

Exploiting the use of induced pluripotent stem cell derived immune cells for immunotherapy

A thesis submitted for the degree of Doctor in
Philosophy at the University of Oxford

Trinity Term 2015



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Immunotherapy traditionally made use of biological agents such as cytokines and monoclonal antibodies. Such first generation therapies lack antigen specificity and fail to induce immunological memory, suggesting that cell therapies may provide the next generation of treatments that are more discerning in their mode of action. Nevertheless, difficulties in obtaining sufficient immunologically-relevant cell types from patients has limited their success. Given that induced pluripotent stem cells (iPSC) may be generated from patients, we have investigated the feasibility of deriving two cell types whose availability is restricted *in vivo*: regulatory T cells (T_{regs}) and $CD141^{+}$ cross-presenting dendritic cells (DCs).

We describe the optimization of protocols for differentiation and purification of $CD141^{+}$ DCs, focussing on their utility as a therapeutic vaccine for HIV-1. We investigate their phenotype, chemotactic capacity, phagocytic ability and propensity to harbour infectious virus. We also assess their immunostimulatory capacity and ability to cross-present exogenous antigen to MHC class I-restricted T cells.

Our findings led us to speculate that iPSC-derived DCs (iPDCs) possess fetal phenotype, which is characterised by excessive secretion of IL-10 and failure to secrete IL-12, under all but the most stringent conditions. We hypothesised that constitutive secretion of IL-10 may be responsible for maintaining the fetal phenotype, a hypothesis we tested by developing an appropriate mouse model. iPSCs were derived from WT and $IL-10^{-/-}$ mice and were shown to differentiate into iPDCs which recapitulate the fetal phenotype observed among human cells. However, loss of the endogenous *Il-10* gene failed to restore full immunogenicity and IL-12 secretion.

Finally, we developed protocols for differentiation of $FoxP3^{+} T_{\text{regs}}$ from iPSCs, a feat that has not previously been achieved. These findings pave the way for the differentiation of T_{regs} from iPSCs reprogrammed from antigen-specific pathogenic T cells, thereby creating a source of T_{regs} with matched specificity for therapeutic intervention.

This DPhil thesis is dedicated to my vivacious twin sister, Nancy.

For being my sunshine.

“Penetration of our mind is our goal, but in the beginning to set things in motion, there is no substitute for sweat.”

B. K. S. Iyengar

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Abbreviations

2ME	2, mercapto-ethanol
7AAD	7-aminoactinomycin D
APC	Antigen presenting cell
BMDC	Bone marrow derived dendritic cell
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CD40L	CD40 ligand
CM	Cardiomyocytes
CO ₂	carbon dioxide
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene Plasticizer Xylene
EB	Embryoid bodies
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERAP	Endoplasmic reticulum aminopeptidase
ES	Embryonic stem
FACS	Flow assisted cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
Flt3L	FMS-like tyrosine kinase 3 ligand
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft versus host disease
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM	Intercellular Adhesion Molecule 1
ICOS	Inducible costimulator
ICOSL	Inducible costimulator ligand
IFN	Interferon
IL	Interleukin
iPSC	Induced pluripotent stem cell
KCl	Potassium chloride
KO	Knockout
LC	Langerhans Cells
LFA	Lymphocyte function-associated antigen
LIF	Leukemia inhibitory factor

LN	Lymph nodes
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
M-CSF	Macrophage colony-stimulating factor
mDC	Myeloid dendritic cells
MEM	Minimum essential media
MHC	Major histocompatibility complex
MMC	Mitomycin C
moDC	Monocyte derived dendritic cells
MS	Multiple sclerosis
NEAA	Non-essential amino acids
NHS	National Health Service
NK	Natural killer
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Programmed death
pDC	Plasmacytoid dendritic cell
PDL	Programmed death ligand
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PGE	Prostaglandin E
PRR	Pattern recognition receptors
PSC	Pluripotent stem cell
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SLE	Systemic Lupus Erythematosus
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	T helper (cells)
TLR	Toll like receptor
TNF	Tumour necrosis factor
Tr1	Type 1 regulatory T (cells)
T _{reg}	Regulatory T (cells)
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
VSVG	Vesicular stomatitis virus
WT	Wild type

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Chapter 1

Introduction

1.1 The spectrum of immunotherapy

The immune system is an interactive network of lymphoid organs, cells, molecules and cytokines that exists to protect the host from infectious agents while maintaining minimum collateral damage to the individual [1]. Many components of the immune system are involved in maintaining the fine balance between immunity and tolerance, resulting in immune homeostasis. Dysregulation of immune homeostasis leads to the development of immuno-pathology, which manifests due to excessive activity of the immune system or its inappropriate suppression.

The global burden of diseases with an immuno-pathological basis is immense. More than 80 autoimmune diseases have been identified, with the most common diseases being systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes. The National Institute of Health (NIH) has estimated that approximately 14–22 million people are currently affected by autoimmune diseases in the United States alone, with the healthcare cost estimated to be in the range of \$100 billion per annum [2, 3]. At the other end of the spectrum, HIV-1 infection illustrates the consequences of the loss of immune function. Worldwide, 36.9 million people were estimated to be living with HIV-1 by the end of 2014. The annual cost of HIV-1 treatment in the United Kingdom was estimated to be as high as £758 million in 2013 [4].

Despite advances in our understanding of the immune system over the last 30 years and the advent of various treatments for these disorders, immune-related diseases still remain a substantial burden, reinforcing the need for the development of novel targeted therapies for the treatment of immune-related disorders.

1.1.2 First generation immunotherapies

As mentioned above, maintaining the delicate balance between tolerance and immunity is critical for optimal functioning of the immune system, the fine tuning of which is the ultimate goal of immunotherapy. The first generation of immunotherapies has harnessed biological molecules, such as monoclonal antibodies and cytokines for the treatment of diseases [5].

1.1.2.1 Suppressing excessive immune responses

General immunosuppression, which has been historically used for reducing excessive immune responses, has been successful in decreasing the incidence of graft rejection and in alleviating the clinical symptoms of autoimmune disease. Prolonged immunosuppression, however, has been shown to have many deleterious side effects and has been correlated with the increased susceptibility to infection with opportunistic pathogens and a propensity towards malignancy [6, 7]. Advances in the understanding of the immunology of rejection over the last three decades have led to the development of first generation of immunotherapeutic approaches to suppress excessive immune responses.

CAMPATH-1 (alemtuzumab), a humanised rat monoclonal antibody (mAb) specific for CD52 molecule expressed in all lymphocytes [8, 9], has been shown to be useful in the treatment of multiple sclerosis (MS), a T cell-mediated autoimmune diseases [10]. A short treatment of CAMPATH-1 is sufficient to deplete mature T and B lymphocytes from the patient, resulting in reconstitution of the immune system with a reformed lymphocyte

population, with increased numbers of regulatory T cells (T_{regs}) [11]. CAMPATH-1 has also been shown to be effective in preventing graft-versus-host disease (GVHD) and bone marrow rejection during transplantation [12, 13].

In 1992, blocking of tumour-necrosis factor α (TNF α), a potent pro-inflammatory cytokine, was shown to markedly reduce joint inflammation in patients suffering with rheumatoid arthritis (RA). This seminal discovery heralded a new generation of targeted drugs for autoimmune diseases. The first clinical trial using anti-TNF α antibody, infliximab, to target RA was initiated in the same year and demonstrated the potent anti-inflammatory effects of anti-TNF α greatly improving the pathology of RA and retarding joint damage [14]. Other monoclonal antibodies such as rituximab, which targets antibody-producing B cells, and tocilizumab, which targets the activity of pro-inflammatory cytokine IL-6, have also been successfully employed for the treatment for RA as well as SLE [15, 16].

1.1.2.2 Stimulating protective immune response

Failure of the immune system to respond effectively to pathogens or neoplasms gives rise to severe infections and cancer. Various methods of stimulating protective immune responses have been exploited including cytokine modulation and the use of antigen-specific mAbs that function as immune checkpoint inhibitors [17].

Therapeutic mAbs that target tumour associated antigens (TAA) and tumour specific antigens (TSA) or block pathways involved in tumour function have also been exploited for the treatment of cancer [17, 18]. Although these antibodies have been shown to be

highly specific, clinical trials have proven disappointing, with an objective response rate of only 8-10% [19-21]. Specific mAbs have also been developed to block immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death-1 (PD-1) and programmed-death ligand (PD-L1), which are exploited by tumour cells to inhibit T cell or DC functions, enhancing their ability to evade immune surveillance [22, 23]. CTLA-4 is normally expressed by regulatory T cells (T_{regs}) and binds to CD80 or CD86 co-stimulatory molecules expressed on DCs, inducing a negative signaling cascade that results in tolerance. Antibodies against CTLA-4 have been shown to enhance survival in patients with advanced melanoma and were approved as a therapy by the US Food and Drug Administration (FDA) in 2010 [22]. These mAbs have also been shown to be successful in the treatment of lung cancer and hepatocellular carcinoma (HCC). PD-1 is expressed by T cells, which, on ligation with its ligand, PD-L1 expressed by DCs and tumour cells, suppresses T cell activation [24-27]. Anti-PD-1 mAbs, such as nivolumab, has shown impressive results for the treatment of metastatic melanoma and squamous cell lung cancer, and has been recently approved by the FDA for the treatment of the latter [28, 29].

Cytokines, such as IL-2, interferon- α (IFN- α) and tumour necrosis factor (TNF- α), among others, have also been used to stimulate non-specific immune responses against cancer. They act either by directly inhibiting the growth of tumour cells or by promoting the proliferation and cytokine production by T cells and natural killer (NK) cells, enhancing their ability to target tumour cells [17, 30]. A number of cytokines, used singly or in combination, have been proven to be effective in enhancing the immunogenicity of tumours and have been exploited in clinical trials for the treatment of advanced cancer

[31]. IL-2 has, for example, been approved for the treatment of metastatic melanoma and renal cell carcinoma [32].

1.1.2.3 Limitations of first generation immunotherapies

Some success has been achieved through the use of first generation immunotherapeutics, nevertheless, these approaches focus on systemic stimulation or suppression of the immune system, which leads to deleterious side effects. Long term suppression of TNF- α , which plays a critical role in the defence of immune system against pathogens, has been associated with increased incidences of infections, malignancies and cardiac disease [33]. The use of CAMPATH-1 has also been associated with the high incidence of infection and thyroid diseases, in particular, Graves's hyperthyroidism, a type of autoimmune disease [11, 34, 35]. Similarly, cytokines and mAbs do not target antigen-specific responses, having instead, a systemic impact, and have been correlated with the excessive activation of the immune response, leading to autoimmune disease [36].

Although the use of mAbs and cytokines for modulating the immune response has been shown to be promising, response rates remain disappointingly low when used as a single therapy. Furthermore, since the administration of cytokines and therapeutic mAbs is inherently passive, they fail to generate a long-lasting memory response, requiring repeated administration [19-21], which may elicit neutralizing anti-idiotypic responses. Such responses can potentially inhibit the binding of the therapeutic mAbs to their targets, greatly limiting the efficacy of the treatment [37]. Furthermore, long term use of mAbs and cytokines may have unprecedented side effects, as demonstrated by the

thromboembolic complications observed in early clinical trials investigating the use of mAbs targeting the CD40-ligand, CD154, for blockade of T cell stimulation [38].

The ultimate aim of immunotherapy is to either activate the immune system to recognize infection by generating a specific and durable effector T cell response or to suppress unwanted T cell responses either by inducing T_{reg} cells or causing T cell anergy. After decades of research, it is now accepted that the interaction between DCs and T cells lies at the heart of the immune system, the outcome of which is responsible for skewing the nature of the immune response towards either tolerance or immunity [39, 40]. Intervening in this fundamental interaction permits the fine tuning of subsequent responses for therapeutic purpose. The dynamics of T cell and DC interactions have been exploited in the second generation of immunotherapies, which will be described later in the chapter.

In the sections below, we described the interaction between DCs and T cells and discuss what approaches have been explored in immunotherapy. We further outline how induced pluripotent stem cells (iPSCs) can be exploited to address the current obstacles to immunotherapy. Since DCs are highly heterogeneous and different subsets have been shown to perform different roles in the immune response [41], we first describe the various subsets of DCs that have been identified in humans.

1.2 Dendritic cells (DCs) and their subsets

Dendritic cells (DCs) are the sentinels of the immune system, specializing in surveillance of the tissue microenvironment for antigens, their acquisition and presentation to naive T cells, which plays a critical role in the initiation of the immune response [40]. DCs are central in coordinating immune responses to pathogens and providing a link between innate and adaptive immunity [42]. DCs are the most efficient antigen presenting cells (APCs), owing to their high levels of MHC class II expression and costimulatory capacity. They present endogenous antigen via MHC class I to CD8⁺ T cells and exogenous antigen either directly via MHC class II to naive CD4⁺ T cells, a process termed conventional presentation, or via MHC class I to naive CD8⁺ cytotoxic T lymphocytes (CTL), known as cross-presentation [43].

Although united by their function as professional APCs, DCs are extremely heterogeneous population of cells with various subsets defined by a range of cell surface molecules, resident locations and functional specialisation [41, 44]. All DCs are derived from the bone marrow, express MHC class II and lack the expression of lineage markers of T cells (CD3), B cells (CD19) and NK cells (CD56). DCs can be classified either based on their expression of cells-surface molecules or on the basis of function and anatomical location. Five major types of DCs have been described, the CD11c⁺ CD123^{lo} myeloid DCs, the CD14⁺ DCs, the CD207⁺ Langerhans cells, CD1a⁺ dermal DCs and the CD11c⁻ CD123^{hi}, plasmacytoid DCs [41, 45, 46]. These subsets are further described below. In addition, we also describe the properties of *in vitro* generated monocyte derived DCs, given that it is this population that is most commonly used for therapeutic intervention.

1.2.1 Myeloid DCs (mDCs)

mDCs, also known as classical DCs, express cell surface molecules CD11c and CD11b, and are analogous to the mouse CD11c⁺ conventional DC subset. They lack the expression of CD14, which distinguishes them from monocytes and CD14⁺ DCs. mDCs can be further classified into two populations, the CD1c⁺ and CD141⁺ subsets, which are analogous to the mouse CD11b and CD8/CD103 subsets, respectively.

CD1c⁺ mDCs:

CD1c⁺ mDCs, also known as the BDCA-1⁺ DCs, are the major population of human mDCs, comprising around 1% of mononuclear cells [47]. They also express myeloid markers, CD11b and CD11c [48]. CD1c⁺ mDCs are found in peripheral blood as well as spleen, lymph nodes and other tissues that serve as interface with the environment, such as skin and gut mucosa. It is postulated that CD1c⁺ DCs from blood migrate into the tissues to give rise to tissue resident DCs, although these claims have been debated [49]. CD1c⁺ DCs have been found to express toll-like receptors (TLRs) 1-8 and have been shown to respond well to stimulation through TLR3, TLR4 and TLR8 by poly(IC), lipopolysaccharide (LPS) and R848, respectively [50]. CD1c⁺ DCs are potent stimulators of naive CD4⁺ T cells but have a limited capacity to cross-present antigen via MHC class I to CD8⁺ T cells [51-54].

CD141⁺ mDCs:

CD141⁺ mDCs, also known as the BDCA-3⁺ DCs, comprise around 0.1% of mononuclear cells [47]. They are found in blood as well as other lymphoid tissues, although to a lesser extent than the CD1c⁺ subset [52]. CD141⁺ DCs have high expression of TLR3 and TLR10 and have been found to express TLRs 1, 2, 6, and 8. However, they lack the expression of TLRs 4, 5, 7 and 9 [50, 52]. They respond strongly to TLR3 stimulation, producing Type I IFN, suggesting their involvement in anti-viral immunity [52]. CD141⁺ mDCs are homologous to mouse CD8 α ⁺ DCs and have an enhanced capacity to take up dead or necrotic cells via CLEC9A and to cross-present antigens via MHC class I to CD8⁺ T cells [51-55]. This subset is identified by their lower level expression of CD11b and CD11c, as compared to CD1c⁺ mDCs. They also express CLEC9A and XCR1, which are also markers of the mouse cross presenting subset [48, 56-58].

1.2.2 Plasmacytoid DCs (pDCs)

pDCs are a recently identified, small subset of DCs that, like mDCs, originate from the common DC progenitor (CDP) [59, 60]. They are the most abundant DCs found in blood but have also been observed in lymphoid organs and lymph nodes (LN). They resemble plasma cells in morphology and express B cell marker B220/CD45RA and do not express CD11c [61, 62]. In the steady state, pDCs have been found to express low levels of MHC class II and costimulatory molecules but were not able to stimulate the proliferation of naïve CD4⁺ T cells. On maturation, pDCs acquire DC-like interdigitating morphology, upregulate MHC class II and co-stimulatory molecules and are able to prime naïve T cells towards either a Th1 or Th2 phenotype [61-63]. They express high levels of TLR7 and

TLR9, which, on recognizing viral nucleic acids, induce the production of large amounts of type-I IFN. pDCs can be distinguished from mDCs by the lack of CD11c and the constitutive expression of CD123 (IL-3 Receptor) and BDCA-4 (neuropilin) [47].

1.2.3 Langerhans Cells (LCs)

LCs are a subset of DCs found as a reticular network in the supra-basal layer of the epidermis and other stratified squamous epithelia, accounting for 3–5% of total epidermal cells [39, 64]. They are characterised by the expression of MHC class II and high levels of the C-type lectin, Langerin (CD207). LCs can also be identified by the presence of birbeck granules in the cytoplasm, whose function is poorly understood, although it is speculated to be involved in antigen presentation [65].

LCs maintain a steady state population in the skin and are unable to self-renew but can be replenished by circulating monocytes when required [66]. On capturing antigens, LCs upregulate MHC class II and co-stimulatory molecules such as CD40 as well as the chemokine receptor, CCR7, which is known to be involved in DC migration. These CD11c⁺ MHC Class II^{hi} CD207⁺ CD40^{hi} LCs subsequently migrate to the draining LNs where they present antigen to CD4⁺ T cells to elicit an immune response [64]. LCs have also been shown to cross present exogenously-derived antigen to CD8⁺ T cells via MHC class I, to stimulate a cytotoxic response [67]. LCs act as sentinels in the skin, maintaining overall epidermal health by providing tolerance to commensal microorganisms and initiating an immunogenic response to pathogens invading the skin [41].

1.2.4 CD14⁺ DCs

CD14⁺ DCs are found in non-lymphoid tissues in small numbers. They express CD11c and MHC class II but do not express CD1c, CD141, CCR7 and co-stimulatory molecules [67, 68]. They were initially referred to as 'dermal type-DCs' as they were first observed in the dermis of the skin. However, this term is misleading as the dermis also contains CD1a⁺ DCs. These DCs also express macrophage markers such as CD163, making them difficult to differentiate from tissue macrophages [69].

CD14⁺ DCs have been shown to be tolerogenic in the steady state and are inefficient at stimulating the proliferation of naive CD4⁺ T cells, even upon maturation [70, 71]. They express very low levels of CCR7 and no chemotactic capacity in response to the chemokine CCL7, making their ability to migrate towards the LNs questionable [41, 68, 72]. Since CD14⁺ DCs have been shown to possess the capability to differentiate into LC [73] or macrophages [70], it has been suggested that they may be quiescent 'tissue monocytes' [41]. However, other groups have shown CD14⁺ DCs to have a role in priming follicular helper T cells, which can induce class switching in B cells [41, 67, 68, 74].

1.2.5 CD1a⁺ Dermal DCs

CD1a⁺ DCs are found near lymphatic vessels in the upper layers of the dermis [68, 75]. These DCs express MHC Class II, CCR7 and costimulatory molecule such as CD40 alongside CD1a but do not express CD207 and CD14, distinguishing them from LCs and CD14⁺ DCs. Unlike CD14⁺ dermal DCs, they have been shown to have a chemotactic response to CCR7 ligands and are capable of migrating to LNs. They have been shown to produce

significant quantities of IL-12 upon stimulation and are potent in stimulating the proliferation of naïve T cells in the allogeneic mixed leukocyte reaction (MLR) [68, 75].

1.2.6 In vitro-derived DCs or monocyte derived DCs

DCs have been obtained *in vitro* by differentiating CD14⁺ monocyte precursors obtained from peripheral blood mononuclear cells (PBMCs) using granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 [76]. These DCs, called monocyte-derived DCs (moDCs), express surface molecules typical of mDCs, CD11c, MHC class II and co-stimulatory molecules CD86, CD80 and CD40. Like mDCs, moDCs also possess the capacity to secrete cytokines IL-1 β , IL-6, TNF- α , IL-12 and IL-23 [77]. They upregulate the expression of MHC class II and costimulatory molecules on maturation with inflammatory stimuli and have been shown to possess the ability to potently stimulate the proliferation of naïve CD4⁺ T cells in an allogeneic MLR [41, 77]. Given their potent immunostimulatory capacity, moDCs have been extensively exploited in order to prime immune responses to various pathogens and tumour associated antigens and have, therefore, been used as potential DC vaccines, as elaborated below.

DCs can also be classified as non-lymphoid/migratory DCs, lymphoid/resident DC and blood/precursor DCs on the basis of their function and anatomical location [41]. The anatomical location of the above described DC subsets is depicted in Figure 1.1.

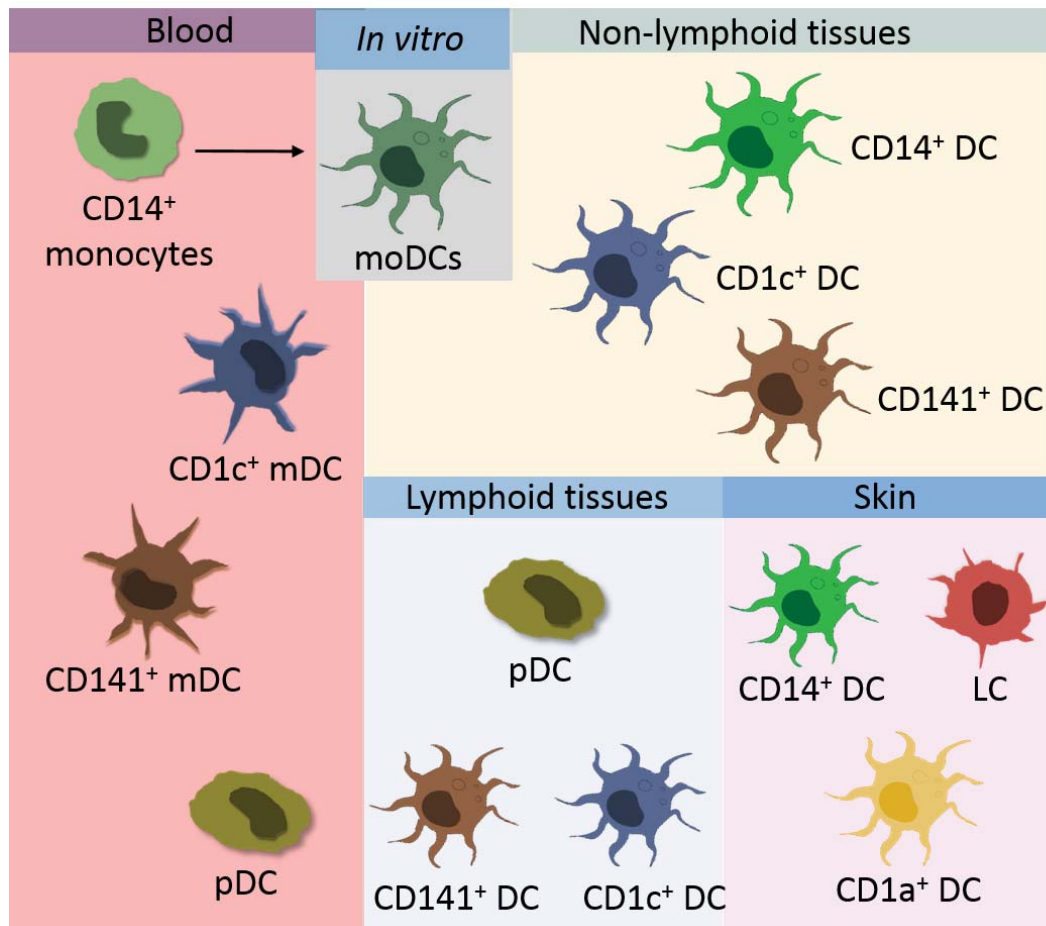


Figure 1.1- Distribution of DC subsets in blood, skin, lymphoid tissues and non-lymphoid tissues. CD141⁺ and CD1c⁺ mDCs (myeloid DCs) are found in blood, lymphoid and non-lymphoid tissues, whereas plasmacytoid DCs (pDCs) are found in blood and lymphoid tissues. Skin DCs consist of Langerhans Cells (LC), CD1a⁺ and CD14⁺ dermal DCs. CD14⁺ DCs are also found in non-lymphoid tissues. CD14⁺ monocytes present in blood give rise to monocyte-derived DCs (moDCs) *in vitro* in the presence of GM-CSF and IL-4.

1.3 The interaction between DCs and T cells

The interactions between DCs and T cells are central to the immune system, orchestrating the balance between tolerance and immunogenicity. To initiate an immune response, T cells must be activated by DCs, which provide the necessary signals required for the activation of naïve T cells that have not previously encountered their cognate antigen. For a long time, it was believed only two signals were required for the activation of T cells [78, 79]. The first signal is provided through the engagement of T cell receptor (TCR) with epitopes from antigens presented on MHC molecules expressed by DCs, leading to the formation of the immune synapse [80]. The second signal is provided by the subsequent engagement of co-stimulatory molecules within the immune synapse, a prime example being the interaction between CD80 or CD86 on DCs with CD28 molecules on T cells. This signal is critical for inducing immunogenicity, without which T cells become anergic. Subsequently, costimulation was also found to be required for the development of T cell anergy [81] and for the induction of T_{reg} cells [82]. Activation of T cells by DCs stimulates the production of a variety of cytokines by both the interacting cells. Many studies have demonstrated that the balance of these cytokines provides an essential third signal that determine the outcome of the T cell activation [83-85]. This led to the 'three-signal' theory of T cell activation that is well established today. The lack of one of the signals tips the balance of the immune response towards tolerance [78]. In the sections that follow, we elaborate on the role of each of the three signals in controlling the balance of immunity and tolerance.

1.3.1 Antigen processing and presentation

All nucleated cells express MHC class I and present endogenously-derived antigens to CD8⁺ T cells. Exogenously-derived antigens are presented by DCs, either directly via MHC class II to naive CD4⁺ T cells, a process termed 'conventional presentation', or via MHC class I to naive CD8⁺ T cells, termed 'cross-presentation' [43]. Cross-presentation plays a critical role in the recognition of tumours and tissue-specific viruses [43, 86, 87] and has also been implicated in central and peripheral tolerance to self-antigens (Figure 1.2) [88, 89].

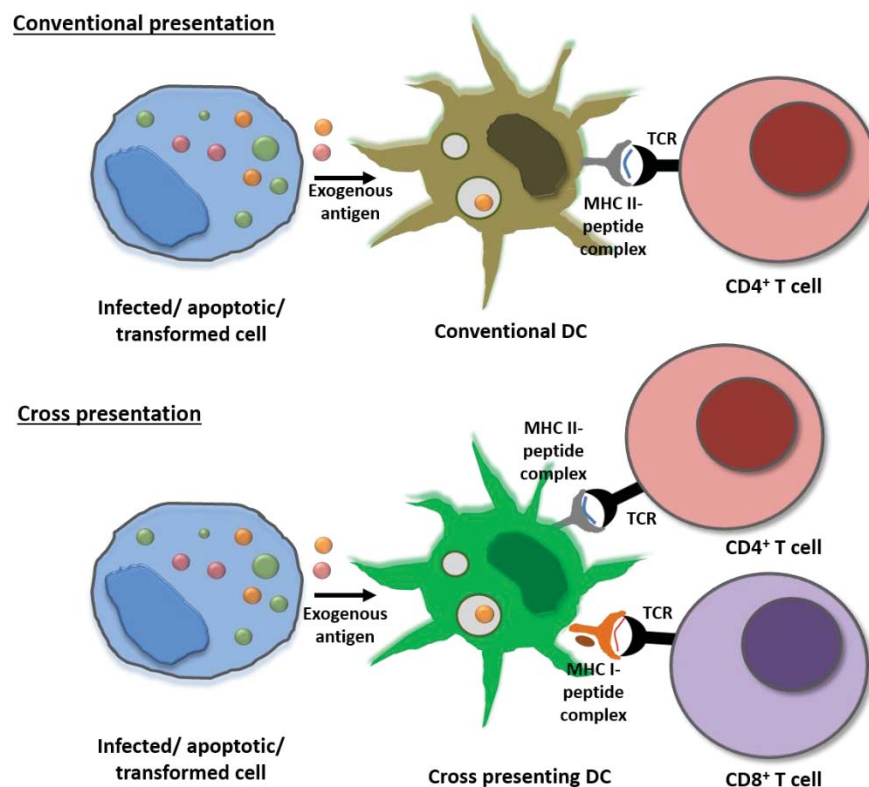


Figure 1.2 Presentation of exogenously-derived antigen by DCs. Top: Direct/Conventional presentation-dendritic cell acquires exogenous antigen from apoptotic/ infected or transformed cells and display the processed antigen in complex with MHC class II molecules to T helper cells. Bottom: Cross presentation: dendritic cells acquire exogenous antigen from apoptotic/ infected or transformed cells and display the processed antigen in complex with MHC class I molecules to cytotoxic T cells.

Mechanism of antigen presentation and cross-presentation

Endogenous proteins are degraded into peptides in the cytosol with the help of proteasomes. These peptides are translocated to the endoplasmic reticulum (ER) with the help of the transporter associated with antigen presentation (TAP), where they are trimmed into the right size with the help of ER aminopeptidases (ERAP). Peptides with appropriate binding motif then bind to MHC class I molecules that have been assembled in the ER and are stabilised by chaperone proteins, calreticulin, Erp57 and tapasin. On binding the peptide, the chaperone proteins are released, allowing the peptide-MHC class I complex to translocate to the surface of the cell, where the peptide is presented to CD8⁺ T cells [43, 90].

Exogenously-derived foreign proteins and peptides are internalized by either macropinocytosis, micropinocytosis, phagocytosis, receptor-mediated endocytosis or via gap junctions into endosomes, where they are cleaved by lysosomal proteases to give rise to peptides 10-16 amino acid long, suitable for binding to MHC class II [90]. The MHC class II molecules are assembled in the ER as a complex with the invariant chain (CD74), which protects them from prematurely binding high-affinity peptides from ubiquitous self-proteins. The MHC class II-CD74 complexes are transported to the antigen-containing endosomes, where CD74 undergoes lysis, allowing peptides generated from exogenous antigens to bind to MHC class II [90]. The MHC class II-peptide complex is then transported to the cell surface and presented to CD4⁺ T cells [90].

Cross-presentation is believed to occur by two pathways, vacuolar and cytosolic. In the vacuolar pathway, the exogenously derived antigen is processed by proteases in the endosomes, much like in MHC class II presentation. The peptides are then loaded onto

the MHC class I molecule which have been recycled from the cell surface into endosomes. The MHC class I-peptide complex is transported to the cell surface for presentation to CD8⁺ T cells [43, 91]. In the cytosolic pathway, the exogenously derived antigen, which has been internalised by endocytosis, is transported from the endosome to the cytosol, where it is degraded by proteasomes into peptides, in a manner reminiscent of the processing of endogenous antigen. These peptides are translocated to the ER via TAP and loaded onto nascent MHC class I molecules by the canonical endogenous pathway [43, 91]. There is strong evidence supporting both the cytosolic and vacuolar pathways for cross-presentation. The existence of the cytosolic pathway is supported by the discovery that cross-presentation of certain antigens does not occur in the absence of one of the proteasome subunits [43, 91, 92]. Conversely, the vacuolar pathway for cross-presentation is supported by the discovery that recruitment of ER-resident proteins to phagocytic compartments is critical for efficient cross-presentation [93]. Some researchers view the distinction of the mechanism of cross presentation into two distinct compartments as an oversimplification and have suggested that cross presentation can take place in either of the two compartments [43, 91]. According to the modular view of cross presentation, each step of the cross-presentation pathway (antigen processing, loading and transport), can occur in either the vacuolar, cytosolic or even a hybrid of these two compartments. The final pathway is thought to be dependent on the method by which the antigen has been internalized, as well as the nature and size of the antigen [43, 91].

Recent discovery of human cross presenting cells

The CD8 α^+ DCs have long been identified as the cross presenting DC subset in mice [94-96]. They express cell surface markers typical of DCs such as CD11c, CD86 and MHC class II and have also been shown to express c-type lectin-like receptor, CLEC9A and high levels of TLR3 as well as the chemokine receptor, XCR1 [56, 97]. CD8 α^+ DCs possess the potential to cross present exogenously-derived antigen to CD8 $^+$ T cells [43, 98]. The human counterpart of mouse cross presenting DC has been recently identified as the CD141 $^+$ DC subset [52-54]. These cells express cell surface marker CD141 (BDCA-3) and are found at low frequency in blood, tonsils and bone marrow [52]. The CD141 $^+$ DCs, like mouse CD8 α^+ DCs, also express XCR1, CLEC9A and high levels of TLR3. Like their mouse counterpart, CD141 $^+$ DCs have also been shown to secrete high levels of the pro-inflammatory cytokine IL-12 when activated [43]. CD141 $^+$ DCs have been shown to be more efficient at cross-presentation of antigen to CD8 $^+$ T cells than other human DC subsets [52-54] and are now recognized as the primary cross presenting DC subset in humans.

Cross-presentation allows DCs to acquire exogenous antigens, often from necrotic or apoptotic cells, and present it via MHC class I to CD8 $^+$ T cells. This process plays a critical role in the recognition of tumours and tissue-specific viruses that can bypass the antigen presentation pathway [43, 86, 87, 89, 99]. However, cross presentation also plays an important role in the induction of tolerance. Self-antigens cross presented by DCs in steady state, in the absence of co-stimulation or inflammatory stimuli, leads to deletion of autoreactive CTLs, a process termed cross-tolerance [43, 88, 100]. The recent identification of the human counterpart of murine cross-presenting dendritic cells

promises to facilitate the translation of the knowledge accrued from the study of mouse DC biology into clinical practice, rejuvenating interest in the use of DCs as vaccines for the induction of immunity against viruses and tumours [52-54].

1.3.2 Co-stimulation and inhibition

The interactions between T cells and DCs have been shown to be crucial for the initiation of adaptive immunity and for determining the balance between immunity and tolerance. As mentioned above, the interaction between T cells and DCs provides the context for T-cell receptor (TCR) ligation via peptide–MHC complexes on the DC surface. This leads to the formation of an immunological synapse and constitutes the first signal required for immune activation of T cells [78, 101]. However, the interaction between MHC and TCR alone is insufficient for the initiation of T cell responses. In addition to recognition of antigen, the formation of immunological synapse also results in various receptor–ligand interactions or co-stimulation, which helps regulate the outcome of T-cell activation. Co-stimulation involves reciprocal signals between T cells and APCs and is critical for the activation of naïve T cells, to prevent T cell anergy and to induce differentiation of naïve T cells into effector and memory subsets [102]. In addition to interactions required for the activation of a lymphocyte, inhibitory signals or negative costimulatory signals have been identified.

A number of co-stimulatory pathways have been identified including CD28-CD80/CD86, ICOS-ICOSL, CD40-CD40L and ICAM-1/LFA-1, to name a few [103]. The interaction between CD28 molecules present on naïve T cells with either one of its ligands, CD80 (B7.1) and CD86 (B7.2) expressed by DCs, is one of the best characterised costimulatory

pathways [104, 105]. This interaction is the primary signal required for the activation of naïve T cells and results in increased secretion of multiple cytokines, including IL-2, increased expression of anti-apoptotic proteins (such as bcl-xL) and increased chemokine production [104]. Immature DCs express low levels of CD80/CD86, which are upregulated on maturation with pro-inflammatory cytokines or through ligation of TLRs. The upregulation of CD80/CD86 enhances the costimulatory capacity of DCs and leads to more efficient activation of T cells. CD80/CD86 co-stimulation also upregulates CD40L expression, permitting T-cell help for B cells (18, 19). Thus CD28 co-stimulation by CD80/CD86 enhances and sustains T cell responses initiated by antigen recognition by promoting T cell survival and enabling their activation [106, 107].

Despite its importance in T cell activation, CD28 does not account for all co-stimulation in T cells. Inducible costimulatory (ICOS) receptor has been identified as an additional costimulatory axis. ICOS binds ICOS-Ligand (ICOSL) expressed on the surface on DCs. Unlike CD28, which is involved in the activation of naïve T cells, ICOS is expressed at very low levels in naïve T cells and has been shown to be primarily involved in the co-stimulation of memory and effector T cells, inducing the production of the effector cytokines such as IFN- γ , TNF- α and IL-4, but little or no IL-2 [102, 108].

Both CD80 and CD86 also bind cytotoxic T lymphocyte-associated molecule-4 (CTLA4), a receptor closely related to CD28, which has been shown to deliver inhibitory signals to T cells, down-regulating IL-2 production and aborting cell cycle advancement [109, 110]. CTLA-4 is not expressed on the surface of resting T cells but is up-regulated following CD4⁺ and CD8⁺ T cell activation and is involved in the negative regulation of T cell responses [111, 112]. Binding of CD80/CD86 to CTLA-4 inhibits T cell proliferation and IL-2

synthesis and is responsible for setting the activation threshold of T cells, which is crucial for the down-regulation of immune responses when the pathogen in question has been cleared [102]. T cells also express inhibitor receptor, programmed cell death-1 (PD-1), which like CTLA4, is closely related to the CD28 receptor. PD-1 interacts with its ligands, PD-L1 and PD-L2, expressed by DCs, regulating the maintenance of peripheral tolerance and attenuating infectious immunity. The PD-1- PDL-1 interaction has been shown to play a critical role in T cell anergy and induction of T_{reg} s [113]. Other inhibitory molecules such as Immunoglobulin-like transcript (ILT) 3 and ILT4, expressed primarily by tolerogenic DCs, have also been shown to play a role in regulating the balance between immunity and tolerance, as depicted in Figure 1.3 [114].

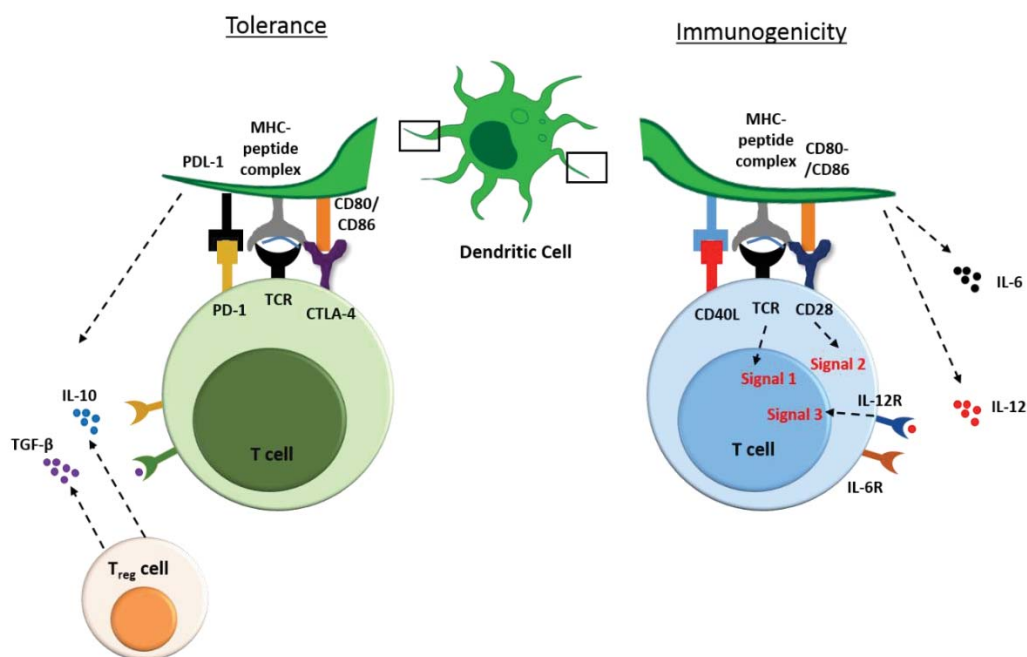


Figure 1.3 DC interactions with T cells in immunity and tolerance. RHS depicts interaction leading to immunogenicity. These included the TCR-mediated recognition of antigen presented on MHC molecule (Signal 1), co-stimulatory interactions between CD28- CD80/CD86 and CD40-CD40L (Signal 2) and presence of pro-inflammatory cytokines such as IL-6 and IL-12 (Signal 3). LHS: TCR-mediated antigen presentation in the presence of tolerogenic signals such as interaction of CTLA4 on T cells with CD80/CD86, presence of anti-inflammatory cytokines such as IL-10 and TGF- β and the interactions of PD-1 and PDL-1 lead to the induction of tolerance.

In addition to costimulatory and inhibitory signals, CD40-CD40 ligand (CD40L) interaction has been shown to be important in the bidirectional crosstalk between DCs and T cells. Both CD40 and its ligand, CD40-Ligand (CD40L or CD154) can be expressed by DCs as well as T cells. Ligation of CD40 on DCs has been shown to upregulate expression of costimulatory molecules and adhesion molecules as well as increase the production of IL-12, a cytokine involved in Th-1 polarization of naïve T cells [115-117]. The CD40-CD40L interaction has also been shown to be important in the 'licensing' DCs to break cross tolerance. When CD4⁺ T cells are activated, they up-regulate CD40L, which binds to CD40 receptor expressed on DCs, inducing the up-regulation of co-stimulatory molecules and MHC molecules, 'licensing' them to cross present antigen via MHC class I to CD8⁺ T cells to elicit an immunogenic response [118].

It has also been demonstrated that some of these co-stimulatory pathways work in a sequential manner and mutually reinforce one another. Interaction of CD80/CD86 on DCs with CD28 on the T cells has been shown to upregulate CD40L expression, which, in turn, binds to CD40 receptor on DCs, enhancing CD80/CD86 expression and reinforcing the CD28/CD40 positive feedback loop. Similarly, co-stimulation of CD28 induces the expression of ICOS, which interacts with ICOSL on DC, providing a second level of co-stimulation [102]. In this way co-stimulatory and inhibitory interactions between DCs and T cells shape the specific outcome of the interaction, and the nature of the subsequent immune response.

1.3.3 The cytokine environment

Cytokines are important mediators of the immune response, responsible for the communication between various immune cells. Although they can be secreted by almost any cell in the body, T cells and APCs are the two principal producers of cytokines [119]. The cytokines acting on a target cell can have either a synergistic or antagonistic effect. A cytokine can also be pleiotropic, having different biological roles in various tissues. The balance of pro-inflammatory and anti-inflammatory cytokines present in the local microenvironment during DC-T cell interaction determines whether antigen presentation and co-stimulation leads to the induction of tolerance or immunity, thereby providing the 'third signal' required for immune activation [85, 120].

DCs are known to have the capacity for production of a large repertoire of cytokines [121]. Immature DCs specialize in capturing and processing antigen, but possess limited capacity to stimulate T cells. In this state, DCs produce anti-inflammatory cytokine Transforming growth factor (TGF)- β and pro-inflammatory cytokines IL-1 α , IL-1 β , TNF- α , and low levels of IL-6 [39, 121]. DCs mature on exposure to microbial products such as lipopolysaccharide (LPS), CpG containing DNA, and double-stranded RNA. These microbial agents, called pathogen-associated molecular patterns (PAMPs) are recognized by various pattern-recognition receptors (PRR) including TLRs, leading to a cascade of signalling, eventually resulting in the upregulation of costimulatory and MHC molecules. This activates DCs into a state ready for T cell activation [122]. Maturation of DCs also leads to the change in their cytokine profile. On maturation with LPS and CpG containing DNA, DCs have been shown to up-regulate the production of IL-2, which plays an important role in T cell proliferation, immune activation and maintenance of homeostasis [123].

Maturation of DCs also leads to the increased secretion of pro-inflammatory cytokine such as IL-12, TNF- α , IL-1 β , IL-6, and type I IFN [39] which, along with antigen presentation and costimulation, leads to the activation of naïve T cells. It should be noted that the secretion of pro-inflammatory cytokines by DCs has been shown to reverse T_{reg}-mediated immune suppression. IL-12 in particular, is an important cytokine secreted by mature DCs and is critical in the polarisation of naïve CD4⁺ T cells towards a Th1 phenotype and activation of CD8⁺ T cells [124, 125]. Indeed, the balance of cytokines has been shown to be the deciding factor for the polarisation of naïve CD4⁺ T cells, also known as T helper (Th) cells to different phenotypes [71]. IL-12 skews the naïve CD4⁺ T cells towards Th1 phenotype whereas an excess of IL-4 results in a Th2 response [126]. TGF- β , IL-6 and IL-21 have been shown to co-operate to enhance Th17 commitment while the presence of TGF- β and IL-2 drives the differentiation of T_{regs}. These different Th cells produce cytokines characteristic of their subset. Th1 cells are known to secrete IFN- γ , TNF- α and IL-2, which are important in activation of macrophages [127]; Th2 cells secrete IL-4, IL-5 and IL-13 and play an important role in allergic responses and the eradication of extracellular pathogens. Th17 cells secrete IL-17, IL-21 and IL-22, which have a role in the recruitment of neutrophils to the site of infection and in the defence against extracellular bacteria and fungi [128]. T_{reg} cells are critical in the maintenance of peripheral tolerance, preventing autoimmunity and regulation of immune homeostasis [129]. They produce immunosuppressive cytokines such as IL-10 and TGF- β and up-regulate inhibitory molecules CTLA-4, which has been shown to suppress the ability of DCs to activate naïve T cells [130]. The polarisation of T helper cell subsets is illustrated in Figure 1.4.

CD8⁺ T cells also require signals provided by cytokines to develop optimal effector functions and undergo clonal expansion [78, 131]. In the absence of pro-inflammatory

cytokines, CD8⁺ T cells fail to expand, possess limited lytic ability and fail to produce a memory subset [132]. Several studies suggest that alongside antigen presentation and co-stimulation, the presence IL-12, IL-6 or Type I IFN are sufficient to provide the necessary signals for induction of efficient T cell immunity [131] [124].

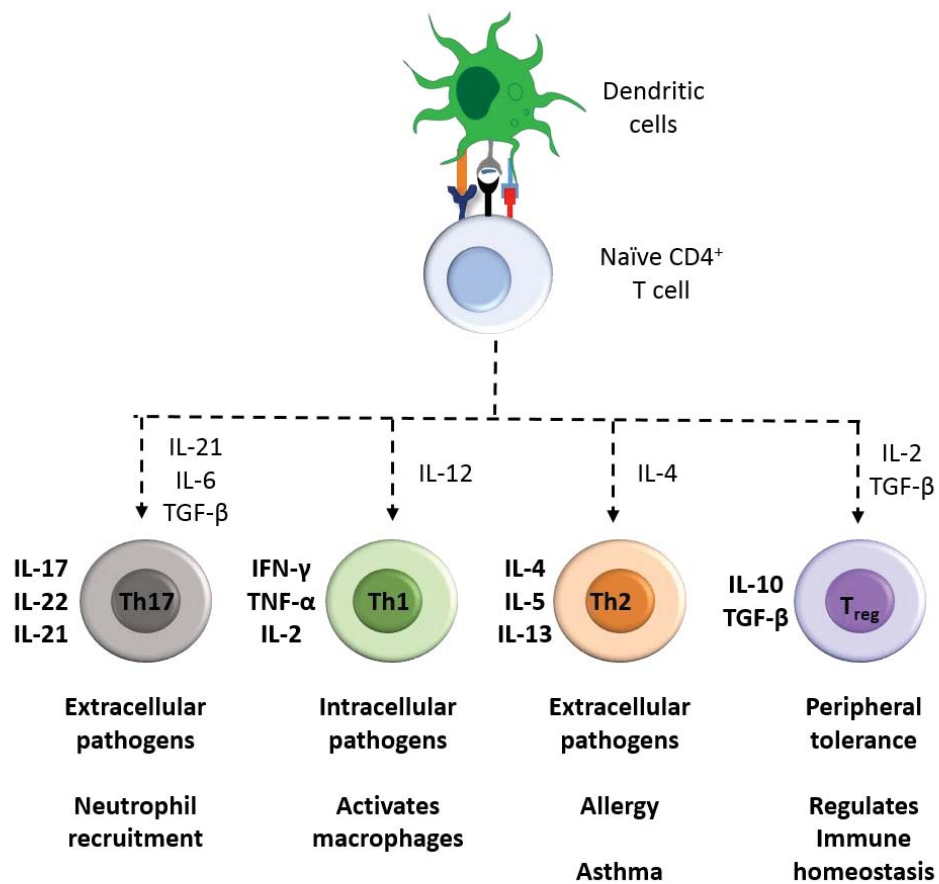


Figure 1.4 Polarization of CD4⁺ T cells and cytokines involved. Activation of CD4⁺ T cells by DCs in the presence of different cytokines can polarize the T cells into different effector subsets. T helper 1 (Th1) subset is obtained in the presence of IL12 whereas Th2 is obtained in the presence of IL-4. In the presence of IL-6, IL-21 and TGFβ, Th17 cells are obtained, whereas T_{regs} are obtained in the presence of IL-2 and TGF-β. The cytokines produced by each Th subset and their function is illustrated in the figure.

1.4 Exploitation of DCs in Immunotherapy

The ability of DCs to present exogenously-derived antigen to CD4⁺ and CD8⁺ T cells makes them an attractive target for immunotherapy. However, their trace numbers in peripheral blood and the inaccessibility of tissue resident DCs makes harnessing them for immunotherapy difficult. The first clinical trial utilising DCs was only made possible by the discovery of the method to isolate monocytes from peripheral blood and their *in vitro* differentiation into DCs [133, 134]. The result of this clinical trial, which involved the re-infusion of TAA-pulsed moDCs into the LNs of melanoma patients was promising, with 5 out of 16 recipients showing regression of metastasis in various organs [134]. A considerable number of clinical trials harnessing moDCs for therapeutic vaccination have been explored. In this section, we give an overview of the developments in use of moDCs for immunotherapy against melanoma and HIV-1 and outline the limitations hindering their success in these therapeutic approaches.

1.4.1 DCs in cancer immunotherapy

Despite the presence of tumour immunosurveillance, cancer cells still manage to evade the immune response by a variety of mechanisms [135]. Some cancer cells, being mutated self-cells, express tumour specific antigens (TSA), which can be recognized by CD8⁺ T cells. However, in many cases cancer cells down-regulate their expression of neo-antigens and MHC molecules, which allows them to escape detection by immune system [136, 137]. Furthermore, many tumours have been known to create a tolerogenic

environment through the secretion of immunosuppressive cytokines, such as IL-10 and TGF- β , or the up-regulation of immune checkpoint molecules such as PD-L1 [138], facilitating the development of T_{reg} cells or inhibiting the maturation of DCs.

Given the relative abundance of monocytes in peripheral blood and their ease of differentiation *in vitro*, moDCs have been the main source of DCs for cancer immunotherapy [17]. Over 54 clinical trials have been conducted in the 16 years since the first report of the use of moDCs for the treatment of metastatic melanoma [139] [134, 140]. Most of these trials involve pulsing moDCs with TAA or TSA, in the presence of a maturation signal, and their reinfusion into the patient, with or without an adjuvant. Early studies showed that injection of moDCs pulsed with antigen in the absence or maturation signals often resulted in the formation of IL-10 producing T_{reg} cells, confirming the importance of maturation of DCs in tipping the balance between tolerance and immunity [141, 142]. A statistical review of the 54 clinical trials conducted for melanoma led to the conclusion that moDC-based vaccines are safe, well tolerated and can induce the proliferation of CD4⁺ and CD8⁺ T cells specific to tumour antigens [143]. Although the induction of antigen-specific immune responses and tumour regression have been reported in a subset of the treated patients, the overall clinical outcome of these trials has, however, been disappointing, with less than 10% of patients demonstrating an objective response to these treatments [139] [140]. Although no significant correlations were made between trial parameters and final clinical outcome, a few trials showed a link between survival of patients and immunological response [139] [140]. The low efficacy of moDC-based therapeutic approaches is further illustrated by the use of Provenge, the first DC-based immunotherapy to be approved by the US FDA. Provenge, marketed by Dendreon, harnesses peripheral blood DCs obtained by leukapheresis for the treatment

of prostate cancer [144]. These DCs were pulsed with the prostate TAA, prostatic acid phosphatase (PAP) in the presence of GM-CSF and re-infused into the patients with two subsequent booster doses. Although enhanced T cell responses were observed in some patients, the treatment regime resulted in an average increased survival of 4.1 months as compared to placebo [144, 145].

1.4.2 DCs in HIV-1 immunotherapy

HIV-1 infection cripples the immune system due to its ability to diminish the numbers of CD4⁺ T cells, which are crucial for the immune response against exogenous antigens. [146]. Although combination anti-retroviral therapy (CART), the current therapy for HIV-1, has been shown to substantially alleviate the viral load and ameliorate disease symptoms, it fails to eliminate sanctuary reservoirs of HIV-1 in memory CD4⁺ T cells, tissue macrophages and microglia and does not mitigate the risk of transmitting the HIV-1 to uninfected individuals [147-149]. The best hope of curbing the spread of HIV-1 is, therefore, through the development of a prophylactic vaccine. Despite advancements in our understanding of the HIV-1 pathology since its identification 30 years ago, an effective vaccine for HIV-1 continues to elude us [150], the efficacy of antibody-based and T cell-based vaccines having proven disappointing [151-153]. The major challenges in the development of an effective vaccine for HIV-1 include high mutation rate of the virus and its rapid establishment of sanctuary reservoirs invisible to the immune system [150].

Although prophylactic vaccines have failed to live up to early expectations, therapeutic vaccines utilizing moDCs pulsed with SIV antigens have been shown to be effective in the Macaque-SIV model, stimulating a robust CD8⁺ T cell response [153, 154], suggesting that

antigen loaded moDCs may have the potential to activate T cell responses during HIV-1 infection in humans. Accordingly, autologous moDCs have been investigated for their efficacy for treatment of HIV-1 in many clinical trials. Although results from these trials suggest that moDC-based immunotherapy is feasible, safe and able to elicit HIV-1-specific immunological responses, only 2 clinical trials reported a reduction in the viral load of HIV-1 in patients [155]. These studies made use of autologous moDCs pulsed with either heat or chemically-inactivated autologous HIV-1 and administered to patients either subcutaneously or intravenously at 2 week intervals. [156][157, 158] [159]. Although vaccinations with moDCs were found to be feasible and well-tolerated, the results obtained were, once again, disappointing. Only a modest decrease in plasma viral load was observed in patients who received the vaccine as compared to those who received the control [155, 156, 159]. Any increase in HIV-1 specific T cells was also observed in the control group and correlated directly to increase in plasma viral load [155, 156, 159].

1.4.3 Limitation to the use of moDCs in immunotherapy

Most clinical trials utilizing moDCs exhibit significant inter-trial variability between outcomes, [140, 160] which may be, in part, due to the donor-to-donor variability in yield and quality of moDCs. In the case of cancer vaccination, this is further compounded by long-term exposure to chemotherapeutic agents [17], while in HIV-1, moDCs have been shown to be susceptible to viral infection, possibly compromising their functional integrity [145]. Furthermore, the limited capacity of moDC to cross-present antigen to CD8⁺ T cells, which is essential for an effective immune response against viruses and tumours, may also explain the marginal success of using moDCs in these clinical trials.

The ability of the recently identified CD141⁺ XCR1⁺ human DC subset to cross present exogenously supplied antigen to CD8⁺ T cells make them ideal for application in immunotherapy. However, their trace numbers in peripheral blood (less than 0.1% of peripheral blood monocytic cells [52]) preclude the clinical translation of this approach. Alternative sources of obtaining this cross presenting DC subset, such as differentiation from haematopoietic progenitors and humanised spleen, have been reported. However, the yield from these methods is still extremely low, hindering the full exploitation of DCs in clinical trials [51, 161].

1.5 Exploitation of T_{reg} in immunotherapy

Regulatory T cells (T_{regs}) are a subset of CD4⁺ T lymphocytes characterised by the expression of IL-2 receptor α chain (CD25) and the transcription factor Forkhead box Protein 3 (FoxP3) [162]. They secrete high levels of immunosuppressive cytokines, TGF- β and IL-10 and suppress the proliferation and functions of a variety of immune cells including T cells, natural killer (NK) cells, B cells and APCs [163]. There are three major subsets of T_{reg} cells, the thymus-derived natural T_{reg} (nT_{regs}), peripherally-induced, iT_{reg} and type 1 regulatory T (Tr1) cells. nT_{regs} are selected during T cell development in the thymus and continue to persist in the periphery where they play a critical role in maintaining tolerance to self-antigens. iT_{regs} are derived from conventional CD4⁺ T cells, which have up-regulated the expression of CD25 and FoxP3 following TCR engagement in the

presence of environment factors such as suppressive cytokines or tolerogenic DCs. iT_{regs} are thought to restrict responses to harmless foreign antigens and have been shown to play an important role in fetal-maternal and oral tolerance [164]. Tr1 cells, like iTregs, are also induced in the periphery, but do not express FoxP3 and secrete large amounts of IL-10 [165-168].

The role of CD4⁺ CD25⁺ FoxP3⁺ T_{reg} cells in maintaining immune homeostasis, inducing tolerance to self-antigens and preventing excessive immune responses makes them ideal for exploitation in immunotherapy for human autoimmune diseases, chronic inflammation and in graft versus host disease (GVHD) [163, 169]. In the thirty years since the first description of suppressor cells [170], many advancements have been made in the understanding of T_{regs} biology as well as their exploitation in the induction of tolerance. Various studies have investigated the induction of tolerance via the adoptive transfer of T_{regs} or their endogenous activation *in vivo*.

1.5.1 Adoptive transfer of Tregs

Adoptive transfer of T_{regs} involves the isolation of naturally occurring autologous T_{regs} (nT_{regs}), their expansion *ex vivo* and subsequent reinfusion into the patient to induce tolerance to autoimmune diseases or to organ allograft. T_{reg} cells have been isolated from banked umbilical cord blood (UCB) as well as peripheral blood using magnetic beads or by flow cytometry assisted cell sorting [36, 171]. Since T_{regs} make up a very small proportion of peripheral blood (about 5–10% of CD4⁺ T cells), they need to be expanded for adoptive transfer [172]. T_{reg} cells are usually expanded *ex vivo* using the anti-CD3/CD28 microbeads or cell-based artificial APCs, in the presence of recombinant IL-2 [173, 174]. These T_{regs}

are analysed for phenotype and function prior to reinfusion back into the patient. The successful application of adoptive transfer of nT_{regs} has been hindered by the lack of specific cell surface markers uniquely identifying T_{reg} cells. The expression of FoxP3 in humans is not restricted to the T_{reg} population [175] and its identification requires permeabilization of the cells which renders them non-viable and is, therefore, unsuitable for clinical applications. CD25, another marker for T_{reg} cells is also transiently expressed by naïve CD4⁺ conventional T cells and is upregulated by all T cells upon activation [176]. The contamination of adoptively transferred cells with effector T cells may undermine any beneficial effects. To bypass this issue, T_{regs} have also been induced *ex vivo* from CD4⁺ CD25⁻ naïve T cells with the help of TGF- β in the presence of IL-2 and IL-10. Vitamin D3, indoleamine 2, 3-dioxygenase (IDO) and retinoic acid have also been used instead of IL-10 [176, 177]. Although some studies have indicated that iT_{regs} are more tolerogenic than nT_{regs}, the induction of T_{regs} *in vitro* is rarely 100% efficient and some conventional CD4⁺ T cells may still be present.

Despite the potential concerns with T_{reg} purity and the lack of consensus regarding clinical considerations of dose, timing and site of T_{reg} administration during adoptive transfer [178], many clinical trials to test the therapeutic potential of T_{regs} have been conducted. In the first clinical trial, bead purified nT_{regs} were adoptively transferred to five HSC transplant recipients in an attempt to suppress GVDH [13]. Although the number of cells infused were later found to be too low to have a profound effect on the clinical outcome of the patients, this trial demonstrated that *ex vivo* purified T_{regs} can be administered safely and are well-tolerated [179]. Larger Phase I/II trials employing UCB-derived and peripheral blood-derived T_{regs}, which were expanded using anti-CD3/CD28-coated beads and IL-2, were subsequently investigated. Although these expanded nT_{regs} were well

tolerated and reduced the incidence of GVHD (from 61 to 43%), the overall clinical outcome was not found to be significantly different [180, 181]. Furthermore, *in vivo* expanded T_{regs} have been shown to be unstable, possessing the potential to revert to conventional effector T cells [36, 177], which would result in rejection of the cells or aggravation of GVHD [36]. Adoptive transfer of large numbers of non-antigen specific *in vitro* expanded T_{regs} may result in systemic immunosuppression leading to opportunistic infections and increase in incidence of neoplasia [36].

1.5.2 *In vivo* stimulation of T_{regs}

In the past, most approaches to inducing tolerance *in vivo* focused on generalised immunosuppression, usually with the help of mAbs, such as CAMPATH-1 and anti-CD3, for the depletion of T cells [182]. Recent data suggests that these antibodies selectively deplete effector T cells and also induce the generation of $CD4^+ CD25^+ FoxP3^+ T_{regs}$ [182-184]. Although treatment with these antibodies result in transient depletion of T cells, the effect on autoimmune disease and graft tolerance persists, suggesting they can restore immune homeostasis [182].

Anti-CD3 mAbs have been shown to induce cell death of effector T cells and inactivate helper T cells while sparing nT_{regs} , altering the balance of the immune system towards tolerance [182, 185]. Various anti-CD3 mAbs have been investigated for the treatment for type 1 diabetes and other autoimmune disorders. Recent clinical trials investigating the use of anti-CD3 mAbs, such as oteelixumab and teplizumab, for the treatment for type-1 diabetes showed an increase in T_{reg} cell function and a decrease in the production of the proinflammatory cytokine, IFN- γ , resulting in improved clinical parameters for 1-2 years

[186-188]. Treatment with anti-CD4 antibody which targets Th cells has also been shown to increase the survival of cardiac allografts, an effect which has been correlated with the induction of T_{regs} *in vivo* [189, 190]. Other mAbs such CAMPATH-1 (anti-CD52) and anti-CD45RO have been shown to alter the balance of effector and regulatory T cells, likewise leading to the induction of tolerance [191] [192].

A soluble CTLA-4-Fc fusion protein, Abatacept, has been shown to competitively bind to CD28 receptor on T cells, disrupting its interaction with CD80/CD86, which is critical for T cell the activation, depriving effector T cells of one of the three critical activation signal required for its full T cells activation. Abatacept has been shown to successfully alter the balance of effector and regulatory T cells and has been approved by the US FDA for the treatment of RA [182, 193].

Targeting cytokines, which is the 'third signal' required for the full activation of T cells, has also been shown to be effective in re-stabilising immune homeostasis. mAbs blocking pro-inflammatory cytokines such as TNF- α have been shown to be effective in the treatment of RA [5]. Human recombinant cytokines known to have an immunomodulatory fuction, such as IL-10 (Ilodecakin), TGF- β and low dose IL-2 have also been investigated for their use in immunotherapy [182].

Although the results from *in vivo* stimulation of nT_{regs} and induction of iT_{regs} have been shown to be promising, the therapeutic responses using these approaches are not durable, and require long-term continuation of therapy [194]. The re-establishment of immune homeostasis after targeting T cells with depleting mAbs may lead to skewed development of certain T cell clones and give rise to other immune disorders. Furthermore, the use of anti-TNF- α therapy for the treatment of RA has been shown to,

in some cases, lead to the development of multiple sclerosis [195]. Taken together, these drawbacks demonstrate the need for the development of a method to induce antigen-specific T_{regs} either *in vitro* or *in vivo*.

1.6 iPSCs as a novel sources of immune cells

Pluripotent stem cells have unlimited potential to self-renew and can differentiate into all somatic cell types. Since the first derivation of human ES cells in 1998, there has been much interest in their potential to produce diverse cell types for use in regenerative medicine, drug discovery and immunotherapy [196]. However, the ethical issues surrounding their derivation from the inner cell mass of the human blastocysts, as well as the constraints of using an allogeneic source of cells hinders their application in the clinic [17].

In Nobel prize-winning work, published in 2006, Takahashi and Yamanaka described the potential to derive pluripotent stem cells (PSCs) from adult mouse fibroblasts by retroviral transduction with genes encoding Oct3/4, Sox2, c-Myc and Klf4 [197]. A year later, these findings were replicated using human fibroblasts by two independent groups [198, 199]. These stem cells, referred to as induced pluripotent stem cells (iPSCs), were found to be similar to ES cells, possessing indefinite self-renewing capacity and unrestricted differentiation potential. Like ES cells, they developed into teratomas when transplanted under the kidney capsule of immune-compromised mice and were able to give rise to germline competent-chimeras. iPSCs offers a means of harnessing

pluripotency for regenerative medicine and immunotherapy without the ethical concerns that confound the use of ES cells [17, 197-199]. They also offer a way of bypassing the issue of immunological rejection of allogeneic stem cell-derived tissues faced by ES cells. Furthermore, the prospect of deriving these cells in an autologous fashion allows for pluripotency to be personalised, providing the opportunity to develop patient-specific treatments [17].

Since their initial discovery, many advances have been made in this field. iPSCs have been reprogrammed from a myriad of somatic cells including dermal fibroblast cells, pancreatic beta cells, mature B lymphocytes, DCs, and cord blood cells, to name a few [200]. Many tissues have also been successfully differentiated from iPSCs including cardiomyocytes, neurons, T cells, DCs and retinal pigmented epithelium. Furthermore, iPSCs have been successfully reprogrammed from patients with various diseases and subsequently re-differentiated into the cell types that are compromised by the disease in question, providing a method to study the mechanism and treatments of diseases that are difficult to recapitulate in animal models. iPSCs have great potential for use in regenerative medicine, modelling diseases, to screen candidate drugs and in immunotherapy. In this section, we outline the current challenges facing the field of iPSCs and the possibility of harnessing iPSCs for immunotherapy, focussing on their differentiation into T cells and DCs.

1.6.1 Limitations to the application of iPSCs

Despite the promise of iPSCs in the clinic, a few challenges still need to be addressed. Retroviral and lentiviral transduction, which are routinely used for the reprogramming of

somatic cells to iPSCs has been shown to lead to insertional mutagenesis which has the potential to cause leukemia and tumours [201]. Many efforts have been made to optimise the reprogramming process to increase the safety of the resulting cell lines. Non-integrating vectors, such as adenoviruses and episomal vectors, have been employed for the transient expression of reprogramming factors, Oct3/4, Sox2, c-Myc and Klf4. These vectors have been shown to successfully reprogram somatic cells into iPSCs, although with much lower efficiency than integrating viral vectors [202, 203]. iPSCs have also been successfully generated by the direct delivery of re-programming proteins into somatic cells [204] and transfection with synthetic mRNA [205], albeit at very low efficiency [17]. Small molecules, such as the histone deacetylase inhibitor and valproic acid have been shown to increase the reprogramming efficiency by up to 100-fold [206]. Recent work has demonstrated the successful reprogramming of adult fibroblast into iPSCs utilizing a combination of seven small molecules added in cell culture medium, suggesting that induced pluripotency may be achievable without genetic intervention [207].

The ability to generate iPSCs in an autologous fashion was expected to overcome the issues of immunological rejection hindering the application of ES cells in the clinic. In 2011, Zhao *et al* demonstrated the immune rejection of iPSCs in mice by syngeneic recipients [208], causing a huge impact to the pluripotent stem cell field. Work by another group suggested that the ectopic upregulation of *Hormad1* and *Zg16* genes, normally expressed only during embryonic development, may subsequently render the iPSCs immunogenic [209]. However, the reports of autologous iPSC rejection utilized undifferentiated iPSCs for the subcutaneous injection into mice. Any use of iPSCs in the clinic would require the *in vitro* differentiation of iPSCs into the relevant cell type prior to

its introduction into the patient. It is speculated that fully differentiated tissues would possess different immunological properties to their undifferentiated counterparts. The claim of rejection of autologous iPSCs was refuted eighteen months after its publication by Araki *et al*, who demonstrated the negligible immunogenicity of terminally differentiated cells derived from autologous iPSCs [210]. The issue of immunogenicity of iPSCs remains controversial in the field and is currently being investigated in our laboratory.

In addition to the issues of tumorigenicity and immunogenicity confounding the use iPSC, there is now an acknowledgment that cells differentiated from iPSCs are mostly immature and display neonatal or fetal-like characteristics [211-214], which may have an implication on their clinical applicability in regenerative medicine and immunotherapy. Many groups investigating the differentiation of iPSCs into red blood cells (RBCs) have reported that iPSC-derived RBCs express fetal hemoglobins and do not undergo efficient enucleation, which may impair their ability to efficiently bind oxygen molecules [215, 216]. iPSC-derived cardiomyocytes (CM) were also found to be similar to neonatal CM, expressing β -myosin heavy chain, a neonatal CM marker, which calls into question their potential for treatment of congestive heart failure and myocardial infarction [213]. Whether iPSC-derived fetal-like cells can undergo maturation within their site of engraftment remains to be determined [214]. The development of a method to enhance the maturation of iPSC-derived cells *in vitro* will be critical for their successful translation into the clinic.

1.6.2 Harnessing iPSCs for immunotherapy

Although much of the interest in iPSCs is focussed on applications in regenerative medicine and disease modelling, the translation of these approaches into the clinic has been slow, which may be explained by the difficulty faced in the precise delivery of iPSC-derived cells to the required site as well as the numerous safety issues, not least of which is their potential tumourigenicity and immunogenicity, described above [217].

Over the last few years, the successful differentiation of iPSCs into cells of the haematopoietic lineage, such as DCs [218], T cells [219, 220] and NKT cells [221] has been achieved. The opportunity to derive iPSCs in an autologous fashion, together with their propensity for genome editing make them attractive candidates for application in immunotherapy. Furthermore, the capacity for self-renewal greatly facilitates the scale-up of cell production, which may overcome many obstacles encountered using conventional sources of cells for immunotherapy [17]. The exploitation of iPSCs in immunotherapy is considered the 'low hanging fruit' of iPSC application since the success of immune mediation is not contingent on the prolonged survival of iPSC-derived tissues but on the immunological effect they cause [145]. For instance, once DCs have been administered, they interact with the appropriate T cells, resulting in the induction of either immunogenicity or tolerance, after which the demise of the DC is inconsequential to the success of the therapy [145]. In fact, the short life span of immune cells may prove to be beneficial from a safety perspective, minimizing the risk of potential tumorigenicity.

1.6.2.1 Differentiation of DCs from iPSCs

The recent discovery of the human cross presenting DC subset has rejuvenated interest in harnessing DCs for the induction of immunity against viruses and tumours as well as for induction of tolerance in organ transplantation and autoimmune disease [52, 53]. However, their trace numbers in peripheral blood (less than 0.1% of peripheral blood mononuclear cells) limits the clinical translation of this approach [52]. Several groups have demonstrated the successful differentiation of DCs from both murine and human ES cells, through formation of embryoid bodies (EBs) [222, 223]. These DCs were found to be functional, with the capacity to up-regulate co-stimulatory and MHC molecules on stimulation with inflammatory cytokines. Since the use of animal products is unsuitable for clinical applications, this protocol was later optimized for the differentiation of DCs in an animal product-free manner, compatible with their downstream use *in vivo* [224]. Work by other groups have demonstrated that functional DCs can also be terminally differentiated by co-culturing ES cells with OP9 bone marrow stromal cells with the addition of GM-CSF, although the use of OP9 cells, which were originally derived from mice, precludes the application of this method in the clinic [225]. Although ES cell-derived DCs were shown to be functional, possessing the capacity to process and present exogenously-derived antigen to CD4⁺ T cells, their clinical applicability is restricted by their differentiation from allogeneic sources, requiring the matching of HLA haplotypes before their potential use in the clinic.

Recent work in our laboratory has shown that CD141⁺ DCs can be successfully derived from human iPSCs using a cocktail of GM-CSF, stem cell factor (SCF), vascular endothelial growth factor (VEGF) and bone morphogenetic protein-4 (BMP4), in an approach

compatible with their downstream clinical application [218]. These DCs were found to be functional, capable of up-regulating co-stimulatory and MHC molecules on stimulation with a cocktail of pro-inflammatory cytokines and possessing the ability to stimulate the proliferation of naive allogeneic T cells in a dose-dependent manner [226]. iPSC-derived DCs were also shown to cross-present the exogenously supplied TAA, Melan A, to CD8⁺ T cells *in vitro*, stimulating a primary Melan A-specific cytotoxic T cell response [226]. Since iPSCs have indefinite capacity for self-renewal, this approach provides a potentially unlimited source of autologous DCs that may potentially bypass the issue of patient-to-patient variability [17].

1.6.2.2 Differentiation of T cells from iPSCs

As discussed above, adoptive transfer of T_{reg} cells has shown promise for treating autoimmune diseases such as SLE and RA and for inducing tolerance during organ transplantation. A few clinical trials investigating the therapeutic efficacy of this approach are currently underway [178, 227-229]. Although the results from clinical trials employing adoptive T cell transfer have been encouraging, a few challenges still hinder the widespread application of this technique. The lack of specific markers for identification of T_{reg} cells and their low frequency in the blood makes the purification of uncontaminated T_{reg} cells challenging [230]. Furthermore, in many cases, the adoptively transferred T cells have been shown to undergo exhaustion due to the rapid expansion regimes, and fail to persist *in vivo* [17] [231]. Given the drawbacks of current T cell-based immunotherapy approaches, the differentiation of potentially unlimited numbers of T cells from pluripotent stem cells has proven an attractive goal.

The first report of differentiation of T cells from pluripotent stem cells *in vitro* made use of ES cell-derived haematopoietic progenitors, cultured in fetal thymus organ cultures (FTOC). Subsequently, The successful differentiation of T cells were achieved using mouse ES cells co-cultured with the OP9 stromal cell line constitutively expressing delta-like ligand 1 (OP9-DL1) in the presence of Flt3 ligand (Flt3L) and IL-7. [83, 232]. However, these T cells were found to be immature and required a thymic microenvironment for TCR rearrangement and positive selection to give rise to mature CD4⁺ and CD8⁺ T cell repertoire. The FTOC-matured T cells were able to reconstitute the immune system of RAG2^{-/-} mice, suggesting that ES cell-derived T cells generated in this manner were functional *in vivo* [221, 233].

These findings suggest that stem cells may potentially serve as an unlimited source of naïve T cells for adoptive transfer. However, the requirement for a thymic microenvironment to support TCR rearrangement and positive selection poses a significant barrier to the translation of this technique to the clinic [17]. To address this hurdle, several groups have reported the successful reprogramming of antigen-specific human T cells into iPSCs and their successful re-differentiation into effector T cells [219, 220, 234]. These T cells were found to capture the V(D)J recombination of the T cell of origin, bypassing the need for thymic microenvironment for positive selection and pre-defining their antigen specificity [219, 220, 234]. Using this technique, tumour infiltrating CD8⁺ T cells, which can be readily obtained from tumour biopsies, can be reprogrammed into iPSCs and re-differentiated into unlimited numbers of antigen specific effector T cells for adoptive transfer for the treatment of cancer.

In 2012, T_{reg} cells were successfully differentiated from murine iPSCs transduced with FoxP3 expressing retroviral transduction, demonstrating the possibility of generating T_{regs} for adoptive T_{reg} transfer [230]. Effector T cells specific to antigens involved in graft rejection can be isolated and reprogrammed into iPSCs, providing an opportunity for their phenotypic reassignment into T_{reg} cells, this technique can be harnessed for immunotherapy of autoimmune diseases such as SLE, RA and MS as well as to provide tolerance to organ transplants. The prospects for generating iPSCs from patient-specific T cells and their re-differentiation into either effector or T_{reg} cells may provide a comprehensive approach to immunotherapy. Furthermore, the potential to scale-up production of iPSC-derived T cells and their tractability for genome editing may herald a new era in immunotherapy, in which treatments are tailored to the individual needs of the patient [17].

1.7 Aim of the thesis

Clinical trials investigating the use of DCs and T cells for immunotherapy have demonstrated the immune cell-based therapeutic approaches are well tolerated and effective. However, harnessing moDCs for use in the treatment of chronic infections like HIV-1 have proven disappointing due to their inability to cross present antigen. Similarly, harnessing *in vitro* expanded T_{reg} cells for adoptive transfer in autoimmune disease has

been hindered by the difficulty in obtaining large numbers of antigen-specific T_{regs} for administration.

The ever-improving technology of deriving iPSCs in a autologous fashion, their indefinite self-renewal, together with their tractability for genome editing make them attractive candidates for application in immunotherapy. In this project, we investigate two approaches of harnessing iPSCs in immunotherapy: firstly, in differentiating DCs for therapeutic vaccination against HIV-1; and secondly, in the generation of antigen specific T_{regs} for adoptive transfer to induce tolerance.

Differentiating DCs for HIV-1 immunotherapy

HIV-1 infection progressively diminishes the number of CD4⁺ T cells, crippling the ability of the immune system to eradicate the virus. Therapeutic vaccines are an attractive approach for elimination of residual reservoirs of infectious virus that remain following anti-retroviral therapy. Nevertheless, the use of moDCs has shown marginal success in humans, possibly due to their limited capacity to cross-present exogenous antigens from HIV-1 to naïve CD8⁺ T cells. Previous work in our laboratory has demonstrated that iPSCs derived from human dermal fibroblasts may be differentiated *in vitro* into DCs, we now investigate the potential of this novel source of DCs for vaccination to HIV-1. We characterise these cells with respect to their phenotype and investigate their suitability for therapeutic vaccination against HIV-1 by exploring their capacity to cross present exogenously derived antigen, including HIV-1 antigens, to CD8⁺ T cells. Given the immunosuppressive role of IL-10 in DC immunogenicity, we also investigate whether the

capacity of iPSC-derived DCs to secrete high levels of interleukin-10 might limit their immunogenicity.

Harnessing iPSC for the phenotypic reassignment of T cells

Given that use of T_{regs} for immunotherapy in autoimmune disease and GVHD is limited by the failure to obtain sufficient antigen-specific T_{reg} cells for adoptive transfer, we exploit the potential of iPSCs to generate antigen-specific T_{regs} for immunotherapy.

Given that mouse and human iPSCs have been successfully differentiated into T cells and work by many groups has shown the successful reprogramming of iPSCs from antigen-specific effector T cells. Building on these protocols, we finally aim to investigate the feasibility of immune-modulation through the reprogramming antigen-specific mouse T cells to iPSCs and their re-differentiation into antigen-specific T_{reg} cells. Here, we lay the groundwork for the above aim by investigating the feasibility of differentiating mouse T cell-derived iPSCs into $CD4^+ CD25^+ FoxP3^+ T_{regs}$.

Chapter 2

Materials and Methods

2.1 General protocols

2.1.1 Cell culture details

Biosafety cabinet and incubator: All tissue culture was conducted in a sterile biological containment level 2 safety cabinet. All cells were incubated at 37°C, 95% humidity and 5% CO₂, unless otherwise indicated.

Cell bank: All cells were placed for long term storage in cryovials in the vapour phase of a liquid nitrogen cylinder in the cell bank facility at the Dunn school of Pathology.

Culture media: All media were used at room temperature and stored at 4°C, unless otherwise indicated. All media was filter sterilized through a 0.22µm filter system.

Centrifugation: Cells were centrifuged at 300g for 5 minutes in a Sorvall Legend RT centrifuge, unless otherwise indicated.

Thawing cells: Cells were thawed by gently swirling the cryovial in a 37°C water bath until almost fully thawed. The appropriate media is added to the cryovial drop-by-drop and the contents were transferred to a 15ml falcon tube containing the appropriate media, centrifuged and then plated out in the required media on an appropriate dish, plate or flask.

Freezing cells: Cell pellet was resuspended in FBS (Life Technologies, 10828-028) supplemented with 10% DMSO (Sigma, D2650), unless otherwise stated to give a required density appropriate for the cell line, aliquoted into cryovials (1.8mls, Thermo Fisher, 375418) and snap frozen in dry ice for 30 minutes. The cells were stored overnight in -80°C and then transferred into liquid nitrogen for long term storage.

2.1.2 Medium for cell culture

The media used for cell culture are summarized below:

R10

RPMI (Lonza, BE12-702F/12) supplemented with 10% FBS, 50 U/ml penicillin (PAA laboratories, P11-010), 50 µg/ml streptomycin (PAA laboratories, P11-010), 2mM L-glutamine (PAA laboratories, M11-004), 1 mM Sodium Pyruvate (PAA laboratories, S11-003), 50 µM 2-mercaptoethanol (2ME; Sigma, M7522) and 0.1 mM MEM non-essential amino acids (NEAA; PAA, M11-003).

mTeSR-1

mTeSR-1™ Kit for the Maintenance of hESCs and hiPSCs (STEMCELL technologies, 05850).

MEF media

Dulbecco's Modified Eagle's Medium (Life Technologies, 10565-018) ,10% FBS, 1 mM Sodium pyruvate, 0.1 mM NEAA, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 mM 2ME.

Alpha MEM

Alpha MEM Powder (Invitrogen, 12000-014), 950ml distilled water, 2 g/L Sodium bicarbonate (Sigma, S5761), pH to 7.0-7.4 with 1N NaOH or 1N HCl.

OP9 media

Alpha MEM (as above), 20% FBS, 1 mM Sodium Pyruvate, 0.1 mM NEAA, 2 mM L-glutamine.

X6310 media

Iscove's Modified Dulbecco's Medium (Biowhitaker, BE12-722F), 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 50 µM 2-ME.

Mouse iPSC medium

Dulbecco's Modified Eagle's Medium, 15% KO-SR, 1 mM Sodium Pyruvate, 0.1 mM MEM non-essential amino acid, 2 mM L-glutamine, 50 mM 2-ME, 1000 U/ml leukemia inhibitory factor (LIF; Chemicon International, ESG1107).

Mouse embryoid body medium

Dulbecco's Modified Eagle's Medium, 15% Knockout Serum replacement, 1 mM Sodium Pyruvate, 0.1 mM NEAA, 2 mM L-glutamine, 50 mM 2-ME.

Mouse iPDC medium

Dulbecco's Modified Eagle's Medium, 15% FCS, 1 mM Sodium Pyruvate, 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 4% GM-CSF supernatant from X6310 cell line (equivalent to ~50U/mL of recombinant mouse GMCSF), 1000 U/ml recombinant mouse IL-3.

XVIVO -15 differentiation medium

XVIVO-15 (Lonza, BE04-418Q), 1 mM Sodium Pyruvate, 0.1 mM NEAA, 2 mM L-glutamine, 50M 2-mercaptoethanol.

R5h media

RPMI containing 5% human male AB serum (Sigma, H4522), 50 U/ml penicillin, 50 μ g/ml streptomycin, 2mM L-glutamine, 1 mM Sodium Pyruvate, 0.1 mM NEAA and 50 μ M 2-ME.

2.1.3 Flow cytometry

The buffers used for flow cytometry are detailed below:

- Washing Buffer- 5% heat-inactivated normal rabbit serum (Jackson ImmunoResearch, 011-000-120), 1 % BSA 0.1% NaN₃, 0.02% Ethylenediaminetetraacetic acid (EDTA, Sigma, E6635) in PBS.
- Blocking Buffer- 5% heat-inactivated normal rabbit serum, 1 % BSA, 0.1 % NaN₃ in PBS.

Method:

T cells and DCs were harvested by gentle pipetting. Human iPSCs were harvested by dissociation using 0.02% EDTA for 2 minutes at room temperature. All other cells were harvested using enzyme-free Cell dissociation buffer (Gibco, 13150-016). Cells were centrifuged, washed twice with washing buffer and passed through a 70 µm cell strainer to remove clusters. Cells were then incubated for 15 min on ice in blocking buffer + 1:1000 Fcγ Receptor blocking antibody (for mouse- AbD Serotec, BUF041B; for human- Cambridge bioscience, 422302). Cells were resuspended in washing buffer with antibody (-ies) at desired dilution (see antibody list- 2.4.1) for 30 minutes on ice. 7-AAD (Sigma-Aldrich, A9400) was added at a concentration of 250 ng/ml for the final 10 min of antibody incubation, if required. Cells were washed twice with washing buffer and fixed in 1% formaldehyde (Sigma-Aldrich, F8775) in washing buffer for at least 30 minutes before running on the FACSCalibur flow cytometer (BD Biosciences).

Intracellular staining for nuclear and cytoplasmic proteins were performed using commercial permeabilisation and fixation buffers (eBioscience, 00-5521), according to the manufacturer's instructions. In summary, cells were stained for surface markers as above and permeabilised using the Fix/Perm buffer at 4°C for minimum 1 hour and up to 24 hours. Cells were then washed in permeabilisation buffer (supplied in kit) followed by intracellular staining with antibodies diluted in permeabilisation buffer for 30 minutes on ice. Cells were then washed twice in permeabilisation Buffer and resuspended in 200 µl of PBS and fixed in 1% formaldehyde in washing buffer.

For the detection of intracellular cytokines, cells were treated with 10 µg/ml Brefeldin A (Sigma, B7651) and incubated for 3-5 hours at 37°C and 5% CO₂, after which cells were permeabilized and stained for intracellular cytokines, as above.

2.1.4 Cytokine release and ELISA (Enzyme Linked Immunosorbent Assay)

Mouse or human iPDCs (1×10^5 - 2×10^5 cells) were cultured for 18-24 hours in a 96 well round bottomed plate with or without stimulants. Supernatants were harvested and stored at -20°C until an ELISA assay was performed to detect the presence of cytokines. ELISAs were performed according to the manufacturer's instructions. Briefly, ELISA plates (costar, 9018) were coated with capture antibody and incubated overnight at 4°C . The wells were washed 5 times with washing buffer consisting of 0.05% Tween-20 (sigma, P137-9) in PBS. 100 μl of supernatants and standards (diluted as required) were added to each well for the detection of cytokine and incubated for 2 hours or overnight, followed by 5 washes with wash buffer. Detection antibody was added for 1 hour and washed, followed by the addition of Avidin bound to horse radish peroxidase enzyme (Avidin-HRP) for 30 minutes at room temperature. Plates were washed 8-10 times with wash buffer, followed by the addition of substrate for HRP, which was incubated at room temperature for 15 minutes (or until a range of colours developed in the standard wells). Stop solution (2N H_2SO_4) was added to each well and the absorbance at 450 nm was measured using a BioTek ELX808 plate reader.

The following kits were used in this thesis:

- Mouse IL-6 - Mouse IL-6 ELISA Ready-set-go (eBioscience, 88-7064-86)
- Mouse IL-10- Mouse IL-10 ELISA Ready-SET-Go! (eBioscience, 88-7104-22)
- Mouse IL-12 - Mouse IL-12 p70 ELISA Ready-Set-Go (eBioscience, 88-7121-86)
- Human IL-6 - Human IL-6 ELISA Ready-SET-Go (eBioscience, 88-7066)
- Human IL-8 Human IL-8 ELISA Ready-SET-Go (eBioscience, 88-8086-22)
- Human IL-10- Human IL-10 DuoSet ELISA Development Kit (R&D Systems, DY217B)

- Human IL-12- Human IL-12p70 ELISA Ready-SET-Go (eBioscience, 88-7126)

2.1.5 Magnetic sorting of cells

The buffers used for all these extractions are detailed below:

- Running Buffer- 2 mM EDTA, 0.5% BSA (Sigma, A3311) in PBS.
- Rinsing Buffer- 2 mM EDTA in PBS.

Method:

Miltenyi MACs technology was used for the enrichment or purification of cell subsets from iPDCs cultures, PBMCs or mouse spleens, according to manufacturer's instruction. Briefly, cells were counted, washed in PBS and resuspended in running buffer. Cells were then incubated with saturating concentration of the appropriate antibody cocktail for 15 minutes at 4°C, washed and either incubated with a secondary antibody if required. Cells were resuspended at a final concentration of upto 10^8 cells in 500µl and passed through an MS (Miltenyi Biotech, 130-042-201) or LS column (Miltenyi Biotech,130-042-401), which is placed in the magnetic field of a QuadroMACSTM manual separator. The bead-bound cells are retained in the column and the unbound cells can be collected in the eluent. Bead-bound cells can be collected by removing the column from the magnetic field and flushing out the bound cells using a syringe plunger (provided). This method can be used for enrichment of target cells type (positive selection) or depletion of unwanted cells (negative selection). Any variations to protocol for each cell type are recorded in relevant sections.

The different kits used are listed below:

- Pan T cell isolation kit II, human (Miltenyi Biotech,130-091-156))
- Naïve CD4⁺ T cell selection kit II, human (Miltenyi Biotech,130-094-131)
- CD14 microbeads, human (Miltenyi Biotech,130-050-201)
- Anti- FITC microbeads (Miltenyi Biotech, 130-048-701)
- CD141 DC isolation kit, human (Miltenyi Biotech, 130-090-633)

2.1.6 Proliferation assay - Mixed Leukocyte reaction

Mixed Lymphocyte Reaction (MLR) is an *in vitro* assay measuring the proliferation of lymphocytes from a donor (responder) in response to co-culture with allogeneic lymphocytes from another individual (the stimulator). As CD4⁺ T cells proliferate on recognizing foreign MHC II molecules, this assay is often used to determine if DCs can present antigen via MHC-class II to allogeneic CD4⁺ T cells. We assess the proliferation of T cells by staining CD4⁺ T cells with carboxyfluorescein succinimidyl ester (CFSE) prior to co-culture. Since CFSE is divided equally among progeny cells, it served a good marker for cell proliferation.

CFSE staining:

Naïve CD4⁺ T cells (or T cells from spleen in the case of mice) were counted and resuspended to a concentration of 2×10^6 cells per ml of pre-warmed PBS. 1 μ l of 10mM CFSE (eBioscience, 65-0850-85) was added to 10 mls of warm PBS. CFSE solution and cell suspension was mixed at the ratio of 1:1 and incubated at room temperature, away from light for 10 minutes. Equal volume of cold R10 was added and cells were incubated on ice in the dark for 5 minutes. Cells were washed twice with cold R10 and resuspended to 2×10^6 in R10.

Co-culture set up:

2×10^5 CFSE-stained T cells were plated in 100 μ l of R10 per well of 96-well round bottom plates. DCs were resuspended in R10 media and plated at top concentration of 5×10^4 cells/well to give the top ratio of DC: T cells of 1:4. DCs were serially diluted to give DC: T cells ratios of 1:4, 1:8, 1:16, 1:32, 1:64 and in some experiments 1:128. Co-cultures were plated in triplicates in a total volume of 200 μ l per well and incubated for 4 days at 37°C.

Flow cytometry analysis of proliferation:

The 96 well U-bottomed plate was centrifuged for 5 minutes at 300g and washed twice with FACS washing buffer. Cells were stained with CD3 antibody and corresponding isotype control for 30 minutes on ice (see section 2.1.3 for details of flow cytometry protocols). Cells were then washed twice in washing buffer and fixed with 1% formaldehyde in washing buffer for 30 minutes and analyzed by flow cytometry. CD3⁺ cells were gated to mark the T cell population. T cell cultured in the absence of DCs were used to determine the level of CFSE-staining of unproliferated T cells. T cells showing staining below that level were gated on in order to determine the percentage of proliferated cells. The gating strategy is shown in figure 2.1.

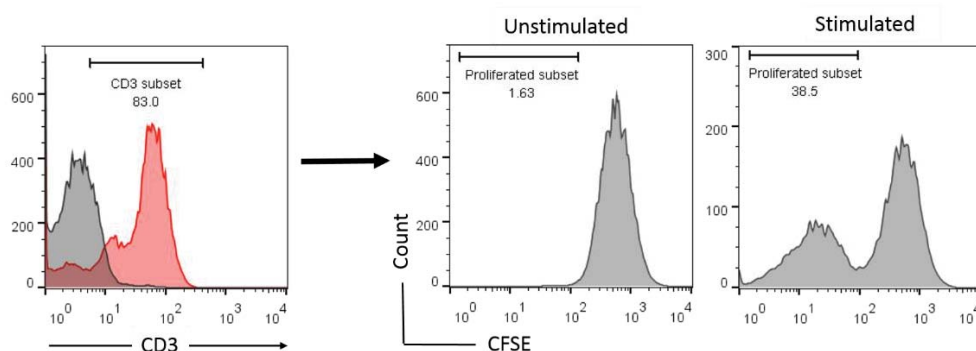


Figure 2.1 CFSE gating strategy. T cells were gated on using CD3 expression as compared to isotype control. Cells with level of CFSE staining below that detected by unstimulated T cell control populations (not co-cultured with DCs) were gated on to determine the percentage of proliferated cells.

2.1.7 Photomicrographs

Photographs of the cell cultures were taken with a SP-500UZ digital camera (Olympus, Shinjuku, Japan) on a CKX41 inverted microscope (Olympus), with 10X, 20X or 40X objective lens.

2.2 Human Protocols

2.2.1 Cell lines and cell culture

2.2.1.1 iPSC C15

The human iPSCs line C15 was a kind donation from Dr. Lee Carpenter at the Stem Cell Research Laboratory, National Health Service Blood and Transplant, Oxford, UK [235].

C15 cells were cultured in six-well plates coated with matrigel, with mTesR-1 media, as described below.

Matrigel coating:

1 vial of MatrigelTM (BD Biosciences, 356231) was thawed on ice and diluted 1:60 using KO-DMEM (Invitrogen, 10829-018). 2mls of this solution was used to coat each well of five six-well plates (Corning, BC010) for at least one hour. Plates can be used after one hour or stored in the fridge for up to 2 weeks.

Maintenance:

C15 iPSCs were cultured in six-well plates coated with matrigel (as above) with 2ml of mTeSR-1 (StemCell technologies, 05850) per well. Medium was replaced daily and cells were passaged every 6-7 days or at 80% confluency at 1:12 dilution using 0.02% EDTA in PBS (Sigma, E6635). Media was removed from wells and iPSCs were incubated with 0.02% EDTA in PBS for 30-60 seconds, before EDTA was removed and 1ml of mTeSR1 containing rock inhibitor (Y27632; Calbiochem, 688001) at a final concentration of 10 μ M was added to each well. The colonies were scraped into small clusters using a 25cm cell scraper (Corning, CLS3010) and transferred to a 15 or 50ml Falcon tube. Wells were washed with 1ml of mTeSR-1 to collect any remaining cluster, mTeSR-1 was added to give final volume enough for 1:12 split and 2mls of cell clusters were gently plated out into each well of six-well plates coated with matrigel. Cells were incubated at 37°C with 5% CO₂.

Freezing:

iPSCs were incubated for 2 hours with 1ml/well of mTeSR-1 containing 10 μ M of Y27632. The cells were then passage as stated above and frozen at the concentration of 1 well/vial in mTeSR-1 containing 10 μ M of Y27632 with 10% DMSO in 1.8ml cryovials.

2.2.1.2 Jurkat cells lines

CD8 1G4 Jurkat and unmodified Jurkat lymphoblast cell lines were obtained from Prof. Oreste Acuto's lab at the Sir William Dunn school of Pathology. Jurkat cells grow in suspension and were maintained in R10 in T75 flasks (Corning, 431464) at a cell concentration between 1×10^5 and 1×10^6 viable cells/ml. Cells were passaged every 2-3 days either by the addition of fresh R10 or by replacement of media. Cell density was not allowed to exceed 3×10^6 /ml. When required, 5×10^5 - 1×10^6 cells were frozen in R10 supplemented with 10% DMSO.

2.2.1.3 T2 GFP

T2 GFP cells was also obtained from Prof. Oreste Acuto. It is a GFP expressing HLAA2⁺ B lymphoblast cell line, deficient in transporter associated with antigen processing (TAP). T2 cells can be efficiently loaded with exogenous peptides and is useful for studying antigen presentation. T2 GFP cells were grown in suspension and were maintained in R10 in T75 flasks between density of 3×10^5 and 1×10^6 viable cells/ml. Cells passaged every 2-3 days either by the addition of fresh R10 or by replacement of media. When required, 5×10^5 - 1×10^6 cells were frozen in R10 supplemented with 10% DMSO.

2.2.1.4 CD4⁺ CCR5⁺ TZM-BL

CD4⁺ CCR5⁺ TZM-BL, a HeLa derivative cell line that expresses high levels of CD4 and CCR5, was a kind gift from Dr. Michael Moore, Sir William Dunn school of Pathology, Oxford. TZM-BL cells were cultured in MEF media in T25 flasks. Cells were passaged when 80% confluent, which is usually every 2-3 days by washing the flasks with PBS to remove residual serum and incubating the cells with 1ml of TrypLE express (Gibco, 12604) for 5 minutes at 37°C. Flasks were agitated to dislodge adherent cells which were transferred to a 15ml falcon tube, supplemented with MEF media and centrifuged for 5 minutes at 300g and plated into a fresh T25 flask at 1:10 dilution.

2.2.2 Isolation and maintenance of Primary Blood Mononuclear Cells (PBMCs)

Leukocyte cones were obtained from NHS-blood transfusion service. Peripheral blood (usually around 50-60 mls) was drawn from HLA-A2:01⁺ individuals by a trained phlebotomist. PBMCs were obtained from leukocyte cones or fresh blood by ficoll gradient centrifugation. Leukocyte cones were diluted 1 in 2 in PBS whereas fresh blood was not diluted. 15mls of blood was carefully overlayed on top of 5 mls of Ficoll-Paque Plus (GE Healthcare, 17-1440-02) and centrifuged at 900g for 20 minutes at room temperature with breaks set on low. The white PBMC interface was harvested carefully by pipetting using a 1 ml Gilson. PBMCs were transferred to a 50ml falcon tube and washed repeatedly with 50 ml of cold PBS, spinning at 300g for 5 minutes in between, until supernatants appeared clear.

2.2.2.1 Isolation of monocytes from PBMCs and generation of monocyte derived DCs (moDCs)

CD14⁺ monocytes were isolated by magnetic separation from PBMCs using human CD14 microbeads, according to manufacturer's protocol. Briefly, cells were labeled with CD14 microbeads and isolated by positive selection using protocol described in section 2.1.5. CD14⁺ monocytes were frozen for long term storage in FBS supplemented in 10% DMSO at the density of 10⁷/ vial.

moDCs were obtained by culturing CD14⁺ monocytes in R10 media containing 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 ng/ml IL-4 in 6-well plates at 37°C with 5% CO₂. moDCs are semi-adherent and were harvested by gentle pipetting with a 10mls pipette after 6-8 days.

2.2.2.2 Isolation of naïve T cells and pan T cells from PBMCs

Naïve CD4⁺ T cells or Pan T cells were obtained from the negative fraction of the above CD14⁺ cell separation, using the naïve CD4⁺ T cell selection kit II or pan T cell isolation kit, according to manufacturer's protocol. Briefly, cells were resuspended in running buffer after CD14 selection. T cells were isolated by depletion of other cells present in PBMCs. All other cells were labeled with a cocktail of biotinylated monoclonal antibodies for 10 minutes at 4 °C, washed, followed by incubation with anti-biotin microbeads for 15 minutes at 4 °C. T cells were enriched by negative selection using method described in section 2.1.5. The T cells eluted from the column were used in the MLR or frozen for long term storage in FBS supplemented in 10% DMSO at the density of 10⁷/ vial.

2.2.3 Differentiation of iPSC into iPDCs

DCs were differentiated from human iPSCs using protocols developed in the Fairchild laboratory with minor variations described below [218].

Set up:

Cells were expanded until approximate number of plates required for differentiation was achieved. To estimate total number of iPSCs in culture, one well of iPSCs was dissociated into single cells using TrypLEExpress and counted using a hemocytometer. Cells were then passaged using 0.02% EDTA, as stated in section 2.2.1.1, but without the use of rock inhibitor. Cell fragments equivalent to 3×10^6 cells were plated in each well of ultra-low attachment 6-well plates (Corning, CLS347) containing 4mls/well of mTeSR-1 supplemented with 50 ng/ml GM-CSF, 20 ng/ml stem cell factor (SCF), 50 ng/ml vascular endothelial growth factor (VEGF) and 50 ng/ml bone morphogenetic protein-4 (BMP4). The plates were gently rocked back and forth and side to side to ensure even dispersal of clusters across the well and incubated at 37°C and 5% CO₂.

Feeding:

Cells were fed every 2-3 days by replacing 2mls of supernatant with 2mls of XVIVO-15 differentiation medium supplemented with the above cytokines. The cytokines were successively removed from culture until only GM-CSF remained. BMP4 was removed from day 5 onwards, VEGF from day 14 and SCF from day 19. On appearance of monocytes (day 17-20), the medium was supplemented with 25 ng/ml of recombinant human IL-4. The concentration of IL-4 in the medium was increased to 100ng/ml as DCs accumulated in the cultures.

Harvesting iPDCs and Maturation:

DCs are semi-adherent and were harvested 24-28 days after the setup of differentiation by gentle pipetting using a 10ml pipette. Harvested iPDCs were passed through a 70 μ m cell strainer (BD Falcon, 352350) to remove embryoid bodies and plated at 1×10^6 cells per well of a six-well plate in XVIVO-15 differentiation medium supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4. After 3-4 days, DC were matured, if required for 48 hours with a cocktail of inflammatory cytokines consisting of tumour necrosis factor (TNF)- α (50 ng/ml), prostaglandin E₂ (PGE) (1 μ g/ml), interleukin (IL)-1 β (10 ng/ml) and interferon (IFN)- γ (20 ng/ml).

2.2.4 Phagocytosis assay

A fraction of DCs were fixed using 2% paraformaldehyde in PBS and incubated for 30 minutes at room temperature. 3% glycine was added and cells were centrifuged and washed 3 times in R10. Live and Fixed DCs were resuspended in 2 mls of R10, counted and plated into the wells of a 6 well cell bind plate at 4×10^5 per well. 5×10^5 FluoSpheres[®] carboxylate-modified microspheres with 2.0 μ m diameter and yellow-green fluorescence (505/515) (molecular probes, F8827) were added to each well, leaving some wells without beads as negative control, and incubated at 37°C, 5% CO₂. After 2 hours, DCs were harvested, washed twice in cold PBS and stained for CD11c, CD141 and XCR1 with reporter molecules at different wavelengths to microspheres as described in section 2.1.3 and analyzed for percentage of cells that have taken up microspheres, by flow cytometry.

2.2.5 Antigen processing assay

DCs were plated at 10^5 cells per well in R10 media into two 96-well U-bottomed plates (Nunc, 163320). Plates containing cells were either incubated at 37°C, 5% CO₂ or kept on ice. 100 µg/ml of DQOvalbumin[™] (Life Technologies, D12053) was added to each well and further incubated either at 37°C or on ice for 30 minutes. Some wells were left without DQovalbumin[™] as negative control. Cells were then washed twice in washing buffer and stained with CD11c, CD141 and 7AAD as described in section 2.1.3 and analyzed for percentage of DQovalbumin[™] positive cells by flow cytometry.

2.2.6 Antigen cross presentation to Jurkats

Mature iPDCs and T2GFP cells were resuspended in R10 to give final concentration of 1×10^6 per ml. 10^5 cells were plated in each well of 96-well U-bottomed plates and pulsed with 20mM or 90mM cancer testis antigen NYESO peptide, NY

NYESO-9V (NYESO-1₁₅₇₋₁₆₅-SLLMWITQV; from Dr. Oreste Acuto at University of Oxford) or 20mM NYESO recombinant protein (Thermo Fisher, 10283204) for 8 hours at 37°C, 5% CO₂ to allow antigen processing. 10^5 Jurkats (CD8 1G4 or normal) were added to each well as required and incubate for 12 hours at 37°C, 5% CO₂ after which supernatants were collected for human IL-8 ELISA (see section 2.1.4). Cells from wells spun down at 300g for 5 minutes, washed twice with PBS and stained for CD3, CD69, CD11c, and intracellular IL-8 following protocol in section 2.1.3 and analyzed by flow cytometry. Experiments were set up in triplicate.

2.2.7 Cytokine release assay

Immature moDCs or iPDCs (2×10^5) were plated in 96-well U-bottomed plates in triplicates in R10 media. Cytokines or TLR agonists, 50 ng/ml TNF, 1 μ g/ml PGE₂, 10 ng/ml IL-1 β and 20 ng/ml IFN- γ , 3 μ g/ml ODN2216, 10 μ g/ml poly IC, 500ng/ml soluble CD40L and 5 μ g/ml R848) were added singly or in combination, as required. The final volume of each well was made up to 200 μ l with R10. Cells were incubated for 18 hours at 37°C, 5% CO₂ after which 150 μ l supernatants were collected for human IL-10, IL-12 and IL-6 ELISA (see section 2.1.4). Cells were pelleted, washed twice with wash buffer at room temperature and stained for CD11c, CD141, CD40 and/or CD80, as described in section 2.1.3 and analyzed by flow cytometry.

2.2.8 Cross presentation to CD8⁺ T cells

Immature HLA-A*02:01⁺ moDCs and iPDCs were harvested and resuspended in R5h media. DCs were plated at 4×10^4 per well of a 96-well plate (flat bottomed) and were either left untreated or pulsed with 1 μ M HIV-gag peptide (SLYNTVATL; Proimmune, p010-0A-G) or 1 μ M whole HIV-Gag p17 (Amsbio, HI1A-710-5). HLA-A*02:01-restricted naïve T cells were added to the culture at a ratio of 1:10 DC: T cells. The total volume of a well in the 96 well plate was made upto 200 μ l with R5h media and cultured at 37 °C, 5% CO₂ for 14 days. On day 5-6, cells may be transferred to 48 well plate if media turns yellow or cells are proliferating too quickly. Recombinant human IL-2 was added at 10 U/ml from days 4–7 (add everyday), and 500 U/ml for the remainder of the culture period (add every day). After 14 days, cells were harvested into falcon tubes, centrifuged, washed twice with PBS and stained with phycoerythrin (PE) conjugated HLA-A2:01 SLYNTVATL pentamer (Pro5[®] Recombinant MHC I Pentamer; Proimmune, F010-2A-G) at

1:5 dilution (10 μ L in total 50 μ L) at 37 degrees for 30 minutes. Cells were then washed with wash buffer at room temperature and stained for CD8 and CD11c, following protocols described in section 2.1.3 and analyzed by flow cytometry.

2.2.9 Chemotaxis assay

Experiments were carried out in a 16 well plate (CIM-16; Acea biosciences, 368032) on an xCELLigence Real-Time Cell Analyzer (RTCA) instrument (Acea Biosciences, 00380601050). Chemokines were diluted to desired concentration in 160 μ L final volume of chemotaxis buffer (RPMI + 0.5% BSA + 25 mM HEPES buffer) and loaded into the bottom of the well. The upper chamber was assembled with the lower chamber. 50 μ L chemotaxis buffer was added to the upper chamber and left to pre-equilibrate at room temperature for 30 minutes. Cells were resuspended in chemotaxis buffer at a concentration of 8x10⁶/ml. 50 μ L of cell suspension was added to the top chamber so that each well contains 4x10⁵ cells. The assembled plate was transferred to the RTCA instrument and data were collected every 5 seconds for 3000 sweeps [236]. DCs adhere to the underside of the filter as they move through the 8 μ m pores towards the bottom well containing the chemoattractant, producing a signal of impedance which is measured as cell index, which is an arbitrary unit, correlating to cell status. Cell Index (CI) is calculated based on the equation $CI = \text{Max}_{(i=1..N)} * [(R_{\text{cell}(fi)} / R_{\text{b}(fi)}) - 1]$, where N is the number of the frequency points at which the impedance was measured, $R_{\text{cell}(fi)}$ and $R_{\text{b}(fi)}$ are the frequency-dependent electrode resistances with cells present and without cells, respectively [237]. Increase in cell index correlates to increasing numbers of migrated DCs adhering to the underside of the filter [236]. Wells with no chemokine were used as negative control. Data was analysed using the RTCA software 1.2.1. The net migration of the cells was

measured in delta cells index, which correlates to the maximum CI- minimum CI during the migration period.

2.2.10 Gene expression – RT-PCR

RNA was isolated from 10^6 cells using SV Total RNA Isolation System (Promega, Z3105), according the manufacturer's instructions, with DNase digestion. RNA concentration was measured using a Nanodrop spectrophotometer. The quality of RNA was determined by the 260:280 ratio of RNA, which should be around 2.0. Reverse transcription of total RNA into cDNA was performed using the EZ-first strand cDNA synthesis kit (GeneFlow, 20-800-50) as per manufacturer's instructions. 300µg of total RNA from each sample was used for each cDNA synthesis with Oligo-dT primers. To determine expression of CCR5 and housekeeping gene, GAPDH, 0.2mM dNTP, 2mM MgCl₂, 250nM forward primer, 250nM reverse primer, 2µL of cDNA and 0.2 µL 1x GoTaq DNA polymerase (promega, M8301) was made up to volume of 25 µL using deionized water and run on the thermocycler using the following parameters: 94 °C for 3 min, 40 cycles of 94 °C for 40 seconds, 62 °C for 40 seconds, 72°C for 30 seconds; followed by 5 minutes of final extension at 72 °C. PCR products were separated on a 1.5% agarose gel containing 0.5µL/mL ethidium bromide (Sigma, E1385) at 70V for 1 hour, alongside 100bp DNA ladder (Thermo Scientific, SLL-100S-LD). The sequence of primers are given in the table below.

Primer	Sequence 5'-3'
CCR5 forward	ACATCCGTTCCCCTACAAGA
CCR5 reverse	CTGCATTTGCTTCACATTG
GAPDH forward	AAGGTGAAGGTCGGAGTCA
GAPDH reverse	AATGAAGGGGTCATTGATGC

Table 1: Sequence of primers for CCR5 and GAPDH

2.2.11 MLR with IL-10 neutralizing antibody

MLR was performed following protocol set out in section 2.1.6 except with the addition of 10µg/ml of LEAF™ Purified IL-10 antibody (Azide-Free, 0.2 µm filtered; Biolegend, 506810) for the neutralization of IL-10 bioactivity. Anti-dog CD4 with Rat IgG2a isotype, a kind gift from Prof. Herman Waldman, was used as negative control at the same concentration.

2.2.12 Infection of cells with HIV-1 virus

To detect the ability of HIV-1 to infect DCs and TZMBL cells, we used two non-replicating GFP reporter viruses, R5 tropic HIV-1 with a JRFL envelope (HIV-1 HIG-GFP-JRFL) and HIV-1 containing vesicular stomatitis virus envelope glycoprotein, (HIV-HIG-VSVG). Vesicular stomatitis virus envelope glycoprotein (VSV-G) has a distinct advantage over other retroviral envelopes, allowing the virus to infect most cells, serving as a positive control for infection [238]. We also investigated the presence of luminescence in the cells after being treated with R5 tropic virus, HIV- NL4.3 Luc, which has been engineered to express luciferase enzyme. All three viruses were kind donations from Dr. Kenny Moore (Sir

William Dunn School of Pathology, Oxford). Cells were spinoculated in 96-well plates with 100µL of virus at 2000g for 90 min at 37°C and incubated for 2hrs in the incubator, before the media containing virus was removed and replaced with 200µL of media. Infection was detected after 3 days by analysing GFP expression by flow cytometry or luminescence (in arbitrary units termed relative light units; RLU) using SpectraMax platereader (Molecular Devices).

2.3 Mouse experiments

2.3.1 Experimental mice

All mice used in this thesis were maintained in the specific pathogen-free facility at the Sir William Dunn School of Pathology, Oxford, in accordance with the Animal (Scientific Procedures) Act of 1986. The project license number is PPL 303049.

Mouse strain	MHC I haplotype	MHC II haplotype	Modification
CBA/Ca	H-2K ^k	I-A ^k	-
CBA.RAG1 ^{-/-}	H-2K ^k	I-A ^k	Do not possess mature B and T lymphocytes.
C57BL/6	H-2K ^b	I-A ^b	-
C57BL/6 RAG1 ^{-/-}	H-2K ^b	I-A ^b	Do not possess mature B and T lymphocytes.
C57BL/6J(B6).FoxP3 hCD2	H-2K ^b	I-A ^b	Cells expressing FoxP3 co-express hCD2 fusion protein at the cell surface
B6.129P2-il10tm1Cgn/J	H-2K ^b	I-A ^b	<i>Il10</i> -deficient mice

Table 2.2: Strains of mice used in thesis.

2.3.2 Derivation of Mouse embryonic fibroblasts (MEFs)

MEFs were prepared from day 13 to 14 embryos obtained from CBA/Ca pregnant female mice. The embryos were decapitated and the heart, liver, reproductive and digestive tracts (red tissue) were removed. The white tissues were washed in PBS, placed in 5mls of Trypsin-EDTA for 5 minutes and finely minced with a scalpel. The tissues were repeatedly disaggregated using a 5ml pipette. The resulting cells were incubated at 37°C in trypsin-EDTA for 15 minutes and passed through a 70µm cell strainer. The cell suspension was plated out into T75 tissue culture flasks and cultured until confluency and passaged for expansion. The cells are harvested at P2, and frozen down in MEF media with 10% DMSO and stored long term in liquid nitrogen. When thawed, MEFs were cultured with MEF media, expanded and mitotically inactivated through exposure to 1 µg/mL solution of mitomycin C (MMC; Sigma M0503) for 3 hours for use as feeders.

2.3.3 Reprogramming of cells into iPSCs

Mouse iPSCs were generated with the help of Timothy Davies in the Fairchild laboratory (Sir William Dunn School of Pathology, Oxford). Cells were reprogrammed by introduction of the three transcription factors required for pluripotency, OCT4, SOX2 and KLF4. The transcription factors were packaged and delivered as separate retroviruses. Viral transduction was carried out in advanced DMEM+ 2% KO-serum replacement (SR), while continued culture was carried on in DMEM+ 15% KO-SR. pMX-based retroviral vectors in VSVG envelope individually containing Oct3/4, Sox2 and Klf4 transcriptional factors (kind donation from Dr. Sally Cowley, Sir William Dunn School of Pathology) were added to cultures of cells to be reprogrammed (at around 70% confluency) at the ratio of 3:1:1 (Oct3/4:Sox2:Klf4) and incubated for 24 hours, after which the infection was repeated.

Cells were incubated for 48 hours, after which medium in wells was replaced with NHDF to remove all viral particles. Cells were disaggregated (if required) to single cells using TrypLEExpress, as described earlier, and plated onto layers of mitotically inactivated mouse embryonic fibroblasts (MEFs) at a density of 8,000 cells per well of a 6 well plate. After 24hrs, Medium was replaced by mouse iPSC medium supplemented with 50µg/ml Vitamin C and 0.5µM valproic acid (VPA). On day 16, cells were passaged onto fresh 6 well plates coated with inactivated MEFs with by mouse iPSC medium supplemented with 50µg/ml Vitamin C. The cells were maintained at 37°C and 5% CO₂ until colonies of cells with a stem cell-like morphology appeared (around 21-25 days after first transduction). The colonies were individually picked, mechanically dispersed and plated onto fresh feeder cells for expansion into individual cells lines.

2.3.4 Teratoma assay

Teratoma assays were performed with the help of Timothy Davies in the Fairchild lab. The ability of iPSCs to give rise the 3 primary germ layers was determined by the ability of the iPSC line to give rise to a teratoma following transplantation under the kidney capsule of immune-compromised adult CBA.RAG1^{-/-} mice. 14 day old EBs were washed in PBS and kept on ice prior to implantation. The kidney capsule of the anaesthetised mouse was gently lifted away from the kidney and perforated with sharp forcep, and 3-4 EBs are pushed under the capsule using the side of the forceps. Teratomas were recovered after 21 days, rinsed in PBS, embedded in optimum cutting temperature (OCT) compound (VWR, 25608-930), cryosectioned and H&E stained for analysis.

2.3.5 Hematoxylin and eosin (H&E) staining

Teratomas were sectioned into 7µm slices by Richard Stillion at the Sir Willaim Dunn School of Pathology's Histology facility. The sections were stained for 20s with 0.5% Harris haematoxylin (Leica, 3803815E), washed in running tap water for 5 minutes and counterstained with 1% Eosin Y (Leica, 3801600) for 20s. The stained sections was dehydrated through an alcohol gradient from 70% to 100% ethanol and cleared in HistoClear II (National Diagnostics, HS-202) for 30s each. The slides were then mounted in dibutyl phthalate xylene (DPX) (Merck, 100579) and dried overnight at 37°C. H&E stained cryosections were viewed and photographed on a Nikon Coolscan system using 5X, 10X, and 20X objective lenses.

2.3.6 Cell lines and cell culture

2.3.6.1 MEFs

Mouse embryonic fibroblasts (MEFs) were cultured in T75 flasks in MEF media and passaged every 2-3 days using trypLEExpress, into T25 flasks at a dilution of 1:5. MEFs were mitotically inactivated by treatment with 10µg/ml MMC for 3 hours at 37°C to generate feeder cells for the culture of ESCs and iPSCs.

2.3.6.2 Mouse iPS and ES cell lines

Mouse iPSC and ESCs were cultured in T25 flasks coated with mitotically inactivated MEFs in mouse iPSC medium at 37° C incubator at 5% CO₂. Cells were passaged every 3 days using TrypLEExpress, usually at 1:10 dilution into T25 flask coated with inactivated MEFs or with 2% gelatin. Lines used are listed below, with comments regarding their origin.

Line name	Type	Comments
miPB6/2	iPS cells	Reprogrammed from MEFs of C57BL/6 WT mice
miPIL/6	iPS cells	Reprogrammed from MEFs of C57BL/6 IL-10 ^{-/-} mice
miPRT2-1	iPS cells	Reprogrammed from T cells of C57BL/6 mice
ESF116	ES cells	Derived from blastocyst of male CBA/Ca WT mice

Table 2.3: Mouse iPS or ES cell lines used in thesis

2.3.6.2 OP9 and OP9 DL1

OP9 is a mouse bone marrow stromal cell line that has been isolated by Kodama *et al.* from macrophage colony-stimulating factor (M-CSF)-deficient (osteopetrotic) mice [239]. The OP9DL1 cell line was generated via retroviral transduction of OP9 cells with a DL1-green fluorescent protein (GFP) [83]. These lines were kindly donated by Prof. Juan Carlos Zuñiga-Pflücker (Toronto, Canada) and are cultured on 10cm cell culture dishes in OP9 medium. Cells were grown to 80% confluency and split 1:4, every 2 days using 0.25% trypsin.

2.3.6.3 X6310

X6310, a kind donation from Prof. David Gray (University of Edinburgh), is a cell line that has been stably transfected with a vector to produce GM-CSF. They were grown in T75 or T175s in X6310 medium containing 1µg /ml G418 and passaged at a dilution 1:10 when 90% confluent using TrypLEExpress.

2.3.7 Primary cell isolation

2.3.7.1 Isolation of T cells from spleen:

Spleens were removed from mice and teased apart using forceps, in R10 and crushed through a 70µm filter using the blunt end of 2ml syringe. The cell suspension was added to an equilibrated 20ml syringe column containing 2 grams of nylon wool (Polysciences, 18369). The column was incubated at 37°C for 1 hour. Stromal cells and B cells selectively adhered to the nylon wool and non-adherent T cells were then eluted from the column after 1 hour using 35-50mls of warm R10. Red blood cells were subsequently lysed using Red Blood Cell lysis buffer (Sigma, R7757) and washed two times in R10, before being ready to use.

2.3.7.2 Isolation of bone marrow and generation of BMDCs:

Mouse femur and tibia were collected, cleared of tissue with the help of forceps and sterilized in 70% ethanol for 1-2 minutes. The bones were rinsed twice in PBS and the epiphyses were cut using a sharp scissor. The bone marrow was flushed out with R10 medium using a 25 gauge needle (Terumo, NN2516R) and a 20ml syringe (Terumo, SS20ES1). The cell suspension obtained was strained through a 70µm cell strainer and centrifuged. RBCs were lysed using red blood cell lysis buffer for 3 minutes, 2-3 volumes of R10 was added to the tube and centrifuged as above. The cells were resuspended in BMDC media and plated in a 10cm cell culture dish (Corning, 430167) (one leg was plated per dish), and incubated at 37° C and 5% CO₂. BMDC medium was replaced on day 3 and day 6 after plating and cells were ready for use on day 7. If required, BMDCs can be matured on day 6 using 1µg/ml lipopolysaccharide (LPS; Sigma, L3880).

2.3.8 Making GMCSF

X6310 cell line was grown in T75 containing X6310 media containing 1µg /ml G418 (Gibco, 11811-031) and expanded gradually in T175 flasks. Once expanded, G418 was removed from the media and the cells were allowed to proliferate until overconfluent. The supernatant in the flasks was harvested, filter sterilized and used as a source of GM-CSF. The conditioned medium can be stored at -80°C for long term storage. New batches of conditioned media were tested against old batches for ability to give rise to BMDCs.

2.3.9 Differentiation of mouse iPSC into DCs

Mouse iPSCs were passaged as mentioned above, onto T75 flasks, pre-coated with 2% gelatin for 30 minutes, usually at a dilution of 1:10. Cells were cultured for 2-3 days in fully supplemented DMEM medium containing 1000U/ml LIF, until sub-confluent. At least 2 passages of cells on gelatin were performed before the cells were differentiated into EBs. Cells were washed with PBS, trypsinized with TrypLEExpress for 5 minute and centrifuged. The pellet obtained was resuspended in 40ml of medium without LIF and plated in two 10 cm bacteriological culture dishes to allow formation of embryoid bodies (EBs). Medium was replenished every 7 days. After 14 days, 15-25 EBs were transferred to 10 cm cell culture dishes in 25mls of mouse iPDC medium. Medium was replenished every 7 days or when medium was exhausted, as judged by yellow coloration. DCs appeared around day 14-21 and were harvested by gentle pipetting. iPDCs were matured overnight with 1µg/ml LPS, if required, with a change in medium the next day and used after 48 hours.

2.3.10 Differentiation of iPS or ES cells into T cells

iPSCs were differentiated into T cells using the OP9 and OP9DL1 co-culture system established by the Zuñiga-Pflücker laboratory (Toronto, Canada) [240]. ESF116 or iPTR2-1 cell lines were grown on MEFs until 80% confluent and dissociated using 0.25% trypsin-EDTA, washed in PBS and resuspended in OP9 medium. Dissociated cells (5×10^4 to 10^5) were plated onto 10cm tissue culture dishes with 70-80% confluent OP9 cells in 9mls of OP9 medium and cultured at 37°C and 5% CO₂. The medium in the co-culture was replenished after 3 days. On day 5, the cells were trypsinized using 0.25% trypsin-EDTA as above for 4-5 minutes, resuspended in OP9 medium and re-plated onto culture dish for 30 minutes to allow stroma to adhere. The non-adherent cells were collected and filtered through a 40µm cell strainer (BD falcon, 352340), centrifuged at 4°C and resuspended in OP9 medium. 5×10^5 to 10^6 cells were plated onto 10cm dishes containing 70-80% confluent OP9 cells in 9mls of OP9 medium with 5ng/ml recombinant mouse Flt3-Ligand (rmFlt3-L). On day 8, the semi-adherent cells were harvested, strained through a 40µm cell strainer and centrifuged at 400g for 5 minutes at 4°C. Cells from each 10cm dish were resuspended in 3mls OP9 medium and seeded onto one well of 6-well plate containing 80% confluent OP9-DL1 cells with 5ng/ml rmFlt3-L and 1ng/ml mouse IL-7. The media was changed every 2 days and OP9-DL1 stromal layer replenished every 4 days using the method employed on day 8. By day 16, lymphocyte-like cells should be visible. The cells were harvested at day 20 onwards, without disrupting the stromal layer and stained for T cell markers as described in section 2.1.3.

2.3.11 Cytokine release assay

Immature BMDCs or iPDCs (10^5 cells) were plated in a 96-well U-bottomed plates in triplicates in R10 media. 1 $\mu\text{g/ml}$ LPS, 300ng/ml surface coated CD40-L or maturation cocktail (50ng/ml TNF- α , 1 $\mu\text{g/ml}$ PGE₂, 10 ng/ml IL-1- β , 20 ng/ml IFN- γ) were added, as required. Volume was made up to 200 μl with R10. Cells were incubated for 18 hours at 37°C, 5% CO₂ after which 150 μl supernatants were collected for mouse IL-10, IL-12 and IL-6 ELISA (see section 2.1.4).

2.3.12 Induction of Regulatory T cells

For iPDCs:

10^5 mouse iPDCs or BMDCs were plated in each well of 24-well plates with 10^6 allogeneic T cells (derived from spleen) in final volume of 2 ml of R10 supplemented with 100U/ml human IL-2, with the addition of 100nM Marilyn (male antigen DBY peptide₅₈₉₋₆₀₃ (NAGFNSNRANSSRSS)) peptide (a gift from Dr. Elizabeth Adams, Prof. Herman Waldman's laboratory, Oxford). 10ng/ml TGF β were added to every well except the negative controls. After 7 days, cells were harvested and stained for CD4, CD25 and FoxP3 to detect T_{reg} cells (See section 2.1.3) and analysed by flow cytometry.

For iPSC-derived T cells (IPT):

10^5 mouse IPT-derived T cells were plated in each well of 48-well plates with 1ml of R10 media supplemented with 100U/ml IL-2 and 10ng/ml TGF- β and CD3/CD28 coated beads (Life Technologies, 11452D) at 1:1 ratio to number of cells. Wells without TGF- β and CD3/CD28-coated beads were used as negative controls. After 5 days, cells were

harvested and stained for CD3, CD4, CD8, CD25 and FoxP3 to detect the presence of T_{regs} (details in section 2.1.3) and analysed by flow cytometry.

2.3.13 Genomic DNA extraction and gene expression

Four to Six EBs from each iPSC line were washed in PBS and digested with 50µL of 200µg/mL Proteinase K in digestion buffer (50mM KCl in 10mM Tris-HCl, pH 8.3, plus 0.1mg/mL gelatin, and 0.45% Tween 20) at 55°C for 5 minutes in a Techne TC-412 thermocycler. Proteinase K enzymatic activity was stopped by subjecting the samples to 95°C for 6 minutes. Digested EBs were added to an equal volume of master-mix of JumpStart REDTaq Ready Mix PCR Reaction mix (Sigma, P0982) and supplemented with 2µl of 10µM primers or autoclaved water. After an initial denaturing step of 4min 30s at 95°C; 35 cycles of denaturing at 95°C for 35s, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute was performed, followed by a final extension of 7 minutes at 72°C. PCR products were separated on a 2% agarose gel containing 0.5µL/mL ethidium bromide at 70V for 1 hour, alongside a 100 bp DNA ladder. The primers used for are described in the table below. Note that the primers for wild type IL-10 and mutant IL-10 have a common forward primer*, resulting in a band of 137 bp in the wild type, 312 bp in the KO and both bands in the cells obtained from heterozygous mice [241]. SRY primers for used for determination of sex and control primers IL3 and MyoG were used [242].

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Band size
Wild type	GTTATTGTCTTCCCGGCTGT	CTTGCACTACCAAAGCCACA*	137bp
MyoG	TTACGTCCATCGTGGACAGC	TGGGCTGGGTGTTAGTCTTA	250bp
Mutant	CCACACGCGTCACCTTAATA	CTTGCACTACCAAAGCCACA*	312bp
SRY	TGGGACTGGTGACAATTGTC	GAGTACAGGTGTGCAGCTCT	402bp
IL3	GGGACTCCAAGCTTCAATCA	TGGAGGAGGAAGAAAAGCAA	544bp

Table 2.4: Sequences of primers for genomic PCR

2.3.14 Karyotyping

Karyotyping was performed with the help of Dr. Edward Evans at the Department of Zoology, Oxford. iPSCs at day 3 of passage were dissociated with TrypLEExpress for 4-5 minutes, and centrifuged. The pellet was resuspended in fresh miPSC medium and plated in T25 flasks coated with fresh MEFs in 7mls medium and cultured overnight. A 40µL drop of 10µg/mL colcemid (Sigma, D1925) was added to the flask and incubated for 30 min. The cells were scraped off and briefly exposed to a hypotonic solution, and then fixed in methanol/acetic acid. Chromosome spreads were prepared and Giemsa stained for karyotype analysis. At least 30 G-banded mitotic cells were routinely scored for chromosome number, together with the sex chromosomes and the normality of the autosomes.

2.3.15 Enrichment of CD4⁺ T cells for reprogramming

T cells were derived from spleens of C57BL/6 Marilyn hCD2-FoxP3 KI RAG^{-/-} mice using method described in section 2.3.7.1, including enrichment using nylon wool column. The

cells obtained were enriched for CD4⁺ T cells using the EasySep™ Mouse CD4⁺ T Cell Isolation Kit (STEMCELL, 19852) according to manufacturer's instructions. In summary, cells were counted and resuspended in PBS with 2% FBS and 1 mM EDTA. Normal rat serum and CD4⁺ T Cell Isolation antibody cocktail was added to the cells as instructed and incubated for 10 minutes at room temperature. The RapidSpheres™ (provided in kit) were vortexed and added to the sample and further incubated at room temperature for 2.5 minutes. R10 was added to the polystyrene tube to make volume upto 3mls. The tube was placed in an EasySep™ magnet for 5 minutes. The negatively selected T cells were pipetted out carefully without disturbing the cells with beads attached the side of the tubes.

2.3.16 Fetal thymus organ culture

2.3.16.1 Depleting thymocytes from thymus:

Day 13 embryos were obtained from CBA/Ca pregnant mice with the help of Dr. Paul Fairchild. The embryos were dissected and thymus lobes were obtained and placed in a 10cm culture dish in a few drops of R10. Each thymus lobe was resuspended in 20µl of R10 containing 1.35mM of 2' deoxyguanosine (dGuo, sigma, D7145) in inverted Terasaki plates (Greiner, 653102) and cultured at 37°C 5% CO₂ for 7 days to deplete thymocytes. Small piece of sterile sponge soaked in R10 was added to the base of the Terasaki plates to maintain a humidified environment. The thymus was transferred to fresh drops in R10 and cultured for 2 days to remove traces of dGuo.

2.3.16.2 Repopulation of thymocyte depleted thymus:

iPSC-derived T cells were resuspended in R10 to a concentration of 2.5×10^6 /ml. 20 μ l of cell suspension was aliquoted to each well of Terasaki plates, so that each well contains 5×10^4 T cells. One thymocyte-depleted thymus was added to each well and the plates were incubated at 37°C, 5% CO₂, with the media being topped up every 2 days so that the thymus does not dry out. After 2 days, the thymus lobes were recovered and transferred to a piece of sterile 7 μ m filter disk, placed on top of a sterile sponge soaked in R10, in a 10cm dish containing 10mls of R10, to form an air-liquid interface which most closely recapitulates the thymic *in vivo*. The thymus was incubated for 7 days.

2.3.16.3 Harvesting repopulated organ cultured thymus

Thymus was harvested from the foam disk and placed in a 10cm dish with 2-3 mls of warm 1mg/ml Collagenase (Sigma, C2139) in R10 for 25 minutes at 37°C. The lobes were then disaggregated using a 26 gauge syringe needle. The thymocyte were then washed with R10 three times to remove any trace of collagenase and plated on a 10cm tissue culture plate for 1 hour to get rid of stromal cells. The non-adherent thymocytes were stained and analysed by flow cytometry as described in section 2.1.3.

2.4 List of antibodies and cytokines used

2.4.1 List of antibodies

Antibody Target	Target Species	Label	Isotype	Company	Catalog no.	Clone
CCR7	Human	PE	mouse IgG2a	Biolegend	353203	G043H7
CD11b	Human	PE	mouse IgG2a	Immunotools	21279114	MEM-174
CD11c	Human	FITC	mouse IgG1	AbD SeroTec	MCA2087F	BU15
CD11c	Human	APC	mouse IgG1	Immunotools	21487116	BU15
CD123	Human	PE	mouse IgG1	BD Pharmingen	555644	9F5
XCR1	Human	FITC	goat IgG	R&D systems	FAB857F	polyclonal
XCR1	Human	biotin	goat IgG	R&D systems	BAF857	polyclonal
CD14	Human	PE	mouse IgG1	Immunotools	21270144	MEM-18
CD141	Human	PE	mouse IgG1	R&D systems	FAB3947P	501733
CD207	Human	PE	mouse IgG1	Immunotech	IM3577	DCGM4
CD4	Human	FITC	mouse IgG1	eBioscience	11-0049-41	RPA-T4
CD40	Human	FITC	mouse IgG2a	AbD SeroTec	MCA1590F	LOB7/6
CXCR4	Human	APC	mouse IgG2a	eBioscience	17-9999-41	12G5
CD69	Human	APC	mouse IgG1	Biolegend	310909	FN50
CCR5	Human	PE	mouse IgG2b	R&D systems	FAB182P	45531
CD80	Human	FITC	mouse IgG1	SeroTec	MCA2071F	MEM-233
CD86	Human	FITC	mouse IgG1	AbD SeroTec	MCA1118F	BU63
HLAII DP DQ DR	Human	PE	mouse IgG2a	AbD SeroTec	MCA477PE	WR18
Nanog	Human	PE	goat IgG	R&D systems	IC1997P	polyclonal
Oct3/4	Human	FITC	mouse IgG2b	R&D systems	IC1759F	240408
SSEA-4	Human	PE	mouse IgG3	R&D systems	FAB1435P	MC-813-70
TLR3	Human	PE	mouse IgG1	eBioscience	12-9039-80	TLR3.7
TLR4	Human	FITC	mouse IgG2a	AbD SeroTec	MCA2061F	HTA125
TLR7	Human	PE	mouse IgG2a	R&D systems	IC5875P	533707
TLR8	Human	AF488	mouse IgG1	imgenex	IMG321AF488	44C143
TLR9	Human	PE	rat IgG2a	eBioscience	12-9099-80	eB721665
TRA-1-60	Human	FITC	mouse IgM	Millipore	FCMAB115F	TRA-1-60
HLAA2	Human	FITC	mouse IgG2b	AbD SeroTec	MCA2090F	BB7.2

Clec9A	Human	APC	mouse IgG1	R&D	FAB6049A	683409
CD8	Human	APC	mouse IgG2a	eBioscience	17-0086-42	OKT-8
Nanog	Mouse	AF488	mouse IgG1	BD Biosciences	560278	M55-312
Oct3/4	Mouse	AF647	mouse IgG1	BD Biosciences	560329	40/Oct-3
SSEA-1	Mouse	PE	mouse IgM	BD Biosciences	560142	MC480
I-A(b)	Mouse	PE	mouse IgG2a	BD Biosciences	553552	AF6-120.1
H2A(b)	Mouse	FITC	mouse IgG2a	BD Biosciences	553569	AF6-88.5
DL1	Mouse	PE	Arm hamster IgG1	eBioscience	12-5767-80	HMD1-5
FoxP3	Mouse	APC	rat IgG2a	eBioscience	17-5773-82	FJK-16s
CD3	Mouse	AF647	rat IgG2a	AbD Serotech	MCA500A6 47T	KT3
CD3	Mouse	PE	rat IgG2b	BD Biosciences	555275	17A2
CD4	Mouse	PE	rat IgG2a	BD Biosciences	553652	H129.19
CD4	Mouse	Biotin	rat IgG2b	R&D Systems	BAM554	Gk1.5
CD4	Mouse	FITC	rat IgG2a	eBioscience	11-0042-82	RM4-5
CD4	Mouse	APC	rat IgG2a	eBioscience	17-0042-82	RM4-5
CD8	Mouse	PE	rat IgG2a	BD Biosciences	553033	53-6.7
CD8α	Mouse	PE	rat IgG2b	EuroBioSciences	M22126P	YTS 169.4
CD11b	Mouse	FITC	rat IgG2b	eBioscience	11-0112-81	M1/70
CD11c	Mouse	APC	Arm hamster IgG1	BD Biosciences	550261	HL3
CD25	Mouse	APC	rat IgG1	eBioscience	17-0251	PC61.5
CD25	Mouse	FITC	rat IgG1	Abd Serotech	MCA1260F	PC61.5
CD40	Mouse	FITC	rat IgG2a	BD Biosciences	553790	3/23
CD45	Mouse	PE	rat IgG2b	Southern Biotech	1660-09	I3/2.3
CD86	Mouse	PE	rat IgG2b	eBioscience	12-0861-81	PO3.1
CD80	Mouse	PE	Arm Ham IgG2	BD Biosciences	553769	16-10A1

Table 2.5: List of antibodies use in thesis

2.4.2 List of cytokines, chemokines and TLR agonists

Cytokine/Chemokine/Stimulants	Company	Catalogue number
GMCSF	Immunotools	11343127
VEGF	Immunotools	11343127
SCF	Immunotools	11343665
BMP-4	Immunotools	11345043
IFN- γ	R&D systems	285-IF/CF
TNF- α	R&D systems	210-TA/CF
IL-1 β	R&D systems	201-LB/CF
Prostaglandin E2	Sigma	6532
ODN 2216	Invivogen	tlrl-2216
Poly (I:C)	Invivogen	tlrl-picw
sCD40 ligand	R&D systems	6245-CL
R848	Invivogen	tlrl-r848-5
IL-4	Peprtech	200-04-500
CCL19	R&D systems	361-MI
XCL1	R&D systems	695-LT/CF
CCL5	R&D systems	278-RN
Vitamin C	Sigma	A4544
Valproic acid	Signam	P4543
Mouse Flt3-L	308-FK-005	308-FK-005
Mouse IL-7	Immunotools	12340073
Human IL-2	Immunotools	11340025
TGF β	Peprtech	100-21C

Table 2.6: List of cytokines, chemokines and TLR agonists use in thesis

2.5 Statistical analysis

Sample size has been shown in the legends of each figure and is denoted by 'n'. Data in this thesis have been presented as mean or median (where appropriate) $\pm$ standard deviation (SD). Statistical significance of more than 2 conditions was determined by one-way analysis of variance (ANOVA) using GraphPad Prism 6. Paired and unpaired non-parametric T T tests were also performed using GraphPad Prism 6 when comparisons were done directly between 2 conditions. Differences have been reported as statistically significant if $p > 0.05$ (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Chapter 3:

Differentiation of iPSCs into DCs and investigating their use in HIV-1 immunotherapy

3.1 Introduction

HIV-1 and CART

HIV-1 infects more than 35.3 million people worldwide, with 2.3 million new cases of infections reported every year. It has also been recognised by the World Health Organization (WHO) as the sixth greatest cause of mortality [243]. HIV-1 infection progressively diminishes the number of CD4⁺ T cells, which are crucial for the immune response against exogenous antigens [43], crippling the immune system and resulting in the high morbidity and mortality associated with the disease [146, 244]. Combination anti-retroviral therapy (CART), the current treatment for HIV-1, involves using a combination of anti-retroviral drugs like reverse transcriptase inhibitors and protease inhibitors to inhibit viral replication [245]. CART has been shown to substantially alleviate the viral load of HIV-1 in infected individuals and ameliorate disease symptoms, converting it from a fatal illness to a chronic, yet manageable disease [147]. However, CART cannot restore HIV-1-specific immune responses and fails to eliminate sanctuary reservoirs of HIV-1, creating lifelong dependency on the treatment [159]. The anti-retrovirals used for CART have been known to cause side effects such as diarrhoea, nausea, headaches, pancreatitis as well as metabolic disorders such as lipodystrophy [246]. CART treated individuals still carry the risk of onward transmission of the virus to successive partners, propagating the disease, as evident from the unchanged incidence of new HIV-1 cases in the United States in the last few years [148, 149, 243]. Furthermore, lifelong treatment of CART has significant economic implications to the healthcare costs. It has been estimated that CART costs the UK National Health Service more than £1 billion

per annum. Therapeutic vaccines for the treatment of HIV-1, which would have the potential to eliminate all sanctuary reservoirs of the virus, are being explored [159]. These vaccines have the potential to provide a ‘functional cure’, for the disease, that is, the control of HIV-1 infection without the need for lifelong CART treatment [247].

DCs in HIV-Immunotherapy

The unique property of DCs to modulate the outcome of both adaptive and innate immune responses makes them an attractive target for use in immunotherapy [39]. *In vivo*-derived moDCs that have been pulsed *ex vivo* with antigens have been shown to elicit an antigen-specific T cell response in many murine models of human tumours and chronic infections [39, 248]. Since mononuclear cells from the patient’s blood provide an easily accessible source of autologous monocytes that may be differentiated into DCs, this approach has been extensively explored for the treatment of HIV-1 [159].

The use of autologous moDCs as therapeutic vaccines has been explored in the simian immunodeficiency virus (SIV)-macaque model [153, 154]. In this study, autologous moDCs pulsed with aldrithiol-2-inactivated SIV were subcutaneously injected into SIV-infected rhesus macaques, with four boosters administered every other week. Six weeks after the first immunization, 50-fold decrease of SIV cDNA and a 1,000-fold decrease of SIV RNA were observed in peripheral blood, while CD4⁺ T cells counts were found to increase 13 weeks after immunization [154]. The decrease in viral load and increased CD4⁺ T cells counts were maintained for the remaining 34 weeks of the study, suggesting that moDCs pulsed with inactivated whole-virus are capable of mounting an effective immune response against SIV in macaques [154]. More than 13 clinical trials utilizing autologous

moDC for HIV-1 immunotherapy have been reported [247]. Although these trials varied widely in the design, type of HIV-1 antigen used, method of loading and maturation stimuli, the vaccines were found to be well tolerated and a degree of immunological response was observed in many trials, however, none of them curative [159, 247]. Most of these trials were, however, non-controlled and not randomized, making the interpretation of the results difficult. In 2013, a blinded, placebo-controlled clinical trial investigating the use of autologous moDCs pulsed with heat-inactivated autologous HIV-1 for the treatment of HIV-1 was conducted [247]. The vaccine was found to be well tolerated and resulted in significant decrease in viral loads which correlated with the increase in HIV-1 specific T cell response [247]. However, these effects were maintained only for 24 weeks and did not correlate with increase in CD4⁺ T cell counts, suggesting that although the vaccine was able to elicit an immunological response, it was not able to functionally cure HIV-1 [247].

The disappointing results obtained in harnessing moDCs for HIV-1 immunotherapy may be, in part, due to the donor to donor variability observed in moDCs and in part, due to the inadequacies of the T cell repertoire in HIV-1 infected individuals. Since CD4⁺ T cells are required to 'license' DCs to cross present antigen to CD8⁺ T cells, their depletion in HIV-1 infected individuals may limit the efficacy of moDCs in stimulating HIV-1 specific CTL response [43]. Furthermore, moDCs possess a limited capacity to cross-present antigen to CD8⁺ T cells, which is essential for eliciting an effective cytotoxic T lymphocyte response against exogenous antigen [43].

Cross Presenting DCs in HIV-1 Immunotherapy

The recently identified cross presenting subset of DCs, namely, the CD141⁺ myeloid DCs (mDCs), would be ideal for application in HIV-1 immunotherapy, due to its ability to process exogenously derived antigen and present them to CD8⁺ T cells, [52, 53] and may overcome many limitations associated with the use of moDCs for immunotherapy. However, their trace numbers in peripheral blood (less than 0.1% of peripheral blood monocytic cells) preclude their use in the clinic [52]. Alternative sources of generating cross-presenting DCs such as from humanised mouse spleen have failed to overcome these limitations [51, 161]. Several groups have reported the successful differentiation of functional DCs from both murine and human embryonic stem cells (ESCs) [222, 223, 225] (discussed in chapter 1.6.2.1). Furthermore, recent work in our laboratory has shown the successful differentiation of DCs from human induced pluripotent stem cells (iPSCs) using an approach compatible with their downstream clinical application [249]. These DCs were found to express cell surface molecules similar to mDCs, such as CD11c and HLA-DR and were found to upregulate co-stimulatory molecule expression on maturation with a cocktail of pro-inflammatory cytokines [218]. A subset of these DCs were found to express CD141 and XCR1, which are markers of the recently described human cross presenting DC subset. Furthermore, unlike moDCs, these human iPSC-derived DCs (hiPDCs) possessed the capacity to cross present melanoma tumour associated antigen, melan A to CD8⁺ T cells, eliciting an antigen-specific CTL response and lytic capacity [218]. Since iPSCs have indefinite capacity for self-renewal this approach provides a potentially unlimited source of autologous DCs that might bypass the issue of patient-to-patient variability and lack of cross-presentation capacity inherent in the use of moDC for vaccination purposes.

HIV-1 reservoir

DCs express cell surface molecules, CD4 and chemokine receptors CCR5 and CXCR4, through which HIV-1 can enter the cells and have been shown to be readily infectable with the virus [250]. DCs have been shown to play an important role in the dissemination of the virus and are thought to be the first cell type to be infected through intravaginal inoculation [251, 252]. Although HIV-1 replication is inefficient in DCs and does not affect its viability and function, DCs can act as a viral reservoir for the virus, which despite treatment with CART, persist for long periods of time [253]. On maturation, these DCs migrate into LNs, where they *trans*-infect CD4⁺ T cells, contributing to viral persistence [254]. Furthermore, mDCs have been shown to preferentially transfer HIV-1 to antigen-specific CD4⁺ T cells, impairing the development of immune response against HIV-1 [255]. Like mDCs, moDCs derived *in vitro* have also been shown to be infectable with HIV-1 upon administration into patients and serve in extending the reservoir for HIV-1 [256]. It has been shown that individuals who possess the CCR5Δ32 homozygous mutation are not susceptible to HIV-1 infection [257], since HIV-1 cannot enter the cell. Given that it is impossible to cure HIV-1 without eliminating the latent reservoirs of the virus, we investigate whether hiPDCs express co-receptors for HIV-1 entry into the cell, such as CCR5, CXCR4 and CD4 and whether they can be infected by HIV-1 virus *in vitro*.

Aims:

In light of the recent identification of a protocol to differentiate human iPSCs into CD141⁺ DCs and the demonstration that these hiPDCs possess the capacity to cross-present

antigen to CD8⁺ T cells, we propose to investigate the suitability of these hiPDCs as a therapeutic vaccine for HIV-1.

To this end, we make use of HLA-A*0201⁺ iPSC line, which has been previously derived in the laboratory, to differentiate hiPDCs. We will further characterise these hiPDCs for phenotype and function to determine the extent to which this subset resemble the cross presenting DC found *in vivo*. Given that moDCs may themselves become infected with HIV-1 upon reinfusion into the patient and may serve as another reservoir of HIV-1, we investigate whether hiPDCs express co-receptors for HIV-1 and whether they can be infected by the virus. Finally, we shall investigate the capacity of hiPDCs to cross present exogenously-derived HIV-1 Gag protein to naïve CD8⁺ T cells from a HLA-A*0201⁺ individual and determine whether they are able to clear latent reservoirs of HIV-1 in the absence of CD4⁺ T cell help, recapitulating the immune scenario in AIDS patients.

3.2 Results

3.2.1 Morphology and pluripotency of iPSC colonies

For the purpose of differentiating dendritic cells from stem cells, we utilized the human iPSC line C15, a kind gift from Prof. Susanne Watt and Dr. Lee Carpenter at the Stem Cell Research laboratories, John Radcliffe Hospital, Oxford. This iPSC line was derived from the dermal fibroblasts of an HLA-A*0201⁺ donor and has been previously shown to be pluripotent by virtue of its expression of markers of pluripotency and its ability to give rise to teratomas consisting of all three germ layers upon implantation under the kidney

capsule of immunocompromised mice [235]. The C15 cell line has also been shown to be karyotypically normal.

To confirm that the iPSCs maintained pluripotency during long term culture, we characterised them for cell surface makers of pluripotency SSEA-4 and Tra 1-60 alongside transcriptional markers of pluripotent stem cell status, Oct3/4 and Nanog [258]. The C15 iPSC cell line was thawed and cultured on matrigel in mTeSR-1 medium. Once established, the line displayed characteristic morphology of human pluripotent stem cells, growing in well-defined colonies with the cells having a high nucleus to cytoplasm ratio (Figure 3.1a). The cell line was found to express all four markers of pluripotency, Tra 1-60, SSEA-4, Nanog and Oct3/4 (Figure 3.1b), consistent with earlier reports [235]. C15 iPSCs were cultured in six-well, matrigel coated plates with 2ml of mTeSR-1 per well. Medium was replaced daily and cells were passaged at 80% confluency by incubating with 0.02% EDTA in PBS followed by cell scraping to break colonies into small clusters.

3.2.2 Optimization of protocol for differentiation of iPSCs into DCs

Having shown that the C15 iPSC line expresses markers of pluripotency, we proceeded to investigate its differentiation into dendritic cells (DCs), following the protocol previously described in the laboratory [218]. iPSCs were expanded to 80-85% confluency and harvested using 0.02% EDTA and cell scraping, as described in the methods section. Cell clusters were plated into low attachment 6 well plates containing XVIVO-15 based differentiation medium supplemented with 50ng/ml GMCSF, 50ng/ml VEG-F, 20ng/ml SCF and 50ng/ml BMP-4 [218]. Differentiation cultures were fed every 2-3 days with XVIVO-15 supplemented with the above cocktail of growth factors. Cytokines were

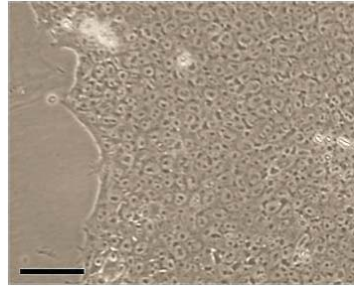
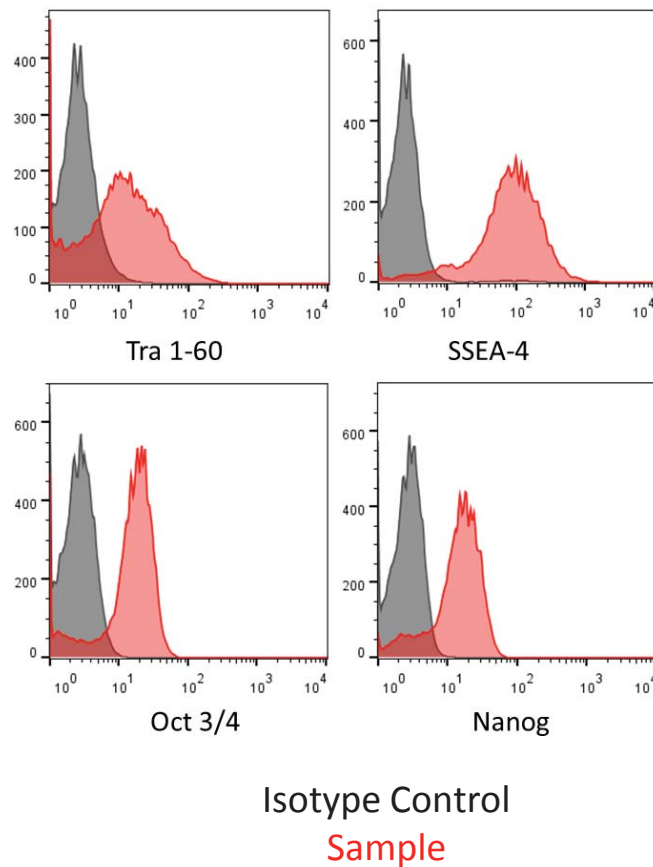
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Figure 3.1 Morphology of iPSC and expression of pluripotency markers. iPSC line C15 was stained for markers of pluripotency and analysed by flow cytometry. **(A)** Photomicrograph of C15 human iPSC colonies grown on matrigel coated 6 well plates. Scale bar=100 μ m. **(B)** iPSC line C15 were stained for cell surface markers of pluripotency, SSEA4 and Tra1-60 and intracellular markers OCT3/4 and Nanog and analysed by flow cytometry. One representative FACS plot for each marker is shown (red) overlaid upon the expression of corresponding isotype controls (black). These data are representative of 3 independent experiments.

serially depleted from the differentiation cultures on days 5 (BMP-4), 14 (VEG-F) and 19 (SCF) respectively, as described in the methods section.

Our early experiments revealed that, although small cell clusters were visible 2-4 days after setting up differentiation, they failed to develop into the embryoid body (EB)-like structures described in previously established laboratory protocols [218]. By day 14-16, when EBs had been previously reported to give rise to small monocyte-like cells, we observed that most cells in our cultures were non-viable (Figure 3.2 A), which was confirmed by trypan blue exclusion.

We investigated plating the C15 cell clusters into 6 well plates containing mTeSR-1, the medium in which the stem cells were maintained, instead of XVIVO-15, to minimize the impact of sudden media change. Figure 3.2-B illustrates the timeline of the differentiation process along with the cytokines used. Cell clusters were observed on day 3, which later gave rise to well-formed EBs structures (Figure 3.2 C- Day 3). Around day 14-16, viable monocyte-like cells were observed in the differentiation, consistent with our previous findings [218]. The monocyte-like progenitors (Figure 3.2 C-Post Harvest) were harvested around day 24-28 and plated in XVIVO-15 supplemented with IL-4 and GM-CSF on 6 well cell bind plates for 7 days. During the last 2 days of culture, hiPDCs were matured with a cocktail of cytokines, if required. Irregularly shaped cells with multiple cytoplasmic projections, distinctive of DCs were observed at the end of the 7 days in both mature and immature cultures. A proportion of these cells were found to be CD11c⁺ HLA-DR⁺, as will be demonstrated in latter sections. This optimized protocol (Figure 3.2 B) for the differentiation of iPSC into dendritic cells was used for all subsequent experiments.

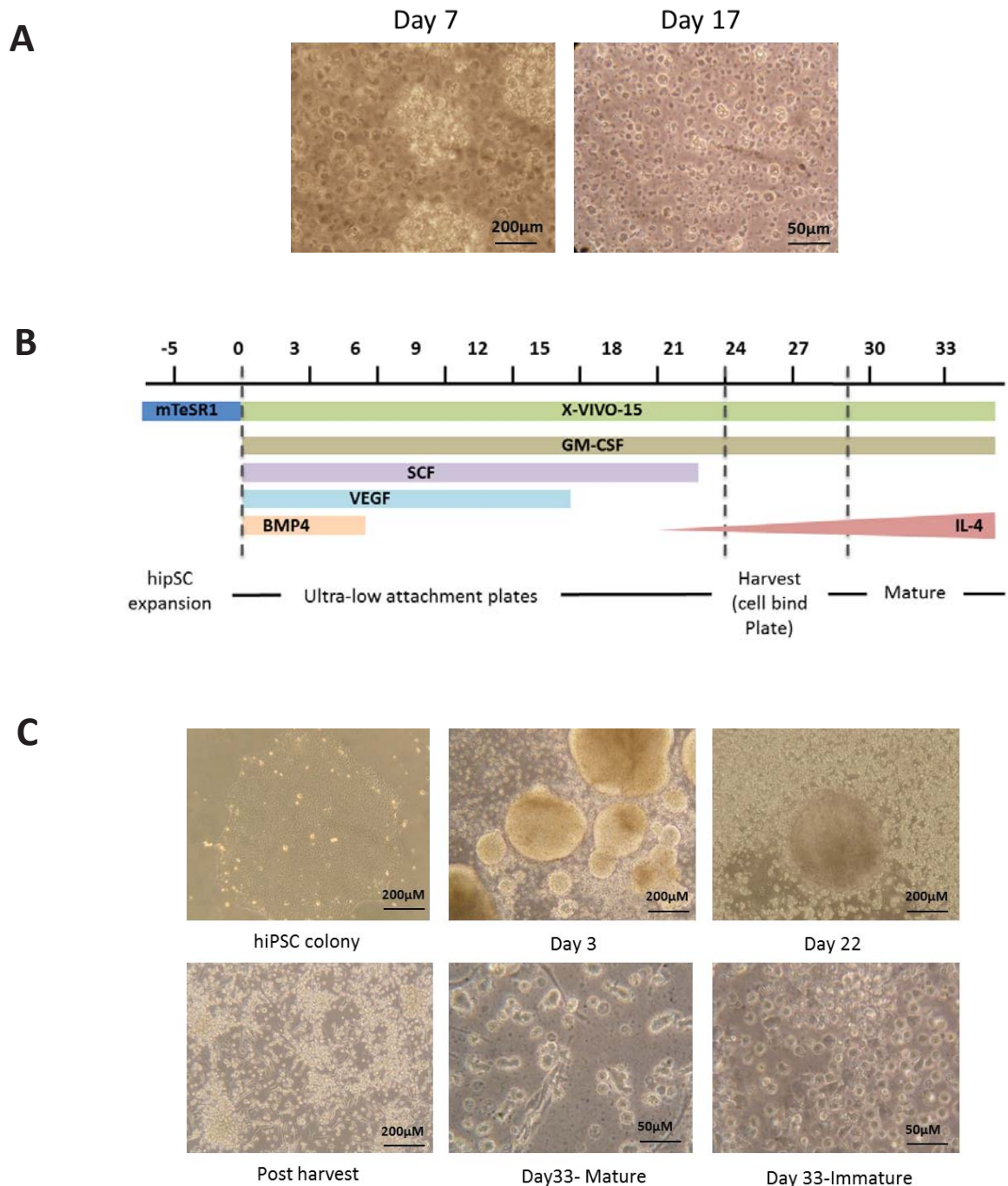


Figure 3.2 Modification of protocol for the differentiation of DCs from iPSCs. The protocol for the differentiation of hiPDCs was modified following excessive cell death in differentiation cultures. **(A)** Photomicrograph of iPS cell differentiation cultures using the original Silk *et al* protocol on days 7 and 17 of differentiation process. **(B)** Illustration of the timeline of the new protocol for differentiation of iPS cells into hiPDCs. iPS cells were expanded on matrigel in mTeSR-1 and were made into EBs on low attachment plates with the same media with the addition of GFs. Cells were fed every 1-2 days, with GFs being gradually withdrawn to give rise to monocyte like precursors, which are harvested into GM-CSF and IL-4 containing media to give rise to DCs. **(C)** **Representative** photomicrographs illustrating the morphology and appearance of cells during the differentiation process.

3.2.3 Yield of DC progenitors and hiPDCs

The yield of monocyte-like progenitors from independent hiPDC differentiations, as determined by counting the number of cells before plating onto 6-well cell bind plates for the final 7 days of differentiation into DCs, was found to be variable, ranging from 20% to 100% of the original number of iPSCs plated (median $\pm$ SD, 68.25 ± 26.17 ; n=8). The low yield in some experiments is explained by the observation of significant cell death or apoptosis that occurs during the differentiation process (Figure 3.3 C).

After the 7 day differentiation of monocyte-like precursors with GM-CSF and IL-4, many cells in the culture exhibited irregular shape with numerous projections typical of DCs (Figure 3.3 A). These cells also expressed the mDC marker, CD11c, confirming their DC-like nature. The yield of these cells was found to vary extensively between experiments (median $\pm$ SD, 39.51 ± 32.63 ; n=10), with the yield relative to numbers of input iPSCs ranging from 10-100% (Figure 3.3 C). Since CD11c is considered to be one of the most consistent markers of myeloid DCs [259], the percentage of CD11c⁺ cells was used to determine the final yield of hiPDCs obtained at the end of the final 7 day culture period. The yield of CD11c⁺ hiPDCs ranged from 3.73% to 87% (Figure 3.3 C) with the median being 26.28% ($\pm$ SD 28.82; n=8). These findings suggest significant variability between different experiments, which has been reported for many tissues differentiated from iPSCs and may be explained by random variation in the differentiation propensities possessed by different lines and by the same line at different passages [260-262].

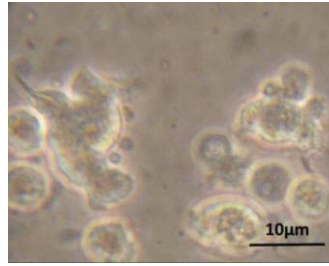
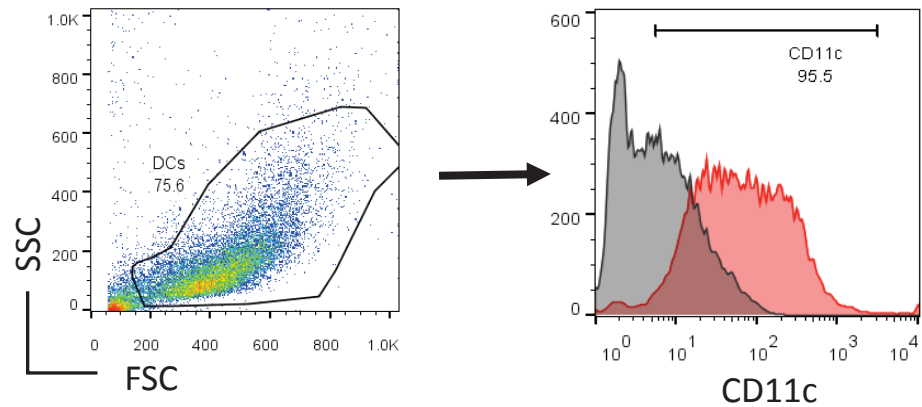
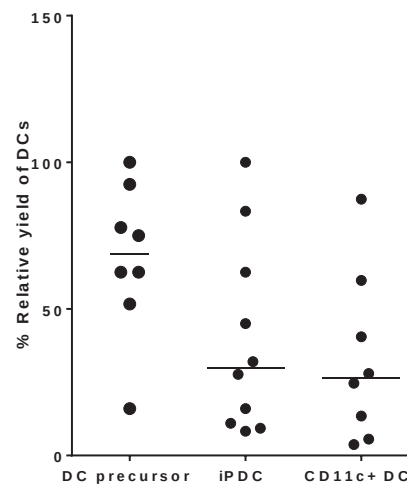
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Figure 3.3 Morphology and yield of hiPDCs. Cells obtained at the end of the differentiation were harvested, stained for CD11c, analysed by flow cytometry and investigated for the yield relative to iPSC input. **(A)** Photomicrograph of DCs obtained from iPSCs at the end of the differentiation. **(B)** FACS dot plot depicting the cells obtained at the end of the differentiation shown on FSC vs. SSC and the gating strategy used. These cells were stained for CD11c, a markers of mDC. Isotype control shown in black, CD11c stain shown in red. Plot is representative of all differentiations **(C)** Yield of DC precursors (or monocyte-like precursors, final number of hiPDCs and number of CD11c⁺ hiPDCs relative to numbers of iPSCs used for the differentiation. Each symbol represents an individual experiment with the black line denoting the median. (DC precursors- n=8; median- 68.25; SD- 26.17); (DC n=10; median-39.51 ; SD- 32.63); (CD11c⁺ DC n=8; median- 26.28; SD- 28.82).

3.2.4 hiPDCs have DC phenotype

Having established that cells with a distinctive morphology similar to that of DCs were successfully differentiated from iPSCs, we proceeded to characterise these cells for typical markers expressed by DCs, using moDCs for comparison. As previously mentioned, hiPDCs were found to express CD11c, a marker of mDCs. The percentage of CD11c⁺ DCs varied between experiments, with the median being 85.5% of total yield of cells ($\pm$ SD 11.93; n=16) (Figure 3.4 A). A subset of these cells, like moDCs, was found to co-express HLA-DR (HLA class II), a hallmark of antigen presenting cells (Figure 3.4 B). Although around 85% of hiPDCs expressed CD11c, only around 30-40% expressed HLA-DR, unlike moDCs where most of the population co-expressed the two markers (Figure 3.4 B). Since both moDCs and hiPDCs were found to express high levels of CD11c, the cells were gated on this population for further analysis of their surface phenotype.

3.2.4.1 hiPDCs express co-stimulatory molecules

Priming of naïve antigen-specific T cells requires presentation of cognate antigen in an MHC-restricted manner together with the provision of critical co-stimulatory signals [104]. CD80 (B7.1) and CD86 (B7.2) are costimulatory molecules expressed by mature DCs that interact with CD28 on T cells during antigen presentation (Section 1.3.2), resulting in the production of IL-2, activating the T cells and resulting in their increased survival and clonal expansion. CD40 is constitutively expressed on all DCs and binds CD40L on T cells. This molecular interaction has an important role in T cell priming and differentiation [263], since it increases the expression of other costimulatory molecules on DCs and plays a role in the secretion of IL-12 by DCs [264, 265]. The CD40-CD40L interaction also

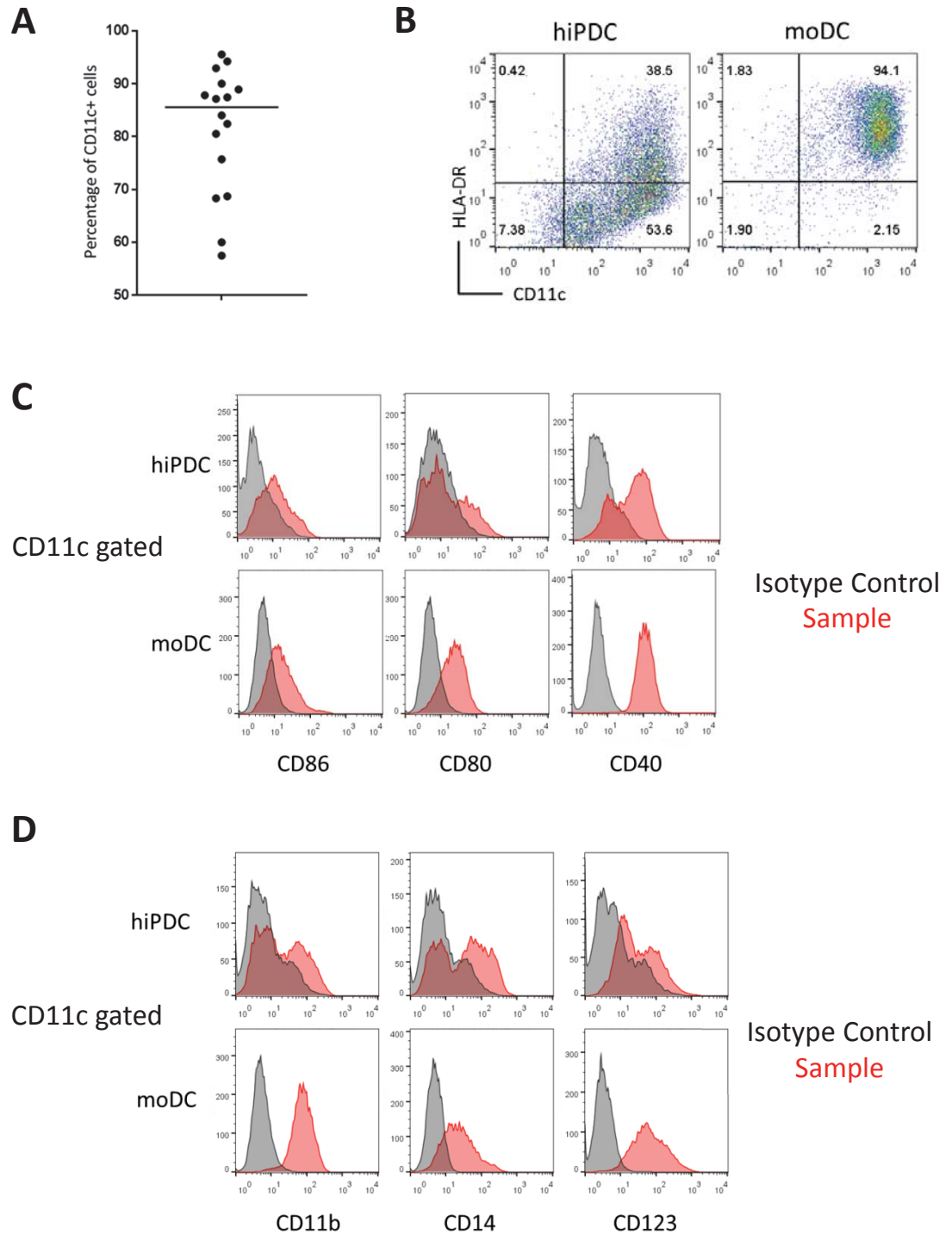


Figure 3.4 hiPDCs express markers of DC and co-stimulatory markers. Cells obtained at the end of the differentiation were stained for CD11c alongside other markers of DC. **(A)** Percentage of CD11c⁺ cells obtained at the end of differentiation. Each symbol represents an individual experiment with the black line denoting the median. (DC precursors- n=16; median- 85.55; SD- 11.94). **(B)** CD11c⁺ hiPDCs and moDCs were stained for MHC II, HLA-DR and CD11c, markers of the mDC subset. **(C)** Histograms depict representative FACS staining of hiPDCs and moDCs with co-stimulatory markers CD86, CD80 and CD40. **(D)** Histograms depict representative FACS staining of hiPDCs and moDCs with DC subset markers, CD11b, CD14 and CD123. All FACS plots are representative of at least 4 independent experiments. Isotype control in histograms are shown in black, CD11c pre-gated cells shown in red; gates in dot plots are set based on expression of isotype control.

'licenses' the DCs to cross present exogenously-derived antigen to CD8⁺ T cells, which is important in the immune response to tumours and viruses [266].

Since these molecules play a critical role in the ability of DCs to stimulate an effective immune response, we investigated whether hiPDCs expressed these co-stimulatory molecules. CD11c⁺ hiPDCs, like moDCs were found to express CD40, CD80 and CD86 (Figure 3.4 C), although it must be noted that the expression of these molecules is lower in hiPDCs than in moDCs and only a subset of these DCs express co-stimulatory molecules. The expression of these molecules on maturation is discussed in further sections.

3.2.4.2 CD11b and CD123

A subset of hiPDCs, like moDCs were found to express CD14, which is usually upregulated in monocytes and acts as a co-receptor for LPS along with TLR-4, suggesting incomplete differentiation and the presence of population of monocyte-like markers in harvested cells. A subset of hiPDCs and moDCs was also found to express CD11b, which is found at low levels in mDC subsets. Both moDCs and hiPDCs were also found to express CD123, which although usually a marker of pDCs, has also been found to be expressed in mDCs that have been freshly isolated or generated *in vitro* (Figure 3.4 D).

3.2.4.3 HLA-A2 and CCR7

Both hiPDCs and moDCs expressed HLA-A2 (Figure 3.5 A), consistent with the origin of the DCs (C15 cells line is known to be HLA-A*0201⁺ and moDCs in these experiments were

derived from an HLA-A*02⁺ donor). They also express chemokine receptor 7 (CCR7) (Figure 3.5 A), which binds chemokines CCL19 and CCL21 secreted by T cells and is responsible for the migration of DCs to secondary lymphoid organs [133].

3.2.4.4 Toll-Like Receptors (TLRs)

To characterise the likely responsiveness of hiPDCs to external stimuli, we investigated the expression of TLRs both on the cell surface and intracellularly in CD11c⁺ cells (Figure 3.5B).

hiPDCs were shown to express TLR3, which recognizes intracellular double stranded RNA during viral infection. This is consistent with the hiPDC population previously described in our laboratory [218] and is characteristic of cross presenting subset of DCs [53]. The expression of TLR3 in hiPDCs was higher than that observed in moDCs.

hiPDCs also express low levels of TLR8, which recognizes guanine-rich oligonucleotides. However, unlike moDCs, hiPDCs were not found to express TLR9 or TLR7, which are intracellular PRR and recognize CpG-rich DNA found in bacterial genomes and single stranded RNA. Work by other members of the laboratory has established that hiPDCs express low levels of TLR2 and TLR4, which have been shown to be involved in the detection of Gram-positive and Gram-negative bacteria (via LPS), respectively [267].

3.2.5 hiPDCs mature in response to inflammatory cytokines

DCs exist in two distinct states *in vivo* [268]. In the steady state, DCs are referred to as immature, characterized by their low expression of surface HLA class II and co-stimulatory

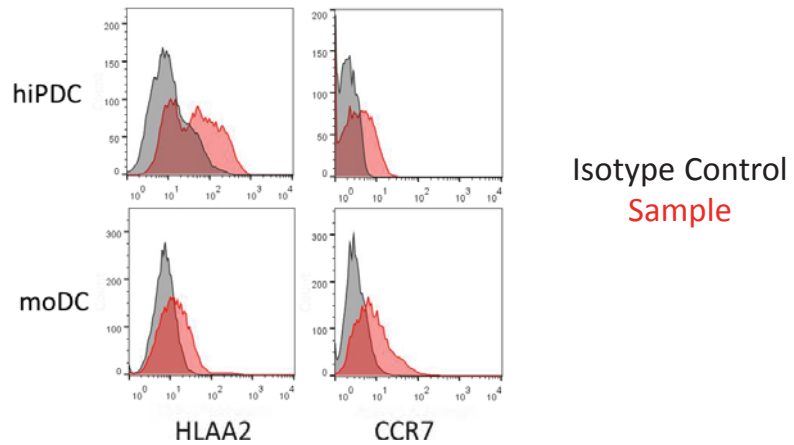
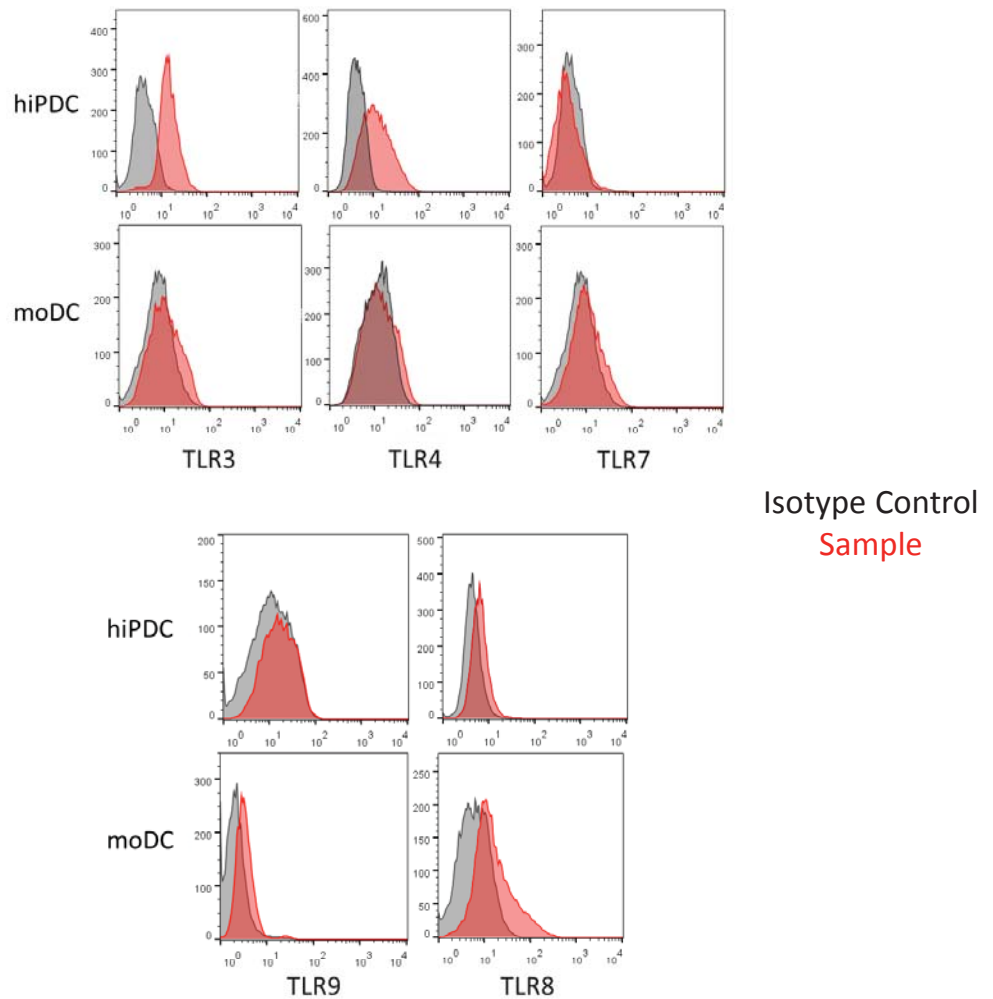
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Figure 3.5 Expression of MHC I, CCR7 and TLR markers. hiPDCs and moDCs were stained and analysed by flow cytometry. **(A)** hiPDCs and moDCs were stained for MHC class I and chemokine receptor CCR7. **(B)** hiPDCs and moDCs were permeabilized and stained for expression of TLRs 3,4,7,8 and 9. All FACS plots are representative of at least 4 independent experiments. Isotype control shown in black, CD11c pre-gated cells shown in red.

molecules CD80, CD86 and CD40. Maturation of DCs may be induced either through exposure to inflammatory stimuli or ligation of one or more TLRs. [269]. Maturation of DCs is a complex process which has a critical impact on DC function, in particular, in determining the balance between inducing tolerance or immunity. Maturation of DCs is known to upregulate HLA class II and co-stimulatory molecules. It increases the ability of DCs to migrate in response to the chemokines CCL19 and CCL21, stimulates the secretion of inflammatory cytokines and enhances the ability of DCs to activate T cells, eliciting an immune response [270].

hiPDCs and moDCs were cultured with a maturation cocktail consisting of TNF- α , PGE₂, IL-1 β and IFN- γ , which has previously been shown to stimulate the maturation of ES cell derived DCs [218], and were studied to determine the effect on co-stimulatory molecule expression, migration capacity and cytokine secretion.

3.2.5.1 Effect of maturation on HLA class II and co-stimulatory molecule expression

We have previously established, using flow cytometry, that both immature hiPDCs and moDCs express HLA class II and the co-stimulatory molecules CD80, CD86 and CD40. On maturation with the above mentioned cocktail of cytokines, the level of expression of all these molecules was found to be upregulated, consistent with the potential of DC to initiate primary T cells responses. The results from various independent experiments are summarised in the figures 3.6 and 3.7.

Although the levels of expression are variable across different experiments, which may be a reflection of variability in independent differentiations, we consistently observed

upregulation of all the markers upon maturation with inflammatory cytokines. However, it must be noted that the level of expression of CD40 and CD80 in both hiPDC and moDC, does not increase as much on maturation as compared to that of CD86 and HLA class II.

3.2.5.2 Effect of maturation on cytokine production

DCs are pivotal in orchestrating the adaptive immune response to invading pathogen. They are known to produce cytokines which are capable of polarising naive T cells towards a Th1, Th2, T_{reg} or Th17 phenotype. On maturation, DCs produce a host of pro-inflammatory cytokines, which are instrumental in their ability to modulate the immune system [120].

We investigated the levels of IL-6, IL-10 and IL-12 produced by hiPDCs and moDCs, both in steady state and on maturation. moDCs and hiPDCs were cultured for 18 hours, with or without inflammatory stimuli, after which supernatants were collected. The levels of cytokines secreted were determined using standard ELISA, as discussed in chapter 2.

We observed that in steady state, hiPDCs, like moDCs, secrete IL-6, (Figure 3.8 A), a pro-inflammatory cytokine known play an important role in T cell priming [121]. On maturation with inflammatory cytokines, a significant increase in the levels of IL-6 secretion was observed in both hiPDCs and moDCs, which is a reflection of the successful maturation of these cells.

IL-10 is an anti-inflammatory cytokine which has been shown to be secreted by DCs and to have an autocrine effect on DC function [271]. IL-10 has been shown to hamper the ability of DCs to initiate Th1 response [271] and has also been shown to induce a

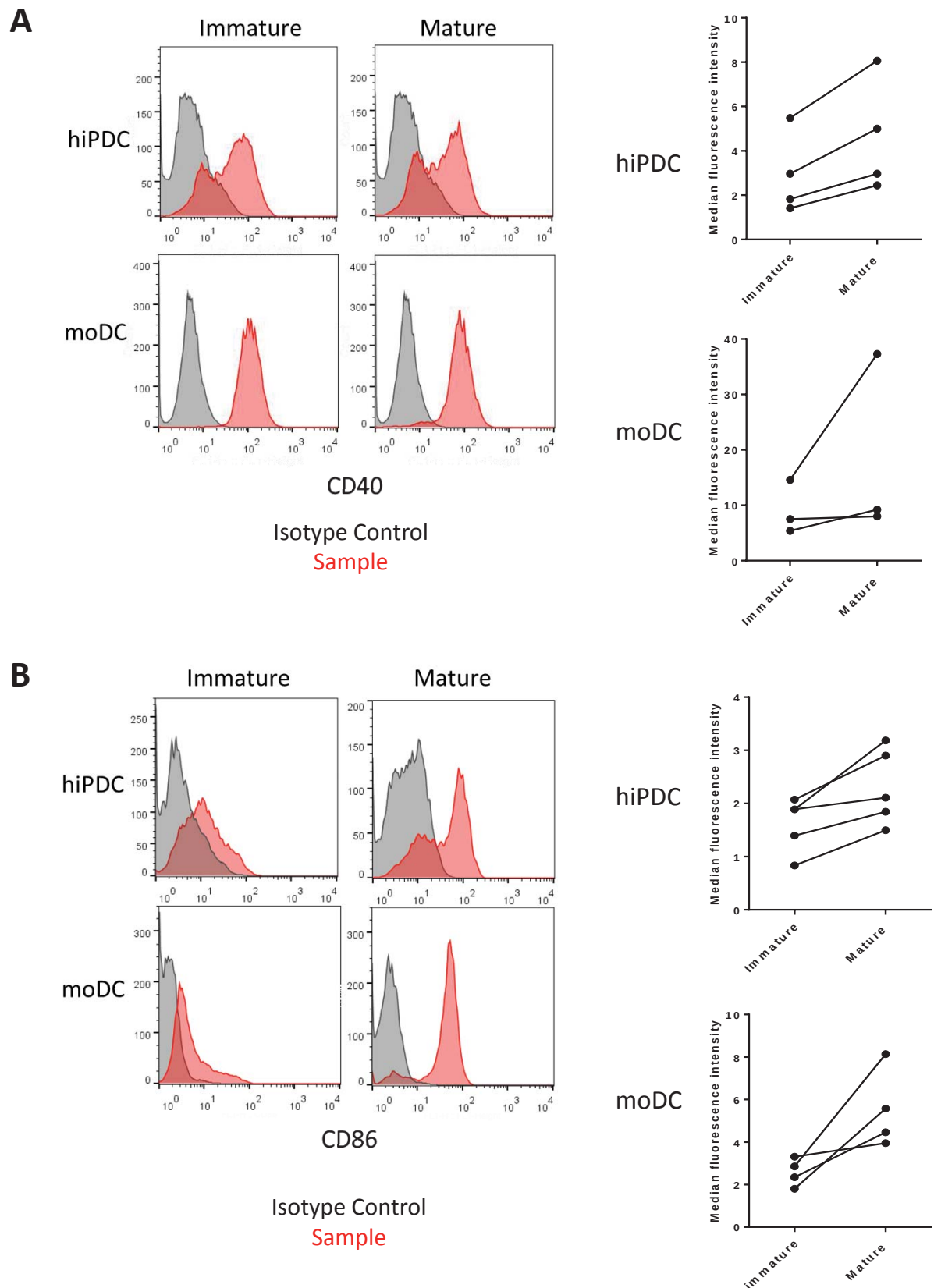


Figure 3.6 Expression of co-stimulatory markers in response to maturation. Immature and inflammatory cytokine-cocktail matured hiPDCs and moDCs were stained for (A) CD40 and (C) CD86 and analysed by flow cytometry. All FACs plots are representative of 4 independent experiments. Isotype control shown in black, CD11c pre-gated cells are shown in red. Graphs (B) and (D) depict the relative median fluorescence intensity of CD40 and CD86, respectively in four independent experiments. Each line depicts an independent experiment and the levels of MFI is plotted for immature and mature DCs

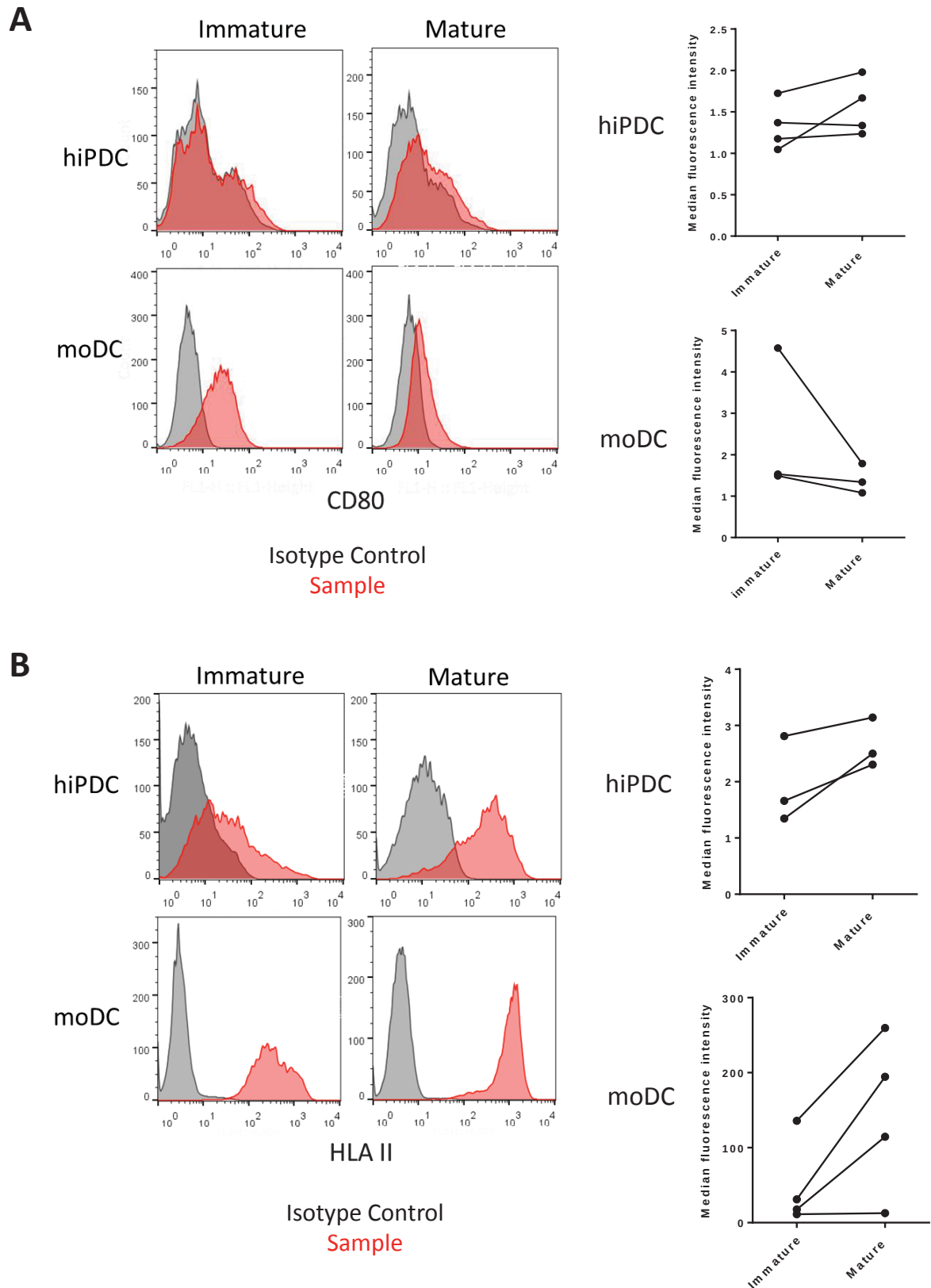


Figure 3.7 Expression of co-stimulatory markers in response to maturation. Immature and inflammatory cytokine-cocktail matured hiPDCs and moDCs were stained for **(A)** CD80 and **(C)** MHC II and analysed by flow cytometry. All FACS plots are representative of 4 independent experiments. Isotype control shown in black, CD11c pre-gated cells are shown in red. Graphs **(B)** and **(D)** depict the relative median fluorescence intensity of CD80 and MHC II, respectively in four independent experiments. Each line depicts an independent experiment and the levels of MFI is plotted for immature and mature DCs

tolerogenic state among DCs, which is able to polarize naïve T cells towards a T_{reg} phenotype [272, 273]. Both hiPDCs and moDCs were found to secrete IL-10, the levels of which did not change in moDCs on maturation but decreased in hiPDC, although this decrease was not found to be statistically significant (Figure 3.8 B). Immature hiPDCs was found to secrete significantly more IL-10 than immature moDCs, leading us to speculate whether these iPSC-derived DCs may be more tolerogenic in nature.

IL-12 (IL12p70) is an immune-regulatory cytokine produced by APCs in response to stimulation [274]. It has been shown to play a vital role in both innate and adaptive immune response and has been shown to be critical in steering the differentiation of naïve T cells towards a Th1 phenotype. IL-12 has been shown to be important in HIV-1 infection, where the loss of effective Th1 is attributed to the suppression of IL-12 by HIV-1. [275]. IL-12 was not detected in immature and mature hiPDC, but was detected in moDCs upon maturation by inflammatory cytokines (Figure 3.8 C), although the levels of this are found to be very low as compared to that reported in the literature [276]. In various studies, IL-12 is found to be secreted in response to TLR3, TLR7/8 and TLR9 stimulation, which may not be sufficiently achieved by the cytokine cocktail used [276]. CD40 ligation has also been shown to be important in regulating the production of IL-12 [118]. Since, IL-12 has been shown to be critical in polarising Th1 response [277], we further investigated if hiPDCs can secrete IL-12 in response to stimulation via TLR3, TLR7, TLR 8 and soluble CD40 ligand (sCD40L), singly and in combination with TNF- α , IL-1 β and IFN- γ .

When stimulated with a cocktail of TNF- α , IL-1 β and IFN- γ in addition to TLR8 agonist R848, hiPDCs were found to secrete IL-12p70, demonstrating that under stringent

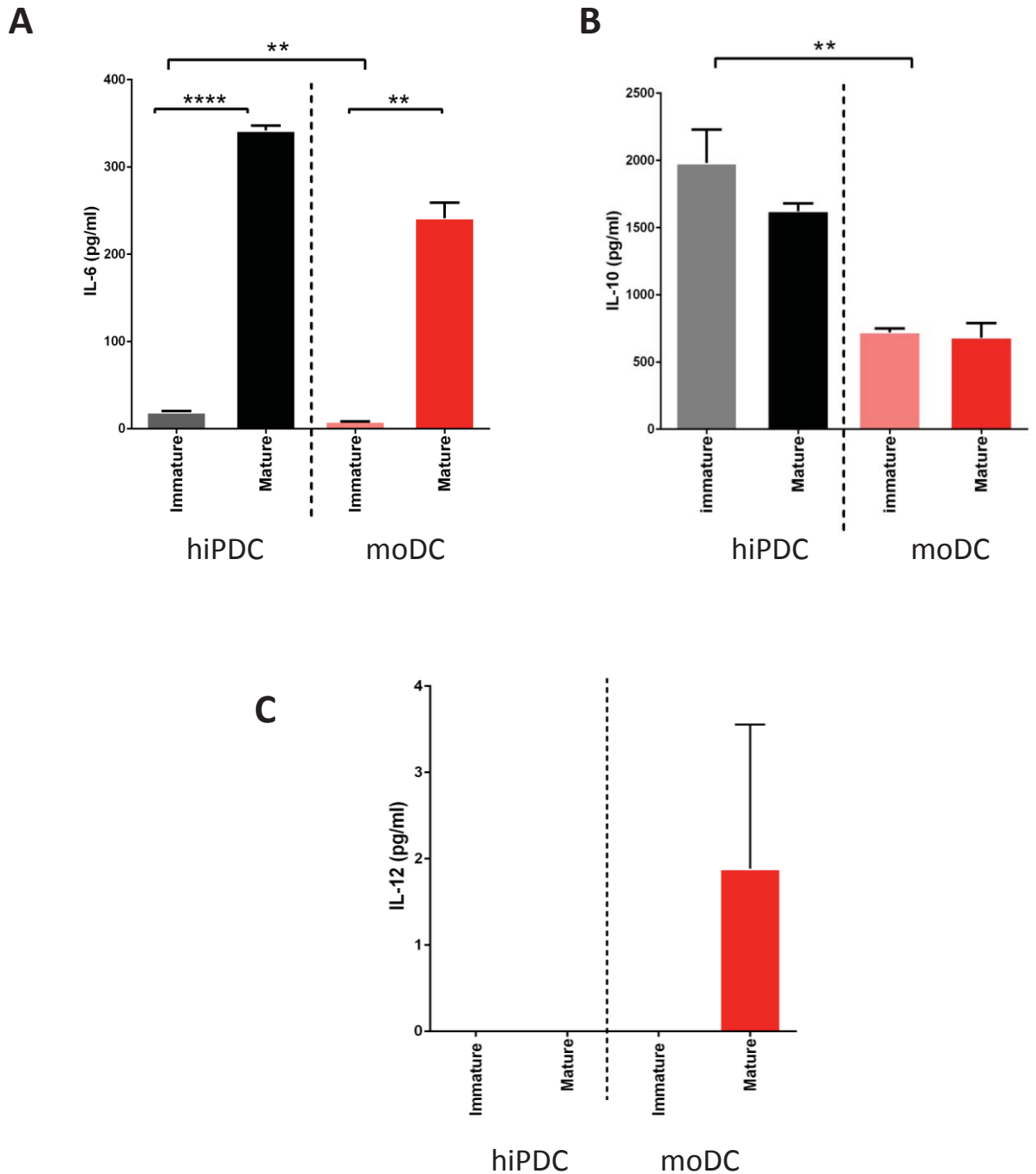


Figure 3.8 Secretion of IL-6, IL-10 and IL-12 in Immature and mature hiPDCs and moDCs. Immature hiPDCs and moDCs were harvested and cultured for 18 hours with or without maturation cocktail consisting of TNF- α , IL-1 β and IFN- γ and PGE₂. Supernatants were collected the amounts of IL6 **(A)**, IL-10 **(B)** and IL-12p70 **(C)** were determined by standard ELISA. Black bars indicate hiPDC and red bars indicate moDCs. Plots are representative of at least 3 independent experiments, with n=3 in each experiment. Statistical analysis was performed using parametric T test with Welch's correction. (* $p \leq 0.05$, ** $p \leq 0.01$).

conditions, hiPDCs are able to produce IL-12 (Figure 3.9 A). When TNF- α , IL-1 β and IFN- γ were combined with agonists of TLR7/8 (R848), TLR9 (ODN2216), TLR3 (Poly IC) and sCD40L, a significantly higher level of IL-12 was secreted indicating that the cytokines induced a synergistic effect, which has been observed previously in the case of combinations of TLR9 and TLR3 agonists [278].

As observed in the previous section, moDCs were able to secrete low levels of IL-12p70. This level increased slightly with the withdrawal of PGE₂ from the maturation cocktail, although this increase was not found to be significant (Figure 3.9). The levels of IL-12p70 secreted by moDCs was also found to increase with addition of TLR3 and TLR9 agonist to the basic maturation cocktail and also in the presence sCD40L. The levels of IL12p70 significantly increased upon addition of TLR7/8 agonist R848. Like hiPDCs, the levels of IL12p70 was further augmented upon addition of agonists of TLR7/8, TLR9, TLR3 and soluble CD40L, in combination with TNF- α , IL-1 β and IFN- γ (Figure 3.9 A), reaching levels of IL-12p70 that have been reported in the literature [276].

3.2.6 Functional properties of hiPDCs

The primary function of DCs is to capture antigen, migrate to the secondary lymphoid organs and present it via HLA molecules to naive T cells to elicit an immune response. We determined the functionality of hiPDCs by investigating their ability to capture and process exogenously-supplied antigen and their ability to stimulate allogeneic T cell proliferation in a mixed leukocyte reaction.

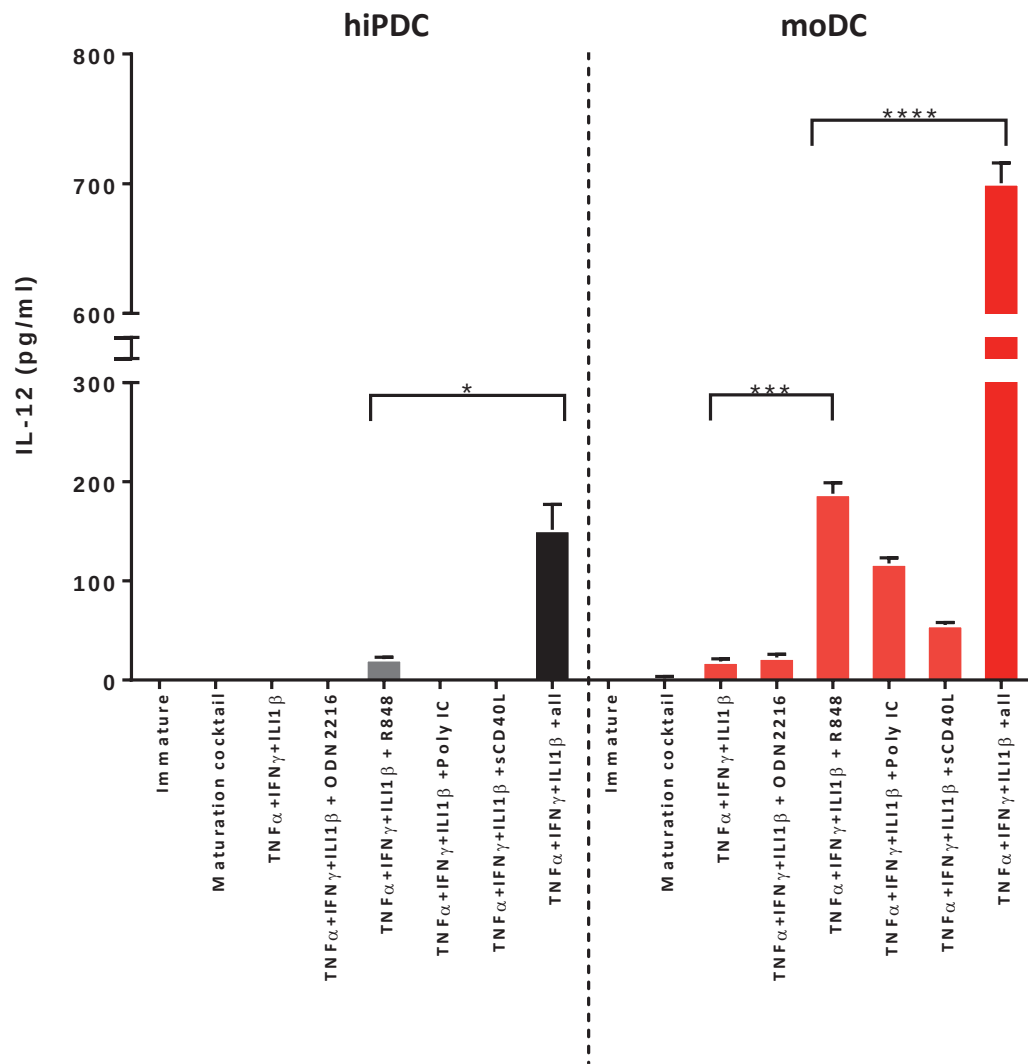


Figure 3.9 Secretion of IL-12 in hiPDCs and moDCs matured using different combination of cytokines and TLR stimulants. Immature hiPDCs and moDCs were harvested and cultured for 18 hours either without maturation cocktail or with a maturation cocktail that consists of of TNF- α , IL-1 β and IFN- γ with the addition of PGE₂, R848 , ODN 2216, sCD40L and PolyIC, either singly or in combinations. Supernatants were collected the amounts of IL-12p70 were determined by standard ELISA. Black bars indicate hiPDC and red bars indicate moDCs. Plot is representative of 3 independent experiments, with n=3 in each experiment. Statistical analysis was performed using parametric T test with Welch's correction. (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3.2.6.1 hiPDCs are able to phagocytose antigen

Like macrophages and neutrophils, DCs are professional phagocytes, constantly sampling the local microenvironment for pathogens and their constituent antigens and specialized in capturing them. The ability of DCs to phagocytose antigen is therefore a critical aspect of their function. To determine the functionality of the DCs differentiated from iPSCs, we investigated their ability to phagocytose FITC-labelled beads of 2µm diameter, using moDCs for comparison. Fixed DCs were used as negative controls and the cells were treated with trypan blue after incubation with beads to quench the fluorescence from beads that had adhered non-specifically to the outer surface of the cell.

As seen in Figure 3.10A, more than 50% of live immature hiPDCs and moDCs phagocytosed more than one FITC-labelled bead over the 3 hour incubation period (flow cytometry plots coloured red). DCs fixed with 4% PFA were used as a negative control. We notice that some fixed cells also seemed to phagocytose beads (around 10% for all cell types), which may be due to incomplete quenching of the fluorescence of the beads on the surface of the cells or endogenous fluorescence which is known to be high in some populations of DCs. A significant difference was, however, consistently observed between fixed and live cells in the case of immature hiPDCs and immature and mature moDCs.

The results from the flow cytometry staining are summarised in Figure 3.10B. The ability of the mature hiPDCs to phagocytose beads was significantly less than that of immature hiPDCs (Figure 3.10B). This is consistent with the known ability of DCs to downregulate their phagocytic capacity on maturation, while enhancing the ability to present antigen [279, 280]. Although a difference was observed in the ability of immature and mature moDCs to phagocytose beads, the decrease was not found to be significant.

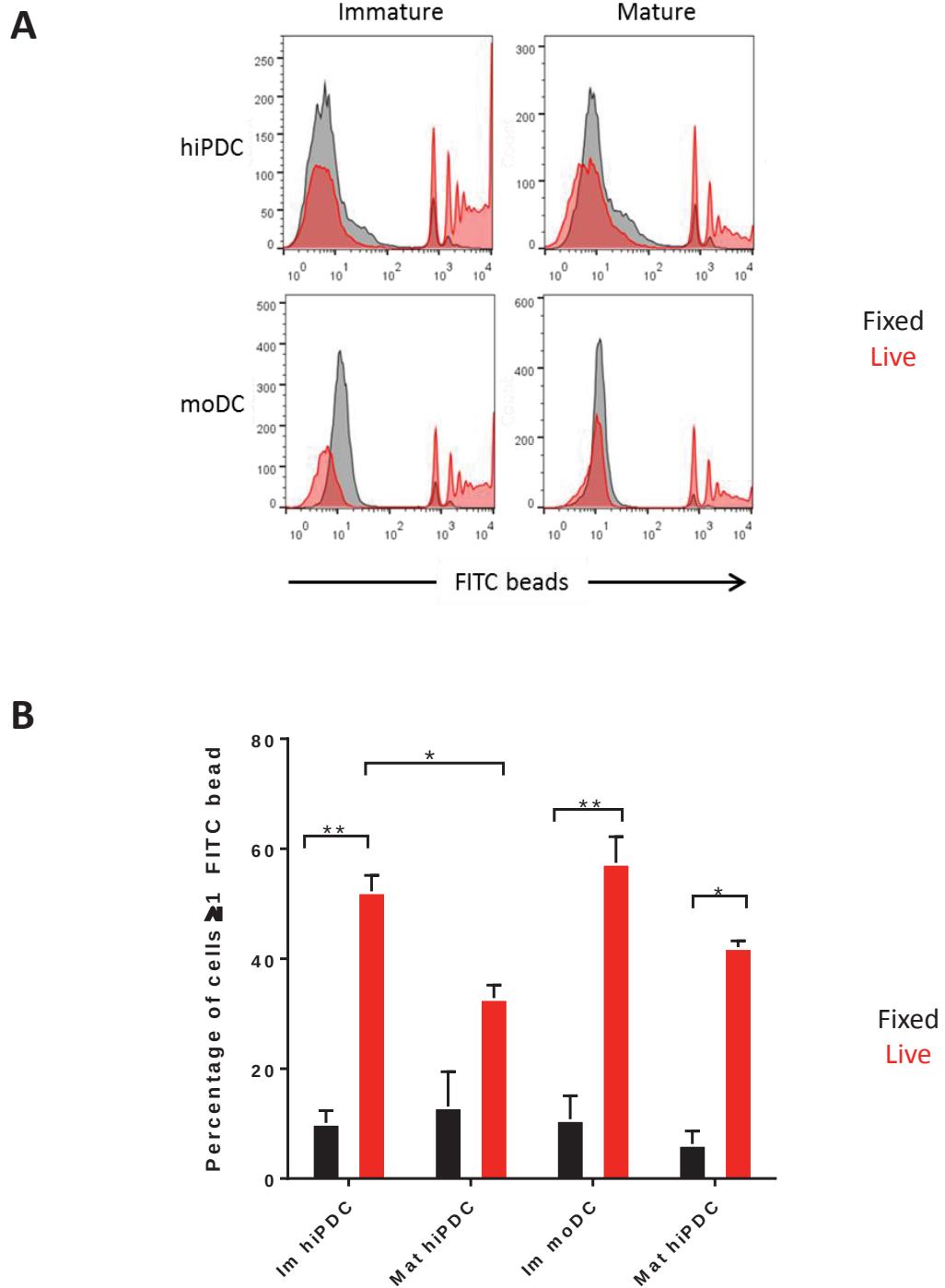


Figure 3.10 The phagocytic capacity hiPDCs and moDCs. 3×10^5 hiPDCs or moDCs were incubated with $2 \mu\text{m}$ FITC-labelled beads for 3 hrs at 37°C . Fixed cells (black bars) were used as negative control for non-specific binding. Red bars represent live cells. Cells were treated with Trypan to quench non-specific binding of beads to cell surface, washed, stained for CD11c and analysed by flow cytometry. The histograms are data from CD11c gated populations. Plot is representative of 3 independent experiments, with $n=3$ in each experiment. Statistical analysis was performed using parametric T test with Welch's correction. (* $p \leq 0.05$, ** $p \leq 0.01$).

3.2.6.2 hiPDCs are able to process antigen

Unlike macrophages and neutrophils, DCs are professional antigen presenting cells and process antigen via exogenous or endogenous pathways and present them to naive T cell repertoire. We investigated the capacity of hiPDCs to process antigen using DQOvalbuminTM, an ovalbumin protein conjugated with boron-dipyrromethene (BODIPY), a photostable, pH insensitive dye which fluoresces in the FITC range (FL1 channel) once the probe has undergone proteolytic cleavage [281]. BODIPY, unlike FITC, is stable, even in the acidic pH of DC endosomes, making DQOvalbuminTM superior to FITC-BSA or FITC-Ovalbumin traditionally used for assessing the ability of DCs to process antigen [282].

We incubated immature and mature moDCs and hiPDCs with DQOvalbuminTM for 3 hours at 37°C. As the proteolytic capacity of enzymes is highly temperature dependent and is reduced at low temperature, cells incubated with DQOvalbuminTM at 4°C were used as negative controls. We detected positive fluorescence in the FL1 channel, corresponding to proteolytically cleaved DQOvalbuminTM, in both immature and mature hiPDCs and moDCs (Figure 3.11A- red flow cytometry histograms). The ability of both moDCs and hiPDCs to process DQOvalbuminTM was found to increase on maturation, and this increase was found to be significant in the case of hiPDCs. This is consistent with the ability of DCs to upregulate their capacity to process and present antigen on maturation [283] .

We observed low levels of positive fluorescence in the FL1 channel in both hiPDCs and moDCs incubated with DQOvalbuminTM at 4°C, suggesting some residual activity of proteolytic enzymes, even at low temperatures (Figure 3.11A- black flow cytometry histograms). The difference between the percentage of fluorescent cells at 37°C and 4°C

was found to be statistically significant in both immature and mature hiPDCs suggesting that DCs were able to process DQOvalbuminTM more efficiently at 37°C (Figure 3.11B).

3.2.6.3 hiPDCs can stimulate allogeneic T cells

Having shown that hiPDCs are able to acquire and process foreign antigen, we next tested the ability of these DCs to induce proliferation of naïve T cells in an allogeneic Mixed Leukocyte Reaction (MLR), which is commonly used for the evaluation of DC immunogenicity [284]. An MLR involves the co-culture of two cells types, a stimulant, in this case the DCs, and a responder, the naïve allogeneic T cells. In an MLR, DCs have been shown to cause T cell clustering and activation and to stimulate their subsequent proliferation.

CFSE-stained naïve T cells were cultured for 4 days, alone or with increasing numbers of immature and mature hiPDCs and moDCs. Once it has entered the cytosol, CFSE is cleaved by esterases and binds to amino groups of macromolecules, rendering it membrane impermeable. CFSE divides equally among daughter cells during cell division, making it ideal for tracking proliferation of T cells. Proliferation is therefore measured as a percentage of cells that have undergone CFSE dilution (section 2.1.6).

As seen in Figure 3.12A, hiPDCs, like moDCs, stimulate a potent proliferative responses among naïve allogeneic T cells in a dose-dependent manner, similar to moDCs, indicating that hiPDCs are, indeed, immunostimulatory. The percentage of naïve T cells stimulated by mature hiPDCs was found to be significantly higher than that of immature cells as well as was also observed for moDCs (Figure 3.12B), which is consistent with the literature,

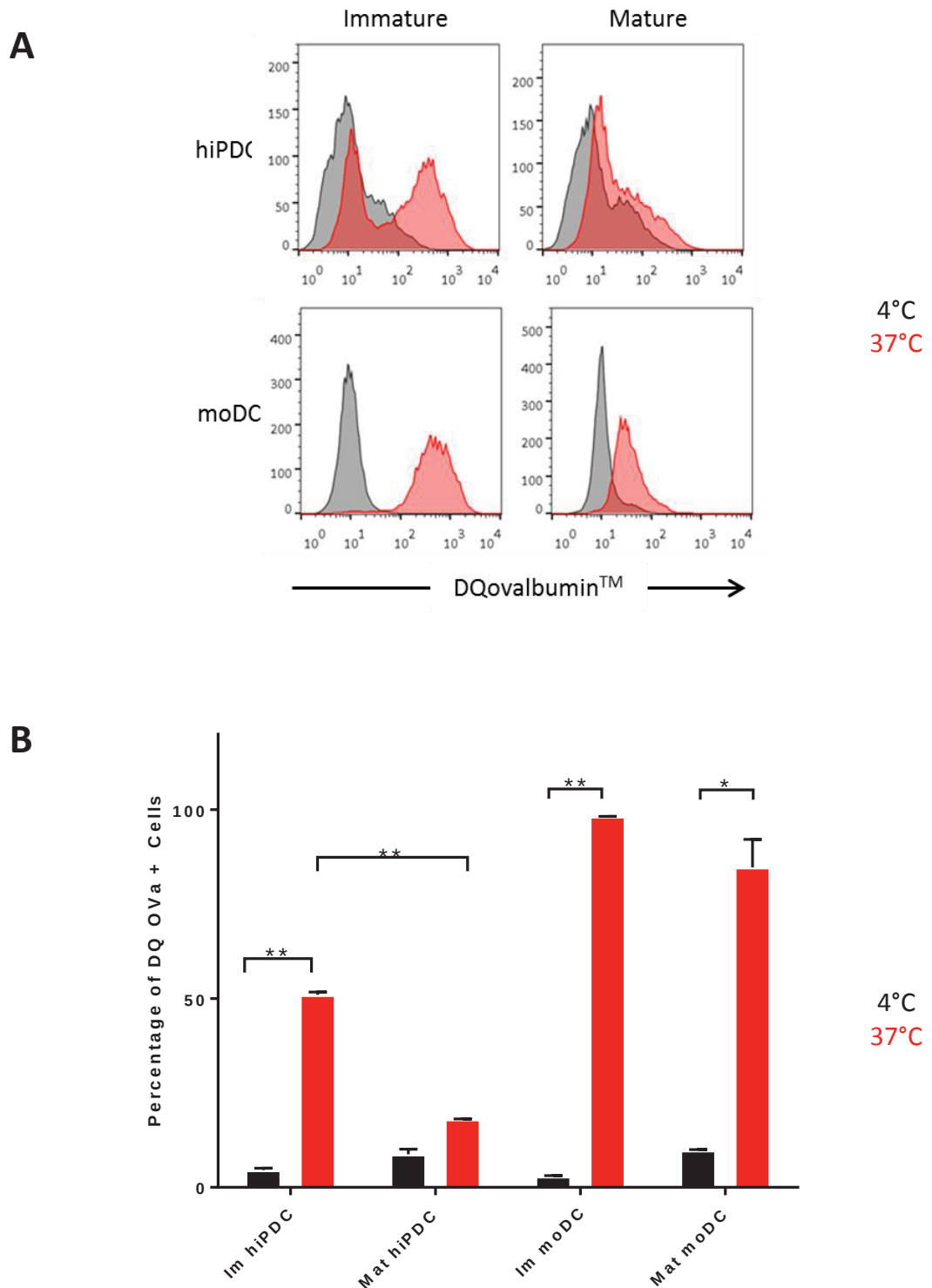


Figure 3.11 The capacity hiPDCs and moDCs to process antigen. 10^5 cells were incubated with with 100 μ g/ml of DQovalbuminTM at 37°C. Samples incubated at 4°C were used as negative controls. Cells were harvested after 30 minutes, washed, stained for CD11c and analysed by flow cytometry for the levels of fluorescence of cleaved DQovalbuminTM, which fluoresces in the FL-1 channel. The histograms displayed were gated on CD11c⁺ cells. Plot is representative of 3 independent experiments, with $n=3$ in each experiment. Statistical analysis was performed using parametric T test (* $p \leq 0.05$, ** $p \leq 0.01$).

confirming that the maturation of hiPDCs by inflammatory cytokines produces a potent immunostimulatory phenotype, an essential property of any therapeutic vaccine.

3.2.6.4 hiPDCs are able to migrate along a chemokine gradient

The ability of DCs to elicit an effective immune response depends on their ability to migrate, both to the sites of inflammation during pathogen invasion and towards the T cells in the secondary lymphoid organs on capturing antigen. DCs have been shown to migrate in response to chemokines CCL19 and CCL21, which are secreted by T cells.

Having already demonstrated that hiPDCs express chemokine receptor CCR7, we investigated the capacity of hiPDCs, as well as moDCs, to migrate along a gradient of CCL19. To this end, we made use of the xCELLigence Real-Time Cell Analyser (RTCA) instrument, which is able to measure the chemotaxis of cells along an increasing gradient of chemokine in real time. Chemokines were loaded in the lower chamber of wells and cells were introduced to the upper chamber. These two chambers are separated by a filter with pores of 8µm diameter, in which is embedded a gold micro-electrode. When the cells pass through this filter, the electrical charge is broken, generating a signal of electrical impedance, whose change is measured in arbitrary units known as cell index [236]. The measurement of cell index is therefore a reflection of the number of cells migrating through the filter with respect to time. Wells with no agonist were used as negative controls and the net chemotaxis was determined as the maximum vs. minimum cell index, termed as Delta Cell Index.

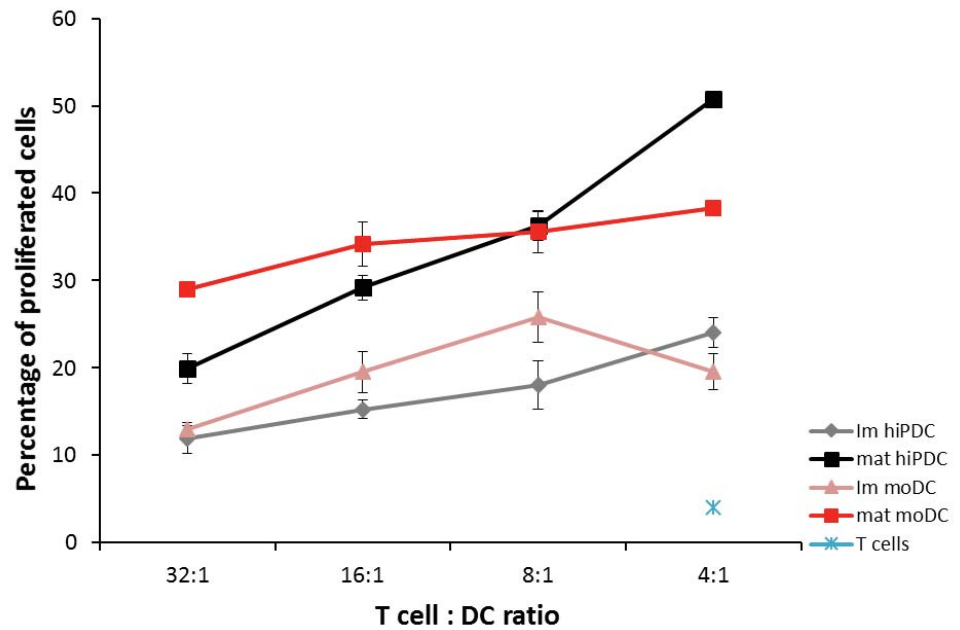
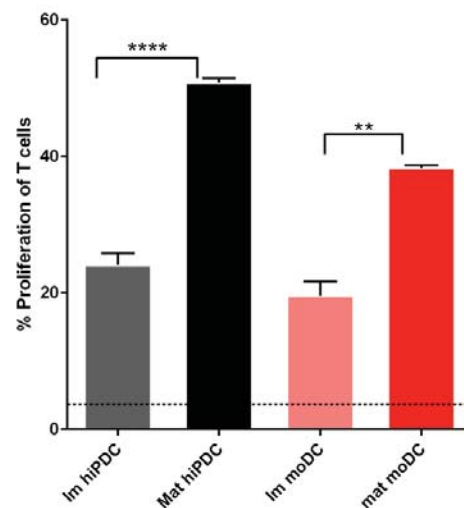
A**B**

Figure 3.12 The allostimulatory capacity of hiPDCs and moDCs. Varying numbers of Immature and Mature hiPDCs and moDCs were co-cultured with 10^5 CFSE-labelled naïve $CD4^+$ T cells per well of a 96-well plate to give different ratios of stimulant to effector cells. Cells were harvested after 5 days and analysed for the percentage of proliferated cells using flow cytometry. **(A)** depicts percentage of proliferated T cells when co-cultured with Immature or mature hiPDC or moDCs. **(B)** Comparison of percentage of proliferated T cells at 4:1 T cells: DC ratio. Plots are representative of 3 independent experiments, with $n=3$ in each experiment. Statistical analysis was performed using parametric Mann Whitney U test. (** $p \leq 0.001$, **** $p \leq 0.0001$).

In the absence of any chemokine, a low background signal was observed for hiPDCs, suggesting that these cells are largely static in the absence of an appropriate stimulus. In the presence of 100ng/ml of CCL19, a rapidly rising cell index was observed in hiPDCs for the first 1.5 hours of the experiment (figure 3.13A), the maximum cell index being achieved after 2.5 hours (at 0.77), after which the signal was found to plateau. The chemotaxis of moDCs was low compared to that of hiPDCs, with the maximum cell index of 0.21 being attained at 0.7 hours (Figure 3.13A). The cell index of moDCs in absence of agonist was found to fall below zero at several time points, the biological relevance of which has yet to be elucidated. However, significant donor to donor variability was observed with respect to the migration of moDCs in response to chemokines. This plot is therefore representative of 3 independent experiments with moDCs being obtained from different donors in each experiment. The delta cell index of both moDCs and hiPDCs are seen in Figure 3.13B and C. A significant difference was observed between the delta cell index of hiPDCs migrating in the presence of CCL19 compared to cells in the absence of chemokine, indicating that hiPDCs are able to respond to local chemotactic stimuli.

3.2.7 Subsets of hiPDCs

As mentioned in the introduction, the four major subsets of human DCs are plasmacytoid, expressing CD123; CD14⁺ DCs, which are more monocyte and macrophage-like and express CD11c and CD14; Langerhans cells (LCs), which express CD207 and possess birbeck granules; and mDCs, which express CD11c. In humans both CD14⁺ DCs and mDCs express CD11c, but mDCs lack CD14. mDCs can be further subdivided into CD1c⁺ and CD141⁺ fractions [41].

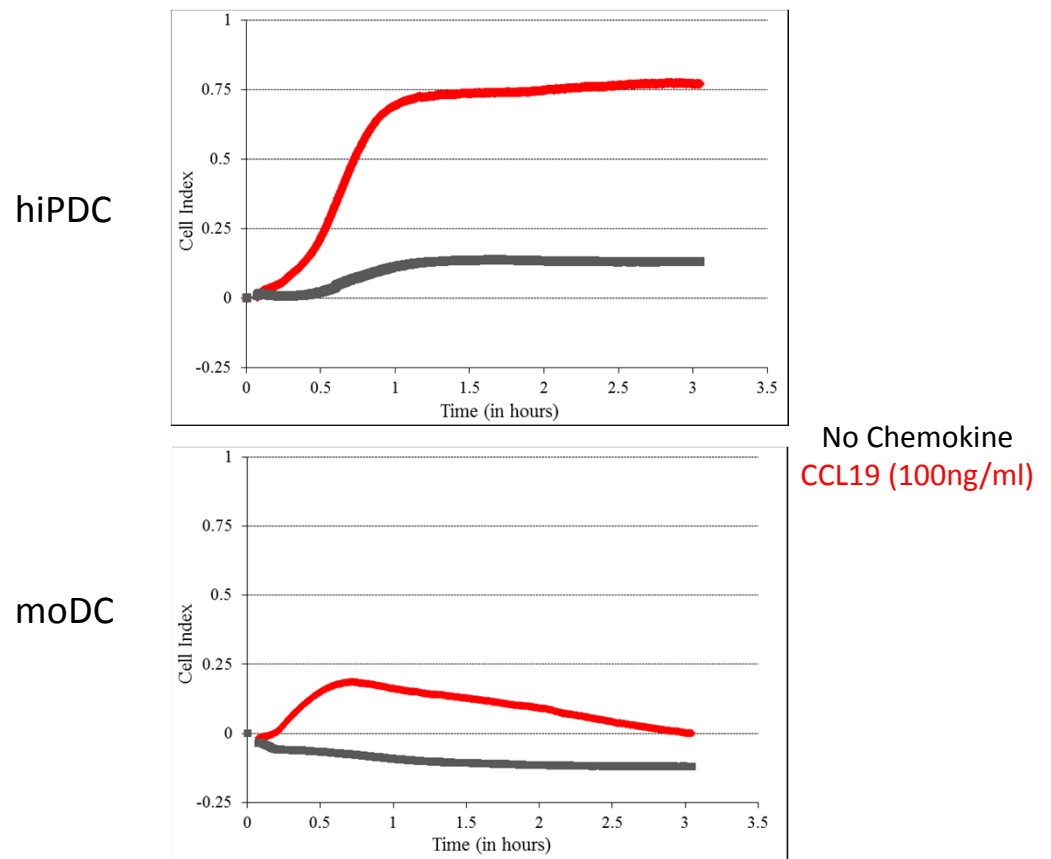
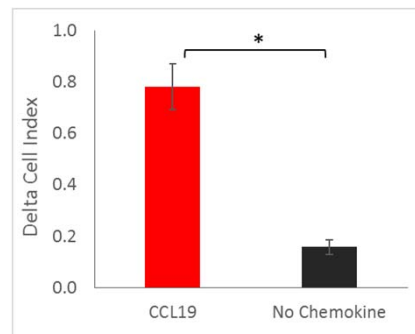
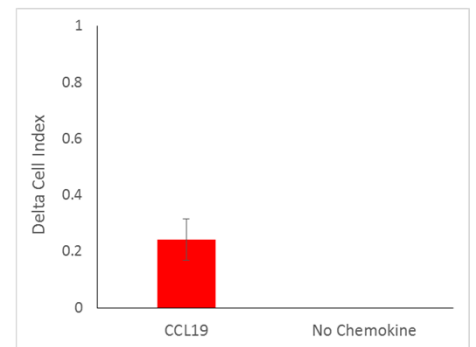
A**B****C**

Figure 3.13 hiPDCs can migrate in response to CCL19. 4×10^5 hiPDCs or moDCs were placed in the top chamber with CCL19 (100ng/ml) (red) or no chemokine (black) in the bottom chamber of the 16 well plate. Chemotaxis was measured using xCELLigence RTCA-DP instrument for 4 hours. **(A)** Depicts the plot of arbitrary unit, cell index, used to measure impedance on the y axis vs. time on the x-axis. **(B)&(C)** represents the comparison of delta cell index, the max-min of cell index achieved in the experiment for CCL19 and no chemokine control for hiPDC **(B)** and moDC **(C)**. Plot is representative of 3 independent experiments, with n=2 in each experiment. Result is depict as Mean $\pm$ SD; Statistical analysis was performed using non-parametric T test with Welch's correction. (* $p \leq 0.05$)

We have previously shown that hiPDCs express cell surface molecules CD11c, CD11b and HLA class II, which identify the mDC population [41]. However, as demonstrated earlier, they were also found to express pDC marker, CD123 and monocyte marker, CD14. We further investigated the cell surface expression of CD11c alongside CD14, CD123 and CD207 on hiPDCs by flow cytometry to identify the different subsets of hiPDCs, using moDCs by way of comparison.

As shown in Figure 3.4A, on average 85% of cells in all hiPDC differentiations express CD11c, indicating a significant proportion of hiPDCs may be myeloid-like. However, this is variable and in some differentiations, may reach as high as 95%. In experiments described here, 80% of hiPDCs were found to express CD11c (Figure 3.14 A). A subset of these cells, however, co-expressed CD123, a marker of pDCs (approx. 10-46% of total cells, varied between experiments). A subset of CD11c⁺ hiPDCs and moDCs (approx. 26% and 31% of total cells, respectively; varied between experiments), was also found to co-express the monocyte marker CD14, a possible vestige of their incomplete differentiation. No expression of CD207 was found in either hiPDCs or moDCs, confirming the absence of cells with the features of Langerhans cells.

As mentioned earlier, a majority of hiPDCs express CD11c, a marker of human myeloid DC (mDC) subset identified *in vivo*. Two subsets of myeloid DCs have been described in humans, the majority CD1c⁺ fraction and a minority, CD141⁺ cross presenting fraction. To further investigate whether the CD11c⁺ myeloid-like hiPDCs contain these two subsets observed *in vivo* [41], we stained hiPDCs with CD11c alongside CD1c and CD141. As shown in Figure 3.14, 35% of CD11c⁺ hiPDCs were found to co-express CD141 and CD1c, while 22% expressed CD141 alone. However, a major subset of CD11c⁺ hiPDCs (almost

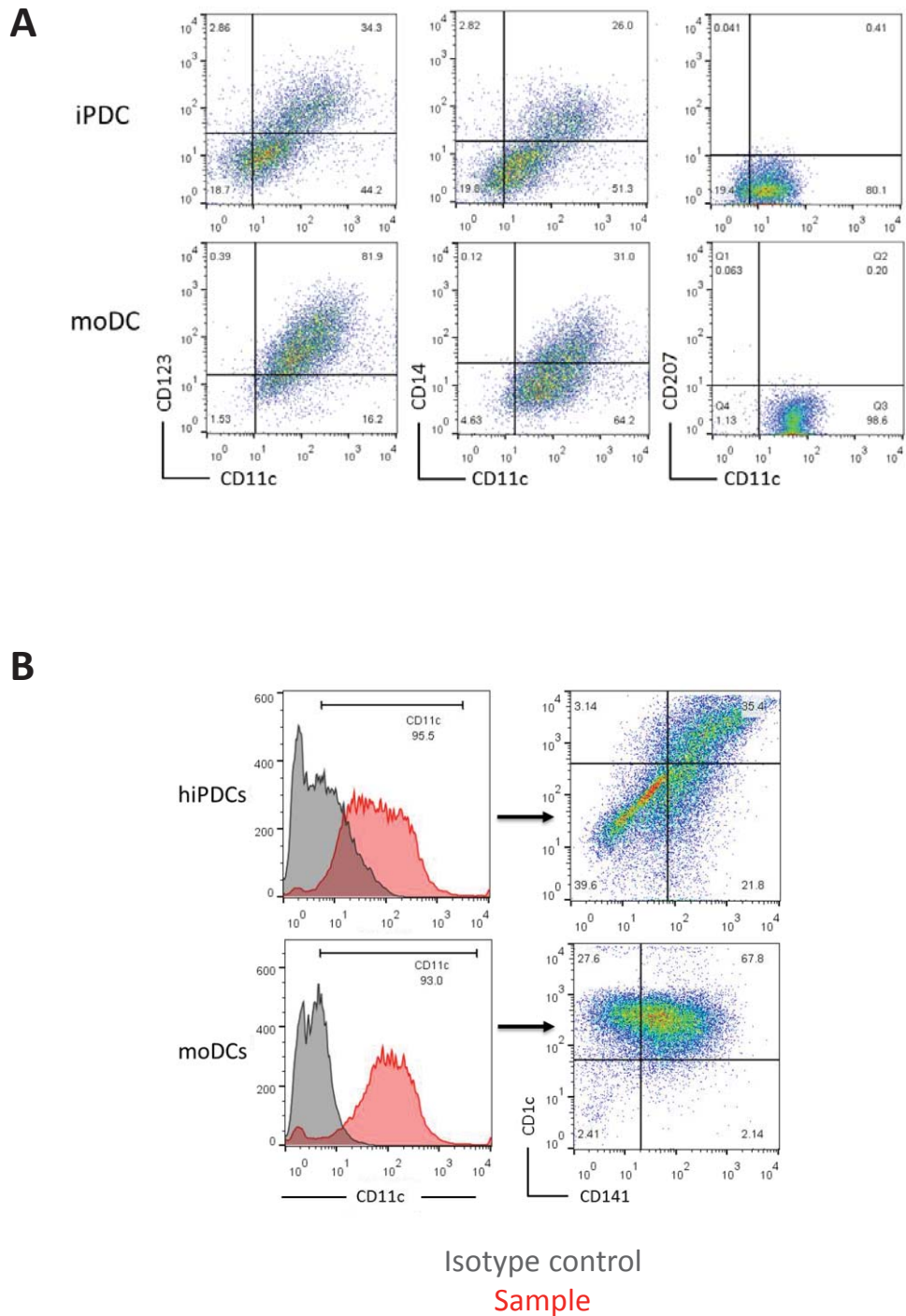


Figure 3.14 Subsets of hiPDCs. Immature hiPDCs and moDCs were stained for various markers to determine the subset of DCs differentiated from iPSC **(A)** DCs were stained for CD11c, shown on x-axis, alongside markers of pDC (CD123), Langerhans Cells (CD207) and monocytes (CD14), shown on the y-axis **(B)** Cells were stained for co-stained for CD11c, CD1c and CD141 to determine subsets of mDCs. CD11c⁺ cells were gated to show the expression of CD141 (x-axis) vs. CD1c (y-axis). All FACs plots are representative of 3 independent experiments; Isotype control in histogram overlays are shown in black, antibody stained cells are shown in red; gates in dot plots are set based on expression of isotype control.

40%) did not express either of these markers. In moDCs, almost 70% of the CD11c⁺ moDCs were found to co-express CD141 and CD1c, while the remainder expressed CD1c alone. No double negative and CD141 single positive subsets were observed. These results suggest that hiPDCs consist of a heterogeneous population of DCs.

3.2.8 hiPDCs express markers of antigen cross presentation

As mentioned in the introduction, the CD141⁺ subset of mDCs was recently identified as the cross presenting subset of DCs in human. Since this subset would be ideal for application in immunotherapy and since our hiPDCs express CD141, we investigated their expression of other markers of the cross-presenting subset, namely XCR1 and CLEC9A [51-53].

Around 45% of differentiated cells in culture were found to co-express CD141 and CD11c, although this number varied significantly between experiments. Around 6% of hiPDCs (in this particular experiment) co-expressed cross presentation markers CD141 and XCR1, although this percentage also varied extensively between independent experiments (Figure 3.15A). A proportion of moDCs, like hiPDCs was also found to co-express CD11c and CD141, with a small subset expressing XCR1. No expression of CLEC9A was observed, either in moDCs or hiPDCs although reports suggest that its expression is not always reproducible and is highly dependent on culture conditions [285, 286].

The percentage of XCR1⁺ cells ranged from 0-20% across 9 experiments, with the median being 3.99%. When found to be present, the mean fluorescence intensity (MFI) of XCR1 was found to be relatively low with the average MFI being 1.47. It should be noted,

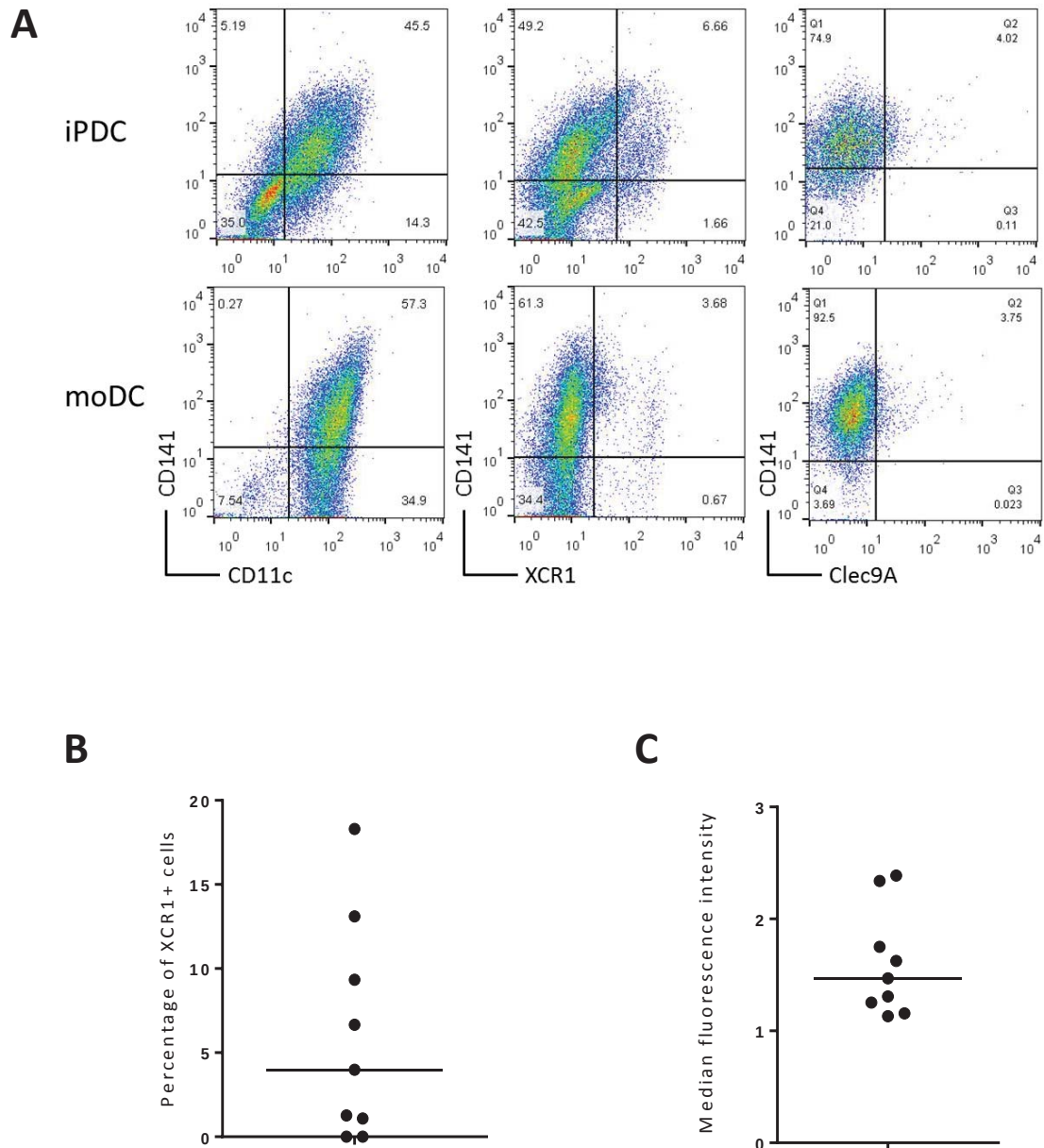


Figure 3.15 Expression of markers of cross presenting DC subset. Immature hiPDCs and moDCs were stained for markers used to identify human cross presenting DCs, CD141, XCR1 and CLEC9A. **(A)** Dot plots showing the expression of CD11c vs. CD141; CD141 vs. XCR1; and CD11c vs. CLEC9A; respectively. **(B)** Plot showing percentage of CD11c⁺ CD141⁺ XCR1⁺ hiPDCs over 9 experiments. Median=3.99% $\pm$ SD 6.47; n=9. **(C)** Plot showing the relative median fluorescence intensity of XCR1 across different experiments. Median=1.47% $\pm$ SD 1.6; n=9. All FACS plots are representative of at least 3 independent experiments; gates in dot plots are set based on expression of isotype control. Each symbol in graphs represents an individual experiment with the black line denoting the median.

however, that flow cytometric analysis of chemokine receptor expression has been shown to have poor sensitivity and low signal to noise ratio, since their highly conserved nature between species has made it difficult to produce high affinity mAbs [287]. Given that XCR1 was found to be expressed only in some experiments and the percentage of XCR1⁺ cells was found to be variable, we proceeded to confirm the expression of functional XCR1 by investigating the ability of hiPDCs and moDCs to migrate in response to an increasing gradient of its ligand, the chemokine XCL1. *In vivo*, XCL1 is secreted by CD8⁺ T cells and acts as a chemo-attractant for enhancing their cross presentation of antigen to the MHC class I-restricted T cell repertoire [288].

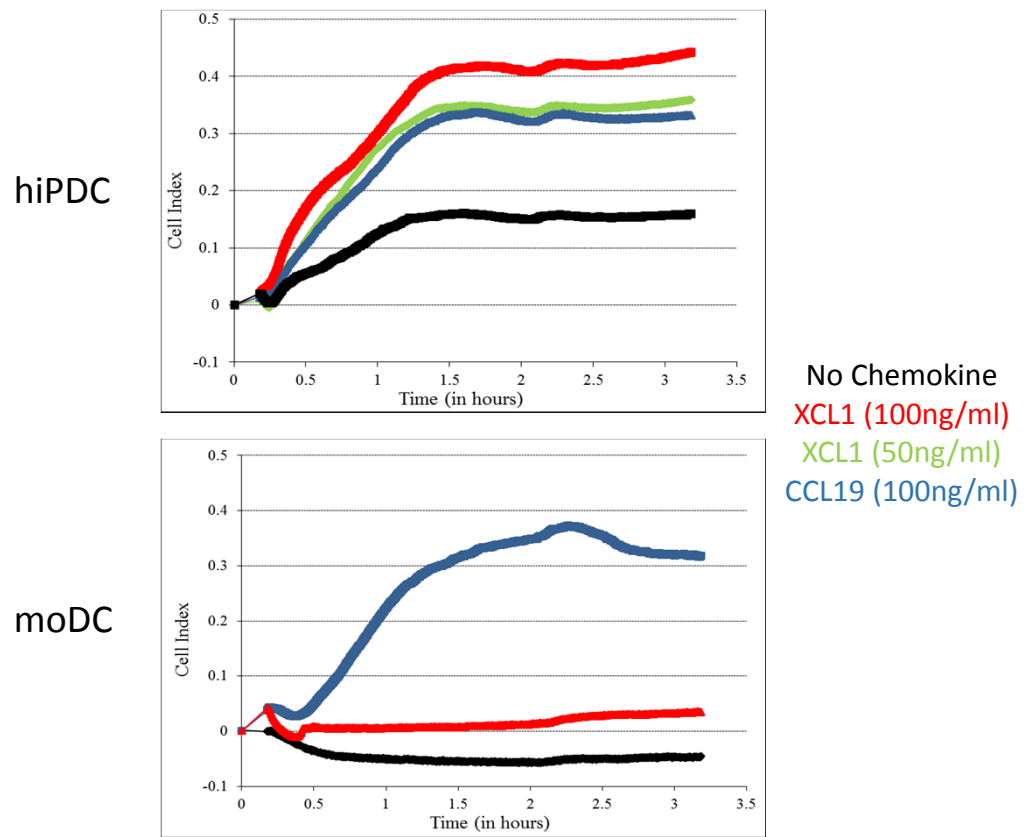
To investigate the capacity of hiPDCs, as well as moDCs, to migrate along a concentration gradient of XCL1, we made use of the xCELLigence RTCA instrument used earlier for investigating the ability of hiPDCs to migrate in response to CCL19. XCL1 was introduced into the lower chamber of the wells and cells were loaded in the upper chamber. Since hiPDCs and moDCs were both found to migrate along a gradient of CCL19 (Figure 3.13), this chemokine was used as a positive control for chemotaxis. In the absence of any chemokine, a very low background signal was observed in both moDCs and hiPDCs. A rapidly rising cell index was observed for the first 1.5 hours of the experiment in response to the presence of 100ng/ml of CCL19, 100ng/ml of XCL1 and 50ng/ml of XCL1 (Figure 3.16A), with the maximum cell index being achieved after 3 hours. The delta cell index of cells migrating to XCL1 was found to be significantly higher than CCL19. These data indicate the expression of functional XCR1 by hiPDCs and suggests that the issues surrounding its detection by FACS may be due, in part, to the low affinity of mAbs used.

moDCs were found to migrate strongly to CCL19 (Figure 3.16A), with the delta cell index being significantly higher than that of wells containing no cytokine, consistent with results described in section 3.6.4. However, the delta cell index of moDCs migrating in response to XCL1 was found to be low suggesting that moDCs may not express functional XCR1 receptor, consistent with reports in the literature [53]. It should be noted that the cell index of moDCs in the no chemokine control (similar to figure 3.13A) falls below zero at several time points, an occurrence that has yet to be elucidated.

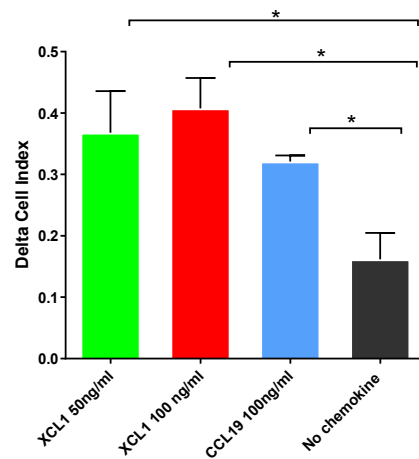
3.2.9 Ability of hiPDCs to process and cross present exogenously-derived antigen

Given that hiPDCs have been shown to express makers of cross presentation, CD141 and functional expression of XCR1, we investigated whether iPSC derived DCs can cross present exogenously-derived antigen via HLA class I. We pulsed hiPDCs with the well-characterised cancer testis antigen, NYESO₍₁₅₇₋₁₆₅₎ 9V peptide or NYESO recombinant protein for 8 hours before co-culturing these cells with Jurkat cells genetically modified to express the 1G4 TCR specific for NYESO peptide, presented in the context of HLA-A*0201. T2 GFP, an HLA-A*0201⁺ B lymphoblast cell line deficient in transporter associated with antigen processing -1 (TAP-1), was co-cultured with 1G4 Jurkat in the presence of NYESO 9V peptide as a positive control for activation. Unmodified Jurkat cells were used as negative controls. After 12 hours of co-culture, cells were harvested and stained for CD3, CD11c and T cell activation marker, CD69. We observed the upregulation of CD69 expression, consistent with activation of 1G4 Jurkat cells, in response to NYESO 9V peptide presentation on the surface of hiPDCs and T2-GFP cells, while no upregulation was observed when DCs or T2 cells were not pulsed with peptide (Figure 3.17A), suggesting that CD69 expression may serve as a surrogate marker

A



B



C

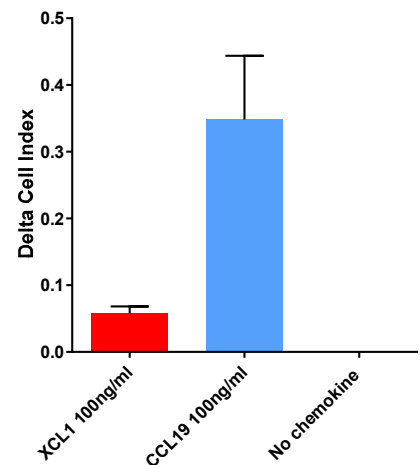


Figure 3.16 hiPDCs can migrate in response to XCL1. 4×10^5 hiPDCs or moDCs were placed in the top chamber with XCL1 (green-100ng/ml; red-50ng/ml); CCL19 (blue-100ng/ml) or no chemokine (black) in the bottom chamber of the 16 well plate. Chemotaxis was measured using RTCA-DP instrument for 4 hours. **(A)** Depicts the plot of cell index (used to measure impedance) with respect to time. Graphs **(B) & (C)** represents the comparison of delta cell index (max-min of cell index over 4 hours) for each chemokine and no chemokine control for hiPDCs **(B)** and moDCs **(C)** Plot is representative of 3 independent experiments, with $n=2$ in each experiment. Result is depicted as Mean $\pm$ SD; Statistical analysis was performed using non-parametric T test with Welch's correction. ($*p \leq 0.05$).

for T cell activation. We therefore used this system to investigate activation of Jurkat cells by iPDCs pulsed with whole NYESO protein. We observed upregulation of CD69 expression when 1G4 Jurkat cells were co-cultured with hiPDCs pulsed with either peptide or NYESO protein applied in equimolar concentrations, indicating that hiPDCs can indeed process exogenously derived protein antigens and present them via HLA class I (Figure 3.17B). However, despite repeated attempts with both immature and mature hiPDCs, optimising both the concentration of peptide and protein used and adjusting the period of time over which the cells were co-cultured, these results were only observed once. Since CD69 is an early marker of T cell activation and is only transiently expressed, we investigated alternative readouts for the activation of Jurkats.

Since IL-8 is known to be produced by T cells when stimulated by APCs via the CD28 receptor [289], we investigated the secretion of IL-8 during the co-culture of 1G4 Jurkat or unmodified Jurkat cells with hiPDCs in the presence of either 9V NYESO peptide or NYESO protein for 16 hours as an alternative readout to CD69 upregulation. Supernatants were collected and the levels of IL-8 secretion were determined by standard ELISA. Although IL-8 was found to be produced by 1G4 Jurkat cells co-cultured with hiPDCs in the absence of antigen, the level of IL-8 was significantly higher in presence of NYESO protein suggesting cross-presentation of the antigen (Figure 3.17C). However, hiPDCs cultured alone were found to secrete moderate levels of IL-8 which was significantly upregulated in the presence of NYESO 9V peptide. Although the level of IL-8 produced by hiPDCs in the presence of protein was not investigated, these data suggests that the high levels of IL-8 observed in co-culture may be, in part, secreted by hiPDCs, thereby confounding interpretation of the results. This is supported by the fact that supernatants from unmodified Jurkat cells, which do not express the 1G4 TCR, were also found to

contain high levels of IL-8 when co-cultured with hiPDCs (Figure 4.17C). The production of IL-8 by hiPDCs makes this assay unsuitable for determining their capacity to process exogenously-derived NYESO protein and present the peptide via HLA class I to 1G4 Jurkat.

Although the upregulation of CD69 expression observed in 1G4 Jurkat co-cultured with hiPDCs that have been pulsed with protein suggests that hiPDCs possess the capacity to process and present exogenously derived antigen via HLA class I, more evidence is required before a definitive conclusion can be drawn.

3.2.10 Capacity of hiPDCs to cross present HIV-1 antigens to CD8⁺ T cells

To determine the feasibility of exploiting hiPDCs as a therapeutic vaccine for HIV-1, we investigated the capacity of hiPDCs to process exogenously-derived HIV-1 antigen and present it via HLA class I to naïve CD8⁺ T cells. Immature HLA-A*0201⁺ moDCs and hiPDCs were either left untreated or pulsed with HIV-1-Gag peptide (SLYNTVATL) or recombinant HIV-1-Gag protein and co-cultured with HLA-A*0201-restricted naïve T cells at a ratio of 1:10. T cells appeared activated and started clustering after 2-3 days. Recombinant IL-2 was added to the cultures from day 4 onwards to promote proliferation of antigen specific T cells. By day 5-6, we observed a rapid expansion of T cells in the well. After 14 days, cells were harvested and stained with HLA-A*0201 SLYNTVATL tetramer alongside CD8 and CD11c.

We observed the presence of a population of CD8⁺ cells (3.09%) which stained positively for HLA-A*0201 SLYNTVATL tetramer when hiPDCs pulsed with HIV-1 Gag protein were co-cultured with HLA-A*0201 T cells isolated from PBMCs (Figure 3.18). Unexpectedly, no

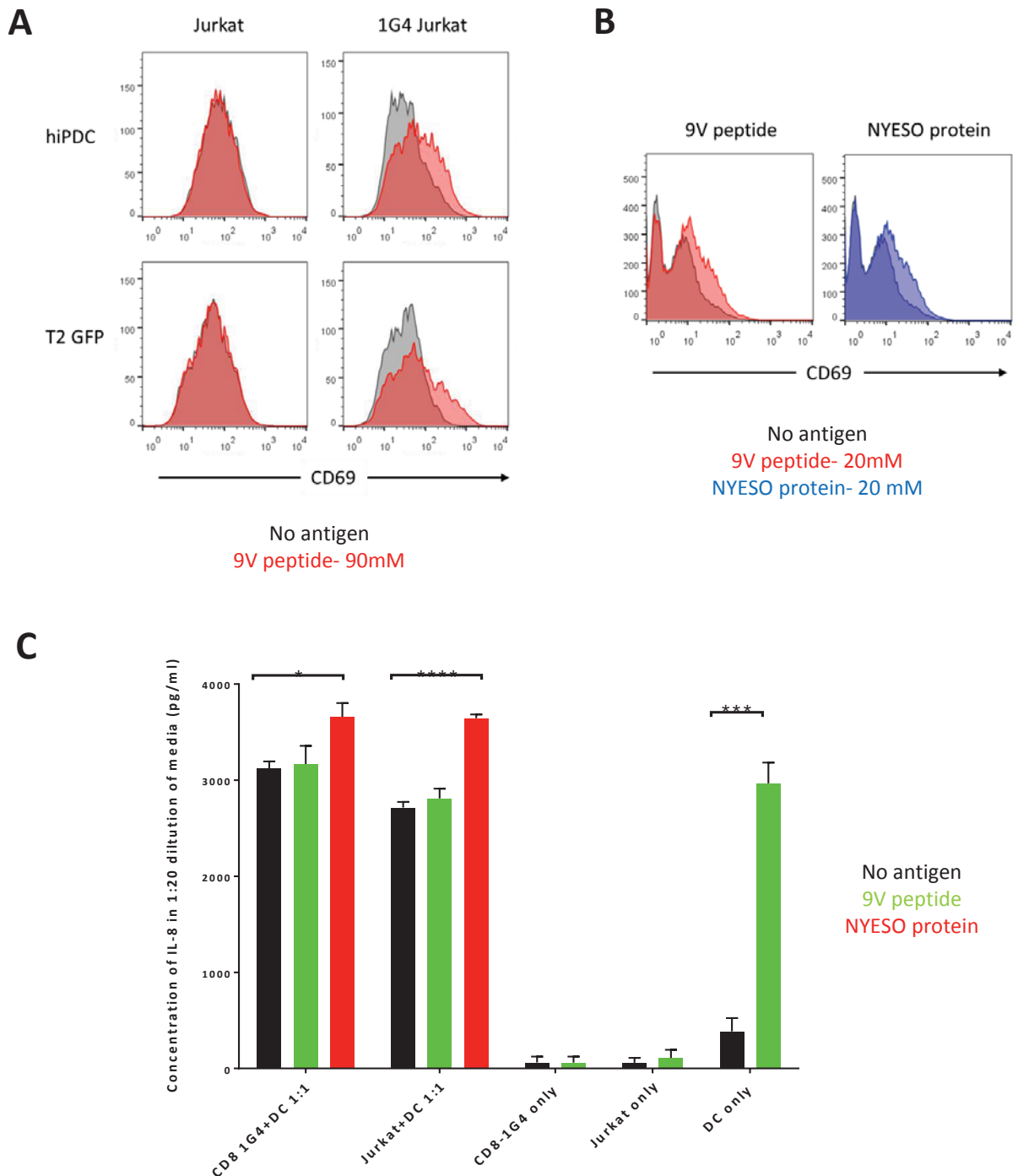


Figure 3.17 Capacity of hiPDCs to process and present exogenous antigen. T2 GFP cells or hiPDCs were pulsed with 9V ESO₁₅₇₋₁₆₅ peptide or NYESO recombinant protein, incubated for 8 hours and co-cultured with 10⁵ Jurkat cells or HLA-A2-specific 1G4 Jurkat cells for 12 hours. **(A)&(B)** Cells were harvested and stained for T cell activation marker, CD69 alongside CD3 and CD11c and analysed by flow cytometry. **(A)** CD69 expression in CD3⁺ Jurkats and 1G4 Jurkats cultured with either T2 GFP or hiPDCs pulsed with 90mM 9V peptide. FACS plots represent best data from 15 independent experiments **(B)** CD69 expression in CD3⁺ 1G4 Jurkats cultured with hiPDCs pulsed with either 20mM 9V peptide or NYESO protein. **(C)** IL-8 secretion was determined by ELISA. Black bars indicate cells not pulsed with antigen, red bars indicate cells pulsed with NYESO protein while green bars indicate cells pulsed with 9V peptide. Plot is representative of 2 independent experiments, with n=3 in each experiment. Error bars depict standard deviation. Statistical analysis was performed using parametric T test with Welch's correction. (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

staining with tetramer was detected in T cells co-cultured with hiPDCs or moDCs pulsed with HIV-1 SLYNTVATL peptide. These results were, however, only observed in one instance despite repeated attempts with both immature and mature hiPDCs at various DC:T cell ratios.

Since we expected the presentation of HIV-1 SLYNTVATL peptide by autologous moDCs to HLA-A*0201⁺ T cells to serve as a positive control in these experiments, the lack of tetramer staining of the CD8⁺ T cells from these cultures was unanticipated. Due to low numbers of T cells obtained from PBMCs of HLA-A*0201⁺ donors, we were unable to deplete the memory T cell subset (CD45RO) before co-culturing the cells with DCs. This may have led to the expansion of alloreactive memory T cells in a manner similar to an allogeneic MLR, thereby diluting the antigen-specific subset. This would explain the rapid expansion of T cells observed in co-culture around day 5-6.

Although the positive tetramer staining observed in CD8⁺ T cells co-cultured with hiPDCs pulsed with HIV-1-Gag protein suggests that hiPDCs possess the ability to process and present exogenously derived HIV-1 antigen to CD8⁺ T cells, further experiments with CD45RO depleted T cells will need to be performed in order to confirm this finding.

3.2.11 Purification of cross-presenting subset of DCs

We have previously shown that the percentage of CD11c⁺ cells obtained by differentiating iPSCs into DCs is highly variable (Figure 3.3C). Given that the final aim of the project is to

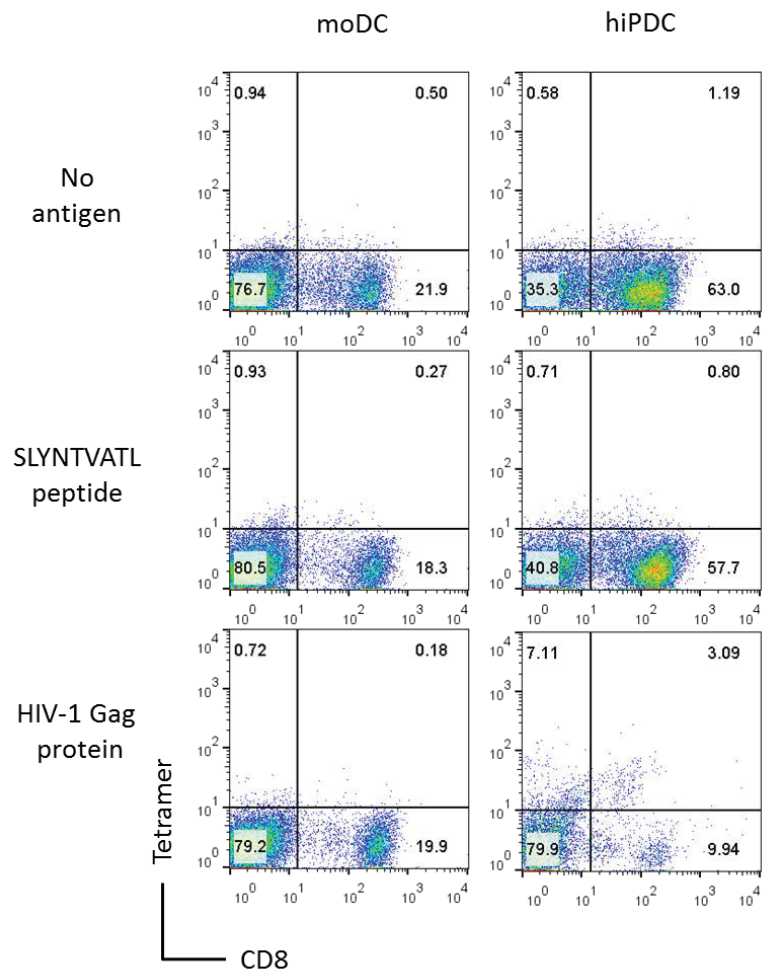


Figure 3.18 Capacity of hiPDCs to cross present HIV antigen. Immature hiPDCs or HLA-A*02:01⁺ moDCs were either left unpulsed or pulsed with HIV-1 SLYNTVATL peptide or HIV-1 Gag protein and co-cultured with HLA-A*02:01⁺ CD8⁺ T cells for 14 days. Cells were harvested and stained for expression of tetramer specific for HIV-1 SLYNTVATL bound HLA-A*02:01 (y axis) with respect to CD8 expression to identify CD8⁺ antigen specific cells that have been primed by DCs against HIV-1 Gag. FACS dot plots represent best data from 6 independent experiments; gates are set based on expression of appropriate isotype control.

harness hiPDCs for use as a therapeutic vaccine for HIV-1, we developed a robust method for purifying the cross-presenting subset of hiPDCs. Early attempts at purifying this subset by fluorescence activated cell sorting (FACS) using XCR1 antibody appear to induce widespread apoptosis. Furthermore, iPDCs were found to be sensitive to the high pressure applied on them during the sorting process.

An initial purification of hiPDCs is achieved at the harvesting stage, where semi-adherent cells are removed from the surface of the tissue culture plastic by gentle pipetting whereas macrophages adhere strongly to the surface [290]. We have established that hiPDCs contain both subsets of myeloid DCs, namely, the CD141 and CD1c subsets, although the markers have been shown to be co-expressed by a proportion of cells (Figure 3.14B). We have also observed that, although hiPDCs express functional XCR1, this marker is not reliable for identifying the cross presenting subset of hiPDCs. Given these findings, we investigated three methods of purification of hiPDCs: purifying the CD11c⁺ population, depleting the CD1c⁺ subset and enriching the CD141⁺ subset.

Having observed the presence of small subsets of contaminating CD14⁺ and CD123⁺ cells harvested from hiPDC differentiations, we proceeded to purify hiPDCs using mDC marker CD11c, which was found to be highly expressed in hiPDCs (Figure 3.3B). hiPDCs were stained with CD11c biotin and purified using anti-biotin microbeads. To detect the expression of CD11c by flow cytometry, cells were counterstained with streptavidin-FITC, which, having a significantly higher affinity than anti-biotin antibodies, competitively replaced anti-biotin beads. Before purification, 55% of hiPDCs were found to express CD11c (Figure 3.19A). After purification, the percentage of CD11c⁺ cells was enriched to

90.1%. The negative fraction obtained from the purification was found to contain only 30.9% CD11c⁺ cells, suggesting a successful enrichment.

We further investigated purifying the CD141⁺ subset of hiPDCs by either depleting CD1c⁺ cells using anti-CD1c microbeads or enriching the CD141⁺ cells through positive selection with anti-CD141 microbeads. After purifying hiPDCs by depleting CD1c⁺ cells, the percentage of CD11c⁺ CD141⁺ increased from 60.4% to 74.8%. On purifying the cells using CD141 enrichment, the percentage of CD11c⁺ CD141⁺ cells increased from 23.4% to 40.5%, which was unexpectedly low. This may be due to the inability to effectively stain for CD141 after enrichment since most surface molecules have probably been occupied by anti-CD141 microbeads.

These results show that purifying the mDC subset using anti-CD11c beads followed by depleting with anti-CD1c beads is an efficient method of enriching the CD11c⁺ CD141⁺ subset, if only because it allows for subsequent staining for CD141 post-purification.

3.2.12 hiPDCs express receptors for HIV-1 entry

In vivo, mDCs have been shown to express HIV-1 receptor CD4 and co-receptors, CCR5 or CXCR4, and have been shown to be infected by HIV-1 and serve as a reservoir of the virus. Reservoirs of HIV-1 can be latent for a long period and can later trans-infect responding CD4⁺ T cells, causing recurrence of HIV-1 infection. Since the final aim of the project was to utilize hiPDCs to cross present HIV-1 antigen to CD8⁺ T cells in the patient, we investigated, whether hiPDCs express co-receptors of HIV-1 infection.

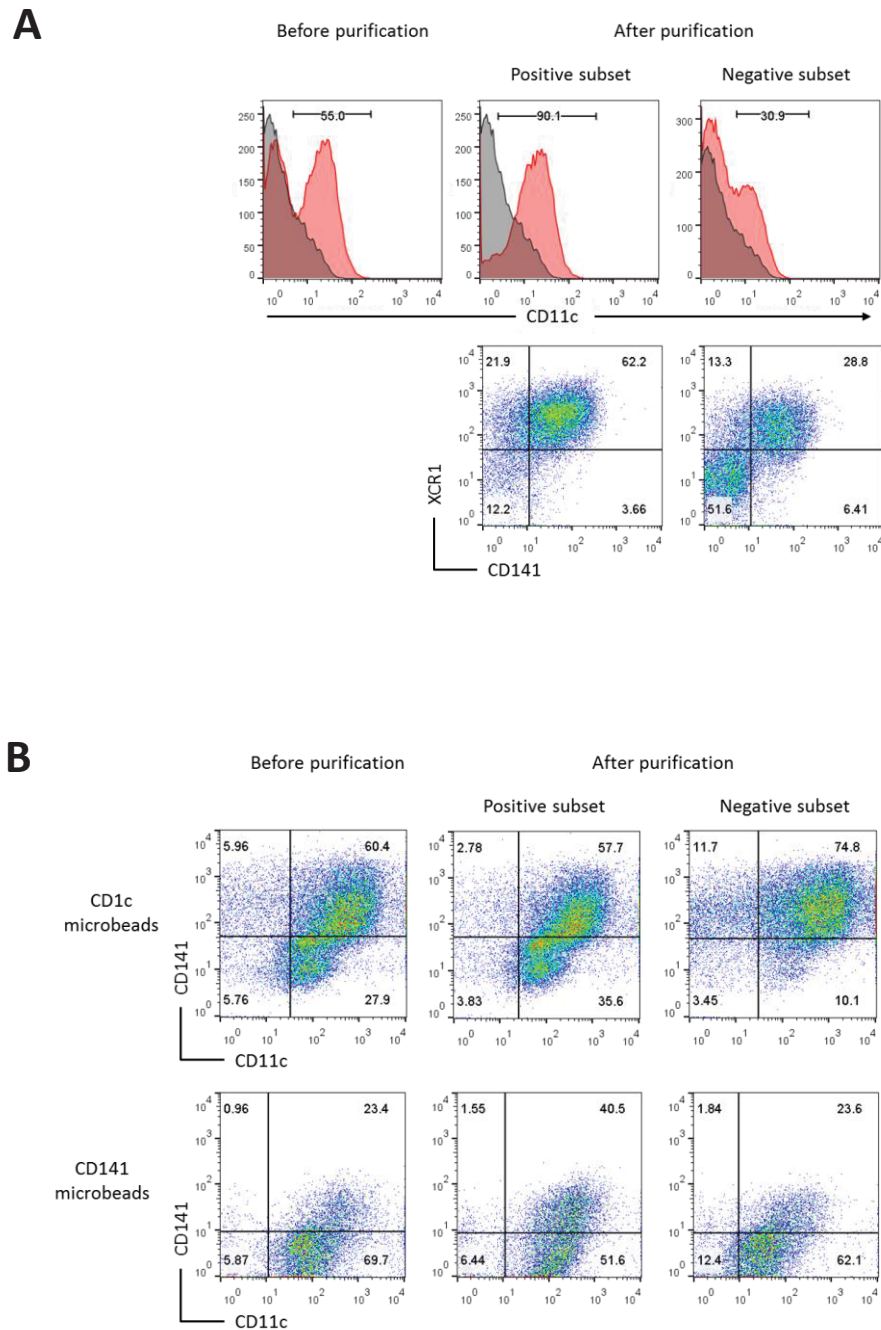


Figure 3.19 Purification of CD11c⁺ and CD141⁺ subset of hiPDCs. hiPDCs were harvested and purified for CD11c⁺ or CD141⁺ subset using the Miltenyi MACs microbead system. (A) hiPDCs were stained with CD11c biotin antibody and purified using anti-biotin microbead. FACS plots show CD11c expression and CD141 vs. XCR1 expression of DCs before purification and of negative and positive subsets, after purification. (B) hiPDCs were purified using anti-CD1c microbeads (top panel) to get rid of CD1c or anti-CD141 microbeads (bottom panel) to positively select for CD141 subset. FACS plots show CD11c expression and CD141 vs. XCR1 expression of DCs before purification and of negative and positive subsets, after purification. FACS dot plots are representative of 4 (for A) and 2 (B) independent experiments; gates are set based on expression of appropriate isotype control.

In the immature state, hiPDCs, like moDCs were not found to express HIV-1 co-receptors CD4, CCR5 and CXCR4. However, on maturation, they were both found to express CD4 and CXCR4 (Figure 3.20A). No expression of CCR5 was detected in moDCs, in contrast to various reports of its expression [291]. Very low expression of CCR5 was observed in mature hiPDCs and this expression varied between experiments and may, like XCR1, be an artefact of the mAbs commercially available.

Using standard RT-PCR, we further investigated the expression of the *CCR5* gene in immature and mature hiPDCs and moDCs, using HeLa cells line, TZMBL (a kind of gift from Dr. Kenny Moore at the Sir William Dunn School of Pathology), which has been transfected with CD4 and CCR5 making it susceptible to HIV-1 infection, as a positive control. We also investigated the expression of the *CCR5* gene in heterogeneous PBMCs, since such a preparation contains DCs, T cells and macrophages, which are known to express CCR5 [292]. *GAPDH* housekeeping gene was used as a positive control for gene expression. We observed the gene expression of *CCR5* as well as *GAPDH* in both immature and mature moDCs and hiPDCs as well as in both positive controls, PBMCs and TZMBL cells transfected with CD4 and CCR5, but not in the water negative control, indicating the presence of CCR5 at the mRNA level in both sources of DCs (Figure 3.20B). Unlike all other cells, three bands were observed at 189 bp for mature moDCs. We speculate that this may be three splice variants of CCR5, however, there are no reports of such splice variants of *CCR5* in the literature and this needs to be addressed in future studies.

To further investigate the expression of functional CCR5, we examined the ability of hiPDCs and moDCs to migrate in response to a gradient of CCL5, the ligand of CCR5, using

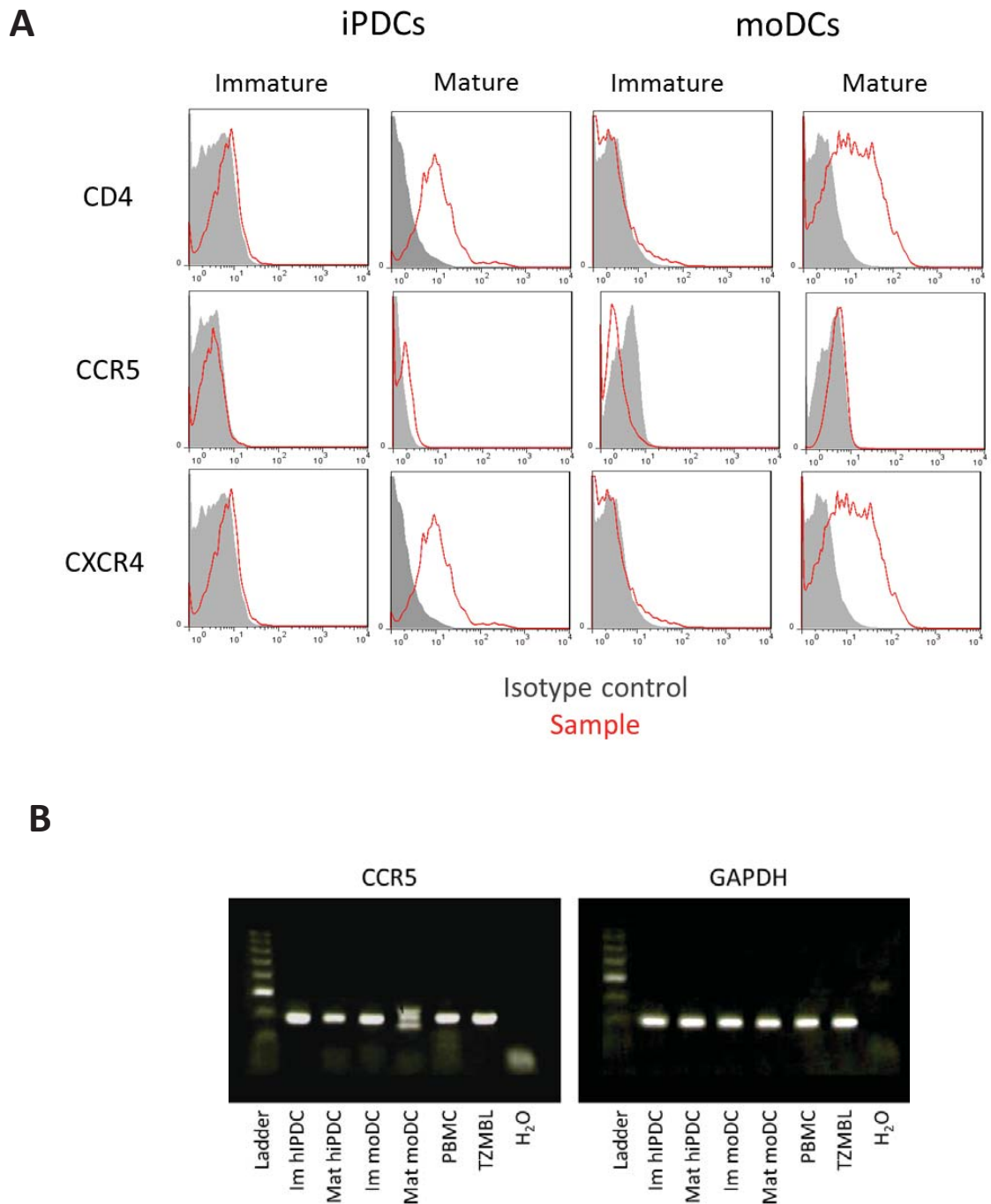


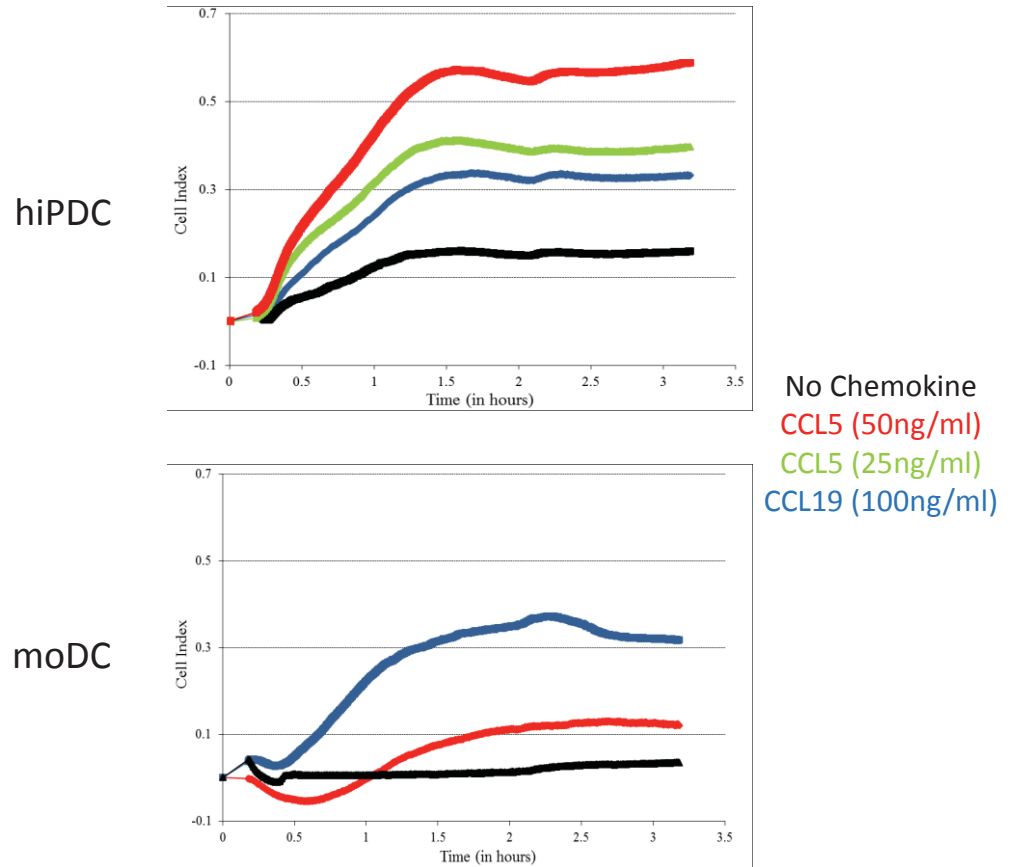
Figure 3.20 Expression of co-receptors for HIV-1 entry. Immature and mature hiPDCs and moDCs were investigated for receptors used for HIV-1 infection **(A)** DCs were stained with HIV-1 co-receptors CD4, CCR5 and CXCR4 and analysed by flow cytometry; All FACs plots are representative of at least 3 independent experiments; Isotype control in histogram overlays are shown in black, antibody stained CD11c gated DCs are shown in red **(B)** mRNA was extracted from cell lysate of each sample. RT-PCR was carried out with as described in methods section. SYBR safe staining of PCR products show the presence of bands for CCR5 (189bp) and housekeeping gene GAPDH (201bp) in samples separated in 1.5% agarose gel. Water was used as negative control; Ladder=100bp.

the xCELLigence RTCA instrument. Since we earlier established that hiPDCs and moDCs are able to migrate to CCL19, we again used CCL19 in these experiments as a positive control for chemotaxis. Both moDCs and hiPDCs were found to migrate in the presence of 100ng/ml of CCL19, with the delta cell index of CCL19 being significantly higher than that of negative control samples, consistent with earlier data. hiPDCs were also found to migrate along a chemokine gradient of 50ng/ml and 25ng/ml of CCL5 in a dose-dependent manner (Figure 3.21A), the maximum cell index being achieved after around 1.5 hours. The delta cell indices of both concentrations of CCL5 were found to be significantly higher than that of the no chemokine control (Figure 3.21B), indicating the presence of functional CCR5. The delta cell index of moDCs migrating to CCL5 was found to be low in comparison and not significantly different from the no cytokine control (Figure 3.21C), despite our earlier finding that CCR5 was present at the mRNA level in these cells and confirming the need for functional studies to formally determine chemokine receptor expression.

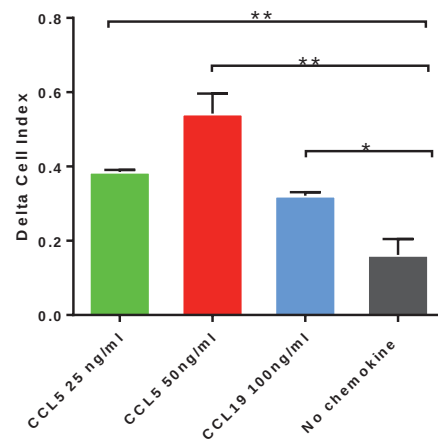
3.2.13 hiPDCs can be infected by HIV-1

Given that hiPDCs were found to express co-receptors for HIV-1 entry, CD4, CCR5 and CXCR4, we further investigated the ability of these cells to be productively infected by HIV-1. For these purposes, we infected the cells with an R5 tropic clone of HIV-1 which contains a JRFL glycoprotein envelope (HIV-1 HIG-GFP-JRFL) and the positive control virus, HIV-HIG-VSVG, which contains an envelope allowing it to infect most cells. The R5-tropic viruses should only be able to infect cells expressing CD4 and CCR5. Since moDCs and TZMBL cell line (expressing CD4 and CCR5) has been previously shown to be infected by

A



B



C

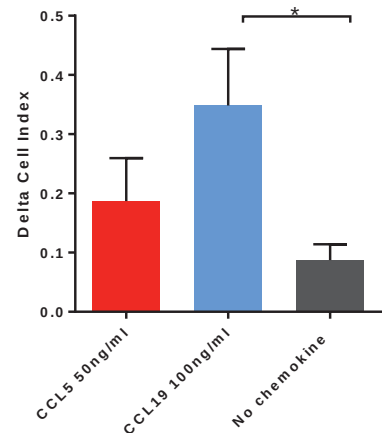


Figure 3.21 hiPDCs can migrate in response to CCL5. 4×10^5 hiPDCs or moDCs were placed in the top chamber with CCL5 (green-50ng/ml; red-25ng/ml); CCL19 (blue-100ng/ml) or no chemokine (black) in the bottom chamber of the 16 well plate. Chemotaxis was measured using RTCA-DP instrument for 4 hours. **(A)** Depicts the plot of cell index (used to measure impedance) on the y axis vs. time on the x-axis. **(B) & (C)** represents the comparison of delta cell index (max-min of cell index over 4 hours) for each chemokine and no chemokine control for hiPDCs **(B)** and moDCs **(C)**. Plot is representative of 3 independent experiments, with n=2 in each experiment. Result is depicted as Mean $\pm$ SD; Statistical analysis was performed using non-parametric T test with Welch's correction. (* $p \leq 0.05$, ** $p \leq 0.001$).

these viruses (Dr. Kenny Moore, personal communication), these cells were used as a positive control.

The percentage of cells infected by the GFP reporter viruses HIV-HIG-JRFL and HIV-HIG-VSVG was measured by the number of GFP⁺ cells present after 3 days of infection with the virus. Using HIV-HIG-JRFL, very little GFP expression could be detected among hiPDCs, moDCs and TZMBLs cells, indicating either no infection or very low infection by the virus, which may be an indication of the low titre of virus present (Figure 3.22A). However, on infection with HIV-HIG-VSVG, all cells were found to express GFP, although the percentage of hiPDCs and moDCs infected (24% and 38% respectively) was much lower than that of TZMBL cells (85%), consistent with observations by Dr. Kenny Moore that TZMBL transfected with CD4 and CCR5 are readily tractable targets for infection by HIV-HIG-VSVG and serve as a good positive control (Figure 3.22A).

To confirm that hiPDCs can indeed be infected with HIV-1 virus, we further infected the hiPDCs, moDCs and TZMBL cells with HIV-NL4.3 Luc, a non-replicating HIV-1 virus expressing luciferase enzyme. Cells were infected with either neat virus, 1:10 dilution or no virus, to determine whether the amount of luciferase (and hence, viral load) is dependent on the number of virus particles present in the surrounding medium. Three days after infection, substrate for luciferase was added and the amount of chemiluminescence was detected using a standard plate reader.

hiPDCs, moDCs and TZMBL cells were all found to be infected with HIV-NL4.3 Luc, with the amount of chemiluminescence detected (in relative light units) decreasing concomitantly with virus titre (Figure 3.22B). TZMBL, being highly susceptible to HIV-1 infection was found to have the highest viral load, with cells infected with the virus at

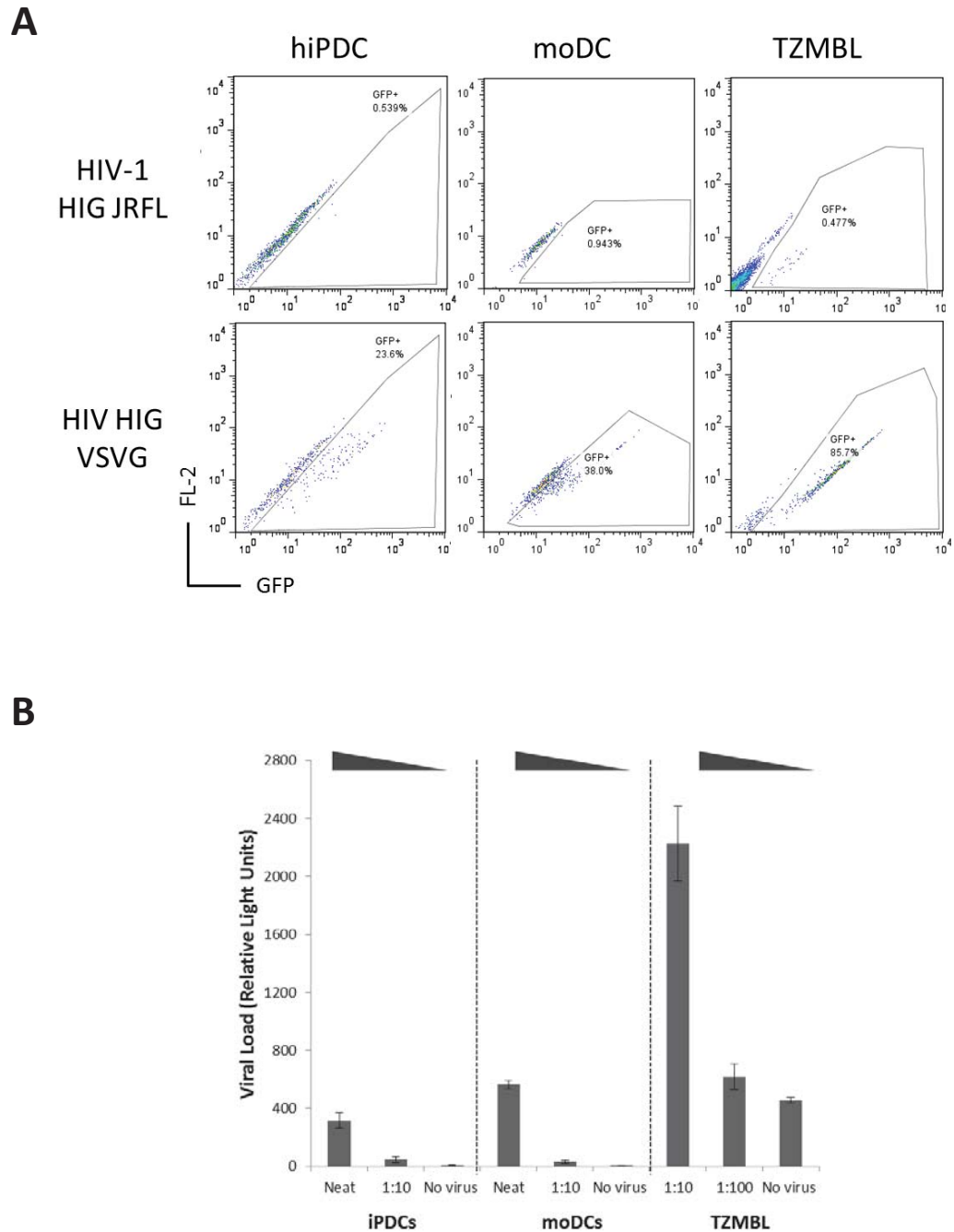


Figure 3.22 hiPDCs can be infected with HIV-1. 5×10^5 immature hiPDCs and moDCs and 10^5 CD4⁺ CCR5⁺ TZMBL line cells were infected with non-replicating HIV-1 for 3 days to determine whether HIV-1 can infect these cells. **(A)** Infection of cells with GFP reporter viruses, HIV-HIG-JRFL and HIV-HIG-VSVG. Cells were fixed and analysed by flow cytometry; All FACs plots are representative of 2 independent experiments with $n=3$ in each experiment; Cells are gated on GFP⁺ fraction **(B)** Cells were infected with Luciferase⁺ HIV-NL4.3 Luc; Luciferase substrate was added at the end of the incubation and relative levels of chemiluminescence was determine using a plate reader. Plot is representative of 2 independent experiments with $n=3$ for each experiment. Error bars = SD.

1:10 titre having five times the viral load observed in moDCs. hiPDCs were least readily infected with HIV-1, although the viral load was also found to be dose-dependent.

3.3 Discussion

Previous work in the lab has demonstrated the feasibility of differentiating DCs from human iPSCs. We sought to build upon these findings by further characterising this novel source to determine their suitability for downstream use as a therapeutic vaccine for HIV-1. An ideal therapeutic vaccine for HIV-1 should be able to induce cytotoxic immune response against HIV-1 and be able to clear all sanctuary reservoirs of the virus, while producing minimal side effects.

To investigate this, we differentiated DCs utilizing an iPSC line that has been previously shown by our laboratory to give rise to DCs *in vitro* [218]. Our early experiments revealed significant cell death using the initial protocol, in which stem cell clusters were plated into a low binding plate in XVIVO-15 based medium. We therefore modified the protocol to harvest cell clusters into mTeSR-1, the medium in which iPSCs were cultured, with the addition of growth factors, GM-CSF, VEG-F, SCF and BMP-4 (Figure 3.2B). The initial formation of EBs in the medium in which iPSCs were cultured, corroborates most protocols for the generation of EBs from stem cells for the derivation of monocytes or macrophages [293, 294]. Although we have not established why this modification led to less cell death in the differentiation cultures, it has since been successfully used to differentiate DCs not only from the C15 line but also from two other iPSC lines derived

from a Pompe disease patient in our laboratory (Alison Leishman, unpublished thesis) (Figure 3.2C).

Although the differentiation of DCs from iPSCs takes up to 5 weeks and requires the addition of many growth factors, it does not make use of the mouse stromal OP9 cell line, which has been used in other protocols of DC differentiation, precluding their downstream clinical application [295, 296]. Other work in the lab has shown that matrigel extracellular matrix used to coat plates for iPSC culture can be replaced by fully-defined, xeno-free Corning Synthemax culture plates, making this protocol amenable for application in the clinic. In 2013, following the publication of the protocol for differentiation of DCs from iPSC by our laboratory, Yanagimachi *et al*, published a protocol for the derivation of DCs and macrophages from iPSC. Their protocol involved a 5-step method where the iPSCs grown on matrigel-coated plates were differentiated in StemPro34 medium with the addition of BMP-4, VEGF, SCF, bFGF, IL-3, Thrombopoietin, M-CSF, GM-CSF, IL-4 and Flt-3 ligand [294]. Although the time taken to obtain pluripotent stem cell derived-DCs (PS-DCs) at the end of the 5 step process was roughly similar to ours (22-35 days), the protocol required sorting of CD14⁺ cells at the end of day 15-22 and involves the use of many more growth factors than our protocol, making the process more expensive and cumbersome. The yield of CD11c⁺ hiPDCs differentiated from our protocol was found to vary extensively from experiment to experiment, ranging from as little as 3.7% to as high as 87.4% of the starting iPSC numbers (Figure 3.3 C). We have not yet established the reason for the extensive variability between experiments, however, this variability has been observed for the differentiation of iPSC into many different cell types and may reflect the chaotic nature of the differentiation pathway from a pluripotent source [260-262].

Most of the cells obtained at the end of our differentiation display irregular shape with long processes, characteristic of DCs (Figure 3.3A). On average, 85% of cells produced at the end of the differentiation process expressed CD11c, a marker of mDCs. They also expressed other cell surface molecules typical of mDCs, including HLA-A2, HLA-DR, CD86, CD80 and CD40 (Figure 3.4, 3.5). In comparison to our iPDCs, DCs obtained by Yanagimachi *et al* expressed CD11c, CD11b, CD80, CD86 and HLA-ABC but very low HLA-DR (class II), which is fundamental to the antigen presenting functions of DCs. The low HLA-DR brings into question the ability of these cells to present antigen to T cells [294]. Importantly, Yanagimachi *et al* did not investigate their cultures for other DC subsets and did not report the percentage of CD11c⁺ cells obtained in their cultures [294].

Surprisingly, we observed expression of the pDC marker, CD123 (IL-3R) in moDCs and hiPDCs, which was found to be co-expressed with CD11c (Figure 3.14), in contrast to its pattern of expression on pDCs, which are known to be CD11c⁻ CD123⁺ [297]. However, CD123 has been shown to be expressed in some subsets of *in vitro* generated moDCs [298]. We also observed the expression of CD14 in CD11c⁺ hiPDCs (26% of cells), which, together with CD11c, could be a marker of residual monocytes or the CD14⁺ DC subset [41]. The majority of moDCs were found to co-express CD11c and either CD123 or CD14, indicating hiPDCs might bear some similarity to moDCs, upregulating markers not observed in *in vivo* subsets of DCs (Figure 3.14).

hiPDCs, like moDCs, were found to express chemokine receptor, CCR7, consistent with reports of mDCs in the literature (Figure 3.5) [299]. CCR7 acts to direct the migration of DCs towards secondary lymphoid organs and is critical to the ability of DCs to induce an

immune response [300]. Accordingly, CCR7^{-/-} DCs fail to migrate to the draining LNs in response to *in vivo* stimuli [300].

hiPDCs have been shown to express TLR3 and TLR8 and low levels of TLR4, but no TLR9 and TLR7, consistent with reports of TLR expression in the cross-presenting CD141⁺ subset of mDCs in the literature (Figure 3.5) [50]. Since dsRNA has been shown to stimulate TLR3 in DCs, inducing CD86 upregulation and the increased secretion of IL-12 and IL-6, enhancing cross-presentation of exogenously-derived antigen to CD8⁺ T cells, its expression by hiPDCs suggests its similarity to the cross-presenting DC subset [301]. It should also be noted that the expression of TLR3 in hiPDCs is higher than that in moDCs, which may explain the inability of moDCs to cross present antigen to CD8⁺ T cells.

When immature, DCs are specialized in sampling the local microenvironment for microbial pathogens and their constituent antigens. Once mature, they are capable of processing and presenting antigen to T cells, initiating the immune response. The maturation of DCs also plays an important role in regulating the balance between tolerance and immunity [279]. To assess the capacity for hiPDCs to mature, we cultured them with a cocktail of inflammatory cytokines consisting of TNF- α , IL-6, IL-1 β and PGE₂, which has been shown to be effective for the maturation of DCs derived from PBMCs as well as ES and iPS cell-derived DCs [218, 224, 302]. Following 48 hours of exposure to the maturation cocktail, both hiPDCs and moDCs were found to upregulate HLA class II and co-stimulatory molecules CD86, CD80 and CD40, consistent with earlier reports of hiPDC maturation in the laboratory as well as other reports of moDC maturation in the literature (Figure 3.6, 3.7). The upregulation of HLA class II and co-stimulatory molecules

enhance the ability of DCs to present antigen and to interact with naïve T cells to elicit an immune response.

hiPDCs, like moDCs, significantly upregulated the secretion of IL-6 upon maturation. IL-6 is a potent pro-inflammatory cytokine which has been shown to have an important role in T cell priming (Figure 3.8). The upregulation of IL-6 on maturation is consistent with that observed in iPSC-derived DCs described by Yanagimachi *et al* (2013) [294]. We also investigated the secretion of IL-12, a pro-inflammatory cytokine known for its role in priming naïve T cells towards a Th1 phenotype and critical for eliciting potent CTL responses desirable in the treatment of HIV-1 [274]. moDCs were able to produce low levels of IL-12p70 on maturation but no IL-12 secretion was detected from hiPDCs (Figure 3.8).

hiPDCs, were also found to secrete the anti-inflammatory cytokine IL-10, which has been shown previously to be secreted by DCs (Figure 3.8). Since IL-10 has been demonstrated to steer DCs to a more tolerogenic phenotype and to induce the differentiation of T_{reg} cells, the high levels of IL-10 secreted by hiPDCs made us question the capacity of these DCs to elicit an immune response. DCs in steady state have been shown to be tolerogenic, however, with the right maturation signals, can prime T cells for immunogenicity. We found that the level of IL-10 decreased on maturation, consistent with reports of moDCs, although this decrease was not statistically significant. In contrast to our findings, the PS-DCs produced by Yanagimachi's group produced more IL-10 on maturation, which led them to deduce the need for further improvement of the differentiation and maturation protocols [294]. The high levels of IL-10 and low levels of IL-12 have been observed in neonatal DCs [303]. Similarly, semi-mature DCs have also

been shown to secrete more IL-10 when matured and do not produce pro-inflammatory cytokines like IL-6 or IL-12 [272]. These results are also consistent with levels of cytokine production observed in mouse iPDCs, leading us to speculate whether iPSC-derived DCs are more akin to neonatal or semi-mature DCs and whether this might have an effect on their function.

Given the demonstration that the stimulation of DCs via TLR 3 and/or TLR 8, alongside TNF- α , IL-1 β and IFN- γ produces mature DCs which can secrete high levels of IL-12p70 [304], we further investigated whether we were able to elicit IL-12 secretion from hiPDCs by maturation with a different cytokine cocktail or in combination TLR stimulation. We observed low level secretion of IL-12 by hiPDCs on stimulation with TNF- α , IL-1 β and IFN- γ with the addition of TLR8 agonist, R848. However, the level of IL-12 secretion increased almost 10-fold on the addition of TLR3 agonist, TLR9 agonist and CD40L to the above cocktail, demonstrating that hiPDCs possess the capacity to secrete IL-12 when provided with the right combination of stimuli (Figure 3.9). The increased secretion of IL-12 in response to a mixture of cytokine cocktail in combination with TLR agonists is supported by reports that TLR agonists can act in synergy to increase the secretion of IL-12 with no effect on the level of expression of co-stimulatory markers [278]. Furthermore, since Dectin-1 stimulation has been shown to increase IL-12 expression by unlocking the transcription control of IL-12p35 subunit in neonatal moDCs, resulting in increased ability to induce Th1 polarization, we further propose to investigate the effect of Dectin-1 on IL-12 expression in hiPDCs [305].

Having shown that hiPDCs exhibit a phenotype similar to mDCs and can be matured effectively, upregulating HLA class II and co-stimulatory markers and cytokine production,

we proceeded to investigate whether hiPDCs behave like functional DCs. We observed that hiPDCs, like moDCs, are able to phagocytose FITC-labelled latex beads. We also detected FITC beads associated with a small proportion of fixed DCs, both in hiPDCs and moDCs, which may be due to incomplete quenching of beads that had adhered non-specifically to the cell surface. However, live DCs were found to phagocytose significantly more beads than fixed DCs, leading us to conclude that hiPDCs possess the capacity to phagocytose particles such as whole pathogens. Furthermore, we also demonstrated that immature DCs phagocytose significantly more FITC-labelled beads cells than mature DCs (Figure 3.10). This difference is consistent with the specialized ability of immature DCs to constantly sample the environment for antigens and ingest them by fluid-phase or receptor mediated endocytosis. This ability is downregulated as DCs mature and home to the LNs to present the acquired antigen to T cells.

We further demonstrated that hiPDCs, like moDCs, were able to process DQOvalbuminTM added exogenously in the medium. As mentioned in section 3.6.2, DQOvalbuminTM consists of ovalbumin protein conjugated with a BODIPY dye which, upon proteolytic degradation, emits fluorescence that may be detected at 515 nm [281]. DCs incubated with DQOvalbuminTM at 4°C were used as negative controls, since the proteolytic capacity of enzymes decreases at low temperature. A significantly higher percentage of hiPDCs incubated with DQOvalbuminTM at 37°C emitted positive fluorescence in the FL1 channel than those incubated at 4°C, suggesting that hiPDCs can indeed process exogenously derived antigen, a hallmark of DC function (Figure 3.11). The number of hiPDCs able to process DQOvalbuminTM at 37°C significantly increased following maturation, consistent with the enhanced ability of DCs to process and present peptide upon maturation [279, 280, 283].

To test the ability of hiPDCs to activate naïve T cells, we co-cultured allogeneic naïve T cells with hiPDCs and moDCs in a MLR. As seen in Figure 3.12, mature hiPDCs and moDCs had the most potent capacity to stimulate allogeneic T cell proliferation and this capacity was found to correlate positively with the dose of DCs in the co-culture. Although immature hiPDCs and moDCs were also found to stimulate proliferation of naïve T cells in a dose-dependent manner, the percentage of T cells undergoing proliferation was found to be significantly lower than those stimulated by matured cells. These results suggest that hiPDCs possess the capacity to stimulate the activation of naïve T cells, a critical function of a potential vaccine.

We have previously shown that hiPDCs express chemokine receptor CCR7, which is crucial to the ability of hiPDCs to migrate to a gradient of chemokines secreted in lymphatic vessels and the T-cell areas of lymph nodes [306]. However, it has been demonstrated that even when expressed, CCR7 can exist in an uncoupled form, hindering the migration of DCs to its ligand CCL19 and CCL21 [307, 308]. We confirmed the functional expression of CCR7 by demonstrating that both moDCs and hiPDCs were able to migrate in response to its ligand, CCL19.

Having established that hiPDCs are functional, possessing the ability to take up and process antigen as well as the ability to stimulate naïve allogeneic T cells and migrate towards a chemokine gradient, we further investigated whether hiPDCs contain the cross presenting, CD141⁺ (XCR1⁺ Clec9A⁺) subset and whether they possess the ability to present exogenously derived antigen via MHC class I. We earlier established that an average of 85% of hiPDCs express the mDC marker CD11c, of this subset of hiPDCs, some cells were found to express CD141 alone whereas some co-expressed CD141 and CD11c

(Figure 3.14B). mDCs *in vivo* consist of a majority CD1c subset and minority CD141 subset [46, 47, 309], however, the markers are not distinct. The CD141⁺ subset of mDCs have been shown to express low to intermediate expression of CD1c and have been described as 'CD141^{hi} DCs' whereas the CD11c⁺ CD1c^{hi} subset has also been found to express low levels of CD141 expression and are referred to as 'CD1c^{hi} DCs' [72]. hiPDCs were found to contain a population akin to the CD141^{hi} DCs and another population which unlike blood mDC subsets, shows a high expression of both CD141 and CD1c. These findings suggest that hiPDCs may not yet be terminally differentiated.

Although it has been shown that the CD1c⁺ subset has negligible capacity to cross present antigen, the major cross presenting subset of DCs in humans has been identified to be the CD141⁺ XCR1⁺ Clec9A⁺ subset of mDCs [52-54, 310, 311]. CD11c⁺ hiPDCs were found to contain a subset of cells that co-expresses CD141 and XCR1, which are markers that have been used to identify the cross-presenting subset of human DCs [53]. XCR1 has been shown to be specifically expressed in cross presenting DCs in mouse CD8 α ⁺, human CD141⁺, and sheep CD26⁺ DCs and has been identified as the first specific marker of the cross presenting subset, which is conserved across these species [54]. Although a proportion of CD11c⁺ CD141⁺ hiPDCs expresses XCR1, this expression was not found to be consistent. In differentiations where XCR1⁺ DCs were identified, the percentage was found to be highly variable, ranging from 1.2% to 18% of the CD11c⁺ subset (Figure 3.15). Additionally, the relative MFI of XCR1 was found to vary extensively between experiments. It should be noted that FACS analysis of chemokine receptors has been shown to have poor sensitivity and low signal to noise ratio due to their highly conserved nature and therefore, lack of high affinity mAbs [287].

To establish the presence of XCR1 definitively, we investigated whether hiPDCs would migrate towards a gradient of XCL1, the only known ligand for XCR1. *In vivo*, XCL1 is produced by CD8⁺ T cells after encountering antigen on DCs; and acts as a potent chemoattractant for cross-presenting DCs [56]. In mice, XCL1 has also been shown to induce prolonged interactions between CD8⁺ T cells and CD8 α ⁺ DCs [56]. Using a real-time chemotaxis assay, hiPDCs, but not moDCs, were found to migrate to XCL1 in a dose-dependent manner. This was found to be true even for differentiations where XCR1 expression was not detected by FACS, indicating that although the expression of XCR1 is highly variable, hiPDCs are able to migrate to its sole ligand, XCL1. These data lead us to conclude that a subset of hiPDCs expresses markers of cross-presentation, CD141, TLR3 and functional expression of XCR1. Since we have yet to identify the parameters that lead to variability among the independent differentiations, future experiments will seek to modify the protocol of DC differentiation to obtain a consistent yield of the XCR1⁺ subset.

C-type lectin CLEC9A has been reported to be expressed by the cross presenting DC subset in humans. However, this marker is also found on other human myeloid cells and is not specific to the cross-presenting subset [57, 58, 312]. CLEC9A has been shown to be involved in the recognition of signals displayed by necrotic cells, in particular filamentous actin (F-actin), and acts as an endocytic receptor for DCs, aiding in the delivery of antigens to the endocytic compartment for cross-presentation [55, 285, 286, 313]. No expression of CLEC9A was detected in hiPDCs or moDCs, intracellularly (Figure 3.15) or cell surface-bound (not shown). CLEC9A has been shown to be internalized on binding F-actin, which has been found to be expressed by necrotic cells. Since large numbers of dead cells have been observed during the hiPDC differentiation process, we speculate

that this may result in the constant ligation to CLEC9A, leading to its continuous internalization, which might explain its lack of expression on hiPDCs.

Since the ability of hiPDCs to cross present antigen is critical to its function as a vaccine for HIV-1 immunotherapy, we investigated the ability of these cells to process a defined exogenous antigen, in this case the testis cancer antigen, NYESO and present it via HLA class I to 1G4 Jurkat T cells specific for the 157-169 epitope of NYESO bound to HLA-A*0201. We observed the upregulation of the T cell activation marker, CD69 in 1G4 Jurkat cultured with hiPDCs that had been pulsed with NYESO protein and 9V NYESO peptide, suggesting that hiPDCs may, indeed, possess the capacity to cross-present antigen. However, despite numerous attempts and improvements, this result was only observed once. Since CD69 upregulation is known to be transient and could be easily missed in co-culture experiments (Dr. Omer Dushek, personal communication), we investigated using IL-8 secretion as a surrogate readout for 1G4 Jurkat activation. This method has been shown to be effective in detecting 1G4 activation on ligation to surface bound peptide-HLA complex (Dr. Omer Dushek, personal communication). Although IL-8 production was significantly upregulated in 1G4 Jurkat co-cultured with hiPDCs in the presence of cognate protein and peptide, hiPDCs were found to constitutively produce high levels of IL-8, making this assay difficult to interpret.

Although the upregulation of CD69 expression observed in 1G4 Jurkat co-cultured with hiPDCs pulsed with protein suggests that hiPDCs possess the capacity to cross present antigen, more experiments need to be performed to substantiate this finding. We propose to use 1G4 Jurkat that has been genetically modified to express luciferase enzyme on recognition of NYESO derived peptide bound to HLA-A*0201 to further

investigate the ability of hiPDCs to cross present exogenously derived antigen via HLA class I.

Given the importance of the ability of hiPDCs to cross present HIV-1 antigen to CD8⁺ T cells in determining their utility for HIV-1 immunotherapy and the inconclusive results obtained in the antigen presentation assay to 1G4 Jurkats, we co-cultured immature HLA-A*0201⁺ moDCs and hiPDCs pulsed with HIV-1-Gag peptide or protein with HLA-A*0201 T cells. We were able to detect a population of antigen-specific CD8⁺ T cells in one instance, suggesting that hiPDCs possess the ability to process and present exogenously derived HIV-1 antigen to CD8⁺ T cells, however, we were unable to confirm this single observation. Furthermore, no such population was observed in the positive control sample of autologous moDCs pulsed with HIV-1 peptide, making the positive result difficult to interpret.

We speculate that the failure to detect expansion of antigen-specific T cells in co-cultures may be due to the presence of memory of T cells, which tend to be alloreactive, resulting in potent stimulation of T cell proliferation, which would have diluted the presence of antigen-specific T cells. We were, however, unable to obtain sufficient blood from HLA-A*0201⁺ donors to purify naïve CD45RA⁺ T cells for use in this assay. The absence of CD8⁺ T cells with positive tetramer staining in positive control samples may also be explained by the low precursor frequency of antigen-specific naïve CD8⁺ T cells. It is estimated that only 1 in 2×10^5 CD8⁺ T cells or roughly 1 in 6×10^5 pan T cells is antigen specific [314]. Since each co-culture only contains 4×10^5 T cells, the probability of the presence of HIV-1-Gag peptide specific CD8⁺ T cells is low. To confirm the capacity of hiPDCs to cross present HIV-1 antigen to CD8⁺ T cells, we propose to acquire more blood from HLA-A*0201⁺

donors and repeat these experiments with CD45RO depleted T cells. We also propose to increase the numbers of T cells in the co-culture, thus increasing the probability of including HIV-1 Gag specific naïve CD8⁺ T cells.

Given that the ultimate aim is to use hiPDCs for therapeutic vaccination against HIV-1 in the clinic, there is a need for the purification of the cross presenting subset that would be pulsed with antigen and introduced into the patient. To this end, we developed protocols for their purification using CD11c enrichment, which was found to be very effective. Although the purification only yielded 90.1% CD11c⁺ cells, which is not sufficiently pure for exploitation in the clinic, the bead:cell ratio can be optimized to give better purification of the CD11c⁺ subset.

CD11c⁺ hiPDCs have been shown to contain both CD1c⁺ and CD141⁺ subsets, although some cells were found to co-express both markers. Since CD141⁺ cells are the most efficient cross presenting DCs, we proceeded to purify this subset by either depleting CD1c⁺ or enriching CD141⁺ cells. CD1c depletion was found to be more effective at purifying the CD11c⁺ CD141⁺ subset than CD141 enrichment, which may be due to the inability to effectively stain for CD141 post-purification. However, the CD1c depletion yielded 74.8% pure population of CD11c⁺ CD141⁺ hiPDCs, suggesting that the bead:cell ratio used for this depletion also needs to be further optimized.

Given the failure of previous attempts to purify the cross-presenting subset using XCR1 antibody by FACS and the successful enrichment of hiPDCs using CD11c microbeads, we propose to use this method for the purification of myeloid subset of hiPDCs followed by CD1c depletion to obtain an enriched CD11c⁺ CD141⁺ population.

One of the major hurdles to eradicating HIV-1 is the ability of the virus to remain latent in the body in certain sanctuary sites, called reservoirs. DCs have been shown to act as a reservoir for HIV-1 and can transinfect CD4⁺ T cells upon interacting with them. Since we aim to introduce hiPDCs into the body for immunotherapy, we investigated whether they can be infected by the virus, using moDCs as a control. We demonstrated that hiPDCs, when matured, express co-receptors for HIV-1 entry, CD4 and CXCR4. We also detected the low level expression of CCR5 by flow cytometry in mature hiPDCs but not in moDCs, contradictory to reports in the literature. It has been shown that the level of CCR5 on the surface may influence the ease with which cells are infected with HIV-1. Individuals with the CCR5Δ32 mutation do not express the protein on their cell surface, making them resistant to HIV-1 infection [257, 315]. We were able to detect the CCR5 mRNA by standard RT-PCR in both immature and mature moDCs and hiPDCs. The functional expression of CCR5 was confirmed by the observation that hiPDCs are able to migrate in response to a cytokine gradient of CCL5, the ligand of CCR5 in a dose-dependent manner, indicating that hiPDCs express functional CCR5. moDCs were also found to migrate to CCL5 although the delta cell index of the chemotaxis was not found to be significantly different from the negative control.

We were able to demonstrate that, despite low level expression of CCR5 in hiPDCs and lack of expression in moDCs, both cell types can be infected by non-replicating R5 tropic-HIV-1 *in vitro*. This suggests that DCs differentiated from iPSCs could serve as a reservoir of HIV-1, and participate in the transmission of HIV-1 by either harbouring and transporting the virus (trans-infection) or allowing the replication of new virus which can be transmitted to CD4⁺ T cells (cis-infection) [316]. Although immature mDCs have been shown to survive *in vitro* for 45 days [317], on maturation these cells can only survive for

2-3 days, potentially persisting long enough for them to cross-present HIV-1 antigen to CD8⁺ T cells without acting as a long-lasting reservoir for HIV-1. Since CRISPR/Cas9 technology has been shown to be successful in engineering an iPSC line with the CCR5Δ32 mutation, making it resistant to HIV-1, it may prove feasible to employ the technology to generate iPSC lines with a similar CCR5Δ32 mutation and differentiate them to give rise to HIV-1 resistant hiPDCs [318].

To summarize, we have adapted the protocol for the generation of DCs from iPSCs and have shown that, phenotypically, most of these DCs are similar to mDCs found *in vivo*, expressing typical markers of mDCs alongside co-stimulatory markers. We have also demonstrated that hiPDCs are functional, can be matured upon stimulation with inflammatory cytokines and can secrete IL-6 and IL-12, albeit under stringent conditions. We have also shown that hiPDCs have the capacity to process and present exogenously-supplied antigen, stimulate proliferation of naïve allogeneic T cells and migrate in response to appropriate chemokine gradients. A subset of these hiPDCs express cross-presentation markers CD141 and XCR1. Although we were not able to prove their ability to cross-present HIV-1 antigen within the timeframe of this project, other work in the laboratory has shown that hiPDCs can cross-present TAA to CD8⁺ T cells [218]. The ability of hiPDCs to efficiently take up and process antigen suggests that they would have the capacity to process HIV-1 antigen and present it to CD8⁺ T cells *in vivo*, activating them to elicit an HIV-1 specific cytotoxic immune response.

Using cross-presenting DCs is an attractive route for immunotherapy against HIV-1, since CD4⁺ T cells are depleted in HIV-1 infected individuals. Cross-presentation activates CD8⁺ T cells, eliciting an antigen-specific cytotoxic immune response, which should be able to

eliminate cells actively infected by HIV-1 and its reservoirs, following reactivation of the virus with appropriate latency reversing agents. Since the numbers of cross-presenting DCs present in peripheral blood are very low, hiPDCs can potentially be used as a viable alternative for this form of therapy. iPSCs are immortal and form an infinite source of hiPDCs. iPSCs can also be generated from the patient for autologous immunotherapy or an HLA-matched iPSC line can be utilized for the purpose.

hiPDCs generated using our method may potentially be better at priming T cells than the PS-DCs generated by the five-step protocol described by Yanagimachi *et al* [294]. The PS-DCs had a high level of IL-10 secretion, which was upregulated on stimulation and a low level expression of MHC class II, HLA-DR as compared to moDCs. hiPDCs generated using our protocol have a normal expression of HLA-DR and although they secrete IL-10, this level was not upregulated on stimulation. In the next chapter, we investigate the effect of IL-10 on the immunogenicity of mouse hiPDCs and whether knocking out *IL-10* will increase the ability of DCs to prime T cells.

Chapter 4

The effect of IL-10 on the immunogenicity of iPSC-derived DCs

4.1 Introduction

Previous work has demonstrated that human iPSC-derived DCs (iPDCs) express low levels of costimulatory molecules and MHC class II, which are critical for the ability of DCs to stimulate effector responses in T cells. These findings suggest that iPSC-derived DCs may possess a more fetal or primitive phenotype. This is supported by the unprecedented low levels of IL-12 and high levels of IL-10 secreted by human iPDCs which is maintained even upon maturation, much like that observed in neonatal DCs [303, 319].

HIV-1 infected moDCs have also been shown to produce high levels of IL-10 [320]. Immature DCs and DCs producing IL-10 stimulate impoverished effector T cell responses, promoting instead, the development of regulatory T cells (T_{regs}), which is one of the mechanisms employed by the virus to evade the immune system. This suggests that despite possessing the capacity to stimulate an efficient proliferation of allogeneic T cells, iPDCs, like autologous moDCs, may not be potent in inducing an HIV-1 specific effector T cells response to HIV-1. moDCs from HIV-1⁺ individuals have also been shown to have reduced capacity to polarize naïve T cells toward a Th1 phenotype, reminiscent of the impact of IL-10 on DC function.

Given the role played by IL-10 in promoting a tolerogenic microenvironment, we hypothesized that IL-10 secretion by iPDCs may be responsible for maintaining the fetal phenotype. To address this hypothesis, we developed a murine model using iPSC from WT and IL-10^{-/-} mice. We investigated whether murine iPDCs, like their human

counterparts, display a more fetal phenotype, and whether this can be reversed by targeting the *Il-10* gene to prevent any autocrine effect on DC function.

Discovery of Interleukin-10

Interleukin-10 (IL-10), also known as cytokine synthesis inhibitory factor (CSIF), is an immuno-modulatory cytokine that plays a critical role in suppressing the immune response and in preventing autoimmunity [321-323]. It has been shown to have a critical role in limiting the immune response *in vivo* and protecting the host from immune reactivity to self. Mice deficient in IL-10 develop inflammatory bowel disease (IBD) upon microbial challenge and have been found to be susceptible to other autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [323, 324]. Recent studies have identified IL-10 as one of the susceptibility loci for ulcerative colitis in humans [325].

IL-10 was first found to be produced by Th2 cells and acted by dampening the Th1 immune response [321]. It is now known that IL-10 is secreted by a variety of leukocytes such as B cells, macrophages, DCs, T_{reg} cells and several other subsets of T cells. IL-10 has a profound effect on APCs, NK cells and lymphocytes and plays an important role in the balance between Th1 and Th2 dependent immune responses. [322, 326, 327].

Direct effect of IL-10 on T cells

IL-10 was first described as a product of Th2 cells, which was found to antagonise the induction of Th1 immune responses [321]. However, this effect of IL-10 on T cells has

since been found to be indirect, taking place via its effect on monocytes, DCs and macrophages [322]. IL-10 was also found to have an effect on the proliferation of CD4⁺ T cells and their ability to produce cytokines such as IL-2, IL-4, TNF α and IFN γ , limiting their proliferation, differentiation and activation. IL-10 has also been shown to suppress the ability of CD4⁺ T cells to elicit a pro-inflammatory response in tissues. [322, 328, 329].

In contrast to its effect on CD4⁺ T cells, IL-10, in the presence of low doses of IL-2 and absence of APCs, was found to stimulate recruitment and proliferation of CD8⁺ T cells. However, activation of T cells in the presence of IL-10 has been shown to result in their anergy suggesting that IL-10 may have a role in induction of tolerance [322, 330]. In the absence of IL-2 and presence of APCs, IL-10 suppresses the proliferation of CD8⁺ T cells and decreases their ability to respond to allo-stimulation. However, this effect is mediated indirectly by the impact that IL-10 has on APCs, elaborated below [330].

Although IL-10 has been found to inhibit Th1 immune responses, Th1 cells have also been identified as a potential source of IL-10. The equilibrium of IL-10 and IFN γ secreted by Th-1 cells is thought to regulate the fine balance between immunity and tolerance in the immune system. [331]

IL-10 and DCs

IL-10 maintain DCs in an immature state, irrespective of the presence of inflammatory stimuli or TLR agonists which usually results in their maturation [322, 326, 332, 333]. Ligation of the IL-10 receptor triggers signalling through the JAK/STAT3 pathway resulting in the suppression of DC maturation [334]. When matured in the presence of IL-10, DCs

have been found to possess a reduced capacity to stimulate Th1 responses. This effect has been attributed to the reduced expression of co-stimulatory molecules CD40, CD80 and CD86 by DCs matured in the presence of IL-10 [335]. Co-stimulatory molecules are crucial for eliciting effective activation and differentiation of T cells following TCR engagement with peptide-MHC complexes presented by DCs. IL-10 has also been shown to down-regulate the expression of cell adhesion molecule CD54 (ICAM-1) and MHC molecules, which interact with LFA-1 and TCR receptors, respectively, on T cells at the DC-T cell synapse during antigen presentation. Furthermore, IL-10 reduces the production of Th1-inducing pro-inflammatory cytokines including IL-12, IL-6, IFN γ and TNF α by DCs, leading to the preferential induction of Th2 instead of Th1 responses [326, 329, 335]. It has also been shown that treatment of human DCs with IL-10 impairs their ability to prime naïve CD4⁺ T cell proliferation in the allogeneic MLR, and that this inhibition is dose-dependent [336]. It should be noted that DCs themselves have been known to produce IL-10 *in vivo*, on ligation of their pathogen recognition receptors (PRR). The IL-10 produced has an autocrine effect on the DCs, leading to their impaired maturation and reduced ability to polarise naïve T cells into Th1 effector cells [337].

IL-10 has been shown to play an important role in the induction of peripheral tolerance by DCs. Semi-mature DCs, which upregulate co-stimulatory molecules and MHC expression but produce high levels of IL-10 and in the absence of IL-12, have been shown to induce the development of CD4⁺ CD25⁺ FoxP3⁺ T_{reg} cells, which are capable of inducing tolerance to self-peptides in the periphery [338]. Human DCs differentiated from CD14⁺ monocytes in the presence of GM-CSF, IL-4 and IL-10, the so-called 'DC-10', have been found to have a mature phenotype, expressing CD86 and HLA-DR but have a high IL-10 to IL-12 ratio, even on maturation. These DCs have been shown to be efficient at inducing

T_{reg} cells [339, 340]. IL-10 has also been shown to inhibit the development of human DCs from PBMCs or CD14⁺ precursors when cultured in the presence of GM-CSF and IL-4 and instead steer the differentiation towards a macrophage phenotype [341] .

IL-10 and immunogenicity

It has been demonstrated that the capacity of DCs to present antigen is enhanced when endogenous *Il-10* gene expression is suppressed, which, in turn, enhances the Th1 response to pathogens [342]. IL-10^{-/-} mice were found to be more resistant to *Chlamydia* infection as compared to their WT counterparts, which was attributed to the capacity of IL-10^{-/-} mice to stimulate more potent Th1 response to the pathogen. DCs from IL-10^{-/-} mice were found to secrete higher levels of pro-inflammatory cytokines, IL-6 and IL-12, and were found to be more effective at stimulating the production of IFN-γ as compared to their WT counterparts [342].

Work in the Huang laboratory at Imperial College, London has shown that BMDCs generated from IL-10^{-/-} C57BL/6 mice are highly immunogenic and have enhanced capacity to stimulate T cells *in vivo* as compared to those derived from WT C57BL/6 mice. Likewise, BMDCs in which IL-10 expression has been knocked down by siRNA have been shown to superior at eliciting T cells response as compared to their conventional counterparts. Although knocking out IL-10 was only shown to have a marginal effect on the expression of co-stimulatory molecules, CD80, CD86 and CD40, these DCs were also found to upregulate expression of MHC class I and II as compared to WT BMDCs. The IL-10^{-/-} BMDCs also produced significantly more IL-12 and IFNγ than WT BMDCs, which are the cytokines involved in polarising naïve T cells towards a Th1 phenotype [343]. Chen *et*

al further utilized DCs generated from IL-10^{-/-} and WT mice loaded with antigens to vaccinate mice to hepatocellular carcinoma. They concluded that DCs from IL-10^{-/-} mice were superior to those generated from WT mice in triggering immune response against tumours [343].

Aim

Given our aim to utilize iPDCs for HIV-1 immunotherapy and the high levels of IL-10 in human iPDCs which might favour the induction of T_{reg} cells, known to pose a barrier to vaccination to chronic HIV-1 infection [320], we explored whether knocking out *Il-10* has an effect on the immunogenicity of iPSC-derived DCs. To investigate this, we reprogram MEFs from IL-10^{-/-} and WT mice to generate iPSC lines, which we further differentiated into iPDCs. These iPDCs were compared for their cell surface expression of co-stimulatory molecules and MHC determinants and for their ability to produce cytokines. We also investigated their ability to stimulate proliferation of naïve T cells and to induce differentiation of CD4⁺ CD25⁺ FoxP3⁺ T_{reg} cells from naïve mice.

4.2 Results

4.2.1 Generation of iPSC lines from WT and IL-10^{-/-} mice

In order to develop a model to investigate the role of IL-10 on the immunogenicity of DCs, day 13 embryos were obtained from IL-10 knockout (strain B6.129P2-Il10tm1Cgn/J, stock

no. 002251), and WT control (strain C57BL/6, stock no. 000664) mice, a kind gift from Dr. Fang-Ping Huang (Imperial College, London). Fibroblast cells harvested from the embryos were reprogrammed into iPSC lines using lentiviral transduction for the introduction of the pluripotency associated transcription factors, Oct3/4, Klf4 and Sox2, following the Yamanaka protocol, with the help of Timothy Davies (Fairchild lab, Sir William Dunn School of Pathology) as described in section 2.3.3. Figure 4.1 shows the timeline of the reprogramming process. Briefly, sub-confluent MEFs derived from day 13 embryos cells were infected with pMX-based retroviral vectors in VSVG envelope individually containing Oct3/4, Sox2 and Klf4 transcriptional at 3:1:1 ratio and incubated for 24 hours, after which the infection was repeated. Cells were incubated for 48 hours, after they were passaged onto layers of inactivated fibroblasts. After 24hrs, valproic acid and vitamin C were added to the medium. On day 15 after first viral transduction, cells were passaged onto 6 well plates coated with freshly inactivated MEFs, along with withdrawal of valproic acid from the medium. From day 23 onwards, domed, phase-bright colonies consisting of round cells with a high nucleus to cytoplasm ratio, typical of mouse pluripotent stem cells, were detected. Individual colonies were picked, expanded on inactivated feeders and frozen at passage 1 or 2. Each colony was expanded to give rise to an iPSC line.

4.2.2 Characterisation of reprogrammed IL-10^{-/-} and WT iPSC lines

iPSC lines reprogrammed from IL-10^{-/-} and WT C57BL/6 MEFs were characterised to determine their pluripotency, gender, and karyotype. 5 KO and 2 WT iPSC lines, obtained at the end of the reprogramming, were analysed for gender by means of genomic PCR amplification of the male-specific *Sry* gene, which is located on the Y chromosome [344].

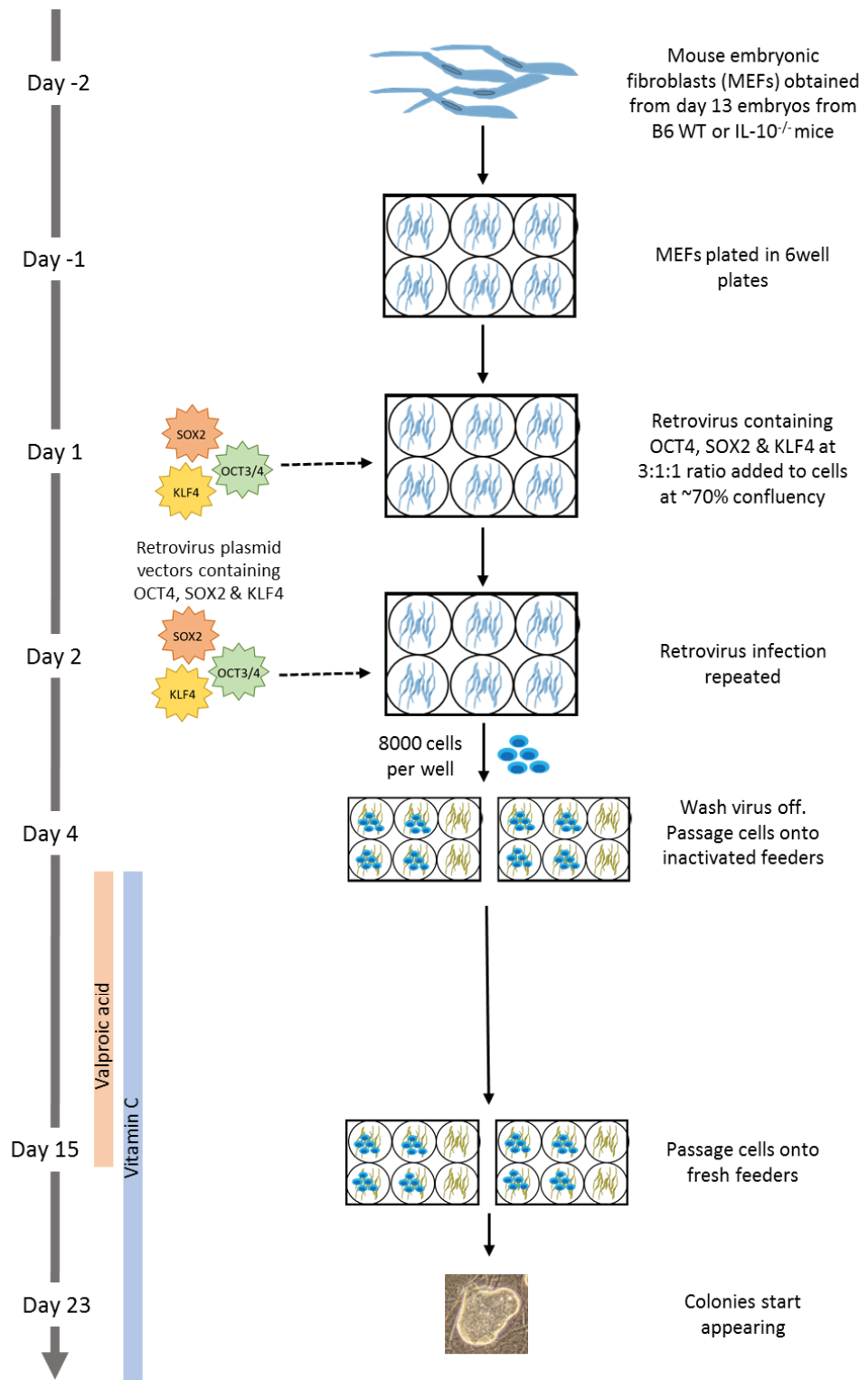


Figure 4.1 Timeline of reprogramming of MEFs into iPSCs. MEFs derived from day 13 embryos of either C57BL/6 WT or IL-10^{-/-} mice were plated into 6 well plates a day prior to infection. Sub-confluent cells were infected with pMX-based retroviral vectors in VSVG envelope individually containing Oct3/4, Sox2 and Klf4 transcriptional at 3:1:1 ratio and incubated for 24 hours, after which the infection was repeated. Cells were incubated for 48 hours, after which medium in wells was replaced to remove viral particles. Cells were passaged onto layers of inactivated fibroblasts. After 24hrs, valproic acid and vitamin C were added. Cells were passaged onto fresh 6 well plates with inactivated MEFs on day 15 after first viral transduction, along with withdrawal of valproic acid from the medium. stem cell-like colonies appeared around 21-25 days after first transduction.

Autosomal genes *Myogenin* (*Myo G*) and *il-3*, found on chromosomes 1 and 11, respectively, were used as positive controls. The genomic PCR confirmed that both WT lines and 2 out of the 5 IL-10^{-/-} lines assayed showed the presence of *Sry* band of anticipated 402 bp, indicating they are male.

Male IL-10^{-/-} line, IPIL/6 and male WT line, iPB6/2, were chosen for our experiments. These lines will be further referred to as KO and WT mouse iPSCs, respectively. The selected lines are marked with white boxes in Figure 4.2B. The karyotype of the KO and WT iPSC lines were determined by G-banding of iPSCs that had been passaged 5 times on gelatin, with the help of Dr. Edward Evans (Department of Zoology, Oxford). Both the KO and WT lines chosen were found to be normal diploid karyotype, 40XY, confirming the sex to be male (figure 4.2C).

4.2.2.1 Pluripotency of IL-10^{-/-} and WT mouse iPSCs

The KO and WT iPSC lines were found to express cell surface markers of pluripotency, SSEA-1 (Figure 4.3A), which plays an important role the migration of the cells in the preimplantation embryo. Both lines also expressed and intracellular transcription factors, Nanog and Oct3/4, which are involved in self-renewal, characteristic of pluripotent mouse ES and iPS cell lines (Figure 4.3A)[258]. The levels of expression of all three markers are consistent with other pluripotent stem cell lines grown in our laboratory [242].

To investigate the capacity of the mouse iPSC lines to differentiate and give rise to tissues from distinct embryonic germ layer, we differentiated both KO and WT iPSCs into

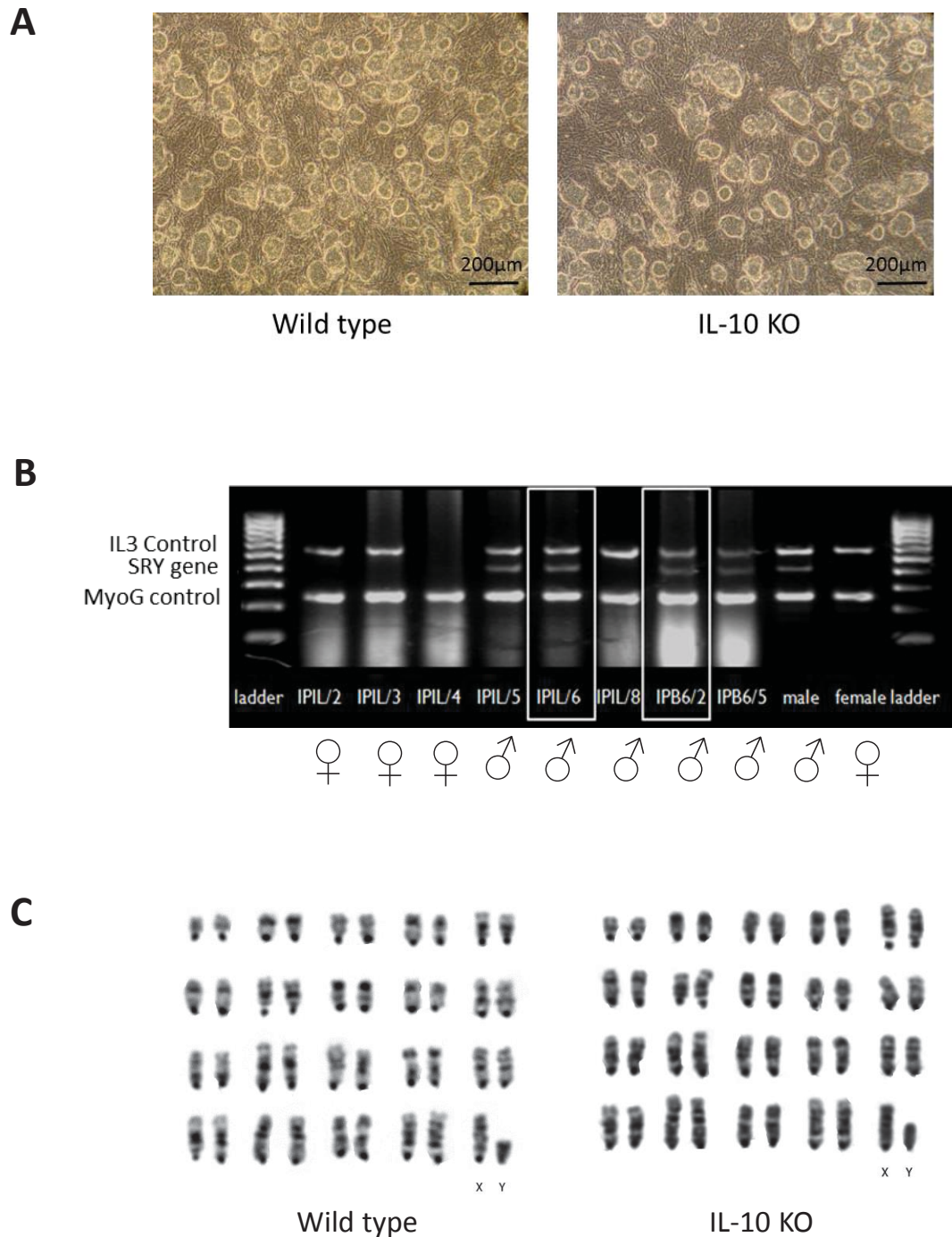


Figure 4.2 Morphology , Karyotype and Sex of mouse iPSC line. Reprogrammed C57BL/6 mouse iPSC WT and KO lines were studied for their morphology, karyotype and sex. **(A)** Photomicrographs showing typical morphology of iPSC lines reprogrammed from IL-10^{-/-} and B6 WT MEFs grown on a layer of inactivated feeders **(B)** gel from PCR of genomic DNA to determine the sex of various lines reprogrammed from B6 WT and IL-10^{-/-} MEFs. SRY band (402 bp); IL3 band (544 bp) and Myo G (250 bp). The lines finally used for this thesis were marked with white boxes, namely, iPIL/6 (KO) and iPB6/2 (WT). ♀- Female; ♂- Male. **(C)** Karyotypic analysis of the WT and IL-10 KO lines chosen for analysis

embryoid bodies (EBs) *in vitro*, following a protocol previously established in our laboratory. Both KO and WT iPSC line were able to form well-defined EBs after a few days in suspension culture. (Figure 4.3B). Day 14 EBs were transplanted under the kidney capsule of CBA.RAG1^{-/-} mice to investigate their ability to give rise to teratomas, which remains the gold standard assay for pluripotency. After 21 days, teratomas were found to develop from both WT and KO iPSC-line derived EBs (Figure 4.4A). These teratomas were harvested, sectioned and stained with H&E to investigate the presence of all three germ layers. The stained cryosections revealed that the teratomas from both WT and KO iPSC line possess a variety of tissues including epithelium (ectoderm), muscle (mesoderm) and stomach lining (endoderm) (Figure 4.4B), confirming both KO and WT mouse iPSC lines to be pluripotent.

4.2.3 Differentiation of IL-10^{-/-} and WT mouse iPSCs into DCs

Previous work in our laboratory has shown that DCs can be routinely differentiated from mouse ES cells via an intermediate EB stage in medium containing IL-3 and GM-CSF [222]. A similar protocol was used to differentiate DCs from our novel mouse iPSC lines. The timeline of this differentiation is shown in Figure 4.5, with photomicrographs illustrating critical stages in this process.

KO and WT iPSCs were cultured on inactivated feeder layers in T25 flasks, until sub-confluent, and passaged sequentially onto gelatin to progressively remove feeder cells. After at least two passages on gelatin, iPSCs were harvested and plated onto bacteriological culture plates to allow the formation of EBs. After 14 days, EBs were plated onto cell culture dishes with medium supplemented with 50U/ml GM-CSF and

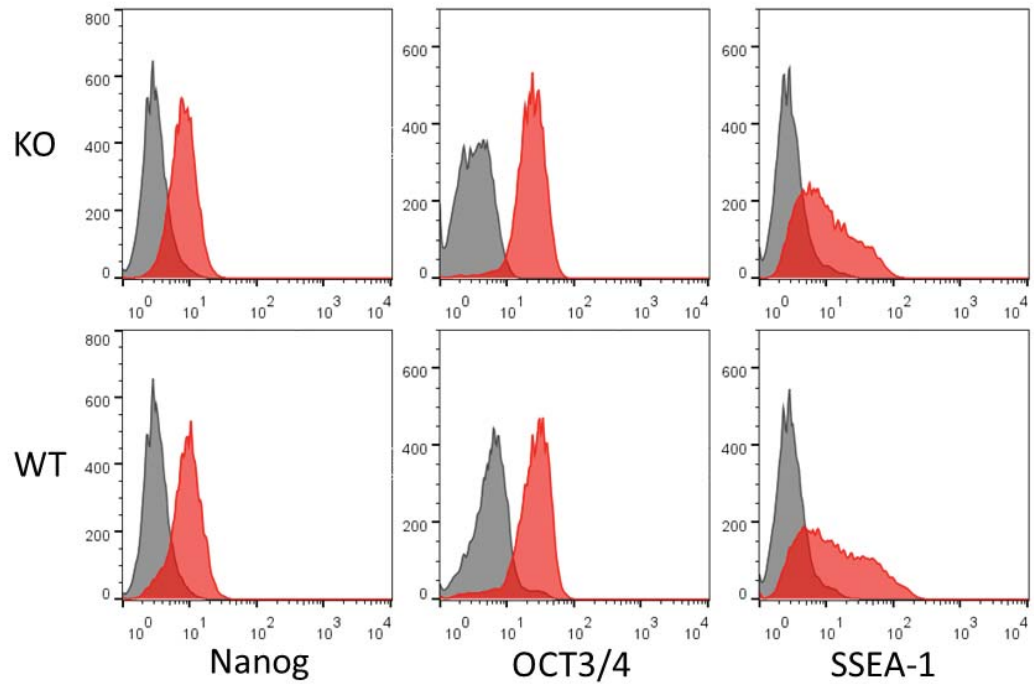
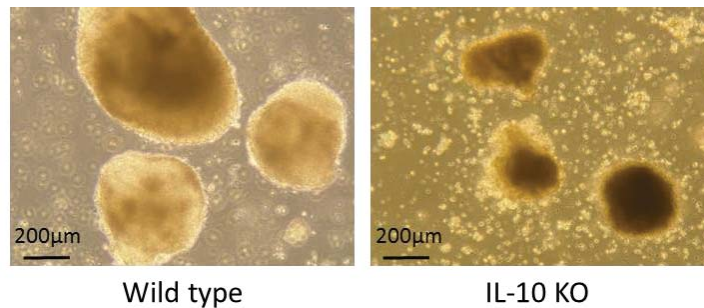
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Figure 4.3 Pluripotency of mouse iPSC lines- Pluripotency markers and EB formation. Reprogrammed C57BL/6 mouse iPSC WT and KO lines were stained for markers of pluripotency and investigated for their ability to differentiate into EBs **(A)** mouse iPSC lines KO and WT lines were stained for cell surface markers of pluripotency, SSEA-1 and intracellular markers OCT3/4 and Nanog and analysed by flow cytometry. One representative FACS plot for each marker is shown (red) overlaid upon the expression of corresponding isotype controls (black). Plots are representative of three independent experiments. **(B)** Photomicrograph of EBs derived from mouse iPSC lines KO and WT lines.

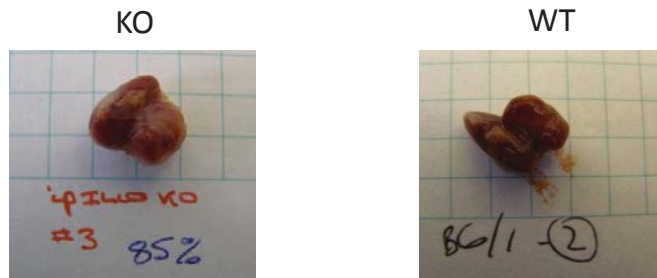
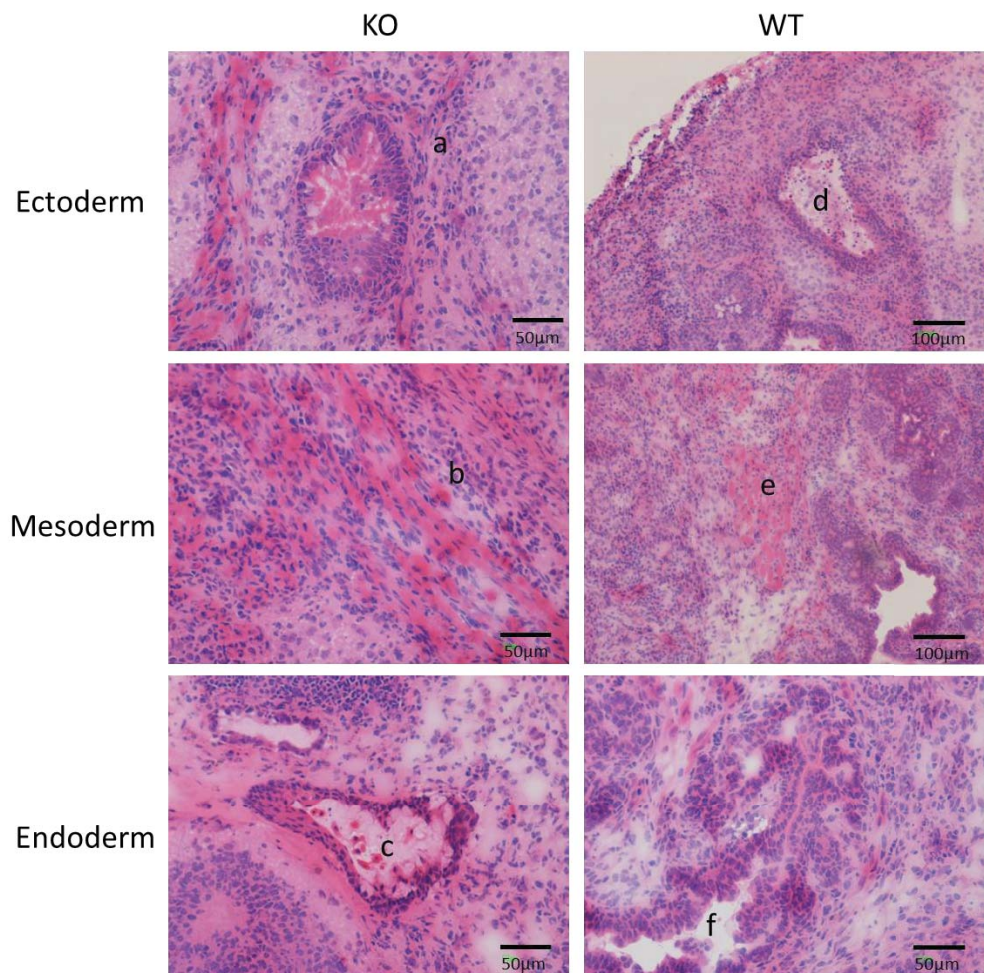
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Figure 4.4 Pluripotency of mouse iPSC lines- Teratoma assay. Reprogrammed C57BL/6 mouse iPSC WT and KO lines were investigated for their ability to form teratomas containing all three germ layers **(A)** Representative photomicrograph of teratomas obtained from transplanting EBs from KO and WT lines under the kidney capsule of RAG^{-/-} mice. **(B)** H&E stained sections of teratomas derived from KO and WT lines showing all tissues derived from all three germ layers. a&d-epithelium , b&e-muscle, c&f-stomach lining. Scale bar= 50µm. Data are representative of at least three teratomas.

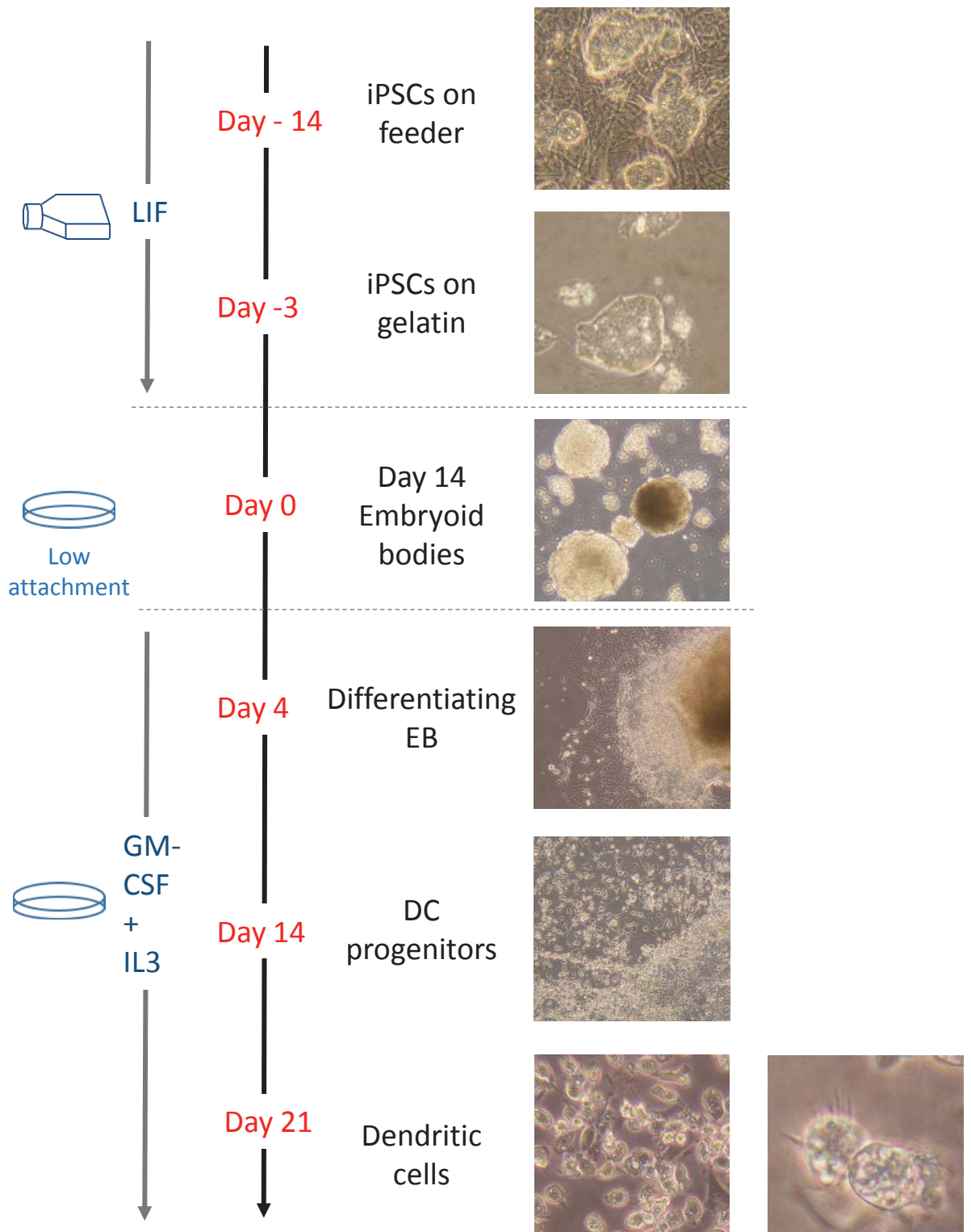


Figure 4.5 Differentiation of mouse iPS into DCs. Illustration of the timeline for differentiation of s into iPDCs. iPS cells were grown on MEFs and transferred to gelatin for at least 2 passages and were made into EBs, which were grown on low attachment dishes for 14 days. EBs were then transferred to tissue culture plastic and grown in media supplemented with GM-CSF and IL-3 for 14-21 days until DCs start appearing. DCs can be matured 3 days before use with the help of 1 μ g/ml LPS. Photomicrographs are representative of differentiation of both KO and WT mouse iPS line.

1000U/ml IL-3. 10-12 days after plating, round cells were found to accumulate around the edges of EBs that had adhered to the surface of the dish. After 14-16 days, cells with irregular shape and long processes, similar to that of BMDCs were observed in the culture, suggesting the formation of DCs (Figure 4.6A).

To confirm this, the DC-like cells were harvested after 21-24 days by gentle pipetting and stained for cell surface markers used for identifying mouse DCs and analysed by flow cytometry. Immature mouse iPSC-derived DCs (miPDCs) derived from both KO and WT lines expressed CD11c, a markers typical of DCs in both mouse and human (Figure 4.6A). Although the level of CD11c expression was found to be slightly less than BMDCs, they were found to be consistent with that of iPDCs derived by other members of the lab in established protocols (Timothy Davies, personal communication). Both KO and WT iPDCs were found to express other DC markers, MHC I (H2K^b), MHC II (IA^b) and low levels of CD11b (Figure 4.6A), similar to that observed in BMDCs derived from C57BL/6 mice and consistent with previous reports. These data suggests that, consistent with our studies of ES cells, both KO and WT iPSCs were able to differentiate into DCs, via an EB stage.

4.2.4 IL-10 expression in IL-10 KO and WT mouse iPSCs and iPDCs

To confirm that the DCs derived from KO iPSCs do not produce IL-10, both KO and WT iPSC lines and the iPDCs differentiated from them were investigated for the expression of IL-10, using PCR for detection of genomic *il-10* gene expression in iPSCs and ELISA for detection of IL-10 secretion from iPDCs. Genomic DNA was extracted from both KO and WT iPSCs grown for at least two passages on gelatin and amplified by PCR to investigate the presence of the *il-10* gene. We used a three-primer system recommended for the

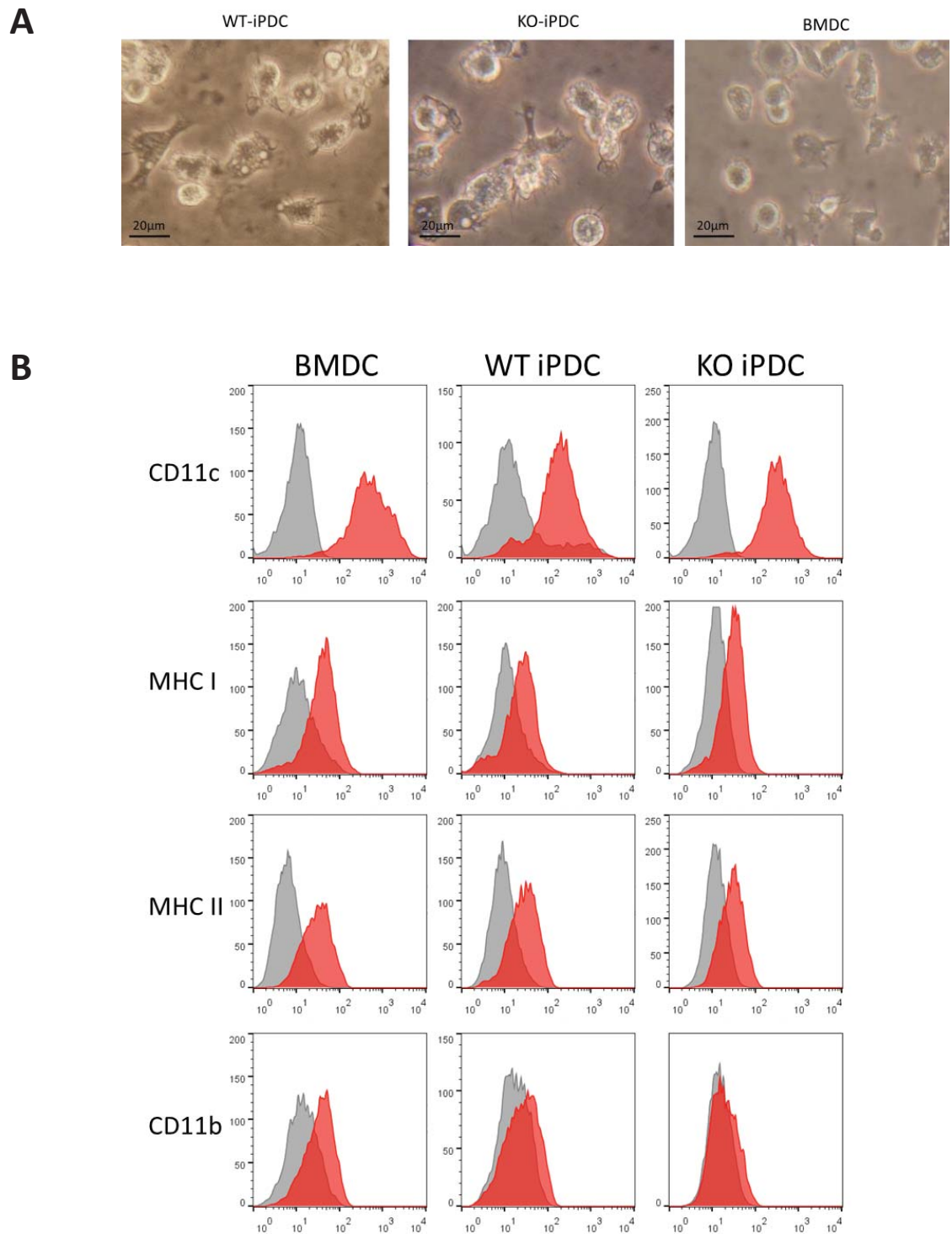


Figure 4.6 mouse iPDCs have DC phenotype. Cells obtained at the end of the differentiation were stained for markers of DC. **(A)** Photomicrograph of BMDCs and DCs differentiated from B6 WT and IL-10 KO mouse iPSCs **(B)** Flow cytometry analysis of cell surface expression of DC markers MHC I, MHC II, CD11c and CD11b by iPDCs and BMDCs. All FACS plots are representative of at least 4 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red.

genotyping of IL-10^{-/-} mice by Jackson Laboratories [241], with a common forward primer and distinct reverse primers for WT and mutant sequences. A 137-bp band was detected in the IL-10^{+/+} (WT) and 312-bp band was detected in IL-10^{-/-} (KO) samples, as anticipated. A band of 250-bp for *Myo G* control was observed in all samples (Figure 4.7A). This confirms the lack of functional *Il-10* gene in iPSCs reprogrammed from IL-10^{-/-} mice-derived MEFs.

To determine levels of IL-10 protein secreted by iPDCs differentiated from these novel lines, immature KO iPDCs, WT iPDCs and BMDCs were harvested and cultured with or without DC maturation cocktail (TNF- α , PGE-2, IL-1 β and IFN- γ) or 1 μ g/ml LPS. Supernatants were collected after 18 hours and the level of IL-10 from each sample was determined by standard ELISA. Low levels of IL-10 were produced by immature BMDC and WT iPDCs, which increased on maturation with LPS and maturation cocktail, although the increase was only found to be significant in the case of BMDCs matured with LPS. KO iPDCs did not produce any IL-10 under any condition, consistent with the homozygous disruption of the *Il-10* gene.

Both genomic DNA data from iPSCs and IL-10 secretion levels from iPDCs confirm the lack of expression of IL-10 at both the genomic and protein levels, suggesting that our iPSC lines provide a good model to investigate the functional impact of IL-10 of iPDC function, and the utility of targeting the gene in order to enhance vaccination strategies to HIV-1.

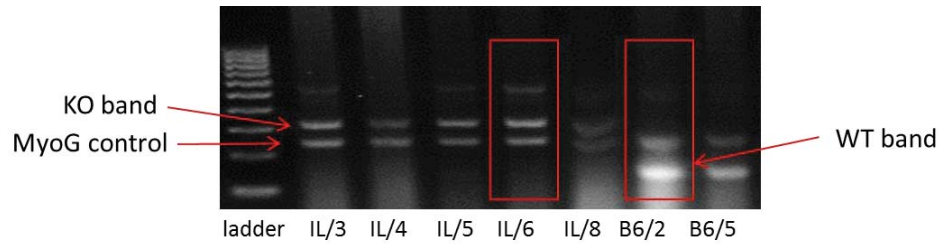
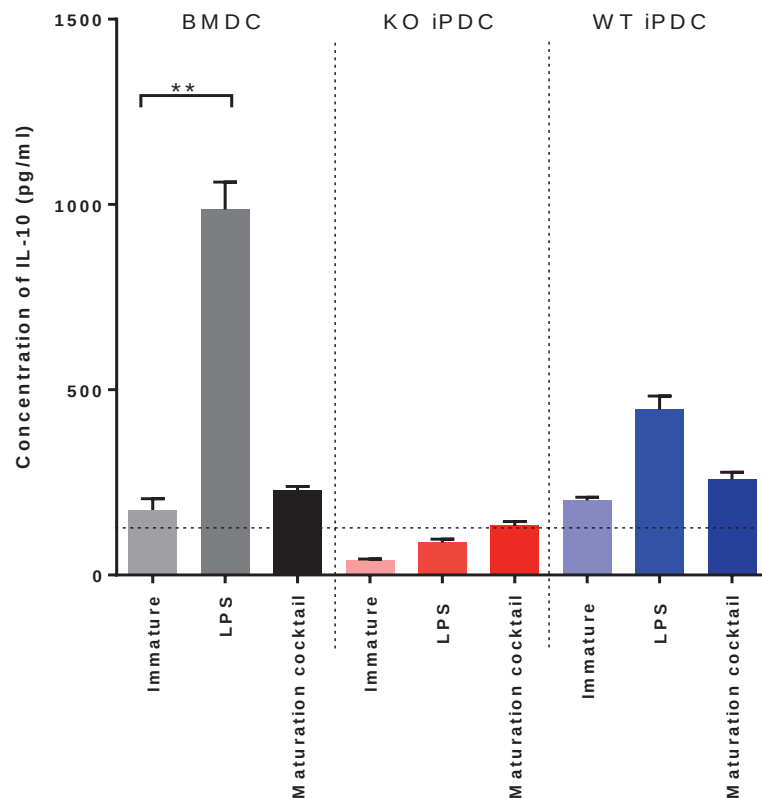
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Figure 4.7 IL-10 expression by IL-10 KO and WT mouse iPSC lines. Reprogrammed C57BL/6 mouse iPSC WT and KO lines were studied for their ability to express IL-10, both on genomic level by mouse iPSC and on protein level by iPDCs. **(A)** Genomic DNA gene expression analysis by PCR of the various lines that were reprogrammed. The lines finally used for this thesis were marked with red boxes. Bands-137bp (WT), 312 bp (KO), both 137bp & 312bp (heterozygous) and 250 bp (MyoG) **(B)** Immature iPDCs and BMDCs were harvested and cultured for 18 hours with or without maturation cocktail or 1µg/ml LPS. Supernatants were collected the amounts IL-10 was determined by standard ELISA. Black bars indicate BMDC, red bars indicate KO miPDCs and blue bars indicate WT iPDCs. Plots are representative of at least 3 independent experiments, with n=3 in each experiment Statistical analysis was performed using parametric T test with Welch's correction (** $p \leq 0.001$).

4.2.5 Effect of IL-10 on the immunobiology of mouse iPDC.

IL-10 has been shown to have an inhibitory effect on the development of DCs from their progenitors and to have an important role in the immunogenicity of DCs [341]. Furthermore, given that BMDCs from IL-10^{-/-} mice have been shown to be more immunogenic and to have greater potential for vaccine delivery than their WT counterparts [343], we investigated the effect of autocrine IL-10 production on the differentiation of iPDCs, their phenotype and their function. We compared WT and KO iPSCs for their efficiency in sustaining iPDC differentiation and compared the WT and KO iPDCs for their expression of MHC molecules, co-stimulatory markers, their ability to stimulate allogeneic T cells, their secretion of pro-inflammatory cytokines and their ability to induce T_{reg} cells.

4.2.5.1 Yield of viable DCs

Previous work in our laboratory (Victoria Strong, unpublished thesis) has shown that the addition of IL-10 to differentiating cultures of mouse ES cells profoundly inhibits their ability to give rise to DCs. To investigate the effect of endogenous IL-10 on the ability of iPSCs to give rise to DCs, we generated EBs from KO or WT iPSCs differentiated in the presence of 50U/ml GM-CSF and 1000U/ml IL-3, according to protocols described in section 4.4 [222]. After 21 days, DCs were observed in the wells plated with both KO and WT EBs. Since macrophages adhere strongly to the tissue culture plastic, DCs were harvested by gentle pipetting and the number of viable cells was determined by trypan blue exclusion. The number of DCs derived from each EBs varied extensively for both WT and KO samples, with the range resembling a normal distribution. Results are cumulative

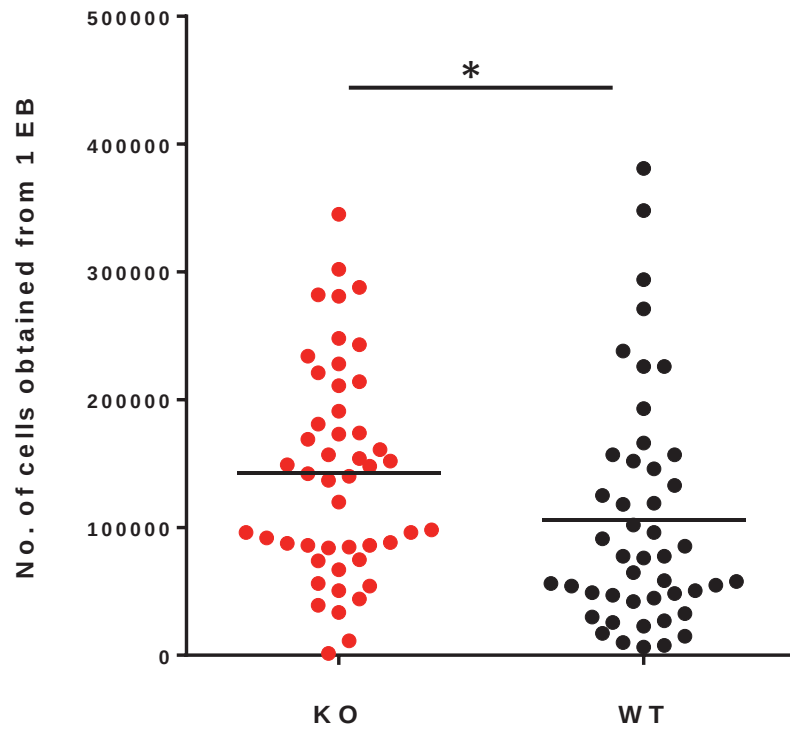


Figure 4.8 Comparison of yield of iPDCs from WT and KO mouse iPSC. One EB was plated in each well of a 24 well plate at differentiated into DCs. The number of viable DCs obtained from each EB of WT and KO mouse iPSC line was compared. Each dot in graph represents number of cells obtained from 1 EB. Black and red line represents mean of all dots. ; Statistical analysis was performed using parametric T test with Welch's correction. ($*p \leq 0.05$). Results are cumulative from 2 experiments with $n=24$ in each experiment. KO (mean $\pm$ SD- 142705 $\pm$ 11965, $n=48$); WT (mean $\pm$ SD -106043 $\pm$ 13523, $n=46$).

from 2 independent experiments with n=24 in each experiment. KO (mean $\pm$ SD: 142705 $\pm$ 11965, n=48); WT (mean $\pm$ SD: 106043 $\pm$ 13523, n=46).

Interestingly, KO iPSC line derived EBs gave rise to significantly higher numbers of DCs than the WT line, suggesting that knocking out IL-10 improves the yield of iPDCs differentiated from iPSCs, consistent with earlier reports of the effect of IL-10 in inhibiting DC differentiation from its progenitors [341].

4.2.5.2 Comparison of co-stimulatory markers and MHC expression

Immature and LPS-matured KO and WT iPDCs were harvested after 21 days and stained for cell surface MHC molecules and co-stimulatory molecules and analysed by flow cytometry. Surface co-stimulatory molecules CD80 and CD86 are involved in enhancing the interaction between T cells and DCs during antigen presentation [345]. They interact with CD28 molecules on T cells and play a crucial role in providing activation signals to T cells to initiate their proliferation and differentiation [346, 347]. Both CD80 and CD86 were found to be expressed in KO and WT iPDCs, as well as BMDCs, and were up-regulated on maturation with LPS in all populations, correlating with the increased capacity of mature DCs to present antigen to T cells (Figure 4.9) The expression of CD86 was found to be higher in mature KO as compared to mature WT iPDCs, however, this difference was not found to be significant.

CD40 is a transmembrane surface receptor that binds to CD40L, a member of the TNF family, expressed on activated CD4⁺ T cells. This binding 'licenses' the DCs to produce more cytokines, upregulate co-stimulatory markers and renders DCs more efficient at

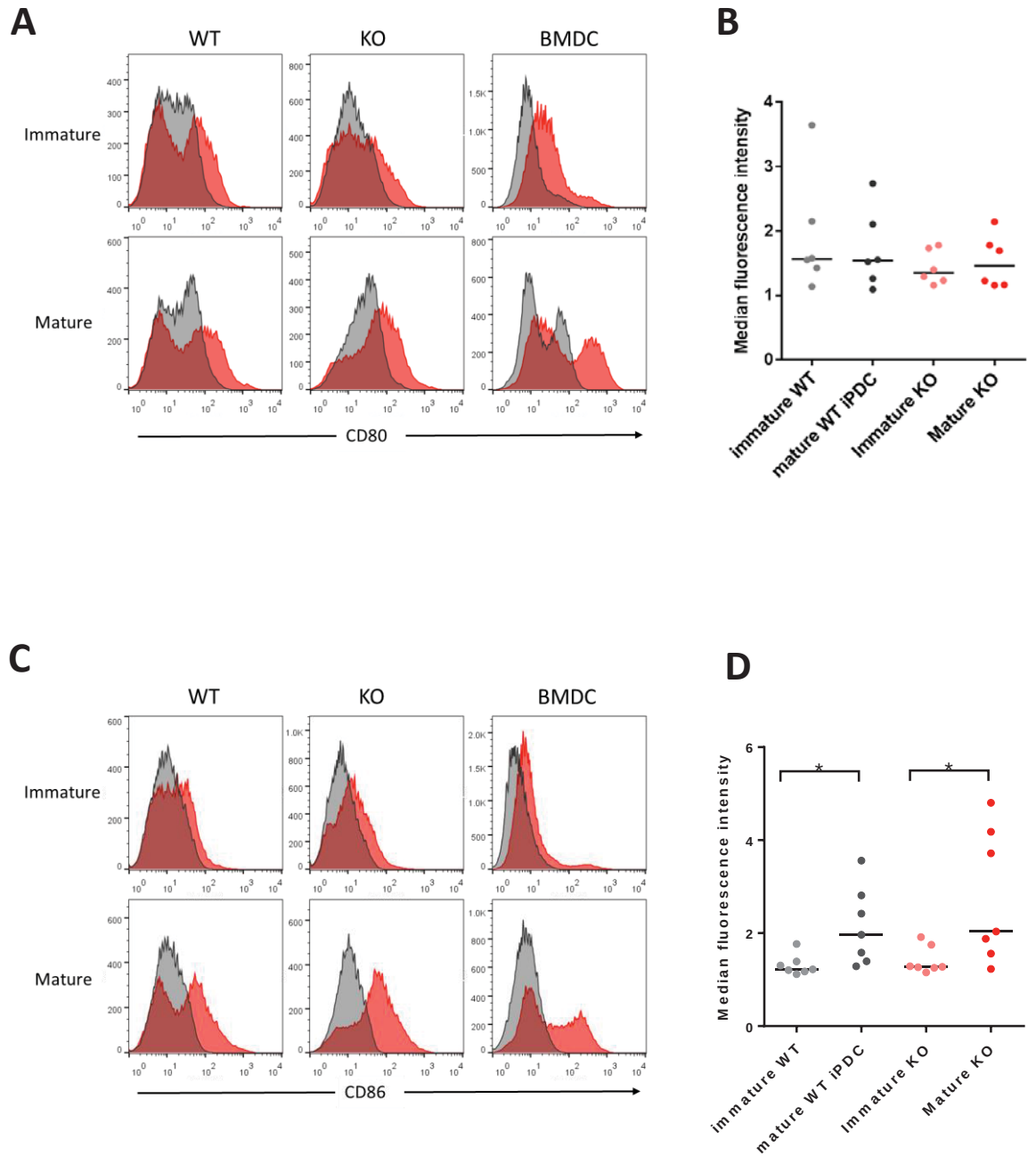


Figure 4.9 Comparison of co-stimulatory marker expression in KO and WT iPDCs. DCs obtained at the end of the differentiation were stained for **(A)** CD80 and **(C)** CD86 and analysed by flow cytometry. All FACs plots are representative of at least 6 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red. Plots **(B)** and **(D)** shows the relative median fluorescence intensity of CD80 and CD86, respectively, across 6 independent experiments. Each symbol in graphs represents an individual experiment with the black line denoting the median. Statistical analysis was performed using parametric T test with Welch's correction ($*p \leq 0.05$).

antigen presentation, in particular, at priming CD8⁺ T cell response [266]. *In vivo*, CD40 plays an important role in converting the outcome of antigen presentation from tolerance to immunity [118, 348]. No CD40 expression was observed in immature or mature KO and WT iPDCs. Immature BMDCs were also found not to express CD40, however its expression was upregulated on maturation with LPS, consistent with reports in the literature (Figure 4.10A, C). The lack of CD40 expression in iPDCs is consistent with its expression in mouse ES and iPS cell derived DCs reported by other members of our laboratory (Simon Hackett and Alison Leishman, personal communication). Low CD40 levels have also been observed in neonatal-derived DCs [303], which leads us to speculate whether iPDCs possess a fetal phenotype.

CD54 or ICAM-1 is a cellular adhesion molecule expressed on DCs that binds leukocyte integrin complex, LFA-1 expressed on T cells [349]. This binding strengthens the immune synapse and is thought to act as a co-stimulatory signal for CD8⁺ T cells and is also considered to be a surrogate marker of activation of APCs [350]. CD54 was found to be expressed in immature and mature DCs derived from both WT and KO iPSCs (Figure 4.10B). The level of CD54 was upregulated slightly on maturation on both KO and WT iPDCs. No significant difference was observed in the level of CD54 between either immature or mature KO and WT iPDCs (Figure 4.10D), suggesting that disruption of IL-10 secretion does not have an effect on the subsequent expression of CD54.

Both WT and KO iPDCs, like BMDCs, express MHC class I and MHC class II on their surface (Figure 4.11A, C). MHC molecules are responsible for the presentation of peptide by DCs to T cells and are the first level of signal required by T cells for activation, as discussed in the introduction. The levels of both MHC class I and II expression were upregulated on

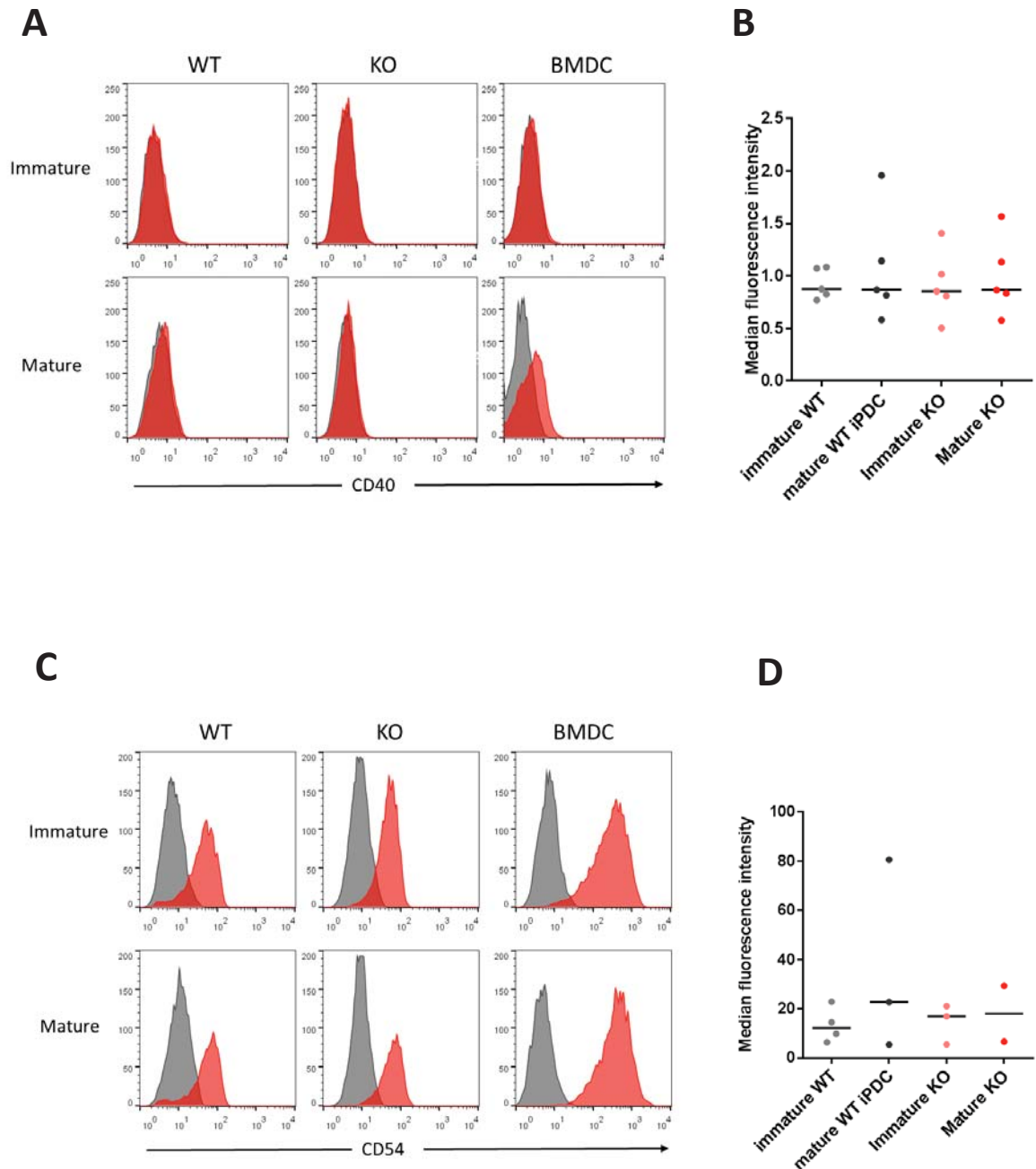


Figure 4.10 Comparison of CD40 and CD54 expression in KO and WT iPDCs. DCs obtained at the end of the differentiation were stained for **(A)** CD40 and **(C)** CD54 and analysed by flow cytometry. All FACs plots are representative of at least 3 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red. Plots **(B)** and **(D)** shows the relative median fluorescence intensity of CD40 and CD54, respectively, across 6 independent experiments. Each symbol in graphs represents an individual experiment with the black line denoting the median. Statistical analysis was performed using parametric T test with Welch's correction.

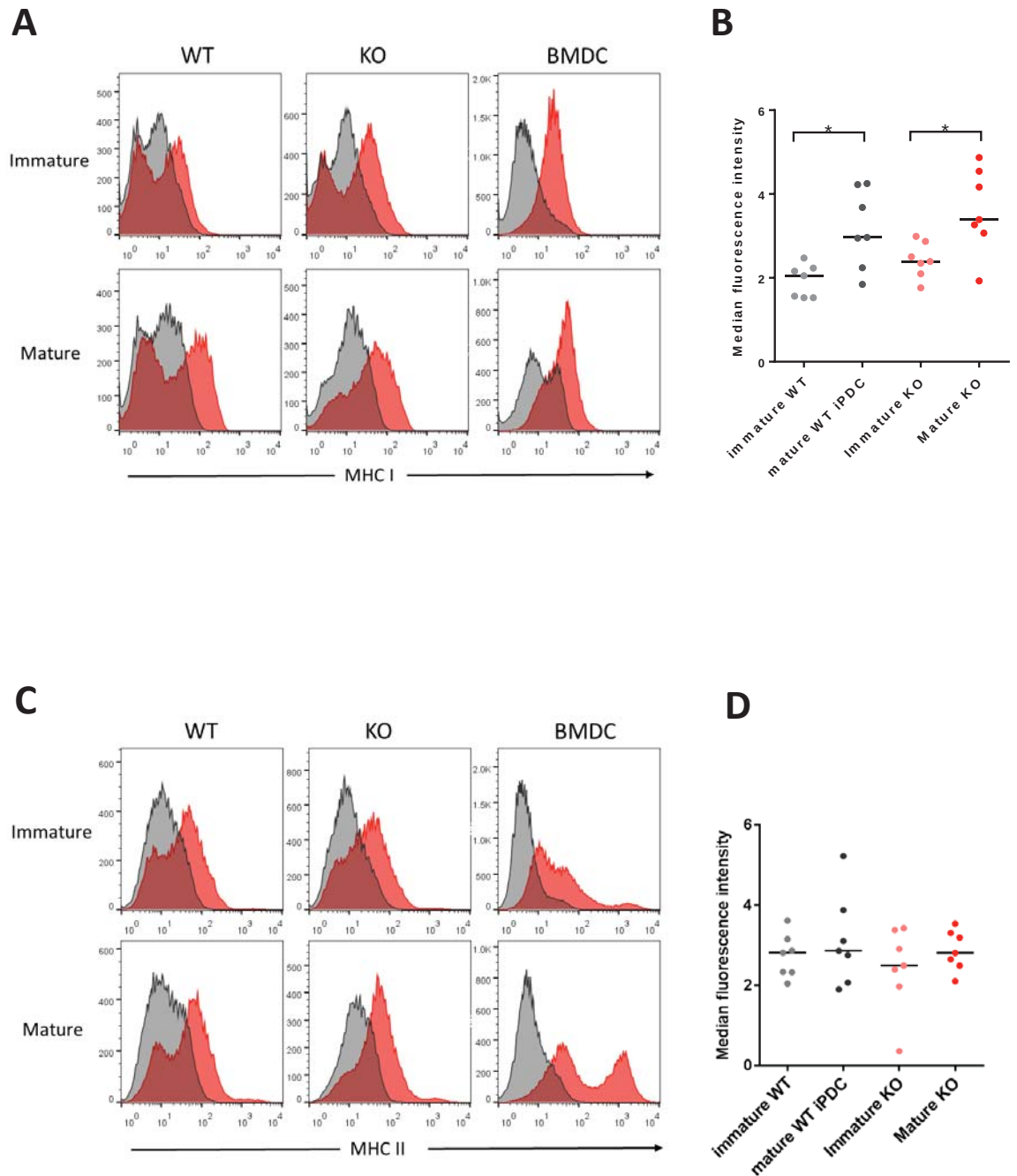


Figure 4.11 Comparison of MHC expression in KO and WT iPDCs. DCs obtained at the end of the differentiation were stained for **(A)** MHC I and **(C)** MHC II and analysed by flow cytometry. All FACs plots are representative of at least 3 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red. Plots **(B)** and **(D)** shows the relative median fluorescence intensity of MHC I and MHC II, respectively, across 7 independent experiments. Each symbol in graphs represents an individual experiment with the black line denoting the median. Statistical analysis was performed using parametric T test with Welch's correction ($*p \leq 0.05$).

maturation with LPS in all populations of DCs, however, this increase in expression was only found to be significant in the case of MHC class I, for both KO and WT iPDCs. The expression of MHC class I was found to be slightly higher in mature KO iPDCs as compared to mature WT iPDCs, however, this difference was not found to be significant. These data demonstrate that knocking out IL-10 has an effect on the levels of MHC class I and co-stimulatory molecule CD86, whereas it does not change the levels of CD80, CD54 and MHC class II, which are known to be involved in co-stimulation and antigen presentation to T cells.

4.2.5.3 Comparison of allostimulatory capacity

DCs are unique in their potential to stimulate proliferation of naïve T cells. The T cell stimulatory capacity of iPDCs was compared in a mixed leukocyte reaction (MLR) involving the co-culture of immature and LPS-matured iPDCs and BMDCs with CFSE-labelled allogeneic T cells for 5 days. The percentage of T cells that had proliferated was determined as a function of CFSE-dilution. [284].

Both immature and mature KO and WT iPDCs, like BMDCs were able to stimulate naïve allogeneic T cell proliferation in a dose-dependent manner, suggesting that the DCs generated from iPSCs are capable of stimulating naïve T cells (Figure 4.12A, C). Both KO and WT iPDCs were significantly better than BMDCs in stimulating the proliferation of naïve allogeneic T cells, in both the immature and mature state. We also observed that KO iPDCs had a greater capacity to stimulate T cell proliferation than WT iPDCs, although this difference was only found to be significant in the case of immature iPDC. (Figure 4.12B,D).

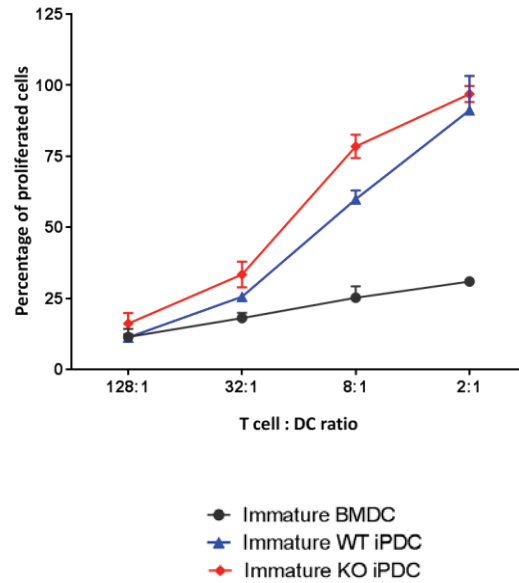
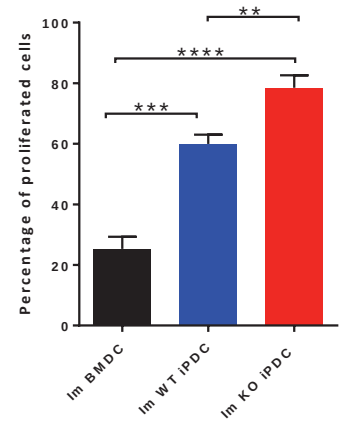
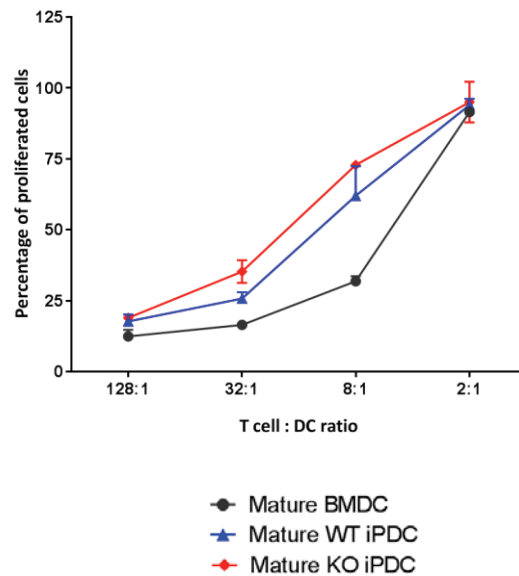
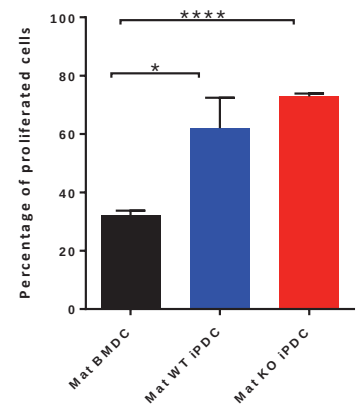
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Figure 4.12 Comparison of allostimulatory capacity of KO and WT miPDCs. Increasing numbers of Immature and Mature iPDCs and BMDCs were co-cultured with 10^5 CFSE-labelled splenic-T cells per well of a 96-well plate to give different ratios of stimulant to effector cells. Cells were harvested after 5 days and analysed for the percentage of proliferated cells using flow cytometry. Plot (A) depicts comparison of allostimulatory capacity of immature DCs, whereas plot (C) depicts that of mature DCs. Plots are representative of 3 independent experiments, with $n=3$ in each experiment. Plots (B) and (D) Percentage of cells proliferated in Immature (B) and Mature (D) iPDCs at 8:1 T cells: DC ratio. Statistical analysis was performed using parametric T test with Welch's correction (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

These data suggests that knocking out IL-10 slightly enhances the ability of iPDCs to stimulate naïve T cells, consistent with the observation by Chen *et al* that IL-10^{-/-} BMDCs display enhanced immunogenicity as compared to WT BMDCs [343]. Since the ability of DCs to potently stimulate naïve T cell activation and clonal expansion is pivotal to their success as a vehicle for vaccine delivery, knocking out IL-10 from human iPDCs may have a beneficial effect to their ability to initiate efficient T cell responses against HIV-1.

4.2.5.4 Comparison of levels of inflammatory cytokines secreted

The ability of DCs to polarize naïve T cells is dependent on their ability to upregulate co-stimulatory and MHC molecules, and their capacity to produce pro-inflammatory cytokines in response to maturation [121]. IL-6 is a pleiotropic cytokine involved in the survival and differentiation of naïve T cells and in T cell priming [351-354]. IL-6 is known to be produced at low levels by immature DCs and is upregulated on maturation with LPS [351]. IL-12 is the key cytokine that drives the differentiation of naïve T cells to a Th1 phenotype and is also essential for the priming of CD8⁺ CTL [355]. IL-12 is produced on maturation of DCs with microbial agents like LPS and in response to pro-inflammatory signals [356].

Since it has been shown that DCs from IL-10^{-/-} mice secrete more pro-inflammatory cytokines such as IL-6 and IL-12 than their WT counterparts [342], we compared the levels of these cytokines produced by immature and matured KO and WT iPDCs, using BMDCs as controls. Interestingly, IL-6 was not found to be produced by immature BMDCs but was secreted by immature WT and KO iPDCs, although these results varied between experiments. On maturation with LPS or cytokine cocktail, BMDCs produced large

amounts of IL-6, with DCs matured in response to LPS being significantly better at secreting IL-6 than those matured with a cocktail of pro-inflammatory cytokines (Figure 4.13A). Mature WT iPDCs also upregulated the secretion of IL-6, although this increase was not found to be significant. No significant differences were observed in the levels of IL-6 between KO and WT iPDCs (Figure 4.13A).

IL-12 was not detected in mouse KO or WT iPDC, either in immature state or on maturation with LPS, cocktail of pro-inflammatory cytokines (Figure 4.13B) or CD40L (data not shown). No IL-12 was detected in immature BMDCs or when matured with cytokine cocktail but it was detected upon their maturation with LPS (Figure 4.13B). These data are similar to those obtained for human iPDCs and moDCs stimulated with a similar cytokine cocktail as used in this experiment, suggesting that mouse iPDCs are a good model for studying the fetal-like phenotype of human iPDCs.

These data suggests that, contrary to the observed effect of knocking out IL-10 on the ability of BMDCs to secrete pro-inflammatory cytokines, knocking out IL-10 in iPDCs did not have a profound effect on the levels of pro-inflammatory cytokine production.

4.2.5.5 Comparison of T_{reg} induction

DCs producing IL-10 are known to have a greater propensity for the induction of tolerance by enhancing the development of IL-10 secreting CD4⁺ CD25⁺ FoxP3⁺ T_{reg} cells, capable of inducing peripheral tolerance to antigens [338]. Since inadvertent T_{reg} induction has been shown to interfere with HIV-1 vaccination [357], we investigated the impact on T_{reg} induction by disruption of IL-10 secretion.

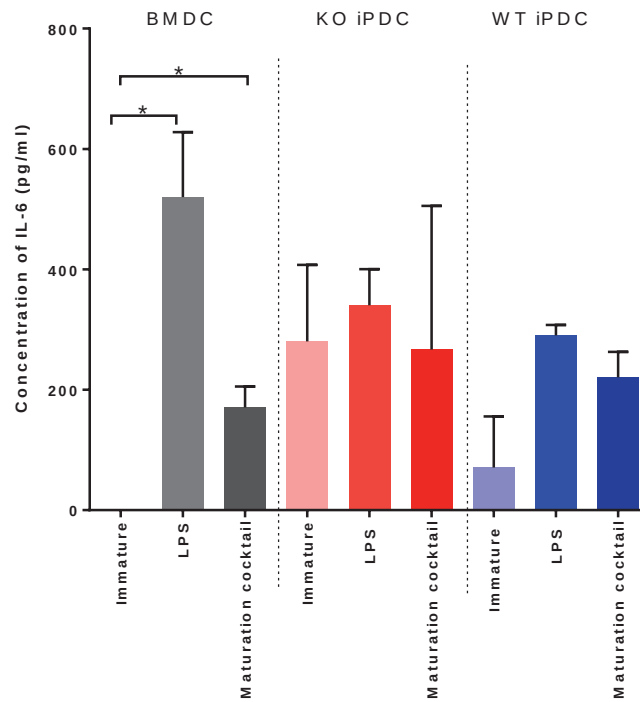
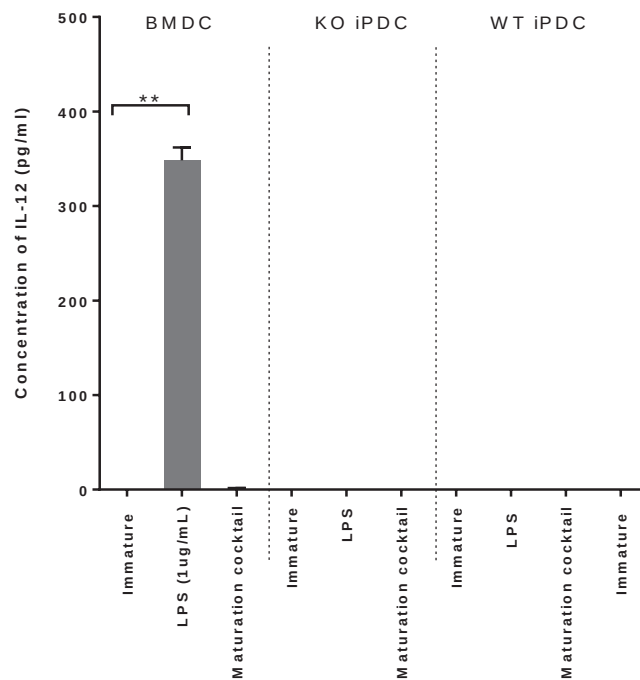
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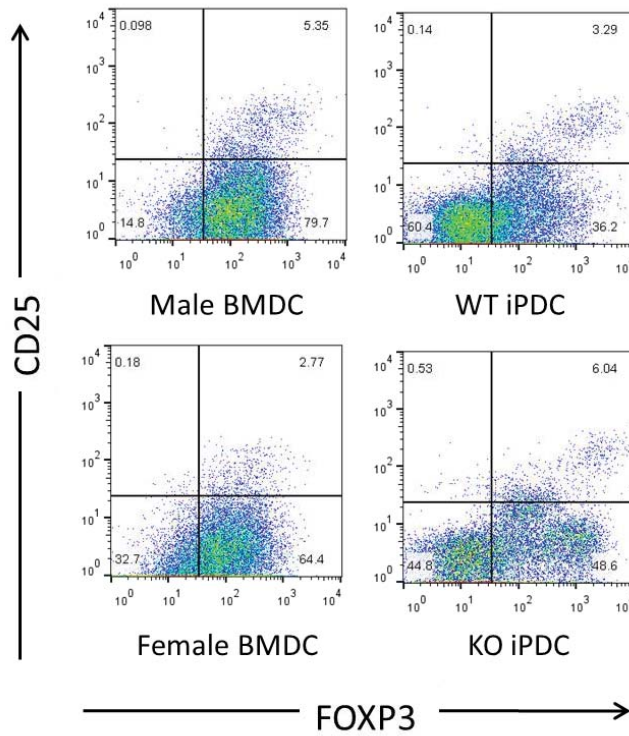
Figure 4.13 Comparison of cytokine secretion by KO and WT miPDC. Immature iPDCs and BMDCs (10^5) were harvested and cultured for 18 hours with or without maturation cocktail or $1\mu\text{g/ml}$ LPS. Supernatants were collected the amounts IL-16 **(A)** and IL-12 **(B)** secreted was determined by standard ELISA. Black bars indicate BMDC, red bars indicate KO miPDCs and blue bars indicate WT iPDCs. Plots are representative of at least 3 independent experiments, with $n=3$ in each experiment. Statistical analysis was performed using parametric T test with Welch's corrections (** $p \leq 0.001$).

A T_{reg} induction assay was performed to investigate the effect of IL-10 on the ability of iPDCs to induce the differentiation of T_{reg} cells from naïve T cells in the presence of TGF β and IL-2. Naïve T cells from female B6 Marilyn $RAG1^{-/-}$ mice, a kind gift from Dr. Elizabeth Adams (Prof. Herman Waldmann's laboratory) at Sir William Dunn School of Pathology, Oxford, were used for this assay. Due to the $RAG1^{-/-}$ phenotype, B6 Marilyn $RAG1^{-/-}$ mice do not contain mature T and B cells and only female mice contain $CD4^{+}$ T cells with transgenic TCRs (V β 6) specific for the male antigen, DBY peptide₅₈₉₋₆₀₃ bound to H-2A^b [358]. They were especially selected as a source of $CD4^{+}$ T cells for T_{reg} induction assay as they carry no natural T_{regs} usually found in WT animals.

Immature iPDCs, male BMDCs and female BMDCs were harvested and 10^5 cells cultured with naïve T cells from female B6 Marilyn $RAG1^{-/-}$ mice for 7 days with 100U/ml IL-2, 10ng/ml TGF- β and male DBY peptide to investigate the differentiation of T_{reg} cells. Cells were harvested after 7 days and stained for CD4, CD25 and FoxP3 to detect the presence of T_{reg} cells.

Both male and female BMDCs, as well as KO and WT iPDCs were able to induce the differentiation of naïve T cells from female B6 Marilyn $RAG1^{-/-}$ mice into $CD4^{+}$ $CD25^{+}$ FoxP3⁺ T_{regs} in the presence of IL-2, Dby peptide and TGF- β . Male BMDCs were found to induce more T_{reg} cells than female BMDCs, on account of the presentation of endogenous DBY peptide bound to H-2A^b. KO iPDCs were found to have a greater capacity to induce T_{reg} cells than WT iPDC (6.04% vs 3.29%, respectively), as seen in Figure 4.14A. This difference was found to be consistent across four T_{reg} induction experiments. Figure 4.14B shows the average percentage of $CD4^{+}$ $CD25^{+}$ FoxP3⁺ T_{reg} from four independent experiments. We note that although the percentages of T_{reg} cells induced varied

A



B

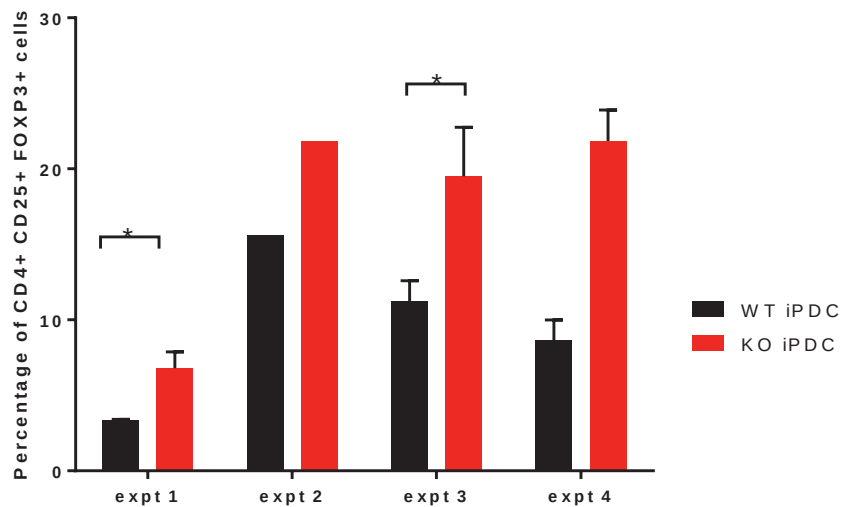


Figure 4.14 T_{reg} induction by KO and WT miPDC. 10^5 immature iPDCs, male BMDCs and female BMDCs were harvested and cultured with naïve T cells from B6 Marilyn RAG^{-/-} mice for 7 days with IL-2, TGF- β and Marilyn peptide to induce the differentiation of T_{reg} cells. After 7 days, cells were harvested and stained for CD4, CD25 and Foxp3 to detect Treg cells. **(A)** Flow cytometry dot plots of CD4 gated cells showing the expression of CD25 vs. FOXP3. FACS plots are representative of at least 4 independent experiments; gates in dot plots are set based on expression of isotype control. **(B)** Data summarising the average percentage of CD4⁺ CD25⁺ FOXP3⁺ cells from four different T_{reg} induction experiments.

significantly between experiments, KO iPDCs were paradoxically found to be consistently better at inducing T_{reg} differentiation from naïve T cells than WT iPDCs. These results were unexpected given the documented capacity of IL-10 to enhance the differentiation of T_{regs} from naïve T cells. However, there are no reports of the effect of autocrine IL-10 in DCs on DC function and their ability to induce T_{regs} . We therefore speculate that endogenous IL-10 in DCs may not have a profound effect on T_{reg} induction in the presence of TGF- β , as IL-10 and TGF- β have been found to be redundant in suppressing inflammation under certain conditions [359].

4.3 Discussion

We have previously shown that human iPDCs secrete high levels of IL-10 and low levels of IL-12. This, alongside the low expression of co-stimulatory molecules CD40 and CD80, leads us to speculate whether iPDCs possess a fetal-like phenotype that has been described in various other iPSC-derived cell types such as cardiomyocytes and neurons [211, 212, 360, 361]. moDCs derived from HIV-1 infected individuals have also been shown to produce high levels of IL-10 and low IL-12, which have been demonstrated to impede the immune response to the virus [320]. Given that BMDCs from IL-10^{-/-} mice have been shown to be more effective at eliciting a potent immune response than their WT counterparts [343], we investigated the effect of knocking out IL-10 on the immunogenicity of DCs, with the possibility of applying this knowledge to enhance the

potency of iPDCs as a vaccine for HIV-1. To model this scenario, we reprogrammed MEFs derived from IL-10 KO and WT mice into iPSCs, and differentiated them into DCs. These DCs were studied to determine the effect of autocrine IL-10 on their immunogenicity and function.

We successfully reprogrammed MEFs from C57BL/6 WT and IL-10^{-/-} mice into iPSCs using retroviral vectors, using a method similar to that originally employed by Yamanaka *et al* [197]. Three weeks after transduction, stem cell-like colonies were picked and expanded individually to give rise to distinct mouse iPSC lines. These lines were tested for their pluripotency, IL-10 expression and karyotype. One representative iPSC line with normal karyotype was picked for each of the WT and IL-10^{-/-} strains. Using gene expression analysis of genomic DNA, we confirmed that the iPSCs derived from the KO strain did not express the *Il-10* gene, whereas its WT counterpart did.

The KO and WT iPSC lines picked for this project were found to display typical stem cell morphology of domed colonies with cells possessing a high nucleus to cytoplasm ratio. Both iPSC lines were also found to be pluripotent, expressing markers of pluripotency, SSEA-1, Nanog and Oct3/4 and possessing the potential to develop into teratomas containing all three germ layers, when transplanted in the form of EBs under the kidney capsule of RAG1^{-/-} mice.

To investigate the effect of IL-10 on the immunogenicity of DCs, we successfully differentiated KO and WT iPSCs into DCs, using protocols previously established in our lab. These iPDCs, like BMDC controls, displayed typical DC morphology and expressed cell surface markers typical of DCs, such as CD11c, MHC class II and CD11b. These results were consistent with other reports of DCs differentiated from iPS and ES cells observed in

our laboratory, confirming that both KO and WT iPSC lines established were able to give rise to DCs [222]. Using standard ELISA, we observed that WT iPDCs secreted IL-10 whereas KO iPDCs did not, consistent with their differentiation from WT and IL-10 KO iPSC line, respectively and confirming their utility as a model of iPDCs derived from human iPSCs.

The yield of DCs was found to be significantly higher when differentiated from KO iPSCs as compared to their WT counterpart. This corroborates the finding that in humans, IL-10 inhibits the differentiation of DCs from PBMCs or CD14⁺ monocytes and promotes the formation of macrophage-like cells which upregulate the macrophage markers CD68 and CD16 [341, 362]. It also adds weight to the previous observation that addition of IL-10 to ES cells cultures profoundly inhibits ES-derived DC differentiation. We did not observe any marked differences in the morphology of cells differentiated from WT and KO iPSC-derived EBs but did not stain the cells for macrophage markers. When IL-10 was added to already differentiated DCs, it caused a slight downregulation of MHC class II but no acquisition of macrophage markers [362]. The pathway by which IL-10 inhibits DC differentiation has yet to be elucidated. Since IL-3 and IL-4 have been known to inhibit macrophage differentiation and are also known to hinder IL-10 secretion, it has been speculated that IL-10 may redirect the differentiation of monocytes along macrophage lineage. No effect of IL-10 on development of DCs in mice has, until now, been reported.

Since IL-10 has been known to have an effect on the antigen presentation capacity of DCs, we investigated the effect of IL-10 on co-stimulatory molecules, CD80, CD86 and CD40, MHC class I and II and activation marker CD54 by comparing their expression in KO and WT iPDCs.

Both CD80 and CD86 were upregulated on maturation in KO and WT iPDCs, consistent with reports in literature of mouse DCs matured with LPS [363]. However, this upregulation was only found to be significant in the case of CD86, whereas the upregulation of CD80 on maturation was found to be negligible, consistent with reports of CD80 expression in human iPDCs. Although the mean fluorescence intensity (MFI) of CD86 staining of mature DC was found to vary substantially in both WT and KO iPDCs, no significant differences were observed in the MFI of CD86 and CD80 in both immature and mature KO or WT iPDCs, in contrast to results reported for IL-10^{-/-} BMDCs, where both CD80 and CD86 was found to be upregulated as compared to WT-BMDCs [343].

No cell surface expression of the costimulatory molecule CD40 was observed in either immature or mature WT and KO iPDCs. BMDCs did not express CD40 in their immature state but upregulated the molecules on maturation with LPS for 24 hours, in contrast with reports in the literature where CD40 expression was observed even in immature BMDCs, albeit at lower levels than after maturation. The lack of expression of CD40 in iPDCs is consistent with reports of low expression of CD40 in human iPDCs as well as in mouse iPDCs and ES-DCs in previous work in the laboratory (Simon Hackett, PhD Thesis, 2013). Low expression of CD40 has been observed in human cord-blood derived neonatal DCs [303], which were also found to have low expression of IL-12, similar to that observed in human and murine iPDCs and were found to be less efficient at stimulating IFN- γ production from CD4⁺ T cells [303, 319]. These findings are therefore consistent with the observation that iPSC-derived DCs closely resemble DCs in neonatal state. This may have a profound effect on the function of the DCs *in vivo* since CD40-CD40L interaction is responsible for the 'licensing' of DCs by CD4⁺ T cells to allow DCs to prime CD8⁺ T cell to become effector CTLs [118].

The level of CD54, an adhesion molecule which is known to facilitate leukocyte endothelial transmigration and is commonly used as a surrogate marker for APC activation, was analysed for differences between WT and KO iPDCs. CD54 was highly expressed in both WT and KO iPDCs as well as in BMDC control. However, the maturation of iPDCs and BMDCs with LPS only resulted in a marginal increase in CD54 expression, in contrast to reports of mouse DC maturation in the literature, where CD54 was found to be significantly upregulated on maturation with LPS [363].

MHC class I and MHC class II were found to be expressed in both KO and WT iPDCs and were upregulated on maturation with LPS for 24 hours, although this increase was only found to be significant in the case of MHC class I. The level of MHC class I was also found to be higher in KO iPDC as compared to WT iPDCs, consistent with previous observations of BMDCs in this same strain [343], however this difference was not found to be significant. The level of MHC class II appeared to be higher in immature WT iPDCs as compared to that in immature KO iPDCs, but not significantly different. The level of MHC II in mature WT and KO iPDCs was found to be roughly equivalent, suggesting that targeting IL-10 expression has little effect on the ability of DCs to stabilize MHC II for antigen presentation [364]. These results are in contrast with results observed in BMDCs derived from IL-10^{-/-} mice, where the expression of MHC II was found to be significantly upregulated, where expression of MHC I which was found to be only slightly upregulated as compared to their WT counterpart [343]. Since upregulation of MHC class I has been associated with enhanced potential of DCs to cross present antigen to CD8⁺ T cells and facilitate the stimulation of virus specific CTLs, knocking out IL-10 in iPDCs may enhance their effectiveness as a vaccine for HIV-1 [72, 364].

To assess their functional capacity, we further compared KO and WT iPDCs for their ability to stimulate proliferation of naïve T cells in an allogeneic MLR. Immature KO iPDCs had a greater capacity to stimulate the proliferation of naïve T cells than their WT counterparts, although this difference was only found to be significant for immature iPDCs at 8:1 ratio of T cells: DCs. The increase in stimulatory potential of knocking out IL-10 is consistent with observations by Chen *et al* that IL-10 KO-BMDCs are more potent at stimulating T cell proliferation as compared to their WT counterparts [343]. However, only immature BMDCs were used for their experiment and the difference was not found to be significant [343]. Interestingly, we observed that both KO and WT iPDCs were more potent at stimulating the proliferation of naïve T cells as compared to BMDCs. This is in contrast to data shown in human iPDCs, where the ability of iPDCs to stimulate T cell proliferation was found to be only marginally higher than that of moDCs, and only observed in some experiments.

We compared WT-iPDCs and KO-iPDCs for their ability to produce pro-inflammatory cytokines, IL-6 and IL-12, which are known to be involved in the polarisation of naïve T cells towards a Th1 phenotype. Both WT and KO iPDCs were found to secrete IL-6 whilst immature as well as on maturation with LPS or a pro-inflammatory cytokine cocktail. No significant upregulation of IL-6 secretion was observed on maturation, in contrast to reports in the literature where IL-6 secretion was found to be significantly upregulated on maturation with LPS [121]. We did not observe any significant differences in levels of IL-6 secreted between KO and WT iPDCs, in contrast to reports in the literature of increased IL-6 production by BMDCs derived from IL-10 KO mice [342].

It has been reported that IL-10^{-/-} BMDCs secrete more Th1-polarizing cytokine, IL-12p70 as compared to WT-BMDCs [342, 343]. We did not observe any IL-12p70 secretion in immature KO or WT iPDCs or on maturation with 1µg/ml LPS or with a cocktail of TNFα, IFNγ, IL-1β and PGE-2 for 24 hours. This is consistent with our observation in human iPDCs, where secretion of IL-12 was not detected in immature iPDCs as well as iPDCs matured by a cocktail of cytokines or with single TLR stimulants. These results lead us to conclude that iPDCs more closely resemble neonatal DCs, which have also been found to secrete lower levels of IL-12 than adult DCs [303]. Surprisingly, no IL-12p70 secretion was detected in immature BMDCs, although they produced high levels of IL-12 on stimulation with LPS, consistent with previous reports [356].

Both immature WT and KO iPDCs were able to induce differentiation of CD4⁺ CD25⁺ FoxP3⁺ T_{reg} cells from naïve T cells, in the presence of TGF-β and IL-2, consistent with reports in the literature of immature DCs inducing the differentiation of T_{regs} [365]. Although the percentages of induced T_{regs} varied from experiment to experiment, KO iPDCs were found to be consistently superior at inducing the polarization of naïve T cells towards a regulatory phenotype. Our results contradict the reports of IL-10 function in the literature, where the production of IL-10 leads to the formation of an immunosuppressive environment, conducive to the formation of T_{reg} cells [366, 367].

To correlate this work back to human iPDCs, we neutralised the endogenous IL-10 in immature and mature iPDCs using IL-10 neutralising antibody and studied the effects on the ability of DCs to stimulate proliferation of naïve CD4⁺ T cells. The addition of IL-10 neutralizing antibody was found to have no effect on the ability of either immature or mature iPDCs to prime naïve T cells (data not shown). These findings were in contrast to

observations in mouse iPDCs, where knocking out IL-10 leads to increased ability of iPSCs-derived DCs to stimulate naïve T cells. These data suggest that autocrine IL-10 may not have a significant impact on the immunogenicity of human iPDCs and may not serve as an appropriate target for enhancing the immunogenicity of Dc-derived vaccines.

In summary, we have shown that endogenous IL-10 has a role in the differentiation of mouse iPDCs, consistent with its known ability to inhibit DC differentiation. We also demonstrated that knocking out IL-10 upregulated the expression of MHC class I and class II and co-stimulatory molecule CD86 in iPDCs, consistent with its effects on BMDCs [343]. Furthermore, knocking out IL-10 enhanced the ability of iPDCs to stimulate the proliferation of naïve T cells, substantiating results obtained by Chen *et al* using IL-10^{-/-} mice. Unexpectedly, IL-10^{-/-} iPDCs were found to be better at inducing the differentiation of T_{reg} cells from naïve T cells, contrary to the well-established role played by IL-10 in enhancing tolerance. Knocking out IL-10 in iPDCs had no effect on the levels of IL-6, unlike results obtained from BMDCs, in which IL-6, along with other pro-inflammatory cytokines, were upregulated on knocking out IL-10 [342].

Although the enhancement of stimulatory potential and increase in CD86 and MHC expression points to an increase in immunogenicity in iPDCs on knocking out IL-10, the lack of effect on pro-inflammatory cytokine IL-6 and enhancement of T_{reg} induction makes it difficult to conclude whether targeting the *IL-10* gene in human iPSCs would overcome the deficiencies of a fetal-like phenotype of iPDCs and will help enhance its ability to cross present HIV-1 antigen to CD8⁺ T cells. Nevertheless, the enhanced expression of MHC class I and increased yield of DCs following targeting of endogenous IL-10 may prove beneficial when considering clinical applications of human iPDCs.

Chapter 5:

Harnessing iPSC for the phenotypic reassignment of T cells

5.1 Introduction

Regulatory T cells and self-tolerance

Regulatory T cells (T_{regs}) have been shown to be indispensable in the inhibition of excessive immune response and the maintenance of immunological self-tolerance [163]. Individuals lacking functional T_{regs} have been shown to suffer from a range of autoimmune diseases such as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, rheumatoid arthritis (RA), autoimmune liver disease, systemic lupus erythematosus (SLE) and Kawasaki disease [162, 368-370]. T_{regs} have also been shown to moderate excessive immune responses to non-self-antigens and have been shown to be involved in conferring tolerance to organ transplants *in vivo* [371-374]. T_{regs} can suppress the function of $CD4^{+}$ and $CD8^{+}$ T cells either directly, by cell to cell contact or indirectly, via the secretion of immunosuppressive cytokines, TGF- β and IL-10 [178]. They can also mediate their function through DCs, by downregulating the cell surface expression of co-stimulatory molecules and reducing their ability to present antigen to T cells [178].

Current T_{regs} -based therapeutic strategies

The role of T_{regs} in suppressing excessive immune responses against alloantigens and induction of tolerance to self-antigens makes them ideal for exploitation in immunotherapy. T_{regs} have the potential to influence the outcomes of numerous conditions where clinical tolerance is required, such as autoimmunity, the lysosomal

storage diseases, hematopoietic stem cell transplantation and allograft rejection. In the case of tissue transplantation in particular, prolonged use of immunosuppression to prevent rejection of grafted tissues leads to a decrease in the long-term survival of patients due to infection and malignancy [178].

Various studies have investigated stimulating the expansion of nT_{regs} as well as induction of iT_{regs} from conventional T cells *in vivo*. Low doses of IL-2, a potent T cells growth factor, has been shown to preferentially expand nT_{regs} *in vivo* without expanding the numbers of effector T cells. Although the numbers of nT_{regs} returned to baseline levels after the treatment was withdrawn, the transient rise in nT_{regs} resulted in a slight improvement of inflammation among patients with graft-versus host disease (GVHD) [375]. The stimulation of mouse T cells *in vivo* with a strong alloantigen in combination with IL-2 and TGF- β has been shown to successfully induce the differentiation of iT_{regs} from conventional $CD4^+$ T cells. As an important modulator in the immune system, DCs have also been harnessed to induce the T_{reg} differentiation *in vivo*. In this approach, DCs were treated with modulatory agents such as rapamycin, Vitamin D3, IL-10 or dexamethasone to generate a stable tolerogenic phenotype. These tolerogenic DCs were found to express PD-L1, which has been shown to be required for iT_{reg} development *in vivo*, and were shown to induce the expansion of nT_{regs} *in vivo*. However, these *in vivo* induced T_{regs} were found to be unstable and could revert back to effector T cell phenotype [177]

In 1995, Sakaguchi *et al* first demonstrated that the transfer of $CD4^+$ T cells which have been depleted for CD25 into nude mice resulted in development of many autoimmune diseases, which was found to be reversed by the co-administration of $CD4^+ CD25^+ T_{regs}$, indicating that $CD4^+ CD25^+ T_{regs}$ play a role in self-tolerance [162]. Adoptive transfer of

T_{regs} has since been shown to treat many autoimmune diseases and prevent graft rejection and GVHD in mouse models [36, 171, 376-378]. Adoptive transfer of T_{regs} involves the isolation of antigen specific autologous or donor-derived T_{regs} , their *ex vivo* expansion and subsequent reinfusion into the patient to induce tolerance [177]. Adoptive transfer of T_{regs} isolated from peripheral blood show great promise for inducing tolerance during organ transplant and for treating various autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. A few clinical trials investigating the therapeutic potential of T_{regs} are underway. [178, 227-229]. Although the preliminary results from the first clinical trials have been encouraging, a few problems still exist which hinder the application of adoptive T_{reg} transfer as a therapy. Due to the lack of specific markers for T_{regs} and their low frequency in peripheral blood, purification of T_{regs} has proven to be challenging and to date, no protocol has achieved a 100% pure T_{reg} population [230]. Contamination of T_{regs} with effector T cells during adoptive transfer may aggravate autoimmune disease or graft rejection when the cells are expanded and reinfused into the patient [177, 230]. Furthermore, adoptive transfer of T_{regs} requires a high number of cells which has proven to be problematic since T_{regs} have also been found to be refractory to extrinsic expansion [177, 230]. Furthermore, it has been speculated that excessive T_{reg} activity may tip the balance of the immune system towards tolerance, leading to impaired immune responses to infection and potentially higher incidences of malignancy [177].

Differentiation of T cells from iPSCs

Given the difficulty in obtaining sufficient numbers of T_{regs} for adoptive transfer *in vivo*, the advent of induced pluripotency offers unrivalled opportunities. The proven ability to produce iPSCs from individuals in a patient-specific manner with the capacity for indefinite self-renewal and unrestricted differentiation potential, may facilitate the scale-up in production of T_{regs} [17].

In 2003, work in the Zuniga-Pflucker lab showed the successful differentiation of T cells from mouse embryonic stem (ES) cell-derived pre-hematopoietic progenitors in fetal thymus organ cultures (FTOCs) [379]. A year later, T cells were successfully differentiated from ES cells co-cultures with the OP9 stromal cell line expressing notch ligand, delta-like ligand 1 (DL1), which has been shown to be involved in the lineage specification and commitment of T cells [83, 232, 380, 381]. These cells were found to be double positive for CD4 and CD8 and were introduced into FTOCs to provide a microenvironment conducive to TCR rearrangement and positive selection. Following this, T cells were successfully differentiated from murine iPSCs when co-cultured with either OP9DL1 or with an initial co-culture on OP9 cells followed by OP9-DL1 [382, 383]. The T cells differentiated in this way were shown to be functional, with the ability to produce IFN γ and IL-2 when stimulated. Adoptive transfer of these iPSC-derived T cells into RAG1^{-/-} mice was shown to successfully reconstitute the T cell pool [382, 383]. Subsequently, CD4⁺ CD8⁺ T cells were shown to be generated from human ES cells via co-culture with OP9 cells followed by OP9-DL1 in the presence of Flt3L and IL-7, as described in the murine systems [384]. These T cells were found to proliferate in response to stimulation and secrete immune-modulatory cytokines [384].

These findings suggest that pluripotent stem cells have the potential to provide an unlimited source of T cells for adoptive transfer. However, the requirement for culturing iPSC or ESC-derived T cells in FTOC to allow for V(D)J recombination and positive selection greatly hinders the translation of these protocols to the clinic [17].

Derivation of iPSCs from antigen-specific T cells

Since iPSCs can be produced from various types of tissues, several groups have tried to overcome the need for thymic selection by generating iPSC lines from T cells or antigen specific T cells that have already undergone V(D)J recombination to capture a particular antigen specificity [17].

In 2010, iPSCs were successfully reprogrammed from terminally differentiated murine [221] as well as human T cells [233, 385, 386]. In 2012, Vizacardo *et al* were able to demonstrate the successful differentiation of CD4⁺ CD8⁺ T cells from an iPS cell line reprogrammed from CD8⁺ T cells specific for melanoma antigen MART-1 [219]. On stimulation with anti-CD3 antibody, these DP cells were able to generate large numbers of CD8⁺ T cells which were shown to respond to MART-1, demonstrating the antigen specificity of the cells of origin [17]. This result has been substantiated by Nishimura and colleagues, who reprogrammed iPSCs from HIV-1 specific CD8⁺ T cells and were able to demonstrate their successful re-differentiation into functional CD8⁺ T cells. These results show that T cells re-differentiated from antigen specific T cell-derived iPSCs were able to retain the V(D)J recombination of their cell of origin, suggesting that this method can be used to generate antigen-specific T cells for all types of antigens.

Following the successful reprogramming of antigen-specific T cells into iPSCs, chimeric antigen receptor (CAR) technology has been used to genetically engineer T cell-derived iPSCs with TCR specific for the CD19 antigen expressed by many leukaemia cells [234]. These CAR-modified iPSCs have been subsequently shown to differentiate into CD19 antigen-specific functional T cells. These T cells had the advantage of being specific for the chosen antigen irrespective of the HLA type, suggesting they could be used as a potential source for off-the-shelf T cell therapies. However, these iPSCs were found to develop into $\gamma\delta$ T cells rather than $\alpha\beta$ T cells, despite the expression of endogenous $\alpha\beta$ TCR on their surface. The T cells differentiated from CAR-modified iPSCs prematurely express $\alpha\beta$ TCR, much like T cells during fetal development. This suggests that T cells derived from CAR-modified iPSCs may be intrinsically skewed towards an embryonic phenotype, which is unsuitable for T cell immunotherapy. [234].

Generating T_{regs} from iPSC

In 2012, Haque and colleagues demonstrated the successful differentiation of functional CD4⁺ CD25⁺ FoxP3⁺ T_{regs} from murine iPSCs that had been transduced with FoxP3 expressing retroviral vector using adult thymic organ culture [230]. They also showed the successful differentiation of T_{reg} precursors by co-culturing FoxP3-transduced iPSCs on OP9-DL1 for 7 days. These precursors were adoptively transferred into RAG^{-/-} mice for 6 weeks, after which CD4⁺ CD25⁺ FoxP3⁺ T_{regs} were detected in the lymph node and spleens of the mice. These results demonstrated that mouse iPSCs transduced with FoxP3 are capable of producing functional T_{reg} both *in vitro* and *in vivo*. T_{regs} generated by both these methods were found to be functional, capable of producing immuno-suppressive

cytokines such as TGF- β and IL-10 and able to significantly reduce autoimmune arthritis in mice [230]. Although the ability to differentiate functional T_{regs} from iPSCs holds great promise for adoptive cell transfer for immunotherapy, T_{regs} differentiated by the above method were non-antigen specific, which may explain their inability to cure autoimmune arthritis in mouse models of collagen-induced arthritis. It was also noted that a significant proportion of T_{regs} were not viable at the end of the differentiation process, suggesting the need for survival signals during the last stages of co-culture with OP9-DL1 [230]. Since IL-2 has been shown to play a crucial role in T_{reg} homeostasis and TGF- β has been shown to promote T_{reg} development, both these cytokines can be used to enhance the survival of iPS-derived T_{regs} [387]. Furthermore, the use of retroviral transduction prohibits the application of this process in human immunotherapy due to the potential of retroviruses to give rise to insertional mutations, which have the potential to be oncogenic [388-390].

Aim

Given that adoptive cell transfer of T_{regs} for immunotherapy is limited by the failure to obtain sufficient numbers of antigen-specific T_{regs} for *ex-vivo* expansion as well as the inability to adequately purify the subset, there is a need for a method of generating large numbers of pure antigen-specific T_{regs} that can be reinfused back into the body. We propose to exploit the capacity of iPSCs to self-renew and their potential to differentiate into almost all cell types to generate antigen-specific T_{regs} for immunotherapy.

The final aim of the project is to investigate immune-modulation through the reprogramming of antigen specific T cells to iPSCs and their re-differentiation into antigen-specific T_{regs}. As mentioned earlier, the production of iPSC from antigen-specific T

cells captures the V(D)J recombination and hence the TCR specificity of the T cells, eliminating the need for a thymic microenvironment for the positive selection of T cells. Since iPSCs have been shown to have an epigenetic memory of their cells of origin, we reasoned that T cell-derived iPSCs may favour re-differentiation along the T cell lineage [391].

Here, we lay the groundwork for the above aim by investigating the feasibility of differentiating mouse T cell-derived iPSCs into $CD4^+ CD25^+ FoxP3^+ T_{regs}$ without retroviral transduction with the gene *FoxP3*, using the OP9/OP9-DL1 co-culture system that has been used in the approaches mentioned.

5.2 Results

5.2.1 Pluripotency of T cell-derived iPS cells.

T cells were harvested from the spleens of C57BL/6 (H-2^b) mice and purified using nylon wool column, following the protocol outlined in section 2.3.7. These T cells were reprogrammed into iPSC lines using retroviral transduction for the introduction of the pluripotency associated genes Oct4, Klf4 and Sox2, following the original Yamanaka protocol, with the help of Timothy Davies (Fairchild lab, Sir William Dunn School of Pathology) as described in section 2.3.3. Individual colonies obtained at the end of the reprogramming process were individually picked, expanded on feeders and frozen at passage 1 or 2. Each colony was expanded to give rise to an iPSC line.

Figure 5.1A shows a photomicrograph of a colony of the ipT cell line cultures on a layer of mitotically inactivated feeders. The line was found to have morphology typical of mouse pluripotent stem cells, with domed, bright colonies consisting of round cells with high nucleus to cytoplasm ratio.

iPSC lines were grown on gelatin until 70% confluent and karyotyped by fluorescence in situ hybridization (FISH) analysis by the Chromosome Dynamics Core at the Wellcome Trust Centre for Human Genetics (Oxford). The line chosen for these experiments, ipTR2-1, hereafter referred to as ipT, was found to be male and karyotypically normal, possessing the chromosome number 40XY (Figure 5.1B).

5.2.1.1 Expression of pluripotency markers

ipT cells were stained for markers of pluripotency alongside B6 WT iPSC line, which has been previously shown to be pluripotent (Section 4.3), and the T cells from which it had been derived as negative controls. The ipT and B6 WT iPSCs were found to express intracellular transcription factors, Nanog and Oct3/4, and cell surface marker of pluripotency SSEA-1, while T cells were not found to express any of these markers. The expression of these markers is characteristic of pluripotent mouse ES or iPS cell lines, suggesting that the ipT line is indeed pluripotent.

5.2.1.2 Ability to differentiate into all three germ layers

To further confirm the pluripotency of the reprogrammed ipT line, we investigated its ability to differentiate into embryoid bodies (EBs) *in vitro*, following a protocol previously

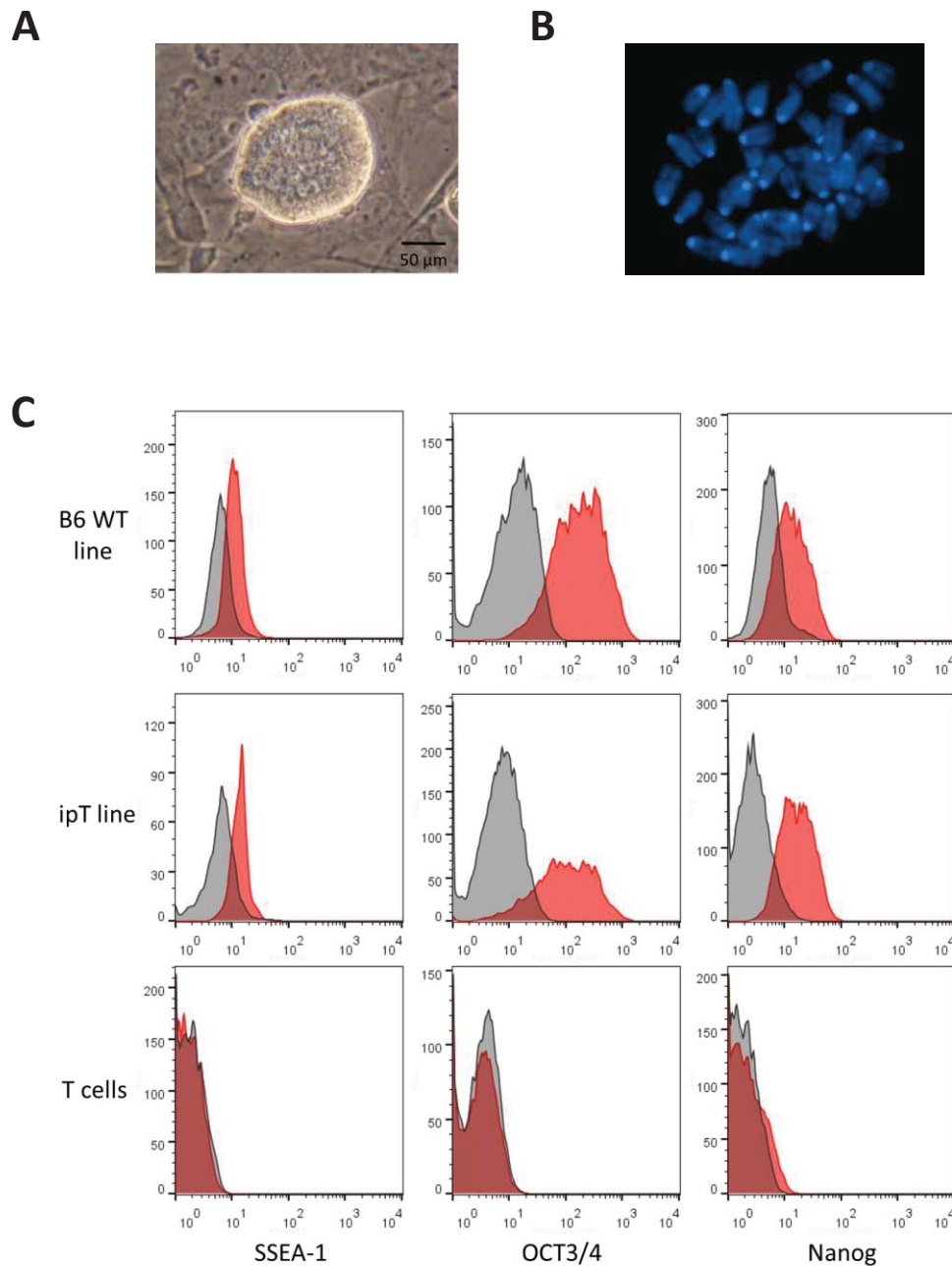


Figure 5.1 Morphology, karyotype and pluripotency of ipT mouse iPSC line. Mouse ipT iPSCs were studied for their morphology, karyotype and expression of pluripotency markers **(A)** Photomicrograph showing the typical morphology of ipT line grown on a layer of inactivated feeders **(B)** Chromosome spread of ipT line as obtained by fluorescence in situ hybridization (FISH) analysis showing the line to be male and karyotypically normal (40XY) **(C)** ipT cells were stained for cell surface markers of pluripotency, SSEA-1 and intracellular markers OCT3/4 and Nanog and analysed by flow cytometry. One representative FACS plot for each marker is shown (red) overlaid upon the expression of corresponding isotype controls (black). Plots are representative of three independent experiments.

established in our laboratory [222]. The ipT cells was able to form well-defined EBs after a few days of differentiation in suspension when LIF was withdrawn from the medium (Figure 5.2A). Since the ability of iPSCs to give rise to teratomas is one of the standard measures of pluripotency of a stem cell line, we transplanted day 14 EBs generated from the ipT line under the kidney capsule of CBA.RAG1^{-/-} mice to investigate their ability to give rise to teratomas. After 21 days, teratomas were detectable under the kidney capsule of the mice that have been transplanted with EBs (Figure 5.2B). These teratomas were harvested, sectioned and stained with H&E to investigate the presence of tissues from each of the three embryonic germ layers. The H&E stained sections revealed that the teratomas from ipT line possessed tissues originating from all three germ layers, namely the endoderm (epithelium), mesoderm (muscle) and ectoderm (gut lining) as seen in Figure 5.2C, confirming the ipT line to be pluripotent.

5.2.1.3 Ability to give rise to cells of the haematopoietic lineage

Given that the T cell-derived iPSC line, ipT has been shown to be pluripotent, we investigated its ability to give rise to cells of the haematopoietic lineage since DCs were first differentiated from ES and iPSCs in our laboratory and therefore have been well characterised previously, [222] (section 4.4). The timeline of this differentiation has been outlined in Figure 4.5 in chapter 4, with photomicrographs illustrating the process.

ipT cells were cultured on feeders and transferred onto gelatin for at least 2 passages to remove all remnant feeder cells. Cells were then harvested and plated onto bacteriological culture plates to allow the formation of EBs. After 14 days, EBs were plated onto tissue culture dishes with R10 medium supplemented with GM-SCF and IL-3.

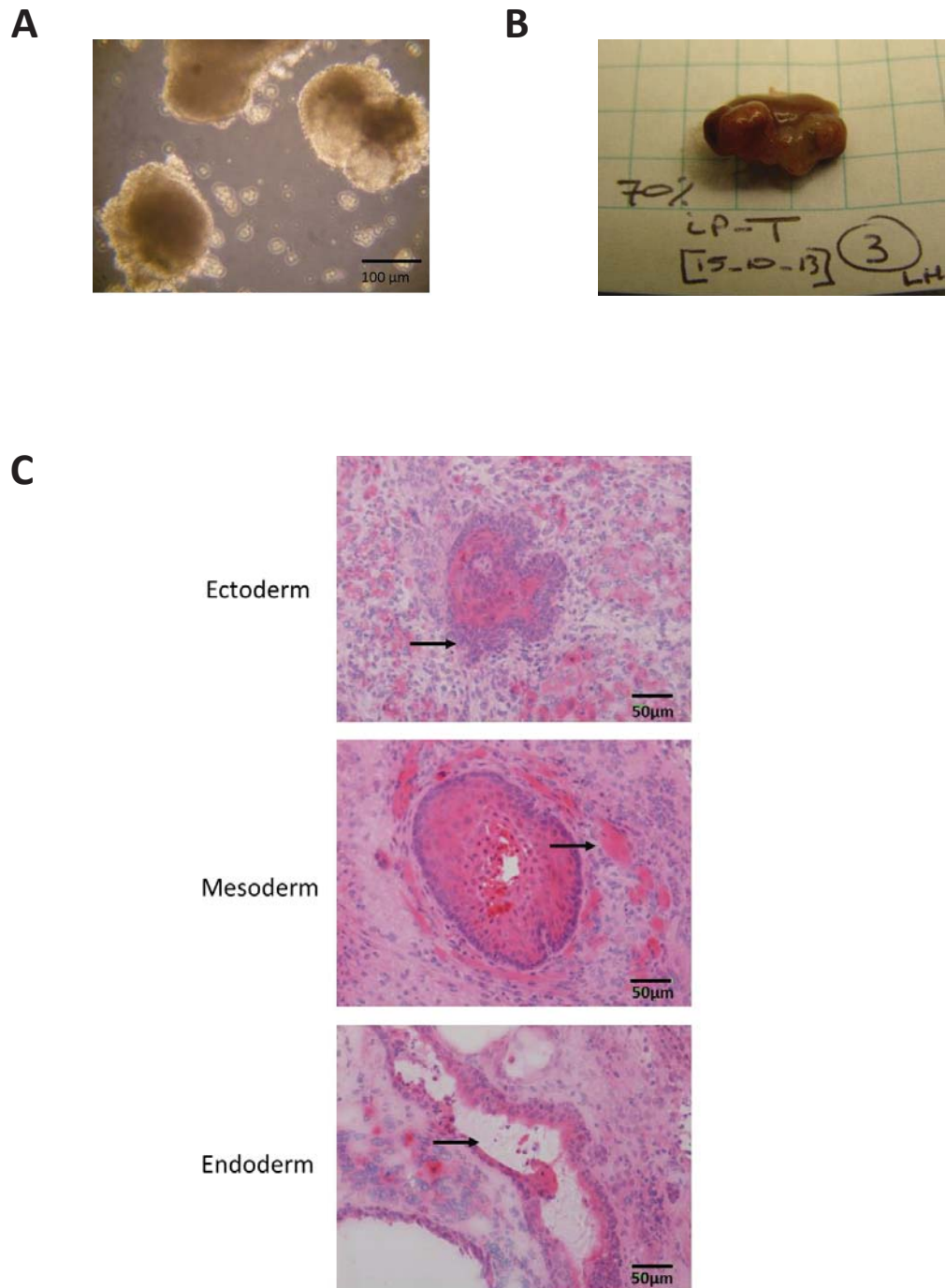


Figure 5.2 Pluripotency of ipT line- Teratoma assay. Reprogrammed ipT iPSC line was investigated for its ability to form teratomas containing all three germ layers **(A)** Photomicrograph of EBs derived from miPSC line ipT. **(B)** Representative photomicrograph of teratoma obtained from transplanting day 14 EBs from ipT line under the kidney capsule of $\text{RAG}^{-/-}$ mice. **(C)** H&E stained sections of teratomas derived from ipT line showing all tissues derived from all three germ layers. ectoderm-epithelium, mesoderm-muscle, endoderm-stomach lining. Scale bar= 50 μm . Data are representative of at least three teratomas.

Irregularly shaped cells with long processes, reminiscent of BMDCs were observed in the culture 14-16 days after plating. These cells were harvested after 21-24 days, stained for cell surface markers typical of murine DCs and analysed by flow cytometry, using BMDCs as a comparison. Mouse iPSC-derived DCs (miPDCs) derived from ipT lines were found to express CD11c, a marker typical of DCs in both mouse and human (Figure 5.3A). Although the level of expression was found to be slightly lesser than BMDCs, it is consistent with that of CD11c expression seen in iPDCs derived from other mouse iPSC lines (Section 4.3). iPDCs derived from ipT iPSC line were also found to express other DC markers including CD86, MHC I (H-2K^b) and MHC II (H2-A^b), like BMDCs, demonstrating that ipT line possessed the capacity to differentiate into representative components of the haematopoietic system (figure 5.3). Since both DCs and T cells are derived from hematopoietic stem cells (HSCs), these data suggest that the ipT line has the potential for differentiating into other cells of the haematopoietic lineage.

5.2.2 T cell re-differentiation from T cell-derived iPSCs

Since we aim to differentiate T_{regs} from iPSCs without retroviral transduction with *FoxP3*, we first sought to demonstrate the differentiation of primary T cells using protocols already established in the literature. As mentioned in the introduction to this chapter, T cells have been shown to be differentiated from mouse ESCs, HSCs as well as iPSCs, using many different protocols. The most widely used protocol involved co-culturing ES cells with OP9 stromal cells in the presence of Flt-3 ligand (Flt3L) to stimulate the differentiation of haematopoietic progenitors, which were shown to have the capacity to give rise to T cells, B cells and NK T cells [83, 221, 392]. T cell specification was achieved

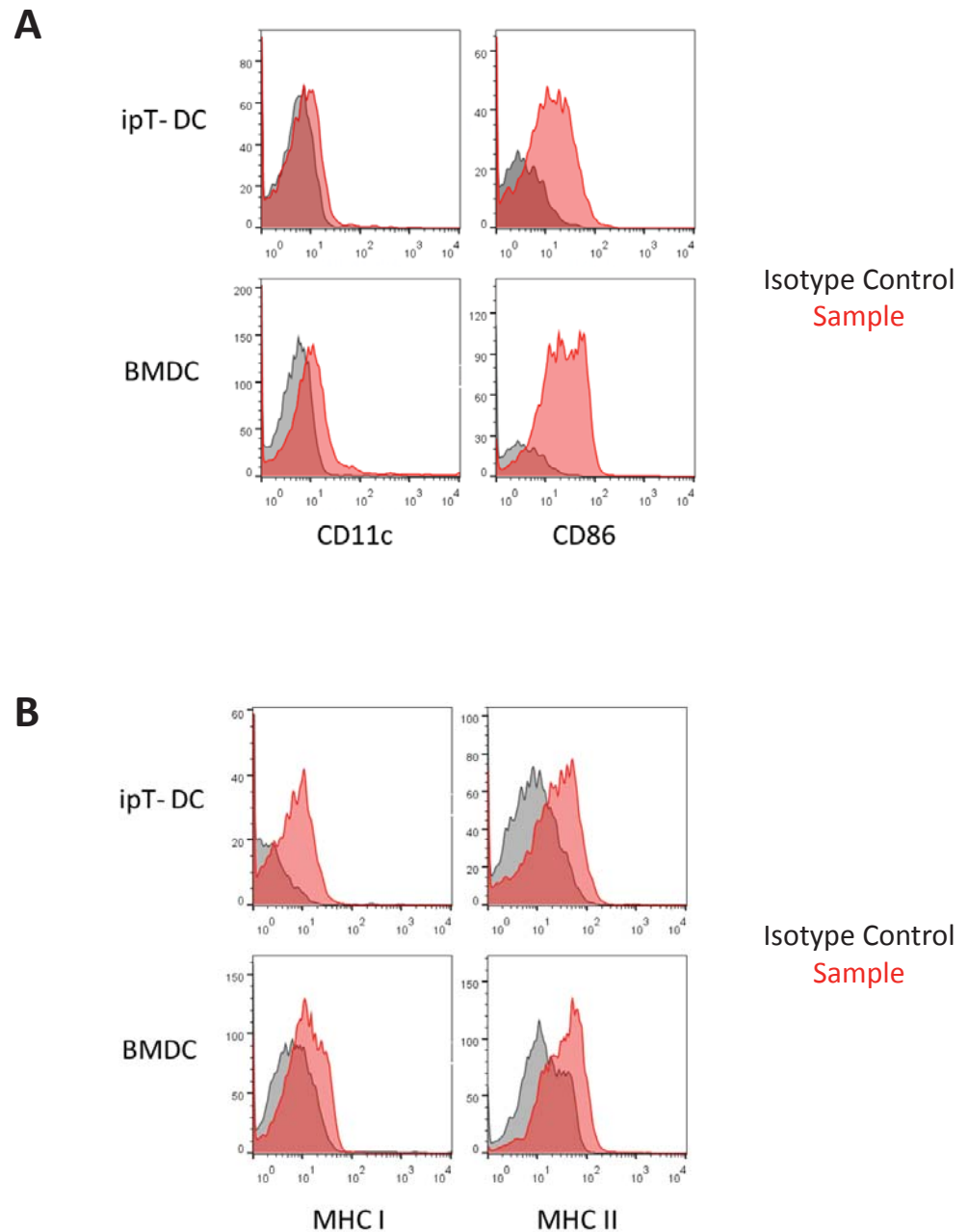


Figure 5.3 Differentiation of ipT cells into iPDCs. ipT cells were differentiated into DCs with the help of GMCSF and IL-3. Cells obtained at the end of the differentiation were stained for markers of DCs, using BMDCs as positive control. **(A)** Flow cytometry analysis of cell surface expression of DC markers CD11c and CD86 by ipT-derived DCs and BMDCs. **(B)** Flow cytometry analysis of cell surface expression of MHC I and MHC II, by ipT-derived DCs and BMDCs; All FACS plots are representative of 3 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red.

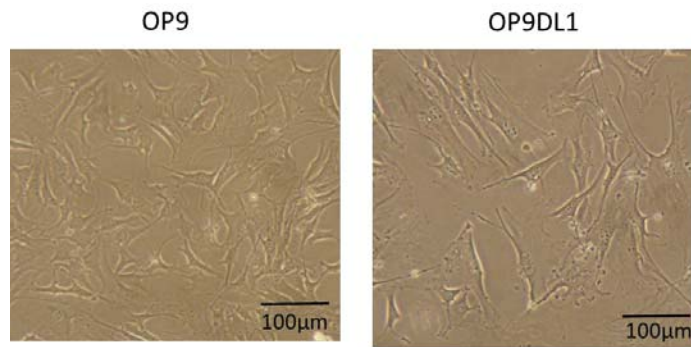
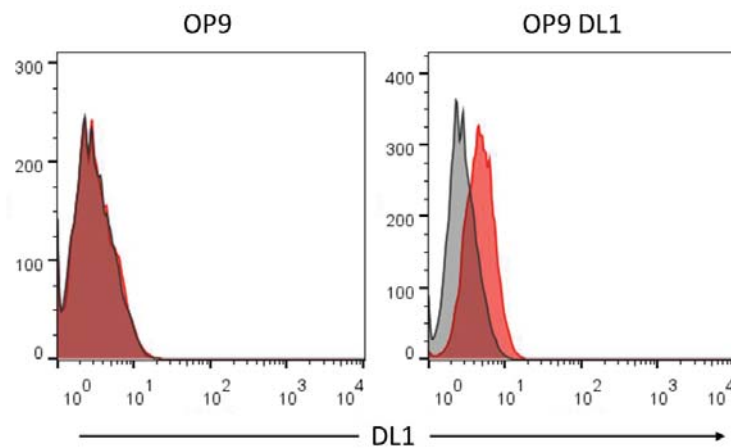
by subsequent co-culture with notch ligand-expressing OP9-DL1 stromal cells in the presence of Flt3L and IL-7 [83, 232, 240]. We employed the use of OP9 and OP9-DL1 for the differentiation of T cells from iPSC line ipT following protocols published by Holmes *et al* in 2009 [240].

5.2.2.1 Expression of notch ligand by OP9DL1 cells

OP9 and OP9-DL1 cells were a kind gift from Prof. Juan Carlos Zuñiga-Pflücker (Toronto, Canada). OP9 is a mouse bone marrow stromal cell line isolated from M-CSF-deficient (osteopetrotic) mice [239]. The lack of M-CSF suppresses the formation of macrophages in the co-cultures [393]. OP9-DL1 cells were generated via retroviral transduction of OP9 cells with a vector expressing the notch ligand, Delta like-1 (DL1) [83]. Since notch signalling has been shown to be critical for the differentiation of T cells from their progenitors, we investigated the expression of DL-1 on the OP9-DL1 stromal cells using OP9 cells as a negative control [394-396].

Both OP9 and OP9-DL1 cells were found to possess fibroblast-like morphology consistent with reports in the literature (Figure 5.4A) [239]. No adipocyte-like cells were observed in culture. Spontaneous adipogenesis has been observed in overgrown OP9 cells, corresponding to the loss of hematopoiesis-inducing potential of these cells [295].

Both OP9 and OP9-DL1 cells were cultured on 10cm tissue culture dishes in OP9 medium, until 80% confluent, harvested, stained with DL1 antibody and analysed by flow cytometry. OP9-DL1 cells were found to consistently express notch ligand DL1, whereas OP9 cells showed no staining above background level (Figure 5.4B). This confirms that

A**B**

Isotype Control
Sample

Figure 5.4 Expression of notch ligand by OP9DL1 cells. OP9 and OP9-DL1 cells were harvested, stained with DL1 antibody and analysed by flow cytometry **(A)** Photomicrograph showing the typical morphology of OP9 and OP9DL1 stromal cells. **(B)** Flow cytometry analysis of cell surface expression of notch ligand, DL1 by OP9 and OP9DL1 cells. All FACS plots are representative of 3 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red.

OP9-DL1 stromal cells express the notch ligand DL1 on the cell surface, making them suitable for T cell differentiation.

5.2.2.2 Establishing the protocol for T cell differentiation

Since the most widely-characterised protocols for differentiation of T cells from pluripotent stem cells employ ES cells, we initially established the differentiation of T cells from ES cells alongside our ipT iPSCs. Mouse ES cell line, ESF116 was used for this purpose. ESF116 is an ES cell line derived from the epiblast of a delayed-implanting CBA/Ca blastocyst [222, 397] and has been previously shown to be male, karyotypically normal and pluripotent. This was also the first line employed for the original differentiation of DCs from mouse ES cells, and has been demonstrated to have the potential to differentiate along the haematopoietic lineage [222].

ESF116 or ipT cells were differentiated into T cells using the OP9 and OP9-DL1 co-culture system established by the Zuñiga-Pflücker laboratory (Toronto, Canada) [240] as described in section 2.3.10 of chapter 2 and diagrammatically represented in Figure 5.5. Briefly, 5×10^4 to 10^5 pluripotent stem cells (PSCs) grown on MEFs were dissociated into single cells and plated onto a layer of sub-confluent OP9 cells. OP9 cells were used to initiate the differentiation as more mesoderm colonies have been reported to develop on OP9 cells compared to OP9-DL1 cells [240].

After 3 days, small ES and iPS cells were observed on the stromal layer. These colonies were found to have less defined borders than typical PSCs, suggesting the beginning of the differentiation process. The medium in the co-culture was replenished at this point. After 5 days, mesoderm-like colonies resembling a 'wagon wheel' appearance were

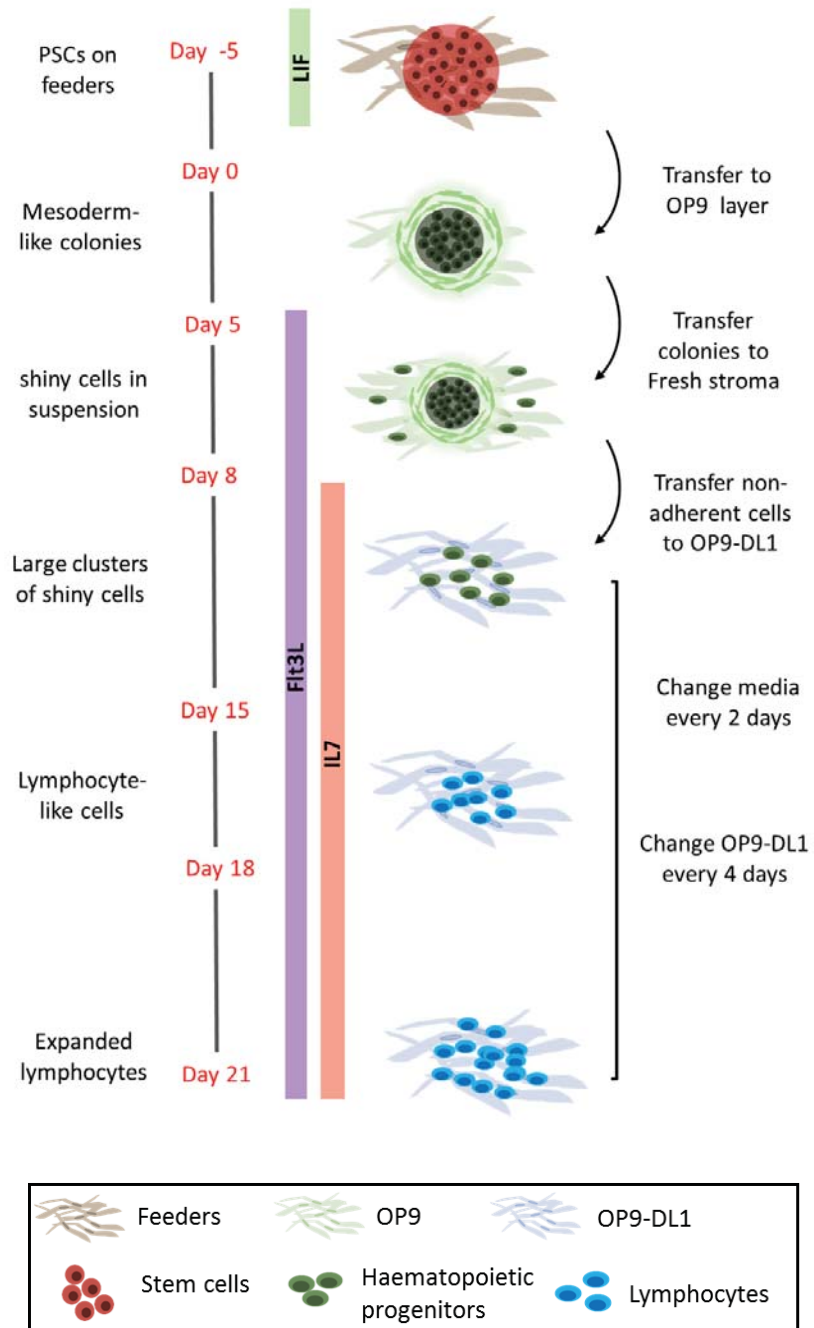


Figure 5.5 Differentiation of stem cells into T cells – Timeline. Illustration of timeline for differentiation of mouse iPSCs and ES cells into T cells. iPSCs were grown on MEFs and transferred onto OP9 cells. After 3 days, the medium in the co-culture was replenished. On day 5, the cells were dissociated and re-plated onto fresh OP9 cells with Flt3L. On day 8, colonies with semi-concentric circle of cells with clusters of round, shiny cells in suspension were observed. These non-adherent cells were harvested and seeded onto OP9-DL1 with Flt3-L and IL-7. The medium was changed every 2 days and OP9-DL1 stromal layer replenished every 4 days. Lymphocyte-like cells were observed around day 19. These cells were harvested after 20-24 days.

observed on a layer of confluent stroma. These colonies were visible to the naked eye as white plaques on the dish (Figure 5.6-Day 5). The cells were dissociated and re-plated onto 10 cm culture dishes for 30 minutes to remove adherent stromal cells. Non-adherent cells (5×10^5 to 10^6) were plated onto 10cm dishes containing sub-confluent OP9 cells in medium supplemented with Flt3L. On day 8, colonies with semi-concentric circle of cells were observed (Figure 5.6-Day 8) with clusters of round, refractile cells either in suspension or lightly attached to the stromal layer. These cells were harvested and seeded onto 6-well plate containing sub-confluent OP9-DL1 cells in medium supplemented with Flt3L and IL-7. At this stage, some cells were stained with mAbs specific for CD45 to ascertain the presence of hematopoietic progenitors.

The medium was changed every 2 days and OP9-DL1 stromal layer replenished every 4 days. On day 12, many clusters of round, refractile cells were observed throughout the dish ((Figure 5.6-Day 12). By day 19, lymphocyte-like cells were visible and started to expand (Figure 5.6-Day 19). Between days 20-24, rapid expansion of lymphocyte-like cells was observed. These cells possess a morphology similar to that of T cells, namely small, round cells which do not adhere to the surface of the plastic (Figure 5.6-Day 24). The cells were harvested for use in experiments by gentle pipetting so as not to disrupt the stromal layer.

It must be noted that this protocol was successfully established after many experimental trials where the type of media used, source of stromal cells and numbers of cells seeded were optimized. We empirically observed that, for successful differentiation of T cells from PSCs, OP9 medium made using α MEM derived from powder is superior to pre-made α MEM in liquid form. It is also important to note that, when adipocyte-like cells were

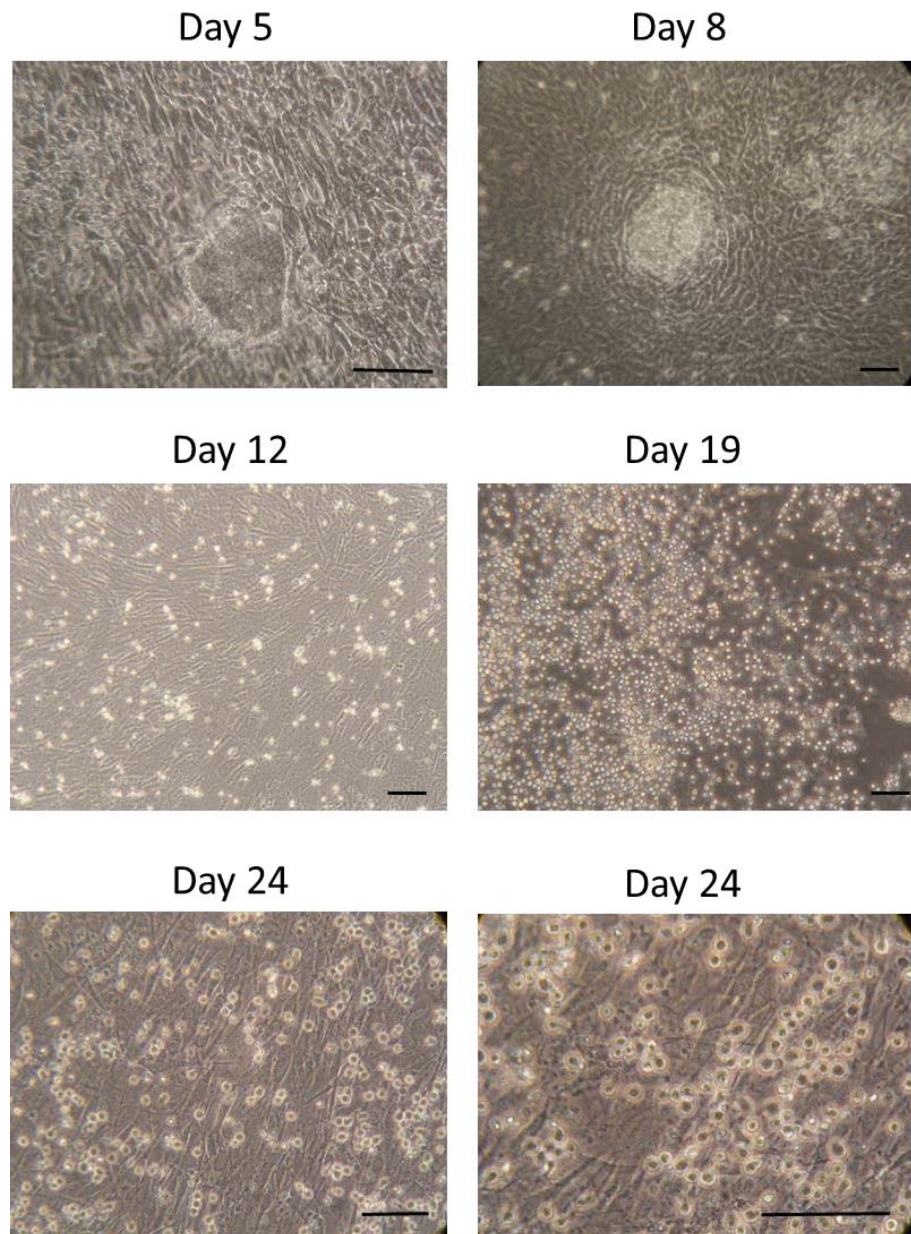


Figure 5.6 Differentiation of stem cells into T cells – Photomicrographs. Photomicrographs of typical morphology of cells during differentiation of mouse iPSCs and ES cells into T cells. On **day 5**, small ES/iPS cell colonies were observed on stromal layer. On **day 8**, colonies with semi-concentric circle of cells with clusters of round, shiny cells in suspension were observed. These cells were harvested and seeded onto OP9-DL1 with Flt3-L and IL-7. On **day 12**, many clusters of round, shiny cells were observed. By **day 19**, lymphocyte-like cells were visible and start to expand. Between **days 20-24**, rapid expansion of lymphocyte-like cells were observed.

observed in OP9 and OP9-DL1 cultures, the stromal cells had already lost their ability to support haematopoietic differentiation.

5.2.3 Presence of haematopoietic progenitors at day 8 of differentiation

Non-adherent cells harvested on day 8 were stained with CD45 to ascertain the appearance of hematopoietic progenitors. CD45 is expressed by all cells of hematopoietic origin. Co-culture of mouse ES and iPS cells with OP9 stromal cells has been shown to induce the expression of CD45 [232, 240].

Cell surface expression of CD45 was observed in non-adherent cells harvested 8 days after the co-culture of both ES and iPS cells with OP9 stromal cells in the presence of Flt3L, whereas no expression of CD45 was observed in OP9 cells co-cultured alone (Figure 5.7A and 5.8A). This indicates the development of hematopoietic progenitors from ES and iPS cells on co-culture with OP9 stroma, consistent with reports of the ability of OP9 cells to support the differentiation of cells of the hematopoietic lineage [219, 240, 383, 398].

It should be noted that small, non-adherent cells were also observed in cultures containing OP9 cells alone, making it difficult to differentiate if these cells were derived from OP9 cells or from iPSCs. However, the OP9-derived non-adherent cells did not give rise to CD45⁺ cells on co-culture with OP9-DL1.

5.2.4 Characterization of iPS and ES cell-derived T cells

ES and iPS cell derived lymphocyte-like cells harvested on or after day 20 were stained for T cell markers CD3, CD4, CD8 and CD45 and analysed by flow cytometry. Lymphocyte-like

cells derived from both ESF116 and ipT cells lines were shown to express pan T cell marker CD3 as well as hematopoietic cell marker, CD45, suggesting the successful differentiation of T cells (Figure 5.7A & 5.7B). No CD3 or CD45 expression were detected in the OP9-DL1 only controls for either experiment, confirming that these CD45⁺ CD3⁺ cells were indeed derived from ESF116 and ipT cells.

The majority of T cells derived from both ES and iPS cells were found to express both CD4 and CD8, corresponding to immature double positive T cells subset, consistent with results obtained from the differentiation of ES cells using the OP9/OP9DL1 co-culture system (Figure 5.7A & 5.7B) [83, 232, 240, 380, 382, 383]. A small percentage of CD8 single positive (SP) cells were observed in T cells derived from ESF116 whereas T cells derived for iPSCs were found to contain a small population of both CD4 and CD8 SP cells. This is consistent with observations by Schmitt *et al* on the presence of small numbers of CD4 and CD8 SP cells when ES cells were differentiated on OP9-DL1 stroma.

5.2.5 Maturation of iPSC-derived T cells in fetal thymus organ cultures

Since the thymus is critical for the development of T and their positive selection [399], fetal thymus organ culture (FTOC), a technique where the fetal thymus lobes are explanted and cultured *in vitro*, has often been harnessed to investigate the development of T cells as well as to elucidate the mechanisms involved in positive and negative selection of T cells [400]. The first successful differentiation of mature T cells from mouse ES cells required the reconstitution of pre-hematopoietic progenitors into FTOCs [379]. The ES cell-derived T cells obtained by this method were shown to contain normal CD4 and CD8 thymic subsets, suggesting that thymic selection is required for the successful

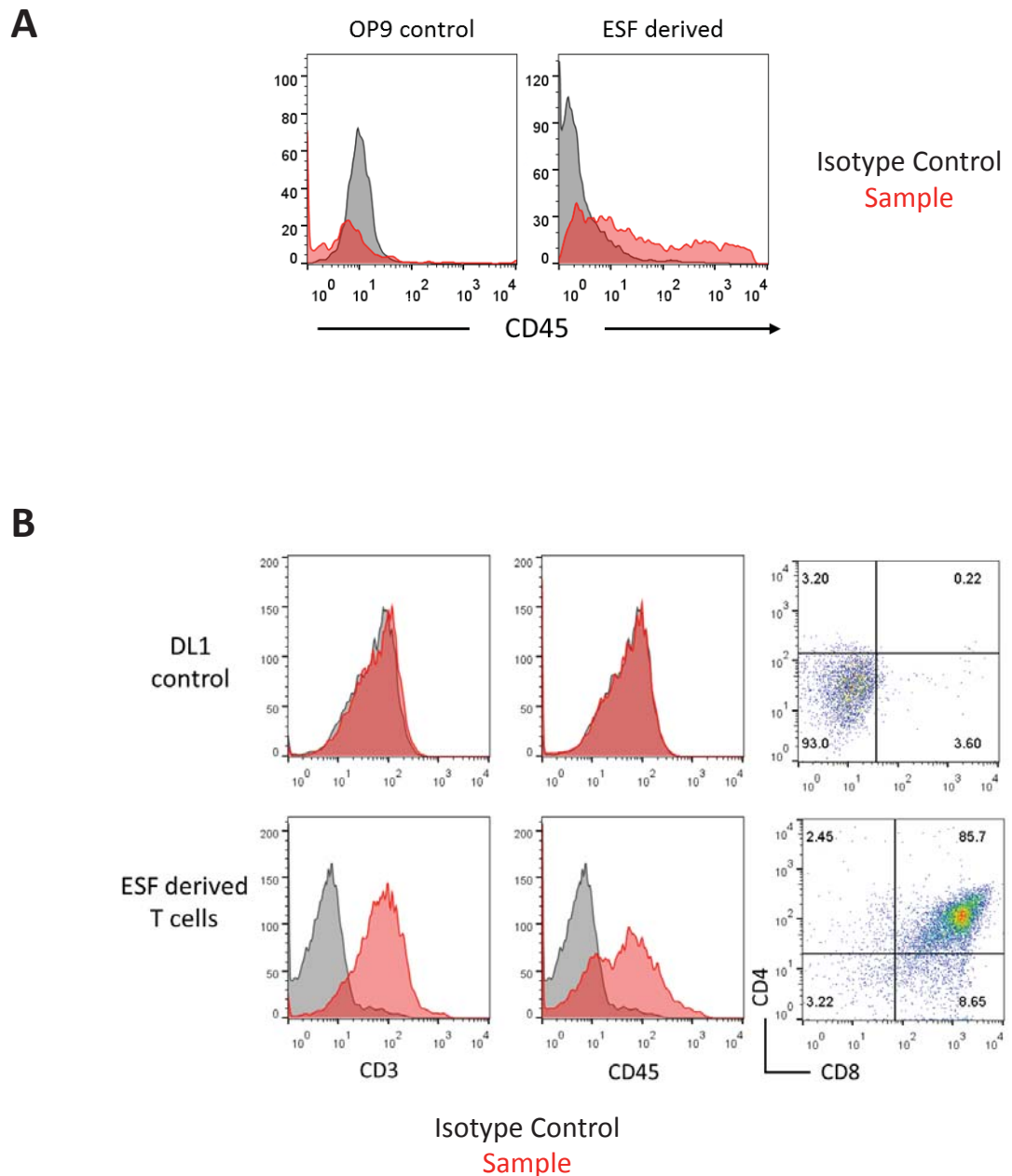


Figure 5.7 Characterization of ES cell-derived T cells. ESF116 ES cells were differentiated into T cells using a method of co-culture with OP9 and OP9DL1. Cells obtained on day 8 were analysed for cell surface expression of haematopoietic progenitors. Cells obtained at the end of the differentiation were stained for markers of T cells **(A)** Flow cytometry analysis of cell surface expression of haematopoietic cells marker, CD45 on progenitor cells from co-culture and OP9 stroma only control. **(B)** Flow cytometry analysis of lymphocyte-like cells obtained at the end of co-culture for cell surface expression of T cell markers, CD3, CD4, CD8 and CD45, using OP9DL1 only cells as a negative control. All FACS plots are representative of 3 independent experiments. Isotype controls in histograms are shown in black; antibody staining is shown in red. Gates in dot plots are set based on expression of isotype control

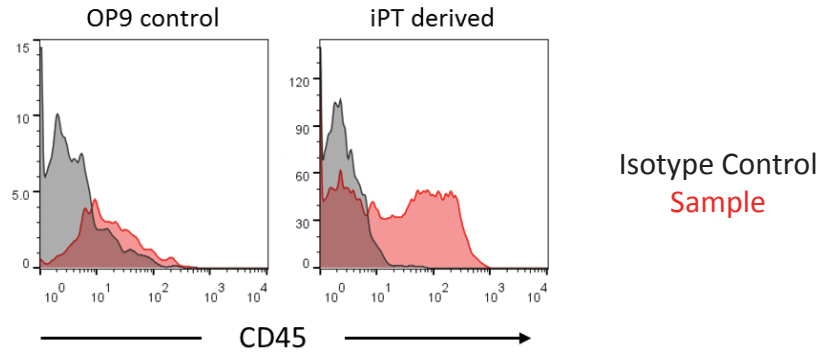
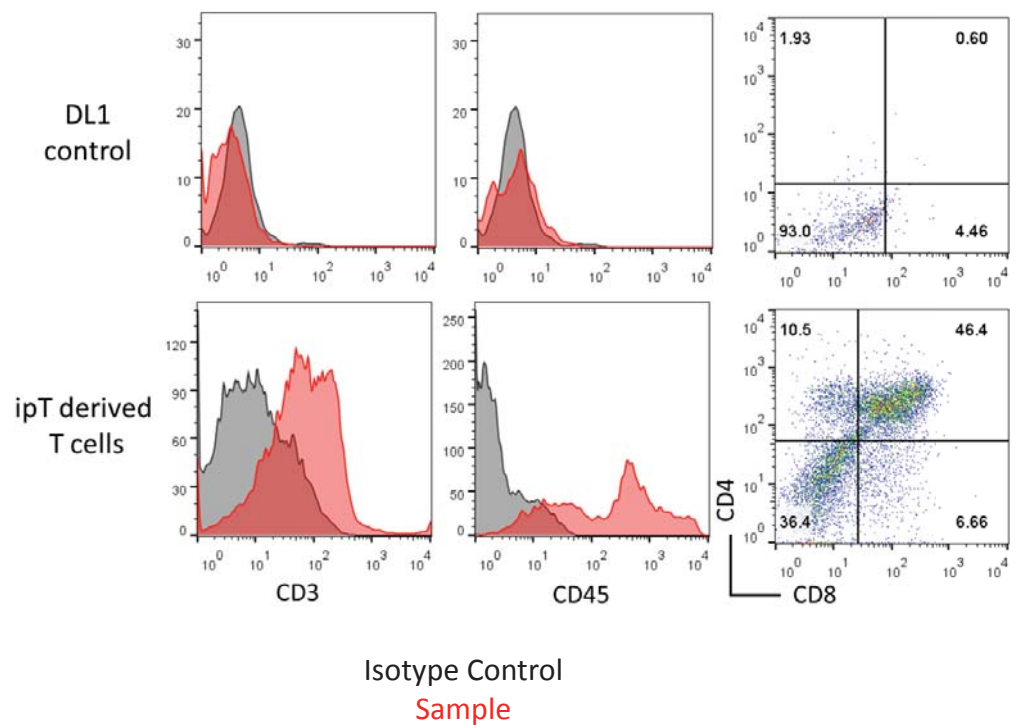
A**B**

Figure 5.8 Characterization of iPSC cell-derived T cells. ipT iPSCs were differentiated into T cells using a method of co-culture with OP9 and OP9DL1. Cells obtained on day 8 were analysed for cell surface expression of haematopoietic progenitors. Cells obtained at the end of the differentiation were stained for markers of T cells **(A)** Flow cytometry analysis of cell surface expression of haematopoietic cells marker, CD45 on progenitor cells from co-culture and OP9 stroma only control. **(B)** Flow cytometry analysis of lymphocyte-like cells obtained at the end of co-culture for cell surface expression of T cell markers, CD3, CD4, CD8 and CD45, using OP9DL1 only cells as a negative control. All FACS plots are representative of 3 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red. Gates in dot plots are set based on expression of isotype control

maturation of CD4⁺ CD8⁺ DP T cells into SP subsets. Given these findings, we investigated whether the reconstitution of iPSC-derived T cells in FTOCs will result in maturation of the CD4⁺ CD8⁺ DP T cells into SP subsets.

To achieve this we depleted thymocytes from thymi obtained from day 13 CBA/Ca embryos (H-2^k) using 1.35mM 2' deoxyguanosine (dGuo) in hanging drop cultures. CBA/Ca thymi were used so that cells derived from ipT could be distinguished from any residual thymocytes on the basis of H-2K^b expression. After 7 days of culture, these thymocyte-depleted fetal thymi were repopulated with iPSC-derived T cells. After 2 days, the thymus lobes were recovered and transferred to a sterile porous filter and placed on top of a sterile sponge soaked in R10 to form an air-liquid interface which most closely recapitulates the thymic *in vivo*. After 7 days, the thymus lobes were harvested, digested with collagenase and disaggregated by repeated passage through a 26G syringe needle. The cells obtained were stained for pan TCR, H-2K^b, CD4 and CD8 to ascertain the maturation of iPSC-derived T cells. This process is illustrated in Figure 5.9A.

Around 50% of cells obtained after FTOC were found to express pan TCR, a pan T cell marker (Figure 5.9B). The TCR⁺ subset of cells were found to express H-2K^b, the MHC class I haplotype expressed by C57BL/6 mice, suggesting that all TCR⁺ cells obtained had differentiated from the C57BL/6-derived ipT line rather than representing residual endogenous thymocytes that had survived the 2'dGuo treatment. This confirms the successful depletion of intrinsic thymocytes from fetal thymus lobes using dGuo.

Furthermore, we investigated the expression of CD4 and CD8 in iPSC-derived T cells as well as fetal thymocytes before and after FTOC. Before FTOC, iPSC-derived T cells were found to contain cells that were either double negative (DN) or DP for CD4 and CD8. After FTOC, we observed a decrease in numbers of DN cells and increase in numbers of CD8⁺

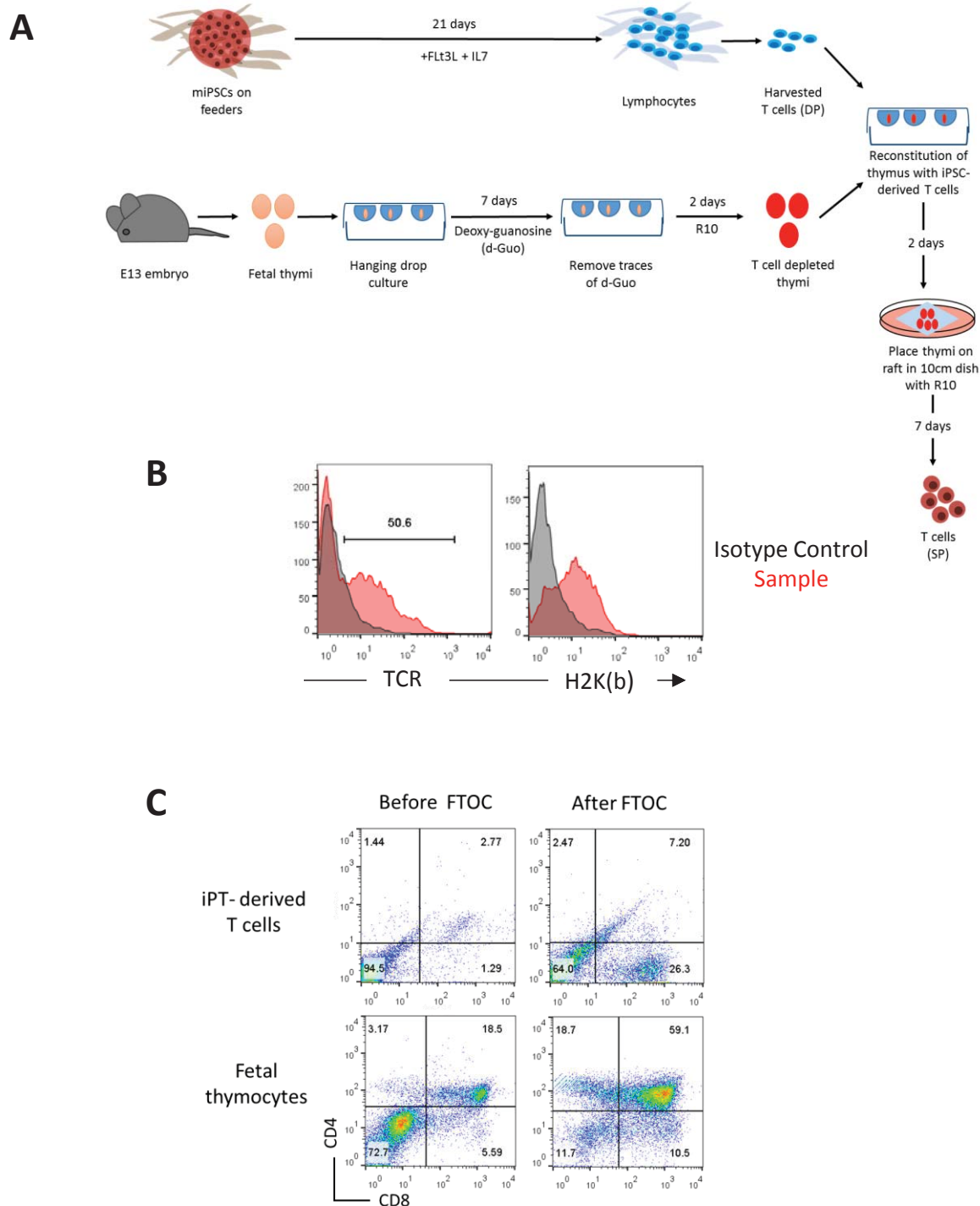


Figure 5.9 Maturation of stem cell-derived T cells using fetal thymus organ culture (FTOC) Naïve DP T cells were reconstituted in FTOC to allow maturation of T cells **(A)** Illustration of process of maturation of naïve stem cell-derived T cells in FTOC. Thymocytes were depleted from fetal thymi using 2' deoxyguanosine (dGuo) in hanging drop cultures for 7 days after which the thymi were transferred into fresh medium to remove all traces of dGuo. The thymi were repopulated with iPSC-derived T cells in hanging drop cultures for two days after which they were recovered and transferred to a sterile porous disk on top of a sponge soaked in R10 to grow on a media/air interface. After 7 days, the thymi were digested with collagenase and disaggregated to form single cells. The cells obtained were stained for pan TCR, H2Kb, CD4 and CD8 **(B)**, using fetal thymocytes **(C)** as positive control. All FACS plots are representative of 2 independent experiments. Isotype control in histograms are shown in black; antibody staining is shown in red. Gates in dot plots are set based on expression of isotype control.

cells. Very few CD4⁺ CD8⁺ T cells were detected and unexpectedly, no expression of CD4⁺ cells was observed (Figure 5.9C). The presence of CD8⁺ cells suggests that the iPSC-derived T cells have undergone a process of positive selection in the FTOCs, however, the absence of any CD4⁺ T cells leads us to speculate whether the ipT line may be derived from CD8⁺ T cells, favoring the re-differentiation along the CD8⁺ T cell lineage.

5.2.6 Differentiation of T_{regs} from mouse iPS and ES cells

Given that we have established the successful differentiation of CD4⁺ CD8⁺ DP T cells from ES cells as well as T cell-derived iPSCs, we next investigated the feasibility of differentiating these T cells into T_{regs} without the transduction of *Foxp3* gene by retrovirus, as was shown by Haque *et al* [230]. CD4⁺ CD25⁺ FoxP3⁺ T_{reg} differentiation has been achieved *in vitro* by co-culturing naïve T cells with anti-CD3/CD28 coated beads and IL-2 for the activation and expansion of T cells, respectively, in the presence of immunosuppressive cytokine TGF-β [401-403].

We plated mouse ESF116 or ipT-derived T cells in 48-well plates in medium supplemented with IL-2 and TGF-β and stimulated the cells with anti-CD3/CD28 coated beads. Cells supplemented with IL-2 alone were used as negative controls. After 5 days, cells were harvested and stained for CD3, CD4, CD8, CD25 and FoxP3 to detect the presence of T_{regs} and analysed by flow cytometry. We detected the presence of CD25⁺ FoxP3⁺ cells in samples where iPS and ES cells derived T cells were stimulated with CD3/CD28 beads in the presence of IL-2 and TGF-β, whereas negligible numbers of CD25⁺ FoxP3⁺ cells were observed in samples where TGF-β and CD3/CD28 were absent (Figure

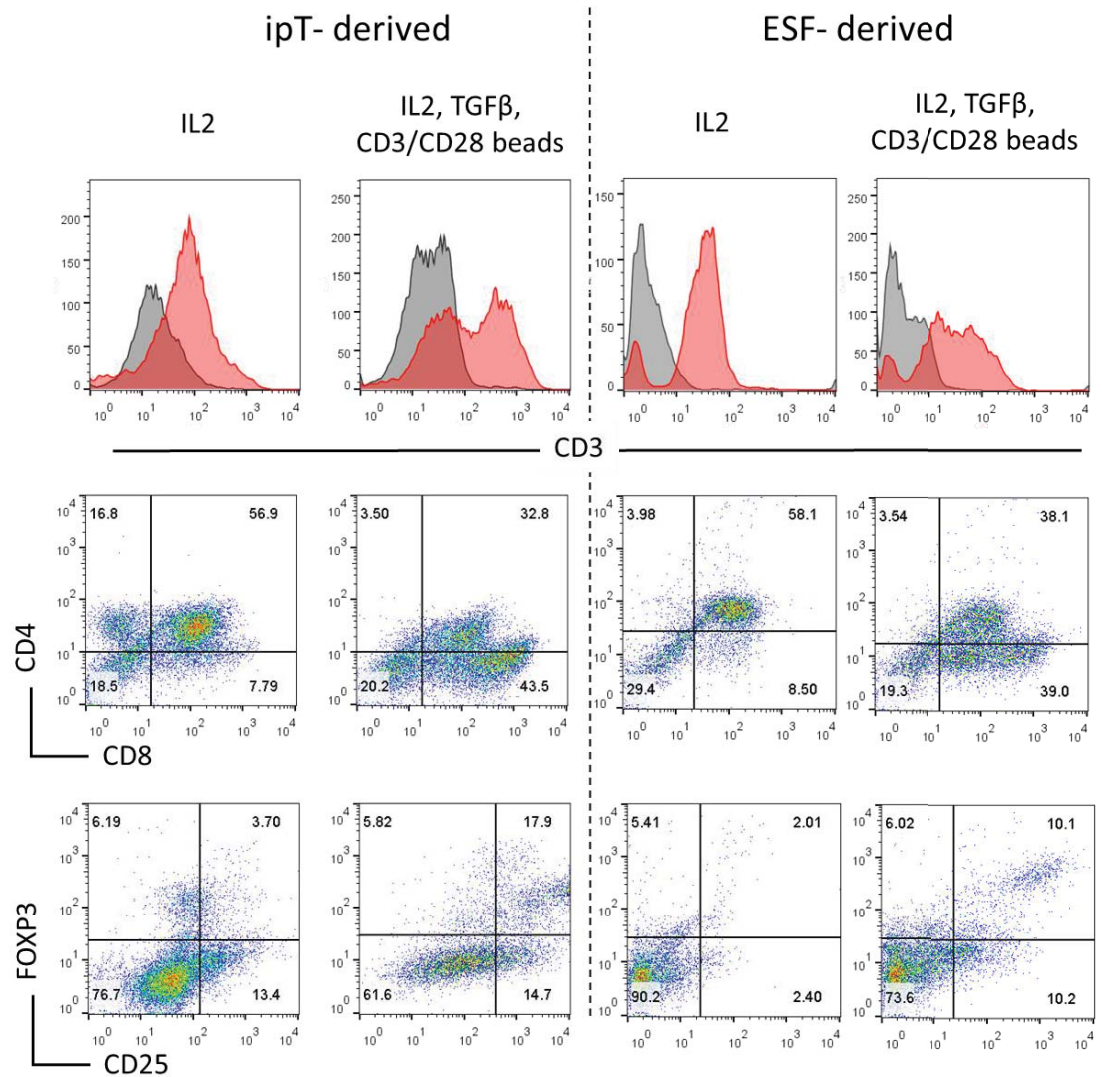


Figure 5.10 Differentiation of T_{reg} cells from mouse iPSC and ES cells. Naïve $CD4^{+} CD8^{+}$ T cells differentiated from ipT iPSCs and ESF 116 ESCs were differentiated into T_{reg} cells by stimulating cells with 1:1 CD3/CD28 beads, 100U/ml IL-2 and 10ng/ml TGF- β . After 5 days, cells were harvested and stained for CD3, CD4, CD8, CD25 and FOXP3 to detect Treg cells and analysed by flow cytometry. Flow cytometry dot plots of CD3 gated cells showing the expression of CD4 vs CD8 and CD25 vs. FOXP3. FACS plots are representative of 2 independent experiments; gates in dot plots are set based on expression of isotype control.

5.10), suggesting the successful differentiation of T_{regs} from naïve iPS and ES cell-derived T cells.

Interestingly, T cells cultured with IL-2, TGF- β and CD3/CD28 coated-beads were found to contain a population of CD8 single positive T cells alongside the CD4⁺ CD8⁺ subset, whereas the T cells cultured with IL-2 alone did not. No CD4 single positive cells were observed in either ES or iPS cell-derived T cells. This result is reminiscent of the expression of CD4 and CD8 obtained when iPSC-derived T cells were matured in FTOC. The unexpected differentiation of CD8⁺ T cell subset, from naïve CD4⁺ CD8⁺ iPSCs – derived T cells, leads us to speculate whether the CD25⁺ FoxP3⁺ cells obtained are a subset of CD8⁺ T_{regs}, which are known to be present in small numbers in the thymus and secondary lymphoid organs of mice and have been shown by a few groups to express FoxP3, although this is still controversial [404-406]

5.2.7 Isolation of antigen specific CD4⁺ cells for reprogramming

Given that the final aim of the project is to investigate immune-modulation through reprogramming antigen specific T cells to iPSCs and their re-differentiation into antigen-specific T_{regs}, we sought to reprogram CD4⁺ T cells from C57BL/6 Marilyn hCD2-FoxP3 KI RAG1^{-/-} mice into iPSCs. Marilyn RAG1^{-/-} mice contain TCR transgenic T cells which are able to recognise an epitope of male antigen, DBY₅₈₉₋₆₀₃ presented by H-2A^b. The female mice promptly reject male skin grafts, providing a convenient readout for T cell pathogenicity. Expression of the human CD2 gene under the FoxP3 promoter results in surface expression of hCD2 upon upregulation of FoxP3, providing a convenient marker for the quantification and purification of T_{regs}.

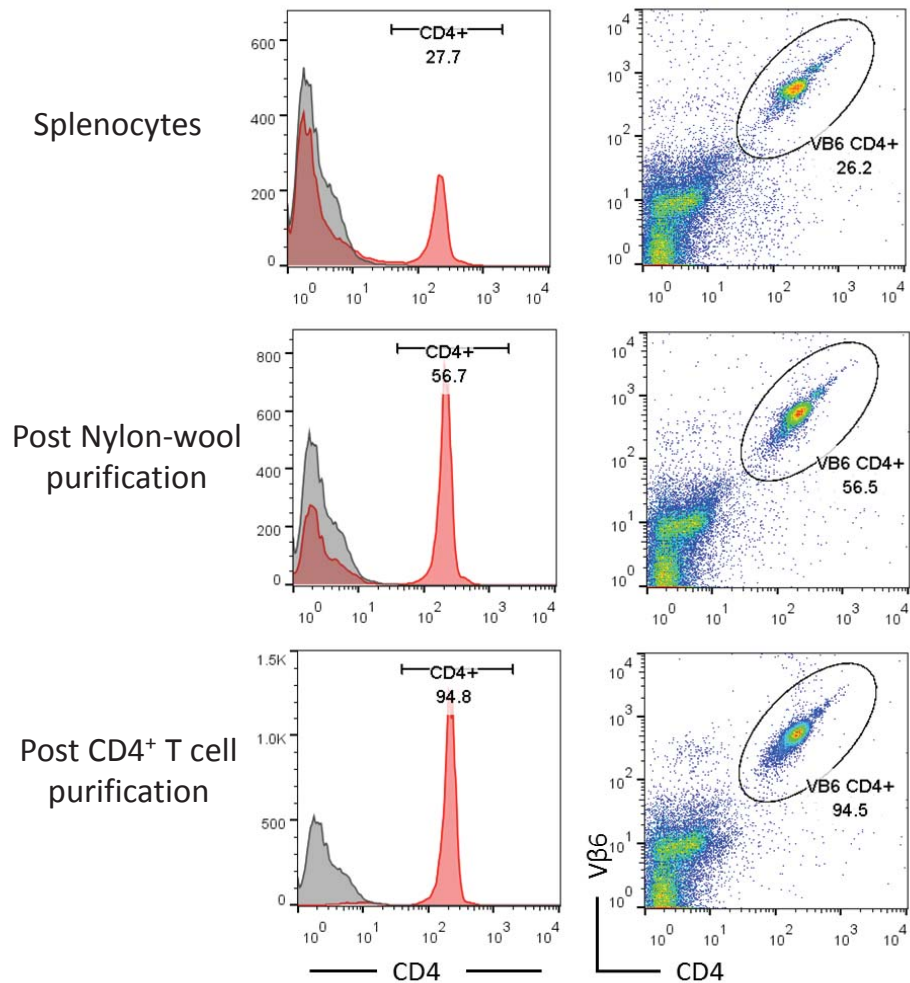


Figure 5.11 Purification of antigen specific CD4⁺ cells for reprogramming. Spleens of female C57BL/6 Marilyn hCD2-FOXP3 KI RAG^{-/-} mice were harvested and purified using the nylon wool column followed by red blood cell lysis. The T cells obtained were further enriched for CD4⁺ T cells using EasySep™ Mouse CD4⁺ T Cell Isolation Kit. The negatively selected T cells obtained were stained for CD4 and Vβ6 alongside splenocytes and nylon-wool purified T cells and analysed by flow cytometry. FACS plots show the expression of CD4 alone or with respect to Vβ6.

Spleens of female C57BL/6 Marilyn hCD2-FoxP3 KI RAG1^{-/-} mice were harvested and purified by passage over nylon wool columns followed by red blood cell lysis. The cells obtained were further enriched for CD4⁺ T cells using the EasySep™ Mouse CD4⁺ T Cell isolation kit according to manufacturer's instructions. The negatively selected T cells obtained were stained for CD4 and Vβ6 alongside splenocytes and nylon-wool purified T cells to ascertain the purity of the CD4⁺ T cells subset.

The percentage of CD4⁺ Vβ6⁺ T cells was found to increase from 26.2% to 56.5% on purification of splenocytes using nylon wool column. Further purification of T cells using EasySep™ Mouse CD4⁺ kit increased the purity of the CD4⁺ Vβ6⁺ subset to 94.5% (Figure 5.6). These cells were used for reprogramming into iPSCs using method described in section 2.3.3 and will form the focus of future efforts to reprogramme pathogenic T cells into T_{regs} with matched antigen specificity, a process we have dubbed 'phenotypic reassignment' of T cells.

5.3 Discussion

The use of regulatory T cells holds great promise for immunotherapy of many autoimmune diseases as well as tolerance induction for tissue and organ transplantation. However, current T_{reg}-based therapies are limited by the difficulty in obtaining pure T_{regs} and the failure to generate sufficient numbers of stable, antigen-specific T_{regs} for adoptive transfer. The ability to reprogram antigen specific T cells to iPSCs offers the possibility to

re-differentiate them into antigen-specific T_{regs} that can be exploited for adoptive T_{reg} transfer.

We have laid the groundwork for the above goal by investigating the derivation of T_{regs} from iPSCs without need for retroviral transduction with *FoxP3*. To this end, we used an iPSC line that has been reprogrammed from the T cells of C57BL/6 mice. The iPSC line obtained, ipT, was found to be pluripotent, displaying typical stem cell morphology and expressing markers of pluripotency, such as SSEA-1, Nanog and Oct3/4. The ipT iPSC line was also found to possess the potential to develop into teratomas containing tissues derived from all three embryonic germ layers, when implanted under the kidney capsule of immune-compromised RAG1^{-/-} mice. We were able to demonstrate the successful differentiation of CD11c⁺ MHC class II⁺ DCs from the ipT iPSCs, confirming their capacity to differentiate into cells of the haematopoietic lineage.

Many protocols for the differentiation of T cells from mouse ES cells have been described [221, 232, 240, 380, 383]. However, many of these protocols have been shown to be slight alterations from the original protocol pioneered by Schmitt *et al* in 2002, where only notch ligand-expressing, OP9-DL1 was used [83]. Notch ligand has been shown to be critical for the commitment of lymphoid progenitors to the T cell lineage and has been shown to function with other signals to regulate subsequent T cell development [394, 395]. However, subsequent reports from the same group showed more efficient mesodermal specification and subsequent haematopoietic commitment when ES cells were co-cultured with OP9 prior to seeding on OP9-DL1 stromal cells [240]. OP9 differs from OP9-DL1 cells in lacking the notch ligand, DL1 and has been shown to support the differentiation of ES cells into many haematopoietic lineages. [379, 392, 398]. The

concentration of growth factors used and the numbers of cells seeded at each stage of the differentiation has also been optimized over the years. Following personal communication with members of the Zuniga-Pflucker laboratory who pioneered the technique, we adopted the protocol described by Holmes *et al* in 2009 for the directed differentiation of the ipT line, as well as ES cell line, ESF116, into T cells [240].

ES cells have been shown to follow a similar pattern of lineage commitment and differentiation to the one observed *in vivo* for functional lymphocytes [240]. Consistent with these observations, we observed the formation of mesoderm-like colonies by day 5 of the differentiation on a monolayer of OP9 cells seeded with either ES or iPSCs, suggesting the specification of these PSCs down the mesoderm lineage. At this stage, Flt3L, which is known to play an important role in the proliferation, survival, and differentiation of haematopoietic precursors, was added to the differentiation medium [407]. By day 8, we observed the appearance of non-adherent round, refractile haematopoietic progenitor cells, which were found to express the pan-hematopoietic marker, CD45. These progenitors were co-cultured with OP9-DL1 stroma, which expresses notch ligand critical for lymphoid specification of cells, alongside IL-7 and Flt3L, which have been shown to work synergistically to promote growth of lymphoid progenitors [408]. By day 19, lymphoid cells with morphology similar to T cells were observed. These cells were found to express lymphocyte marker CD3 and CD45, suggesting the successful differentiation of T cells from ES and iPS cells. They were also found to co-express CD4 and CD8, markers of different T cell subsets, consistent with previous reports in the literature. The co-expression of CD4 and CD8 corroborates the fact that the thymus microenvironment is required for the positive selection of T cells into SP cells [232, 240, 383]. We also observed the presence of small numbers of both CD4⁺ and CD8⁺ cells in ipT

derived T cells, consistent with the profile of surface markers expression by ES cell-derived T cells reported by Schmitt *et al*, suggesting that iPSCs, like ES cells, have a capacity to give rise to T cells [232].

Although we were finally able to demonstrate the differentiation of T cells from both ES and iPSCs, our initial attempts at the differentiation proved unsuccessful. Despite the formation of mesoderm-like colonies in our initial differentiation, no appearance of non-adherent refractile cells were detected on day 8, or even on further incubation of PSCs on OP9 cells. These results were consistent with attempts by other members of our laboratory (Philip Kruger, Personal Communication). During this process we optimized many aspects of the differentiation protocol, with guidance from the pioneers of the technique (Dr. Patrick Brauer, Personal Communication). Since OP9 cells have been known to lose their ability to support robust haematopoiesis when cultured for long periods, we obtained new batches of both OP9 and OP9-DL1 cells lines [240]. Although different batches of FBS have been shown to have a profound effect of the success of the differentiation of T cells from iPSCs [240], modifying the origin and batch of the serum had no discernible effect on our differentiation protocol. Since the source of α MEM has been known to have an effect on the growth and differentiation of the co-cultures (Dr. Patrick Brauer, Personal Communication), we switched from using commercially available liquid α MEM media to reconstituting our own medium from α MEM powder. Following this subtle change in protocol, we observed an increased number of non-adherent haematopoietic progenitors in our cultures at day 8 which finally led to the successful differentiation of T cells from both ES as well as iPS cells.

One of the major hurdles of differentiation of T cells from PSCs *in vitro* is the need for thymic microenvironment to facilitate the positive selection of T cells. T cells obtained from both iPS and ES cells, consistent with reports in the literature, have been shown to be double positive for CD4 and CD8, indicating that they have yet to navigate the final stage of development. Fetal thymic organ culture (FTOC) has been used to facilitate the positive selection of ES cell-derived CD4⁺ CD8⁺ T cells [379, 409]. During positive selection, CD4⁺ CD8⁺ T cells interact with cortical thymic epithelial cells (cTEC), which express self-peptides presented on MHC class I and class II. The DP T cells that preferentially bind self-peptide associated with MHC class I with low affinity become CD8⁺ T cells, whereas those that bind self-peptide associated with MHC class II become CD4⁺ T cells according to the instructive model of T cell lineage commitment [410-412].

After maturing, the CD4⁺ CD8⁺ T cells obtained from iPSCs in FTOC, we observed the presence of CD8 single positive T cells but no CD4 single positive population, in contrast to reports in the literature, which show the successful positive selection of both CD4⁺ and CD8⁺ single positive T cell subsets when CD4⁺ CD8⁺ ES cell-derived T cells were matured using FTOC [379]. Given that the iPSC line was derived from purified T cells, we speculate that the cell of origin was CD8⁺, defining its TCR as MHC class I-restricted, strongly favouring re-differentiation along the CD8⁺ T cell lineage. These cells were found to express H-2K^b expressed by C57BL/6 but not CBA/Ca mice, confirming that these cells were indeed derived from T cells that had originated from the ipT line. The appearance of CD8⁺ T cells indicates the presence of cTEC cells involved in positive selection of DP T cells through presentation of self-peptide in the context of MHC class I. The absence of CD4⁺ T cells after maturation has also been observed in human iPSC differentiation into T cells, where the stimulation of the CD4⁺ CD8⁺ T cells with a α CD3 antibody led to the rapid

proliferation of T cells followed by maturation of these cells into both CD4⁺ and CD8⁺ T cells [219]. However, after a few days, the CD4⁺ T cells disappeared and the number of CD8⁺ T cells increased. Although, it is unclear why the CD4⁺ T cells disappeared from the culture, we speculate that a similar mechanism might be occurring with the ipT-derived T cells in FTOC. It must be noted that we don't know if the iPSC line used for this differentiation was derived from CD8⁺ T cells, which leads us to speculate whether iPSCs derived from antigen specific CD4⁺ T cells might have a propensity to re-differentiate into CD4⁺ T cells.

Given the successful establishment of the protocol of differentiation of ES and iPS cells into T cells, we differentiated the naïve CD4⁺ CD8⁺ T cells into T_{regs} following a protocol that has been described by Fantini *et al* in 2007 [413], which involves the CD3/CD28 stimulation of naïve T cells in the presence of 10ng/ml TGF-β and 100U/ml IL-2 [413]. CD3/CD28-coated beads mimic the activation of T cells by APCs by utilising anti-CD3 and anti-CD28 antibodies bound to an inert surface for the stimulation of TCR/CD3 complex as well as CD80 or CD86 co-stimulation, which has been shown to be required for activation of naïve T cells [414]. TGF-β is an anti-inflammatory cytokine, which has been shown to induce the expression of FoxP3 and the differentiation of naïve T cells to iT_{regs} [165, 415]. IL-2 is a potent T cells growth factor and has been shown to be essential of T_{reg} function consistent with their constitutive expression of CD25 [416, 417]. Although the cells obtained at the end of the T_{reg} induction expressed CD25 and FoxP3, which are known markers of T_{regs}, they did not co-express CD4. We also observed the presence of CD8 single positive T cells when samples were stimulated with CD3/CD28-coated beads, consistent with reports by Vizcardo *et al* and reminiscent of the results obtained on maturation of naïve ipT derived T cells in FTOC, leading us to speculate whether the

expression of CD4 has been downregulated in culture. Expression of CD8 and lack of expression of CD4 in these *in vitro* induced putative T_{regs} leads us to speculate whether the cells obtained were CD8⁺ T_{regs}, another suppressor T cell subset, which have been found to express CD25 and in some instances, FoxP3. [404, 406]. The expression of FoxP3 in CD8 T_{regs} is still controversial and differentiating CD8⁺ T_{regs} from conventional CD8⁺ T cells have been difficult because of the lack of a reliable marker [405, 406]. Despite the lack of consensus on expression of FoxP3 by CD8 T_{regs}, it is agreed that its expression in either CD4⁺ or CD8⁺ T cell subset confers regulatory capacity [406]. We propose to use flow cytometry to investigate whether the CD4⁻ CD25⁺ FoxP3⁺ putative T_{regs} co-express CD8. We also propose to purify the CD25⁺ FoxP3⁺ subset from these cultures and investigate the secretion of anti-inflammatory cytokines IL-10 and TGF-β associated with T_{reg} function.

The only other report of T_{reg} differentiation from stem cells involved the introduction of *Foxp3* gene into iPSCs by retroviral transduction and their subsequent differentiation into T_{regs} using adult thymic organ culture [230]. The risk of insertional mutagenesis and need for thymic organ culture, makes the application of this technique to the clinic highly unlikely. The differentiation of functional T_{regs} *in vitro* from iPSCs without the recourse to the use of retroviral transduction would have major implications for the treatment of autoimmune disease and organ transplantation. Furthermore, iPSCs have been successfully reprogrammed from antigen-specific T cells, capturing the V(D)J recombination of the TCR. These iPSCs have been shown to differentiate into T cells, maintaining the antigen specificity of the source T cell [219, 220, 234]. Given these findings, this method can be harnessed for immune modulation through reprogramming of pathogenic T cells to pluripotency and their re-directed differentiation to antigen-

specific T_{regs} thereby producing an abundant source of T_{reg} with matched specificity to the pathogenic T cells. This approach has the potential to bypass many issues hindering the clinical application of adoptive T_{reg} transfer. The reprogramming of iPSC from antigen-specific T cells captures the relevant V(D)J gene combination and TCR specificity, bypassing the need for a thymic microenvironment for positive selection. Furthermore, since iPSCs can self-renew, this technique can potentially be harnessed to differentiate unlimited T_{regs} of homogenous specificity on demand. These T_{regs} will be specific to the antigen in question and can be adoptively transferred into the patient to induce tolerance to the antigen. Furthermore, current T_{reg} adoptive transfer techniques rely on the expansion of polyclonal T_{regs} , which can potentially lead to systemic immunosuppression, increasing the risk of opportunistic infection and spontaneous malignancy [174, 178]. The generation of antigen-specific T_{regs} *in vitro* has the potential to overcome this problem, providing tolerance specific to the antigen in question without effecting the systemic balance of immunity and tolerance.

As proof of principle of the capacity of this approach to induce tolerance to a specific antigen *in vivo*, we further aim to investigate the phenotypic reassignment of $CD4^+$ T cells from female C57BL/6 Marilyn hCD2-FoxP3 KI RAG1^{-/-} mice into T_{regs} . These mice contain TCR transgenic T cells which recognise male antigen, Dby presented by H-2A^b. The female mice promptly reject male skin grafts, providing a convenient readout for T cell pathogenicity. We propose to reprogram $CD4^+$ T cells, into iPSCs and re-differentiate them into T_{regs} and reinfuse them into these mice to investigate their ability to tolerate male skin grafts. Expression of the human CD2 gene under the FoxP3 promoter results in surface expression of hCD2 upon up-regulation of FoxP3, providing a convenient marker for the quantification and purification of T_{regs} . We can also harness this marker to

subsequently eliminate T_{regs} using cytotoxic mAbs, which can help confirm that tolerance to skin graft is indeed due to the iPSC-derived T_{regs} . To this end, we have purified $CD4^{+}$ T cells from female C57BL/6 Marilyn hCD2-FoxP3 KI $RAG1^{-/-}$ mice and are currently attempting to reprogram these cells into iPSCs.

Chapter 6:

Discussion

Many strategies to restore immune homeostasis by either suppressing or stimulating the immune response have been exploited. However, many of these approaches, such as the use of monoclonal antibodies and cytokines, are non-specific, do not generate a memory response and have proven disappointing [17]. Since the interaction between DCs and T cells has been shown to be instrumental in directing the immune response towards either tolerance or immunity, many approaches harnessing immune cells for immunotherapy have been explored. DCs have been exploited in vaccination approaches for the treatment of cancer and other chronic infectious agents such as HIV-1, however, their low numbers in peripheral blood and the inability of *in vitro* derived moDCs to cross present antigen have hindered their success in immunotherapy [17, 43]. T cells have been exploited for adoptive transfer either to target tumours or for the induction of tolerance in autoimmune diseases and organ allografts. The difficulty in obtaining pure antigen-specific T cell subsets has, however, hindered their success in mediating immune responses [230].

iPSCs offer a means of harnessing pluripotency without the ethical concerns associated with the use of ES cells. iPSCs have unlimited potential to self-renew and have been shown to have the capacity to differentiate into all somatic cells types, including many cells of the haematopoietic lineage [218, 219, 221]. The ability to derive iPSCs in an autologous fashion, together with their tractability for genome editing makes them attractive targets for addressing unmet medical needs [145]. The demonstration that DCs and T cells can be differentiated from iPSCs has sparked new interest in exploring their potential in immunotherapy. Furthermore, the exploitation of iPSCs in immunotherapy is considered the 'low hanging fruit' of iPSC application since the success of immune intervention is not contingent on the prolonged survival of iPSC-derived tissues required

for conventional cell replacement therapy but on the immunological legacy they leave behind [145].

We investigated two approaches of harnessing iPSCs in immunotherapy; firstly, in differentiating DCs for therapeutic vaccination against chronic infectious agents, using HIV-1 as a model system; and secondly, in the generation of T_{regs} for the induction of tolerance.

6.1 Harnessing iPDCs for HIV-1 immunotherapy

Given the recent development of protocols in our laboratory to differentiate human iPSCs into DCs and the demonstration that these iPDCs possess the capacity to cross present antigen to CD8⁺ T cells [218], we investigated the suitability of these iPSC-derived DCs (iPDCs) as a therapeutic vaccine for HIV-1. An ideal therapeutic vaccine for HIV-1 should be able to induce cytotoxic immune responses against the virus and be able to clear all of its latent reservoirs, while producing minimum side effects. Given the absence of CD4⁺ T cells help in patients with HIV-1, the capacity of iPDCs to cross present antigen to CD8⁺ T cells may bypass the need for CD4⁺ T cell help usually required for stimulating a cytotoxic T lymphocyte response. We investigated the differentiation of DCs from human iPSC and characterised these iPDCs for phenotype and function to determine the extent to which this subset resembles the cross presenting DC subset found *in vivo*. We also investigated the capacity of these iPDCs to cross present exogenously-derived HIV-1 Gag protein to naïve CD8⁺ T cells.

We were able to demonstrate the successful differentiation of DCs from HLA-A*0201⁺ iPSCs using a slightly modified protocol to the one previously published by our laboratory. These iPDCs expressed typical myeloid DC (mDC) phenotype, expressing cell surface molecules such as CD11c, CD86 and HLA-DR, and were able to upregulate their expression of costimulatory molecules on exposure to pro-inflammatory stimuli [41, 249]. We further demonstrated that these DCs possessed the capacity to phagocytose and process exogenously supplied antigen and were able to potently stimulate the proliferation of naïve CD4⁺ T cells, which are the hallmarks of DC function. These iPDCs also possess the ability to migrate along a chemokine gradient of CCL19, which is essential for its successful homing to secondary lymphoid tissues [300].

Although these DCs were found to secrete high concentrations of the pro-inflammatory cytokine IL-6, their inability to secrete IL-12 when stimulated by cocktail of pro-inflammatory cytokines, raises the question of whether they will be effective at cross priming CD8⁺ T cells. We were, however, able to detect low level secretion of IL-12 when iPDCs were stimulated with cytokine cocktail (IL1 β , TNF- α and IFN γ) in the presence of either TLR8 agonist or with a combination of TLR3,8 and 9 agonists and CD40L. Given the role of IL-12 in driving Th1 development and activating CD8⁺ T cells, this highlights the fact that, despite their similarity to mDCs, more research needs to be done into investigating the most efficient method for maturing iPDCs for clinical use.

In addition to the low levels of IL-12, iPDCs were found also to secrete high levels of anti-inflammatory cytokine, IL-10, even on maturation, leading us to speculate whether, like many iPSC-derived cell types, iPDCs exhibit a neonatal-like phenotype, which has been demonstrated by Goriely *et al* to correlate with an impaired ability to secrete functional

IL-12 and failure to stimulate primary T cells responses [303]. Moreover, HIV-1 infected DCs have been shown to exhibit a similar cytokine profile and have been shown to stimulate impoverished T cell responses against HIV-1 [418]. Given the implications of the high levels of IL-10 secreted by DCs on their intended use as a vaccine for HIV-1 and the observation that BMDCs from IL-10^{-/-} mice are more immunogenic than their WT counterpart [343], we investigated whether knocking out IL-10 gene would enhance the immunogenicity of iPDCs. Although mouse iPDCs were shown to be a good model for this investigation, recapitulating the high levels of IL-10 and lack IL-12 secretion, knocking out *Il-10* gene was found to have only a marginal effect on the overall immunogenicity of the iPDCs with respect to MHC class II, co-stimulatory molecules and IL-12 expression. The neonatal-like phenotype of iPDCs and its impact on their ability to function as a potent vaccine for HIV-1 still remains to be addressed. These findings suggest that iPDCs are qualitatively different from BMDCs and that endogenous IL-10 expression is not responsible for maintaining the neonatal phenotype.

A subset of iPDCs was found to express CD141 and XCR1, markers of the CD141⁺ XCR1⁺ mDC subset observed *in vivo* [51-53] and were also found to express TLR3, unlike moDCs, suggesting they may be akin to the cross presenting mDC subset recently identified in humans. Although the expression of XCR1 was found to be highly variable and absent in some instances, we were able to demonstrate the migration of iPDCs to its ligand, XCL1, confirming the functional expression of the chemokine receptor. Having demonstrated that iPDCs display characteristics of the cross presenting subset, we further investigated their ability to take up, process and present exogenously supplied cancer-testis antigen, NYESO to Jurkat cells transgenically expressing the 1G4 TCR specific for NYESO₁₅₇₋₁₆₅ peptide presented in the context of HLA*A0201. Although promising results were

obtained in one instance, we were unable to draw any conclusions from these experiments due to the difficulty in obtaining a reliable readout for activation of 1G4 jurkat cell line, a problem identified by other laboratories making use of the line. We are currently exploring the use of 1G4 jurkat cell line that has been genetically modified to express luciferase upon recognition of the HLA*A2-restricted NYESO epitope which would enable us to confirm the ability of iPDCs to process exogenous antigen and present it via HLA-class I.

Given the vagaries of the Jurkat system, we further investigated the ability of iPDCs to process and cross present exogenously derived HIV-1 Gag protein on HLA class I to donor derived HLA-A*0201⁺ T cells. Although we were able to detect CD8⁺ T cells specific to HIV-1 peptide in one instance, suggesting the ability of iPDCs to cross present HIV-1 derived antigen to CD8⁺ T cells, we were not able to confirm this finding. The difficulty in obtaining HLA typed blood and inability to obtain enough cells to deplete the memory T cell subset has hindered our ability to fully investigate this assay. Previous work in our laboratory has shown that iPDCs possess the capability to cross present exogenously supplied tumour antigen, Melan A to CD8⁺ T cells, eliciting a Melan A-specific cytotoxic response [218], which augurs well for the ability of iPDCs to cross present HIV-1 antigen. We are currently working on optimizing the assay to detect cross presentation of HIV-1 antigen by iPDCs. Furthermore, to recapitulate the aspects of HIV-1 pathogenicity, we propose to purify CD8⁺ T cells from HLA-A*0201⁺ donor for cross presentation experiments to investigate the ability of iPDCs to elicit a CTL response in the absence of CD4⁺ T cells help thereby mimicking, more closely, the likely scenario encountered in chronically-infected HIV-1⁺ patients.

A challenge in the potential clinical application of iPDCs in immunotherapy is the variable differentiation efficiencies across independent experiments, which has also been observed in iPSC-derived neurons and cardiomyocytes [260, 419]. We speculate that this may be due to conventional embryoid body (EBs) formation methods, which involve scraping adherent iPSCs colonies to generate clumps of cells, leading to development of EBs of heterogeneous size and resulting in high numbers of dead cells. The protocol could perhaps be improved by the use of AggreWell™ plates (STEMCELL technologies, Canada), which contain many 400µm microwells to allow the aggregation of single cells to form EBs of uniform size and shape. The percentage of CD11c⁺ iPDCs obtained from independent experiment was also found to be highly variable. It will be necessary to purify these subsets prior to use in the clinic, to increase the efficiency of the cells *in vivo* and to reduce the risk of teratoma formation. We have demonstrated the enrichment of the CD11c⁺ CD141⁺ cross presenting subset using positive selection by CD11c and CD1c magnetic beads. However, this purification only resulted in a 75% pure population of cross presenting iPDCs, which is too low for clinical applications. Although this protocol needs to be optimized to obtain purer population of iPDCs for vaccination purposes, it must be noted that while successive magnetic enrichments may increase the purity of the cells, it will, inevitably, decrease the final yield of iPDCs. Given our observation that knocking out *Il-10* in mouse iPSCs results in significant increase in the numbers of iPDCs generated, this strategy may be employed to increase the efficiency and yield of iPDC differentiation in the clinic.

Another potential challenge to the clinical application of iPDCs in HIV-1 immunotherapy is the susceptibility of iPDCs to virus infection, which suggests that iPDCs administered for immunotherapy could be infected by HIV-1 *in vivo*, act as latent reservoirs of the virus

and trans-infect CD4⁺ T cells, re-establishing the disease. It has, however, been shown that the normal life span of DCs following antigen capture and maturation is restricted to a few days [145], during which it is anticipated they would have interacted with the T cell repertoire and elicited HIV-1-specific CTL response. Their short life-span may, therefore, reduce the risk of their posing as sanctuary reservoirs of the virus. Furthermore, since iPSCs are amenable to genetic manipulation, it might be feasible to genetically modify them to express the CCR5Δ32 mutation, which has been shown to render cells refractory to HIV-1 infection [257].

The ability to differentiate potentially unlimited numbers of functional DCs in an autologous fashion shows great promise for DC-based immunotherapy. Although we have shown that iPDCs express markers of cross presenting DCs, technical issues have impeded the demonstration of their capacity to cross present HIV-1 antigen to CD8⁺ T cells. The previously-reported ability of iPDCs to cross present tumour antigens to elicit an antigen-specific CTL response bodes well for their application in HIV-1 immunotherapy; however, more research needs to be done to prove their expediency as a vaccine for HIV-1. To this end, we have begun a collaboration with Dr. John Frater (Nuffield Department of Medicine, Oxford), to reprogram iPSCs from dermal fibroblasts obtained from HIV-1 patients. These iPSCs will be differentiated into iPDCs, which can be harnessed for the cross presentation of autologous HIV-1-derived antigen to CD8⁺ T cells obtained from the same donors, recapitulating the immune response to autologous iPSCs-derived immunotherapy. If successful, we further propose to make use of human immune system (HIS) mouse model to investigate the efficacy of therapeutic iPDC vaccination in the clearance of viral reservoirs *in vivo*. HIS mice are generated by reconstituting the immune-deficient RAG2^{-/-} IL-2Rγ^{-/-} mice with human fetal bone marrow,

liver and thymus cells, permitting the generation of positively selected, mature T cells with human MHC determinants [420]. HIV-1 infection of these mice recapitulates the HIV-1 pathogenesis in human, making them an ideal model to test the efficacy of iPDCs as a vaccine for HIV-1.

Conclusions

Thirty years after its first identification, an effective prophylactic vaccine for HIV-1 still eludes us due to the hyper-variability of the virus and the persistence of its latent reservoirs. Although a few vaccines have entered phase III trials, no vaccine has been able to prevent HIV-1 infection. The failures in these approaches have prompted the development of therapeutic vaccines aimed at eradicating the virus and the clearance of its latent reservoirs. The recent report of the eradication of HIV-1 in the 'Berlin patient' following HSC transplantation from a CCR5 Δ 32 homozygous individual has demonstrated that, in principle, a cure for HIV-1 is possible [421]. However, the translation of this approach, which requires HSC transplantation, may not be practical on a large scale given the need for many appropriate CCR5 Δ 32⁺ donors [422]. Since T cell immunity is a vital element of protective immune responses in HIV-1, immunotherapy approaches to enhance or inhibit immune response as well as therapeutic vaccines have recently been explored [422]. Given the promise of DC-based therapeutic vaccination and the limitations faced by the use of moDCs, harnessing autologous iPSC-derived DCs for the treatment of HIV-1 shows immense potential. If successful, a therapeutic vaccine for HIV-1 should clear all latent reservoirs of the virus and will overcome the need for lifelong treatment with ART and its associated side effects and economic implications.

Furthermore, clearance of latent reservoirs of the virus will eliminate the risk of transmission of the disease and help to move towards eradication of HIV-1.

6.2 Phenotypic reassignment of T cells

The adoptive transfer of T_{regs} has recently gained traction as a promising immunotherapeutic strategy to treat conditions that require the induction of tolerance to improve outcomes, such as autoimmune disease, solid organ transplantation, and HSC transplantation. Current approaches for the adoptive transfer of T_{regs} are limited by the difficulty in isolating pure T_{regs} and the failure to generate sufficient numbers of stable, antigen-specific T_{regs} for adoptive transfer. We exploited the capacity of iPSCs for indefinite self-renewal and their potential to differentiate into all somatic cell types to generate antigen-specific T_{regs} for immunotherapy. The final aim of the project is to investigate immune-modulation through the reprogramming pathogenic T cells to iPSCs and their re-differentiation into T_{regs} with matched antigen-specificity, a process we have dubbed 'phenotypic reassignment'. To lay the groundwork for the above aim, we investigated the feasibility of differentiating mouse T cell-derived iPSCs into T_{regs} .

We demonstrated the successful differentiation of mouse T cell-derived iPSCs into T cells, following a protocol that has been well established for use in ES cells. These T cells were found to be double positive for CD4 and CD8, and their exposure to a thymic microenvironment demonstrated their capacity for maturation. We also demonstrated the subsequent differentiation of these T cells into $CD25^+ FoxP3^+$ putative T_{regs} with the help of CD3/CD28 activation in the presence of TGF- β and IL-2, which is the first report of

differentiation of FoxP3⁺ cells from pluripotent stem cells without the need for retroviral transduction with *Foxp3* gene [230].

Despite the successful re-differentiation of T cells from T cell-derived iPSCs, the majority of CD3⁺ T cells were found to express both CD4 and CD8 and on maturation, using fetal thymus organ culture, developed into CD8 single positive T cells alone. This leads us to speculate whether these iPSCs were initially reprogrammed from CD8⁺ T cells, which might explain their propensity to re-differentiate along the CD8⁺ T cell lineage. We are currently exploring the functional capacity of these T cells by investigating their ability to proliferate when stimulated by allogeneic DCs and their ability to reconstitute the immune system of immune-deficient mice.

Furthermore, despite the successful differentiation of CD25⁺ FoxP3⁺ putative T_{regs} from iPSCs without the need for retroviral transduction of *Foxp3* gene, these putative T_{regs} did not express CD4, which leads us to speculate whether they are CD8⁺ T_{regs}, reported by some investigators to express FoxP3 and have immuno-suppressive properties [405], although their expression of FoxP3 is still controversial. We are currently investigating whether these putative T_{regs} co-express CD8 alongside CD25 and FoxP3 and whether they possess any other characteristics of CD8⁺ T_{regs}. To confirm their immunosuppressive capacity, we are also investigating the ability of these CD25⁺ FoxP3⁺ putative T_{regs} to secrete immunosuppressive cytokines IL-10, TGF-β and to suppress the proliferation and IFNγ production of effector CD4⁺ T cells in a dose-dependent manner.

We are currently in the process of reprogramming CD4⁺ T cells derived from female C57BL/6 Marilyn hCD2-FoxP3 KI RAG1^{-/-} mice into iPSCs. We speculate that the reprogramming of iPSCs from antigen-specific T cells would capture the relevant V(D)J

gene combination and TCR specificity, bypassing the need for positive selection of T cells differentiated from these iPSCs. Furthermore, we propose to investigate whether their derivation from a CD4⁺ T cells would enhance their propensity to re-differentiate into CD4⁺ T cells rather than CD8⁺ T cells.

The C57BL/6 Marilyn hCD2-FoxP3 KI RAG1^{-/-} mice express a transgenic TCR specific for the 589-603 epitope of the male antigen, DBY, allowing female mice to promptly reject male skin grafts, providing a convenient readout for T cell pathogenicity. We propose to investigate the re-differentiation of T_{regs} from the CD4⁺ T cell derived-iPSCs and investigate whether their reinfusion back into the mice will result in tolerance to male skin grafts. Furthermore, these mice express the human CD2 gene under the FoxP3 promoter, providing a convenient marker for monitoring the success of the differentiation protocol and for quantification and purification of T_{regs}. The expression of cell surface human CD2 also allows for the ablation of these iPSC-derived T_{regs} (ipT_{regs}) *in vivo* using cytotoxic anti-CD2 mAbs, which should break the tolerance to male skin grafts, confirming the role played by the ipT_{regs} in conferring tolerance. Given their derivation from iPSCs, this technique can potentially be harnessed to differentiate unlimited homogenous antigen-specific T_{regs}, bypassing the issue of obtaining large numbers of pure T_{regs} currently hindering the clinical application of their adoptive transfer. Furthermore, the potential of obtaining antigen specific T_{regs} by 'phenotypic reassignment' of effector CD4⁺ T cells may bypass the problem of systemic immunosuppression faced by conventional adoptive transfer techniques [14, 75].

Challenges and future direction

Despite its potential in immunotherapy, there are a few challenges that need to be resolved for the successful application of this approach in the future. The use of OP9-DL1, a mouse stromal cell line to provide notch ligand signalling for T cell commitment is not compliant with translation into the clinic. Plate-bound notch ligand [423-425] (and notch ligand-coated microbeads [426] have been successfully used for the differentiation of T cells from HSCs and might be investigated for the differentiation of T cells from iPSCs. Additionally, the efficiency of T cell differentiation from iPSCs has been universally found to be highly variable between independent experiments and needs to be further optimized to allow a more consistent yield for translation into the clinic.

If successful, the 'phenotypic reassignment' of T cells has the potential to treat many autoimmune diseases and to provide tolerance to organ and HSC transplants. Furthermore, it can also be harnessed to provide tolerance for recombinant enzymes and proteins being introduced into the body to treat the lysosomal storage diseases and other single gene disorders in which the delivery of a single protein would have therapeutic benefit if it were not for the induction of neutralising immune response.

6.3 Potential and challenges in the clinical translation of iPSC-based immunotherapy

Although the use of iPSC-derived immune cells for immunotherapy is still in its infancy, the successful differentiation of T cells and functional DCs from iPSCs suggests that harnessing the power of induced pluripotency for the treatment for immune-related

diseases may soon become a reality. The properties of iPSCs offer an attractive strategy to address the challenges currently hindering the application of cell-based immunotherapeutic approaches. The ability to generate autologous iPSCs provides opportunities for the development of patient-specific immune cells, which bypasses the issues of rejection and the need for immunosuppression. Furthermore, the unlimited capacity of iPSCs to self-renew greatly facilitates the scale-up of cell production. Additionally, since the success of immune mediation is not contingent on the prolonged survival of iPSC-derived tissues but on the immunological effect caused by immune cells, immunotherapy may be considered the 'low-hanging fruit' of the iPSC field.

Despite the potential of harnessing iPSCs for immunotherapy, many challenges still need to be addressed for the successful translation of this approach to the clinic, one of which is the neonatal-like phenotype of iPSC-derived immune cells. Strategies that allow the maturation of iPSC-derived tissues *in vitro* need to be developed to allow for the clinical translation of iPSC-based immunotherapeutic approaches. Additionally, the reprogramming of iPSCs and their validation is still an inefficient and cumbersome process. Advancements in non-integrating reprogramming techniques, such as the use of small molecules and microRNA, need to be further explored to allow for efficient generation of autologous iPSCs from patients. Furthermore, the cost of reprogramming iPSCs from each patient and the time involved in the generation and validation of a new line may be prohibitive to the use of iPSCs for personalised immunotherapy. It has been suggested that until the reprogramming process has been streamlined to reduce time and cost, iPSC banks can be used as a pragmatic source of off-the shelf HLA-matched iPSCs lines [427].

Current methods of iPSC differentiation are still relatively time consuming and cumbersome. In our laboratory, for example, it takes 5-6 weeks for the successful differentiation of DCs from human iPSCs and 5 weeks for the differentiation of T_{regs} from mouse iPSCs. Furthermore, differentiation of immune cells, like many other iPSC progeny, have been shown to be relatively inefficient and expensive, requiring the use of many recombinant human growth factors and cytokines. Given that a small molecule synthetic replacement of VEGF has already been identified [428], the cost and efficiency of differentiation can be streamlined by investigating a high throughput screening platform for the identification of small molecule replacements for growth factors and cytokines required for the differentiation of DCs and T_{regs} from iPSCs. Other issues impeding the use of iPSCs in the clinic include the need to purify iPSC-derived cells to minimize contamination of undifferentiated iPSCs in inocula for re-infusion to the patients. Undifferentiated iPSC have a propensity to give rise to teratomas *in vivo* and pose a substantial risk to the patient. Recently, suicide gene therapy has been explored to address the safety concerns for clinical translation of pluripotent cells by the introduction of inducible caspase-9 (iC9) into iPSCs. Activation of iC9 with a chemical inducer of dimerization (CID) has been shown to initiate a caspase cascade that eliminates iPSCs and tumours originating from iPSCs, suggesting that suicide gene therapy may be a promising tool for future iPSC-mediated therapy [429].

Although challenges undoubtedly still exist, recent advances in the differentiation of immune cells from iPSCs and the drive to streamline the cost, safety and efficiency of generating iPSCs, may herald a new era in immunotherapy in which treatments are tailored to the individual needs of the patient.

References

1. Parkin, J. and B. Cohen, *An overview of the immune system*. Lancet, 2001. **357**(9270): p. 1777-89.
2. asociation, A.a.r.d. *Autoimmune Statistics*. 2015; Available from: <https://www.aarda.org/autoimmune-information/autoimmune-statistics>.
3. committee, N.-A.d.c., *Autoimmune Diseases Research Plan* 2015.
4. Mandalia, S., et al., *Rising population cost for treating people living with HIV in the UK, 1997-2013*. PLoS One, 2010. **5**(12): p. e15677.
5. Feldmann, M. and L. Steinman, *Design of effective immunotherapy for human autoimmunity*. Nature, 2005. **435**(7042): p. 612-9.
6. Ruiz, P., et al., *Transplant tolerance: new insights and strategies for long-term allograft acceptance*. Clin Dev Immunol, 2013. **2013**: p. 210506.
7. Fairchild, P.J., *Transplantation tolerance in an age of induced pluripotency*. Curr Opin Organ Transplant, 2009. **14**(4): p. 321-5.
8. Hale, G., et al., *Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement*. Blood, 1983. **62**(4): p. 873-82.
9. Xia, M.Q., et al., *Characterization of the CAMPATH-1 (CDw52) antigen: biochemical analysis and cDNA cloning reveal an unusually small peptide backbone*. Eur J Immunol, 1991. **21**(7): p. 1677-84.
10. Mathieson, P.W., et al., *Monoclonal-antibody therapy in systemic vasculitis*. N Engl J Med, 1990. **323**(4): p. 250-4.
11. Coles, A.J., *Alemtuzumab therapy for multiple sclerosis*. Neurotherapeutics, 2013. **10**(1): p. 29-33.
12. Hale, G., et al., *CAMPATH-1 antibodies in stem-cell transplantation*. Cytotherapy, 2001. **3**(3): p. 145-64.
13. Kottaridis, P.D., et al., *In vivo CAMPATH-1H prevents GvHD following nonmyeloablative stem-cell transplantation*. Cytotherapy, 2001. **3**(3): p. 197-201.
14. Brennan, F.M., et al., *Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis*. Lancet, 1989. **2**(8657): p. 244-7.
15. Gonzalez-Vacarezza, N., et al., *Rituximab and tocilizumab for the treatment of rheumatoid arthritis*. Int J Technol Assess Health Care, 2014. **30**(3): p. 282-8.
16. Ramos-Casals, M., et al., *Rituximab in systemic lupus erythematosus: A systematic review of off-label use in 188 cases*. Lupus, 2009. **18**(9): p. 767-76.
17. Sachamitr, P., S. Hackett, and P.J. Fairchild, *Induced pluripotent stem cells: challenges and opportunities for cancer immunotherapy*. Front Immunol, 2014. **5**: p. 176.
18. Kohler, G. and C. Milstein, *Continuous cultures of fused cells secreting antibody of predefined specificity*. 1975. J Immunol, 2005. **174**(5): p. 2453-5.
19. Rasul, K.I. and D.J. Kerr, *Targeted therapies: Cetuximab plus chemotherapy in patients with advanced NSCLC*. Nat Rev Clin Oncol, 2009. **6**(9): p. 499-500.
20. Adams, G.P. and L.M. Weiner, *Monoclonal antibody therapy of cancer*. Nat Biotechnol, 2005. **23**(9): p. 1147-57.
21. Winter, M.C. and B.W. Hancock, *Ten years of rituximab in NHL*. Expert Opin Drug Saf, 2009. **8**(2): p. 223-35.
22. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy*. Nat Rev Cancer, 2012. **12**(4): p. 252-64.
23. Sharma, P., et al., *Novel cancer immunotherapy agents with survival benefit: recent successes and next steps*. Nat Rev Cancer, 2011. **11**(11): p. 805-12.

24. Sandner, S.E., et al., *Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo*. J Immunol, 2005. **174**(6): p. 3408-15.
25. Suss, G. and K. Shortman, *A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis*. J Exp Med, 1996. **183**(4): p. 1789-96.
26. Akdis, C.A. and K. Blaser, *IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy*. FASEB J, 1999. **13**(6): p. 603-9.
27. Annunziato, F., et al., *Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes*. J Exp Med, 2002. **196**(3): p. 379-87.
28. Weber, J.S., et al., *Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma*. J Clin Oncol, 2013. **31**(34): p. 4311-8.
29. Mahoney, K.M., G.J. Freeman, and D.F. McDermott, *The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma*. Clin Ther, 2015. **37**(4): p. 764-82.
30. Murphy, K.P., et al., *Janeway's immunobiology*. 7th ed. 2008, New York ; London: Garland Science. xxi, 887 p.
31. Lee, S. and K. Margolin, *Cytokines in cancer immunotherapy*. Cancers (Basel), 2011. **3**(4): p. 3856-93.
32. Dutcher, J., *Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic melanoma*. Oncology (Williston Park), 2002. **16**(11 Suppl 13): p. 4-10.
33. Hyrich, K.L., et al., *Anti-tumour necrosis factor alpha therapy in rheumatoid arthritis: an update on safety*. Ann Rheum Dis, 2004. **63**(12): p. 1538-43.
34. Coles, A.J., et al., *Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial*. Lancet, 2012. **380**(9856): p. 1829-39.
35. Tsourdi, E., et al., *Graves' disease after treatment with alemtuzumab for multiple sclerosis*. Hormones (Athens), 2015. **14**(1): p. 148-53.
36. Riley, J.L., C.H. June, and B.R. Blazar, *Human T regulatory cell therapy: take a billion or so and call me in the morning*. Immunity, 2009. **30**(5): p. 656-65.
37. Zarour, H.M. and S. Ferrone, *Cancer immunotherapy: Progress and challenges in the clinical setting*. Eur J Immunol, 2011. **41**(6): p. 1510-5.
38. Kawai, T., et al., *Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand*. Nat Med, 2000. **6**(2): p. 114.
39. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity*. Nature, 1998. **392**(6673): p. 245-52.
40. Steinman, R.M. and J. Banchereau, *Taking dendritic cells into medicine*. Nature, 2007. **449**(7161): p. 419-26.
41. Collin, M., N. McGovern, and M. Haniffa, *Human dendritic cell subsets*. Immunology, 2013. **140**(1): p. 22-30.
42. Idoyaga, J. and R.M. Steinman, *SnapShot: Dendritic Cells*. Cell, 2011. **146**(4): p. 660-660 e2.
43. Sachamitr, P. and P.J. Fairchild, *Cross presentation of antigen by dendritic cells: mechanisms and implications for immunotherapy*. Expert Rev Clin Immunol, 2012. **8**(6): p. 547-55.
44. Abu-Sanad, A., et al., *Simultaneous inhibition of ATR and PARP sensitizes colon cancer cell lines to irinotecan*. Front Pharmacol, 2015. **6**: p. 147.
45. Robinson, S.P., et al., *Human peripheral blood contains two distinct lineages of dendritic cells*. Eur J Immunol, 1999. **29**(9): p. 2769-78.
46. Ziegler-Heitbrock, L., et al., *Nomenclature of monocytes and dendritic cells in blood*. Blood, 2010. **116**(16): p. e74-80.
47. Dzionek, A., et al., *BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood*. J Immunol, 2000. **165**(11): p. 6037-46.

48. Cella, M., et al., *Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon*. Nat Med, 1999. **5**(8): p. 919-23.
49. Wu, L. and Y.J. Liu, *Development of dendritic-cell lineages*. Immunity, 2007. **26**(6): p. 741-50.
50. Hemont, C., et al., *Human blood mDC subsets exhibit distinct TLR repertoire and responsiveness*. J Leukoc Biol, 2013. **93**(4): p. 599-609.
51. Poulin, L.F., et al., *Characterization of human DNCR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells*. J Exp Med, 2010. **207**(6): p. 1261-71.
52. Jongbloed, S.L., et al., *Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens*. J Exp Med, 2010. **207**(6): p. 1247-60.
53. Bachem, A., et al., *Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells*. J Exp Med, 2010. **207**(6): p. 1273-81.
54. Crozat, K., et al., *The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells*. J Exp Med, 2010. **207**(6): p. 1283-92.
55. Sancho, D., et al., *Identification of a dendritic cell receptor that couples sensing of necrosis to immunity*. Nature, 2009. **458**(7240): p. 899-903.
56. Dorner, B.G., et al., *Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells*. Immunity, 2009. **31**(5): p. 823-33.
57. Huysamen, C., et al., *CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes*. J Biol Chem, 2008. **283**(24): p. 16693-701.
58. Caminschi, I., et al., *The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement*. Blood, 2008. **112**(8): p. 3264-73.
59. Merad, M., et al., *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting*. Annu Rev Immunol, 2013. **31**: p. 563-604.
60. Facchetti, F., et al., *The plasmacytoid monocyte/interferon producing cells*. Virchows Arch, 2003. **443**(6): p. 703-17.
61. Reizis, B., et al., *Plasmacytoid dendritic cells: recent progress and open questions*. Annu Rev Immunol, 2011. **29**: p. 163-83.
62. Colonna, M., G. Trinchieri, and Y.J. Liu, *Plasmacytoid dendritic cells in immunity*. Nat Immunol, 2004. **5**(12): p. 1219-26.
63. O'Doherty, U., et al., *Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature*. Immunology, 1994. **82**(3): p. 487-93.
64. Merad, M., F. Ginhoux, and M. Collin, *Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells*. Nat Rev Immunol, 2008. **8**(12): p. 935-47.
65. Birbeck, M.S., A.S. Breathnach, and J.D. Overall, *An Electron Microscope Study of Basal Melanocytes and High-Level Clear Cells (Langerhans Cells) in Vitiligo*. Journal of Investigative Dermatology, 1961. **37**(1): p. 51-63.
66. Ginhoux, F., et al., *Langerhans cells arise from monocytes in vivo*. Nature Immunology, 2006. **7**(3): p. 265-273.
67. Klechevsky, E., et al., *Functional specializations of human epidermal langerhans cells and CD14(+) dermal dendritic cells*. Immunity, 2008. **29**(3): p. 497-510.
68. Angel, C.E., et al., *Cutting edge: CD1a+ antigen-presenting cells in human dermis respond rapidly to CCR7 ligands*. J Immunol, 2006. **176**(10): p. 5730-4.

69. Ochoa, M.T., et al., *"Dermal dendritic cells" comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages.* J Invest Dermatol, 2008. **128**(9): p. 2225-31.
70. de Gruijl, T.D., et al., *A postmigrational switch among skin-derived dendritic cells to a macrophage-like phenotype is predetermined by the intracutaneous cytokine balance.* J Immunol, 2006. **176**(12): p. 7232-42.
71. Penel-Sotirakis, K., et al., *Differential capacity of human skin dendritic cells to polarize CD4+ T cells into IL-17, IL-21 and IL-22 producing cells.* PLoS One, 2012. **7**(11): p. e45680.
72. Haniffa, M., et al., *Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells.* Immunity, 2012. **37**(1): p. 60-73.
73. Larregina, A.T., et al., *Pattern of cytokine receptors expressed by human dendritic cells migrated from dermal explants.* Immunology, 1997. **91**(2): p. 303-13.
74. Matthews, K., et al., *Potent induction of antibody-secreting B cells by human dermal-derived CD14+ dendritic cells triggered by dual TLR ligation.* J Immunol, 2012. **189**(12): p. 5729-44.
75. Nestle, F.O., et al., *Skin immune sentinels in health and disease.* Nat Rev Immunol, 2009. **9**(10): p. 679-91.
76. Romani, N., et al., *Proliferating dendritic cell progenitors in human blood.* J Exp Med, 1994. **180**(1): p. 83-93.
77. Ebner, S., et al., *Production of IL-12 by human monocyte-derived dendritic cells is optimal when the stimulus is given at the onset of maturation, and is further enhanced by IL-4.* J Immunol, 2001. **166**(1): p. 633-41.
78. Cools, N., et al., *Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells.* J Leukoc Biol, 2007. **82**(6): p. 1365-74.
79. Greenfield, E.A., K.A. Nguyen, and V.K. Kuchroo, *CD28/B7 costimulation: a review.* Crit Rev Immunol, 1998. **18**(5): p. 389-418.
80. Cunningham, A.J. and K.J. Lafferty, *A simple conservative explanation of the H-2 restriction of interactions between lymphocytes.* Scand J Immunol, 1977. **6**(1-2): p. 1-6.
81. Greenwald, R.J., et al., *CTLA-4 regulates induction of anergy in vivo.* Immunity, 2001. **14**(2): p. 145-55.
82. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-40.
83. Schmitt, T.M. and J.C. Zuniga-Pflucker, *Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro.* Immunity, 2002. **17**(6): p. 749-56.
84. Hernandez, J., et al., *Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens.* J Exp Med, 2002. **196**(3): p. 323-33.
85. Curtsinger, J.M., et al., *Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells.* J Immunol, 1999. **162**(6): p. 3256-62.
86. Heath, W.R. and F.R. Carbone, *Cross-presentation, dendritic cells, tolerance and immunity.* Annu Rev Immunol, 2001. **19**: p. 47-64.
87. Van Kaer, L., *Major histocompatibility complex class I-restricted antigen processing and presentation.* Tissue Antigens, 2002. **60**(1): p. 1-9.
88. Merckenschlager, M., et al., *Intrathymic deletion of MHC class I-restricted cytotoxic T cell precursors by constitutive cross-presentation of exogenous antigen.* Eur J Immunol, 1999. **29**(5): p. 1477-86.
89. Kurts, C., et al., *CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity.* J Exp Med, 1997. **186**(12): p. 2057-62.
90. Blum, J.S., P.A. Wearsch, and P. Cresswell, *Pathways of antigen processing.* Annu Rev Immunol, 2013. **31**: p. 443-73.

91. Segura, E. and J.A. Villadangos, *A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells*. Traffic, 2011. **12**(12): p. 1677-85.
92. Palmowski, M.J., et al., *Role of immunoproteasomes in cross-presentation*. J Immunol, 2006. **177**(2): p. 983-90.
93. Kovacsics-Bankowski, M. and K.L. Rock, *A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules*. Science, 1995. **267**(5195): p. 243-6.
94. den Haan, J.M., S.M. Lehar, and M.J. Bevan, *CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo*. J Exp Med, 2000. **192**(12): p. 1685-96.
95. Iyoda, T., et al., *The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo*. J Exp Med, 2002. **195**(10): p. 1289-302.
96. Schulz, O. and C. Reis e Sousa, *Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells*. Immunology, 2002. **107**(2): p. 183-9.
97. Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines*. Eur J Immunol, 2003. **33**(4): p. 827-33.
98. Reis e Sousa, C., et al., *In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas*. J Exp Med, 1997. **186**(11): p. 1819-29.
99. Heath, W.R. and F.R. Carbone, *Cross-presentation in viral immunity and self-tolerance*. Nat Rev Immunol, 2001. **1**(2): p. 126-34.
100. Yamazaki, S., et al., *Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells*. J Exp Med, 2003. **198**(2): p. 235-47.
101. Paul, W.E. and R.A. Seder, *Lymphocyte responses and cytokines*. Cell, 1994. **76**(2): p. 241-51.
102. Frauwirth, K.A. and C.B. Thompson, *Activation and inhibition of lymphocytes by costimulation*. J Clin Invest, 2002. **109**(3): p. 295-9.
103. Broeren, C.P., et al., *Costimulation light: activation of CD4+ T cells with CD80 or CD86 rather than anti-CD28 leads to a Th2 cytokine profile*. J Immunol, 2000. **165**(12): p. 6908-14.
104. Harris, N.L. and F. Ronchese, *The role of B7 costimulation in T-cell immunity*. Immunol Cell Biol, 1999. **77**(4): p. 304-11.
105. June, C.H., et al., *Role of the CD28 receptor in T-cell activation*. Immunol Today, 1990. **11**(6): p. 211-6.
106. de Boer, M., et al., *Ligation of B7 with CD28/CTLA-4 on T cells results in CD40 ligand expression, interleukin-4 secretion and efficient help for antibody production by B cells*. Eur J Immunol, 1993. **23**(12): p. 3120-5.
107. McAdam, A.J., A.N. Schweitzer, and A.H. Sharpe, *The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells*. Immunol Rev, 1998. **165**: p. 231-47.
108. Hutloff, A., et al., *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28*. Nature, 1999. **397**(6716): p. 263-6.
109. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation*. J Exp Med, 1995. **182**(2): p. 459-65.
110. Blair, P.J., et al., *CTLA-4 ligation delivers a unique signal to resting human CD4 T cells that inhibits interleukin-2 secretion but allows Bcl-X(L) induction*. J Immunol, 1998. **160**(1): p. 12-5.
111. Linsley, P.S., et al., *CTLA-4 is a second receptor for the B cell activation antigen B7*. J Exp Med, 1991. **174**(3): p. 561-9.

112. Waterhouse, P., et al., *CTLA-4, a negative regulator of T-lymphocyte activation*. Immunol Rev, 1996. **153**: p. 183-207.
113. Jin, H.T., R. Ahmed, and T. Okazaki, *Role of PD-1 in regulating T-cell immunity*. Curr Top Microbiol Immunol, 2011. **350**: p. 17-37.
114. Manavalan, J.S., et al., *High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells*. Transpl Immunol, 2003. **11**(3-4): p. 245-58.
115. Cella, M., et al., *Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation*. J Exp Med, 1996. **184**(2): p. 747-52.
116. Macatonia, S.E., et al., *Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells*. J Immunol, 1995. **154**(10): p. 5071-9.
117. Kawabe, T., et al., *The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation*. Immunity, 1994. **1**(3): p. 167-78.
118. Ma, D.Y. and E.A. Clark, *The role of CD40 and CD154/CD40L in dendritic cells*. Semin Immunol, 2009. **21**(5): p. 265-72.
119. Khan, M.M., A.C. Tran, and K.M. Keaney, *Forskolin and prostaglandin E2 regulate the generation of human cytolytic T lymphocytes*. Immunopharmacology, 1990. **19**(3): p. 151-61.
120. Blanco, P., et al., *Dendritic cells and cytokines in human inflammatory and autoimmune diseases*. Cytokine Growth Factor Rev, 2008. **19**(1): p. 41-52.
121. Morelli, A.E., et al., *Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation*. Blood, 2001. **98**(5): p. 1512-23.
122. Gi, M., W. Im, and S. Hong, *Dendritic cells as danger-recognizing biosensors*. Sensors (Basel), 2009. **9**(9): p. 6730-51.
123. Granucci, F., et al., *Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming*. J Immunol, 2003. **170**(10): p. 5075-81.
124. Curtsinger, J.M., et al., *Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation*. J Immunol, 2005. **174**(8): p. 4465-9.
125. Langrish, C.L., et al., *IL-12 and IL-23: master regulators of innate and adaptive immunity*. Immunol Rev, 2004. **202**: p. 96-105.
126. Seder, R.A. and W.E. Paul, *Acquisition of lymphokine-producing phenotype by CD4+ T cells*. Annu Rev Immunol, 1994. **12**: p. 635-73.
127. Romagnani, S., *T-cell subsets (Th1 versus Th2)*. Ann Allergy Asthma Immunol, 2000. **85**(1): p. 9-18; quiz 18, 21.
128. Zhu, J. and W.E. Paul, *CD4 T cells: fates, functions, and faults*. Blood, 2008. **112**(5): p. 1557-69.
129. Swain, S.L., K.K. McKinstry, and T.M. Strutt, *Expanding roles for CD4(+) T cells in immunity to viruses*. Nat Rev Immunol, 2012. **12**(2): p. 136-48.
130. Chattopadhyay, G. and E.M. Shevach, *Antigen-specific induced T regulatory cells impair dendritic cell function via an IL-10/MARCH1-dependent mechanism*. J Immunol, 2013. **191**(12): p. 5875-84.
131. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation*. Curr Opin Immunol, 2010. **22**(3): p. 333-40.
132. Mescher, M.F., et al., *Signals required for programming effector and memory development by CD8+ T cells*. Immunol Rev, 2006. **211**: p. 81-92.
133. Sallusto, F., C.R. Mackay, and A. Lanzavecchia, *The role of chemokine receptors in primary, effector, and memory immune responses*. Annu Rev Immunol, 2000. **18**: p. 593-620.
134. Nestle, F.O., et al., *Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells*. Nat Med, 1998. **4**(3): p. 328-32.

135. Kim, R., M. Emi, and K. Tanabe, *Cancer immunoediting from immune surveillance to immune escape*. Immunology, 2007. **121**(1): p. 1-14.
136. Maeurer, M.J., et al., *Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen*. J Clin Invest, 1996. **98**(7): p. 1633-41.
137. Kim, U., et al., *Immunological escape mechanism in spontaneously metastasizing mammary tumors*. Proc Natl Acad Sci U S A, 1975. **72**(3): p. 1012-6.
138. Gajewski, T.F., J. Louahed, and V.G. Brichard, *Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy*. Cancer J, 2010. **16**(4): p. 399-403.
139. Nakai, N., et al., *Dendritic cell vaccination in human melanoma: relationships between clinical effects and vaccine parameters*. Pigment Cell Melanoma Res, 2010. **23**(5): p. 607-19.
140. Engell-Noerregaard, L., et al., *Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters*. Cancer Immunol Immunother, 2009. **58**(1): p. 1-14.
141. Dhodapkar, M.V. and R.M. Steinman, *Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans*. Blood, 2002. **100**(1): p. 174-7.
142. Dhodapkar, M.V., et al., *Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells*. J Exp Med, 2001. **193**(2): p. 233-8.
143. Palucka, K. and J. Banchereau, *Cancer immunotherapy via dendritic cells*. Nat Rev Cancer, 2012. **12**(4): p. 265-77.
144. Kantoff, P.W., et al., *Sipuleucel-T immunotherapy for castration-resistant prostate cancer*. N Engl J Med, 2010. **363**(5): p. 411-22.
145. Fairchild, P.J., et al., *Dendritic cells and pluripotency: unlikely allies in the pursuit of immunotherapy*. Regen Med, 2015. **10**(3): p. 275-86.
146. McCune, J.M., *The dynamics of CD4+ T-cell depletion in HIV disease*. Nature, 2001. **410**(6831): p. 974-9.
147. Durand, C.M., J.N. Blankson, and R.F. Siliciano, *Developing strategies for HIV-1 eradication*. Trends Immunol, 2012. **33**(11): p. 554-62.
148. Letvin, N.L. and B.D. Walker, *Immunopathogenesis and immunotherapy in AIDS virus infections*. Nat Med, 2003. **9**(7): p. 861-6.
149. Kiem, H.P., et al., *Hematopoietic-stem-cell-based gene therapy for HIV disease*. Cell Stem Cell, 2012. **10**(2): p. 137-47.
150. Smed-Sorensen, A. and K. Lore, *Targeting dendritic cells for improved HIV-1 vaccines*. Adv Exp Med Biol, 2013. **762**: p. 263-88.
151. Walker, B.D., R. Ahmed, and S. Plotkin, *Moving ahead an HIV vaccine: use both arms to beat HIV*. Nat Med, 2011. **17**(10): p. 1194-5.
152. Flynn, N.M., et al., *Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection*. J Infect Dis, 2005. **191**(5): p. 654-65.
153. Buchbinder, S.P., et al., *Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial*. Lancet, 2008. **372**(9653): p. 1881-93.
154. Lu, W., et al., *Therapeutic dendritic-cell vaccine for simian AIDS*. Nat Med, 2003. **9**(1): p. 27-32.
155. Garcia, F. and J.P. Routy, *Challenges in dendritic cells-based therapeutic vaccination in HIV-1 infection Workshop in dendritic cell-based vaccine clinical trials in HIV-1*. Vaccine, 2011. **29**(38): p. 6454-63.
156. Lu, W., et al., *Therapeutic dendritic-cell vaccine for chronic HIV-1 infection*. Nat Med, 2004. **10**(12): p. 1359-65.

157. Garcia, F., et al., *Therapeutic immunization with dendritic cells loaded with heat-inactivated autologous HIV-1 in patients with chronic HIV-1 infection*. J Infect Dis, 2005. **191**(10): p. 1680-5.
158. Ide, F., et al., *Peptide-loaded dendritic-cell vaccination followed by treatment interruption for chronic HIV-1 infection: a phase 1 trial*. J Med Virol, 2006. **78**(6): p. 711-8.
159. Garcia, F., et al., *A therapeutic dendritic cell-based vaccine for HIV-1 infection*. J Infect Dis, 2011. **203**(4): p. 473-8.
160. Robson, N.C., et al., *Presentation of tumour antigens by dendritic cells and challenges faced*. Curr Opin Immunol, 2010. **22**(1): p. 137-44.
161. Cravens, P.D., et al., *Development and activation of human dendritic cells in vivo in a xenograft model of human hematopoiesis*. Stem Cells, 2005. **23**(2): p. 264-78.
162. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases*. J Immunol, 1995. **155**(3): p. 1151-64.
163. Sakaguchi, S., et al., *Regulatory T cells and immune tolerance*. Cell, 2008. **133**(5): p. 775-87.
164. La Rocca, C., et al., *The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus*. Immunol Lett, 2014. **162**(1 Pt A): p. 41-8.
165. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3*. J Exp Med, 2003. **198**(12): p. 1875-86.
166. Bilate, A.M. and J.J. Lafaille, *Induced CD4+Foxp3+ regulatory T cells in immune tolerance*. Annu Rev Immunol, 2012. **30**: p. 733-58.
167. Francisco, L.M., et al., *PD-L1 regulates the development, maintenance, and function of induced regulatory T cells*. J Exp Med, 2009. **206**(13): p. 3015-29.
168. Schmitt, E.G. and C.B. Williams, *Generation and function of induced regulatory T cells*. Front Immunol, 2013. **4**: p. 152.
169. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance*. Cell, 2000. **101**(5): p. 455-8.
170. Gershon, R.K. and K. Kondo, *Infectious immunological tolerance*. Immunology, 1971. **21**(6): p. 903-14.
171. June, C.H. and B.R. Blazar, *Clinical application of expanded CD4+25+ cells*. Semin Immunol, 2006. **18**(2): p. 78-88.
172. Ferrer, I.R., et al., *Induction of transplantation tolerance through regulatory cells: from mice to men*. Immunol Rev, 2014. **258**(1): p. 102-16.
173. Earle, K.E., et al., *In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation*. Clin Immunol, 2005. **115**(1): p. 3-9.
174. Golshayan, D., et al., *In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance*. Blood, 2007. **109**(2): p. 827-35.
175. Allan, S.E., et al., *Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production*. Int Immunol, 2007. **19**(4): p. 345-54.
176. Singer, B.D., L.S. King, and F.R. D'Alessio, *Regulatory T cells as immunotherapy*. Front Immunol, 2014. **5**: p. 46.
177. Hippen, K.L., et al., *Clinical perspectives for regulatory T cells in transplantation tolerance*. Semin Immunol, 2011. **23**(6): p. 462-8.
178. Safinia, N., et al., *Promoting transplantation tolerance; adoptive regulatory T cell therapy*. Clin Exp Immunol, 2013. **172**(2): p. 158-68.
179. Hoffmann, P., et al., *Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion*. Blood, 2006. **108**(13): p. 4260-7.

180. Hippen, K.L., et al., *Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells*. Blood, 2008. **112**(7): p. 2847-57.
181. Di Ianni, M., et al., *Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation*. Blood, 2011. **117**(14): p. 3921-8.
182. Brusko, T.M., A.L. Putnam, and J.A. Bluestone, *Human regulatory T cells: role in autoimmune disease and therapeutic opportunities*. Immunol Rev, 2008. **223**: p. 371-90.
183. Minamimura, K., W. Gao, and T. Maki, *CD4+ regulatory T cells are spared from deletion by antilymphocyte serum, a polyclonal anti-T cell antibody*. J Immunol, 2006. **176**(7): p. 4125-32.
184. Lopez, M., et al., *A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells*. J Am Soc Nephrol, 2006. **17**(10): p. 2844-53.
185. Smith, J.A., Q. Tang, and J.A. Bluestone, *Partial TCR signals delivered by FcR-nonbinding anti-CD3 monoclonal antibodies differentially regulate individual Th subsets*. J Immunol, 1998. **160**(10): p. 4841-9.
186. Herold, K.C., et al., *A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes*. Diabetes, 2005. **54**(6): p. 1763-9.
187. Sherry, N., et al., *Teplizumab for treatment of type 1 diabetes (Protege study): 1-year results from a randomised, placebo-controlled trial*. Lancet, 2011. **378**(9790): p. 487-97.
188. Sprangers, B., et al., *Otelixizumab in the treatment of type 1 diabetes mellitus*. Immunotherapy, 2011. **3**(11): p. 1303-16.
189. Darby, C.R., et al., *Nondepleting anti-CD4 antibodies in transplantation. Evidence that modulation is far less effective than prolonged CD4 blockade*. Transplantation, 1994. **57**(10): p. 1419-26.
190. Bushell, A., P.J. Morris, and K.J. Wood, *Induction of operational tolerance by random blood transfusion combined with anti-CD4 antibody therapy. A protocol with significant clinical potential*. Transplantation, 1994. **58**(2): p. 133-9.
191. Noris, M., et al., *Regulatory T cells and T cell depletion: role of immunosuppressive drugs*. J Am Soc Nephrol, 2007. **18**(3): p. 1007-18.
192. Gregori, S., et al., *An anti-CD45RO/RB monoclonal antibody modulates T cell responses via induction of apoptosis and generation of regulatory T cells*. J Exp Med, 2005. **201**(8): p. 1293-305.
193. Huurman, V.A., et al., *Differential inhibition of autoreactive memory- and alloreactive naive T cell responses by soluble cytotoxic T lymphocyte antigen 4 (sCTLA4), CTLA4Ig and LEA29Y*. Clin Exp Immunol, 2007. **150**(3): p. 487-93.
194. Smilek, D.E., M.R. Ehlers, and G.T. Nepom, *Restoring the balance: immunotherapeutic combinations for autoimmune disease*. Dis Model Mech, 2014. **7**(5): p. 503-13.
195. Sicotte, N.L. and R.R. Voskuhl, *Onset of multiple sclerosis associated with anti-TNF therapy*. Neurology, 2001. **57**(10): p. 1885-8.
196. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts*. Science, 1998. **282**(5391): p. 1145-7.
197. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors*. Cell, 2006. **126**(4): p. 663-76.
198. Nakagawa, M., et al., *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts*. Nat Biotechnol, 2008. **26**(1): p. 101-6.
199. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors*. Cell, 2007. **131**(5): p. 861-72.
200. Walia, B., et al., *Induced pluripotent stem cells: fundamentals and applications of the reprogramming process and its ramifications on regenerative medicine*. Stem Cell Rev, 2012. **8**(1): p. 100-15.

201. Jiang, Z., Y. Han, and X. Cao, *Induced pluripotent stem cell (iPSCs) and their application in immunotherapy*. Cell Mol Immunol, 2014. **11**(1): p. 17-24.
202. Stadtfeld, M., et al., *Induced pluripotent stem cells generated without viral integration*. Science, 2008. **322**(5903): p. 945-9.
203. Yu, J., et al., *Human induced pluripotent stem cells free of vector and transgene sequences*. Science, 2009. **324**(5928): p. 797-801.
204. Kim, D., et al., *Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins*. Cell Stem Cell, 2009. **4**(6): p. 472-6.
205. Warren, L., et al., *Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA*. Cell Stem Cell, 2010. **7**(5): p. 618-30.
206. Huangfu, D., et al., *Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds*. Nat Biotechnol, 2008. **26**(7): p. 795-7.
207. Hou, P., et al., *Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds*. Science, 2013. **341**(6146): p. 651-4.
208. Zhao, T., et al., *Immunogenicity of induced pluripotent stem cells*. Nature, 2011. **474**(7350): p. 212-5.
209. Chen, Y.T., et al., *Identification of CT46/HORMAD1, an immunogenic cancer/testis antigen encoding a putative meiosis-related protein*. Cancer Immun, 2005. **5**: p. 9.
210. Araki, R., et al., *Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells*. Nature, 2013. **494**(7435): p. 100-4.
211. Robertson, C., D.D. Tran, and S.C. George, *Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes*. Stem Cells, 2013. **31**(5): p. 829-37.
212. Batalov, I. and A.W. Feinberg, *Differentiation of Cardiomyocytes from Human Pluripotent Stem Cells Using Monolayer Culture*. Biomark Insights, 2015. **10**(Suppl 1): p. 71-6.
213. Yu, T., et al., *In vivo differentiation of induced pluripotent stem cell-derived cardiomyocytes*. Circ J, 2013. **77**(5): p. 1297-306.
214. Wu, S.M. and K. Hochedlinger, *Harnessing the potential of induced pluripotent stem cells for regenerative medicine*. Nat Cell Biol, 2011. **13**(5): p. 497-505.
215. Chang, C.J., et al., *Production of embryonic and fetal-like red blood cells from human induced pluripotent stem cells*. PLoS One, 2011. **6**(10): p. e25761.
216. Kobari, L., et al., *Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model*. Haematologica, 2012. **97**(12): p. 1795-803.
217. Lee, A.S., et al., *Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies*. Nat Med, 2013. **19**(8): p. 998-1004.
218. Silk, K.M., et al., *Cross-presentation of tumour antigens by human induced pluripotent stem cell-derived CD141(+)XCR1(+) dendritic cells*. Gene Ther, 2012. **19**(10): p. 1035-40.
219. Vizcardo, R., et al., *Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells*. Cell Stem Cell, 2013. **12**(1): p. 31-6.
220. Nishimura, T., et al., *Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation*. Cell Stem Cell, 2013. **12**(1): p. 114-26.
221. Watarai, H., et al., *Murine induced pluripotent stem cells can be derived from and differentiate into natural killer T cells*. J Clin Invest, 2010. **120**(7): p. 2610-8.
222. Fairchild, P.J., et al., *Directed differentiation of dendritic cells from mouse embryonic stem cells*. Curr Biol, 2000. **10**(23): p. 1515-8.
223. Zhan, X., et al., *Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro*. Lancet, 2004. **364**(9429): p. 163-71.
224. Tseng, S.Y., et al., *Generation of immunogenic dendritic cells from human embryonic stem cells without serum and feeder cells*. Regen Med, 2009. **4**(4): p. 513-26.

225. Senju, S., et al., *Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells*. Blood, 2003. **101**(9): p. 3501-8.
226. Silk, K.M., et al., *Differentiation of dendritic cells from human embryonic stem cells*. Methods Mol Biol, 2011. **767**: p. 449-61.
227. Bluestone, J.A. and Q. Tang, *Therapeutic vaccination using CD4+CD25+ antigen-specific regulatory T cells*. Proc Natl Acad Sci U S A, 2004. **101 Suppl 2**: p. 14622-6.
228. Scalapino, K.J. and D.I. Daikh, *Suppression of glomerulonephritis in NZB/NZW lupus prone mice by adoptive transfer of ex vivo expanded regulatory T cells*. PLoS One, 2009. **4**(6): p. e6031.
229. Wright, G.P., et al., *Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis*. Proc Natl Acad Sci U S A, 2009. **106**(45): p. 19078-83.
230. Haque, R., et al., *Programming of regulatory T cells from pluripotent stem cells and prevention of autoimmunity*. J Immunol, 2012. **189**(3): p. 1228-36.
231. Warren, E.H., et al., *Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens*. Blood, 2010. **115**(19): p. 3869-78.
232. Schmitt, T.M., et al., *Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro*. Nat Immunol, 2004. **5**(4): p. 410-7.
233. Loh, Y.H., et al., *Reprogramming of T cells from human peripheral blood*. Cell Stem Cell, 2010. **7**(1): p. 15-9.
234. Themeli, M., et al., *Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy*. Nat Biotechnol, 2013. **31**(10): p. 928-33.
235. Carpenter, L., et al., *Human induced pluripotent stem cells are capable of B-cell lymphopoiesis*. Blood, 2011. **117**(15): p. 4008-11.
236. Iqbal, A.J., et al., *A real time chemotaxis assay unveils unique migratory profiles amongst different primary murine macrophages*. PLoS One, 2013. **8**(3): p. e58744.
237. Sun, M., et al., *A dynamic real-time method for monitoring epithelial barrier function in vitro*. Anal Biochem, 2012. **425**(2): p. 96-103.
238. Bartz, S.R. and M.A. Vodicka, *Production of high-titer human immunodeficiency virus type 1 pseudotyped with vesicular stomatitis virus glycoprotein*. Methods, 1997. **12**(4): p. 337-42.
239. Kodama, H., et al., *Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells*. Exp Hematol, 1994. **22**(10): p. 979-84.
240. Holmes, R. and J.C. Zuniga-Pflucker, *The OP9-DL1 system: generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro*. Cold Spring Harb Protoc, 2009. **2009**(2): p. pdb prot5156.
241. *Genotyping protocol database- B6.129P2-Il10tm1Cgn/J*. 2009; Available from: http://jaxmice.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JR_S_CODE:2631,002251.
242. Davies, T.J. and P.J. Fairchild, *Optimization of protocols for derivation of mouse embryonic stem cell lines from refractory strains, including the non obese diabetic mouse*. Stem Cells Dev, 2012. **21**(10): p. 1688-700.
243. Fauci, A.S., G.K. Folkers, and C.W. Dieffenbach, *HIV-AIDS: much accomplished, much to do*. Nat Immunol, 2013. **14**(11): p. 1104-7.
244. Cooper, A., et al., *HIV-1 causes CD4 cell death through DNA-dependent protein kinase during viral integration*. Nature, 2013. **498**(7454): p. 376-9.
245. Maenza, J. and C. Flexner, *Combination antiretroviral therapy for HIV infection*. Am Fam Physician, 1998. **57**(11): p. 2789-98.

246. Martinez, E., et al., *Risk of lipodystrophy in HIV-1-infected patients treated with protease inhibitors: a prospective cohort study*. Lancet, 2001. **357**(9256): p. 592-8.
247. Garcia, F., et al., *A dendritic cell-based vaccine elicits T cell responses associated with control of HIV-1 replication*. Sci Transl Med, 2013. **5**(166): p. 166ra2.
248. Banchereau, J., et al., *Dendritic cells: controllers of the immune system and a new promise for immunotherapy*. Ann N Y Acad Sci, 2003. **987**: p. 180-7.
249. Silk, K.M., et al., *Cross-presentation of tumour antigens by human induced pluripotent stem cell-derived CD141(+)XCR1(+) dendritic cells*. Gene Ther, 2011.
250. Cameron, P.U., et al., *Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells*. Science, 1992. **257**(5068): p. 383-7.
251. Spira, A.I., et al., *Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques*. J Exp Med, 1996. **183**(1): p. 215-25.
252. Alexaki, A., Y. Liu, and B. Wigdahl, *Cellular reservoirs of HIV-1 and their role in viral persistence*. Curr HIV Res, 2008. **6**(5): p. 388-400.
253. Nicholson, J.K., et al., *In vitro infection of human monocytes with human T lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV)*. J Immunol, 1986. **137**(1): p. 323-9.
254. Coleman, C.M., C.S. Gelais, and L. Wu, *Cellular and viral mechanisms of HIV-1 transmission mediated by dendritic cells*. Adv Exp Med Biol, 2013. **762**: p. 109-30.
255. Smed-Sorensen, A., et al., *Differential susceptibility to human immunodeficiency virus type 1 infection of myeloid and plasmacytoid dendritic cells*. J Virol, 2005. **79**(14): p. 8861-9.
256. Gorry, P.R. and P. Ancuta, *Coreceptors and HIV-1 pathogenesis*. Curr HIV/AIDS Rep, 2011. **8**(1): p. 45-53.
257. Samson, M., et al., *Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene*. Nature, 1996. **382**(6593): p. 722-5.
258. Nichols, J., et al., *Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4*. Cell, 1998. **95**(3): p. 379-91.
259. Hellman, P. and H. Eriksson, *Early activation markers of human peripheral dendritic cells*. Hum Immunol, 2007. **68**(5): p. 324-33.
260. Hu, B.Y., et al., *Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency*. Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4335-40.
261. Cahan, P. and G.Q. Daley, *Origins and implications of pluripotent stem cell variability and heterogeneity*. Nat Rev Mol Cell Biol, 2013. **14**(6): p. 357-68.
262. Acimovic, I., et al., *Human pluripotent stem cell-derived cardiomyocytes as research and therapeutic tools*. Biomed Res Int, 2014. **2014**: p. 512831.
263. MacDonald, A.S. and E.J. Pearce, *Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells*. J Immunol, 2002. **168**(7): p. 3127-30.
264. Lapteva, N., et al., *Enhanced activation of human dendritic cells by inducible CD40 and Toll-like receptor-4 ligation*. Cancer Res, 2007. **67**(21): p. 10528-37.
265. Haenssle, H., et al., *CD40 ligation during dendritic cell maturation reduces cell death and prevents interleukin-10-induced regression to macrophage-like monocytes*. Exp Dermatol, 2008. **17**(3): p. 177-87.
266. Hernandez, M.G., L. Shen, and K.L. Rock, *CD40-CD40 ligand interaction between dendritic cells and CD8+ T cells is needed to stimulate maximal T cell responses in the absence of CD4+ T cell help*. J Immunol, 2007. **178**(5): p. 2844-52.

267. Akira, S. and K. Takeda, *Toll-like receptor signalling*. Nat Rev Immunol, 2004. **4**(7): p. 499-511.
268. Dalod, M., et al., *Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming*. EMBO J, 2014. **33**(10): p. 1104-16.
269. Mellman, I. and R.M. Steinman, *Dendritic cells: specialized and regulated antigen processing machines*. Cell, 2001. **106**(3): p. 255-8.
270. Probst, H.C., et al., *Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression*. J Immunol, 2003. **171**(10): p. 5415-22.
271. Corinti, S., et al., *Regulatory activity of autocrine IL-10 on dendritic cell functions*. Journal of Immunology, 2001. **166**(7): p. 4312-4318.
272. Svajger, U. and P. Rozman, *Tolerogenic dendritic cells: molecular and cellular mechanisms in transplantation*. Journal of Leukocyte Biology, 2014. **95**(1): p. 53-69.
273. Huang, F.P., Y.X. Chen, and C.K. To, *Guiding the "misguided" - functional conditioning of dendritic cells for the DC-based immunotherapy against tumours*. Eur J Immunol, 2011. **41**(1): p. 18-25.
274. Trinchieri, G., *Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity*. Annu Rev Immunol, 1995. **13**: p. 251-76.
275. Villinger, F. and A.A. Ansari, *Role of IL-12 in HIV infection and vaccine*. Eur Cytokine Netw, 2010. **21**(3): p. 215-8.
276. Langenkamp, A., et al., *Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells*. Nat Immunol, 2000. **1**(4): p. 311-6.
277. Boullart, A.C., et al., *Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration*. Cancer Immunol Immunother, 2008. **57**(11): p. 1589-97.
278. Krummen, M., et al., *Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy*. J Leukoc Biol, 2010. **88**(1): p. 189-99.
279. Tan, J.K. and H.C. O'Neill, *Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity*. J Leukoc Biol, 2005. **78**(2): p. 319-24.
280. Drutman, S.B. and E.S. Trombetta, *Dendritic cells continue to capture and present antigens after maturation in vivo*. J Immunol, 2010. **185**(4): p. 2140-6.
281. Jones, L.J., et al., *Quenched BODIPY dye-labeled casein substrates for the assay of protease activity by direct fluorescence measurement*. Anal Biochem, 1997. **251**(2): p. 144-52.
282. Polyak, S., et al., *Impaired class II expression and antigen uptake in monocytic cells after HIV-1 infection*. J Immunol, 1997. **159**(5): p. 2177-88.
283. Platt, C.D., et al., *Mature dendritic cells use endocytic receptors to capture and present antigens*. Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4287-92.
284. Tourkova, I.L., et al., *Mechanisms of dendritic cell-induced T cell proliferation in the primary MLR assay*. Immunol Lett, 2001. **78**(2): p. 75-82.
285. Zhang, J.G., et al., *The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments*. Immunity, 2012. **36**(4): p. 646-57.
286. Ahrens, S., et al., *F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells*. Immunity, 2012. **36**(4): p. 635-45.
287. Ford, L.B., C.A. Hansell, and R.J. Nibbs, *Using fluorescent chemokine uptake to detect chemokine receptors by fluorescent activated cell sorting*. Methods Mol Biol, 2013. **1013**: p. 203-14.
288. Kroczyk, R.A. and V. Henn, *The Role of XCR1 and its Ligand XCL1 in Antigen Cross-Presentation by Murine and Human Dendritic Cells*. Front Immunol, 2012. **3**: p. 14.

289. Wechsler, A.S., et al., *Induction of IL-8 expression in T cells uses the CD28 costimulatory pathway*. J Immunol, 1994. **153**(6): p. 2515-23.
290. Steinman, R.M. and M.C. Nussenzweig, *Dendritic cells: features and functions*. Immunol Rev, 1980. **53**: p. 127-47.
291. Pashenkov, M., et al., *Elevated expression of CCR5 by myeloid (CD11c+) blood dendritic cells in multiple sclerosis and acute optic neuritis*. Clin Exp Immunol, 2002. **127**(3): p. 519-26.
292. Fukada, K., et al., *Functional expression of the chemokine receptor CCR5 on virus epitope-specific memory and effector CD8+ T cells*. J Immunol, 2002. **168**(5): p. 2225-32.
293. van Wilgenburg, B., et al., *Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions*. PLoS One, 2013. **8**(8): p. e71098.
294. Yanagimachi, M.D., et al., *Robust and highly-efficient differentiation of functional monocytic cells from human pluripotent stem cells under serum- and feeder cell-free conditions*. PLoS One, 2013. **8**(4): p. e59243.
295. Choi, K.D., M. Vodyanik, and Slukvin, II, *Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells*. Nat Protoc, 2011. **6**(3): p. 296-313.
296. Choi, K.D., M.A. Vodyanik, and Slukvin, II, *Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors*. J Clin Invest, 2009. **119**(9): p. 2818-29.
297. McKenna, K., A.S. Beignon, and N. Bhardwaj, *Plasmacytoid dendritic cells: linking innate and adaptive immunity*. J Virol, 2005. **79**(1): p. 17-27.
298. Shi, J., et al., *Identification of CD123+ myeloid dendritic cells as an early-stage immature subset with strong tumoristatic potential*. Cancer Lett, 2008. **270**(1): p. 19-29.
299. Seth, S., et al., *CCR7 essentially contributes to the homing of plasmacytoid dendritic cells to lymph nodes under steady-state as well as inflammatory conditions*. J Immunol, 2011. **186**(6): p. 3364-72.
300. Forster, R., et al., *CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs*. Cell, 1999. **99**(1): p. 23-33.
301. Jelinek, I., et al., *TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection*. J Immunol, 2011. **186**(4): p. 2422-9.
302. Li, D.Y., et al., *Maturation induction of human peripheral blood mononuclear cell-derived dendritic cells*. Exp Ther Med, 2012. **4**(1): p. 131-134.
303. Goriely, S., et al., *Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes*. J Immunol, 2001. **166**(3): p. 2141-6.
304. Zobywalski, A., et al., *Generation of clinical grade dendritic cells with capacity to produce biologically active IL-12p70*. J Transl Med, 2007. **5**: p. 18.
305. Lemoine, S., et al., *Dectin-1 activation unlocks IL12A expression and reveals the T1 potency of neonatal dendritic cells*. J Allergy Clin Immunol, 2015.
306. Cyster, J.G., *Chemokines and cell migration in secondary lymphoid organs*. Science, 1999. **286**(5447): p. 2098-102.
307. Scandella, E., et al., *Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells*. Blood, 2002. **100**(4): p. 1354-61.
308. Luft, T., et al., *Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets*. Blood, 2002. **100**(4): p. 1362-72.
309. MacDonald, K.P., et al., *Characterization of human blood dendritic cell subsets*. Blood, 2002. **100**(13): p. 4512-20.

310. Segura, E., et al., *Characterization of resident and migratory dendritic cells in human lymph nodes*. J Exp Med, 2012. **209**(4): p. 653-60.
311. Hoeffel, G., et al., *Antigen crosspresentation by human plasmacytoid dendritic cells*. Immunity, 2007. **27**(3): p. 481-92.
312. Sancho, D., et al., *Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin*. J Clin Invest, 2008. **118**(6): p. 2098-110.
313. Schreibelt, G., et al., *The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells*. Blood, 2012. **119**(10): p. 2284-92.
314. Blattman, J.N., et al., *Estimating the precursor frequency of naive antigen-specific CD8 T cells*. J Exp Med, 2002. **195**(5): p. 657-64.
315. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection*. Cell, 1996. **86**(3): p. 367-77.
316. Dong, C., et al., *Characterization of human immunodeficiency virus type 1 replication in immature and mature dendritic cells reveals dissociable cis- and trans-infection*. J Virol, 2007. **81**(20): p. 11352-62.
317. Popov, S., et al., *Long-term productive human immunodeficiency virus infection of CD1a-sorted myeloid dendritic cells*. J Virol, 2005. **79**(1): p. 602-8.
318. Ye, L., et al., *Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Delta32 mutation confers resistance to HIV infection*. Proc Natl Acad Sci U S A, 2014. **111**(26): p. 9591-6.
319. Petty, R.E. and D.W. Hunt, *Neonatal dendritic cells*. Vaccine, 1998. **16**(14-15): p. 1378-82.
320. Granelli-Piperno, A., et al., *HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation*. Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7669-74.
321. Fiorentino, D.F., et al., *IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells*. J Immunol, 1991. **146**(10): p. 3444-51.
322. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor*. Annu Rev Immunol, 2001. **19**: p. 683-765.
323. Kuhn, R., et al., *Interleukin-10-deficient mice develop chronic enterocolitis*. Cell, 1993. **75**(2): p. 263-74.
324. Berg, D.J., et al., *Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance*. J Clin Invest, 1995. **96**(5): p. 2339-47.
325. Franke, A., et al., *Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility*. Nat Genet, 2008. **40**(11): p. 1319-23.
326. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes*. J Exp Med, 1991. **174**(5): p. 1209-20.
327. Ding, L. and E.M. Shevach, *IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function*. J Immunol, 1992. **148**(10): p. 3133-9.
328. Joss, A., et al., *IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway*. Eur J Immunol, 2000. **30**(6): p. 1683-90.
329. Couper, K.N., D.G. Blount, and E.M. Riley, *IL-10: the master regulator of immunity to infection*. J Immunol, 2008. **180**(9): p. 5771-7.
330. Groux, H., et al., *Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells*. J Immunol, 1998. **160**(7): p. 3188-93.
331. O'Garra, A. and P. Vieira, *T(H)1 cells control themselves by producing interleukin-10*. Nat Rev Immunol, 2007. **7**(6): p. 425-8.
332. Lang, R., et al., *Shaping gene expression in activated and resting primary macrophages by IL-10*. J Immunol, 2002. **169**(5): p. 2253-63.

333. Maldonado, R.A. and U.H. von Andrian, *How tolerogenic dendritic cells induce regulatory T cells*. Adv Immunol, 2010. **108**: p. 111-65.
334. Murray, P.J., *Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response*. Curr Opin Pharmacol, 2006. **6**(4): p. 379-86.
335. De Smedt, T., et al., *Effect of interleukin-10 on dendritic cell maturation and function*. Eur J Immunol, 1997. **27**(5): p. 1229-35.
336. Steinbrink, K., et al., *Induction of tolerance by IL-10-treated dendritic cells*. J Immunol, 1997. **159**(10): p. 4772-80.
337. Demangel, C., P. Bertolino, and W.J. Britton, *Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production*. Eur J Immunol, 2002. **32**(4): p. 994-1002.
338. Lutz, M.B. and G. Schuler, *Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?* Trends Immunol, 2002. **23**(9): p. 445-9.
339. Gregori, S., M.G. Roncarolo, and R. Bacchetta, *Methods for in vitro generation of human type 1 regulatory T cells*. Methods Mol Biol, 2011. **677**: p. 31-46.
340. Gregori, S., et al., *Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway*. Blood, 2010. **116**(6): p. 935-44.
341. Buelens, C., et al., *Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor*. Eur J Immunol, 1997. **27**(3): p. 756-62.
342. Igietsme, J.U., et al., *Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development*. J Immunol, 2000. **164**(8): p. 4212-9.
343. Chen, Y.X., et al., *A crucial role for dendritic cell (DC) IL-10 in inhibiting successful DC-based immunotherapy: superior antitumor immunity against hepatocellular carcinoma evoked by DC devoid of IL-10*. J Immunol, 2007. **179**(9): p. 6009-15.
344. Lambert, J.F., et al., *Quick sex determination of mouse fetuses*. J Neurosci Methods, 2000. **95**(2): p. 127-32.
345. Lim, T.S., et al., *CD80 and CD86 differentially regulate mechanical interactions of T-cells with antigen-presenting dendritic cells and B-cells*. PLoS One, 2012. **7**(9): p. e45185.
346. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone, *CD28/B7 system of T cell costimulation*. Annu Rev Immunol, 1996. **14**: p. 233-58.
347. Acuto, O. and F. Michel, *CD28-mediated co-stimulation: a quantitative support for TCR signalling*. Nat Rev Immunol, 2003. **3**(12): p. 939-51.
348. van Kooten, C. and J. Banchereau, *CD40-CD40 ligand*. J Leukoc Biol, 2000. **67**(1): p. 2-17.
349. Van Severen, G.A., et al., *The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells*. J Immunol, 1990. **144**(12): p. 4579-86.
350. Sheikh, N.A. and L.A. Jones, *CD54 is a surrogate marker of antigen presenting cell activation*. Cancer Immunol Immunother, 2008. **57**(9): p. 1381-90.
351. Dienz, O. and M. Rincon, *The effects of IL-6 on CD4 T cell responses*. Clin Immunol, 2009. **130**(1): p. 27-33.
352. Vanden Bush, T.J., et al., *Cutting Edge: Importance of IL-6 and cooperation between innate and adaptive immune receptors in cellular vaccination with B lymphocytes*. J Immunol, 2009. **183**(8): p. 4833-7.
353. Teague, T.K., et al., *IL-6 rescues resting mouse T cells from apoptosis*. J Immunol, 1997. **158**(12): p. 5791-6.
354. Takeda, K., et al., *Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice*. J Immunol, 1998. **161**(9): p. 4652-60.

355. O'Garra, A., *Cytokines induce the development of functionally heterogeneous T helper cell subsets*. Immunity, 1998. **8**(3): p. 275-83.
356. Jiang, H.R., et al., *Secretion of interleukin-10 or interleukin-12 by LPS-activated dendritic cells is critically dependent on time of stimulus relative to initiation of purified DC culture*. J Leukoc Biol, 2002. **72**(5): p. 978-85.
357. Holmes, D., et al., *Foxp3 and Treg cells in HIV-1 infection and immuno-pathogenesis*. Immunol Res, 2008. **41**(3): p. 248-66.
358. Lantz, O., et al., *Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation*. Nat Immunol, 2000. **1**(1): p. 54-8.
359. Herbert, D.R., et al., *IL-10 and TGF-beta redundantly protect against severe liver injury and mortality during acute schistosomiasis*. J Immunol, 2008. **181**(10): p. 7214-20.
360. Marchetto, M.C., et al., *A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells*. Cell, 2010. **143**(4): p. 527-39.
361. Patani, R., et al., *Investigating the utility of human embryonic stem cell-derived neurons to model ageing and neurodegenerative disease using whole-genome gene expression and splicing analysis*. J Neurochem, 2012. **122**(4): p. 738-51.
362. Allavena, P., et al., *IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages*. Eur J Immunol, 1998. **28**(1): p. 359-69.
363. Winzler, C., et al., *Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures*. J Exp Med, 1997. **185**(2): p. 317-28.
364. Cella, M., et al., *Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells*. Nature, 1997. **388**(6644): p. 782-7.
365. Gad, M., et al., *Characterization of T-regulatory cells, induced by immature dendritic cells, which inhibit enteroantigen-reactive colitis-inducing T-cell responses in vitro and in vivo*. Immunology, 2004. **113**(4): p. 499-508.
366. Gabrilovich, D., *Mechanisms and functional significance of tumour-induced dendritic-cell defects*. Nat Rev Immunol, 2004. **4**(12): p. 941-52.
367. Chang, W.L., et al., *Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality*. J Virol, 2004. **78**(16): p. 8720-31.
368. Crispin, J.C., A. Martinez, and J. Alcocer-Varela, *Quantification of regulatory T cells in patients with systemic lupus erythematosus*. J Autoimmun, 2003. **21**(3): p. 273-6.
369. Furuno, K., et al., *CD25+CD4+ regulatory T cells in patients with Kawasaki disease*. J Pediatr, 2004. **145**(3): p. 385-90.
370. Cao, D., et al., *CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease*. Arthritis Res Ther, 2004. **6**(4): p. R335-46.
371. Long, S.A. and J.H. Buckner, *CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game*. J Immunol, 2011. **187**(5): p. 2061-6.
372. Hara, T., et al., *Thymus-dependent modulation of Ly49 inhibitory receptor expression on NK1.1+gamma/delta T cells*. Immunology, 2001. **102**(1): p. 24-30.
373. Graca, L., et al., *Donor-specific transplantation tolerance: the paradoxical behavior of CD4+CD25+ T cells*. Proc Natl Acad Sci U S A, 2004. **101**(27): p. 10122-6.
374. Sakaguchi, S., et al., *Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance*. Immunol Rev, 2001. **182**: p. 18-32.
375. van Amelsfort, J.M., et al., *CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid*. Arthritis Rheum, 2004. **50**(9): p. 2775-85.
376. Bluestone, J.A., Q. Tang, and C.E. Sedwick, *T regulatory cells in autoimmune diabetes: past challenges, future prospects*. J Clin Immunol, 2008. **28**(6): p. 677-84.

377. Tang, Q., et al., *In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes*. J Exp Med, 2004. **199**(11): p. 1455-65.
378. Joffre, O., et al., *Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes*. Blood, 2004. **103**(11): p. 4216-21.
379. de Pooter, R.F., et al., *In vitro generation of T lymphocytes from embryonic stem cell-derived prehematopoietic progenitors*. Blood, 2003. **102**(5): p. 1649-53.
380. Liang, H.C., R. Holmes, and J.C. Zuniga-Pflucker, *Directed differentiation of embryonic stem cells to the T-lymphocyte lineage*. Methods Mol Biol, 2013. **1029**: p. 119-28.
381. Ikawa, T., et al., *E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment*. J Exp Med, 2006. **203**(5): p. 1329-42.
382. Lei, F., et al., *T lineage differentiation from induced pluripotent stem cells*. Cell Immunol, 2009. **260**(1): p. 1-5.
383. Wada, H., et al., *Successful differentiation to T cells, but unsuccessful B-cell generation, from B-cell-derived induced pluripotent stem cells*. Int Immunol, 2011. **23**(1): p. 65-74.
384. Timmermans, F., et al., *Generation of T cells from human embryonic stem cell-derived hematopoietic zones*. J Immunol, 2009. **182**(11): p. 6879-88.
385. Seki, T., et al., *Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells*. Cell Stem Cell, 2010. **7**(1): p. 11-4.
386. Brown, M.E., et al., *Derivation of induced pluripotent stem cells from human peripheral blood T lymphocytes*. PLoS One, 2010. **5**(6): p. e11373.
387. Ouyang, W., et al., *Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development*. Immunity, 2010. **32**(5): p. 642-53.
388. Hacein-Bey-Abina, S., et al., *LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1*. Science, 2003. **302**(5644): p. 415-9.
389. Prochownik, E.V., *c-Myc: linking transformation and genomic instability*. Curr Mol Med, 2008. **8**(6): p. 446-58.
390. Rowland, B.D. and D.S. Peeper, *KLF4, p21 and context-dependent opposing forces in cancer*. Nat Rev Cancer, 2006. **6**(1): p. 11-23.
391. Kim, K., et al., *Epigenetic memory in induced pluripotent stem cells*. Nature, 2010. **467**(7313): p. 285-90.
392. Cho, S.K., et al., *Functional characterization of B lymphocytes generated in vitro from embryonic stem cells*. Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9797-802.
393. Yoshida, H., et al., *The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene*. Nature, 1990. **345**(6274): p. 442-4.
394. Deftos, M.L. and M.J. Bevan, *Notch signaling in T cell development*. Curr Opin Immunol, 2000. **12**(2): p. 166-72.
395. Deftos, M.L., et al., *Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes*. Immunity, 2000. **13**(1): p. 73-84.
396. Benveniste, P., et al., *Notch signals are required for in vitro but not in vivo maintenance of human hematopoietic stem cells and delay the appearance of multipotent progenitors*. Blood, 2014. **123**(8): p. 1167-77.
397. Brook, F.A. and R.L. Gardner, *The origin and efficient derivation of embryonic stem cells in the mouse*. Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5709-12.
398. Nakano, T., H. Kodama, and T. Honjo, *Generation of lymphohematopoietic cells from embryonic stem cells in culture*. Science, 1994. **265**(5175): p. 1098-101.
399. Bevan, M.J., *In thymic selection, peptide diversity gives and takes away*. Immunity, 1997. **7**(2): p. 175-8.
400. Anderson, G. and E.J. Jenkinson, *Review article: thymus organ cultures and T-cell receptor repertoire development*. Immunology, 2000. **100**(4): p. 405-10.
401. Hoffmann, P., et al., *Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation*. Eur J Immunol, 2009. **39**(4): p. 1088-97.

402. Brunstein, C.G., et al., *Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics*. Blood, 2011. **117**(3): p. 1061-70.
403. Baron, U., et al., *DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells*. Eur J Immunol, 2007. **37**(9): p. 2378-89.
404. Mayer, C.T., et al., *CD8+ Foxp3+ T cells share developmental and phenotypic features with classical CD4+ Foxp3+ regulatory T cells but lack potent suppressive activity*. Eur J Immunol, 2011. **41**(3): p. 716-25.
405. Kim, H.J., et al., *Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance*. Nature, 2010. **467**(7313): p. 328-32.
406. Robb, R.J., et al., *Identification and expansion of highly suppressive CD8(+)FoxP3(+) regulatory T cells after experimental allogeneic bone marrow transplantation*. Blood, 2012. **119**(24): p. 5898-908.
407. Wodnar-Filipowicz, A., *Flt3 ligand: role in control of hematopoietic and immune functions of the bone marrow*. News Physiol Sci, 2003. **18**: p. 247-51.
408. Sitnicka, E., et al., *Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool*. Immunity, 2002. **17**(4): p. 463-72.
409. Kingston, R., E.J. Jenkinson, and J.J. Owen, *A single stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations*. Nature, 1985. **317**(6040): p. 811-3.
410. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision*. Nat Rev Immunol, 2002. **2**(5): p. 309-22.
411. Robey, E. and B.J. Fowlkes, *Selective events in T cell development*. Annu Rev Immunol, 1994. **12**: p. 675-705.
412. von Boehmer, H., H.S. Teh, and P. Kisielow, *The thymus selects the useful, neglects the useless and destroys the harmful*. Immunol Today, 1989. **10**(2): p. 57-61.
413. Fantini, M.C., et al., *In vitro generation of CD4+ CD25+ regulatory cells from murine naive T cells*. Nat Protoc, 2007. **2**(7): p. 1789-94.
414. Trickett, A. and Y.L. Kwan, *T cell stimulation and expansion using anti-CD3/CD28 beads*. J Immunol Methods, 2003. **275**(1-2): p. 251-5.
415. Nakamura, K., et al., *TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice*. J Immunol, 2004. **172**(2): p. 834-42.
416. Nelson, B.H., *IL-2, regulatory T cells, and tolerance*. J Immunol, 2004. **172**(7): p. 3983-8.
417. de la Rosa, M., et al., *Interleukin-2 is essential for CD4+CD25+ regulatory T cell function*. Eur J Immunol, 2004. **34**(9): p. 2480-8.
418. Buisson, S., et al., *Monocyte-derived dendritic cells from HIV type 1-infected individuals show reduced ability to stimulate T cells and have altered production of interleukin (IL)-12 and IL-10*. J Infect Dis, 2009. **199**(12): p. 1862-71.
419. Narsinh, K.H., et al., *Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells*. J Clin Invest, 2011. **121**(3): p. 1217-21.
420. Nischang, M., et al., *Modeling HIV infection and therapies in humanized mice*. Swiss Med Wkly, 2012. **142**: p. w13618.
421. Hutter, G., et al., *Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation*. N Engl J Med, 2009. **360**(7): p. 692-8.
422. Smith, P.L., H. Tanner, and A. Dalgleish, *Developments in HIV-1 immunotherapy and therapeutic vaccination*. F1000Prime Rep, 2014. **6**: p. 43.
423. Dallas, M.H., et al., *Enhanced T-cell reconstitution by hematopoietic progenitors expanded ex vivo using the Notch ligand Delta1*. Blood, 2007. **109**(8): p. 3579-87.
424. Varnum-Finney, B., et al., *Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling*. J Cell Sci, 2000. **113 Pt 23**: p. 4313-8.

425. Angyal, A., *Autoimmune Disorders - Current Concepts and Advances from Bedside to Mechanistic Insights*, ed. F.-P. Huang. 2011.
426. Taqvi, S., L. Dixit, and K. Roy, *Biomaterial-based notch signaling for the differentiation of hematopoietic stem cells into T cells*. J Biomed Mater Res A, 2006. **79**(3): p. 689-97.
427. Solomon, S., F. Pitossi, and M.S. Rao, *Banking on iPSC--is it doable and is it worthwhile*. Stem Cell Rev, 2015. **11**(1): p. 1-10.
428. Peterson, R.T., et al., *Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation*. Nat Biotechnol, 2004. **22**(5): p. 595-9.
429. Ando, M., et al., *A Safeguard System for Induced Pluripotent Stem Cell-Derived Rejuvenated T Cell Therapy*. Stem Cell Reports, 2015.

“In the end only three things matter: how much you loved; how gently you lived, and how gracefully you let go of things not meant for you.”

Lord Buddha