and CLEC4A were all confirmed to be expressed at the protein level in moDC with different variations between conditions.

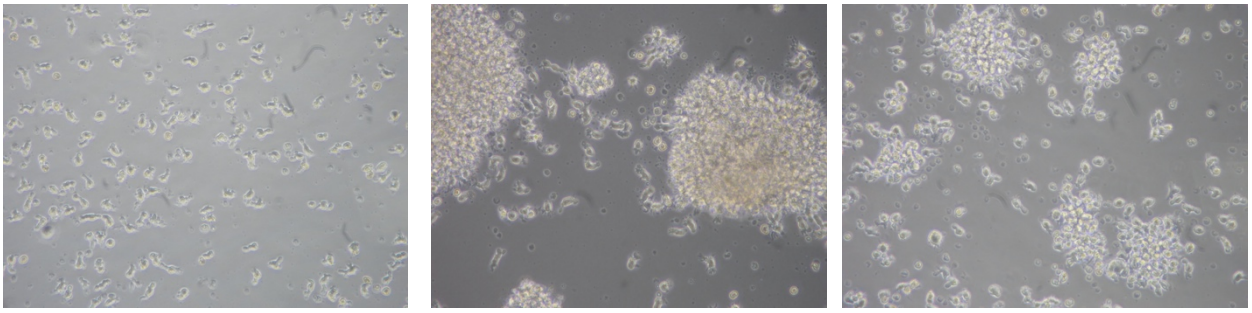


Figure 3.14: Microscopic picture of different moDC conditions after 7 days of culture. A representative of 3 independent experiments.

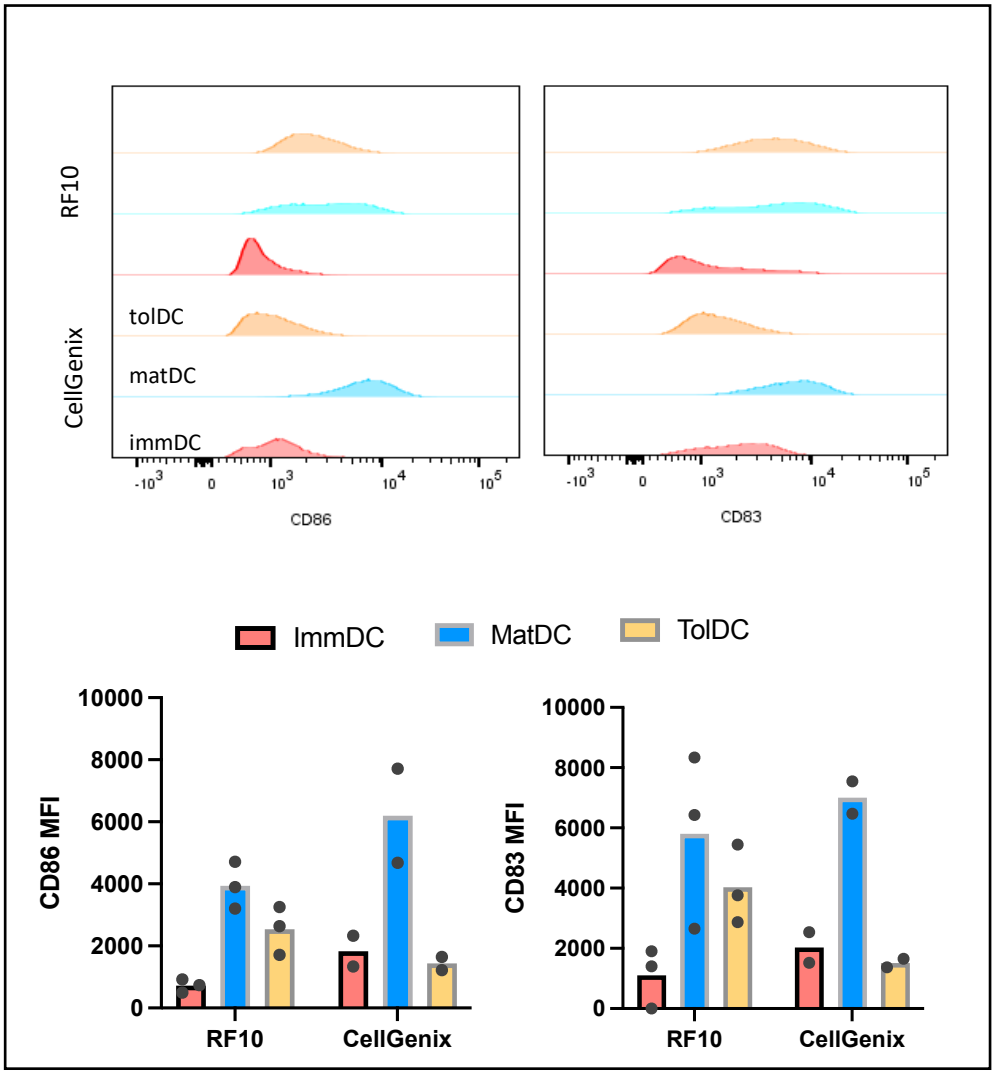


Figure 3.15: Expression of CD86 and CD83 in tolDC cultured in two different media. Representative histogram demonstrate an effect of culture media (RF10 and CG) on the phenotype of moDC red (immDC), blue (matDC) and yellow (tolDC). Bar graph demonstrate the mean of three (N=3) and two (N=2) unpaired independent experiment in RF10 and CG, respectively.

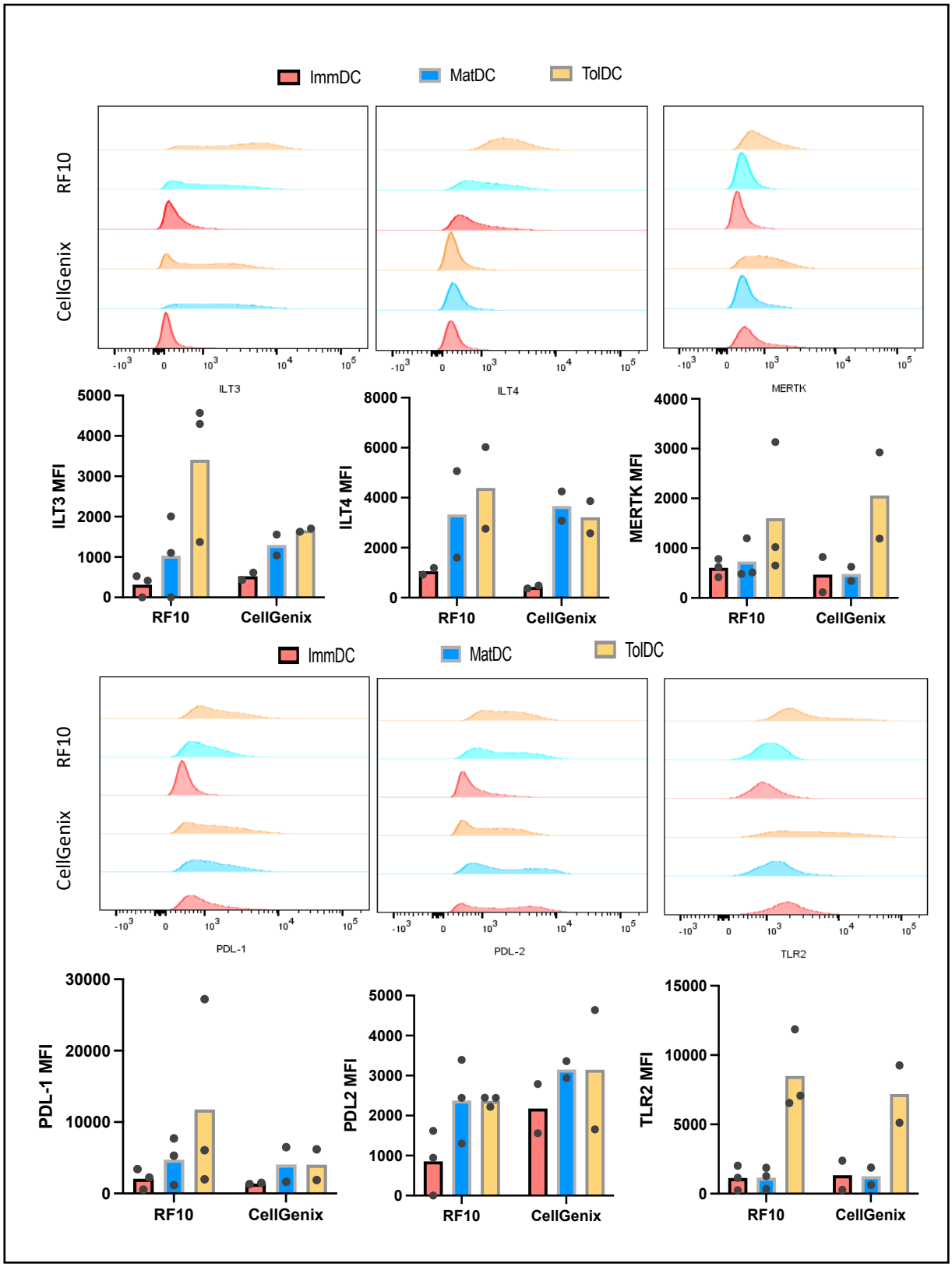


Figure 3.16: Expression of tolerogenic marker (ILT3, ILT4, MERTK, PDL-1 and PDL-2) and QC marker TLR2 in tolDC in the two-culture media. Representative histograms demonstrate an effect of culture media (RF10 and CellGenix) on the phenotype of moDC: red (immDC), blue (matDC) and yellow (tolDC). Bar graphs demonstrate the mean of three (N=3) and two (N=2) unpaired independent experiment in RF10 and CG, respectively.

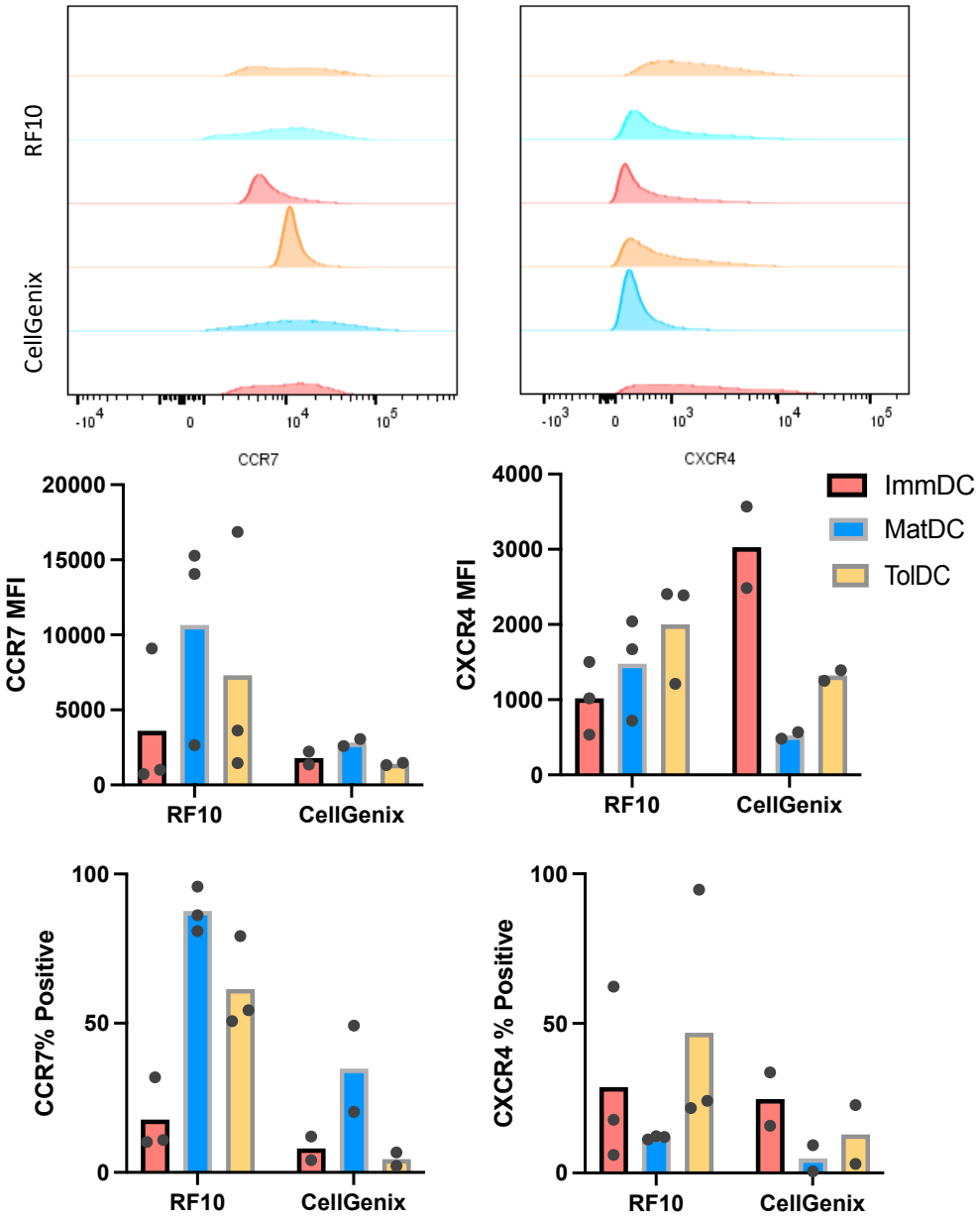


Figure 3.17: Expression of migratory molecules in tolDC in two culture media. Representative histograms demonstrate an effect of culture media (RF10 and CellGenix) on the phenotype of moDC: red (immDC), blue (matDC) and yellow (tolDC). Bar graphs demonstrate the mean of three (N=3) and two (N=2) unpaired independent experiment in RF10 and CG, respectively. MFI shown is from entire cell population.

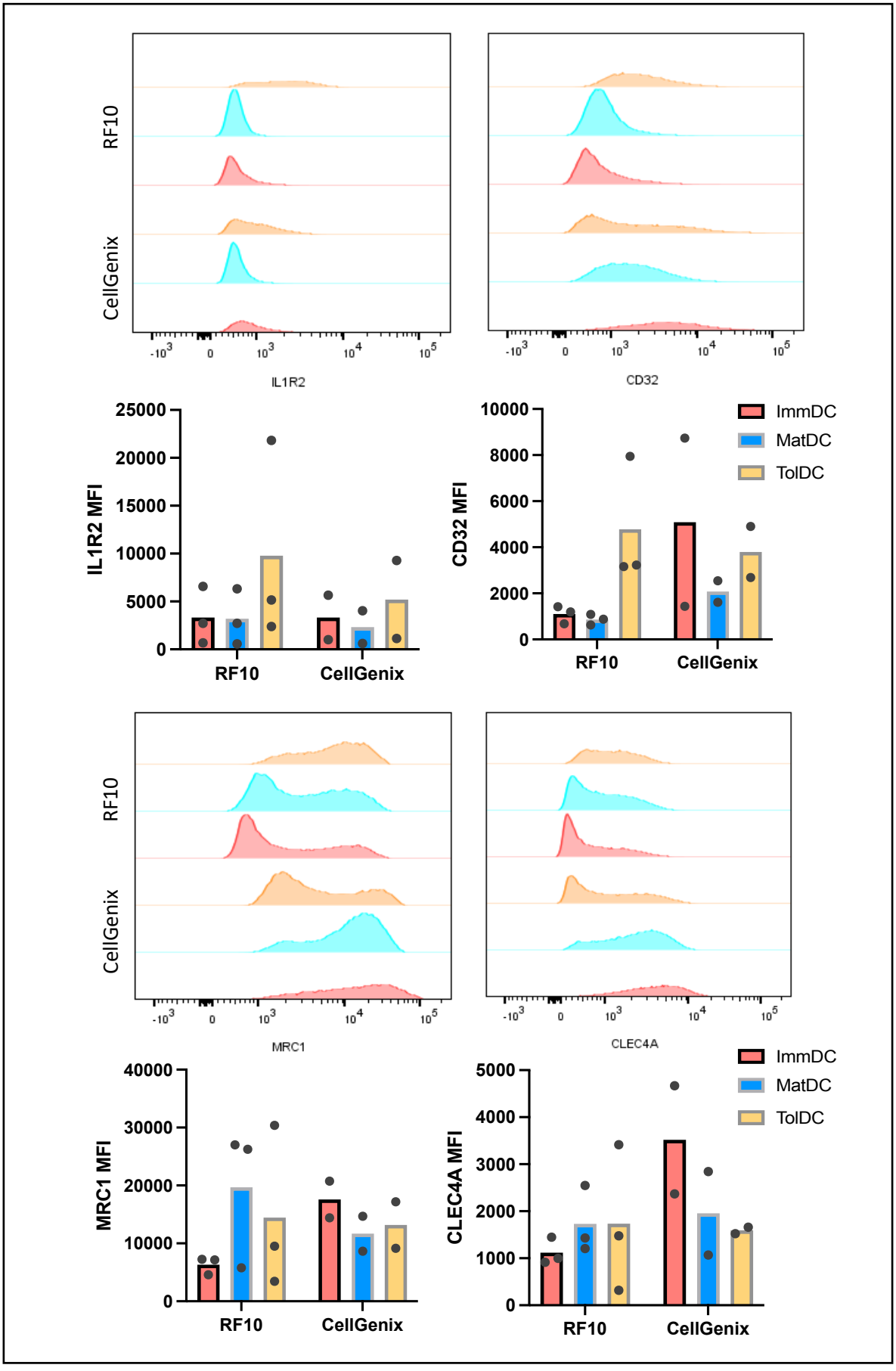

RF10
CellGenix
IL1R2
CD32
IL1R2 MFI
CD32 MFI
ImmDC
MatDC
TolDC
RF10
CellGenix
MRC1
CLEC4A
MRC1 MFI
CLEC4A MFI

Figure 3.18: Validation and expression of markers of interest in tolDC at protein level in tow culture media. Representative histogram demonstrate an effect of culture media (RF10 and CG) on phenotype of moDC red (immDC), blue (matDC) and yellow (tolDC). Bar graphs demonstrate the mean of three (N=3) and two (N=2) unpaired independent experiment in RF10 and CG, respectively.

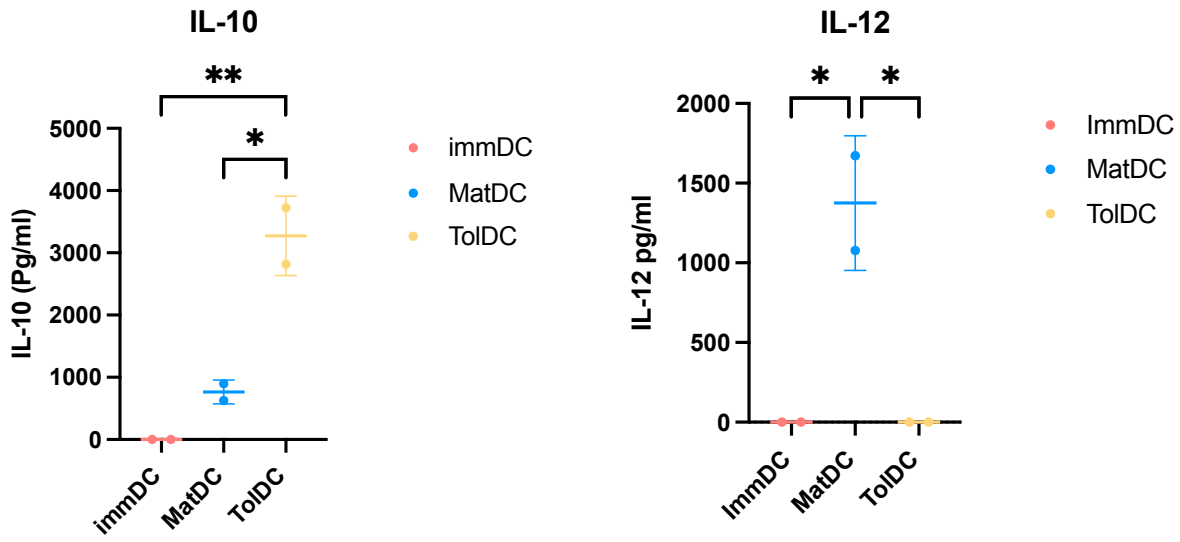


Figure 3.19: Cytokines secretion profiles of tolDC, matDC and immDC. Data represent the mean of 2 independent experiments (N=2). matDC was stimulated with MPLA+ IFNγ while tolDC stimulated with MPLA only. The results represent the mean and SD error bars.

3.5 Discussion

The aim of this chapter was to identify the transcriptional signature genes for tolDC and compare it with the conventional DC that either derived from peripheral blood or cultured from CD34+ cells in order to find out the most closely related cDC to monocyte derived tolDC.

3.5.1 Tolerogenic signature genes in tolDC

As demonstrated in several studies, different tolDC types that were generated using different protocols share key phenotypic and functional characteristics. Conversely, many

transcriptomic and proteomic studies showed that every immunomodulatory agent produces tolDC with a distinct transcriptomic expression profile (Schinnerling et al., 2015).

Identification of tolerogenic signature genes and proteins in tolDC could increase our understanding of how these cells exert their tolerogenic function, contribute to the identification of novel therapeutic targets for either cancer or autoimmune diseases, and provide appropriate markers for quality control of therapeutic tolDC in clinical trials.

Typically, tolDC shows a semi-mature state with lower expression of HLA-DR, co-stimulatory molecules e.g., CD86 and the maturation marker CD83, while having a higher expression of immune regulatory and inhibitory molecules such as ILT3, PDL1 and MERTK at both gene and protein levels. However, down regulation and upregulation of these important tolerogenic genes mainly depend on the protocol used to generate tolDC (Ritprajak et al., 2019). For example, it identified that Dexamethasone treated tolDC has higher upregulation of genes that are related to immune function such as *C1QB* and *C1QC*, while vitD3 treated tolDC highly upregulate the genes that are related to metabolism and cell differentiation including *MAP7* and *MUCL1*. Hence, both immune modulatory agents can lead to tolerance induction but in different ways through activating different immunological pathways. In addition, maturation of DC has an extraordinary degree of plasticity, meaning that differentiated matDC could switch to have some tolerogenic features, which mainly depends on the stimulus used for maturation.
An example of this is shown in two studies by Mimura et al., and Švajger and Rožman, who found that pro-inflammatory IFN-γ stimulation of DC led to maturation of DC and upregulation of a number of immune inhibitory molecules, including PDL-1, ILT3 and ILT4, which may give some tolerogenic potential (Mimura et al., 2018; Švajger and Rožman, 2019). This observation is consistent with my findings in (figure 3.16) demonstrating the expression of these immune inhibitory molecules (PDL-1, ILT3 and ILT4) in matDC stimulated with IFN-γ and MPLA. This stimulation cocktail has shown to lead to better maturation in DC with migratory capacity (Spiering et al., 2019; Ten Brinke et al., 2007).

Therefore, manipulating DC with a combination of stimuli may produce a mature state with some regulatory capacity. However, the balance between the stimulatory and regulatory capacity in these cells should be taken into account, for example, by assessing the PDL-1/CD86 ratio. Furthermore, an anti-inflammatory cytokine profile of tolDC with high IL-10 and low

IL12p70 is essential for determining the tolerogenic state of these cells. In agreement with that, our gold standard tolDC shows high production of IL-10 and low production of IL12p70 reflecting the tolerogenic profile of these cells (Figure 3.19).

Besides the characteristic phenotype and cytokines profile of tolDC, defining the tolerogenic signature genes involved in tolerance induction could provide a better understanding and manipulation of tolDC generation. Several important immune regulator genes have been reported in the literature in relation to tolerance induction in DC. A summary of these important genes is listed in Table 3.5 and 3.6 for down- and up-regulated genes, respectively. An example of an important gene that is associated with tolerance induction is the autoimmune regulator (*AIRE*), which is involved in thymic negative selection by enforcing the expression of tissue-restricted antigens (central tolerance) (Li et al., 2015; Perry et al., 2014). In addition, this gene is also observed by the same group in peripheral blood DC and macrophages, suggesting a contribution to peripheral tolerance induction.
Interestingly, in our study, I found that cul-cDC2 has a significantly higher expression of *AIRE* than cul-cDC1 (figure 3.11). At the same time, bl-cDC2 shows no significant difference in the expression of this gene as compared to bl-cDC1 (not shown). Notably, *AIRE* was similar in tolDC compared to matDC with no significant difference (not shown). This finding is consistent with Crossland et al., (2016), who suggested that *AIRE* is not essential for generating and functioning tolDC *per se*. However, whether *AIRE* plays a role in the immunoregulatory actions of cDC2 remains to be established.

Another interesting finding in this chapter is the striking and significant upregulation of *IL1R2* in tolDC compared to matDC (figure 3.1C& 3.2). This gene encodes IL1R2, a decoy receptor that binds IL-1β with a high affinity (Molgora et al., 2018). A possible explanation of this striking upregulation is the dexamethasone treatment used to induce tolDC, as it has been shown that *in-vitro* glucocorticoid administration increases the expression of this marker in neutrophil (Peters et al., 2013; Re et al., 1994). This result was previously also observed in our group by PhD student Jennifer Dahlstrom (Thesis December 2021), suggesting a need for further validation at the protein level, which could contribute to identifying an additional mechanism of action of tolDC as well as a potential QC marker for therapeutic tolDC. Hence, IL1R2 was further validated in tolDC at the protein level in two different media, which showed higher expression in tolDC compared to other conditions with slightly higher expression in tolDC cultured in RF10 medium. This confirms the expression of these molecules at gene and

protein level. Therefore, further study is needed to explore the functional capacity of this molecule in tolDC.

TLR2 is one of the markers that was reported to be a QC marker in the AuToDeCRA trial (Cooke et al., 2022; Harry et al., 2010a) and I also observed high expression of TLR2 in tolDC compared to matDC in both RF10 and CellGenix DC culture media (figure 3.16), signifying the continued use of this marker for QC. However, its role in the tolDC function remains unknown and requires further investigation.

To conclude, in agreement with previous studies in the literature, it remains challenging to define a 'universal' tolerogenic gene signature for tolDC, which is predominantly due to heterogenous tolDC generation methods.

3.5.2 Tolerogenic signature pathways

To gain further insight into the mechanism of action of tolDC, pathway enrichment analysis was performed to define the highly enriched pathway in tolDC compared to matDC. Our results were in accordance with the recent observation in the study by Malinarich et al., (Malinarich et al., 2015) that the 'immunometabolism' pathway is one of the crucial pathways that is highly enriched in tolDC with high mitochondrial activity and glycolytic capacity (figure 3.7).

Recently, immunometabolism has been demonstrated to play a critical role in the pathogenesis of some diseases, including autoimmune diseases, suggesting a potential therapeutic target to re-establish tolerance. For example, the review by Suwa et al., stated that synovium of RA patients has elevated hypoxia, which induces the elevation of hypoxia-inducible factor alpha (HIFα). This factor promotes glycolytic metabolic activity in the synovium, leading to a pro-inflammatory environment (Suwa et al., 2023). Hence, understanding the underlying metabolic state and pathway of tolDC would play an important part in immune therapeutic interventions.

Generally, metabolism plays an essential role in DC activation where anabolism induces maturation and a proinflammatory state in DC through glycolysis, in contrast to catabolism that promotes a tolerogenic state mainly through activation of oxidative phospholeration (OXPHOS) pathway (Everts et al., 2012; Suwa et al., 2023). Hence, regulation of metabolism could support the induction of tolerogenic DC.

FKBP5 (FK506 binding protein 5) is a co-chaperone immunophilin that is one of the significantly upregulated genes in tolDC and where it is found to be mainly related to metabolism (Table 3.2 and Figure 3.7D). In support of our results, Schinnerling et al., in their review, observed an upregulation of this gene in tolDC treated with dexamethasone alone or in combination with VitD3 and was associated mainly with the immunometabolism pathway as well (Schinnerling et al., 2015). Additionally, this gene was also identified to be involved in numerous different signaling pathways. For example, *FKBP5* is involved in the glucocorticoid receptor signaling pathway, mainly in relation to stress. It is also engaged in NF-κB and AKT signaling pathways in relation to radiation and chemotherapy, respectively. Moreover, the *FKBP5* gene is considered to be a target receptor for both FK506 and rapamycin (immunosuppressant agents), resulting in calcineurin reduction in vitro (Li et al., 2011). In addition, in agreement with our finding that immunometabolism is a highly enriched pathway in tolDC, high expression of genes that are related to other metabolic pathways, specifically the OXPHOS pathway that relates to induction of tolDC was observed by Mohammadnezhad et al., (2022), in contrast to mature DC.

To this end, metabolic reprogramming could be the target for either the generation of tolDC or the development of new therapeutics that target the pathways related to tolerance induction. For instance, inhibiting the glycolysis pathway and promoting oxidative phosphorylation can induce DC with tolerogenic phenotype and function. Hence, understanding the gene sets and pathways related to tolerance is needed.

3.5.3 Natural pro-tolerogenic genes in conventional DC

One of the crucial aims of this chapter was to identify the 'natural' tolerogenic genes in cDC to select the most appropriate cDC subset for the generation of tolerogenic cDC. It is previously confirmed in the literature that cDC2 are phenotypically and functionally heterogenous and two distinct subpopulations have been identified: DC2 and DC3 (Cytlak et al., 2020; Dutertre et al., 2019). In contrast, cDC1 is a homogenous population with a defined phenotype and function.

In this chapter, I found that both cultured and peripheral blood cDC subsets (cDC1 and cDC2) naturally express some important tolerogenic or immune regulatory molecules at different levels (figures 3.11 & 3.13). For example, *IDO*, a well-known gene in relation to tolerance

induction, was expressed at higher levels in cDC1 than cDC2 in both cultured and peripheral blood cells (figures 3.11 & 3.13). This observation is in agreement with Gargaro et al., who also found that after LPS stimulation, only cDC1 expresses IDO protein with no expression in cDC2 (Gargaro et al., 2022). Similarly, the expression level of another gene known to have a tolerogenic function, *BTLA*, was higher in cDC1 than in cDC2, suggesting a potential tolerogenic ability in cDC1. In contrast, I found that in comparison to cDC1, cDC2 expressed significantly higher levels of other crucial immune regulatory and inhibitory molecules including *PDL-1, LILRB4, LILRB2,* as well as a trend in higher levels of *MERTK* in both cultured and peripheral blood cells (figures 3.11& 3.13). My results are consistent with other studies Binnewies et al., and Navarro-Barriuso et al., that also mentioned the expression of these genes in cDC2 (Binnewies et al., 2019; Navarro-Barriuso et al., 2018a).

It is also interesting to note that in comparison to cDC1, cDC2 shows more similarity in the expression of genes of interest with tolDC, including *IL1R2*, *CLEC4A* (in bl-cDC2), *FKBP5* (in cul-cDc2) and *MRC1* (in both cDC2) that could contribute to its tolerogenic function. One possible explanation of this similarity between cDC2 and tolDC is that cDC2 is phenotypically more similar to monocytes than cDC1 with expression of CD1c and CD163 in both cell types. In addition CD14+ subset of cDC2 (DC3) shares features with monocyte including expression of CD163, S100A8 and S100A9 (Heger et al., 2020a). However, bl-cDC2 used in this analysis was sorted as CD1c+CD14- cells to ensure removing DC3 subsets. Therefore, further phenotypical and functional comparison between cDC2 and tolDC could contribute to understanding their differences.

3.5.4 Similarity between cDC2 and tolDC at the gene level

This study's initial and critical objective is to identify an alternative cell source to monocytes for the generation of tolerogenic DC. Therefore, finding the similarities and differences between our gold-standard monocyte-derived tolDC and human conventional DC subsets is a reasonable and informative starting point.

Hence, although cDC1 shows a greater expression of a crucial tolerogenic gene such as *IDO* and *BTLA*, cDC2 was more closely aligned than cDC1 with tolDC with regard to the expression of a number of tolerogenic genes including *PDL-1*, *PRDM1* and *MRC1*. In addition and consistent with (Binnewies et al., 2019), cDC2 but not cDC1 express the *LILRB4* gene. Taken

together, the cDC2 subset was chosen to generate cDC-derived tolDC, as a possible alternative approach to the currently used therapeutic tolDC, which is primarily based upon monocyte-derived DC.

3.6 Summary and future work

In summary, this study has found a potential QC marker for monocyte-derived tolDC including IL1R2 at both the gene and protein levels and *FKBP5* at the gene level, which needs to be further validated at the protein level. In addition, another encouraging finding is the immunometabolism pathway that was found to be one of the highly enriched pathways in the tolDC, which could help to further our understanding of how tolDC functions and provide possibilities to define novel therapeutic targets. Finally, the similarity in the DEGs between cDC2 and tolDC, specifically in relation to the expression of immune inhibitory genes encoding *ILT3*, *MERTK* and *PDL-1* as well as the expression of *CCR7* migratory molecules at a high level suggests that cDC2 could be used as a new alternative approach for generation of tolDC. Therefore, I will investigate the use of cDC2 to generate tolDC at the phenotypical and functional level in the following chapters. This work will help identify the most closely related cDC subsets to tolDC. Also, it will support the selection of conventional DC2 as an alternative approach to monocytes in the generation of a tolDC-based therapy.

Chapter 4: Generation of tolerogenic cDC2 – phenotype and migratory activity

4.1 Introduction

Conventional DC type 2 (cDC2) have an excellent ability to prime naïve $CD4^+$ T cells (Collin and Bigley, 2018). They also have a prominent migratory ability to T cell area of lymphoid tissues, (thought to be better than the migratory abilities of moDC and cDC1), where they prime and educate naïve $CD4^+$ T cells (Granot et al., 2017; Segura, 2022). Although most of the research in the literature that has been performed on cDC2 is in association with cancer immunotherapy, it is also possible that these cells could be used in relation to immunotherapy for autoimmune diseases, because they naturally express some immune regulatory genes ((Mair and Liechti, 2021) and Chapter 3 of this Thesis). In addition, it is conceivable that a single DC subset could play various immunogenic or tolerogenic functions based on external factors in their environment (Bourdely et al., 2020b). Moreover, as already mentioned in Chapter 1, there are at least two different subtypes of cDC2 (including DC2 and DC3), with each subtype known to have distinctive functions; $CD5^+$ DC2 have migratory potential (Collin and Bigley, 2018), whereas $CD163^+$ DC3 possess pro-inflammatory function (Bourdely et al., 2020b). There are several reasons for selecting cDC2 as an alternative cellular approach for tolDC generation. Firstly, the unique ability to prime naïve $CD4^+$ T cells could help in educating the cells for peripheral tolerance induction. Secondly, in Chapter 3, I observed that cDC2s are more aligned with Mo-tolDC than cDC1s in terms of their gene expression profile, which could mean that these cells may be able to exert some tolerogenic function. Moreover, cDC2s are practically more feasible to isolate from peripheral blood than cDC1, as they are the dominant population of cDC in the blood that account for around 0.3-1% of total PBMC (Collin and Bigley, 2018).

Although peripheral blood cDC2 has some regulatory potential with the expression of some regulatory molecules, including BTLA, their plasticity to induce stimulatory activity upon encountering inflammatory stimuli should be taken into consideration when generating tolerogenic cells for autoimmune disease. Therefore, to generate a stable (meaning resistant to inflammatory stimuli) tol-cDC2 with the tolerogenic phenotype (i.e., a semi-mature state and anti-inflammatory cytokine profile), which could be translated to the clinic, I performed an investigation on the suitable pharmacological or tolerizing agents that could be used. As

determined in the literature, several protocols can induce a tolerogenic state in moDC, including dexamethasone, vitamin D3 (VitD3), rapamycin (Rapa) and certain cytokines (e.g., IL-10). However, to the best of my knowledge, there is only one study that was performed by the Di Blasio et al., group, which used a cocktail of cytokines, including IL-6 and PGE-2 for the generation of tolerogenic $CD14^+$ cDC2 (Di Blasio et al., 2020; van Wigcheren et al., 2021). Hence, in reviewing the literature, no data was found on using the pharmacological agents, including dexamethasone and VitD3 in the generation of tolerogenic cDC2 (tol-cDC2). Therefore, because these two immunomodulatory agents were extensively used as a gold standard protocol for Mo-tolDC generation used in clinical trials (Harry et al., 2010b; Hilkens et al., 2023), I conducted an investigation of their immunomodulatory effects on cDC2. Furthermore, as mentioned before in Chapter one, rapamycin is a well-known immunosuppressant agent that induces the expression of the CCR7 migratory molecule in moDC (Sordi et al., 2006), making it a promising candidate to induce tolerogenic function in cDC2, whilst not inhibiting their migratory capacity. The present study was designed to determine the optimal culture conditions to generate cDC2 with tolerogenic function without compromising their migratory ability.

4.2 Chapter aim & objectives

The general aim of this chapter is to design a stable tol-cDC2 with migratory ability that could be further developed as a new cell therapy for autoimmune disease. The specific objectives are:

1. To determine the phenotype and subtype of freshly isolated and cultured cDC2.
2. To evaluate the expression of selected tolerogenic markers in freshly isolated cDC2.
3. To optimize cDC2 culture conditions.
4. To assess the effect of different tolerogenic agents on the generation of tol-cDC2.
5. To compare the impact of tolerogenic agents on migratory ability of tol-cDC2.

4.3 Experimental approach

cDC2 were isolated from LRS cones of healthy individuals as described in Chapter 2. Then, the cells were cultured in either CellGenix DC medium or X-VIVO15 + 2% human serum (HS) supplemented with GM-CSF. The cells then were primed with immunomodulatory agents dexamethasone, VitD3, rapamycin or a combination of them for 1 hour to ensure activation of immune-regulatory pathways before stimulation with LPS for 16-20 hours. Untreated and

unstimulated cDC2 (Un-cDC2) were also cultured as a negative control. The cells then were harvested and analyzed by flow cytometry to assess viability, purity, phenotype and cDC2 subtypes. For cDC2 analysis, FlowJo was used, and the cells were gated as described in Chapter 2, after which assessment of marker expression in bulk CD1c+ cDC2 was performed. Markers to define CD1c+ cDC2 populations and subtypes are described in figure 4.1. The nomenclature of cDC2 conditions is listed in table 4.1 below.

cDC2 condition	Abbreviation	Treatment protocol
Freshly isolated cDC2	cDC2	None
Cultured un-treated cDC2	Un-cDC2	None*
Cultured LPS stimulated cDC2	Stim-cDC2	Stimulated with LPS for 16-20 hours
Cultured dexamethasone primed and LPS stimulated cDC2	Dex-cDC2	Primed with dexamethasone for 1hr then stimulated with LPS for 16-20 hours.
Cultured VitD3 primed and LPS stimulated cDC2	VitD3-cDC2	Primed with VitD3 for 1hr then stimulated with LPS for 16-20 hours.
Cultured Rapamycin primed and LPS stimulated cDC2	Rapa-cDC2	Primed with Rapamycin for 1hr then stimulated with LPS for 16-20 hours.
Cultured VitD3 + dexamethasone primed and LPS stimulated	VitDex-cDC2	Primed with VitD3 and dexamethasone for 1hr then stimulated with LPS for 16-20 hours.
Cultured VitD3 + rapamycin primed and LPS stimulated	VitRapa-cDC2	Primed with VitD3 and Rapamycin for 1hr then stimulated with LPS for 16-20 hours.

Table 4.1: Nomenclature of cDC2 conditions that have been used in this Chapter. *Note: all cultured conditions are supplemented with GM-CSF.

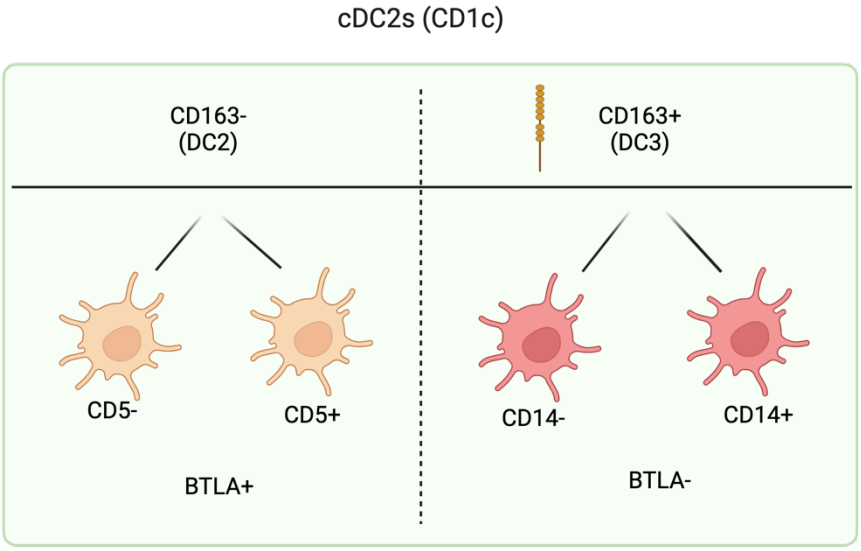


Figure 4.1: Marker to define cDC2 population and subtype. As described in introduction, cDC2 have heterogenous subtypes that are mainly phenotypically characterized as follows: CD1c^{+} CD5^{+} CD163^{-} (DC2) and CD1c^{+} CD5^{-} CD163^{+} and either CD14^{-} or CD14^{+} DC3. Hence, for defining cDC2 phenotype and for purity check I used CD3^{+}CD19^{+} and CD16^{+} as a dump channel to exclude the lineage marker-positive cells. Then, HLA-DR^{+} to gate on the cells of interest. CD1c^{+} was used to define the cDC2 bulk population after depletion of CD14^{+} monocyte and CD19^{+} B cells that also could express CD1c marker.

4.4 Results

4.4.1 Phenotypical characterization of cDC2 subsets before and after culture

As previously mentioned, the CD1c marker was used to define the 'bulk' cDC2 population after the exclusion of lineage-positive cells to exclude lymphocytes and monocytes as well as circulating CD14^{+} DC3. As shown in Chapter 2, after isolation, the majority of linage-negative and HLA-DR-positive cells are CD1c^{+} cells, with a purity of around 95-99 % (figure 2.6). In addition, irrespective of the culture environment, three subpopulations could be identified within the bulk cDC2 population: CD163^{-} cells, which are further subdivided into CD5^{+} and CD5^{-} (DC2) and CD163^{+} cells, which could be further subdivided according to CD14 expression into CD163^{+}/CD14^{-} cells (the majority of the CD163^{+} population) and CD163^{+}/ CD14^{+} (DC3), see figure 4.2 for the gating strategy. The newly identified subset of circulating CD14^{+} CD163^{+} DC3 was possibly depleted during the CD14-depletion step. In addition, to

confirm that the CD14 mAb was working, PBMC were stained with the same master mix, showing that $CD14^+$ cells could be readily identified (data not shown).

Notably, after culture, I found that CD163 expression by cDC2 varied according to the culture medium used. As shown in figure 4.3 A, the majority of the bulk cDC2 cells that were cultured in CellGenix (CG) DC medium expressed lower levels of CD163 in comparison to freshly isolated cDC2. This could mean either that the medium decreased the expression of this marker or that the $CD163^+$ marker was lost after overnight culture.

However, expression of $CD5^+$ was consistently around 17-25 % in freshly isolated cDC2, while this proportion was slightly increased after culture in X-VIVO15 medium to around 30-45 % in both culture conditions (figure 4.3B). Notably, there was no significant difference between the frequency of $CD5^+$ or $CD5^-$ cells between the freshly isolated and cultured cDC2 within both cultured media (figure 4.4). There was only a slight increase in the frequency of $CD5^+$ subset after culture in both Un-cDC2 and Stim-cDC2 compared to freshly isolated cDC2, but it does not reach statistical significance (figure 4.4).

Next, in order to investigate the reason for the different expression of CD163 by cDC2 after culture, another culture medium was used: X-VIVO15 supplemented with 2% HS (hereafter simply referred to as X-VIVO15). As shown in Figure 4.3 B, the majority of cDC2 cultured overnight in X-VIVO15 media had shifted to a $CD163^+$ phenotype compared to freshly isolated cDC2. This observation means that the culture medium used may have an impact on the classification of cDC2 into subsets. In addition, this observation revealed that variations in the expression of CD163 in bulk cDC2 after culture may mean that this marker is not robust enough for defining cultured cDC2 subsets.

As shown in figure 4.5A, Un-cDC2 shows a typical morphology of immature DC after overnight culture in both culture media (CellGenix and X-VIVO15), i.e. no clusters of cells were present. This morphology gives an idea of the state of maturation of cells, which helps in the further generation of tolerogenic cDC2. In contrast, the morphology of the Stim-cDC2 under the microscope after overnight culture demonstrates a sign of maturation and activation where the cells clustered together (figure 4.5B). Notably, it appears that the size and granularity of cultured cDC2 are different according to culture media, with a bigger size in cDC2 cultured in X-VIVO15 media than cDC2 cultured in CG media (figure 4.5C).

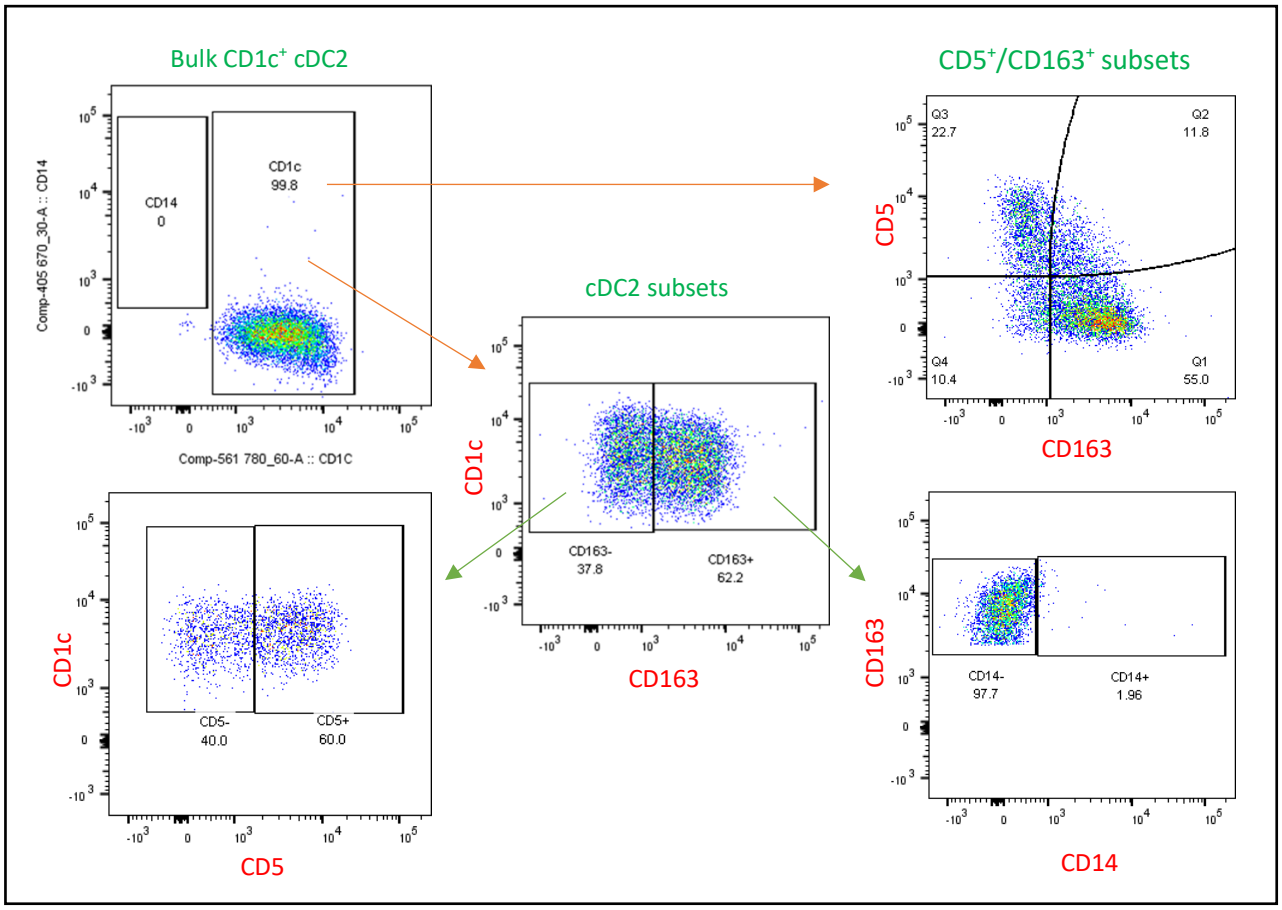


Figure 4.2: Representative example for gating strategy for cDC2 subtype in freshly isolated cDC2 as a control. This gating strategy was also applied to other cDC2 conditions.

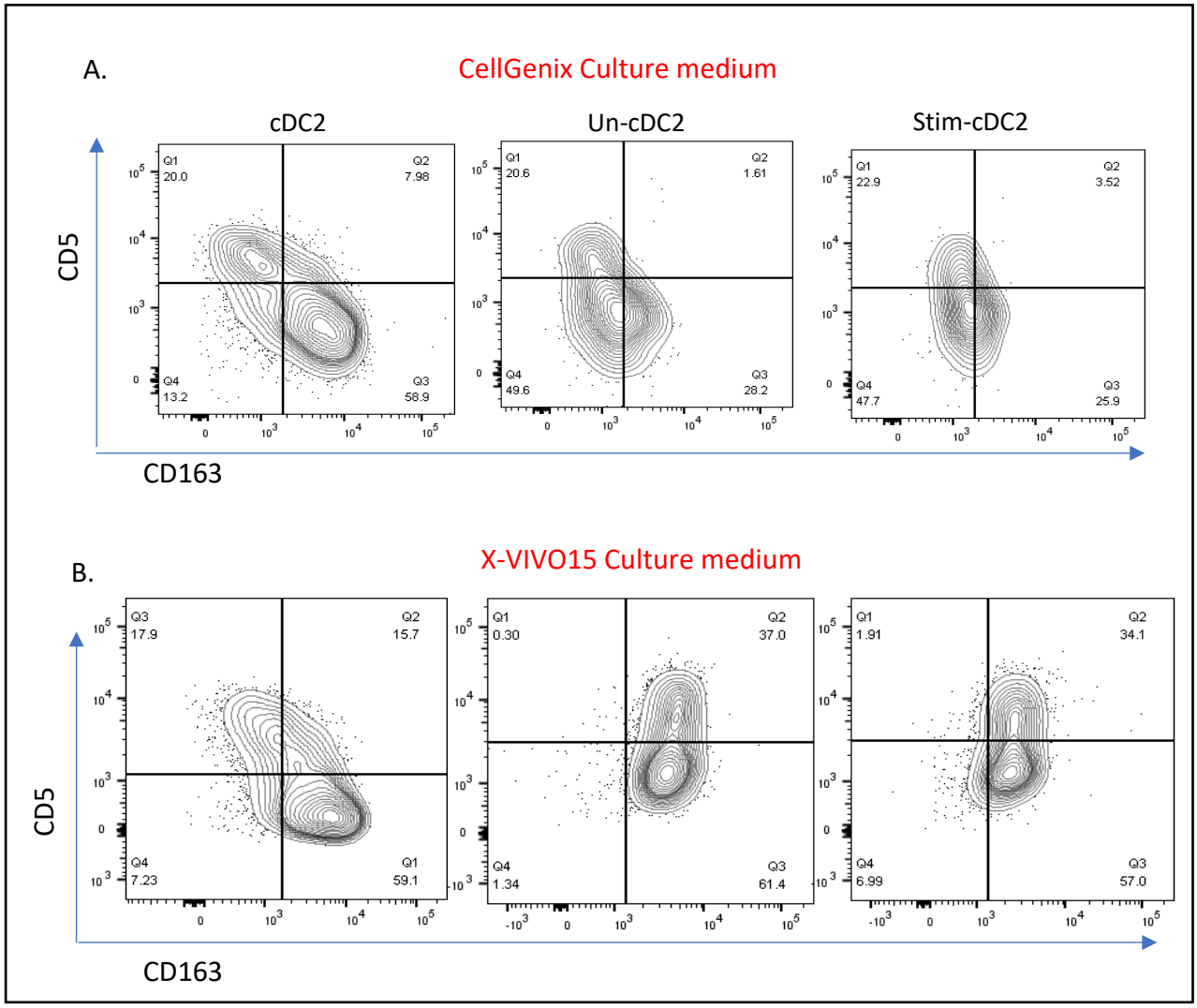


Figure 4.3: Flow plot of cDC2 subtype in freshly isolated and cultured cDC2 in CellGenix and X-VIVO15 medium after gating on the bulk CD1c+ population. Data representative of 3 and 8 independent experiments for CellGenix and X-VIVO15, respectively.

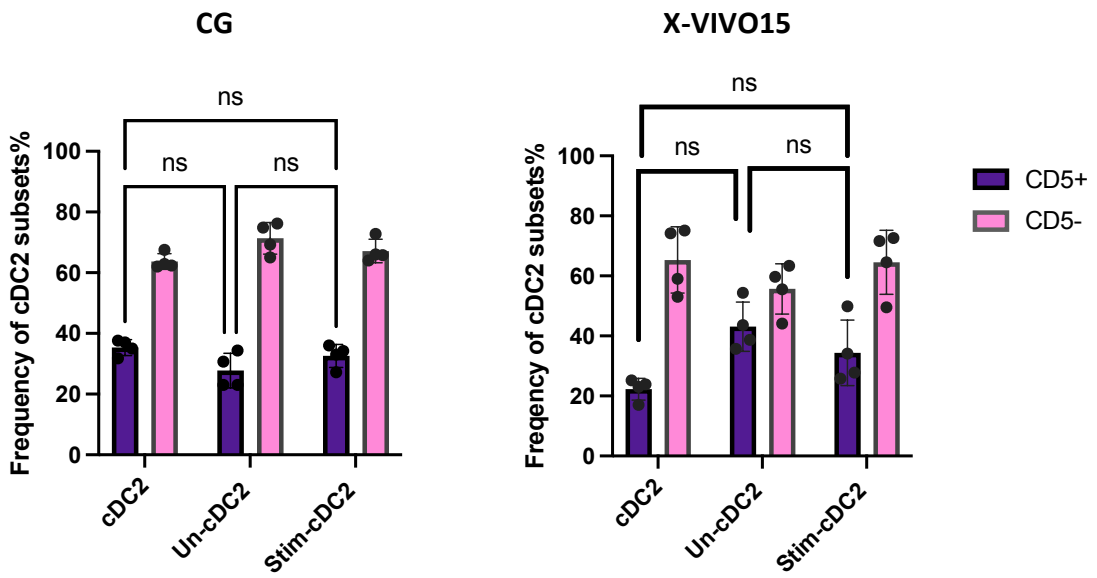


Figure 4.4: Frequency of CD5+ and CD5- in freshly isolated cDC2 and cultured cDC2 in CellGenix (CG) and X-VIVO15 medium. Data represents the mean and SEM (error bars) of four independent experiments. Statistically significant difference was based on the Two-way ANOVA test between all conditions and cDC2 subsets. * = p <0.05, ** = p <0.01, *** = p <0.0001, **** = p <0.00001.

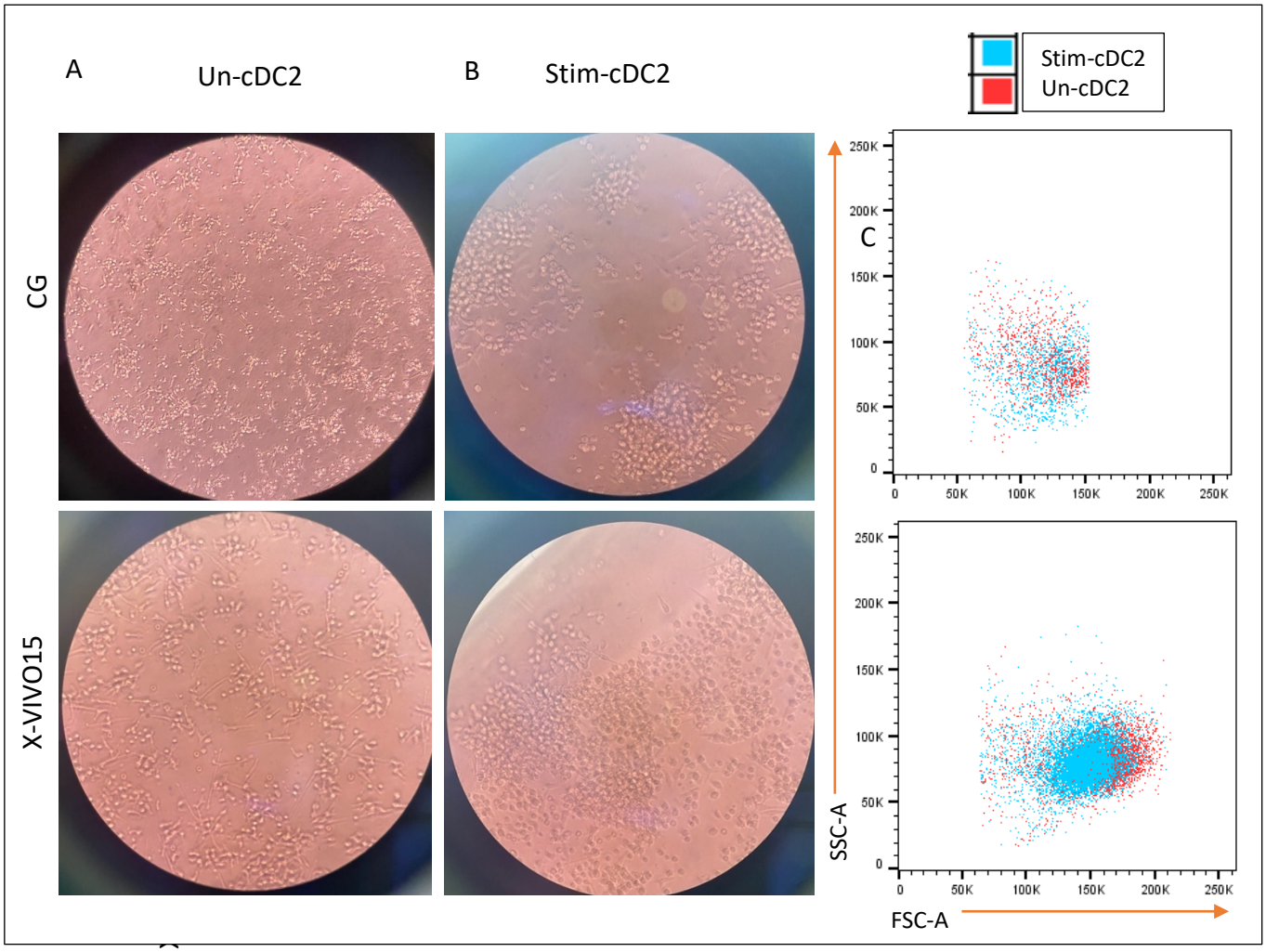


Figure 4.5: Representative microscopic picture of cDC2 after overnight culture in CellGenix and X-VIVO15 DC medium. A) Un stimulated cDC2 in both media. B) LPS stimulated cDC2 in both media. C) Flow plot of cultured cDC2 in both media demonstrate the FSC-A and SSC-A for size and granularity.

4.4.1.1 Freshly isolated cDC2 naturally express ILT3 immune inhibitory molecule

As found in Chapter 3, both cultured and peripheral blood cDC2 naturally express *LILRB4* (ILT3 gene) compared to cDC1 and other immune cell types. Therefore, I wanted to validate the expression of this immune inhibitory molecule (ILT3) at the protein level in bulk cDC2. Besides that, the expression of maturation and costimulatory molecules, as well as other tolerogenic markers, were assessed in freshly isolated cDC2 as a control to compare with the cultured conditions. Hence, as demonstrated in figure 4.6 and 4.7, freshly isolated cDC2 do not express CD86 and CD83 compared to cultured cDC2, while both Un-cDC2 and Stim-cDC2 displayed significantly higher expression of CD86. Surprisingly, cDC2 appeared to have matured 'spontaneously' after culture, without the addition of LPS (see Un-cDC2 condition; figure 4.7). Remarkably, freshly isolated cDC2 demonstrates significantly higher expression of ILT3 marker compared to Stim-cDC2 (figure 4.7). However, freshly isolated cDC2 do not express other tolerogenic markers of interest (MERTK and PDL-1) compared to cultured conditions. To ensure that ILT3 expression was a correct finding, I included an FMO (Fluorescence Mines One) plus isotype-matched control for ILT3, which shows that the observed ILT3 expression was not caused by non-specific binding/background fluorescence (figure 4.6D).

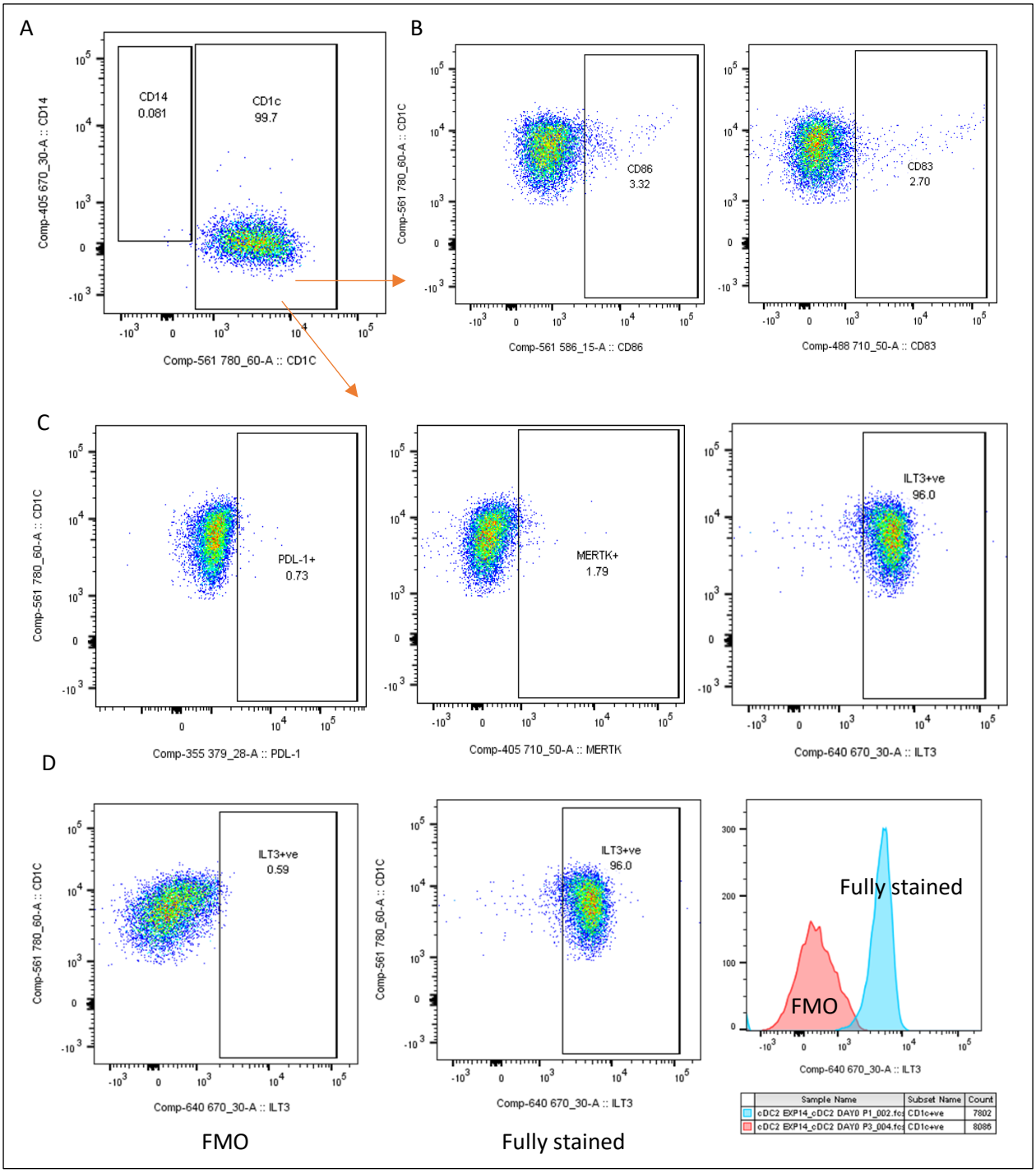


Figure 4.6: Representative flow plot of freshly isolated cDC2 showing the bulk CD1c^{+} (A) and the expression of maturation marker (B) and tolerogenic marker (C) after gating on CD1c+ population. D) The FMO + isotype-matched control for ILT3 is used to remove any background.

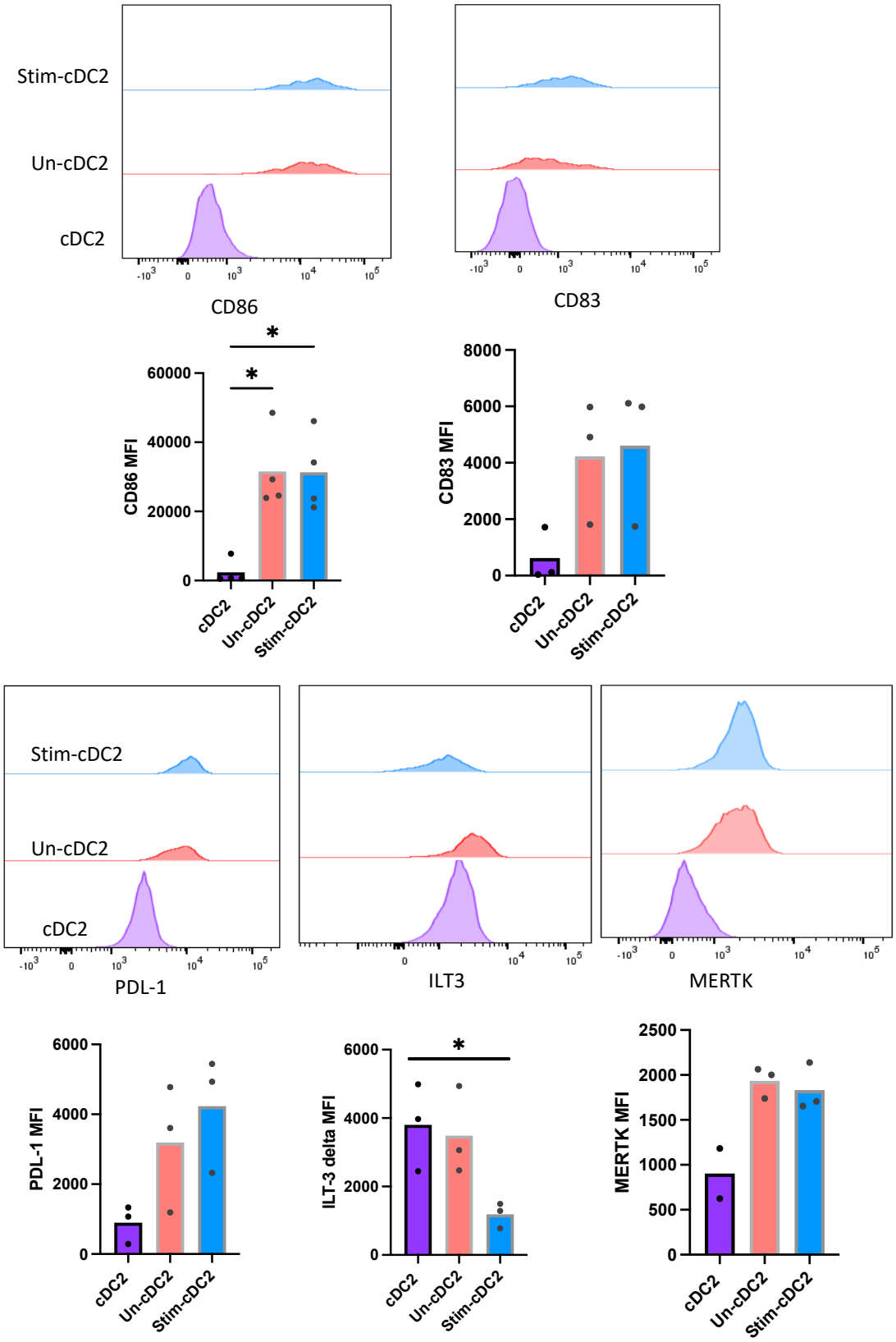


Figure 4.7: Phenotype of freshly isolated cDC2 and cultured cDC2 in CG medium. Bar graphs demonstrate expression of different marker of interest in freshly isolated cDC2 (purple) compared to Un-cDC2 (red) and Stim-cDC2 (blue). Data represent the mean of three

independent experiment. Statistically significant difference based on one-way ANOVA for parametric test between all group. * = p <0.05, ** = p <0.01, *** = p <0.0001

4.4.2 Generation of tolerogenic cDC2 (tol-cDC2)

In order to generate a stable tol-cDC2, various well-known immune modulatory agents were examined including dexamethasone, rapamycin and the active form of vitamin D3 (VitD3) as well as various combinations. At the start of these cDC2 culture experiments, CellGenix DC culture medium was used as a standard GMP-compatible medium, also used for the AuToDeCRA clinical trials with monocyte-derived tolDC. In addition, because it is well-established that cDC2 needs GM-CSF for survival, this cytokine was added to all cDC2 cultures. Different morphologies were found after the treatment of cDC2 with different immune modulatory agents (figure 4.8). After overnight culture, the Dex-cDC2 demonstrated an immature-like appearance with lower aggregation and clustering of the cells, which likely reflects reduced activation as compared to rapa-cDC2 and VitD3-cDC2, which both showed more cluster formation (figure 4.8).

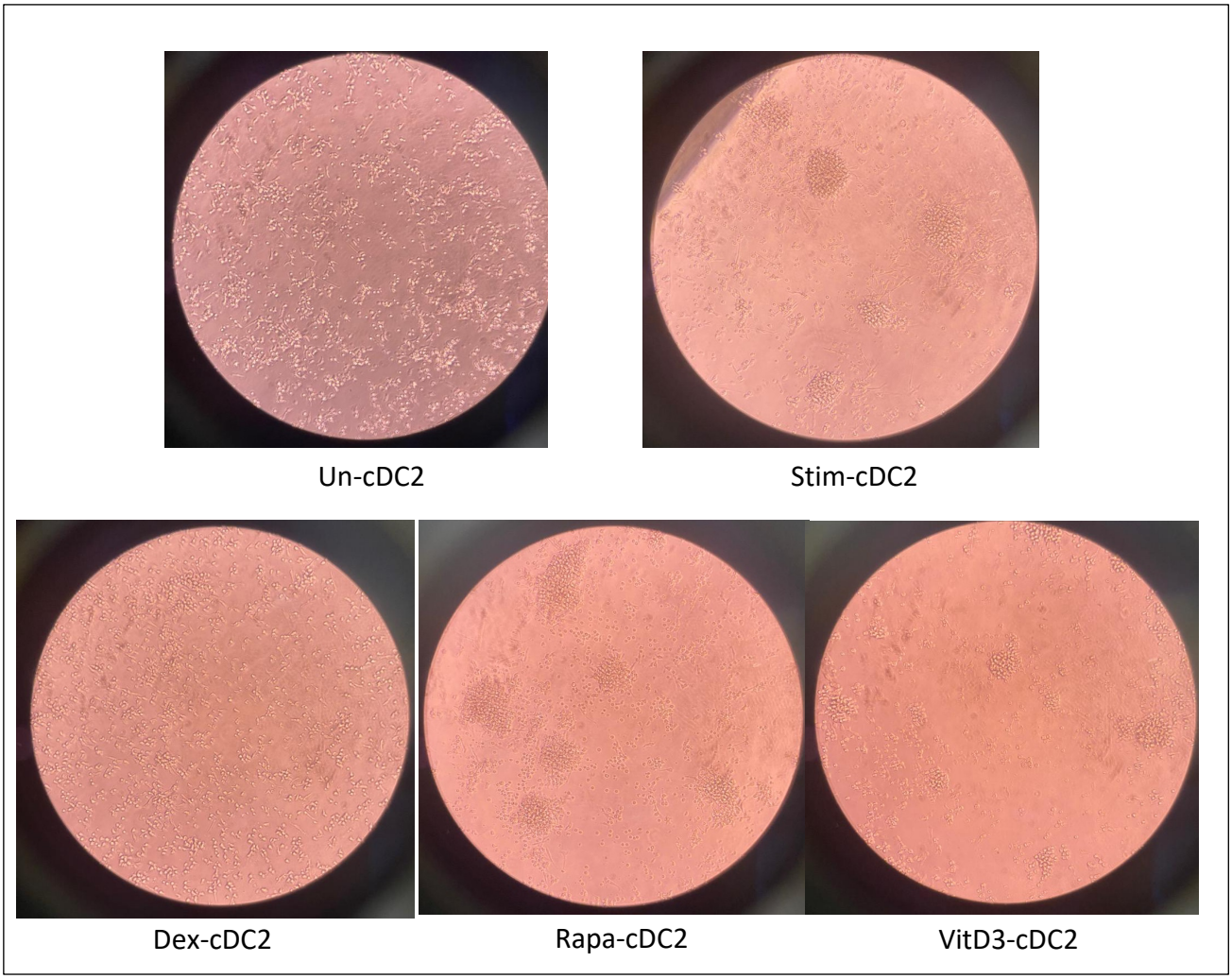


Figure 4.8: Microscopic morphology of cultured cDC2 treated with different immune modulatory agents after overnight culture in CellGenix medium. Data representative of four independent experiments. Magnification 20X.

4.4.3.1 Phenotype of tol-cDC2

To examine the effect of each tolerizing agent on the phenotype of cDC2, after harvesting the cultured cDC2, I flow stained them with the panel of marker of interest as mentioned in Chapter 2 table 2.3. As shown in figure 4.8, different immune modulatory agents induced different tol-cDC2 phenotypes. For example, VitD3 treatment alone or in combination with other immune modulatory agents promoted the induction of CD14 molecule in the $CD1c^+$ population (figure 4.9E, F &G). Notably, after gating on these double-positive $CD1c^+CD14^+$ cells (green box) to assess which cDC2 subset they were part of, we found that most of them were part of the $CD163^+$ $CD5^-$ population (figure 4.10). However, the $CD1c^+$ $CD14^-$ cells (Orange box) were found to be part of both subsets of cDC2 ($CD5^+$ and $CD5^-$) (figure 4.10).

Moreover, vitD3 treatment was found to significantly increase the expression of PDL-1 compared to other conditions, whereas no significant differences were found for the expression of ILT3 and MERTK between all conditions (figure 4.11). There was also a trend of increased expression of CD86, with increased expression in VitD3-cDC2 as compared to Dex-cDC2 and Rapa-cDC2. Furthermore, it is noteworthy that the expression of CD83 exhibited a pronounced elevation in both VitD3-cDC2 and Rapa-cDC2, surpassing the level observed in Stim-cDC2. However, Dex-cDC2 seems to have lower expression of both CD86 and CD83 compared to Stim-cDC2 and other immunomodulatory treated cDC2. However, this does not reach a statistical significance. Finally, rapa-cDC2 and VitD3-cDC2 showed a trend of enhanced CCR7 expression compared to other conditions, although these differences did not reach statistical significance.

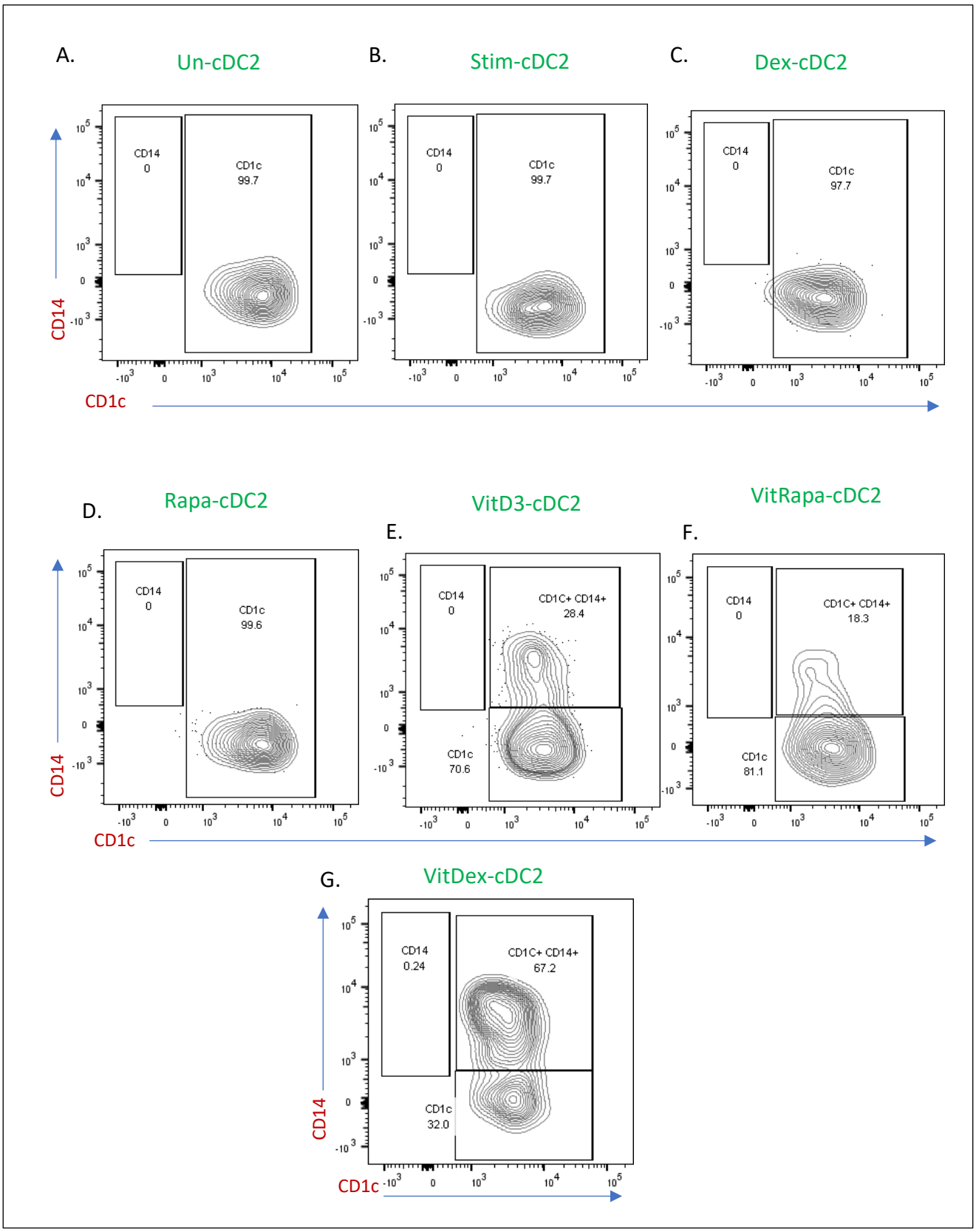


Figure 4.9: Re-expression of CD14 molecule in the bulk cDC2 after treatment with vitD3. Representative flow plot of three independent experiments after overnight culture in X-VIVO15 medium plus immunomodulators. A) Untreated and unstimulated cDC2. B) LPS

stimulated cDC2. C) dexamethasone only treated cDC2. D) rapamycin only treated cDC2. E) VitD3 only treated cDC2. F) VitD3 and rapamycin treated cDC2. G) VitD3 and dexamethasone treated cDC2. Data representative of three independent experiment.

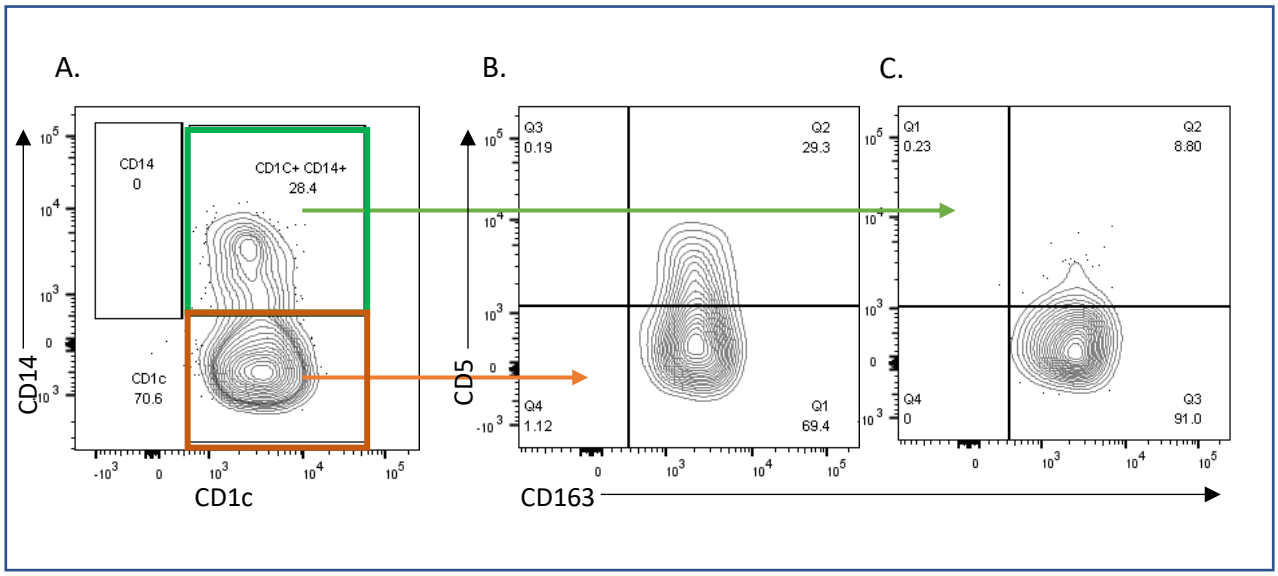


Figure 4.10: Contour plot demonstrate cDC2 subset on each gate. A) VitD3-cDC2 (Bulk CD1c) B) Gated on orange box ($CD1c^{+}$ $CD14^{-}$) for cDC2 subset assessment. C) Gated on green box ($CD1c^{+}CD14^{+}$) for cDC2 subset assessment. Data representative of three independent experiment.

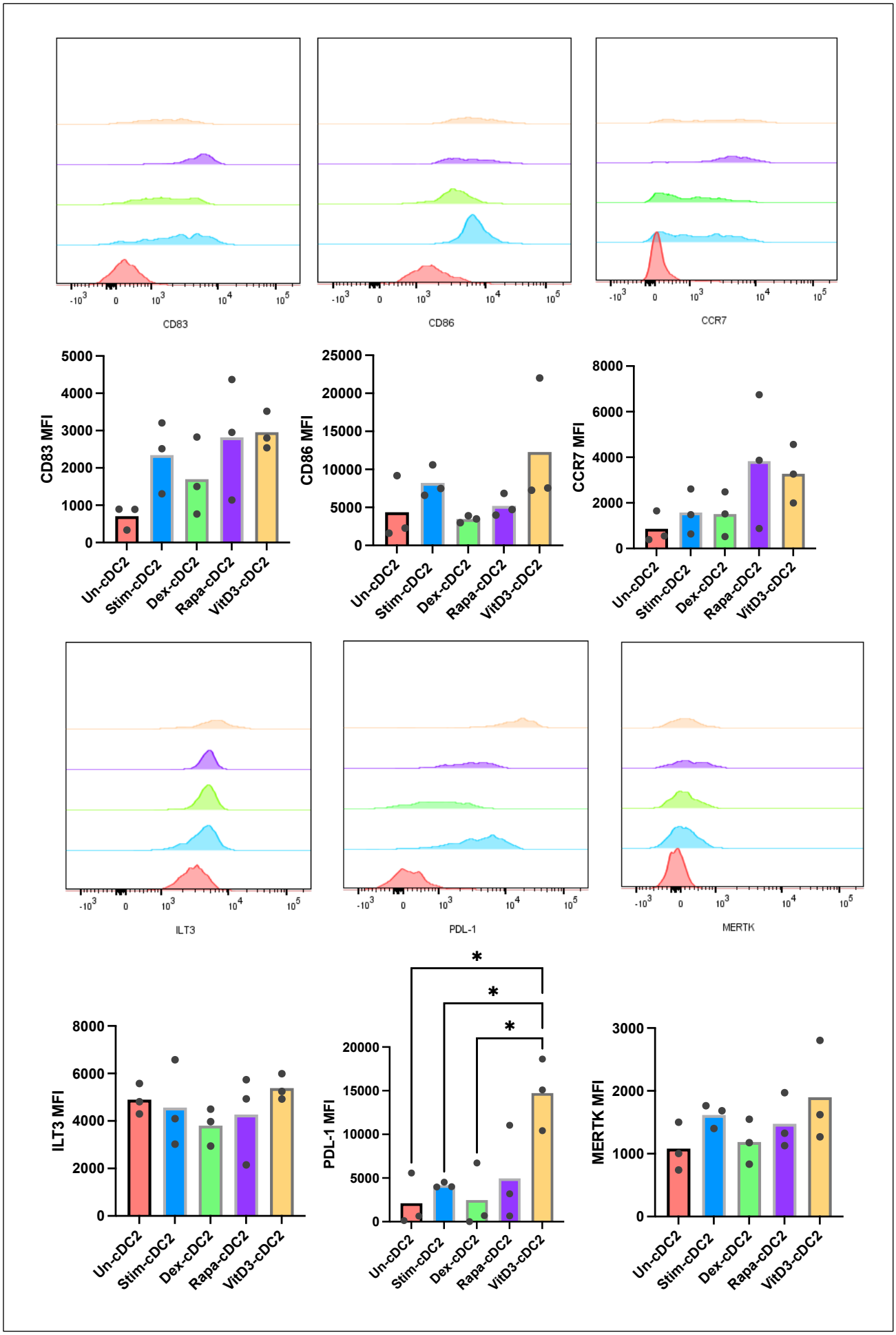

CD83
CD86
CCR7
CD83 MFI
CD86 MFI
CCR7 MFI
Un-cDC2
Stim-cDC2
Dex-cDC2
Rapa-cDC2
VitD3-cDC2
ILT3
PDL-1
MERTK
ILT3 MFI
PDL-1 MFI
MERTK MFI
*
*
*

Figure 4.11: Phenotype of cDC2 in CG medium after treatment with various tolerogenic agents, which are dexamethasone (green), Rapamycin (purple) and VitD3 (yellow). Red represents the negative control Un-cDC2 and blue represent the positive control Stim-cDC2. Data represents the MFI of three independent experiments N=3. Error bar represents SEM. Bars represent the mean of the results of the three individual experiments. Statistically significant difference demonstrates the result of one-way ordinary ANOVA (parametric test) between all groups. * = p <0.05, ** = p <0.01, *** = p <0.0001

4.4.3.2 Cytokine secretion profile of tol-cDC2

Surprisingly, VitD3-cDC2 strikingly secreted profound levels of the anti-inflammatory cytokine IL-10 as compared with all other conditions (figure 4.12). However, VitD3-cDC2 also produced significantly high level of the pro-inflammatory cytokines IL-6 and TNF-α compared to other cDC2 conditions, whilst there were no differences in IL12p70 secretion compared to Stim-cDC2 (figure 4.12).

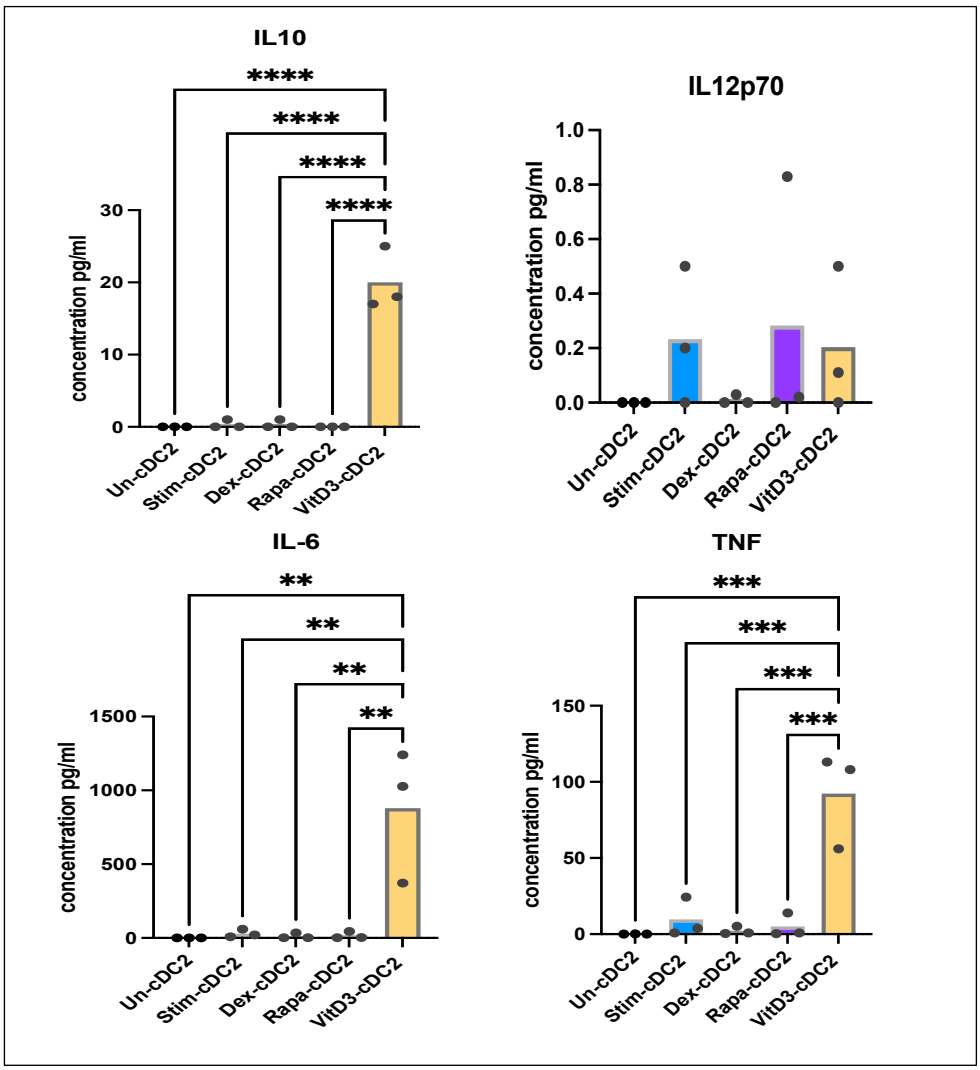


Figure 4.12: Cytokines secretion profile of CellGenix-cultured cDC2 in different conditions. Bar graphs demonstrate the mean of three independent experiments. Error bar represents the SEM. Statistically significant difference demonstrate the result of one-way ordinary ANOVA (parametric test) between all groups. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$, **** = $p < 0.00001$.

4.4.3 Optimization of culture environments for proper tol-cDC2 generation

In order to obtain an optimal culture condition for *in-vitro* tol-cDC2 generation, I investigated the effect of two well-known media that have been used previously in the generation of therapeutic DC: CellGenix GMP DC medium (used for Mo-TolDC therapy (Harry et al., 2010b)) and X-VIVO15+2 % HS (used for anti-cancer therapy in both moDC (De Vries et al., 2003) and cDC2 (Schreibelt et al., 2016)). Furthermore, the effect of supplementation with different GM-CSF concentrations as well as effect of adding human serum to the culture media were also assessed in different cDC2 conditions. Additionally, the timing for priming cDC2 with tolerogenic agent (vitD3) was also evaluated, in order to reach an optimal practical time for generation of tol-cDC2.

4.4.3.1 Impact of culture media on generation of phenotypically stable tolerogenic cDC2

Firstly, CellGenix and X-VIVO15+ 2% HS culture media were both used to assess their impact on the expression of (PDL-1, ILT3 and MERTK), (CD86 and CD83), CCR7 and subtyping markers (CD5 and CD163). Viability of cDC2 was assessed and found to be similar and acceptable in both culture media with the range of live cells between 80-90% (Table 4.2). In addition, percentage of cDC2 yield after culture was assessed showing adequate outcome in all culture conditions, except for the Dex-cDC2 condition, which demonstrates a lower percentage of yield (Table 4.3). However, as observed before in figure 4.5, cDC2 that were cultured in CellGenix were found to be morphologically different with adherent elongated cells in contrast to cDC2 that were cultured in X-VIVO, which were more rounded and smaller in size with only few elongated cells.

As shown in figure 4.13A, after performing an unsupervised hierarchical clustering the cells were clustered into two main divisions, which were mainly (but not entirely) based on culture media. Interestingly, both VitD3-cDC2 that were either cultured in CG or X-VIVO15 were

clustered in the same group with Un-cDC2 in X-VIVO15 (Red cluster), which may provide an idea of the semi-mature state of these cells (figure 4.13A). However, Rapa-cDC2 were always clustered with Stim-cDC2 irrespective of the culture media used (figure 4.13B), which could be due to the increase in expression of CCR7, as shown in figure 4.14.

Remarkably, all cDC2 conditions that were cultured in CG medium yielded lower expression of all regulatory markers of interest as well as maturation markers compared to cDC2 that were cultured in X-VIVO15 medium (figure 4.14). However, VitD3-cDC2 cultured in CG is the only exception where the cells expressed PDL-1 and ILT3 tolerogenic markers in addition to CD83 maturation marker, compared to all other conditions cultured in CG media. (figure 4.14).

Moreover, to further approve that selecting VitD3-cDC2 as a tol-cDC2 of choice to continue further functional analysis, evaluating the balance between co-inhibitory and co-stimulatory molecules (PDL-1/CD86 MFI ratio) was performed. As demonstrated in figure 4.15, VitD3-cDC2 demonstrated a significant increase in the PDL-1/CD86 ratio compared to other immune modulatory agents in cells that were cultured in CG. Similarly, in X-VIVO15 cultured VitD3-cDC2 demonstrated a higher PDL-1/CD86 ratio compared to other conditions, although this does not reach statistical significance (figure 4.15).

Finally, after deciding to focus on VitD3-cDC2 as tol-cDC2 of choice to continue the further functional analysis, MSD assays were performed as described in chapter 2 to assess the cytokine secretion differences between the two paired culture media used for cDC2 culture. As shown in figure 4.16, the secretion of IL-10 was statistically significantly enhanced in VitD3-cDC2 compared to other conditions, with no significant differences between the culture media. In addition, there were no significant differences in the secretion of IL-12p70, IL-6 and TNF-α in VitD3-cDC2 between the two different media (figure 4.16).

Therefore, to this end different culture media have an impact on the phenotype of cDC2 after culture. X-VIVO15 culture medium could have a better expression of tolerogenic marker of interest as well as CCR7, although CD86 and CD83 were also more highly expressed in cDC2 that were cultured in X-VIVO15 medium. Hence, further functional experiments may need to help in selection of the optimal culture medium for generation of tol-cDC2. Lastly, VitD3-cDC2, irrespective of culture medium, have greater expression of the tolerogenic marker PDL-

1, ILT3 and MERTK, as well as appropriate expression of CCR7 and expression of co-stimulatory molecules for stimulation of T cells (including regulatory T cells).
Hence, all further phenotypical optimization and functional experiments will be focused on VitD3 as the treatment of choice to generate tolerogenic cDC2.

Cultured cDC2	CellGenix culture medium	X-VIVO15 culture medium
Un-cDC2	88%	89%
Stim-cDC2	87%	91%
Dex-cDC2	89%	91%
Rapa-cDC2	89%	83%
VitD3-cDC2	91%	92%

Table 4.2: Viability of cultured cDC2 in different culture media. Data represent the percentage mean of three independent experiments in both culture media except Dex-cDC2 and Rapa-cDC2 two independent experiments in X-VIVO15. The viability was assessed by Zombie Aqua (ZA) viability dye by flow cytometer as percentage of live cells.

Cultured cDC2	CellGenix culture medium	X-VIVO15 culture medium
Un-cDC2	86%	78%
Stim-cDC2	79%	76%
Dex-cDC2	67%	65%
Rapa-cDC2	82%	82%
VitD3-cDC2	79%	77%

Table 4.3: Percentage of yield in cultured cDC2 in different culture media. Data represent the percentage mean of three independent experiments in both culture media, except for Dex-cDC2 and Rapa-cDC2, for which two independent experiments were performed in X-VIVO15. Yield was calculated according to the following formula:

Yield % = (Number of cells obtained / total number of seeded cells) X100

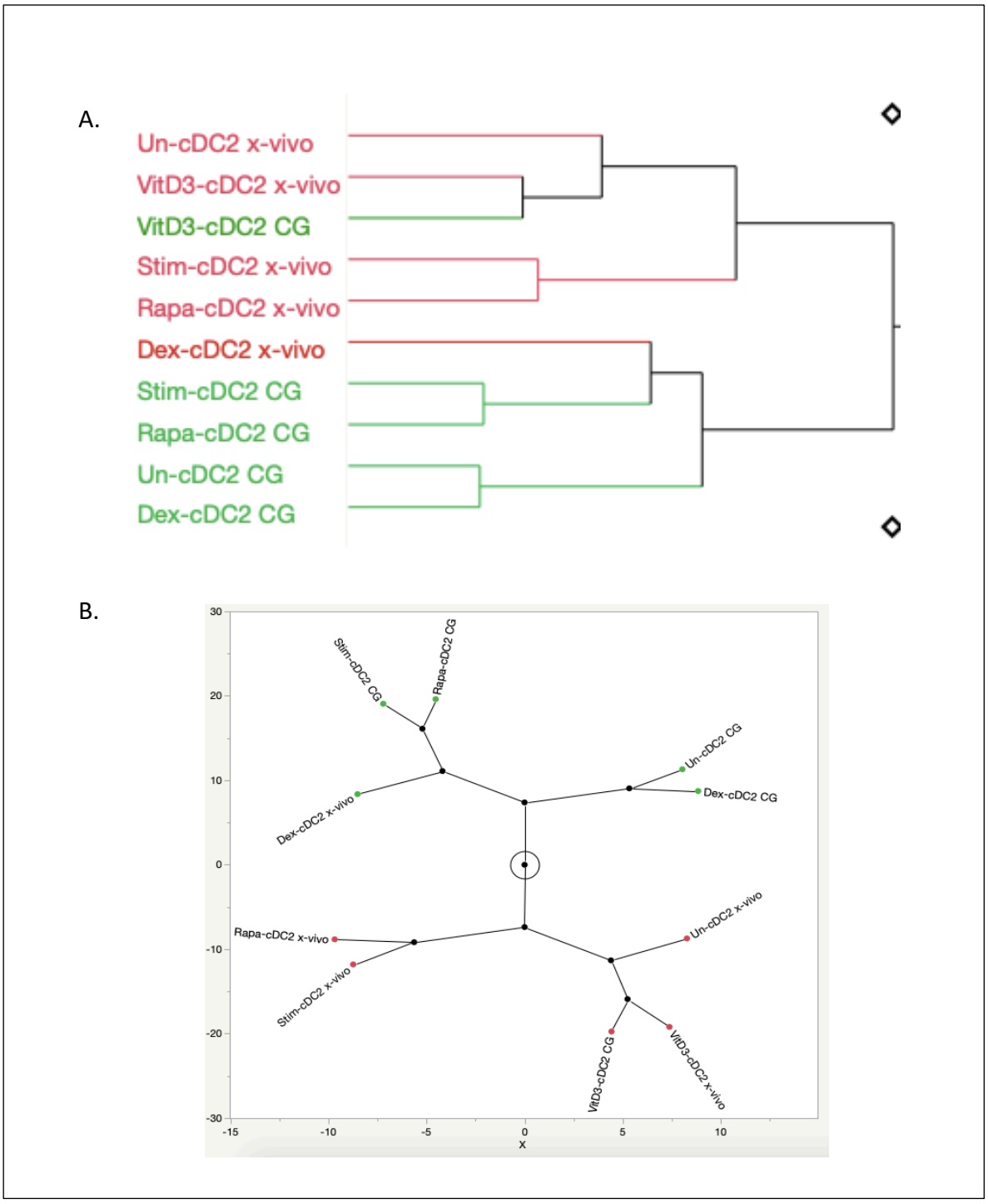


Figure 4.13: Impact of different culture media on clustering of cDC2 conditions. A. Hierarchical clustering of different cDC2 condition in CellGenix (CG) and X-VIVO15 culture media. B. Constellation plot demonstrate the division in different cDC2 conditions after clustering. These results are based on three independent experiments N=3.

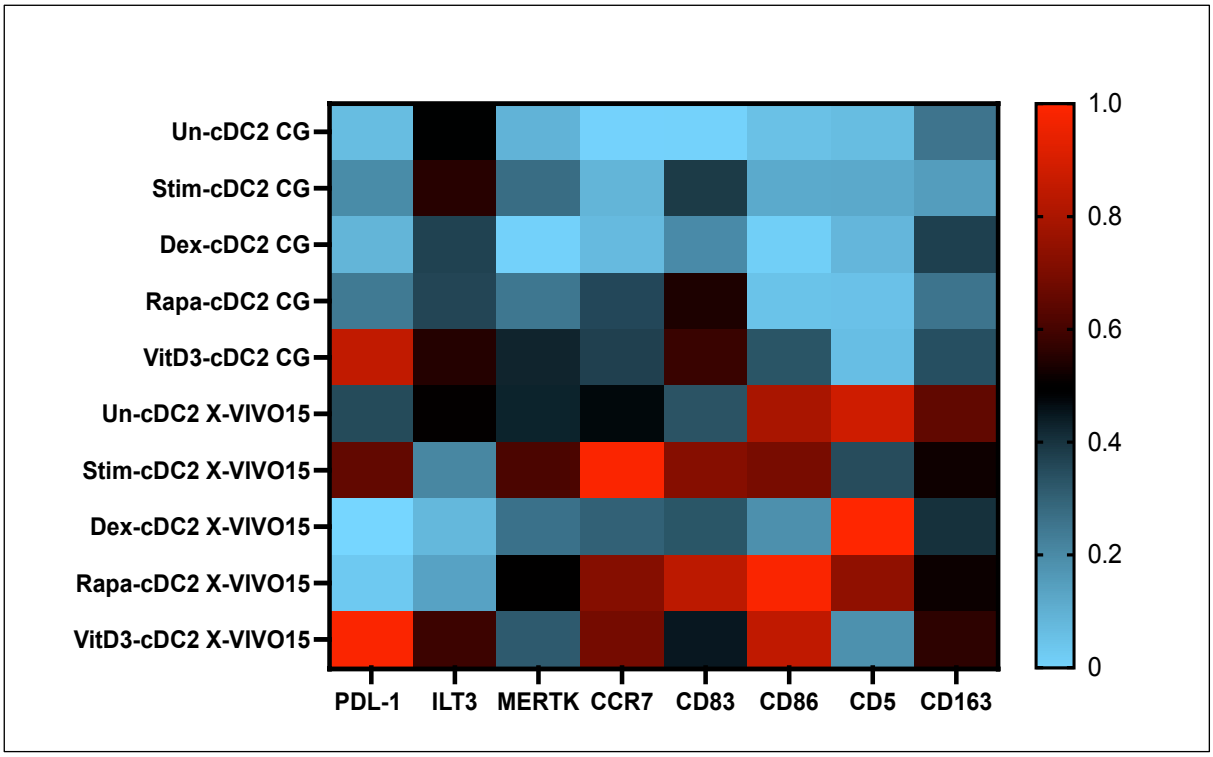


Figure 4.14: Expression of various markers of interest in different cDC2 conditions. Heatmap demonstrates the MFI expression of normalized data in different cDC2 conditions. Red represents the highest score of expression while blue represent the lowest expression. These results are based on three independent experiments.

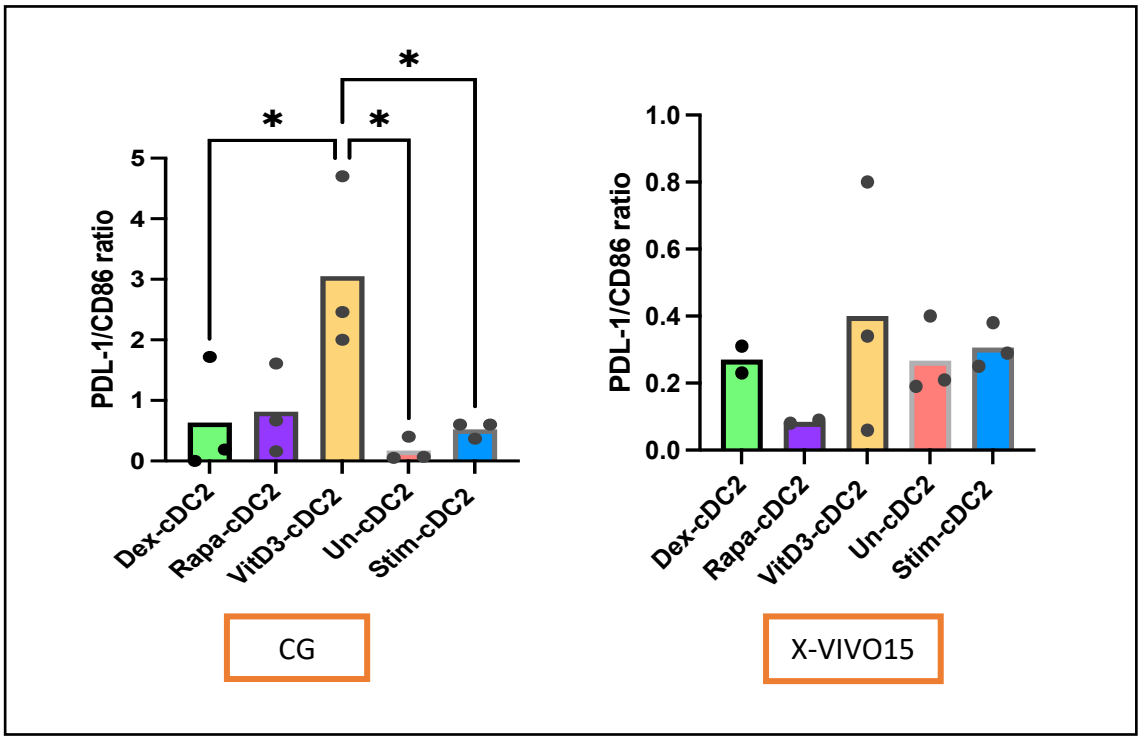


Figure 4.15: PDL-1/ CD86 MFI ratio in VitD3-cDC2 in the two different culture media (CellGenix (CG) and X-VIVO15). Data on the bars represent the mean of the ratio of three independent experiments in each culture medium and error bars represents the SEM. Statistically significant differences were based on ordinary one-way ANOVA parametric test between all groups. * = p <0.05, ** = p <0.01, *** = p <0.0001.

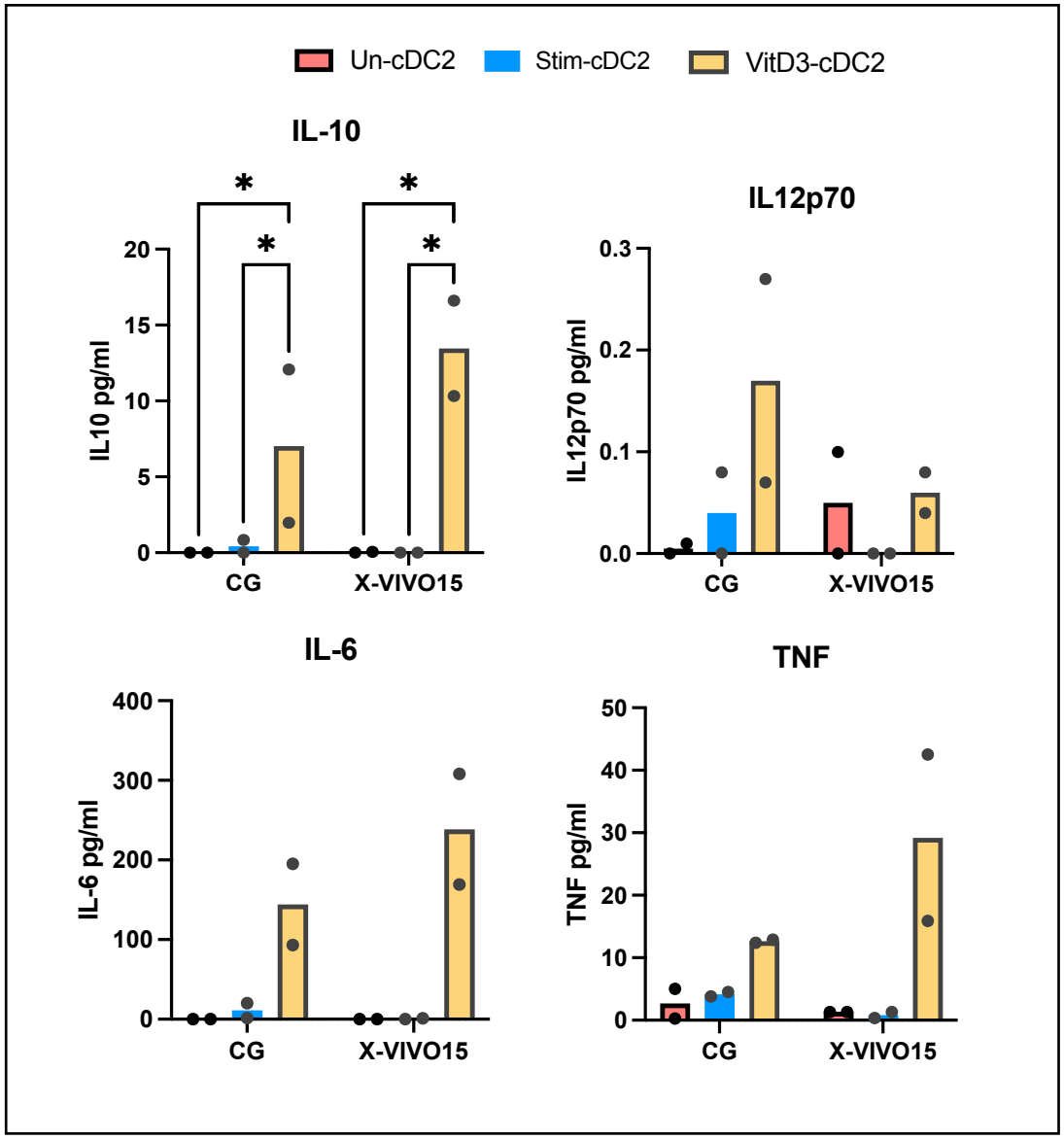


Figure 4.16: Cytokine secretion profile differences in cultured cDC2 between two culture media, namely CellGenix (CG) and X-VIVO15. Data on bars represent the mean of two paired independent experiments (N=2) and error bars demonstrate SEM. Statistically significant differences were based on a Two-way ANOVA parametric test between all conditions in the two different media. * = p <0.05, ** = p <0.01, *** = p <0.0001.

4.4.3.2 Expression of markers of interest in different cDC2 subsets

In order to investigate whether there is any difference in the expression of the tolerogenic marker of interest (ILT3, PDL-1 and MERTK), CCR7 and (CD86 and CD83) in the different subsets of cDC2 ($CD5^+$ DC2 and $CD163^+$ DC3), a heatmap statistics on the flow plots that shows the expression (saturation) of each marker in the two gated cDC2 subsets was performed. As shown in figure 4.17, in freshly isolated cDC2, there was no obvious difference in the expression of ILT3, PDL-1, MERTK, CD86 and CD83 between the $CD5^+$ and $CD163^+$ cDC2 subsets. Remarkably, as I previously found that freshly isolated cDC2 have significantly higher expression of ILT3, here I also showed higher expression of this tolerogenic marker in both cDC2 subsets (yellow saturation) compared to other tolerogenic markers of interest (figure 4.17A). However, expression of CCR7 was found to be higher in the $CD5^+$ population in freshly isolated cDC2 (figure 4.17B).

As we previously mentioned, the CD163 marker is not useful for distinguishing between cDC2 subsets after culture in X-VIVO15, thus in this section, I defined the subsets by the expression of CD5. Interestingly, after culture the expression of (ILT3, PDL1 and MERTK) and (CD86 and CD83) between $CD5^+$ and $CD5^-$ cells remains the same with no clear difference between the two cDC2 subsets in all cultured conditions (figure 4.18 and 4.19). However, as I mentioned before, it seems that the only difference was the expression of CCR7 molecules with higher expression in the $CD5^+$ than the $CD5^-$ subset in Un-cDC2 condition, although $CD5^-$ subsets also started to express CCR7 after LPS activation in both Stim-cDC2 and VitD3-cDC2 (figure 4.19).

To further demonstrate the statistically significant difference in the expression of CCR7 between $CD5^+$ and $CD5^-$ subsets, a bar graph was performed for four independent experiments. As demonstrated in figure 4.20A, there was a significant difference in the frequency of CCR7 between the $CD5^+$ and $CD5^-$ subsets, with higher frequency in the CD5+ population in all cultured conditions. Interestingly, the frequency of CCR7 in both CD5+ and CD5- subsets of Un-cDC2 was significantly lower than both Stim-cDC2 and VitD3-cDC2 (figure 4.20A). Moreover, the MFI of CCR7 in the $CD5^+$ subset was also significantly higher than in the $CD5^-$ subset in all cultured cDC2 conditions (figure 4.20B).

To this end, expression of markers of interest appears to not be differentially regulated on the $CD5^+$ or $CD5^-$ cDC2 subsets. The only exception was for CCR7, which was expressed mainly in the $CD5^+$ population in freshly isolated cDC2. However, after stimulation, the $CD5^-$ subset was also found to express CCR7.

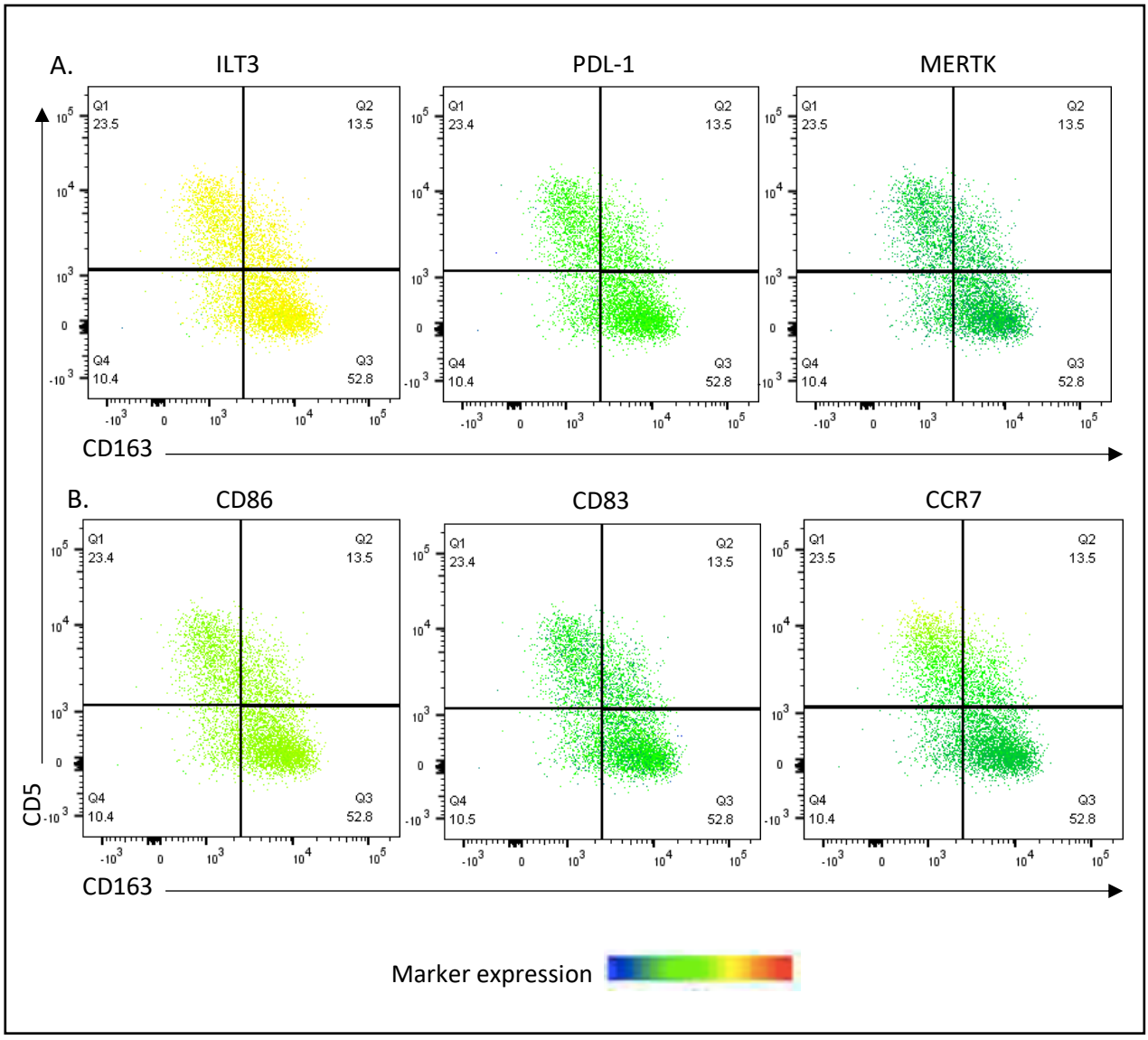


Figure 4.17: Expression of markers of interest in the freshly isolated cDC2 subsets. Flow plot demonstrate the heatmap statistics of expression of marker of interest in the two subsets of cDC2 namely $CD5^+$ (DC2) upper left quadrant and $CD163^+$ (DC3) lower right quadrant. Closer to red color demonstrate high expression while closer to blue color demonstrate low expression. Data are representative of 6 independent experiment.

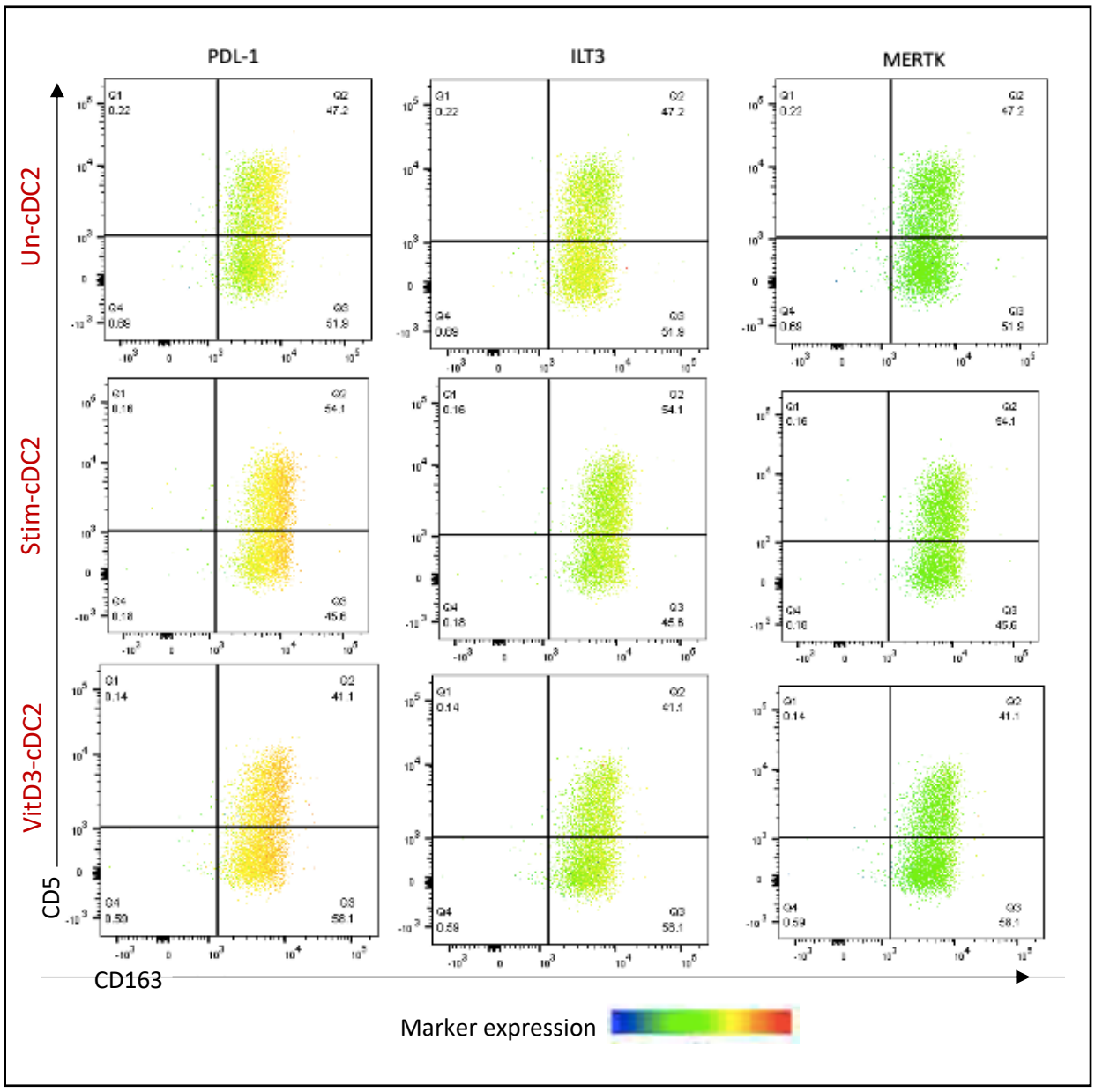


Figure 4.18: Expression of marker of interest in X-VIVO15-Cultured cDC2 subsets. Flow plots demonstrate the heatmap statistics of (PDL-1, ILT3 and MERTK) among the cDC2 subsets $CD5^{+}$ and $CD5^{-}$ population in different cDC2 condition. Closer to red color demonstrate high expression while closer to blue color demonstrate low expression. Data are representative of four independent experiments.

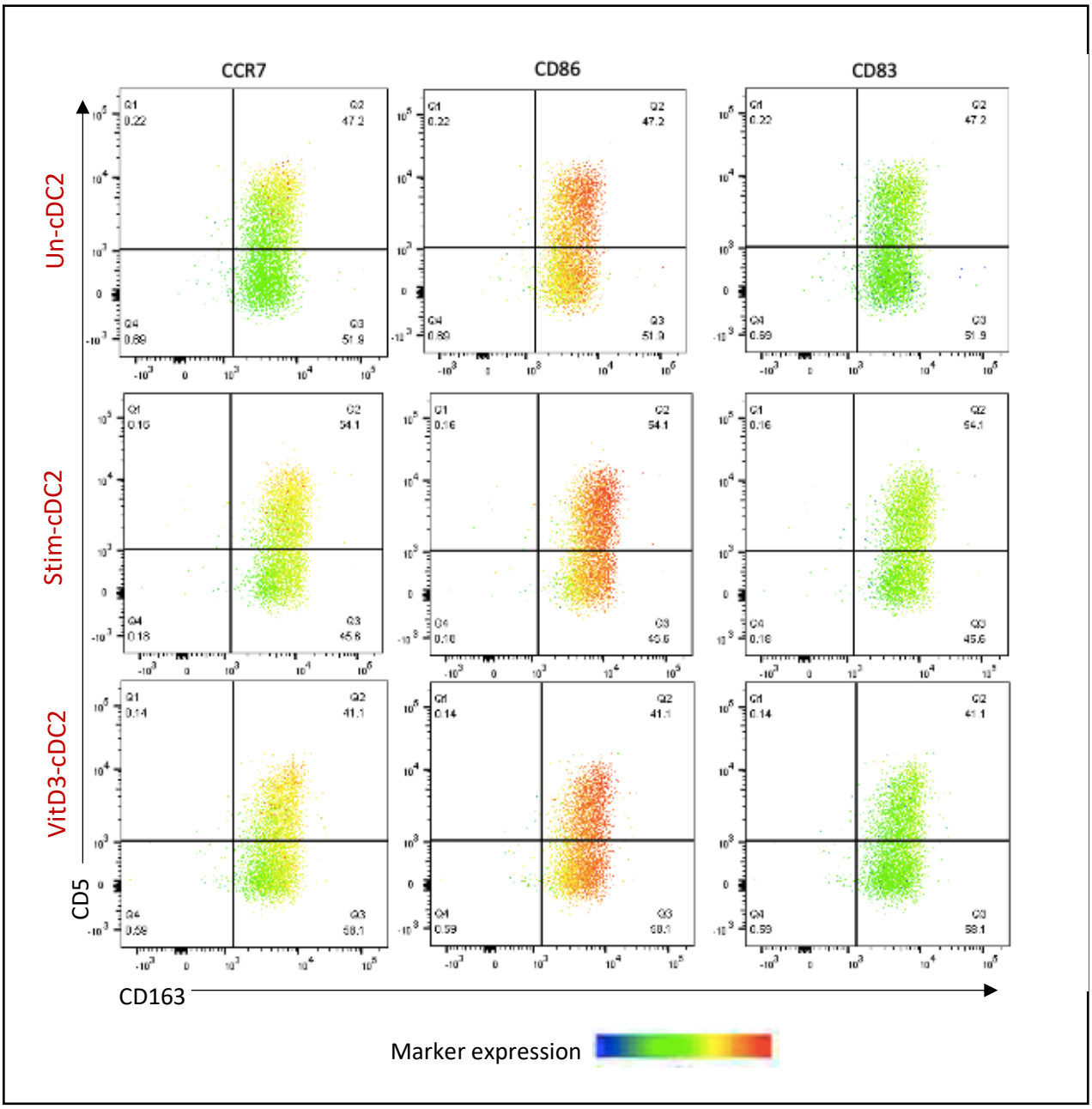


Figure 4.19: Expression of marker of interest in X-VIVO15-Cultured cDC2 subsets. Flow plots demonstrate the heatmap statistics of CCR7 and (CD86 and CD83) among the cDC2 subsets CD5+ and CD5- populations in different cDC2 conditions. Closer to red colour demonstrates high expression, while closer to blue colour demonstrates low expression. Data are representative of four independent experiments.

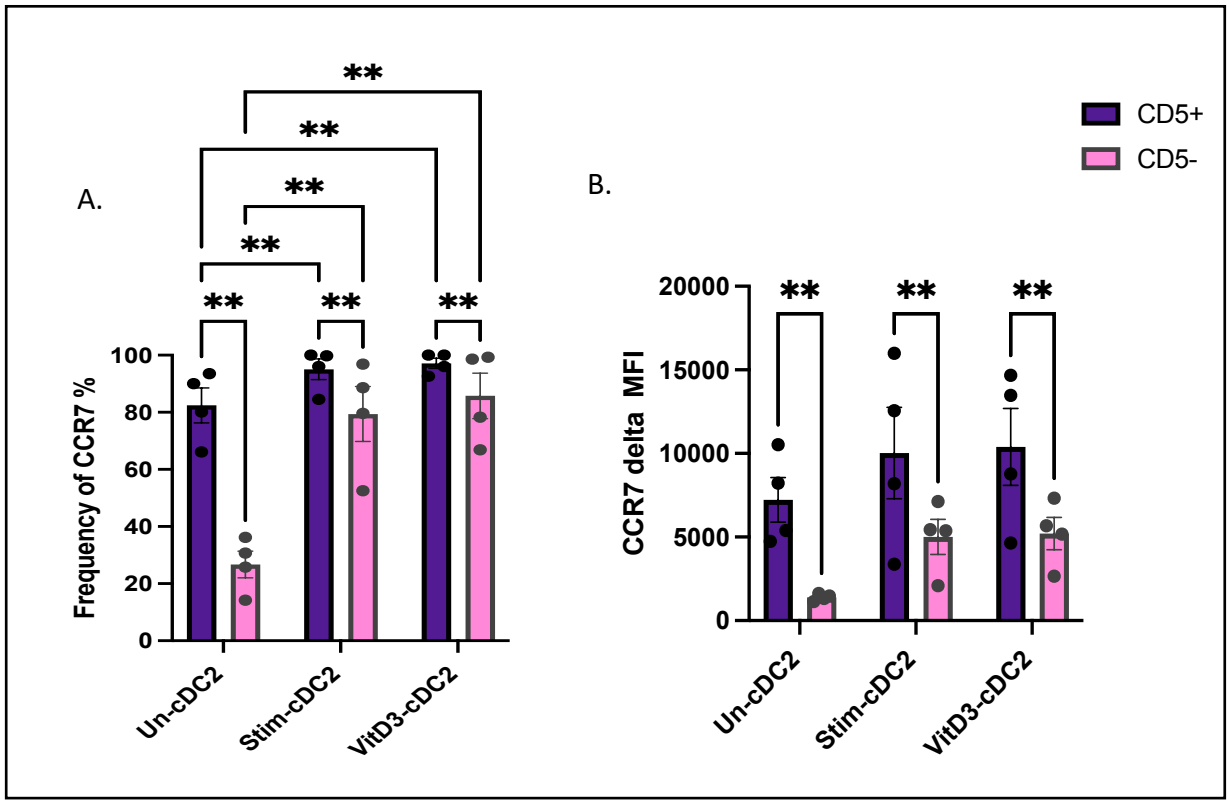


Figure 4.20: Expression of CCR7 in the $CD5^-$ (pink bar) and $CD5^+$ (purple bar) cDC2 in different culture conditions. A) Frequency of parents of CCR7 in $CD5^+$ (purple bar) and $CD5^-$ (pink bar) cDC2 subset. B) Delta Mean fluorescent intensity (MFI) of CCR7 in different cDC2 subsets. Delta MFI was calculated by subtraction of fully stained MFI from the FMO MFI of CCR7. Data represent the mean, and the error bar demonstrates SEM. Statistically significant difference based on two-way ANOVA test between all cDC2 conditions for four independent experiments N=4. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$.

4.4.3.3 Different GM-CSF concentration does not have a huge impact on the expression of markers of interest

GM-CSF is known to be important for cDC2 survival after culture. Therefore, I examined the effect of different GM-CSF concentrations on the viability of cDC2 after culture, the phenotype of tol-cDC2 as well as their cytokine profile.

Interestingly, as demonstrated in figure 4.21, the overlay histogram of FSC of different GM-CSF concentrations does not appear to have any difference in the size and granularity of cultured cDC2. However, in the Un-cDC2 condition, it seems that without GM-CSF supplementation (the red histogram) there was more debris and dead cells than in the cell

supplemented with GM-CSF. Nevertheless, I did not observe this difference in Stim-cDC2 that could be due to stimulation with LPS increasing the survival of the cells (figure 4.21).

As shown in figure 4.22, there were no obvious differences in the viability and yield of the cells with or without GM-CSF supplementation in X-VIVO15 for the majority of the culture conditions. The only exception was Un-cDC2 for which viability was slightly lower but still at acceptable levels (NB commonly accepted QC for DC therapies is 70% viability). Moreover, it appears that the cell surface and cytokine secretion profiles were overall quite similar between the different GM-CSF concentrations (figure 4.22 and 4.23). However, we observed a slight increase in the expression of ILT3 in VitD3-cDC2 that was supplemented with 10ng/ml GM-CSF in the two optimization experiments. Thus, different concentration of GM-CSF does not appear to have a huge impact on the expression of different makers of interest. Therefore, we chose the lower concentration (10 ng/ml) to save on costs and it has also been used by other groups.

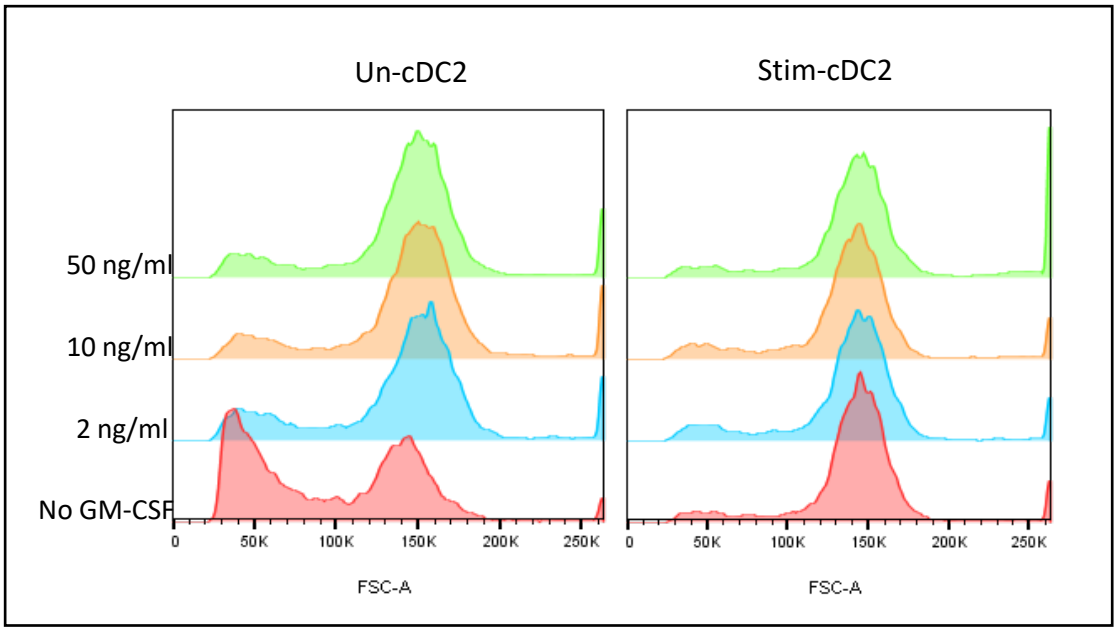


Figure 4.21: Effect of different GM-CSF concentrations on the size and granularity of cDC2. The histogram represents the overlay of FSC and SSC of (A) Un-cDC2 and (B) Stim-cDC2 with supplementation of different GM-CSF concentrations.

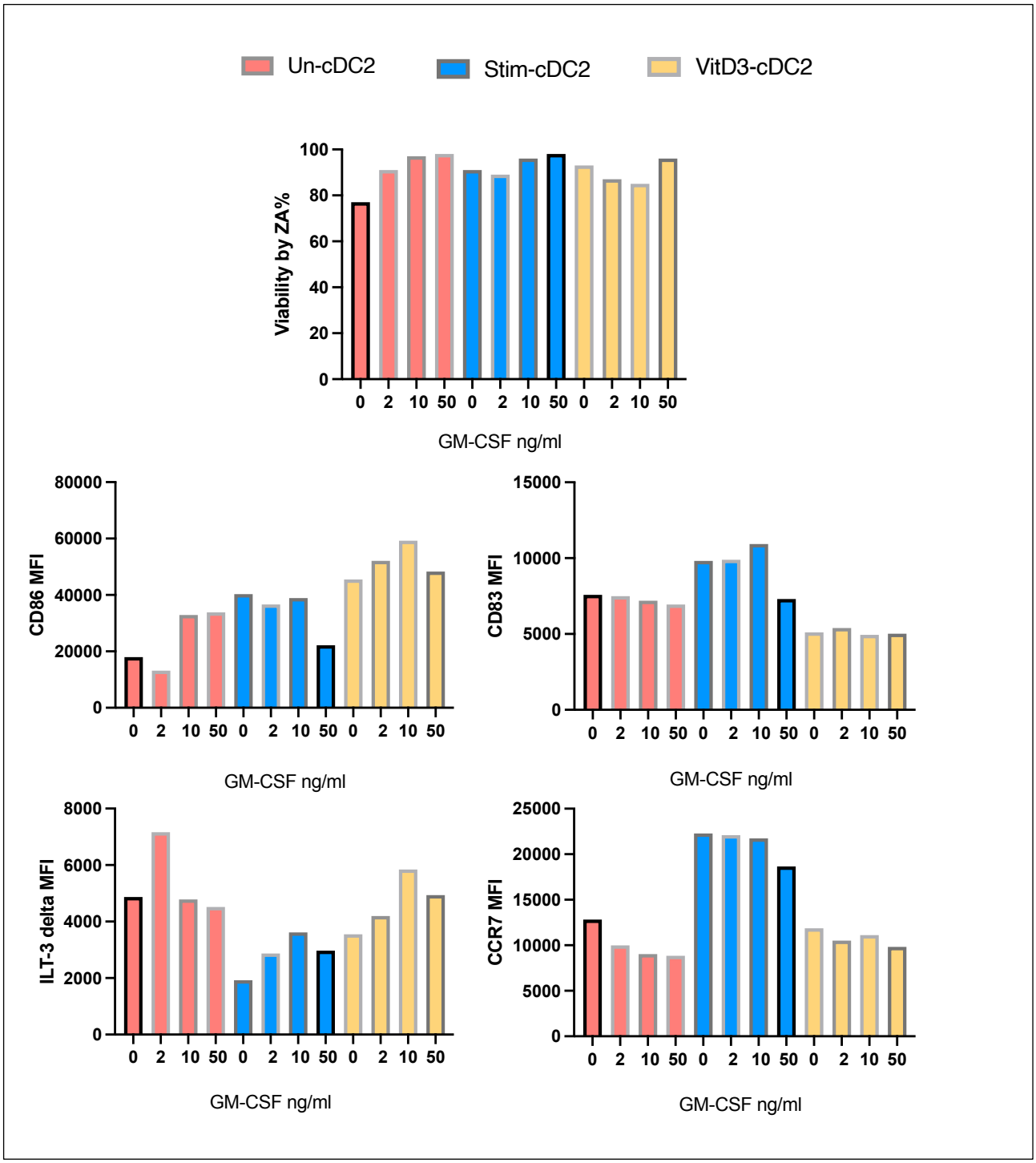


Figure 4.22: Effect of different GM-CSF concentration in the expression of marker of interest. Representative bar graph of two independent experiments demonstrates effect of different GM-CSF concentration on viability of cDC in different conditions as well as expression of co-stimulatory molecules and tolerogenic markers. cDC2 were cultured in X-VIVO15.

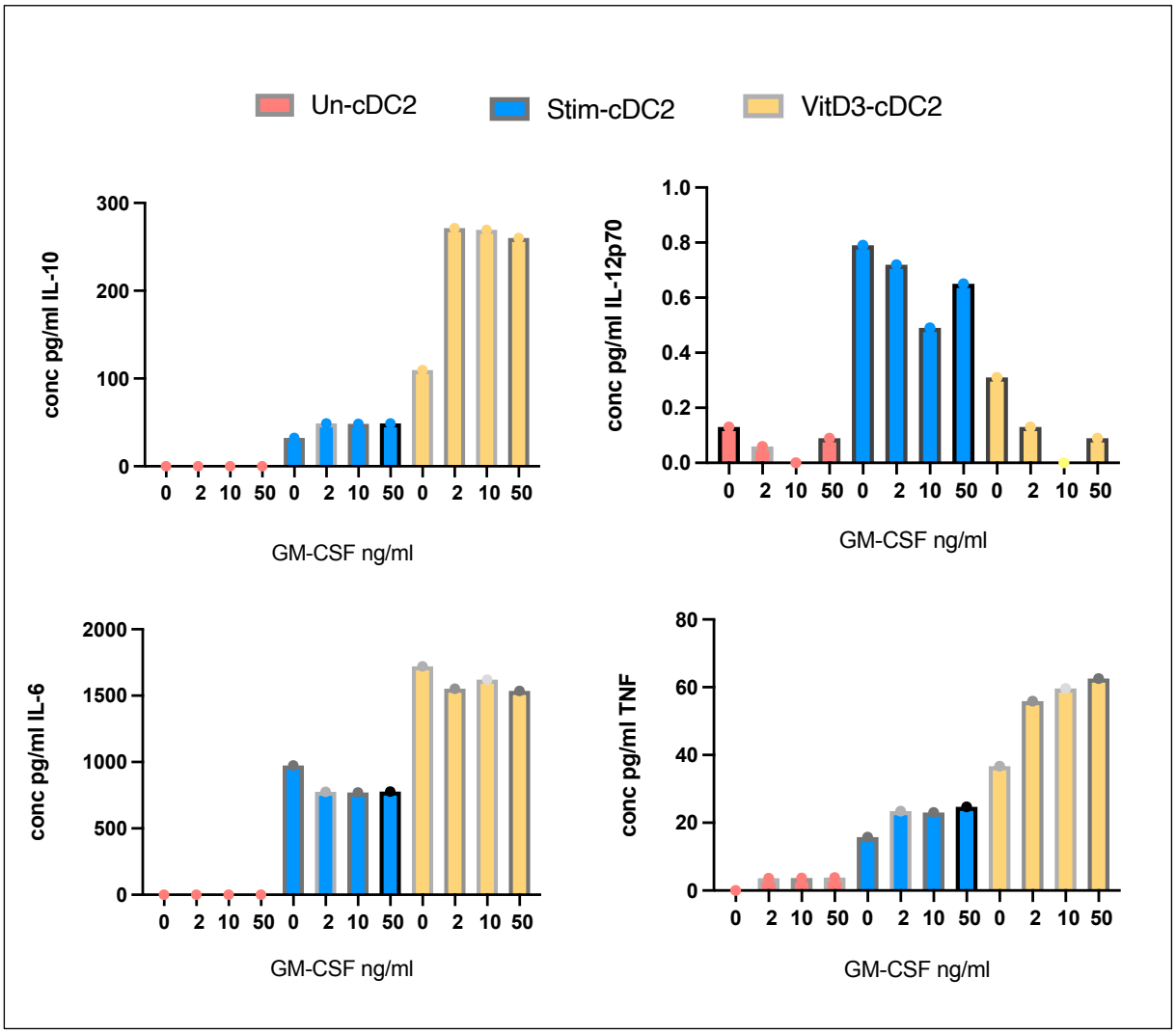


Figure 4.23: Bar graph demonstrate effect of different GM-CSF concentration on the cytokine profile of cDC2 in different conditions. Data represent the result of one experiment for optimization purpose.

4.4.3.5 Timing of priming with VitD3 affects the expression of tolerogenic marker.

As previously observed in the context of Mo-tolDC generation in our group (data not shown), timing for prime with immunomodulatory agents may exert distinct effect on the expression of tolerogenic marker. Specifically, my colleagues Emma Jackson and Gabrielle Barran found that priming of moDC with either dex or VitD3 before LPS stimulation improved the generation of cells with a tolerogenic phenotype (data not shown). Hence, to assess this concept in the context of cDC2, we decided to prime cDC2 for 1 hour with VitD3 prior to stimulation

with LPS for 16-20 hr. The rationale for this decision was to keep the culture period as short as possible, to avoid the risk of excess cell death.

The two optimization experiments that I carried out (figure 4.24) showed a clear trend: priming of cDC2 with vitD3 for 1 hour increased the expression of all tolerogenic markers, maturation markers as well as CCR7 as compared to cDC2 that were treated with VitD3 and LPS at the same time. Overall DC activation was enhanced by vitD3 priming. Hence, more repeats will be necessary to calculate the statistical significance of this observation. Nevertheless, taken together, these data strongly suggest that cDC2, like moDC, need to be primed with a tolerogenic agent for suitable period of time to ensure the activation of immune regulatory pathways, before stimulation with LPS. Hence, for all further experiments cDC2 were primed with vitD3 for 1hr before stimulation.

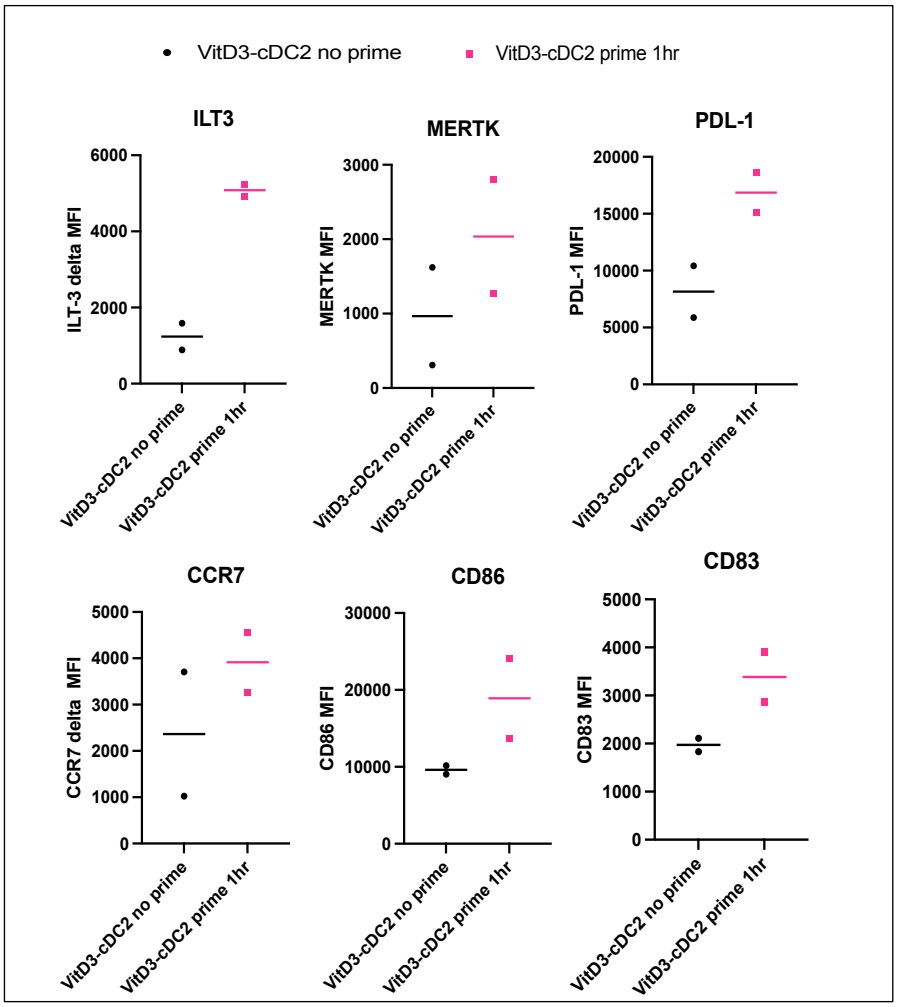


Figure 4.24: Priming of cDC with vitD3 for 1 hour improves expression of tolerogenic markers. cDC2 were primed with VitD3 for 1 hour before LPS activation or were activated with LPS and vitD3 at the same time ('no prime'). Expression of surface markers was

determined by flow cytometry. Data are displayed as MFI (for PDL-1, MERTK, CCR7 and CD86) or as delta MFI (for ILT-3). Delta MFI were calculated as follow: MFI of fully stained cDC2 - MFI of ILT3 FMO. The line between the data represents the mean. N=2 for each priming experiment.

4.4.3.5 Effect of different VitD3 concentrations on generation of tolerogenic cDC2

In order to generate an optimal VitD3-cDC2 with higher expression of tolerogenic markers as well as CCR7, three different concentrations of VitD3 were examined for optimization. As shown in figure 4.25, there are no clear differences between different doses of VitD3 in the expression of all markers of interest, with a slightly higher expression of PDL-1 and CCR7 molecules at 10^{-8} M VitD3.

To this end, we decided to continue use 10^{-8} M VitD3 as an optimal dose for tol-cDC2 generation and also to perform all further functional analysis. In addition, this is also the most commonly used vitD3 concentration in the literature.

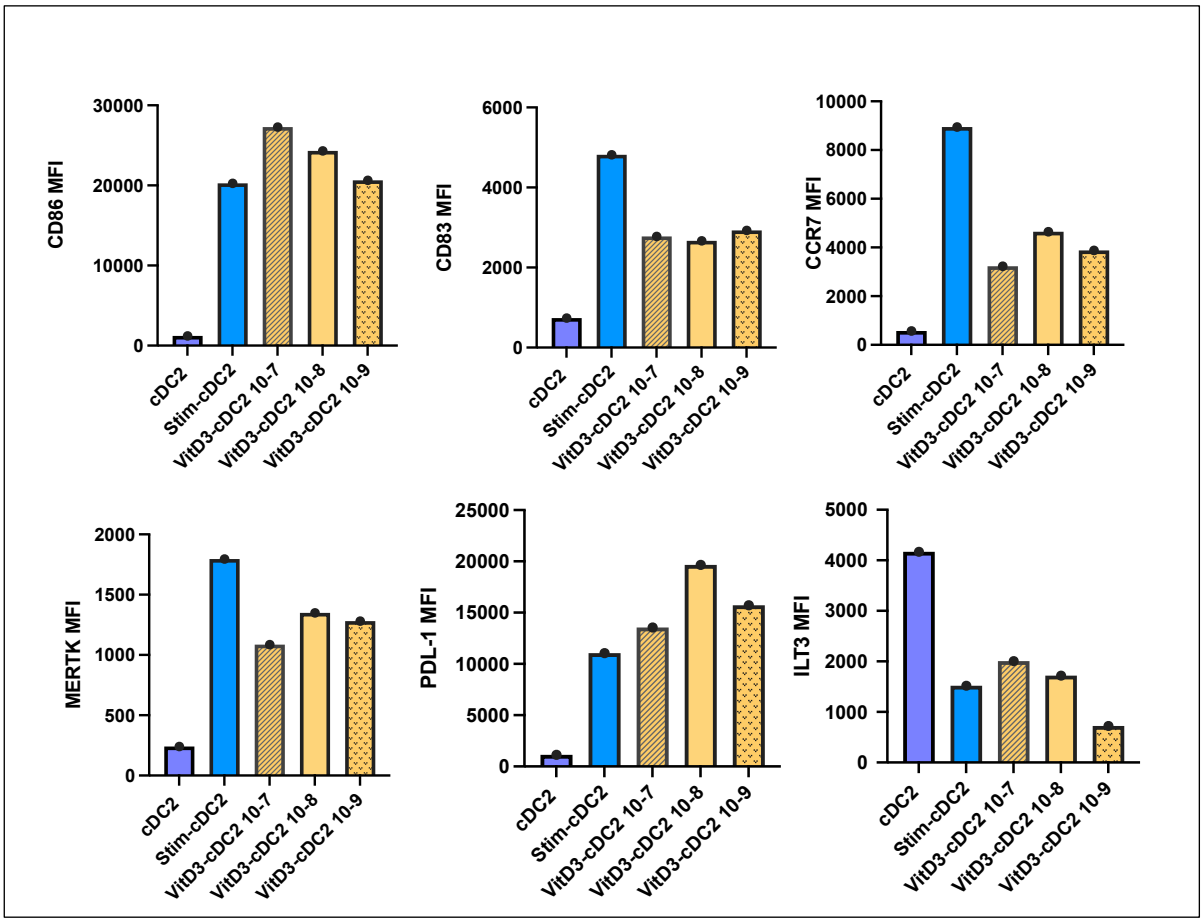


Figure 4.25: The effect of different VitD3 concentrations (10^{-7}, 10^{-8} and 10^{-9} M) on the expression of marker of interest and co-stimulatory molecules. Blue is the positive control stim-cDC2. N=1

4.4.4 Effect of different culture media and different tolerizing agents on the migratory ability of cDC2

Freshly isolated cDC2 have been shown to express some CCR7 migratory molecules. Remarkably, most of the CCR7 expressed by cDC2 was from the $CD5^{+}$ population as shown in t-SNE visualization (figure 4.26). As previously mentioned, one of the crucial aims of my project is to generate a tol-cDC2 with high migratory ability. Hence, to address this aim, I first investigated the effect of various tolerizing agents and culture media on the expression of CCR7. Then, functional capacity for migration of selected tol-cDC2 was also assessed in the two different media.

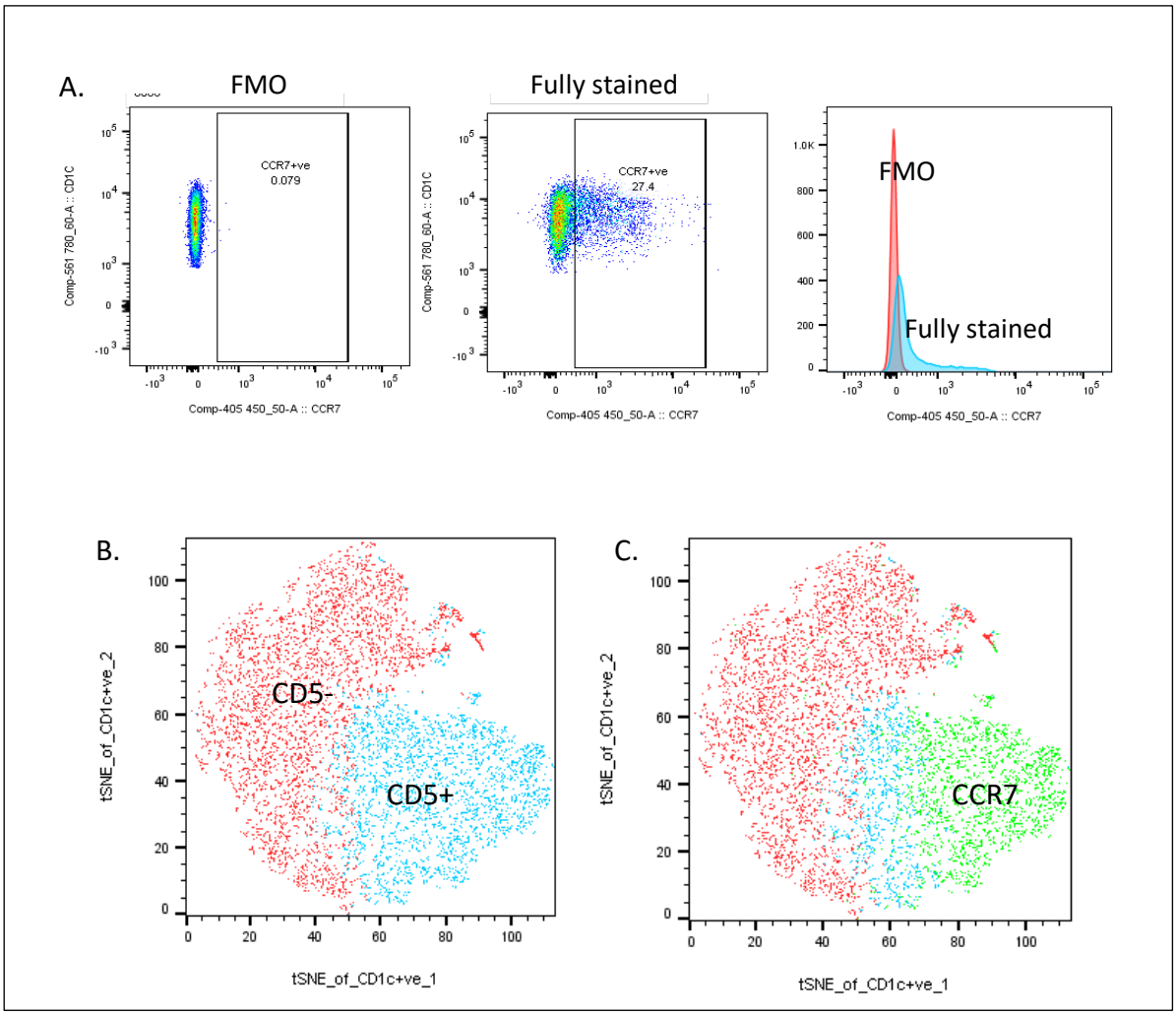


Figure 4.26: Expression of CCR7 migratory molecule in freshly isolated cDC2. A) FMO control for CCR7 to remove any background. t-SNE visualization demonstrate B) cDC2 subtype in which red is the $CD5^-$ population and blue is the $CD5^+$ population. C) Expression of CCR7 in $CD5^+$ population (green).

4.4.4.1 Phenotype expression of CCR7 in tol-cDC2

As previously shown (figure 4.27A), CCR7 was found to be expressed at higher levels in cDC2 that were cultured in X-VIVO15 medium than CG medium, irrespective of the immunomodulatory treatment. To have a closer look into CCR7 expression in the selected tol-cDC2 type, which is VitD3-cDC2, its expression was compared in both culture medium with the positive control Stim-cDC2. As shown in figure 4.27B, in general there is a trend of increased CCR7 expression in cDC2 that were cultured in X-VIVO15, which could give an enhanced migratory ability to tol-cDC2, although this does not reach statistical significance.

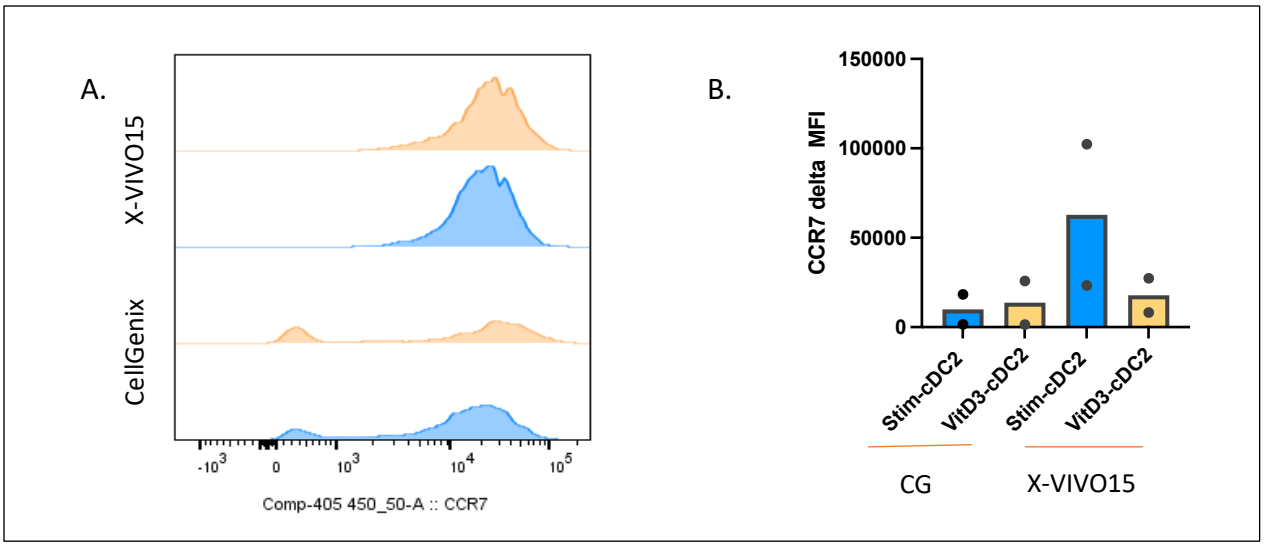


Figure 4.27: Expression of CCR7 in cultured VitD3-cDC2 in two different culture media. Yellow is VitD3-cDC2 and blue is the positive control Stim-cDC2. A) A representative histogram of paired experiment in CG and X-VIVO15. B) Bar graph demonstrates the mean MFI of CCR7 of two independent experiments (N=2) in VitD3-cDC2 in both CG and X-VIVO15 culture medium.

4.4.4.2 Migration of VitD3-cDC2 at functional level

To finalize this chapter, I also assessed the migratory ability of VitD3-cDC2 at functional level. Therefore, CCR7-dependent migration towards CCL19 and CCL21 was assessed using trans-well plates as described in Chapter 2. In addition, I also tested the effect of CG and X-VIVO15 culture media on the migration efficiency of VitD3-cDC2 in order to assist in selecting the optimal culture media for further functional analysis. As shown in figure 4.28A, irrespective of cDC2 condition, cDC2 that were cultured in X-VIVO15 medium demonstrated significantly higher migration efficacy compared to CG medium, which is approximately around 17% in Stim-cDC2 (control) and 13% in VitD3-cDC2. Beside all previous results, these interesting results support the use of X-VIVO15 as an optimal culture medium for generation of tolerogenic cDC2 with high migratory capacity. Moreover, to assess the relationship between migration efficacy and CCR7 expression, both parameters were plotted in figure 4.28B. Although care should be taken with the interpretation of these data (because migration efficacy and CCR7 expression were not determined within the same experiments), it appears that there may be a positive correlation between CCR7 expression and migration efficacy for stim-cDC2. However, this was less apparent for VitD3-cDC2. More data will be needed to establish the extent of the relationship between these parameters in these cultured cell types, although it would be reasonable to expect that a positive correlation does exist.

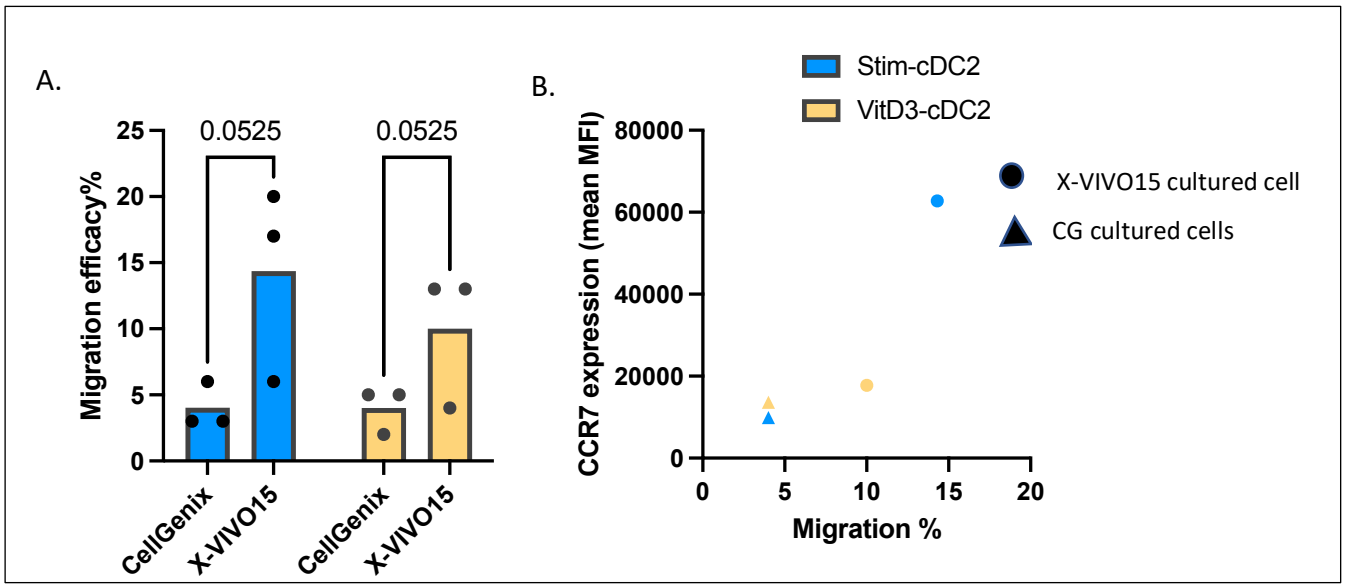


Figure 4. 28: Migration efficacy of VitD3-cDC2 in two culture media. A) Data on bars represents the mean of three independent experiments (N=3), while error bars demonstrate the

SEM. Statistically significant differences were based on Two-way ANOVA test between two cDC2 conditions in two different culture media. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$. B) Graph represent the correlation between CCR7 expression and migration percentage in Stim-cDC2 and VitD3-cDC2 in both media. Data are based on mean MFI of CCR7 expression (N=3) and Mean of migration percentage (N=3). CCR7 expression and migration efficacy data were unpaired. Blue is Stim-cDC2 and Yellow is VitD3-cDC2.

4.5 Discussion

A significant constraint associated with tolerogenic moDC is their limited migratory capacity to T cell region, even following LPS stimulation. This will further affect on stimulation of T cells either in a regulatory or stimulatory manner. Therefore, the aim of this chapter was to generate a phenotypically optimal tolerogenic cDC2 with intermediate expression of maturation markers and high expression of immune inhibitory molecules and anti-inflammatory cytokines. Hence, various immunomodulatory agents were tested to examine their effect on the generation of phenotypically tol-cDC2 with suitable migratory capacity. In addition, to select the suitable culture environment for the generation of tol-cDC2, culture media and supplementations were also examined. All these optimizations were performed first to identify the best culture environment as well as to select one favorable treatment for the generation of tol-cDC2 based on the expression of tolerogenic markers of interest and migratory capacity. The chosen tol-cDC2 will then be taken forward for further functional analysis (Chapter 5).

4.5.1 cDC2 subtype after culture

cDC2 is known to be a heterogenous type of conventional DC with two main subtypes present in the peripheral blood: $CD163^+$ $CD5^-$ (majority of cDC2) and $CD5^+$ (minority of cDC2). However, there are many factors that could affect this subtype proportion after culture. For example, as I observed in figure 4.3, different culture media appear to have an impact on the proportion of cDC2 subtype with a low proportion of $CD163^+$ cells in CG culture medium with no effect on the $CD5^+$ population, whereas the majority of cells were shifted to be $CD163^+$ in X-VIVO15 culture medium, even in the $CD5^+$ population. This observation could mean that CD163 is not a useful marker for in vitro cultured cDC2 subtypes, but whether this has changed the overall functionality of the cells is not known. However, CD5 could be more appropriate

to distinguish between the cDC2 subtype as it demonstrates approximately consistent proportion before and after culture (figure 4.3 and 4.4).

With respect to the $CD5^+$ population, I found that the MFI of this marker was generally increased after culture in the X-VIVO15 medium compared to cells that were cultured in the CG medium (heatmap figure 4.14). Interestingly, as shown in figure 4.26 B&C, most of the $CD5^+$ freshly isolated cDC2 naturally express CCR7, which reflects the bona fide cDC2 with migratory capacity. In addition, in-vitro cDC2 stimulation was found to increase expression of CCR7 not only in the $CD5^+$ population but also in $CD5^-$ subset. This observation is similar to what was described in the literature; that freshly isolated $CD5^+$ DC2 naturally express low levels of CCR7 (Yin et al., 2017). Therefore, considering all these findings concerning the difference between cDC2 subsets in the expression of a marker of interest, it may endorse the utilization of bulk cDC2 for tol-cDC2 therapy. However, whether these two different subsets could have any different functions for developing tol-cDC2 is not known. Therefore, further studies would need to be conducted to address this point. For example, a study with sorted cell subsets rather than magnetic isolation could help in identifying and understanding any functional differences between the subsets, but this would have a consequence such as the complexity and expensive cost of the procedure for the translation into a GMP product for clinical application.

Interestingly, another new subtype of cDC2 which has been reported in the literature as a distinct type of DC, is called DC3. This type is phenotypically and functionally intermediate between cDC2s and monocytes and is characterized by the expression of $CD163^+$ $CD5^-$ $CD14^+$ $CD1c^+$ (Villani et al., 2017). This unique subset was also found to be accumulated in the blood of systemic lupus erythematosus (SLE) patients (Dutertre et al., 2019; Villani et al., 2017). Hence, this means that DC3 could contribute to the pathophysiology of some autoimmune diseases. Therefore, we need to address what could induce its development. (Bourdely et al., 2020b; Cytlak et al., 2020) determined that DC3 depends on GM-CSF cytokines in their development. However, in my findings, we do not see any induction of DC3 ($CD14^+$) from cDC2 after culture, even though we supplemented it with GM-CSF. This could have two explanations: first, DC3 has a distinct developmental pathway that is not related to cDC2 as determined by (Bourdely et al., 2020). Second, I already depleted $CD14^+$ cells during cDC2 isolation, therefore most of DC3 was depleted as well. This explanation also confirmed that the $CD14^+$ $CD1c^+$ population that appears after treatment with VitD3 is not a bona fide DC3

population. DC3 is thought to be mainly present in response to inflammatory stimuli and considered to be an inflammatory subtype (Heger et al., 2020).

To this end, markers that distinguish freshly isolated cDC2 may not be very useful for cultured cDC2, such as the CD163 marker. Hence, we may have to apply techniques like single-cell RNAseq to really understand what is going on during the cultures.

4.5.2 Natural pro-tolerogenic markers in freshly isolated cDC2

cDC2s are professional APCs that can efficiently prime and educate $CD4^+$ T cells either in a stimulatory or regulatory fashion based on environmental factors. As we previously determined in Chapter 3, both peripheral blood- and $CD34^+$-derived cDC2 express some important tolerogenic genes including *LILRB4* (the gene encoding ILT3 immune inhibitory molecule). We also found that freshly isolated cDC2 from peripheral blood express high levels of ILT3 protein on their surface. This finding was also confirmed by (Dutertre et al., 2019; Mair and Liechti, 2021), who identified that CD85K (another name for ILT3) is expressed at high levels by cDC2, DC3 and pDC, but not by cDC1. This also makes cDC2 a promising alternative for generating tolerogenic DC based immunotherapy. In addition, Mair and Liechti, 2021 also found that both subtypes of cDC2 have high expression of ILT3 (CD85K). These findings agreed with our finding that there is no difference in the expression of ILT3 between the $CD5^+$ and $CD5^-$ cDC2 subsets. Nevertheless, there could be differences in the expression of other markers between the subsets that are not known yet.

Although we found that expression of ILT3 was decreased after culture, particularly in LPS-stimulated conditions, this appeared not to be the case in VitD3-treated cDC2, where the ILT3 expression remained high compared to treatment with other immune modulatory agents. However, freshly isolated cDC2 has the highest expression compared to all cultured conditions. This could be explained mainly due to their steady state condition that needs high expression of immune inhibitory molecules to prevent the induction of an immune response. However, in response to danger signals or any inflammatory signal, cDC2 downregulates ILT3 to become a mature cDC2 that can induce an immune response. Similar to our finding, (Penna et al., 2005b) discovered that LPS stimulation downregulates ILT3 expression in moDC, while vitD3 treatment can induce ILT3 expression even with LPS stimulation. Thus, further studies are

needed to investigate the effect of neutralizing ILT3 antibody on the regulatory mechanism for tolerance induction in cDC2.

4.5.3. An optimal culture environment for tol-cDC2 generation

There have been a limited number of studies that assess the phenotype of cDC2 after culture, especially in the context of the effect of culture media and other supplementations into the generation of tol-cDC2 for autoimmune disease immunotherapy. Therefore, comparing my results with published data from other groups is very difficult. However, some studies investigated the impact of culture environment on moDC, focusing on the CellGenix GMP DC medium and X-VIVO15 medium, both commonly used for DC culture. For example, Calmeiro et al., found that different culture media and environments can affect the phenotype, metabolic activity and cytokines profiles of cultured moDC (Calmeiro et al., 2021). For example, in agreement with my findings for cDC2, they found that moDC cultured in X-VIVO15 medium expressed higher levels of CD86 and CCR7 than other serum-free media such as DendriMACS and AIM-V. These findings were also confirmed at the functional level in my results when I showed the higher migratory efficacy of cDC2 cultured in X-VIVO15 compared to the CellGenix medium (figure 4.28).

In addition, to help interpret the comparison between the two culture media with respect to the various immune modulatory agents used to induce regulatory function in cDC2, hierarchical clustering was performed (figure 4.13 & 4.14) and to help find the relationship between the conditions. At the same time, the heatmap showed an overview of the effect of culture media on each condition. Hence, we found VitD3-cDC2 always clustered next to Un-cDC2, which may give an idea of the semi-maturation state of these cells. Moreover, VitD3-cDC2 was also found to have high expression of CCR7 with higher expression in cells cultured in X-VIVO15 medium. Consistent with our findings, several groups have found similar semi-mature states in moDC that were treated with VitD3 (Barragan et al., 2015; Švajger and Rožman, 2019).

Another critical quality control to look at for DC that are cultured for therapeutic use is their viability. Hence, I investigated the effect of supplementation with different concentrations of GM-CSF on the viability and yield of cDC2 after culture. I observed only minor effects of GM-CSF supplementation on the viability of cDC2 after overnight culture. However, various studies confirmed the impact of GM-CSF presence on the viability of moDC and cDC type

1&2. For instance, (Lellahi et al., 2023) determined that GM-CSF alone and in combination with other cytokines improves the viability of cDC1 and cDC2 after 24 or 48 hours of culture. This dissimilarity to my results could be due to the different concentrations of cultured cells/well as they seeded 30,000 cDC2/ well in a 96-well plate, while I seeded around 150,000 cDC2/ well in a 96-well plate. I observed that this higher concentration of cells/well improved the viability and yield after overnight culture compared to a lower concentration of cells (50,000 cells/well) that I used at the start of my project (data not shown). Hence, a higher concentration of cells/well could improve the viability of cDC2 even without any GM-CSF supplementation.

Moreover, as GM-CSF is considered one of the pro-inflammatory cytokines, I wanted to investigate its effect on the phenotype and cytokine secretion profile of cultured cDC2. Consistent with Lellahi et al., I found that different concentrations of GM-CSF had no impact on the expression of co-stimulatory molecules, migratory molecule and tolerogenic marker of interest except a marginal difference in ILT3 expression and IL-10 with the use of 10ng/ml GM-CSF. In conclusion, many factors in the culture environment could affect the generation of optimal tol-cDC2. Therefore, selecting a suitable culture medium, as well as cytokines and serum supplementation, will impact the phenotype and therefore the function of these cells.

4.5.4 VitD3 treatment for generation of tol-cDC2 with optimal migratory capacity

VitD3 is well-known to exhibit pleiotropic immunomodulatory effects on moDC for immunotherapy for autoimmune diseases. However, to the best of my knowledge, no previous study assessed the impact of vitD3 treatment on cDC2 for generations of tolerogenic cDC for autoimmunity. Hence, comparing my findings with other groups who either used vitD3 in the context of moDC or cDC2 for cancer immunotherapy is very challenging as each cell type and generation protocol has different effects, respectively.

Nevertheless, there is some similarity with respect to the impact of VitD3 treatment on moDC and cDC2. For example, I found that vitD3 treatment induces re-expression of CD14 in cDC2 either alone or in combination with other immune regulatory agents. This finding is also supported by (Švajger and Rožman, 2019) and (L Bishop et al., 2021), who observed high expression of CD14 in moDC after treatment with vitD3 alone or in combination with IFN-γ. Whether this induction of CD14 has any regulatory function on these VitD3-treated DC is

unknown. However, the tolerogenic impact of $CD14^+$ cDC2 is also mentioned by Van Wigcheren et al, (2021), who stated in their review that $CD14^+$ cDC2 that were generated by IL-6 and PGE2 have tolerogenic features with the secretion of IL-10, expression of MERTK and PDL-1 and finally inhibition of antigen-specific T cell proliferation, which makes them an interesting candidate for generation of immunotherapy for autoimmune diseases. Interestingly, (Bakdash et al., 2016) identified a population of $CD1c^+$ $CD14^+$ cells that expanded in the microenvironment of melanoma patients with high expression of PDL-1 and suppressed antigen-specific $CD4^+$ T cell responses. Altogether, this observation could contribute to either targeting these cells in-vivo or generating tol-cDC2 with similar features in-vitro for autoimmune interventions.

Another important feature to look at with regard to the effect of VitD3 treatment of cDC2 is the expression of CCR7 as well as migratory capacity towards the chemokines CCL21 and CCL19. There are a number of studies that have previously reported the in-vivo prominent migratory ability of cDC into lymphoid tissue either constantly, at steady-state conditions, to induce tolerance (Hong et al., 2022) or in inflammatory or cancer diseases, to induce immunogenic responses. In contrast, moDC demonstrated poor migratory capacity (Liu et al., 2021). Thus, in addition to our finding in chapter three that cDC2 shows higher *CCR7* gene expression compared to other immune cells, we also found in this chapter that freshly isolated peripheral blood cDC2 expressed CCR7, alongside the immune inhibitory molecule ILT-3, which supports the idea of steady-state induction of tolerance.

In addition, Mo-TolDC generated with our gold standard protocol (VitD3 and dex) was found to maintain lower expression of CCR7 after LPS stimulation, which may not promote migration to T cell area (Amy E Anderson et al., 2008; Cooke et al., 2022). In my investigation, I found that the CCR7 expression and migratory ability of VitD3-cDC2 was only slightly reduced compared to Stim-cDC2. Noteworthy, VitD3-cDC2 also exhibited a tolerogenic phenotype with high expression of PDL-1 and ILT3 and high secretion of IL-10 with lower secretion of IL-12p70. However, it should also be noted that VitD3-cDC2 secreted high levels of IL-6 and TNF-α; this could be decreased using anti-TNF and anti-IL-6 antibodies but may lead to a huge immunosuppressive effect. The observation of elevated IL-6 and TNF-α after treatment with VitD3 was also described in the context of moDC by (Švajger and Rožman, 2019). They found

an increase in the secretion of IL1β, IL-6, IL-8 and TNF-α after treatment with VitD3 alone or in combination with IFN-γ.

Another important point to address concerning this high pro-inflammatory cytokine secretion by VitD3-cDC2 is whether these cytokines are produced by specific cDC2 subsets or not. (Heger et al., 2020b) recognized that the $CD1c^+$ $CD14^+$ subset has a trend to produce higher secretion of TNF and IL-10 compared to $CD1c^+$ $CD14^-$ cells. Thus, this could be the case for our double positive $CD1c^+CD14^+$ population that appears after treatment with VitD3. Therefore, further studies are needed to sort this minor population to understand their functional attributes.

Furthermore, VitD3 is well-known to regulate DC maturation by reducing the expression of CD86 and CD80 (Barragan et al., 2015; Švajger and Rožman, 2019). Conversely, in the context of cDC2, I found that treatment with VitD3 then stimulation with LPS increased expression of CD86 even more than the LPS stimulated alone (figure 4.11). In agreement with that, one study that treated moDC with vitD3 observed the same high expression level of CD86 as found in the control mature DC (Unger et al., 2009). Surprisingly, we also found that VitD3-cDC2 expresses high levels of CD83 (figure 4.11). Recently, several studies have reported the immune regulatory function of CD83 in the DC (Grosche et al., 2020). Therefore, further studies are needed to address the regulatory function of CD83 in cDC2.

Two studies reported that DC had a dose-dependent response to vitD3 treatment where the co-stimulatory molecules decreased after treatment with a high concentration of vitD3 (10^{-7} or 10^{-8} M). Nevertheless, this impact was lost when treated with a low dose of vitD3 (10^{-10} M) (Ferreira et al., 2013; Švajger and Rožman, 2019). Inconsistent with these findings, and in the context of cDC2, I observe no significant differences between the doses of vitD3 treatment in the expression of co-stimulatory molecules and tolerogenic markers. These different outcomes could be due to different DC origins, which vary in response to VitD3.

Together, X-VIVO15 culture medium, GM-CSF and human serum supplementation, as well as the dose of vitD3 treatment, have a broad impact not only on the morphology and phenotype of VitD3-cDC2 but also affect VitD3-cDC2 function with increased migration towered CCL21 and CCL19. Thus, the next chapter will test further functional analysis for selecting VitD3-cDC2 in the context of T cell stimulatory capacity.

4.6 Conclusion

In conclusion, cDC2 comprises heterogeneous subtypes that could be affected after culture with various factors. X-VIVO15 culture medium could be superior to GC medium in terms of facilitating the generation of tolerogenic cDC2 that maintains CCR7-dependent migratory ability. In addition, cDC2 naturally expresses the inhibitory molecule ILT3 at gene and protein levels. Thus, this could contribute to the tolerogenic function of cDC2. Treatment of cDC2 with vitD3 was selected to perform further functional analysis based on the strong tolerogenic phenotype with high production of IL-10 and remarkable migratory ability. Together all these findings make VitD3-cDC2 a promising approach for tol-cDC2 generation.

Chapter 5: Functional characterization of VitD3-treated cDC2 for tolerance induction

5.1 Introduction

cDC2 are distributed across various lymphoid organs and can also be found circulating in the peripheral blood. (Segura, 2022). The diverse locations of cDC2 play a crucial role in carrying out a wide range of functions, including the activation of naïve $CD4^+$ T cells into various subtypes of T cells, either promoting stimulation or regulation. Polarizations of T cells are dependent on the cDC2 phenotype (i.e. expression of either stimulatory or regulatory marker), the cytokine microenvironment and other soluble mediators in the microenvironment such as PGE2 (Kalinski, 2012; Sreeramkumar et al., 2012). A summary of the different T cell subsets that can be polarized in response to cDC2 stimulation is illustrated in figure 5.1. Interestingly, different types of tissue-resident or circulating cDC2 have been shown to induce different types of Tregs and promote suppression of effector T cells under various microenvironmental conditions (Ritprajak et al., 2019a). In addition, number of studies found accumulation of cDC2 with immunosuppressive function in the tumor microenvironment of advanced cancer patients, leading to poor prognosis (Bakdash et al., 2016; Saito et al., 2022). Thus, together these findings make cDC2 a promising target for the generation of therapeutic tolerogenic DC.

Furthermore, as determined in the previous chapters, cDC2 upregulates the expression of important immune inhibitory molecules and tolerogenic markers at gene and protein levels. Specifically, VitD3-treated cDC2 showed a tolerogenic phenotype with high PDL-1, ILT3 and IL-10 expression. In addition, VitD3-cDC2 has also demonstrated proper CCR7 expression, accompanied by a remarkable migratory ability towards CCL21 and CCL19 chemokines. Therefore, an investigation into the capacity of VitD3-cDC2 to activate and polarize naïve $CD4^+$ T cells is necessary to confirm their promise as an alternative approach for the generation of therapeutic tolDC.

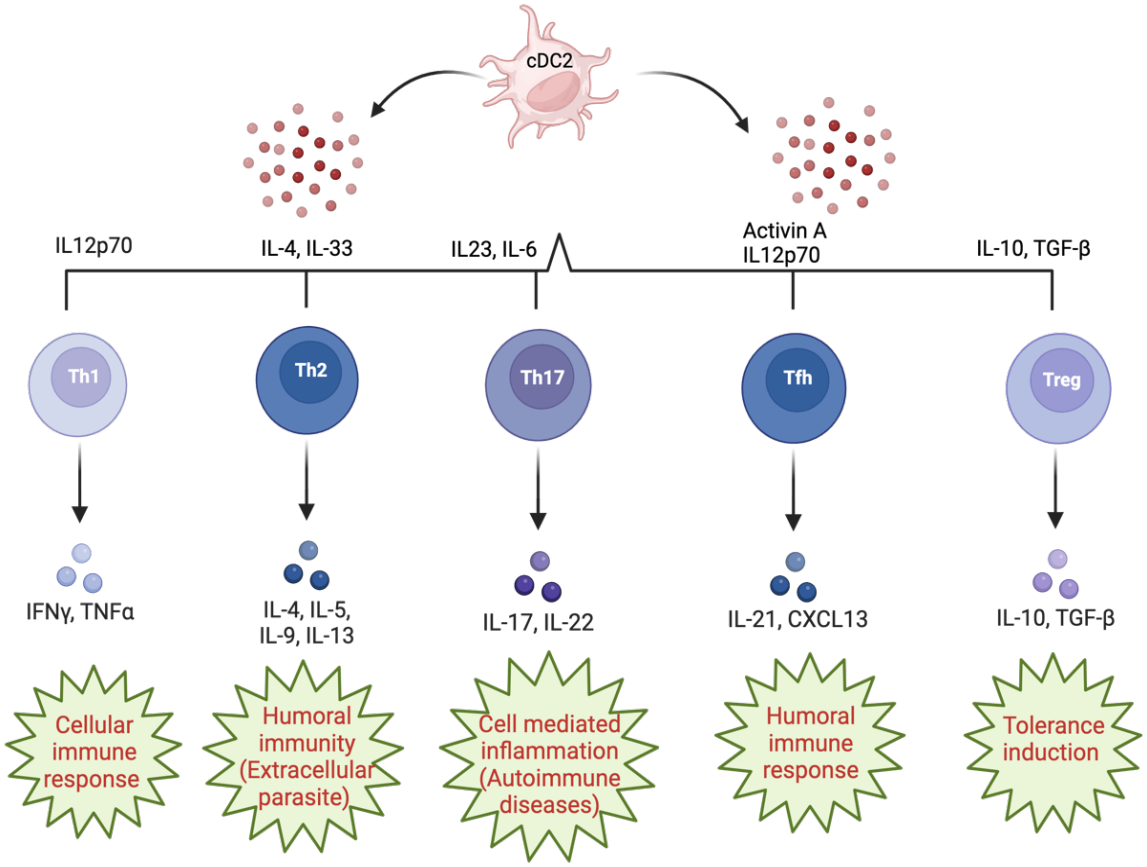


Figure 5.1: Summary of T cell subsets after stimulation with cDC2. Each subset is polarized according to the specific cytokines and other molecules in the microenvironment as well as the phenotype of cDC2 stimulator. Therefore, each T cell subset will induce a distinct immune response. Th1 (T helper 1) produces IFN-γ and TNF-α inducing various cellular immune responses. For example, it activates macrophages and cytotoxic T cells (CTL) to exert its function and enhance B cell function as well. On the other hand, Th2 (T helper 2) is responsible for orchestrating immune response against extracellular parasites and allergens by secretion of IL-4, IL-5 and IL-13. These cytokines help to activate other immune cells, particularly B cells to produce antibodies, which is important in defending against extracellular parasite and allergic response. cDC2 can also promote the differentiation of $CD4^+$ T cells into Th17 (T helper 17), which produces IL-17 that is involved in response against extracellular bacteria and fungi and is also involved in various autoimmune diseases. Finally, cDC2 can also induce the development of regulatory T cells (Tregs) that are involved in immune tolerance

and regulation. The information in this figure was collected from these references (Durand et al., 2019; Kumar et al., 2019; Leal Rojas et al., 2017). Created with BioRender.com.

5.2 Chapter aims

The aim of this chapter is to evaluate the basic T cell stimulatory and polarizing activity of VitD3-cDC2. Specific objectives are:

1. To test the ability of VitD3-cDC2 to stimulate naïve $CD4^+$ T cells.
2. To characterize the T cell polarization effect of VitD3-cDC2.

5.3 Experimental approach

VitD3-cDC2 were co-cultured with allogenic CTFR-labelled naïve $CD4^+$ T cells, also referred to as a MLR assay. Proliferation of T cells was assessed between days 4-6, as well as the expression of activation markers and secretion of cytokines to obtain the full picture of VitD3-cDC2 stimulatory capacity. Next, in order to investigate the VitD3-cDC2 polarization effect on $CD4^+$ T cells, a re-stimulation assay was performed after resting the primed T cells for 3 to 4 days with stimulation by either Stim-cDC2 or CD3/CD28 daynabeads. Plus, a small amount of IL-2 (10 U/ml) is added to support their viability. After that, the expression of activation markers, FOXP3 and the cytokine secretion profiles were determined. Detailed information on both MLR and re-stimulation assay can be found in Chapter 2 methodology. See table 5.1 below for the T cell nomenclature used in all figures of this chapter.

T cells condition	Abbreviation	Treatment protocol
T cell only	T cell	Negative control: T cells that were cultured without any stimulation or any labelling
T cell + CTFR	T labelled	Positive control: T cells that were labelled with cell trace far red (CTFR) without any stimulation
T cell+ Un-cDC2	$T_{un\text{-}cDC2}$	CTFR-labelled T cell that were co-cultured with Un-cDC2.
T cell+ Stim-cDC2	$T_{stim\text{-}cDC2}$	CTFR-labelled T cell that were co-cultured with Stim-cDC2.
T cell+ VitD3-cDC2	$T_{vitD3\text{-}cDC2}$	CTFR-labelled T cell that were co-cultured with VitD3-cDC2.

Table 5.1: Nomenclature of T cells that were co-cultured with different cDC2 types.

5.4 Results

5.4.1 VitD3-cDC2 exhibits low T cell-stimulatory potential

Because MLRs with cDC2 were not previously tested in our laboratory, and also due to variable 'strengths' of allogeneic T cell responses (dependent on the HLA-mismatch), a cDC2/T cell titration was performed. In addition, the co-culture time duration was also assessed to optimize the appropriate co-culture system to perform all further analyses.

5.4.1.1 Optimization and titration of cDC2/ T cell co-cultures

To identify the appropriate co-culture period for cDC2 with naïve $CD4^+$ T cells, I initially started with our usual co-culture duration for moDC/T cell co-cultures, which is six days. Three cDC2/T cell ratios were assessed: 1:10, 1:20 and 1:40. As shown in figure 5.2, the 1:10 cDC2/T cell ratio demonstrated the highest aggregation and clustering of T cells. However, the 1:40 ratio also showed a prominent morphological appearance of T cell activation, especially in T cells co-cultured with Stim-cDC2. From now on, the 1:10 cDC2/ T cell ratio was selected for further analysis as this is the most commonly used ratio in the literature and in our laboratory.

However, as demonstrated in the representative histogram of 1:10 cDC2/ T cell ratio (figure 5.3), most T cells had proliferated after six days of co-culture, even the T cells stimulated with VitD3-cDC2. These findings were also identified in other cDC2/ T cell ratios after six days of co-culture (data not shown), suggesting that the duration of the co-culture was too long to observe any differences in the stimulatory activity of the different cDC2 types. Therefore, it was decided to reduce the co-culture time to 4 days for further experiments.

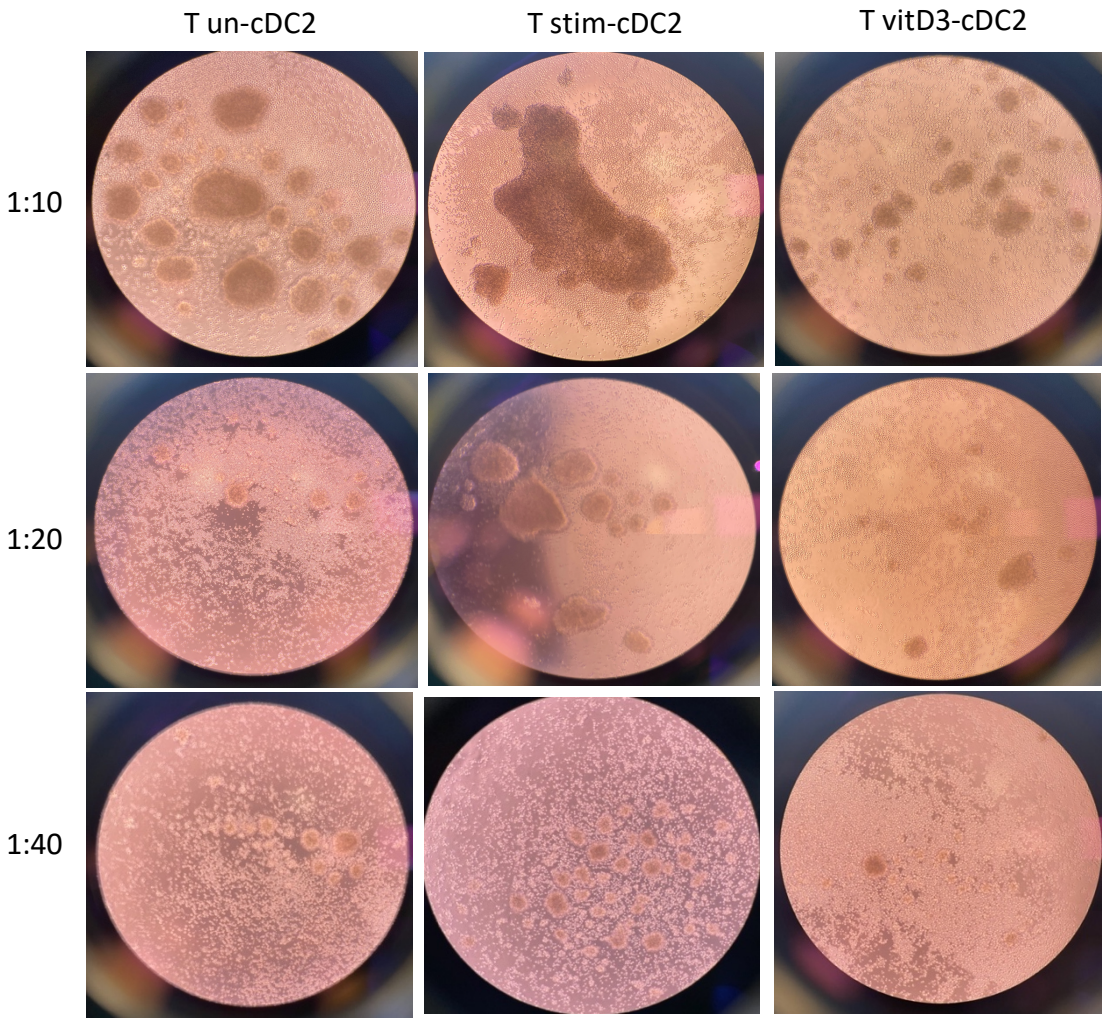


Figure 5.2: Microscopic appearance of cDC2/ T cell co-cultured at different ratios for six days. The number of cDC2 is titrated while the number of naïve $CD4^+$ T cells remains the same (1x105 per well in a 96-well plate). T cells were labelled with CTV prior to co-culture with different cDC2 types. Magnification is 20X.

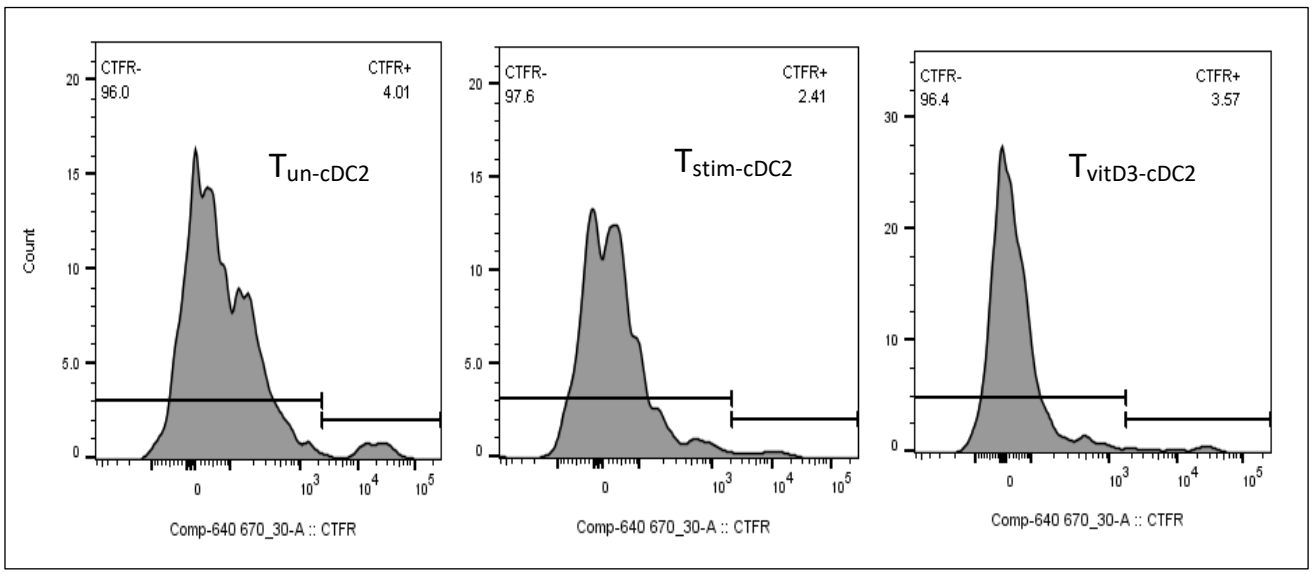


Figure 5.3: A representative histogram of 1:10 cDC2 / T cell ratio demonstrates the percentage of proliferated T cells after six days of co-culture with different cDC2 types. CTFR- is the percentage of T cells with negative CTFR dye stain, indicating that they have proliferated.

5.4.1.2 T cell proliferation capacity

As shown in figure 5.4, T cells stimulated with VitD3-cDC2 demonstrated lower activation and aggregation of the cells under the microscope than T cells stimulated with other cDC2 types. In addition, T cells stimulated with VitD3-cDC2 showed lower percentages of proliferated cells in all cDC2/T cell ratios compared to the other T cells, although this did not reach statistical significance (figure 5.5A&B).

A proliferation index was calculated to look closer into the T cell stimulatory capacity with regard to the number of cell divisions. While a proliferation percentage shows the proportion of cells that have proliferated in response to a certain stimulus, a proliferation index also specifies the number of divisions that have occurred. Additionally, a proliferation index provides a more comprehensive understanding of how fast the responding cells are growing.
As shown in figure 5.5D, $T_{VitD3\text{-}cDC2}$ exhibited a lower proliferation index than the other T cell cultures, although this did not reach statistical significance. This lower trend is consistent with the lower proliferation percentage (figure 5B) and the lower number of peaks induced in these T cell cultures by VitD3-cDC2 (data not shown). Thus, it appears that $T_{VitD3\text{-}cDC2}$ has a lower stimulatory capacity towards naïve $CD4^+$ T cells, although more experimental repeats will be necessary to confirm this.

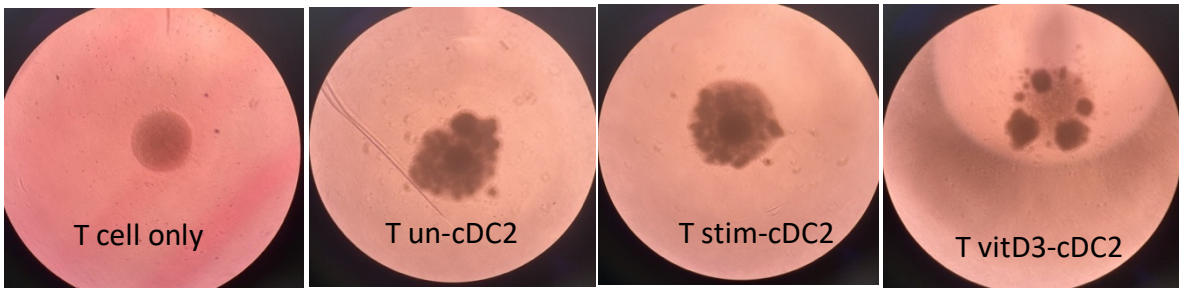


Figure 5.4: Microscopic appearance of cDC2/ T cell co-culture in a 1:10 ratio after four days of incubation. Magnification is 40X.

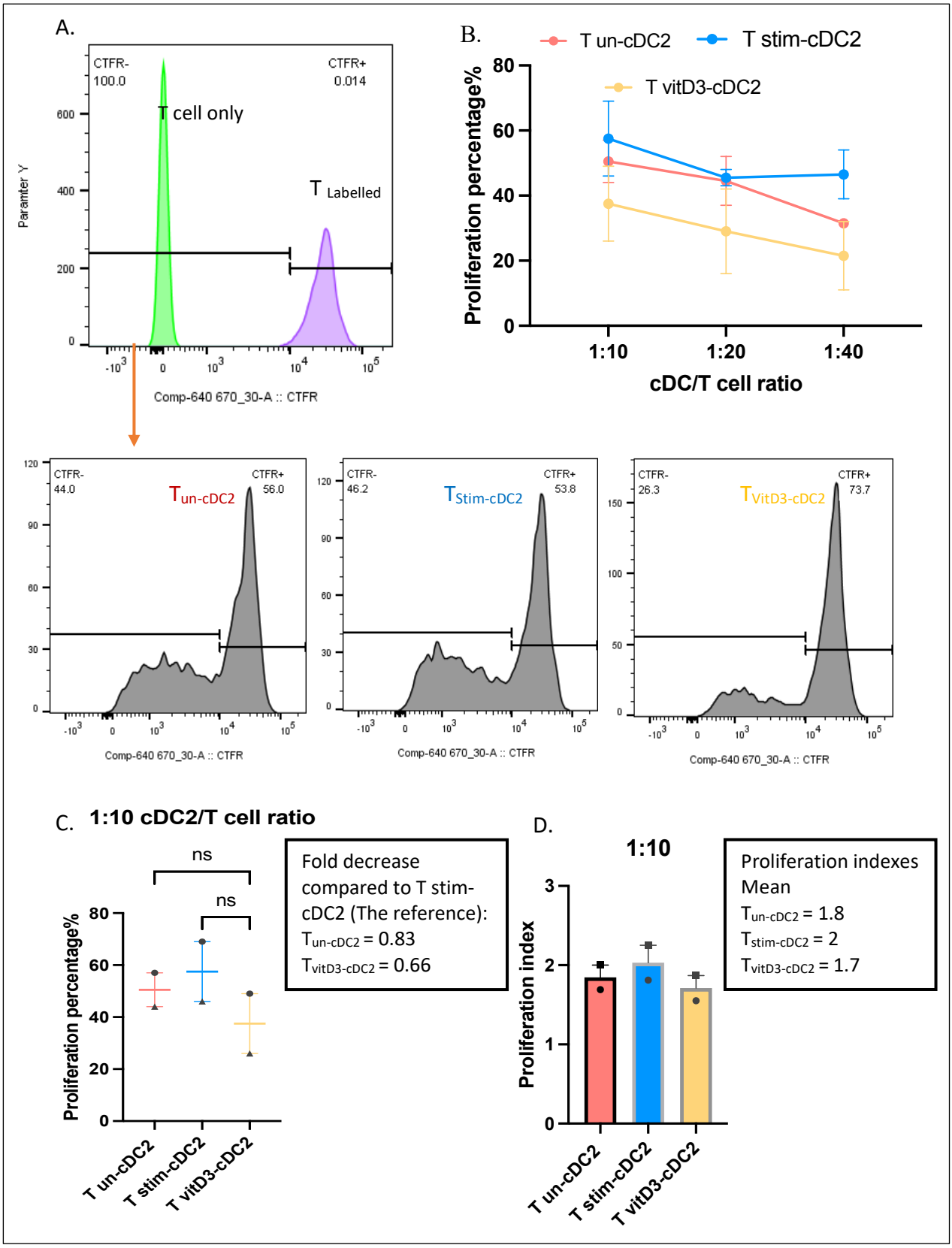


Figure 5.5: T cell stimulatory capacity after stimulation with VitD3-cDC2 for four days of co-culture. A) A representative histogram of negative control (T cell only without CTFR labelling) and positive control (T labeled). B) Proliferation percentage of T cells reflects the

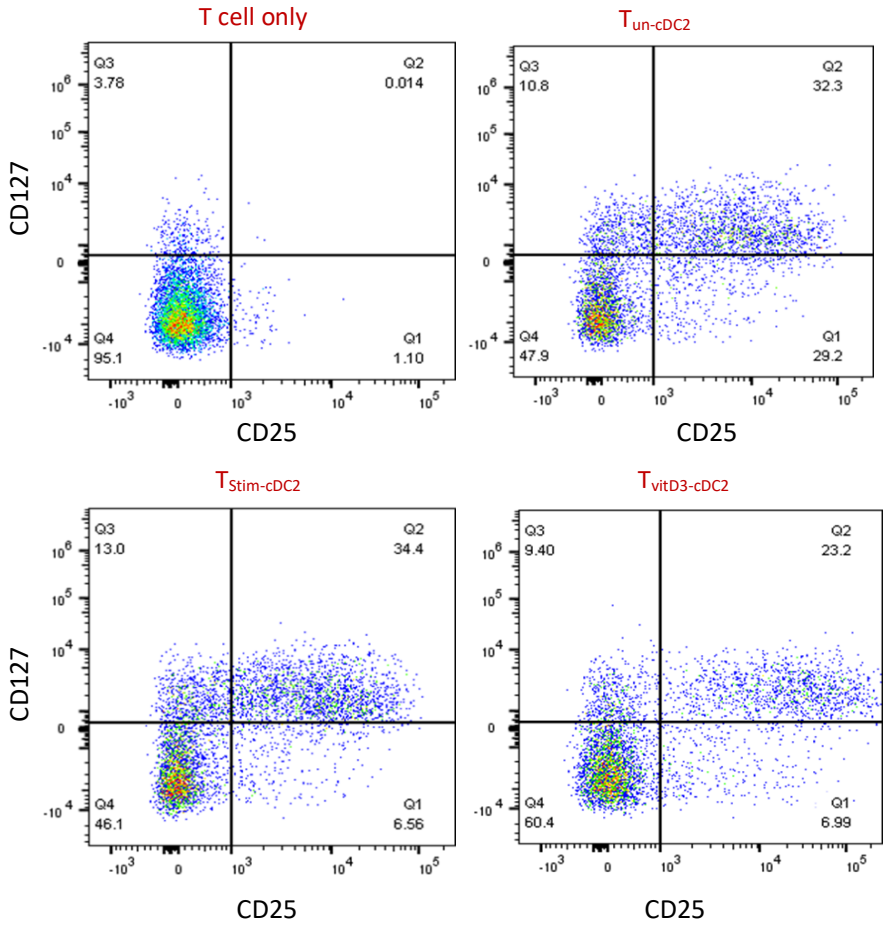
proportion of divided cells in all three different cDC2/T cell ratios. C) The proliferation percentage of T cells in 1:10 cDC2/T cell ratio and the fold decrease value compared to the reference value (Tstim-cDC2) . D) Proliferation index of different T cells. Data represent the mean of two independent experiments N=2. Fold Decrease = sample value / reference value. Value less than 1 indicate a decrease in proliferation percentage compared to the reference condition.

5.4.1.3 T cell activation markers

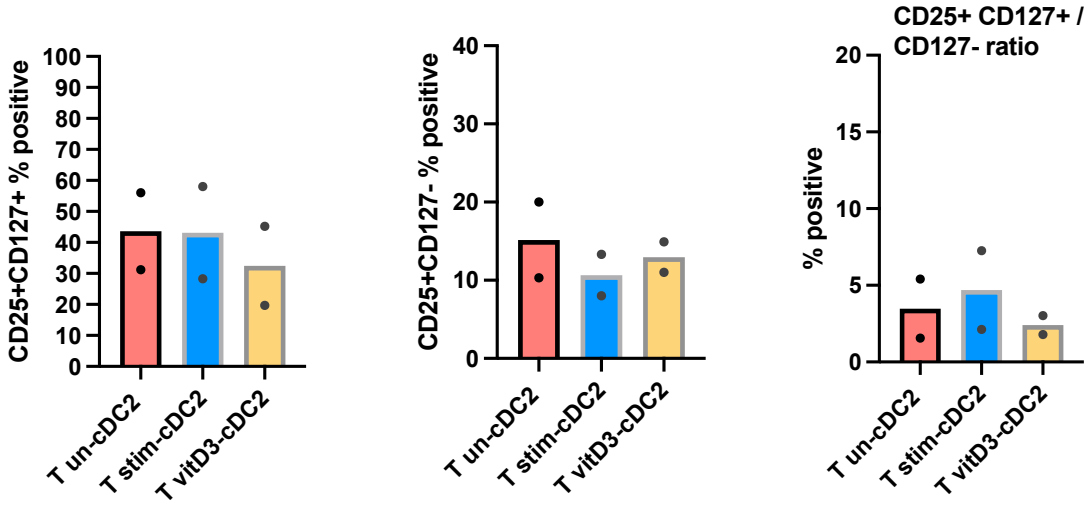
To identify the phenotype of T cells that were stimulated with different cDC2 types, we selected the 1:10 ratio to assess the expression of activation markers of interest: CD25 and CD127 to determine the T cell effector subset ($CD25^{+}CD127^{high}$ T cells) (Liu et al., 2006), regulatory T cells subset ($CD25^{+}CD127^{low}$ T cells) and IL-2 producing naïve and memory T cells ($CD25^{-/low}$ $CD127^{+}$) (Dunham et al., 2008; Yu et al., 2012). In addition, CD45RO was also used to identify the memory T cell population ($CD45RO^{+}$) (Machura et al., 2008).

As shown in figure 5.6Aand B, the % positive of $CD25^{+}CD127^{+}$ effector T cells was less than around 16% (mean) in $T_{vitD3\text{-}cDC2}$ compared to both $T_{un\text{-}cDC2}$ and $T_{Stim\text{-}cDC2}$ with approximately 22% (mean) and 18% (mean), respectively. Additionally, the % positive of $CD25^{+}CD127^{-}$ Treg was higher in $T_{un\text{-}cDC2}$ and $T_{vitD3\text{-}cDC2}$ than in $T_{Stim\text{-}cDC2}$ (figure 5.6B). Notably, $CD25^{+}$ $CD127^{+}$ to $CD25^{+}CD127^{-}$ ratio was slightly lower in $T_{vitD3\text{-}cDC2}$ compared to both $T_{un\text{-}cDC2}$ and $T_{Stim\text{-}cDC2}$ (figure 5.6B).In addition, as shown in the representative histogram (figure 5.6C), there were no obvious differences in the expression of all CD25, CD127 and CD45RO markers between different T cell types except for a slight spread in the CD25 marker in $T_{vitD3\text{-}cDC2}$. However, when we look closer at the % positive of these markers as shown in figure 5.6D, it appears that $T_{vitD3\text{-}cDC2}$ demonstrates lower CD25 and CD45RO % positive cells compared to both $T_{un\text{-}cDC2}$ and $T_{Stim\text{-}cDC2.}$ However, we observed a slight increase in the MFI of this % positive CD25 population in $T_{vitD3\text{-}cDC2}$ compared to both $T_{un\text{-}cDC2}$ and $T_{Stim\text{-}cDC2}$, but this does not reach a significant difference (figure 5.6E). No obvious difference between all T cell types was found in the MFI of positive CD127 and CD45RO populations (figure 5.6E). These results could be due to the short duration of the primary MLR, where all these activation markers are expected to be high at that activation stage. A re-stimulation assay could be widely beneficial to confirm the regulatory capacity of VitD3-cDC2 on T cells.

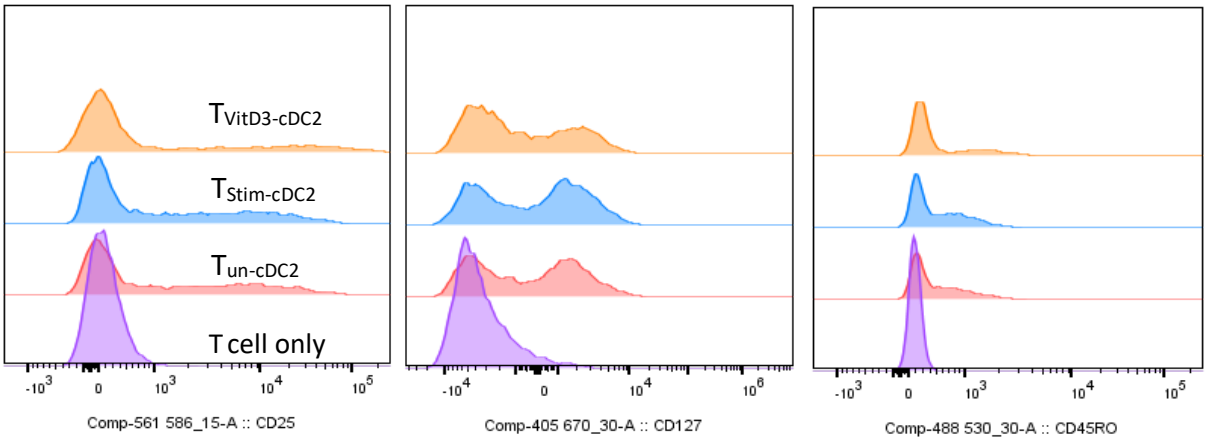
A.


T cell only
T un-cDC2
T Stim-cDC2
T vitD3-cDC2
CD127
CD25
Q3 3.78
Q2 0.014
Q4 95.1
Q1 1.10
Q3 10.8
Q2 32.3
Q4 47.9
Q1 29.2
Q3 13.0
Q2 34.4
Q4 46.1
Q1 6.56
Q3 9.40
Q2 23.2
Q4 60.4
Q1 6.99

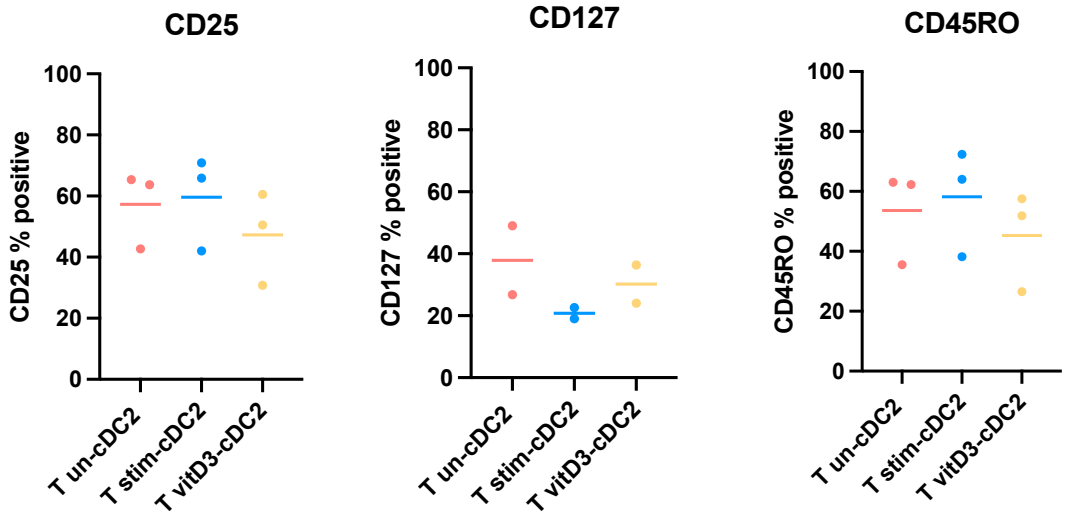
B.


CD25+CD127+ % positive
CD25+CD127- % positive
CD25+ CD127+ / CD127- ratio
% positive
T un-cDC2
T stim-cDC2
T vitD3-cDC2

C.


T VitD3-cDC2
T Stim-cDC2
T un-cDC2
T cell only
Comp-561 586_15-A :: CD25
Comp-405 670_30-A :: CD127
Comp-488 530_30-A :: CD45RO

D.



E.

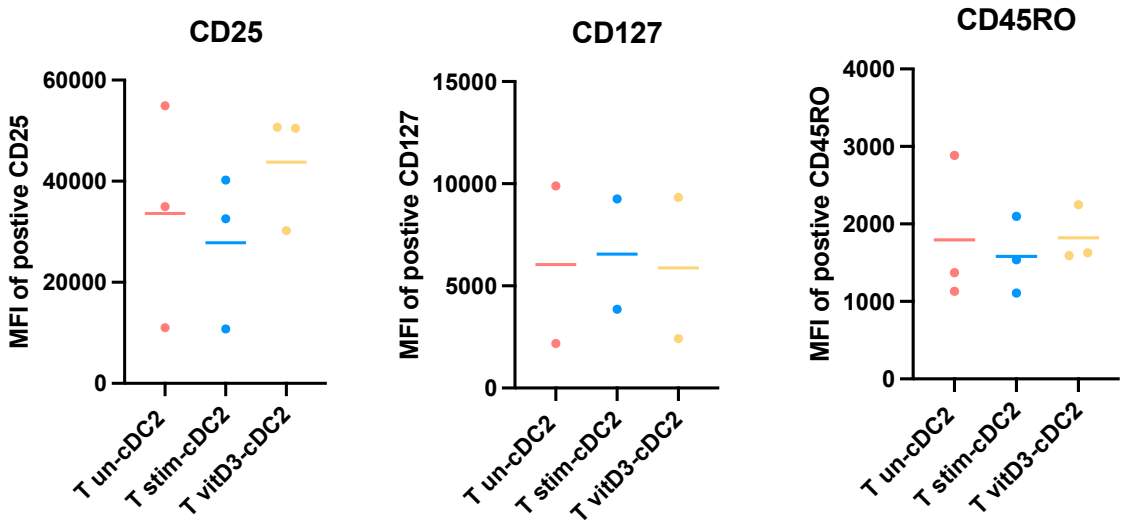


Figure 5.6: Expression of activation markers on T cells after stimulation with different cDC2 types after 4 days of co-culture. A) Representative flow plot demonstrates the expression of CD25 and CD127 molecules in CD4+ T cell only, $T_{un\text{-}cDC2}$, $T_{Stim\text{-}cDC2}$ and $T_{VitD3\text{-}cDC2}$. B) Bar graphs represent the % positive of the CD25+CD127+ population in $T_{un\text{-}cDC2}$, $T_{Stim\text{-}cDC2}$ and $T_{VitD3\text{-}cDC2}$. C) a representative histogram of CD25, CD127 and CD45RO expression by T cells primed by different cDC2 types. D) Data on the graph represent the % positive of CD25, CD127 and CD45RO markers in $T_{un\text{-}cDC2}$, $T_{Stim\text{-}cDC2}$ and $T_{VitD3\text{-}cDC2}$. E) Data on the graph demonstrate the MFI (mean fluorescent intensity) of cells that positively expressed CD25, CD127 and CD45RO population in $T_{un\text{-}cDC2}$, $T_{Stim\text{-}cDC2}$ and $T_{VitD3\text{-}cDC2}$. Data in the graphs

represents the mean of three independent experiments N=3 except CD127 N=2 and the error bar represent the SD.

5.4.1.4 T cell cytokine profile

To determine a fuller picture of the VitD3-cDC2 stimulatory capacity, the cytokines secreted during the cDC2/T cell co-cultures were assessed after 4 days. As shown in figure 5.7A and 5.7B, $T_{VitD3\text{-}cDC2}$ have significantly lower IFN-g and TNF-a secretion compared to other cDC2/T cell co-cultures. However, the secretion of IL-10 was generally low with no significant difference between T cells that were stimulated with different cDC2 types (figure 5.7C), which could be due to consumption or breakdown of this cytokine in the 4-day culture. In addition, to assess the balance in the secretion of pro-inflammatory and anti-inflammatory cytokines, IFN-g/ IL-10 ratio were also measured. $T_{VitD3\text{-}cDC2}$ demonstrated lower IFN-g/IL-10 ratio compared to other T cell types, although this doesn't reach the significance (figure 5.7D). This cytokine profile suggests that VitD3-cDC2 -activated T cells to produce low levels of IFN-g.

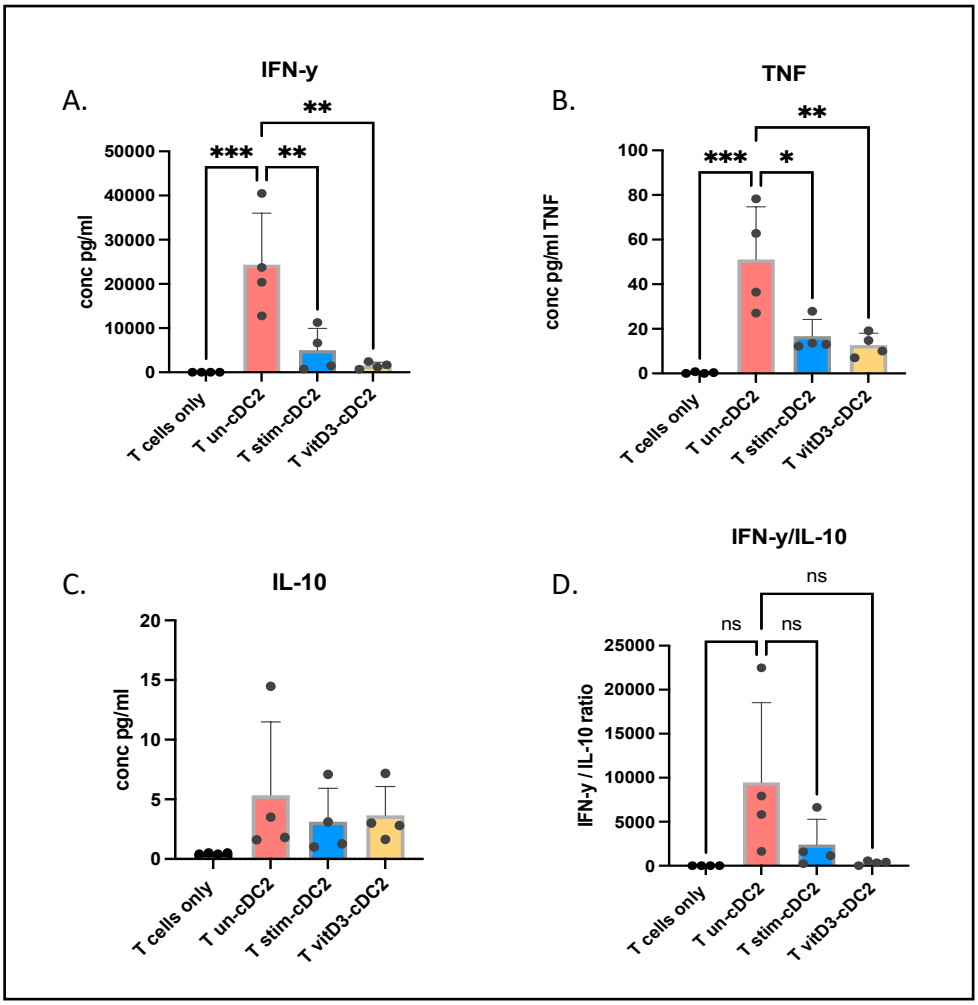


Figure 5.7: T cell cytokine profile after 4 days of co-culture with different cDC2 types. Red is T cell that stimulated with Un-cDC2, blue is T cell that stimulated with Stim-cDC2 and yellow is T cell that stimulated with-VitD3-cDC2. Significance is dependent on one-way ANOVA test of four independent experiments N=4. Error bars demonstrate the SEM. * = *p<0.05 ** = p<0.01 *** = p<0.0001.*

5.4.2 VitD3-cDC2 induce a regulatory T cell phenotype

To address the regulatory capacity of VitD3-cDC2, previously primed T cells were rested for 3 to 4 days then stimulated with either Stim-cDC2 (from the original donor of cDC2) or anti-CD3/CD28 beads as described in chapter 2. As demonstrated in figure 5.8A, $T_{VitD3\text{-}cDC2}$ have slightly higher FOXP3 expression after re-stimulation with Stim-cDC2 compared to $T_{Stim\text{-}cDC2}$ and $T_{Un\text{-}cDC2}$. In addition, this increase in FOXP3 expression was observed only in $CD25^+$ T cells, which was around 30% (mean) $CD25^+$ $FOXP3^+$ T cell in $T_{vitD3\text{-}cDC2}$ after re-stimulation (figure 5.8B&D). Furthermore, as shown in figure 5.8C, we observed slightly higher MFI in the FOXP3 marker in $CD25^+/FOXP3^+$ cells in $T_{VitD3\text{-}cDC2}$ compared to other T cell types. This could explain the regulatory function of VitD3-cDC2 through induction of $CD25^+$ $FOXP3^+$ Tregs. However, this observation was made in only two independent experiments. Hence, further experiments and analysis is necessary to confirm these promising results.

In addition, after re-stimulation with CD3/CD28 beads, $T_{VitD3\text{-}cDC2}$ showed minor spread and increase in CD137 expression compared to $T_{stim\text{-}cDC2}$ and $T_{Un\text{-}cDC2}$ (figure 5.9A). The percentage positive of $CD137^+FOXP3^+$ Treg were slightly higher in $T_{VitD3\text{-}cDC2}$ (the mean is 20%) compared to $T_{stim\text{-}cDC2}$ (16%) and $T_{Un\text{-}cDC2}$ (13%) (figure 5.9B &D). However, The MFI of CD137 in $CD137^+/FOXP3^+$ cells were higher in $T_{stim\text{-}cDC2}$ compared to both $T_{VitD3\text{-}cDC2}$ and $T_{Un\text{-}cDC2}$ (figure 5.9C). Furthermore, as shown in figure 5.9D, the percentage of $CD137^+$ $FOXP3^-$ cells in $T_{VitD3\text{-}cDC2}$ were also slightly higher compared to other T cell types. Hence, assessing the balance between regulatory and stimulatory capacity of these cells is essential to determine its functional capacity.

Interestingly, IL-10 secretion by $T_{VitD3\text{-}cDC2}$ increased after re-stimulation with Stim-cDC2 while IFN-g secretion decreased, compared to $T_{Stim\text{-}cDC2}$ and $T_{Un\text{-}cDC2}$ (figure 5.11A). In addition, the ratio of IFN-γ/IL-10 cytokines secretion was significantly lower in $T_{ViD3\text{-}cDC2}$ compared to $T_{un\text{-}cDC2}$ (figure 5.11B).

To this end, all these observations could support the regulatory capacity of VitD3-cDC2.Thus, further research is necessary to understand and identify the mechanism of naïve $CD4^+$ T cell regulation by VitD3-cDC2.

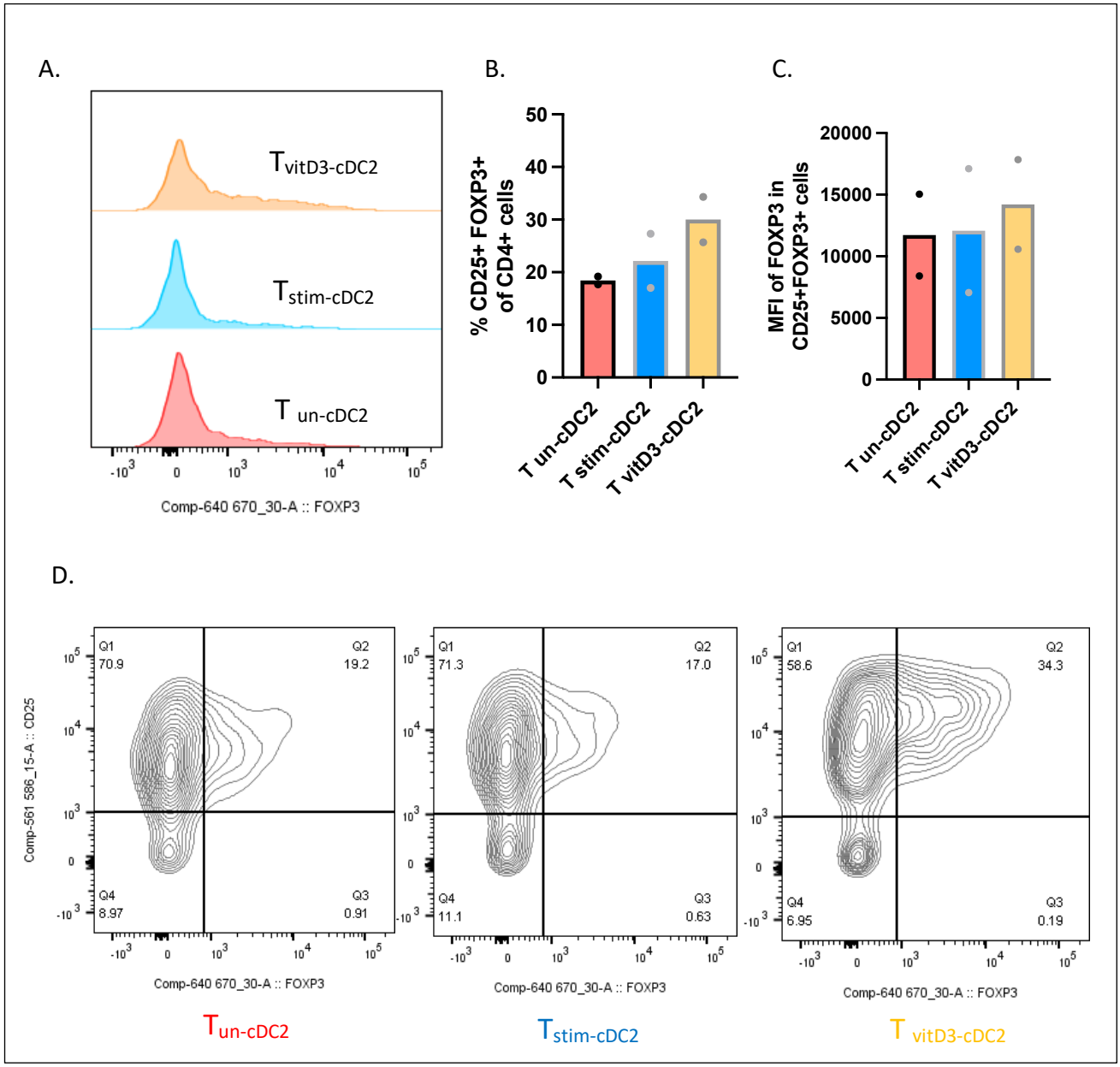


Figure 5.8: Expression of FOXP3 in T cells primed with VitD3-cDC2 after re-stimulation with either Stim-cDC2 or anti-CD3/CD28 Dynabeads for 3 days. A) Representative histogram of FOXP3 expression in T cell after re-stimulation. B) Bar graph demonstrated the % positive of $CD25^+$ $FOXP3^+$ in different T cell types after re-stimulation. C) Bar graph demonstrate the MFI of FOXP3 in $CD25^+$/$FOXP3^+$ cells in different T cell types of two

independent experiment N=2. D) Representative flow plot displayed $CD25^+$ $FOXP3^+$ expression in different primed T cell after re-stimulation with Stim-cDC2.

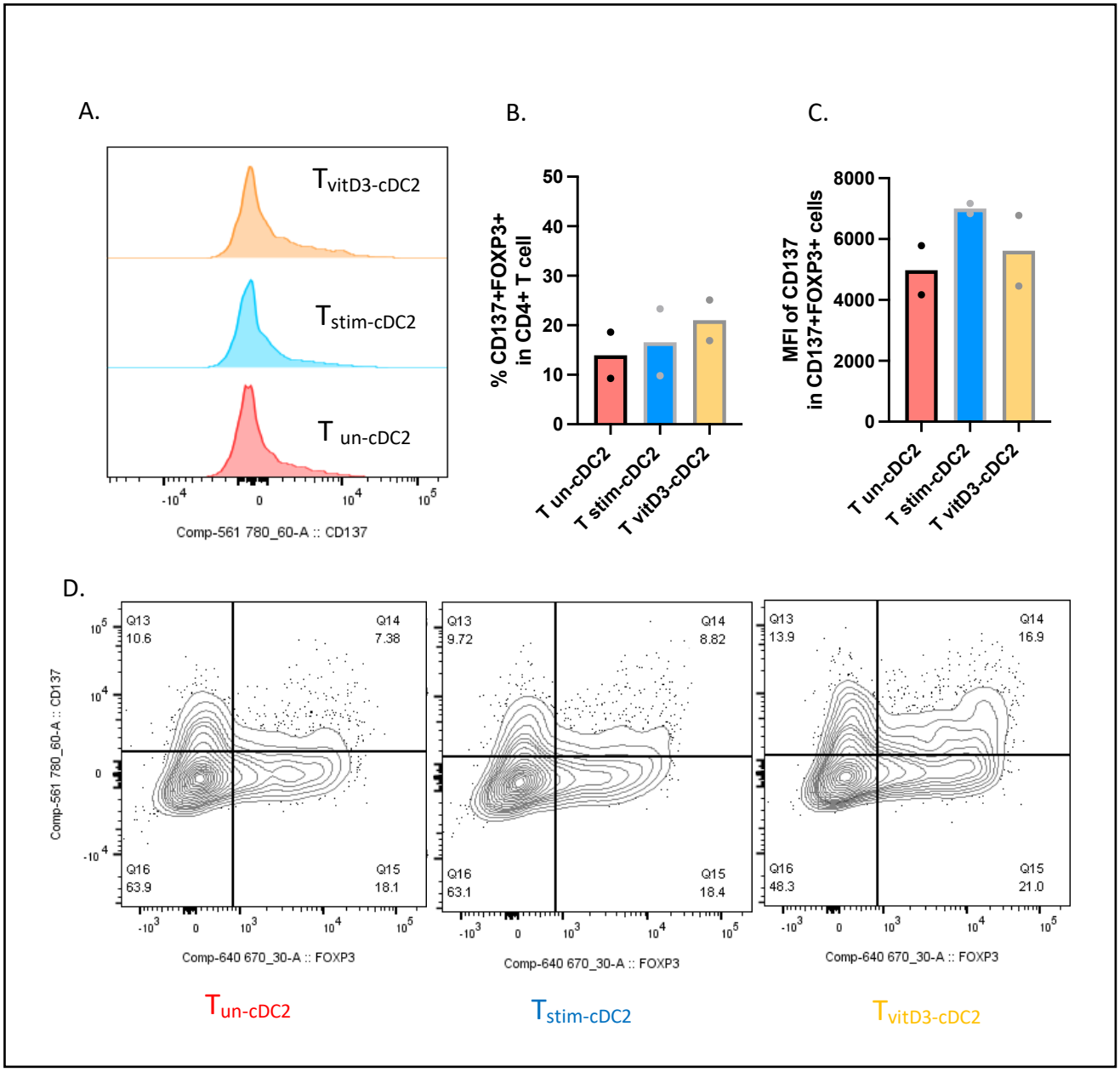
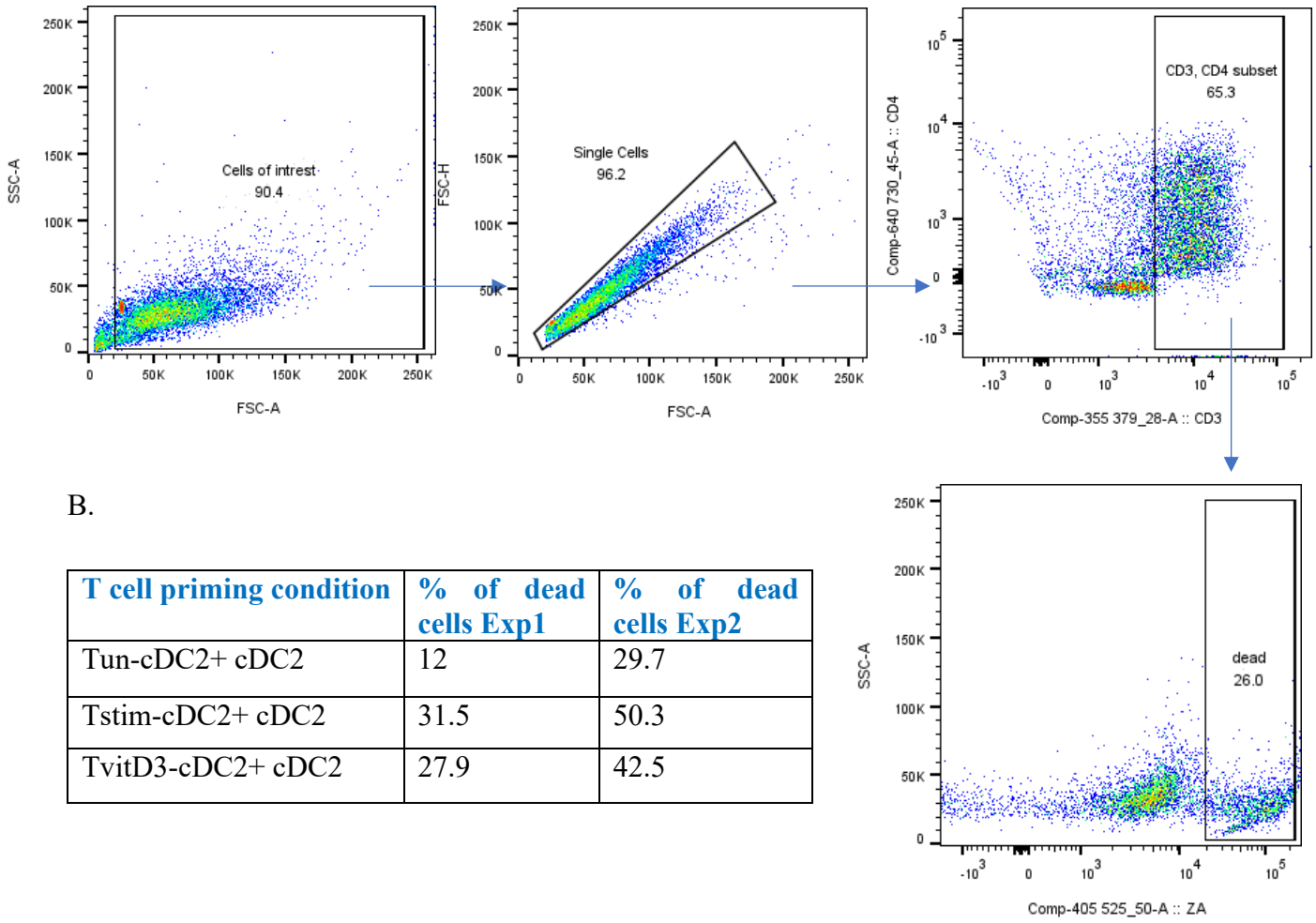


Figure 5.9: Expression of CD137 marker in different primed T cell after re-stimulation with anti-CD3/CD28 beads for 3 days. A) representative histograms demonstrate the expression of CD137 marker in different $CD4^+$ T cell types. B) Bar graphs represent the % positive CD137/FOXP3 in different T cell types. C) Bar graphs represent the MFI of CD137 marker in $CD137^+/FOXP3^+$ cells in different T cell types. Data represents the mean of two independent experiment and error bar show SD (N=2). D) Representative flow plot showing the CD137/ FOXP3 expression in different T cell types.

Moreover, in order to assess if T cell apoptosis or deletion could be a mechanism of tolerance induction by VitD3-cDC2, percentages of dead cells in the $CD3^{+}CD4^{+}$ T cell population after re-stimulation assay were calculated. As shown in figure 5.10, $T_{un\text{-}cDC2}$ demonstrates the lowest percentage of dead cells, while $T_{stim\text{-}cDC2}$ have higher percentage of dead cells that most likely reflects activation-induced cell death. Hence determining the percentage of dead cells does not give much of insight into the possibility of the induction of T cell apoptosis. Ideally, to assess T cell apoptosis, active caspase 3 and/or annexin V measurements should be performed.

A.



B.

T cell priming condition	% of dead cells Exp1	% of dead cells Exp2
Tun-cDC2+ cDC2	12	29.7
Tstim-cDC2+ cDC2	31.5	50.3
TvitD3-cDC2+ cDC2	27.9	42.5

Figure 5.10: Percentage of dead cells after re-stimulation assay in the CD3+CD4+ population. A) representative flow plot demonstrates an example of gating strategy to assess the T cell apoptosis or deletion. B) Table showing the percentage of dead cells after re-stimulation in two independent experiments. N=2

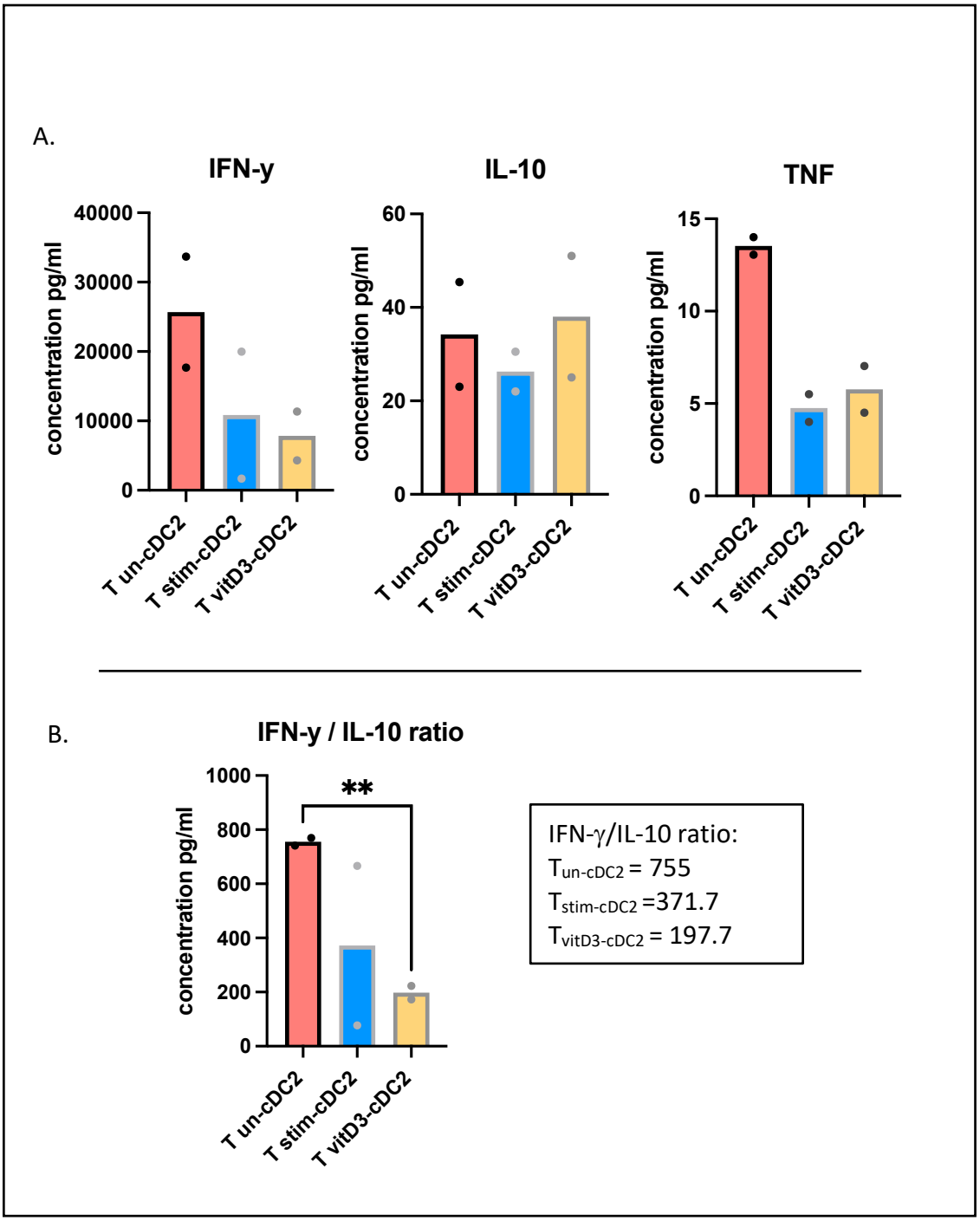


Figure 5.11: Cytokine secretion profile of different T cell types after re-stimulation. A) Representative bar graphs of two independent experiment (N=2) demonstrate the cytokines secretion profile of primed T cells after re-stimulation with Stim-cDC2 for 3 days. B) Bar graphs demonstrate the mean+SEM of IFN-g/IL-10 ratio in different T cell types. Significant difference is a result of unpaired t-test between each T cell type. * = $p<0.05$ ** = $p<0.01$

5.4.3 Summary of the phenotypical and functional characteristic features of VitD3-cDC2

To summarize our finding in the context of both phenotypical and functional characterization of VitD3-cDC2, a radar plot demonstrating the fold-change between VitD3-cDC2 and the control Stim-cDC2 was performed. As shown in figure 5.12, PDL-1 expression on the surface of VitD3-cDC2 and IL-10 cytokine secretion by VitD3-cDC2 displays the highest difference compared to Stim-cDC2 with a 3.5- and 5-fold change, respectively. It is also worth noting that, although the expression of CCR7 and migration efficacy of VitD-cDC2 is less than that of Stim-cDC2, VitD3-cDC2 still showed an appropriate migration efficacy towards CCL21 and CCL19. In addition, despite that VitD3-cDC2 were found to express high levels of the co-stimulatory molecule CD86, they exhibited an overall lower $CD4^+$ T cell stimulatory capacity compared to Stim-cDC2. This is according to the combination of lower proliferation percentage and strong downregulation of IFN-γ secretion in both primary allo-MLR and after re-stimulation with Stim-cDC2. Finally, we observed higher FOXP3 expression in $T_{VitD3\text{-}cDC2}$ after re-stimulation compared to Stim-cDC2. All together these findings suggest that these cells could be promising as an alternative approach for moDC in the generation of a tolerogenic cDC2-based immunotherapy.

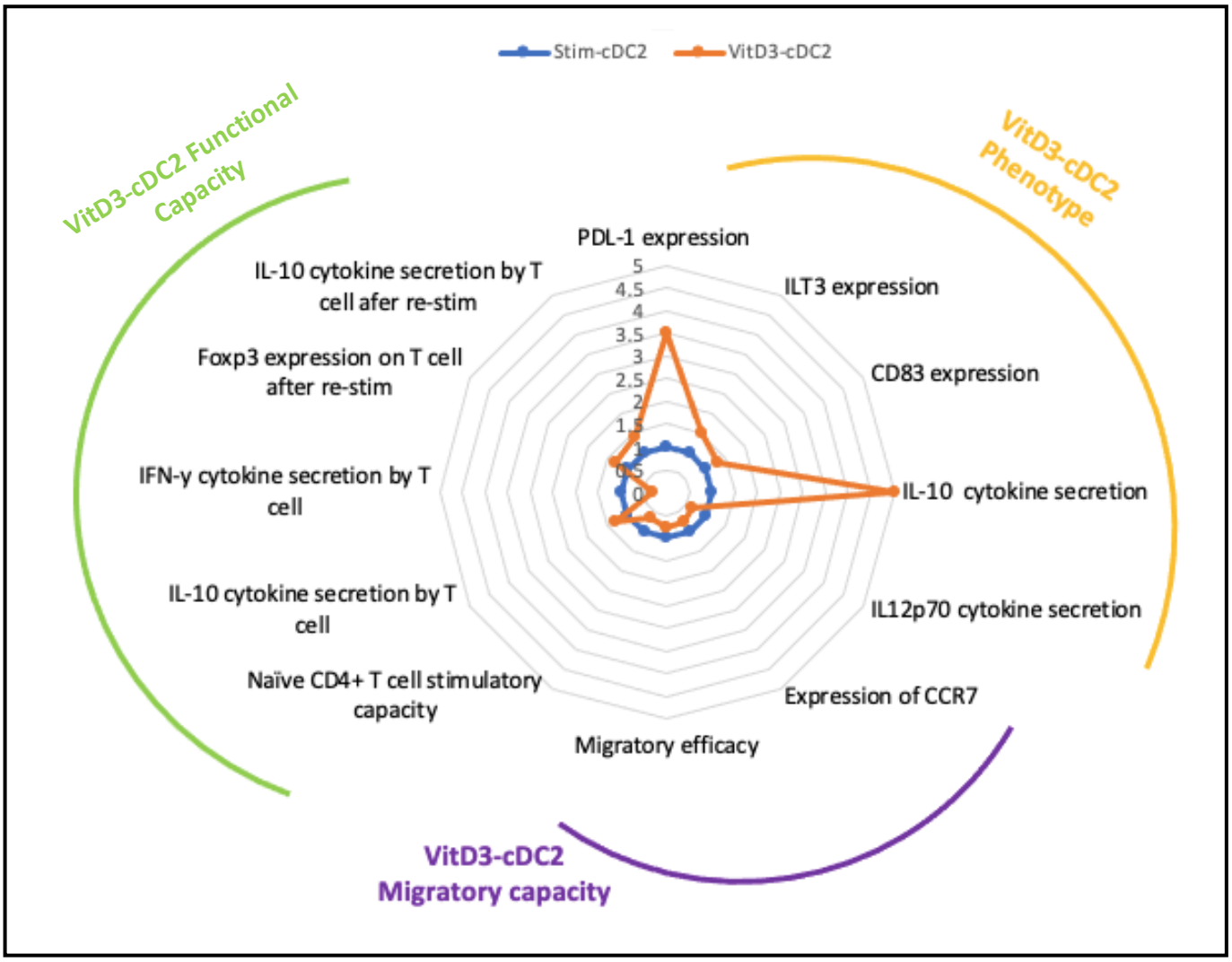


Figure 5.12: Radar plot demonstrates fold change in VitD3-cDC2 with the control Stim-cDC2 in selected features of interest. Yellow arch demonstrates phenotypical features and cytokine profile of VitD3-cDC2. Purple arch illustrates the migratory efficacy and CCR7 expression in VitD3-cDC2. Green arch shows the stimulatory capacity and cytokines profile of VitD3-cDC2 on naïve CD4+ T cells.

5.5 Discussion

The aim of this chapter was to determine whether VitD3-cDC2 exerted regulatory effects on naïve $CD4^+$ T cells. The most promising finding was that priming by VitD3-cDC2 appeared to skew T cells toward a more regulatory phenotype with enhanced levels of FoxP3 and IL-10 observed after restimulation of the primed T cells. Further repeats will be needed to show

whether these findings are statistically and also biologically significant, i.e. by showing that the VitD3-cDC2 primed T cells exert regulatory function on other immune cells.

It was very challenging to compare our findings with other groups as to the best of our knowledge this is the first study that uses VitD3-treated cDC2 for induction of tolerance. For those reasons, we tried to select the most appropriate studies that either used VitD3-treated moDC for tolerance induction or cDC2 that were produced for cancer immunotherapy. There are several studies that identified the presence of tolerogenic $CD14^{+}$cDC2 ('DC3') in the tumor microenvironment (TME) of cancer patients (Bakdash et al., 2016; Di Blasio et al., 2020; Van Ee et al., 2018). From our perspective it was encouraging that cDC2 could be modulated by its surroundings to induce immunosuppressive effects on T cells. Since this could mean that tolerogenic cDC2 could be generated in the laboratory for autoimmune disease interventions. Beside that, our findings on chapter 4 reveled that we could indeed induce tolerogenic features in cDC2 by modulation with VitD3.

5.5.1 VitD3-cDC2 and T cell priming

As previously mentioned, T cell activation and priming requires two initial signals that depend on ligation of MHCII/peptide on DC with TCR on the T cell (signal 1) and co-stimulation via binding of CD80/CD86 on DC with CD28 on the T cell (signal 2)(Tai et al., 2018). After that according to the expression of either immune stimulatory or inhibitory molecules as well as the cytokine microenvironment (signal 3), the T cell polarization will change to either T effector cell to produce immunity or T cell apoptosis or induction of Tregs to induce tolerance, respectively. Several reports have shown that vitD3-treated moDC have suppressive effect on T cell proliferation and IFN-g secretion (Lee et al., 2016; Navarro-Barriuso et al., 2021; Švajger and Rožman, 2019; Unger et al., 2009). This suppressive effect could be further augmented with combination with other immune modulatory agent such as IFN-γ (Švajger and Rožman, 2019) or Dexamethasone (Amy E. Anderson et al., 2008; Harry et al., 2010a). As expected, and in agreement with these previous studies, we found that VitD3-cDC2 also have a low stimulatory capacity for naïve $CD4^{+}$ T cells and low IFN-γ and TNF-α cytokine secretion. Interestingly, while in chapter four we demonstrated that VitD3-cDC2 expressed high levels of the co-stimulatory molecule CD86, this CD86 expression could also lead to produce a negative signal when interacts with CTLA-4 on T cells (Horton et al., 2017; Yu et al., 2019). Furthermore, when it comes to DC/T cell interactions, multiple interactions take place. Thus,

the balance between co-stimulatory molecule CD86 with inhibitory molecules is essential for producing the desired immune response.

5.5.2 Mechanism of tolerance induction by VitD3-cDC2

There are various mechanism for tolerance induction by tolerogenic dendritic cells (Castenmiller et al., 2021). This is mainly dependent on the immunomodulatory pathways that are involved during interaction between DC and T cells (Bourque and Hawiger, 2018). Several studies and reviews determined that treatment of DC with VitD3 were found to produce various immunomodulatory pathway during interaction with T cells leading to peripheral tolerance (Unger et al., 2009). Of note, majority of these mechanism is related to high IL-10 secretion and expression of PDL-1 and ILT-3 immunoinhibitory molecules in the context of moDC (Nagy et al., 2023; van Wigcheren et al., 2021) However, it is not known yet if these immunomodulatory pathways could be the same in context of VitD3-treated cDC2. Nevertheless, according to our finding in figure 5.11, high levels of PDL-1 expression and IL-10 cytokine secretion by VitD3-cDC2 could be the two main immunomodulatory pathway that can explain the tolerogenic effects on naïve $CD4^+$ T cells.

Another molecule that is expressed by our VitD3-cDC2 that could potentially have a role in the immunosuppressive activity of these cells is CD14 (See Chapter 4, figure 4.9). Interestingly, this molecule was always found to be expressed in vitD3-treated DC (L Bishop et al., 2021; Švajger and Rožman, 2019). This induced $CD14^+$ in VitD3-cDC2 could be equal to what is specified in Di Blasio et al., in which induced $CD14^+$ cDC2 after treatment with IL-6 and PGE2 have promising immunosuppressive activity (Di Blasio et al., 2020). In addition, several studies in context of cancer immunotherapy identified accumulation of $CD14^+$ cDC2 in TME of different cancer patient including melanoma and breast cancer, which exerted tolerogenic effects on anti-tumor T cells (Di Blasio et al., 2020; Schreibelt et al., 2016; Van Ee et al., 2018). Intriguingly, this $CD14^+$ cDC2 that was induced in response to TME could be equivalent to both our VitD3-cDC2 and IL-6/PGE-induced $CD14^+$cDC2. These $CD14^+$ cDC2 could be of great potential for antigen- specific tolerance induction. Hence, extensive research in this area is needed to discover the mechanism in which these $CD14^+$ cDC2 exert their tolerogenic effect on T cells.

5.5.2.1 Induction of Regulatory T cells (Tregs)

One of the crucial mechanisms leading to peripheral tolerance is the induction of Tregs. cDC2 can induce Treg in the periphery as well as increase the expansion of naturally existing Tregs (Bourque and Hawiger, 2018; Price et al., 2015). As our second aim is to assess the regulatory capacity of $T_{vitD3\text{-}cDC2}$, we found that vitD3-cDC2 could potentially promote $CD25^{+}FOXP3^{+}$Tregs. This observation is consistent with Unger et al., who demonstrated that moDC treated with VitD3 induced Tregs from naïve $CD4^{+}$ T cells as compared to dexamethasone-treated moDC and mature DC (Unger et al., 2009).

There are different immunomodulatory axes that could contribute to the induction of Tregs. For instance, PDL-1/PD-1 axis is crucially important, specifically in relation to vitD3-treated DC (Švajger and Rožman, 2019). Engagement of PDL-1 to either PD-1 or in competition with co-stimulatory molecules for CD28 both lead to a negative effect on TCR signaling and induce Tregs (Iberg and Hawiger, 2020). In addition,(Švajger and Rožman, 2019 and Unger et al., 2009) in their studies identified that PDL-1/CD86 ratio is high on vitD3-treated moDC and induction of Tregs by these cells is dependent on PDL-1 immune inhibitory molecule. Consequently, this could be also the case in our VitD3-cDC2 as the PDL-1/CD86 ratio was also high compared to Stim-cDC2. In addition, we found that the fold change in PDL-1 was 3.5 higher in vitD3-cDC2 compared to Stim-cDC2 (figure 5.11), which could also explain the regulatory effect of these cells.

Another vital immunomodulatory axis for Treg induction is the anti-inflammatory cytokine IL-10 (Horton et al., 2017). IL-10 and TGF-b are both secreted by tolerogenic dendritic cells, providing an anti-inflammatory environment that induces $CD25^{+}FOXP3^{+}$ Tregs (Raker et al., 2015). Notably, several studies demonstrated that IL-10-modulated moDC that produces high amounts of IL-10 promotes Tr1 regulatory T cells (Gregori et al., 2010; Hafkamp et al., 2021). Similarly, dexamethasone-treated moDC was found to induce Tr1 regulatory T cells in a contact-dependent manner (Raker et al., 2015). However, interestingly VitD3 treatment has been shown to have a different outcome on different DC types. For example, Van Der Aar et al., identified that targeting epidermal Langerhans cells and dermal DC with VitD3 led to induction of either $CD25^{+}FOXP3^{+}$ Treg or $CD25^{+}FOXP3^{-}$ (IL-10 producing)Tregs (Tr1), respectively (Van Der Aar et al., 2011). Unlike that, treatment of moDC with VitD3 led to the induction of IL-10-producing Treg (Hafkamp et al., 2021). Excitingly, we observed an increase in $CD25^{+}FOXP3^{+}$ Tregs, which is very promising in relation to VitD3-cDC2 regulatory activity.

CD137 (4-BB1, member of the TNFR superfamily) is another interesting molecule that was initially identified as a potent marker of effector T cells. Surprisingly, this molecule was recently found to be also expressed by Treg and involved in its regulatory function (Luu et al., 2021; Nowak et al., 2018). Interestingly, we observed a slight increase in the percentage of positive CD137/FOXP3 cells in $T_{VitD3\text{-}cDC2}$ compared to other T cell types suggesting the potential contribution of CD137/CD137L axis in Treg regulatory function in relation of VitD3-cDC2. Further research is necessary to assess the contribution of this potential regulatory axis in VitD3-cDC2 regulatory capacity.

To this end, VitD3-cDC2 could have various immunomodulatory activities on T cells to induce tolerance. Further investigation is necessary to address the exact contribution of each surface molecule or cytokine of VitD3-cDC2 into T cell polarization or tolerance induction.

5.5.2.2 Induction of T cell anergy or apoptosis

Another mechanism of peripheral tolerance induction by tolerogenic DC are T cell anergy and apoptosis induction. moDC treatment with VitD3 was found to also induce peripheral tolerance through these mechanisms (Horton et al., 2017; Van Halteren et al., 2004). For example, TGF-b immunomodulatory axis is found to induce peripheral tolerance through its immunosuppressive effect that either induces T cell apoptosis or Treg differentiation (Zhuang et al., 2020). Hence, assessing all regulatory cytokines and molecules will be one of the crucial aims for future research in our VitD3-cDC2.

Of note, CTLA-4 is one of the pivotal immune inhibitory molecules that is expressed on the surface of activated T cells, which competes with CD28 to bind with co-stimulatory molecules, including CD86, with high affinity. This interaction will then lead to T cell anergy and hyporesponsiveness (Horton et al., 2017). In line with that, our VitD3-cDC2 could potentially employ its lower stimulatory effect through binding of its high CD86 molecule with CTLA-4 inhibitory molecule on T cell.

5.6 Summary and future work

Overall, our VitD3-cDC2 has a lower stimulatory capacity to naïve $CD4^+$ T cells with low IFN-g secretion. In addition, VitD3-cDC2 could have a regulatory capacity through expression of high PDL-1 and production of high IL-10 leading to potential induction of $CD25^+FOXP3^+$ Treg. Altogether, these promising findings could contribute to our understanding of the functional capacity of cDC2 as well as provide the basis for future tol-cDC2 generation for autoimmune diseases. However, a proper modification into the balance between expression of co-stimulatory and immune inhibitory molecules as well as secretion of some of pro-inflammatory cytokines including IL-6 and TNF-a and anti-inflammatory IL-10 by VitD3-cDC2 should be considered. Further investigation and studies are necessary to identify the mechanism of regulation and tolerance induction by VitD3-cDC2.

Chapter 6: General discussion, conclusion and future work

6.1 General discussion

The generation of tolerogenic dendritic cells (tolDC) has emerged as a promising avenue for immune tolerance induction, holding great potential for the treatment of autoimmune diseases and the regulation of immune responses. The most cellular approach used for generation of tolDC in the literature is moDC. However, this cellular approach appears to have limited success in clinical trials, which may be due to poor migratory capacity to the T cell area of lymphoid tissue, leading to not producing the desired immune response (Adema et al., 2005). Therefore, in this thesis, we investigated an alternative approach for generating tolDC from conventional dendritic cells (cDC). The rationale for that is mainly due to the strong ability to prime and educate naïve $CD4^+$ T cells as well as the great capacity to migrate to the T cell area of lymphoid tissues.

Therefore, to help in selecting the most appropriate cDC for tolDC generation, transcriptomic comparisons between monocyte-derived tolDC and both peripheral blood-derived and cultured $CD34^+$-derived conventional dendritic cells (cDC1 and cDC2) were performed. Based on the results of this comparative analysis, cDC2 were more closely aligned with tolDC than cDC1 in terms of their gene expression profile and because cDC2 is the dominant human DC subtype in the peripheral blood, we select cDC2 as a potential alternative approach for tolDC generation.

We next examined the impact of different immunomodulatory agent on the generation of tol-cDC2 with specific emphasis on their migratory capacity and T cell stimulatory capacity. Specifically, we investigated the impact of VitD3 treatment on the generation of tol-cDC2.

6.1.1 Transcriptomic analysis in Mo-tolDC and conventional DC

My first aim on this project was to perform a comparative transcriptomic analysis between tolDC and cDC. This was in order to identify the unique gene expression profile in each type of DC as well as determining the more closely related subset of cDC to tolDC in terms of their gene expression profile**.** Therefore, I used an existing Nanostring data set of different moDC and cDC types that generated in Hilkens and Bigely laboratory, respectively. Then, Nanostring

nCounter analysis platform were performed to obtain the differential gene expression profile of both tolDC and cDC. My analyses showed that Mo-tolDC express some important tolerogenic genes such as *MERTK*, *LILRB4* and *PDL-1*. They also remarkably expressed *IL1R2* at gene level and we also validated that at the protein level. These markers could have a role in the tolDC mechanism of tolerance induction and may also be suitable as QC markers for therapeutic tolDC.

In addition, my finding demonstrates that both cultured- and peripheral blood-derived cDC (cDC1 and cDC2) naturally express varying levels of some immune regulatory molecules, including *BTLA and IDO*. This may explain the contribution of these DC in tolerance induction during steady state. However, these circulating cDC, after encountering any stimulation or pathogen, have a great ability to change into mature cDC that can strongly stimulate T cells to induce a suitable immune response. Hence, generation of tol-cDC that are treated with immunomodulatory agent is important to insure the resistance to any inflammatory stimuli.

Additionally, my results showed that cDC2 is more closely aligned with Mo-tolDC than cDC1 with regard to their gene expression profile, which might be because they both depend on the same transcription factor IRF4. Finally, similar to Mo-tolDC, I found that peripheral blood cDC2 naturally upregulate *LILRB4* gene compared to cDC1, which could contribute to the natural tolerogenic function of cDC2 at steady state. All together these findings facilitate the selection of cDC2 as an alternative potential source for tolDC generation.

6.1.2 Generation of tolDC from cDC2

The general aim of my project was to generate a cDC2-based tolDC with suitable migratory capacity. So, after we investigated various immune modulatory agents for generation of tol-cDC2, I selected VitD3-cDC2 as a potential tol-cDC2. There were several reasons behind this choice. First, from the phenotypical standpoint, VitD3-cDC2 showed higher expression of all the three tolerogenic marker of interest, namely ILT3 and MERTK (non-significant high level) and significantly higher expression of PDL-1 compared to other immunomodulatory agents.

Secondly, VitD3-cDC2 were the only condition that demonstrated a striking production of IL-10. However, they also produced high level of IL-6 and TNF-α. Thirdly, expression of CCR7 was notably higher compared to other conditions, with appropriate migratory capacity towards

CCL21 and CCL19, only slightly reduced as compared to Stim-cDC2. Finally, VitD3-cDC2 showed low T cell stimulatory capacity with strong downregulation of IFN-γ production and a slight increase in IL-10 production. Of note, after re-stimulation with mature cDC2, T cells that had been previously primed with VitD3-cDC2 showed a slight increase in the proportion of $CD25^{+}FOXP3^{+}$ T cells with high production of IL-10.

However, there are two important features of concern that need to be addressed in relation to VitD3-cDC2, and that raises the question of whether **these cells are indeed tolerogenic?**

First, the high expression of the co-stimulatory molecule CD86 and the maturation marker CD83 by VitD3-cDC2. However, a different conclusion could be drawn from this higher expression of CD86. For example, CD86 expression could contribute to strong T cell interaction with CTLA-4 inhibitory molecule as a highly competitive molecule to CD86 with CD28 leading to T cell hyporesponsiveness rather than stimulation. Hence, investigating the expression of CTLA-4 on the surface of T cells could facilitate into understanding the impact of high CD86 expression on VitD3-cDC2 in producing the desired immune response. Another interesting assumption of high CD86 expression is the contribution of this co-stimulatory marker in the proliferation and preservation of Treg regulatory phenotype in the presence of CTLA-4 marker as well (Halliday et al., 2020). Furthermore, although, the expression of CD86 was high, the PDL-1/CD86 ratio was also high in VitD3-cDC2 that could explain the balance into an immune inhibitory and immune stimulatory response. This high PDL-1/CD86 ratio was also observed as one of the important results in the context of DCreg that was generated for clinical testing in organ transplantation by (Zahorchak et al., 2018).

With regard to the high expression of CD83, there are several studies that point to a regulatory function of this molecule. For instance, Kryczanowsky et al., in their study of IL-10 generated tolDC identified that two subpopulations were developed: $CD83^{low}CCR7^{-}$ and $CD83^{high}CCR7^{+}$ with superior immunosuppressive function compared to $CD83^{low}$ (Kryczanowsky et al., 2016). In addition, a study by Wild et al., identified that the knockdown of CD83 on DC in mice leads to enhanced immune response and a decrease in the number of Treg in peripheral lymphoid organ (Wild et al., 2019). They also found that knockdown of CD83 on DC mice in an experimental autoimmune encephalitis model will lead to worsening disease progression. Thus, in line with our findings, high CD83 expression could contribute to VitD3-cDC2

regulatory function. Further research is necessary to understand the mechanism of CD83 regulation by DC.

The second feature of concern in VitD3-cDC2 is the high production of the pro-inflammatory cytokines IL-6 and TNF-α. This could mean a careful safety procedure should be done before using this cell type in the clinical setting. For example, determining the safe and effective dosage for VitD3-cDC2 cells is necessary to ensure that the concentrations used are within a therapeutic range that minimizes adverse reactions. Notably, this higher pro-inflammatory cytokine secretion was also observed in the context of Mo-tolDC generated with VitD3 (Lee et al., 2016; Unger et al., 2009). Therefore, evaluating the balance between pro-inflammatory and anti-inflammatory cytokines is of crucial importance to ensure the effectiveness of tolerance induction.

Collectively, it is difficult to draw firm conclusions about the tolerogenic state of VitD3-cDC2, but all their immune inhibitory characteristic features as well as their low T cell stimulatory capacity with strong downregulation of IFN-γ could give a picture of a semi-mature state of VitD3-cDC2 with potential to induce tolerance.

6.1.3 Migratory capacity of VitD3-cDC2

Another crucial aim of my project is to generate tol-cDC2 with good migratory capacity. Therefore, an important question to answer is whether **VitD3-cDC2 has a suitable migratory capacity.** Interestingly, VitD3-cDC2 showed an interesting shift in migratory behavior with appropriate CCR7 expression as well as migratory capacity towards CCL21 and CCL19. However, this migratory ability of VitD3-cDC2 appears to be affected by culture media with superior migration capacity in X-VIVO15 cultured VitD3-cDC2 compared to cellGenix cultured cells. Hence, yes, the VitD3-cDC2 showed appropriate migratory capacity similar to Stim-cDC2, which does support their choice as an alternative cell source to Mo-tolDC.

6.2 Summary of the key findings and limitations of this study

The main limitation of this study was the small sample size, especially in chapter 5, in which the number of replicates was limited to 2 or 3. This was due to limited laboratory time, due to the Covid-19 pandemic, as outlined in the Covid-19 Impact Statement. This limitation could

lead to low certainty with regard to the outcomes in chapter 5. Therefore, future research efforts should prioritize conducting follow-up studies with larger sample sizes. Increasing the sample size would not only enhance the statistical power and reliability of the findings but also allow for a more comprehensive validation and extension of the results obtained with the current dataset.

Additionally, there are some other limitations in this study in relation to the generation of tolDC from cDC2 as an alternative cellular approach. First, as the cDC2 are known to be a dominant subset of DC in the peripheral blood (Collin and Bigley, 2018), their isolation is believed to be feasible and reliable for the generation of tolDC. However, the number of cDC2 that could be isolated from the blood is relatively small, which limited the number of replicates as well as the number of culture conditions that I could do in one single experiment.

Secondly, the presence of diverse subsets of cDC2s, along with the emergence of newer populations like DC3, which share certain phenotypical characteristics with cDC2, could potentially complicate the creation of distinctly pure, stable, and functionally consistent tolerogenic cDC2 which could affect the efficacy and specificity of the intended therapeutic outcomes. In addition, these heterogenous subsets could also potentially impacting cDC2 reliability for therapeutic use.

Finally, fluctuations in the expression of defining markers for cDC2 subsets, particularly CD163, pre- and post-culture, may hinder the ability to differentiate between various cDC2 subsets and accurately identify potential contamination from the DC3 population.

Another fundamental limitation of this study was in relation to the Nanostring data analysis, in which different culture conditions were used for the moDC populations and the cultured cDC subsets. However, a culture signature removal was performed to help in reducing the culture effect on that comparison.

On the other hand, there are several important findings and contributions to knowledge in this thesis in relation to the generation of tolDC from cDC2. The key findings of this thesis are as follows:

1. cDC2 naturally expresses ILT3 immune inhibitory molecule at gene and protein levels, which could reflect the importance of these cells in tolerance induction during steady state.

2. There are no obvious differences in the expression of tolerogenic markers of interest between the $CD5^+$ and $CD5^-$ cDC2 subsets, except the expression of CCR7, which is mainly expressed in the $CD5^+$ subset. However, CCR7 is increased in the $CD5^-$ subset after activation with LPS.

3. Different culture media can have an impact on the phenotype and function (e.g., migration) of cDC2, hence selecting an appropriate culture medium that supports the desired phenotype/function is of great importance when developing cDC2-based cell therapies.

4. X-VIVO15 culture medium could be superior in inducing the expression of CCR7 in cDC2, which therefore leads to suitable migratory capacity for these cells even after treatment with the immune modulatory agent VitD3.

5. VitD3 could be the favorable immune modulatory agent to use for the generation of tolerogenic cDC, as it is the only treatment that showed high secretion of IL-10 and a higher PDL-1/CD86 ratio compared to the other agents that were tested. It also supported appropriate CCR7 expression by cDC2 and their ability to migrate towards CCL21 and CCL19.

6. VitD3-cDC2 demonstrated a low stimulatory capacity for naïve $CD4^+$ T cells with strong downregulation of IFN-γ secretion.

7. T cells that were primed with VitD3-cDC2 exhibited slight increase in $CD25^+$ $FOXP3^+$ T cell with high secretion of IL-10 after re-stimulation assay. This result is very promising in the role of VitD3 treated cDC2 for polarization and induction of Treg.

Overall, all these promising results may highlight the potential use of cDC2 in the context of tolerance induction and immunotherapy for autoimmune diseases. Specifically, VitD3-cDC2 were characterized by semi-tolerogenic phenotype, efficient migratory ability as well as low stimulatory capacity to naïve CD4+ T cell with strong downregulation of IFN-γ secretion. This aspect is encouraging for pre-clinical grade consideration to use for tolerance induction.

Strength	Challenge	Opportunities
1. Strong ability of cDC2 to prime and educate naïve $CD4^+$ T cells. 2. High migratory ability towards CCL21 and CCL19. 3. Anti-inflammatory cytokine profile with high IL-10 secretion. 4. Short priming time with VitD3, so short in culture duration.	1. Heterogeneity of cDC2 subsets. 2. Heterogenous cytokine secretion with anti-inflammatory and pro-inflammatory cytokine profile. 3. limited number of cDC2 in the peripheral blood. 4. Minimal literature exists on use of cDC2 in the context of autoimmune disease. 5. Short in-vitro life span of cDC2.	1. Potential for targeted therapy as cDC2 have a great ability for Ag presentation to naïve $CD4^+$ T cells. 2. Synergistic combination therapy with cDC1 or pDC for cancer or autoimmune disease immunotherapy as each one has specific immune function that could be coordinated to regulate immune response.

Table 6.1: Overview of strengths, challenges and opportunities for VitD3-cDC2 in the application as immunotherapy for autoimmune disease.

6.3 Conclusions and future implications

Overall, I believe that cDC2 could be used as an alternative approach for generating tolDC therapy for autoimmune diseases. In addition, the closer gene expression profile of cDC2 to Mo-tolDC than cDC1 also suggests the potential use as an alternative therapy for tolDC generation (detailed in Chapter 3). Moreover, in this thesis, I present evidence highlighting the importance and impact of culture media in generating appropriate cDC2-based tolerogenic DC (detailed in Chapter 4). Treatment of cDC2 with VitD3 encourages semi-tolerogenic phenotype, appropriate migratory capacity (Chapter 4) and low stimulatory ability to naïve $CD4^+$ T cell (Chapter 5).

However, there are still several crucial questions that will need to be addressed in future work in relation to the use of cDC2 as a tolDC therapy. One of the crucial questions is related to the heterogeneity of cDC2 and whether DC3 is completely depleted during the $CD14^+$ depletion step or not? To answer this question, carefully defining marker procedure should be done. For

example, an FMO to ensure the presence of $CD163^+$ subset or not should be used. It is also possible to use the BTLA marker, which appears from the literature that DC3 does not express this marker while DC2 does.

Another important question to answer is whether there are any differences in the stimulatory capacity of the $CD5^+$ and $CD5^-$ cDC2 subsets after treatment with VitD3 and whether either subset could be more reliable for generating a tolerogenic immunotherapy. Although part of this question was already answered by my findings (Chapter 4), which show no obvious differences in the expression of tolerogenic markers of interest, their functional properties or inhibitory capacity after treatment with VitD3-cDC2 remain to be investigated. Therefore, performing a cell sort (i.e., sorting $CD5^+$ and $CD5^-$ cDC2) prior to cell culture could help answer these questions. However, this will be much more complicated to translate to clinical sitting due to several reasons, including the higher cost of cell sorting in terms of both equipment and operation.

Furthermore, in order to understand the molecular mechanism underlining the VitD3-cDC2 low stimulatory capacity, as well as comparing the DEG of these cells with Mo-tolDC, transcriptomic analysis could be applied. For example, single cell RNAseq or Nanostring nCounter platform could be performed in order to achieve the gene expression profile of these cells. Furthermore, proteomics to explore the protein-protein interaction could also be used to discover the molecular interaction between VitD3-cDC2 and T cells.

Finally, another important challenge that should be addressed in generating tolDC form cDC2 is the difference in cDC2 derived from autoimmune patients and those from healthy controls, which could impact on the therapeutic application. There are some studies that identified phenotypical variation and functional discrepancies in cDC2 between autoimmune patients and healthy controls. For instance, the difference in phenotypic and transcriptional expression of cDC2 was determined in the synovial fluid and peripheral blood of RA patients compared to healthy controls. Therefore, comparative studies focused on delineating the molecular, functional, and phenotypic distinctions can help assess the suitability of using cDC2 from individuals with autoimmune conditions for therapeutic interventions.

Overall, this thesis has provided the basis for future work on the generation of tol-cDC2 and its application for the treatment of autoimmune diseases.

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