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Where, when and what do tolerogenic dendritic cells do to T cells?

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Abstract

Rheumatoid arthritis (RA) is the most common autoimmune disease which affects up to 1% of the population. During RA there is a systemic loss of immunological tolerance which results in inflammation in the joints. This leads to progressive and irreversible cartilage and bone erosion. The current treatments for RA involve broad immunosuppression which leave patients at risk of developing infections and cancer. There is a need for more targeted treatments which suppress the pathological immune response while leaving protective immunity intact. Tolerogenic dendritic cells (tol-DCs) have gained interest in recent years as a potential treatment of autoimmunity as they allow a targeted antigen (Ag) specific suppression of the immune response. Tol-DCs have been investigated in multiple clinical trials for the treatment of RA, multiple sclerosis, diabetes type 1, Crohn's disease and transplantation. Tol-DCs are thought to mainly mediate their effects through CD4+ T cells.

The main barriers in advancing this treatment are; tol-DCs exact mechanisms of action on CD4+ T cells still needs to be elucidated, quality control (QC) markers which inform tol-DCs potency as a treatment need to be identified, as well as biomarkers of successful tol-DC therapy in CD4+ T cells which would confirm tol-DC therapy had been successful and the injection route which maximises this treatment is unknown. Intradermal, intravenous, intra-articular, intraperitoneal and intranodal injection routes of tol-DCs have been tested in clinical trials. Subcutaneous injection of tol-DCs has not been tested in human clinical trials. It is thought tol-DCs need to migrate to the draining LN (dLN) to induce tolerance. Choosing an injection route which maximises migration to the dLN could therefore enhance the effects of tol-DC treatment.

In this thesis, I investigated potential mechanisms of action of tol-DCs, QC markers of tol-DCs and markers of tolerogenicity in CD4+ T cells in human Dexamethasone + Vitamin D3 tol-DCs. In a murine study, Dex+VitD3 tol-DCs were shown to migrate to the dLN after subcutaneous footpad injection. This tol-DC type's ability to modulate Ag-specific CD4+ T cell activation *in vivo* was measured and finally subcutaneous footpad injection of tol-DCs was tested in an acute model of inflammatory arthritis. This work will help inform and improve future clinical trials with tol-DCs.

Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

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Abbreviations

ACPA	Anti-citrullinated Protein Antibody
Ab	Antibody
Ag	Antigen
AIA	Antigen Induced Arthritis
APC	Antigen Presenting Cell
AUTODECRA	Autologous Tolerogenic Dendritic Cells for Rheumatoid Arthritis
BM-DC	Bone marrow-derived dendritic cell
Bregs	Regulatory B cells
CCL	Chemokine Ligand
CCR	Chemokine Receptor
cDC	Conventional/classical Dendritic Cells
CDP	Common Dendritic Cell Progenitor
CFH	Complement Factor H
CFA	Complete Freund's Adjuvant
CFSE	Carboxyflurescein Succinmidyl Ester
CIA	Collagen Induced Arthritis
ConA	Concanavalin A
CRP	C-Reactive Protein
CTLA-4	Cytotoxic T-Lymphocyte Associated protein 4
CTV	Cell Trace Violet
DAMP	Danger Associated Molecular Pattern
DAS-28	Disease Activity Score 28
DC	Dendritic Cell
DDA	Dimethyl-dioctadecylammonium
Dex	Dexamethasone
dLN	Draining Lymph Node
DMARD	Disease Modifying Anti-Rheumatic Drug
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescent Activated Cell Sorting

Fas-L	Fas Ligand
FCS	Fetal Calf Serum
FcγRIIb	Fc-gamma Receptor 2 b
FRC	Fibroblastic Reticular Cells
FoxP3	Forkhead box P3
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Good Manufacturing Practices
GWAS	Genome Wide Association Studies
HAO	Heat Aggregated OVA
HBSS	Hank's Balanced Salt Solution
HEV	High Endothelial Venules
HLA	Human Leukocyte Antigen
IBD	Inflammatory Bowel Disease
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
ILT	Immunoglobulin-like Transcript
IP	Intraperitoneal
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
IV	Intravenous
KO	Knock Out
LAIR-1	Leukocyte Associated Immunoglobulin-like Receptor 1
LAL	Late Apoptotic Lymphocyte
LAP	Latency Associated Peptide
LN	Lymph Node
LPS	Lipopolysaccharide
mAbs	Monoclonal Antibodies
MAP	Mitogen Activated Protein
mBSA	Methylated Bovine Serum Albumin
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex

MLR	Mixed Leukocyte Reaction
MMP	Matrix Metalloproteinases
Mo-mac	Monocyte-derived Macrophage
Mo-DC	Monocyte-derived Dendritic Cell
MPLA	Monophosphoryl Lipid A
MRC-1	Mannose Receptor C-like 1
MS	Multiple Sclerosis
NF-KB	Nuclear Factor Kappa light chain enhanced of activated B cells
NK	Natural Killer
OVA	Ovalbumin
PAD	Protein Arginine Deiminases
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCA	Principal Component Analaysis
PD-1	Programmed cell Death protein 1
pDC	Plasmacytoid Dendritic Cell
PD-L1/2	Programmed Death Ligand 1/2
PGE2	Prostaglandin E2
PRR	Pathogen Recognition Receptor
QC	Quality Control
Q-PCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear factor Kappa b
RANK-L	Receptor Activator of Nuclear factor Kappa b Ligand
RBC	Red Blood Cell
RF	Rheumatoid Factor
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SC	Subcutaneous
SCS	Subcapsular Sinus
siRNA	Short interfering Ribonucleic Acid
SLE	Systemic Lupus Erythematosus
TCR	T Cell Receptor
Tfh	T follicular helper cell

TGF-β	Transforming Growth Factor beta
Th	T helper cell
TIR	Toll-interleukin receptor
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor alpha
Tol-DC	Tolerogenic Dendritic Cell
Tr1	T regulatory 1 cell
TRAIL	TNF-related Apoptosis Inducing Ligand
TRAIL-R	TNF-related Apoptosis Inducing Ligand Receptor
Treg	Regulatory T cell
WT	Wild Type
UV	Ultra Violet
VitD3	Vitamin D3
YFP	Yellow Fluorescent Protein

Chapter 1 Introduction

1.1 The Immune system

The main function of the immune system is to maintain tolerance to self-tissues and harmless antigens (Ags) while protecting against pathogens and tumours which cause damage. The immune system is broadly split into two parts: the innate and adaptive immune system. The innate immune system evolved first and is rapid and Ag non-specific. Whereas the adaptive immune system takes longer to develop but is Ag-specific. Dendritic cells (DCs) link innate and adaptive immunity and are referred to as professional Ag-presenting cells (APCs), as they can prime naïve T cells to become effector cells. DCs play a key role in the decision to induce tolerance to self and harmless antigens or initiate immune responses against pathogens. This duality has made DCs an attractive target for immunotherapy.

1.1.1 Dendritic cell subsets

DCs are known as the sentinels of the immune system, they are strategically placed throughout the body to respond to pathogens. They are found at higher levels in the skin, lung and gastrointestinal tract. DCs display a wide range of functional heterogeneity and specialisation. This is reflected in the number of subsets of DCs and the sensitivity of DCs to respond to environmental cues. Pre-cDCs develop in the bone marrow from common DC precursors (CDPs) before circulating in the blood and seeding lymphoid and non-lymphoid tissue[1]. Plasmacytoid DCs (pDCs) have been found to be lymphoid not myeloid derived[2]. pDCs are specialised to respond to viral Ags and produce high amounts of type I interferons (IFN) in response to viral infections[3].

cDCs are further split into 2 groups: cDC1s and cDC2s. In humans these groups are identified by BDCA-3+/CD141+ and BDCA-1+/CD1c+ respectively. In mice they are split by their expression of CD8α+/CD103+ and CD11b+/CD172a+[1]. cDC1s are important for cross-presentation of Ags to CD8+ T cells whereas cDC2s are specialised to prime CD4+ T cells[4].

More recently additional subsets of DCs have been identified in humans[5]. DC1 are CLEC9A+, DC2 and DC3 are both CD1c+. DC1-3 are derived from common cDC precursors. However, DC3 can also be derived from CD14+ monocytes. DC4 are CD1c-CD141-CD11c+ and are derived from CD16+ monocytes. Finally, DC5 are Axl+Siglec6+.

Monocyte-derived DCs (mo-DCs) are derived from circulating monocytes and are sometimes referred to as inflammatory DCs as they are present in high numbers during inflammation however they are also maintained in certain tissues in the steady state e.g. the spleen, lung, dermis and intestinal lamina propria[6–9]. Mo-DCs are transcriptionally more similar to monocyte derived macrophages (mo-macs) than cDCs[10].

In addition to subsets, DCs can also be defined by the environment they inhabit. For example, non-lymphoid and lymphoid tissue cDCs. For cDC1s, CD8α+ cells are found in lymphoid tissues and CD103+ cells are found in non-lymphoid tissue[11]. This distinction is not clear for cDC2s and CD11b+ DCs can reside in lymphoid or non-lymphoid tissue.

1.1.2 Dendritic cell maturation

DCs can undergo immunogenic or homeostatic maturation. DC maturation is a complex process which relies on many environmental cues. DCs express multiple pattern recognition receptors (PRRs) which can recognise PAMPs and DAMPs (Pathogen/Danger Associated Molecular Patterns). Upon immunogenic or homeostatic maturation the PRRs expressed by DCs can change[12]. Toll-like receptors (TLRs) are the most studied PRR.

The expression levels of different TLRs vary depending on the DC subset.
For example, the TLRs expressed by CD8α+ DCs and pDCs suggest they are more equipped to deal with intracellular pathogens whereas CD11b+ cDCs and mo-DCs seem more specialised to recognise extracellular pathogens. pDCs express high levels of TLR-7 and TLR-9. TLR-7 recognises single stranded RNA and TLR-9 recognises unmethylated CpG DNA which is commonly found in viruses and bacteria[12]. TLR-7 and TLR-9 are located on endosomes inside the cell so

therefore respond to internalised pathogens[13]. CD8α+ DCs express high levels of TLR-3[12]. TLR-3 recognises viral double stranded RNA and is located intracellularly[13]. In contrast, CD11b+ cDCs and mo-DCs express TLR-1, 2, 4, 5 and 6 located on the cell surface which recognise extracellular bacteria[12].

In the steady state, which is characterised by the absence of inflammation, non-lymphoid tissue cDCs are described as immature. They display low levels of peptide-MHC and co-stimulatory molecules. Immature DCs continuously sample Ag in their environment. The Ag is broken down into peptides and is presented on MHC molecules[14].

As a result of infection, injury, inflammation or vaccination DCs can become activated through their PRRs. This results in upregulation of peptide-MHC, co-stimulatory molecules (e.g. CD80/86) and the chemokine receptor CCR7 so they can then migrate to the T cell zone of the dLN and prime naïve T cells to become effector cells[15,16].

During the steady state, a proportion of cDCs undergo homeostatic maturation and become semi-mature. These cells upregulate peptide-MHC and CCR7 and migrate to T cell zones of dLN. Instead of inducing effector T cells they delete or anergise self-reactive T cells and induce Tregs to harmless Ag[17,18]. Whether a DC will induce tolerance or not depends on the signals and environmental cues received during maturation.

The idea that mature DCs induce an immune response and semi-mature DCs induce tolerance is perhaps too oversimplified. There is evidence that DCs that look mature (express high levels of co-stimulatory molecules) can still induce tolerance[19,20].

1.1.3 Dendritic cell migration

Lymph node resident DCs can either present soluble Ag which flows through the lymphatics or they can present Ag transported into the LN by migratory DCs[21]. The mode of entry of Ag to the LN depends on Ag size, Ag <200nm can enter the LN directly via the lymphatics whereas Ag larger than this will need to be transported into the LN by migratory DCs[22,23].

DCs in the periphery upregulate CCR7 in response to PAMPs/DAMPs and can migrate through the afferent lymph to the nearest LN by following CCL19/21 gradients. CCL21 is thought to be more important for DC migration than CCL19 as CCL21 alone is sufficient for DC migration to the LN[24]. Lymphatic endothelial cells display CCL21 on heparin sulphates constitutively, although the expression of CCL21 is upregulated during inflammation[25].

Once in the lymphatic capillaries, these vessels become larger collecting lymph vessels and DCs can be transported passively via shear flow to the nearest dLN[26]. The afferent lymphatics deposit lymph and DCs into the subcapsular sinus (SCS) which is the space below the fibrous cap of the LN[27].

Fibroblastic reticular cells (FRCs), which are stromal cells found in the LNs, are the source of CCL19/21[28]. FRCs also provide a structure for DCs and T cells to migrate on and conduits for small Ag and lymph to flow through[29]. Lymph node resident DCs can sample Ag directly from conduits[30].

After entering the LN, migratory DCs continue to follow CCL19/21 gradients to the paracortex (the T cell area) and can prime naïve T cells there or pass on Ag to lymph node resident DCs[21]. In contrast, immature DCs express inflammatory chemokine receptors which allows them to traffic to sites of inflammation[31].

1.1.4 DC-T cell interaction

For a naïve T cell to become an effector T cell it requires three signals from a DC. Naïve T cells require peptide-MHC signal through their T cell receptor (TCR) (signal 1) and co-stimulation via CD80/86-CD28 interactions (signal 2) to become effector T cells. Production of cytokines from the DC (signal 3) can influence the polarisation of the subsequent differentiation of effector T cells, tailoring the immune response to combat a specific threat[32]. The DC-T cell interaction can be seen in Figure 1-1.

The expression of inhibitory receptors limits T cell co-stimulation. For example, CTLA-4 on T cells can block T cell co-stimulation directly by binding CD80/86 on

DCs with greater affinity than CD28[33]. PD-L1 or PD-L2 on DCs can interact with PD-1 on T cells resulting in suppression of T cell responses[34].

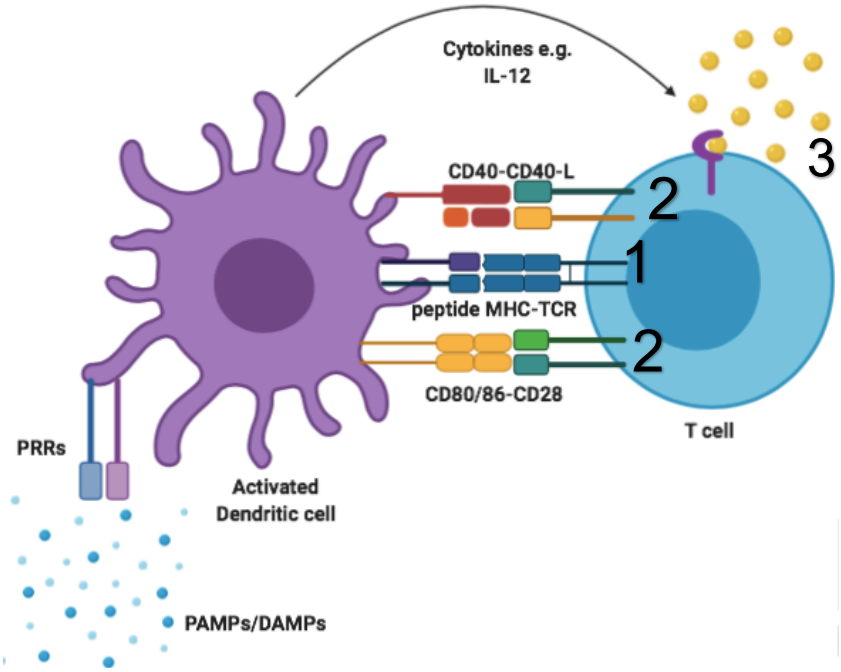


Figure 1-1 DC-T cell interaction.

Signal 1-3 for a naïve CD4+ T cell to become a Th1 cell. Signal 1 is peptide presented on MHC Class II which signals through the TCR. Signal 2 is co-stimulation via CD80/86-CD28 and CD40-CD40-L. Finally, signal 3 is cytokines produced by the DC which influences the differentiation of the naïve T cell. In this example, IL-12 promotes Th1 differentiation.

1.1.5 T cell subsets

The main CD4+ T cell subsets and the cytokines associated with each one can be seen in Figure 1-2.

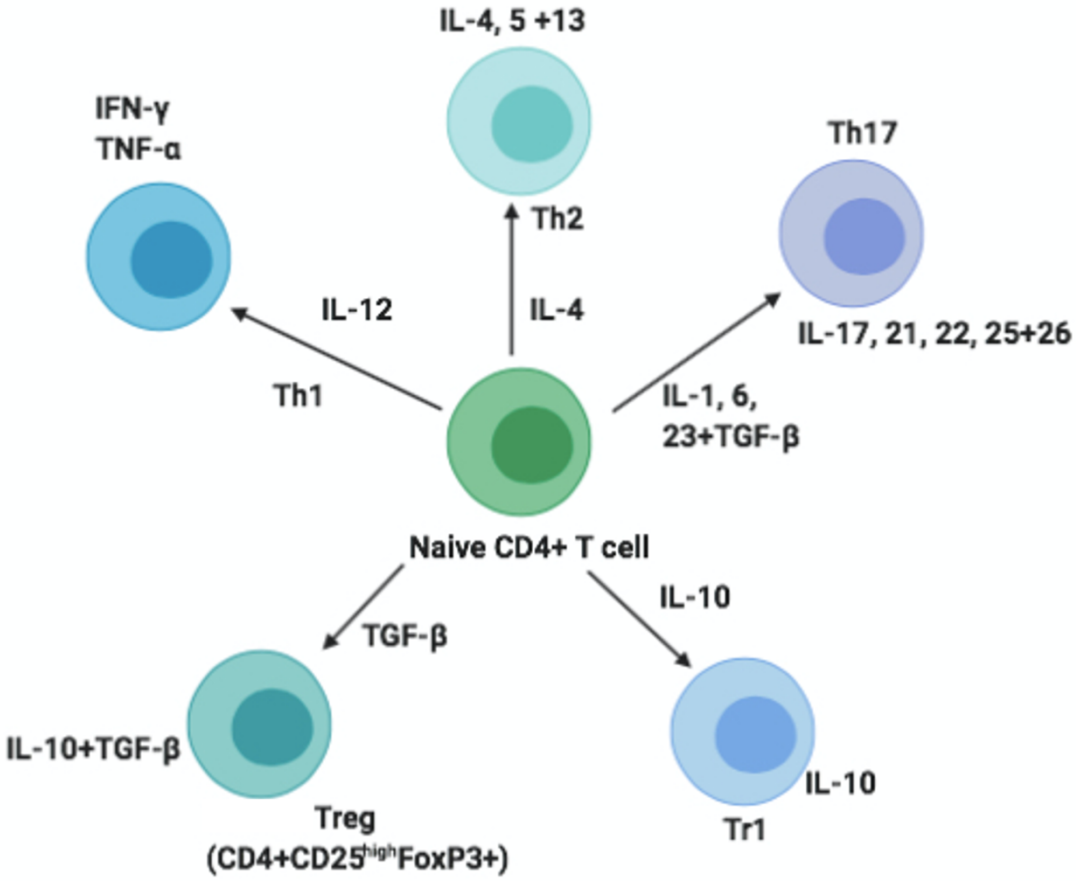


Figure 1-2 T cell subsets and associated cytokines.

A naïve CD4+ T cell can differentiate into any of the Th/Treg subsets depending on the cytokines it is exposed to. IL-12 induces Th1 cells which then produce IFN-γ and TNF-α. IL-4 induces Th2 cells which produce IL-4, 5+13. IL-1, IL-6, 23 and TGF-β induces Th17 cells which then produce IL-17,21,22,25+26. IL-10 induces Tr1 cells which produce IL-10. TGF-β induces (CD4+CD25^{high}FoxP3+) Tregs which produce IL-10+TGF-β.

Th1 cells have evolved to clear intracellular pathogens and tumour cells[35]. However, Th1 cells have been shown to be involved in autoimmune conditions such as RA. Th2 cells are important in the clearance of extracellular bacteria and parasitic worms. Although, Th2 cells have been found to be detrimental in allergy[35]. Th17 cells are involved in the clearance of extracellular bacteria or fungi but have been shown to be involved in autoimmune conditions such as RA, multiple sclerosis (MS) and inflammatory bowel disease (IBD)[36].

Natural regulatory T cells (Tregs), which are CD4+CD25^{high}FoxP3+, are induced in the thymus during central tolerance. Tregs induced in the periphery are either inducible CD4+CD25^{high}FoxP3+ Tregs or T regulatory type 1 (Tr1) cells. TGF-β production is required to induce CD4+CD25^{high}FoxP3+ Tregs whereas high IL-10 production is required to induce Tr1 cells. CD4+CD25^{high}FoxP3+ Tregs express

high levels of CD25 which is the alpha subunit of the IL-2 receptor. For this reason, FoxP3+ Tregs require more IL-2. Tr1 cells are CD49+LAG-3+ in humans and mice[37]. Tr1 cells secrete high levels of IL-10 and low levels of pro-inflammatory cytokines.

1.1.6 Dendritic cells in peripheral tolerance

DCs play a key role in both central tolerance in the thymus and peripheral tolerance. During central tolerance, DCs can delete autoreactive T cells via negative selection and positively select for natural Tregs[38]. T cells which have high affinity for self-Ags are deleted. Tregs have moderate affinity for self-Ags but this is lower than the threshold of activation required for deletion. Other selected T cells have low affinity for self-Ags[39].

In individuals with autoimmunity, autoreactive T cells may have escaped negative selection in the thymus and are not controlled by peripheral tolerance mechanisms.

Using myeloid-lineage specific deletion of cDCs in mice, cDCs were found to be crucial in dietary antigen tolerance and induction of Tregs[40]. Furthermore, XCR1+ DCs in the intestine were shown to be essential in promoting tolerance and mice lacking XCR1+ DCs were more susceptible to colitis[41].

In contrast, transfer of DCs pulsed with autoantigen is sufficient to trigger autoimmunity in recipient mice[42]. These results show that DCs are crucial in maintaining self-tolerance but are also involved in breaking tolerance and triggering autoimmunity.

Steady state migratory DCs which express CCR7 and not lymphoid resident DCs are essential in maintaining peripheral tolerance and inducing FoxP3+ Tregs[18]. The upregulation of CCR7 on DCs and migration to the dLNs is essential for tolerance to inhaled and ingested Ags in the lung and gut respectively[43,44]. Feeding mice OVA results in tolerance to OVA when it is subsequently given subcutaneously or intravenously. Tolerance to OVA could not be induced in mice which had their mesenteric LNs removed or in CCR7 deficient or plt/plt mice. Plt

mice lack CCL19 and CCL21, the ligands for CCR7. The location of tolerance induction was found to be the draining mesenteric LNs[44].

Similarly, when OVA is given through inhalation or intratracheally, there is usually OVA specific unresponsiveness[43]. CCR7 deficient mice were found to have impaired migration of DCs to the bronchial LN. In addition, when OVA loaded DCs or CCR7 deficient DCs were given intratracheally to WT mice, the CCR7 deficient DCs were unable to induce tolerance to OVA. Tolerance induction was found to be dependent on CCR7 dependent migration of DCs from the lung to the bronchial LN[43].

In the absence of co-stimulation, or if the majority of signals received are inhibitory rather than stimulatory, T cell anergy is induced. Anergy is defined as a lack of responsiveness and T cells are unable to proliferate even when Ag is presented by an APC expressing adequate levels of costimulatory molecules[45]. Anergic T cells have been shown themselves to be able to suppress proliferation of naïve T cells mainly via cytotoxic T-lymphocyte-associated protein (CTLA-4)[46,47].

In addition to CTLA-4 and PD-L1/PD-L2 which suppress T cell responses. Other inhibitory receptors include immunoglobulin like transcripts (ILTs) which can interact with HLA-G molecules on T cells. This has been found to be an important interaction for the induction of Tr1 cells[48,49]. Additionally, the expression of Fas or TNF-related apoptosis inducing (TRAIL) on the cell surface of DCs interacts with Fas-L/TRAIL-R on T cells and results in T cell apoptosis[50,51].

The interaction between DCs and Tregs is likely to be two-way. Studies have shown that interactions with Tregs can reduce co-stimulatory molecule expression and Ag presentation capabilities of DCs and increase their production of IL-10[52,53]. This downregulation of co-stimulatory molecules on DCs appears to be dependent on Tregs expression of CTLA-4 which they express constitutively[54]. Furthermore, DCs previously modulated by FoxP3+ Tregs were found to be able to induce Tr1 cells[55].

Other mechanisms of peripheral tolerance include blocking of T cell clonal expansion and skewing of T cell cytokine profile towards less inflammatory and more immunoregulatory cytokines.

1.1.7 Mo-DCs

Mo-DCs develop from circulating classical monocytes. Classical monocytes are defined as CD14+CD16- in humans and Gr-1+Ly6C^{high} in mice[56]. Classical monocytes can extravasate into tissues and differentiate into mo-DCs or monocyte-derived macrophages (mo-macs)[8,57,58]. The decision for a monocyte to become a mo-DC or mo-mac is controlled by transcription factors. IRF4 was found to promote mo-DCs whereas MAFB promoted mo-mac development. Additionally, activation of the aryl hydrocarbon receptor induced mo-DC differentiation while inhibiting mo-macs[59].

Mo-DCs are transcriptionally more closely related to inflammatory macrophages (mo-macs) than cDCs[10]. However, they were termed mo-DCs as they display some DC-like features. Murine mo-DCs and cDCs share many markers such as CD11b, CD11c and MHC Class II. Murine mo-DCs are separated from cDCs in mice by their expression of CCR2, CD64 and Ly6C[8]. In humans, mo-DCs also share many markers with cDCs such as HLA-DR, CD11c, CD1a, CD1b and CD1c[10]. Human mo-DCs express macrophage markers such as CD206, CD14 and CD11b but lack CD16 and CD163 macrophage markers[10].

Studies *in vivo* have suggested that mo-DCs are not as effective at Ag presentation or migration to the dLNs as cDCs. In a dust mite allergen model, mo-DCs were found to be able to migrate to the dLN and induce Th2 cells but only when high doses of allergen were given[60]. cDCs were the main DC type which migrated to the dLN. Similarly, in a model of skin inflammation, mo-DCs had less migratory capacity and reduced ability to activate T cells compared with cDCs[8]. When trying to induce tolerance, reduced Ag presentation is not necessarily a hindrance as immature DCs express lower levels of MHC Class II and can still induce tolerance[14], although it is important for the mo-DCs to reach the dLNs to interact with T cells there.

Another study found that when comparing mo-DCs and cDCs effects on naïve CD4+ T cells, mo-DCs were more likely to induce T cell polarisation to Th1 and Th17 whereas cDCs promoted more T cell proliferation[61]. This indicates a potential division of labour between mo-DCs and cDCs. This study also found that mo-DCs could suppress cDCs ability to stimulate T cell proliferation *in vitro* and *in vivo* partially through nitric oxide production.

The best evidence for mo-DCs having cDC like features is from *in vitro* culture systems. Bone marrow cells are cultured with Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) to induce their differentiation into DCs[62]. These cells were originally considered to be mo-DCs and had high CCR7 expression, Ag presentation and T cell stimulation capacity. However, on further investigation a small population of the most mature DC like cells in bone marrow-derived dendritic cell (BM-DC) cultures with the highest expression of CCR7 and Ag presentation capabilities were found to be contaminating cDC2s[63].

However, a study which isolated monocytes from mouse bone marrow and induced their differentiation into mo-DCs, found that mo-DCs displayed a DC-like phenotype, were efficient Ag presenters and could migrate to the T cell area of the LNs[64]. This study found that mo-DCs were the most efficient at inducing T cell proliferation in co-cultures when compared to splenic cDCs. Additionally, this study showed mo-DCs could be efficient Ag presenters *in vivo* after injection of Lipopolysaccharide (LPS).

Mo-DCs generated *in vitro* may be better at activating T cells and migrating than mo-DCs found *in vivo*. This could be due to mo-DCs generated *in vitro* being exposed to strong inflammatory stimuli such as GM-CSF and LPS. When mice are given LPS or high doses of dust mite allergen, mo-DCs are effective at presenting Ag, activating T cells, and migrating to the dLN *in vivo*[60,64].

Monocyte-derived cells are particularly plastic and are very sensitive to their environment. This is why the modular spectrum model vs the discrete polarisation model (M1 vs M2) has been suggested[65]. The modular spectrum model identifies monocyte-derived cells by their function e.g., Ag presentation. Mo-macs were stimulated with multiple cytokines and TLR ligands and transcriptionally compared to each other. An unbiased bioinformatics clustering

approach found that a spectrum model fitted the available data better rather than the M1 vs M2 polarisation model[66].

Guilliams *et al*. propose that instead of defining mo-DCs and mo-macs, it would be better to group them together and term them monocyte-derived cells[65]. This is due to the overlapping and similar features between the two, including many shared markers. Mo-DCs and mo-macs are mainly separated by their morphology and mo-DCs ability to activate naive CD4+ T cells and migrate to the dLN[8,10,67]. However, mo-macs have been shown to be able to activate memory CD4+ T cells[68].

1.1.8 Tolerogenic DCs *in vivo*

Tolerogenic DCs can be induced *in vivo* after the administration of certain immunosuppressive drugs such as rapamycin, glucocorticoids and vitamin D3[69]. Rapamycin prevents IL-2 dependent T cell proliferation and is often used to treat patients who have received transplants to prevent graft rejection. In addition to its effects on T cells, rapamycin treatment was found to increase the numbers of circulating DCs in patients which expressed high levels of ILT-3 and ILT-4 and this was found to correlate with the number of FoxP3+ Tregs[70]. Furthermore, deficiency in vitamin D has been linked to multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and type 1 diabetes development due to correlations with Vitamin D levels with the incidence and severity of disease[71].

The gut is continuously exposed to food and microbial Ags as well as commensal micro-organisms. For this reason, it is an immunosuppressive environment which favours the induction of tolerogenic responses. In mice, CD103+ DCs have been identified in mesenteric LNs which can induce FoxP3+ Tregs via TGF-β[72]. A similar population in the mouse lung has been identified. CD103+ DCs in the lung can induce FoxP3+ Tregs to harmless Ags[73]. Similarly, to the gut, the lung is continuously exposed to foreign Ag and allergens as well as commensal micro-organisms.

The skin is a barrier site, like the gut and lung, which is continuously exposed to foreign Ag and commensal micro-organisms. In the skin, Rel-b+TGF-β+Langerin+

dermal DCs have been identified which can induce FoxP3+ Tregs in the skin draining LNs[74]. Interestingly in the skin, Langerhans cells, a population of macrophages found in the epidermis, are naturally tolerogenic. Even after adjuvant stimulation and upregulation of co-stimulatory molecules they can still induce FoxP3+ Tregs[75]. This may be because they are inefficient at Ag presentation and express lower levels of MHC Class II[76].

In the mouse spleen, a population of DCs which are CD8α+CD205+ are specialized to induce FoxP3+ Tregs in the presence of low doses of Ag[77]. This population is thought to be important in cross-presenting self-Ags to T cells and inducing tolerance.

A circulating monocyte derived DC population has been found to have immunoregulatory effects in humans. This population has been termed DC-10 and was found to express CD14+CD16+CD11c+CD11b+HLA-DR+CD83+CD1a-CD1c-[49]. DC-10 can induce Tr1 cells through IL-10 production and this was found to be dependent on ILT-4/HLA-G interactions[48].

Mo-DCs have been shown to have additional tolerogenic effects *in vivo*. In humans, a population of CD14+CD141+ cells in the dermis of the skin have been identified[78]. These resident DCs produced high amounts of IL-10, could induce regulatory T cells, T cell anergy, cross-present self-antigens to autoreactive T cells and additionally had LN homing capacity. However, a human dermal CD14+ population which had variable expression of CD141 has since been shown to be mo-macs, highlighting the cross over and similarities between these cells[68]. In this study, the CD14+ cells were shown to not be able to migrate via CCR7 and the lymphatics suggesting that the CD14+ population found in the human dermis may be heterogenous and contain mo-DCs and mo-macs.

The liver is an immunosuppressive environment. In humans, a population of CD1c+ (BDCA-1+) DCs were found in the liver which could promote tolerance. This population was found to be the dominant DC population in the liver. After isolation of this subset and stimulation with TLR-4 agonists high amounts of IL-10 were produced. Furthermore, this population was found to be able to induce T cell anergy and FoxP3+ Tregs[79].

Certain conditions have been associated with an induction of a tolerogenic DC phenotype such as cancer and transplantation. In metastatic melanoma, a population of BDCA-1+CD14+ DCs were found to be expanded. These cells were found to express high levels of PD-L1 to suppress T cell responses. Interestingly, this population expresses a mixture of markers associated with the BDCA-1+ DC subset and monocyte derived cells[80]. The tumour microenvironment itself can promote immature myeloid DCs to differentiate into a regulatory subset which produces high amounts of TGF-β and induces FoxP3+ Treg proliferation[81].

Transplantation studies in mice have identified DC populations associated with graft acceptance. After bone marrow transplantation, CD49+CD200R3+ DCs could prevent graft rejection through the induction of T cell anergy and FoxP3+ Tregs. Depletion of CD49+CD200R3+ DCs before bone marrow transplantation resulted in faster rejection[82]. In a mouse model of kidney transplantation, the presence of IDO+ DCs was associated with longer graft acceptance and these cells were found to localise with FoxP3+ Tregs[83].

The stroma of the spleen, liver and lung itself has been shown to induce differentiation of a regulatory DC subset which are $CD11b^{high}I\text{-}A^{low}$ (MHC Class II) and produce high levels of IL-10 and TGF-β[84-86]. This highlights the importance of the microenvironment on DC phenotype and function.

In summary, there are multiple tolerogenic DC subsets which have been reported *in vivo*. It is unclear whether these tolerogenic DC subsets found *in vivo* are resistant to further stimulation by inflammatory stimuli. They could be a result of their environment rather than a stable tolerogenic population.

1.1.9 Dendritic cells in autoimmunity

DCs are crucial in the development of autoimmunity. DCs become activated and present self-Ags to T cells, priming autoreactive responses. MS patients have been shown to have an increased number of circulating myeloid DCs which express higher co-stimulatory molecules and produce more inflammatory cytokines than circulating myeloid DCs from healthy controls[87]. During RA there

is a decrease in circulating myeloid DCs but there is an increase in myeloid DCs in the synovial fluid. Furthermore, DCs in synovial fluid displayed a more mature phenotype than peripheral blood DCs[88]. DCs secrete a range of inflammatory chemokines and cytokines which tip the balance towards inflammation and attract further immune cells. DCs are involved in the loss of tolerance to self-Ags and the perpetuation of inflammation related to autoimmunity.

There is a theory that a failure to clear apoptotic cells could lead to autoimmunity. Exposure to apoptotic cells promotes tolerogenic responses in DCs[89]. Failure to clear apoptotic cells increases the availability of intracellular self-Ags to be presented by DCs. Additionally, an accumulation of apoptotic cells can lead to secondary necrosis and release of DAMPs. In autoimmune conditions such as Sjorgen's syndrome and SLE there is an increase in the frequency of apoptotic cells[90].

1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common autoimmune condition, affecting up to 1% of the population[91]. RA involves a systemic loss of immune tolerance which localises in the joints. This leads to irreversible and progressive cartilage and bone erosion, high rates of pain and disability and has a huge impact on the economy. Due to the on-going long term inflammation during RA, patients have increased risk of developing cardiovascular disease and cancer[92].

From Genome Wide Associate Studies (GWAS) it appears RA is an immune mediated disorder with the biggest genetic risk factor being HLA-DRB alleles (MHC Class II molecules). Up to 90% of RA patients have one of the DRB*0401, DRB*0404, DRB*0101 and DRB*1402 risk alleles[93]. RA can be seropositive or seronegative. Only seropositive RA can be associated with these HLA-DRB risk alleles. Much less is known about seronegative RA, but it is likely to involve distinct genetic and environmental factors.

Seropositive RA accounts for 80-90% of RA patients and is associated with more joint destruction[91]. Seropositive RA is characterised by the presence of autoantibodies such as Anti-Citrullinated Peptide Autoantibody (ACPA) or rheumatoid factor (RF). RF binds the Fc portion of IgG. These autoantibodies can

be present in healthy individuals, as well as T cells specific to citrullinated peptides, for months to years before arthritis onset[94]. This phase of disease is known as the pre-RA phase. During the pre-RA phase there is also an increase in circulating inflammatory cytokines and chemokines.

In individuals with HLA-DRB risk alleles and seropositive RA there is an immune response against citrullinated self-peptides. ACPA binds citrullinated peptides and the HLA-DRB risk alleles bind citrullinated peptides with higher affinity[95]. This is due to the HLA-DRB alleles conferring a positive charge at peptide binding groove 4, so they bind citrulline more effectively than positively charged arginine[95]. As the disease progresses, additional post-translational modifications can generate neo-epitopes. This is termed epitope spreading and this is when the initial immune response is directed against one or two dominant epitopes, then there is diversification of the epitopes recognised as the immune response progresses[96].

Although the GWAS studies have highlighted the genetic component of susceptibility, the low rate of concordance between monozygotic twins (12-15%) suggests environmental factors are also important for the development of RA[91]. There are environmental risk factors strongly associated with the development of seropositive RA in individuals with HLA-DRB risk alleles such as smoking[97]. Smoking increases the presence of the peptidyl arginase deiminase (PAD) enzyme in the lung which catalyses citrullination, citrullinated peptides then bind HLA-DRB molecules with greater affinity[98]. Similarly, in the gums, periodontitis has long been linked to RA development and the presence of the bacteria *P. gingivalis,* which has been shown to contain the enzyme PAD, could catalyse citrullination of self-peptides[99]. Additionally, changes in the microbiome have been shown to be associated with RA and these changes can partially normalise after treatment[100].

Post-translational modifications at mucosal barrier sites such as the lung, gum or gastrointestinal tract could cause a loss in immune tolerance. This then localizes in the joints and the pre-RA phase develops into early RA (where there are signs of articular localisation) followed by established disease. See Figure 1-3. During established disease there is on-going inflammation and associated tissue re-

modelling and damage. Theories of how a systemic loss of tolerance could then localise in the joints include autoantibodies becoming stuck in the circulation in joints or autoantibodies activating joint resident cells directly. Microtrauma or a viral infection could increase joint vasculature allowing circulating autoantibodies to enter.

RA is thought to mainly be an immune-mediated disease due to GWAS studies but there are non-immune cells involved in RA pathogenesis too. These include fibroblasts, osteoclasts and chondrocytes which are joint resident cells. These cells could become activated through the presence of autoantibodies and release inflammatory mediators and cytokines, such as TNF-α and IL-8, which then attract immune cells[101,102].

Synovial fibroblasts and macrophages are found constitutively in the synovial membrane of a healthy joint. The synovial fibroblasts produce extracellular matrix which provides structure for the synovium[103]. In contrast, lymphocytes, mast cells and DCs are rare in a normal joint synovium[104]. During RA, synovial fibroblasts proliferate and there is increased blood flow to the joint[105]. This could allow ACPAs to enter, activating synovial fibroblasts and osteoclasts. ACPAs can also bind and activate osteoclasts directly leading to bone reabsorption[101].

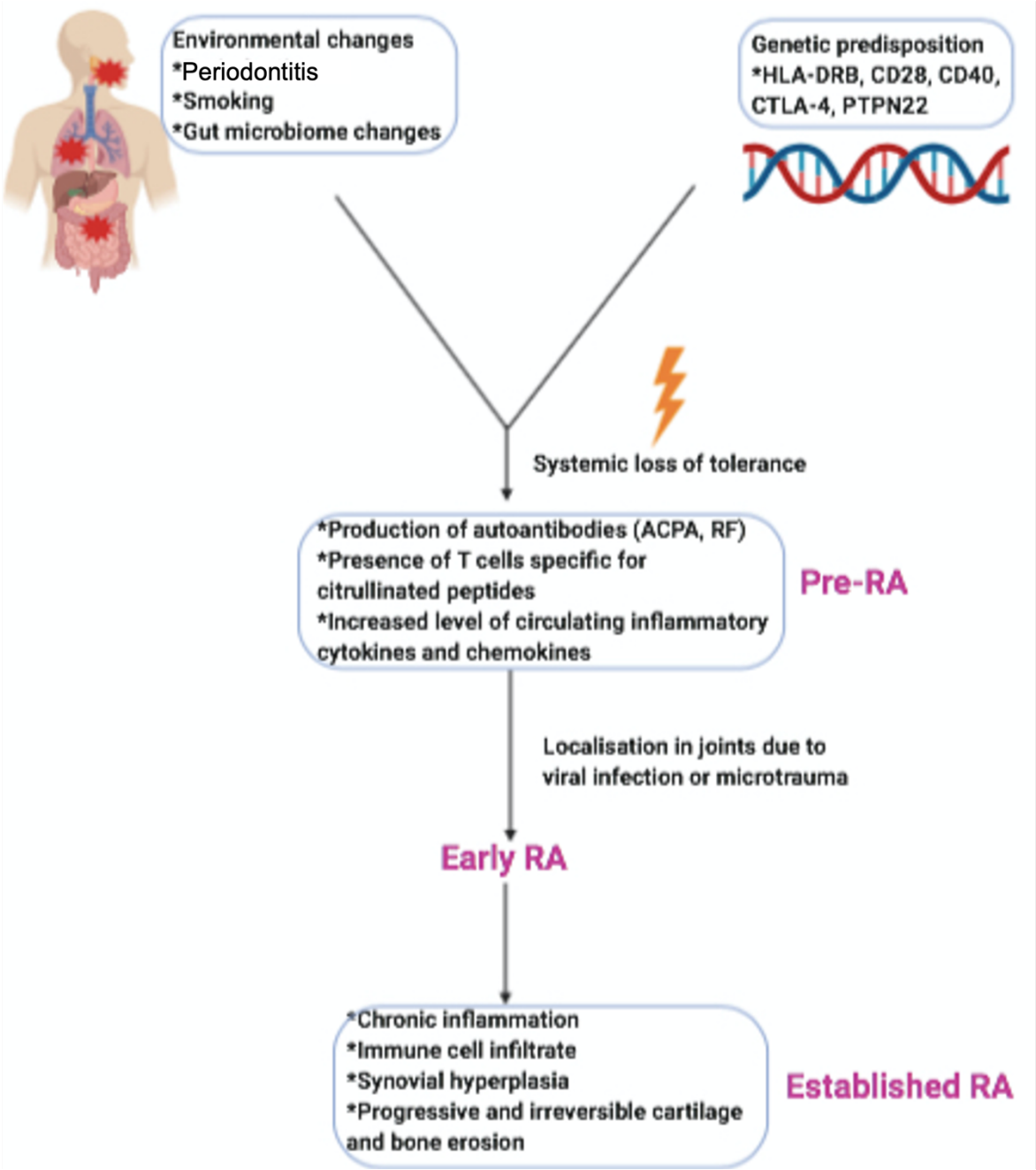


Figure 1-3 The progression of RA from pre-RA to established disease.

Individuals with a genetic predisposition for developing RA are then exposed to environmental factors such as periodontitis, smoking and gut microbiome changes. This results in a systemic loss of tolerance and the pre-RA phase. The pre-RA phase can last for months-years and consists of the presence of circulating autoantibodies, T cells specific for citrullinated peptides and an increase in circulating inflammatory cytokines. There is then a localisation in the joints due to viral infection or microtrauma and this is characterised as early RA. Early RA develops into established RA which is characterised by chronic inflammation, a large immune cell infiltrate, synovial hyperplasia and progressive and irreversible cartilage and bone erosion.

During RA there is a massive immune cell infiltrate into the synovium, including innate (mast cells, macrophages, dendritic cells, NK cells, innate lymphoid cells) and adaptive cells (T+B cells and plasma cells)[91]. B cells produce autoantibodies,

inflammatory cytokines and can present autoantigen to T cells. Figure 1-4 shows all the immune cells involved in the pathogenesis of RA. Compared to a healthy joint there is a huge increase in the number and types of immune cells present. During RA there is an increase in angiogenesis and ectopic lymphoid structures can form allowing new responses to autoantigens to be primed in the joint rather than the LN[106]. Although there is an increase in angiogenesis, the new blood vessels that form are dysregulated which leads to an overall hypoxic environment which can further activate cells[107].

In general, immune cells during RA are more activated and inflammatory, producing many inflammatory mediators and cytokines which can further activate and recruit more immune cells. This is a positive feedback loop and there are not sufficient regulatory mechanisms to stop this once it starts. The bone and cartilage are damaged from the continual exposure to an inflammatory environment. Osteoclasts (bone re-absorbing cells) are activated by autoantibodies and inflammatory cytokines[108,109]. Synovial fibroblasts and macrophages produce matrix metalloproteases (MMPs) which contribute to joint damage[110,111].

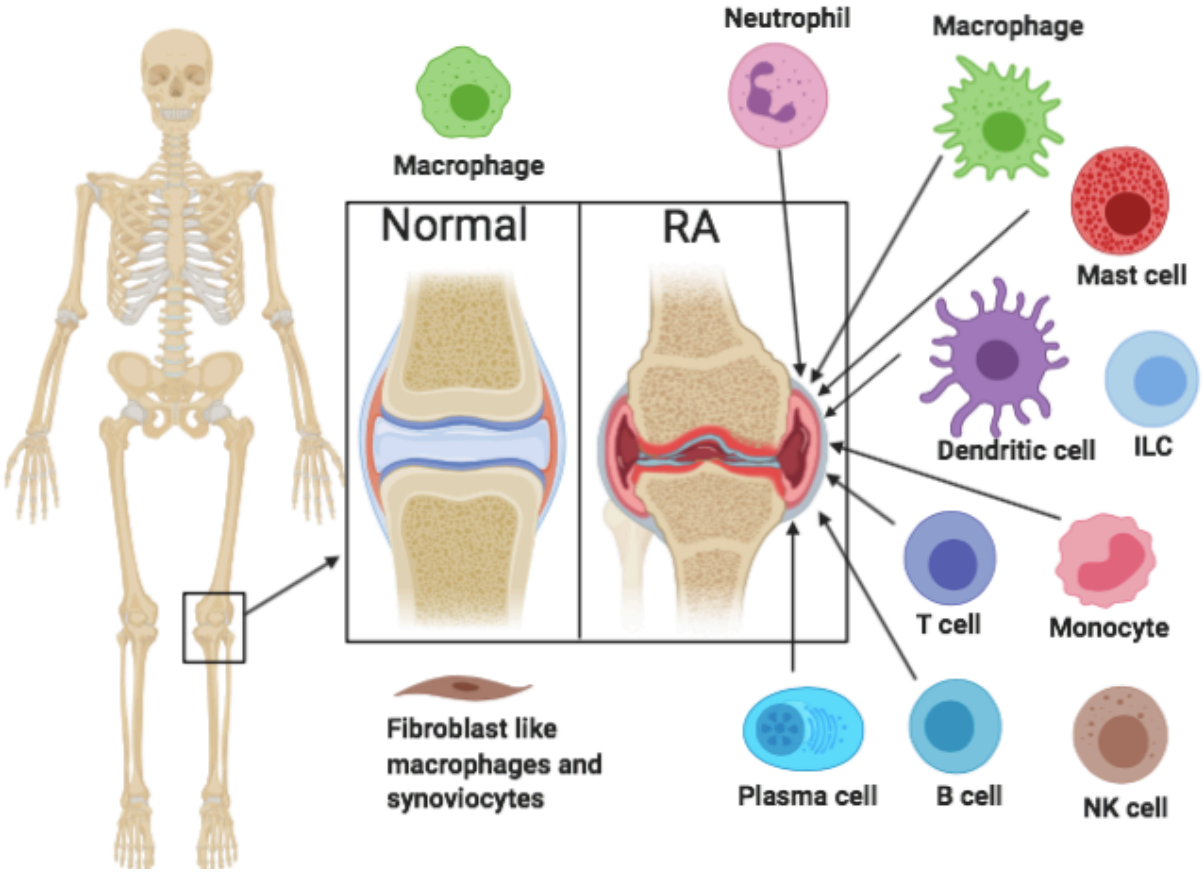


Figure 1-4 Immune cells involved in the pathogenesis of RA.

1.2.1 Monocyte and macrophage involvement in RA pathogenesis

Monocytes are continuously recruited to the joint by inflammatory chemokines and differentiate into mo-macs or mo-DCs there[112-114]. In contrast, monocytes are not found in synovial fluid of healthy controls or RA patients in remission[103].

Mo-DCs from RA patients were found to produce more IL-6 and IL-23, making them better at inducing Th17 cells. Not only this but mo-DCs from RA patients were found to be defective in inducing Tregs[115]. Furthermore, *in vitro* studies have shown that mo-DCs stimulated with CD40-L can degrade collagen via production of TNF-α[116].

Inflammatory mo-macs are thought to be pathogenic whereas resident tissue macrophages could be protective[117]. In a mouse model of immune complex driven, sterile inflammatory arthritis, mo-macs were found to express high levels of co-stimulatory molecules CD80/86 and inflammatory cytokines (IL-1β+IL-12)

when compared with resident macrophages. Resident tissue macrophages were found to be required for the resolution of inflammation in this model.

In humans, inflammatory mo-macs contribute to the synovial tissue macrophage population in active RA. These macrophages are susceptible to anti-TNF-α treatment whereas CD163 macrophages which are typically found in healthy joints are not[118]. During active RA, CD163 tissue-resident macrophages are found in much lower numbers than inflammatory mo-macs.

Macrophages are thought of as being M1-polarised or M2-polarised. LPS and IFN-γ stimulated macrophages which are inflammatory and secrete high levels of inflammatory cytokines are described as M1-like. Whereas, M2 macrophages which are anti-inflammatory, pro-resolution and repair are induced by Th2 cytokines and secrete IL-10 and TGF-β[119]. During RA, macrophages can be activated by auto-antibodies, immune complexes or by citrullinated peptides[120]. Macrophages in RA are generally more pro-inflammatory (M1-like) and produce high quantities of inflammatory mediators such as TNF-α, IL-1 and IL-6[110].

The number of CD68+ macrophages in the synovial sub-lining layer of the joint has been found to correlate with Disease Activity Score 28 (DAS-28). DAS-28 is the main way of scoring RA activity. The number of macrophages present in the synovial sub-lining layer has been found to be the best predictor of treatment response for RA[121].

1.2.2 Dendritic cell and T cell involvement in RA pathogenesis

From GWAS studies the risk alleles for developing RA such as HLA-DRB alleles (MHC Class II molecules), CD28, CD40, CTLA-4 (involved in T cell co-stimulation) and PTPN22 (involved in the threshold of T cell activation) point at the importance of DCs and T cells in RA pathogenesis[122]. Additionally, the use of the biologic drug Abatacept has been found to be a successful treatment for RA[123]. Abatacept is the extracellular domain of CTLA-4 linked to the Fc portion of human IgG. Abatacept blocks DC-T cell co-stimulation as it binds to CD80/86 with higher affinity than CD28. This treatment has been found to effectively inhibit T cell activation, proliferation and production of inflammatory

mediators[124]. As well as this, Abatacept can also prevent CD4+ T cells from becoming follicular T helper cells (Tfh) and providing B cell help[124].

DCs are greatly increased in the RA synovium and synovial fluid compared to healthy controls[125]. This increase could be due to DCs being attracted to the inflamed joint by cytokines or chemokines or DCs could differentiate from myeloid precursors in the synovial fluid directly[126]. DCs are generally more activated during RA, expressing more co-stimulatory molecules and presenting auto-antigen to T cells[127]. DCs can secrete a range of chemokines which can attract macrophages, monocytes and neutrophils to the joint. The secretion of the chemokines CCL3, CCL17, CXCL9 and CXCL10 specifically attracts T cells to the synovium[127]. Intra-articular CD1c+ DCs from RA patients were found to be better at inducing CD4+ T cell proliferation and IFN-γ, IL-17 and IL-4 production by T cells compared to DCs from the peripheral blood[127].

CD4+ T cells are another cell type which is greatly enriched in RA synovium. Effector and memory CD4+ T cells recognising citrullinated peptides are increased in seropositive RA[128]. The majority of CD4+ T cells which recognised citrullinated peptides had a Th1 memory phenotype. Memory T cells which have been previously activated can become re-activated when entering the joint and interacting with APCs there. Th1 cells can activate macrophages through production of IFN-γ. This in turn enhances macrophages ability to present Ag and activate autoreactive memory T cells[103].

A peripheral T cell population expressing PD-1^{high}CXCR5-CD4+ T cells has been found to be expanded in RA[129]. This subset has been found to provide B cell help in producing autoantibodies.

RA is thought to be predominantly a Th1/Th17 condition. DCs can produce IL-12 and IL-23 which skews CD4+ T cells towards a Th1/Th17 phenotype[127]. Circulating Th17 cells and levels of IL-17 are increased in early RA patients and this has been found to strongly correlate with DAS-28 and C-reactive protein (CRP)[130]. The frequency of Th17 cells was found to be even higher in the synovial fluid than the peripheral blood of these patients. In established RA the number of Th17 cells was found to correlate with response to treatment and

DAS-28. An additional study found IL-17 to be upregulated in the joint tissue of early disease and for this to be predictive of joint damage[131]. In contrast, some studies have shown Th1 cells dominate in the joint of RA patients rather than Th17 cells in established synovitis[132].The results of blocking IL-17A or the IL-17R in humans have been disappointing but would perhaps be more effective if patients were stratified by IL-17 levels[133].

IFN-γ may be protective in early disease. Patients with higher levels of IFN-γ were found to have resolving synovitis compared to patients who went on to develop RA[134]. Clinical trials testing recombinant IFN-γ have had mixed results. One showed no improvement of symptoms compared to the placebo whereas the other showed improved disease symptoms[135,136]. Unfortunately attempts to treat patients with anti-IL-12/IL-23p40 and anti-IL-23 to target Th1 and Th17 cells have been unsuccessful[137].

In humans CD4+ T cells have been found which can have both Th1 and Th17 characteristics. T cells which can produce IL-17 and IFN-γ at the same time have been found in patients with seropositive arthralgia[138]. Furthermore, numbers of IL-17+IFN-γ+CD4+ T cells were increased in patients with seropositive arthralgia compared to healthy controls.

The most commonly studied model of arthritis in mice is collagen-induced arthritis (CIA). CIA has similarities with human disease as certain strains of mice are susceptible to developing CIA due to their MHC haplotype. There is a large immune cell infiltrate during CIA and progressive joint damage. Additionally, there are collagen type II specific autoantibodies and rheumatoid factor present in CIA mice. Collagen type II is the dominant collagen type present in the joint[139].

Evidence from mouse models suggests Th17 cells may be more important in pathogenesis than Th1 cells. CIA was found to be accelerated in IFN-γR deficient mice[140]. Although paradoxically, treatment with anti-IL-12 antibody significantly reduced CIA in wild type (WT) and IFN-γR deficient mice[141]. This suggests a protective role for IFN-γ but a potentially pathogenic role for IL-12. In contrast, mice lacking IL-17 had suppressed arthritis and mice treated with IL-17 blocking

antibodies had significantly reduced severity of CIA[142,143]. Conversely, overexpression of IL-17 in the joint was found to promote arthritis and increase joint destruction[144]. Furthermore, IL-6 has been found to be essential for the induction of CIA[145]. IL-6 is a critical cytokine in generating Th17 cells[146].

Using genetically modified mice which specifically lack either IL-12 or IL-23, authors show that IL-23 deletion is protective in CIA whereas IL-12 deletion exacerbates arthritis[147]. The IL-23p19 KO mice were completely resistant to developing CIA. These mice had a decrease in Th17 cells but an increase in Th1 cells. Conversely, IL-12p35 KO mice had an increase in Th17 cells but a decrease in Th1 cells. This suggests that Th17 cells may be more involved in pathogenesis when compared to Th1 cells. Additionally, the timing of IL-12 treatment seems to be important as complete deletion exacerbates arthritis whereas treating with an anti-IL-12 antibody was protective. The loss of Th1 could shift the balance towards a Th17 phenotype.

Not only do Th17 cells contribute to the overall inflammation in the joint, but they also contribute to joint destruction directly. IL-17, which is produced in abundance by Th17 cells, activates macrophages and synovial fibroblasts. The activation of these cells leads to the expression of RANK-L which can interact with RANK on osteoclast precursors (osteoblasts) that then differentiate into osteoclasts[148].

Treg cells are present during RA but are thought to be defective. Treg ($CD4+CD25^{high}$) cells isolated from RA patients were found to be anergic and were unable to suppress activated T cells. Treg suppressive capabilities were found to be restored in patients who responded to anti-TNF-α treatment (infliximab)[149].

In humans, peripheral circulating Tregs which can produce IL-17 have been identified and these cells are increased in RA patients. The number of IL-17 producing Tregs was increased in the synovial fluid compared to the peripheral blood. These Tregs maintained their suppressive capabilities in the blood but these were lost in the synovial fluid maybe due to the dominant inflammatory environment there[150]. An *in vitro* study has shown FoxP3+ Tregs can produce IL-

17 when activated by inflammatory cytokines IL-1β and IL-6[151]. Alternatively, Tregs in the joint could be exhausted.

In mice, using a reporter system for the fate mapping of cells, FoxP3+ Tregs have been shown to be able to lose their FoxP3 expression and differentiate into pathogenic Th17 cells in a mouse model of CIA[152]. This conversion was mediated by IL-6. These ex-Tregs were more osteoclastogenic than Th17 cells that had developed normally from T cells. This cross over between Tregs and Th17 cells could be due to the similarities in their developmental pathways. Tregs develop in the presence of TGF-β alone, whereas Th17 cells develop in the presence of TGF-β and IL-6[146]. Hence, Tregs could develop into Th17 in an inflammatory environment.

During RA, DCs skew CD4+ T cells towards a Th1/Th17 phenotype and there is a defect in Tregs which contributes to pathogenesis.

1.2.3 Current treatments for RA

Early diagnosis and treatment of RA is essential in preventing joint damage, increasing the chances of remission and reducing mortality[153]. Current treatments for RA involve broad immunosuppression. This leaves patients at risk of developing infections[154]. Treatments for RA include Disease Modifying Anti-Rheumatic Drugs (DMARDs), that range from methotrexate to biologics. These treatments are expensive, life-long and rarely achieve long-term remission.

Biologics targeting TNF-α and IL-6 have been found to be relatively successful treatments in RA but there remains a high rate of non-responders. Rituximab which targets B cells (anti-CD20) and Abatacept which targets DC-T cell co-stimulation (CTLA-4:Ig) have been found to be similarly successful[123,155].

Abatacept has been found to reduce synovial IFN-γ and the immune cell infiltrate in synovial biopsies from patients[156,157]. Abatacept not only blocks interactions between DCs and T cells but it also stops interactions between other cells which express CD80/86 such as B cells, osteoclasts, macrophages and endothelial

cells[158]. This was found to reduce TNF-α and IL-12p70 production by macrophages[159] and inhibit osteoclast differentiation[160].

Regardless of the treatment chosen, 30-50% of patients will not respond adequately highlighting the need for more effective treatments[161]. Furthermore, half of patients will relapse when treatments stop and very few will achieve long-term remission[162]. The ultimate goal is to achieve remission through Ag-specific treatment which targets pathogenic responses while leaving protective responses intact. This would reduce the side effects seen with broad immunosuppression such as increased susceptibility to developing infections. The current treatments for RA treat disease symptoms but do not target the underlying causes of autoimmunity and do not reinstate tolerance. Therefore, when treatments stop a high proportion of patients will relapse.

1.3 Antigen specific tolerogenic therapies

Multiple Ag-specific tolerogenic therapies are being investigated for the treatment of autoimmunity. These include peptide therapy, nanoparticles, DNA vaccines, antibody (Ab) mediated Ag targeting to DCs *in vivo,* Treg therapy and tolerogenic dendritic cells (tol-DCs).

When the Ag is known, peptide therapy can be very effective. Peptide therapy has been investigated mainly as a treatment for allergy. Administering low doses of peptide or peptide epitopes promotes tolerance to that peptide. DCs engulf the peptide and promote expansion of Tregs or T cell deletion[163]. In the case of allergy, the antigen is external and not an autoantigen. However, trials testing peptide therapy have been completed for type 1 diabetes[164,165], MS[166], and RA[167]. One type I diabetes trial found reduced IFN-γ production by T cells and a bias towards Th2 with peptide therapy whereas another diabetes type I trial with the same peptide from the insulin β chain found no effect on β-cell function[164,165]. A clinical trial testing heat shock protein dnaJP1 peptide therapy in RA patients found a significant decrease in TNF-α producing T cells and an increase in IL-10 producing T cells with peptide therapy. Myelin basic protein peptide therapy delayed disease progression in a subset of MS patients and there were decreased levels of autoantibodies in the cerebrospinal fluid[166].

Nanoparticles, like peptide therapy, work by delivering Ag to APCs. Nanoparticles are small delivery systems which range from 50nm to 1μm in size and are readily phagocytosed by APCs. Nanoparticles can contain Ag alone, which in the absence of inflammatory signals promote tolerance, or they can contain Ag and a tolerising signal. Nanoparticles made of liposomes rather than metals are favourable as liposomes will degrade and not build up within the host causing toxicity[168]. Liposomes have been shown to be able to suppress arthritis in mice in an Ag-specific manner[169].

DNA vaccines can be utilised which result in the transient expression of a protein in non-inflamed tissues e.g., insulin. DNA vaccines have been investigated for type 1 diabetes and MS[170–172].

Ag coupled to Abs which target DC-specific receptors such as DEC-205 and DC-SIGN is an additional strategy to promote tolerance. DEC-205 and DC-SIGN are endocytic receptors and when targeting these receptors on steady state DCs will favour tolerogenic responses. This approach has been shown to be effective in preventing mouse models of arthritis, diabetes and MS[173–175].

Tolerising cellular therapies such as tolerogenic dendritic cells and Tregs are being investigated as potential treatments for autoimmunity. Tregs specific to an autoantigen can be expanded *in vitro* then administered to a patient. Unfortunately, there are problems relating to Treg therapy including the product being unstable[176]. Additionally, Ag-specific Tregs are rare, and it is not yet possible to expand only Ag-specific clones, the Tregs are generated through polyclonal stimulation. This means that there is a risk pathogenic clones are also expanded which could exacerbate autoimmune diseases.

1.4 Tol-DCs

The idea to use *in vitro* generated mo-DCs therapeutically was first developed for cancer treatment[177]. Monocytes can be isolated from a patient's blood and induced to become mo-DCs by being cultured in the presence of IL-4 and GM-CSF[178]. Mo-DCs generated *in vitro* were found to be transcriptionally very similar to mo-DCs found *in vivo*[10]. For cancer immunotherapy these mo-DCs are reprogrammed to induce strong T cell responses against tumour Ags. These cells

are then administered to the patient to try and stimulate an immune response against the tumour.

This idea was then adapted for the treatment of autoimmunity. Monocytes can be isolated from a patient's blood and reprogrammed to be immunoregulatory. These cells are known as tolerogenic DCs (tol-DCs). Tol-DCs have been investigated as a potential treatment for rheumatoid arthritis, lupus, type I diabetes, allergy, transplantation, multiple sclerosis and Crohn's disease[179–187]. For this thesis I am going to focus on tol-DCs as a potential treatment for RA as that is our intended use for them.

Tol-DCs allow for an Ag-specific suppression of the immune response while keeping protective immunity intact. Due to this reason, tol-DC treatment would reduce the side-effects seen with current medication for autoimmunity.

Tol-DCs can be generated from mo-DCs *in vitro* by treating with certain immunosuppressive drugs (e.g. glucocorticoids, NF-KB inhibitors)[188,189], reagents (vitamin D3)[190] or cytokines (IL-10 or TGF-β)[191,192]. Alternatively, tol-DCs can be genetically engineered from DCs either by over-expressing immunomodulatory molecules such as Fas-L, TRAIL, IDO or CTLA-4 or by silencing immune-stimulatory molecules such as IL-12p70, IL-23p19, CD40 or CD80/86[193–199]. Additionally, immature DCs are tolerogenic but are unstable and can mature *in vivo*. Similarly, short exposure to LPS or TNF-α can generate tol-DCs but there are conflicting reports whether these can be immunogenic *in vivo*[200,201].

The NF-KB signalling pathway is essential in the maturation of DCs and the majority of mechanisms to generate tol-DCs work by inhibiting NF-KB induced maturation. For example, Dex+VitD3 have been shown downregulate components of the NF-KB pathway such as Rel-b[190]. The addition of Dex+VitD3 together stops the desensitisation and downregulation of glucocorticoid receptors and results in increased production of IL-10 by tol-DCs[202].

Maturation of tol-DCs with LPS, MPLA or an inflammatory cytokine mixture enhances their Ag presentation capacity, ability to migrate to LNs via CCR7 and

makes them resistant to further maturation[203,204]. MPLA is a non-toxic homolog of LPS which is approved for use in humans[205].

Immature or mature DCs can be genetically engineered to generate robust tol-DCs. In contrast, the addition of immunosuppressive cytokines or drugs must occur before maturation to generate tol-DCs[206,207]. However, when corticosteroids (e.g. Dexamethasone) are added too early (within the first 48hrs of culture) their differentiation from monocytes into immature DCs is inhibited[208].

The comparison of different tol-DC types is confounded by the differences in protocols. For example, IL-10 can be added throughout the whole culture or at the same point as maturation[49,209]. DC-10 are a subset found *in vivo* but they appear to be identical to IL-10 tol-DCs generated *in vitro* so the terms are sometimes used interchangeably[49]. Interestingly, IL-10 tol-DCs were found to express higher levels of soluble and membrane bound CD25[191]. The soluble form of CD25 is thought to scavenge IL-2 from T cells which could be an additional mechanism for inducing T cell anergy.

Although tol-DCs can be generated in a variety of different ways there are general features which are thought to be common to all tol-DC types. These include a reduced expression of co-stimulatory molecules such as CD40, CD80/86 when compared to mature mo-DCs[203]. Tol-DCs are sometimes referred to as semi-mature. Rapamycin tol-DCs are the exception to this rule, being the only tol-DC type, which exhibits a mature phenotype.

Importantly, tol-DCs have an altered cytokine profile with reduced production of pro-inflammatory cytokines and an increased production of anti-inflammatory cytokines such as IL-10 and TGF-β[210]. Tol-DCs are thought to mediate their effects mainly through T cells. Depending on the tol-DC type T cell anergy, T cell deletion or the induction of Tregs (FoxP3+ or Tr1) can be achieved[49,194,211,212]. A summary of the main tol-DC types, phenotypes, migration and mechanisms of action can be seen in Table 1-1. A figure summarising tol-DCs mechanisms of action can be seen in Figure 1-5.

Tol-DCs genetically engineered to express Fas-L or TRAIL delete autoreactive T cells and are sometimes referred to as killer DCs[194,213]. High expression of co-stimulatory molecules may be required to induce FoxP3+ Tregs[214]. Tol-DCs genetically engineered to express CTLA-4 or IDO, Rel-b inhibited or rapamycin tol-DCs are the only tol-DC types shown to induce FoxP3+ Tregs[195]. High production of IL-10 is thought to be required to induce Tr1 cells and the majority of tol-DC types are able to induce Tr1 cells.

Tol-DCs can induce other regulatory cells other than Tregs. For example, Dex+VitD3 tol-DCs have been shown to be able to induce regulatory B cells (Bregs). Bregs themselves have been found to be able to suppress Th1/Th17 responses and to induce FoxP3+ Tregs[215].

Memory T cells are more resistant to tolerance inducing mechanisms when compared to naïve T cells. This is due to memory T cells being able to respond rapidly when they recognise their Ag and having low requirements for costimulation[211]. It is reassuring that multiple studies have shown tol-DCs can induce anergy in memory T cells[211,216,217].

Rapamycin and TGF-β tol-DCs are the only tol-DC types which show CCR7 expression similar to mature mo-DCs after stimulation. This will have implications for the ability of different tol-DC types to migrate to the LNs after administration.

Table 1-1 A summary of the main tol-DC types, phenotype, migration and mechanisms of action.

Type of Tol-DC	Phenotype	Migration	Mechanism of Action
IL-10	↓CD40, 83, 86 and similar MHC Class II when compared to mature mo-DCs[203] ↑Inhibitory receptors (ILT2,3+4+HLA-G)[209] ↓IL-6, IL-12+IL-23, TNF-α+IL-1β ↑IL-10[203,218]	↓CCR7 expression and reduced migration[203] $CD83^{high}$ CCR7+ population show strong migration towards CCL21 after stimulation with a maturation mixture[191]	Induction of T cell anergy in naïve and memory T cells[46,211] ↓T cell proliferation, IL-2+IFN-γ[211] Induction of IL-10 secreting Tr1 cells[49]
TGF-β	↓CD40, 83, 80+86 and similar MHC Class II when compared to mature mo-DCs[203] ↓IL-6, IL-12, IL-23, TNF-α and IL-1β[203,219]	CCR7 expression similar to mature mo-DCs[203]	↓T cell proliferation[219]
Dex	↓CD40,80,83+86 and similar MHC Class II when compared to mature mo-DCs[220,221] ↓IL-6, IL-12, IL-23, TNF-α[203] ↑IL-10[220]	Showed high migration towards CCL5 a ligand of CCR5 without any stimulation ↓CCR7 and migratory capacity ↓CXCR4 expression[203,220] Showed high migration towards CXCL10 a ligand of CXCR3 after LPS stimulation[222]	↓T cell proliferation, IFN-γ+IL-17 Induction of anergy in naïve and memory CD4+ T cells[216,220] Induction of IL-10 secreting Tr1 cells[222]
VitD3	↓CD80/86 and MHC Class II when compared to mature mo-DCs[221] ↑ILT3[223] ↑PD-L1 expression[222] ↓IL-6, IL-12, IL-23 and TNF-α ↑IL-10[210]	↓CCR7 expression and migratory capacity[203]	↓T cell proliferation+IFN-γ[221] Induction of T cell apoptosis[224] Induction of IL-10 secreting Tr1 cells[225]
Dex+VitD3	↓CD40, 83+86 molecules and similar CD80+MHC Class II when compared to mature mo-DCs ↓IL-6, IL-12 and TNF-α ↑IL-10 and TGF-β production[217,226]	↓CCR7 and CXCR4 expression and migratory capacity Express CCR1, CCR2+CCR5[204]	Induction of anergy in memory T cells ↓T cell proliferation+IFN-γ[217] Induction of IL-10 secreting Tr1 cells Induction of CD19+ IL-10 producing Breg cells[227]
Rapamycin	↑co-stimulatory molecules (display a mature phenotype), similar MHC Class II when compared to mature mo-DCs ↓TNF-α, IL-6 and IL-23[203]	CCR7 levels similar to mature mo-DCs[203]	↓T cell proliferation+IFN-γ[221] Induction of T cell anergy[228] Induction of FoxP3+ Tregs[221]
NF-KB inhibitors	↓ CD40,80+86 Similar MHC Class II when compared to mature mo-DCs[229] ↓IL-12 ↑IL-10[230]		↑% FoxP3+Treg ↓ T cell proliferation+ proinflammatory cytokines[229] T cell anergy[231]

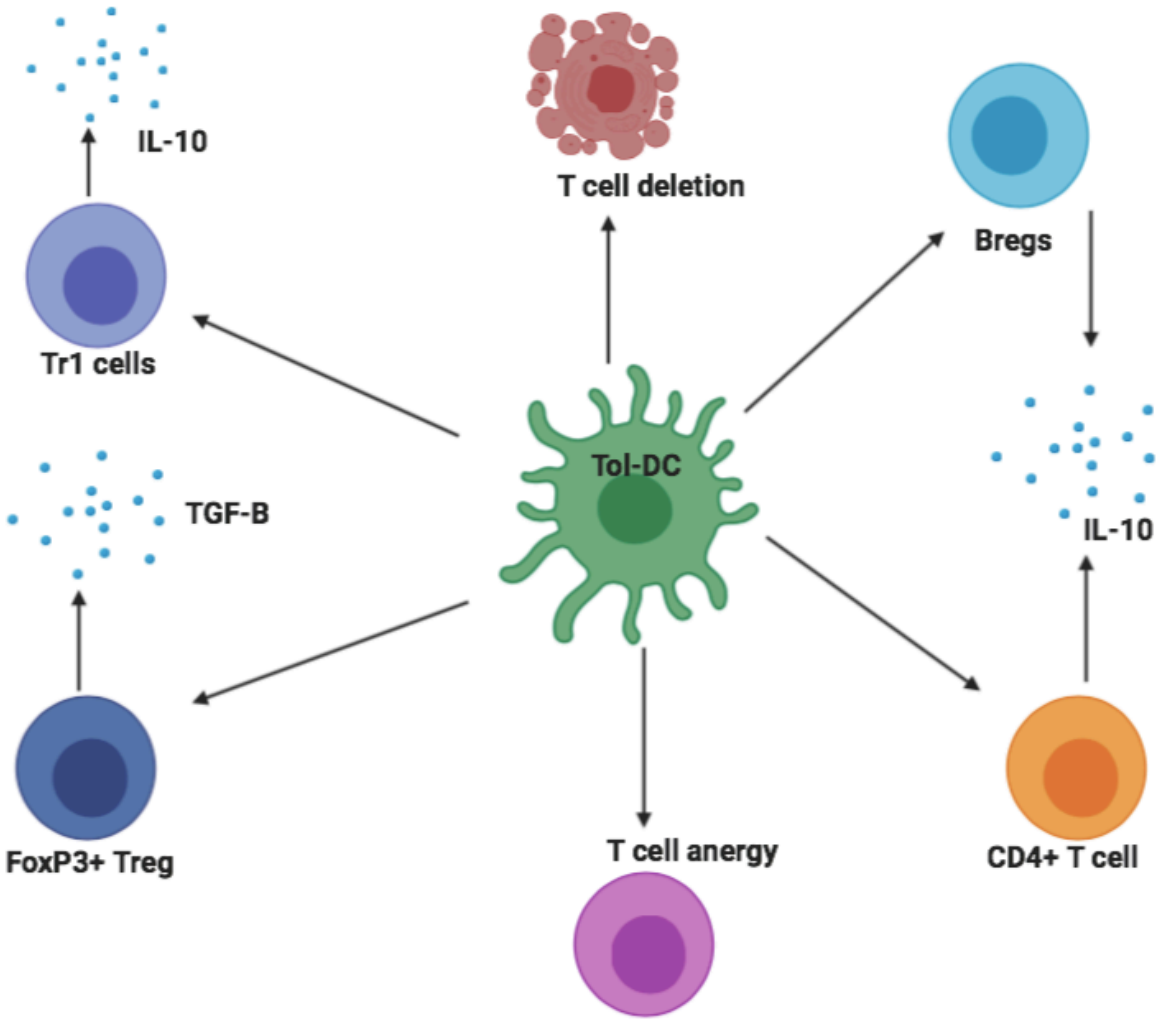


Figure 1-5 A summary of tol-DCs mechanisms of action.

Tol-DCs mainly mediate their effects on T cells. Tol-DCs can induce T cell deletion, anergy or skew T cell cytokine profile towards IL-10. Tol-DCs can induce FoxP3+ Tregs, Tr1 cells or Bregs.

1.4.1 Bone marrow-derived dendritic cells

Due to cDCs being rare in tissues and blood, many studies have relied on generating and expanding DCs using *in vitro* systems. A common method for generating DCs from mice is from bone marrow as bone marrow contains monocytes and common DC precursors (CDPs). GM-CSF is added to induce DC differentiation and growth[62]. BM-DC cultures will contain a mixture of mo-DCs, mo-mac and DCs from CDPs[63]. Tol-DCs generated from mice are induced from BM-DCs. This differs from the human tol-DC cultures which are generated from monocytes and are a mixture of mo-DC and mo-mac. The differences in production between human and mouse tol-DCs can be seen in Figure 1-6.

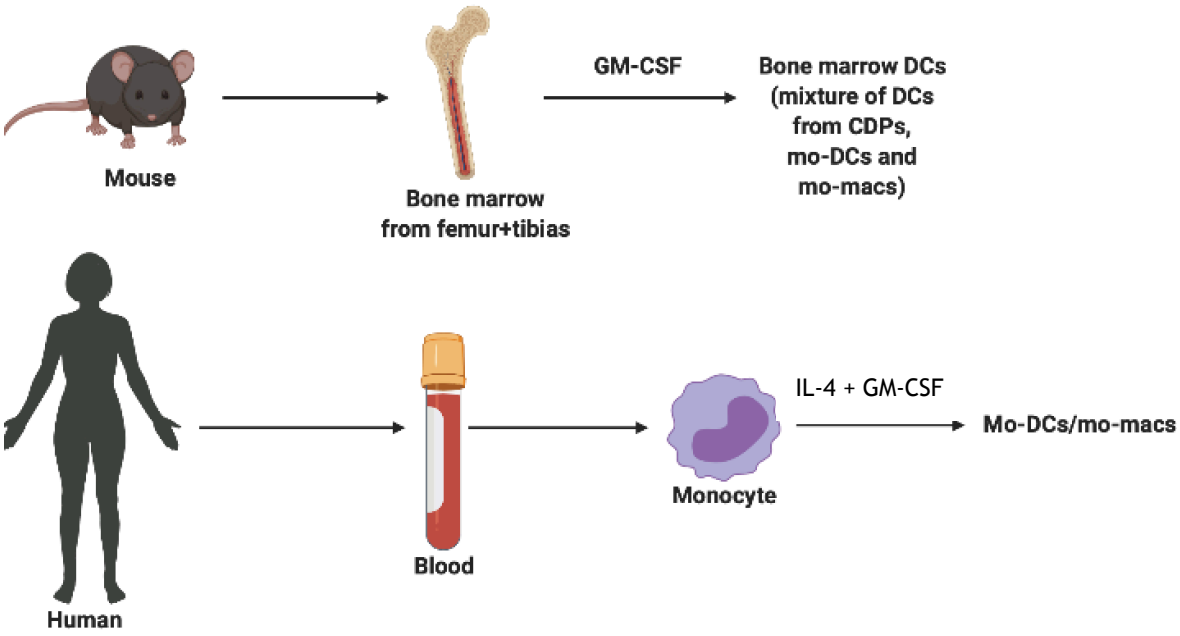


Figure 1-6 Differences in production of tol-DC from mice and humans.

1.4.2 Tol-DCs in mouse models of arthritis

Tol-DCs have been tested multiple times in different mouse models of arthritis, these studies have been summarised in Table 1-2. The most common arthritis model tol-DCs have been tested in is CIA. During CIA, mice are injected with collagen type II, the antigen the autoimmune response is directed against, and a strong immunostimulatory adjuvant. The mice are usually injected once with collagen type II and Complete Freund's adjuvant (CFA) then given a booster injection to stimulate arthritis induction. LPS can be administered to try and synchronise the onset of arthritis.

Other mouse models of arthritis include antigen induced arthritis (AIA), humanised proteoglycan induced arthritis and the ovalbumin (OVA) induced arthritis model which is sometimes referred to as the OVA induced breach of tolerance model. To induce AIA, mice are immunised with methylated bovine serum albumin (mBSA) and adjuvant then mBSA is injected intra-articularly to induce arthritis in the joint[189]. The humanised proteoglycan induced model involves injecting BALB/c mice twice with human proteoglycan and dimethyl-dioctadecylammonium (DDA) as an adjuvant[232]. The OVA induced arthritis model utilises OT-II, TcR transgenic CD4+ T cells with a TCR specific for OVA peptide (aa 323-339). OT-II CD4+ T cells are polarised *in vitro* towards a Th1 phenotype and adoptively transferred into C57BL/6 mice. C57BL/6 mice are then

challenged with OVA and CFA. Heat aggregated OVA is then injected intra-articularly or in the footpad to induce a loss of tolerance in the joint[233].

Table 1-2 A summary of the studies testing tol-DCs in mouse models of arthritis.

Tol-DC type	Model	Number of cells	Injection routes	Time-points	Outcome	Effects on T cells
Dex+VitD3 +LPS Humanised proteoglycan loaded	Humanised proteo-glycan induced model	1 million	IV	Day 17 before the 2nd hPG/DDA injection	Loaded or unloaded tol-DCs ameliorate arthritis.	Tol-DCs can reduce naïve and effector CD4+ T cell activation and proliferation ↑ FoxP3+ Tregs[232]
Dex+VitD3 +LPS Collagen type II loaded	Established CIA	1 million, 200,000 or 40,000 IV 2.5 million IP	IV or IP	Injections IV given 1 or 3x Injections IP given 3x. Days 3, 7 and 11 after arthritis onset (day 24).	Loaded not unloaded tol-DCs reduced clinical score of CIA. 1 or 2.5 million tol-DCs equally as good. 200,000 or 40,000 not effective. Need multiple injections not just one. IP injection not effective.	↓ Th17 cells ↑ IL-10 producing CD4+ T cells[234]
Tacrolimus tol-DCs +LPS+IFN-γ No Ag loading	Established CIA	10^4 10^5 10^6	IP	Days 27, 31 or 35 or solely on day 27	10^6 cells injected 3x had the best effect. CIA progression and severity significantly inhibited. Less immune cell infiltrate and damage.	Suppressed CD4+ T cell proliferation ↓inflammatory cytokines and Th17[235]
Tacrolimus tol-DCs + TNF+PGE2 Collagen type II loaded	CIA	500,000	IV	Day 7 and 14 after 1st immunisation	Reduces severity of arthritis in an Ag-specific manner	Autoreactive T cells deleted[236]
IL-10+TGF-β No stimulation Collagen type II loaded	CIA	1 million	IV	3 weeks after 1st immunisation	Suppressed CIA	Suppressed memory T cell response ↑ Tregs[237]
IL-10+TGF-β tol-DCs +LPS Collagen type II loaded	Established CIA	5 million-50,000	IV	Injected 4 weeks after induction of CIA	500,000 the best. CIA progression inhibited.	↓ inflammatory cytokines ↑ anti-inflammatory cytokines+ Treg:Th17 ratio[238]
TNF, IL-10 or Dex -No further stimulation	CIA	2.5 million	IV	-7, -5 or -3 before immunisation	Able to inhibit the development of arthritis	Th2 skewing for TNF and IL-10 tol-DCs[239]

for TNF, LPS stimulation for IL-10+Dex Collagen type II loaded						
Short term LPS stimulation No further stimulation Collagen type II loaded	Established CIA	500,000	IP	Day 35 after 1st immunisation	Significantly reduced CIA severity. Reduced synovitis and cartilage damage.	↓ IFN-γ by splenocytes stimulated with collagen type II[240]
Short term LPS stimulated No further stimulation	Established CIA	500,000	IP	Day 35 after 1st immunisation	Inhibit CIA in an IL-10 and TGF-β dependent manner	↑IL-10+TGF-β producing CD4+ T cells ↓ Th17[241]
TNF No further stimulation Collagen type II loaded	Established CIA	200,000	SC	Once a week starting at day 44 after 1st immunisation	Semi-mature DCs loaded with collagen type II and methotrexate most effective in inhibiting arthritis	↑FoxP3+ Tregs+IL-10 ↓Autoreactive T cells, IFN-γ+IL-17[242]
TNF No further stimulation Collagen type II loaded	CIA	200,000 or 2 million	SC abdominal area	Day 21+31 after 1st immunisation	Low doses anti-arthritic whereas high doses exacerbated arthritis	Low dose induced FoxP3+ Tregs, Th2 cytokines and TGF-β[201]
TNF No further stimulation Collagen type II loaded	CIA	1 million	SC base of tail	3 days before immunisation then 3 days before booster	Delayed onset and reduced severity	↓ IgG2a[243]
TNF No further stimulation Collagen type II loaded	CIA	2.5 million	IV	-7, -5, -2 prior to immunisation	TNF-DCs + collagen type II lowered arthritis score the most	↓ Th1 response[244]
Knock down of IL-12p35 No stimulation Collagen type II loaded	CIA	5 million	IP	7 days before and/or 12 days after 1st immunisation	Inhibited progression of arthritis, reduced clinical scores, decrease immune cell infiltrate	Suppressed T+B cell responses to collagen type II[197]
Knock down of CD40, CD80+CD86 No stimulation	CIA	5 million	IV	Day 7+14 after 1st immunisation	Reduced disease severity	↓ inflammatory cytokines+Ab response ↑FoxP3+ Tregs[245]

Collagen type II loaded						
Rel-b silenced tol-DCs No stimulation or Ag loading	CIA	2 million	IV	7 and 14 days after 1st immunisation	Inhibited progression of CIA	Suppression of collagen type II T cell responses. ↓proinflammatory cytokines ↑% of FoxP3+Treg[229]
NF-KB inhibitor (BAY-11-7085) No stimulation mBSA loaded	Established AIA	500,000	SC base of the tail	2, 4 or 6 days after mBSA was injected intra-articularly	Day 2 and 4 better. Effect even after 10 days. Inflammation and damage reduced in mice given tol-DCs.	Beneficial effect dependent on IL-10. Change in Ab isotype.[189]
DCs treated with anti-NF-KB agent (LF-15-0195) +LPS+TNF No Ag loading	CIA	5 million	IP	Day 12 after 1st immunisation	Improved CIA clinical score. ↓immune cell infiltrate in the joints.	↓T cell proliferation, Ab+IFN-γ[230]
Genetically engineered to express IDO or CTLA-4 No stimulation or Ag loading	Established CIA	1 million	IV	4 days after LPS injection on day 32	Improval of arthritis	Induction of FoxP3+ Treg[246]
Engineered to express TRAIL +LPS Collagen type II loaded	CIA	5 million	IP	2 weeks after 1st immunisation. 2x per week for 2 weeks.	Reduced incidence of arthritis and infiltration of T cells	Depletion of autoreactive T cells ↓ IFN-γ[194]
Genetically engineered to express Fas-L No stimulation or Ag loading	Established CIA	1 million	IV	28 days mice received LPS. Mice received tol-DCs 4 days after.	Substantial disease improvement	Depletion of autoreactive T cells. Reduction in IFN-γ and T cell proliferation[213]
Genetically engineered to express IL-4 No stimulation or Ag loading	Established CIA	1 million	IV	4 days after LPS injection to stimulate CIA (on day 32 after 1st immunisation)	Suppressed disease	Th2 skewing[193]
Genetically engineered	CIA	300,000	SC, IV or IP	15 days after 1st	Reduced incidence and severity of CIA	Th2 skewing[247]

to express IL-4 No stimulation or Ag loading				immunisation		

The results of testing tol-DCs in various mouse models of arthritis conclude that tol-DCs have a beneficial impact on disease. The number of tol-DCs injected in these studies varies from 40,000 to 5 million. The tol-DCs were either administered once or multiple times. Two studies which compared multiple injections of tol-DCs with one injection of tol-DCs concluded that multiple injections of tol-DCs are required to maximise tol-DCs effects[234,235]. The dose and number of injections needs to be optimised for each tol-DC type and model used.

Some studies have shown loading tol-DCs with a relevant autoantigen is essential for their therapeutic effect[234]. In contrast, other studies have shown tol-DC treatment can be effective even without Ag loading. For example, one study found IL-10 and TGF-β tol-DCs could still be beneficial in CIA when left unloaded (no collagen type II) and unstimulated (no LPS). Although, tol-DC treatment was found to have more of a beneficial effect on arthritis when loaded with relevant autoantigen and stimulated with LPS[238].

The answer to whether tol-DCs need to be loaded/unloaded and stimulated/unstimulated will depend on the tol-DC type and the model. One study found stimulation is required for Dex+VitD3 tol-DCs to have a tolerising effect but not Ag loading[232].

Stimulating tol-DCs before transfer will reduce their Ag uptake *in vivo* but will also ensure stability of the tol-DC product. When the Ag is not known, Ag uptake *in vivo* may be required. However, it is then hard to ensure the tolerising effect is directed against the antigen driving the pathogenic immune response.

In summary, it seems that when tol-DCs are stimulated prior to transfer, Ag loading is required to see a therapeutic effect[194,230,234,240]. In contrast, if tol-DCs are left unstimulated Ag loading is not required. This is probably due to unstimulated tol-DCs being able to take up Ag *in vivo*[193,213,246,247].

The injection route chosen for tol-DC transfer is important in determining the success of treatment. For example, Dex+VitD3 tol-DCs were found to be effective via intravenous injection but not intraperitoneal[234]. Subcutaneous, intravenous and intraperitoneal injection of tol-DCs have been tested in mouse models of arthritis with intravenous being the most common. Very few studies have tested more than one injection route at a time. The route of injection will have to be optimised for each tol-DC type and model.

Tol-DCs have been tested at different time-points in the CIA model prophylactically and during disease. When given prophylactically tol-DCs can prevent CIA development and some tol-DCs have been found to be effective even in established disease (see table 1-2).

Not all tol-DCs types would be effective prophylactically and the tol-DCs mechanism of action needs to be taken into account. For example, Fas-L tol-DCs which act by killing autoreactive T cells would probably not be useful prophylactically as no autoreactive T cells would be present. As disease progresses and epitope spreading occurs Fas-L tol-DCs may not be as effective as other tol-DC types which induce Tregs. Tregs can act via bystander suppression and induce DCs to express less co-stimulatory molecules and produce more IL-10.

Tol-DCs have been shown to be an effective treatment in mouse models of arthritis and can have effects on T cell populations such as an increase in Tregs and a decrease in Th1/Th17. Additionally, they can alter the cytokines produced by T cells with a decrease in IFN-γ and an increase in IL-10+TGF-β. The dose, number of injections and route of injection needs to be optimised for each tol-DC type and model.

1.4.3 Clinical trials with tol-DCs

Table 1-3 Clinical trials with tol-DCs

Adapted from: Phillips, B.E. et al. Clinical tolerogenic dendritic cells: exploring therapeutic impact on human autoimmune disease *Frontiers in Immunology* (2017) 8:1279.

Clinical trial/disease	Type 1 Diabetes[181]	Rheumavax[180] RA	Autodecra[179] RA	Crohn's disease[184]	Multiple Sclerosis+ Neuromyelitis[182]	Multiple Sclerosis[183]	Liver transplant recipients[248]
How tol-DCs were generated	Anti-sense CD40, CD80+CD86	BAY 11-7082 (NF-KB inhibitor)	Dex+vitD3 Stimulated with MPLA	Dex+vitA Stimulated with cytokines	Dex Stimulated with cytokines	VitD3 Stimulated with cytokines	VitD3+IL-10 Unstimulated
Antigen	None	4 citrullinated peptides	Synovial fluid	None	7 myelin peptides +/- AQP4	7 myelin peptides	None but tol-DCs donor derived
Injection concentration	$1.0x10^7$	0.5-1.0 or 2-4.5x10^6 (depending on weight of patients)	1.0, 3.0 or 10.0x10^6	2.0, 5.0 and 10.0x10^6	50, 100, 150 or 300 x10^6	5.0, 10.0 or 15.0x10^6	2.5-10x10^6 per kg
Dose	1 injection	1 injection	1 injection	1 or 3 injections	1 injection	1 injection	1 injection
Frequency	2 weeks apart (4x)	1x	1x	1 or 3 injections every 2 weeks, escalating doses (3x or 9x)	3x, 2 weeks apart	First 4 injections every 2 weeks, injection 5+6 at monthly intervals	1x 7 days before transplant
Injection site	Intradermal	Intradermal	Intra-articular (knee joint)	Intraperitoneal	Intravenous	Intradermal or intranodal	Intravenous
Study length	12 months	6 months	91 days (~3 months)	3 months	6 months	12 months	Only until transplant
Outcomes	Increase in Breg population	Decrease in CRP and DAS28	Stabilisation of symptoms in 2	Crohn's disease activity index decreased, 1	PBMCs stimulated with peptide produced less IL-	Not published yet	Intact tol-DCs cross dressing

	Increase in serum IL-4+IL-10	Decrease in IL-15, IL-29, CX3CL1 + CXCL11	patients receiving the highest doses	patient reached clinical remission and 2 clinical response	10. Decrease in NK cells		host DCs in blood and LNs PD-L1 co-localisation with donor HLA at higher levels than with recipient HLA
Changes to T cells	Slight increase in $CD25^{high}$ FoxP3+cells	Significant reduction in effector T cells Increased ratio of regulatory to effector T cells Reduction in IL-6 T cell response to $vimentin_{447-455}$–Cit450	No systemic effects observed	% of circulating FoxP3+ Tregs significantly increased Reduced IFN-γ production after *ex vivo* CD3 stimulation of T cells	Increase in Tr1 cells. Decrease in memory CD8+ T cells	Not published yet	Decrease in memory CD8+ T cells Increase in CD25+FoxP3+ Treg/memory CD8+ T cell ratio

The results of the clinical trials completed to date with tol-DCs can be seen in Table 1-3. The overall finding was tol-DC treatment is safe and well tolerated. As can be seen from the table, the trials completed differ in many aspects. Firstly, all the tol-DCs were generated in different ways. Secondly, the tol-DCs were stimulated with cytokines or MPLA or left unstimulated. Thirdly, the tol-DCs were either loaded with a relevant autoantigen for the disease or left unloaded.

AUTODECRA (Autologous Tolerogenic Dendritic Cells for Rheumatoid Arthritis) was a clinical trial completed at Newcastle University in 2017. The aim of AUTODECRA was to test the feasibility and safety of intra-articular injection of Dex+VitD3 tol-DCs in inflammatory arthritis patients. The treatment was found to be safe and well tolerated. Although there were no systemic effects observed with tol-DC treatment, there was a stabilisation of symptoms in patients receiving the highest doses. The second AUTODECRA trial is scheduled and currently recruiting, and this trial aims to test the immunomodulatory effect of citrullinated peptide loaded tol-DC treatment in HLA-DRB risk allele positive arthritis patients. This trial also aims to test different injection routes of tol-DCs.

For RA the dominant autoantigen is not known and will vary from patient to patient. That is why for the AUTODECRA trial tol-DCs were loaded with synovial fluid from the joint, although this is a highly invasive procedure and not always possible. Loading tol-DCs with synovial fluid ensures that tol-DCs are loaded with relevant autoantigens for each individual. Synovial fluid from RA patients contains autoantigens such as type II collagen and human cartilage gp39[249].

In RA ~70% of patients have an immune response against citrullinated peptides but this is to a range of different peptides. Rheumavax may have been more successful in its outcomes when compared to AUTODECRA due to choosing to load with citrullinated peptides. Rheumavax only included patients with seropositive RA and HLA-DRB risk alleles whereas AUTODECRA included different inflammatory arthritis conditions such as seronegative and psoriatic arthritis.

For Crohn's disease the autoantigen is not known but the immune response is thought to be directed against commensal microflora. Tol-DCs were not loaded with any Ag for the type I diabetes, Crohn's disease and transplantation trials. This suggests that tol-DCs can take up Ag *in vivo* which has been confirmed in a mouse study using Dex+VitD3 tol-DCs[232]. The authors of the Crohn's disease trial suggest that the presence of inhibitory receptors on their tol-DCs and the production of IL-10 and TGF-β may have overcome the need for Ag. However, for the transplantation trial tol-DCs were generated from donor cells so will express donor HLA.

Loading with disease associated Ags (if known) should reduce the chance of suppressing non-pathogenic immune responses and keeping protective immunity intact. Unfortunately, due to the nature of autoimmunity and epitope spreading, there are immune responses to new autoantigens as the diseases progress. The type 1 diabetes trial and Rheumavax tested T cell responses against non-disease associated Ags and these remained robust confirming tol-DC therapy leaves protective immunity unaffected and patients are not globally immunosuppressed.

The number of cells injected varied from 1-300 million cells and the injection number ranged from 1-9x. Data from animal models suggests multiple injections of tol-DCs may be required to have the most impact on disease[234,235]. The majority of trials chose the frequency of injections to be every 2 weeks, with only one trial choosing every 3 days then monthly.

Most trials chose intradermal injection route, as this is thought increase migration of DCs to the LN compared to subcutaneous and intravenous injection[250,251]. The Crohn's disease trial chose intraperitoneal to target mesenteric LNs. Similarly, the second MS trial chose intranodal to inject directly into the cervical LN in the neck, the dLN of the central nervous system. Although this will deliver tol-DCs directly into the LN there are concerns that intranodal injections could damage LN architecture and therefore compromise function[252].

The first AUTODECRA trial chose intra-articular injection to inject tol-DCs straight into the knee joint. The reason for this was firstly to deliver the tol-DCs

straight into the diseased area but also because if there were any adverse effects the joint could be irrigated. There were no systemic effects of tol-DCs observed in the first AUTODECRA trial. Tol-DCs were radiolabelled and 24 hours after injection they remained in the joint (Hilkens & Isaacs, unpublished). This suggests tol-DCs cannot migrate to dLNs following intra-articular administration. The stabilisation of symptoms in two patients receiving the highest doses of tol-DCs and the lack of flares relating to tol-DC therapy suggests tol-DCs can maintain their immunosuppressive phenotype even in the dominant inflammatory environment found in the joint.

The length of time patients were observed for after treatment varied from 3-12 months. The problem with tolerising therapies is there might not be an immediate improvement of symptoms and it could take a long time for any effects to be observed. The first six tol-DC trials which have been completed did see effects of tol-DC treatment within 12 months but the effects beyond this are unknown. Patients from the Crohn's disease and MS + neuromyelitis trial were checked at year 2 but only for safety reasons. Two patients out of nine from the Crohn's disease trial maintained corticoid-free clinical remission 12 months after tol-DC administration which is a promising sign.

In some of the trials tol-DCs did appear to have a systemic effect. For example, an increase in serum IL-10 was observed in the diabetes trial, a reduction in DAS-28 and CRP in Rheumavax and an improvement of Crohn's disease activity index. Four trials found an increase in FoxP3+ Tregs and one found an increase in Tr1 cells. Some changes to effector T cells were also observed. For example, a decrease in the number of effector T cells or a decrease in the production of IL-6 or IFN-γ by effector T cells in response to stimulation. In the MS and neuromyelitis trial there was an increase of IL-10 production by effector T cells stimulated with peptide. Furthermore, a decrease in memory CD8+ T cells was observed in two clinical trials.

1.4.4 Questions remaining relating to tol-DC therapy

- Choosing the Ag tol-DCs are loaded with. This is difficult due to epitope spreading and not one known dominant Ag is known for many of these complex diseases. Although more expensive, personalizing the Ags used

for each patient would be most effective. Alternatively, a cocktail of different peptides could be used.

- Timing of treatment. Usually for clinical trials patients are quite far into disease process, tol-DC therapy may be more effective at earlier stages.

- Number of injections (one vs multiple)

- Route of injection. Choosing an injection route which increases tol-DC migration to the dLN could improve tol-DC therapy.

- No biomarkers of successful tol-DC therapy in CD4+ T cells

1.5 Aims of project

The aim of this thesis was to inform and improve AUTODECRA 2. Therefore, Dex+VitD3 tol-DCs are used in this project as this is the tol-DC type used for AUTODECRA.

Tol-DCs are thought to need to migrate to the dLN to induce tolerance. Therefore, choosing an injection route which maximises tol-DC migration to the dLN could improve this treatment. Many injection routes have been tested in human clinical trials with tol-DCs such as intravenous, intradermal, intranodal, intra-articular and intraperitoneal. Subcutaneous injection of tol-DCs has not been tested in human clinical trials before. Furthermore, subcutaneous footpad injection of tol-DCs has never been tested in arthritis models. Subcutaneous footpad injection is a useful injection route to test as it is located near the knee joint. Additionally, in the acute breach of tolerance model, inflammation is localised to the foot and ankle joint so the dLN (popliteal LN) is the main disease associated LN in this model. We can measure migration from the footpad to the draining popliteal LN located near the knee joint. It is unclear whether tol-DCs can migrate to the dLN (popliteal LN) or joint after subcutaneous footpad injection.

Tol-DCs have to date not been tested in the OVA-induced breach of tolerance model of arthritis. This will give us more insight of the ability of tol-DCs to

modulate a breach in tolerance and autoimmunity. We can also determine whether subcutaneous injection of tol-DCs can be beneficial in altering disease.

Human Dex+VitD3 tol-DCs have previously been shown to work in part through TGF-β[226]. However, blocking TGF-β did not completely prevent their immunoregulatory effect. Therefore, there must be additional mechanisms of action of tol-DCs. In Chapter 3, we aimed to identify other potential mechanisms of action of tol-DCs as well as quality control (QC) markers for the next AUTODECRA clinical trial. There are currently no biomarkers of successful tol-DC therapy in CD4+ T cells. Identifying these would make comparisons of different tol-DC clinical trials easier and would inform whether tol-DC treatment had been successful. In Chapter 3, potential biomarkers of successful tol-DC therapy in CD4+ T cells were identified.

More generally this project will add to our knowledge of tolerance induction mechanisms *in vivo* and will determine how tol-DCs mediate their effects on T cells. This study will help determine where, when and what tol-DCs do to T cells.

The aims of this thesis are summarised below:

1. To determine whether tol-DCs can migrate to the dLN after subcutaneous (footpad) injection

2. To test subcutaneous injection of tol-DCs in the breach of tolerance mouse model of arthritis

3. Identify other potential mechanisms of action of human tol-DCs (other than TGF-β) and QC markers for AUTODECRA 2

4. Identify potential biomarkers of successful tol-DC therapy in CD4+ T cells

Chapter 2 Material and Methods

2.1 Material and Methods for Chapter 3

2.1.1 Sample collection

Leukocyte Reduction System (LRS) Cones from healthy volunteers were collected from the blood donation centre (NHS Blood and Transplant, Newcastle Blood Donor Centre). Ethical approval for using these LRS Cones was granted by the Ethics Committee of the Faculty of Medicine of Newcastle University (ref 1659/10369/2019). Previous studies have shown tol-DCs generated from healthy controls or RA patients are comparable[253].

2.1.2 Cell separation

Blood was diluted 1:2 with HBSS (LONZA, BioWhittaker) + 2mM EDTA (Fisher BioReagents). Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. 15-20ml of diluted blood was layered onto 15ml of Lymphoprep (Axis-Shield PoC, Oslo, Norway) and centrifuged at 900g for 30 minutes. PBMCs were recovered with a sterile Pasteur pipette and washed with HBSS and 1% heat inactivated FCS (Lab Tech) before being passed through a 70μm filter and counted. Monocytes were isolated using CD14+ microbeads following manufacturer's instructions (MACS, Miltenyi Biotec, Germany). Purity was routinely 95% when checked by flow cytometry.

For naïve T cell isolation, CD4+ T cells were isolated from LRS Cones using EasySep human CD4+ T cell enrichment cocktail (Stem Cell). After 20 minutes incubation with the T cell enrichment cocktail at room temperature, blood was diluted 1:2 with PBS (Sigma Aldrich) and 2% FCS, layered onto Lymphoprep and centrifuged at 900g for 30 minutes. CD4+ cells were recovered from the interface with a sterile Pasteur pipette. After washing naïve CD4+ T cells were negatively isolated using CD45RO microbeads and MACs magnetic cell separation as per manufacturer's instructions (MACS, Miltenyi Biotec, Germany). Naïve CD4+ CD45RO- T cells were frozen in 10% DMSO (Sigma Aldrich) in FCS. Purity was routinely 90-95% when checked by flow cytometry.

2.1.3 Mo-DC generation

Monocytes were resuspended in CellGro (CellGenix), this is a serum-free Good Manufacturing Practices (GMP) medium. Monocytes were cultured at 37ºC 5% CO_2 for 7 days. Monocytes were seeded in 24 well plates at 0.5 million cells per ml with 50 ng/ml IL-4 and 50 ng/ml GM-CSF (both from Immunotools). After 3 days the medium was refreshed with IL-4 and GM-CSF (both 50 ng/ml), for tol-DCs Dexamethasone (Sigma Aldrich) was also added to the medium at 10^{-6} M per well. On day 6, cells were either stimulated with MPLA (Avanti Polar Lipids) at 1μg/ml to generate mature mo-DCs or MPLA, Dexamethasone at 10^{-6}M and Vitamin D3 (R+D Tocris) at 10^{-10} M to generate tol-DCs. MPLA is a non-toxic analog of LPS and is used to induce mature mo-DCs and to stabilise the tol-DC product making it resistant to further maturation.

2.1.4 DC and allogenic naïve CD4+ T cell co-cultures

After harvesting and washing DCs were resuspended in RF10 (RPMI (Sigma Aldrich) +100U/ml Penicillin+100μg/ml Streptomycin+2mM Glutamine+10% FCS) and counted. T cells were thawed in a 37°C water bath, then resuspended in RF10 and counted.

For Mixed Leukocyte Reaction (MLR) kinetics experiments mature mo-DCs and tol-DCs were cultured in 96 flat bottom plates at a ratio of either 1:10 (10,000 DCs to 100,000 T cells), 1.5:15 (15,000 DCs to 150,000 T cells) or 2:20 (20,000 DCs to 200,000 T cells) in 200μl per well. MLRs were cultured for 10 days at 37ºC 5% CO_2, with wells being harvested each day for flow cytometry staining. The wells were split when the medium turned yellow (usually on day 6). Wells were given RF10 supplemented with 10IU/ml IL-2.

For subsequent MLRs, mature and tol-DCs were cultured 1:10 (100,000 to 1 million) with naïve T cells in 24 well plates. T cells were cultured on their own as a control. Cells were harvested on day 3 and day 6 for flow cytometry staining and sorting.

2.1.5 Flow cytometry

After culture DCs were washed and resuspended in FACs buffer 3% FCS, 1mM EDTA and 0.01% sodium azide prepared in PBS without Ca^{2+} or Mg^{2+} (Sigma Aldrich). Antibody staining was carried out in a 50μl volume on ice for 30 minutes. Human IgG (Octagom) was included in the antibody staining to prevent non-specific binding at 4μg/ml per reaction. Cells were then washed and resuspended in 200μl of FACs buffer. The following antibodies were used for flow staining.

Marker	Fluorochrome	Clone	Concentration	Manufacturer
CD1c	Pe-Cy7	L161	1:50	Biolegend
CD11c	BV421	3.9	1:50	Biolegend
CD11c	PE-Dazzle594	3.9	1:10	Biolegend
CD14	PE	M5E2 (RUO)	1:20	BD
CD2	PerCP eFluor	RPA-2.10	1:20	eBioscience
CD3	APC	UCHT1	1:20	Biolegend
CD4	AF700	SK3	1:100	Biolegend
CD25	Pe-Cy7	BC96	1:50	Biolegend
CD45-RO	FITC	UCHL1 (RUO)	1:20	BD
CD69	PE	FN50 (RUO)	1:10	BD
CD82 (TLR-2)	APC	TL2.1	1:10	eBioscience
CD83	PE	HB15e (RUO)	1:10	BD
CD86	V480	2331 (FUN-1)	1:50	BD
HLA-DRB	APC	G46-6 (RUO)	1:100	BD
LAP	PE	27232	1:10	R&D Systems

For live/dead staining, DAPI was added to samples diluted 1:2000 in PBS and incubated for 20 minutes.

2.1.6 Sorting

Antibody staining was carried out as above. CD2 PerCP eFluor (eBioscience), CD3 APC (Biolegend), CD4 Alexa Fluor 700 (Biolegend) and CD11c BV421 (Biolegend) mAbs were used to sort T cells from DCs. Before sorting cells were passed through a 30μm filter. T cells were sorted on the BD FACS Aria into RF10 before pelleting, performing the first step of the RNA isolation and then freezing at -80°C.

2.1.7 ELISAs

ELISAs were performed on supernatants from cell culture in 96 well EIA/RIA flat bottom plates. Purified rat anti-human IL-6, purified rat anti-human IL-10 and purified mouse anti-human IFN-γ capture Abs were used to test for IL-6, IL-10 and IFN-γ production (all from BD Pharmingen Biosciences). Biotin rat anti-human IL-6, biotin anti-human IL-10 and biotin mouse anti-human IFN-γ secondary Abs were used (all from BD Pharmingen Biosciences). Extravadin peroxidase and OPD tablets were used for detection (both from Sigma Aldrich). The reaction was stopped using 3M sulphuric acid and the plates were read at an OD of 490nm.

2.1.8 RNA isolation

RNA isolation was carried out on 250,000-300,000 mature or tol-DCs using the Qiagen RNeasy Microkit following manufacturer's instructions. For RNA isolation on T cells; 500,000-1 million cells were used.

2.1.9 Nanostring

RNA concentrations were measured using Qubit (Life technologies). RNA was diluted to 30ng/μl for each DC sample with a final volume of 6μl. For T cells, samples were diluted to 10ng/μl in a final volume of 8μl. Samples were run on the Nanostring nCounter assay (Nanostring®, Seattle, USA) using the Human V2 Immunology Panel.

Nanostring utilises labelled molecular probes which attach to the mRNA of interest. Each gene has a unique molecular barcode which hybridises with mRNA and activates a reporter probe. The number of bound probes is directly proportional to the expression level of that gene in each sample. This allows for the relative expression of genes to be measured without having to amplify or convert mRNA to cDNA. This technique has been found to be highly reproducible and sensitive[254].

2.1.10 Pathway analysis

Pathway analysis was performed using DAVID software and R. Differentially expressed genes were imported into the Database for Annotation, Visualisation and Integrated Discovery (DAVID). The gene lists were analysed using the functional annotation tool to find the most significant gene clusters according to their gene ontology terms (GO biological process, cellular component and molecular function). The results from DAVID were then used to make graphs in R statistical software.

2.1.11 Graphs and statistical analysis

Nanostring results were analsyed on nSolver software. All graphs and statistical analysis apart from Nanostring and pathway analysis were performed on Graph Pad Prism (Graph Pad software, San Diego, California). Flow cytometry analysis was performed with FlowJo. Figures for this thesis were made with Biorender.com (Canada).

2.2 Material and Methods for Chapters 4 and 5

2.2.1 Animals

5-6-week-old C57BL/6 mice were purchased from Envigo. OT-II/CD45.1 and CD11c-YFP[255] mice were bred in house. All animals were specific pathogen free and maintained under standard animal house conditions at the University of Glasgow in accordance with UK Home Office Regulations. All procedures were conducted under licenses issued by the UK Home Office under the Animals (Scientific Procedures) Act of 1986 and approved by the University of Glasgow Ethical Review Committee.

2.2.2 Mature BM-DC and tol-DC generation

Tibia and femur bones were collected. After flushing out the bone marrow with medium, cells were centrifuged at 400g for 5 minutes (all centrifuge runs unless otherwise stated were completed at this speed). Red blood cell lysis was performed by adding 1ml of RBC lysis buffer (Invitrogen) to the cell pellet for 2 minutes. Cells were then re-suspended in medium and washed by centrifugation.

BM-DCs were grown in complete RPMI 1640, (10% FCS, 100IU/ml Penicillin, 100μg/ml Streptomycin+2mM L-Glutamine) (all from Gibco) with GM-CSF as previously described[62]. Briefly, 1.5 million cells per well were grown at 37°C 5% CO_2 in 3ml of complete RPMI supplemented with 5% GM-CSF (from x63 cell line[256]). On day 3 and 6, the medium was refreshed with complete RPMI supplemented with 5% GM-CSF. On day 7 cells were stimulated with LPS at 100ng/ml (*E. coli* 055:B5, Sigma Aldrich) to generate mature BM-DCs. To generate tol-DCs, cells were stimulated with LPS, Dexamethasone at 10^{-6}M (Sigma) and Vitamin D3 at 10^{-10}M (R&D Tocris). Cells were harvested on day 8 using PBS with no $CaCl_2$ or $MgCl_2$ (Gibco) containing 2mM EDTA (Invitrogen).

For adoptive transfers and arthritis experiment, 6 hours before harvesting, mature BM-DCs and tol-DCs had their medium refreshed and supplemented with 1μg/ml OVA peptide (aa323-339) from Sigma-Aldrich.

2.2.3 Flow cytometry

Cells were centrifuged and re-suspended in FACs buffer. FACs buffer consisted of PBS with no $CaCl_2$ or $MgCl_2$ (Gibco) + 2% FBS (Gibco) and 2mM EDTA (Invitrogen). Flow cytometry Abs were added to each sample in 100μl of Fc block (2.4G2 hybridoma supernatant) at 2x concentration and stained for 30 minutes on ice. A table showing the Abs used in flow cytometry experiments is shown below. The live/dead stain was performed for 20 minutes in 100μl of PBS and 2mM EDTA.

Marker	Fluorochrome	Clone	Concentration	Manufacturer
CCR7	E450	4B12	1:200	eBioscience
CD4	BV605	RM4-5	1:200	Biolegend
CD11b	AF488	M1/70	1:200	eBioscience
CD11b	AF700	M1/70	1:200	eBioscience
CD11c	FITC	I-IL3	1:200	BD Pharmingen
CD11c	PerCPCy5.5	N418	1:200	eBioscience
CD24	PE	30-F1	1:200	Biolegend
CD25	AF700	PC61.5	1:200	eBioscience
CD25	BV711	PC61	1:200	Biolegend
CD40	APC	3/23	1:200	Biolegend
CD40	PE	3/23	1:200	BD Pharmingen
CD44	PerCPCy5.5	IM7	1:400	Invitrogen
CD45.1	E450	A20	1:200	eBioscience
CD45.1	APC	A20	1:200	Invitrogen
CD45.1	PE	A20	1:200	Invitrogen
CD62-L	FITC	MEL-14	1:200	Invitrogen
CD62-L	PE	MEL-14	1:200	BD Pharmingen
CD64	BV711	X54-5/7.1	1:200	Biolegend
CD69	Pe-Cy7	H1.2F3	1:200	eBioscience
CD80	FITC	16-10A1	1:200	BD Biosciences
CD86	Pe-Cy7	GL-1	1:800	Biolegend
CD115	Pe-Cy7	AFS98	1:200	Biolegend
CD117	APC-Cy7	ACK2	1:200	eBioscience
CD135 (Flt-3)	PE	A2F10	1:200	Invitrogen

CD205 (DEC-205)	APC	205yekta	1:200	Biolegend
CD206 (Mannose Receptor)	AF647	CO68C2	1:200	eBioscience
F4/80	V450	BM8	1:200	Invitrogen
MerTK	PE	2B10C42	1:200	Biolegend
MHC Class II	E450	M5/114.15.2	1:800	Invitrogen
MHC Class II	BV785	M5/114.15.2	1:800	Biolegend
T-bet	FITC	4B10	1:200	Invitrogen
Vα2	Pe-Cy7	B20.1	1:200	BD Pharmingen
Vβ5.1/2	PE	MR9-4	1:200	BD Pharmingen
Vβ5.1/2	E450	MR9-4	1:200	Invitrogen
Viability e506	E506		1:1000	Invitrogen
Viability e780	E780		1:1000	Invitrogen

2.2.4 DC-T cell co-cultures

Lymph nodes and spleens were collected from OT-II/CD45.1, TCR transgenic mice. These mice have a transgenically expressed TCR that specifically recognises OVA peptide (aa 323-339)[233]. CD45.1 is an allelic variant of the haemopoietic cell marker CD45, it allows separation of cells from CD45.2 recipients when transferring cells/bone marrow. The LNs were passed through a 70μm filter. The spleen was passed through a 70μm filter. The cells were resuspended in complete RPMI and centrifuged. After removing the supernatant, red blood cells were lysed in spleen samples by adding 2ml of RBC lysis buffer for 5 minutes (Invitrogen). The cells were topped up with complete RPMI and centrifuged again. The splenocytes and LN cells were then combined. CD4+ isolation was performed using a mouse CD4+ T cell isolation kit (MACS) from Mitenyi Biotec following manufacturer's instructions. MACS buffer consisted of PBS with no $CaCl_2$ or $MgCl_2$ (Gibco) + 2% FBS (Gibco) and 2mM EDTA (Invitrogen).

CD4+ T cells were co-cultured in complete RPMI with mature BM-DCs or tol-DCs at a ratio of 10:1 (100,000:10,000) in 200μl in 96 well round bottom plates. OVA peptide (aa 323-339) and ConA, both from Sigma-Aldrich, were added at a final concentration of 0.5 and 1μg/ml respectively. Cells were cultured for 3 days at

37°C 5% CO_2 with cells being harvested at 24, 48 or 72hrs for flow cytometry staining. Day 3 was found to be the optimal time-point for further investigation, so all subsequent DC-T cell co-cultures were analysed on Day 3.

2.2.5 Labelling

Cells were labelled with Cell Trace Violet (Invitrogen) following manufacturer's instructions. The optimal labelling concentration was determined to be 0.25μM.

2.2.6 Migration experiments

CD11c-YFP mature BM-DCs and tol-DCs were harvested on day 8 and labelled with Cell Trace Violet (Invitrogen). Cells were filtered through 100μm filter then centrifuged and re-suspended in PBS (1 million cells per 25μl). 25μl of PBS containing 1 million mature BM-DCs were injected into the left footpad and 25μl containing 1 million tol-DCs were injected into the right footpad of 9 WT mice. One mouse was used as non-injected WT control. At 12, 16 and 24 hours after injection, 3 mice were culled at each time-point and popliteal and inguinal LNs harvested. LNs were passed through a 70μm filter and digested with Collagenase D (Sigma) at a 1:10 dilution. Plates were incubated for 25 minutes at 37°C and 80rpm. Cells were then washed with FACs buffer and stained with flow cytometry Abs.

2.2.7 Adoptive transfers

T cells from OT-II/CD45.1 mice were isolated as described previously in Section 2.2.4. 1 million cells were stained with CD4, CD45-1, Vα2 and Vβ5.1/2 and analysed on the flow cytometer. The rest of the cells were Cell Trace Violet labelled then counted. One million CD4+ OT-II T cells in 200μl of PBS was injected intravenously into WT mice. 24 hours later, OVA loaded mature BM-DCs or tol-DCs were injected into both footpads in 25μl of PBS. A control mouse received T cells only and no DCs. At days 3 and 5 after transfer of DCs, draining (popliteal) LNs were harvested and passed through a 70μm filter before flow cytometry staining. After analysis, day 3 was picked as the most promising time-point for future investigation.

On one occasion, spleens were also harvested for re-stimulation with PMA/Ionomycin or OVA peptide (aa323-339) to measure IFN-γ production. Spleens were passed through a 70μm filter and RBC lysed before being re-suspended in complete RPMI. Cells were plated up at 1 million/per ml per well. Cells were stimulated with PMA (20ng/ml) and Ionomycin (1μg/ml) for 6 hours at 37°C before being harvested for flow cytometry staining. Intracellular staining was performed using a BD Cytofix/Cytoperm kit (with BD GolgiStop) following manufacturer's instructions.

2.2.8 Breach of tolerance model of arthritis

The breach of tolerance model of arthritis has been previously described[233]. In this model OT-II CD4+ T cells which express a transgenic TCR specific for OVA peptide (aa323-339) are polarised to a Th1 phenotype *in vitro* then transferred to C57BL/6 recipients. The mice are then challenged with OVA/CFA subcutaneously and 10 days later heat aggregated OVA (HAO) is injected into the footpad to localise inflammation to the joint. There is an immune response directed first against OVA but an immune response against self-antigens such as collagen type II also develops[257].

2.2.8.1 Preparation of Th1 polarised CD4+ OT-II cells

Lymph nodes and spleens were harvested from OT-II/CD45.1 mice and passed through a 70μm filter. After centrifuging the cells and removing the supernatant, RBC lysis was performed on spleens (500μl per spleen for 5 minutes). After washing the spleens with complete medium, the splenocytes and LN cells were combined and re-suspended in MACs buffer (PBS with no $CaCl_2$ or $MgCl_2$ (Gibco) + 2% FBS (Gibco) and 2mM EDTA (Invitrogen)). CD4+ T cell isolation was performed following manufacturer's instructions (Miltenyi Biotec). The CD4- fraction was also collected to use as APCs.

Spleens from C57BL/6 mice were harvested. These were passed through a 70μm filter and RBC lysed as described previously. These cells were used as the APCs to stimulate the CD4+ OT-II T cells.

500μl of 1mg/ml Mitomycin C (Sigma Aldrich) was added to 10ml of APCs and the cells were incubated at 37°C for 45 minutes. APCs were washed twice with complete medium.

APCs:CD4+ T cells were cultured at a 5:1 ratio with 25 million CD4+ T cells per 75cm^3 flask in 50ml of complete medium. OVA peptide (aa323-339) was added at 1μg/ml (Sigma-Aldrich), anti-IL-4 at 2μg/ml (InVivoMab, clone 11B11) and recombinant IL-12 at 10ng/ml (R+D). The cells were cultured for 72 hours at 37°C and 5% CO_2.

2.2.8.2 Cell transfers

The Th1 polarised cells were harvested and checked by flow cytometry for the % of CD4+ Vα2/Vβ5.1-2 double positives. The equivalent of 2.5 million Vα2/Vβ5.1-2 double positive cells were adoptively transferred per C57BL/6 mouse in 200μl of PBS (Day 0 of experiment). T cells were checked by flow cytometry using FoxP3 nuclear staining kit (Invitrogen) for levels of T-bet. 91% of live CD4+ cells were T-bet+.

Days 1-3 after Th1 adoptive transfer, 1 million OVA peptide (aa323-339) loaded mature BM-DCs or tol-DCs were subcutaneously injected into both footpads in 25μl of PBS. 25μl of PBS was given to 5 mice as a control and 5 mice received no treatment. DCs were checked after transfer by flow cytometry to confirm they were mature and tolerogenic.

2.2.8.3 Preparation of OVA in Complete Freund's Adjuvant (CFA)

The equivalent of 1μg/μl of OVA protein (Worthington Biochem) was added to CFA (Sigma-Aldrich) diluted 1:1 in PBS. The mixture was quickly taken up and down in a 1ml syringe until emulsified, (this was when the mixture became solid and no longer floated on top of water). 100μl (equivalent to 100μg of OVA protein) was injected subcutaneously into the scruff of all mice in the experiment.

2.2.8.4 Preparation of Heat Aggregated OVA (HAO)

HAO was prepared as 20mg/ml stocks in PBS using OVA protein. 200μl of 20mg/ml OVA protein/PBS mixture was incubated at 100°C for 2 hours. The eppendorfs were centrifuged at 13,300 rpm for 5 minutes and the supernatant was removed. Aggregated OVA was washed with PBS and centrifuged again. Supernatant was removed and 200μl of PBS was added to the pellet. Aliquots were frozen before use in the model.

Stock aliquots were thawed and placed in a Gentle-MACS c-tubes (Mitenyi Bio). 1.8ml of PBS was added. HAO was homogenized using a gentle MACS Dissociator (Mitenyi Biotec) using a customised program. The programme was run 12x and HAO was ready to be injected when it passed through a 25 gauge needle. 100μl (containing 100μg of HAO) was injected into 15 mice in both footpads.

2.2.8.5 Acute arthritis model

An overview of the experiment can be seen in Figure 2-1. On day 0, the equivalent of 2.5 million Vα2/Vβ5.1-2 double positive OT-II cells were adoptively transferred in 200μl of PBS to 20 C57BL/6 mice. On days 1-3 mice received PBS, OVA peptide (aa323-339) loaded mature or tol-DCs subcutaneously into both footpads and 5 mice received no treatment. On day 4, 100μg OVA/CFA was injected subcutaneously into the scruff. Ten days later, 50μl of 100μg Heat Aggregated OVA (HAO), or PBS was injected subcutaneously into the footpad. The experimental groups can be seen in Table 2-1.

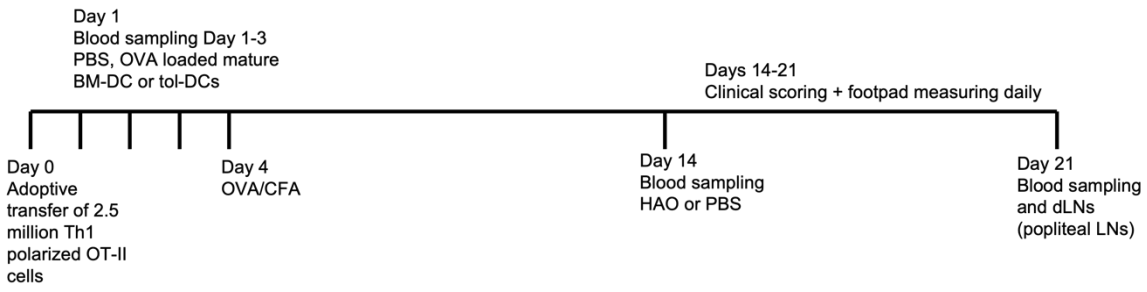


Figure 2-1 Arthritis experiment overview.

Table 2-1 Experimental groups in arthritis experiment.

Group	Immunisation	Treatment	Challenge
1	OVA/CFA	None	PBS
2	OVA/CFA	PBS	HAO
3	OVA/CFA	Mature DCs	HAO
4	OVA/CFA	Tol-DCs	HAO

For 7 days after HAO, the mice were clinically scored by a blind observer and footpad thickness measured daily. The clinical scoring criteria is outlined below.

0= no reaction normal
1= Mild but definite redness and swelling of the ankle or apparent redness and swelling limited to individual digits
2= Moderate redness and swelling of the ankle
3= Severe redness and swelling of the entire paw including digits
4= Maximally inflamed limb with involvement of multiple joints

2.2.9 Imaging

2.2.9.1 InCell

CD4+ T cells were isolated from OT-II mice as previously described in section 2.2.4 and labelled with CFSE at a concentration of 7.5μM. Mature BM-DCs, tol-DCs or unstimulated DCs were labelled with CMPTX at a concentration of 7.5μM (both Invitrogen). The cells were cultured together in a 384 well plate (for imaging) at 8000 T cells to 8000 DCs per well. The wells were either left unstimulated, stimulated with ConA (1μg/ml) or different concentrations of OVA peptide (10 or 1μg/ml). The plate was incuabted at 37°C and 5% CO_2 for 24 hours before being analysed on the InCell 2000 analyser. This machine measures the total CFSE and total CMPTX area then calculates the % overlap between CFSE and CMPTX positive cells.

2.2.9.2 Immunofluorescence

24 hours after subcutaneous footpad injection of CD11c-YFP mature BM-DCs or tol-DCs the dLNs were harvested and frozen in OCT (Tissue Tek) and stored at -80°C. Tissue sections were prepared on the cryotome, and slides were stored at -20°C until immunofluorescence staining was performed. Tissue sections were

fixed in acetone for 10 minutes then washed with PBS. Sections were then blocked with Fc block for 30 minutes then stained with CD3 AF594 (Biolegend), anti-GFP rabbit polyclonal AF488 (Invitrogen) and B220 APC (eBioscience) Abs at 1:100 concentrations in Fc block at -4°C overnight. After washing with PBS and allowing the slides to dry, antifade mounting media (Vectashield) was added, a coverslip was placed on top and sealed with nail varnish. Slides were imaged on the Zeiss LSM880 confocal microscope and images were analysed using Fiji (ImageJ).

2.2.10 ELISAs

ELISA MAX Deluxe Set from Biolegend were used to measure IL-6, IL-10, TNF-α and IFN-γ production. Cat numbers (431304, 431414, 430904 and 430804 respectively). Manufacturer's guidelines were followed.

Anti-OVA IgG1 and anti-OVA IgG2c ELISAs were performed by first coating high binding plates (Corning) with OVA protein (20μg/ml) in carbonate bicarbonate buffer (Sigma-Aldrich). Plates were washed with PBS-0.05% Tween and then blocked with Animal-free blocker (Vector). Serum was added at 1:200 dilution, a serial dilution was then performed. Biotin SP-conjugated AffiniPure Goat Anti-mouse IgG Fcγ Subclass 1 specific and Biotin SP-conjugated AffiniPure Goat Anti-mouse Subclass 2c specific (both from Jackson ImmunoResearch) were used as detection antibodies and added at a 1:10,000 dilution. ExtrAvidin peroxidase (Sigma-Aldrich) was added at 1:10,000 dilution. OPD tablets were used to develop the colour (Sigma-Aldrich). 10% H_2SO_4 was added to stop the reaction.

Anti-collagen IgG analysis was performed similarly. Plates were coated with Collagen from bovine tracheal cartilage type II (Sigma-Aldrich) at 4μg/ml. Serum was added at 1:50 dilution, a serial dilution was then performed. DAKO polyclonal rabbit anti-mouse Immunoglobulin HRP was used (Sigma-Aldrich) at a 1:5000 dilution.

2.2.11 Q-PCR

5 million mature BM-DCs or tol-DCs were collected, and RNA isolation was performed using RNeasy Mini Kit (74104). cDNA was synthesised using QuantiTect

Reverse Transcription Kit (205310). Q-PCR was performed using QuantiNova SYBR green PCR kit (208052) in 384 well plates on Quant Studio 7 Flex (Applied Biosystems). All kits were from Qiagen and manufacturers guidelines were followed. The primer sequences shown below in Table 2-2 were used.

Table 2-2 Primer sequences used for Q-PCR.

Name	Sequence 5'-3'
GADPH forward	TCACCACCATGGAGAAGGC
GADPH reverse	GCTAAGCAGTTGGTGGTGCA
Complement factor H forward	TGCCAAAATGCAAAAGCAGT
Complement factor H reverse	GGTATCCAGGAAAATCTGAGAAAGT
C1QA forward	TCACCAACCAGGAGAGTCCA
C1QA reverse	CACCTGAAAGAGCCCCTTGT
C1QB forward	CAAAGGCGATTCTGGGGACT
C1QB reverse	TAGTAGAGGCCAGGCACCTT
CD24 forward	TTCGCATGGTCACACACTGA
CD24 reverse	ACACACACAGTAGCTTCGGG
IL-1R2 forward	AAGGACTCGATCACACGCTG
IL-1R2 reverse	AGCAGATGCCCAGACATCAA
FcgR2B forward	ATCTGGACTGGAGCCAACAAG
FcgR2B reverse	TTCTTCATCCAGGGCTTCGG
LAIR-1 forward	AGGAGGGTTCTCTGCCTGAT
LAIR-1 reverse	TTCCATAAAGGTGCTGCCGT
CD86 forward	GCAAGGTCACCCGAAACCTA
CD86 reverse	CACACACCATCCGGGAATGA

2.2.12 Graphs, statistical analysis and figures

All graphs and statistical analysis were performed on Graph Pad Prism (Graph Pad software, San Diego, California). Flow cytometry data was analysed with FlowJo. Figures for this thesis were made with Biorender.com (Canada).

Chapter 3 An Investigation into Human Tolerogenic Dendritic cells and their Action on Naïve T cells

3.1 Introduction

AUTODECRA 2 is the next clinical trial investigating tol-DCs as a treatment for inflammatory arthritis and is scheduled and currently recruiting. There remain unanswered questions relating to tol-DC therapy. Answering these could significantly inform and improve AUTODECRA 2. The main aim of this chapter is to identify additional mechanisms of action of tol-DCs, as well as potential quality control (QC) markers and biomarkers of successful tol-DC therapy.

Previous human *in vitro* work has shown that Dex+VitD3 tol-DCs work in part through production of TGF-β, although blocking TGF-β does not completely prevent tol-DCs immunosuppressive effect on T cells[226]. This suggests there must be additional mechanisms of action of this tol-DC type which need to be identified. Comparing mature and tol-DCs, 24-hours post-stimulation allowed us to identify additional immunoregulatory genes upregulated in tol-DCs which could be important for their function. We included tol-DCs 6-hours post stimulation in this analysis as there could be genes upregulated 6-hours post stimulation which could then be downregulated by 24-hours post stimulation.

Validating some of these immunoregulatory molecules upregulated in tol-DCs would be useful for identifying functional QC markers. There is a need for QC markers which help inform about tol-DCs potency as a treatment. TLR-2 was used previously as a QC marker for the first AUTODECRA clinical trial. TLR-2 is induced as a result of Dexamethasone treatment and is highly expressed on Dexamethasone tol-DCs, although it is also expressed at lower levels on Vitamin D3 and IL-10 tol-DCs[210]. QC markers should ideally be highly and stably expressed by tol-DCs alone and involved in their function, allowing detection by flow cytometry.

In this chapter a Nanostring nCounter Assay was performed, comparing mature mo-DCs and tol-DCs at 6- and 24-hours post-stimulation. This allowed us to

identify potential additional mechanisms of action of tol-DCs and potential QC markers for the next clinical trial.

Identifying functional QC markers of tol-DCs is useful for determining their potency as a treatment but expression of these would not tell us if tol-DC treatment has actually been successful. There are currently no biomarkers of successful tol-DC therapy. Identifying biomarkers could distinguish responders and non-responders to treatment. Due to the fact tol-DCs are thought to primarily mediate their effects through their action on T cells and naïve T cells circulate in the blood we decided to look for a potential biomarker in naïve T cells. Previous human *in vitro* work has shown that Dex+vitD3 tol-DCs have differential effects on naïve and memory T cells[217]. This tol-DC type induces naïve T cells to become Tr1 cells and renders memory T cells anergic.

Tol-DCs induce naïve T cells to become Tr1 cells which produce high amounts of IL-10[217]. IL-10 is not a suitable biomarker as it is transiently and not stably expressed. To try and identify a potential biomarker other than IL-10, we co-cultured mature mo-DCs or tol-DCs with naïve T cells and then performed a Nanostring nCounter assay to identify which genes were upregulated in T cells as a result of interaction with tol-DCs.

Identifying additional modes of action of tol-DCs as well as potential functional QC markers and biomarkers of successful tol-DC therapy would greatly improve the next AUTODECRA clinical trial. The aims of this chapter are summarised below.

- Identify other potential mechanisms of action of tol-DCs other than TGF-β. If validated these could be used as functional QC markers for AUTODECRA 2.

- What genes are induced in naïve T cells as a result of interaction with tol-DCs? This could lead to identification of a biomarker of successful tol-DC therapy.

3.2 Results

3.2.1 Phenotype of mature mo-DCs and tol-DCs by flow cytometry

Representative flow cytometry plots of mature and tol-DCs can be seen in Figure 3-1. The leukocyte cell population was identified, avoiding debris using forward scatter and side scatter. Doublets were then excluded using side scatter area vs side scatter width. Following culture with IL-4 and GM-CSF, monocytes differentiate into DCs and lose expression of the monocyte marker CD14 and gain expression of CD1c (a marker for monocyte to mo-DC conversion induced by GM-CSF) as shown in Figure 3-1A. There are slightly more CD14 positive cells and lower levels of CD1c expression in the tol-DC group compared with the mature mo-DC group. This could be due to adding Dexamethasone on day 3 or Vitamin D3 on day 6 as both are known to inhibit DC differentiation from monocytes[188,258].

TLR-2 should be high on tol-DCs and low on mature mo-DCs as Dexamethasone induces TLR-2 expression[210]. TLR-2 has previously been used as a quality control marker for the first AUTODECRA clinical trial[179]. LAP (latency associated peptide) is the membrane bound form of TGF-β1 before it is cleaved into its active form. Expression of LAP is variable between donors but should be higher in tol-DCs as previous work has shown tol-DCs work in part through TGF-β production[226]. Figure 3-1A shows that tol-DCs have higher TLR-2 and LAP expression than mature mo-DCs.

Figure 3-1B shows histograms comparing the expression of CD83, a classical DC maturation marker; CD86, a co-stimulatory molecule and HLA-DR (MHC Class II) between mature and tol-DCs. Compared with mature mo-DCs, tol-DCs have lower expression of CD83 and CD86 and comparable expression of HLA-DR (MHC Class II). This phenotype is consistent with previous findings of the group[217].

3.2.2 Tol-DCs produce significantly higher levels of IL-10 and significantly lower levels of IL-6 than mature mo-DCs

Supernatants were collected on day 7 of mature mo-DC and tol-DC generation and the concentrations of IL-6 and IL-10 were measured by ELISA. The results can be seen in Figure 3-2. Tol-DCs produce significantly more IL-10 and

significantly less IL-6 than mature mo-DCs. This is in line with previous studies that found tol-DCs produce lower levels of inflammatory cytokines and more anti-inflammatory cytokines than mature mo-DCs[217].

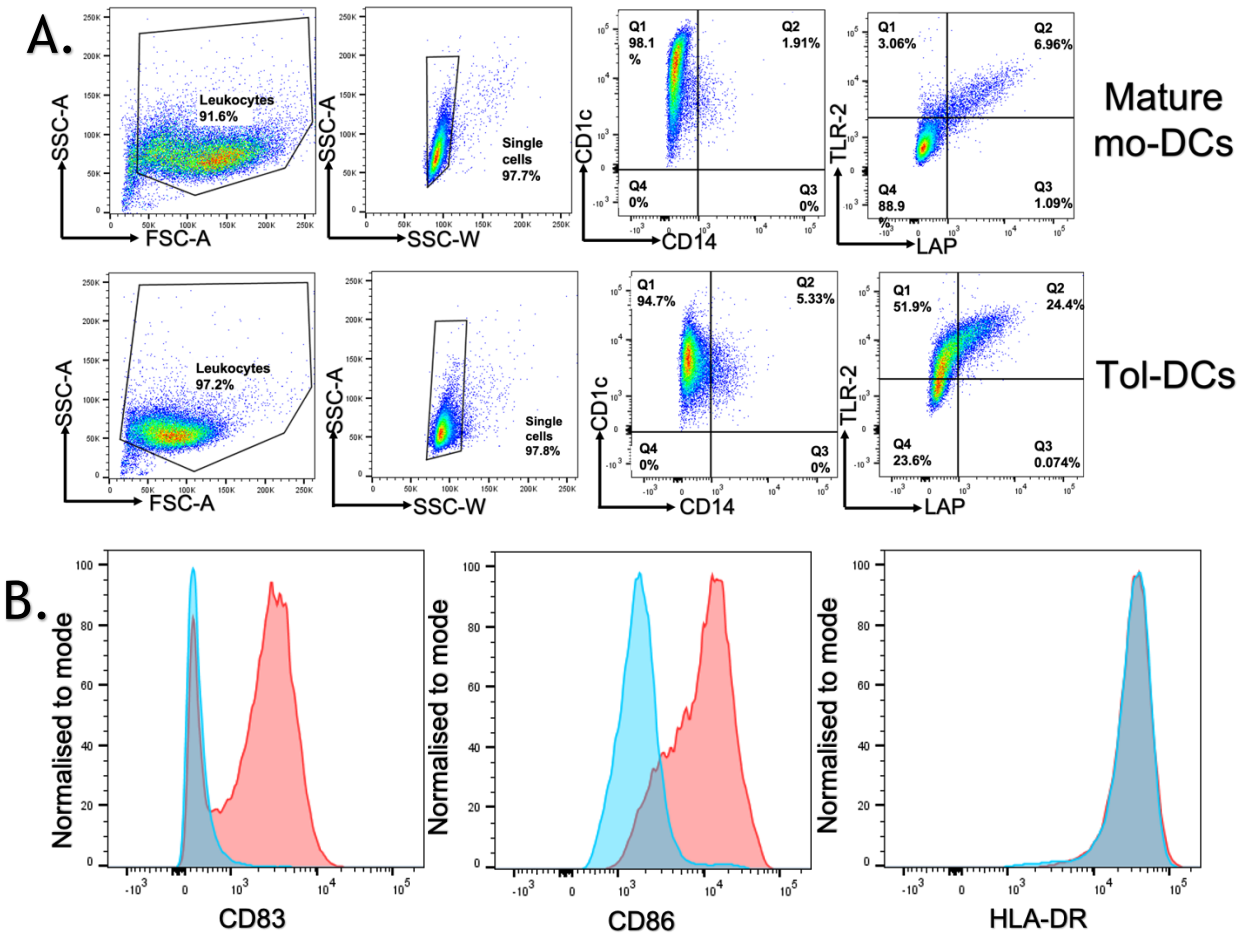


Figure 3-1 Representative phenotypic analysis of mature mo-DCs and tol-DCs by flow cytometry.

DCs were generated from monocytes by culturing with IL-4 and GM-CSF. MPLA was added to generate mature mo-DCs and Dex, VitD3 and MPLA were added to generate tol-DCs. After culturing DCs were harvested and resuspended in FACS buffer then stained with flow cytometry Abs. In the histograms tol-DCs are shown in blue and mature mo-DCs in red. Representative of 30 experiments.

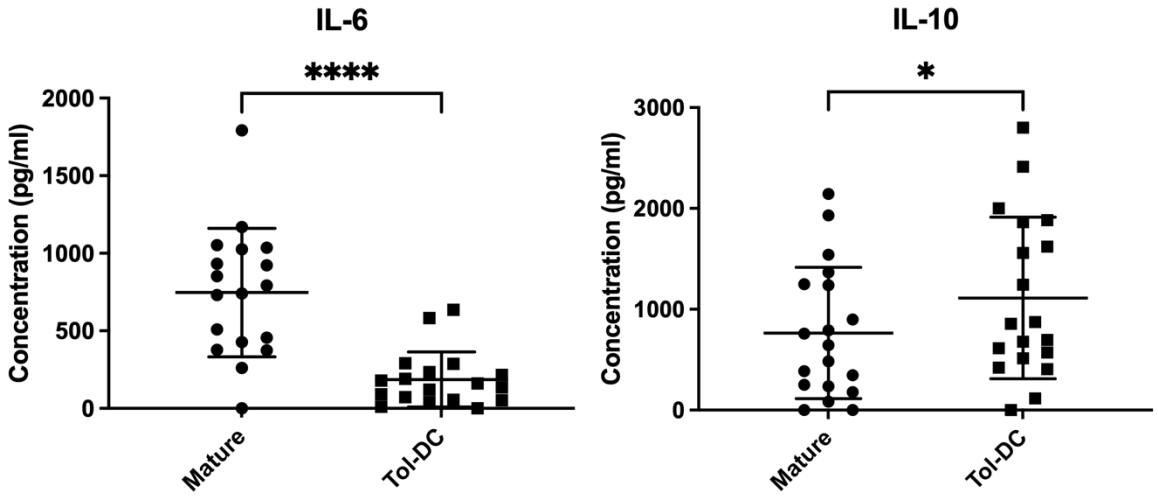


Figure 3-2 Tol-DCs produce significantly higher IL-10 and lower levels of IL-6 when compared to mature mo-DCs.

Supernatants were collected before harvesting DCs on day 7 and tested by ELISA for IL-10 and IL-6 concentrations. Data passed an Anderson-Darlington normality test. A paired student's t test was performed. * = p-value <0.05 **** = p-value <0.0001.

3.2.3 24-hour tol-DCs are transcriptionally distinct from the other groups tested

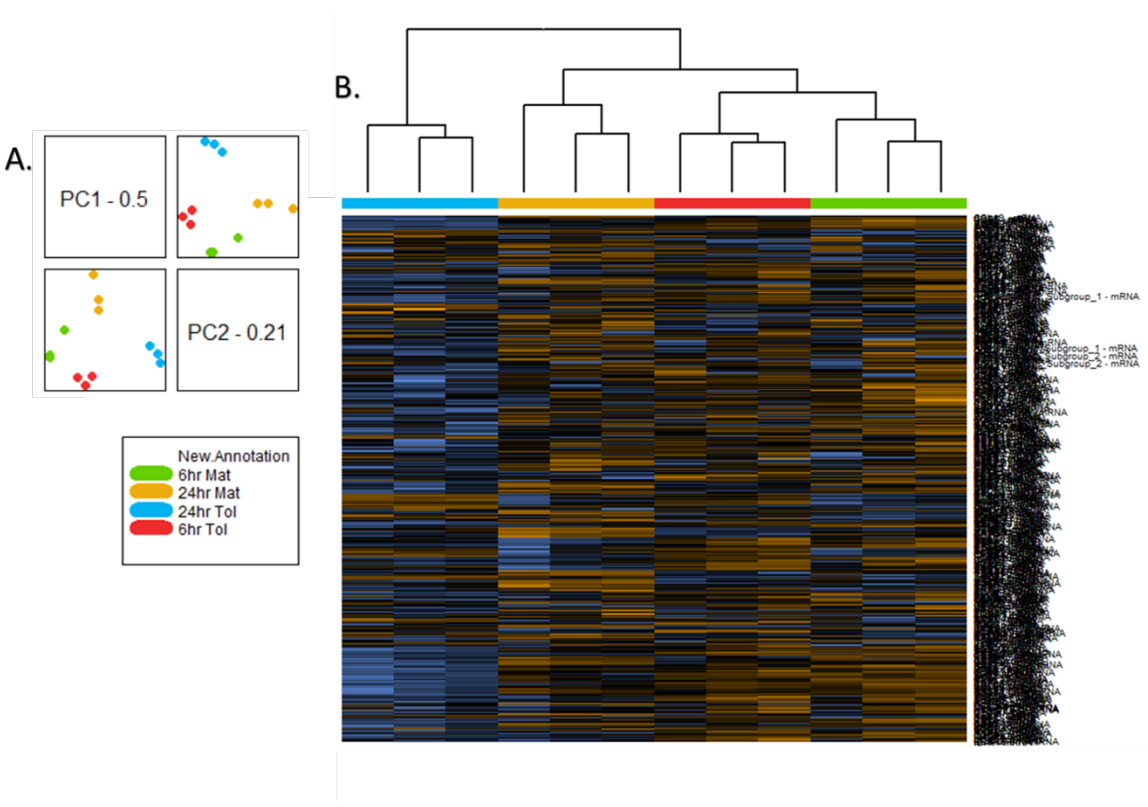
A Nanostring nCounter assay was performed to compare gene expression in mature mo-DCs and tol-DCs at 6- and 24-hours post-stimulation. We used the Human Immunology V2 panel which contains 579 genes.

The normal time point for harvesting mature mo-DCs and tol-DCs is 24 hours after stimulation, including an earlier time-point, 6 hours post-stimulation, allowed us to determine whether these cells diverge earlier and if there are major differences between the two cell types earlier than 24 hours. Three donors were used and there were four different conditions for each donor (6-hour mature, 6-hour tolerogenic, 24-hour mature and 24-hour tolerogenic). The results from this experiment can be seen in Figure 3-3.

Figure 3-3A shows the principal component analysis of principal component 1 vs principal component 2, which account for 50% and 21% of the separation respectively. This demonstrates that the different cell type replicates from different donors group together in clusters meaning the variation between donors is less than the variation between treatment groups. When examining the primary component (50% variance) 24-hour tol-DCs are the most distant cell type from the others, suggesting broad gene expression differences. Initially, 6-hour

tol-DCs and 6-hour mature mo-DCs cluster in two distinct but close clusters. Curiously, when only considering this first component, 6-hour tol-DCs and 6-hour mature mo-DCs show little separation; however, the second principal component (21%) demonstrates vast distance suggesting some dissimilarities exist between the two cell types. Ultimately, 24-hour mature mo-DCs and 24-hour tol-DCs have a greater relative separation than at their respective 6-hour marks.

Figure 3-3B shows the heat-map of all data, showing which genes are upregulated or downregulated in the different groups after normalisation. Twenty-four-hour tol-DCs are the most distinct population, branching off first and there is a general trend for downregulation of genes in this group compared with the others. Six-hour mature mo-DCs and 6-hour tol-DCs are the most similar branching off together and separately from 24-hour mature mo-DCs. Figure 3-3C-F show a breakdown of the data by grouping genes by function. There is a general trend for a downregulation of genes involved in cell activation, cell development, cell proliferation and T cell proliferation in 24-hour tol-DCs compared with other groups. This fits with what we already know about tol-DCs as Dexamethasone and Vitamin D3 inhibit DC development and differentiation and tol-DCs induce lower levels of T cell proliferation[188,190].



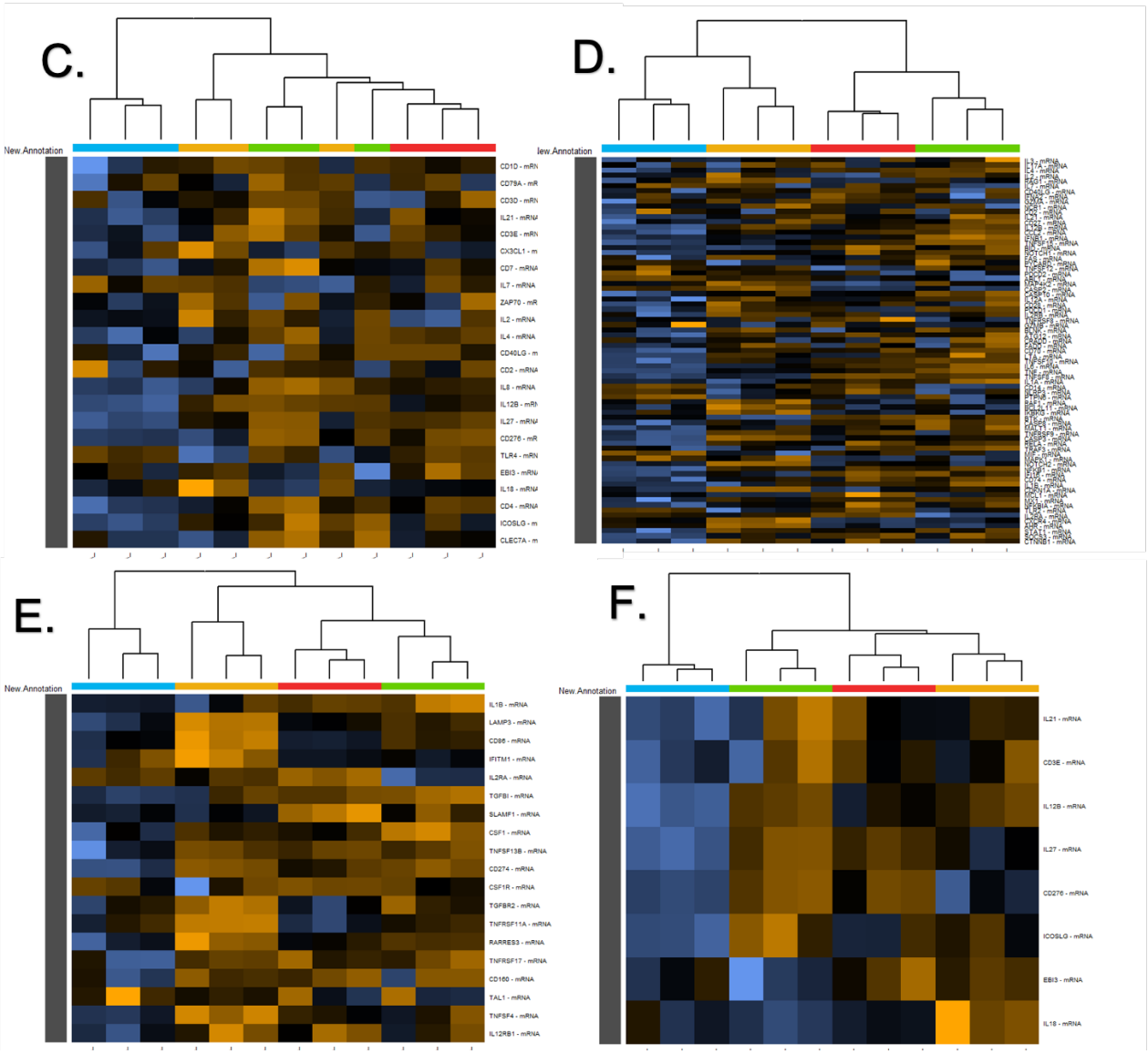


Figure 3-3 24-hour tol-DCs are transcriptionally distinct from 6-hour mature mo-DCs or tol-DCs and 24-hour mature mo-DCs.

A. shows the principal component analysis, PC1 vs PC2. **B.** shows the heatmap of all data after normalisation. Blue shows genes that have been downregulated and orange shows genes that have been upregulated. **C.** cell activation. **D.** cell development. **E.** cell proliferation. **F.** T cell activation.

3.2.4 Differentially expressed genes when tol-DCs and mature mo-DCs are compared at 6-hours post-stimulation

Volcano plots of differentially expressed genes were generated using nSolver software. Figure 3-4 shows a volcano plot where 6-hour tol-DCs were compared with 6-hour mature mo-DCs (the baseline). In total there were 75 genes which were differentially expressed (p-values less than 0.05). Genes which had a log2 fold change of more than 2 up or downregulation and a p-value of less than 0.05 were considered important. A table of these genes can be seen in table 1 and 2 (shown in appendix).

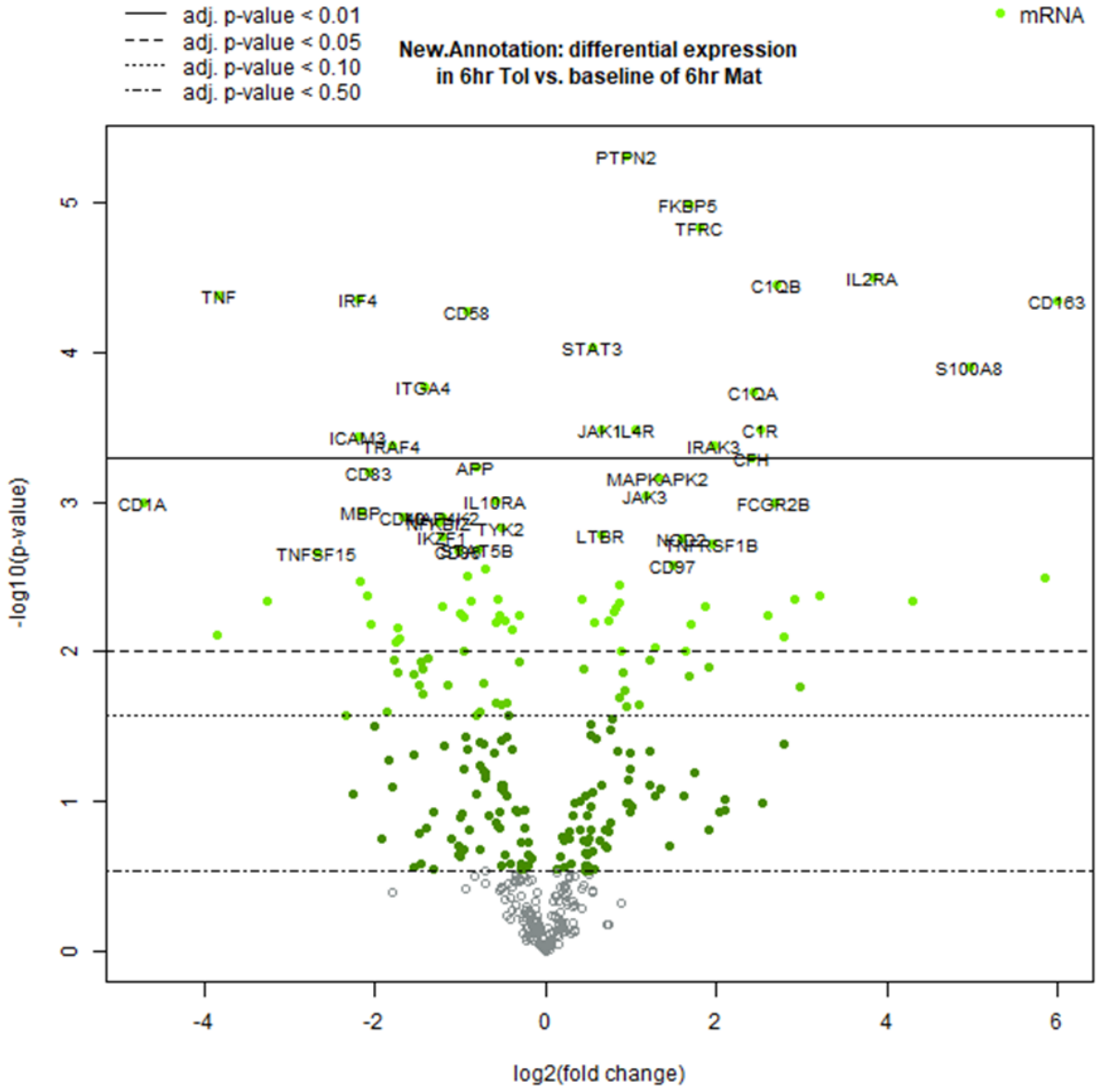


Figure 3-4 Volcano plot of differentially expressed genes when 6-hour tol-DCs are compared to a baseline of 6-hour mature mo-DCs.
A Nanostring assay was performed to compare mRNA expression of 570 human immunology genes. Differentially expressed genes when 6-hour tol-DCs are compared to 6-hour mature mo-

DCs are shown here. Genes upregulated in 6-hour tol-DCs are shown on the right and genes upregulated in 6-hour mature mo-DCs are shown on the left.

Certain inflammatory molecules, such as TNF and IL-12β are upregulated in 6-hour mature mo-DCs when compared with 6-hour tol-DCs. As well as these, CCR7 a chemokine receptor involved in cell trafficking to the lymph nodes and CCL3 are also upregulated in 6-hour mature mo-DCs compared with 6-hour tol-DCs. CCL3 is an inflammatory chemokine which attracts monocytes and macrophages to sites of inflammation. CD83, a DC maturation marker is upregulated in mature mo-DCs whereas CD14 a monocyte marker and CD163 a macrophage marker are upregulated in 6 hour tol-DCs indicating that even at 6 hours post stimulation, mature mo-DCs are more DC like and tol-DCs are less mature and more monocyte/macrophage like.

Some anti-inflammatory molecules are upregulated in 6-hour tol-DCs such as IL-10 and some regulatory complement proteins, however 6-hour tol-DCs still express certain inflammatory molecules such as CXCL12, TNF receptor 2 and C1R which activates complement.

3.2.5 Differentially expressed genes when tol-DCs are compared at 6 and 24-hours post-stimulation

We next compared 24-hour tol-DCs to a baseline of 6-hour tol-DCs. The results can be seen in Figure 3-5 and tables 3 and 4 (shown in appendix). There were 78 differentially expressed genes when tol-DCs were compared at these two time-points (p-values of <0.05 and a log2 fold change of >2 up or downregulation).

Six-hour tol-DCs still express many inflammatory chemokines (CCL2, CCL3, CCL4, CCL8, CXCL9, CCL20, CXCL10, CXCL11) as well as inflammatory cytokines (TNF, IL-6, IL-12β, IL-15, IL-23α, IL-27) and co-stimulatory molecules (CD40+CD80). Furthermore, TRAF1 and NF-KB1, molecules involved in TNF and NF-KB signalling, were upregulated in 6-hour tol-DCs when compared to 24-hour tol-DCs. Interestingly, IL-10 was upregulated in 6-hour tol-DCs when compared to 24-hour tol-DCs.

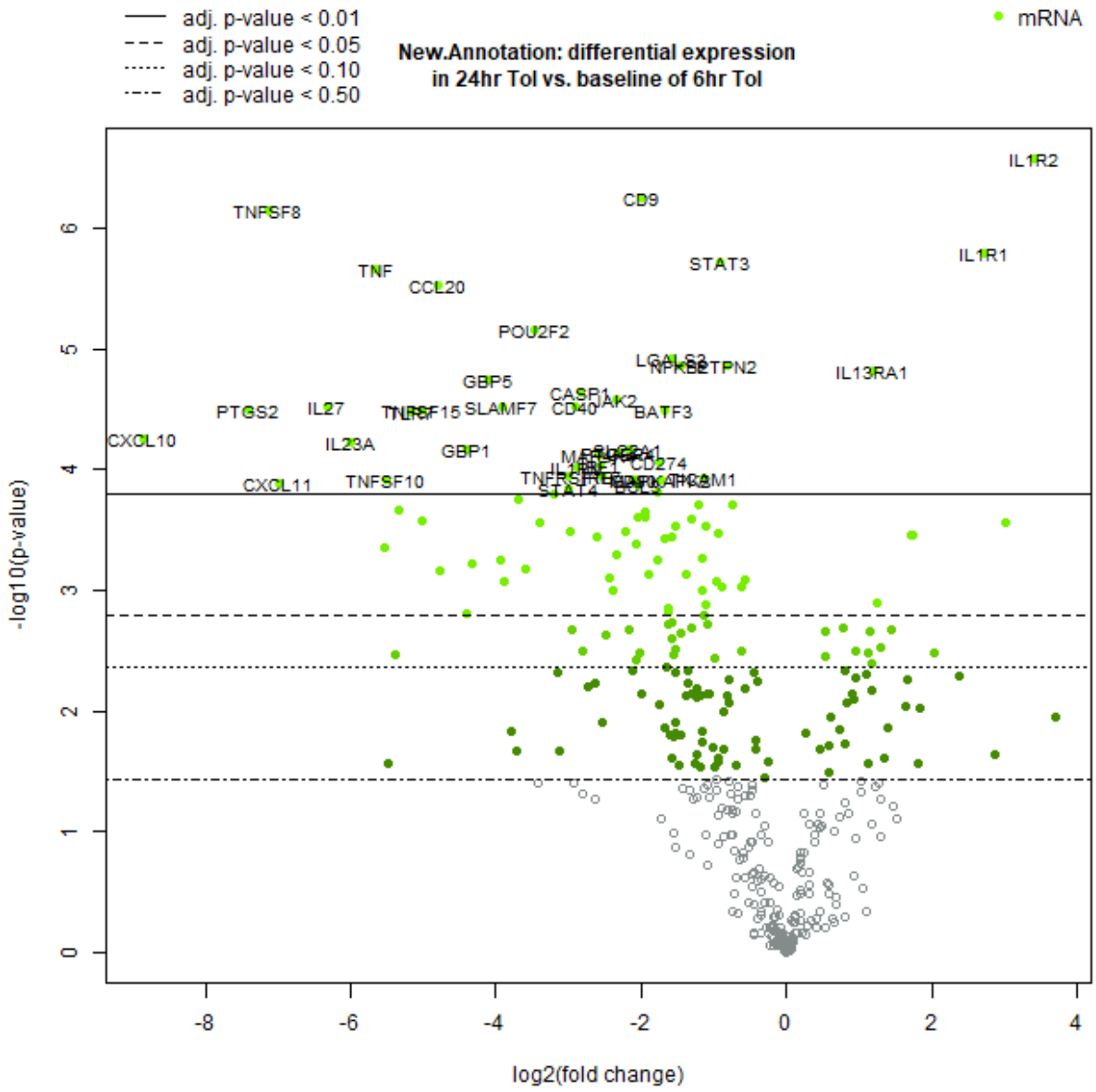


Figure 3-5 Volcano plot of differentially expressed genes when 24-hour tol-DCs are compared to a baseline of 6-hour tol-DCs.
A Nanostring assay was performed to compare mRNA expression of 570 human immunology genes. Differentially expressed genes when 24-hour tol-DCs are compared to 6-hour tol-DCs are shown here. Genes upregulated in 24-hour tol-DCs are shown on the right and genes upregulated in 6-hour tol-DCs are shown on the left.

3.2.6 Differentially expressed genes when mature mo-DCs and tol-DCs are compared at 24-hours post-stimulation

Subsequently, 24-hour tol-DCs were compared with 24-hour mature mo-DCs. The results can be seen in Figure 3-6 and table 5 (shown in appendix). There were 186 differentially expressed genes (p values <0.05). There were more than double the number of differentially expressed genes when the two cell types

were compared at 24-hours than at 6-hours. This confirms that gene expression patterns in mature and tol-DCs diverge with time.

When 24-hour mature mo-DCs are compared with 24-hour tol-DCs, there are more inflammatory genes upregulated in mature mo-DCs such as co-stimulatory molecules (CD80, CD86 and CD40), inflammatory cytokines (IL-12β, TNF, IL-23α, IL-27), inflammatory chemokines (CCL2, CCL3, CCL8, CCL13, CXCL9, CXCL10+CXCL11) and the inflammatory transcription factor NF-KB1. CCL19, CXCR4 and CCR7 are involved in migration to secondary lymphoid tissue and are upregulated in mature mo-DCs when compared to tol-DCs, suggesting a reduced lymph node homing capacity of tol-DCs.

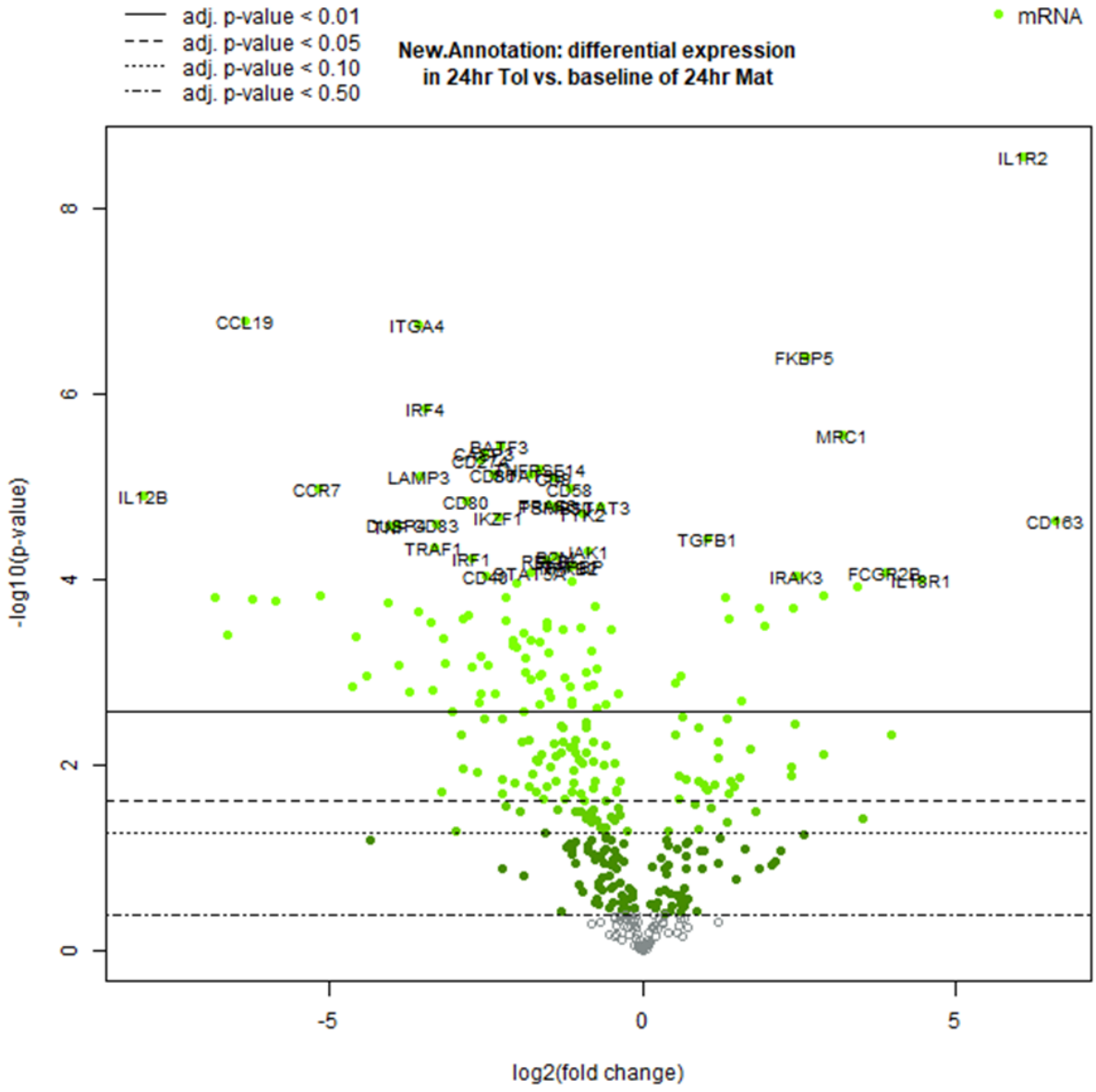
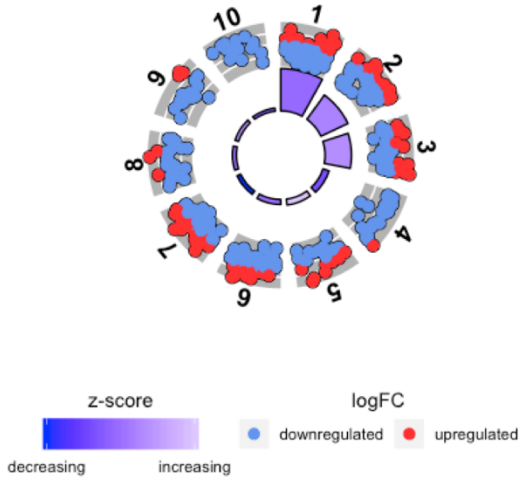


Figure 3-6 Volcano plot of differentially expressed genes when 24-hour tol-DCs are compared to a baseline of 24-hour mature mo-DCs.

A Nanostring assay was performed to compare mRNA expression of 570 human immunology genes. Differentially expressed genes when 24-hour tol-DCs are compared to 24-hour mature mo-DCs are shown here. Genes upregulated in 24-hour tol-DCs are shown on the right and genes upregulated in 24-hour mature mo-DCs shown on the left.

3.2.7 24-hour tol-DCs upregulate fewer genes in inflammatory pathways than 24-hour mature mo-DCs

Pathway analysis was performed to compare the differential pathway expression in mature and tol-DCs at 24 hours post-stimulation. The results can be seen in Figure 3-7.



ID	Description
1	immune response
2	inflammatory response
3	innate immune response
4	defense response to virus
5	positive regulation of NF-kappaB transcription factor activi
6	signal transduction
7	protein binding
8	external side of plasma membrane
9	positive regulation of inflammatory response
10	type I interferon signaling pathway

Figure 3-7 Pathway analysis comparing pathways upregulated or downregulated in 24-hour tol-DCs when compared to 24-hour mature mo-DCs.

Each dot corresponds to a gene. The numbers correspond to pathways which can be seen in the table. Blue represents genes which are downregulated in tol-DCs (so up-regulated in mature mo-DCs). Red are genes upregulated in tol-DCs so down regulated in mature mo-DCs.

The top three differentially expressed pathways are immune response, inflammatory response and the innate immune response. 24-hour mature mo-DCs upregulated many genes in these pathways, tol-DCs upregulated fewer and different genes in these pathways compared to mature mo-DCs.

3.2.8 Immunoregulatory molecules upregulated in 24-hour tol-DCs

Table 3-1 Genes upregulated in 24-hour tol-DCs when compared with 24-hour mature mo-DCs.

Genes with a log2 value of >2 and a p-value <0.05 were considered important.

Upregulated in 24hr tol-DCs	Log2 fold change	p-value	Description
CD163	6.58	0.000373	Monocyte/macrophage marker, scavenger receptor. Associated with tissue resident macrophages.
IL1R2	**6.07**	**1.09E-06**	**Decoy receptor for IL-1 family cytokines**
IL18R1	4.44	0.00099	Part of the IL-1R family, IL-18 receptor
CD24	**3.97**	**0.0147**	**Binds DAMPs, can signal and activate MAPK pathway. Can negatively regulate homeostatic T cell proliferation**
FCGR2B	**3.89**	**0.000892**	**CD32b, inhibitory receptor contains ITIMs. Can inhibit cell activation and NF-KB.**
TLR4	3.42	0.00108	PRR
MRC1	**3.2**	**0.000183**	**Mannose receptor C like 1, endocytic receptor which cycles between the plasma membrane and endosomal compartment. Involved in the phagocytosis of dead cells.**
CFH	**2.89**	**0.00128**	**Complement factor H, regulates the alternative pathway**
TLR2	2.88	0.0206	PRR
FKBP5	2.59	3.86E-05	Plays a role in immunoregulation and protein folding and trafficking
IRAK3	2.47	0.000929	IL-1R associated kinase 3
CLEC4E	2.42	0.0119	PRR which binds carbohydrates
C1QA	**2.41**	**0.00147**	**Complement protein, deficiency in C1Q has been shown to be associated with lupus**
S100A8	2.38	0.027	Calcium binding protein involved in cell cycle progression and differentiation
LAIR1	**2.37**	**0.0317**	**Leukocyte associated immunoglobulin like receptor 1, inhibitory receptor prevents cell activation**
C1QB	**1.96**	**0.00199**	**C1Q deficiency associated with lupus**

Twenty four-hour tol-DCs in comparison to 24-hour mature mo-DCs, demonstrated upregulation of very few genes, however some immunoregulatory genes were increased (highlighted in bold in Table 3-1).

IL-1R2 is a decoy receptor for IL-1 inflammatory cytokines which can be expressed on the cell surface or secreted as a soluble form[259]. This receptor can bind IL-1α and IL-1β with high affinity but has a short cytoplasmic tail and no TIR domains so is unable to signal. IL-1R2 deficient mice were found to be more susceptible to collagen type II induced arthritis (CIA)[260]. In humans, IL-1R2 levels were found to be elevated in rheumatoid arthritis patients and this was found to negatively correlate with severity of disease[261]. Furthermore, higher levels of IL-

1R2 production by monocytes in RA patients was found to be predictive of a good response to anti-TNF-α[262].

Mannose receptor c-like 1 or CD206 expression is associated with M2-like anti-inflammatory macrophages and is involved in the phagocytosis of apoptotic cells. CD206 can also be expressed by dendritic cells. A recent study found that there was an increase in MerTK+CD206+ synovial macrophages in healthy controls and RA patients in remission when compared to active RA[263].

FcγRIIb or CD32b is an inhibitory receptor. Human mo-DCs and circulating conventional DCs express two isoforms of the FcγRII, CD32a and CD32b. The balance of these receptors determines the threshold of DC activation. Binding of CD32a induces activation of DCs leading to increased production of pro-inflammatory cytokines and T cell stimulation. Co-ligation of CD32b limits DC activation through CD32a whereas ligation of CD32b alone keeps DCs in an immature state and reduces their T cell stimulatory ability. Binding of IgG immune complexes to CD32b on DCs increases their production of IL-10 and improves their Ag presentation capacity[264]. Additionally, CD32b on DCs has been shown to promote CD4+ and CD8+ T cell tolerance and enhance the generation of Ag specific Tr1 cells[265,266].

CD32b has been shown to be important in mouse models of arthritis. Deletion of the CD32b gene in B6 and DBA/1 mice renders them more susceptible to CIA[267,268]. Furthermore, co-ligation of CD32b was found to reduce immune complex mediated joint inflammation and facilitate the uptake of immune complexes[269]. The balance of activatory and inhibitory FcγRs has been shown to be pivotal in the outcome of experimental arthritis[268,270].

In humans there was found to be increased expression of FcγRII (CD32) on circulating monocytes in RA patients with active disease as well as those in remission[271]. Expression of the inhibitory receptor CD32b however was found to be similar on monocytes from RA patients or healthy controls[272]. Moreover, a single nucleotide polymorphism in CD32b in RA patients was found to be the biggest predictor of joint damage during the first 6 years of RA[273]. This suggests that there is an increase in the activatory FcγRII on immune cells in RA but the

inhibitory receptor CD32b is either defective or does not increase to the same levels so is unable to control cell activation. This is the case in systemic lupus erythematosus (SLE) patients, circulating DCs from these patients have a higher ratio of activatory to inhibitory Fcγ receptors as compared to healthy controls[274].

Interestingly, two complement components were upregulated in tol-DCs, complement factor H and C1Q. Complement factor H is a negative regulator of the alternative pathway and protects self-cells from being lysed. Activation of the alternative pathway results in the formation of the membrane attack complex and the lysis of cell membranes[275]. Dexamethasone tol-DCs have been found to produce significantly more complement factor H than mature mo-DCs. Blocking complement factor H through RNA interference was found to increase tol-DCs immuno-stimulatory capacity on T cells suggesting complement factor H is important for tol-DC function[276]. Additionally, complement factor H on its own is sufficient to generate tol-DCs with reduced expression of co-stimulatory molecules and low production of inflammatory cytokines and increased production of anti-inflammatory cytokines (IL-10+TGF-β). Complement factor H generated tol-DCs were found to reduce CD4+ T cell proliferation, inhibit IFN-γ production and to induce FoxP3+ Tregs[277].

Complement factor H inhibits C3a and C5a, which are components of the alternative pathway. C3a and C5a have been shown to be important in the DC-T cell immune synapse[278,279]. The absence of signalling of C3aR and C5aR on the surface of CD4+ T cells was found to induce Tregs *in vitro* and *in vivo* which produced high amounts of IL-10. Similarly, TGF-β1 was found to prevent C3aR and C5aR signalling in CD4+ T cells[280]. By inhibiting activation of the alternative pathway, tol-DCs could induce Tregs through this mechanism.

C1q is a complement molecule which can activate the classical complement pathway. C1q has additional roles outside of this such as binding to immunoglobulin and labelling apoptotic cells for clearance[281]. C1q promotes FcγR phagocytosis in the absence of C1r and C1s[282]. DCs treated with C1q either by internalising C1q-bound late apoptotic lymphocyte (LAL) or by interacting with immobilised C1q were found to be tolerogenic and could suppress Th1 and Th17 responses *in vitro*[283,284]. C1q can inhibit the production of inflammatory

cytokines by mo-DCs, increase the production of anti-inflammatory cytokines such as IL-10 and additionally induce increased uptake of apoptotic cells[284].

C1q deficiency has long been linked to SLE development[285]. SLE is a chronic autoimmune disease which is characterised by the presence of autoantibodies and the formation of immune complexes. In SLE there is thought to be a failure to clear apoptotic cells due to the lack of C1q and these apoptotic cells provide a source of auto-antigen[286].

C1q and LAIR-1 have both been implicated in preventing mo-DC differentiation as well as being involved in SLE. Due to C1q containing collagen-like sites and LAIR being a collagen receptor Son, M. *et al*. hypothesised that C1q binds LAIR-1[287]. They proved this in cell free binding assays and using cell lines transfected with LAIR-1. C1q phosphorylates LAIR-1 ITIMs on monocytes which has an immune inhibitory effect.

LAIR-2 was found to be an inhibitor of LAIR-1 in a concentration dependent manner as it competes to bind C1q. C1q increased LAIR-1 and CD14 expression on mo-DCs and the addition of LAIR-2 reversed this decreasing LAIR-1 and CD14 expression, lifting the block on monocyte to DC differentiation. DCs treated with C1q secrete decreased levels of inflammatory cytokines such as IL-6 and TNF-α and this is reversed when LAIR-1 is inhibited[287]. Interestingly, increased levels of LAIR-2 are found in rheumatoid arthritis suggesting the immunomodulatory role of C1q and LAIR-1 could be lost. Activated CD4+ T cells were found to be the main source of LAIR-2[288].

The results of this Nanostring assay have identified additional mechanisms by which tol-DCs could function which need to be further validated at the protein level by flow cytometry or ELISA.

3.2.9 Time course of T cell activation during MLRs

Human DC-T cell co-cultures typically run for 10 days but can run longer. To decide what time-points to pick for further genetic analysis, T cell activation marker expression was measured throughout 10 days by flow cytometry.

A mixed-leukocyte reaction (MLR)-type model was employed to investigate the time course of T cell activation. In this model, DCs from one donor are cultured with allogenic T cells from a different donor and there is an immune response against the opposite donor's MHC/peptide complexes. This is due to differences in MHC haplotype between donors[289]. As mature mo-DCs and tol-DCs have similar MHC expression this was an appropriate technique to use. Cultures were maintained for 10 days with the wells being split when the medium turned yellow (indicating the nutrients are depleted), this was usually on day 6.

The gating strategy for gating on T cells can be seen in Figure 3-8. Within the T cell population, the expression of the T cell activation markers CD25, CD69 and CD45-RO were measured. These markers were compared across the different days and the results can be seen in Figure 3-9. CD25 and CD45-RO expression was found to peak on day 10 in both mature and tol-DC groups. CD69 expression peaked on day 6 in T cells co-cultured with tol-DCs and mature mo-DCs. T cells co-cultured with tol-DCs had similar numbers of CD25 and CD45-RO % positive cells as T cells co-cultured with mature mo-DCs until day 5 when T cells co-cultured with tol-DCs had lower numbers. The number of % positive CD69 T cells were similar between the groups until day 7, when T cells co-cultured with tol-DCs express higher numbers than T cells co-cultured with mature mo-DCs.

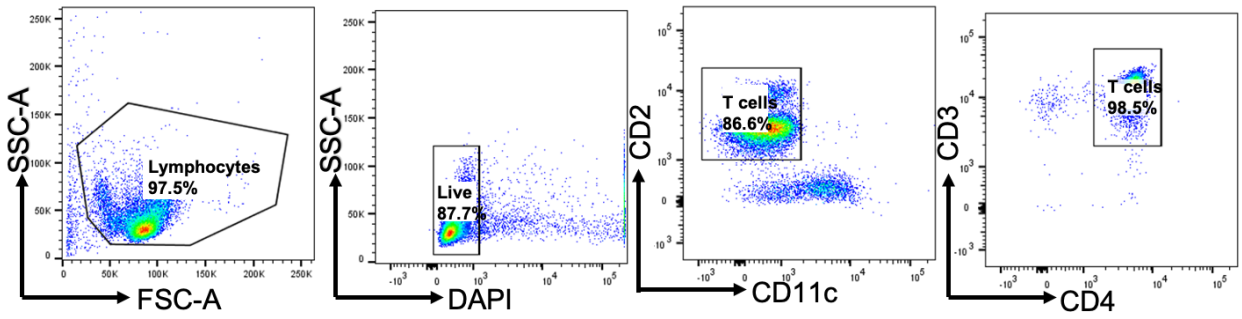


Figure 3-8 T cell flow cytometry gating strategy.

Lymphocytes were gated using SSC-A vs FSC-A. Dead cells were excluded using DAPI. CD2+ CD3+ CD4+ T cells were gated excluding CD11c+ cells.

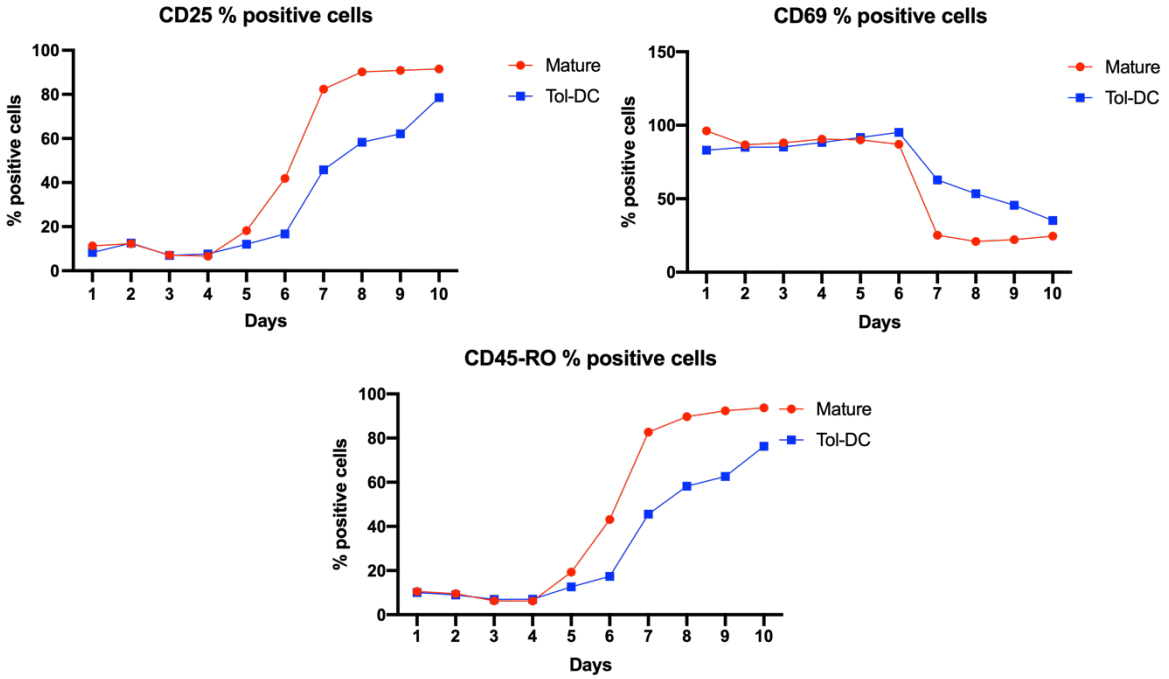


Figure 3-9 Tol-DCs induce lower levels of CD25 and CD45-RO expression than mature mo-DCs.

T cells either co-cultured with mature mo-DCs or tol-DCs were gated on using the gating strategy seen in Figure 3-8. The expression of T cell activation markers CD25, CD69 and CD45-RO were then measured and compared across 10 days of the MLR.

The median fluorescent intensity (MFI) values were comparable for the different T cell activation markers throughout two separate MLRs. The results from these two MLRs can be seen in Figure 3-10. CD25 peaks on day 7 and CD45-RO peaks on day 10 in both experiments. CD69 seems to remain high till day 5 or 6 after which it drops off to low levels.

For the next Nanostring assay we decided to choose day 3 and day 6 as the time-points to compare gene expression of T cells co-cultured with mature mo-DCs or

tol-DCs. We wanted to include an early and a later time-point. Although some of the activation markers peak later than day 6, after day 6 we would have had to split the wells which could affect RNA expression on days 7-10.

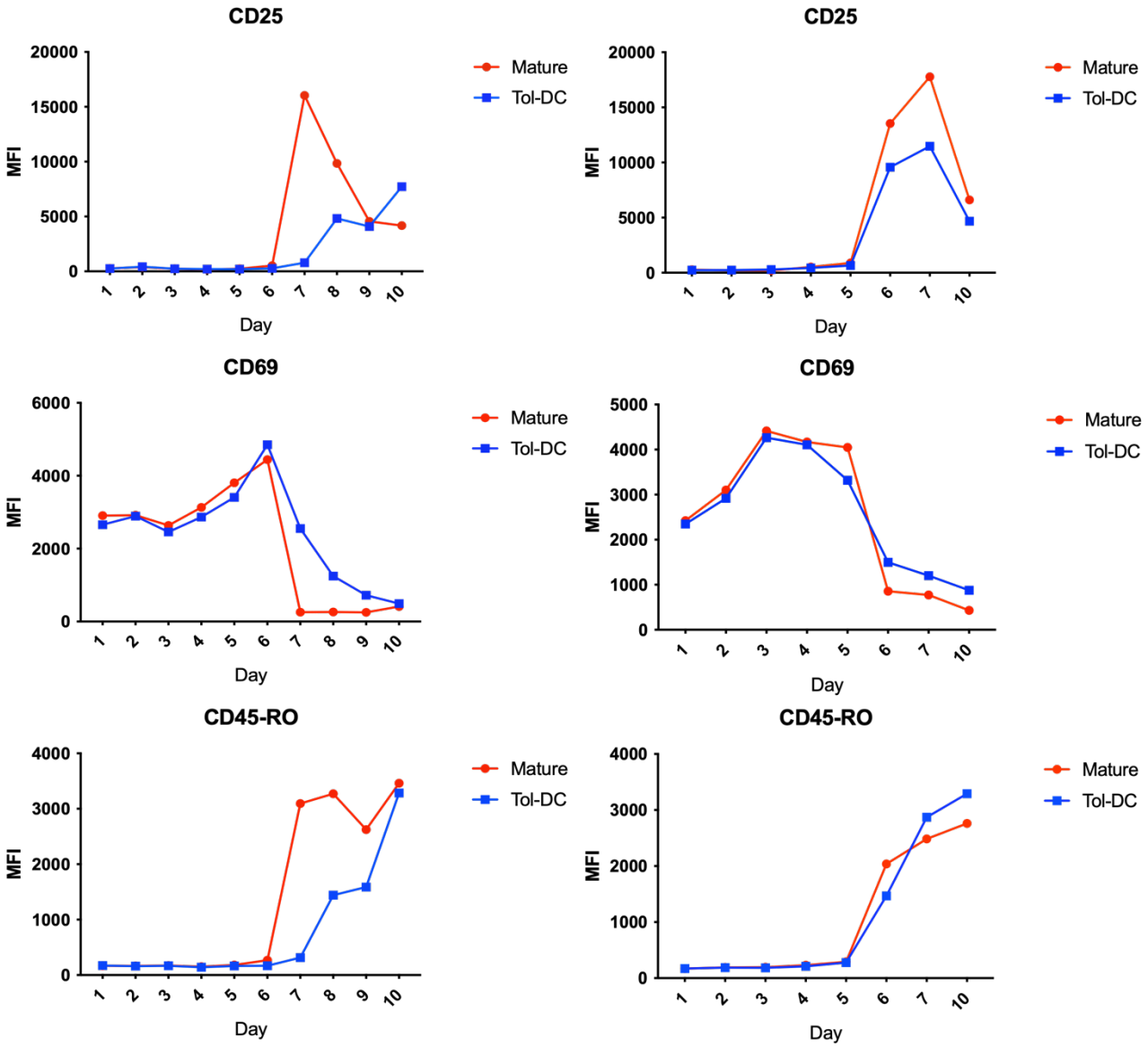


Figure 3-10 Graphs of median fluorescent intensity of the T cell activation markers throughout two comparable MLRs.

The results from two MLRs can be seen side-by-side for the different T cell activation markers.

3.2.10 Tol-DCs induce smaller clusters and lower levels of IFN-γ production from T cells

A representative picture of the wells of an MLR on day 6 can be seen in Figure 3-11. Wells containing mature mo-DCs and T cells typically had larger clusters than wells containing tol-DCs and T cells.

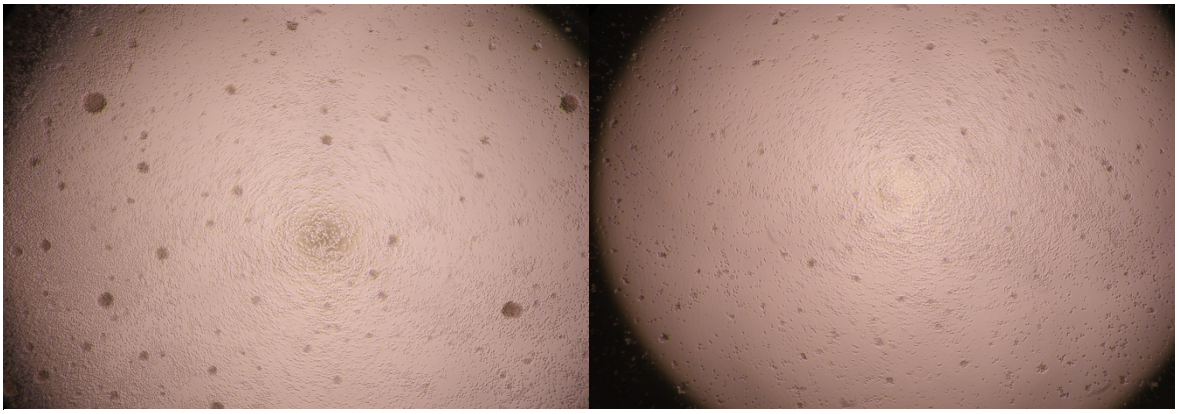


Figure 3-11 Mature mo-DCs and T cells form larger clusters than tol-DCs and T cells. Representative pictures of the wells of a MLR on day 6. Mature mo-DCs and T cells are shown on the left and tol-DCs and T cells on the right.

Supernatants from day 6 of MLRs were collected and analysed for IFN-γ production. The results can be seen in Figure 3-12. T cells co-cultured with mature mo-DCs produced significantly more IFN-γ than T cells co-cultured with tol-DCs.

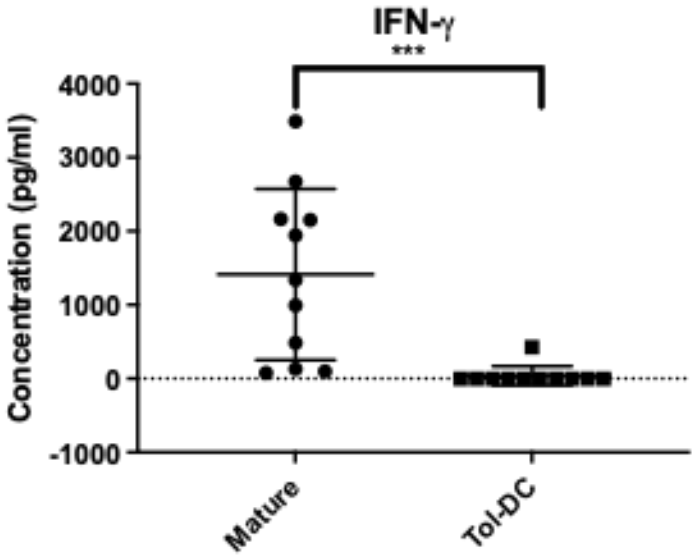


Figure 3-12 Mature mo-DCs induce significantly higher IFN-γ production from T cells than tol-DCs.

Supernatants were collected on day 6 of MLRs and analysed by ELISA for IFN-γ expression. Data failed normality tests therefore a Wilcoxin signed-rank test was performed. *** =p-value <0.001. n=11

Naïve T cells were cultured with mature or tol-DCs for 3 or 6 days and then analysed for T cell activation marker expression by flow cytometry and the remaining cells were sorted for genetic analysis.

CD25, CD69, and CD45-RO expression was measured by flow cytometry. The percentage of positive cells and the MFIs were plotted for 10 separate experiments, the results can be seen in Figure 3-13. T cells cultured alone on day 0, 3 and 6 were used as controls. All of the T cell activation markers MFI values and percentage positive cells were significantly increased in the mature mo-DC-T cell group from day 3 to day 6.

The T cells co-cultured with tol-DCs significantly upregulated the number of CD25 and CD45-RO positive cells from day 3 to day 6. The MFI values were only significantly different between the two time-points for CD69. Although the number of CD25 and CD45-RO positive cells significantly increased from day 3 to day 6 in the tol-DC group, these values never reached the same levels as for the mature mo-DC group.

CD69 expression was similar between T cells treated with mature mo-DCs or tol-DCs. The MFI values and proportion of positive cells were low for CD69 compared with the other markers. As CD69 is an early T cell activation marker, the biggest difference may be earlier, such as within the first 24 hours. Day 3 and day 6 may be too late to see a significant difference in CD69 expression between the mature mo-DC and tol-DC groups.

On days 3 and 6, T cells were sorted from MLRs for genetic analysis. A representative sort can be seen in Figure 3-14.

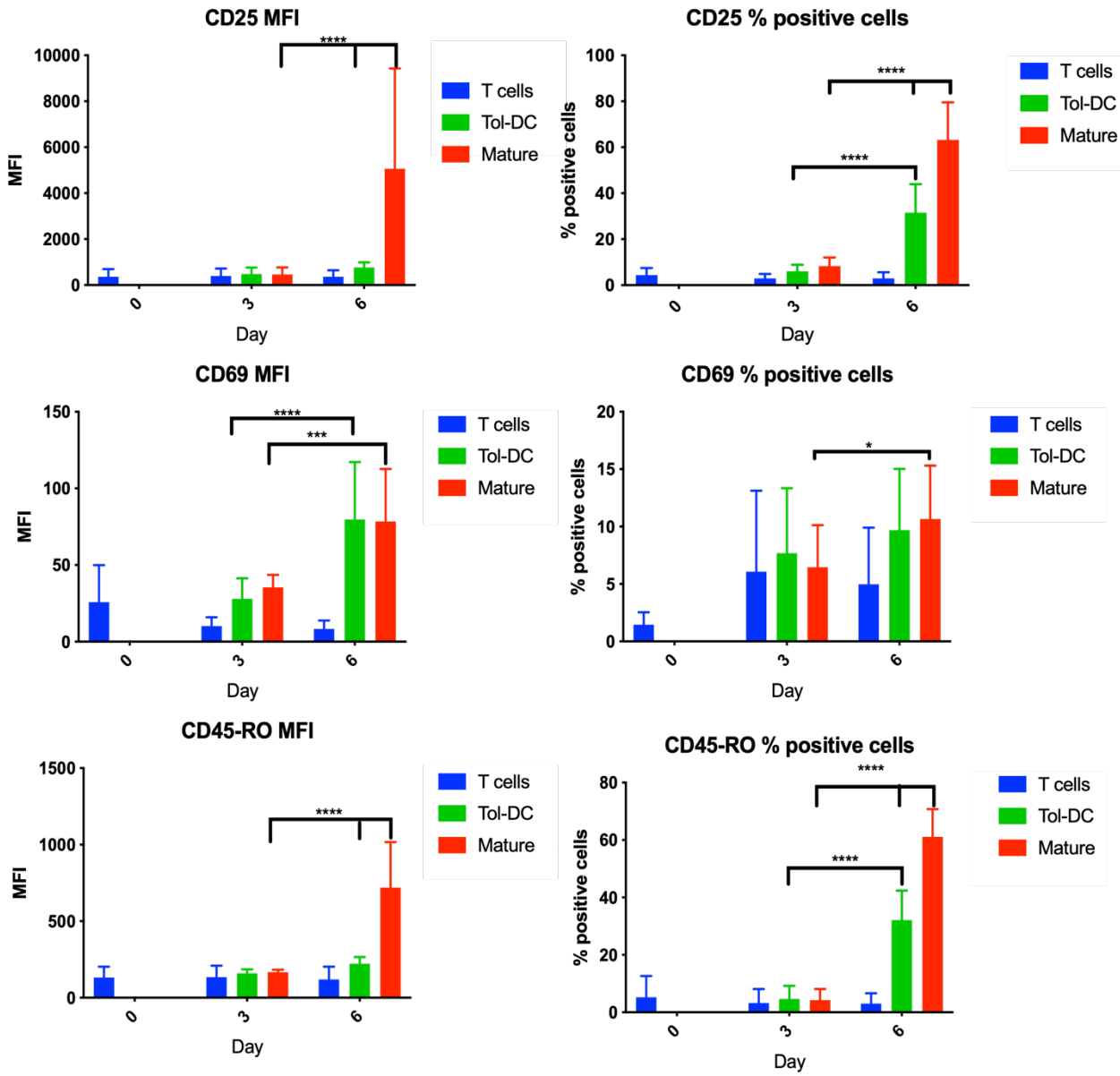


Figure 3-13 Tol-DCs induce significantly lower levels of T cell activation than mature mo-DCs on day 3+6 of MLRs.

T cells co-cultured with mature or tol-DCs were harvested on day 3 and day 6 and expression of CD25, CD69 and CD45-RO were measured by flow cytometry. T cells cultured alone were measured on day 0, 3 and 6 as a control. Data was found to be normally distributed by a Shapiro-Wilk test. A 2-way ANOVA with a post-correctional Tukey test was performed * = p-value <0.05 ** = p-value <0.01 *** = p-value <0.001 **** = p-value <0.0001. n=10.

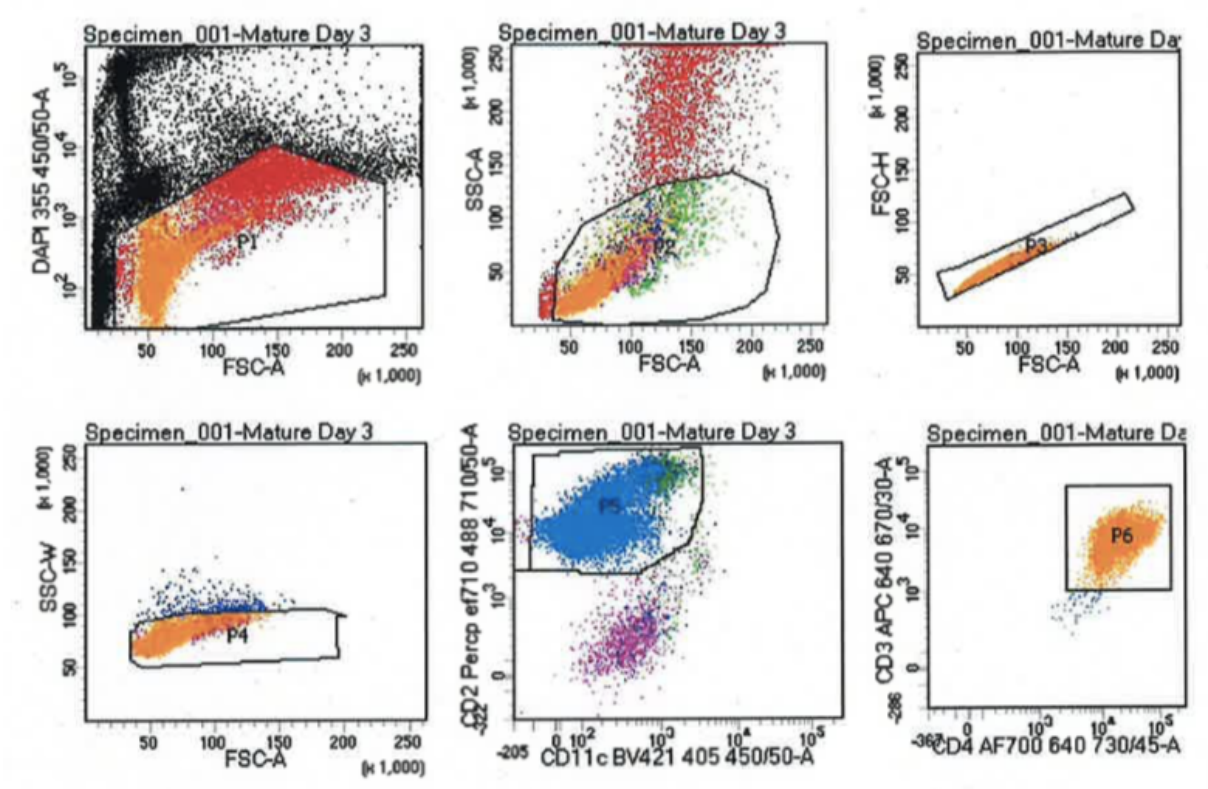


Figure 3-14 Representative sort of CD2+CD3+CD4+ T cells on Day 6 of MLR.

Dead cells were excluded using DAPI, lymphocytes were gated using SSC-A vs FSC-A. Doublets were then excluded and T cells were gated using CD2, CD3 and CD4. CD11c was used to distinguish DCs from T cells.

3.2.11 T cells separate by day of MLR rather than treatment

Using the same Nanostring nCounter assay as described previously, we compared 579 human immune genes between T cells cultured with mature or tol-DCs at day 3 and day 6. The principal component plots (PC1 vs PC2) and the heat maps of all data after normalisation can be seen in Figure 3-15.

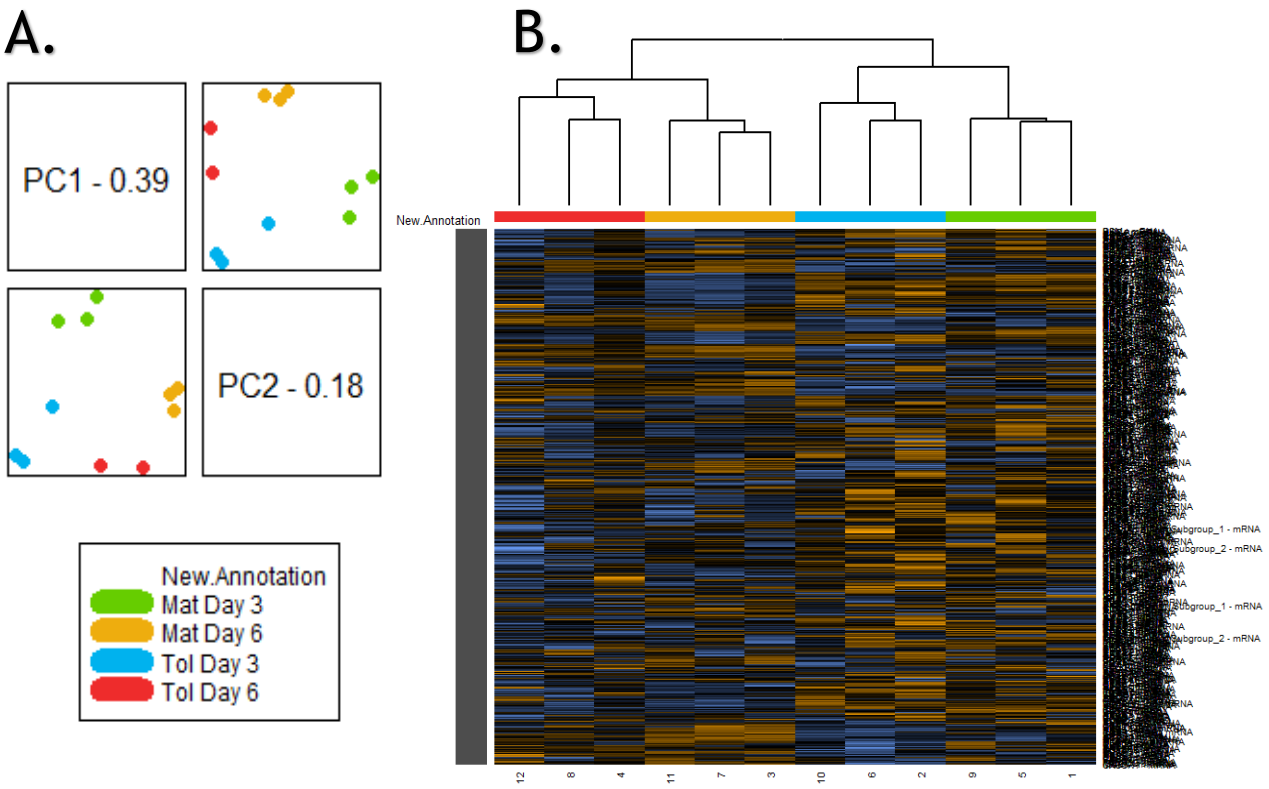


Figure 3-15 Nanostring data comparing T cells co-cultured with mature or tol-DCs on day 3+6 of MLRs.

A. shows the principal component analysis, PC1 vs PC2. **B.** shows the heatmap of all data after normalisation. Blue shows genes which have been downregulated and orange shows genes which have been upregulated.

Figure 3-15A shows the principal component analysis of principal component 1 vs principal component 2, which account for 39% and 18% of the separation respectively. This demonstrates that the different cell type replicates from three different donors group together in clusters.

Figure 3-15B shows the heatmap of all data after normalisation. T cells treated with mature mo-DCs or tol-DCs at day 3 branch off together but separately. The same is true for the groups at day 6 and compared to the day 3 time-point there seems to be more of a downregulation of genes.

3.2.12 Differentially expressed genes on Day 3 of MLRs

Figure 3-16 shows the volcano plot of differentially expressed genes when T cells treated with mature or tol-DCs are compared at day 3. When the groups are compared at this time-point there are 49 differentially expressed genes (p-values <0.05), shown in table 6 and 7 in the appendix. IFN-γ is upregulated in T cells co-cultured with mature mo-DCs when compared to T cells co-cultured with tol-DCs (log2 fold change of 6.96). This confirms the ELISA result shown previously that tol-DCs induce low IFN-γ production in T cells.

There are very few genes upregulated in T cells co-cultured with tol-DCs. IL-16 and LAIR-1 are the genes with the highest upregulation in T cells co-cultured with tol-DCs (log2 fold change of 0.919 and 1.24 respectively).

SLAMF6 was upregulated in T cell co-cultured with tol-DCs but with a lower log2 fold change of 0.786. Interestingly SLAMF6 is a transmembrane protein which has been shown to be important in preventing autoimmunity. Adoptive transfer of SLAMF6 deficient CD4+ T cells were shown to induce a SLE-like phenotype in recipient mice[290].

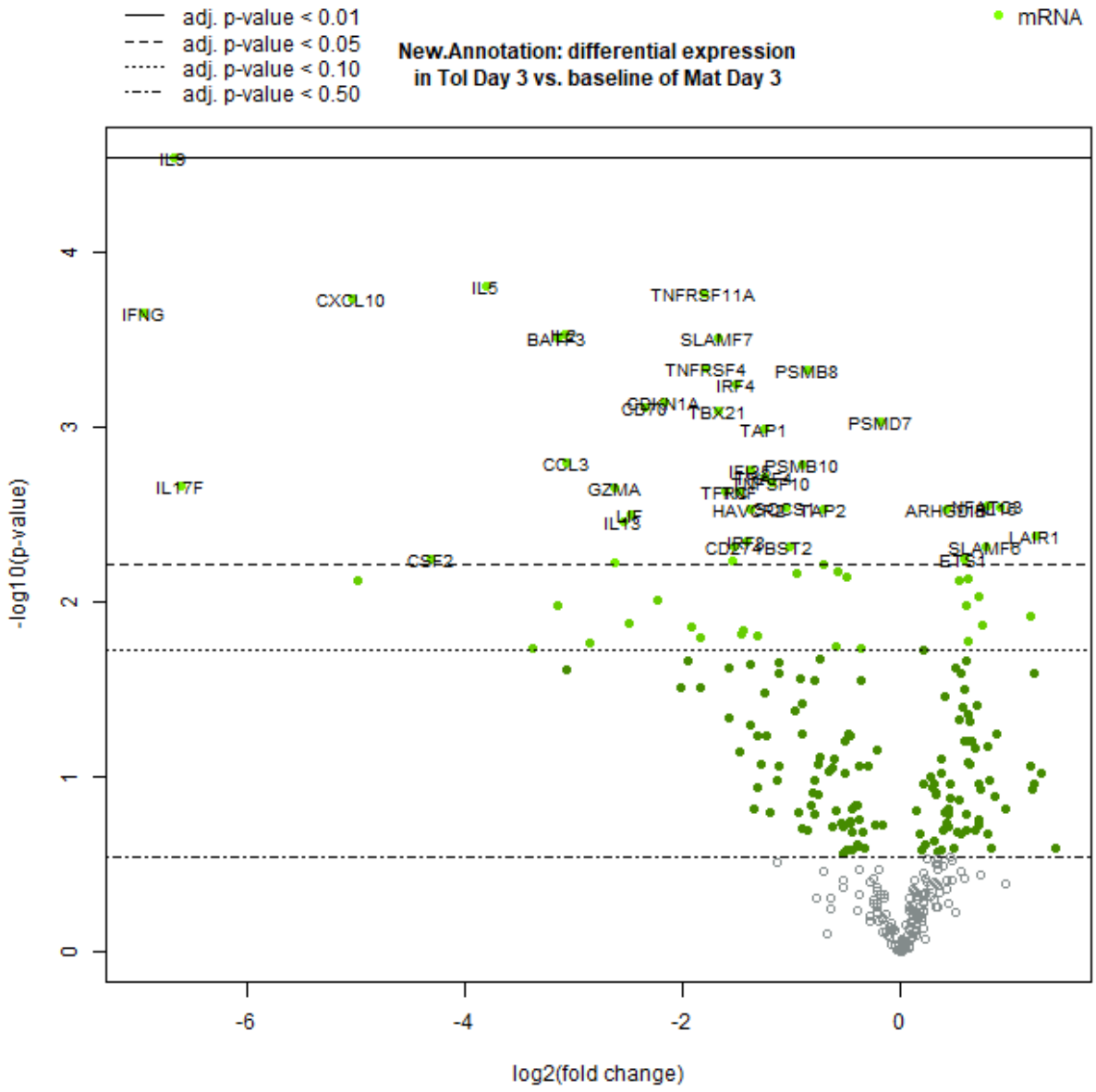


Figure 3-16 Volcano plot of differentially expressed genes when T cells co-cultured with mature or tol-DCs are compared on day 3 of MLR.
A Nanostring assay was performed to compare mRNA expression of 570 human immunology genes. Differentially expressed genes when T cells cultured with tol-DCs on Day 3 of MLRs when compared to T cells cultured with mature mo-DCs on Day 3 of MLRs are shown here. Genes upregulated in T cells cultured with tol-DCs are shown on the right and genes upregulated in T cells co-cultured with mature mo-DCs are shown on the left.

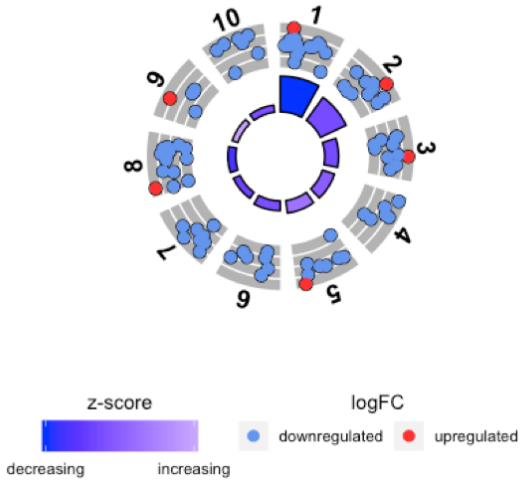
IL-16 is a cytokine which can have pro- or anti-inflammatory effects. IL-16 can induce an upregulation of IL-2R and CD4 cross-linking on T cells[291]. However, IL-16 can also inhibit TCR/CD3 dependent activation and proliferation leading to T cell anergy[292]. Additionally, IL-16 is a chemoattractant for CD4+ T cells and could preferentially attract Tregs[293]. There is no IL-16R, IL-16 instead binds directly to CD4 so testing for IL-16 would have to be through an ELISA[294].

LAIR-1 is an inhibitory receptor which contains ITIM motifs and can prevent cell activation. Naïve T cells have been found to express the highest levels of LAIR-1, suggesting T cells co-cultured with tol-DCs are more naïve-like[295]. LAIR-1 can crosslink on the cell surface and prevent TCR mediated signals. TCR activation leads to upregulation of LAIR-1 on the cell surface. Mitogen activated protein kinase 14 (p38α) was also found to be upregulated in T cells co-cultured with tol-DCs. MAP kinase or p38 signalling triggered after TCR activation has been shown to increase LAIR-1 as MAP kinase inhibitors decreased LAIR-1 expression on T cells[295].

LAIR-1 sufficient CD4+ T cells were found to produce lower levels of IL-2, IL-17 and IFN-γ after stimulation with anti-CD3 and collagen than LAIR-1 deficient CD4+ T cells[296]. Similarly, treating CD4+ T cells with LAIR-1 stimulating Abs was found to reduce their production of cytokines after CD3 and collagen stimulation. In arthritis models, LAIR-1 KO mice were found to develop a more severe form of CIA than WT mice and treatment with LAIR-1 stimulating Abs was found to suppress CIA[296]. In humans, LAIR-1 has been found to be significantly decreased on circulating CD4+ T cells in RA patients compared to osteoarthritis patients and healthy controls [297].

3.2.13 T cells co-cultured with tol-DCs upregulate fewer inflammatory pathways on Day 3 of MLRs

Pathway analysis was performed and compared between T cells co-cultured with mature or tol-DCs on day 3. The results can be seen in Figure 3-17.



ID	Description
1	immune response
2	cytokine activity
3	inflammatory response
4	positive regulation of B cell proliferation
5	adaptive immune response
6	tumor necrosis factor-mediated signaling pathway
7	cell-cell signaling
8	extracellular space
9	positive regulation of interferon-gamma production
10	external side of plasma membrane

Figure 3-17 Pathway analysis comparing pathways upregulated or downregulated in T cells co-cultured with tol-DCs when compared to T cells co-cultured with mature mo-DCs on day 3.

Each dot corresponds to a gene. The numbers correspond to pathways which can be seen in the table. Blue represents genes which are downregulated in T cells co-cultured with tol-DCs so up-regulated in T cells co-culture with mature mo-DCs. Red are genes upregulated in T cells co-cultured with tol-DCs so down regulated in T cells co-cultured with mature mo-DCs.

Very few genes are upregulated in T cells co-cultured with tol-DCs when compared with T cells co-cultured with mature mo-DCs on day 3. The top two differentially expressed pathways are immune response and cytokine activity. T cells co-cultured with tol-DCs only upregulate one gene in these pathways whereas T cells co-cultured with mature mo-DCs upregulate many.

3.2.14 Lower numbers of differentially expressed genes between T cells co-cultured with mature mo-DCs or tol-DCs when compared on Day 6 rather than Day 3 of MLRs

Next, T cells co-cultured with mature or tol-DCs were compared at the day 6 time-point, the results can be seen in Figure 3-18. There were only 7 differentially expressed genes at this time-point (p-values <0.05), significantly fewer than when the groups are compared at day 3. The 7 differentially expressed genes were upregulated in the mature DC-T cell group (shown in table 8 in appendix). The genes that were upregulated in T cells co-cultured with tol-DCs did not reach statistical significance. CD83 and IL-16 were the genes with the highest log2 fold change with the lowest p-values of 0.07 and 0.09 respectively. CD83 is classically thought of as a DC maturation marker but it can

also be expressed on T cells. There is some literature to suggest CD83 can have an immunoregulatory role in T cells in humans and mice[298,299].

From this study LAIR-1 or IL-16 appear to be upregulated in T cells co-cultured with tol-DCs and warrant further investigation. The next step would be to perform RNA sequencing as this includes a much larger range of genes. This study suggests choosing an earlier time-point for RNA sequencing experiments would be beneficial as there were more differentially expressed genes at day 3 than at day 6.

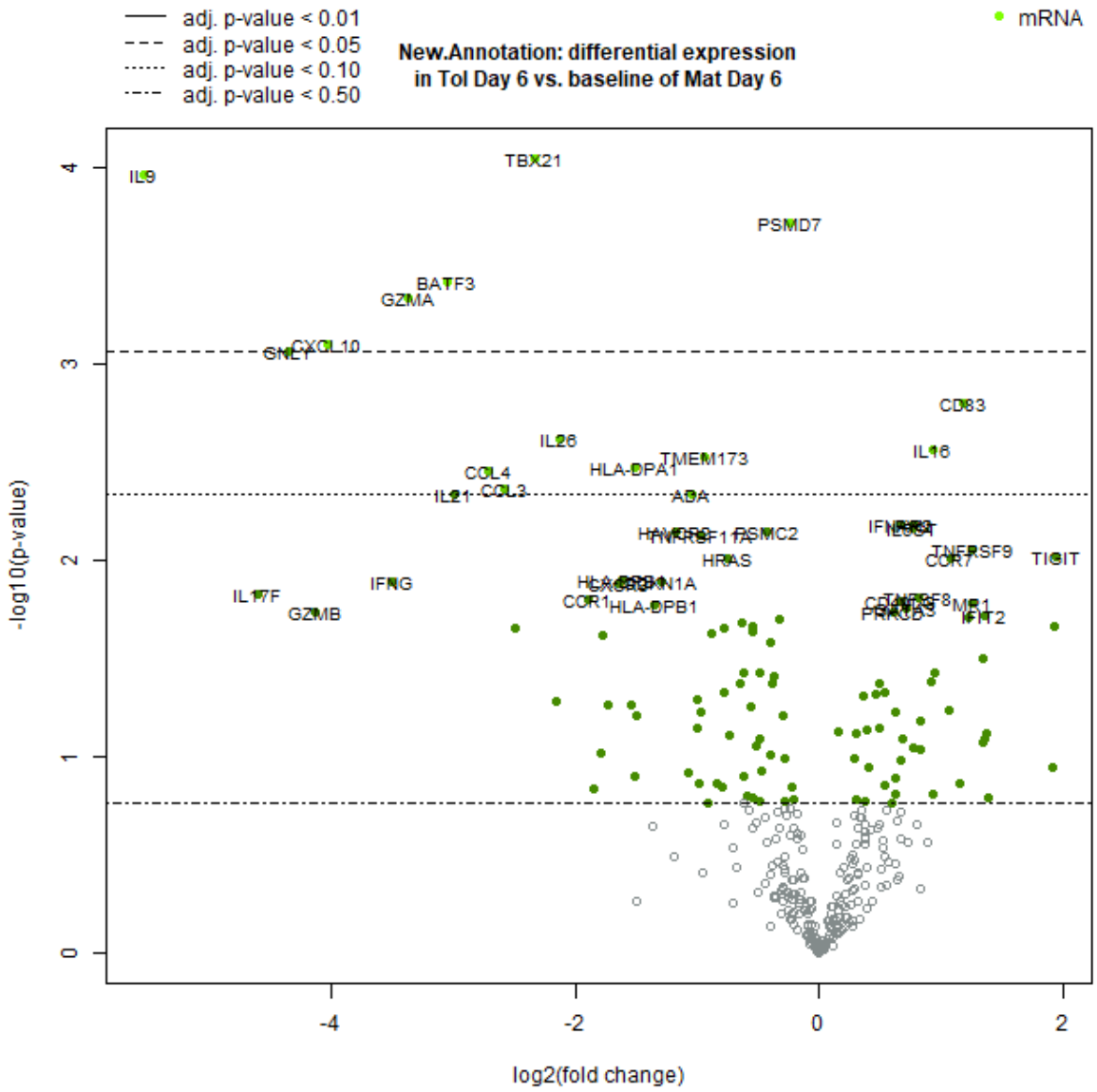


Figure 3-18 Volcano plot of differentially expressed genes when T cells co-cultured with mature or tol-DCs are compared on day 6.
A Nanostring assay was performed to compare mRNA expression of 570 human immunology genes. Differentially expressed genes when T cells cultured with tol-DCs on Day 6 of MLRs when compared to T cells cultured with mature mo-DCs on Day 6 of MLRs are shown here. Genes upregulated in T cells cultured with tol-DCs are shown on the right and genes upregulated in T cells co-cultured with mature mo-DCs are shown on the left.

3.3 Discussion

Comparing gene expression of mature and tol-DCs at 24-hours post-stimulation has identified potential mechanisms of action of tol-DCs other than TGF-β. These include pathways involved in regulation of IL-1α and IL-1β inflammatory cytokines through the IL-1 decoy receptor, inhibitory receptors such as LAIR-1 and FcγRIIb (CD32b) preventing cell activation, increased phagocytosis of apoptotic cells and complement regulation. These genes and pathways will need to be validated at the protein level either by flow cytometry or ELISA and shown to be functionally relevant for tol-DCs mechanism of action on T cells. FcγRII (CD32) and IL-1R2 have since been validated at the protein level by flow cytometry (data not shown) and are being investigated as potential QC markers of tol-DCs for AUTODECRA 2.

To test whether these molecules are important for tol-DCs immunoregulatory effects on T cells, they could be blocked in tol-DCs either by using monoclonal Abs (mAbs) or short interfering RNA (siRNA) before culturing with CD4+ T cells. The effect on T cell activation, proliferation and cytokine production could then be assessed.

These results are consistent with a study comparing gene expression between different tol-DC types which found C1q and IL-1Rα (an IL-1R antagonist) to be the only genes consistently upregulated in several different tol-DC types[300]. C1QA was found to be upregulated in TGF-β, IL-10, Dexamethasone and Dex+VitD3 tol-DCs[300–303,211]. Similarly, C1QC was upregulated in Dexamethasone, IL-10 and rapamycin tol-DCs[304]. As C1q is a common gene upregulated in multiple tol-DC types it suggests C1q is an important molecule for tol-DCs mechanism of action.

C1q is thought to be important in promoting the clearance of apoptotic cells and immune complexes[281]. Similarly, mannose receptor c-like 1 (MRC-1) is involved in the phagocytosis of dead cells and FcγRIIb (CD32b) has been shown to promote the clearance of immune complexes[305,270]. MRC-1 has previously been shown to be upregulated in Dexamethasone and IL-10 tol-DCs[304]. Additionally, Dexamethasone treatment on DCs has been shown to increase mannose receptor

endocytosis[306]. FcγRIIb (CD32b) has been found to be upregulated in Dexamethasone, Dex+VitD3, IL-10 and IL-10+TGF-β tol-DCs previously[211,303,307].

The fact that three molecules involved in the phagocytosis of apoptotic cells and immune complexes are upregulated in tol-DCs suggests this may be an important function of tol-DCs. This could be tested by labelling cells with a fluorescent dye such as CFSE and inducing apoptosis by irradiating cells with UV or camptothecin[308,309]. These cells could then be added to mature mo-DC or tol-DC cultures and their uptake of the fluorescent apoptotic cells could be measured by flow cytometry.

There is a lot of evidence to suggest that a failure to clear apoptotic cells leads to autoimmunity[310]. Delayed clearance of apoptotic cells can lead to secondary necrosis and the release of DAMPs[311].

Immature dendritic cells which ingest apoptotic cells become tolerogenic and fail to activate T cells, instead inducing FoxP3+ Tregs. They produce more anti-inflammatory cytokines, express reduced levels of co-stimulatory molecules and are resistant to further stimulation[89]. Tol-DCs can be generated through exposure to apoptotic cells *in vivo*. Adoptive transfer of tol-DCs generated in this way were found to inhibit effector memory CD4+ T cells in an EAE mouse model[312].

Dendritic cells in the steady state, continuously sample apoptotic cells which gives a tolerogenic signal to intracellular and self-antigens. Other forms of cell death can lead to new self-antigens being seen by the immune system. The immune system may not have been tolerised to these new self-antigens before and this could trigger new immune responses and autoimmunity.

3.3.1 Identification of a biomarker of successful tol-DC therapy

The choice of naïve T cells in this study assumes tol-DCs reach the LN after injection, where naïve T cell-DC interactions take place. Previous studies in a CIA model have shown tol-DCs reach the lymph nodes following intravenous injection[234]. Although tol-DCs did not migrate in great numbers to the popliteal LN, they mainly migrated to the liver and the arthritic feet. The change in

injection route, from intravenous to subcutaneous could increase tol-DC migration to the dLN but this has not yet been proven for Dex+VitD3 tol-DCs.

It is important to know whether tol-DCs act on naïve T cells, activated T cells or both as this would change the location the tol-DCs need to be. Naïve T cells are found in the lymph nodes whereas activated T cells are found in the tissues.

Nonetheless, this study has still given us interesting insights into what tol-DCs do to naïve T cells. This study found that tol-DCs induce lower levels of T cell activation than mature mo-DCs. Additionally, from the ELISA results mature mo-DCs induce significantly more IFN-γ production by T cells than tol-DCs. This is confirmed in the gene expression analysis as T cells co-cultured with mature mo-DCs differentially expressed IFN-γ. Similarly, BATF3, Tbx21, IL-2, IL-5, IL-13 and IL-17F are differentially expressed in T cells co-cultured with mature mo-DCs compared with T cells co-cultured with tol-DCs. BATF3 is a Th17 specific transcription factor and IL-17F is a Th17 specific cytokine. Tbx21 is a Th1 specific transcription factor and IL-5 and IL-13 are Th2 cytokines. IL-2 is important in preventing T cell anergy. These results together suggest tol-DCs induce fewer Th1, Th2, and Th17 cells than mature mo-DCs. This is consistent with previous studies that demonstrate tol-DCs induce lower IFN-γ and IL-17 production from T cells than mature mo-DCs during co-cultures[217,253]. These studies conclude that tol-DCs suppress CD4+ and CD8+ T cell responses.

The few genes that are upregulated in naïve T cells as a result of tol-DC interaction are immunosuppressive such as LAIR-1 an inhibitory receptor which prevents cell activation. LAIR-1 and C1q have been shown to be able to interact, LAIR-1 on T cells could interact with C1q on tol-DCs. C1q phosphorylates ITIMs on LAIR-1 which has an immune inhibitory effect[287]. LAIR-1 was found to be upregulated on tol-DCs as well as T cells co-cultured with tol-DCs. LAIR-1 can interact homotypically and LAIR-1 has been shown to crosslink on T cells[295]. This suggests LAIR-1 on tol-DCs and LAIR-1 on T cells could interact.

IL-16 is another gene upregulated in T cells co-cultured with tol-DCs. IL-16 is a cytokine which can have pro or anti-inflammatory functions. IL-16 can preferentially attract Tregs and induce T cell anergy[293]. There is no IL-16R so the only way to test for IL-16 would be through an ELISA which would perhaps not

make it a suitable biomarker as this would be more time-consuming than testing for a biomarker by flow cytometry. Additionally, the cells would have to be sorted before performing an ELISA to ensure the IL-16 was produced by the T cells. LAIR-1 could be a potential biomarker at day 3 and could be tested for by flow cytometry.

The next step is to perform RNA sequencing. The Nanostring assay only included 579 immune genes. RNA sequencing would sequence every RNA molecule present in the sample. There might be a more differentially expressed gene if a higher number of genes were included. From this work choosing an earlier time-point rather than a later time-point would be advisable as there were more differentially expressed genes at day 3 rather than day 6. RNA sequencing would be more difficult on day 3 however as the T cells have not proliferated much by that point so RNA yields would be lower. By day 6 there is a substantially higher number of T cells.

This study has identified other potential mechanisms of action of tol-DCs other than TGF-β. These will need to be further validated at the protein level and shown to be important for tol-DCs suppressive capacities on T cells. Some of these molecules could be used as functional QC markers for AUTODECRA 2, such as CD32b (FcγRIIb) or IL-1R2 which have already been validated at the protein level by flow cytometry. This study has confirmed tol-DCs induce lower levels of T cell activation and lower IFN-γ production than mature mo-DCs which is consistent with previous findings. LAIR-1 and IL-16 were genes induced in naïve T cells as a result of tol-DC interaction. LAIR-1 could be a possible biomarker of successful tol-DC therapy as it can be detected by flow cytometry. RNA sequencing needs to be performed to confirm these findings or to detect a gene which is differentially expressed to a higher degree by T cells co-cultured with tol-DCs other than LAIR-1.

Chapter 4 Characterisation of Murine Dexamethasone and Vitamin D3 Tolerogenic Dendritic cells

4.1 Introduction

Due to the difficulties with performing tol-DC migration studies in humans, the remainder of this project was carried out on murine cells. Before these migration studies could be performed, it was first important to verify that human and murine Dex+VitD3 tol-DCs were comparable.

There are differences in the procedures for generating human and murine tol-DCs. Murine tol-DCs are generated from bone marrow by adding GM-CSF and will contain DCs from CDPs as well as mo-DCs and mo-macs[63]. Whereas human tol-DCs are generated only from monocytes by adding GM-CSF and IL-4. Murine Dex+VitD3 tol-DCs are defined phenotypically in this chapter when compared to mature BM-DC. This is to confirm murine Dex+VitD3 tol-DCs are immunoregulatory when compared to mature BM-DCs and are comparable to human Dex+VitD3 tol-DCs.

Historically, tol-DCs are thought to be more macrophage-like than mature BM-DCs[227]. Recent studies have found that BM-DC cultures contain DC-like and macrophage-like cells[63]. To assess if there were any differences in the relative contributions of DCs and macrophages to mature BM-DC and tol-DC cultures DC and macrophage specific markers were tested and levels compared between the two groups. This is important as the differences we see between mature BM-DCs and tol-DCs could be due to differing contributions of DCs or macrophages. Mo-macs are thought to be poorer at Ag presentation, T cell stimulation and migration to the dLN than mo-DCs[8,10,67].

To further confirm that murine Dex+VitD3 tol-DCs were similar to human Dex+VitD3 tol-DCs the immunoregulatory genes found to be upregulated in human tol-DCs in Chapter 3 were tested in murine tol-DCs by Q-PCR.

Before carrying out migration studies and testing this tol-DC type in the breach of tolerance model of arthritis the ability of murine Dex+VitD3 tol-DCs to

modulate T cell activation *in vitro* was assessed. Murine Dex+VitD3 tol-DCs were tested for their ability to suppress an Ag-specific OVA response in CD4+ OT-II T cells which have a transgenic TCR specific for OVA peptide. This differs from the human MLRs performed in Chapter 3 as in MLRs the immune response is directed against differing MHC haplotypes between the different donors which could vary from culture to culture depending on the donors. Using mouse models allows us to measure the effect of tol-DCs on an Ag-specific CD4+ T cell response.

The main aims of this chapter are summarised below:

- Characterise the murine Dex+VitD3 tol-DCs phenotype compared with mature BM-DCs

- Determine relative contributions of DCs and macrophages to mature BM-DC and tol-DC cultures

- To assess whether tol-DCs can modulate an Ag-specific immune response to OVA peptide using DC-T cell co-cultures with OVA-specific CD4+ T cells

- Compare the interaction between mature BM-DCs and T cells and tol-DCs and T cells in co-cultures

4.2 Results

4.2.1 Phenotype of mature BM-DCs and tol-DCs by flow cytometry

Murine Dex+VitD3 tol-DCs were characterised by flow cytometry. Representative flow cytometry plots of the gating strategy for mature BM-DCs and tol-DCs can be seen in Figure 4-1. First, leukocytes are gated avoiding debris, then live cells are identified and doublets are excluded using FSA-A and FSC-H. There are similar numbers of CD11c and CD11b positive cells between mature BM-DCs and tol-DCs. Histograms showing the representative phenotype of mature BM-DCs and tol-DCs can be seen in Figure 4-2. CD80 expression is similar between mature BM-DCs and tol-DCs. MHC class II expression is either similar or slightly lower in tol-DCs. However, tol-DCs consistently express lower levels of CD86 and CD40 than mature BM-DCs.

The only marker upregulated in tol-DCs when compared with mature BM-DCs is MerTK. MerTK is a tyrosine kinase receptor and is often used as a macrophage marker. MerTK has previously been shown to be upregulated in human Dexamethasone tol-DCs and has been found to be essential for their immunoregulatory effect on T cells[313]. MerTK is induced by Dexamethasone treatment.

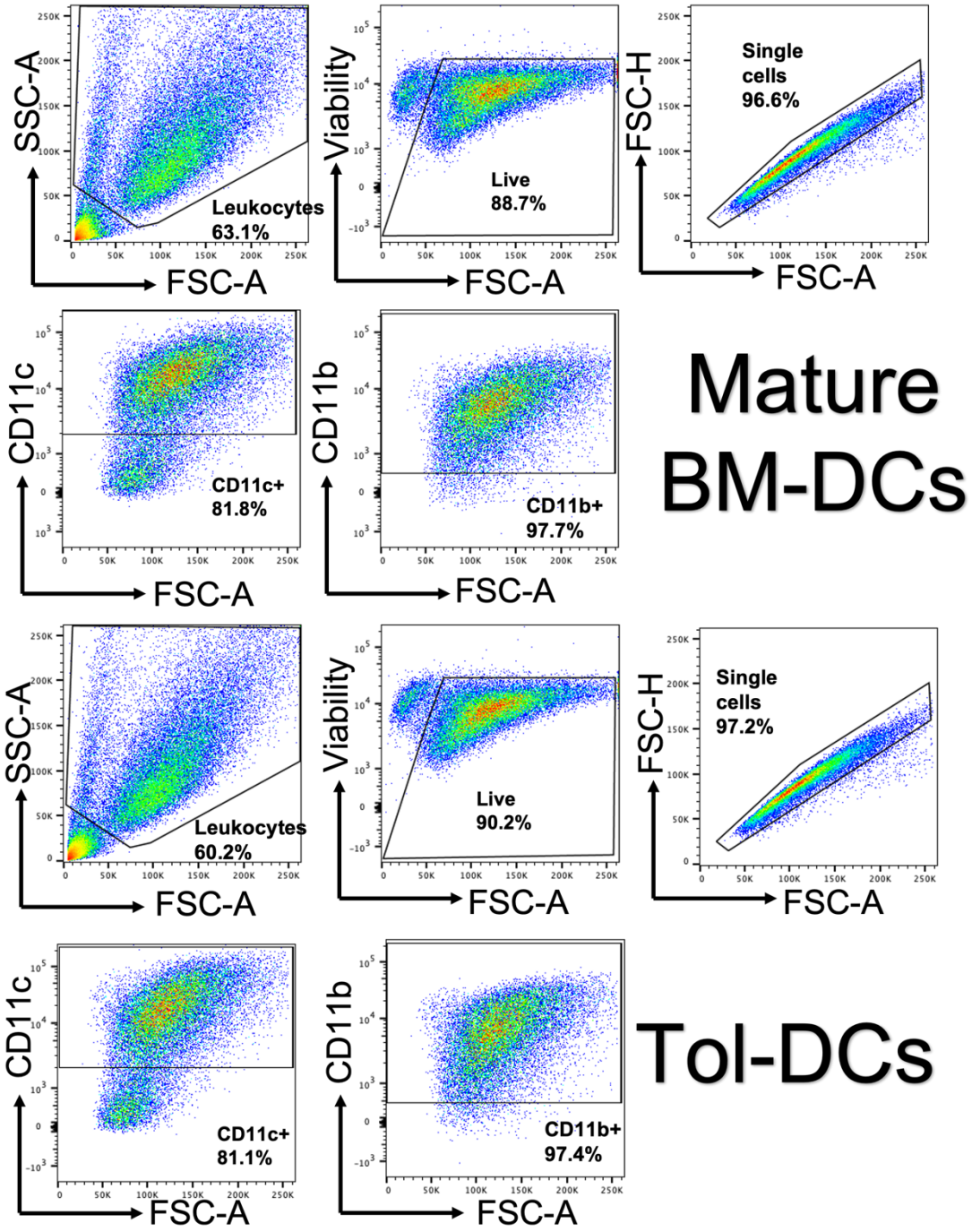


Figure 4-1 Flow cytometry gating strategy for mature BM-DCs and tol-DCs.

Bone marrow cells are cultured for 6 days with GM-CSF to induce their differentiation into DCs. On Day 7 mature BM-DCs are stimulated with LPS and tol-DCs are stimulated with LPS, Dexamethasone and Vitamin D3. On Day 8 cells are harvested and stained with flow cytometry Abs. First leukocytes are gated, excluding debris, then live cells are identified and doublets are excluded using FSC-H and FSC-A. CD11c and CD11b positive cells are then identified.

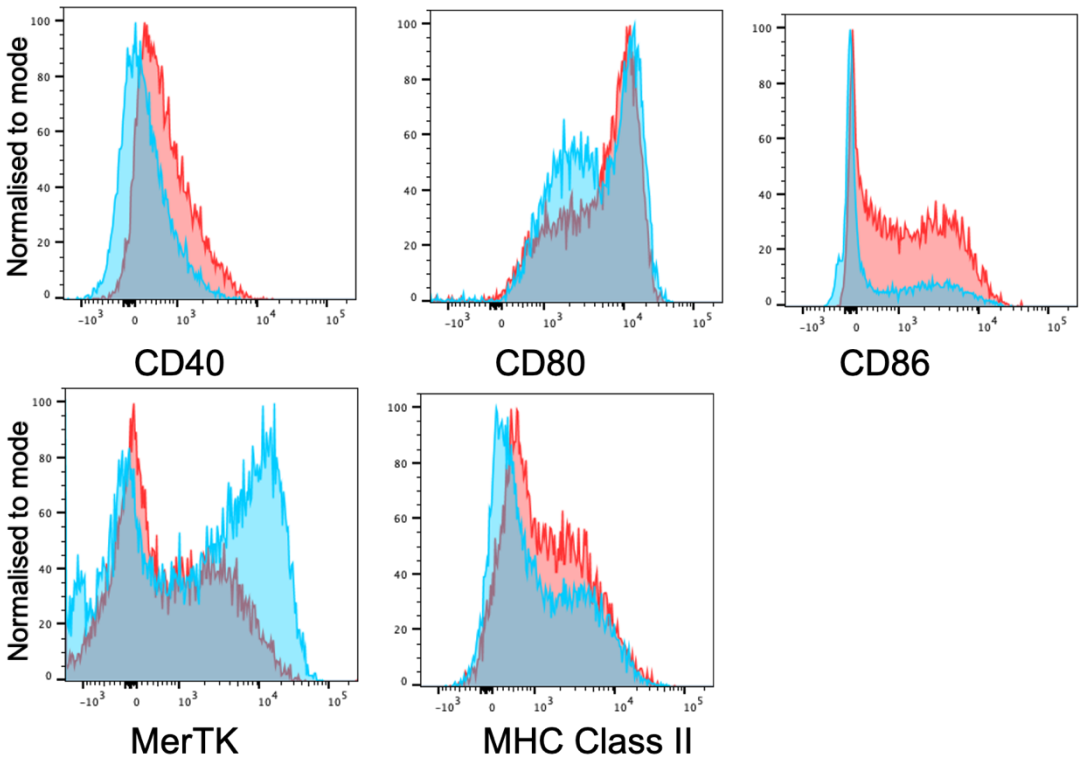


Figure 4-2 Representative phenotype of mature BM-DCs and tol-DCs by flow cytometry. Red shows mature BM-DCs and blue shows tol-DCs.

4.2.2 Tol-DCs produce significantly lower levels of cytokines than mature BM-DCs

Supernatants were collected on day 8 of culture of mature BM-DCs and tol-DCs. ELISAs were performed to test the concentration of TNF-α, IL-6 and IL-10. The results can be seen in Figure 4-3. Tol-DCs produce significantly lower levels of TNF-α and IL-6 than mature BM-DCs (inflammatory cytokines). However, they also produce significantly lower levels of IL-10 (an anti-inflammatory cytokine). This result differs from human Dex+VitD3 tol-DCs which produce more IL-10 than mature mo-DCs[217] (also shown in Chapter 3).

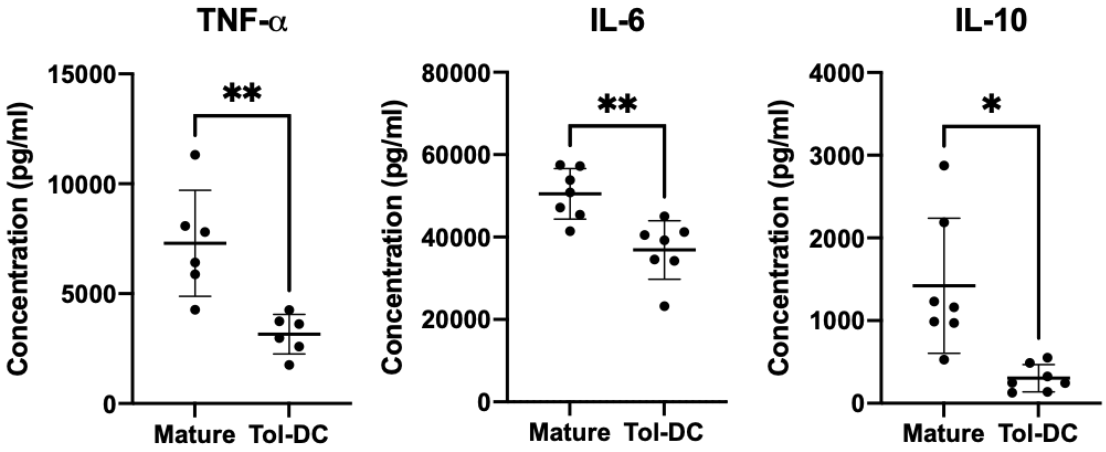


Figure 4-3 Tol-DCs produce significantly lower levels of TNF-a, IL-6 and IL-10 when compared to mature BM-DCs.

Supernatants were collected on day 8 before harvesting and tested for the concentration of TNF-α, IL-6 and IL-10 by ELISA. The TNF-α and IL-6 data was found to be normally distributed by a Kolmogorov-Smirnov test. On this data a student's t test was performed. The IL-10 data failed the Kolmogorov-Smirnov normality test therefore a Wilcoxin matched pair test was performed. ** = p-value <0.01 * = p-value <0.05.

4.2.3 The relative contribution of DCs and macrophages to mature BM-DC and tol-DC cultures is similar

A study by Helft *et al*. showed that BM-DCs are comprised of two distinct populations[63]. Tol-DCs are thought to be more macrophage-like. To see if there was a difference between the relative contributions of DCs and macrophages to BM-DC and tol-DC cultures, the gating strategy from Helft *et al*. was replicated. The results can be seen in Figure 4-4. First leukocytes are gated, excluding debris, then live cells are gated and doublets excluded using FSC-H and FSC-A. CD11c and MHC Class II positive cells are then identified, and two distinct populations of cells start to emerge. These were more separated when looking at the CD11b vs MHC Class II gate. $CD11b^{intermediate}$MHC Class II^{high} cells are thought to be more DC-like, whereas $CD11b^{high}$MHC Class $II^{intermediate}$ are thought to be more macrophage-like. When comparing mature BM-DCs and tol-DCs at this level, mature BM-DCs had ~10% more DC-like cells than tol-DCs.

The expression of DC-specific and macrophage-specific markers was then compared between the groups and the results can be seen in Figure 4-5. These markers were chosen from the Helft *et al*. study[63]. There were no differences between DC and macrophage specific markers between mature BM-DCs and tol-

DCs. This was when gating on DCs and macrophages or one population specifically.

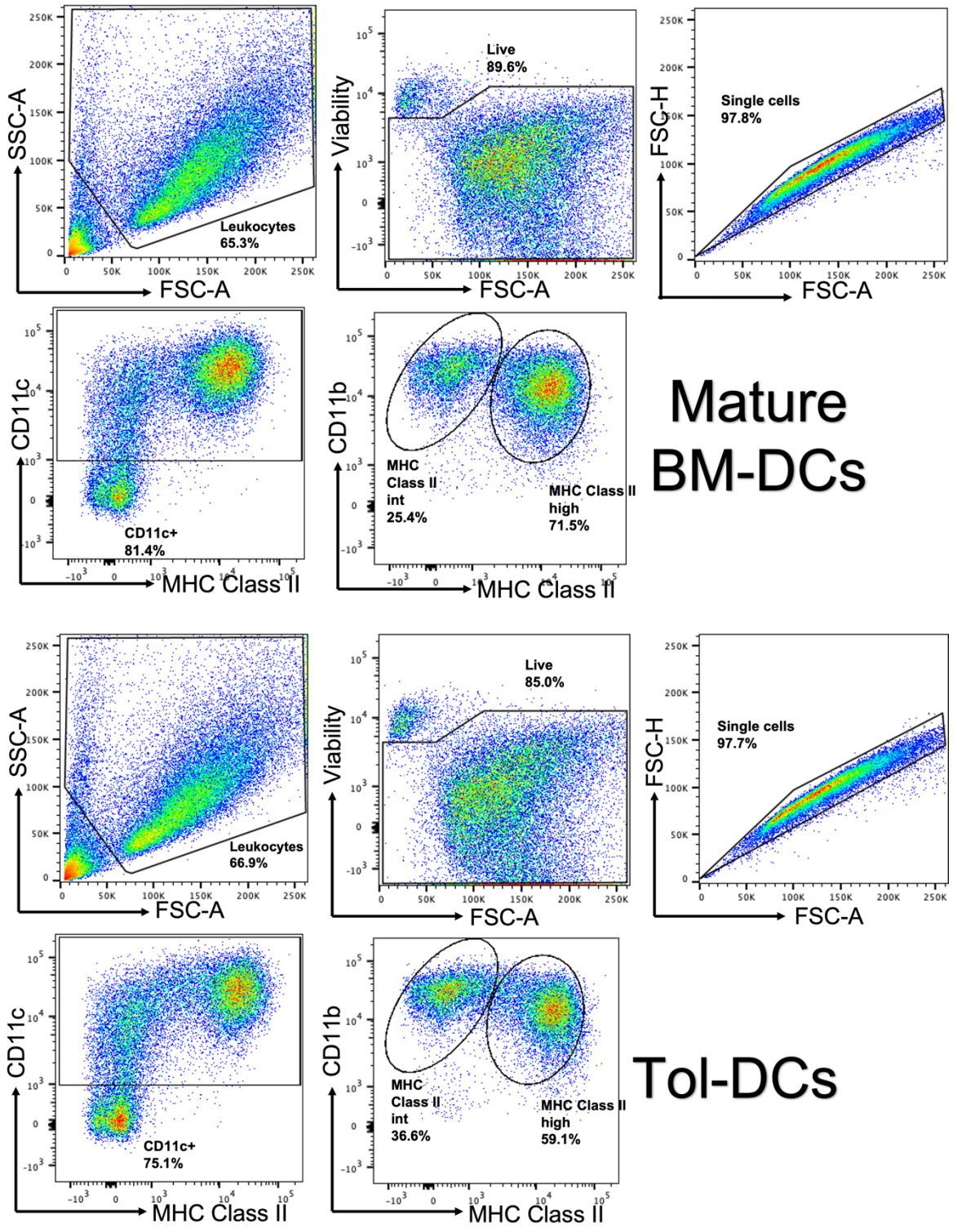


Figure 4-4 Gating strategy to determine the relative contribution of DCs and macrophages to mature BM-DC and tol-DCs.

On Day 8 of culture mature BM-DCs and tol-DCs were harvested and stained with flow cytometry Abs. Leukocytes are gated excluding debris, then live cells are gated and doublets are excluded using FSC-H and FSC-A. CD11c and MHC Class II positive cells are identified and then separated by their expression of CD11b and MHC Class II. Representative of 3 separate experiments.

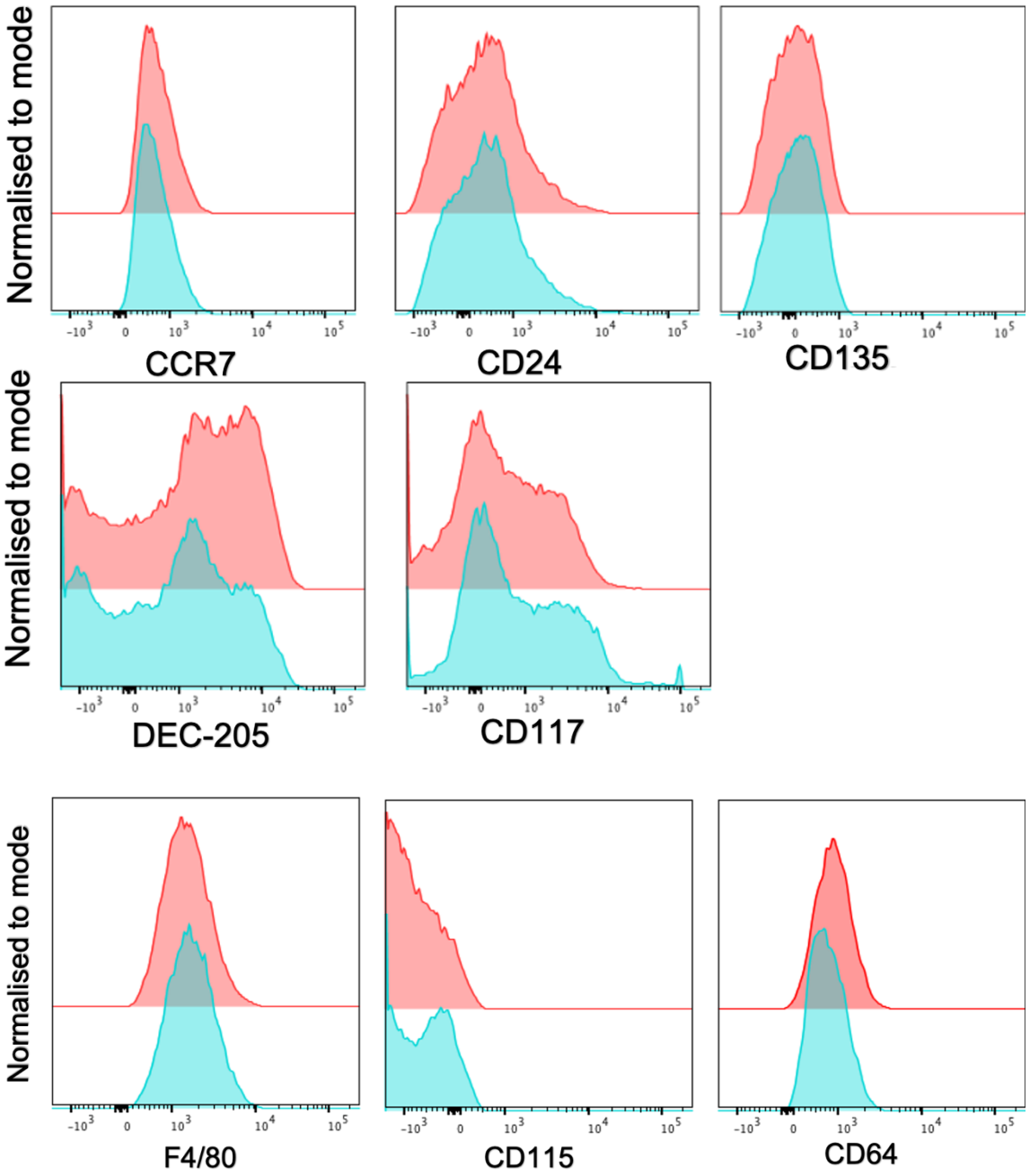


Figure 4-5 There are no differences in DC-specific or macrophage-specific markers between mature BM-DC and tol-DC cultures.

Mature BM-DCs are shown in red and tol-DCs in blue. CCR7, CD24, CD135, DEC-205 and CD117 are DC-specific markers. F4/80, CD115 and CD64 are macrophage-specific markers. Representative of 3 separate experiments.

4.2.4 IL-1R2, LAIR-1, C1QA and C1QB are significantly upregulated in tol-DCs

From the Nanostring experiment in Chapter 3, some of the immunoregulatory genes that were identified as being upregulated in tol-DCs when compared to mature mo-DCs were tested by Q-PCR to see if they were upregulated in murine tol-DCs. The expression of complement factor H, C1QA, C1QB, CD24, IL-1R2, FcγRIIb and LAIR-1 was tested by Q-PCR in mature BM-DCs and tol-DCs. CD86 was used as a quality control marker as we know this molecule should be downregulated in tol-DCs.

The only genes found to be consistently upregulated in tol-DCs when compared to mature BM-DCs were IL-1R2, LAIR-1, C1QA and C1QB. The results can be seen in Figure 4-6. C1QA and C1QB are significantly upregulated in tol-DCs when compared to mature BM-DCs. Deficiency in C1Q has been linked to lupus development and C1Q is involved in the uptake of apoptotic cells[281,285]. This suggests uptake of apoptotic cells could be a potential mechanism of action of murine tol-DCs, like human tol-DCs shown in Chapter 3. IL-1R2 is a decoy receptor for IL-1 inflammatory cytokines and LAIR-1 is an inhibitory receptor preventing cell activation.

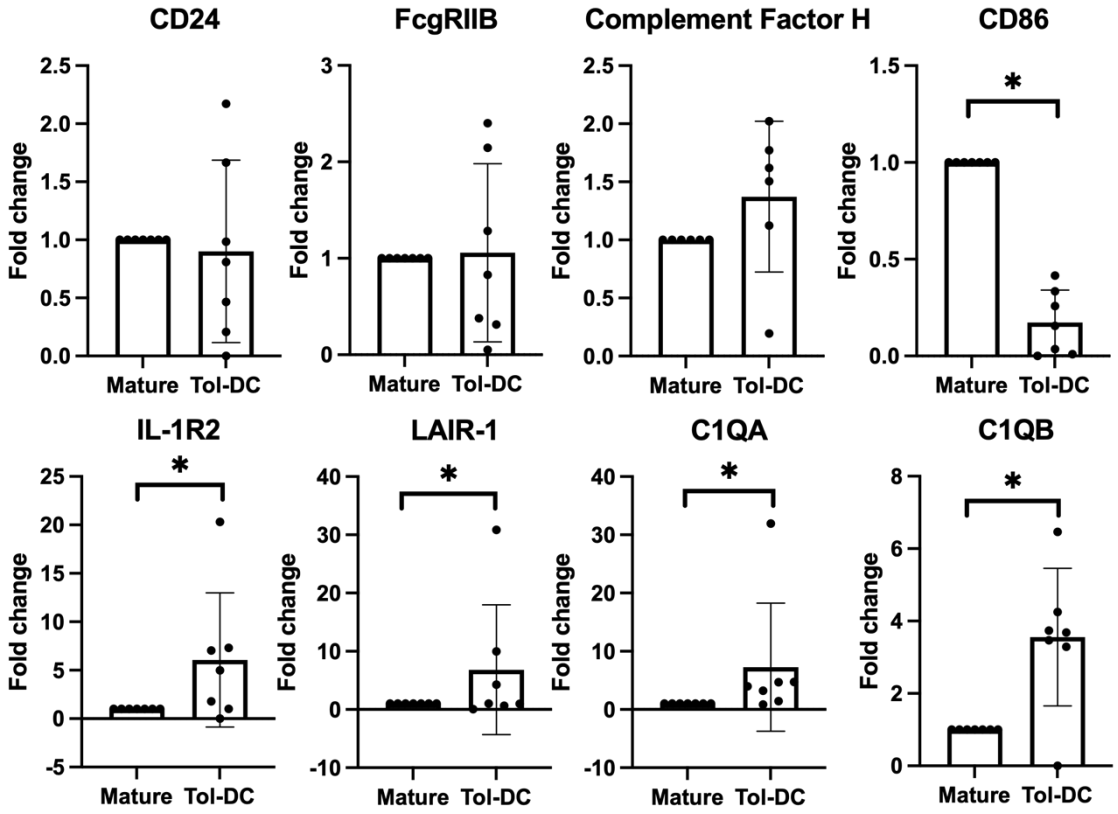


Figure 4-6 IL-1R2, LAIR-1, C1QA and C1QB are significantly upregulated in tol-DCs when compared to mature BM-DCs.

After harvesting cells, cells were counted and 5 million mature BM-DCs and 5 million tol-DCs were used for RNA isolation. RNA was then converted to cDNA and Q-PCR was performed following manufacturers guidelines (Qiagen). The data was found to be not normally distributed. A Wilcoxin matched pairs signed-rank test was then performed. * = p-value <0.05.

4.2.5 Tol-DCs induce significantly lower levels of CD4+ OVA-specific T cell activation *in vitro* than mature BM-DCs

CD45.1/OT-II T cells have a transgenic TCR which is specific for OVA peptide. This allows us to test tol-DCs action on an Ag-specific CD4+ T cell response *in vitro*. Mature BM-DCs or tol-DCs were cultured with OVA-specific CD4+ T cells. Concanavalin A (ConA) was added as a positive control as this non-specifically activates T cells by crosslinking the TCR[314]. No OVA peptide was the negative control as T cells should not become activated in the absence of Ag. The gating strategy for T cells can be seen in Figure 4-7. Gates were applied to leukocytes, live cells and single cells. CD4+CD45-1+ T cells were then gated. CD45-1 allows the OVA-specific T cells to be identified. Finally, CD11c+ cells are excluded which are the DC population.

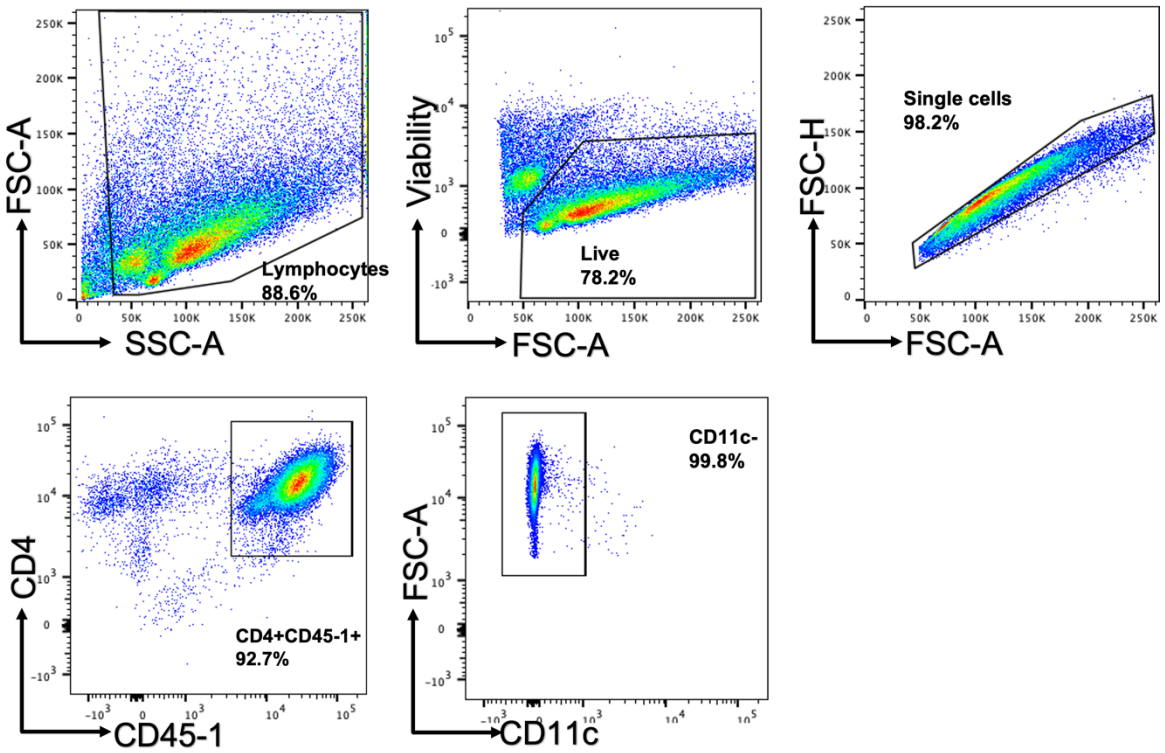


Figure 4-7 T cell flow cytometry gating strategy.
After co-culture with mature BM-DCs or tol-DCs for 3 days cells were then harvested and stained with flow cytometry Abs. Lymphocytes are gated avoiding debris, then live cells and single cells. CD4+CD45-1+ cells are gated to identify the OVA-specific CD4+ T cells. CD11c cells are excluded as these are DCs.

After using this gating strategy T cell activation markers (CD25, CD44 and CD62-L) were compared between the groups on Day 3 of co-culture. The results from two separate experiments ran in triplicate can be seen in figure 4-8. The experiments were ran in triplicate to ensure there were no plate to plate variations. CD25 and CD62-L expression was significantly lower in the Tol+ConA and Tol+OVA groups when compared with Mature+ConA and Mature+OVA groups. CD44 was only significantly lower when comparing Tol+ConA to Mature+ConA.

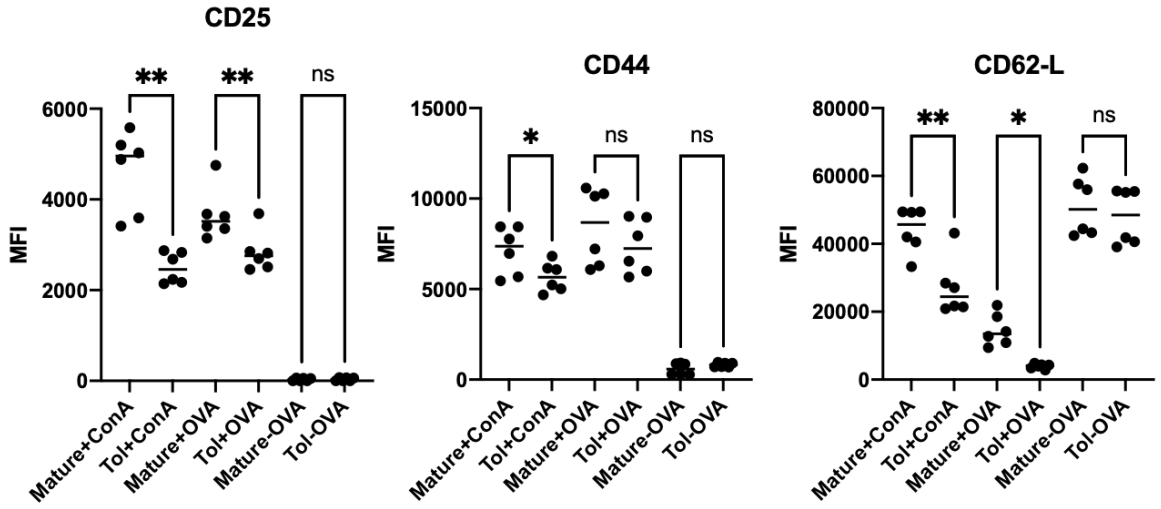


Figure 4-8 Tol-DCs induce significantly lower levels of T cell activation than mature BM-DCs.

T cells were analysed by flow cytometry on Day 3 of DC-T cell co-cultures. The median fluorescence intensities of CD25, CD44 and CD62-L were plotted for each of the groups. The data was found to be normally distributed with a Kolmogorov-Smirnov test. A one-way ANOVA with a Tukey's multiple comparisons test was performed. Two separate experiments ran in triplicate were pooled. Representative of 4 separate experiments. ** = p-value <0.01 * = p-value <0.05 ns=not significant.

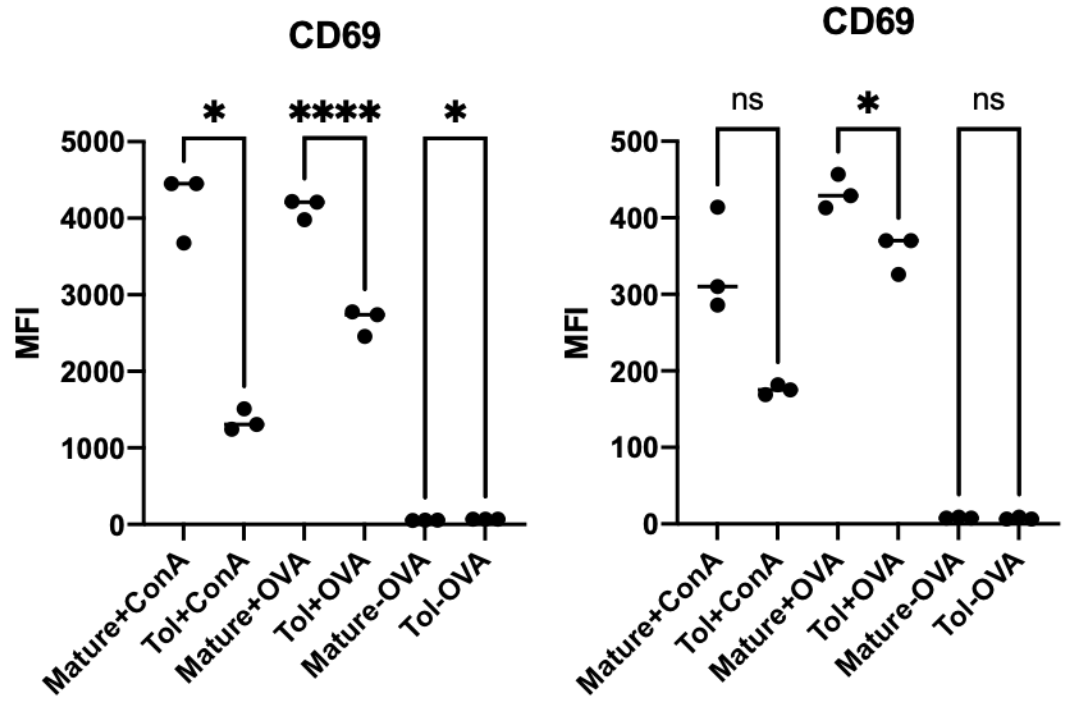


Figure 4-9 Tol-DCs induce significantly lower levels of T cell CD69 expression than mature BM-DCs.

T cells were analysed on Day 3 of DC-T cell co-cultures. The median fluorescence intensities of CD69 are shown separately for 2 experiments ran in triplicate. Representative of 4 separate experiments. The data was found to be normally distributed with a Kolmogorov-Smirnov test. A one-way ANOVA with a Tukey's multiple comparisons test was performed. Representative of 4 separate experiments. **** = p-value <0.0001 * = p-value <0.05 ns=not significant.

The results of CD69 expression in two separate experiments can be seen in Figure 4-9. In one experiment CD69 was significantly lower in the Tol+ConA and the Tol+OVA group when compared to the Mature+ConA and the Mature+OVA group. In the second experiment, CD69 was only significantly lower in the Tol+OVA group when compared to the Mature+OVA group. The differences in this result could be due to CD69 being an early activation marker which could be less relevant by Day 3 of co-culture. Although there is some variation in the response to ConA, in the presence of antigen specific activation, lower levels of CD69 expression were consistently seen in the tol-DC group when compared with the mature BM-DC group.

T cell proliferation was measured by Cell Trace Violet label. As the T cells proliferate the levels of Cell Trace Violet gradually reduce consistent with rounds of proliferation. The results can be seen in Figure 4-10. In the first experiment tol-DCs induced lower levels of T cell proliferation than mature BM-DCs in the presence of OVA peptide, whereas in a repeat experiment tol-DCs induced similar levels of T cell proliferation to mature BM-DCs. Without OVA peptide being present (the -OVA controls) there was no T cell proliferation induced.

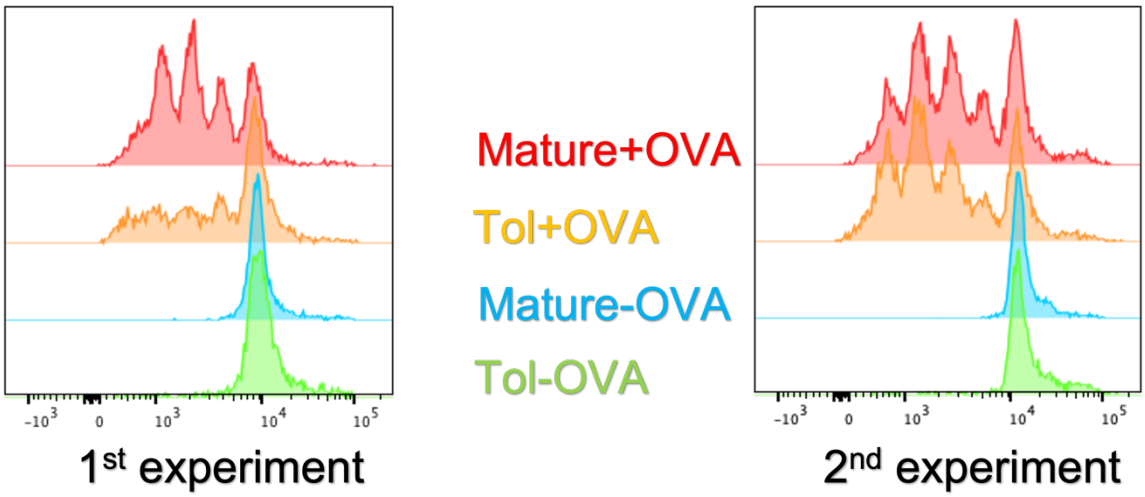


Figure 4-10 T cell proliferation responses induced by tol-DC are inconsistent.

Proliferation of T cells was analysed on Day 3 of co-culture with mature BM-DCs or tol-DCs using Cell Trace Violet. In the first experiment, tol-DCs appeared to induce lower levels of T cell

proliferation. However, in the second experiment proliferation was similar between Mature+OVA and Tol+OVA groups.

4.2.6 No significant differences in IFN-γ or IL-10 production by T cells co-cultured with tol-DCs

Next, T cell supernatants from Day 3 of co-culture were tested for IFN-γ and IL-10, the results can be seen in Figure 4-11. This was to determine whether tol-DCs induced similar T cell cytokine production as mature BM-DC. Previously it has been shown that human tol-DCs induce lower levels of IFN-γ and higher levels of IL-10 production by T cells when compared to mature DCs[217]. Although T cells co-cultured with tol-DCs produced slightly lower levels of IFN-γ than T cells co-cultured with mature BM-DCs, this was not statistically significant. The IFN-γ concentration was lower in the ConA groups than the OVA groups which was unexpected. IFN-γ is acid labile and by Day 3 of co-cultures the medium had turned yellow indicating low pH levels which could have affected the assay[315].

IL-10 concentrations were low in all the groups tested. This was consistent with the results from the ELISAs on DC supernatants which showed murine tol-DCs from BM-DCs produce significantly lower levels of IL-10 than mature BM-DCs. This differs from human tol-DCs which produce higher levels of IL-10 than mature mo-DCs.

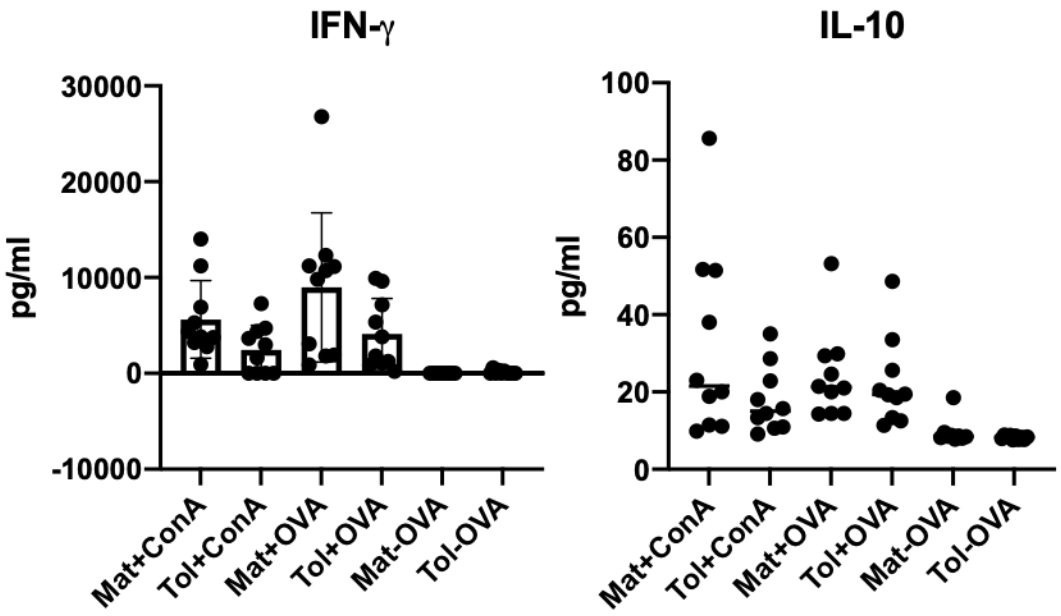


Figure 4-11 No significant differences in cytokines production by T cells co-cultured with tol-DCs.

IFN-γ and IL-10 ELISA was performed on supernatants from Day 3 of DC-T cell co-cultures. Data was found to be not normally distributed. A Kruskal-Wallis test with a Friedman multiple comparisons test was then performed. The results were not statistically significant indicating the groups were not significantly different from each other.

4.2.7 Tol-DCs and mature BM-DCs have similar levels of interaction with OVA-specific CD4+ T cells at 24 hours

To determine whether the differences seen in T cell activation with mature BM-DCs or tol-DCs was due to differences in the levels of interaction an InCell analyser was utilised. Tol-DCs could perhaps have lower levels of interaction with T cells which could lead to reduced activation.

For this experiment, mature BM-DCs, tol-DCs or unstimulated DCs were labelled with CMPTX and CD4+ OVA-specific T cells were labelled with CFSE. The differing labels were used so the different cells could be identified. The InCell Analyser images the wells then measures the total CMPTX signal and total CFSE signal before calculating the percentage overlap between the differently labelled cells. The percentage overlap of CMPTX and CFSE signal is a measure of how much the cells are interacting. The results can be seen in Figure 4-12.

The percentage overlap between mature BM-DCs and T cells and tol-DCs and T cells was not significantly different for any of the conditions tested. However,

unstimulated DCs had significantly higher percentage overlap with T cells in the ConA and 10μg OVA groups. CD4+ T cells have been shown previously to have more interactions with DCs before they are activated[316]. After they become activated, they then have transient interactions with DCs which is more like non-specific interactions. CD4+ T cells co-cultured with mature BM-DCs and tol-DCs may have become activated earlier than CD4+ T cells co-cultured with unstimulated DCs. This could explain why unstimulated DCs had significantly higher percentage overlap with T cells in the ConA and 10μg OVA groups.

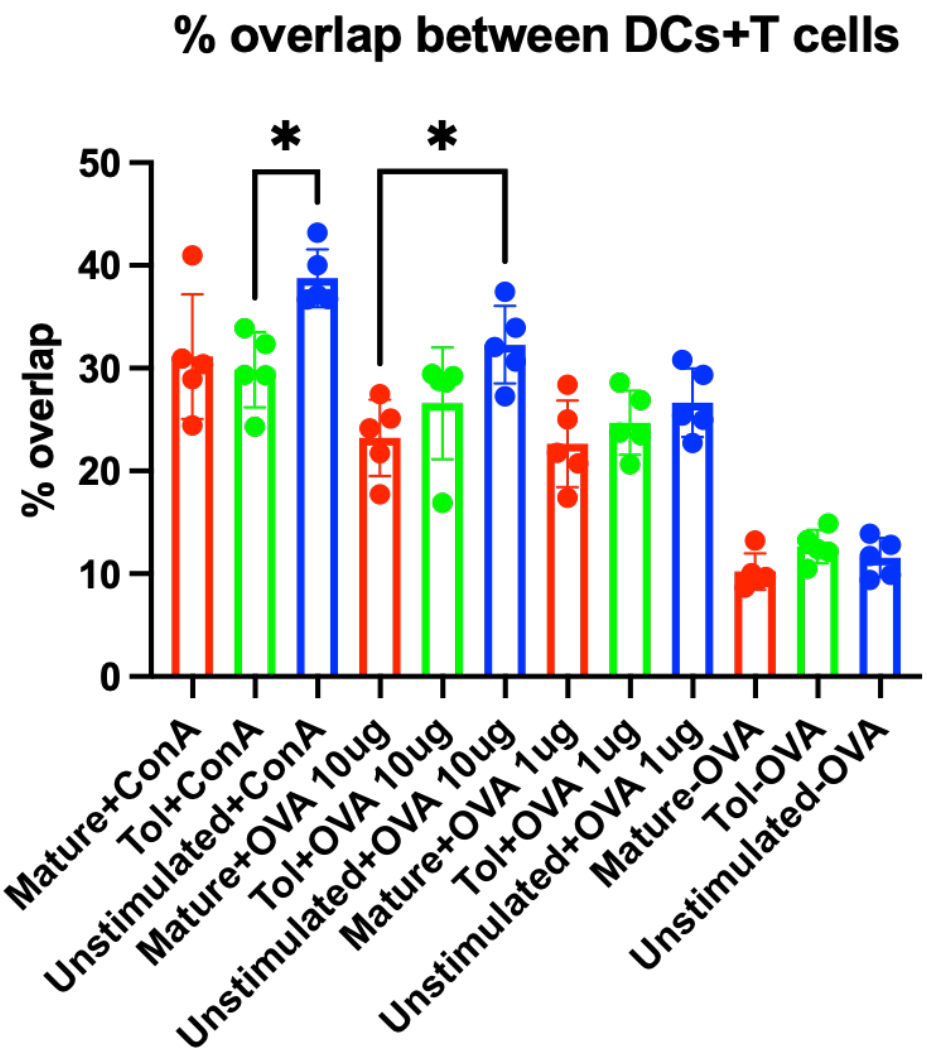


Figure 4-12 Mature BM-DCs and tol-DCs have similar levels of interaction with CD4+ OVA-specific T cells.

CD4+ OVA-specific T cells were labelled with 7.5μM CFSE. After harvesting unstimulated, mature and tol-DCs were labelled with 7.5μM CMPTX. 8000 T cells were cultured with 8000 DCs in 384 well plates for imaging. ConA was added at 1μg/ml, OVA peptide at 10 or 1μg/ml and some wells (the -OVA groups) contained no treatment. After 24 hours in culture the plate was imaged on an InCell 2000 analyser. The data was found to be normally distributed by a Shapiro-Wilk test. A one-way ANOVA was then performed with a Tukey's multiple comparisons test. * = p-value <0.05.

4.3 Discussion

Murine Dex+VitD3 were characterised by flow cytometry and compared to mature BM-DCs. Similar to human Dex+VitD3 tol-DCs, murine tol-DCs expressed similar levels of MHC Class II and CD80 to their mature counterparts and lower levels of CD40 and CD86[253].

MerTK was found to be upregulated on murine Dex+VitD3 tol-DCs. Previously, MerTK has been shown to be upregulated on human Dexamethasone tol-DCs and found to be essential for their immunoregulatory effect on T cells[313]. MerTK is important for the uptake and regulation of the immune response to apoptotic cells[317]. The absence of MerTK leads to the accumulation of apoptotic cells and autoantibody production in mice. This is consistent with the human data from Chapter 3 which suggests tol-DCs could phagocytose apoptotic cells due to the upregulation of genes involved in this process (e.g., MRC-1+C1q). Additionally, a population of synovial macrophages which are MerTK+CD206+ were found to be associated with healthy controls and RA patients in remission[263]. This confirms MerTK immunoregulatory effect in human RA patients and this could be an additional benefit of using MerTK+ tol-DCs as a treatment for RA.

The only genes consistently upregulated in murine Dex+VitD3 tol-DCs were found to be IL-1R2, LAIR-1 and C1QA and C1QB. C1QA has previously been found to be upregulated in multiple human tol-DC types and C1QA, C1QB and C1QC were found to be upregulated in human Dexamethasone tol-DCs[211,301]. Similarly, to MerTK, C1q is involved in the uptake of apoptotic cells and impaired clearance of apoptotic cells could lead to autoimmunity[310]. This suggests that similar to the human tol-DCs outlined in Chapter 3, that murine tol-DCs mechanism of action could include increased uptake of apoptotic cells. Additionally, when other DCs and macrophages interact with C1q it has an immunosuppressive effect on these cells[283].

IL-1R2 is a decoy receptor for inflammatory IL-1 cytokines and LAIR-1 is an inhibitory receptor. IL-1Rα (another IL-1R antagonist) has been found to be consistently upregulated in multiple tol-DC types[300]. Furthermore, IL-1R2 deficient mice were found to have increased susceptibility to CIA[260]. LAIR-1 is a receptor which has been shown to be able to interact with C1q which resulted in

an immune inhibitory effect, preventing cell activation[287]. Like human tol-DCs, additional mechanisms of action of murine tol-DCs could be preventing cell activation via LAIR-1 and reducing IL-1 inflammatory cytokines through the IL-1R2 decoy receptor.

Murine tol-DCs were found to produce significantly lower levels of TNF-α, IL-6 and IL-10 than mature BM-DCs. This is consistent with a study on murine Dex+VitD3 tol-DCs which found tol-DCs to produce significantly lower levels of TNF-α and IL-10[234]. This result differs from human tol-DCs which produced significantly more IL-10 than mature mo-DCs[217]. This difference could be explained by human tol-DCs being exclusively mo-DCs and mo-macs, whereas murine tol-DCs also include cDCs from CDPs. Mo-DCs are thought to produce more cytokines than cDCs[61]. The cDCs could respond differently than mo-DCs/mo-macs to the Dexamethasone and Vitamin D3 given in tol-DC cultures. Alternatively, there could be differences in how human and murine cells respond to Dexamethasone and Vitamin D3.

The relative components of DCs and macrophages to mature BM-DC and tol-DC cultures were determined to see if they differ. Tol-DCs were found to contain ~10% more CD11b^{high} MHC Class II^{intermediate} macrophage-like cells and ~10% fewer CD11b^{intermediate} MHC Class II^{high} DC-like cells than mature BM-DCs. However, when comparing DC-specific markers and macrophage-specific markers there were no differences in levels of expression. This was when gating on DC or macrophage populations specifically or including both at the same time. Although, tol-DCs express more CD11b than mature BM-DCs and slightly lower levels of MHC Class II (which has also been observed when analysing tol-DCs phenotype by flow cytometry), there are no differences in DC-specific or macrophage-specific markers. For this reason, mature BM-DCs and tol-DCs are comparable and tol-DCs do not appear to be more macrophage-like. The differences then seen between mature BM-DCs and tol-DCs are not due to the presence of more macrophages in tol-DC cultures. Mo-DCs are thought to be more efficient at Ag presentation, T cell stimulation and migrating to the dLN than mo-macs[8,10,67].

In the DC-T cell co-culture results presented here, there was significantly reduced expression of CD25, CD44, CD62-L and CD69 in the tol-DC groups when

compared to the mature BM-DC groups. There were no significant differences observed between IFN-γ and IL-10 production by T cells co-cultured with mature BM-DCs or tol-DCs.

A recently completed murine study testing Dex+VitD3 tol-DCs in a humanised proteoglycan-induced model of arthritis used a similar Ag-specific *in vitro* system to test tol-DCs ability to modulate T cell activation[232]. Naïve T cells from mB29b mice were isolated. These T cells have a transgenic TCR specific for human proteoglycan. Mature BM-DCs or tol-DCs were pulsed with the relevant peptide and co-cultured with mB29b T cells for 3 days. Similar to the results shown in this chapter, there was significantly reduced expression of CD25 in the T cells co-cultured with tol-DCs. However, in contrast to the results shown here, they found a significantly higher expression of CD62-L on T cells co-cultured with tol-DCs. This is perhaps due to reduced downregulation and a more naïve phenotype. CD62-L expression allows naïve T cells to home to LNs and T cells have to lose expression of CD62-L to be able to leave the LN[318].

Additionally, tol-DCs were found to significantly reduce IFN-γ production by T cells and although no significant differences were observed for IL-10, the concentrations were much higher than the ones found in this chapter (500-1000pg/ml compared to 10-80pg/ml). There was also an increase in CD25+FoxP3+ expression in T cells co-cultured with tol-DCs which suggests in this system Dex+VitD3 tol-DCs induce FoxP3+ Tregs.

The difference in results shown here and the proteoglycan study could be due to the different strains of mice and Ags used. In this study, naïve T cells were not isolated before co-cultures, however the mice were not exposed to OVA and OVA is not present endogenously so the OVA specific T cells should be naïve.

Similarly, to human Dex+VitD3 tol-DCs, murine tol-DCs induced significantly lower levels of T cell activation than mature BM-DCs (Chapter 3). The IFN-γ production from T cells however was not significantly reduced in T cells co-cultured with tol-DCs. The IL-10 was low in all groups tested and there were no significant differences between groups. This differs from human tol-DCs which were shown to induce significantly lower levels of IFN-γ and significantly higher

levels of IL-10 production from T cells[217]. The T cell proliferation result was inconsistent, some cultures showed tol-DCs to induce lower levels of T cell proliferation and some cultures showed similar T cell proliferation between T cells co-cultured with tol-DCs or mature BM-DCs. Previously, human tol-DCs have been shown to induce significantly lower levels of T cell proliferation than mature mo-DCs[253].

In this study, the results from *in vitro* T cell activation assays suggest tol-DCs do not induce Tregs. There was no increase in IL-10 in T cells co-cultured with tol-DCs when compared to mature BM-DCs, suggesting murine Dex+VitD3 tol-DCs are not inducing Tr1 cells which produce high amounts of IL-10. This was confirmed by including some Tr1 specific markers (CD49b+LAG-3) in flow cytometry experiments on DC-T cell cultures, there was no increase in these markers in T cells co-cultured with tol-DCs. This tol-DC type does not induce T cell anergy as the T cells become activated when compared with the no OVA control. The most likely mechanism of action of this tol-DC type is to skew the cytokine production of T cells towards less inflammatory cytokines. Although the results from the IFN-γ ELISA were not significant, there does look like there is a slight reduction in IFN-γ production by T cells co-cultured with tol-DCs when compared to T cells co-cultured with mature BM-DCs.

To test whether the lower levels of T cell activation induced by tol-DCs was due to decreased levels in interaction between these cells an InCell Analyser was utilised. This measures the interaction between DCs and T cells. Stable DC-T cell interactions are thought to occur only during the first 24 hours of the activation process[316,319]. From the 24-hour time-point there was no significant difference between the percentage interaction between mature BM-DCs and T cells and tol-DCs and T cells suggesting that the decrease in T cell activation seen with tol-DCs is not due to reduced interaction. CD4+ T cells have been shown to interact with DCs more before they are activated[316]. After activation CD4+ T cells then have transient interactions with DCs which are more like non-specific interactions. Stable DC-T cell interactions are thought to mainly occur during T cell priming[320]. This could explain the result observed here that there are significantly higher levels of interaction between unstimulated DCs and T cells. The T cells co-cultured with mature BM-DCs or tol-DCs could have been

activated sooner than the T cells co-cultured with unstimulated DCs and hence have significantly less interaction.

This chapter has confirmed murine Dex+VitD3 tol-DCs are comparable to human Dex+VitD3 tol-DCs by flow cytometry phenotypic analysis. IL-1R2, LAIR-1, C1QA and C1QB genes found to be upregulated in human tol-DCs in Chapter 3 were also found to be upregulated in murine tol-DCs. Murine tol-DCs were found to produce lower levels of inflammatory cytokines but also lower levels of IL-10 than their mature counterpart. This differs from human tol-DCs which produce more IL-10 than mature mo-DCs. This could be due to the presence of DCs from CDPs in murine cultures. There is a trend for lower levels of IFN-γ production from T cells cultured with murine tol-DCs however this is not significant and murine tol-DCs induce low production of IL-10 from T cells which also differs from human tol-DCs. Importantly though, murine tol-DCs still induce lower levels of T cell activation than mature BM-DCs.

Although murine tol-DCs differ from human tol-DCs in some respects, they appear to overall be immunoregulatory and reduce T cell activation when compared to mature BM-DCs. After confirming this, we could now go on to the *in vivo* part of the project in Chapter 5.

Chapter 5 Migration of Murine Tolerogenic Dendritic cells after Subcutaneous Footpad Injection and Ability to Modulate Arthritis

5.1 Introduction

The first AUTODECRA clinical trial aimed to test the safety and feasibility of tol-DCs as a treatment for inflammatory arthritis and the treatment was found to be safe and well tolerated[179]. The second AUTODECRA trial, which is scheduled and currently recruiting, aims to test different injection routes of tol-DCs and the immunomodulatory effect of this treatment on T cells. The injection route chosen can influence the effectiveness of treatment[234]. DCs are thought to have to migrate to the LN to induce tolerance[321,322]. A murine transplantation model, found that co-expressing both CCR7 and viral IL-10 in immature DCs before transfer was essential in prolonging graft survival[323]. Therefore, to optimise tol-DC therapy, an injection route which increases the migration of tol-DCs to the LN is an important consideration. However, tol-DC migration studies carried out to date are limited.

Intradermal administration is becoming a preferred injection route for human tol-DC trials as it is thought to increase migration to the dLN. A study carried out in human cancer patients, found intradermal injection of mature DCs resulted in three times higher migration to the LNs than subcutaneous injection (0.95 or 1.02% maximum uptake for intradermal vs 0.30 or 0.37% maximum uptake for subcutaneous[250]). The DCs were radiolabelled before transfer and maximum uptake was calculated by measuring maximum lymph node activity and dividing by inoculation site activity at 0 hours after injection.

Intravenous and not intraperitoneal injection of Dex+VitD3 tol-DCs was found to be effective in an established mouse model of CIA[234]. Intravenous injection overcomes the need for tol-DCs to migrate to the dLN via CCR7, although the cells mainly go to the lungs, liver, and spleen[324]. The spleen is a secondary lymphoid organ, like the LN, but it is thought tol-DC therapy would be most effective when targeting the disease dLNs as there is a high proportion of central memory T cells there[325]. After intravenous injection of two million CFSE labelled

tol-DCs the migration rate to the LNs was found to be ~1000 cells[234]. Interestingly, this study showed there were no differences between mature BM-DC and tol-DC migration after intravenous injection. However, this study measured migration rates during CIA so under inflammatory conditions which could have increased migration of tol-DCs to the dLN.

Intranodal injection would deliver cells straight into the LN but this injection route could damage LN architecture[252] and all of the tol-DC product would be in the LN. Previously human tol-DCs which expressed $CD83^{high}CCR7+$ were found to induce more suppressive Tregs than tol-DCs which were $CD83^{low}CCR7-$[191]. This suggests that intranodal injection would remove the selection pressure of the best tol-DCs to migrate and reach the LN. All the tol-DC product would be in the LN after intranodal injection rather than just the most tolerogenic cells.

Subcutaneous footpad injection has not been tested as an injection route of tol-DCs. It is a useful injection route to test in murine arthritis models as the dLN is near the knee joint. In the acute breach of tolerance model, inflammation is localised to the foot and ankle joint so the main disease associated dLN is the popliteal LN. Experiments injecting dye into the footpad have shown the popliteal LN is the main dLN of the footpad[326]. Furthermore, BM-DCs injected into the footpad mostly migrate to the popliteal LN[324].

BM-DC migration to the dLN after subcutaneous footpad injection has been reported previously. The best migration rates were found to be 20% of total cells analysed by flow cytometry. However, this study injected two million cells in 100μl which is a very high volume for the footpad[327]. One explanation of why the migration rate was so high could be because the cells were forced out of the footpad due to hydrostatic pressure. The study did not specify if the cells were dead or alive. In contrast, another study found a lower migration rate of 0.3% after injecting three million cells in 40μl[328]. This is still a high volume for the footpad. In this study, we injected one million cells in 25μl. Local regulations recommend this volume for footpad injections.

Tol-DCs have been found in the dLN following intravenous and subcutaneous injection routes[234,329,330]. However, the area of the LN (T cell vs B cell area) has not been established. In this study we set out to determine what area of the LN

tol-DCs were in after migration using immunofluorescence and confocal microscopy.

Previously, intravenous injection of humanised proteoglycan loaded Dex+VitD3 tol-DCs have been shown to significantly reduce CD4+ T cell Ag-specific proliferation[232]. There was additionally a reduction in CD25 and significantly less of a downregulation of CD62-L in the tol-DC group. In this chapter, using a similar adoptive transfer system, the effect of subcutaneous footpad injection of tol-DCs on OVA-specific CD4+ T cell activation and proliferation was measured.

To determine whether subcutaneous footpad injection of tol-DCs could modulate arthritis progression, an acute model of inflammatory arthritis was utilised. This model uses OVA-specific CD4+ T cells which are first polarised towards a Th1 phenotype *in vitro* before being adoptively transferred into WT mice. OVA/Complete Freund's Adjuvant is injected to initiate the immune response against OVA. Ten days later Heat Aggregated OVA (HAO) is injected in the footpad to localise inflammation to the ankle joint and digits of the foot. In this model there is an immune response first against OVA which is an irrelevant Ag which is not present in normal mice but then an autoimmune response against collagen type II spontaneously develops which is more like human disease[257]. Therefore, this model is often referred to as the breach of tolerance model. In contrast, CIA is induced by injecting exogenous collagen type II and an adjuvant which stimulates an autoimmune response against collagen type II which is present constitutively in mice.

In the breach of tolerance model, the number of Ag-presenting cDCs were found to increase in the popliteal LN before autoreactive T and B cell responses[331]. In contrast, no significant difference in Ag-presenting pDCs in the popliteal LN was observed. Furthermore, transient depletion of CD11c+ cells was found to prevent the breach of tolerance. In contrast, specific depletion of pDCs was found to enhance the severity of arthritis suggesting pDCs have a protective role[332]. OVA-pulsed BM-DCs injected subcutaneously into the footpad were found to be sufficient to induce arthritis and anti-collagen type II responses. This highlights the role of DCs in the breach of tolerance model.

The aims of this chapter are summarised below:

- Characterise the migration of tol-DCs to the dLN after subcutaneous footpad injection

- Test the ability of tol-DCs to modulate T cell activation after this injection route using adoptive transfers

- Test the ability of subcutaneously transferred tol-DCs to modulate disease progression in an acute model of inflammatory arthritis (the breach of tolerance model)

5.2 Results

5.2.1 Peak migration of mature BM-DCs and tol-DCs to the dLN is at 24-hours

The aim of these migration studies was to determine whether tol-DCs could migrate to the dLN (popliteal LN) after subcutaneous footpad injection. A time-course was completed to determine when peak migration of the injected cells to the dLN occurred.

For this experiment, mature and tol-DCs were generated from CD11c-YFP mice then labelled with Cell Trace Violet (CTV). The reason for using the two different labels is that YFP is thought to be degraded when the cells die or are taken up by other cells as it is a protein whereas CTV can be followed even after the cells die and are taken up by other cells.

Nine WT mice were injected with one million double labelled mature BM-DCs into the left footpad and one million double labelled tol-DCs into the right footpad. Pilot experiments had shown that there was no migration of cells from the right hand-side to the left hand-side and vice versa. Three mice were culled at 12-, 16- and 24-hours post-injection and one WT mouse was culled as an un-injected WT control (the time 0 time-point). The popliteal LNs (dLNs) were harvested and inguinal LNs were harvested as a control. These time-points were chosen as previous studies have shown that peak migration of BM-DCs is 48 hours after subcutaneous footpad injection[324]. A pilot experiment showed that there was a higher number of live YFP+ cells at 24 hours post-injection when compared to 48 hours post-injection.

The results from this experiment can be seen in Figure 5-1. The results show that after subcutaneous footpad injection, mature BM-DCs and tol-DCs migrate to the popliteal LNs and not the inguinal LNs. Mature BM-DC and tol-DC migration from the footpad to the dLN is similar until 24 hours and the time-point with peak migration to the dLN was found to be 24 hours post-injection. Although there was a dip in cell migration at 16 hours in this experiment this was not observed in other experiments. There were significantly fewer live YFP+ cells in the tol-DC group than the mature BM-DC group at 24 hours.

To confirm this result, two experiments were combined, and a similar result was observed (Figure 5-2). There was only a significant difference between mature BM-DC and tol-DC migration at 24 hours and peak migration to the dLN was at 24 hours post-injection.

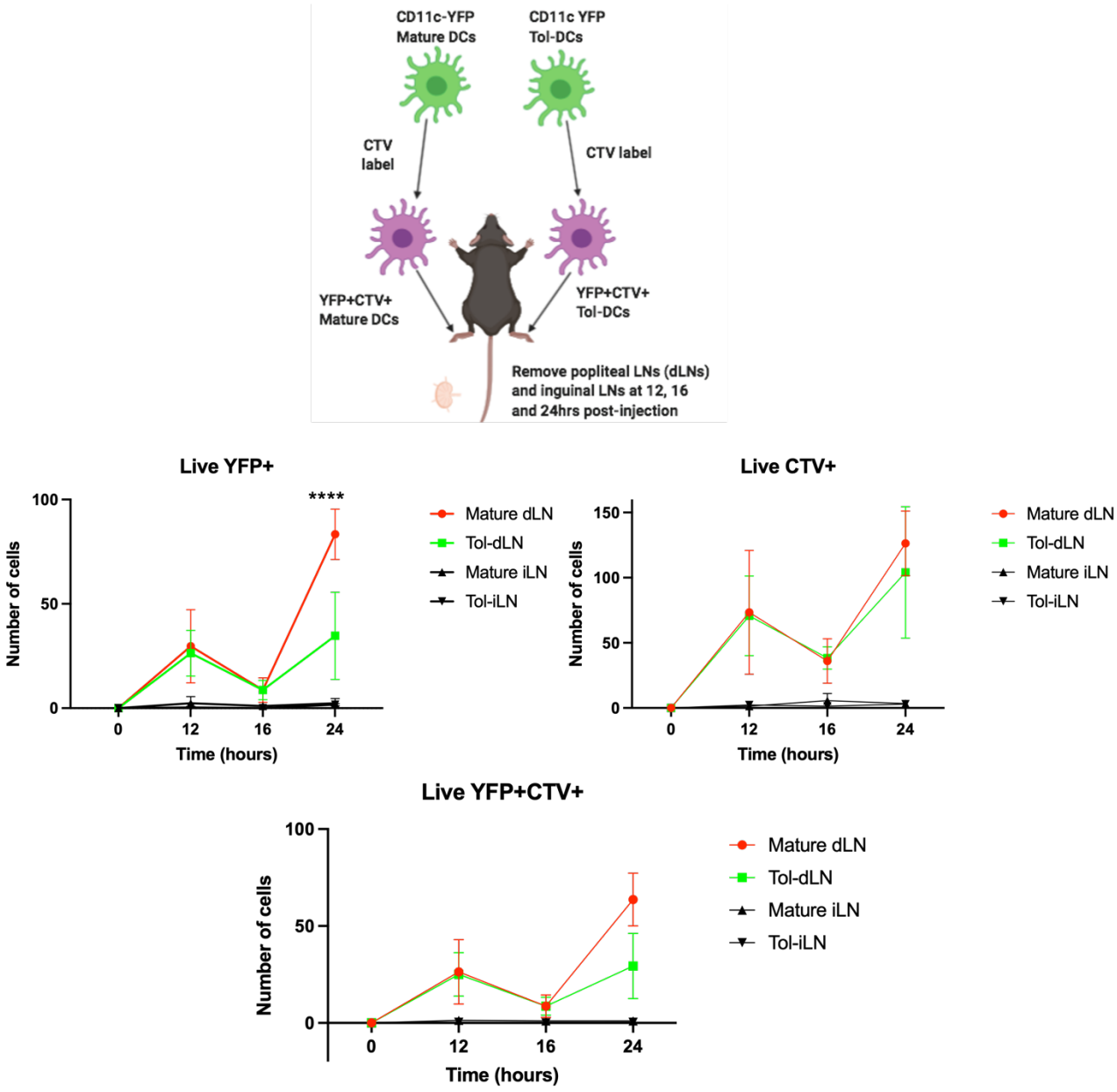


Figure 5-1 Peak migration of injected mature BM-DCs and tol-DCs to the dLN after subcutaneous footpad injection was found to be at 24 hours.

Mature BM-DCs and tol-DCs were generated from CD11c-YFP mice. After harvesting these cells were labelled with CTV at 0.25μM. 9 WT mice were injected with 1 million mature BM-DCs into the left footpad and 1 million tol-DCs in the right footpad in 25μl of PBS. 3 mice were culled at each time-point and the number of live YFP+, live CTV+ and live YFP+CTV+ cells were determined. An un-injected WT control was used as the time 0 time-point. Data passed a Shapiro-Wilk normality test. Therefore a 2-way ANOVA was performed with a Tukey's multiple comparisons test. Live YFP+ graph, mature dLN compared to Tol-dLN at 24 hours post-injection ****. **** = p-value <0.0001.

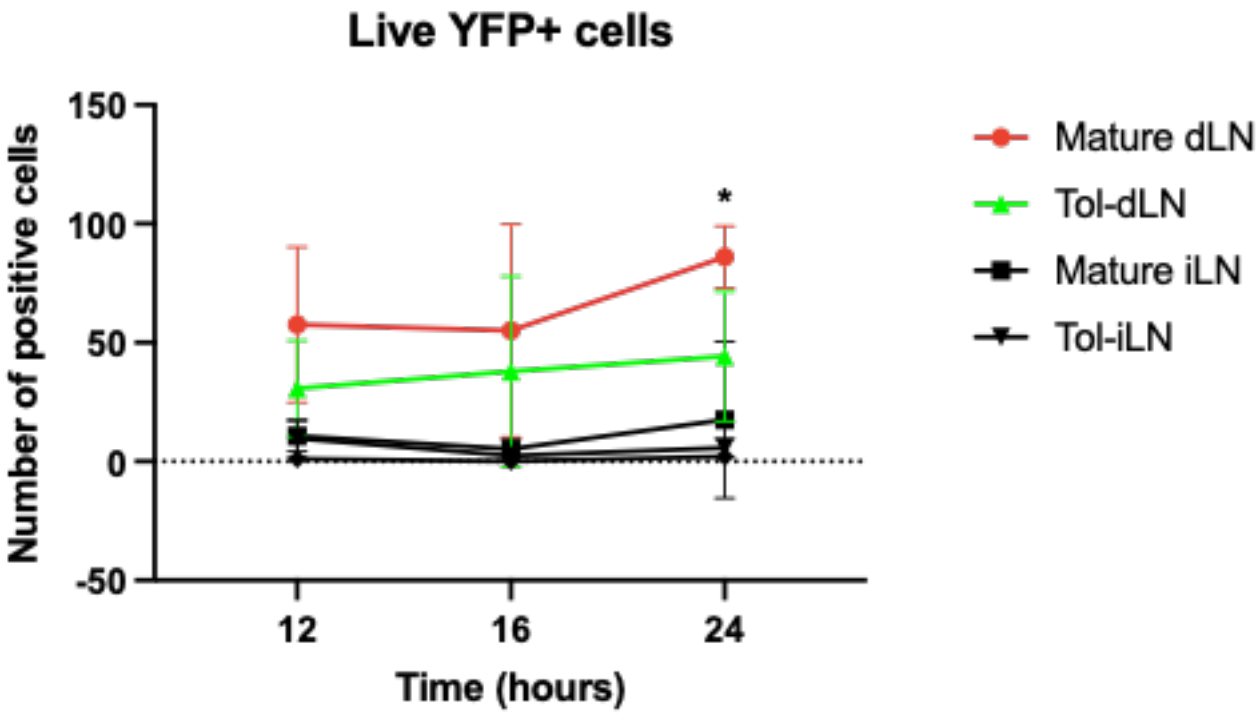


Figure 5-2 Tol-DC migration to the dLN after subcutaneous footpad injection is significantly lower 24 hours post-injection when compared to mature BM-DCs.

The results of 2 separate migration experiments were combined and a similar result was observed. Data was found to be normally distributed with a Shapiro-Wilk test. A two-way ANOVA was then performed with a Tukey's multiple comparisons test. Mature dLN compared to tol-dLN at 24 hours post-injection *. * = p-value <0.05.

5.2.2 YFP+ tol-DCs can be found in the B and T cell area of the dLN after subcutaneous footpad injection

To determine what area of the dLN tol-DCs localise in, immunofluorescence and confocal microscopy was performed. Samples were stained with CD3 (to determine T cell area), B220 (to determine B cell area) and anti-GFP (to amplify YFP signal). A control LN was taken from an un-injected mouse. Representative images from two separate experiments can be seen in Figure 5-3. Tol-DCs could be found in the B and T cell areas of the dLN following subcutaneous footpad injection. When looking at x40 objective there appears to be intact YFP+ cells.

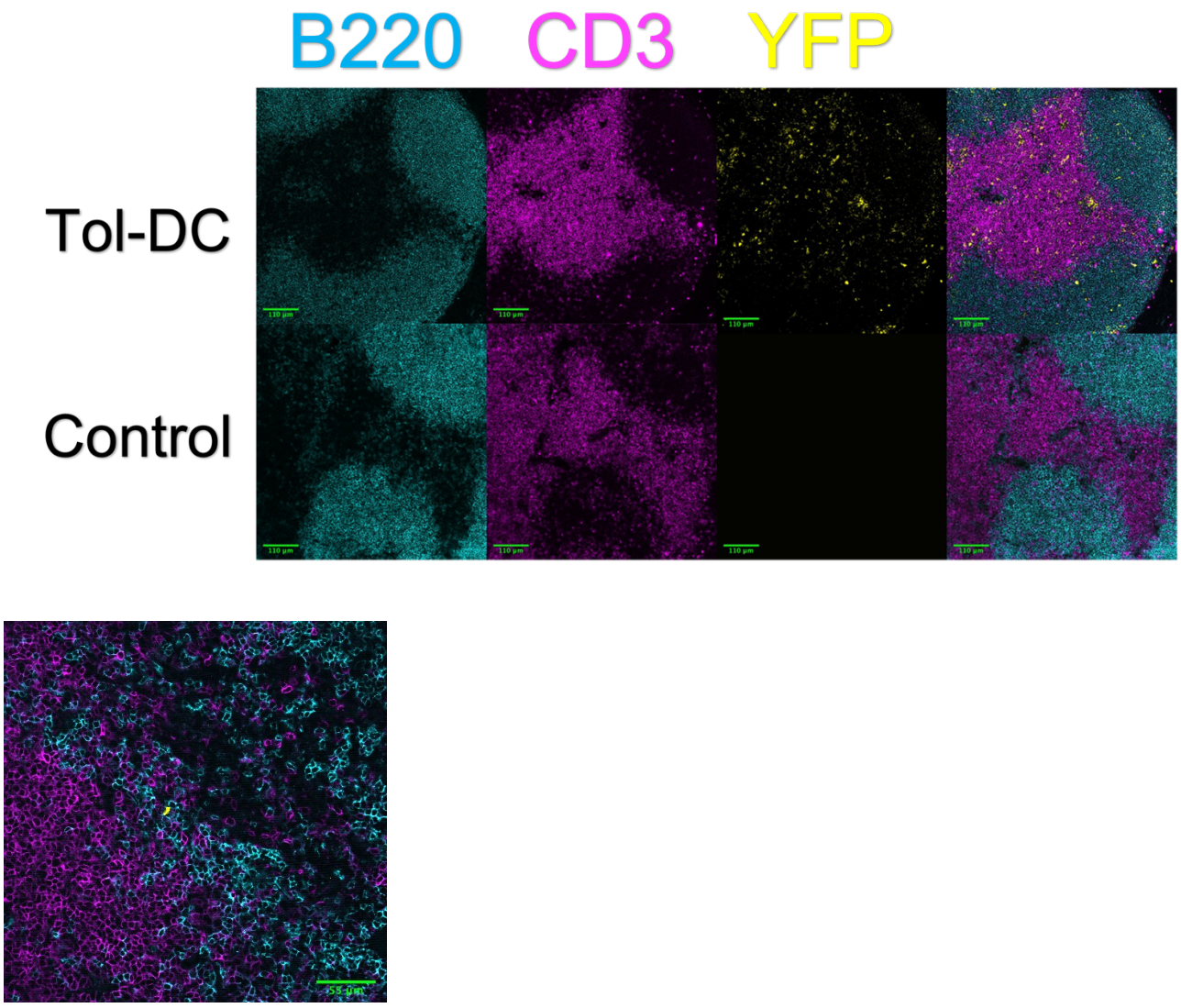


Figure 5-3 Tol-DCs can be found in the T cell and B cell areas of the dLN following subcutaneous footpad injection.

Tol-DCs were grown from a CD11c-YFP mouse. After harvesting on Day 8, 1 million tol-DCs were injected into the footpad in 25μl of PBS. 24 hours later the dLN was harvested before being frozen down in OCT and stored at -80°C. Tissue sections were prepared on the cryotome and slides were stained with CD3 (T cell area), B220 (B cell area) and anti-GFP (to amplify YFP signal). When

looking at x40 objective, there appears to be intact cells. Representative images of 2 separate experiments are shown.

5.2.3 No significant differences between CD4+ T cell activation and proliferation *in vivo* following subcutaneous injection of mature BM-DCs or tol-DCs

To test the ability of tol-DCs to modulate T cell activation *in vivo* after subcutaneous footpad injection, adoptive transfers were utilised. The equivalent of one million CTV labelled CD4+ OVA-specific T cells were injected intravenously into WT mice. 24 hours later one million OVA peptide loaded mature BM-DCs or tol-DCs were injected into both footpads. The control mouse received T cells but no DCs. An outline of this experiment can be seen in Figure 5-4. Day 3 was chosen as peak expansion of CD4+ T cells has previously been observed at this time after subcutaneous injection of Ag[333].

Three days after injection of DCs, the dLNs (popliteal LNs) were harvested and the number of cells counted before flow cytometry analysis. The results of cell counts can be seen in Figure 5-5. The dLNs from mice which received tol-DCs macroscopically looked smaller than dLN from mice which received mature BM-DCs. However, there was no significant difference in the cell numbers.

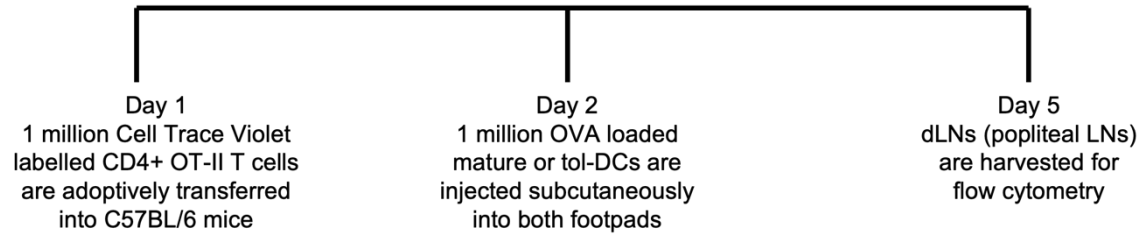


Figure 5-4 Adoptive transfers experimental overview.

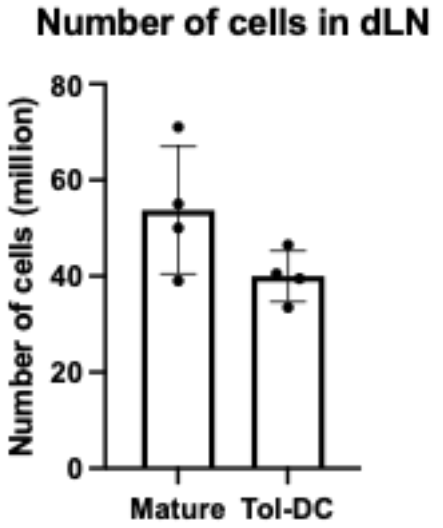


Figure 5-5 No statistically significant difference in the number of cells in the dLNs of mice which received subcutaneous footpad injection of mature BM-DCs or tol-DCs.

3 days after injection of OVA loaded mature BM-DCs or tol-DCs, dLNs (popliteal LNs) were harvested for flow cytometry. Before flow cytometry staining the number of cells in the dLNs were counted. The data passed a Shapiro-Wilk normality test therefore a student's t-test was performed. There were no statistically significant differences between mature BM-DC and tol-DC groups.

A representative gating strategy for each of the experimental groups can be seen in Figure 5-6. Lymphocytes, live cells then single cells are gated. CD4+CD45-1+ cells are gated to identify the OVA-specific T cells. The number of CD4+CD45-1+ cells was plotted for each mouse and can be seen in Figure 5-7. There were significantly fewer OVA-specific CD4+ T cells in the tol-DC group when compared to the mature BM-DC group.

After gating, CD4+ CD45-1+ OVA-specific T cells the MFI of CD25, CD44 and CD62-L was assessed, and the results can be seen in Figure 5-8. These results are representative of three separate experiments. There was no significant difference in T cell activation markers between the mice that received mature BM-DCs or tol-DCs. The CD4+ OVA-specific T cells did appear to be activated in the mature BM-DC and tol-DC groups when compared to the control.

Similarly, to the *in vitro* data in Chapter 4, there were no significant differences in proliferation of T cells between mice that received mature BM-DCs or tol-DCs. The results can be seen in Figure 5-9. The majority of the adoptively transferred T cells had proliferated by Day 3. The OVA-specific CD4+ cells in the control mouse did not proliferate as there was no OVA present.

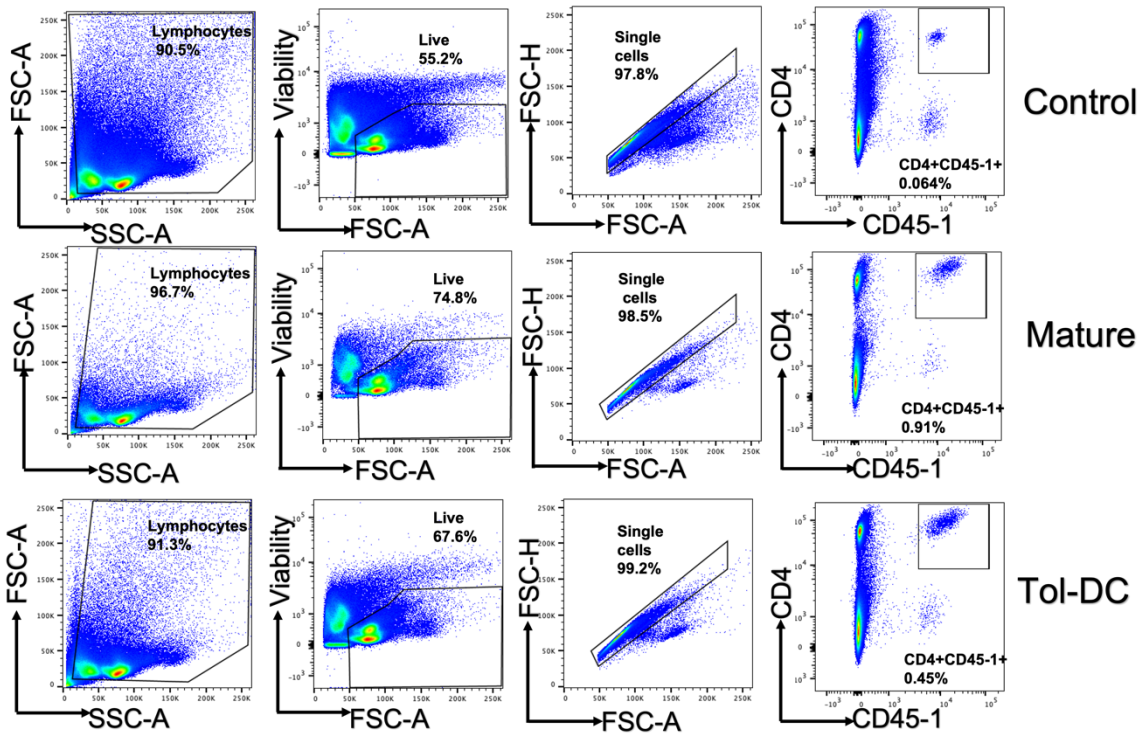


Figure 5-6 Flow cytometry gating strategy to identify OVA-specific T cells.

The dLNs were harvested, passed through a 70μM filter and counted before flow cytometry staining. Representative gating for each experimental group is shown. To identify OVA-specific T cells, first lymphocytes, live cells and single cells were gated. CD4+CD45-1+ cells were then identified. CD45-1 identifies the transgenic OVA-specific T cells.

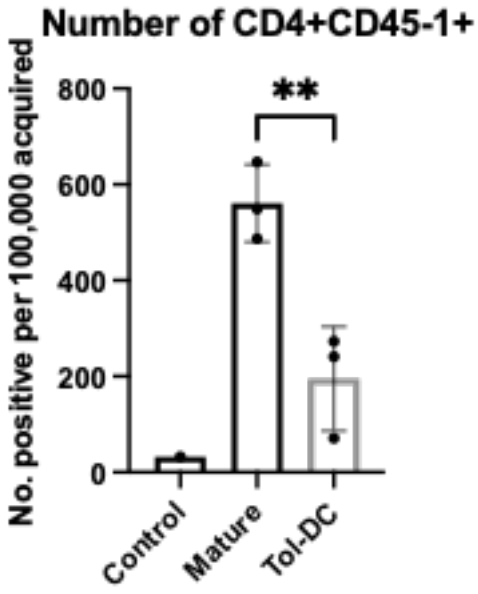


Figure 5-7 Significantly fewer CD4+CD45-1+ cells in the tol-DC group when compared to the mature BM-DC group.

The number of CD4+CD45-1+ cells per 100,000 events acquired was plotted for each of the mice. The results shown are from one experiment containing 3 mice in the mature BM-DC and tol-DC group and 1 control mouse. Data passed Shapiro-Wilk normality test therefore a student's t-test was performed. ** = p-value <0.01.

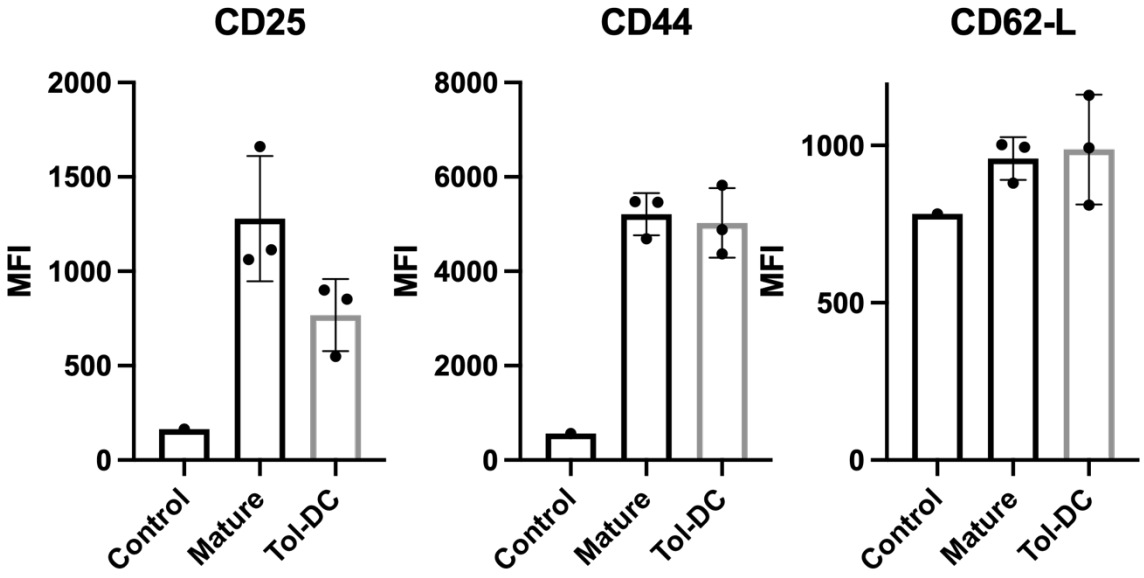
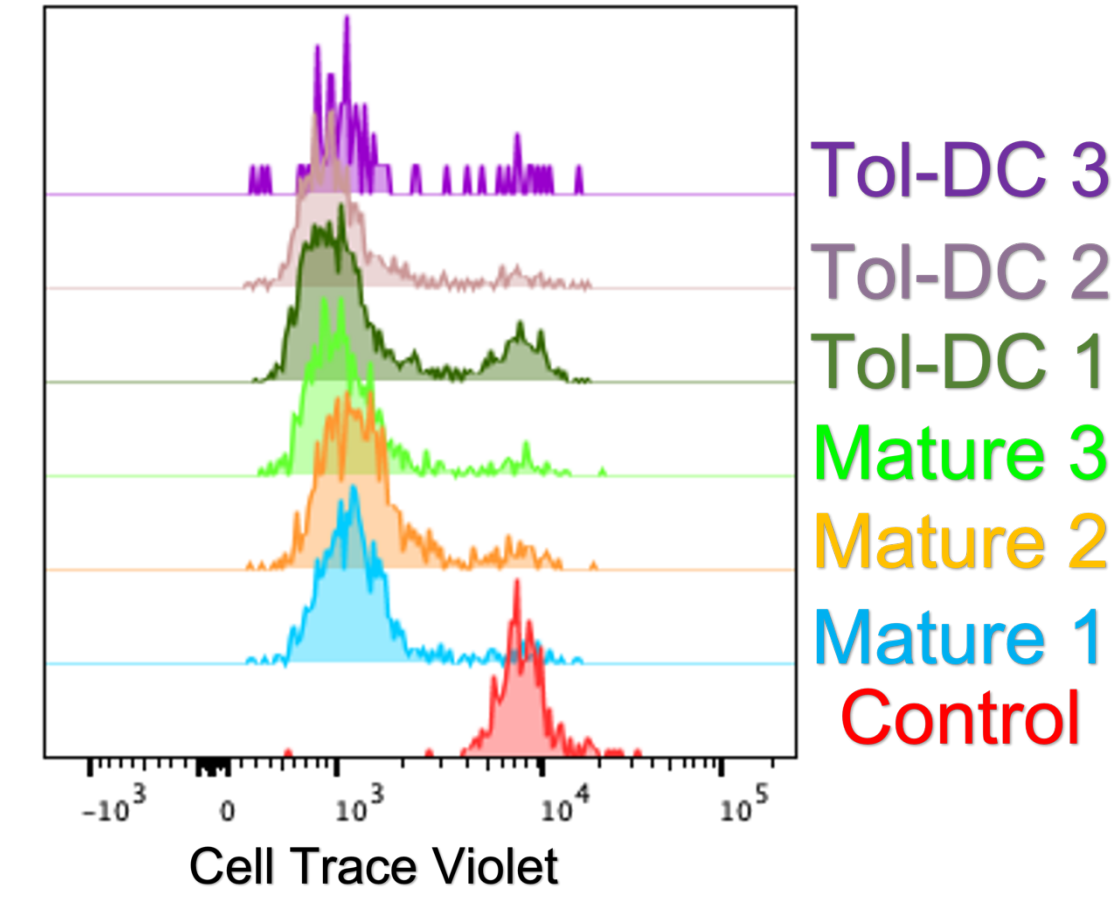


Figure 5-8 No significant difference in OVA-specific CD4+ T cell activation in vivo after tol-DC treatment.

After following the gating strategy shown in Figure 5-6 the Median Fluorescent Intensities (MFIs) of CD25, CD44 and CD62-L were plotted. The results shown above are from 1 experiment which is representative of 3 separate experiments. Data passed a Shapiro-Wilk normality test therefore a student's t-test was performed, and the results were not statistically significant.



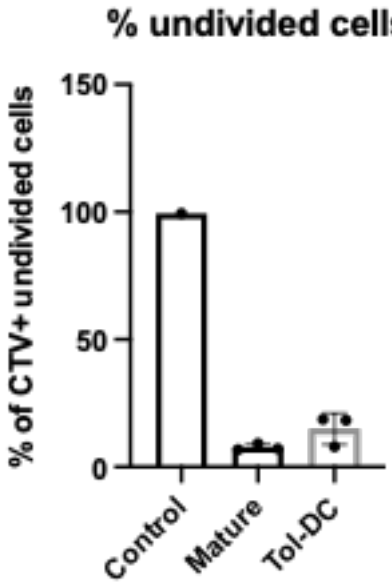


Figure 5-9 No significant difference in OVA-specific CD4+ T cell proliferation with mature BM-DC or tol-DC treatment.

After following the gating strategy shown in Figure 5-6, Cell Trace Violet expression in T cells was assessed. The results shown are from one experiment containing 3 mice in the mature BM-DC and tol-DC group and 1 control mouse. The % of undivided CTV+ cells was plotted for each of the mice. Data passed a Shapiro-Wilk normality test therefore a student's t-test was performed, and the results were not statistically significant.

After adoptive transfers, on one occasion, the spleens were harvested for re-stimulation with PMA/Ionomycin to measure IFN-γ production. There was not much of a cytokine response observed after re-stimulation with OVA peptide. PMA/Ionomycin bypasses the need for TCR stimulation and activates T cells, resulting in their cytokine production[334]. The results can be seen in Figure 5-10. After following the gating shown in Figure 5-6, when looking at number of cells there were fewer IFN-γ positive cells in the tol-DC group than the control or mature BM-DC group.

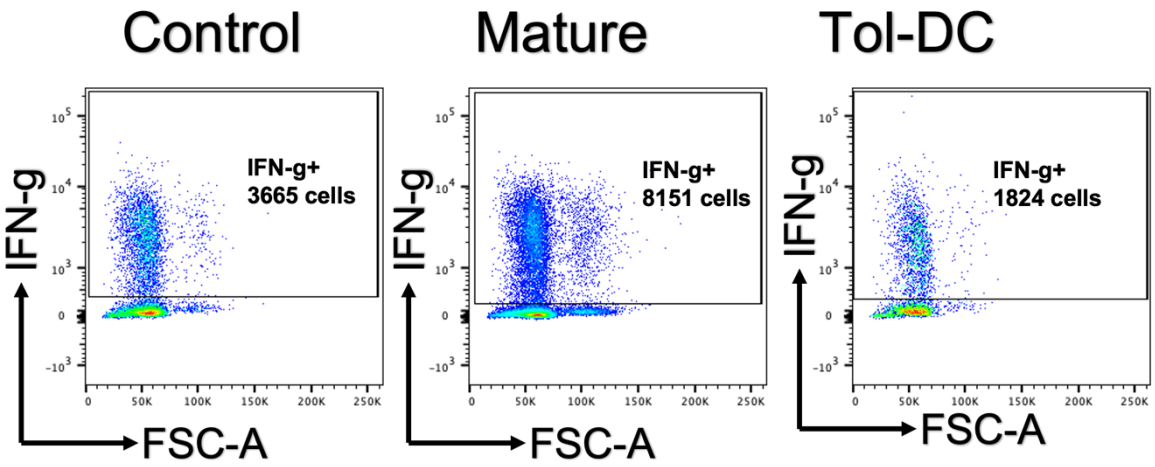


Figure 5-10 There were fewer IFN-g positive cells in the tol-DC group after re-stimulating splenocytes with PMA/Ionomycin.

Spleens were harvested in one adoptive transfer experiment. After passing spleens through a 70μM filter and lysing red blood cells, cells were re-suspended at 1 million per ml in complete RPMI and stimulated with PMA/Ionomycin for 6 hours at 37°C. Cells were harvested for flow cytometry and stained using an intracellular staining kit. After following the gating strategy shown in Figure 5-6, the number of IFN-γ cells is shown for each of the experimental groups.

5.2.4 Testing subcutaneous footpad injection of tol-DCs in an acute inflammatory mouse model of arthritis

To determine whether subcutaneous footpad injection of tol-DCs could modulate arthritis, an acute inflammatory arthritis model was utilised. An overview of the experiment and experimental groups can be seen in the results section (2.2.8.5).

Tolerance inducing treatments are most likely to be beneficial earlier in the disease process. For this reason, we decided to test tol-DC treatment as early as possible prophylactically (before Abs against OVA are generated). T cells specific to OVA peptide have been adoptively transferred into WT mice however mice

have not received Ag (OVA/CFA) to initiate an immune response yet. We decided to test tol-DC treatment in this period. Testing tol-DC treatment before OVA-specific T cells have been transferred would not make sense as there would be no OVA-specific CD4+ T cells present for the tol-DCs to interact with.

All mice received Th1 polarised OVA-specific T cells and were immunised with OVA/CFA. Group 1 received no treatment and were challenged with PBS instead of HAO. This group did not display any symptoms of arthritis.

5.2.5 Tol-DCs had similar total arthritis clinical scores to the PBS control and had no effect on footpad swelling

The total clinical score and footpad measurements (left+right) were recorded daily for a week after HAO or PBS injection. The results can be seen in Figure 5-11. The mice which received OVA-peptide loaded mature DCs had the highest total clinical score. The mice which received OVA-peptide loaded tol-DCs or PBS had a similar total clinical score. Mice which received no treatment and PBS, rather than HAO did not develop arthritis, and had a total clinical score of 0. The increase observed from Day 4-5 was probably due to a change in scorers.

There were no significant differences in total clinical score between mature DC, tol-DC and PBS groups. However, there was a significant difference between the no arthritis control group (None/PBS) and the mature DC group on Day 2+3. There was a significant difference between the None/PBS group and tol-DCs on Day 2+3 and a significant difference between the None/PBS group and PBS on Day 5. This confirms the experiment worked as the experimental groups had significantly higher arthritis clinical scores than the no arthritis control and is consistent with previous findings[233].

We measured left and right footpad thickness as previous studies have shown the inflammation is limited to each side and does not transfer to the other footpad[332]. Footpad thickness as well as footpad swelling was measured. Footpad swelling was the increase from the measurement taken on Day 0 for each mouse. Left footpad thickness and swelling was similar for PBS, mature DCs and tol-DCs. The right footpad thickness was higher in mice which received mature DCs (Day 3-5), and right footpad swelling was higher in this group (Days 4+5) than the PBS

and tol-DC group. This was due to two mice in in mature DC group having very swollen right footpads. However, this difference was not significant.

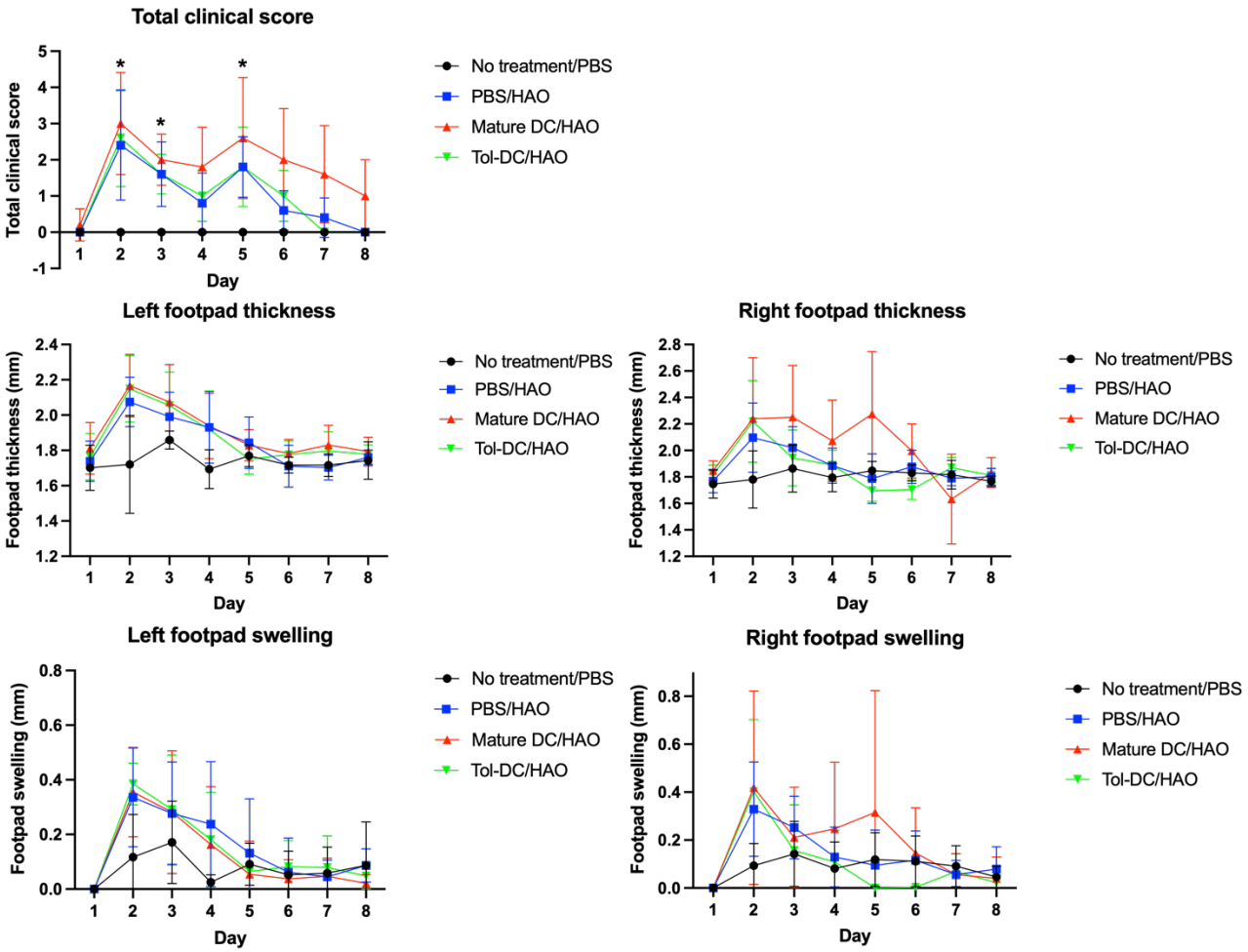


Figure 5-11 Tol-DCs had similar arthritis clinical scores as the PBS control and had no effect on footpad swelling.

After HAO or PBS injection, mice were clinically scored and footpads measured daily for one week. Total clinical score, left footpad thickness, right footpad thickness, left footpad swelling and right footpad swelling are plotted here. A 2-way ANOVA and Tukey's multiple comparisons test was performed on this data. There were only significant differences for total clinical score data, outlined below. Day 2 No treatment/PBS when compared to Mature DC/HAO and Tol-DC/HAO * Day 3 No treatment/PBS when compared to Mature DC/HAO * and Tol-DC/HAO ** Day 5 No treatment/PBS when compared to PBS/HAO *. * = p-value <0.05 and ** = p-value <0.01.

5.2.6 Mature DC groups had significantly higher numbers of OVA-specific CD4+ T cells and CD44 expression

After the final cull on Day 21 of the experiment, the dLNs (popliteal LNs) were harvested for flow cytometry. Following the gating strategy shown in Figure 5-12, the number of CD4+CD45-1+ OVA-specific T cells were plotted for each of the groups and the results can be seen in Figure 5-13. The highest number of OVA-specific CD4+ T cells was observed in two mice in the mature DC group and the numbers of these cells were significantly higher than those found in the None/PBS group. One mouse in the tol-DC group had a high number of OVA-specific T cells. However, the other mice in the tol-DC group had lower numbers of OVA-specific T cells like the PBS group.

CD44 MFI and % positive cells was plotted for the total CD4+ population. The results can be seen in Figure 5-14 as well as representative gating of CD44+ cells for each of the experimental groups. The mature DC group had significantly higher CD44 % positive cells than the PBS/None and PBS/HAO groups. The tol-DC group did have significantly higher CD44 % positive cells from the PBS/None group but were not significantly different from the PBS/HAO group.

Similarly, the mature DC group had a significantly higher values for CD44 MFI than the None/PBS and PBS/HAO group. The CD44 MFI for the tol-DC group was not significantly different from the None/PBS control or PBS/HAO group.

Although there are no significant differences between the Mature DC and tol-DC group directly, the Mature DC group was significantly different from the None/PBS and PBS/HAO control groups for CD44 MFI and CD44 % positive cells. Whereas the tol-DC group was only significantly higher than the None/PBS control for CD44 % positive cells.

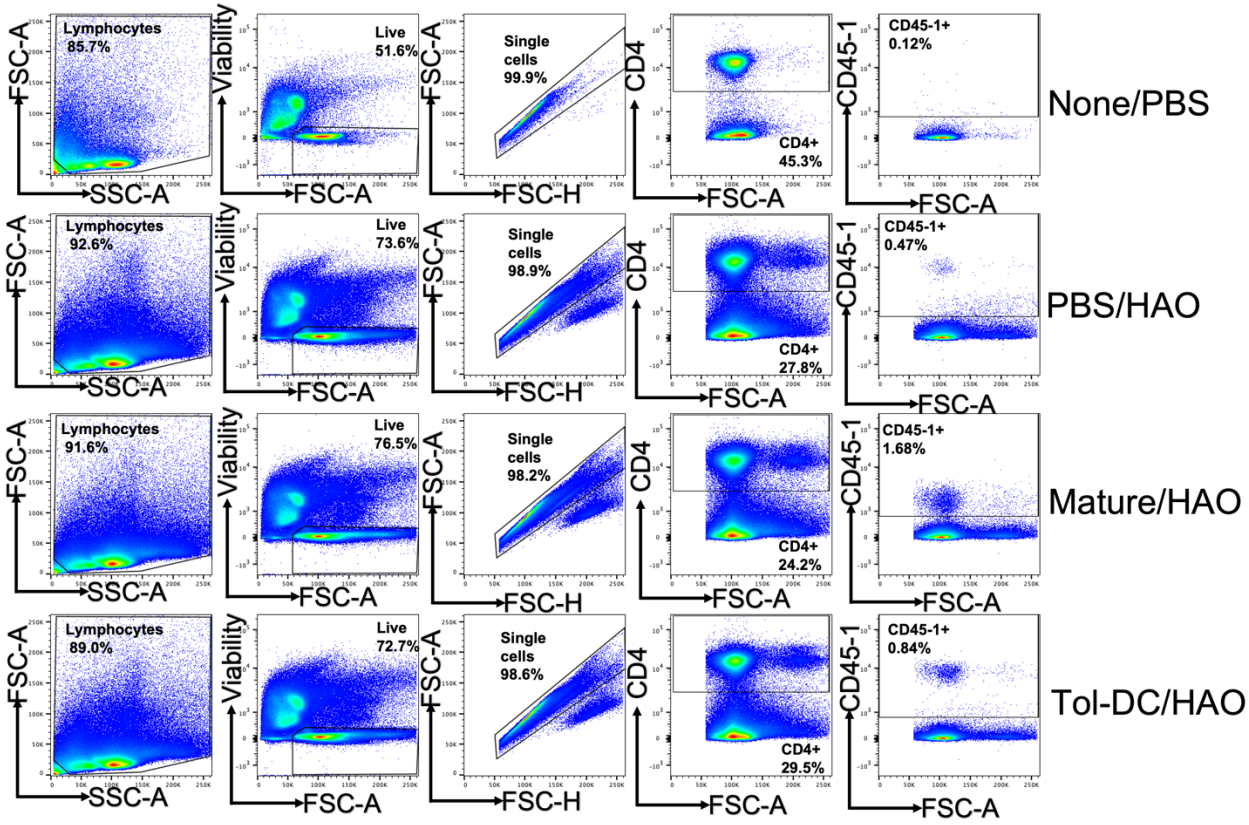


Figure 5-12 Flow cytometry gating strategy to identify OVA-specific CD4+ T cells.

Representative gating for each of the experimental groups can be seen above. First lymphocytes were gated using FSC-A and SSC-A. Then live and single cells were identified. CD4+ cells were gated and then CD45-1+ cells to identify the OVA-specific T cells.

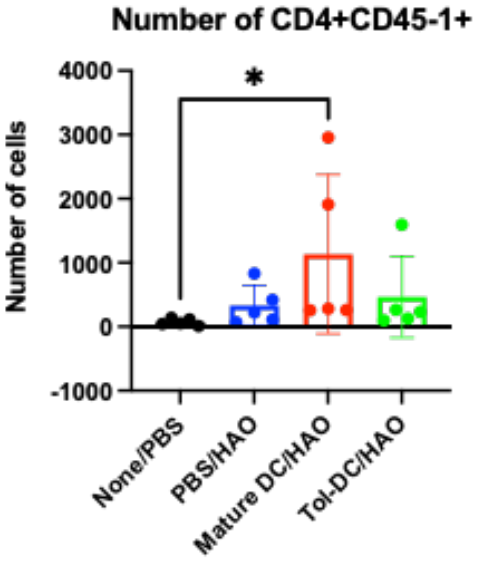


Figure 5-13 The mature BM-DC group had a significantly higher number of OVA-specific CD4+ T cells than the no arthritis control.

After following the gating strategy outlined in Figure 5-12, the number of CD4+CD45-1+ cells were plotted for each of the groups. The data failed a Shapiro-Wilk normality test so a Kruskal-Wallis test was performed. * = p-value <0.05.

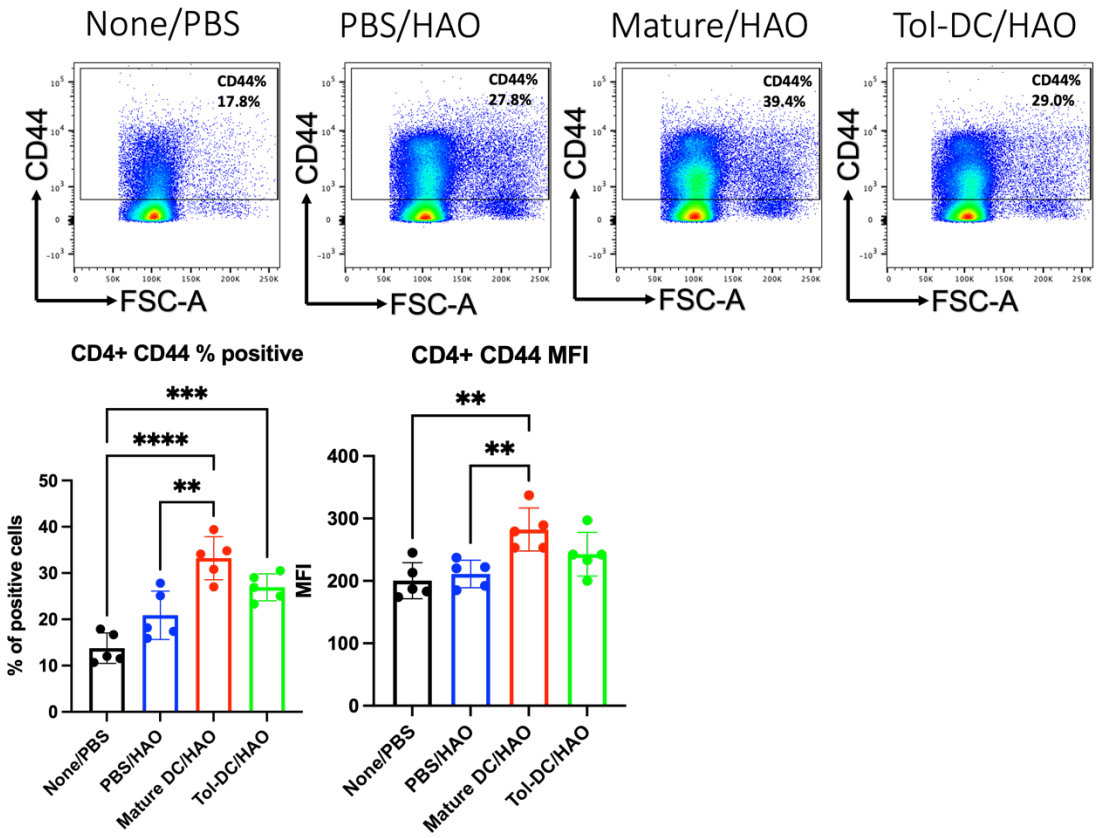


Figure 5-14 Mature BM-DCs have significantly higher numbers of CD4+CD44+ cells and CD44 MFI than None/PBS and PBS/HAO control groups.

The representative gating of CD44 positive cells for each of the groups is shown above. The number of CD4+CD44+ cells and CD44 MFI is plotted for each of the groups. The data passed a Shapiro-Wilk normality test therefore a one-way ANOVA with a Tukey's multiple comparisons test was performed. ** = p-value <0.01, *** = p-value <0.01 and **** = p-value <0.001.

5.2.7 Tol-DCs did not significantly alter OVA-specific Ab production

Blood sampling was performed on Day 14 (after OVA/CFA but before HAO challenge) and on the final day of the experiment. Anti-OVA IgG1 and anti-OVA IgG2c ELISAs were performed on the serum. The results of these ELISAs can be seen in Figure 5-15.

There was no significant difference in anti-OVA IgG1 and IgG2c between the groups at the final time-point. This could be because all mice in the experiment received Th1 polarised OVA specific T cells and OVA/CFA so had a similar Ab response against OVA. Tol-DC treatment did not seem to alter this response.

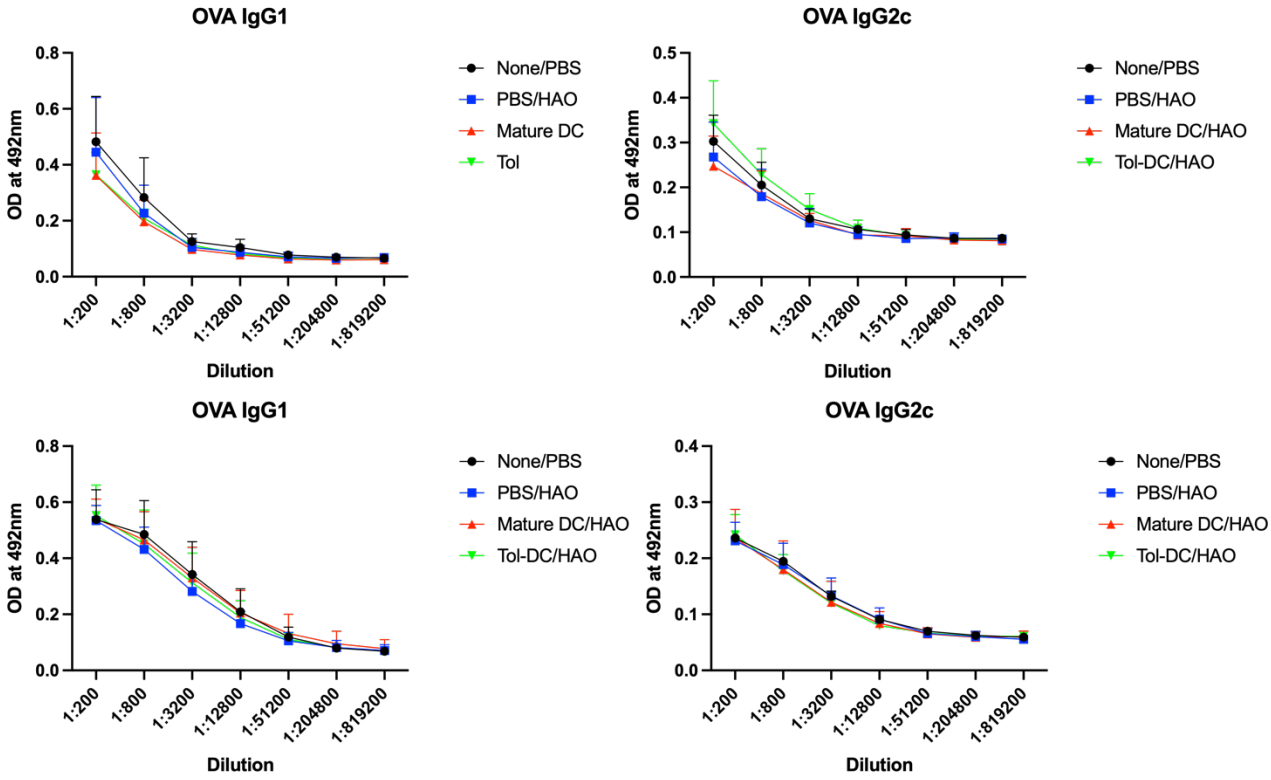


Figure 5-15 No significant differences in anti-OVA IgG1 or IgG2c production with tol-DC treatment.

Blood sampling was performed after OVA/CFA challenge but before HAO challenge on Day 14 and at the final time-point of the experiment on Day 21. Anti-OVA IgG1 and IgG2c ELISAs were performed on the serum. A 2-way ANOVA with a Tukey's multiple comparisons test was performed. There were no statistically significant differences between the groups.

5.3 Discussion

To my knowledge, subcutaneous footpad injection of tol-DCs has not been tested in any mouse models before. This is a useful injection route to test in arthritis models as the dLN is near the knee joint. Additionally, with the acute breach of tolerance model the popliteal LN will be the main disease-associated dLN as the inflammation is mainly localised to the ankle/foot in this model.

Mature BM-DCs and tol-DCs were both found to be able to migrate to the dLN after subcutaneous footpad injection. There was more of a CTV signal than a YFP signal which could suggest the injected cells are taken up by endogenous cells as YFP is a protein and will degrade after the YFP+ cells die or are taken up by other cells. Whereas the CTV signal can be tracked through different cells. Additionally, there was only a significant difference between mature BM-DC and tol-DC numbers when looking at live YFP+ cells and the numbers of live CTV+ cells were similar between the groups. This could be due to the YFP marker measuring migration rates whereas the CTV marker is potentially measuring uptake rates of injected cells or cell debris by endogenous dLN cells.

The numbers of mature BM-DCs and tol-DCs found in the dLN were low (50-100 live cells) and this was consistent with previous studies which found 90 BM-DC injected cells in the dLN after subcutaneous footpad injection of 600,000 cells[324]. Although the numbers of mature BM-DCs or tol-DCs which reach the dLN after subcutaneous footpad injection are low, it has been reported that only 85 DCs are required in the dLN to stimulate an immune response[335].

Tol-DC migration is thought to be lower than mature mo-DC migration and human tol-DCs express significantly lower levels of CCR7 than mature mo-DCs[204,220]. From the *in vitro* work in Chapter 4, murine mature BM-DCs and tol-DCs were found to express similar levels of CCR7. CCR7 is important for DCs to express as it allows them to follow CCL19/CCL21 gradients and enter the lymphatics and migrate to the dLN[336]. However, as shown in this chapter, tol-DCs were found to migrate significantly less than mature BM-DCs at 24 hours. To my knowledge, mature BM-DC and tol-DC migration after subcutaneous injection has not been directly compared *in vivo* before. To improve tol-DC migration, CCR7 could be enhanced on tol-DCs by low dose radiation[327], adenovirus mediated

over-expression[337] or by treating the cells with prostaglandin E2[338]. Although, low dose radiation could potentially activate tol-DCs.

This study found that tol-DCs can be found in the B and T cell area of the dLN following subcutaneous footpad injection. This is reassuring to know as it is thought tol-DCs would need to migrate to the dLN and interact with T cells there to induce tolerance. Previous imaging studies have found tol-DCs in the dLNs after intravenous and subcutaneous injection[234,329,330]. Murine studies which have examined tol-DC migration, mostly do so by fluorescently labelling the cells then digesting the dLNs for flow cytometry. A limited number of murine studies have looked at tol-DC migration to the dLN using imaging techniques such as near infrared and two-photon live imaging[329,330]. However, it is hard to identify which area of the LN the tol-DCs are in using these techniques. BM-DCs have previously been shown to be able to migrate to the T cell area of the dLN following subcutaneous footpad injection using immunofluorescence and confocal microscopy[324].

Dex+VitD3 tol-DCs have previously been tested in a humanised proteoglycan induced model of arthritis. In this study they also performed adoptive transfers and found that after adoptive transfer of Ag-specific CD4+ T cells and intravenous tol-DC treatment 24 hours later, tol-DCs could significantly reduce T cell proliferation and CD25 expression was decreased although not significantly[232]. There was additionally significantly less of a downregulation of CD62-L in the T cells of mice receiving tol-DCs. In the data shown above, there were no significant differences observed between *in vivo* CD4+ T cell activation and proliferation between mature BM-DC and tol-DC groups. The experimental design of adoptive transfers was similar. However, the proteoglycan study analysed Ag-specific CD4+ T cells from spleens after adoptive transfer experiments whereas in this study the Ag-specific CD4+ T cells were analysed in the popliteal LN (dLN). After adoptive transfer of CD4+ T cells they will circulate to many LNs, intravenous injection of tol-DCs could be more effective at delivering tol-DCs to these LNs rather than subcutaneous footpad where the tol-DCs will migrate mainly to one LN (the popliteal).

Importantly, tol-DCs have been shown to be able to suppress previously activated effector T cells *in vivo*. There was significantly reduced proliferation

and CD25 expression, CD62-L was less downregulated and there was a significant increase in CD4+CD25+FoxP3+ Treg cells with tol-DC treatment[232].

However, T cell activation markers might not be the best read out of effective tol-DC treatment. From the *in vitro* and *in vivo* work outlined in this thesis, it appears T cells stimulated by tol-DCs do become activated (when compared to the control) and Tr1 cells did not appear to be induced due to the lack of IL-10. There could be an alteration in cytokine production, with a reduction in IFN-γ or other inflammatory cytokines with tol-DC treatment. However, this was not significant from the ELISAs of supernatants from DC-T cell co-cultures in Chapter 4. In this chapter, after adoptive transfers the splenocytes were re-stimulated with PMA/Ionomycin on one occasion. From this data, it did appear there were fewer IFN-γ positive cells in the tol-DC treated group when compared to the control and mature DC group. Although, this will need to be repeated to be confirmed.

Alternatively, the T cells stimulated by tol-DCs could become activated then exhausted or then become anergic on subsequent rechallenge. Human Dex+VitD3 tol-DCs have been shown to induce anergy in memory T cells[217]. This was not evaluated in this study; however, it would be useful to investigate in future work.

Murine Dex+VitD3 tol-DCs have been tested in two different models of arthritis and have been found to be able to significantly reduce arthritis disease severity and progression in each one. Dex+VitD3 tol-DCs are thought to induce Tr1 cells in established CIA as there was a significant increase in IL-10 production by splenic CD4+ T cells after tol-DC treatment[234]. In contrast, there was a significant increase in CD25+FoxP3+ Tregs in the popliteal LN in mice receiving tol-DCs in the humanised proteoglycan induced model[232]. Differences in the Treg induced could be due to different models or different mouse strains as both used one million Dex+VitD3 tol-DCs and injected intravenously.

In this study, subcutaneous injection of mature DCs exacerbated arthritis clinical scores whereas the tol-DC group had similar total clinical scores as the PBS control group. The reason tol-DC treatment did not reduce arthritis below the control is perhaps because PBS is not the best control in this experiment. The

mice receiving mature BM-DCs or tol-DCs saw OVA peptide three times more than the mice that received PBS. Immature BM-DCs loaded with OVA peptide could have been used as a control instead but this could introduce more variation into the system as immature BM-DCs could then mature *in vivo*. Alternatively, soluble OVA could be used as a control, endogenous DCs could present this Ag *in vivo*.

Tol-DC treatment was found to reduce arthritis clinical scores below the control of PBS in an established model of CIA[234]. This could be because the CIA model is quite an extreme model as it utilises exogenous collagen type II to direct the immune response against endogenous collagen. Although, in the OVA-model, we polarise CD4+ T cells to a Th1 phenotype before transfer and like the CIA model use CFA to stimulate the immune response. The CIA model was established rather than acute. Tol-DC treatment is maybe more effective, or the improvement is easier to see in this setting. Alternatively, the differences observed could be due to the different injection routes chosen or mouse strains.

Tol-DCs did not have a significant effect on footpad thickness or anti-OVA IgG1 and IgG2c Ab production. Similarly, no differences in anti-collagen Abs were observed in the established CIA model with tol-DC treatment[234]. An anti-collagen IgG ELISA was performed on serum from the final time-point of this experiment. There was either very low levels of anti-collagen IgG suggesting there was not a breach of tolerance or there was a technical issue with performing the ELISA.

In this study, there was a decrease in OVA-peptide specific T cells and CD44+ CD4+ T cells in the dLNs of mice in the tol-DC group when compared to the mature DC group. Although this was not significant. This suggests tol-DCs can alter Ag-specific CD4+ T cell numbers as well as CD44 expression in the total CD4+ population.

In the humanised proteoglycan induced model tol-DC treatment was given on Day 17 (after the 1st humanised proteoglycan and DDA injection on Day 0 but before the 2nd injection on Day 21)[232]. This was termed as the pre-clinical stage as Abs were present but there were no signs of disease. In this study, we tested tol-DC treatment before the presence of Abs against OVA, however OVA-specific T cells were present.

Tolerance inducing therapy is most likely to be beneficial earlier in disease process. Therefore, in this study, we tested tol-DC treatment as early as possible. In future experiments tol-DC treatment could be tested after the breach of tolerance (after OVA/CFA injection but before HAO injection to localise inflammation to the joint). However, unpublished results from another member of the lab found trying to tolerise with OVA peptide orally after HAO challenge made arthritis worse. Tol-DC treatment has already been tested in established CIA and was found to be able to significantly inhibit disease severity and progression[234]. This coincided with a significant decrease in IL-17 producing CD4+ T cells and an increase in IL-10 producing Tr1 cells.

This chapter has established that tol-DCs can migrate to the T cell area and B cell area of the dLN following subcutaneous footpad injection. After adoptive transfer of OVA peptide specific CD4+ T cells and subcutaneous footpad injection of OVA peptide loaded mature BM-DCs or tol-DCs, there were no significant differences in T cell proliferation or CD25, CD44 or CD62-L expression. Testing subcutaneous injection of tol-DCs in an acute model of inflammatory arthritis found that mature DCs exacerbated arthritis clinical scores. Whereas tol-DC groups had a similar total clinical score as PBS control groups. However, tol-DC treatment did not reduce clinical scores below the control. Tol-DCs had no effect on footpad swelling or Ab production. Although there was not a significant difference in the number of OVA-peptide specific T cells or CD4+ CD44 MFI between the tol-DC group and control groups.

I think there is sufficient evidence that subcutaneous injection of tol-DCs is a promising injection route to include in future clinical trials. Tol-DCs can migrate to the dLN after subcutaneous footpad injection and there is evidence to suggest that they can modulate Ag-specific CD4+ T cells and reduce CD44 expression. Choosing the best injection route for each tol-DC type and disease is essential to maximise the effect of this treatment. The dose and frequency of injections will also need to be optimised. Increasing the number of cells to a point could be effective. However, a study testing tol-DC treatment in CIA found 500,000 tol-DCs more effective than 5 million in reducing arthritis[238].

Chapter 6 General Discussion

The current treatment of RA employs broad suppression of the immune response which leaves patients at risk of developing infections and cancer[92]. Additionally, there is a high rate of non-responders due to the non-specific nature of the current treatments[161]. More specific and effective treatments for RA are required, which target the pathogenic immune response while leaving protective immunity intact. Tol-DCs have gained a lot of interest in recent years as a potential treatment of autoimmunity. They induce a targeted Ag-specific immune suppression and would reduce the side effects seen with current treatments. Some of the main barriers in progressing clinical trials with tol-DCs is that there is a lack of quality control (QC) markers which would inform tol-DCs potency as a treatment, there are no markers of tolerogenicity in CD4+ T cells which would confirm if tol-DC treatment had been successful and the injection route which maximises the effect of tol-DC treatment is unknown. This thesis aimed to answer some of these questions.

6.1 Summary of key findings

- Identification of potential QC markers of tol-DCs. IL-1R2 and FcγRII since been validated by flow cytometry (Hilkens & Isaacs, unpublished)

- Identified other potential mechanisms of action of tol-DCs (increased uptake of apoptotic cells/immune complexes, inhibitory receptors, IL-1R2 decoy receptor for inflammatory cytokines)

- Identified LAIR-1 as a potential biomarker of induction of tolerance in CD4+ T cells

- After subcutaneous footpad injection of tol-DCs in mice, these cells can migrate to the B and T cell area of the dLN

- Tol-DCs significantly alter OVA-specific CD4+ T cell activation *in vitro* but not *in vivo*

- Subcutaneous footpad injection of tol-DCs in an acute model of inflammatory arthritis had no effect on clinical scores, footpad thickness/swelling or Ab production. However, the mature DC group had a significantly higher number of OVA-specific CD4+ T cells and CD4+CD44 MFI when compared to the controls whereas the tol-DC group was not significantly different from the controls in these parameters.

6.2 Differences and similarities between human and murine tol-DCs

A summary of the differences and similarities between human and murine Dex+VitD3 tol-DCs in different contexts can be seen in Table 6-1.

Table 6-1 Differences and similarities between human and murine Dex+VitD3 tol-DCs.

	Human	**Murine (this study)**	**Murine established CIA**[234]	**Murine hPG**[232]	**Murine colitis**[339]
Generation	Monocytes	Bone marrow	Bone marrow	Bone marrow	Bone marrow
Phenotype by flow cytometry	Lower CD83+CD86 Similar CD80[253] and MHC Class II	Lower CD40+CD86 Similar CD80 and MHC Class II Higher MerTK	Lower CD40, CD80, CD86 Similar MHC Class II	Lower MHC Class II+CD86	Lower CD40, CD86, MHC Class II Similar CD80
Genes transcriptionally upregulated	CFH, MRC-1, FcγRIIb, C1QA, C1QB, IL-1R2 and LAIR-1	C1QA, C1QB, IL-1R2 and LAIR-1	N/A	N/A	N/A
Cytokine production	Reduced IL-6 and increased IL-10	Reduced TNF-α, IL-6 and IL-10	Reduced IL-1β, IL-10, IL-12, IL-23 and TNF-α	No significant differences with Mature BM-DCs	N/A
Effect on T cells *in vitro*	Reduced activation (CD25, CD45-RO) Reduced proliferation[253] Reduced IFN-γ production, increased IL-10[217] Naïve T cells induced to be Tr1 and induce memory T	Reduced activation (CD25, CD44, CD62-L and CD69) No differences in proliferation or T cell cytokine production observed	Reduced proliferation	Reduced activation (CD25, increased CD62-L) Reduced IL-2, IL-6, IL-17 and IFN-γ	Reduced proliferation Reduced IL-2, IL-4, IL-5, IL-10, IL-12, IL-17 and IFN-γ

	cells to be anergic[217]				
Effect on T cells *in vivo*	No significant differences observed in AUTODECRA 1 will be further investigated in AUTODECRA 2	No effects on T cell proliferation or activation observed Reduction in number of Ag-specific T cells and CD44 expression although not significant	Increased IL-10 (Tr1) and reduced IL-17 production by splenic CD4+ T cells	Reduced T cell proliferation Reduced CD25, increased CD62-L Increase in CD25+FoxP3+ Tregs	No significant changes in Th1, Th17 or CD25+FoxP3+ Tregs

In this study, MerTK was found to be upregulated on murine tol-DCs when compared to mature BM-DCs by flow cytometry. FcγRII and IL-1R2 have since been found to be upregulated in human tol-DCs when compared to mature mo-DCs by flow cytometry. C1QA, C1QB and LAIR-1 were found to be upregulated transcriptionally in human and murine tol-DCs. This suggests similar mechanisms of action between human and murine tol-DCs. Potential mechanisms of action of tol-DCs can be seen in Figure 6-1. As discussed in Chapter 3, LAIR-1 on tol-DCs could interact with LAIR-1 on CD4+ T cells resulting in an immune inhibitory effect. Furthermore, C1q has been shown to be able to interact with LAIR-1 which would prevent activation of T cells. Removing apoptotic cells before they become necrotic would prevent endogenous DC and macrophage activation which could have a downstream effect on T cells and prevent T cell activation.

Showing a molecule is upregulated transcriptionally (at the mRNA level) does not necessarily mean it is upregulated at the protein level. That is why it is important to validate if this upregulation is still observed at the protein level. MerTK, FcγRII and IL-1R2 have been validated at the protein level. LAIR-1 and C1q will also have to be validated at the protein level. As C1q is secreted it will need to be tested for by ELISA and the cells will need to be cultured in serum free medium to ensure the C1q measured is being produced by the cells. LAIR-1 expression could be tested by flow cytometry. To test these molecules' role in tol-DC function, they could be blocked on tol-DCs *in vitro* using mAbs or siRNA

and then these cells could be co-cultured with CD4+ T cells. The effect on T cell activation, proliferation and cytokine production could then be assessed.

Molecules involved with uptake of apoptotic cells were found to be upregulated in tol-DCs (C1q, MerTK and FcγRIIb). To determine whether tol-DCs have increased uptake of apoptotic cells when compared to their mature counterparts an *in vitro* experiment could be performed. Cells could first be labelled with a fluorescent dye such as CFSE and then apoptosis could be induced either by irradiating the cells with UV light or treating the cells with camptothecin[308,309]. These cells could then be added to mature or tol-DC cultures and their uptake of the fluorescent apoptotic cells could be measured by flow cytometry.

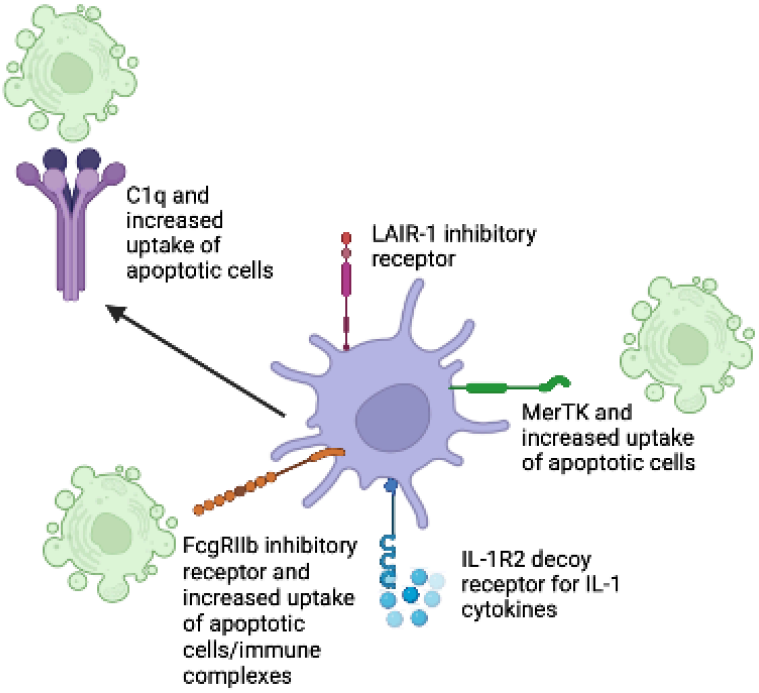


Figure 6-1 Potential mechanisms of action of human and murine Dex+VitD3 tol-DCs.

6.3 Tol-DC migration and injection route

As discussed previously, mo-DCs have been shown to have lower migration rates than cDCs and human tol-DCs lower migration rates than mature mo-DCs[8,60,204,220]. This study also showed murine tol-DCs had significantly lower migration rates than mature BM-DCs at 24 hours post injection. The numbers of

tol-DCs found in the dLN were low. Enhancing tol-DC migration to the dLN could improve the effectiveness of this treatment.

Although circulating cDCs are rare in human blood, clinical trials with CD141+ and CD1c+ cDCs (myeloid/conventional DCs) may be more effective than mo-DCs in cancer immunotherapy[340,341]. Tol-DCs have been generated from cDCs before but have never been directly compared with tol-DCs from mo-DCs[342]. cDCs could migrate better than mo-DCs but it is unclear whether tol-DCs generated from cDCs would be as tolerogenic as tol-DCs generated from mo-DCs. In cancer the aim is to stimulate the immune response against the tumour whereas for autoimmunity the aim is to dampen the abhorrent immune response against self. Although mo-DCs might have less T cell stimulatory abilities than cDCs this might not be an obstacle when trying to induce tolerance.

The best injection route will need to be optimised for each disease and the type of tol-DC chosen will affect this. The tolerogenic signal given to induce tol-DCs can reduce the subsequent CCR7 expression which would affect the cells ability to migrate to LNs. Alternative strategies to generate tol-DCs may overcome this issue, for example, rapamycin tol-DCs have been shown to express higher levels of CCR7 and show better migration rates than other tol-DC types[343]. Strategies to try and increase CCR7 expression such as low dose radiation have been considered but this could potentially activate tol-DCs[327]. Alternatively, adenovirus mediated over-expression of CCR7 in immature DCs was found to not activate the cells[337]. Prostaglandin E2 expression has also been shown to be important in affecting CCR7 expression and subsequent migration of human mo-DCs[338]. Intranodal injection would overcome the need for CCR7 mediated migration however it could destroy LN architecture and remove the selection of the most tolerogenic cells[191,252]. Similarly, intravenous injection would overcome the need for CCR7 mediated migration and tol-DCs have been found in LNs after this injection route[234,329,330]. However, tol-DCs mostly go to the spleen, liver and lungs after this injection route.

Choosing the best injection route for tol-DC therapy is difficult for RA as multiple joints are affected, therefore multiple dLNs might need to be targeted. In other diseases it is easier to target the disease-specific dLNs such as for MS

can target cervical LN, for Crohn's disease can target mesenteric LNs and for transplantation can target the dLN of the transplanted organ.

During RA there is likely to be on-going immune response in the joints including Ag-presentation and T cell activation. In an antigen-induced model of arthritis, articular DCs have been shown to reactivate Ag-specific CD4+ T cells in the joint[344]. Therefore, injecting tol-DCs straight into the affected joint was considered. Tol-DCs were injected intra-articularly in AUTODECRA 1[179] and the tol-DCs were found to not migrate from the joint to the dLN after 24 hours (Hilkens & Isaacs, unpublished). A major question in advancing tol-DC therapy is what T cells tol-DCs need to tolerise, circulating or tissue resident T cells. It is likely tol-DCs need to migrate to the LN for long lasting effects rather than the diseased site however whether this needs to be the disease associated dLNs or any LN is yet to be established. Furthermore, targeting both the diseased site and the dLN could enhance the effects of tol-DCs.

This study showed that after subcutaneous footpad injection of tol-DCs in mice, they can migrate to the dLN. However, it remains unclear if after this injection route tol-DCs can migrate to the knee or ankle joint. This is probably unlikely due to the size of the footpad and injection volume. It is also unknown whether there is any movement of cells between the dLN and joint. Tol-DCs are unlikely to leave the dLN after migrating there but CD4+ T cells could possibly migrate to the joint after interacting with tol-DCs in the dLN. In an antigen-induced model of arthritis CD4+ endogenous T cells and adoptively transferred OT-II T cells were shown to accumulate in the joint[344]. This thesis has shown tol-DCs can reach the dLN but whether tol-DCs interact with CD4+ T cells in the dLN has still to be established. These questions could be answered using available mouse models and two-photon techniques.

Ds-Red OVA-specific CD4+ T cells could be adoptively transferred into CD11c-YFP mice, 24 hours before subcutaneous injection of CTV labelled OVA-peptide loaded tol-DCs. It would be possible to image whether CTV tol-DCs and ds-Red OVA-specific CD4+ T cells interact in the dLN or joint. Endogenous DCs would be labelled with YFP therefore whether endogenous DCs play a role in Ag presentation after tol-DC transfer could be determined. Whether endogenous YFP+ DCs interact with CTV tol-DCs and dsRed CD4+ T cells could be observed. If

there were any YFP+CTV+ double positive cells, this would suggest the endogenous DCs are phagocytosing apoptotic tol-DCs. It would also be possible to measure CD4+ T cell egress to the joint from the dLN to determine whether tol-DC treatment could affect this. These experiments would first have to be performed in healthy mice then it could be adapted to arthritic mice to see if the interactions were the same or different during an inflammatory context. The inflammatory environment could increase migration of tol-DCs and interactions of tol-DCs with CD4+ T cells in the dLNs. However, paradoxically in an inflammatory context it might be harder for tol-DCs to mediate their effects as they may have to compete with endogenous DCs.

A limitation of this study is that subcutaneous footpad injection in mice might not be comparable to subcutaneous injection in humans. The subcutaneous footpad space in mice is very small so the cells could be forced out due to hydrostatic pressure rather than actively migrating[22]. Tol-DCs have previously been shown to migrate to the inguinal LN after injection in the subcutaneous flank[247]. This injection route is probably more comparable to subcutaneous injection in humans.

The injection route which maximises tol-DC effects is only going to be answered with more clinical trials. AUTODECRA 2 will test multiple injection routes of tol-DCs such as intradermal, intranodal or intra-articular. The immunomodulatory effects on T cells will be measured as well as effect on traditional disease markers (DAS-28 etc.) Although it is technically more challenging to measure tol-DC migration in humans it is possible through labelling techniques such as MRI[345].

6.4 Clinical implications for the future of tol-DC therapy

Selection of the autoantigen to load tol-DCs with is difficult for RA. This is due to the autoimmune response being first directed against one peptide (which can differ between patients). Then there is an increase in the autoantigens recognised with no dominant epitope spreading pattern. Epitope spreading occurs years before clinical onset of RA[346]. Loading tol-DCs with citrullinated peptides has been trialled for treating HLA-DRB risk allele positive RA[180]. Heat shock proteins have also been investigated as a potential Ag to load tol-DCs with

for the treatment of RA[347]. Choosing an Ag to load tol-DCs with is easier for transplantation or allergy where the Ag is known.

The type of tol-DC used for each disease would need to be chosen based on the best tolerance inducing mechanism (e.g., Tregs vs killing of autoreactive T cells). Tregs can induce bystander suppression which might be the best strategy for treating autoimmunity[348]. Inducing T cell anergy or deletion might not be as effective as epitope spreading which can occur years before the clinical onset of RA can generate multiple autoantigens[96].

Tol-DC therapy would be most effective when the Ag is known and treatment can be administered as early in the disease process as possible, for example in transplantation, tol-DCs could be given prophylactically before transplant. A clinical trial testing donor derived tol-DCs in liver transplant recipients has recently been completed[248]. Patients included in RA clinical trials have symptomatic and on-going disease which has failed other treatments therefore it might be harder for tol-DCs to be beneficial in this setting. Tol-DC treatment could potentially be used prophylactically for RA by identifying individuals at risk of developing RA (presence of genetic risk alleles, autoantibodies and family history). Individuals with HLA-DRB risk alleles, RF and CCP have a high risk of developing arthritis[349,350]. Presence of the HLA-DRB risk alleles predicts RA development with 60% sensitivity and 64% specificity. The corresponding figures are 37% and 98% for anti-CCP and 17-42% and 94% for RF. One study showed, combining the presence of HLA-DRB alleles and anti-CCP resulted in the highest odds ratio figure (66.8)[349].

Dex+VitD3 tol-DCs appear to have differing effects on CD4+ T cells depending on the context. See Table 6-1 and Figure 6-2. Tol-DCs reduced inflammatory cytokine production by T cells *in vitro* (humans) and *in vivo* in an established mouse model of CIA. Tr1 cells were induced by tol-DCs in human *in vitro* work and in the established mouse model of CIA. Whereas, CD25+FoxP3+ Tregs were shown to be induced in the humanised proteoglycan induced arthritis model. In this study, tol-DCs were not found to induce Tr1 cells. In a colitis model, tol-DCs had no effects on Th1, Th17 or CD25+FoxP3+ Tregs. This highlights how the same tol-DC type can vary in its function depending on the context. Therefore, each

tol-DC type will need to be carefully chosen for each disease for what outcome is required (the type of Treg induced, T cell anergy, deletion etc.).

In this study, tol-DCs did not seem to effect CD4+ T cell activation or proliferation *in vivo* after the initial activation with Ag. Therefore, tol-DCs could potentially mediate their effects by inducing anergy or exhaustion in CD4+ T cells with subsequent rechallenge. Unfortunately, this was not evaluated in this study but could be a potential mechanism of action of tol-DCs on T cells. This could be tested *in vitro* by restimulating previously activated CD4+ T cells with OVA peptide and mature BM-DCs or tol-DCs. Markers of exhaustion such as PD-1, CTLA-4, TIM-3 and LAG could be evaluated by flow cytometry. As well as T cell activation markers, proliferation and cytokine production. This could also be tested *in vivo* by utilising adoptive transfers of previously activated CD4+ T cells.

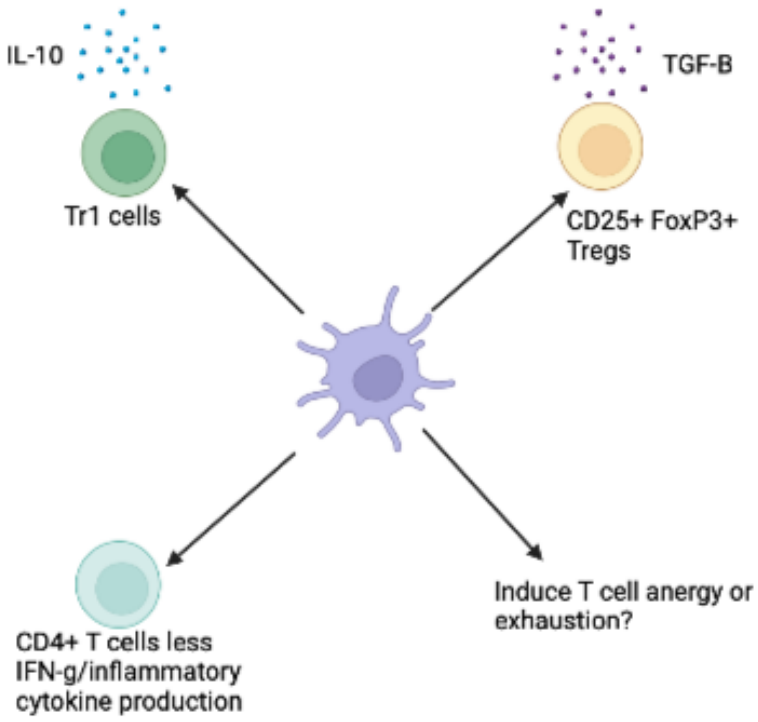


Figure 6-2 Dex+VitD3 tol-DCs effects on CD4+ T cells.

Markers of tolerogenicity in CD4+ T cells as a result of tol-DC treatment have still have not been established. This is important for improving clinical trials and would allow better comparisons between different clinical trials. Identifying markers of tolerogenicity in CD4+ T cells would be an indication that tol-DC

treatment had been successful and had an impact on CD4+ T cells. This study has suggested LAIR-1 could be a potential marker of tolerogenicity. However, LAIR-1 still needs to be validated at the protein level by flow cytometry. Performing RNA sequencing could identify a better biomarker of tol-DC therapy in CD4+ T cells.

There is evidence to suggest that the effect of tol-DCs could be enhanced in combination with other treatments. Such as being co-administered with Tregs[351] or given in combination with CTLA-4:IgG[352]. Co-administering tol-DCs and Tregs is thought to enhance the effects of both as the cells act on each other and reinforce their tolerogenic capacity. For example, IL-10, TGF-β and retinoic acid tol-DCs can stablise FoxP3 expression in Tregs[176]. Furthermore, Tregs can enhance tol-DCs through cell-to-cell contact and production of anti-inflammatory cytokines. Tol-DCs and CTLA-4:IgG co-administration has probably found to be more effective than either alone as CTLA-4:IgG will further block Ag presentation of tol-DCs to T cells. For patients with established RA, tol-DC treatment in combination with another treatment might be the best strategy to increase the chance of a beneficial effect.

6.5 Alternative antigen specific tolerance inducing treatments

This study has shown that tol-DCs can migrate to the dLN following subcutaneous footpad injection. However, most of the injected tol-DCs probably die after injection and are taken up by endogenous migratory DCs which then migrate to the dLN, see Figure 6-3. This could potentially provide a tolerogenic signal to endogenous DCs. There is evidence to support this as endogenous DCs have been shown to be essential for tol-DCs effects[353]. Additionally, tol-DCs have been shown to be short lived and mainly mediate their effects indirectly through endogenous DCs in a transplant model[353]. Furthermore, in the liver transplantation clinical trial, tol-DCs were generated from donor monocytes before being transferred to transplant recipients 7 days before liver transplantation[248]. On the day of transplantation, no intact tol-DCs were found in the dLN. However, DCs in the dLN co-expressed donor HLA and PD-L1.

In a DC transfer model, injected DCs were shown to transfer Ag to endogenous DCs directly in the LN[354]. However, transfer of apoptotic or necrotic DCs was found to not induce a T cell response and this study proposes that viable injected DCs are required to migrate to the dLN and interact with endogenous DCs there for an optimal T cell response.

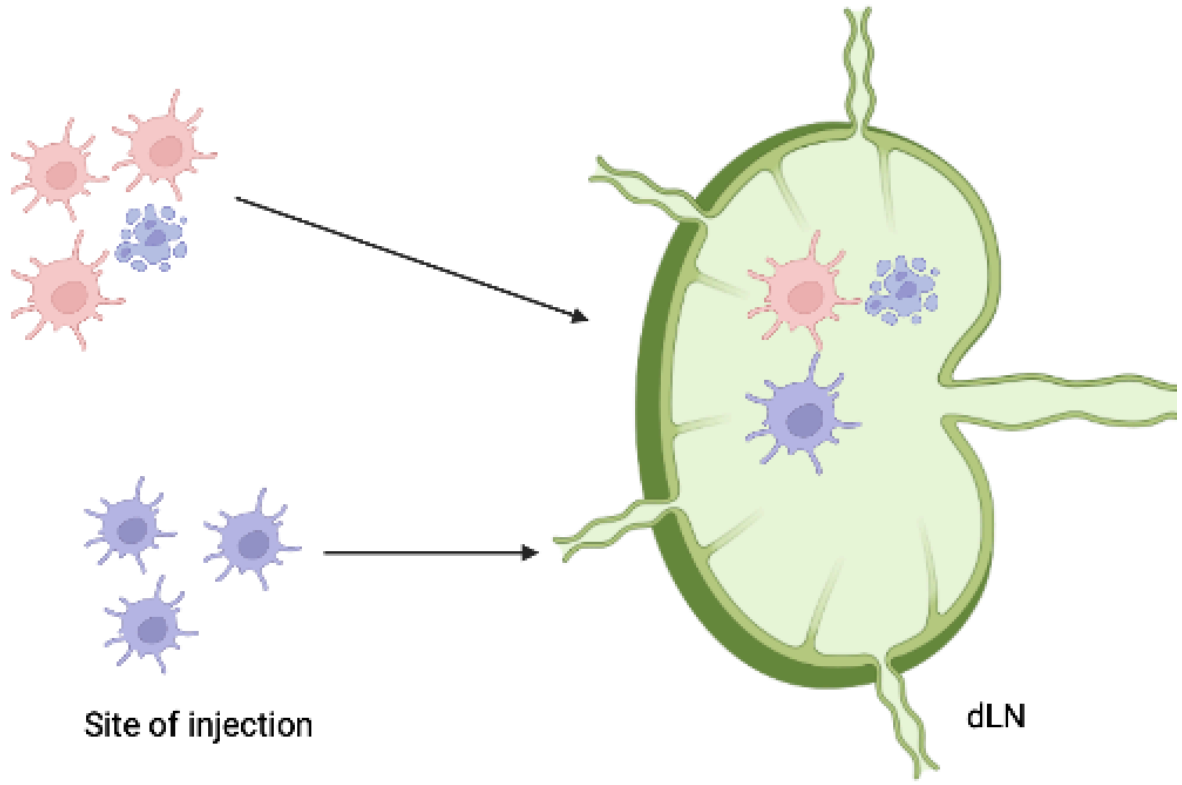


Figure 6-3 Possible routes of entry of tol-DCs to dLN.

After injection tol-DCs can migrate to the dLN directly. Tol-DCs could undergo apoptosis and be taken up by endogenous DCs in the dLN. This process could also happen at the injection site and these endogenous DCs could then migrate to the dLN.

In Chapter 5, there was a higher CTV signal than YFP in the dLN suggesting tol-DCs are taken up by endogenous cells. Unfortunately, many markers are shared between the injected mature BM-DCs/tol-DCs and endogenous DCs so it was hard to elucidate by flow cytometry. Performing an Image Stream which combines imaging and flow cytometry, it would have been possible to look at the morphology of YFP+CTV- cells, YFP+CTV+ and YFP-CTV+ to determine whether there was a particular population of endogenous DCs or macrophages taking up the injected tol-DCs. These populations could be lymph node derived if the tol-DCs migrate there and are then phagocytosed by endogenous populations. There

is a population of macrophages in the T cell area of the LN which has been found to phagocytose apoptotic cells[355].

Alternatively, the migration of endogenous DCs and tol-DCs could be assessed using the Kaede mouse model. The foot could be exposed to UV light before injection of fluorescently labelled tol-DCs. The exposure to UV light would turn all the resident endogenous DCs in the foot from fluorescent green to red[356]. Double positive cells would suggest the endogenous DCs are taking up the injected tol-DCs.

Tol-DCs are bespoke to each patient and expensive to produce so other Ag-specific tolerance inducing therapies which directly target DCs *in vivo* might be more useful. Nanoparticles containing protein or peptide Ag and rapamycin have been shown to be effective in inducing Ag-specific tolerance in mouse models of EAE and allergy[357]. It is important for the Ag to be encapsulated in the nanoparticles rather than being displayed on the surface in case autoantibodies in the circulation could react with the Ag[358]. Nanofibrous hydrogels have also been shown to be effective in the cancer setting. They contain DCs (immature and mature), PD-1 Abs and tumour Ags. The encapsulated DCs were found to have increased viability when compared to DCs injected without the hydrogel. The hydrogel also promoted recruitment of endogenous DCs and there was increased migration of DCs to the dLN[359]. The hydrogel strategy was found to reduce tumour growth and increase survival rates of mice when compared to adoptive transfer of DCs and subcutaneous injection of tumour Ag.

Nanofibrous hydrogels could be adapted to contain autoantigen, a tolerogenic signal and DCs. There is always a concern that supplying an autoantigen peptide to a patient with active autoimmunity could exacerbate the disease. Therefore, providing a tolerogenic signal as well as the autoantigen is essential. Knowing the dominant autoantigen for each patient would still be important when using these strategies and treatment as early as possible in disease process or prophylactically would be advantageous.

6.6 Final conclusions

Tol-DC therapy will prove to be more effective in some diseases than others which will depend on the antigen the tol-DCs are loaded with, the timing of treatment and the injection route chosen. Although we know tol-DCs work mainly through T cells, we still do not know exactly what tol-DCs do to T cells, and this appears to differ in different contexts (human vs different mouse models). Choosing the injection route which maximises the effects of tol-DC therapy and markers of tolerogenicity in CD4+ T cells are the main barriers in advancing this treatment. This study has identified FcγRII and IL-1R2 as potential QC markers of tol-DCs for the next AUTODECRA 2 clinical trial and LAIR-1 as a potential marker of tolerogenicity in CD4+ T cells. Additionally, this study has made a case for including subcutaneous injection route in future clinical trials and has suggested other potential mechanisms of action of tol-DCs which will need to be further validated.

Appendices

Table 1 Genes upregulated in 6-hour mature DCs compared to 6-hour tol-DCs. Genes with a log2 fold change of -2 or less and a Benjamini Hochberg p-value of less than 0.05 were considered important.

Upregulated in 6hr Mat	Log2 fold change	BH p-value	Description
CD1A	-4.72	0.0148	Present lipids to T cells
FCER1A	-3.86	0.0427	High affinity IgE receptor
TNF	-3.82	0.0022	Tumor necrosis factor alpha, inflammatory cytokine
IL12B	-3.27	0.0342	IL-12 subunit β, part of IL-12 and IL-23 receptor
TNFSF15	-2.67	0.0216	Vascular endothelial growth inhibitor, anti-angiogenic. Induced by TNF-α and IL-1α. Can trigger the NF-KB and MAPK signalling pathways and trigger apoptosis in endothelial cells.
IRF4	-2.2	0.0022	TF essential for the development of Th2 and Th17 cells
ICAM3	-2.2	0.00849	Adhesion molecule binds LFA-1 on T cells
CCR7	-2.18	0.0303	Chemokine receptor involved in migration to lymph nodes
MBP	-2.15	0.0163	Major basic protein, similar to lectins
NFATC2	-2.09	0.0342	Nuclear factor of activated T cells
CD83	-2.07	0.0114	DC maturation marker
CCL3	-2.05	0.0375	Chemokine which attracts monocytes and macrophages to sites of inflammation

Table 2 Genes upregulated in 6-hour tol-DCs compared to 6-hour mature DCs. Genes with a log2 fold change of 2 or more and a Benjamini Hochberg p-value of less than 0.05 were considered important.

Upregulated in 6hr tol	Log2 fold change	BH p-value	Description
CD163	5.99	0.0022	Scavenger receptor for hemoglobulin-haptoglobulin complex
CD14	5.86	0.0288	Monocyte marker
S100A8	4.97	0.0044	Calcium binding protein involved in cell cycle progression and differentiation, binds TLR-4
S100A9	4.3	0.0342	Calcium binding protein involved in cell cycle progression and differentiation, binds TLR-4
IL2RA	3.84	0.0022	CD25, part of IL-2 receptor
FCGR3A/B	3.21	0.0342	Low affinity immunoglobulin gamma Fc region receptor III-A
LAIR1	2.91	0.0342	Leukocyte associated immunoglobulin like receptor 1, inhibitory receptor prevents the lysis of cells recognized as self
IL10	2.79	0.0428	Anti-inflammatory cytokine
C1QB	2.7	0.0022	C1Q deficiency associated with lupus
FCGR2B	2.69	0.0148	Low affinity immunoglobulin Fc region 170receptor II-b
CXCL12	2.61	0.0364	Chemokine involved in migration to SLT
C1R	2.51	0.00808	Complement protein which activates C1s
C1QA	2.44	0.0055	Complement protein, deficiency in C1Q has been shown to be associated with lupus
CFH	2.41	0.00986	Complement factor H, regulates complement
IRAK3	1.97	0.00867	IL-1R associated kinase 3
TNFRSF1B	1.96	0.0206	TNF receptor 2

Table 3 mRNA upregulated in 6-hour tol-DCs compared to 24-hour tol-DCs. Genes with a log2 fold change of -2 or less and a Benjamini Hochberg p-value of less than 0.05 were considered important.

Upregulated in 6hr tol	Log2 fold change	BH p-value	Decription
CXCL10	-8.86	0.0061	Chemokine produced in response to IFN-γ, binds CXCR3
PTGS2	-7.41	0.00382	Cox-2
TNFSF8	-7.14	0.000603	CD30
CXCL11	-6.99	0.00883	Induced by IFN-γ and IFN-β. Attracts activated T cells
IL27	-6.31	0.00382	IL-1 cytokine, promotes Th1+Tr1 and suppresses Th2+Th17 development
IL23A	-5.99	0.00635	IL-23 subunit alpha, induces Th17 cells
TNF	-5.66	0.000929	Inflammatory cytokine
CCL2	-5.54	0.0177	Attracts monocytes, DCs and memory T cells to sites of inflammation
TNFSF10	-5.5	0.0084	TRAIL (TNF related apoptosis inducing ligand)
IL12B	-5.33	0.0122	IL-12 subunit β, part of IL-12 and IL-23 receptor
TLR7	-5.16	0.00382	PRR
TNFSF15	-5.02	0.00382	Vascular endothelial growth inhibitor, anti-angiogenic. Induced by TNF-α and IL-1α. Can trigger the NF-KB and MAPK signalling pathways and trigger apoptosis in endothelial cells.
IL6	-5.02	0.0132	Inflammatory cytokine
CCL20	-4.8	0.0011	Chemokine involved in the migration of cells to the LN
CXCL9	-4.77	0.0247	Attracts T cells, induced by IFN-γ
GBP1	-4.41	0.0068	Interferon-induced guanylate-binding protein 1
CD1A	-4.4	0.0462	Present lipids to T cells
IL10	-4.33	0.0223	Anti-inflammatory cytokine
GBP5	-4.1	0.00361	Guanylate binding protein 5
CCL8	-3.94	0.0211	Attracts multiple cell types to sites of inflammation
SLAMF7	-3.92	0.00382	Surface antigen CD319
SMAD3	-3.89	0.0279	Mediates signals initiated by the TGF-β cytokine family
CCL3	-3.7	0.0103	Chemokine which attracts monocytes and macrophages to sites of inflammation
CCL4	-3.59	0.0237	Chemokine which attracts NK cells and monocytes
POU2F2	-3.48	0.00219	TF
CD70	-3.41	0.0132	Ligand for CD27 on T cells, important for generation and maintenance of T cell immunity
IL1RAP	-3.21	0.0097	IL-1 receptor accessory protein
TNFRSF1B	-3.01	0.0084	TNF receptor 2
STAT4	-3	0.00914	TF required for Th1 differentation
CD36	-2.97	0.0145	Found on the cell membrane, imports fatty acids into cells
CD40	-2.91	0.00382	Co-stimulatory molecule
IL1RN	-2.89	0.00781	IL-1 receptor antagonist
CASP1	-2.84	0.00382	Caspase-1, involved in cleavage of IL-1β and IL-18. Also involved in programmed cell death.
IFIH1	-2.6	0.015	RIG-1-like receptor (intracellular PRR)
MAP4K4	-2.59	0.007	Mitogen activated protein kinase 4
IRF1	-2.57	0.00781	Interferon regulatory factor 1, TF
IRF7	-2.53	0.0084	Interferon regulatory factor 7, TF
IL7R	-2.43	0.0273	IL-7 receptor
HLA-DOB	-2.38	0.0314	MHC Class II molecule located in intracellular vesicles
JAK2	-2.35	0.00382	Signalling molecule
TRAF1	-2.34	0.0196	Signalling molecule involved in TNF signalling and NF-KB activation. Can also mediate anti-apoptotic signals induced by TNF signalling.
PTGER4	-2.31	0.007	Prostaglandin EP4 receptor
SLAMF1	-2.22	0.0145	Signalling lymphocytic activation molecule 1
SLC2A1	-2.18	0.0068	Glucose transporter

ADA	-2.18	0.0573	An enzyme in purine metabolism
CD80	-2.09	0.0084	Co-stimulatory molecule
NFKB1	-2.06	0.0165	Part of NF-KB signalling pathway
STAT2	-2.05	0.0132	Signalling molecule
BCL3	-2.04	0.00883	Regulates apoptosis
CD9	-2	0.000603	Cell surface glycoprotein which can bind integrins. Can mediate cell adhesion and migration. Also found on the surface of exosomes.
IL15	-1.96	0.0131	Secreted in response to viruses. Induces proliferation of NK cells.

Table 4 mRNA upregulated in 24-hour tol-DCs compared to 6-hour tol-DCs. Genes with a log2 fold change of 2 or more and a Benjamini Hochberg p-value of less than 0.05 were considered important.

Upregulated in 24hr tol	Log2 fold change	BH p-value	Description
CARD9	2.02	0.078	Adaptor protein which mediates signals from PRRs
IL1R1	2.72	0.000929	Receptor for IL-1α, IL-1β and IL-1R antagonist
CLEC4A	3.02	0.0132	C-type lectin domain family 4 member A
IL1R2	3.4	0.000603	Decoy receptor for IL-1 family cytokines

Table 5 mRNA upregulated in 24-hour mature DCs compared to 24-hour tol-DCs. Genes with a log2 fold change of -2 or less and a Benjamini Hochberg p-value of less than 0.05 were considered important.

Upregulated in 24hr mat	Log2 fold change	BH p-value	Description
IL12B	-7.98	0.000283	IL-12 subunit β, part of IL-12 and IL-23 receptor
CXCL11	-6.82	0.00128	Induced by IFN-γ and IFN-β. Attracts activated T cells
CXCL10	-6.64	0.00227	Chemokine produced in response to IFN-γ, binds CXCR3
CCL19	-6.35	2.35E-05	Chemokine involved in migration to lymph nodes
CD1A	-6.22	0.00131	Present lipids to T cells
CXCL9	-5.87	0.00134	Attracts T cells, induced by IFN-γ
CCR7	-5.17	0.000254	Chemokine receptor involved in migration to lymph nodes
LAG3	-5.16	0.00128	LAG3 binds MHC-Class II, negative regulator of T cell proliferation, activation and homeostasis. Involved in the maturation and activation of DCs.
CCL2	-4.62	0.0057	Attracts monocytes, DCs and memory T cells to sites of inflammation
TNFSF10	-4.58	0.00233	TRAIL (TNF related apoptosis inducing ligand)
PTGS2	-4.42	0.00469	Cox-2
TLR7	-4.07	0.00134	PRR
TNF	-4.05	0.000386	Tumor necrosis factor alpha, inflammatory cytokine
DUSP4	-3.92	0.000381	Dual specificity protein phosphatase 4, an enzyme which negatively regulates members of the MAP kinase superfamily. Signalling involved in cellular differentiation and proliferation.
IL27	-3.9	0.00392	IL-1 cytokine, promotes Th1+Tr1 and suppresses Th2+Th17 development
CCL13	-3.71	0.00636	Chemokine induced by IL-1 and TNF-α can attract monocytes, T cells, eosinophils and basophils to sites of inflammation
RARRES3	-3.59	0.0016	Retinoic acid receptor responder protein 3
ITGA4	-3.58	2.35E-05	Integrin which makes up half of lymphocyte homing receptor
LAMP3	-3.55	0.000224	Also known as DC-LAMP, lysosomal associated protein found on mature DCs
IRF4	-3.48	0.000112	TF essential for the development of Th2 and Th17 cells
ZEB1	-3.38	0.00186	TF that suppresses T cell specific IL-2 gene expression
CCL8	-3.35	0.00623	Attracts multiple cell types to sites of inflammation
TRAF1	-3.33	0.000597	Signalling molecule involved in TNF signalling and NF-KB activation. Can also mediate anti-apoptotic signals induced by TNF signalling.
CD83	-3.3	0.000381	DC maturation marker
FCER1A	-3.21	0.0415	High affinity IgE receptor
CD70	-3.18	0.00237	Ligand for CD27 on T cells, important for generation and maintenance of T cell immunity
TNFSF15	-3.15	0.00384	Vascular endothelial growth inhibitor, anti-angiogenic. Induced by TNF-α and IL-1α. Can trigger the NF-KB and MAPK signalling pathways and trigger apoptosis in endothelial cells.
IDO1	-3.03	0.00907	Immunosuppressive molecule
TNFSF13B	-2.9	0.0146	Also known as CD257, receptor for the cytokine BAFF (B cell activating factor) interaction between BAFF and BAFF receptor triggers NF-KB signalling
SLAMF7	-2.88	0.00176	Surface antigen CD319
LTA	-2.86	0.0277	Lymphotoxin alpha, activates NF-KB signalling
CD80	-2.82	0.000303	Co-stimulatory molecule
TNFSF4	-2.78	0.0017	OX40-L expressed on DC2s, enhances Th2 responses
IRF1	-2.73	0.000723	Interferon regulatory factor 1, TF

NFATC2	-2.73	0.00403	Nuclear factor of activated T cells
MX1	-2.64	0.0295	Interferon-induced GTP-binding protein Mx1, GTPase
GBP1	-2.62	0.00758	Interferon-induced guanylate-binding protein 1
TNFRSF11A	-2.59	0.00336	RANK, receptor for RANK-L
CCL3	-2.59	0.00653	Chemokine which attracts monocytes and macrophages to sites of inflammation
CD274	-2.57	0.000224	PD-L1
CASP3	-2.54	0.000214	Caspase 3
LILRA3	-2.53	0.0106	Leukocyte immunoglobulin like receptor 4, binds to HLA class I
CD40	-2.49	0.000929	Co-stimulatory molecule
HLA-DOB	-2.46	0.00392	MHC Class II molecule located in intracellular vesicles
CD86	-2.37	0.000224	Co-stimulatory molecule
VTN	-2.35	0.00649	Veronectin, cell adhesion molecule
IKZF1	-2.31	0.000356	DNA binding protein ikaros
BATF3	-2.27	0.000198	TF involved in cDC development
TRAF5	-2.25	0.0106	TNF-receptor associated factor 5, TNF signalling molecule
IFITM1	-2.24	0.0346	Interferon-induced transmembrane protein 1 or CD225,
IL23A	-2.24	0.0438	IL-23 subunit alpha, induces Th17 cells
NFKB1	-2.19	0.00186	Part of NF-KB signalling pathway
CXCR4	-2.18	0.00128	Chemokine receptor involved in migration to secondary lymphoid tissue
IFI35	-2.07	0.00244	Interferon-induced 35 kDa protein
PDCD1LG2	-2.06	0.00271	PD-L2
CLEC5A	-2.03	0.0363	A member of the C-type lectin family
CDKN1A	-2.02	0.00101	Cyclin dependent inhibitor 1A
IRF7	-2.01	0.0028	Interferon regulatory factor 7, TF

Table 6 mRNA upregulated in T cells cultured with mature DCs compared to T cells cultured with tol-DCs at day 3 of MLRs. Genes with a log2 fold change of -1 or less and a Benjamini-Hochberg p-value of less than 0.05 were considered important.

Upregulated in T cells co-cultured with mat-DCs Day 3	Log2 fold change	BH p-value	Description
IFNG	-6.96	0.0132	Inflammatory cytokine
IL9	-6.68	0.00983	A cytokine secreted by CD4+ T cells. Stimulates cell proliferation and prevents apoptosis.
IL17F	-6.61	0.0314	Isoform of IL-17, pro-inflammatory cytokine
CXCL10	-5.04	0.0132	Chemokine produced in response to IFN-γ, binds CXCR3
CSF2	-4.32	0.048	GM-CSF, a cytokine and growth factor
IL5	-3.81	0.0132	Th2 cytokine
BATF3	-3.15	0.0132	Transcription factor involved in the generation of Th17 cells
IL2	-3.08	0.0132	Important cytokine for T cells, prevents T cell anergy
CCL3	-3.07	0.031	Inflammatory cytokine, attracts monocytes, macrophages and neutrophils
TNFRSF8	-2.63	0.048	CD30
GZMA	-2.62	0.0314	Granzyme A
IL13	-2.54	0.0354	Th2 cytokine
LIF	-2.47	0.0331	Leukaemia inhibitory factor, inhibits differentiation
CD70	-2.34	0.0194	Highly expressed by activated lymphocytes
CDKN1A	-2.16	0.0194	Cyclin dependent kinase inhibitor
TNFRSF11A	-1.81	0.0132	RANK, member of the TNF receptor family
TNFRSF4	-1.79	0.016	CD134 or OX40 receptor, expressed by activated T cells
TBX21	-1.68	0.0194	Th1 specific TF
SLAMF7	-1.67	0.0132	CD319
TFRC	-1.6	0.0314	Transferrin receptor 1 or CD71, required for iron import
STAT1	-1.55	0.048	TF
CD274	-1.52	0.0426	PD-L1
IRF4	-1.51	0.0177	Interferon regulatory factor 4
TNF	-1.47	0.0314	Inflammatory cytokine
IRF8	-1.41	0.0426	TF
IFI35	-1.37	0.0314	Interferon induced 35kDa protein
HAVCR2	-1.37	0.0321	Cell surface molecule expressed on IFN-γ producing CD4+ T cells. Th17 cells, Tregs and CD8+ Tc1 cells
TRAF4	-1.25	0.0314	Member of the TNF receptor associated factor (TRAF) family
TAP1	-1.24	0.0218	Transporter associated with Ag processing 1
TNFSF10	-1.18	0.0314	TRAIL, a cytokine which causes apoptosis in tumour cells
SOCS1	-1.05	0.0321	Suppressor of cytokine signalling 1
BST2	-1.01	0.0426	Expressed in response to IFN-γ

Table 7 mRNA upregulated in T cells cultured with tol-DCs compared to T cells cultured with mature DCs on day 3 of MLRs. Genes with a Benjamini-Hochberg p-value of less than 0.05 were considered important.

Upregulated in T cells co-cultured with tol-DCs day 3	Log2 fold change	BH p-value	Description
ARHGDIB	0.43	0.0321	Rho GDP-dissociation inhibitor 2
ETS1	0.587	0.048	Transcription factor
SLAMF6	0.786	0.0426	A type 1 transmembrane protein
NFATC3	0.797	0.0321	Nuclear factor of activated T cells cytoplasmic 3
IL16	**0.919**	**0.0321**	**Cytokine, chemo-attractant for $CD4^+$ cells**
LAIR1	**1.24**	**0.0416**	**Inhibitory receptor**

Table 8 mRNA upregulated in T cells co-cultured with mature DCs compared to T cells co-cultured with tol-DCs on day 6. Genes with a Benjamini-Hochberg value of <0.05 were considered important.

Upregulated in T cells co-cultured with mature DCs on day 6	Log2 fold change	BH p-value	Description
IL9	-5.55	0.0184	A cytokine secreted by CD4+ T cells
GNLY	-4.35	0.0423	Granulysin, a protein present in the cytotoxic granules of $CD8^+$ T cells
CXCL10	-4.04	0.0423	Chemokine produced in response to IFN-γ, binds CXCR3
GZMA	-3.37	0.0311	Granzyme A, serum protease present in $CD8^+$ T cells
BATF3	-3.05	0.0311	Transcription factor required for the generation of Th17 cells
TBX21	-2.33	0.0184	Th1 specific TF
PSMD7	-0.234	0.0216	Part of the proteasome

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