Studies of Immune Biology of The Common Marmoset: A Novel Non-Human Primate Transplant Model.

Dr Shilpanjali Prasad, MBBS, FRACP

(Shilpanjali Jesudason)

Transplantation Immunology Laboratory
The Queen Elizabeth Hospital
Department of Medicine, Discipline of Medicine,
University of Adelaide

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THESIS ABSTRACT

Donor-specific immune tolerance is a highly desirable goal in clinical transplantation. Dendritic cells (DC) are potent immune system regulators, and promoting both anti-donor immunity and immune tolerance. DC are therefore an important target for potential tolerance-inducing therapies, which must be validated in non-human primate models before clinical trials. The common marmoset is a small, New World primate which our group is developing as a novel transplant model. The scope of this thesis involves the development of methodology and characterisation of critical aspects of marmoset immune biology pertinent to transplantation and DC immunotherapy.

Chapter 1 discusses the context of this thesis and contains a comprehensive literature review.

Chapter 2 outlines methodology and materials utilised in this thesis.

Chapter 3 describes a new technique for genotyping marmoset major histocompatibility complex (MHC) Class II DRB genes, to facilitate choosing mismatched donor and recipient animals for transplant studies. Genotype-based matching was predictive of *in-vitro* immune reactivity, and therefore validated as a method for selecting immunologically disparate animal pairs. Two new alleles were also identified. This work has given rise to two publications, and the methodology subsequently extended to marmoset MHC Class I and other Class II genes by others in our group.

Chapter 4 describes the first-ever studies of propagation of marmoset DC *in-vitro* from peripheral blood DC precursors (monocytes and stem cells) mobilised by the growth factor G-CSF. These methods enabled large-scale DC production from small volumes of peripheral blood. Marmoset DC were characterised extensively by morphology, phenotype and function, with many similarities to human and NHP DC. As with all animal models, specific differences

were also identified. In particular, marmoset monocyte-derived DC were maturation-resistant, whereas stem-cell derived DC were semi-mature. This work establishes that marmoset DC exist within the paradigm of human and NHP DC systems, and is therefore a feasible model for DC-based tolerance studies.

Chapter 5 describes for the first time the *in-vivo* propagation of marmoset DC following treatment with the growth factor FLT3-Ligand. A three-colour flow cytometry strategy for identifying and sorting marmoset putative peripheral blood myeloid DC was validated. The rare myeloid DC population was expanded massively by FLT3-Ligand, and could be isolated in numbers sufficient for therapeutic use. These DC had typical myeloid DC morphology and were capable of immune stimulation *in-vitro*. In addition, new markers for plasmacytoid DC were evaluated. This work forms the basis for ongoing studies of *in-vivo* marmoset DC.

Chapter 6 describes the culmination of the studies outlined in earlier chapters, with the first-ever studies of DC immunotherapy in marmoset monkeys. Three donor and recipient pairs were chosen on the basis of MHC genotype mismatch. Donor animals were treated with G-CSF and immature monocyte-derived DC propagated *in-vitro*. Recipient animals were treated with intravenous infusion of unmodified immature donor DC, and immune responses monitored. Two animals exhibited reduction in anti-donor (and third party) immune responses, whereas one animal was initially sensitized to donor cells. These preliminary studies establish the feasibility of DC-based immunotherapy in this model, and demonstrate that DC-induced immune modification can occur and be successfully monitored.

Thus, the work presented in this thesis creates a platform from which future studies of DC-based immune tolerance strategies can be developed in this novel transplant model.

DECLARATIONS

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution to Shilpanjali Prasad and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge that the copyright of published works contained within this thesis (as listed below) resides with the copyright holders of those works. Permission to reproduce has been obtained from the publishers as shown below.

1. Prasad S, Humphreys I, Kireta S, Gilchrist RB, Bardy P, Russ GR, Coates PT. MHC Class II DRB genotyping is highly predictive of *in-vitro* alloreactivity in the common marmoset. J Immunol Methods. 2006 Jul 31;314(1-2):153-63. (Publisher: ELSEVIER; License Agreement Date 27.10.08; Number 2056931094502)
2. Prasad S, Humphreys I, Kireta S, et al. The common marmoset as a novel preclinical transplant model: identification of new MHC class II DRB alleles and prediction of *in-vitro* alloreactivity. Tissue Antigens 2007; 69 Suppl 1: 72-75. (Publisher: WILEY-BLACKWELL; permission not required for reproduction of own article in self-edited publication).

PRESENTATIONS AND AWARDS

2008 Australasian Society for Immunology, Annual Scientific Meeting, Canberra (Poster)

2008 XXII International Congress of The Transplantation Society, Sydney (ePoster)
Recipient of TSANZ Young Investigators Award

2008 Postgraduate Research Expo, Faculty of Health Sciences, University of Adelaide, Adelaide (Poster)

2007 TQEH Research Day, Adelaide (Oral Presentation)
Winner, Best Higher Degree Presentation (Medical)

2007 Australia and New Zealand Society of Nephrology, Annual Scientific Meeting, Gold Coast (Oral Presentation)
Finalist, Young Investigators Award

2007 World Congress of Nephrology, Rio de Janeiro (Poster)

2007 Transplantation Society of Australia and New Zealand, Annual Scientific Meeting, Canberra (Mini-Oral Presentation)

2007 Australasian Society for Immunology, Annual Scientific Meeting, Sydney (Oral Presentation)

2006 Transplantation Society of Australia and New Zealand, Annual Scientific Meeting, Canberra (Oral Presentation)
Recipient of TSANZ Young Investigators Award

2006 TQEH Research Day, Adelaide (Oral Presentation)

2005 14th International HLA and Immunogenetics Workshop (IHIWS), Melbourne (Oral Presentation)

2005 Australasian Society for Immunology, Annual Scientific Meeting, Melbourne (Poster)

2004 Australasian Society for Immunology, Annual Scientific Meeting, Adelaide (Oral Presentation)

2004 Australasian and South East Asian Tissue Typing Association (ASEATTA) Annual Meeting, Sydney (Poster)

2004 TQEH Research Day, Adelaide (Poster)

2004 Australia and New Zealand Society of Nephrology, Annual Scientific Meeting, Adelaide (Poster)

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I would like to thank Ian Humphries (Australian Red Cross Blood Service Tissue Typing Laboratory, Adelaide) for his invaluable assistance with sequencing and analysis performed in Chapter 3, Chris Drogemuller (TQEH) for guidance with RelB sequence analysis, John Brealy for electron microscopy assistance, Dr Ian Lewis (Department of Haematology, Royal Adelaide Hospital) for facilitating access to the Allogeneic Stem Cell Transplant Program, and the donors for providing blood samples.

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ADDENDUM

Please note that references 232,236, 237, 239, 240 and 241 have been withdrawn by the authors. This came to my attention following the submission of this thesis. This is a body of work from the University of Alabama (Professor Judy Thomas) relating to the STEALTH protocol for tolerance induction in rhesus monkeys. The data relating to graft and animal survival in these studies have been found to be inaccurate. Full details may be obtained at http://ori.dhhs.gov/misconduct/cases/Thomas_Judith.shtml and http://ori.dhhs.gov/misconduct/cases/Contreras_Juan_Luis.shtml.

I have retained text in Chapter 1 relating to these studies of non-human primate transplant tolerance induction, because at the time of publication and in the years following they were important studies that related to other studies in this area and seemingly advanced the field. Therefore the existence of these studies remains relevant, despite the fact the data is now believed to be misleading. However, I have clearly identified discussion relating to withdrawn publications.

ABBREVIATIONS

AA – alternatively activated DC

APC – antigen presenting cell

BDCA – blood dendritic cell antigen

BM- bone marrow

Caja – Callithrix jacchus

CD – cluster of differentiation

CD40L – CD40 ligand

cDC – conventional DC

CpG – cytosine-guanine oligonucleotide

CTLA-4 – cytotoxic T lymphocyte associated antigen-4

DC – dendritic cell

DC-SIGN - DC-specific intercellular adhesion molecule [ICAM]-3 grabbing non-integrin

DNA – deoxyribonucleic acid

DSG - Deoxyspergualin

EDTA – ethylenediamine tetra acetic acid

ELIspot – enzyme linked immuno-spot assay

FCS – foetal calf serum

FLT3 – fms-like tyrosine kinase 3

FLT3-L – FLT3-ligand

FOXP3 – forkhead box protein 3

G-CSF –granulocyte colony stimulating factor

GM-CSF – granulocyte-macrophage colony stimulating factor

HLA – human leukocyte antigen

HP – haematopoietic precursor

HPDC – DC cultured from haematopoietic precursor cells

iDC – immature DC

IDO – indoleamine 2,3-dioxygenase

IFN – interferon

Ig - immunoglobulin

IL- interleukin

Lin - lineage

LPS - lipopolysaccharide

MDC – myeloid DC

MHC – major histocompatibility complex

MLR – mixed lymphocyte (leukocyte) reaction

MoDC – monocyte-derived DC

NF – nuclear factor

NHP – non-human primate

NK – natural killer

PB – peripheral blood

PBS – phosphate buffered saline

PBMC – peripheral blood mononuclear cell

PCR – polymerase chain reaction

PDC – plasmacytoid DC

RNA – ribonucleic acid

RPMI – Roswell Park Memorial Institute medium

SCF – stem cell factor

Treg – regulatory T-cell

TLR – toll-like receptor

TNF – tumour necrosis factor

TPO – thrombopoietin

TQEH – The Queen Elizabeth Hospital

WCC – White cell count

CHAPTER 1: INTRODUCTION

1. SEEKING TRANSPLANT TOLERANCE

Renal transplantation is the ideal treatment for end-stage kidney disease, conferring a significant survival advantage to patients who are fit for transplant [1]. Outcomes post-transplant are excellent, with 96 % 1-year and 90 % 5-year patient survival for cadaveric kidney recipients in Australia, and 99 % and 95 % respectively for live-donor recipients [2]. Due to advances in immunosuppression in recent decades, graft loss due to acute rejection is almost negligible. The overwhelming causes of graft loss are chronic allograft nephropathy and death with graft function [2,3]. This has shifted focus to the serious, potentially life-threatening complications of long-term immunosuppression which are emerging as the major causes of morbidity and mortality in the transplant population.

Cardiovascular disease, malignancy and to a lesser degree, infection are the leading causes of death with a functioning graft in Australia [2], and all of these complications may be immunosuppression-related. Of special concern, the rate of malignancy at many different sites dramatically increases post-transplant compared to both the general and non-transplanted renal failure populations [4]. In particular, non-melanoma skin cancer and solid organ malignancies with viral aetiology are specifically linked to post-transplant status and net immunosuppressive exposure. Additionally, the potential nephrotoxicity associated with calcineurin inhibition (currently the mainstay of most immunosuppressive regimens) is a serious problem affecting recipients of all types of solid organ and cellular transplants, leading to chronic and even end-stage kidney disease in a significant number of recipients. Other frequently observed immunosuppression-related complications include diabetes,

dyslipidaemia, bone disease including osteoporosis, hypertension, opportunistic infections and a myriad of other less common side-effects.

This significant burden of morbidity has led to substantial interest in strategies for minimising immunosuppressive requirements. The ostensible “holy grail” of transplant management is the concept of transplant tolerance, where there is *donor-specific immune hyporesponsiveness in the absence of ongoing immunosuppression, donor-specific antibody or chronic rejection, with retention of all other standard facets of immune function*. In reality in the clinical situation, it may be that *prope tolerance* (almost tolerance) enabling minimisation of immunosuppression will be more achievable than true operational tolerance[5], and may encompass the same benefits in the long-term.

Several approaches for tolerance induction have been explored with some enthusiasm in murine and non-human primate models, which will be discussed in further detail later in this chapter. Modification of T-cell function to induce donor-specific hypo-responsiveness is central to most of tolerance strategies, either with T-cell depletion, co-stimulation blockade, induction of mixed chimerism, or promotion of regulatory T-cells (Treg). In addition, the importance of dendritic cells (DC) in the establishment and maintenance of naturally occurring central and peripheral tolerance has led to significant interest in DC as a vehicle for transplant tolerance induction, as well as for treating autoimmunity and allergy [6-8]. DC play a critical role in peri-transplant immune processes, possess tolerogenic potential, and have therefore been explored as a therapeutic device for immune modification.

2. DENDRITIC CELL BIOLOGY

2.1 Introduction

First identified in 1973 by Steinman and Cohn [9], DC are a heterogeneous group of migratory antigen presenting cells (APC) that play a critical role in immunosurveillance and regulation. DC provide crucial links between innate and adaptive immune mechanisms [10,11], and therefore the evolution of primary and secondary immune responses. Of all APC (monocytes, macrophages and B cells) DC are the most potent immunomodulators. Despite their importance, they constitute only a tiny fraction (< 0.1%) of the cellular population, making their identification and study challenging.

DC arise from haematopoietic progenitor cells via various developmental pathways that exhibit significant plasticity [12,13]. DC can be migratory cells or resident in tissues, and display a spectrum of maturation and functional characteristics. Immature DC in peripheral tissues are extremely efficient at capturing antigens via phagocytosis, macropinocytosis and endocytosis. They have the ultra-structural machinery for antigen processing, and present peptide fragments of foreign and self antigen bound to major histocompatibility complex (MHC) Class I and II molecules. DC traffic processed antigen to secondary lymphoid organs for stimulation of T-cells in association with MHC (signal 1) and co-stimulatory molecules (signal 2) following DC maturation. This process is facilitated by a wide array of receptors for cytokines and chemokines, toll-like receptors (TLR), C-type lectins, and other receptors involved in inflammatory responses. Under steady state conditions peripheral tolerance to self antigens is maintained through this pathway by the induction of T-cell hyporesponsiveness, whereas when DC are fully activated under the influence of “danger signals” from immunological

insults such as infection or injury, defensive immune responses are initiated. Typical stimuli for DC maturation and activation include tumour-necrosis factor-alpha (TNF-α), T-cell activation through CD40-ligand (CD40L), toll-like receptor (TLR) agonists including bacterial lipopolysaccharide, interleukin-3, cytosine-guanine [CpG] oligonucleotide bacterial DNA motifs or virus particles. DC maturation leads to significant phenotypic and functional changes. The propensity for antigen acquisition is markedly down-regulated, whereas antigen presentation and immunostimulatory capacity are intensified. Co-stimulatory molecule (CD80/86, CD40, OX40L) and MHC expression are enhanced to promote signalling pathways for T-cell activation. The activation and maturation status of DC and the ability to deliver effective co-stimulatory signals influences the final outcome of the DC-T-cell interaction, and dictates whether proliferating T-cells are stimulated or inactivated.

Consequently, DC are responsible for both the suppression of auto-immunity as well as generating adequate responses to pathogens, aberrant or frankly malignant cells and tissue injury. DC have been targeted as vehicles for treating a broad range of immunological issues such as organ and cellular transplantation, auto-immune disease, malignancy and infections [7]. The concept of harnessing specific antigen immunogenicity has led to significant progress in DC vaccination for malignancy and infectious disease. Vaccines employing autologous or cell-line derived DC, or non-cell based vaccines targeting *in-vivo* DC, have now reached the stage of clinical trial for several types of cancer, including advanced stage melanoma [14-16], renal cell carcinoma[17], haematological malignancies[18] and advanced prostate cancer[19]. In addition, human trials of DC vaccines against human immunodeficiency virus (HIV) antigens have also been conducted [20,21]. Such trials are an important precedent for the employment of DC immunotherapy in clinical transplantation, where conversely we seek to exploit the

tolerogenic effect of DC. Most significantly, these studies have established the feasibility and safety of DC propagation, manipulation and subsequent administration to human subjects.

DC are a heterogeneous group, and numerous DC subsets have been well-described in humans and mice [12,22-24]. These subsets have locations throughout the immune network, do not necessarily share a common DC phenotype or functional role, and indeed do not even all arise from the same cell lineage. The complexity of DC subtypes and their evolution reflects their plasticity and ability to perform different functions in different environments, thereby enabling their bipolar effects on the immune response.

DC classification is based on anatomical location, morphology and immunophenotypic characterisation. DC express a myriad of cell surface molecules and receptors. Importantly, some markers are highly DC specific [25], and patterns of surface marker expression allow identification of DC subsets and assessment of DC maturation status [26]. The classification of DC has evolved as various subsets have been distinguished in mice, and this classification has been extrapolated to human DC although direct equivalents do not always exist. The main groups are conventional DC (cDC), (encompassing tissue resident DC, migratory DC, inflammatory DC and 'myeloid' DC arising from lineage negative precursors), monocyte-derived DC (MoDC) and plasmacytoid DC (PDC).

2.2 DC Ontogeny and Precursors

An understanding of DC ontogeny is important when developing techniques for DC propagation and generation. In the steady state, DC have a half-life of only a few days and therefore undergo rapid and constant turnover from circulating and local precursors. DC

originate from $CD34^+$ haematopoietic progenitors in bone marrow and enter the peripheral circulation as precursor DC (pre-DC) before reaching their final locations and differentiation to fully active DC. The original paradigm of conventional DC having 'myeloid' and 'lymphoid' subsets, and plasmacytoid DC being 'lymphoid' in origin is now known to be incorrect. The system of DC ontogeny exhibits significant flexibility and complexity, may be different for lymphoid and non-lymphoid organ DC, or for DC generated during immune insult compared to the steady-state[12].

It is now well established that common myeloid or lymphoid precursors arising from pluripotent stem cells can both give rise to DC. In mice it has been shown that the same lymphoid organ DC subsets may develop from several types of progenitors, regardless of lineage, when cultured under the correct conditions [12,13,27,28]. Even committed DC subsets have the capacity to transform into a different type. For example, it has been demonstrated that lymphocytic choriomeningitis virus infection triggers the conversion of bone-marrow derived plasmacytoid DC into myeloid DC both within bone marrow and *in-vitro*, under the influence of fms-like tyrosine kinase 3 (FLT3) ligand (FLT3-L) and Type I interferons [29]. This tremendous plasticity is a unique feature of DC. It remains unclear exactly at what stage of development the commitment to a certain DC subset is made, although recently evidence of a common, committed and clonal DC precursor (termed CDP or pro-DC) arising downstream from either a common myeloid or lymphoid progenitor has emerged [30,31]. These cells are Lin^- (negative for lineage markers CD14, CD3, CD19, CD20, CD56), $CD11c^-$, and MHC Class II $^-$, but further downstream precursors may express CD11c and this may represent a point of divergence between cDC and PDC pathways [12]. The pathways from these precursors to various DC subtypes are yet to be elucidated, but are influenced by the local

microenvironment, cytokines and transcription factors.

Several cytokines or growth factors are critical for DC development *in-vivo and in-vitro*, and the balance of these cytokines influences the differentiation of certain DC subsets. Most importantly, FLT3 expression appears to be an essential requirement for DC differentiation from precursors of any lineage [13,27,28,30-32]. The fraction of $FLT3^+$ cells within common lymphoid, common myeloid or clonal DC progenitor populations can potentially give rise to either MDC or PDC *in-vivo* or *in-vitro* [28,30,31]. This cytokine mobilises both myeloid and plasmacytoid DC when administered to mice [33-35], rhesus macaques [36-40] and humans [41-43] (discussed in detail in Chapter 5). Conversely, FLT3 deficient mice are also DC deficient, and $FLT3^-$ cells cannot give rise to DC unless transfected to express FLT3. In addition to FLT3, both c-kit (the receptor for stem cell factor (SCF) and the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor are also expressed on DC precursors. In the total absence of GM-CSF, FLT3 preferentially promotes PDC development. In contrast, GM-CSF promotes cDC differentiation even in the presence of FLT3, via STAT-5 dependent mechanisms [13], particularly in the presence of inflammation. GM-CSF also acts on macrophage DC precursors to stimulate differentiation of DC in favour of macrophage production, which occurs in response primarily to macrophage-colony stimulating factor (M-CSF). This pathway is potentially important in the differentiation of DC from monocytes (discussed further in Chapter 4), either in the steady state as a source of cells for DC turnover, or as a response to tissue injury. Interestingly, G-CSF treatment is responsible for preferentially increasing the plasmacytoid $CD11c^-$ DC population in humans[43,44].

DC development, particularly the commitment to a specific DC subset, is also regulated by a

number of transcription factors including Spi-B, NF-κB proteins, Ikaros, interferon regulatory factors (IRF-2, 4 and 8), helix-loop-helix transcription factors (ID2), STAT5 and STAT3 [13]. Disruption of one or more of these pathways leads to deficiencies in DC number, or different patterns of differentiation and maturation. For example, absence of IRF-8 disturbs PDC development in favour of myeloid DC (MDC) subsets [45], and disruption of NFκB / RelB protein up-regulation and nuclear translocation arrests DC maturation (discussed in detail below and in Chapter 4).

In conclusion, the cellular origin of a DC is not necessarily reflected in the final DC phenotype. DC genesis from haematopoietic precursors is a multi-step process and only some of the intermediate stages have as yet been defined. The flexibility of DC development, from diverse cell lineages at various stages of commitment, contributes to a robust and rapid defence response against foreign pathogens and tissue injury.

2.3 Classification of DC Subsets

2.3.1 Murine DC

The murine DC system is the most extensively characterised of any experimental DC model. Although it differs significantly from human DC, and direct DC equivalents between the species are difficult to make, murine DC form the substantial basis of existing knowledge on naturally occurring DC. Murine DC have been characterised by their location (migratory or tissue-resident) and by markers of ontogeny and maturity. This classification has been reviewed in detail previously [12,13,22,46] and is briefly summarised below.

Murine Conventional DC (cDC)

- cDC are found throughout lymphoid and non-lymphoid tissues, and in circulation in blood and lymphatics. They have the classical dendritic cell morphology and function as antigen presenting cells (APC) with a critical role in immunosurveillance.
- Migratory DC are located peripherally in skin (Langerhans cells, dermal DC) and lymphoid interstitium (interstitial DC), and arise from peripheral precursors. These DC constantly and constitutively move via lymphatics between peripheral non-lymphoid tissues and lymph nodes, trafficking antigen, and maturing in the process. These form half of all lymph-node DC in the mouse [46], and are not present in spleen or thymus.
- Tissue-resident DC are found within mouse spleen, thymus, lymph nodes and other organs such as the kidney, liver and lungs. They are infrequently seen in the circulation as they arise and are constantly replenished from bone-marrow derived, tissue-resident precursors and do not traffic through peripheral tissues. They are immature in the steady-state, and mature only in response to pathogens and inflammation [46].
- Thymic DC are unique in that they arise from a thymic precursor, do not migrate and present mostly self-antigen. They have a critical role in the development of self-tolerance via their effects on T-cell selection. The spleen is a DC-rich organ, containing both cDC (several subsets) and PDC in large numbers. Murine splenic DC are the most easily accessible of all *in-vivo* DC models and are therefore the most studied DC.

Administration of the haematopoietic growth factors G-CSF, GM-CSF or FLT3-L mobilises various murine cDC subsets *in-vivo* [33-35]. Murine cDC can also be generated *in-vitro* from bone marrow and splenic precursors using the growth factors GM-CSF, TNF-α and stem-cell factor [12,47]. These DC most closely reflect *in-vivo* migratory DC. In addition, *in-vitro*

generated DC arising from monocyte precursors in the cell population may represent 'inflammatory DC'. *In-vivo*, the capacity for DC to be generated from peripheral monocytes allows for a rapid DC presence at sites of inflammation, particularly when stimulated by the presence of GM-CSF [46]. These inflammatory monocyte-derived DC may not necessarily be found in the steady-state *in-vivo*, but are an invaluable research tool as large numbers of DC may be generated.

Murine cDC express a complex pattern of surface molecules which assist in detailed classification of many DC phenotypes (reviewed in references 9-12). Of most importance in delineating the major subgroups are CD11c (found on all cDC) and/or CD11b, CD8α, CD4 and MHC Class II. The T-cell marker CD8α, found as a homo-dimer on murine DC, is not expressed on human DC, creating a major point of difference between the species. All DC have MHC Class II expression, particularly when mature. Langerhans cells express Langherin, which is not present on other migratory DC. Tissue-resident DC in mice are either $CD4^-CD8^+$ (found in T-cell rich areas of lymph nodes and spleen, and in the thymus) or $CD8^-$ (the majority of lymphoid-resident DC, found in the marginal zones in steady state and T-cell regions when activated). $CD8^-$ DC can be either $CD4^+$ or $CD4^-$, generating three main subsets found in murine LN and spleen: $CD4^-CD8^+$ (which also express CD205), $CD4^+CD8^-$ and $CD4^-CD8^-$. Murine DC that are $CD8^-$ $CD11b^+$ $CD11c^+$ are also known as "myeloid" DC, whereas $CD8^+$ $CD11b^-$ $CD11c^+$ DC are "lymphoid" DC, terms originating from the early paradigm of strict lineages giving rise to DC subsets. Murine DC subsets appear to have differing function. $CD8^+$ DC produce higher levels of IL-12 compared to $CD8^-$ DC, reflecting bias towards Th1-type responses [22]. $CD8^-$ DC are more efficient producers of interferon γ. Other functional differences between these DC subsets such as the capacity for cross-presentation of antigen on

MHC I, tolerogenic capacity and effects on T-cell cytokine production are apparent but not fully elucidated [22].

Murine Plasmacytoid DC

PDC (also known as interferon-producing cells) are so named due to their plasma cell-like appearance when unstimulated. While originally thought be of lymphoid lineage due to their expression of several lymphoid surface markers in the absence of myeloid markers, it has been found they may also arise from non-lymphoid progenitors [13,28]. PDC develop from bone marrow under the influence of growth factors FLT3-L and G-CSF, and can be cultured *in-vitro* from bone marrow precursors with FLT3-L [32]. The use of these growth factors *in-vivo* promotes mobilisation of pre-PDC into peripheral blood (as discussed previously and in Chapter 5). Murine PDC are $CD11c^{low}CD45RA^{+}B220^{+}$MHC Class II^{+}, and also express CD8 when activated. They do not express lineage markers. PDC are found primarily in murine spleen and peripheral lymphoid organs, and exist as pre-DC in the steady-state. PDC are less efficient at antigen presentation and T-cell activation compared with cDC, and require additional (and in humans, possibly multiple) maturation stimuli to attain full activation[42,48]. However, PDC have been shown to participate as essential APC in the immune responses to allografts, phagocytosing antigen within the graft and trafficking it to lymphoid organs, where they can induce regulatory T-cells that are critical for allograft tolerance [49]. Both murine and human PDC are potent, rapid producers of Type I interferons (predominantly α an d β) in response to viral and other microbial stimuli including bacterial CpG oligonucleotide DNA motifs. The ability to respond to these signals is a function of the distinctive expression of TLR 7 and 9 on PDC. Interferon production promotes NK cell-mediated killing of virus-infected and tumour cells, and therefore deficiencies of PDC number and function have been related to viral infection and malignancy [48].

Interferon Producing Killer DC (IKDC)

Recently, a further murine DC subset, the interferon producing killer DC (IKDC) has been described[50]. These cells were originally identified from spleen, are $CD11c^+B220^+NK1.1^+$ and capable of antigen presentation, produce type I and II interferons particularly interferon-γ, and kill in a fashion similar to NK cells. They play an important role in anti-tumour responses.

2.3.2 Human DC

The correlation between mouse and human DC remains unclear, particularly as a greater number of murine DC subsets are recognized using mouse-specific markers such as CD8. Human DC have chiefly been characterised from peripheral blood due to the difficulty in obtaining tissues and identifying mature DC in situ. The DC precursors found in human blood are immature myeloid DC (pre-MDC), pre-PDC, monocytes and haematopoietic progenitors including $CD34^+$ cells. Precursor DC eventually migrate to tissues as immature DC which undergo maturation and activation.

Human Myeloid DC (MDC)

Human MDC (DC1) arise predominantly from myeloid lineage precursors, and are dependent on GM-CSF for development. MDC precursors (pre-MDC) in human blood all express CD11c. Lin^- ($CD14^-$) $CD1^+$ pre-MDC give rise to dermal, non-lymphoid tissue DC or Langerhans DC. Alternatively, monocytes ($CD14^+CD1^-$) can differentiate into immature monocyte-derived DC (MoDC) during inflammation and possibly in the steady state. Pre-MDC are found in the MHC Class II^+ Lin^- $CD11c^+$ fraction of peripheral blood, and this is one strategy for isolation of these rare cells (see Chapter 5). MDC also express other myeloid markers including CD4, CD45RO, CD11b, CD13, and CD33, as well as Fc Receptors (CD16,

CD32, CD64), the receptor for GM-CSF and all TLR types except TLR7 and 9 [24,51,52].

In recent years, specific DC markers have been discovered, and have enabled differentiation of pre-MDC from pre-PDC.

- Discovery of the blood dendritic cell antigen (BDCA) molecules has significantly aided the identification and isolation of human DC [25]. BDCA-1 (CD1c) is involved in antigen presentation, and the majority of myeloid DC are Lin^{-} HLA-DR^{-} BDCA-1^{hi} CD11c^{hi} CD123^{-}. BDCA-1 is also expressed in *in-vitro* propagated DC generated from monocyte or stem cell precursors, and on a small subset of B cells. BDCA-3 (CD141) is expressed most strongly on a subset of myeloid DC that are BDCA-1^{-} HLA-DR^{+} CD11c^{low} CD123^{-} (type 2 MDC). These MDC do not express CD2 or Fc receptors, and are functionally different. CD123 (IL-3 receptor alpha chain [IL-3R-α]), previously thought to be exclusively expressed on PDC, has also been identified on minor subsets of cultured human myeloid DC[53,54].
- CD205 (DEC205) is a multilectin surface receptor that mediates endocytosis [55], and is particularly important in distinguishing apoptotic and necrotic cells. It is DC-specific in mice, but in humans is not exclusively found on DC, but is also present on almost all leukocyte subsets [56].
- Dendritic cell-specific intercellular adhesion molecule [ICAM]-3 grabbing nonintegrin (DC-SIGN/CD209) is a mannose-binding, trans-membrane C-type lectin which facilitates chemokine-induced DC migration and DC binding to ICAM on T-Cells, thereby playing a critical role in T-cell activation [57]. DC-SIGN plays an important role in antigen uptake (particularly microbial carbohydrates) and viral transfection efficiency, and is implicated in the transmission of HIV / SIV [58]. DC-SIGN is expressed in higher levels on immature

tissue and monocyte-derived myeloid DC compared with mature DC, reflecting the antigen uptake capacity of immature DC. It is minimally expressed on freshly isolated blood myeloid DC [54].

Human Plasmacytoid DC

PDC (DC2) pre-DC in human peripheral blood are identified phenotypically by the lack of lineage markers, myeloid markers and Fc receptors, and expression of HLA-DR. PDC also express a high density of CD123, CD45RA, some lymphoid markers (CD4 and T-cell receptor chains), BDCA-2 and 4 (neuropilin-1)[24,25]. Unlike MDC they express TLR 7 and 9 , as well as TLR-1, TLR-6, and TLR-10[51,52]. This enhances their ability to respond to microbial stimuli.

BDCA-2 (CD303) [25] is a C-type lectin involved in antigen uptake. It is specifically expressed on plasmacytoid DC in peripheral blood and lymphoid or non-lymphoid tissues, enabling accurate identification and purification from these sites. BDCA-2 expression is highest on freshly purified PDC, and is subsequently diminished upon culture. BDCA-4 (CD304, neuropilin-1) is a co-receptor for vascular endothelial growth factor (VEGF). It is not exclusively expressed on PDC but may also be found on a subset of MDC (including MoDC), some lymphoid T-cells as well as non-lymphoid tissue such as neurones and vascular endothelium.

Tissue-specific DC in Humans

Renal DC

Although detailed characterisation of human renal DC remains limited, extrapolation from

murine studies suggests they will prove to be critical to understanding many renal pathological processes [59]. Murine renal DC form a contiguous network throughout the renal parenchyma[60], with the vast majority identified as 'myeloid' $CD11c^+$ $CD8^-$ $B220^-$ DC [61]. These DC can be mobilised in mice in high numbers with the administration of FLT3-L [61]. The normal human kidney (assessed in tissue from living transplant donors) also contains both MDC ($BDCA\text{-}1^+ \pm$ $DC\text{-}SIGN^+$) and PDC ($BDCA\text{-}2^+$), with four times more MDC than PDC [62]. PDC are found in higher numbers in humans than in mouse kidneys, where < 10 % are PDC. Renal DC are critical to the immune responses following renal transplantation (discussed below), as well as several other pathological processes. They are located throughout the tubulo-interstitium, minimally in the glomerulus and concentrate in the peri-glomerular area during inflammation [62,63]. These DC traffic renal antigens to draining lymph nodes, and are the primary APC stimulating T-cells in the kidney, particularly after tissue-injury [64]. DC in the kidney are responsible for the early inflammatory response to ischaemia-reperfusion injury (IRI) in mice with the release of pro-inflammatory cytokines including TNF-α [64,65]. IRI induced in pigs leads to an influx of myeloid DC into the kidney, and high DC numbers are also seen in patients with delayed graft function post-transplantation [66]. The number, function and cytokine profile of renal DC has also been shown to alter in patients with glomerulonephritis [67] and SLE [68], as well as murine experimental nephritis models [63]. Renal DC isolated from FLT3-L mobilised mice are immature, poorly allo-stimulatory and capable of inducing regulatory T-cells *in-vitro*. These DC can prolong allogeneic graft survival *in-vivo* [61], reflecting their tolerogenic potential, although this potential is lost upon maturation. They may therefore also play a role in the maintenance of tolerance to renal self-antigens, and tolerise auto-reactive T-cells that have escaped thymic deletion [59]. Renal DC may be valuable targets for therapeutic interventions for the treatment of rejection and other nephro-pathological processes.

Other Tissue DC

- *Skin*: Human skin contains several DC subsets that have been studied: Langerhans cells (LC), dermal DC and plasmacytoid DC. As in mice, LC are recognised as a separate DC subset found in human epidermis. They express langerin (CD207) found within Birbeck granules and CD1a, as well as other markers found on myeloid DC. In addition to GM-CSF, TGF-β is an important cytokine in LC development from precursors located within the epidermis. Dermal DC do not express langerin, but share many features with monocyte-derived DC (see below). Plasmacytoid DC have been found only in low numbers in healthy human skin [69]. DC are recruited into skin in pathological conditions such as skin malignancy, atopic dermatitis, connective tissue disease and other cutaneous inflammatory disorders. However, the type of DC recruited varies depending n the disorder – for example, LC are increased in atopic dermatitis whereas PDC are preferentially recruited in systemic lupus erythematosus (SLE) [69].
- *Liver*: Hepatic DC play an important role in many liver diseases, as well as liver transplantation where they represent a fascinating example of the tolerogenic potential of DC. Murine liver contains both MDC and PDC subsets, and it has been shown in rhesus monkeys that liver DC can be mobilised with FLT3-L administration [39]. Human DC remain poorly characterised, but DC migration studies from biopsies of normal livers reveal a predominantly myeloid DC population with immuno-regulatory properties including immature or semi-mature phenotype, low allo-stimulatory ability, and promotion of IL-10 production rather than IL-12p70 [70]. This may in part explain the apparently lower immunogenicity of liver transplants, and observed cases of tolerance in this setting. In disease states such as chronic viral hepatitis, activated and mature MDC and particularly PDC are recruited into the liver. These DC may have impaired function and

anti-viral effect [71], although their exact role in disease pathogenesis is unclear.

- *Thymus*: Thymic DC most likely arise from local thymic precursors, are located within the medulla and constitute a tiny percentage of the thymic cell population. Thymic myeloid DC are either $CD11c^{+}CD11b^{-}$ or $CD11c^{+}CD11b^{+}$. A smaller plasmacytoid DC population is also present, and these have typical PDC phenotype.

- *Spleen and Lymph Nodes*: Until recently splenic DC have remained poorly described in humans, presumably due to lack of tissue availability. A recent study utilising spleen tissue from deceased organ donors has enabled detailed characterisation of human splenic DC subsets [72]. DC were identified in the sub-capsular, T-cell rich regions and B-cell follicles of the spleen. Using several established surface molecule phenotypes to identify subsets, less than 3 % of spleen cells were MDC and less than 0.5 % were PDC. Interestingly, these estimates were higher when subsets were defined by expression of BDCA molecules. Both immature and mature ($CD83^{+}$) MDC were identified in normal spleen. Splenic DC may differentiate in response to danger signals such as bacterial infection, as suggested in one study where MDC and PDC markers such as MHC-Class II, CD83 and CD123 developed in DC-enriched splenic cell populations after stimulation with bacteria [73]. Human lymph nodes contain both MDC and PDC that have migrated to T-cell rich areas in the parafollicular cortex. Follicular and germinal centre 'DC' in B-cell follicles are not of haematopoietic origin, and are vital to B-cell responses and memory, as opposed to possessing true DC functions.

2.3.3 In-vitro Propagated DC

Due to the rarity of DC *in-vivo*, an array of culture systems for generating DC *in-vitro* from murine, human and primate precursor cells have been developed. The limitation of these

studies is that these cultured DC do not always replicate DC counterparts *in-vivo*, and the *in-vitro* manipulations themselves possibly disturb normal DC function. However, despite these limitations they have become essential to the study of DC.

Monocyte-derived DC (MoDC)

Differentiation of DC from monocytes in peripheral blood and tissues is an important defence against pathogens. Under the influence of GM-CSF, monocytes may be rapidly recruited to sites of inflammation and injury and differentiated to 'inflammatory' DC [12]. The contribution of monocytes to steady-state DC development is less clear. Both human ($CD14^+$) and murine (Ly6c or $Gr\text{-}1^+$) monocytes can be differentiated to MoDC *in-vitro,* most commonly with protocols based on GM-CSF and interleukin (IL)-4, or less commonly IL-13 [74-77]. GM-CSF plays a critical role in DC development as discussed previously, and is the essential component to *in-vitro* myeloid DC generation. IL-4 and IL-13 share the IL-4 receptor α which signals via signal transducer and activation of transcription 6 (STAT-6) variant Y641F [78,79]. Their presence in the culture inhibits macrophage development by inhibiting M-CSF. Data regarding the interchangeable functions of IL-4 and IL-13 has been conflicting, with reports of functional equivalence [75,80], superior DC differentiation with IL-4 [81] or superior DC maturation with IL-13 [82].

The process of monocyte-to-DC differentiation typically takes 4-7 days, involves minimal cell proliferation, and produces immature MoDC which are characterised by low expression of MHC Class II and costimulatory molecules (CD40, CD80, CD86), and some reduction in CD14 expression[76]. MoDC also express DC-SIGN at high levels [58], and may express BDCA-4/Neuropilin-1 and CD1a. MoDC have high migration and antigen-uptake ability. Additional

stimuli are required to produce mature DC in culture; again this occurs without cellular proliferation. Published protocols have reported superior or definitive maturation of MoDC with a wide variety of agents including lipopolysaccharide, TNF-α, IFN-γ, CD40 ligand (CD40L), prostaglandin E2, IL-6, IL-1β, as well as combinations of the above (pro-inflammatory cytokine cocktails) and monocyte-conditioned medium (which may contain variable quantities of TNF-α, IL1β, IL-6, IFN-α and potentially other unknown factors) [54,75,76,83]. DC maturation results in up-regulation of co-stimulatory molecule and MHC Class II expression, and expression of CD83 which is specific to mature DC [76]. Loss of CD14 expression is a recognised feature of mature MoDC, although persistence of CD14 has been observed in some human and non-human primate DC-like subsets (discussed in chapter 4). In addition to the phenotypic changes, monocytes alter function as they differentiate to immature then mature DC. The ability for antigen uptake (as measured by assays of endocytocis / phagocytosis) is markedly reduced in activated MoDC, and ability to induce T-cell proliferation and immune activation through cytokine secretion (e.g. IL-12p70) is increased [74-76].

It is not certain if protocols for MoDC generation *in-vitro* actually replicate the *in-vivo* pathway of myeloid, migratory or inflammatory cDC differentiation from monocytes, which also appears to be GM-CSF dependent [12]. One important issue is that the time-frame of most MoDC culture protocols (up to 1 week) is too lengthy to represent a physiologically relevant DC response. Protocols have been explored for more rapid DC propagation *in-vitro* to better replicate *in-vivo* inflammatory DC. 'FastDC' are functional, mature DC that are generated in short (48 hour) culture with IL-4 / GM-CSF and a pro-inflammatory cytokine cocktail, and have similar morphology and function to standard MoDC [84]. Short term (24 hour) culture in

GM-CSF, followed by calcium signalling with calcium ionophore (calcimycin A23187), has also been shown to produce rapid monocyte conversion to mature DC, although interestingly these DC do not produce IL-12 and skew towards Th2 responses [85]. It is unclear if rapid DC generation is more representative of what occurs physiologically.

Importantly, freshly isolated human blood CD11c^{+} myeloid DC and *in-vitro* cultured MoDC exhibit important differences when directly compared [54]. There is differential expression of DC-specific markers DC-SIGN (expressed on MoDC) and CD123 (expressed on blood MDC after culture). Blood MDC have fewer dendrites, a lobulated nucleus, and less endoplasmic reticulum compared with MoDC, which typically are larger, with an eccentric round nucleus and have more dendrites particularly after activation. Blood MDC are less stimulatory in MLR than MoDC, but generate more IFN-γ producing Th1 effector cells and polarize antigen-specific T-cell responses towards Th1 effectors. Nonetheless, despite the lack of direct *in-vivo* DC correlation, *in-vitro* generated MoDC remain a powerful tool for therapeutic DC research, as large numbers of DC may be readily generated from this relatively common precursor cell available in peripheral blood.

DC Derived from Haematopoietic Precursors

DC can also be generated *in-vitro* from murine or human bone-marrow or blood haematopoietic progenitor cells. These substrates contain not only CD34^{+} stem cells, but various other progenitors at different stages of commitment that can also give rise to DC under the right conditions. Murine DC are most commonly propagated in this way from bone marrow cell suspensions. Multiple protocols are described in mice, humans and NHP for expanding DC precursors from stem cells using combinations of cytokines critical to early bone-marrow DC development, including GM-CSF, stem cell factor (SCF; c-kit ligand),

FLT3-L, TNF-α, and thrombopoietin (TPO) [32,53,86-89]. In particular, FLT3-L based bone marrow cultures are capable of producing DC that are nearly identical to splenic DC subsets in mice [12,32], again indicating the specific importance of this cytokine to DC genesis. In humans, further culture with GM-CSF, IL-4 and TNF-α produces DC that are similar to Langerhans DC (expressing langerin) and interstitial DC (expressing either CD1a or CD14, but not both) [22,88]. FLT3-L and TPO without other cytokines also enables the expansion of human plasmacytoid DC in long-term culture [90].

<u>*DC From Growth Factor-Mobilised Precursors*</u>

Haematopoietic growth factors such as G-CSF and GM-CSF, when administered *in-vivo*, lead to the mobilisation of significant populations of bone-marrow derived DC precursor cells (including monocytes) into peripheral blood. In one study of human G-CSF mobilised donors, 25 % of apheresis product leukocytes were $CD14^+$ monocytes, equating to tens of millions of monocytes per ml of product [91]. Human G-CSF-mobilised monocytes and stem cells can be enriched by leukopheresis (± antibody labelling and cell separation methods), and differentiated *in-vitro* into immature and mature myeloid and plasmacytoid DC in sufficiently large numbers for therapeutic use, with excellent viability and quality [75,87,90,92,93]. G-CSF mobilisation prior to blood sampling can increase final MoDC yield by up to six-fold in human subjects [75]. The magnitude of DC precursors mobilisation induced by G-CSF is significantly enhanced when combined with GM-CSF in human subjects [93]. Similarly, the combination of FLT3-L and G-CSF gives much higher progenitor colony-forming unit and $CD34^+$ cell yields compared to either cytokine used alone in baboons or macaques, and these progenitors continue to proliferate *in-vitro* even in the absence of these growth factors [94].

Both G-CSF and FLT3-L are also useful for *in-vivo* mobilisation of pre-DC, which can then be

harvested from peripheral blood and organs. FLT3-L markedly increases both the functional pre-PDC and pre-MDC populations in humans and mice[35,41,43], whereas G-CSF preferentially mobilises CD11c^{-} plasmacytoid DC or DC2 [43,44]. This is discussed further below and in Chapters 5 and 6.

The use of growth factor mobilisation is also well established in non-human primate models, particularly rhesus macaques. G-CSF, SCF, GM-CSF, FLT3-L, Progenipoietin (ProGP) and the IL-3/G-CSF chimeric molecule myelopoetin have all been used to mobilise primate stem cells, DC precursors or *in-vivo* DC which can potentially be harvested by leukopheresis in larger primate species [36,94-99]. This is explored further in chapters 4 and 5.

G-CSF treatment has effects on the immune system other than promotion of precursors and DC mobilisation, including altered expression of many immunologically relevant genes [100], altered cytokine and chemokine expression and skewing of Th1/Th2 responses[44]. Monocytes from human G-CSF-mobilised donors have been found to have poorer antigen-presentation and response to LPS compared with unmobilised monocytes or monocytes cultured *in-vitro* with G-CSF [101]. Importantly however, G-CSF treatment does not appear to affect the ability to produce standard MoDC *in-vitro* from these precursor cells. Direct comparison reveals that DC generated from G-CSF-mobilised monocytes have similar phenotypic and functional properties compared with steady-state MoDC [75,102]. This remains the case even if monocytes have been subjected to the stress of cryo-preservation and thawing prior to DC differentiation [75,92]. This has important relevance for clinical DC immunotherapy, as it may enable large quantities of donor or recipient DC precursors to be frozen and stored for future use long after transplantation has occurred.

3. DENDRITIC CELLS IN TRANSPLANTATION AND TOLERANCE

3.1 DC and Allo-recognition

The interaction of the recipient and donor immune systems is multi-faceted and bi-directional, involving three distinct but co-existent pathways of allo-recognition, which are central to both immune sensitization *and* tolerogenic immune responses following transplantation [103].

The direct pathway of allo-recognition first described by Larsen and Morris[104] involves "passenger leukocytes" including donor APC (most potently, DC) migrating from the graft and directly presenting donor antigen loaded onto donor MHC molecules to recipient lymphoid tissues. This process is critical to the initiation of allograft rejection, but may also be important to the development of donor-specific tolerance, particularly as it is known that DC of donor origin may persist long-term in recipient tissues (micro-chimerism). The indirect pathway involves recipient migratory DC capturing, processing and presenting donor antigen peptides on self-MHC to antigen-specific recipient T-cells. This process may be additionally stimulated in the context of tissue injury such as surgical trauma, inflammation and post-transplant ischaemia reperfusion injury, but also occurs continuously throughout the life of the graft as recipient DC traffic through the transplant. Indirect allo-recognition therefore sustains anti-donor immunity and contributes to chronic allograft nephropathy / chronic rejection, but also promotes regulatory T-cell responses [103]. The concept of a third, semi-direct pathway proposes the exchange of MHC / antigens between donor and recipient DC, T-cells and endothelium through direct cell contact, cytoplasmic exosomes or vesicles, thereby linking the direct and indirect pathways and enabling recipient DC to present whole donor antigen / MHC complexes directly [105]. In addition, through this mechanism T-cells acquiring MHC

from DC can present donor antigen to other allo-specific T-cells, inducing anergy or apoptosis [106], adding another layer of complexity to mechanisms of tolerance induction. DC clearly play a pivotal role in these complex processes, reinforcing their importance in skewing immune responses, and justifying their utility as a therapeutic target for tolerance induction.

3.2 DC and Tolerance Induction

DC play a critical role in naturally-occurring central and peripheral tolerance to self antigens. In the thymus, DC promote central tolerance through negative selection where clonal auto-reactive thymocytes are deleted by apoptosis. In the steady state, peripheral DC acquire free self-antigen from serum or antigen from apoptotic cells. DC encountering auto-reactive T-cells that have escaped thymic selection cause these cells to undergo anergy, apoptosis, immune deviation towards Th2 cytokines (particularly IL-10 production), or trigger the generation of Treg[6,10,46,107]. This regulatory or inhibitory outcome can be induced by DC that fail to deliver adequate signals for T-cell activation, through lack of co-stimulatory signals, promotion of inhibitory signals, or activation of death-inducing pathways such as Fas (CD95) / Fas Ligand (FasL), TNF/TNF receptor family members and programmed death 1 ligand (PDL1). Several approaches exist for harnessing these antigen-specific inhibitory immune responses in the transplant setting, including using *in-vitro* manipulated tolerogenic DC, delivering allo-peptide to recipient DC *in-situ* and using DC to expand regulatory T-cell populations *ex-vivo.*

3.2.1 Generation of Tolerogenic DC

It has been well-established by numerous *in-vitro* studies that stimulation with a wide variety of tolerogenic DC can induce antigen-specific T-cell apoptosis or hyporesponsiveness [108-114]

and promote the expansion of antigen-specific regulatory T-cell populations[61,115-119]. *In-vitro* generated tolerogenic DC for therapeutic use, as summarised by Morelli and Thomson [6]:

1) should be capable of APC function but have low MHC molecule expression, leading to weaker signal 1;

2) are maturation-resistant immature DC with low co-stimulatory signals compared with inhibitory signals, leading to failure of signal 2;

3) are skewed away from Th1 promoting cytokines (especially IL-12p70) and favour IL-10 and indoleamine 2,3-dioxygenase production;

4) promote regulatory T-cell generation or expansion and apoptosis of antigen-specific effector T-cells;

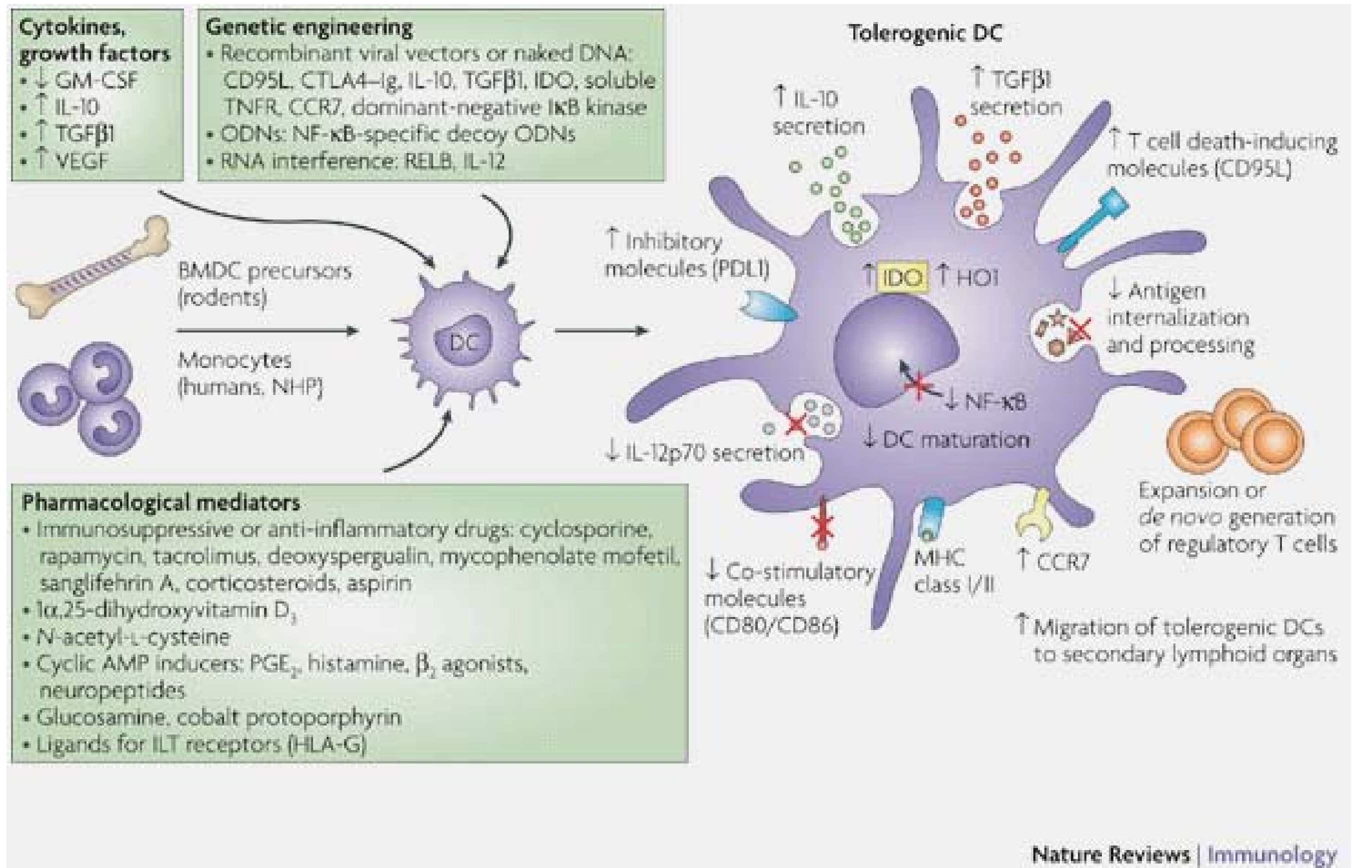
5) should be able to migrate from the site of introduction to secondary lymphoid tissue, but remain resistant to maturation *in-vivo* or NK / T-cell induced death during this journey.

DC with tolerogenic features are not restricted to a specific DC subset, stage of maturation or source of origin, and hence a wide variety of DC types have been explored for pre-transplant immunotherapy.

Stimulation with unmodified, *in-vitro* cultured immature DC alone has been shown to have immunosuppressive outcomes, suppressing mixed lymphocyte culture responses *in-vitro*, and inducing regulatory T-cells that exhibit Th2 cytokine responses (particularly IL-10) [109,120,121]. A highly significant study by Dhodapkar *et al.* reported that basic autologous immature human monocyte-derived DC pulsed with antigen produce antigen-specific effector T-cell inhibition and generate IL-10 producing $CD8^+$ T-cells *in-vivo* in human recipients [120]. In mice, basic immature myeloid DC modestly prolong allograft survival [111]. However, such DC are not capable of producing the enduring inhibitory immune responses which are requisite for stable transplant tolerance.

The tolerogenic capacity of DC may be enhanced through modification of culture conditions or gene expression. These strategies cause DC maturation arrest (via NFkB / Rel B pathways), down-regulation of co-stimulatory molecules (CD80 / CD86, CD40, OX40L), up-regulation of inhibitory molecules (PDL1 or B7-H1), suppression of stimulatory cytokine production (IL-12p70, IFN-γ) and enhancement of inhibitory cytokines (IL-10), or expansion of regulatory T-cells. The vast array of methods for manipulating DC to promote tolerance are summarised in Figure 1.

Figure 1. Mechanisms for Generation of Tolerogenic DCs *In-vitro*.



CCR7, CC-chemokine receptor 7; CTLA4–Ig, cytotoxic T-lymphocyte antigen 4–immunoglobulin fusion protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; HO1, haem oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IκB, inhibitor of NF-κB; IL, interleukin; ILT, immunoglobulin-like transcript; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; PDL1, programmed cell death ligand 1; PGE_2, prostaglandin E_2; TGFβ1, transforming growth factor-β1; TNFR, tumour-necrosis factor receptor; VEGF, vascular endothelial growth factor. Reprinted by permission from Macmillan Publishers Ltd from Morelli and Thomson 2007 [6].

Generation of Tolerogenic DC - Culture Conditions

The cytokine environment in which DC are generated influences DC function and may skew towards immune hyporesponsiveness. Generation of myeloid DC in culture with low-dose GM-CSF and/or in the absence of IL-4 results in immature or maturation-resistant DC that have T-cell inhibitory functions [112,122,123], and may prolong allograft survival (see Table 1). Interestingly, the addition of LPS to GM-CSF at the commencement of DC cultures, (as opposed to later as a maturation stimulus), leads to the generation of immature DC which promote CD4+ T-cell anergy [122]. These cells have not been tested in a transplant model.

Both IL-10 and TGF-β are critically important cytokines involved in inhibitory or regulatory immune responses, and are produced by both DC and regulatory T-cells. DC treated with IL-10 remain immature, with suppressed co-stimulatory molecule expression, and reduced T-cell stimulatory ability [124]. Priming T-cells with IL-10 treated DC leads to antigen-specific anergic effects [124-126], which appear to be mediated by cell cycle arrest due to IL-2 dependent proliferation inhibitors [125]. TGF-β has a similar effect on preventing DC maturation and allostimulatory function [127]. Repetitive monthly injection of rat IL-10 or TGF-β-treated DC over 18 months non-specifically suppresses antigen-stimulated proliferation, and importantly does not provoke malignancy [128]. The combination of IL-10 and TGF-β in culture produces "alternatively activated" DC that possess all the hallmark features of tolerogenic DC *in-vitro*[129]. DC treated with these cytokines have been shown to significantly prolong allograft survival of murine cardiac transplants, in combination with co-stimulation blockade [127,129] (see Table 1).

Generation of Tolerogenic DC - Drug Exposure

A wide array of known immunosuppressants and other drugs have been identified as having important influences on the generation, migration, maturation and immune function of DC Exposing DC to these agents can promote their tolerogenicity.

- *Corticosteroids*: Corticosteroid drugs such as prednisolone and dexamethasone have widespread immunosuppressive effects involving multiple immune pathways and cellular endpoints. They are known to interfere with the differentiation and maturation of DC from precursors[130,131]. Cortisol produced by the adrenals under stress situations has direct effects on T-cell function, but also interferes with the LPS-stimulated maturation of DC, with phenotypic and functional consequences including loss of ability to prime naïve T-cells [132]. Glucocorticoids down-regulate pro-inflammatory cytokine gene transcription (IL-1, IL-6 and TNF-α), suppress IL-12p70 production and increase IL-10 production from CD40-L stimulated DC [133]. Dexamethasone has been shown to reduce overall myeloid and plasmacytoid DC numbers in murine liver and lymphoid tissues [131]. Treatment of DC with dexamethasone renders them tolerogenic and capable of inducing T-cells to become anergic to indirectly presented donor antigen, resulting in prolonged graft survival when administered with a brief course of calcineurin inhibitor[134].
- *Vitamin D*: Vitamin D3 (or its active form, 1α, 25-dihydroxy Vitamin D3 or calcitriol) is a steroid hormone involved in calcium and phosphate homeostasis. However, it has additional effects on immune regulation via actions on DC and T-cells, and may minimise allograft rejection through these pathways. Vitamin D3 treatment of typical monocyte-derived DC leads to impaired differentiation and maturation, poor costimulatory and immuno-stimulatory function leading to T-cell hyporesponsiveness, despite to addition of bacterial lipopolysaccharide to mature DC [135]. Similar effects are observed when DC are treated with both dexamethasone and Vitamin D3 [136]. In a murine islet transplant model,

treatment with Vitamin D3 and mycophenolate mofetil has been shown to produce long-lived islet survival and acceptance of subsequent donor (but not third party) heart allografts [137]. This was associated with the identification of *in-vivo* DC that exhibit a tolerogenic, immature phenotype and regulatory T-cells that promote transplant tolerance when adoptively transferred.

- *Calcineurin Inhibitors:* Cyclosporine and tacrolimus are calcineurin inhibitors which reduce antigen-induced T-cell signalling, IL-2 dependent T-cell proliferation and T-cell activation. They exert inhibitory effects on DC maturation, allostimulatory function, and possibly reduction of co-stimulation molecule expression [138-141]. Calcineurin-treated DC have reduced IFN-γ, TNF-α and IL-12 production, but retain IL-10 secretion [139,140]. Cyclosporine prevents NF-κB-mediated DC maturation when DC are cultured in GM-CSF and TGF-β in the absence of IL-4[142]. Tacrolimus also has been shown to maintain myeloid DC in an immature state [138]. Cyclosporine has been used as an adjuvant immunosuppressant in small animal models of successful DC-based immunotherapy[118,129,134,143] (Tables 1 and 2), where it may act by preventing *in-vivo* DC maturation.
- *Rapamycin:* The receptor for rapamycin is FK506-binding protein 12. This drug-receptor complex inhibits mammalian target of rapamycin (mTOR) protein signalling, primarily affecting IL-2 mediated T-cell proliferation and signalling, but also influencing DC longevity and maturation. Interestingly, the mTOR pathway is essential to GM-CSF-mediated maintenance of DC populations, and mTOR inhibition promotes DC apoptosis [144], which may contribute to abrogation of immune stimulatory signals from DC post-transplantation. DC conditioned with rapamycin are maturation-resistant, have impaired antigen-uptake, produce low levels of IL-12, and produce the expansion of antigen-specific $FOXP3^+$ regulatory T-cells while suppressing effector T-cell function [116,145].

Several small animal transplant studies have now demonstrated that recipient-derived, rapamycin-treated DC (expressing alloantigen through exposure to donor cell lysate) facilitate long-term graft survival (see Table 2) [116,143,146]. This outcome occurs in conjunction with expansion of and graft infiltration by FOXP3$^+$ Treg [116,143].

- *Other drugs:* Mycophenolate mofetil, aspirin, prostaglandin E_2, glucosamine, N-acetyl-cysteine, and histamine have all been used to manipulate DC function *in-vitro* [6]. 15-Deoxyspergualin (DSG) and its analogue LF15-0195 are antibiotic derivatives with significant inhibitory effect on NF-kB / Rel B mediated DC maturation [147], and have been tested in tolerance protocols in non-human primates (discussed below).

Generation of Tolerogenic DC - Genetic Modification

Diverse pathways have been targeted by manipulation of DC gene expression using techniques for DNA delivery or RNA interference, generating DC with immunosuppressive functions. Genes for immuno-modulating cytokines and molcules are an obvious target for manipulation to promote tolerance.

- DC transduced to express high levels of IL-10 gene and product can stimulate regulatory T-cell expansion and enhance T-cell IL-10 secretion [117] , as well as abrogate rejection in a humanised immunodeficient mouse skin transplant model [148]. Genetic over-expression of both IL-10 and TGF-β genes in donor myeloid DC leads to maximal prolongation of kidney graft survival in DC-treated recipients, mediated by impaired effector T-cell responses[149] and enhanced Th2 cytokine skewing[150]. Blockade of TNF-α expression by immature DC virally transduced to express soluble TNF-alpha receptor type I has been shown to prevent DC maturation and antigen presentation[151]. Subsequent tolerogenic effects include T-cell anergy and development of regulatory T-cells producing high levels of IL-10, as well as prolonged cardiac transplant survival.

- Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme involved in tryptophan metabolism, generating protein metabolites such as kynurenine that have immunosuppressive functions [152]. IDO is expressed constitutively in some DC subsets, and can be induced in other DC subsets by CTLA-4-Ig (discussed below) [153]. Increasing IDO gene expression and the presence of IDO positive “regulatory” DC are both associated with renal allograft acceptance [154]. Human and murine *in-vitro* generated myeloid DC transduced with adenoviral vectors encoding IDO gene are capable of producing high levels of IDO *in-vitro*, causing impaired T-cell proliferation and responses[152,155], and proliferation of $CD4^+CD25^+FOXP3^+$ T cells[156] . Conditioning with donor IDO-transduced bone-marrow derived murine DC leads to longer murine skin allograft survival and reduced graft inflammatory infiltrates, an effect which is not observed with third party grafts [156].
- Cytotoxic T Lymphocyte associated antigen-4 (CTLA-4; CD152) is a member of the immunoglobulin family and is expressed by T-cells, particularly Treg subsets [157]. Along with CD28, it acts as the receptor for CD80 / 86 (B7 molecules) on DC and other APC, but it has a higher affinity for these molecules and unlike CD28 it imparts an inhibitory signal to T-cells when activated, inducing anergy. CTLA-4 immunoglobulin (CTLA-4-Ig) is an immunosuppressive fusion protein of CTLA-4 and immunoglobulin that acts by blocking CD28 stimulation by CD80 / 86, thereby reducing T-cell activation. DC exposed to CTLA-4-Ig in soluble form are induced to produce IDO, which in turn inhibits clonal T-cell expansion[153]. Soluble CTLA-4-Ig has been used for tolerance induction in murine and primate transplant models (discussed below). Alternatively, myeloid DC transfected with CTLA4-Ig gene constructs produce T-cell hyporesponsiveness and inhibit IDO up-regulation[113], and enhance transplant survival [158].

- Other genes that have been shown to promote inhibitory responses when manipulated in DC include Fas Ligand (CD95)[159] and TNF-related apoptosis-inducing ligand (TRAIL)[160], although these have not been tested in transplant models. Additionally, blockade of NF-κB pathways by "decoy" oligodeoxyribonucleotides (ODN) [161] or gene transfer of the inhibitor IκB kinase 2 (IKK2)[162], leads to maturation arrest of DC and impairment of subsequent T-cell activation. Conditioning with donor DC modified by both NF-κB ODN and CTLA-4-Ig transfection can produce indefinite allograft survival[161].

3.2.3 Recipient Pre-conditioning with Tolerogenic DC

Small animal transplant studies of recipient pre-conditioning with DC of various origin and phenotype, with or without adjuvant therapy to enhance tolerogenicity, have established the significant potential of DC conditioning by reporting indefinite vascularised allograft survival. Tables 1 and 2 summarise a selection of key papers offering persuasive evidence that both donor and recipient DC infusion pre-transplant can modify donor-specific immunity. The translation of these promising findings to human and primate models has been very limited. In a seminal study of human subjects given a subcutaneous injection of two million autologous DC pulsed with influenza antigen, administration of immature DC led to short term (up to 30 days) antigen-specific effector T-cell dysfunction, and expansion of IL-10 producing CD8+ T-cells whereas mature DC primed immunity [120]. More recently, the effect of stably immature DC modified with vitamin D3 and IL-10, with and without CTLA4-Ig, has been assessed in rhesus macaque recipients [97], but has not yet been tested in a primate transplant model.

The optimal DC subset, phenotype, manipulation and adjunctive treatment for inducing tolerance remains to be determined. Strategies for controlling fate of injected DC require

elucidation, particularly mechanisms for the prevention of *in-vivo* maturation and sensitization of the recipient, avoidance of destruction of DC by NK cells, and enhancement of injected DC homing to lymphoid rather than non-lymphoid tissues. These important issues remain to be resolved in non-human primate models before DC–based therapy can attain clinical application.

Table 1. Summary Of Selected Small Animal Studies Establishing The Benefit Of Donor DC Pre-Treatment On Allograft Survival, Highlighting Different DC Treatments And Protocols. Adapted from Morelli and Thomson, 2007 [6] with additions and modifications. All DC were allogeneic and administered systemically. See Table 2 for definition of abbreviations.

REFERENCE	MODEL	DC TYPE (DONOR)	DC TREATMENT	RECIPIENT TREATMENT	MST (DAYS)
Rastinelli 1995 [163]	Mouse islet	Liver DC	GM-CSF	STZ	30.3 (20 % > 60)
Fu 1996 [111]	Mouse HHT	BM DC precursors[109]	GM-CSF	-	22 [a]
Lu 1997[127]	Mouse HHT	BM iDC	GM-CSF TGF-β	Anti-CD40L	77 (40 % > 100)
Gao 1999 [164]	Mouse HHT	Spleen DC	Long-term Allo-MLC	Anti-CD4	35 25 % > 120
Lutz 2000 [112]	Mouse HHT	BM iDC	GM-CSF Low dose	-	> 100 [b]
Niimi 2001 [165]	Mouse HHT	Spleen mDC	-	Anti-CD40L	> 100
O'Connell 2002 [166]	Mouse HHT	splenic $CD8\alpha^+$mDC	GM-CSF overnight	-	35
Bonham 2002 [161]	Mouse HHT	BM iDC	NF-κB ODN and Ad transfection CTLA4-Ig	-	>100 (40 %)
DePaz 2003 [123]	Rat HHT	BM iDC	GM-CSF IL-4	ALS	>200 (50 %)
Sun 2003 [167]	Mouse HHT	BM iDC	Ad transfection CTLA4-Ig	Anti-CD40L	>100 (50 %)
Coates 2004 [61]	Mouse HHT	FLT3-L mobilised renal DC	Freshly sorted $CD11c^+$ DC	-	19 [c]
Abe 2005 [110]	Mouse HHT	BM pre-PDC	FLT3-L	-	22 [d]
Bjorck 2005 [168]	Mouse HHT	*In-vivo* FLT3-L mobilised Splenic PDC	Freshly sorted $CD11c^+$ PDC	Anti-CD40L	68 (50 % > 100)
Garrod 2006 [169]	Mouse HHT	BM DC	Ad transfection IL-10 and CCR7	-	>100
Lan 2006 [129]	Mouse HHT	BM "AA" DC	GM-CSF, IL-10, TGF-β, LPS	CTLA4-Ig CyA (d 0-9)	> 100
Wang 2006 [151]	Mouse HHT	BM iDC	Ad transfection sTNFRI	-	>100 (50%)

[a] The first study to test immature, co-stimulatory molecule deficient, myeloid *in-vitro* propagated donor DC on allograft survival. Donor BM DC alone, infused one week before transplant, produced only modest prolongation of MST compared with controls and 3rd party BM DC.
[b] Only DC infused 7 days pre-transplant had this effect; DC infused at earlier and later time points were ineffective.
[c] Renal DC from donor animals also prolonged allograft survival of third party grafts (MST 16 days).
[d] MST was also prolonged by donor BM myeloid DC and third party BM pre-PDC.

Table 2. Summary Of Selected Small Animal Studies Establishing The Benefit Of Recipient DC Pre-Treatment On Allograft Survival. Adapted from Morelli and Thomson, 2007 [6] with additions and modifications.

REFERENCE	MODEL	DC TYPE (RECIPIENT)	DC TREATMENT	RECIPIENT TREATMENT	MST (DAYS)
Garrovillo 1999 [170]	Rat HHT	BM iDC (intrathymic)	GM-CSF, IL-4 Donor MHC Class I peptide	ALS	>150 [a]
Garrovillo 2001 [171]	Rat HHT	Thymic DC (iv)	Donor MHC Class I peptide	ALS	> 200 [a]
Mirenda 2004 [134]	Rat Kidney	BM iDC [b]	dexamethasone	CTLA4-Ig (x1) CyA (d 0-10)	> 100
Taner 2005 [146]	Mouse HHT	BM iDC (x3)	GM-CSF, IL-4 Rapamycin, Donor Splenic Lysate	-	>59 (40% > 100) [c]
Peche 2005 [172]	Rat HHT	BM iDC (adherent,x1)	Low dose GM-CSF, IL-4	-	22.5 [d] (20 % > 100)
Beriou 2005 [173]	Rat HHT	BM iDC (> x1)	GM-CSF, IL-4	LF15-0195	>100 (92 % > 100)
Turnquist 2007 [116]	Mouse HHT	BM iDC (x1)	GM-CSF, IL-4 Rapamycin, Donor Splenic Lysate	Rapamycin (d 0-9)	> 100
Horibe 2008 [143]	Rat Skin	BM iDC (x2)	GM-CSF, IL-4 Rapamycin, Donor Splenic Lysate	ALS CyA (d 0-20)	> 113 (50 % > 180)
Kuo 2008 [118]	Rat Hindlimb	BM iDC (x 1)	GM-CSF, Donor Splenic Lysate	ALS CyA (d 0-20)	> 200

[a] Long-term survivors were challenged with a second allograft which was accepted while third party grafts were rejected without rejection of the primary heart graft.
[b] Tolerogenic recipient DC expressed both recipient and donor MHC molecules to induce T-cell regulation via the indirect pathway.
[c] Three doses of DC pulsed with allo-antigen and rapamycin; a single dose of such DC prolonged the MST to only 23.8 days, DC + tacrolimus prolonged the MST to 46.8 days.
[d] Syngeneic and donor allogeneic DC both prolonged allograft survival; only 2 /10 animals in the syngeneic DC group had ST > 100 days

Abbreviations: iDC – immature DC; mDC – mature DC; PDC – plasmacytoid DC; STZ – streptozotocin; HHT-heterotopic heart transplant; BM – bone marrow; MST – median allograft survival time; GM-CSF – granulocyte-macrophage colony stimulating factor; TGF-transforming growth factor; CD40L- CD40 ligand (CD154) monoclonal antibody; allo-MLC – allogeneic mixed lymphocyte culture; IL-4 – interleukin-4; ALS – anti-lymphocyte serum; Ad transfection – DC genetically modified by adenoviral transfection to over-express various genes; CTLA4-Ig - CTLA4-immunoglobulin; FLT3-L- fms-like tyrosine kinase 3 Ligand; IL-10 – interleukin 10; CCR7 – chemokine receptor 7; AA – alternatively activated; CyA – cyclosporine A; Allo-Ag – DC pulsed with allo-antigen; d 0-9 – drug give on days 0-9 post-transplant then ceased; sTNFRI – soluble TNF receptor type I; LF15-0195 – analogue of deoxyspergualin

3.2.4 Targeting Recipient DC In-vivo

In-vivo recipient steady-state DC may be modified to become tolerogenic to donor antigen through a variety of processes. Monoclonal antibodies targeting DC-specific markers such as CD205 (DEC-205) can be used to deliver short alloantigen peptides to quiescent DC which in turn can render reactive T-cells unresponsive [108]. Similarly, MHC-rich exosomes secreted from donor APC (in particular, from DC) also deliver donor antigens which are internalised by DC and presented indirectly. When combined with DC-maturation arrest via deoxyspergualin analogue-mediated NF-κB inhibition, DC exosomes prolong rat cardiac allograft survival [174]. The ability to target DC while minimising DC activation is critical to obtaining a T-cell inhibitory response, therefore adjunctive treatments such as NF-κB or co-stimulation blockade may be necessary.

Recipient DC can also be targeted by systemic administration of apoptotic donor cells. In the steady state, DC capture apoptotic cells (mediated by DEC205), leading to failure of DC maturation due to inhibition of NF-κB pathways [175], subsequent impairment of antigen-specific T-cell activation and proliferative functions, and ultimately T-cell death [176]. This negative effect on immune reactivity induced by donor apoptotic cells translates to prolonged allograft survival of murine heart transplants, particularly when combined with co-stimulation blockade [176].

3.2.5 DC-induced Regulatory T-cell Generation

Regulatory T-cells play a critical role in the induction and maintenance of tolerance both to self and in the transplant setting, and the ability to expand antigen-specific Treg *in-vitro* or *in-vivo* is a hallmark feature of tolerogenic DC. In normal human spleen, DC and $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T-cell subsets are in close association anatomically [72]. It is

well-established that antigen-specific murine, cynomolgus macaque and human Treg can be expanded *ex-vivo* by stimulation with naturally-occurring or *in-vitro* generated antigen-bearing myeloid DC [176-182] and that these expanded T-cells may have superior suppressive functions compared with naturally-occurring Treg. The addition of IL-2 or TGF-β to cultures enhances the percentage of $FOXP3^+$ cells in the expanded T-cell population [179,180]. Plasmacytoid DC also promote the generation of IL-10 producing, $FOXP3^+$ Treg when activated by CpG or CD40-L [115,119]. In *ex-vivo* studies of tolerised and rejecting murine heart allografts, PDC have been shown to mediate tolerance by acquiring allo-antigen through phagocytosis, circulating in blood and migrating to secondary lymphoid organs where they are essential to the generation of $FOXP3^+$ Treg [49].

Importantly, a study of three human myeloma patients has uniquely observed that injection of mature autologous *in-vitro* derived DC enhances the $FOXP3^+$ Treg population *in-vivo* [181]. When adoptively transferred, donor DC-primed and expanded, recipient Treg populations have been shown to reverse rejection and prolong murine cardiac allograft survival [180] and skin graft survival [178]. This is therefore a further potentially useful strategy that harnesses the intrinsic ability of DC to promote immune tolerance.

3.2.6 In-vivo Expansion of DC

DC expanded *in-vivo* using growth factors (most commonly FLT3-L) have been shown to be both phenotypically and functionally immature, requiring additional stimuli to attain maturation *in-vitro* [36,42,43] or mature, promoting T-cell activation [35,183]. In keeping with these divergent findings, the outcomes of transplants performed using growth-factor mobilised donors or recipients, or infusion of growth factor mobilised donor DC into recipients have also

been mixed. Murine donor FLT3-L expanded bone marrow cells (FL-BM)[184] or spleen cells (FL-SC)[185] are capable of inducing significant chimerism when infused in allogeneic recipients, and exhibit tolerogenic potential *in-vitro* [184]. However, results of subsequent allografts into these recipients are conflicting. Donor FL-SC infusion produced long-term and repeated acceptance of MHC-mismatched skin transplants while donor FL-BM treated recipients display accelerated cardiac rejection. Similarly, DC-enriched organs (liver or heart) from FLT3-L treated donors are rapidly rejected due to an aggressive T-Cell and NK cell response, despite donor chimerism evident in recipient tissues [183,186]. In other studies, cardiac and bone marrow recipients treated with FLT3-L exhibit enhanced donor chimerism, however accelerated rejection still occurs in the absence of immunosuppression [187]. These studies suggest that enriching DC populations within donors or recipients by FLT3-L potentially enhances anti-donor immunity. Interestingly however, one study has reported that immature renal DC isolated from FLT3-L mobilised murine kidneys have tolerogenic potential including the ability to stimulate regulatory T-cells *in-vitro*, and prolong allograft survival when systemically infused prior to cardiac transplantation [61]. The outcome of transplanting kidneys enriched with potentially tolerogenic DC from FLT3-mobilised donors is unknown and requires further exploration.

4. NON-HUMAN PRIMATES IN TRANSPLANT TOLERANCE RESEARCH

(Note: Please see addendum on pg 12. References 232, 236, 237, 239, 240 and 241 have been withdrawn by the authors).

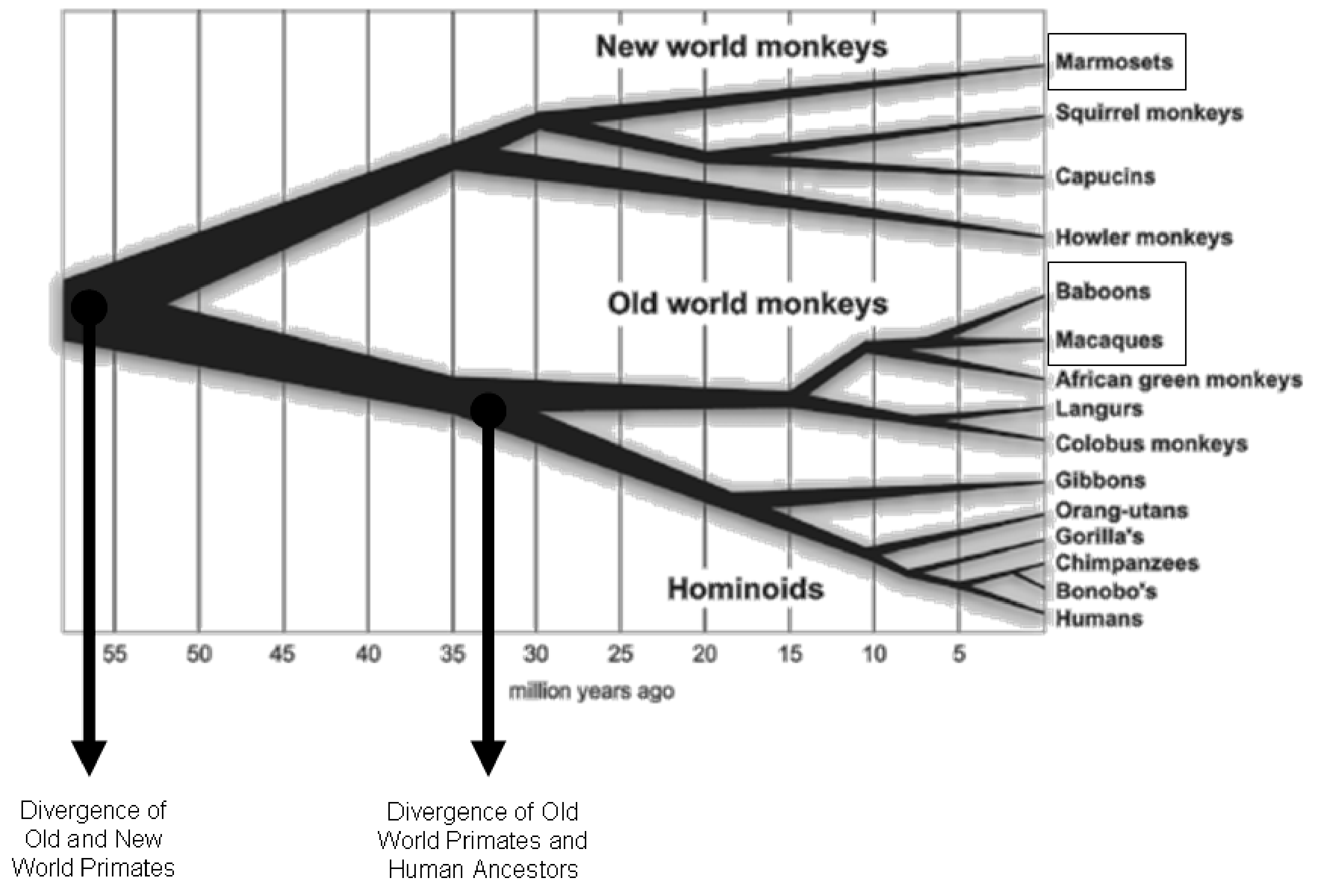
4.1 Introduction

Primates are a diverse mammalian order, with over 350 species. Their phylogenetic classification is based on similarities of morphology, genetics and geographical location, enabling grouping into Old and New World primates (Figure 2). NHP are essential models for biomedical research, and have played crucial roles in the understanding of infectious disease (including prion diseases, malaria and HIV) and autoimmunity, the development of vaccines and organ transplantation [188]. One of the stumbling blocks in advancing the field of organ transplantation and tolerance is that promising outcomes in small animal models often do not translate in the clinical setting. Lower order species have less complex immune systems and a limited immune history, and tolerance is therefore easier to achieve. Intermediate larger animal models (pigs, sheep and NHP) are a necessary step to confirm small animal experimental data. The similarity between NHP and humans is greater than between humans and rodent species, therefore the relevance and applicability of data generated in NHP models is much greater. NHP models are an essential component of research strategies in transplantation and the development of key new therapeutic agents and strategies [189-192].

Old World Primates (Figure 2) such as baboons, rhesus macaques and cynomolgus monkeys are the most commonly utilised animals in transplantation research. Renal, islet and other transplantation procedures are well established in these models, and they share significant biological, immunological and genetic similarities with humans. In particular, their responses

to a large range of diseases, injury and pathogens mimic that observed in humans, enabling study of disease pathogenesis that would not be possible in human subjects. However, due to strict regulation and ethical issues, restricted availability, highly specialised requirements for animal housing and management and associated high financial costs, NHP experiments are infinitely more difficult to conduct. Consequently, less than 0.1 % of animal experiments in biomedical research are conducted on NHP species [188]. Specialised primate facilities are necessarily limited to a small number of research institutions around the world, although collaboration with research groups world-wide is encouraged. Due to the limitations and difficulties of housing, maintaining and utilising large NHP species, alternative species with lesser requirements and of more compact size would be a welcome addition to the array of models available for transplant research.

Figure 2. Simplified phylogenetic tree demonstrating the evolutionary distances and relationships between key non-human primate species used for biomedical research and humans. This tree demonstrates the divergence points of Old and New World Primate species and Hominoid species from which humans evolved.



4.2 The Common Marmoset: A Novel Transplant Model

The common marmoset (*Callithrix jacchus*; Primates suborder *Haplorrhini*; infraorder S*imiiformes*; parvorder *Platyrrhini*; family *Callitrichidae*) is a small arboreal New World Primate naturally found in South America. It is being developed as a feasible and relevant pre-clinical model for transplantation related research by our group. Marmosets retain important similarities to humans in terms of genetic homology, anatomy, disease profile and other biological features, while possessing many of the cost and maintenance advantages of small animal models[193]. Their small size (200-400 grams) promotes ease of animal husbandry, as they can be group housed in relatively small spaces, and do not require specialist animal handlers. Lesser quantities of research pharmaceuticals and other reagents are required compared with large animal models. Marmosets are very easy to breed, having a gestation period of just 144 days. They typically produce multiple (twin or triplet) births, and have no lactational anoestrous. Therefore marmosets are very highly fecund, especially compared to other primate species used in research. The ease of breeding ensures access to large numbers of marmosets without threatening species preservation, as has been the case for other NHP species.

Marmosets, being New World Primates, have an evolutionary distance from humans of over 55 million years, compared with 35 million years between humans and Old World Primates such as baboons and rhesus macaques, and 5 million years between humans and chimpanzees (see phylogenetic tree, Figure 2) [194]. However, considerable genetic similarity between humans and marmosets remains, with an average of 86 % homology between human and marmoset immunity-related proteins, compared to only 60 % (average) between human or marmoset and mice[195]. Homology of co-stimulatory molecules involved in DC-T-cell interactions has been found to be over 90 % [196]. Many human and primate-specific research

reagents such as cytokines and monoclonal antibodies against cell surface receptors are cross reactive with marmosets [197-201], reducing the need to develop species specific reagents in many cases. These features make the marmoset an attractive and relevant pre-clinical model.

Of great interest, this is the only primate species to exhibit naturally-occurring germ-line, somatic and peripheral blood chimerism [202-205], due to placental sharing of foetal cells between twins *in-utero*. Chimerism may have far-reaching consequences for the complex social behaviour observed within marmoset groups and families, because in some instances offspring receive genes arising from their parents' twin sibling, rather than their parent[204,206]. Cousins and uncles or aunts may be more genetically similar to these offspring than their own parents, affecting the usual concepts of relatedness, which may contribute to antagonism or co-operation between family members. From an immunological viewpoint, however, marmosets are a fascinating example of immune tolerance. Sex chromosome studies have confirmed that a large proportion (up to 80%) of peripheral blood lymphocytes in an individual animal may be derived from its co-twin [202,207,208]. This appears to lead to functional immune tolerance, as shown by lack of proliferative response in MLR from co-twin tamarin monkeys [209].

Marmosets are a well-established model in many areas of biomedical and behavioural science. Marmoset experimental autoimmune encephalitis is a robust model for studies of multiple sclerosis pathogenesis and therapy [210-213], due to the similarity of the inducible pathological changes, cytokine expression and response to novel treatments. The species has also been used to study Parkinson's disease[214], as well as in a multitude of neuro-developmental, neuro-cognitive, neuro-hormonal and neuro-behavioural studies. Other areas of research employing marmoset models include pharmacology and drug toxicity studies[215,216], hormonal release studies[217], bone disease[218,219], hypertension[220], immunology and gene therapy[200,221-223] and

fertility research[224,225]. Recently marmoset embryonic stem cell lines have been established[226,227] and the complete marmoset genome is being mapped, indicating their importance as a research model. Marmosets have not yet been used as a model for solid organ transplantation, and characterisation of their DC biology has been very limited[228].

Developing the marmoset for transplantation-related research involves setting up many components essential for a successful kidney (or other organ) transplant model. We have already established a broad range of techniques for assessing transplant-related and dendritic cell biology, including confirming cross-reactivity of many human or NHP specific reagents with marmoset samples[197], establishing normal ranges for key biochemical and haematological parameters, and assessing baseline marmoset renal histology. However, key methodologies, particularly for post-transplant immune monitoring still remain to be adapted to marmoset samples. In particular, techniques have to be modified to accommodate the small blood volume available from these animals (usually 1 ml or less). It is essential to establish reliable methods for donor and recipient selection that will enable choosing animals with maximal immunological disparity. This involves devising a method for accurate MHC genotyping in the marmoset and correlating this with *in-vitro* assays of alloreactivity. The DC biology of this species is thus far minimally described, and methods for large-scale DC production for therapeutic testing require development. Finally, the surgical aspect of renal transplantation in this model are also being finalised by the surgical component of our team. It is anticipated that this model will be sufficiently developed to proceed with transplantation studies in the very near future.

4.3 Transplant Tolerance Strategies Developed in NHP Models

(Note: Please see Addendum on pg 12. References 232, 236, 237, 239, 240 and 241 have been withdrawn by the authors).

Non-human primate models have played a critical role over the last decade in the development of several promising strategies for transplant tolerance induction that are approaching translation to the clinical setting. Although none of these protocols as yet have been shown to produce reliable, reproducible, robust and stable tolerance, they have provided great insight into mechanisms of tolerance and a basis for further studies. In particular, it is likely that dendritic cell-based tolerance strategies will be combined with one or more of the following approaches to maximize the probability of true operational tolerance developing.

4.3.1 T-cell Depletion

Depletion of T-cells at the time of transplantation is believed to assist in avoiding deleterious responses against the allograft at the peak time of immune danger signals. Lymphocyte return to the thymus occurs once a "steady-state" immune environment has been achieved, when the acute trauma, inflammation, and ischaemia-reperfusion injuries associated with transplantation have subsided. This creates a tolerogenic environment and facilitates acceptance of donor-reactive T-cells. Lymphocyte depletion strategies for tolerance induction, with or without immune reconstitution with donor bone marrow to create haematopoietic chimerism, have been widely explored in NHP transplant models [191,192,229-233].

Human polyclonal horse and rabbit anti-thymocyte globulin fail to produce sufficient lymphocyte depletion in rhesus macaques, and may accelerate rejection [191,234]. FN18-CRM9

immunotoxin, a T-cell depleting rhesus anti-CD3 monoclonal antibody developed by the Biomedical Primate Research Centre in the Netherlands, has been shown to be much more effective in this species [230,231]. Treatment of animals results in profound lymphocyte depletion (including memory T-cells) with recovery commencing in one month, with little detrimental immunosuppression [232]. Immunotoxin monotherapy has been shown to significantly prolong rhesus renal allograft survival [231,233,235]. In these studies, almost all transplanted monkeys accepted subsequent donor skin grafts while rejecting third party grafts. These animals had non-specific suppression of MLR and cytotoxic T lymphocyte precursor frequency before skin grafting, but this became donor-specific following the skin graft challenge. This was despite the ongoing presence of intact donor-specific antibody responses in some monkeys. While this data was promising, subsequent histological follow up of the renal grafts showed no changes of acute cellular rejection, but did revealed evidence of T-cell return, plasma cell infiltrates, and glomerular changes of chronic rejection [235]. This was often associated with intact anti-donor IgG responses and positive flow-cytometric cross match. Therefore the T-cell depletion strategy alone did not prevent a delayed humoral response that was detrimental to the allograft. While monotherapy is not tolerogenic, anti-CD3 immunotoxin in conjunction with deoxyspergualin [236,237] has been shown to have a synergistic effect and greater tolerance-promoting potential (discussed below).

4.3.2 Deoxyspergualin and Analogues

The NF-kappa (κ)-B pathway, and in particular up-regulation and nuclear translocation of RelB protein, is critical to dendritic cell differentiation and maturation (reviewed in detail in Chapter 4), as well as other pro-inflammatory processes. RelB blockade can be achieved with the antibiotic derivative deoxyspergualin (DSG) or its analogues (e.g. LF15-0195), significantly prolonging allograft survival when used as monotherapy in primate kidney

transplants [238]. These agents also inhibit immunoglobulin production by B cells. DSG has been used as an adjunctive therapy in tolerance protocols in rhesus monkeys with very promising outcomes, particularly in combination with T-cell depletion with anti-CD3ε immunotoxin (the "STEALTH protocol" developed by Thomas et al. [232]). (PLEASE NOTE THIS WORK HAS SUBSEQUENTLY BEEN WITHDRAWN BY THE AUTHORS.) DSG appears to be most effective when given early post-transplant, skewing the immune response towards tolerance. It has inhibitory effects on lymph node dendritic cell maturation and subsequent activation of T-cells, and promotes high levels Th2 and inhibitory cytokines (particularly IL-10 and IL-4) [232,236-239]. A short induction with immunotoxin, methylprednisolone and aspirin followed by a two week course of DSG induced tolerance in a subset of rhesus macaque kidney recipients, with 87% rejection-free survival and in some cases drug-free survival for over three years without histological chronic rejection [236,240]. (PLEASE NOTE THIS WORK HAS SUBSEQUENTLY BEEN WITHDRAWN BY THE AUTHORS.) Tolerant animals had no anti-donor alloantibody, and exhibited donor-specific immune hyporesponsiveness with acceptance of a second kidney graft and short-term acceptance of skin grafts from the same donor, while retaining third party immunity [232]. (PLEASE NOTE THIS WORK HAS SUBSEQUENTLY BEEN WITHDRAWN BY THE AUTHORS.) Similar success has been seen with this protocol in islet transplantation in rhesus monkeys [241]. (PLEASE NOTE THIS WORK HAS SUBSEQUENTLY BEEN WITHDRAWN BY THE AUTHORS.) The limiting factor in translating these studies to the clinical setting is the lack of cross-reactivity of rhesus anti-CD3 immunotoxin with human CD3, however they provide a very strong argument for ongoing studies of T-cell depletion / NF-κB blockade-based strategies in humans.

4.3.3 Co-stimulation Blockade

This is the best defined and most investigated pathway for tolerance induction. While extremely promising in murine / rodent transplant tolerance studies, co-stimulation blockade alone has been shown thus far in NHP models and human to be insufficient for tolerance induction. This is due in part to the non-deletional nature of the immune response that is elicited where alternate or undefined pathways that may circumvent drug actions in species with more complex immune systems. In addition, species may differ in drug doses required and toxicities observed. Outcomes also vary depending on the type of organ transplanted; it is much more difficult to prevent rejection of skin transplants compared to kidney or heart allografts in primates versus mice. However these studies have led to the development of agents that may be clinically useful for immunosuppression minimisation and may assist in the establishment of tolerance inducing regimens in the future.

CD28 Pathway Blockade

Murine studies employing blockade of CD80 and CD86 (B7.1 and B7.2), ligands for CD28, were very promising for tolerance induction, but this has not been confirmed by NHP data. In early NHP studies, Kirk et al. [242] demonstrated in a rhesus renal transplant model that humanised monoclonal antibodies against CD80 and CD86 only modestly delayed allograft rejection, but did not result in tolerance or prevent donor-specific antibody formation. Further studies revealed co-administration of both anti-CD80 and anti-CD86 antibodies led to a synergistic effect and improved outcome[242-245]. Use of both of these antibodies in combination with cyclosporine[246] or sirolimus[244] in cynomolgus monkeys confirmed prolongation of rejection-free kidney survival but again unsuccessful tolerance induction, with graft failure after treatment withdrawal. Studies assessing the effect of CD80/CD86 blocking antibodies in combination with CD40 pathway antibodies are discussed below.

Cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4-Ig) is an immunoglobulin fusion protein of CTLA4 (CD152), an alternative high affinity receptor for CD80/CD86, bound to the Fc portion of human IgG1. It prevents interaction of these costimulatory molecules with CD28, thereby inhibiting T-cell activation although not in a donor-specific fashion. The first-generation CTLA4-Ig construct successfully prevented rejection in murine studies, but was found to have ineffective binding affinity to CD80/CD86 in NHP, although some prolongation of rejection-free survival was observed when used in combination with anti-CD40-Ligand antibody (see below)[247]. Subsequently, a higher affinity molecule was generated by a two amino-acid substitution (L104E and A29Y), giving rise to belatacept (LEA29Y). This molecule significantly prolonged survival of rhesus islet and renal allografts [190,248] when used as monotherapy. Following NHP studies, commercial preparations of CTLA4 are currently in clinical use. Abatacept (Orencia®, Bristol-Meyers Squibb) is approved for use in moderate-severe rheumatoid arthritis. Belatacept is being used in phase three clinical trials for renal transplantation, with evidence of sparing of renal function and less chronic allograft nephropathy when compared with cyclosporine[249]. However, its role in tolerance induction remains uncertain at present, and it should be considered an immunosuppression-minimising drug.

CD154 / CD40 blockade

CD40-Ligand (CD154) functions as a co-stimulatory signal on T-cells, and interacts with CD40 on B-cells and APC, promoting CD80/CD86: CD28 expression and further T-cell activation. Treatment with various clones of anti-CD154 monoclonal antibody (mAb)in rhesus renal and cardiac transplant models successfully prevented rejection and prolonged graft survival when used as monotherapy [192,250], although true tolerance was not achieved as grafts

had evidence of ongoing anti-donor immunological activity (histological rejection or presence of allo-antibody) and graft failure due to rejection eventually occurred in almost all cases. Interestingly however, even the few long-surviving grafts displayed a cellular infiltrate on histological examination, but not specifically tubulitis, in conjunction with donor-specific antibody, suggesting an immune response promoting graft acceptance[250]. In another rhesus study in which anti-CD154 mAb induction and/or maintenance therapy was given, concurrent standard immunosuppression was successfully withdrawn with long-term renal allograft survival[251]. Combination therapy with anti-CD154 mAb and CTLA4-Ig [191,247] or anti-CD80/CD86 [242,243,245] (thereby blocking multiple co-stimulation pathways) was also shown to promote long-term rejection free survival of rhesus renal transplants, but interestingly was not more effective than anti-CD154 mAb alone. The success of anti-CD154 therapy in rhesus renal transplant models was dose dependent (>10mg/kg induction and maintenance), and greater with longer duration of therapy (> 6 months of monthly doses). Using similar protocols for prevention and reversal of rejection, anti-CD154 therapy was used with benefit in the islet transplant setting in baboons [252], and even successfully promoted long-term survival of rhesus skin transplants, a notoriously difficult model [253]. Anti-CD154 induction (specifically IDEC-131) has also been combined with adjuvant therapies including the mammalian target of rapamycin (mTOR) inhibitor sirolimus and donor-specific transfusion (DST) as a method of delivering donor antigen. The combination of all three treatments followed by drug withdrawal after three months led to a tolerance-like state in three of six rhesus renal transplant recipients, with long-term (> 500 days) graft survival, absence of rejection or donor antibody, and acceptance of subsequent same-donor skin transplants [254]. Interestingly, the same regimen prevented rejection of primary skin grafts in rhesus monkeys but only for the duration of treatment and not after drug withdrawal, and anti-donor immune

responses were not suppressed [255], again confirming the difficulty of achieving tolerance in the skin transplant model in this species.

Despite these extremely promising results utilising anti-CD154 in NHP models, unfortunately subsequent human clinical trials which were complicated by high rates of acute rejection and significant thrombophilia [256]. Thrombotic episodes had been noted in some NHP studies, and were observed with all antibody clones tested in humans, and clinical trials were therefore ceased. The mechanism of thrombosis was most likely due to platelet activation via the Fc region of anti-CD154 mAb [257,258]. Strategies for minimising this serious complication are continuing to be investigated in murine and NHP models. Of some importance to the future of CD154 blockade as a potential therapy, the use of prophylactic anticoagulation with heparin during antibody treatment has been shown to nearly eliminate the risk of thrombosis (clinical or sub-clinical) in cynomolgus monkeys [258]. Future studies may involve a shorter duration of therapy to minimise any toxicity, in conjunction with heparin or other anticoagulation. Anti-CD154 antibodies with variant Fc regions have been tested in mice for tolerogenic potential, but have not been fully assessed in NHP as yet [257].

The limitations of anti-CD154 blockade have led to interest in blockade of CD40 itself, with studies again based on NHP renal transplant models. Anti-CD40 antibody therapy, alone or in combination with CD86 blockade, has shown promising prolongation of rhesus renal allograft survival without alloantibody developing [245,259]. Importantly, induction therapy with anti-CD40 antibody (CH5D12) combined with CD86 antibody followed by a limited (3 month) course of low-dose cyclosporine promoted long-term (> 1290 days) immunosuppression-free survival in two of recipients [259]. Another anti-CD40 antibody (Chi220), which blocks ligand binding to CD40 but also has partial agonist activity and weakly stimulates CD40, led to

greatly prolonged islet allograft survival (172-237 days) in rhesus monkeys when used in conjunction with belatacept [260]. However, in all cases grafts were lost after cessation of treatment, and outcomes in a vascularised allograft model such as renal transplantation are unknown.

In summary, co-stimulation blockade remains a viable component of tolerance induction strategies, although potential side-effects such as thrombosis will need to be mitigated and the optimum combination of antibodies and adjuvant treatments (immunosuppression, DST or mixed chimerism induction – see below) remains to be determined. NHP models will be essential in the future evaluation of these promising regimens.

4.3.4 Mixed Chimerism Induction

Mixed chimerism is the sustained co-existence of allogeneic and host haematopoietic cells in the recipient, and is either micro-chimerism (detectable by PCR) or macro-chimerism (detectable by flow cytometry). Unlike full chimerism where only donor cells are present, mixed chimerism results in both donor and host antigen-presenting cells within the thymus (most potently dendritic cells), leading to deletion of self-reactive *and* donor-reactive T-cells during immune re-constitution. The resulting tolerance due to thymic deletion is considered more robust than peripheral tolerance due to T-cell anergy or hyporesponsiveness that is induced by co-stimulation blockade or other mechanisms that interfere with antigen-presentation at the periphery, although the exact mechanisms are unclear [261]. Cases of spontaneous tolerance occurring in clinical transplantation are characterised by evidence of donor chimerism within the recipient [262-264], and deliberate chimerism induction has attracted much interest.

Haematopoietic macro-chimerism across full MHC barriers may be achieved by an array of total or partial myelo- and lympho-ablative strategies (chemotherapy, antibody-based depletion and/or irradiation) followed by donor bone marrow transplantation. Mixed chimerism induction involves partial or non-myeloablative (therefore significantly less toxic) regimens compared to full chimerism induction. Such regimens include limited thymic irradiation (TI) or reduced fraction total body irradiation (TBI), T-cell depleting antibodies, and immunosuppressants such as calcineurin inhibitors and in particular, co-stimulation blockade. These result in a greater level of immuno-competence in the recipient compared with lethal ablative therapies, and therefore are more feasible and relevant to the clinical transplantation setting.

Sustained mixed macro-chimerism has been achieved quite successfully in murine models, where it has been consistently shown that it enables the acceptance of donor organ transplants, including highly immunogenic tissues such as skin and bowel, as well as composite tissues [261,265]. In humans, combined bone marrow and renal transplantation from HLA-identical siblings following cyclophosphamide / ATG / TI conditioning with or without cyclosporine has resulted in transient multilineage chimerism and long-term graft acceptance without ongoing immunosuppression [266,267]. In the NHP, various approaches have produced chimerism that appears to be transient at best [190]. Despite this, several studies have shown that even transient donor chimerism in NHP recipients facilitates donor allograft acceptance. In early studies, Kawai et al. achieved prolonged immunosuppression-free kidney transplant survival in a large proportion of cynomolgus macaque recipients with a combination of T-cell depletion with ATG, sub-lethal total TBI and TI, donor marrow infusion +/- splenectomy pre-

transplant and limited cyclosporine post-transplant +/- splenectomy [268,269]. The best outcomes were observed in animals that had marrow transplant and splenectomy simultaneously, with kidney transplant at the same time or later. Animals who exhibited chimerism generally did not produce alloantibody [269]. Modifying this regimen by the addition of co-stimulation blockade (anti-CD154) abrogated the need for splenectomy and enhanced donor BM engraftment [270], although durable chimerism again did not ensue and ultimately some animals did succumb to chronic rejection.

More recently, Kean et al. developed a strategy for inducing mixed chimerism in rhesus macaques that involved one non-myelo-ablative dose of busulphan followed by donor stem cell infusion from G-CSF mobilised donors, peri-transplant basiliximab (IL-2 receptor blockade), and maintenance treatment with belatacept (CD28 blockade), anti-CD154 antibody (H106), and sirolimus (rapamycin) for up to 127 days (the CoBBS protocol) [271]. Adjuvant therapy with anti-CD8 antibody, DST, thymectomy and deoxyspergualin were also assessed. This regimen produced one of the best outcomes in terms of chimerism induction in NHP, with reliable development of chimerism in 16/17 animals, peak chimerism of up to 100 %, and median chimerism duration of 36-196 days. Interestingly, none of the adjuvant therapies gave additional benefit, and the regimen had significant toxicity in terms of infectious disease (particularly viral reactivation). The impact of this regimen in the setting of organ transplantation is yet to be determined.

4.3.5 Adoptive Cellular Therapies

In many tolerance studies, long-term surviving renal allografts in NHP histologically exhibit an active regulatory immune process, characterised by mononuclear infiltrates [250,254,259]. These

have been shown to contain regulatory T-cell populations [272,273]. These cells may be a final common pathway for tolerance promotion by various strategies, although they are also found in rejecting allografts [273]. Adoptive transfer of immune regulatory cells as a means of immune modification has not yet been widely explored in NHP models. It has been shown that anergic T-cells have immune suppressive functions *in-vitro* in an antigen-specific and MHC-restricted fashion [274,275]. In rhesus monkeys, Bashuda et al. [276] generated recipient splenic T-cells rendered anergic by co-culture with donor splenocytes in the presence of co-stimulation blockade with anti-CD80/86 antibodies. Adoptive transfer of these T-cells to splenectomized recipient monkeys following brief treatment with cyclophosphamide and cyclosporine led to prolonged renal allograft survival in all and indefinite survival in 3 of 6 of recipients. The long-term survivors had donor-specific hyporesponsiveness and acceptance of skin-grafts. Further studies are warranted to evaluate this approach in NHP.

4.3.6 Transplant Tolerance Strategies in NHP: Conclusions

Existing studies of tolerance induction in NHP highlight the difficulties of achieving durable and stable tolerance in complex species that have a significant immune history and repertoire. The tolerance induced is often transient or easily disrupted (metastable [272,277]). Often processes such as infection or malignancy that trigger memory immune responses can lead to loss of tolerance. This indicates that mechanisms of donor-reactive T-cell silencing are not permanent or that active inhibitory and regulatory processes can be overcome by other aspects of innate and adaptive immunity. Thus far, it appears combination strategies targeting several pathways have been the most successful. Future research will need to explore alternative combinations of therapies, as well as novel mechanisms of immune manipulation such as tolerogenic DC. In

addition, reliable markers of tolerance and methods for assessing and monitoring anti-donor responses and tolerance in NHP are much needed at this stage.

4.4 Non-human Primate Dendritic Cells

4.4.2 Identification and Characterisation of NHP DC

Identification and characterisation of NHP DC subsets, and correlation with both human and murine DC is necessary to enable investigating their therapeutic potential. Important differences exist between human DC and propagated DC from various NHP species, particularly variations in maturation profile, strength of allo-stimulation and expression of surface markers. These specific features of NHP DC are discussed in detail in chapter 4. While no NHP DC model completely resembles the human DC system, they do reflect it more closely than murine DC models, and are therefore essential for translational research.

Strategies for propagating human myeloid (DC1)-like or MoDC *in-vitro* from peripheral blood and bone marrow precursors have been applied to several large NHP species including chimpanzees, baboons, rhesus and pigtailed macaques, cynomolgus monkeys, owl monkeys, and African green monkeys [182,278-288]. One recent study preliminarily assessed bone marrow and monocyte-derived DC in marmosets, however only limited phenotypic and functional data was provided [228]. No other studies of New World monkey DC exist.

Due to their established use as a research model, as well as the availability of rhesus-specific reagents as well as human reagents cross-reactive with rhesus samples, macaque *in-vitro* DC

have been widely utilised. Several protocols for DC generation are described in macaques (see Chapter 4). Monocyte precursors obtained by T-cell depletion or CD14$^+$ cell immunomagnetic selection and cultured in GM-CSF and IL-4 produce immature MoDC which can then be matured with a variety of stimuli. Alternatively, bone marrow precursors cultured in GM-CSF, TNF-α and SCF followed by GM-CSF and IL-4 also produce myeloid DC.

Viral transduction of NHP *in-vitro* propagated DC is feasible and enables manipulation of subsequent immune responses to induce either tolerance or immunity, depending on the nature of the genes introduced. For example, rhesus mature MoDC transduced with adenovirus over-expressing TGF-β1 suppress T-cell responses in an antigen-specific fashion [149], whereas vaccination with rhesus DC transfected to express retroviral antigen [289,290] or EGFP [291] can enhance antigen-specific immunity .

One of the major areas of deficiency regarding NHP DC is the characterisation of DC *in-vivo,* and to date remains largely limited to rhesus macaques. Blood [36,38,40,287,292,293], skin or mucosal surface [294-297], lymph node[236,283,297], liver[39] and kidney[39] DC have been described in healthy or SIV-infected rhesus monkeys. Rhesus circulating DC subsets can be isolated from peripheral blood using monoclonal antibody labelling and cell sorting methods, and possess similarities to human myeloid and plasmacytoid DC [36,40,287]. Rhesus pre-DC1 (pre-MDC) are found in the Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD123$^-$fraction of peripheral blood mononuclear cells, whereas pre-DC2 (pre-PDC) are Lin$^-$ HLA-DR$^+$ CD11c$^-$ CD123$^+$ [36,40,287]. These cells are rare in peripheral blood, but can be mobilised in significant numbers by *in-vivo* administration of FLT3-L which mobilises both MDC and PDC, and / or G-CSF which mobilises PDC only [36,38,40]. Rhesus monkeys are of sufficient weight and size to enable leukopheresis procedures to be performed on and growth-factor mobilised DC or DC precursors including CD34+ stem cells can be

enriched in this manner [38,95,96,98,298]. FLT3-L also mobilises immature DC into parenchymal organs in rhesus monkeys, including liver and kidney [39]. This finding is important to consider when transplanting organs from growth-factor treated donors.

DC have also been identified in healthy and SIV-infected cynomolgus macaque lymph nodes and liver[299-302]. Recently, circulating cynomolgus MDC and PDC have been characterised for the first time and these reflect human and rhesus DC equivalents, although DC numbers and subset proportions vary either due to true disparity or methodological differences [302]. Interestingly, although CD11c staining on cynomolgus monocyte-derived DC was strong, it was found to be much weaker on circulating blood MDC, and was abandoned as an MDC marker in favour of CD1c (BDCA-1). This highlights the pitfalls of extrapolation of DC paradigms from one NHP model to another, and the need to evaluate each species individually.

Data regarding *in-vivo* DC in other NHP species remains very limited. We have identified putative DC in autopsy specimens of marmoset thymus, liver and spleen using immunofluorescent labelling with antibodies including CD11c, CD83, and BDCA-2 (Kireta, Prasad, Coates et al. unpublished data).

4.4.2 DC-based Immune Modification in Non-Human Primate Models

The success of DC manipulation and therapy in murine / rodent models of transplantation as discussed above provides valid justification for proceeding with trials in NHP. However, the evaluation of DC tolerogenic potential in NHP *in-vivo* is currently extremely limited and no studies to date have assessed their influence on NHP solid organ transplant outcomes.

Zahorchak et al. [97] have recently tested for the first time the effect of allogeneic, maturation resistant, *in-vitro* propagated DC on immune responses in recipient rhesus monkeys, and have reported initial sensitisation followed by suppression of donor and third party responses when DC are combined with CTLA4-Ig (discussed further in Chapter 6).

Otherwise, almost all trials of DC-based immunotherapy in NHP have principally occurred in experimental models of human immunodeficiency virus (HIV), using animals infected with simian immunodeficiency virus (SIV). As a result of these pre-clinical studies, preliminary clinical trials using DC vaccines for HIV have now been undertaken with extremely promising early evidence of safety and some efficacy [20,21]. *In-vitro* generated NHP DC can be successfully transduced with HIV/SIV encoding viral vectors or pulsed with viral antigens to elicit anti-viral immune responses *in-vivo* that facilitate clearance of virus [289-291,303-305]. For example, cynomolgus DC transduced with a lentiviral vector[289] or rhesus DC transduced with adenoviral vectors[290] carrying SIV fragments were capable of expanding antigen-specific T-cell populations without inducing immune responses to the viral vector. In addition, SIV-antigen pulsed DC can induce dramatic reductions in blood SIV DNA (up to 50-fold) and RNA (1000-fold) by facilitating T-cell activation in lymph nodes, as well as a specific humoral immune response [304]. Mobilising pre-DC *in-vivo* in infected rhesus monkeys using FLT3-L and G-CSF does not appear to have the same effect on viral clearance [38], indicating that DC need to be matured or manipulated to elicit anti-viral immunity. FLT3-L mobilisation of DC in addition to DNA-based SIV vaccination plus TLR-9 stimulation with CpG to mature DC was found to improve $CD8^+$ T-cell activation and reduce viral load [306]. These studies are important in advancing the field of DC immunotherapy, providing evidence that DC-based

immune manipulation is feasible, safe and effective at influencing immune outcomes in these models.

5. THESIS AIMS AND HYPOTHESES

The focus of the work presented in this thesis is the further development of the marmoset model specifically for transplant studies to assess the potential immuno-modulatory effects of dendritic cells in non-human primates (NHP). This includes the development of MHC genotyping techniques for the selection of donor and recipient animals (Chapter 3), the propagation and characterisation of marmoset DC using haematopoietic growth factors and *in-vitro* culture strategies (Chapters 4 and 5) and the preliminary evaluation of immune response to allogeneic DC infusion in recipient animals (Chapter 6). This work creates a platform from which future studies of DC-based transplant tolerance strategies can be developed.

The hypotheses of this thesis are that:

1. Sequence-based genotyping of common marmoset monkey MHC will facilitate matching of donor / recipient animals pairs that are immunologically disparate and reactive.
2. Common Marmoset monkey *in-vivo* and *in-vitro* propagated DC exist within the paradigm of human and other NHP DC.
3. Marmoset DC and DC precursors can be mobilised with recombinant human growth factors, and propagated *in-vitro* in sufficient numbers for subsequent adoptive DC therapy studies.
4. Treatment of recipient animals with allogeneic *in-vitro* propagated DC is safe and able to stimulate an inhibitory or stimulatory immunological response that can be monitored with accepted methods.

CHAPTER 2: MATERIALS AND METHODS

This chapter describes protocols and procedures related to animal maintenance and laboratory techniques used in the studies in this thesis. Additional comments on specific issues relating to methodology may also be found in the other chapters.

1. ANIMALS

1.1 Marmoset Colony Maintenance

Up to 40 healthy marmosets aged 2-13 years, weight 250-400g, were housed at The Queen Elizabeth Hospital Animal House at any one time. Most marmoset colonies in Australia, including our own, originate from a small number of animals imported in the 1980s from the United Kingdom, most from the MRC colony in Edinburgh. Animals were bred from within the colony and also imported from other colonies within Australia.

This primate colony is closely overseen by the local Animal Ethics Committee (formerly the Queen Elizabeth Hospital AEC, and currently the Institute of Medical and Veterinary Sciences AEC). Maintenance of the colony is in accordance with guidelines set by the National Health and Medical Research Council as described in the NHMRC Policy on the Care and Use of Non-human Primates for Scientific Purposes 2003, which can be viewed via the link http://www.nhmrc.gov.au/publications/synopses/_files/nonhum2.pdf. The projects numbers pertaining to work in this thesis are: 149/07 (General Marmoset Colony Maintenance), 150/07 (Studies of Immune Biology in Marmoset Monkeys) and 61a/07 (The Potential Of Donor Derived Dendritic Cells To Induce Tolerance In Marmoset Monkeys).

The animals are monitored and observed daily, weighed regularly and any signs of illness or abnormal behaviour are identified. They have a diet of water ad libitum, fruit, vegetables, bread, mealworms, egg, marmoset pellets, supplemented with multivitamin and Vitamin D3. Standard operating procedures for enclosure maintenance, diet, health checks and environmental enrichment have been developed by Animal House staff. Animals have access to a purpose-built outdoor enclosure with plants and natural light for several hours each day, weather permitting, to combat Vitamin D deficiency which can affect this species.

1.2 Peripheral Blood Sampling

Between 0.2 to 2ml of PB was obtained via femoral vein venepuncture at any one time. Maximum blood loss was 10mls/kg/month. Animals were weighed regularly during periods of frequent bleeding. In cases of unexplained weight loss, blood sampling was temporarily suspended until a healthy weight was regained. Animals being bled frequently were given supplemental liquid iron daily to prevent anaemia.

The protocol for venepuncture was as follows:

1. Wear gloves, gown, hat and eye protection.
2. Catch marmoset and place in metal transport box. Move to procedure room.
3. Remove monkey and place in harness with legs secured by straps.
4. Feed monkey yoghurt / banana during procedure
5. Swab femoral region with 70 % ethanol
6. Use 27.5 gauge needle with syringe and draw 1 ml blood from femoral vein
7. Remove needle and place pressure on site for 3-5 min

8. Check for bleeding once leg is removed from straps, and again before returning monkey to cage.

2. BUFFERS, MEDIUM AND SOLUTIONS

- **Phosphate-buffered saline (PBS)** was prepared using sodium chloride (May & Baker), sodium phosphate (Amresco) and sodium dihydrogen orthophosphate (Ajax Finechem).
- **Complete medium (CM)** - RPMI-1640 (Gibco BRL) supplemented with 10%-20 % v/v calf serum (FCS; JRH Biosciences), 2mM L-glutamine (MultiCel), sodium pyruvate (ICN Pharmaceuticals), penicillin-streptomycin (MultiCel) and sodium bicarbonate (Amresco). Added cytokines and reagents included recombinant human (rh) interleukin-4 (IL-4; E Biosciences), rh human-cell expressed IL-4 (Apollo Cytokine Research), rh granulocyte macrophage-colony stimulating factor (GM-CSF; Sandoz), rh IL-13 (eBiosciences), rh fetal liver tyrosine kinase 3 ligand (FLT3-L; R&D Systems), rh stem cell factor (SCF, Ancestim; AMGEN Corporation), rh thrombopoietin (TPO; R&D Systems), calcimycin A23187 (Sigma Aldrich), bacterial lipopolysaccharide (LPS; Sigma Aldrich), tumour necrosis factor (TNF-α; R&D Systems), rh CD40 Ligand/TNFSF5 (CD40L; R&D Systems). Human monocyte conditioned medium (MCM) was generated as previously described [280,285].
- **Running buffer** for immunomagnetic bead separation - PBS with 0.5% v/v FCS and 2mM Ethylenediamine tetra-acetate (EDTA; Sigma Aldrich).
- **Rinse Buffer** for immunomagnetic bead separation - PBS with 2mM EDTA.
- **Cell lysis buffer** – 0.15M ammonium chloride (Ajax Finechem), 0.01M sodium bicarbonate, 0.1mM EDTA, in MilliQ water

- **Staining buffer** for flow cytometry studies - PBS with 0.01% FCS, 0.1% w/v sodium azide (Sigma Aldrich)
- **FACS lysing solution** – 10% concentrated FACS lysing solution (BD Biosciences) in distilled water

3. GENE STUDIES

3.1 DNA extraction

Genomic DNA from 49 animals was obtained from whole blood obtained via femoral vein puncture or stored frozen spleen cells from deceased animals. DNA was extracted using QIAamp® DNA Blood Mini kits according to the manufacturer's instructions (QIAGEN). The final DNA preparation was stored at -20ºC in buffer provided with the isolation kit. DNA concentration was measured with optical density spectrophotometry.

3.2 *Caja*-DRB Gene Sequencing Method

3.2.1 Primary PCR

PCR was performed in a 25 µl reaction mix, with 200 ng of genomic DNA, 0.3 mM each of forward and reverse primer (Table 1), 10µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM buffer, 2.0 mM magnesium chloride, 2.5 units of DNA polymerase and sterile water. AmpliTaq Gold® with GeneAmp® (Applied Biosystems) was used for DRB*W16 amplification and TTH Plus® DNA polymerase (Fisher Biotec) for DRB1*03 and DRB*W1201 amplification, along with kit-supplied buffer and magnesium chloride. All reactions were performed in a Perkin Elmer Cetus DNA thermocycler (Perkin Elmer).

DRB*W16 amplification began with 2 minutes pre-heating at 96º C, then 20 s at 95 ºC (denaturation), 40 s at 57ºC (annealing) and 20 s at 72ºC (extension) for 35 cycles, then incubated for 3 minutes at 72ºC. Cycling conditions for DRB1*03 and DRB*1201 amplification were similar, apart from annealing at 57ºC for 40s. Presence of PCR product was confirmed with 2% agarose gel electrophoresis.

Table 1. Primers used for *Caja*-DRB exon 2 amplification.

Primer	Alleles Amplified	Sequence 5'→ 3'
DRB*W16 (1)	All DRB*W16 alleles except DRB*W1606, 1607, 1608, 1611	ACG TTA CTT GGA GCA GG
DRB*W16 (2)	All DRB*W16 alleles except DRB*W1608, 1611	ACG TTW CTT GGA GCA GG
DRB*W16 (3)	DRB*W1608 and DRBW*1611	ACG TTW CTT GGA GCA GC
DRB*W16 (4)	DRB*W1605 (and also 1601, 1613, 1615)	GTT AAG TTT GAG TGT CAT
DRB*W16 (5)	DRB*W1609, 1610 (and also 1604, 1612, 1614)	GCT AAG TGT GAG TGT CAT
DRB1*03	All DRB1*03 alleles	CGT TTC TTG GAG TAT AGC
DRB*W1201	DRB*W1201	AAC GTT TCT TGG AGT TT
DRBREV	All alleles	CTC CGC GGC ACT AGG AAC

3.2.2 Exon 2 Template Fluorescent Dye Terminator Cycle Sequencing

Exon 2 PCR product (10µl) was incubated with 2.5µl ExoSAP-IT™ (Amersham Biosciences) for 15 minutes at 37ºC then 15 minutes at 80ºC to removed excess primers and dNTP. DNA sequencing was performed using ABI PRISM™ Big Dye Terminator v 3.1 Ready Reaction kit (Perkin-Elmer) using -21M13 and M13Rev primers as described by the manufacturer. Product was washed twice with isopropanol (75%) and vacuum dried. Product plus loading buffer (deionised formamide with blue dextran) was heated for 3 minutes at 96ºC to denature DNA,

and was then loaded onto a 50 ml acrylamide gel with 6 M urea (Long Ranger ® Singel pack, Cambrex Bioscience). Nucleotide sequence in both directions was analysed using Assign™ (Conexio Genomics) DNA sequencing analysis software, in comparison with previously published marmoset MHC sequences [307-309].

3.3 Sequencing of Marmoset RelB Gene

3.3.1 RNA Extraction and Reverse Transcription

RNA from cell suspensions was extracted using GE RNA Extraction kits (GE Healthcare) or QIAGEN RNeasy Mini kits (QIAGEN) as per manufacturer's instructions, and quantified using Experion™ RNA StdSens Analysis kit (Bio-Rad Laboratories). Up to 1µg of RNA was reverse transcribed in a 40µl reaction containing 4µl pre-heated oligo-dT (Amersham Biosciences), water, RNA, 8µl RT buffer (Gibco BRL), 200U MMLV reverse transcriptase (Gibco BRL), 40U RNAsin (Promega) and 40uM dNTP (Promega). Samples were incubated for 60 minutes at 37°C then heat-inactivated for 10 minutes at 70°C, and adjusted with water to a cDNA concentration of 0.01µg/µl. Samples were stored at -70°C until use.

3.3.2 Real-time PCR

Primers and standards for human RelB were the kind gift of Dr Ravi Krishnan and Ms Darling Rojas, Transplant Immunology Laboratory, TQEH. Primer sequences were 5' TTTTAACAACCTGGGCATCC 3' and 5' CGCAGCTCTGATGTGTTTGT 3' based on human RelB sequence, NCBI Accession Number NM_006509. Semi-quantitative real-time PCR was performed with either standards for RelB or housekeeping gene GAPDH (in-house standards), non-template control or 10ng cDNA in a reaction mix of 0.5U TthPlus DNA polymerase with 10x Buffer (Fisher Biotec), 2.5mM $MgCl_2$ (Fisher Biotec), 0.5 mM primers,

0.8mM dNTP and 0.8µl Syber-green. Samples were run on a Rotor Gene 2000 Real-time cycler (25s, 95°C; 25s, 55°C; 25s, 72°C x 50 cycles) and analysed with Rotor-Gene v5.0 (Corbett Research). RelB expression (copies/µl) was normalised to GAPDH housekeeping gene expression. PCR product was sequenced by Southpath and Flinders Sequencing Facility, Adelaide, Australia. Sequences were aligned using Vector Nti software (v10, Invitrogen).

4. CELL ISOLATION PROTOCOLS

All washes were performed in 10-50ml volume by centrifuging at 400xg for 7 minutes at 4°C and decanting the supernatant unless otherwise specified.

4.1 Peripheral Blood Mononuclear Cell (PBMC) isolation

Whole blood was transferred into 10ml (for marmoset samples of 300-1500µl volume) or 50ml (for human samples of 10ml volume) centrifuge tubes, diluted with 7 or 35ml PBS, respectively and underlayed with 2 or 12ml of Ficoll-Hypaque (Amersham Biosciences). Samples were centrifuged at 800xg for 25 minutes at room temperature without braking, and the PBMC layer carefully collected, washed 3 times with PBS with 0.5% FCS then re-suspended in CM or running buffer. If persistent red cell contamination was noted, the cell pellet was re-suspended in 2mls of cell lysis buffer and incubated at 37°C for 5 minutes, and washed a further 3 times. Viability and cell count was assessed with trypan blue (Sigma Aldrich) staining and a haemocytometer.

4.2 Isolation of $CD14^+$ Monocytes from PBMC using Automacs ®

PBMC were isolated as per protocol, resuspended 80µl (for maximum 10^7 cells) of cold running buffer and incubated for 15 minutes at 4°C with 20µl of human (or non-human

primate) CD14 microbeads (Miltenyi Biotec). Samples were washed in cold running buffer and resuspended in 500µl of cold running buffer, then processed through the Automated Magnetic Cell Separator (Automacs®, Miltenyi Biotec) using positive selection settings. The Automacs® was set up and maintained in accordance with manufacturer's instructions, using protocols as specified for different bead types. In various experiments either the Positive Selection Sensitive or Positive Selection Double Sensitive settings were used. The positive and negative fraction eluents were collected, washed in running buffer, re-suspended in CM and cell counts performed. In some instances the negative fraction was passed over the magnets a second time. Purity of the positive fraction and extent of monocyte depletion from the negative fraction was assessed by flow cytometry for CD14.

4.3 Isolation of $CD14^+$ Monocytes from Whole Blood Using Automacs ®

Whole blood samples were collected in lithium heparin tubes, and incubated with 25µl of Whole Blood CD14 Microbeads (Miltenyi Biotec) per 500ul whole blood sample for 15 minutes at 4°C. In some instances 25µl of beads were added per 250µl WB to improve the bead concentration. Samples were washed once in cold running buffer. The supernatant was carefully removed by pipette and the pellet re-suspended in running buffer to a final volume of 2mls. Samples were processed through the Automacs® as described above.

4.4 Isolation of $CD14^+$ Monocytes from PBMC Via Plastic Adherence

PBMC were isolated as per protocol. Marmoset PBMC (up to $2x10^6$) were incubated in 6-well plates in 2mls of RPMI-1640 with 1 % FCS for 90 minutes at 37^0C, 5% CO_2. Human PBMC (up to $5x10^7$) were incubated in $75cm^2$ flasks in 10mls of RPMI-1640 with 1 % FCS for 45-60 minutes. Non-adherent cells were removed with extensive PBS washes. Adherent

cells were detached either by cell scraping or overnight culture in CM, or remained in culture for DC differentiation.

4.5 Isolation of $CD34^+$ Peripheral Blood Stem Cells

PBMC were isolated as per protocol and washed once in running buffer. Up to $5x10^6$ PBMC were incubated in 180µl of rinse buffer with 20µl of heat-inactivated rabbit serum for 20 minutes at 4°C. PE-conjugated anti-CD34 antibody was added (20µl per million PBMC) and incubated for a further 20 minutes at 4°C in the dark. After washing once in rinse buffer, 50µl of anti-PE microbeads (Miltenyi Biotec) were added and incubated for 15 minutes at 4°C in the dark. Samples were then washed once in running buffer, re-suspended in 500µl of running buffer and processed through the Automacs® using Positive Selection Sensitive settings. Purity and yield was assessed by flow cytometry for CD34 and lineage markers.

5. IN-VITRO DC PROPAGATION

5.1 Generation of Monocyte-derived Dendritic Cells (MoDC)

Peripheral blood monocytes were isolated either by immunomagnetic bead separation or plastic adherence as described above and cultured for 7 days in CM with IL-4 (40ng /ml) and GM-CSF (800U/ml). CM was refreshed during culture to prevent reversal of differentiation and macrophage formation as has been previously described [75]. The predominant maturation stimulus was TNF-α (10ng/ml) added on day 5; in various experiments LPS (10ng/ml), PGE2 (1µg/ml), Calcimycin (150ng/ml), CD40L (1ug/ml) or human MCM (50% v/v) were used in addition or instead. At the end of culture the non-adherent cell fraction was collected.

5.2 Generation of Haematopoietic Progenitor Derived DC (HPDC)

PBMC were isolated as per protocol. $CD34^+$ haematopoietic progenitor (HP) cells were enriched by immunomagnetic separation using CD34-PE antibody and anti-PE microbeads as described above. Alternatively, non-enriched bulk PBMC were used. Cells ($0.5\text{-}1x10^6$/ml) were cultured in CM with FLT3-L (100ng/ml), SCF (100ng/ml) and TPO (50ng/ml), adapted from human protocols[87,88], for up to 4 weeks. Medium and cytokines were refreshed twice weekly by 50-100 % replacement, and cells adjusted to $1\text{-}2x10^6$/ml. Each week cells were either maintained in cytokine cocktail, or a portion removed and cultured further in IL-4/GM-CSF ± TNFα (as for MoDC).

6. MIXED LEUKOCYTE REACTION (MLR)

6.1 Two-way MLR (Chapter 3)

Allogeneic animals were chosen by *Caja*-DRB genotyping (described in Chapter 3). PBMC were isolated as per protocol and re-suspended in CM with heat-inactivated FCS. PBMC (1 x 10^5 from each animal) were cultured in triplicate wells in a 96 well plate at 37º C in 5% CO_2 for 5 days. To obtain baseline data, 1 x 10^5 PBMC from each animal were cultured alone. In the final 18-24 hours of incubation, each well was treated with 1 μCi of tritiated thymidine ([^{3}H]; Amersham Biosciences). Cells were harvested using a Tomtec Harvester 96 Mach III M. T-cell proliferation via [^{3}H] incorporation was determined in a liquid scintillation counter (Wallac Oy Microbeta® Trilux1450) and expressed as mean (of replicate samples) counts per minute (cpm) ± SD. Alloreactivity was confirmed if there was evidence of T-cell proliferation when cells from animals were cultured together i.e. if the mean cpm (combined cells A and B) was significantly greater than the mean cpm (cells A) + mean cpm (cells B). Statistical

comparison between groups was performed using the Student's t test, with $p < 0.05$ deemed as significant.

6.2 One-way MLR (Chapter 6)

Allogeneic animals were chosen by *Caja*-DRB genotyping (described in Chapter 3). Stimulator PBMC were subjected to irradiation at 30Gy. The remainder of the protocol was identical to two-way MLR. The proliferative response was represented as the stimulation index (SI) = (mean cpm co-cultured cells / mean cpm baseline responder PBMC). Statistical comparison between groups was performed using the Student's t test, with $p < 0.05$ deemed as significant.

6.3 Dendritic Cell MLR (Chapter 4 and 5)

DC were obtained as described above. Allogeneic PBMC from animals chosen by *Caja*-DRB genotyping, or random human buffy coats (Australian Red Cross Blood Service) were used as responder cells. Stimulator DC were subjected to irradiation at 30 Gy. DC were co-cultured with $1x10^5$ allogeneic PBMC in a 1:10 ratio (or other ratio in some experiments) in triplicate for 5 days. PBMC were also cultured alone for baseline data. T-cell proliferation was determined as described above. The proliferative response was represented as the stimulation index (SI) = (mean cpm stimulated PBMC / mean cpm unstimulated PBMC). Statistical comparison between groups was performed using the Student's t test, with $p < 0.05$ deemed as significant.

7. MORPHOLOGICAL ASSESSMENT OF CELLS

7.1 Light Microscopy

Cells were photographed while in culture plates under light microscopy. Cells were also prepared on glass slides by cytospin centrifugation and stained with May-Grunwald Giemsa stain, kindly prepared and processed by the Haematology Laboratory, Institute of Medical and Veterinary Science (IMVS). Cytospin slides were then viewed and photographed.

7.2 Transmission Electron Microscopy

TEM of cell samples (up to $1x10^6$ cells per sample) was kindly performed by Mr John Brealy, Electron Microscopy Department, TQEH using his published protocol [310].

8. FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

8.1 Monoclonal Antibodies

Antibodies were conjugated to Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) or Cychrome (CyC) fluorochromes. Anti-human monoclonal antibodies used for studies in this thesis, unless specified as mouse antibodies.

<u>BD Biosciences</u>: FITC-conjugated CD3 (clone SP34); FITC or PE-conjugated CD14 (clone M5E2); FITC-conjugated CD56 (clone NCAM16.2)

<u>BD Pharmingen</u>: PE-conjugated CD11c (clone S-HCL-3); FITC-conjugated CD86 (clone FUN1); FITC or PE-conjugated CD209/DC-SIGN (clone DCN46); CyC-conjugated HLA-DR

(clone G46-6); FITC-conjugated Isotype control Mouse IgG2b (clone 27-35); PE or CyC-conjugated Isotype control Mouse IgG2a (clone G155-178)

Immunotech: PE-conjugated CD34 (clone 581); PE-conjugated CD40 (clone mAb89); PE-conjugated CD83 (clone HB15A)

eBiosciences: FITC-conjugated CD1a (clone HI149)

DAKO Cytomation: FITC-conjugated CD20 (clone B-Ly-1)

Miltenyi Biotec: PE-conjugated BDCA-2 (clone AC144)

8.2 Sample Preparation and Analysis

Whole blood samples did not require Fc receptor blockade. Other cell samples were re-suspended in staining buffer (50-100µl), blocked with 10 % v/v rabbit serum (ICN Pharmaceuticals, Australia) and incubated for 20 minutes at 4°C. Samples were aliquoted into polypropylene FACS tubes ($0.5x10^5$ - $2x10^6$ cells or up to 200µl of cell suspension per tube) and incubated with appropriate quantities of antibodies (see below) for 20 minutes at 4°C in the dark. Cells were fixed at room temperature with 10 % FACS lysing solution (2 mls per tube) and washed 2-3 times in staining buffer. Samples were analysed on a BD FACScan®" flow cytometer with CellQuest® software (version 3.3). All data are reported in comparison to isotype-matched controls.

8.3 Two and Three Colour Labelling for Dendritic Cell Identification

8.3.1 Compensation Samples

Overlap of fluorescence between detection channels was compensated for at the start of each experiment, using monoclonal antibodies with the highest fluorescence for each channel and a small portion of the sample to be tested.

1. FITC Control (2µL of 1/10 dilution); PE Control (2µL); CyC (1µL of 1/10 dilution)
2. FL1 Compensation: CD14-FITC (2.5µL); PE Control; CyC Control
3. FL2 Compensation: FITC Control; CD11c-PE (5µL); CyC Control
4. FL3 Compensation: FITC Control; PE Control; HLA-DR CyC (5µL)

8.3.2 Monocyte, Stem Cell and DC Identification

Antibody volumes shown are for samples with up to $1x10^6$ cells. The volume was scaled up appropriately if the sample was larger. In some experiments, single colour labelling was used instead of or in addition to the antibody combinations shown. Not all surface marker combinations were analysed in every experiment.

1. CD14-FITC (2.5µL); CD34-PE (40µL); HLA-DR CyC (5µL)
2. Lineage-FITC [CD14 (2.5µL); CD3 (5µL); CD20 (5µL); CD56 (5µL)]; CD11c-PE (5µL); HLA-DR CyC (5µL).
3. Lineage-FITC excluding CD14; CD14-PE (5µL); HLA-DR CyC (5µL).
4. DC-SIGN-FITC (20µL); CD11c-PE (5µL); HLA-DR CyC (5µL)
5. Lineage-FITC; BDCA2-PE (20µL); HLA-DR CyC (5µL)
6. BDCA2-FITC (15µL); CD11c+ PE (5µL); HLA-DR CyC (5µL)
7. CD86-FITC (5µl); CD40-PE (10µl); HLA-DR CyC (5µL)
8. CD14-FITC (2.5µL); CD83-PE (5µl); HLA-DR CyC (5µL)

8.4 FACS of Myeloid DC from Whole Blood (Chapter 5)

Sterile conditions were maintained throughout. Whole blood was stored in lithium heparin tubes then transferred to FACS tubes containing antibodies as listed above. Quantities were doubled for WB volumes up to 150µL and tripled for WB volumes 150-300µL. Cells were

stained as outlined above, and washed in staining buffer, but not fixed in FACS lysing solution. Instead, residual erythrocytes were lysed with ammonium chloride lysis buffer followed by several PBS washes. Cells were sorted on a DiVa instrument using gating strategies as described in Chapter 5. Compensation for signal overlap was performed on each occasion.

8.5 FITC-Dextran Uptake Assay

Cells were collected from culture, washed in PBS and re-suspended in CM. A minimum of $1x10^5$ cells in 2mls of CM containing 1mg/ml FITC-conjugated dextran (Sigma-Aldrich) was incubated at either 0°C (control) or 37°C with for one hour. Cells were washed three times in cold staining buffer, fixed in 1ml of FACS lysing solution for 20 minutes at room temperature in the dark, washed again in staining buffer and immediately analysed by flow cytometry.

8.6 Intracellular Flow Cytometric Staining For Stat-6 Signalling

Marmoset or human PBMC or monocytes ($0.3\text{-}1x10^6$) were cultured in CM with or without IL-4 (10ng/ml) for 60 minutes at 37°C. Cells were fixed in FACS lysing solution as described above, and pelleted. The supernatant was removed and cells were permeabilised in 0.5ml 90% methanol on ice for 30 minutes. After washing twice in PBS and re-suspending in 100µl staining buffer, cells were stained with Alexa Fluor® 488 Anti-Phospho-Stat6 Y641 antibody (clone 18; BD Biosciences; 20µl per 10^6 cells) for 60 minutes at room temperature in the dark. Cells were washed twice in staining buffer, and analysed immediately by flow cytometry (FL-1 channel) using untreated cells as control.

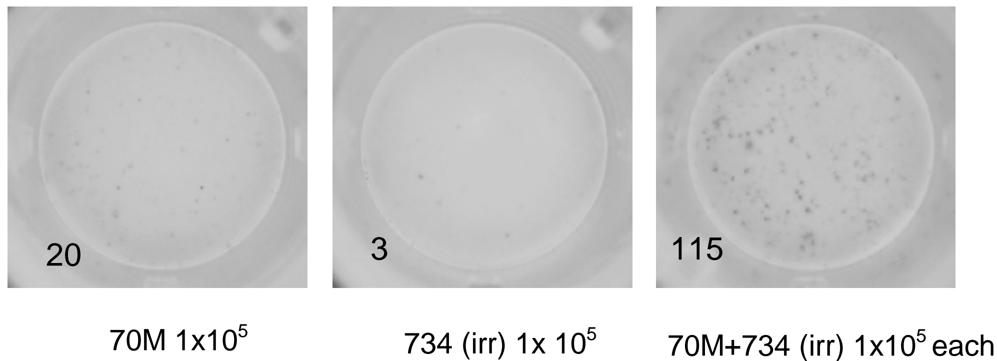
9. ALLOGENEIC PBMC-STIMULATED IFN-γ PRODUCTION

Interferon-γ production was determined by ELISpot (Mabtech) according to the manufacturer's protocol.

- On Day 1, the membrane of a MultiScreen-IP high protein binding 96-well plate (Millipore Corporation) was pre-wet with 35% ethanol, 15µl /well for 1 minute, then washed five times with 200µl/well of sterile water. Capture antibody against human IFN-γ (clone 1-D1K; Mabtech) was diluted to 15µg/ml in sterile PBS and 100µl/well was incubated overnight at 4^0 C. On the same day, freshly isolated PBMC from recipient, donor and third party animals were co-cultured as described above for MLR in a 96-well round bottomed plate to allow for cell-cell contact. Control wells of unstimulated cells from recipients and irradiated cells from donors and third party were also included.
- On Day 2, the plate was washed with PBS, and blocked with 200µl/well of RPMI+10% FCS for 60 minutes at room temperature. Cell suspensions from overnight culture were transferred to wells in duplicate samples. Phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich; 10ng/ml) and Ionomycin (Sigma Aldrich; 5µg/ml) were added to stimulate positive control wells containing $1x10^4$ non-irradiated PBMC. The plate was incubated for 48 hours at 37^0C in 5% CO_2.
- On day 4, the cells were discarded and the plate washed with PBS. 100µl of detection antibody (clone 7-B6-1-biotin; Mabtech) diluted to 1µg/ml in PBS with 0.5% FCS was added to each well and incubated for 2 hours at room temperature. The plate was washed as before, and 100ul of Streptavidin-alkaline phosphatase (Mabtech) diluted 1:1000 with PBS containing 0.5% FCS was added to each well and incubated for 1 hour at room temperature. The plate was washed as before, and 100µl of substrate solution (NBT/BCIP - Nitroblue tetrazolium and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt;

Boehringer Mannheim) was added and developed for 5-20 minutes until spots appeared. Colour development was halted by washing extensively in tap water. After drying, spots were counted in a Mabtech ELISpot reader (example shown in Figure 1). Data is shown as mean of duplicates samples for stimulated recipient PBMC, adjusted for baseline counts.

Figure1. Example of read-out of wells from IFN-γ ELISpot® plate, showing samples 70M (freshly isolated responder PBMC $1x10^5$) and 734 (irradiated stimulator PBMC $1x10^5$) cultured alone for baseline data and in combination for stimulated sample data. The numbers in each well represent absolute spot counts, from which the stimulation index was calculated (stimulated / baseline = 115/23 = 5).



10. BLOOD GROUP TYPING AND CROSS-MATCH

Donor and recipient blood group compatibility was established using human blood group cross-matching methods validated previously by our group for this species (D. Dang, Honours Thesis 2007). Whole blood samples were centrifuged to separate plasma. A 3% cell suspension was made with cold PBS. 150µl of the cell suspension was placed in 4 glass tubes on ice, and 150µl of allogeneic plasma from the donor marmoset, or human anti- A, B or AB serum was added to each cell suspension sample. The samples were centrifuged at > 1000g for

1 minute to generate a hard pellet. The cross-match was considered negative if the pellet could be easily re-suspended.

11. MANUFACTURERS

Ajax Finechem - Seven Hills, NSW, Australia

Amersham Biosciences (currently GE Healthcare) - Brown Deer, WI, USA

AMGEN Corporation – Thousand Oaks, CA, USA

Amresco - Solon, Ohio, USA

Apollo Cytokine Research (now Symansis) – Timaru, New Zealand

Applied Biosystems – Scoresby, Vic, Australia

Australian Red Cross Blood Service – Adelaide, SA, Australia

BD PharMingen and BD Biosciences- San Diego, CA, USA

Beckman Coulter - Hialeah, FL, USA

Bio-Rad laboratories - Hercules, CA, USA

Boehringer Mannheim –Mannheim, Germany

Cambrex Bioscience – East Rutherford, NJ, USA

Conexio Genomics - Perth, WA, Australia

Corbett Research – Mortlake, NSW, Australia

Dako - Glostrup, Denmark

eBioscience – San Diego, CA, USA

Fisher Biotec - WA, Australia

Gibco BRL – Geithersburg, MD, USA

ICN Pharmaceuticals - Costa Mesa, CA, USA

Immunotech - Marseilles, Cedex, France

Institute of Medical and Veterinary Science - Adelaide, SA, Australia

Invitrogen – Melbourne, VIC, Australia

JRH Biosciences - Lenexa, Kansas, USA

Mabtech - Nacka Strand, Sweden

May & Baker Australia - West Footscray, VIC, Australia

Millipore Corporation - Bedford, MA, USA

Miltenyi Biotech - Bergisch Gladbach, Germany

MultiCel Trace Scientific – Clayton, VIC, Australia

Perkin Elmer – Boston, MA, USA

Promega – Madison, WI, USA

QIAGEN – Hilden, Germany

R&D Systems - Minneapolis, MN, USA

Sandoz – Pyrmont, NSW, Australia

Sigma Aldrich - Saint Louis, Missouri, USA

Tomtec – Hamden, CT, USA

Wallac Oy - Turku, Finland

CHAPTER 3: SEQUENCE-BASED GENOTYPING OF MARMOSET MHC DRB GENES

1. INTRODUCTION

The molecular basis of tissue compatibility is predominantly determined by the highly polymorphic genes of the major histocompatibility complex (MHC), located in humans on chromosome 6p21, which encode for human leukocyte antigen (HLA) molecules [311]. These are classified as Class I genes (encoding for the α unit of HLA-A, B C, E, F and G), Class II genes (encoding for both α and β subunits of HLA-DP, DQ, DR) and Class III genes (which are not relevant in transplant matching but encode for a variety of proteins including C2, C4, Factor B, TNF-α). These genes display considerable allelic diversity, especially the HLA-DRB1 locus which is highly polymorphic in humans and other primates such as rhesus macaques and chimpanzees [194]. Genetic similarity at MHC loci is a critical factor in the immunological acceptance and survival of solid organ and cellular allogeneic transplants. HLA-DR matching has been shown to be an important determinant of short and long-term renal transplant outcomes [312]. MHC Class II molecules are also prime determinants of the strength of T-cell stimulation by antigen-presenting cells (APC) in *in-vitro* systems such as the mixed leukocyte reaction (MLR) [313,314].

In non-human primate (NHP) research colonies, animals may originate from a limited pool of families, and exhibit restricted genetic diversity. Thus it is essential to confirm sufficient immunological disparity between the potential donor-recipient pairs. MLR is a standard method of assessing alloreactivity but is time-consuming and must be repeated for each potential donor-recipient pair. Other techniques applied to NHP species to characterise MHC genes or gene expression include the use of typing sera, restriction fragment length

polymorphism (RFLP), polymerase chain reaction (PCR)-based amplification and separation by denaturing gradient gel electrophoresis, or cloning and sequencing[307,315-319].

The common marmoset is emerging as a relevant pre-clinical model for immunological studies, and is also notable for the existence of naturally-occurring chimerism between placental twins (discussed in Chapter 1, section 4.2). The chromosomal location of marmoset MHC is at present unknown. Characterisation of *Caja*-MHC alleles has occurred by methods such as PCR with generic primers based on human or other NHP sequences followed by sub-cloning, or PCR-SSCP (single strand conformation polymorphism) analysis then direct sequencing [307,308,320]. Human serological trays to identify MHC antigens have not been successfully reactive in marmosets [307]. In contrast to other members of the *Callitrichidae* family and Old World monkeys, marmosets have a restricted MHC repertoire [194,321] (full sequence alignments can be found at www.ebi.ac.uk/ipd/mhc/nhp/align.html). Only two Class I genes have been identified, *Caja*-E (2 alleles) and *Caja*–G (5 alleles). No other classical HLA Class I type genes have been found. *Caja*-DRA1 is monomorphic (as in all primates), *Caja*-DQA1 has 2 alleles and *Caja*-DQB1 has 6 alleles, and no functional *Caja*-DP genes have been described. The main site of Class II polymorphism lies within *Caja*-DRB, where only three loci have been identified. Exon 2 of the three known DRB loci – *Caja*- DRB1*03 (11 alleles), *Caja*-DRB*W16 (16 alleles) and *Caja*-DRB*W12 (monomorphic) – have been previously sequenced [307,308,320]. Currently, the gold-standard in human HLA genotyping for the purpose of donor-recipient pairing for transplantation is sequence-based allelic genotyping, using primers specific for polymorphisms unique to different loci and alleles. In this study, the novel application of this technology to marmoset MHC DRB loci, using primers designed for marmoset alleles is discussed. The data presented relates to one of the largest marmoset colonies sequenced thus far, including the identification of two new *Caja*-DRB*W16 alleles,

and the presence of genetic chimerism. This work also demonstrates for the first time in the marmoset that matching at MHC Class II *Caja*-DRB loci accurately predicts the presence of *in-vitro* alloreactivity as assessed in MLR.

2. METHODS

Full methods are described in Chapter 2. Forty nine animals were sequenced. The parentage and sibling relationships of each animal were known, however DNA was not available for all deceased parent animals. There were eleven twin pairs in the colony, six of which were able to be grouped into three families. For two-way mixed lymphocyte reaction (MLR), pairs of marmosets were chosen based on DRB genotype matching of varying degree. One of the fully matched pairs was a set of twins; all other animals within each pair were unrelated.

3. RESULTS

3.1 *Caja*-DRB genotyping

MHC-DRB genotyping was obtained for 49 animals. Fourteen alleles including two previously unreported *Caja*-DRB*W16 alleles were identified in this population (IMGT/MHC-NHP Database nomenclature: *Caja*-DRB*W1623 and *W1624). Full nucleotide sequences for these alleles are available through GenBank (www.ncbi.nlm.nih.gov/Genbank; accession numbers DQ340289 and DQ340290). Deduced amino acid sequence alignments are shown in Figure 1. *Caja*-DRB*W1201 was present in all animals. Allelic frequencies for *Caja*-DRB*W16 and *Caja*-DRB1*03 are shown in Figure 2.

Five upstream primers (see Chapter 2, section 3.2.1, Table 1) were used to sequence all *Caja*-DRB*W16 alleles, spanning codons 5-11 (primers 1-3) and codons 11-16 (primers 4 and 5). Amplification with *Caja*-DRB*W16 primers 1, 2 and 3 resolved all alleles in subsequent sequencing in the majority of cases. However, in some cases sequence analysis and allelic typing was difficult due to the deletion of codon 78 in some alleles (see Figure 1), leading to shift of sequence if the second allele lacked this deletion. This problem most commonly arose if *Caja*-DRB*W1605 was present in combination with either *Caja*-DRB*W1609, *Caja*-DRB*W1610 or *Caja*-DRB*W1623. Two further upstream primers (DRB*W16 primers 4 and 5) were subsequently designed to preferentially amplify *Caja*-DRB*W1605 or *Caja*-DRB*W1609, *W1610 and *W1623 if the presence of these alleles was suspected, thereby clarifying the sequence data. *Caja*-DRB*W16 alleles were not able to be identified in one twin pair, in spite of repeated attempts at sequencing using different primers. *Caja*-DRB*W1605 was the most frequent allele identified, present in 38 of 49 animals.

In total, 26 different genotypes were observed (Table 2), indicating a degree of genetic diversity in this population. Fourteen genotypes were unique to one animal, and 12 were common to more than one animal. Half (18 / 36) of the shared genotypes occurred in twins or siblings from the same family, and the remainder were shared by unrelated animals. Of the 11 pairs of twins in the colony, all had identical *Caja*-DRB1*03 genotype, and only 2/11 pairs had differing *Caja*-DRB*W16 genotype. In contrast, 4 of 9 sibling (but not twin) pairs were different at one of these loci, and one pair differed at both loci.

Figure 1. Alignment of predicted amino-acid sequences for exon 2 of Caja-DRB alleles. (-) denotes identity with HLA-DRB1*010101, (*) denotes codon deletion, and blanks represent unavailable sequence. New allelic sequences identified in this study are indicated in bold type.

```
                          10         20         30         40         50         60         70         80         90
HLA-DRB1*010101  *RFLWQLKFE CHFFNGTERV RLLERCIYNQ EESVRFDSDV GEYRAVTELG RPDAEYWNSQ KDLLEQRRAA VDTYCRHNYG VGESFTVQRR
Caja-DRB1*0301   *---EYSTS- ---------- -F-D-YFF-- --NL------ --F------- -----S---- --F--D---E ---V-----E ILDR-L-P--
Caja-DRB1*0302   *---EYSTS- ---------- -----Y-H-- --NL------ --F------- -----S---- --F--D---- ---F-----E IS-R-L-P--
Caja-DRB1*0303   *---EYSTS- ---------- -F-D-YFF-- --NL------ --F------- -----S---- --F--D---- ---F-----E ILDR-L-P--
Caja-DRB1*0304   *---EYSTS- ---------- -F-D-YFF-- --NL------ --F------- -----S---- ---------E ---F-----E ILDR-L-P--
Caja-DRB1*0305   *---EYSTS- ---------- -----Y-H-- --Y------- ---------- -----S---- --F------E ---------E IS-R-L-P--
Caja-DRB1*0306   *---EYSTS- ---------- ---D-YFF-- --L------- --F------- -----S---- --F--D---- ---F-----E IS-R-L-P--
Caja-DRB1*0307   *---EYSTS- ---------- -F-D-YFF-- --NL------ --F------- ---------- ---------E ---------E IS-R-L-P--
Caja-DRB1*0308          TS- ---------- -F-D-YFF-- --NL------ --F------- -----S---- --F--D---A ---V-----E ILD
Caja-DRB1*0309          TS- ---------- -F-D-YFF-- --NL------ --F------- -----S---- --F--D---E ---F-----E ILD
Caja-DRB1*0310          TS- ---------- -F---Y-H-- --Y------- --Y------- -----S---- --F--D---A ---F-----E ILD
Caja-DRB1*0311          TS- ---------- -F-D-Y-F-- --NL------ --F------- -----S---- ---------A ---F-----E ILD
Caja-DRB*W1201   *---E-F-Y- -LHL----G- ---V-Y---- --Y------- --F------- --A---F--L --YM--T--- ---V------ IF-R-L-***
Caja-DRB*W1601   *-Y-E-V--- --H------- -Y---YF--- --Y------- ---------- ---------- --I---K--- --R*-----E -F-T-L-P--
Caja-DRB*W1604   *-Y-E-A-C- --H------- -Y---YF--- --NL------ ---------- ---------- --I---K--- --R*-----E IS-R-L-P--
Caja-DRB*W1605   *-Y-E-V--- --H------- -----R-H-- --YA------ ---------- ---------- ---------- ---------E ILDR-L-P--
Caja-DRB*W1606   *---E-V-Y- --H------- -F-F-D---- --VL------ --F------- ---------- --I--D---- ---V-----E ILDR-L-P--
Caja-DRB*W1607   *---E-V-Y- --H------- -F-F-D---- --VL------ W-F------- ---------- --I--D---- ---V-----E ILDR-L-P--
Caja-DRB*W1608   *-Y-E-H-A- --H------- -F-D-YF--- --VL------ ---------- --S--KL--- --I---T--E --R*-----E IF-T-L-P--
Caja-DRB*W1609   *-Y-E-A-C- --H------- -Y---YF--- --Y------- ---------- ---------- --I---K--E --R*-----E -S-R-L-P--
Caja-DRB*W1610   *-Y-E-A-C- --H------- -Y---YF--- --Y------- ---------- ---------- --I---K--- --R*-----E -S-T-L-P--
Caja-DRB*W1611   *-Y-E-R-A- --H------- -----YF--- --F------- --F------- --V--S---R --I------- ---F-----E GF-T-L-P--
Caja-DRB*W1612   *-Y-E-A-C- --H------- -Y---YF--- --L------- ---------- --S--KL--- ---------E ---V-----E IS-R-L-P--
Caja-DRB*W1613   *-Y-E-V--- --H------- -----R-H-- --YA------ ---------- ---------- --I------- ---------E ILDR-L-P--
Caja-DRB*W1614   *-Y-E-A-C- --H------- -Y---YF--- --L------- ---------- --S--KL--- ------K--E --R*-----E -S-R-L-P--
Caja-DRB*W1615   *-Y-E-V--- --H------- -----R-H-- --YA------ ---------- ---------- ---------E ---------E IS-R-L-P--
Caja-DRB*W1616   *-Y-E-A-C- --H------- -Y---YF--- --Y------- ---------- ---------- ------T--E --TV-----E IF-
Caja-DRB*W1617   *-Y-E-A-C- --H------- -Y---YF--- --Y------- ---------- ---------- --I---K--- --R*-----E IF-
Caja-DRB*W1618   *-Y-E-V--- --H------- -----R-H-- --YA------ ---------- ---------- --I---K--E --R*-----E -S-
Caja-DRB*W1623         A-C- --H------- -Y---YF--- --Y------- ---------- ---------- --I---K--- --R*-----E -S-R-
Caja-DRB*W1624         A-C- --H------- -Y---YF--- --Y------- ---------- ---------- ---------E ---V-----E ILDR-
```

Figure 2. Frequencies (%) of *Caja*-DRB alleles in the tested cohort of 49 animals.

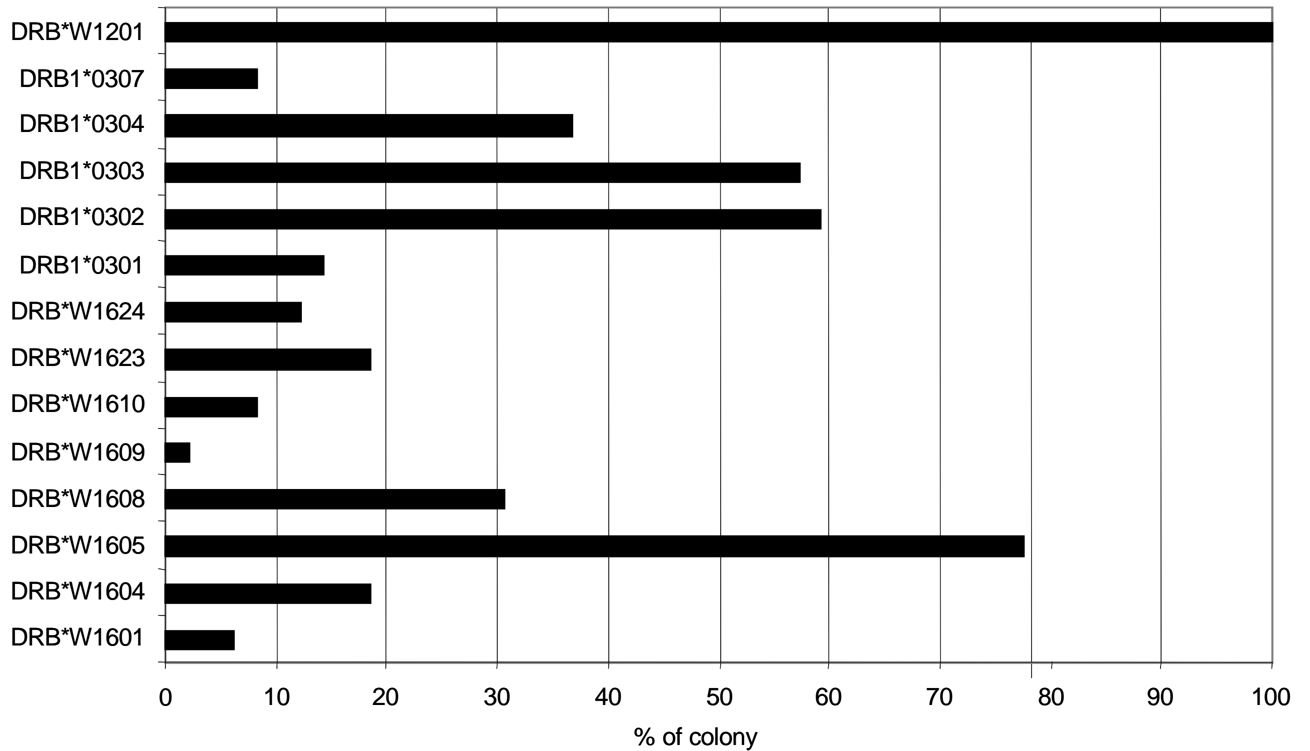


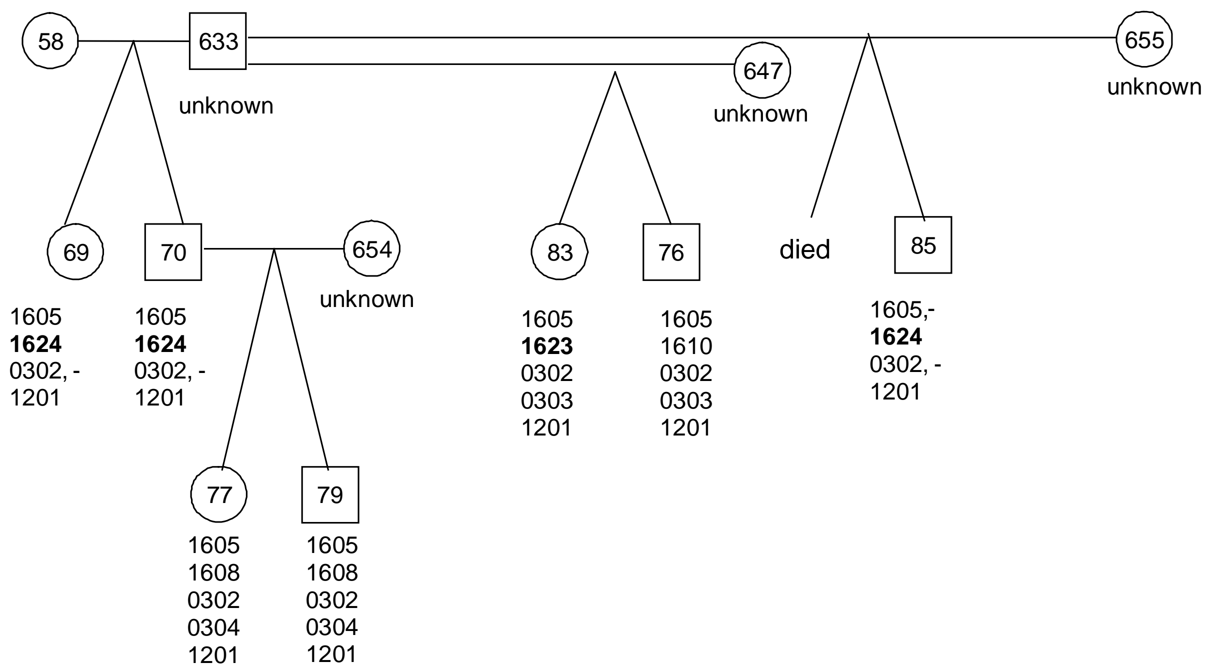
Table 2. Caja-DRB genotypes sequenced from n= 49 animals. Alleles are listed without the appropriate prefixes [*Caja*-DRB*W] or [Caja-DRB1*]. Related animals are either co-twins, non-twins siblings or offspring. The last 14 genotypes listed were each found in one animal only.

Genotype	Related (n)	Unrelated (n)
1605 / 0302 / 0303 / 1201	5	0
1605 / 1624 / 0302 / 1201	3	2
1605 / 1608 / 0302 / 0304 / 1201	2	3
1604 / 0302 / 0303 / 1201	2	2
1605 / 1623 / 0303 / 1201	2	0
1601 / 1605 / 1608 / 0301 / 0304 / 1201	2	0
0303 / 0307 / 1201	2	0
1604 / 1605 / 0302 / 0303 / 1201	0	3
1623 / 0303 / 0304 / 1201	0	2
1605 / 1623 / 0303 / 0304 / 1201	0	2
1605 / 1623 / 0302 / 0303/ 1201	0	2
1605 / 1608 / 0303 / 0304 / 1201	0	2
1608 / 0301 / 0307 / 1201		
1605 / 1610 / 0303 / 1201		
1605 / 1610 / 1608 / 0303 / 0304 / 1201		
1605 / 1610 / 1608 / 0303 / 1201		
1604 / 0303 / 0304 / 1201		
1604 / 0302 / 0304 / 1201		
1605 / 1610 / 0302 / 0303 / 1201		
1605 / 1624 / 0301 / 0302 / 1201		
1601 / 1623 / 0301 / 0303 / 1201		
1605 / 1608 / 0301 / 0307 / 1201		
1605 / 1608 / 0304 / 1201		
1605 / 0302 / 0304 / 1201		
1605 / 0301 / 0302 / 1201		
1605 / 1608 / 1609 / 0302 / 0304 / 1201		

Five animals exhibited chimerism (as seen by the identification of more than two alleles per locus) at *Caja*-DRB*W16. This group consisted of one twin pair (subsequently used in MLR), and three other animals for which sibling genotyping was not available due to lack of DNA. No chimerism at the *Caja*-DRB1*03 locus was noted.

The newly identified alleles *Caja*-DRB*W1623 and *Caja*-DRB*W1624 were confirmed by repeated sequencing, and were identified in twins, non-twin siblings and unrelated animals. An example of a family in which these alleles were identified is shown in Figure 3. *Caja*-DRB*W1623 represents a single nucleotide polymorphism, being one base different from *Caja*-DRB*W1609 at position 208, codon 74 (GAG→ GCG, Glutamic ac id → Alanine) and one base different from *Caja*-DRB*W1610 at position 250, codon 88 (ACG→AGG, Threonine → Arginine). *Caja*-DRB*W1624 is identical to *Caja*-DRB*W1609 (or *W1610) from codon 5 to 66, then is similar to *Caja*-DRB*W1612 except at position 244, codon 86 (TCA →TTA, serine → leucine) and position 248, codon 87 (GAG → GAC, glutamic acid → aspartic acid). This allele is also similar to *Caja*-DRB*W1617 except at positions 199 (codon 71), position 244 (codon 86) and position 248 (codon 87).

Figure 3. Distribution of *Caja*-DRB alleles within members of three families arising from marmoset 633. This is the largest family within the colony for which genotyping data was available. Circles represent females; squares represent males. Alleles identified in this study are shown in bold. Genotype was unknown for animals where genomic DNA was not available due to animal death prior to this project (animals 58, 633, 647, 655, 654).



3.2 Mixed Lymphocyte Reactions

Table 3 summarises the results of MLR for various animal pairs, and Figures 4 and 5 show representative examples of non-alloreactive and alloreactive cells. PBMC from animals matched for alleles at all three *Caja*-DRB loci did not induce T-cell proliferation when combined together in culture, whereas cells from those with partial (1-3 alleles matched) or complete mismatch consistently induced proliferation when co-cultured. Pair 3 of the group with complete matching were twins who exhibited chimerism (genotype *Caja*-DRB*W1601 / *W1605 / *W1608, DRB1*0301 / *0304 and *Caja*-DRB*W1201).

Figure 4 and Figure 5. MLR of PBMC from unrelated animals with identical (Fig. 4) or completely mismatched (Fig. 5) *Caja*-DRB genotyping. In matched pairs, no additional T-cell proliferation (expressed as counts per minute) above baseline was observed, whereas in mismatched animals, a proliferative response indicating alloreactivity was observed. Data are representative of 3 or more experiments.

Figure 4.

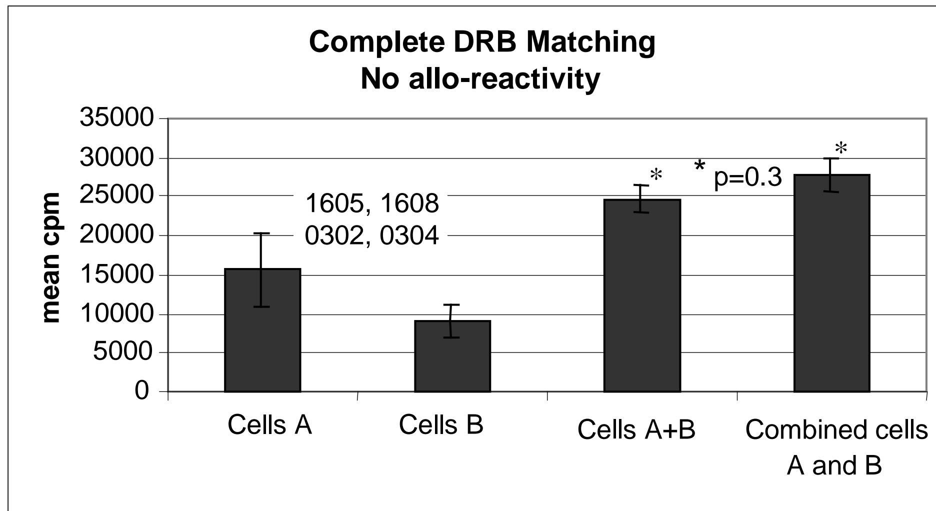


Figure 5.

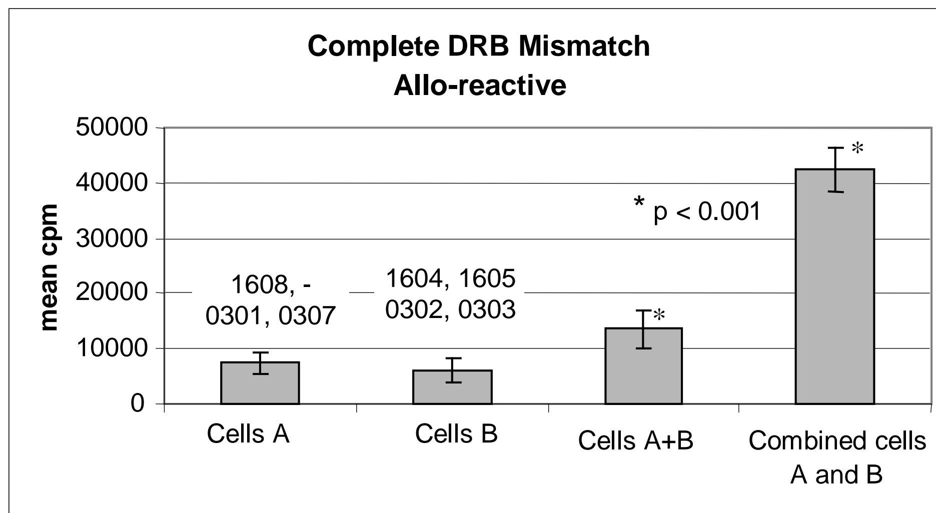


Table 3. Results of lymphocyte culture expressed as mean (of triplicate wells) counts per minute ± SD. The student's t test was applied to determine a significant difference between the sum of single cell counts for animal A and B, and counts for wells with cells from each animal combined together.

	Cells A	**Cells B**	**Cells A + Cells B**	**Combined cells A and B**	**p**
Full match					
1	15646 ± 4609	9035 ± 2102	24681 ± 1647	27824 ± 2208	0.3
2	2560 ± 229	5364 ± 601	7685 ± 464	8808 ± 892	0.13
3	9758 ± 768	8505 ± 2980	18263 ± 3602	22561 ± 2541	0.16
3 Allele match					
1	7778 ± 693	9952 ± 705	18071 ± 193	43132 ± 272	0.004
2	3378 ± 343	8231 ± 1196	10258 ± 1018	17093 ± 637	< 0.001
3	12483 ± 555	6078 ± 841	18561 ± 1390	27942 ± 1142	<0.001
2 Allele Match					
1	12061 ± 4477	8448 ± 1778	20509 ± 5682	45130 ± 9066	0.016
2	7778 ± 693	4262 ± 1020	12039 ± 1703	41919 ± 4311	<0.001
3	7250 ± 422	12483 ± 555	19733 ± 791	34355 ± 3428	<0.001
1 Allele Match					
1	9952 ± 705	4262 ± 1020	14770 ± 238	53644 ± 5591	0.003
2	11789 ± 2669	7474 ± 1996	20371 ± 562	45904 ± 9987	0.04
3	6078 ± 841	7250 ± 422	13328 ± 980	32338 ± 2594	<0.001
Complete Mismatch					
1	12495 ± 1882	11331 ± 1539	24423 ± 684	45459 ± 3428	0.004
2	18747 ± 1942	15376 ± 890	34122 ± 2160	44817 ± 5812	0.04
3	7474 ± 1996	6107 ± 2329	13581 ± 3501	42527 ± 4003	< 0.001
4	9581 ± 1732	29464 ± 1132	40082 ± 2400	73521 ± 6969	0.008
5	7511 ± 1771	4693 ± 817	12204 ± 2503	31915 ± 3770	< 0.001
6	3464 ± 869	9186 ± 157	12561 ± 837	26992 ± 1746	0.002

4. DISCUSSION

This work establishes a rapid method of *Caja*-DRB genotyping, employing a sequence-based technique that is currently used in human tissue typing for allogeneic transplantation. This technique provides high quality sequence data able to accurately differentiate alleles that may differ by only a few bases. Both forward and reverse strands are sequenced from a single PCR amplification of template genomic DNA. In order to maximise the chance of amplifying all *Caja*-DRB*W16 alleles and investigate the presence of new alleles we utilised several upstream primers for this locus. However, for future *Caja*-DRB typing, a single downstream primer and only 4 upstream primers (*Caja*-DRB*W16 primer 2 and 3, DRB1*03 and DRB*W1201) are required to identify all currently known *Caja*-DRB alleles. Exon 2 of *Caja*-DRB1*03 and *Caja*-DRB*W1201 can be amplified using separate reaction mixes within a single PCR run as the cycling conditions are identical. Due to the incorporation of M13-tails in the primary PCR product, universal primers that recognise these tails can be used in the subsequent sequencing PCR, negating the need for locus-specific sequencing primers. This sequencing method facilitates high through-put processing. The system allows analysis of large numbers of samples once optimal conditions have been determined, thereby enabling typing of an entire colony in a relatively short space of time.

In this study we have also shown that *Caja*-DRB allelic typing is an accurate predictor of *in-vitro* alloreactivity as assessed by two-way MLR. In this species, MHC-DRB typing appears to be a reliable substitute for MLR, enabling identification of appropriate donor-recipient pairs for use in transplantation studies. In particular, DRB typing abrogates the need to establish alloreactivity between each different animal pair, saving time and labour. This study is one of

the first to compare Class II matching according to sequence-based allelic typing with MLR measure of allo-responses in the NHP. In human transplantation, the MLR was used as an early assay of histocompatibility, particularly of the Class II DR region which has the predominant influence on allo-stimulated T-cell proliferation [314,322]. Several studies of human donor-recipient pairs (primarily for bone marrow transplantation) undertook DRB matching based on tissue typing techniques such as serotyping [313,314,323,324] or DNA-restriction fragment length polymorphism [324]. These methods failed to accurately predict negative MLR responses in up to 50% of cases, largely due to limitations in identifying immunologically relevant allelic subtypes. Matching according to more specific PCR-based DNA typing techniques (DNA heteroduplex analysis, PCR-sequence specific oligonucleotide typing) has been shown to better correlate with the incidence and degree of proliferative response in MLR, although still do not necessarily predict non-reactive MLR in all cases [314,323,324]. With the advent of highly accurate sequence-based typing techniques which are able to resolve single nucleotide polymorphisms that may lead to significant HLA protein changes, the MLR is no longer clinically applied.

However, MLR remains a useful technique for choosing donor-recipient pairs in traditional NHP transplant models such as rhesus and cynomolgus macaques. The rhesus MHC has been studied in detail. Compared to humans, rhesus macaques have a larger number of DRB loci per haplotype (up to 7), with extensive haplotype polymorphism and less allelic polymorphism [316,317]. In this species, non-DRB1 mismatches in particular lead to higher relative proliferative responses in MLR, as well as a Th-1-skewed cytokine response[325]. Significant advances have recently been made in the characterisation the MHC-DRB of cynomolgus monkeys. Blancher *et al*. used PCR, denaturing gradient gel electrophoresis and direct sequencing to identify 50 *Mafa*-DRB exon 2 sequences, and 20 haplotypes involving 1-6 DRB genes [315]. In animals who

exhibited the ability to respond in MLR, significant proliferation was seen in 98.5 % of pairs with DRB incompatibility. Although the MLR is still routinely used in macaque transplantation studies, advances such as sequence-based typing will facilitate detailed typing of research colonies and improve the accuracy of pre-transplant matching.

Our colony of 49 animals is one of the largest to undergo genotyping of all 3 *Caja*-DRB loci. We confirmed the presence of 12 previously reported and two newly identified alleles forming 26 different genotypes. These results confirm a degree of genetic diversity in our Australian-based marmoset colony, although other studies have found 21 alleles in up to 35 marmosets [307,308]. *Caja*-DRB*W1605 was the most common allelic polymorphism, present in 77.5% of the colony, despite limited inbreeding. This is slightly different to the findings of Antunes *et al.* who also observed *Caja*-DR*W1605 to be the most common allele at this locus, but only in 9 of 24 (37.5%) animals [308]. In contrast, Wu *et al.* identified this allele in only 3 of 18 marmosets [307]. In general, the allelic distribution and frequencies in our population and these two groups differ considerably from each other. This is likely to be a reflection of the distant geographical location of each colony (Australia, The Netherlands and Japan) and different breeding histories and animal sources. Information such as this is useful as it allows colony managers to select animals that will enhance the genetic diversity of their colonies.

Our study confirms the observation that marmosets have a low degree of MHC-DRB polymorphism, particularly in comparison to other New World primates such as the cotton-top tamarin [320,326] and Old World primates, such as the Rhesus macaque [315-317]. Most of the variability lies within the *Caja*-DRB*W16 locus, and is due to single point mutations that almost always lead to amino-acid changes, or recombination between polymorphic regions of alleles. An example of the latter is *Caja*-DRB*W1624, a new allele identified in this study,

where there appears to have been recombination between *Caja*-DRB*W1609 or *W1610 and *Caja*-DRB*W1612 at some point within the highly conserved sequence in codons 39-56.

It has been suggested that marmosets do not exhibit haplotype polymorphism [194] i.e. the number of DRB genes or loci does not vary between individuals, as is the case in other NHP and humans. Antunes *et al.* found that 17 animals tested for all three DRB loci appeared to share the monomorphic *Caja*-DRB*W1201, and possessed alleles for the other genes [308]. Our findings were similar for 47 of 49 animals studied. Interestingly, in one twin pair we could not identify the *Caja*-DRB*W16 locus, despite repeated sequencing attempts using different primers and conditions. In addition, using locus specific primers Wu *et al*. were unable to identify *Caja*-DRB*W1201 in all animals in their study, despite being able to show universal inheritance of this locus in two families where it was present [307]. These findings suggest the possibility that not all marmosets exhibit all three DRB loci. Using our rapid, high-throughput technique it will be relatively straightforward to examine other colonies which may clarify the issue of haplotype polymorphism.

A further interesting feature of *Callitrichidae* is that they are natural bone-marrow chimeras. The majority are born as dizygotic twins, and share the placental circulation via vascular anastamoses, leading to chimerism within bone-marrow and other haematological organs, and possibly the germ line [202,206]. Sex chromosome studies have confirmed that a large proportion (up to 80%) of peripheral blood lymphocytes in an individual animal may be derived from its co-twin [202,207,208]. This appears to lead to functional immune tolerance, as shown by lack of response in mixed culture of lymphocytes from co-twin tamarin monkeys [209], and a similar lack of allo-stimulation in the one twin pair with detectable peripheral blood chimerism from our colony. This chimerism is a mechanism for expansion of the otherwise restricted MHC

repertoire. For example, one animal in the series from Antunes *et al.* [308] possessed eight *Caja*-DRB alleles, when only five should be present. The same study observed chimerism of *Caja*-DRB1*03 in 4 of 27 animals (15%), and of *Caja*-DRB*W16 in 2 of 24 animals (8%). Wu *et al.* found more than two alleles for *Caja*-DRB1*03 in 11 of 18 animals (61%), and for *Caja*-DRB*W16 in 5 of 18 animals (28%). They also found that fraternal twin pairs (n=3) had identical alleles, although data for non-twin siblings was not available. In comparison we found considerably less chimerism, with only 10% of animals having more than two alleles for *Caja*-DRB*W16 and none for *Caja*-DRB1*03. The 11 twin pairs in our study almost universally had identical *Caja*-DRB genotype, whereas sibling pairs were less likely to be identical. Marmosets are most commonly dizygotic twins; therefore the degree of genetic similarity within twin pairs is higher than that expected from Mendelian inheritance of alleles alone. One possible explanation is that some animals are homozygous for one allele, but more than one allelic polymorphism is identified due to the presence of cells originating from the co-twin.

In conclusion, using *Caja*-DRB allele-specific sequence based genotyping, immunologically reactive donor-recipient marmoset pairs can be rapidly and accurately identified. This information will facilitate the development of this preclinical model for future studies of cellular and solid organ transplantation.

5. ADDENDUM

5.1 Additional Data Regarding Marmoset MHC

At the time this study was conducted (2004-2005), 11 *Caja*-DRB1*03 alleles and 16 *Caja*-DRB*W16 alleles had been reported. However, subsequently 6 additional alleles have subsequently been reported to the Immunopolymorphism database (including the two described here) [321]. In addition, a study of marmoset cDNA and exon 2 transcripts has been published by R. Bontrop's group, which has provided additional data regarding marmoset MHC [327]. *Caja*-DRB*W16 is the most polymorphic locus and produces abundant functional protein transcripts. *Caja*-DRB*W12 was present on every haplotype, with moderate gene product detectable. A second *Caja*-DRB*W12 allele was identified, with three single nucleotide polymorphisms, two codon changes and two amino acid changes. *Caja*-DRB1*03 was found to be a pseudogene with low (but often detectable) and imperfect protein transcription of all but two alleles (0307 and 0312) [327]. Exon 2 of these alleles aligns with DRB1*03, but the remaining exons align with DRB*W16, representing exon-shuffling which facilitates functional protein expression. *Caja*-DRB1*03 has similarities to HLA-DRB1*03 due to an EYSTS motif that is highly conserved throughout primate species, and is important for mycobacterial defence. Exon-shuffling and recombination are therefore important mechanisms for increasing the MHC repertoire in this species that has a highly contracted MHC.

5.2 Sequencing of Other *Caja*-MHC Loci

The sequencing method developed in this study has subsequently been extended to characterise marmoset Class I (G and E), DQA1, DQB1 and DQB2 genes. Matching at these

additional loci does not consistently influence *in-vitro* alloreactivity, and is difficult due to the minimal polymorphism observed in our colony (Dieu Dang, Honours Thesis, 2007, University of Adelaide). Therefore DRB matching remains the most practical and reliable method of choosing alloreactive pairs.

CHAPTER 4: *IN-VITRO* PROPAGATION OF MARMOSET DC FROM PERIPHERAL BLOOD PRECURSORS

1. INTRODUCTION

As discussed in Chapter 1, dendritic cells are potent antigen presenting cells capable of directing immune responses towards either immune activation or tolerance. DC manipulation and immunotherapy has been well-demonstrated to promote allograft survival and potentially induce long-term donor-specific tolerance. The rarity of DC *in-vivo* has led to the development of strategies for propagating myeloid DC in large numbers *in-vitro* from human and murine peripheral blood (PB) and bone marrow (BM) precursors[12,32,75-77,328]. Using *in-vitro* culture systems employing an array of cytokines, DC precursors may be differentiated into committed DC subsets that have many similarities (although always not identical) to *in-vivo* DC. Several of these techniques have now been successfully applied to non-human primate (NHP) species [278-286], and a sizeable body of literature describing NHP *in-vitro* DC now exists. However, data regarding common marmoset DC remains limited to one recent study by Ohta *et al* [228]. This study was notably the first description of *in-vitro* propagated DC from marmoset BM and monocyte precursors, however obtaining these precursors required killing of animals to obtain sufficient cell numbers from the entire PB volume or BM.

This issue of low PB cell yields from such a small animal is a major technical hurdle that needs to be overcome in order to develop the marmoset as a successful model for DC therapy in transplantation. In particular, methods for generating sufficient DC from minimally invasive samples such as PB need to be developed to enable the same allogeneic DC donor to survive and be available for repeat DC donation or subsequent organ harvest for transplant. This is

easily achievable in rhesus macaques and other larger primates (weighing 3-10 kg), as 10-40 ml blood samples can yield hundreds of thousands or even millions of DC [278,280]. In marmosets who weigh only 300-400g, the PB volume is at best 10-12mls (as obtained following cardiac puncture at autopsy), and a maximum 1-2ml sample can be safely taken without haemodynamic compromise or anaemia developing. To optimise the yield of DC precursors from such a small sample of PB, haematopoietic growth factors such as G-CSF and GM-CSF can be used to mobilise blood stem cells and monocytes and increase DC yield (reviewed in Chapter 1). G-CSF most likely re-distributes progenitor cells from BM into the circulation, and prolongs progenitor lifespan rather than promoting cell proliferation [94]. G-CSF is highly effective when used alone, but it has been shown to act in synergy when in combination with FLT3-L [94] or GM-CSF [93] to induce even higher yields of monocytes and haematopoietic precursor cells. G-CSF has previously been used successfully in marmosets to induce haematopoietic progenitor cells [221] but has not been used specifically to assist with DC generation.

The aim of this study was to propagate for the first time marmoset myeloid DC *in-vitro* from PB monocytes or haematopoietic precursors (stem cells) mobilised with the haematopoietic growth factor G-CSF. Culture systems were adapted from protocols described for human DC propagation to promote the generation of large numbers of DC-like cells from a small sample of peripheral blood from this small primate species. The phenotype and function of these cells were characterised and compared to human DC from G-CSF mobilised donors.

2. METHODS

Protocols for animal maintenance and blood sampling, reagents and media, cell separation and culture, flow cytometry, endocytocis assay and morphological assessment are described in Chapter 2.

Marmoset PB monocytes and stem cells were mobilised with recombinant human (rh)G-CSF, (Lenograstim, AMGEN Corporation, USA) at a dose of 10-15ug/kg/day given subcutaneously for 5-7 days. Thirty eight mobilisations were conducted in 10 animals over 3 years. During this time, no adverse effect of G-CSF was noted. Repeat mobilisation was well tolerated.

Human PB was obtained after institutional ethics approval from two healthy human donors from the Allogeneic Stem Cell Transplant Program at the Royal Adelaide Hospital, Adelaide, prior to and on Day 5 of G-CSF treatment.

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3. RESULTS

3.1 G-CSF Mobilises Marmoset Monocytes and CD34^{+} Cells

Automated leukocyte counts were performed in 19 healthy animals, 7 animals after G-CSF and 3 animals at multiple time points during G-CSF mobilisation (Table 1 and Figure 1). G-CSF treatment produced a rise in total PB white cell count (WCC) from 5.5 ± 1.09 (mean ± SD) pre-G-CSF to 24.3 ± 6.1 x10^{9}/L post-G-CSF, predominantly due to a rise in neutrophils. G-CSF also caused a 2-26 fold-rise in monocyte count, from 0.1 ± 0.11 to 0.9 ± 0.4 x10^{9}/L, (range 0.0-0.33 pre-G-CSF to 0.3-1.79 x10^{9}/L post-G-CSF), although the contribution of monocytes to the total WCC remained stable (2.7 ± 1.18 %). Peak monocyte numbers

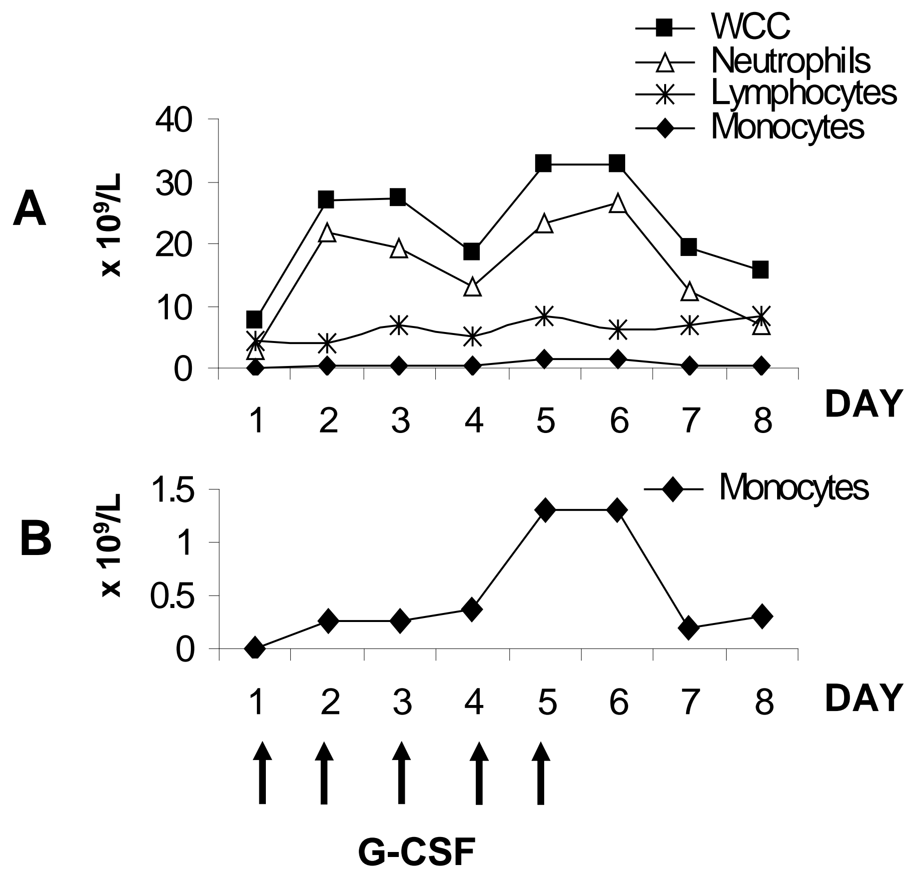
occurred on days 5-7 after starting G-CSF, and therefore we routinely collected monocytes at these time points.

The PB stem cell population was assessed by flow cytometric labeling of $CD34^+$ cells. At baseline < 0.5% of peripheral blood cells (from whole blood) were $CD34^+$, rising to 2.0-8.1% following G-CSF, which represents a 4-16 fold rise. Following processing, 2.9±1.5 % of mononuclear cells were $CD34^+$ (range 1.9-4.6%, n=3).

Table 1. Change in marmoset leukocyte subsets following 5 days of G-CSF administration; cell counts $x10^9$/L by automated cell count.

	Baseline (n=19)		Day 6 (n=7)	
	Mean ± SD	Range	Mean ± SD	Range
WCC	5.5 ± 1.09	3.5-7.5	24.3 ± 6.1	15.6-32.8
Neutrophils	2.2 ± 0.68	1.2-3.85	17 ± 6.3	10.5-26.6
Lymphocytes	3.1 ± 0.70	1.9-4.5	6.7 ± 2.9	4.6-12.8
Monocytes	0.1 ± 0.11	0.0-0.33	0.9 ± 0.4	0.3-1.79

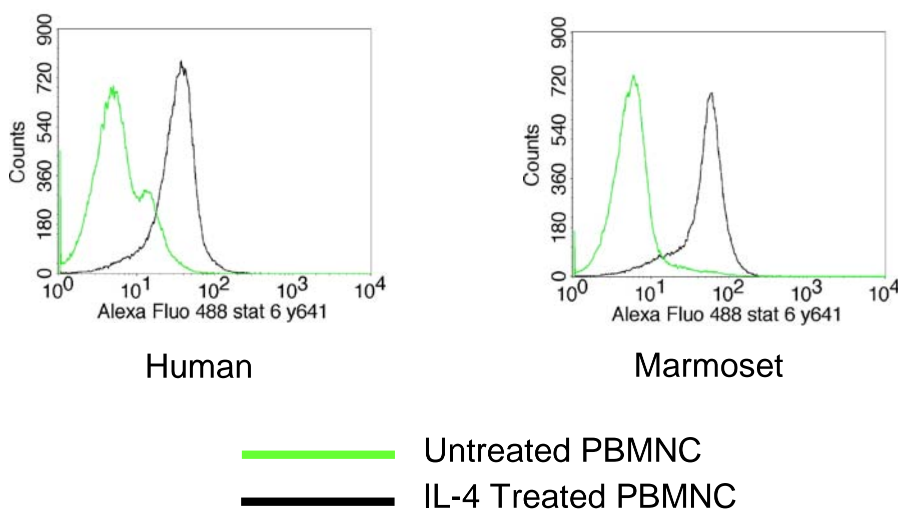
Figure 1. Leukocyte mobilisation with G-CSF treatment (data for one animal, representative of n=3). WCC: peripheral blood white cell count.



3.2 Human IL-4 Leads to Signalling Via STAT-6

Impaired cross-reactivity of human cytokines on NHP biological samples can be responsible for failed experiments. The biological response of marmoset bone marrow progenitor cells to GM-CSF *in-vitro* has been well established[200,221]. Human anti-IL-4 antibody and rhIL-4 have previously been shown to be cross-reactive on marmoset cells and supernatants[329,330]. To confirm the functional activity of rhIL-4 on marmoset cells, we assessed intracellular signaling via the STAT-6 pathway. Both marmoset PBMC and monocytes had STAT-6 signaling response similar to human cells when stimulated by rhIL-4 (Figure 2).

Figure 2. Intracellular staining with Alexa Fluor® 488 Anti-Phospho-Stat6 (Y641) antibody in marmoset and human PBMC with and without IL-4 stimulation. PBMC from both species show signalling in response to rhIL-4. Similar results were obtained using isolated monocytes (not shown).



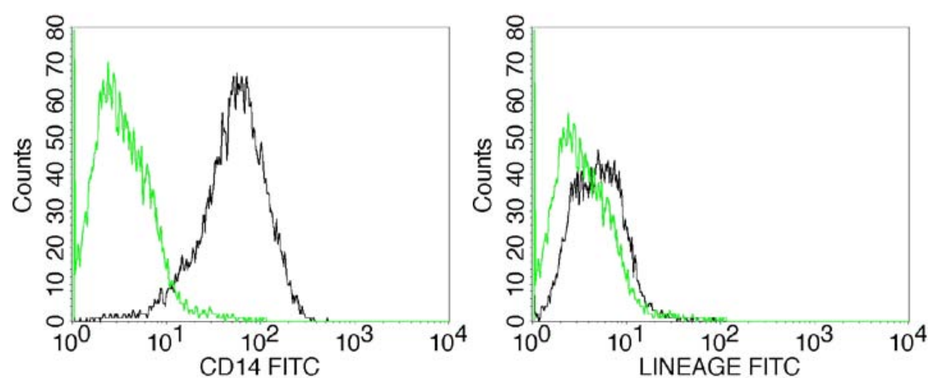
3.3 Marmoset Monocyte-Derived DC

3.3.1 Selection of Marmoset Monocytes for DC Differentiation

The use of anti-$CD14^+$ immunomagnetic beads and Automacs® separation produced unsatisfactory and inconsistent monocyte yield and purity as assessed by cell morphology and flow cytometry. Several types of microbeads and protocols for monocyte selection from PBMC or whole blood were assessed over the course of many experiments (data not shown for brevity). The purity of the positively selected fraction ranged from 50-80%, which was inadequate for the purpose of DC differentiation, without further monocyte purification. Frequently, contamination of the positively selected fraction by lymphocytes and/or erythrocytes or neutrophils was observed, as assessed by flow cytometry forward and side scatter profile, negative CD14 expression and morphology on Giemsa stain of cytospun samples (data not shown). Several explanations for this poor result are possible. Marmoset neutrophils express CD14, and although this expression is weak, some neutrophils may be positively selected. Other factors such as red blood cell haemolysis within whole blood samples may have contributed to poor or non-specific binding of microbeads.

In addition, subsequent survival of immunomagnetically isolated monocytes in culture was variable and overall quite poor. DC did not differentiate from monocytes isolated by this method, despite conducting over 30 experiments with modifications to bead type, separation method, serum type, cytokines and other culture conditions (data not shown). Microbead binding *per se* did not affect cell survival, as monocytes that were first incubated with microbeads then isolated by plastic adherence had excellent survival (data not shown).

In contrast, the plastic adherence method reliably and consistently produced excellent monocyte isolation from PBMC. As assessed by flow cytometry, > 95% of adherent cells were $CD14^{+}$, and < 2% were positive for other lineage markers as shown below: (flow cytometry fluorescence intensity histograms for CD14 and Lineage Cocktail, data representative of a typical isolation, green lines represent isotype controls, black lines represent antibody fluorescence).



Monocytes selected by this method had excellent survival in culture. Therefore, the plastic adherence method was adopted as the best method for monocyte selection in all subsequent experiments.

3.3.2 G-CSF Mobilised MoDC Have Typical DC Morphology

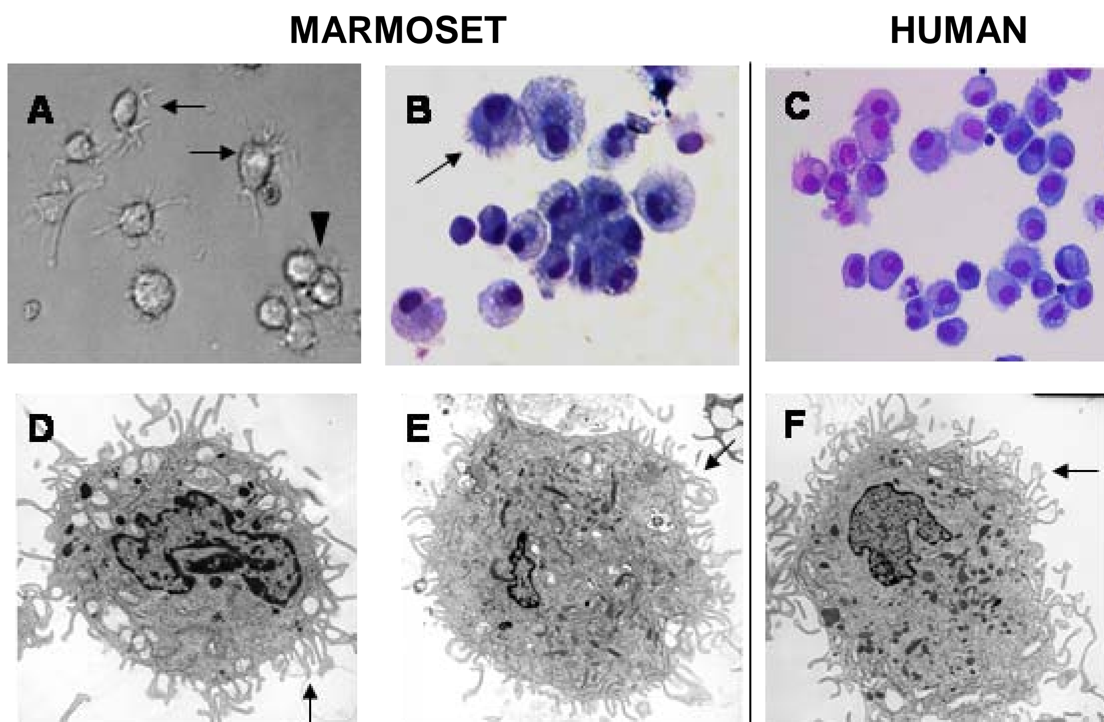
Human MoDC propagated from the same donors before and after treatment with G-CSF were similar and had typical features of MoDC in terms of morphology, phenotype and function (data presented below).

Marmoset MoDC were propagated from both unmobilised (n>20 experiments) and G-CSF mobilised (n>20 experiments) monocytes. However, as the final putative DC yield per ml of

unmobilised PB was less than 1-2x10^5, the majority of experiments used monocytes from G-CSF mobilised blood. This gave a yield of 0.8-1 million putative DC/ml of PB, sufficient for further characterisation studies.

Marmoset monocytes had a uniform round appearance with reniform nucleus and were tightly adherent to plastic. Within 3-4 days of culture in IL-4/GM-CSF, marmoset monocytes developed DC-like morphology, becoming larger, less uniform and non-adherent, with obvious dendritic processes on some cells (Figure 3A). Clumping of stellate cells was also observed. Some cells remained adherent, with branching, spreading appearance; these were presumed to be macrophage-like cells that did not differentiate to DC. Giemsa stain of cytospins of MoDC revealed a large proportion of the non-adherent population had lost their monocytoid appearance and developed typical dendritic morphology with large, eccentric nuclei and hairy cytoplasmic projections (Figure 3B). Marmoset MoDC had a very similar light and electron microscopy appearance to human MoDC propagated under similar conditions, (Figures 3C-F). Marmoset MoDC morphology did not differ appreciably after maturation stimuli (data not shown, similar to Figure 3).

Figure 3. Morphology of marmoset and human MoDC differentiated from G-CSF mobilised monocytes. (A): Light microscopy of marmoset MoDC in culture; 400x magnification. (B): Giemsa stain of cells shown in 2A; 400x. (C): Giemsa stain of human MoDC; 200x. (D-F): Transmission electron microscopy of MoDC; 12-18,000x. Marmoset MoDC are characterised by large eccentric nuclei and cytoplasmic projections or veils (black arrows) and clumping of cells in culture (solid arrow). Immature and maturation-stimuli exposed marmoset MoDC had similar appearances.



3.3.3 Marmoset MoDC Have an Immature Phenotype And Are Maturation Resistant

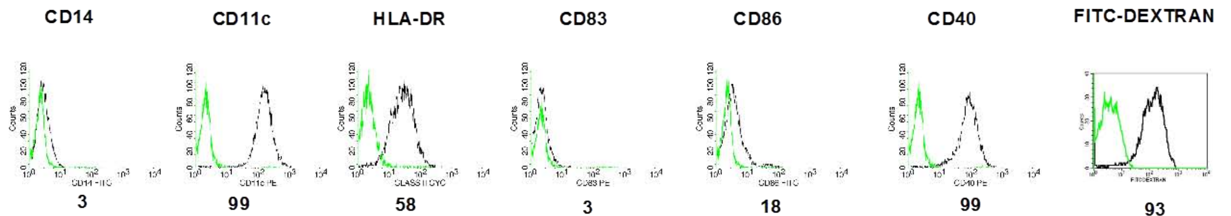
The surface marker expression of marmoset MoDC was compared with human MoDC from G-CSF mobilised donors (Figure 4A and B). Unlike human MoDC, marmoset MoDC had high CD14 expression, which was not down-regulated with maturation stimuli (71.1±19.1 % CD14 positive vs. 66.9±19.6 % after maturation stimuli, $p=0.69$). Over 60% of marmoset MoDC co-expressed CD14 and CD11c at day 7 (not shown). DC-SIGN (CD209) was minimally expressed on marmoset MoDC (<2 %), although we have previously shown DC-SIGN expression on marmoset lymphoid tissue resident DC and freshly isolated BM cells (Kireta, Prasad, Coates *et al*., unpublished data). Marmoset MoDC did not have significant CD1a expression, however, this may be due to lack of cross-reactivity of anti-human antibody with marmoset cells rather than lack of expression.

Marmoset MoDC showed an immature phenotype, with low expression of co-stimulatory molecules CD86 and CD40 and minimal CD83 expression (Figure 4B). This lack of phenotypic maturation was consistently observed despite multiple experiments involving exposure to a variety of maturation stimuli including TNF/ PGE2, LPS, CD40L, calcimycin and MCM. Other strategies tested in this study included shorter (4 day) and longer (11 day) culture periods, higher doses of IL-4 (80ng/ml), rhIL-4 from bacterial-cell expressed and human-cell expressed sources, and rhIL-13 which may have additional maturation effects on DC [82]. None of these various manipulations produced mature DC. In contrast, human G-CSF MoDC showed typical phenotypic changes after exposure to TNF-α. (Figure 4A) including down-regulation of CD14, and up-regulation of HLA-DR, co-stimulatory molecules and CD83. These changes were identical to those observed in MoDC propagated from the same donors prior to G-CSF treatment (data not shown, similar to Figure 4 data).

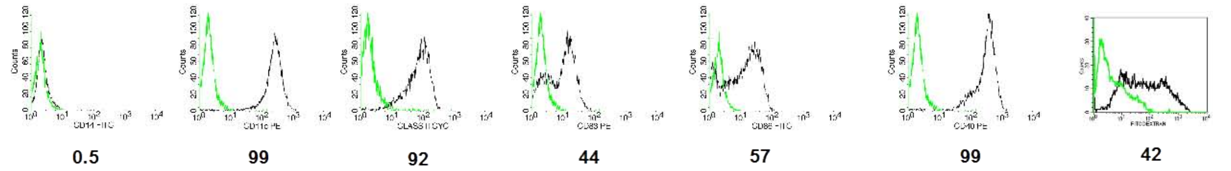
Figure 4. Flow cytometric expression of surface markers on human MoDC (Panel A; n=2), marmoset MoDC (Panel B; without maturation, n=10; with maturation stimulus, n=12), marmoset HP cultured for 2 weeks in FLT3-L, SCF and TPO (Panel C; n=3) and HP after further culture in IL-4/GM-CSF ± maturation (HPDC; Panel C; n=3). Exogenous antigen uptake (FITC-DEXTRAN) is also shown (data from 1-3 experiments). The histograms show representative data from one experiment; numbers below represent the mean % positive cells ± SD from all marmoset experiments, or the mean only where there were less than three experiments. Green lines represent isotype controls, black lines represent antibody fluorescence. The % positive cells for each molecule were not significantly different after maturation of marmoset MoDC with any stimulus (Panel B, all p values > 0.05).

A. HUMAN MODC

IL-4/GMCSF

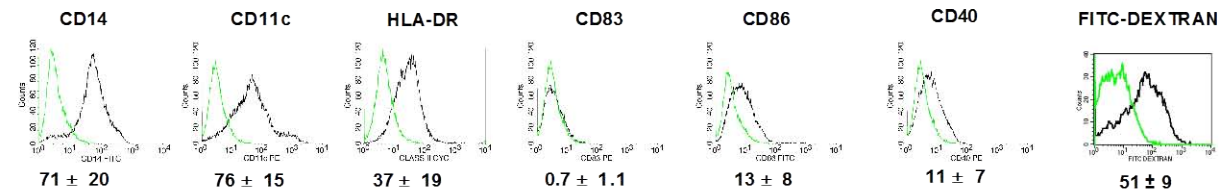


IL-4/GMCSF + TNFa

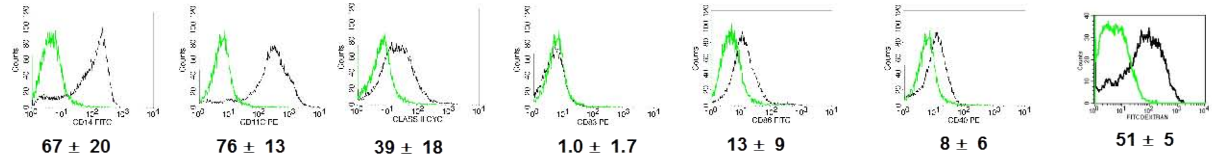


B. MARMOSET MODC

IL-4/GMCSF

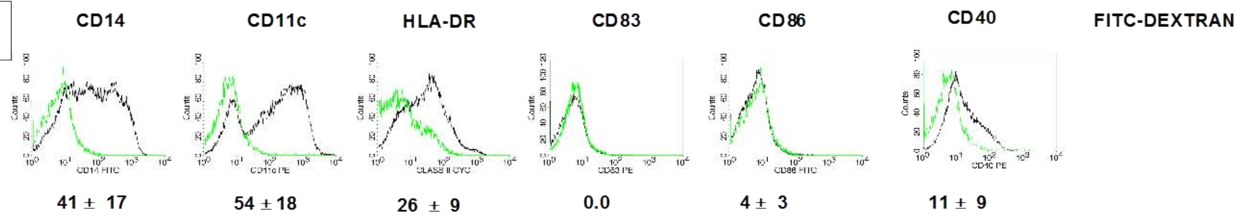


IL-4/GMCSF + TNFa

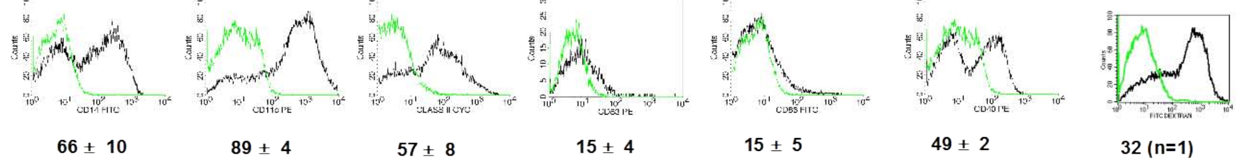


C. MARMOSET HPDC

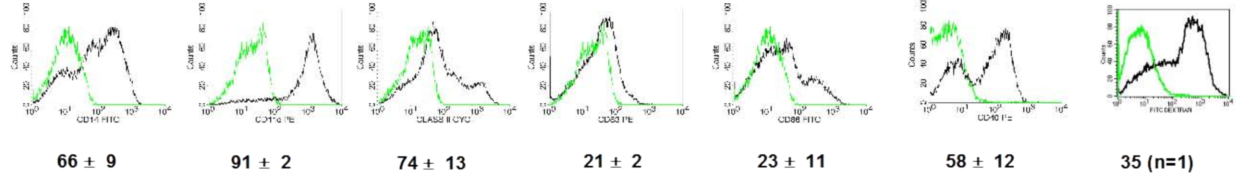
Wk 2 HP



+ IL-4/GM-CSF (HPDC)

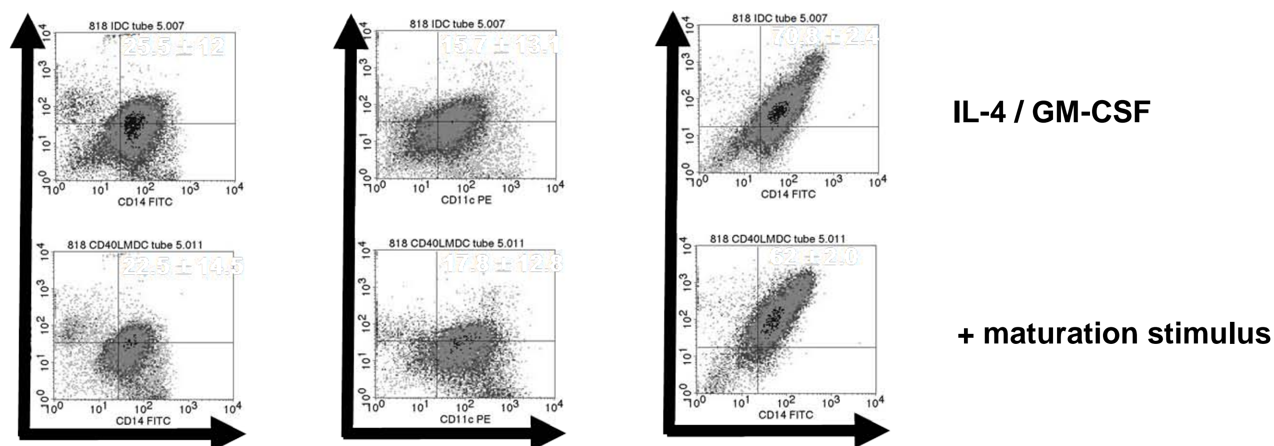


+ IL-4/GM-CSF + TNF-a (HPDC)



Fluorescence Intensity

Figure 5. Co-Expression Of Myeloid Surface Markers On Day 7 Marmoset MoDC Cultured In IL-4 / GM-CSF With Or Without Maturation Stimuli (Various Stimuli Tested). Density plots show data from one representative experiment; numbers represent the mean % of double positive cells ± SD from 12 experiments.



Marmoset MoDC retained moderate ability for antigen (Dextran) uptake, although this was less than undifferentiated monocytes (51.1 ± 9.3 % of DC vs. 70 ± 4.2 % of monocytes; p=0.02). No difference in endocytic capacity was seen after maturation stimuli (Figure 4), unlike human G-CSF MoDC which had a 50 % reduction in antigen uptake with TNF-α. (93 vs. 42%).

Due to the cell yields from small peripheral blood samples in marmosets, MLR using bulk PBMC rather than purified T-cells was performed. 3 day MLR did not allow for sufficient proliferation (data not shown). Four and 5 day MLR did allow time for proliferation. Five day MLR was chosen as this was the standard method used in our laboratory and in other NHP models for DC-stimulated MLR. Responder and stimulator ratios were also chosen based on standard ratios used in DC-stimulated MLR in humans, with the aim being to directly compared marmoset and human DC cultured and handled identically. In MLR against allogeneic PBMC in a 1:10 ratio, marmoset MoDC significantly stimulated proliferation above baseline (p<0.001, data not shown), and produced a higher stimulation index (SI) than undifferentiated monocytes (Figure 6A). However, the allo-stimulatory response was weak, with SI generally 2-3 fold above baseline, and no consistent difference was observed between immature DC and DC exposed to maturation stimuli. At lower DC:PBMC ratios (1:50 or less), marmoset DC had minimal stimulatory effect, with mean SI 1.37 ± 0.29 (Figure 6B). In comparison, human MoDC in allogeneic MLR produced proliferation > 7 fold above baseline, increasing further after maturation with TNF-α (Figure 7). Human MoDC remained allo-stimulatory in a dose-dependent fashion up to 1:1000 ratio. In xenogeneic MLR using human PBMC as responder cells, marmoset MoDC induced significant proliferation above baseline, with higher stimulation produced by TNF-α exposed DC observed in one experiment (Figure 8). However, allostimulation remained lower than that observed in human DC MLR.

Therefore it can be concluded that marmoset MoDC are less potent stimulators in MLR compared with human DC, when stimulating either marmoset or human PBMC.

From these data we conclude that human MoDC from G-CSF mobilised donors have a typical profile, whereas marmoset MoDC propagated under these conditions have an immature surface marker and functional phenotype, and are resistant to standard maturation stimuli.

Figure 6A. Stimulation Index Produced By Marmoset MoDC and HPDC in Allogeneic MLR (mean ± SD).

MoDC data includes MLR data from all experiments with no stimulation (n=12) and with various stimuli: TNF (n=12), TNF+PGE2, LPS, CD40L and Calcimycin (n=1 each). HPDC data includes MLR data from all experiments with no stimulation (n=6) and with various maturation stimuli: TNF (n=5), LPS (n=4), TNF+PGE2 and CD40L (n=1 each).
* $p<0.01$, All DC types had significantly higher SI compared with monocytes.
** $p<0.01$; unstimulated HPDC had higher SI compared with unstimulated MoDC.
*** $p<0.001$; stimulated HPDC had higher SI compared with stimulated MoDC.

Figure 6B. Allostimulation Induced By Marmoset MoDC (representative of n=2 experiments). The DC:PBMC ratio is shown on the x axis.

Figure 6C. Allostimulation Induced By Marmoset HPDC (representative of n=2 experiments).

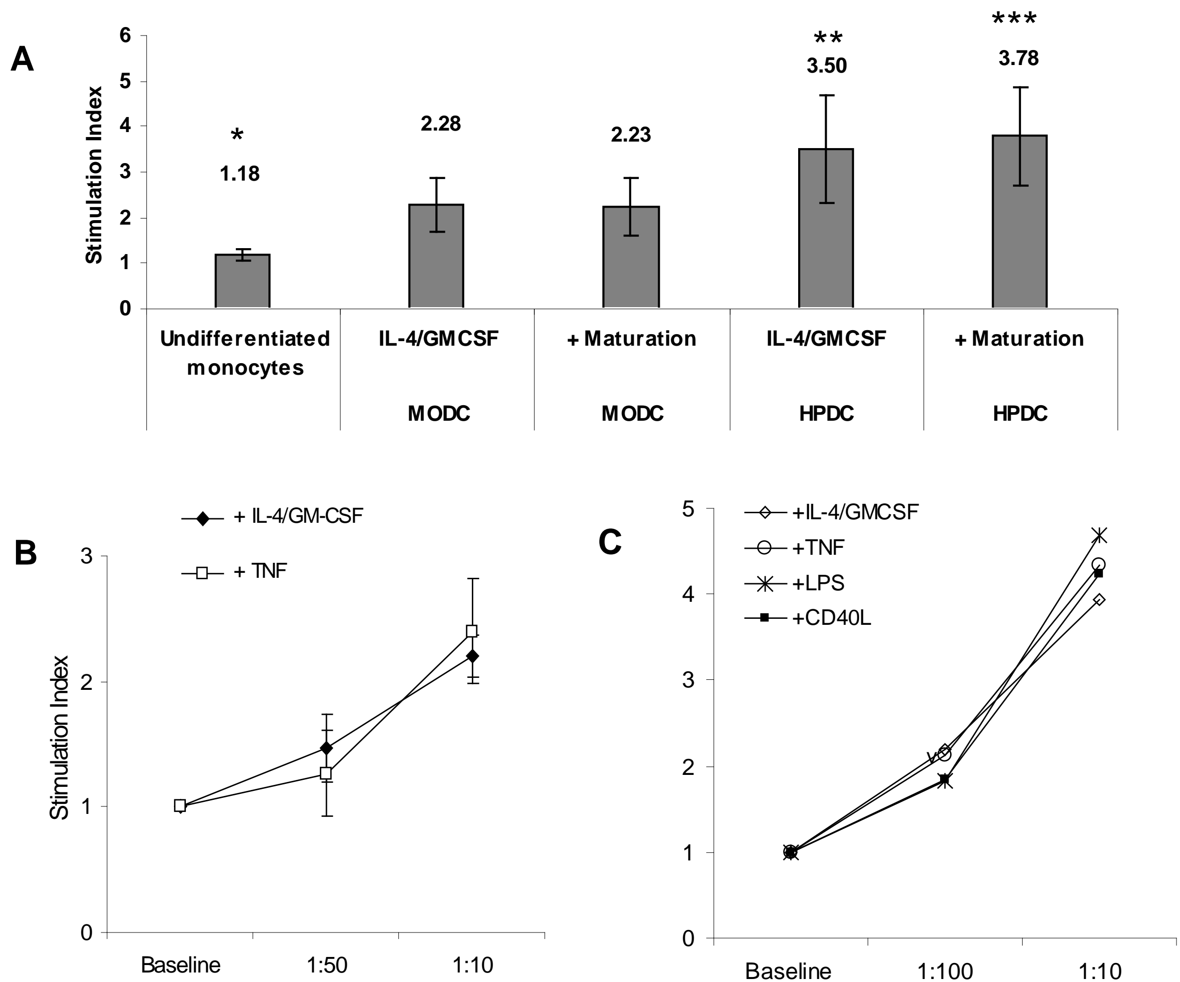
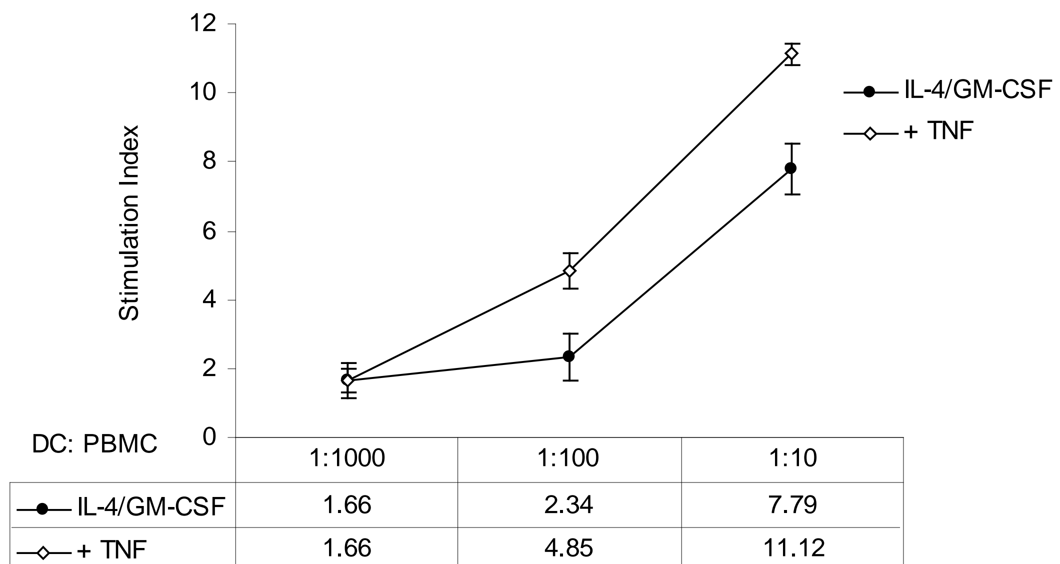
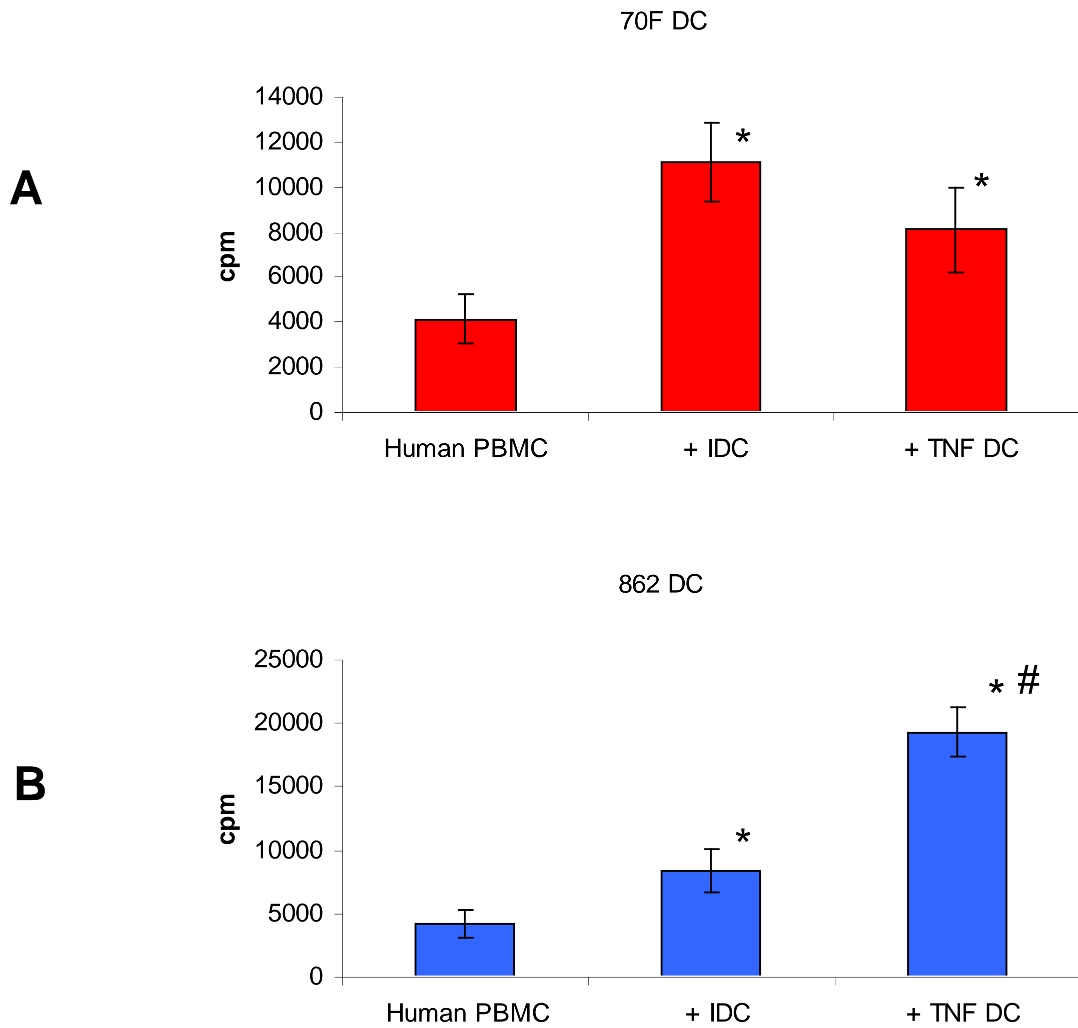


Figure 7. Human MoDC Stimulation Index In Allogeneic MLR. Matured MoDC had higher allostimulation above baseline compared to immature DC ($p<0.05$). Stimulator (DC) to responder (PBMC) ratios are shown on the x axis. Data representative of n=2 experiments.



DC: PBMC	1:1000	1:100	1:10
IL-4/GM-CSF	1.66	2.34	7.79
+ TNF	1.66	4.85	11.12

Figure 8. Xenogeneic MLR Using Marmoset G-CSF Mobilised MoDC (immature DC or TNF-α stimulated DC) as stimulator cells and human PBMC as responders in a 1:10 ratio, showing thymidine incorporation expressed as counts per minute (cpm). Panel A shows DC from marmoset 70F causing significant proliferation above baseline, with no difference in stimulatory ability between immature and TNF-α stimulated DC. Panel B shows allostimulation by DC from marmoset 862, where TNF-α stimulated DC produced higher proliferation. * $p<0.05$ compared to baseline Human PBMC cpm; # $p< 0.05$ compared to immature DC; Student's t-test.



3.4 Haematopoietic Precursor-Derived DC (HPDC)

3.4.1 Culture in Early-acting Cytokines Expands a Myeloid HP Population

The yield and purity of immunomagnetically isolated $CD34^+$ haematopoietic precursors (HP) was low (n=2). Although these cells were able to be expanded and differentiated as outlined below, in view of a recent report suggesting improved DC yield from culture of bulk PBMC rather than purified stem cells[87], bulk PBMC from G-CSF mobilised PB (enriched for HP) were used for all further experiments (n=6).

G-CSF mobilised bulk PBMC were firstly cultured in a cocktail of early-acting cytokines (FLT3-L, SCF and TPO) known to promote the differentiation of DC precursors *in-vitro*[88]. This led to a marked expansion of HP in culture (Figure 9) occurring mainly during the second and third weeks. By week 3, cell numbers had increased 7.7 ± 1.8 fold above baseline (range 5.9-9.6, n=4), providing millions of precursors for further DC differentiation. In four experiments starting with 5 million PBMC or less, 13.25 ± 6.5million cells (range 10-23) were obtained by week 2 and 33.2 ± 15.5 million cells (range 16-46 million) by week 3. Proliferation ceased by the end of week 4, and cultures were discontinued.

The early increase in cell population (weeks 1-3) coincided with the appearance of confluent sheets of uniform, round, small cells (Figure 10A), which were monocytoid (Figure 10B). Few contaminating lymphocytes were identified. By weeks 3-4 in the same cytokines, some cells had developed DC morphology (Figure 10C); however, many undifferentiated precursors also remained (Figure 10D).

Figure 9. Expansion of Cell Numbers under the Influence of Early Acting Cytokines. (A): Cumulative fold-increase (mean ± SD) in cell numbers and (B): Absolute cell numbers (mean ± SD) from bulk PBMC culture in FLT3-L, SCF and TPO (n=4).

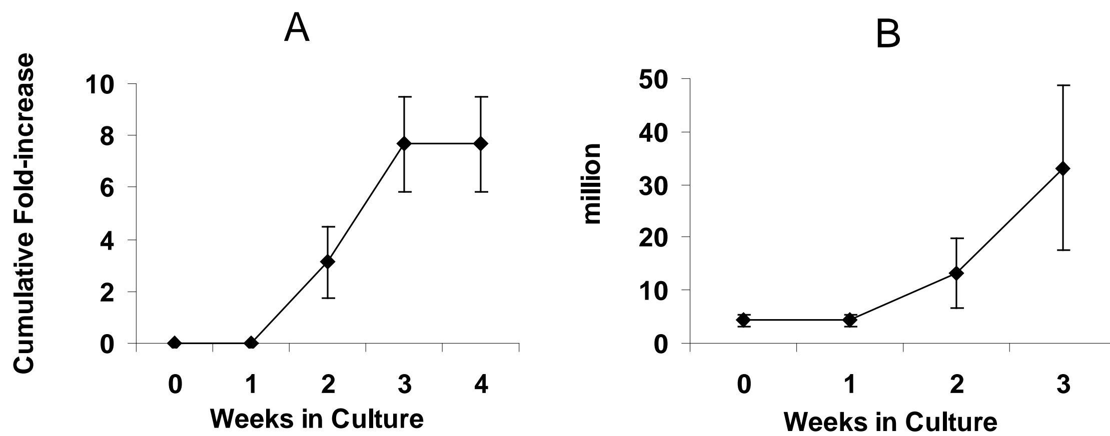
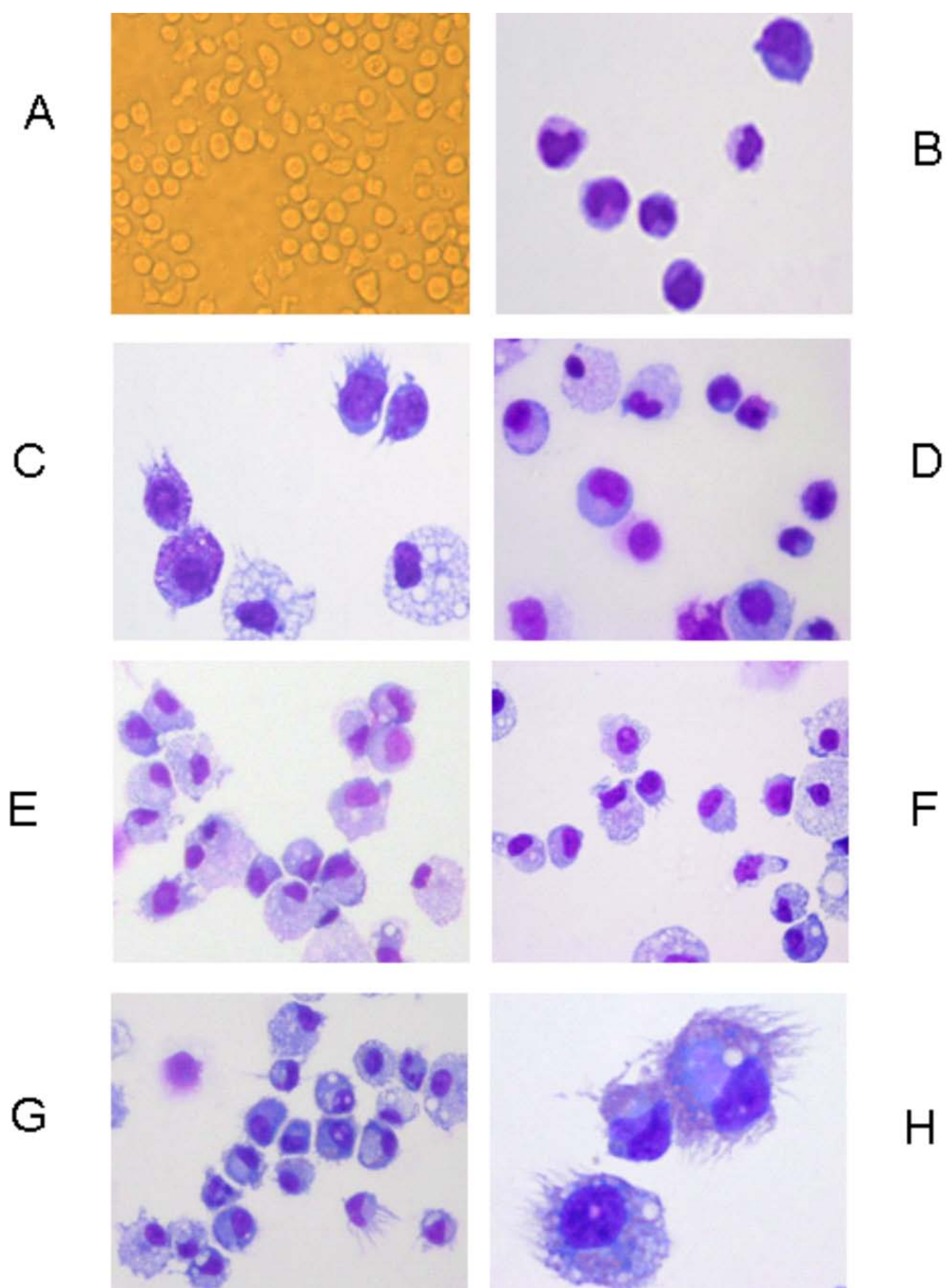
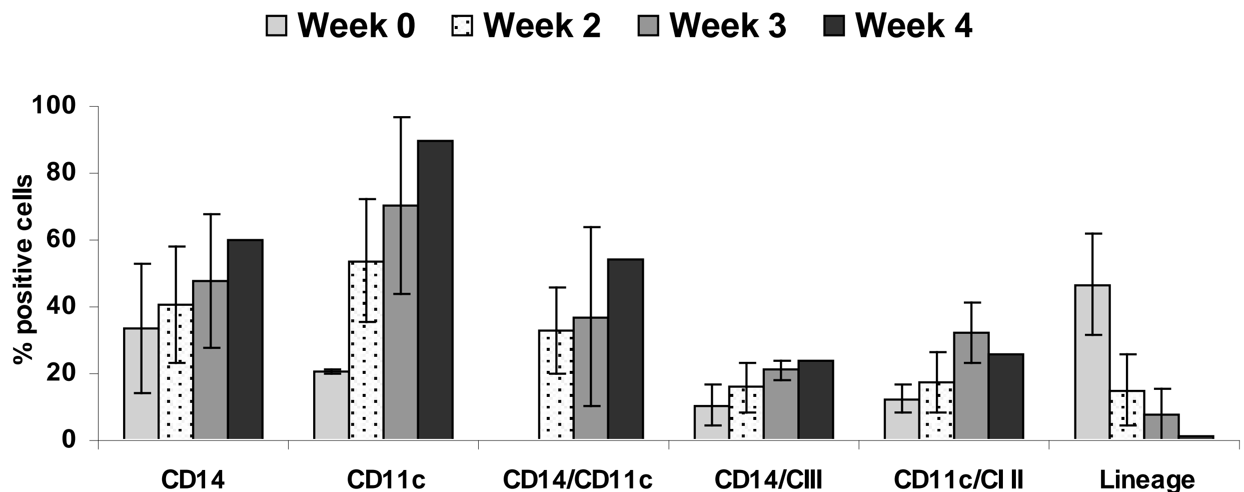


Figure 10. Morphology of G-CSF mobilised HP and HPDC. (A): HP after 2 weeks culture in FLT3, SCF and TPO; light microscopy in culture plate; 20x magnification. (B): Giemsa stain of cells from A; 20x. (C), (D): HP after 3-4 weeks culture; Giemsa stain, 40x. (E), (F): HPDC; Giemsa stain, 20x. (G): HPDC+LPS stimulation; Giemsa stain; 20x. (H): Cells from G; 40x.



The expanding HP cell population was of myeloid lineage, as determined by expression of myeloid markers (Figure 11). By week 3 of culture in early-acting cytokines, over 70 % of cells were $CD11c^+$, rising to 90 % by week 4. Similarly, the percentage of $CD14^+$ cells rose and over half of all cells co-expressed these markers by week four. Importantly, over the same time period cells positive for other lineage markers became negligible.

Figure 11. Expression Of Myeloid And Lineage Markers On HP During Four-Week Culture. Data shown is the mean±SD for n=3 experiments (except week 4, n=1).



3.4.2 HPDC Have a Semi-Mature Phenotype

Expanded myeloid HP were further induced to DC with IL-4/GM-CSF (HPDC) (n=3) and exposed to maturation stimuli TNF-α (n=3), LPS (n=2) and CD40L (n=1). This produced a DC-like appearance in a larger number of cells (Figure 10E-H). In particular, HPDC exposed to maturation stimuli had a more mature DC appearance with extensive hairy dendritic projections. This second differentiation step did not increase cell numbers, but promoted higher expression of myeloid markers (Figure 12A). As with marmoset MoDC, CD14 expression was persistent, the majority of HPDC expressed CD11c and over 50 % co-expressed both of these markers. Exposure to maturation stimuli (TNF-α, LPS or CD40L) did not alter CD14 or CD11c expression.

The mature DC marker CD83 had negligible expression in marmoset HP before exposure to IL-4 / GM-CSF (Figure 12B, Wk 2 HP). Of note, CD83 was expressed in 14.9 ± 3.5% of HPDC, and increased further after maturation with TNF-α (20.7±1.8%) or LPS (19.1±1.3%) (Figure 7B). This indicates a mature DC sub-population can be generated in this culture system.

A trend towards higher costimulatory molecule expression was observed as HP cultures in FLT3-L, SCF and TPO progressed (described in Figure 13). Co-stimulatory molecule expression was increased in HPDC (data displayed in detail in Figure 4, Figure 12B and Figure 13). CD86 was expressed on a higher percentage of HPDC compared with HP (3.9 ± 3.2% vs. 15.3 ± 4.8%; p=0.026,). In individual experiments a definite pattern of CD86 up-regulation in HPDC and further up-regulation with stimulation by TNF-α and LPS was observed, although the absolute percentages varied between experiments (representative data shown in Figure 13). Maturation with CD40L did not increase CD86. HP had low CD40

expression (10.9 ± 8.9%) compared to HPDC (48.7 ± 2.3 %; p=0.002) and HPDC+TNF-α (57.2 ± 11.5%; p=0.005). There was no significant difference in CD40 expression between HPDC and HPDC+TNF-α, however LPS stimulation led to lower CD40 expression (23.5 ± 4.9%; p=0.004 vs. IL-4/GMCSF; p=0.037 vs. TNF-α). The percentage of HLA-DR^+ cells also increased significantly in HPDC (from 26.0 ± 8.7% to 57.0 ± 7.5%, p=0.009), and further in HPDC+TNF-α (73.7 ± 13.1%, p=0.006). LPS maturation had a lesser effect on HLA-DR (45.5 ± 7.8%, p= ns vs. other cell types). HPDC differentiated from HP after 3 weeks in culture in early acting cytokines had a similar profile (data not shown).

Figure 12. Surface marker expression as assessed by flow cytometry on haematopoietic precursors after 2 week culture in FLT3-L, SCF and TPO (Wk 2 HP); after further culture of Wk 2 HP in IL-4 /GM-CSF (+ IL-4/GM-CSF; HPDC), and HPDC after maturation stimuli (+ TNF, + LPS and +CD40L). Panel A shows myeloid markers and Panel B shows co-stimulation molecules and the DC marker CD83. Data shown is the mean % positive cells ± SD for n=3 experiments (except CD40L stimulated cells, n=1).

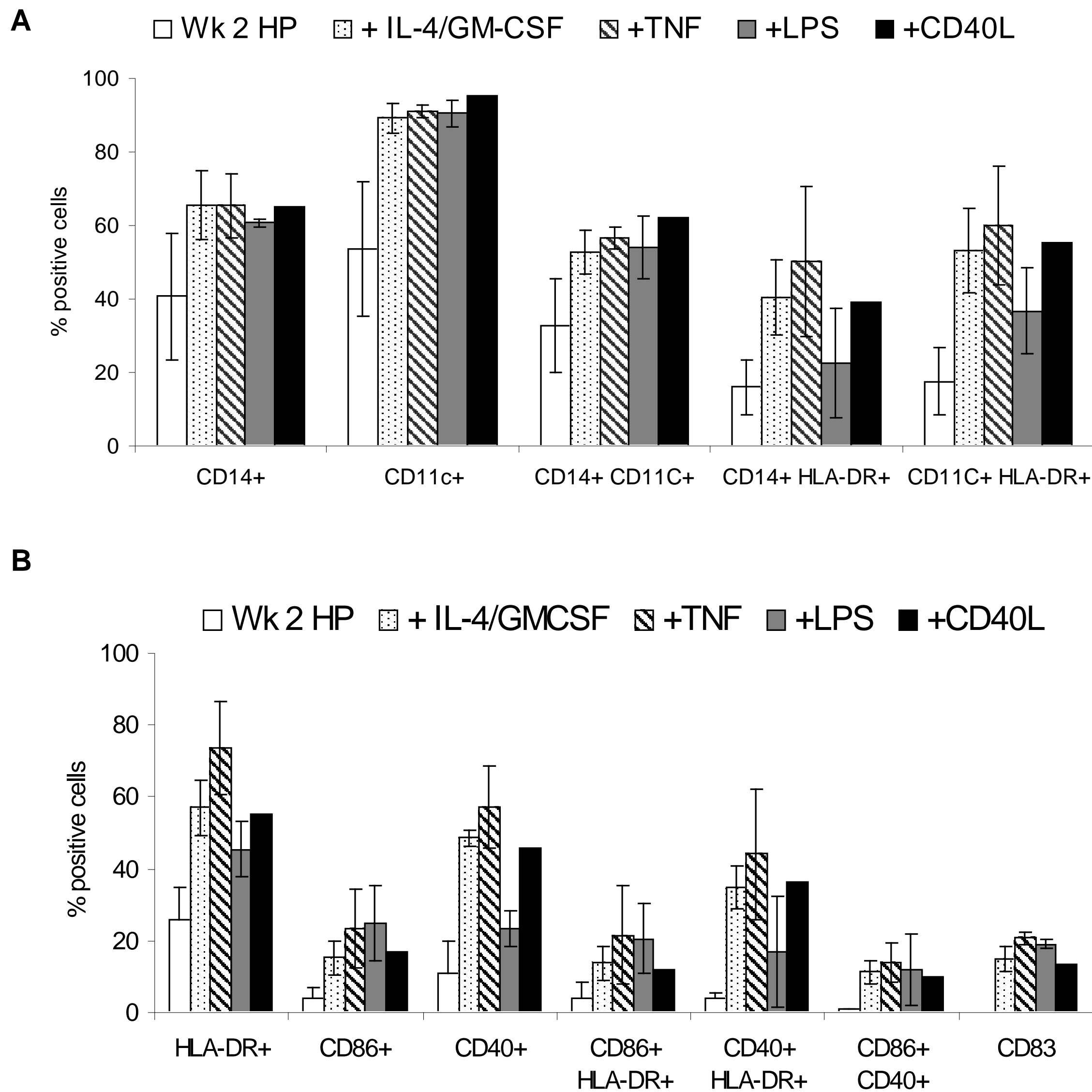
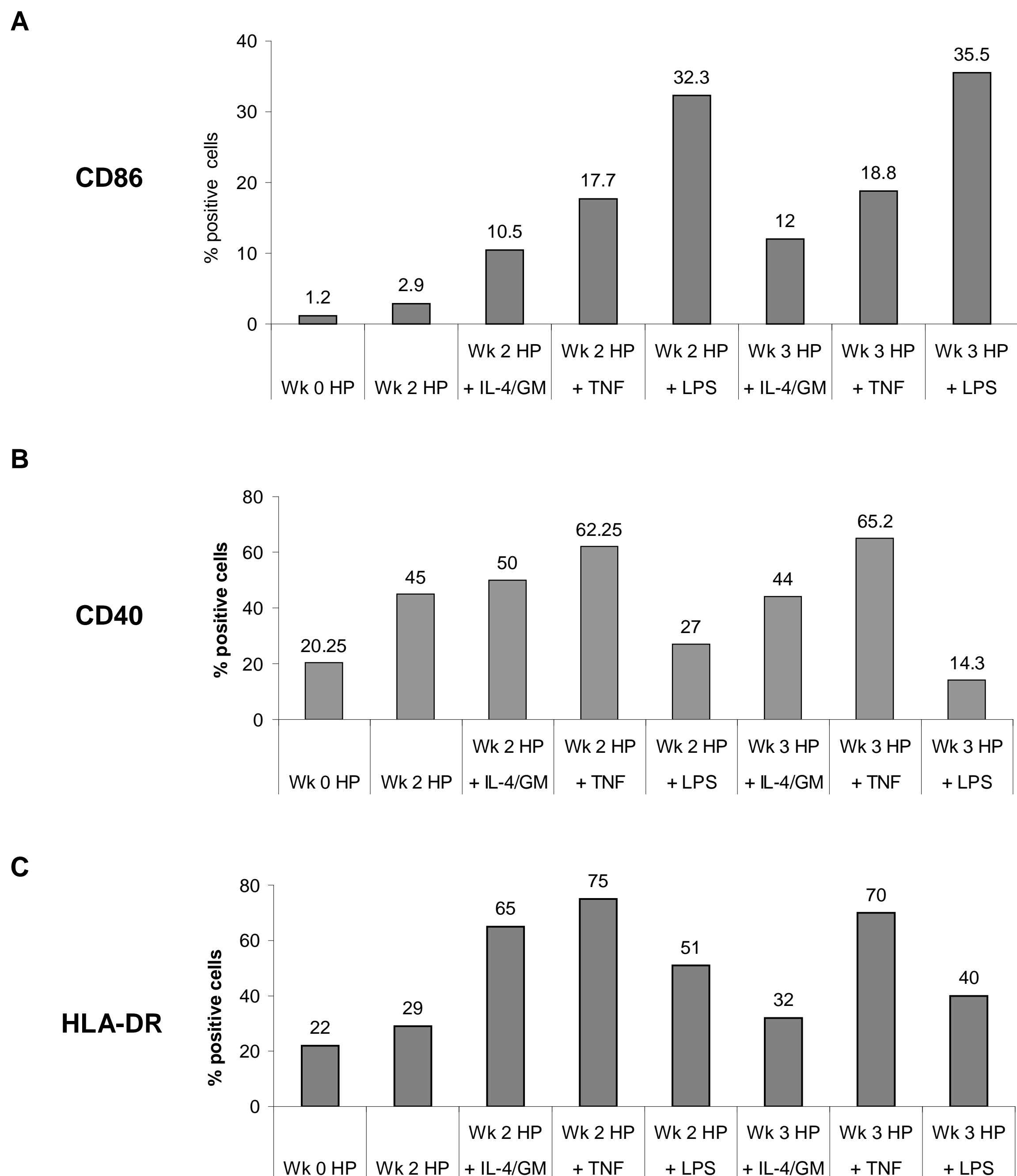


Figure 13. CD86, CD40 and HLA-DR expression on HP and HPDC ± TNF-α or LPS as shown. Data shown is representative of 3 experiments from three different animals.

(A): CD86 was up-regulated in HPDC and further with maturation

(B): CD40 was high in HP and increased further in HPDC. TNF-α produced a minor increase in $CD40^+$ cells in some experiments, whereas LPS stimulation down-regulated CD40.

(C): HLA-DR expression was increased in HPDC, and was particularly stimulated by TNF-α.



HPDC had lesser endocytic capacity compared to marmoset MoDC, with 32.5% of HPDC and 34.5% of HPDC+TNF-α showing FITC-Dextran uptake (Figure 4). This, along with the cell surface phenotype, suggests the cultures may contain populations of immature and mature DC.

In allogeneic MLR, HPDC had significantly higher allo-stimulatory capacity than undifferentiated monocytes and MoDC before and after exposure to maturation stimuli (Figure 6A). No consistent increase in allo-stimulatory capacity was seen in HPDC exposed to maturation stimuli. HPDC remained allo-stimulatory at lower DC: PBMC ratio than MoDC (Figure 6C).

In summary, marmoset HPDC had a more mature DC phenotype compared to MoDC, but complete maturation was not observed with any stimulus. Mature DC markers CD83, CD86, CD40 and HLA-DR were up-regulated by culture of HP in IL-4/GMCSF alone, and variably increased further with maturation stimuli. Antigen uptake was reduced though not completely lost and allo-stimulatory capacity was significantly higher than that observed with marmoset MoDC, but not as high as human DC.

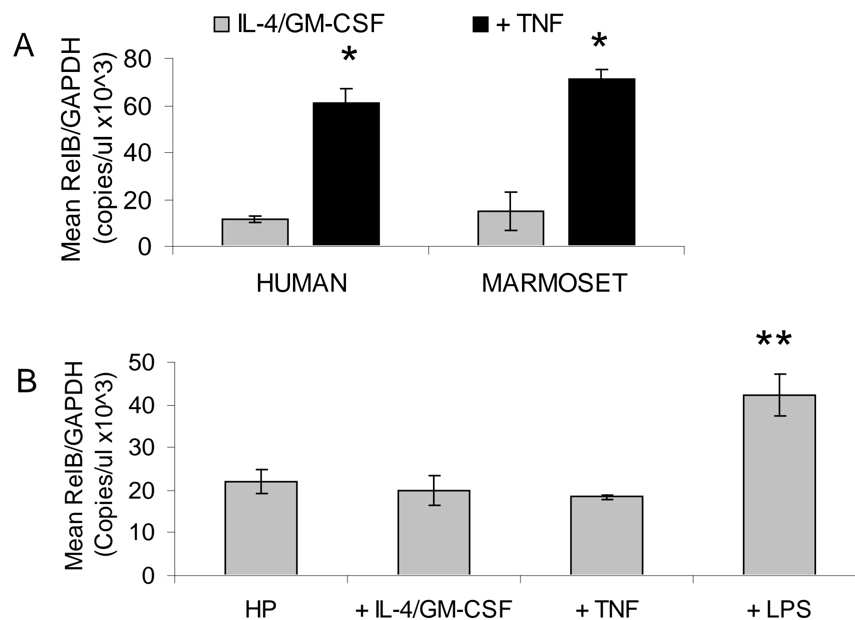
3.5 RelB mRNA Expression is Variably Up-regulated in Marmoset DC

RelB gene PCR product from marmoset DC cDNA was sequenced and aligned with sequences from human DC cDNA. The short marmoset region sequenced in this study had 96% homology with human sequence. Ten single nucleotide differences were consistently observed in DNA from three different animals. This translated to a single amino acid change, reflecting 98.8% amino acid homology for the region sequenced.

RelB expression was much lower than GAPDH expression in human G-CSF mobilised MoDC (n=1), marmoset G-CSF mobilised MoDC (n=5) and marmoset HPDC (n=4). As expected, human MoDC significantly up-regulated expression of RelB in response to maturation (Figure 14), occurring in conjunction with other features of DC maturation as described above. In 2 of 5 experiments, RelB expression was increased in marmoset TNF-α matured MoDC (Figure 14). In other experiments, no change in RelB expression was observed. Up-regulation of RelB expression in HPDC was observed in only one of three experiments. In all other experiments there was no difference between RelB expression in HP, HPDC or HPDC+TNF-α. This may be because HPDC were already partially mature before addition of TNF-α, as shown by other measures of maturity. However, LPS stimulation significantly increased RelB expression in HPDC (Figure 14). Interestingly, DC that had higher RelB expression did not have more mature phenotype or function. We conclude that marmoset RelB expression is not universally up-regulated in response to maturation stimuli, but in cases where up-regulation does occur, this does not translate to functional maturity.

Figure 14. RelB mRNA expression (mean ± SD of four replicates).

(A) Human and marmoset MoDC ± TNF-α. In these experiments stimulated MoDC had higher RelB expression, *p<0.01. (B) Marmoset week 2 HP, HPDC ± TNF-α or LPS. LPS-stimulated cells had significantly higher RelB expression than HP, unstimulated or TNF-α stimulated HPDC, ** p<0.001. Marmoset data is representative of two experiments each.



4. DISCUSSION

In this study we successfully applied two strategies for *in-vitro* propagation of DC from common marmoset precursor cells mobilised with the haematopoietic growth factor G-CSF, enabling large scale DC production from living animals. Marmoset *in-vitro* propagated DC resembled human DC, in particular possessing striking morphological similarity, expressing DC markers such as CD11c, HLA-DR, and CD83 in a small subset, showing antigen-uptake capacity and ability to stimulate allo-proliferation. However, we found DC derived from monocytes (MoDC) and *in-vitro* expanded haematopoietic precursors (HPDC) had phenotypic and functional differences indicating different degrees of maturation.

Marmoset MoDC had a stably immature phenotype despite exposure to stimuli that classically promote DC maturation. Marmoset MoDC had allo-stimulatory capacity above that of non-professional APC but weaker than human MoDC and HPDC. This occurred in conjunction with low levels of costimulatory molecule expression, retention of high antigen-uptake capacity and inconsistent up-regulation of RelB expression, despite maturation stimulation. These cells therefore exhibit some features desirable in tolerogenic DC as defined by Morelli [6], with the ability to efficiently present donor antigen to recipient T-cells without producing immune activation, and resistance to maturation. They are therefore candidate cells for further study of their tolerogenic potential in this species. In contrast, HPDC had a semi-mature phenotype, with stronger allo-stimulatory capacity (although not equal to human DC), higher baseline costimulatory molecule and CD83 expression with variable up-regulation after maturation stimuli, reduced but not absent endocytic capacity, and up-regulation of RelB mRNA expression with LPS stimulation only.

NHP DC have specific characteristics that highlight their similarities and differences to human DC, and several key studies of NHP DC discussed here are summarised in Tables 2 and 3. The variable responses to maturation signals described in this chapter have been observed in other NHP studies. Rhesus MoDC exhibited clear effects of maturation stimuli on DC phenotype and function[280,282,283]. In contrast, in baboon MoDC, functional maturation was not achieved with all stimuli, and baboon CD34-derived DC were phenotypically and functionally semi-mature without maturation stimuli[284]. Similar spontaneous semi-maturity or DC activation has been observed in chimpanzee and African green monkey MoDC[278,285], and in macaque CD34-derived DC [281]. Data regarding DC from New World Primates has been limited, but also suggests differences in maturation profiles. *Aotus* species (owl monkey) MoDC[286] exhibited some response to LPS and Poly I:C stimulation but had variable responses to other stimuli. In the only other study of marmoset propagated DC, Ohta *et al*. observed that marmoset BM CD11c$^+$ cells cultured in IL-4/GM-CSF and stimulated with LPS up-regulated CD80, CD83, CD86 and HLA-DR. Marmoset MoDC stimulated with LPS and IFN-γ increased only CD86 and HLA-DR expression. In both DC types, IL-12 secretion was increased and stimulation in xenogeneic MLR was provoked after stimulation [228], although the magnitude of proliferation was modest. In these experiments, we focussed on initially testing a substantial number of standard stimuli with demonstrated effectiveness in maturing human and NHP DC, and found these had limited effect on marmoset MoDC and some effect on HPDC maturation. The evident higher expression of co-stimulatory molecules in HPDC confirms the cross-reactivity of these antibodies with marmoset cells, and refutes the possibility that poor cross-reactivity is the reason behind lower expression in marmoset MoDC. Many alternative maturation signals remain to be explored including pro-inflammatory cytokine cocktails, IFN-γ, and pre-conditioning of DC cultures with IFN-α prior to maturation stimulation, which has been

shown to enhance the maturation of human MoDC generated rapidly in 48 hour culture [331]. These strategies will form the basis for future planned experiments.

The allo-stimulatory capacity of NHP DC is often less potent than human DC. Data between species is difficult to compare directly due to variations in the MLR technique, in particular whether responder cells are allogeneic or xenogeneic, and whether they are bulk MNC or purified T-cells. It was not possible to obtain sufficient numbers of purified T-cells from the small volumes of marmoset peripheral blood available (< 2 mls), therefore MNC containing T-cell subsets were used in MLR. Preliminary studies confirmed marmoset DC stimulate human MNC in xenogeneic MLR, however, as our aim is to use the allogeneic MLR as means of monitoring anti-donor responses in monkeys receiving donor DC we have preferentially used this method. Other aspects of the MLR such as duration of culture, responder:stimulator ratios and exogenous factors such as serum do play an important role in the MLR outcome. Most studies of NHP DC have replicated the protocols for human DC-stimulated MLR that are well-accepted in the literature. This usually involves a 5 day MLR and stimulator:responder ratios of 1:10 through to 1:1000. We also replicated this for marmoset DC-stimulated MLR to enable direct comparison of marmoset DC to human DC, and under these conditions, marmoset DC were weaker stimulators of allo-proliferation.

Expression of DC-specific markers also varies in NHP DC (summarised in Table 2). Unlike human MoDC, retention of CD14 is a common feature of NHP propagated DC, with 5-70% of MoDC continuing to express CD14. The majority of marmoset MoDC and HPDC were $CD14^+$ even after maturation stimulation. This raises the question of CD14 as a marker for differentiating between monocytes, macrophages and DC. The persistence of CD14 has been associated with endogenous generation of M-CSF in DC cultures, enhanced by the addition of

IL-10, but does not promote macrophage development or prevent DC differentiation [332]. Interestingly, a subset of human monocytes when cultured in GM-CSF, IL-4 and IL-10 evolve into CD14^{+} CD16^{++} CD11c^{+} CD1a^{+} cells [333] . These are not macrophages, as they display features similar to CD14^{-} MoDC, including up-regulation of CD83 upon stimulation with CD40L, and antigen-presenting and allostimulatory ability.

The complement receptor CD11c is an important myeloid DC marker in humans, mice and NHP, although not exclusive to DC. CD11c expression was high on marmoset DC, and co-expressed with CD14 and in some sub-populations with HLA-DR. Marmoset B lymphocytes also express CD11c, although morphology and staining for lineage markers excluded significant lymphocyte contamination of DC cultures. CD1a is expressed on human myeloid DC [54] and subsets of tissues DC, with low[279] or minimal[278,280] expression in NHP DC. CD1a has been reported as a marker of marmoset BM DC but not MoDC [228]. Our preliminary studies also failed to show CD1a expression on marmoset MoDC, although further evaluation is required as a marmoset cross-reactive antibody was only recently identified. DC-SIGN is a C-type lectin strongly expressed on human *in-vivo* myeloid DC and MoDC[57,58]. DC-SIGN has been primarily characterised in macaques, in submucosal, lymphoid and other tissues[296,334], however despite 92 % homology between human and macaque DC-SIGN, macaque MoDC only weakly express this molecule[335]. In African green monkeys, DC-SIGN is strongly expressed in lymph nodes and on the cell surface of MoDC in conjunction with CD11c[285,336]. DC-SIGN^{+} cells have been targeted *in-vivo* with anti-human antibodies in cynomolgus monkeys [300]. We have previously used anti-human DC-SIGN antibody to identify DC-SIGN^{+} cells in marmoset spleen, lymph nodes and thymus, and have shown co-localisation with DC markers including CD11c (Kireta, Prasad, Milton, Coates *et al*., unpublished data). However, no significant DC-SIGN expression was observed on *in-vitro* propagated marmoset DC.

Finally, CD83 is a classical mature DC marker that has been found on NHP DC from several species of Old World Primate, but has only been reported in New World Primate BM-derived DC [228] but not MoDC, and this is consistent with our findings.

These data highlight the importance of choosing appropriate markers to identify and characterise DC in NHP, and being aware of the inter-species differences that exist. The functional implications of these differences are unclear, and may reflect variable cross-reactivity of anti-human antibodies with NHP cells. Expression of these DC and maturation markers may not be reduced, but may be secondary to inefficient cross-reactivity of antibodies. However, the different expression of CD83 and co-stimulatory molecules between marmoset MoDC and HPDC does suggest that the issue lies with expression on the cells rather than antibody cross-reactivity. Never-the less this remains an important issue. In some cases NHP-specific antibodies have been developed, and this may be required in future to obtain additional DC-specific antibodies in marmosets.

The environment in which DC are differentiated may have profound influences on the type of DC produced. The more mature profile of marmoset HPDC has been also observed in human DC cultured with the same cytokines and may be related to spontaneous maturation of cells in long-term culture[87,88]. It is likely that within these cultures there is a spectrum of differentiation and maturation, with subpopulations of more mature DC developing. In contrast, maturation-resistant (and therefore potentially tolerogenic) DC can be generated by manipulating cytokines and other elements of culture systems, as discussed in Chapter 1 (section 3.2.1).

Some of the differences between marmoset and human DC may lie in lower sensitivity or altered response of NHP cells to human cytokines. While the effect of GM-CSF on marmoset cells has previously been established [221] and our studies of STAT-6 signalling clearly confirm similar activity of rhIL-4 on marmoset versus human cells, this may not translate to an equivalent biological effect on DC differentiation. The influence of GM-CSF on DC differentiation is clearly pivotal Murine BMDC exposed to very reduced doses of GM-CSF are maturation-resistant to TNF-α, CD40L and LPS, are poorly allostimulatory, and have been shown to promote tolerance *in-vivo* [112]. Our studies demonstrated no difference in DC phenotype using lower dose (400U / ml) or higher dose (800-1000 U / ml) GM-CSF; both doses produced maturation-resistant MoDC.

Alternatively, cytokines such as IL-10 or TGF-β added to or generated *in-vitro* during DC cultures or MLR may also produce maturation-resistant DC or have immunosuppressive effects[124,125]. The presence of these cytokines has not yet been assessed in marmoset cultures. Other elements of the culture micro-environment such as xenoprotein or other elements in serum may inhibit DC maturation[337]. Fetal calf serum (FCS) is unsuitable when developing protocols for eventual clinical application due to the unwanted immunological effects of xenoprotein. However, most studies investigating DC propagation methods have established protocols using FCS initially, with subsequent modifications such as autologous serum or serum-free culture using medium such as AIM-V. Romani *et al*.[75] suggested 1 % human plasma was optimal for DC culture, after testing several types of serum and serum-free medium. In general it is more difficult to prepare DC in serum-free media, as yield and viability are lower [76]. In summary, multiple factors related to species differences and the culture environment may contribute to variations in DC characteristics from different precursors and in different primate models.

DC development, particularly the commitment to a specific DC subset, is regulated by a number of transcription factors including Ikaros, interferon regulatory factors (IRF-2, 4 and 8), helix-loop-helix transcription factors and STAT3 [13]. To address issues of maturation further, we investigated the NF-κB protein RelB, another transcription factor critical to DC differentiation and maturation. RelB mRNA/protein up-regulation and nuclear translocation is a feature of mature DC produced under the influence of many different standard maturation stimuli [337-339]. RelB blockade is a potential strategy for tolerance induction supported by evidence of tolerogenic features of RelB deficient DC[340,341] and studies of RelB blockade in NHP transplants[236,239]. RelB mRNA expression in marmoset DC may provide clues to the maturation status of marmoset propagated DC. RelB mRNA expression was not consistently increased in marmoset MoDC by stimulation with TNF-α, and was only increased in HPDC by LPS stimulation. It is unclear why the increase in RelB mRNA signal observed in some cases did not translate to phenotypic or functional maturation. It is possible that nuclear translocation of RelB may be impaired in marmoset cells or the increase in RelB signal is insufficient to trigger downstream responses required for RelB to regulate gene transcription and promote DC activation. Due to lack of a cross-reactive anti-RelB antibody, we were unable at this time to evaluate location or cellular expression of RelB protein. LPS and TNF-α activate NF-κB via different preliminary pathways that culminate in a common activation pathway[337] and it may be that these pathways differ in marmosets, accounting for varied responses to LPS and TNF-α. There may be important species-specific differences that underlie these findings, and we are attempting to identify antibodies that are marmoset cross-reactive to enable further investigation of these pathways in marmoset DC.

Many other aspects of DC phenotype and function remain to be defined in this model, and this will occur as cross-reactive commercial assays and kits are discovered and antibodies identified. In particular, our group is now testing assays for measuring IL-12p70 (Human IL-12 Quantikine ELISA Kit, R&D Systems) and Th1 and Th2 cytokines (Cytometric Bead Array kit, BD Biosciences) in addition to ongoing testing of cross-reactivity of multiple antibody clones for DC-specific markers including DC-SIGN and CD1a. These assays will augment the existing data characterising marmoset DC, and may assist in identifying tolerogenic DC in this species.

A major technical hurdle in the use of smaller primate species is their modest circulating blood volume, and low cell yields from PB. In larger NHP species, 40-50 ml of PB can yield up to 1-2x10^6 MoDC [280,284]. In comparison, Ohta *et al.* [228] used all available PB and BM from a killed marmoset to obtain only 1-2 x 10^5 MoDC and 1x10^7 BM-derived DC. Given that our ultimate strategy is to use living donor monkeys for DC transfer then subsequent organ transplant, and continue to use donor leukocytes for post-transplant immune monitoring, this was not a feasible method.

We have overcome this major obstacle by using G-CSF to mobilise precursor cells to peripheral blood. As discussed in Chapter 1 (section 2.3.3), human G-CSF-mobilised monocytes and stem cells can be differentiated *in-vitro* into dendritic cells in sufficient numbers and quality for therapeutic use. In NHP models, G-CSF, SCF, GM-CSF, FLT3-L, and the IL-3/G-CSF chimeric molecule myelopoetin have all been used to mobilise stem cells, DC or DC precursors, which can be enriched with leukopheresis [36,94-98,271].

G-CSF mobilisation has also been previously used successfully in common marmosets by Hibino et al. [221], to obtain progenitor cells for the *in-vitro* expansion of colony-forming units of various lineages and assessment of viral transduction and autologous transplantation. However, this is the first study to use G-CSF mobilisation to improve PB cell yield in New World Primates expressly for mobilisation of DC precursors. We have confirmed the safety of G-CSF in marmosets and demonstrated that repeated doses remain effective over time. We have also demonstrated that a brief course of G-CSF gives predictable monocyte and stem cells mobilisation kinetics that are similar to the kinetics observed in humans. We can routinely obtain $1x10^6$ MoDC from 1ml of mobilised PB and safely take 2-3mls over consecutive days, to obtain adequate numbers of MoDC for extensive characterisation, *in-vitro* manipulations to generate tolerogenic DC, or *in-vivo* administration. This is a significant DC yield given the size of the animal and blood sample.

This study also establishes that NHP haematopoietic precursors can be massively expanded *in-vitro* under the influence of early-acting cytokines. The purification of marmoset stem cells has historically been difficult, with low yield of $CD34^+$ cells by immunomagnetic separation noted by our group and others[221,228]. Given that DC can be successfully propagated from G-CSF mobilised $CD34^+$-enriched PBMC in humans[87], we used this strategy with marmoset G-CSF mobilised blood in which the $CD34^+$ population was shown to be increased. Interestingly, marmoset cells only expanded after week 2 in culture, the peak growth was 5-10 fold and proliferation ceased by week 4. In comparison, human cells in a similar culture system rapidly proliferated within the first week, had a 1,200-fold increase by week 4 and continued proliferating for up to 6-8 weeks[87,88]. Despite differences in responses between species, this strategy provides millions of myeloid HP for DC propagation. Although these HPDC have a

semi-mature phenotype, this is a valuable tool that may be exploited for other DC-based therapeutic manipulations of immunity such as vaccination.

In this study we have, for the first time, propagated marmoset myeloid DC *in-vitro* from PB monocytes or stem cell precursors. The use of G-CSF mobilisation overcomes technical issues related to cell yield, and human protocols can be successfully applied to marmoset precursors to generate DC *in-vitro*. Marmoset DC propagated in this manner have many features similar to human DC, as well as some differing characteristics consistent with DC from other NHP species. Large numbers of DC can be generated with these methods from living animals, enabling further studies to identify potentially tolerogenic DC with or without additional modifications such as gene manipulation. In conclusion, this study confirms the feasibility and relevance of the common marmoset as a pre-clinical model in which to explore DC-based therapeutic strategies.

Table 2. Surface Marker Expression and Response to Maturation Stimuli of NHP DC – Summary of Literature.

The level of molecule expression was categorised as follows: - (0-5% of cells positive), + (< 20 % positive), ++ (20-70 % positive), +++ or ++++ (> 70 % positive). Alternatively, for some studies, each '+' represents 1 log fluorescence intensity. An increase in expression

within a category is denoted by ↑. All monocyte-derived DC in these studies were cultured in IL-4 and GM-CSF. Methods of generating bone-marrow derived DC varied.

Abbreviations: AGM – African green monkey; Cyno – cynomolgus monkey; $CD14^+$ Mo – $CD14^+$ monocytes selected by immunomagnetic separation or cell sorting;   Ad. Mo – monocytes selected by plastic adherence method; BM – bone marrow; $CD34^+$ BM – bone marrow $CD34^+$ cells selected by immunomagnetic separation; TCDMNC – T-cell depleted mononuclear cells; LPS – lipopolysaccharide; IFN – interferon; CC- cytokine cocktail consisting of TNF-α, IL-6, IL-1; PGE2 – prostaglandin E2; MCM – monocyte conditioned medium; n /a - Data not available due to lack of cross-reactive antibodies or not tested / data not reported.

Table 2.

Species	Precursor	Maturation	CD14	CD11c	Class II	CD86	CD40	CD80	CD83	CD1a	DC-SIGN
Marmoset [228]	$CD14^+$ Mo	None	++	++	+	+	n/a	n/a	n/a	n/a	n/a
	$CD14^+$ Mo	LPS+IFNγ	n/a	+	++	++	n/a	n/a	n/a	n/a	n/a
	BM cells	None	++	+/++	+	++	n/a	+	+	+++	n/a
	BM cells	LPS	n/a	+/++	+ ↑	++ ↑	n/a	++	++	n/a	n/a
Aotus[286]	Ad. Mo	LPS	++	n/a	++	n/a	n/a	++	n/a	n/a	n/a
AGM[285]	Ad. Mo	None	+	+++	+++	+++	+	+	++	n/a	+++
	Ad. Mo	CC	+	+++	+++	+++	++	++	++	n/a	+++
Baboon[284]	$CD14^+$ Mo	None	+	n/a	+++	+++	+++	+/++	-/+	n/a	n/a
	$CD14^+$ Mo	CC+ PGE2; LPS; CD40L	-/+	n/a	+++	+++	+++	++/+++	++/+++	n/a	n/a
*	$CD34^+$ BM	None	-/+	n/a	+++	+++	+++	+++	++	n/a	n/a
*	$CD34^+$ BM	CC+ PGE2; CD40L	n/a	n/a	+++ ↑	+++ ↑	++/+++	+++ ↑	++	n/a	n/a
Cyno[279]	Ad. Mo	None	+++	n/a	+++	+++	+++	n/a	-/+	++	n/a
Rhesus[280]	TDMNC	None	n/a	n/a	n/a	+++	n/a	n/a	-	n/a	n/a
	TDMNC	MCM	+/++	++	+++	+++	++	n/a	+	-	n/a
Rhesus[282]	$CD14^+$ Mo	None	+/++	++	+++	++	+++	+	-/+	n/a	n/a
	$CD14^+$ Mo	CC+PGE2; MCM; LPS PolyI:C; CD40L; TNF/PGE2	-/+	++	++++ ↑	++++	+++	++	++	n/a	n/a

Table 3. Functional Studies of NHP DC – Summary of Literature.

IL-12 production by DC under stimulation was categorised as follows: - (no production), + (some baseline production), ↑ (increased production with stimulation). All monocyte-derived DC were cultured in IL-4 and GM-CSF. Methods of generating bone-marrow derived DC varied.

Abbreviations: MLR – mixed leukocyte reaction; SI- stimulation index; AGM – African green monkey; Cyno – cynomolgus monkey; $CD14^+$ Mo – $CD14^+$ monocytes selected by immunomagnetic separation or cell sorting; Ad. Mo – monocytes selected by plastic adherence method; BM – bone marrow; $CD34^+$ BM – bone marrow $CD34^+$ cells selected by immunomagnetic separation; TCDMNC – T-cell depleted mononuclear cells; LPS – lipopolysaccharide; IFN – interferon; CC- cytokine cocktail consisting of TNF-α, IL-6, IL-1; PGE2 – prostaglandin E2; MCM – monocyte conditioned medium; n /a - Data not available due to lack of cross-reactive antibodies or not tested / data not reported; *- data for CD86+ gated DC , day 12 cells only.

Table 3.

Species	Precursor	Maturation	MLR / Proliferation		IL-12	Endocytosis	Conclusions
Marmoset [228]	$CD14^+$ Mo	none	xeno	low	-	n/a	LPS+ IFNγ had some maturation effect
	$CD14^+$ Mo	LPS+IFNγ	xeno	higher	↑	n/a	
	BM cells	None	xeno	very low	-	++	LPS had some maturation effect
	BM cells	LPS	xeno	higher	↑	-/+	
Aotus[286]	Ad. Mo	None	n/a	n/a	-	+++	LPS had some maturation effect
	Ad. Mo	LPS	allo	high	-	-	
AGM[285]	Ad. Mo	None	allo	SI 2.5-3.5	n/a	++	Unstimulated DC were semi-mature Additional stimuli had little effect
	Ad. Mo	CC	allo	SI 2.2-3.7	-	+	
Baboon[284]	$CD14^+$ Mo	none	allo	moderate	+	++/+++	CC had best maturation effect LPS and CD40L had variable effects
	$CD14^+$ Mo	CC+ PGE2; LPS; CD40L	allo	moderate - high	↑	++/+++	
*	$CD34^+$ BM	none	allo	high	+	+	Unstimulated DC were mature Additional stimuli had little effect
*	$CD34^+$ BM	CC+ PGE2; CD40L	allo	high	↑	n/a	
Cyno[279]	Ad. Mo	None	allo	moderate	n/a	+++	Limited data on TNF-α effect
	Ad. Mo	TNF-α	allo	higher	n/a	n/a	
Rhesus[280]	TDMNC	None	allo	moderate	n/a	n/a	MCM produces mature DC
	TDMNC	MCM	allo	higher	n/a	n/a	
Rhesus[282]	$CD14^+$ Mo	None	xeno	low	-	+++	CC and TNF/PGE2 DC were most mature LPS and CD40L DC had incomplete maturation
	$CD14^+$ Mo	CC+PGE2; MCM; LPS PolyI:C; CD40L; TNF/PGE2	xeno	higher	↑↑	-/+	

CHAPTER 5: PROPAGATION OF MARMOSET *IN-VIVO* DC USING FLT3-LIGAND

1. INTRODUCTION

Dendritic cells are specialised and potent antigen-presenting cells constituting less than 1 % of the leukocyte population. Their rarity significantly hampers study of their phenotype and function, particularly in humans and non-human primates (NHP). Consequently they have been mainly studied in murine models where animals can be sacrificed to obtain spleen and bone marrow-derived DC, or in *in-vitro* culture systems designed to expand and differentiate DC from precursors (see Chapter 4). These models have been extremely useful in advancing the field of DC biology, but do not always correlate with the human DC system, as discussed in Chapter 1. The use of haematopoietic growth factor treatment to mobilise *in-vivo* DC in humans and other species has enabled the harvesting of large numbers of DC for characterisation and potentially for DC-based immunotherapy.

Fms-like tyrosine kinase 3 ligand (FLT3-L; Flk2) is a critical cytokine for DC development from bone marrow lymphoid and myeloid precursors *in-vivo*. It also supports the development of other primitive progenitor cells including $CD34^+$ stem cells[94], and its receptor FLT3 is exclusively expressed on immature progenitors. It has been shown to markedly increase myeloid and lymphoid blood and tissue DC subsets when given *in-vivo* to mice[34,35,183], humans[41,43], and rhesus macaques[36,39,40]. Progenipoietin, a chimeric molecule of FLT3-L and G-CSF, also mobilises both MDC and PDC in mice and NHP[33,34,37]. In two separate studies of healthy human volunteers receiving FLT3-L treatment, lineage negative ($CD3^-$ $CD14^-$ $CD19^-$

CD56$^-$) HLA-DR$^+$ CD11c$^+$ (myeloid) and CD11C$^-$ (plasmacytoid) peripheral blood DC were increased on average 44-48 fold and 12-13 fold, respectively [41,43], with the MDC:PDC ratio rising significantly. The greater effect of FLT3-L on the MDC population has also been observed in rhesus macaques, where a 7-57 fold increase in MDC versus a 4.7-5 fold increase in PDC has been reported [36,40]. In all of these studies, FLT3-L mobilised MDC and PDC exhibited typical phenotype, allostimulatory function and responses to maturation and activation stimuli, confirming the value of this strategy for *in-vivo* DC study.

The aim of this chapter was to investigate for the first time the effects of recombinant human FLT3-L on marmoset *in-vivo* circulating DC populations, with particular focus on myeloid DC. The data presented here are from preliminary studies in a small group of animals. The findings establish the safety and feasibility of this strategy for obtaining significant numbers of marmoset peripheral blood MDC for further characterisation and manipulation. This work also paves the way for ongoing studies of the effect of FLT3-L mobilisation on DC in marmoset lymphoid and non-lymphoid organs, particularly the kidney.

2. METHODS

2.1 FLT3-L Mobilisation

Marmosets were maintained as outlined in Chapter 2, section 1. Seven mobilisations were undertaken in one male and four female marmosets (79f, 800, 861, 80m, 813) over a six month period. Two animals (79f and 800) received FLT3-L on two occasions four months apart. Animals received recombinant human FLT3-L expressed in a Chinese hamster ovary cell line (kind gift of AMGEN Corporation, CA, USA under the conditions of Materials Transfer

Agreement No. 200621525), lyophilised powder reconstituted in endotoxin-free PBS, at a dose of 100ug/kg/day subcutaneously for 10 days (designated days 1-10). This dose was chosen based on efficacy in rhesus monkey studies[36,40] although smaller doses have been used effectively in humans[41]. Blood samples in lithium heparin tubes were taken on days 5, 10, 14 and in some cases days 8 and 18 as well, for flow cytometry analysis and leukocyte counts.

2.2 Strategy for Identification and Isolation of Marmoset Myeloid DC

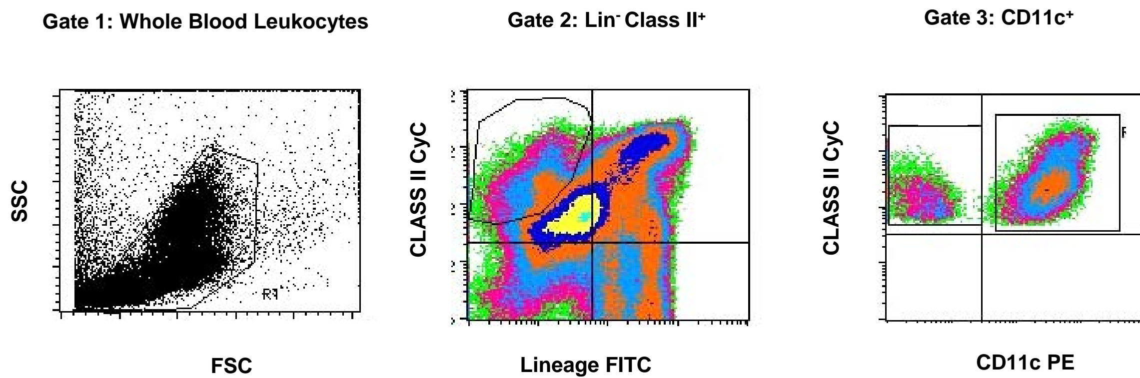
Antibodies and protocols for flow cytometry and fluorescent activated cell sorting are described in Chapter 2, section 8. Whole blood (WB) samples were used for three-colour labelling to minimise cell loss during PBMC isolation procedures. This study therefore differs from others in which PBMC were first isolated by density gradient separation. The gating strategy for identification of myeloid DC is shown in Figure 1. Gate 1 excludes debris and defines cells of interest for analysis. All percentages are expressed as a percentage of cells within Gate 1. Gate 2 defines cells within Gate 1 that are Lin$^-$ HLA-DR$^+$ and Gate 3 defines cells within Gate 2 that are CD11c$^+$. Cells within gate 3 are myeloid DC. Using this gating strategy, Lin$^-$ HLA-DR$^+$ CD11c$^+$ and Lin$^-$ HLA-DR$^+$ CD11c$^-$ cells from animals 861 and 80M (on day 12) and 79f and 800 (on day 14 and 15) were purified by FACS.

2.3 Cell Culture and MLR

Freshly sorted Lin$^-$ HLA-DR$^+$ CD11c$^+$ were washed and cultured overnight in round-bottomed 96-well plates in RPMI +10% FCS with CD40L (3ug/ml), at a concentration of 1-2x10^5 cells per well. In addition, the freshly sorted Lin$^-$ HLA-DR$^+$ CD11c$^-$ populations from animals 79f and 800 were cultured in RPMI +10% FCS with GM-CSF (800U/ml) and IL-4 (40ng/ml). These cells were collected after 48 hours and analysed by flow cytometry for CD11c expression. MLR was performed using irradiated freshly sorted or overnight-cultured CD11c$^+$

DC as stimulators and freshly isolated fully DRB-mismatched allogeneic PBMC as responder cells, as described in Chapter 2, section 6.3.

Figure 1. Three-Colour Gating Strategy For Identification Of Putative Marmoset Myeloid DC. Whole blood leukocytes were analysed by flow cytometry, and selected by forward scatter (FSC) and side-scatter (SSC) profile (Gate 1). The Lin$^-$ ClassII$^+$ population was identified (Gate 2) and CD11c+ cells within this fraction were recognized as myeloid DC (Gate 3). Quadrants are based on isotype-matched negative controls.



3. RESULTS

3.1 Safety and Tolerability

Recombinant human FLT3-L was well tolerated by all animals, who were observed daily during the course of treatment and in the post-treatment period. No injection site or allergic reactions were noted, although some animals did develop inguinal haematomas due to frequent blood sampling from the femoral vein. Lymphadenopathy during FLT3-L treatment has been noted in humans [41], but we did not observe this in marmoset peripherally accessible nodes such as inguinal lymph nodes. One animal (861) was euthanased five months after FLT3-L administration due to the development of lower limb paralysis, the aetiology of which was not evident on autopsy. This animal had a mild lymphocytic infiltrate in the inguinal lymph nodes on histological examination. This event was not attributed to FLT3-L treatment.

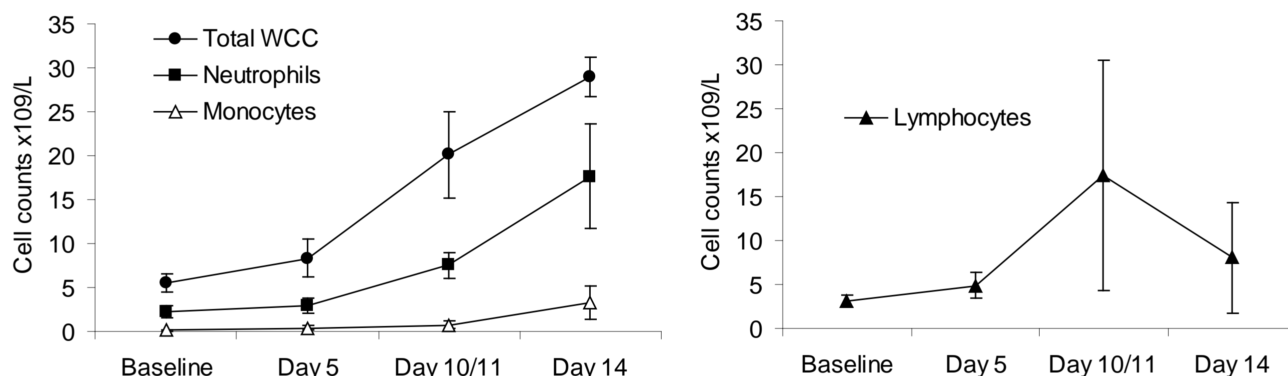
3.2 FLT3-L Increases Circulating Leukocyte and $CD34^+$ Stem Cell Populations

FLT3-L increased the total white cell count (WCC) significantly by day 5 of treatment and at each time point thereafter (Figure 2). Interestingly, this was due to rises in absolute numbers of neutrophils, lymphocytes and monocytes, although percentage contribution of each subset to the total WCC did not change (Table 1). This data is in keeping with human studies where the total WCC, mononuclear cell count and monocyte count was observed to rise, although the lymphocyte count did not alter [41]. In marmosets, the lymphocyte count peaked at day 10 and fell by day 14. In FLT3-L treated rhesus macaques, the lymphocyte percentage also rose significantly by day 10 and dropped by day 14, however no significant change in the total WCC was observed [36]. The significance of these varied responses is uncertain, but may reflect differing sensitivity of various cell lineages to FLT3-L in different species.

Table 1 (and Figure 2). Changes in WCC and leukocyte subsets with FLT3-L treatment of marmosets. Animals were treated with FLT3-L 100ug/kg/day for 10 days (day1-10). Blood samples for automated WCC were taken on days as shown and compared to baseline counts derived from a cohort of healthy untreated animals. Data is expressed as mean ± SD; error bars represent SD. * $p<0.05$ when compared to previous time point using student's t test. # $p<0.05$ when compared to baseline data using student's t test.

	Baseline	Day 5	Day 10/11	Day 14
	n=19	n=7	n=7	n=2
Total WCC	5.5 ± 1.1	8.3 ± 2.2*	20.1 ± 4.9*	29 ± 2.2*
Neutrophils (%)	39.2 ± 7.3	36.3 ± 7.5	37.9 ± 4.6	62 ± 25.5
Neutrophils (x10^9/L)	2.2 ± 0.7	2.98 ± 0.84	7.5 ± 1.4*	17.7 ± 6
Lymphocytes (%)	56.1 ± 6.9	58.1 ± 8.7	51.6 ± 20.6	27± 19.8
Lymphocytes (x10^9/L)	3.1 ± 0.7	4.88 ± 1.4*	17.4 ± 13.0*	8.0 ± 6.3
Monocytes (%)	2.8 ± 2.1	4.71 ± 2.68	4.29 ± 3.7	11 ± 5.7
Monocytes (x10^9/L)	0.15 ± 0.1	0.40 ± 0.3	0.73 ± 0.5#	3.25 ± 1.9

Figure 2.



In untreated animals, the CD34$^+$ population constitutes less than 0.5 % of marmoset blood PBMC. Treatment with FLT3-L induced a rise in the blood CD34$^+$ stem cell population

(Figure 3) as observed in other species[36,43,94]. In all five animals tested, the CD34$^+$ population was increased at day 5 (Table 2). The peak percentage of CD34$^+$ cells varied considerably between experiments, and occurred at day 10 in two animals and day 14 in the other three.

The Lin$^-$ Class II$^+$ fraction of WB leukocytes rose following FLT3-L treatment, peaking during days 10-14 and approaching baseline after day 18, i.e. eight days after ceasing FLT3-L (Table 2). This trend was noted individually in every animal treated, although the absolute percentage varied considerably and therefore did not produce statistical significance due to wide standard deviations in a small cohort of animals. Similarly, the percentage of CD11c$^+$ cells significantly increased from 10.2 ± 7 % on day 5 to 22.4 ± 7.4 % on day 14 ($p=0.019$), returning to 7.8 ± 3.25 % by day 18. The CD14$^+$ population was also mobilised between day 10 and day 14 (5.4 ± 3.7 % to 16.3 ± 8.1; $p=0.03$). This may represent both monocytes and neutrophils as CD14 is expressed on both of these cells type in marmosets.

Table 2. Changes in Cell Populations as Determined by Three-colour Flow Cytometry analysis of peripheral whole blood samples. Data is expressed as mean ± SD. * $p<0.05$ when compared to previous time point using student's t test. # $p<0.05$ when compared to baseline data using student's t test. ** data from previous experiments in another cohort of healthy marmosets (S. Kireta, personal communication)

	Baseline	Day 5	Day 8	Day 10/11	Day 14/15	Day 18
	n=3	n=7	n=2	n=5	n=7	n=2
$Lin^{-}ClassII^{+}$ (% Gate 1)	1.5 ± 0.8	3.8 ± 3.5	2.5 ± 1.4	9.5 ± 7.0	10.2 ± 4.91	1.6 ± 0.3
$Lin^{-}ClassII^{+}$ $CD11c^{+}$ (% Gate 2)	9.27 ± 1.08	21.0 ± 10.5	28.9 ± 3.5	21.7 ± 9.4	$42.9 \pm 22.2^{\#}$	9.96 ± 1.7*
$Lin^{-}ClassII^{+}$ $CD11c^{+}$ (% Gate 1)	0.07 ± 0.01	0.69 ± 0.4*	0.74 ± 0.5*	1.92 ± 0.93*	5.21 ± 0.4*	0.16 ± 0.01*
$Lin^{-}ClassII^{+}$ $^{-}CD11c^{+}$ (%, range)	0.06-0.08	0.25-1.32	0.39, 1.09	0.79-2.84	2.97-6.88	0.16, 0.17
$Lin^{-}ClassII^{+}$ $CD11c^{-}$ (% Gate 2)	59.5 ± 13.4	64.7 ± 12.8	63.15 ± 2.5	62.8 ± 4.4	48.4 ± 18.4	76.36 ± 2.04
	n>3 **	n=5		n=5	n=5	n=2
$CD34^{+}$	< 0.5	1.84 ± 0.48	-	3.77 ± 4.42	1.18 ± 0.94	0.47 ± 0.01

3.3 FLT3-L Mobilises Putative Peripheral Blood Myeloid DC

Presumptive peripheral blood $CD11c^{+}$myeloid DC were identified within the Lin^{-} Class II^{+} population of cells in gate 1. The percentage of whole blood Lin^{-} Class $II^{+}CD11c^{+}$ MDC rose significantly in all animals by five days of FLT3-L treatment (data summarised in Table 2 and Figures 3 and 4), peaking at day 14-15. At this time point, 3-7 % of WB leukocytes were identified as putative MDC, representing a mean 74-fold (range 42-98) increase from baselines determined in untreated animals. Based on this data, MDC purification by FACS cell sorting was performed on samples taken on days 10 or day 15. This enabled a large number of cells to be isolated from small volumes of whole blood, although yields and final cell viability varied (Table 3).

Table 3. Marmoset $Lin^{-}ClassII^{+}$ $CD11c^{+}$ DC yield and Viability following FACS Sorting.

This table shows the animal mobilised, the day of blood sampling and FACS, the volume of whole blood stained with antibodies and processed for FACS, the total number of fluorescence events sorted, the percentage of viable cells within the sorted population after two washes in medium, and the final numbers of viable cells obtained after two washes and re-suspension in 1ml of medium.

Animal	Day	Volume	Events Sorted	% Viability	Final Cell Count
861	10	400ul	223,000	80 %	190,000
80m	10	400ul	127,000	70 %	100,000
79f	14	900ul	346,000	70 %	300,000
800	14	1.2ml	260,000	70 %	215,000
79f	15	1.2ml	847,000	90 %	600,000
800	15	1.2ml	67,000	90 %	400,000

Figure 3. Change in Mean Percentage of $Lin^{-}ClassII^{+}CD11C^{+}$ putative Myeloid DC in Whole Blood Samples From Marmosets Treated With FLT3-L 100ug/kg/day for 10 days (days 0-10; n=7). Error bars represent SD. Data at each time point was significantly different to both baseline and the previous time point (student's t test).

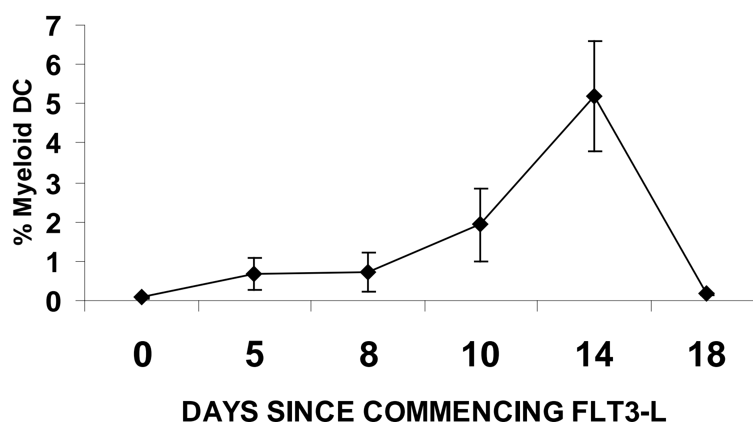
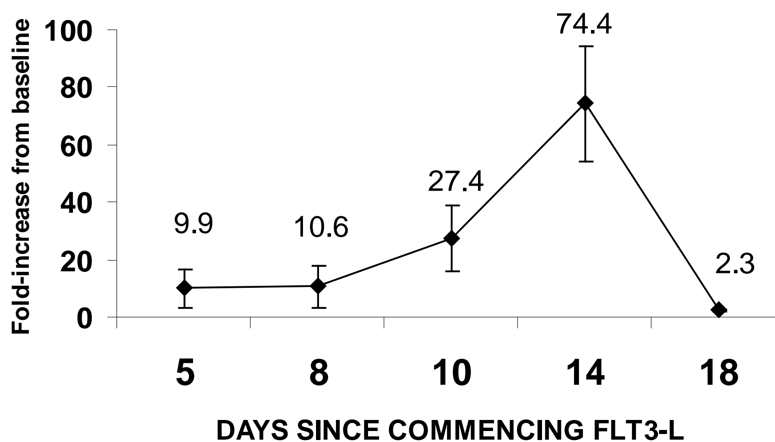


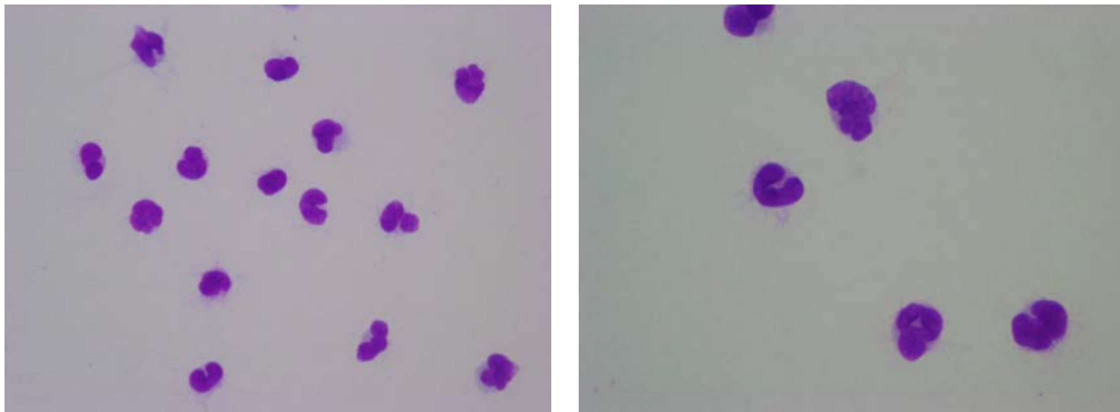
Figure 4. Mean fold-increase in the percentage of $Lin^{-}ClassII^{+}CD11C^{+}$ putative myeloid DC mobilised by FLT3-L (n=7) compared to baseline percentages in untreated animals (n=3). Error bars represent SD.



3.3.1 Morphology of FACS-sorted Marmoset Lin$^-$ClassII$^+$CD11c$^+$ MDC

The morphology of circulating Lin$^-$ClassII$^+$CD11c$^+$ cells isolated by FACS sorting is shown in Figure 5. Freshly isolated cells exhibited a uniform appearance, with multi-lobulated or reniform nuclei and minimal dendritic cytoplasmic processes. After overnight culture in medium containing CD40L, fine dendritic processes were observed.

Figure 5. Morphology of Lin$^-$ClassII$^+$CD11c$^+$ cells isolated by FACS sorting. Giemsa stain; magnification 200-400x.

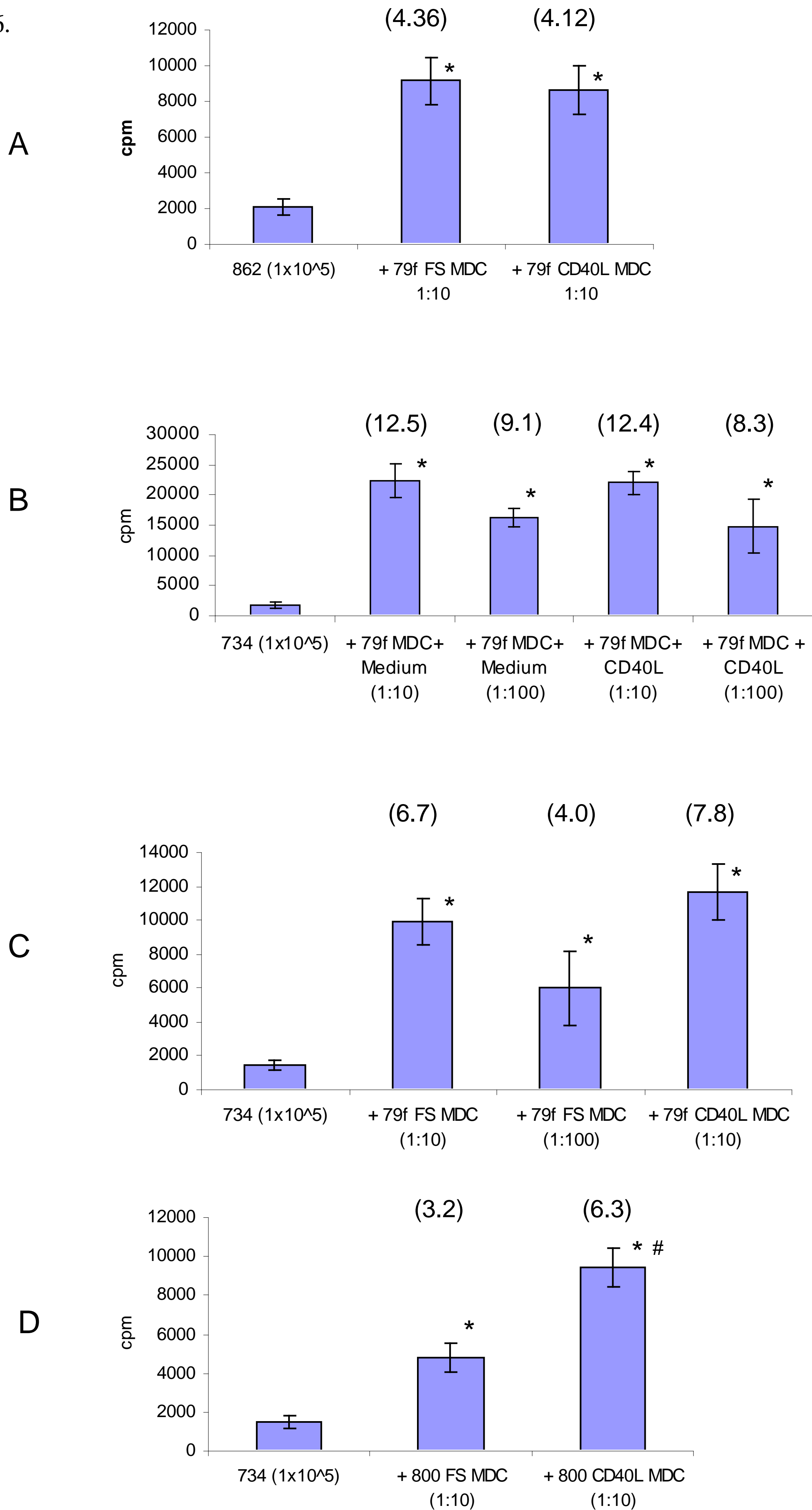


3.3.2 Marmoset Lin⁻ClassII⁺CD11c⁺ MDC have Allostimulatory Ability

Freshly sorted (FS) $CD11c^+$ MDC, and MDC cultured overnight in medium (RPMI + 10% FCS; MDC + Medium) or medium supplemented with CD40L (3ug/ml; CD40L MDC) were used as stimulator cells in MLR with allogeneic PBMC as responders. Complete data from 4 experiments using MDC from two animals (79f and 800) against PBMC from two allogeneic responders is shown in Figure 6 panels A-D. In all cases, marmoset FLT3-L mobilised MDC induced significant T-cell proliferation above baseline counts, with stimulation index ranging from 3.2-12.5. This is substantially higher than that observed for marmoset *in-vitro* propagated DC, where the SI was generally < 4 for immature monocyte-derived DC (Chapter 4, Figure 6). Even a lower MDC:PBMC ratio of 1:100 remained stimulatory (Figure 6B and C), as observed in human and rhesus studies[36,41]. This may represent superior allostimulatory ability of *in-vivo* generated DC, or may reflect activation of DC during the cell sorting or culture process. Interestingly, overnight stimulation of MDC with a substantial concentration of CD40L significantly increased allostimulation in only one experiment (Figure 6D), whereas in three other experiments, CD40L-stimulation did not produce higher proliferative responses (Figure 6 A-C). This raises the possibility that marmoset MDC may have already been activated during the isolation process and could not mature further, although this is speculative at this stage. MDC from animal 79f cultured overnight appeared to induce greater allostimulation of animal 734 PBMC than freshly sorted MDC (Figure 6 and C). However, it must be noted that these MDC originated from two separate blood samples and FACS sorting on two consecutive days.

Figure 6A-D. Flow-sorted marmoset CD11c$^+$ MDC are stimulatory in allogeneic MLR. Data represents four experiments using day 14 and 15 FACS sorted DC from mobilised animals 79f (panels A-C) and 800 (panel D). Freshly sorted (FS) MDC and MDC cultured overnight in RPMI+10%FCS (medium) or with CD40-Ligand (CD40L) were used as stimulator cells in 1:10 or 1:100 ratios as indicated, against allogeneic PBMC from animals 862 (panel A) and 734 (panels B-D). Data is shown as counts per minute (cpm) ± SD. The numbers in parentheses above each bar represent the stimulation index (ratio of stimulated : baseline cpm). * $p < 0.05$ when comparing DC-stimulated cpm against unstimulated responders cells using student's t-test. # $p = 0.003$ when comparing freshly sorted and CD40L-stimulated DC. These results are discussed in detail in the text.

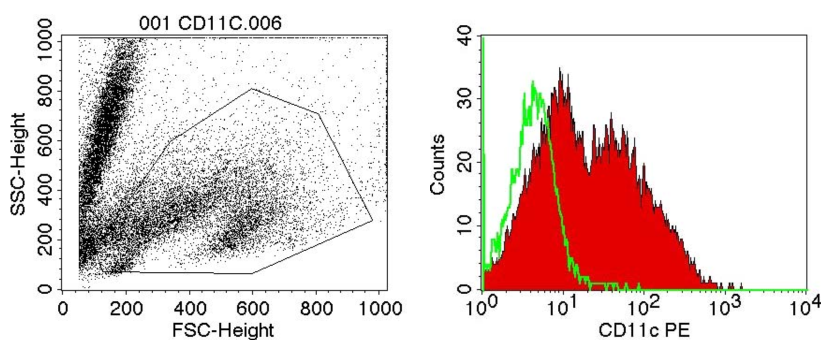
Figure 6.



3.4 FLT3-L Mobilised Lin$^-$ClassII$^+$CD11c$^-$ Cells Acquire CD11c In Culture

The circulating Lin$^-$ClassII$^+$ fraction also contains cells which are negative for CD11c; these constitute the majority of the Lin$^-$ClassII$^+$ population at all time points (Table 2). In rhesus monkeys, these cells are also CD123 negative, and some express CD56 or CD34 [36]. Certain DC subsets may also express CD56. We included CD56 in the lineage cocktail in these preliminary experiments, however, in future studies it will be important to assess the outcome of eliminating this marker. Following FACS-sorting, the Lin$^-$ClassII$^+$CD11c$^-$ fraction was collected and cultured in GM-CSF / IL-4 for 48 hours (n=4). This induced the expression of CD11c in 18.7 – 50.2 % of previously CD11c$^-$ cells (Figure 7). The cells also developed a DC-like morphology in culture when viewed under light microscopy (not shown).

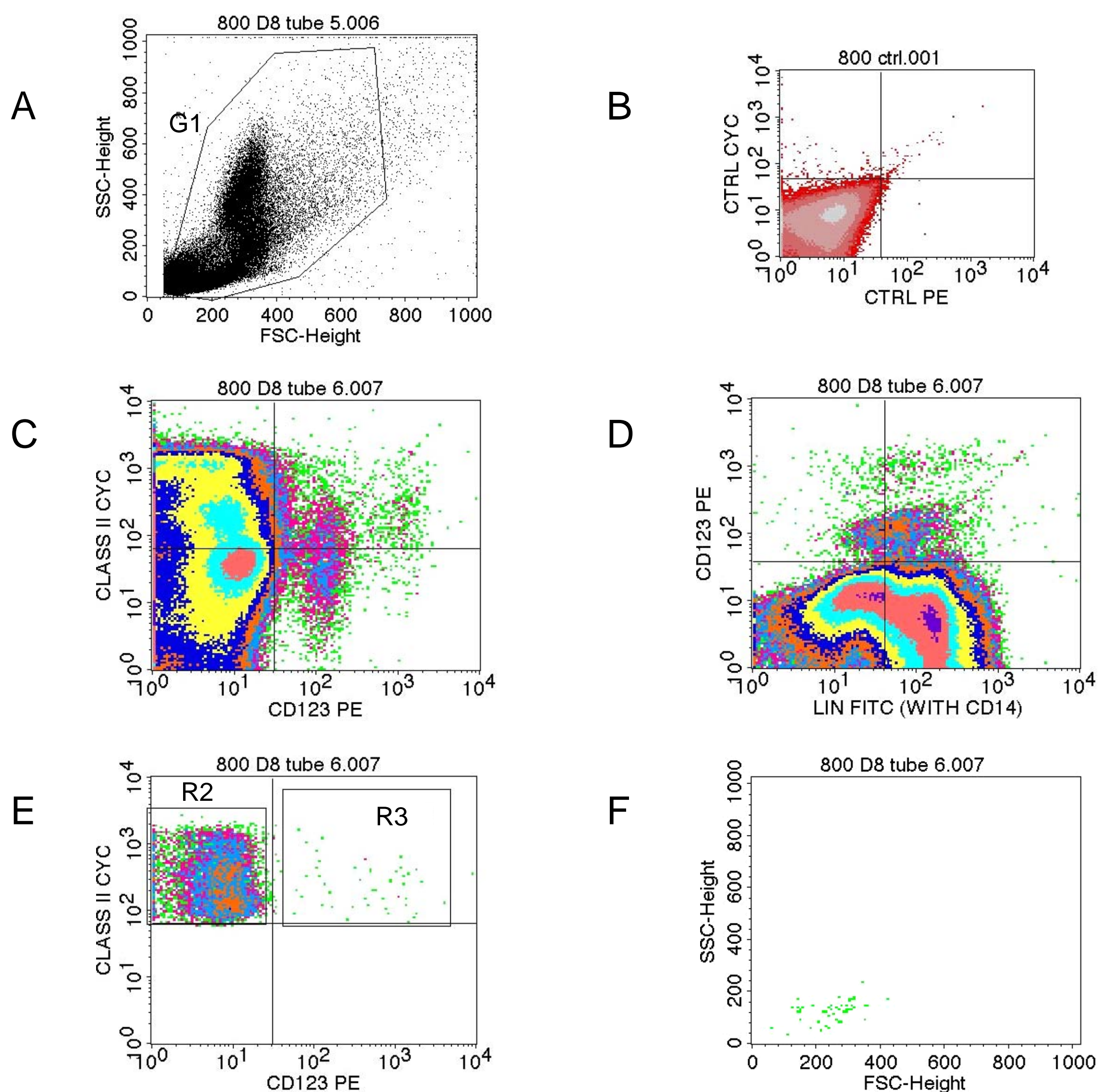
Figure 7. FLT3-L mobilised, FACS isolated, Lin$^-$ClassII$^+$CD11c$^-$ cells acquire CD11c in culture with GM-CSF and IL-4. Data representative of n=4 experiments. The dot plot shows gating of cells of interest, and the histogram shows CD11c-PE fluorescence intensity (red area) compared to isotype-matched PE control (green line), after 48 hours in culture.



3.5 Identification of Lin-ClassII+CD123+ Cells in FLT3-L Mobilised Marmosets

A marmoset cross-reactive anti-human CD123 antibody was identified by our group during the course of this study, and in the last two animals mobilised for this study, staining for CD123 was included at day 8 (animal 800) and day 14 (animals 79f and 800). In animal 800, 3.2 % of cells were $CD123^+$ at day 8 and 3.7 % at day 14. In animal 79f, 0.81 % of day 14 cells were $CD123^+$. While $ClassII^+CD123^+$ cells were identified (0.2 – 1 % of cells), there appeared to be two populations of $CD123^+$ cells with medium and high intensity expression (Figure 8.C). A large proportion of $CD123^+$ cells were also Lin^+ (Figure 8.D), suggesting they were not true plasmacytoid DC. Further analysis of cells within the Lin^- $ClassII^+$ fraction identified a very small population of $CD123^+$ cells (region 3, Figure 8.E), constituting 0.05 – 0.64 % of cells. Although tiny, this population was detectable within the small peripheral blood sample used for these analyses, with forward and side scatter profile similar to PDC (Figure 8F).

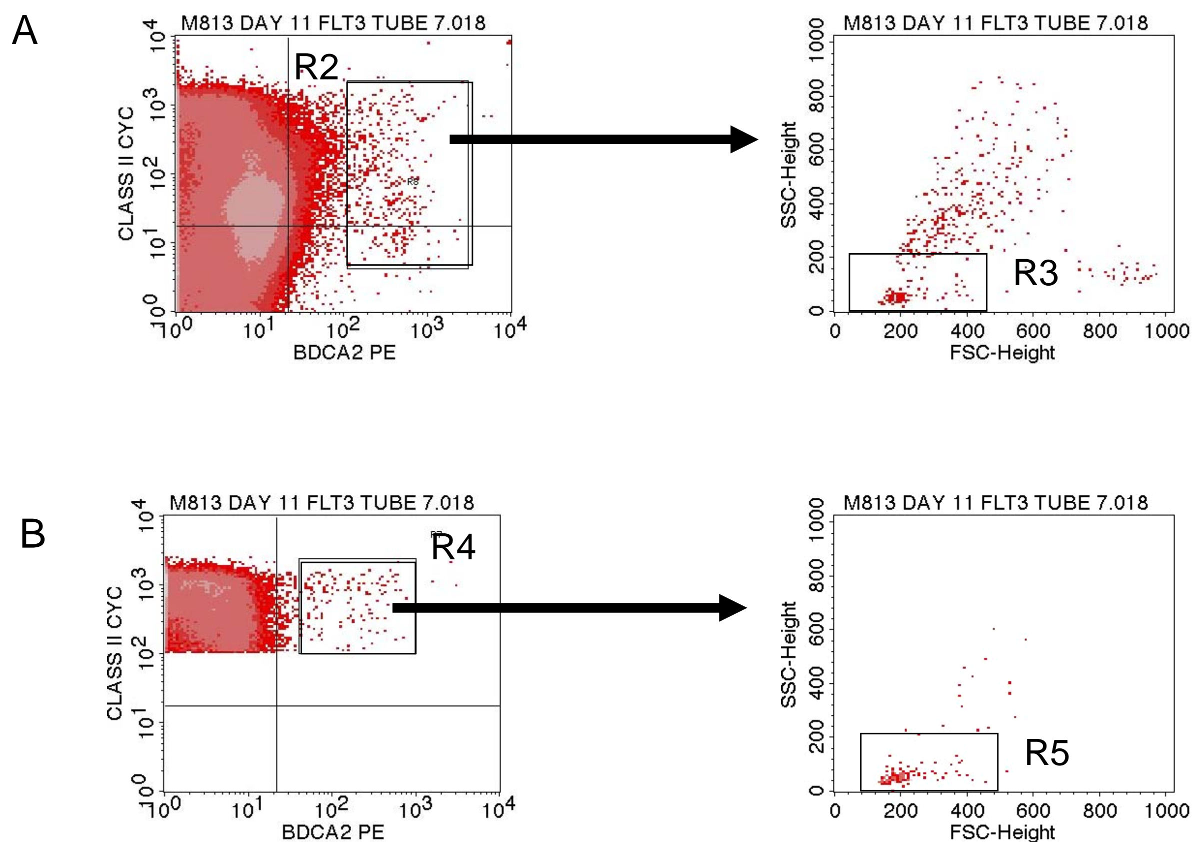
Figure 8. Identification of CD123^{+} cells in marmoset 800 (data representative of two experiments). This animal was treated with FLT3-L for 10 days. Cells of interest were identified within Gate 1 (G1) based on forward (FSC) and side (SSC) scatter profile (panel A). Quadrants were set by isotype-matched controls for each fluorochrome (example shown in panel B). Panel C is a density plot showing Class II (y axis) and CD123 (x axis) expression of cells within G1. Panel D shows CD123 (y axis) and lineage marker (x axis) expression of cells within G1. Panel E shows Class II (y axis) and CD123 (x axis) expression of cells within Gate 2 (Class II^{+} Lin^{-} cells), with Lin^{-}ClassII^{+}CD123^{+} cells within region 3 (R3). Panel F shows the FSC and SSC profile of cells within R3 (putative plasmacytoid DC).



3. 6 Identification of $Lin^-ClassII^+BDCA2^+$ Cells in FLT3-L Mobilised Marmosets

Human BDCA2 monoclonal antibody was tested on mobilised marmoset blood. Virtually no $BDCA2^+$ cells were identified from un-permeabilised samples. Permeabilisation of cells enhanced BDCA2 antibody binding, with a $BDCA2^+$ population identified (Figure 9A and B, regions 2 and 4). Further analysis revealed that that majority of this population was Lin^+, and that excluding lineage cells reduced the Class II^+BDCA2^+ population substantially (Figure 9B), although a Lin-ClassII+BDCA2+ population was identified. However, the forward and side scatter profiles of $BDCA2^+$ cells suggested non-specific staining of cells throughout Gate 1, although a population of cells with lymphoid / PDC-like profile were also noted (regions 3 and 5).

Figure 9. Identification of BDCA2$^+$ cells in marmoset blood (data representative of four experiments). Cells of interest were identified within Gate 1 (G1) based on forward (FSC) and side (SSC) scatter profile (similar to Fig 8, panel A). Quadrants were set by isotype-matched controls for each fluorochrome. Panel A is a density plot showing Class II (y axis) and BDCA2 (x axis) expression of cells within G1. BDCA2$^+$ cells are identified within region 2, and the FSC / SSC profile of these cells shown in the adjacent plot. Panel B shows Class II (y axis) and BDCA2 (x axis) expression of cells within Gate 2 (Class II$^+$ Lin$^-$ cells), with Lin$^-$ ClassII$^+$BDCA2$^+$ cells identified within region 4. Cells within region 3 and 5 are possibly plasmacytoid DC, based on surface markers expression and FSC / SSC profile consistent with PDC. See text for discussion.



4. DISCUSSION

Characterisation of *in-vivo* DC in primate species is difficult due to the rarity of these cells in blood and tissues. In NHP models, sacrificing animals to obtain sufficient DC for study is costly and evokes ethical considerations. The use of haematopoietic growth factors to mobilise DC is a means of overcoming this hurdle. This is the first study to demonstrate the successful use of recombinant human FLT3-L to mobilise peripheral blood myeloid pre-DC in a New World monkey species. A ten-day course of FLT3-L produced a substantial rise in peripheral blood $Lin^{-}ClassII^{+}CD11c^{+}$ putative myeloid DC, that had morphology similar to rhesus and human myeloid DC, and exhibited significant allostimulatory ability in MLR. These preliminary findings confirm the biological relevance of this model for DC–based research.

Peripheral blood MDC rose 73.3 ± 20.3 fold above baseline following 10 days of FLT3-L administration, enabling a large number of MDC to be isolated by FACS. At best, in this study over half a million viable MDC were obtained from a 1ml peripheral blood sample taken at day 14 (animal 79f). This is in keeping with other NHP studies[36,40]. Peak MDC mobilisation was observed at day 14. A similar delayed peak in DC and haematopoietic progenitor populations towards the second week of treatment with FLT3-L has been noted in other NHP studies of DC kinetics where this time-point monitored, and is likely due to the long half-life of CHO-expressed FLT3-L [40,94]. It is an important consideration when deciding the optimal time for peripheral blood sampling to obtain maximum DC yield, particularly when the small size of the marmoset limits to some degree the frequency and volume of blood sampling. Sample volumes in this preliminary study were kept to minimum due to the frequency of blood sampling required to obtain MDC kinetic data. However, in future studies, it is entirely feasible that animals mobilised with FLT3-L will tolerate two larger volume (2-3ml)

venepunctures at day 14-15. This could potentially yield > 2 million MDC from one mobilised animal, without compromising animal health and thereby maintaining the DC donor for future transplant studies. This is a sufficient dose of cells to be used for DC manipulation and DC-based immunotherapy.

Future Investigations Arising from this Preliminary Study

Further studies to fully characterise marmoset FLT3-L mobilised MDC are essential and are planned, but were beyond the time-frame and scope of this thesis, and are discussed below.

In view of the considerable allostimulatory capacity of marmoset MDC and the inconsistent effect of CD40L on enhancing this capacity, additional definition of the maturation status of freshly isolated marmoset MDC is vital. This includes assessment of antigen-uptake ability and surface markers indicative of maturation including co-stimulatory molecules. We anticipate future experiments in which sorted MDC exposed to maturation stimuli will be assessed for CD80, CD86 and CD40 expression using four-colour antibody labeling techniques that are currently being optimized in our laboratory for this purpose. In addition, supernatants from cultures in these experiments have been stored, for analysis of cytokine production that will define maturation status and Th1/Th2 responses elicited by these DC. We are currently identifying reagents that are cross-reactive with marmoset samples for the detection of IL-12, IL-10, IL-6 and other relevant cytokines produced by DC or important to DC function.

The $Lin^{-}ClassII^{+}CD11c^{-}$ population identified in FLT3-L mobilised marmosets also requires further characterisation. In rhesus monkeys, this population has previously been found to expand with FLT3-L administration [40], although this was not observed in marmosets. In

keeping with the findings of Coates *et al.*[36], a large percentage of these cells up-regulated CD11c expression after brief culture in IL-4 and GM-CSF, suggesting that this population may contain DC precursors that are responsive to these cytokines. Other studies have suggested that this population contains $CD34^+$ stem cells, plasmacytoid DC or $CD20^-$ B lymphocytes that were not excluded by the lineage gate[36,40]. The nature of this population remains to be determined in marmosets, although it is probable that plasmacytoid DC are to be found in this population.

A major limitation of our study of marmoset PDC at present is the lack of markers specific for this DC subset. CD123 is present and identifiable on rhesus PDC, although CD123+ cells represent only a small percentage (1-7 %) of the Lin-ClassII+ population, even after FLT3-L treatment. In marmosets we have recently identified a potentially cross-reactive anti-human CD123 antibody, and in this study tested this antibody in mobilised marmosets for the first time. Small numbers of $CD123^+$ cells were identified in two preliminary experiments, however, a proportion of these cells were within the Lin^+ fraction. This data may reflect suboptimal antibody concentration, non-specific binding or other technical factors that require optimising to accurately detect this rare cell type. Clearly further studies are required to improve detection of marmoset CD123 expression and define this population further before concluding they are PDC. Our group has recently cloned marmoset CD123 (N. Rogers, C. Drogemuller, P.T.H. Coates *et al*. unpublished data, personal communication), which may enable development of a marmoset-specific anti-CD123 antibody.

BDCA-2 is another marker weakly but very specifically expressed on human PDC. It has not been found to be cross-reactive with rhesus PDC [36]. We have previously identified BDCA-2 positive cells in marmoset lymphoid tissue after cell permeabilisation (S. Kireta, A. Milton,

P.T.H. Coates *et al*., unpublished data), indicating that the antibody for this molecule is cross-reactive with marmoset samples. As part of this study, small numbers of BDCA-2 positive cells were identified in the $Lin^{-}ClassII^{+}$ fraction of mobilised marmoset peripheral blood after cell permeabilisation. On forward and side-scatter plots these cells were located throughout Gate 1 in most experiments, although some $BDCA\text{-}2^{+}$ cells did have the FSC/ SSC profile of PDC. At this stage BDCA-2 staining is not adequately specific, and methods for using this marker for marmoset PDC remains to be optimised.

The issues of cell yield and viability will also need to be addressed in future experiments. Yield may be affected by first enriching for the PBMC population, or depleting the PBMC population of lineage positive cells to enrich the DC population, although this involves additional manipulation of the sample (including washes) during which cells may be lost, and has not been shown to significantly improve yield from rhesus samples[40]. This becomes relevant given the small blood sample size. The process of cell sorting in itself can affect cell viability. It is also possible that chemical lysis of erythrocytes prior to sorting is detrimental to viability. Samples from animals 79f and 800 on day 15 had reduced exposure to lysis buffer, with residual erythrocytes in the cell suspension. This did not affect the ability to high-speed sort, and viability was improved, therefore this strategy will be employed in future.

Finally, the mobilisation of marmoset tissue DC by FLT3-L remains to be investigated. Peripheral lymph nodes such as inguinal nodes are easy to access and remove with minimal morbidity, and have previously provided insight into the capacity of FLT3-L to promote DC migration into lymphoid tissues in rhesus monkeys[36]. The mobilisation of renal and hepatic myeloid DC by FLT3-L has also been observed in this species [39], and has relevance when using mobilised animals as donors due to the enrichment of the DC population. Whether

FLT3-L mobilised organ DC promote tolerance or rejection when transplanted has not been resolved (discussed in Chapter 1, section 3.2.6).

Conclusions

This preliminary study of recombinant human FLT3-L administration in a small group of common marmosets confirms biological activity of this important DC growth factor *in-vivo* in this species, with significant mobilisation of putative myeloid $CD11c^{+}$ DC into peripheral blood. Using fluorescence activated cell sorting, MDC can be isolated in numbers sufficient for *in-vitro* characterisation, further assessment of any tolerogenic potential and possible use in DC-based tolerance strategies. Marmoset MDC have typical MDC morphology and are allostimulatory, providing the first evidence that marmoset blood DC exist within the paradigm established for human and rhesus DC.

CHAPTER 6: INFUSION OF DONOR-DERIVED IMMATURE DC IN MARMOSET RECIPIENTS

1. INTRODUCTION

Several strategies for DC immunotherapy in solid organ transplantation have had preliminary testing in murine and rodent models with promising outcomes including abrogation of rejection and prolongation of allograft survival in the absence on ongoing immunosuppression (discussed in Chapter 1). Validation of these studies in non-human primate (NHP) species has been extremely limited [97], but is an essential step towards developing and critically evaluating these strategies, and potentially translating them to the clinical setting [190,192,229]. The common marmoset is a New World Monkey species that is being developed by our group as a novel small NHP model for transplant-related research. Chapter 3 of this thesis describes a method for rapid, accurate sequence-based tissue-typing of marmoset MHC DRB for the purpose of selecting adequately mismatched donor-recipient animals pairs[342,343]. Chapters 4 and 5 describe the identification and characterisation of marmoset *in-vitro* and *in-vivo* propagated myeloid DC. From these studies, marmoset immature, maturation-resistant monocyte-derived DC (MoDC) from G-CSF mobilised monocytes were identified as a possible candidate cell for further evaluation of tolerogenic potential *in-vivo*. By using G-CSF to mobilise peripheral blood monocytes, millions of MoDC could be generated from peripheral blood samples in this small NHP, sufficient for therapeutic use.

In this present study marmoset immature MoDC acquired from donor animals were administered to allogeneic recipients, as the first step towards establishing this species as a clinically relevant model for testing DC immunotherapy. This preliminary study enabled us to

assess the feasibility and safety of allogeneic DC infusion in this species, and monitor the subsequent immune response. DC immunotherapy has previously been documented in only one other New World Primate species [286], and these studies have no precedent in the common marmoset.

2. METHODS

2.1 Donor and Recipient Selection

Healthy naïve animals of weight > 300g were used from the local TQEH colony. Three donor and recipient pairs were chosen from several possible pairs on the basis of *Caja*-MHC genotyping as established in Chapter 3, and by D. Dang (Honours Thesis, personal communication, MHC Class I and Class II DQ genotyping). Due to the very restricted polymorphism at Class I loci and Class II loci other than *Caja*-DRB, it was not possible to select completely MHC mismatched animals from within the group of available animals in our colony. As it has been shown by our group that MHC Class I matching does not have a strong influence on allo-reactivity (D. Dang, personal communication), whereas MHC Class II DRB matching reliably predicts allo-reactivity *in-vitro* [342,343], matching was limited to *Caja*-DRB loci (Table 1). The animal pairs chosen also had strong, reproducible and consistent allo-reactivity in baseline one-way MLR which was performed twice at two separate time points prior to DC infusion. All animals chosen for this study had a negative cross-match against donor marmoset red cells, and were deemed to be blood group O on the basis of lack of reactivity with human anti-sera.

The immune response to third party animals was also assessed. Due to technical problems with blood taking and cell yield from the third party animal chosen for Pair 1 (marmoset 70F),

this animal was abandoned after week 3. PBMC from a different third party animal was used in week 4 (marmoset 74M). 70F and 74M had identical MHC genotype. Data must be interpreted considering the lack of baseline data for appropriate comparison in this animal.

Table 1. MHC genotype of donor and recipient pairs. Alleles are shown for *Caja*-E, -G, -DRB*W16, -DRB1*03, -DQA1, -DQB1 and -DQB2. Recipient 1 (828) was exposed to 4 foreign donor antigens. Recipient 2 (70M) was exposed to 5 foreign donor antigens. Recipient 3 (805) was exposed to 6 foreign antigens.

	Animal	G	E	DRB*W16	DRB1*03	DQA1	DQB1	DQB2
Pair 1								
Recipient 1	828	03, 04	01, -	1605, 1608	0304, -	2501, 0101	22012, 2201	0101, 0102
Donor 1	873	03, 04	01, -	1601, 1623	0301, 0303	2501, -	22012, 2201	0101, -
Third Party 1	74M	04,05	01, 02	1604, -	0302, 0303	2501, 2501	22012, 2201	0101, -
Pair 2								
Recipient 2	70M	03, -	01, -	1605, 1624	0302, -	2501, -	22012, -	0101, -
Donor 2	734	03, -	01, -	1623, -	0303, 0304	2501, 0101	22012, 2201	0101, -
Third Party 2	862	03, -	01, -	1608, -	0301, 0307	2501, 0101	22012, 2201	0101, 0102
Pair 3								
Recipient 3	805	03,04	01,-	1604, -	0302, 0304	2501, -	22012, 2201	0101, -
Donor 3	823	03,04	01,-	1605, 1608	0301, 0307	2501, 0101	22012, 2201	0101, 0102
Third Party 3	804	03,04	01,-	1605, 1610	0303, -	2501, -	22012, 2201	0101, -

2.2 Generation and Infusion of MoDC

Naïve donor animals were given recombinant human G-CSF 10ug/kg/day subcutaneously for 5 days (days 1-5). Donors underwent venepuncture to collect 1-1.2ml peripheral blood on days 6 and 7. Immature monocyte-derived DC were generated as described in Chapter 2, section 5.1. DC were collected on day 7 and washed extensively in PBS to remove cytokine and serum. Cell counts were performed using trypan blue exclusion, with viability of > 95 %. Two million immature MoDC were generated from each donor and all cells were used for infusion. DC in 200ul of sterile PBS with 0.1 % heparin sulphate were transferred to a syringe with 27.5 gauge needle attached, and given to un-anaesthetised, restrained recipient animals intravenously as a bolus via the femoral vein. Haemostasis was achieved by pressure on the femoral vein and animals were observed 2-3 times daily for the following week.

2.3 MLR

One-way MLR was performed as described in Chapter 2, section 6.2. Responder cells ($1x10^5$ PBMC) from recipient animals) were stimulated by $1x10^5$ irradiated (30 Gy) PBMC from donor or third party animals.

2.4 Interferon Gamma (IFN-γ) ELISpot Assay

The human IFN-γ enzyme linked immunospot assay (ELISpot; Mabtech) was used to detect IFN-γ production by marmoset responder cells stimulated with irradiated donor PBMC. The final method used is described in Chapter 2, section 9. This method was optimised after trials of several protocols in which the timing and conditions of co-culture of cells as well as the duration of incubation in the ELISpot® plate were varied. The established method [344] of co-culturing $1x10^5$ responder and stimulator PBMC directly in the flat-bottomed ELISpot plate for 24-48 hours did not result in detectable IFN-γ production, although PMA-treated controls

were positive. It was postulated that this cell concentration did not achieve sufficient cell-cell contact for stimulation. Therefore cells were first co-cultured in a round-bottomed plate for 24 hours before being harvested and transferred to the ELISpot plate for a further 24 hours. This method of pre-culturing cells before transfer has been previously described [345], and led to easily detectable spots.

ELISpot experiments conducted in week 6 and 8 for recipients 828 and 70M failed due to technical error related to capture antibody. Additionally, baseline data was unfortunately not available for animal 70M again due a one-off technical issue.

3. RESULTS

3.1 Safety of DC Immunotherapy

DC immunotherapy using blood group-matched, allogeneic donor DC cultured in xenogeneic serum and human cytokines was safe and well tolerated in marmoset monkeys. There were no immediate local complications from the DC infusion, and no adverse events were noticed at any stage during the study. All animals tolerated the frequent blood sampling without adverse events such as weight loss, and minimal change in haemoglobin was observed. Peripheral blood leukocyte counts also remained unchanged.

3.2 Changes in Anti-Donor Immune Responses

Anti-donor immune responses were monitored over a three month period by measuring *in-vitro* allo-reactivity in MLRand the number of IFN-γ-producing T-cells in recipient PBMC cultures stimulated by irradiated donor or third party PBMC. Different responses were

observed in all three recipients, although in general two recipients exhibited transient (but non-specific) immune hyporesponsiveness, and one recipient exhibited donor-specific immune sensitization (Figure 1). In all recipients, IFN-γ secretion was lower in the weeks immediately post-infusion and subsequently rose by weeks 12-22 (Figure 2). The results for each recipient are discussed below. Please refer to Table 1 for *Caja* mismatching between animals.

Recipient 828 showed a marked reduction in donor-specific alloreactivity by the first week following DC infusion (Figure 3A). This was sustained up to week 8 post-infusion, however appeared restored by 12 weeks. IFN-γ secretion was also reduced up to week 4, and also rose to baseline levels by week 12 (Figure 4A). The response to third party PBMC was also measured, although due to technical issues data was only available at week 4, when a different third party animal was selected. The response to third party cells was similar to the anti-donor response, indicating the reduction in immune responsiveness was non-specific.

Recipient 805 also exhibited reduced MLR responses 6 weeks post-infusion (Figure 3C). Anti-donor alloreactivity was higher than anti-third party response at most time points. IFN-γ secretion from recipient PBMC fell one week post-infusion and although variable, returned towards baseline levels by week 22 (Figure 4C). Anti-donor and anti-third party response was similar.

In contrast, Recipient 70M showed progressively heightened anti-donor reactivity in MLR, peaking at week 6 (Figure 3B). Third party responses also increased from weeks 1-4 but to a much lesser degree compared to anti-donor responses, and then returned to levels observed at week 1. In parallel to *in-vitro* alloreactivity, a progressive rise in IFN-γ secretion from

recipient PBMC was observed in response to donor PBMC, whereas the response to third party PBMC remained static throughout (Figure 4B). These findings suggest that donor-specific immune sensitization occurred in this recipient, peaking at week 6 then returning to near baseline levels, and this was greater than the immune response to third party cells.

Figure 1. Anti-donor alloreactivity of recipient PBMC as assessed by one way MLR using irradiated donor or third party PBMC, following donor DC infusion. Stimulation Index = (stimulated proliferation: baseline proliferation). Anti-third party data not shown (see Figure 3). Time-point 0 represents pre-infusion data.

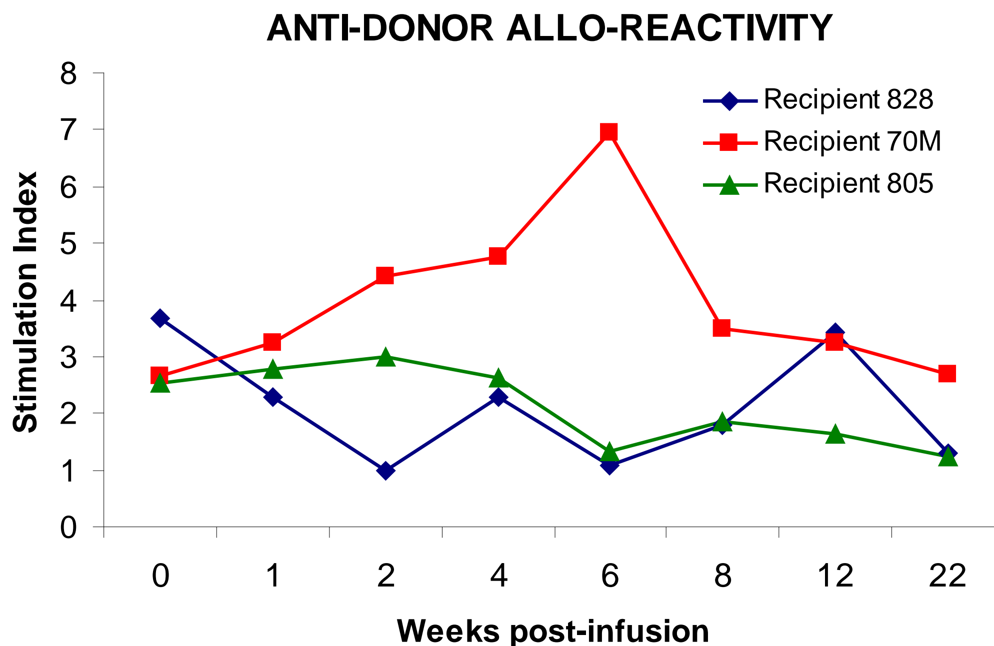


Figure 2. IFN-γ production by recipient PBMC stimulated with donor PBMC following DC infusion. Stimulation index = (IFN-γ production stimulated PBMC: Baseline PBMC). Anti-third party data not shown (see Figure 4). Time-point 0 represents pre-infusion data.

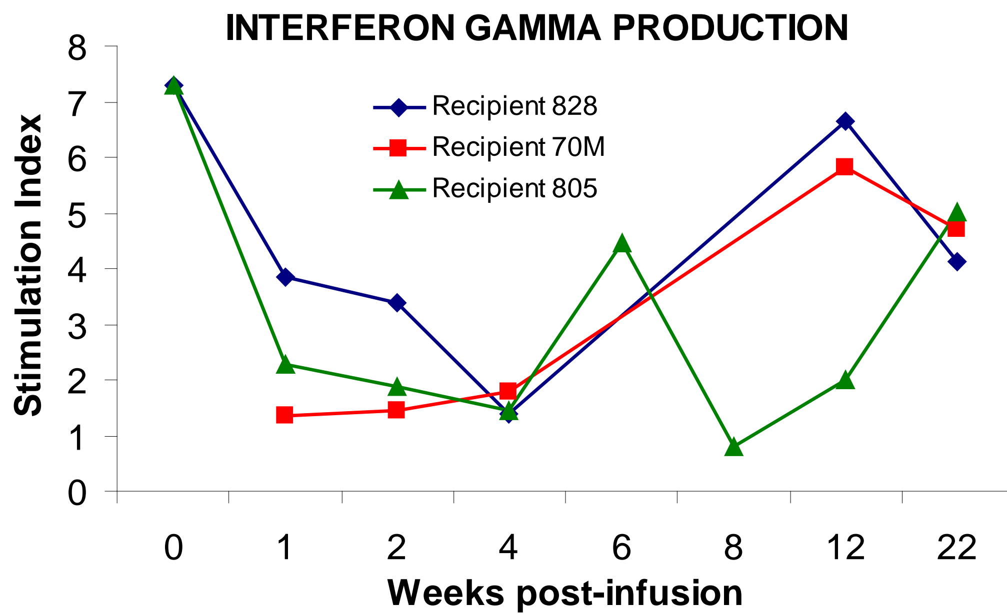
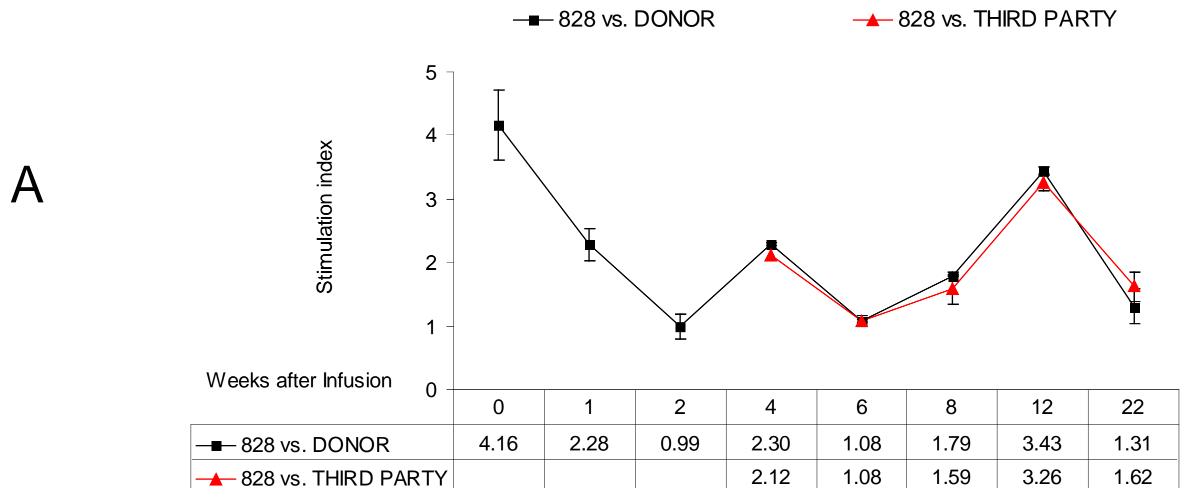
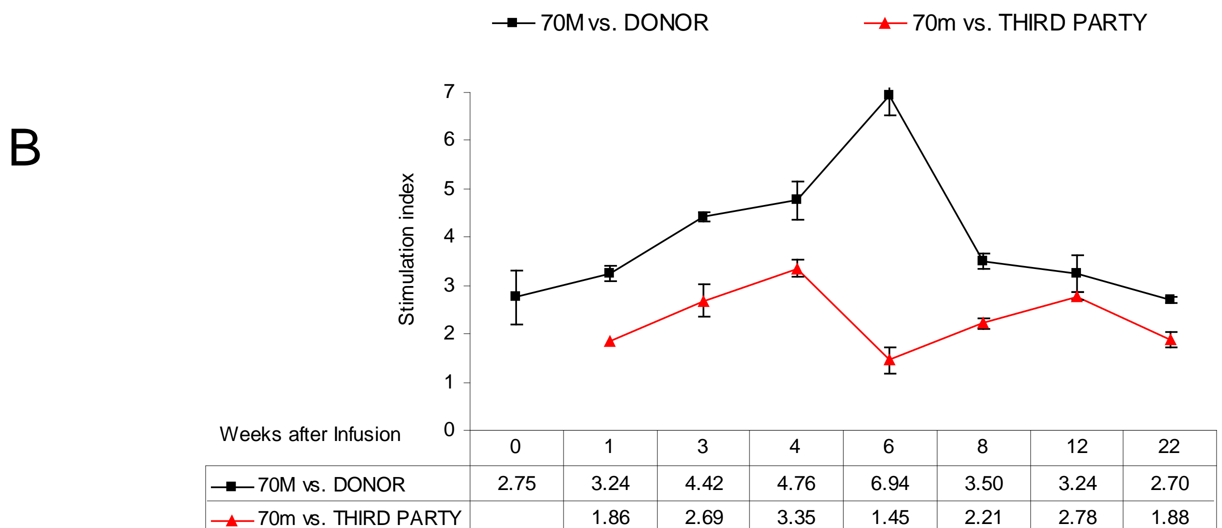


Figure 3. Individual MLR Stimulation Index (stimulated proliferation: baseline proliferation). A. Recipient 828; B. Recipient 70M; C. Recipient 805. Time-point 0 represents pre-infusion data.

A



B



C

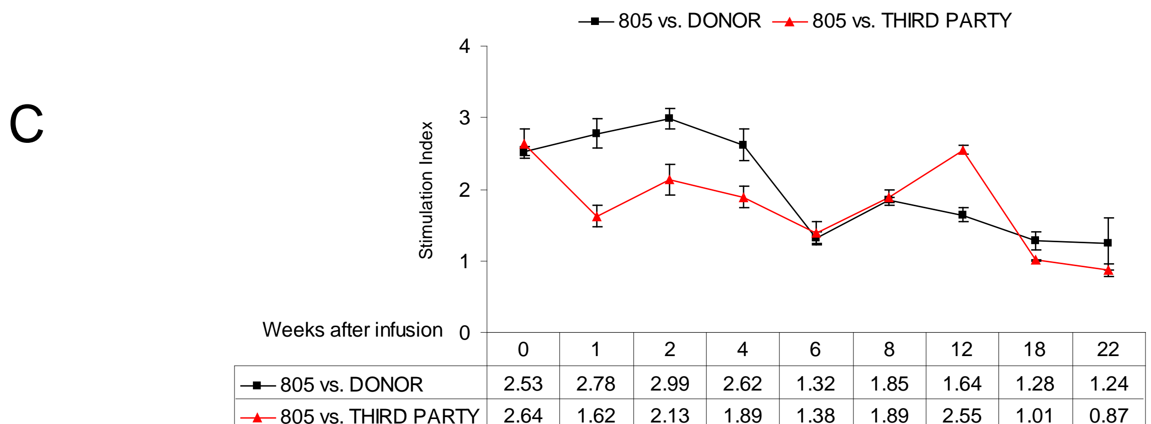
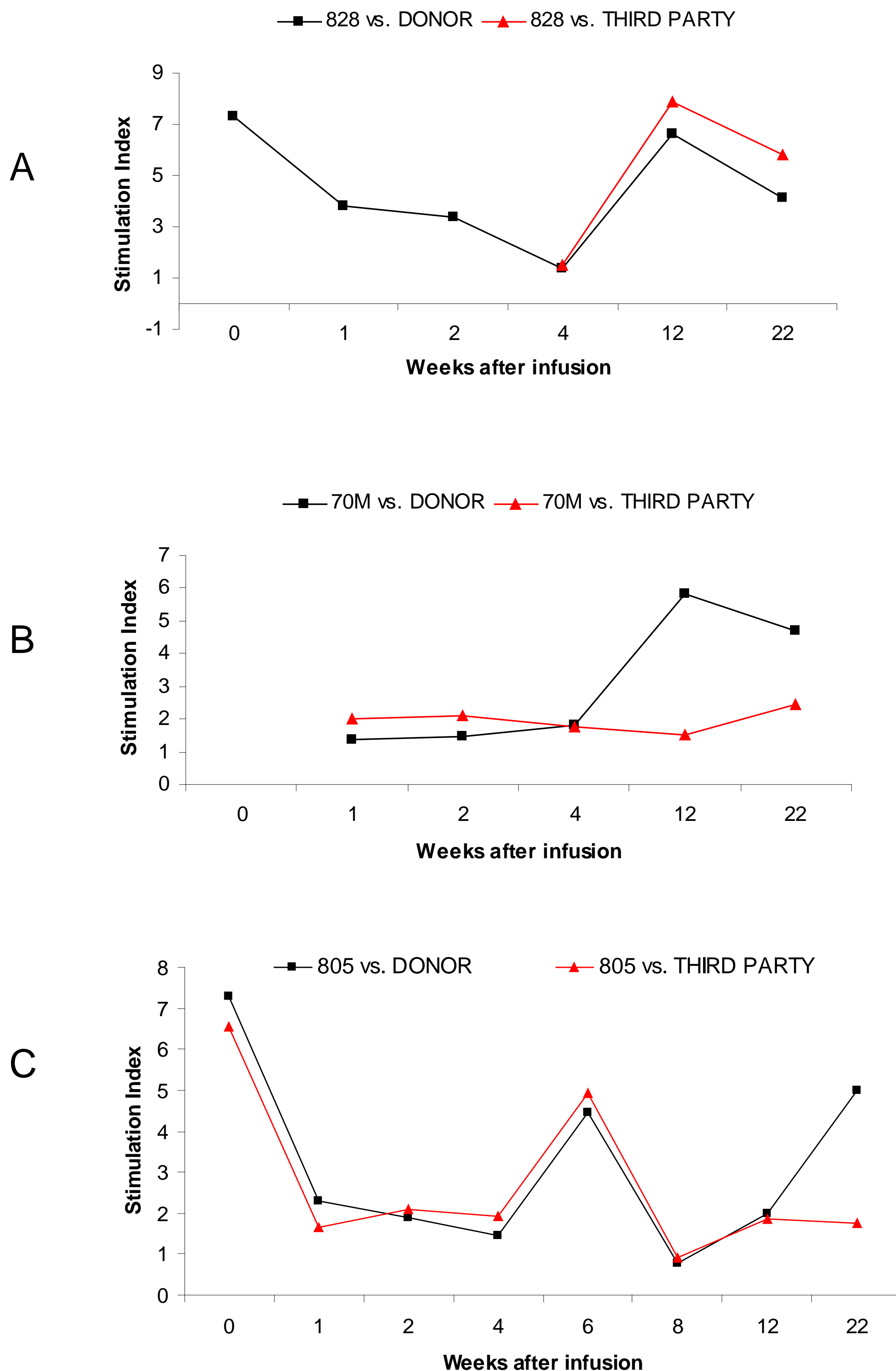


Figure 4. Individual data for ELISpot stimulation index (IFN-γ production stimulated PBMC: Baseline PBMC). A. Recipient 828; B. Recipient 70M; C. Recipient 805. Time-point 0 represents pre-infusion data.



4. DISCUSSION

Although the potential of DC immunotherapy to manipulate immune outcomes has been well-established in rodent and murine models, few previous studies have assessed the effect of DC infusion in a non-human primate model. This study is the first to describe *ex-vivo* anti-donor immune response modification by donor MoDC infusion in a New World Primate species. The tissue-typing data reported in Chapter 3 was used to select animals from the colony. Donor, third party and recipient animals were chosen on the basis of complete *Caja*-DRB mismatching, although mismatches at other loci were present in some instances (Table 1). In addition, there was minimal overlap of *Caja*-DRB alleles between donor and third party animals, to facilitate separate immune responses by recipient cells. Two million G-CSF mobilised, immature donor MoDC were able to be propagated from each donor and safely administered intravenously to recipient animals, equating to a DC dose of 5 million / kg. Two animals (828 and 805) developed a non-specific reduction in anti-donor and anti-third party alloreactivity, which did not return completely to baseline levels by week 22. Allo-PBMC-stimulated IFN-γ production was also reduced transiently, but rose by 2-3 months post-infusion. Based on previous small animal studies, it was unlikely that unmodified, stably immature MoDC alone would be sufficient to promote sustained donor-specific immune hyporesponsiveness; however a finding of transient immune hyporesponsiveness is of interest. One animal (70M) had a transient, donor-specific increase in alloreactivity, associated with a late rise in IFN-γ production in response to donor PBMC compared with third party. This may represent sensitization to donor antigen, or correspond to processes that are important for promoting immune acceptance such as regulatory T-cell proliferation.

These findings confirm that allogeneic immature marmoset MoDC can be generated for therapeutic use, are well-tolerated when administered intravenously, and are capable of inducing definite (albeit transient and variable) immune responses. This validates the ability of these DC to present antigen and influence immune modulation *in-vivo*. In all recipients, the response to re-challenge with donor antigen (*in-vitro* or *in-vivo* with cell infusion or organ transplant) will be extremely informative. Other uncertainties such as the best route of administration of DC (intravenous vs. subcutaneous or dermal), and the ultimate fate of injected DC remain to be resolved. In particular, we do not know the long-term effects of DC therapy on populations of regulatory or effector T cells, and whether detrimental immune responses may be induced. Experiments to resolve these questions are planned but were beyond the time-frame of this thesis.

Two previous studies are relevant to the present work. Only one group has previously described DC immunotherapy in a New World Primate species. Gabriela et al. [286] propagated MoDC from owl monkeys (*Aotus* species), and found that administering autologous DC pulsed with tetanus toxoid primed subsequent *in-vitro* responses to the protein. More recently, Zahorchak *et al*. [97] conducted the first ever study of the effect of treatment with tolerogenic or regulatory DC in rhesus monkeys. Similar to our study, large numbers (up to 100 million/donor) of donor MoDC were generated from peripheral blood monocytes mobilised with G-CSF and GM-CSF, cultured under the influence of IL-4 and GM-CSF. These cells were rendered ‘stably’ immature by treatment with Vitamin D3 and interleukin-10 (VitD3/IL-10), with minimal allostimulatory ability, low co-stimulatory molecule expression and resistance to phenotypic maturation even after exposure to a pro-inflammatory cytokine cocktail. Fifteen million VitD3/IL-10 MoDC were administered intravenously with or without additional co-stimulation blockade with CTLA4-Ig. This equates to a DC dose of 1.5-3 million

/ kg in animals 5-10kg in weight. Following DC infusion, both anti-donor and anti-third party responses were increased for up to 4 weeks. After this time point, only the MoDC administered with co-stimulation blockade led to a reduction in anti-donor and third-party responses up to 100 days post-infusion. Animals treated with VitD3/IL-10 MoDC alone continued to have high responses up to 100 days post-infusion. Responder T-cells were found to up-regulate IL-10 and IFN-γ production, leading to the authors to suggest these findings were due to an "active immuno-regulatory T-cell response" induced by DC. No rise in circulating anti-donor allo-antibodies was observed, indicating lack of a significant humoral response to donor DC infusion. This study signified an important forward step in the *in-vivo* testing of tolerogenic DC.

One of the aims of this work was to address technical issues related to DC immunotherapy in marmosets, to assess the feasibility of this model for DC-based tolerance research. It was established that MoDC could be generated in sufficient numbers from donor animals, cultured in xeno-cytokines and xenoprotein, and safely administered to recipient animals. Post-infusion immune monitoring was limited largely to MLR alloreactivity and IFN-γ production. The MLR was performed twice for each donor-recipient pair at baseline prior to any treatment, and was shown to be reproducible. While inter-assay variability is always an issue with MLR, the consistent baseline data and then the subsequent change from baseline after the infusion does suggest the changes observed in stimulation index are likely to be real. Despite this, it is agreed that MLR is a crude way of monitoring immune responsivity, and other more sophisticated methods need to be developed for the marmoset and other primate models.

This study represents the novel adaptation of the ELISpot method to marmoset samples for the purpose of monitoring immune responses over time. IFN-γ is an important immuno-regulatory

cytokine, with effects on multiple cell types which promote host defenses. It is produced by NK cells, Th1 T-cells, effector T-cells, as well as regulatory T-cells. To a lesser extent it is also produced by DC, macrophages and neutrophils. IFN-γ up-regulates MHC molecules, B7 antigen, Fc receptors and adhesion molecules, and activates macrophages, stimulates B-cell antibody production and promotes Th1 skewing. The ELISpot detects single cells producing IFN-γ, enabling monitoring of antigen specific T-cell responses in response to antigen challenge, before and after a therapeutic intervention such as DC immunotherapy. The advantage of this technique is its sensitivity at detecting low frequency T-cell IFN-γ (<1/1000 cells). This makes it suitable for marmoset work, where cell numbers are limited due to small blood sample size. The ELISpot assay of T-cell IFN-γ production is a surrogate measure of immune reactivity between recipient and donor that has been use in human clinical studies, where a positive or negative ELISpot correlates with acute rejection, chronic allograft nephropathy and renal function in some patient groups [344]. Interestingly it does not necessarily correlate with HLA-matching or panel-reactive antibody level, suggesting it reflects aspects of immune responsiveness that are independent of MHC. This assay was optimised for marmoset samples, and will be a valuable technique for post-transplant immune monitoring in this model.

Additional strategies for immune monitoring include measurement of cytokine levels in plasma and supernatants. Cytokines involved in promoting tolerogenic immune responses are of particular interest, including IL-12p70, IL-10 and TGF-β. At the present time, methods for measuring these cytokines in marmoset samples are being validated in our laboratory. Methods for monitoring anti-donor allo-antibody responses are also being developed. The validation and application of these techniques to this study was beyond the scope and time-frame of this thesis. However, all samples have been stored for future analysis once these

assays are established. This data will be informative and further define the nature of the immune response to allogeneic DC infusion.

In conclusion, this study furthers the development of the marmoset model. The success of these preliminary studies of DC immunotherapy in producing observable immunological consequences validates this model. Future experiments using the marmoset model which further explore the safety, long term immunological consequences, and optimum features of tolerogenic DC therapy will be valuable in progressing this field of research.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

1. SUMMARY AND CONCLUSIONS

Strategies for the use of DC immunotherapy to promote immune tolerance have been well-established in small animal models, but evaluation in non-human primates remains extremely limited. The work presented in this thesis confirms that the common marmoset is a valid, feasible non-human primate model in which to explore DC-based immunotherapeutic strategies and transplant-related research.

The ability to select immunologically disparate or similar animals for *in-vitro* and transplant studies by characterisation of MHC is an essential requirement of any transplant model. Current sequence-based tissue typing methods using marmoset-specific primers have been applied to genotype the most polymorphic marmoset MHC region, *Caja*-DRB, as described in Chapter 3. This has led to the identification of two new alleles, *Caja*-DRB*W1623 and *Caja*-DRB*W1624. The entire local colony has been assessed, with twenty-six genotypes identified. Using selected animal pairs, DRB genotyping has been shown to accurately predict *in-vitro* immune responsiveness in MLR. Our group now uses DRB matching as a rapid, accurate way to select animals for *in-vitro* work, and donor-recipient pairs for DC therapy and transplant studies.

The characterisation of marmoset DC biology has thus far been very limited, and is significantly expanded by the work presented in Chapters 4 and 5. One of the main technical hurdles related to the marmoset model is the small size of the animal, the small blood volume available for sampling, and of course, the rarity of DC and DC precursors. By using G-CSF to

mobilise monocytes and $CD34^+$ stem cells, it has been demonstrated that large numbers of these DC precursors can be obtained from a small volume (< 2mls) of peripheral blood. This negates the need to sacrifice animals to obtain sufficient cells from larger volumes of blood or bone marrow, thereby preserving the same allogeneic donor animal for repeat DC donation, subsequent organ procurement and other studies. This is a very important consideration if this species is to be a feasible model for therapeutic DC manipulation in transplantation.

Optimum methods for marmoset monocyte isolation have been developed; in particular it has been established that plastic adherence selection is superior to immunomagnetic bead separation in terms of monocyte purity, yield, and subsequent survival in culture. Under the influence of IL-4 and GM-CSF, marmoset monocytes develop typical DC morphology, but remain resistant to standard maturation stimuli that act through various pathways including toll-like receptor activation. Marmoset MoDC have low co-stimulatory molecule expression, minimal CD83 expression, weak allostimulation and high endocytic ability, indicating immaturity. The basis of this resistance to maturation is unclear; however, failure of NFκB / RelB up-regulation is possibly implicated, indicating lack of appropriate signals from the stimuli tested. This stable immaturity may be a desirable characteristic of potentially tolerogenic cells, and requires further investigation.

Marmoset G-CSF mobilised, $CD34^+$-enriched populations can give rise to $CD14^+CD11c^+$ myeloid precursors which can be massively expanded under the influence of FLT3-Ligand, SCF and TPO. Further differentiation of these haematopoietic precursors in IL-4 and GM-CSF promotes differentiation of semi-mature DC with typical morphology, moderate co-stimulation expression, some CD83 expression and greater allostimulatory capacity compared with Mo DC. Marmoset DC propagated *in-vitro* from either of the precursor populations

exhibit some differences to human DC, as is the case with DC from other NHP species. Retention of CD14 expression on DC appears to be a feature of NHP DC. The variable response to maturation stimuli and weaker allostimulatory ability compared to human DC has also been described in other NHP DC models. While it is important to recognise species–specific differences (discussed in Chapter 4) it is also important to note that *in-vitro* NHP DC systems, including that of the common marmoset, more closely resemble the paradigm of human DC than other non-primate models.

In addition to *in-vitro* DC propagation, Chapter 5 describes for the first time the mobilisation of *in-vivo* marmoset myeloid DC using FLT3-Ligand. Similar to human and rhesus studies, marmoset myeloid DC can be identified using a three colour flow cytometry strategy detecting $Lin^{-}ClassII^{+}CD11c^{+}$ cells. It has been demonstrated that a ten day course of FLT3-L leads to a mean 74-fold (range 42-98) increase in myeloid DC, representing 3-7% of the peripheral blood leukocyte population by day 14-15. This massive mobilisation enables isolation of significant numbers (minimum $2x10^{5}$ DC /ml of blood) of rare myeloid DC to be obtained by FACS sorting for further study and potential therapeutic manipulation. It has been shown that freshly sorted marmoset myeloid DC have typical morphology and are stronger allo-stimulators in MLR compared with *in-vitro* propagated DC, with stimulation indices approaching that observed for human DC. While further characterisation of this DC subset is required, this data represents the initial description of marmoset *in-vivo* DC.

Finally, the feasibility of DC immunotherapy in this novel model has been established in three experiments described in Chapter 6 evaluating the effect of donor DC infusion on recipient immune responses. Unmodified, stably immature monocyte-derived DC were propagated from G-CSF mobilised donors, and administered as a single dose to allogeneic recipients chosen on

the basis of *Caja*-DRB mismatch. DC infusion was shown to be technically achievable and well-tolerated in this model. Donor-specific immunity as measured by MLR and interferon-γ production was altered in all animals after DC infusion, although the nature of the response varied. In two animals, transient non-specific suppression of immune responses was observed, whereas in the third animal, transient sensitization to donor antigen was noted. This preliminary study is an important step in developing this model for DC-based transplant tolerance studies.

2. FUTURE DIRECTIONS

The work presented in this thesis provides the basis for a number of ongoing studies that will firstly consolidate and supplement the existing characterisation of marmoset DC, and secondly, evaluate strategies of DC immunotherapy for the promotion of beneficial transplant outcomes including tolerance.

2.1 Planned Studies For Further Evaluation Of Marmoset DC:

- Testing additional culture conditions for *in-vitro* propagated DC to promote further maturation of immature DC. This includes serum-free conditions, culture with IFN-α in addition to IL-4 / GM-CSF, and maturation stimulation with IFN-γ, and a pro-inflammatory cocktail containing TNF, PGE2, IL-1β and IL-6.
- Establishing methods for measuring cytokine production by marmoset DC or DC-stimulated T-cells, in particular IL-12 and IL-10.
- Evaluating the feasibility and effect of other strategies for marmoset DC manipulation. DC culture with IL-10 or adenoviral transduction of IL-10 gene is a potential target.

- Identifying additional DC-specific surface markers that are cross-reactive with marmoset cells and optimising protocols for their use in marmoset samples. This includes CD123, CD1a, BDCA molecules and c-type lectins such as DC-SIGN.
- Further characterisation of FLT3-L mobilised myeloid DC, including co-stimulatory molecules expression, endocytic ability, cytokine production and level of maturation.
- Development of a strategy for the isolation and characterisation of FLT3-L mobilised plasmacytoid DC, with functional assessment including responses to viral stimulation.
- Conducting *in-vitro* studies of DC-priming of marmoset T cells to promote specific hyporesponsiveness to allo-antigen.
- Validation of methods for measuring anti-donor alloantibody in recipient animals, to expand the array of tests for post-transplant immune monitoring.

2.2 Planned Studies of DC Immunotherapy In Marmoset Organ Transplant

- The establishment of a marmoset renal transplantation model. The surgical team at TQEH is currently developing the skills required for small animal renal transplantation procedures. Transplants are scheduled to commence this year.
- Treating recipient animals from studies in chapter 6 with a second dose of donor DC ± kidney transplant from the DC donor animal, to assess the immune response to re-challenge with donor antigen.
- Tracking the fate of injected DC using DC-labelling methods and evaluation of lymphoid organ DC populations, to determine whether donor DC reach target regions. The route of administration of DC (intravenous versus subcutaneous) is likely to also be relevant to DC fate.

- Performing infusion of marmoset DC types other than unmodified, immature MoDC to assess their tolerogenic potential.
- Evaluation of immune responses and / or transplant outcomes following DC immunotherapy in conjunction with adjuvant co-stimulation blockade, or other immunosuppression.

In conclusion, there is wide scope for further developing the marmoset as DC and transplant model in which a large number of therapeutic avenues may be explored in the future.

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APPENDIX:

PUBLICATIONS

Prasad, S., Humphreys, I., Kireta, S., Gilchrist, R.B., Bardy, P., Russ, G.R. and Coates, P.T.H. (2006) MHC Class II DRB genotyping is highly predictive of *in-vitro* alloreactivity in the common marmoset.
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