ADDRESSING THE IMMUNOLOGICAL BARRIERS TO REGENERATIVE MEDICINE: DIFFERENTIATION AND CHARACTERISATION OF DENDRITIC CELLS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS

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Abstract

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Induced pluripotent stem cells (iPS cells) have shown great promise in the newly-developing field of regenerative medicine. Recently, however, there have been conflicting reports regarding their immunogenicity, even when derived in an autologous fashion, due to the ectopic expression of certain developmental antigens. Dendritic cells (DC) are professional antigen presenting cells and therefore serve as potential tools for modulating the immune response, particularly with respect to acceptance of stem cell-derived tissues. We hypothesised that DC differentiated from iPS cells, so-called ipDC, may have the capacity to induce tolerance to tissues differentiated from the same parent iPS cell line.

As a first step towards this goal, we have derived iPS cells from both embryonic and adult mouse fibroblasts, developed protocols for their differentiation along the DC lineage and characterised the resulting DC both phenotypically and functionally, using bone-marrow derived-DC (bmDC) for comparison. Furthermore, their potential for inducing tolerance as a function of regulatory T cell induction has also been explored. Our data suggest that, although ipDC are able to function in a similar manner to bmDC both *in vitro* and *in vivo*, they display important differences with respect to cytokine production and MHC class II expression which may reflect their early embryonic origin and render them pro-tolerogenic.

One application for this novel population of DC might be the induction of tolerance to autologous iPS cell-derived tissues. In order to assess the need for tolerance under such circumstances, we have investigated the reported immunological rejection of iPS cells when transplanted syngeneically. Although we were unable to corroborate previous findings showing immunological rejection of iPS cells under such circumstances, we were able to show that the formation of teratomas from iPS cells is a stochastic and very variable process, unlike their generation from ES cells. Importantly, we were able to exclude the involvement of any antigen-specific component to the failure of teratoma survival by inducing blanket tolerance to iPS cell-derived tissues, using a cocktail of non-depleting monoclonal antibodies specific for CD4, CD8 and CD40L.

This is the first reported study deriving dendritic cells from mouse iPS cells and demonstrating their extensive characterisation. Additionally, the work presented in this thesis adds to the growing body of evidence of the immunological properties of iPS cells with our work demonstrating that they appear not to attract an adaptive immune response but, however, show significant heterogeneity with respect to their ability to engraft *in vivo*.

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Abbreviations used in this thesis

3H-TdR Tritiated thymidine
A1.RAG1$^{-/-}$ A1.RAG1$^{-/-}$ mice
Ab Antibody/ies
Ag Antigen(s)
ANOVA Analysis of variance
APC Antigen presenting cell
B6 C57Bl/6
bmDC Bone-marrow derived dendritic cell
BSA Bovine serum albumin
CBA.RAG1-/- CBA/Ca.Rag1-/-
CBA/Ca CBA/Ca mice
d Days
DC Dendritic cell(s)
DMEM Dulbecco's modified eagle's medium
EAE Experimental autoimmune encephalomyelitis
EC Embyronal carcinoma
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme linked immunosorbent assay
EB Embryoid body
EpiSC Post implantation epiblast-derived stem cells
ES cell Embryonic stem cell
FCS Foetal calf serum
Foxp3 Forkhead box P3
GM-CSF Granulocyte macrophage colony stimulating factor
HEL Hen egg lysosyme
hr Hours
i.p. Intraperitoneal
i.v. Intravenous
IFN Interferon
Ig Immunoglobulin
IL Interleukin
IMDM Iscove's modified dulbecco's medium
ipDC Induced pluripotent stem cell-derived dendritic cell
iPS cell Induced pluripotent stem cell
LIF Leukaemia inhibitory factor
LIFR Leukaemia inhibitory factor receptor
LPS Lipopolysaccharide (*E.coli* 0127:B8)
mAb Monoclonal Ab(s)
MAF Mouse adult fibroblast
MEF Mouse embryonic fibroblast
MHC Major histocompatibility complex
min minutes
mTec Medullary thymic epithelial cell
n.s. Not significant

NOS Nitric oxide synthase
OVA Ovalbumin
PBS Phosphate buffered saline
Pen-Strep Penicillin-and Streptomycin antibiotics
RANTES Regulated upon Activation, Normal T-cell Expressed, and Secreted
rhTGFβ1 Recombinant, human TGFβ1
RPMI Roswell Park Memorial Institute
s.c. Subcutaneous
sec Seconds
SSEA Stage-specific embryonic antigen
SR Serum replacement
TBAC Tris-buffered ammonium chloride
TCR T cell receptor
TGFβ Transforming growth factor β (assume TGFβ1, unless otherwise stated)
Th1 T helper 1
Th2 T helper 2
TLR Toll-like receptor(s)
TNFα Tumour necrosis factor
TRA Trafalgar
Tris Tris(hydroxymethyl)aminomethane
v/v Volume/volume
VD3 1 α, 25-dihydroxy vitamin D3
w/v Weight/volume

Chapter One

Introduction

1.0 Introduction

Embryonic stem cells (ES cells) have the ability to self-renew indefinitely and to differentiate into cells from all three embryonic germ layers. There have been to date, a plethora of protocols identified which allow for directed differentiation into cell types as diverse as hepatocytes, pancreatic β-cells and neurones with the prospect of using these differentiated cells in cell replacement therapy. Aside from the ethical issues of using ES cells, one of the major hurdles in overcoming clinical applications of ES cells has been the prospect of immunological rejection upon transplantation.

Following the development of induced pluripotent stem cells (iPS cells) in 2006 (Takahashi *et al.* 2006), it was hoped that these cells would mitigate any immunological rejection, as cells would be derived in an autologous fashion. To date however, conflicting research exists as to the immunogenic properties of these cells and the cell types differentiated from them. This thesis therefore seeks to address the immunological issues associated with transplantation of iPS cells and to develop ways to overcome any potential graft rejection through modulation of the immune system.

1.1 An introduction to regenerative medicine

Over the past century, the advent of antibiotics and therapeutics has drastically increased both standard of living and life expectancy, particularly in developed countries. It is estimated that life expectancy has increased on average by three months per year since 1850 (Oeppen & Vaupel, 2002). The major implication from the development of advanced therapeutics is an ageing population. An ageing population is distinguished by a growing number, and relative proportion of old people, generally defined as 60 years of age or above. It is expected that by 2030 in Europe alone, 21.4% of the population will be over 65, compared to only 8.2% in 1950 (Sherlock, 2000). Worldwide, life expectancy in 2010-2015 is 78 years in developed countries and 68 years in developing countries. By 2040-2050, life expectancy is expected to reach 83 years in developed countries and 74 in developing regions (Ageing in the Twenty-first Century: a celebration and a Challenge, 2012). An ageing population places a variety of demands on society including financial, medical and social. Medically, an ageing population has been shown to correlate with increased incidence of heart disease, diabetes and cancer. The ramifications of these problems are felt in other areas of society: retired households have double the NHS spending as non-retired households and one-seventh of public spending is used on those over the working age (UK Government & Parliament, 2013).

Currently, the three biggest medical, and therefore, financial burdens associated with old age are heart disease, dementia and cancer. These conditions are often chronic diseases that substantially lower the standard of living for the sufferer and caregivers. Current therapeutic options are often supportive and are not able to slow or halt disease progression. As a result, it is in the interests of both patients and society as a whole, to find better ways to address these unmet medical needs.

Many of the aforementioned diseases result from age-related, irreparable damage to tissues. Given the body's limited capacity for rejuvenation, a viable option for regenerating tissues has long been sought by medical research. Much research in the field of cell replacement therapy has, therefore, focused on using stem cells to replace diseased or damaged tissues. Many advances in the field have been made with some efforts to translate research into human clinical trials including treatments for systemic sclerosis (Naraghi and van Laar, *in press*), medulloblastoma (Ajeawung *et al.* 2013) and myocardial infarction (Lim, 2012). However, successes to date have been limited: to improve the clinical outcomes of these treatments, a greater understanding of stem cells and their differentiated progeny is required. In the next sections, I discuss the history of stem cells, and their unique properties of pluripotency, as well as current limitations to their therapeutic application.

1.1.2 What is pluripotency and how can it be applied to regenerative medicine?

The 'potency' of a cell is its ability of a cell to differentiate into different cell types. Several levels of cell potency exist and each cell type has decreasing abilities to differentiate into a wider variety of cells.

Totipotency is the ability of a cell to divide and produce cells from all three germ layers within an organism, including extra-embryonic tissue such as the trophoblast. This latter capability sets these cells aside from pluripotent cells, which are only able to differentiate into cells from all three intraembryonic germ layers and not extra-embryonic tissue. ES cells and iPS cells fall into this category, which will be discussed later.

Multipotency is a term commonly associated with adult stem cells. These cells have limited capacity to differentiate into cell types and are instead restricted to a particular

lineage owing to genetic regulation within the cells. Examples include haematopoietic stem cells, which reside in the bone marrow. These cells have the ability to differentiate only into erythrocytes, myeloid and lymphoid cells (Kondo, 2010). Given that these cells are often resident in anatomical areas of patients that are not easily accessible and often have limited self-renewal capacity that is difficult to sustain *in vitro*, their use is not ideal for cell replacement therapy.

With respect to pluripotency, the genes *Oc3/t4, Sox2* and *Nanog* have been identified as crucial transcription factors for maintaining the state. The loss of *Oct4* expression results in the differentiation of ES cells and the inner cell mass into trophectoderm (Nichols *et al.* 1998). Conversely, over-expression of *Oct3/4* results in differentiation of ES cells or the inner cell mass into primitive endoderm and mesoderm (Niwa *et al.* 2003). As well as *Oct3/4, Nanog* plays an important role in pluripotency and differentiation: loss of *Nanog* results in differentiation of pluripotent cells to primitive endoderm tissues (Mitsui *et al.* 2003). Additionally, *Sox2* has also been shown to play an important role in the maintenance of pluripotency and the regulation of *Fgf4* expression (Avilion *et al.* 2003, Rappollee *et al.* 1994). It is likely that interaction between the pathways by which these genes signal determines the level of pluripotency within a cell.

In the context of cell replacement therapy, it would, therefore, be advantageous to use cells with the highest capacity for differentiation, which is why current clinical efforts are centred on using ES and iPS cells in therapy.

1.1.3 An introduction to ES and iPS cells

Since their isolation, ES cells have been regarded as a 'gold standard' for potential use in regenerative medicine given their pluripotent state, ability to differentiate into any cell type in the body and indefinite capacity for self-renewal. Mouse ES cells were first isolated in 1981 by Martin Evans and Matthew Kaufmann (Evans and Kaufmann, 1981). Following their derivation, Gail Martin published a paper demonstrating *in vitro* culture of ES cells. The group was able to show that the cells remained undifferentiated after culture for 4-5 months and still maintained the developmental potential to form trophoblast and differentiate into all three embryonic germ layers (Martin, 1981). However it was not until 1998 that Thomson *et al.* succeeded in extracting ES cells from human blastocysts. Using fresh or frozen cleavage stage human embryos produced by *in vitro* fertilisation, embryos were cultured until they formed blastocysts. The inner cell mass was extracted and five separate ES cell lines were derived from five embryos. The ES cell lines expressed embryonic markers such as SSEA-3, SSEA-4, TRA-1-60 and alkaline phosphatase. The pluripotency of these lines was investigated by injecting the ES cells into SCID mice and showing their ability to form teratomas containing cell types from all three embryonic germ layers. The authors clearly envisaged the impact that these cells could have on the field of regenerative medicine, remarking "[that] elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardised production of large purified populations of cells such as cardiomyocytes and neurones will provide a potentially limitless source of cells for drug discovery and transplantation therapies." (Thomson *et al.* 1998).

Since the derivation of human and mouse ES cells, several studies compared their gene expression profiles. Ginis *et al.* (2004) showed that by comparing 400 genes in human and mouse ES cells, the two share similar expression of markers in the pluripotent state. One of the most important conclusions the group made was the difference in the expression of the leukaemia inhibitory factor receptor (LIFR): the LIFR was absent or expressed at low levels by human ES cells whilst the LIFR was readily detected in mouse ES cells. This important finding explains the need for the presence of LIF in mouse ES cell cultures compared with human ES cultures which do not require LIF to maintain a pluripotent state.

ES cells have often been described as existing in two states: a naïve state and a primed state. Nichols & Smith (2009) have described that ground-state pluripotency is established in the epiblast of a mature blastocyst which can contribute to all three germ layers. The pre-implantation epiblast is, therefore, described as being in a developmental naïve ground-state. Following implantation, the epiblast is primed for lineage specification which is signalled by extra-embyronic tissue. The *in vitro* counterpart to primed epiblasts are post implantation derived epiblast stem cells (EpiSC) which result upon exposure of ES cells to signalling molecules such as activin A and Fgf (Nichols & Smith, 2009). EpiSC are thought to exist as partially primed cells with weak signalling commitments towards certain lineage differentiation pathways. These cells are thought to mimic EC cells in having limited capacity for differentiation compared to their ES cell counterparts.

Since their first description, there has been much interest in the use of ES cells as a therapeutic source of cells for cell replacement therapy. One of the major limitations to their usage is controlling their differentiation pathways. Given the chaotic differentiation of ES cells *in vitro*, there have been considerable efforts to elucidate

the pathways by which ES cells differentiate into specific cell types and thereby impose conditions upon the cells to differentiate into one particular therapeutic cell type. To date, there are a wide range of protocols which are able to induce directed differentiation into motor neurones (Wicheterie *et al.* 2002), red blood cells (Lu *et al.* 2010) and hepatocytes (Cai *et al.* 2012) to name only a few. Although protocols now permit the generation of cell types according to current good manufacturing practice (cGMP), current efforts are centred around refining these protocols in order to increase the purity and yield of the cells generated.

Although ES cells have many biological limitations, as discussed in the next section, their use has been highly controversial due to the ethical concerns surrounding the use and availability of human blastocysts. However, in 2006, work by Yamanaka *et al.* showed that it was possible to derive pluripotent stem cells from adult somatic cells by retroviral transduction of mouse fibroblasts with the genes *Oct3/4, Sox2, c-Myc* and *Klf4* (Takashahi *et al.* 2006). These cells were dubbed induced pluripotent stem cells (iPS cells) and have broadly the same characteristics as ES cells, namely the ability for indefinite self-renewal, the capacity to differentiate into cells from all three embryonic germ layers and, following injection into mouse blastocysts, the ability to generate germline-competent chimeras. The work was translated to the human in 2007 by two separate groups reporting the derivation of human iPS cells from dermal fibroblasts (Takashai *et al.* 2007 and Nakagawa *et al.* 2007). This seminal work not only greatly facilitated the generation of pluripotent stem cells, but also offered a way of overcoming the ethical concerns of using blastocysts and, in theory, the issues associated with immunological rejection of allogeneic stem cell-derived tissues, given the potential to derive iPS cell lines in an autologous fashion.

Since the work carried out by Takashai *et al.*, there have been many efforts to develop and optimise the reprogramming process. To date, there are several ways of reprogramming somatic cells to iPS cells: by direct delivery of reprogramming proteins into somatic cells (Kim *et al.* 2009) or by administration of synthetic mRNA (Warren *et al.* 2010). Studies have also shown that using small molecules, such as the histone deacetylase inhibitor (HDAC) valproic acid, can also increase the efficiency by which cells are reprogrammed by up to 100-fold (Huangfu *et al.* 2008). Interestingly, recent work by Deng *et al.* (2013) has shown that full reprogramming of somatic cells back to a fully pluripotent state is achievable with the use of seven small molecules alone, at a frequency of 0.2%. This new work suggests that pluripotency may be achieved without the use of virus-based delivery systems. The ways in which somatic cells can be reprogrammed are summarised in Table 1.1.

In addition to the way in which cells are reprogrammed, the number and combination of genes required has also been heavily studied. In addition to the four genes identified by Takashai *et al.*, introduction of other genes such as *LIN28* has been shown to greatly increase the reprogramming efficiency of somatic cells (Balzer *et al.* 2007). Takashai *et al.* identified *Nanog* originally as being dispensable for the reprogramming of cells to pluripotency, although other groups have shown that replacing *Oct3/4* with *Nanog* can also yield reprogrammed iPS cells with a normal ES cell phenotype (Moon *et al.* 2013).

An important factor in translating iPS cells into clinical therapies is their full characterisation. An obvious comparison is ES cells, given their extensive characterisation and full pluripotent capacity. Since the reported derivation of iPS cells, many groups have attempted to compare ES and iPS cells with respect to their gene expression profiles and pluripotency. Although several analyses indicate that iPS

cells share key properties with ES cells such as pluripotency, self-renewal and gene expression profiles, there have also been some important differences highlighted.

To draw upon a few examples, both ES and iPS cells under prolonged culture become aneuploidy due to anaptation to tissue culture conditions (Baker *et al.* 2007). Recent studies have identified that the reprogramming progress can lead to genomic instability especially at specific cancer causative loci (Mayshar *et al.* 2010). Such genome instabilities may hamper their use *in vivo* as their tumorigenicity may be enhanced over time.

There have also been a significant number of studies in assessing the epigenetics of both ES and iPS cells. Broadly speaking, many of the features of the ES cell epigenome are found in iPS cells including their distinctive methylation patterns (Guenther *et al.* 2010). However, studies have shown that iPS cells maintain a degree of 'epigenetic memory.' Kim *et al.* (2010) showed that iPS cells derived by reprogramming using the 'Yamanaka' method maintained DNA methylation signatures of the somatic-cell tissue of origin, which favours their differentiation along lineages related to the 'donor' cell. Clearly, this may mean that it may not be possible to differentiate certain cell types depending upon the somatic cell reprogrammed. The authors hypothesised that these modifications may be overcome using pharmacological agents, which modify chromatin structure and may reverse the epigenetic memory found in many iPS cell lines.

Method of reprogramming	Positive aspects	Negative aspects
Forced expression of genes via retrovirus	Well characterised method, long history of use, arguably a simple approach and low cost, relatively high reprogramming rates of 0.01-0.02%	Integration into the genome may generate immunogenic cells, virus will only enter cells in mitosis, use of oncogenes such as c-Myc
Small molecules	Low cost of compounds, increases the efficiency of reprogramming	Only recent reports of full reprogramming achievable with small molecules alone: further characterisation of lines generated needed.
Synthetic miRNA	No integration within the genome	Very low reprogramming efficiency, miRNA degrade rapidly, modification of miRNA complicated and time-consuming
Forced expression of genes via Sendai virus	No integration into the genome, higher efficiency of reprogramming than using retrovirus, diluted out of culture upon passage rapidly, high reprogramming rate of 0.1%	Difficult to work with, therefore most commonly used as pre-packaged 'kits' which are expensive compared to other viral methods of reprogramming
Episomal plasmid vector system	No genomic footprint	Very low efficiency of reprogramming (0.0002%), loss of episomal plasmid

Table 1.1: Methods of reprogramming and complications associated with derived iPS cell lines. Table 1.1 shows some of the most common ways in which somatic cells are reprogrammed into iPS cells along with issues associated with the method of reprogramming.

Clearly further studies need to be carried out into assessing the suitability for iPS cells in comparison to their ES cell counterparts.

As discussed in the next section, the way in which somatic cells are reprogrammed can account for the variation in lines of iPS cells generated. Until the principles that underpin the reprogramming process are fully understood, the mechanisms by which somatic cells revert back to a pluripotent state will remain elusive. Nevertheless, given the ability for iPS cells to differntiate into any cell type, they hold the potential to supersede ES cells and become the new focus for cell replacement therapy.

1.1.4 Current limitations in the use of pluripotent stem cells

As eluded to earlier, there are several issues, which prevent the therapeutic application of stem cells in cell replacement therapies. An in depth analysis of all of these issues is beyond the scope of this thesis but some of the most salient issues are discussed below.

Tumorigenesis

The propensity for stem cells to form tumours upon transplantation *in vivo* is a highly contentious topic within the field of cell replacement therapy. Much of the early work into pluripotent stem cells focused on embryonic carcinoma cells (EC cells) and concluded that ES cells and EC cells were remarkably similar: they express similar markers, have the ability to differentiate into different cell types and formed teratomas. There are strong phenotypic links between stem cells and tumour and cancer cell lines such as rapid proliferation rates, high levels of genomic instability and gene expression patterns (Sperger *et al.* 2003). Several studies have shown that both ES and iPS cells undergo transformation in culture and can adopt more ‘tumour-like’ capacities such as uncontrolled proliferation. Indeed, when

mouse ES cells have been injected into SCID mice, they have the potential to form teratocarcinomas (Martin, 1981), (Shih *et al.* 2007). Furthermore, iPS cells express the oncogenes *cMyc* and *Klf4*, which contribute to their propensity to form tumours upon transplantation.

There have been some efforts to circumvent tumorigenicity in iPS cells. Work by Vazquez-Martin *et al.* (2012) showed that treating mice with the drug metformin reduced the size and weight of teratoma-like masses after the transplantation of iPS cells into *nude* mice. However, the iPS cells transplanted maintained their full capacity to differentiate into tissues from all three germ layers. The authors hypothesised that this may occur due to the ability of metformin to suppress the Oct3/4-driven compartment of malignant stem cells responsible for teratocarcinoma growth.

In the context of cell replacement therapy, a fine balance must therefore be struck between stem cells having the potential to indefinitely self-renew and maintaining pluripotency and avoiding acquisition of a tumour-like state.

Reprogramming inefficiency and generation of iPS cells

There have been several reports outlining the different methods in reprogramming somatic cells into iPS cells and their relative efficiencies. In practice, it is difficult to compare reprogramming rates as the rates of transfection are often measured indirectly as the constructs lack a reporter gene. Studies are more qualitative than quantitative, however, differences do exist broadly in the methods used. Generally speaking, efficiency rates in reprogramming using the retrovirus method are around 0.1% compared with lentivirus methods at 0.01%. Along with this, the cell type, which is being reprogrammed, also plays

an important role in the efficiency of reprogramming. Mouse embryonic fibroblasts when reprogrammed have an efficiency of around 0.01-0.05% (Takahashi and Yamanka, 2006) compared with 3.6% for neural stem cells (Kim *et al.* 2008). It has been suggested that the method by which somatic cells are reprogrammed may affect their ability to differentiate into certain cell lineages. It is, therefore, apparent that many variables exist when cells are reprogrammed into iPS cells, namely the origin of the cell and the method by which the cells undergo reprogramming to a pluripotent state. These factors could ultimately affect the immunological properties of the iPS cells derived.

Immunogenicity of ES cells

It has been demonstrated that both mouse (Robertson *et al.* 2007) and human (Drukker *et al.* 2002) ES cells express extremely low levels of MHC class I. Additionally, ES cells do not express MHC class II or co-stimulatory molecules such as CD40, CD80 and CD86. However, the differences in MHC expression between undifferentiated and differentiated ES cells was highlighted by Boyd and Wood (2009) who showed that MHC class I and class II were expressed in differentiated insulin precursor cells which was further up-regulated upon treatment with IFN-γ. It is therefore clear that differentiated tissue from ES cells would induce a stronger immune response than that of their undifferentiated counterparts.

Previous work from our lab has shown that mouse ES cell-derived tissues display a fragile level of immune privilege *in vivo.* Using ES cells that differ at defined genetic loci, our lab has been able to show that even expression of different minor histocompatibility antigens is sufficient to induce rejection of differentiated tissues. However, tolerance may be induced using non-depleting antibodies against CD4 and CD8. The way in which tolerance could be

induced was found to be mediated by TGF-β, which polarised infiltrating T cells towards a regulatory phenotype (Robertson *et al.* 2007). It is, therefore, clear that although some level of tolerance can be induced towards ES cells, having a transplantable stem cell line that is truly autologous would be a far better approach. This was thought to have been achieved with the development of iPS cells, however, as discussed in the next section, recent work suggests this may not be the case.

There have been many reports on the prospect of 'banking' human ES cells for therapeutic use with the idea being that a range of ES cell lines could be derived with a variety of MHC haplotypes. However, as reviewed by Fairchild (2010), ES cell banking may not be a practical approach. Fertility clinics, where embryos are harvested, are not well attended by ethnic minorities, which would restrict the MHC range of ES cell lines derived. Additionally, the MHC haplotype of the embryo is not known prospectively until an ES cell line has been derived. A more attractive alternative to ES cell banking is the banking of iPS cells. The MHC haplotype of the donor can be evaluated in advance which would allow for a wider recruitment of donors. Alongside recruitment of 'regular' donors, it is hypothesised that 'super donors', individuals homozygous at MHC loci, may also be recruited which would allow for a wider range of potential recipients (Nakatsuji *et al.* 2008). The degree of immunological matching required between the donor and recipients of ES cells has not yet been determined in humans, although, work from our laboratory has shown that even full matching at MHC loci is not sufficient to ensure acceptance, with differences at minor histocompatibility loci sufficient to provoke rejection. As discussed in the following sections, it is likely that iPS cells would also require similar levels of donor-recipient matching to prevent rejection.

1.2 The need for tolerance in regenerative medicine

Although many barriers exist in the translation of stem cell therapies to the clinic, one of the most pressing, as discussed above, is the issue of rejection of transplanted tissues by the host immune system. Following the development of iPS cells in 2006 (Takashi, 2006), it was hoped that this would mitigate the need for any immune modulation, as the cells derived would be autologous and would, therefore, express the same antigens as the host. However, as discussed in the next section, recent work by several groups has shown that this may not be the case, which, if found to be true, would suggest that immune modulation would be required to prevent the rejection of iPS cells and their differentiated progeny.

1.2.1 Recent identification of iPS cell rejection

The discovery of iPS cells created much excitement in the field of cell replacement therapy as not only do iPS cells overcome the ethical objections associated with ES cells, but they were also thought to mitigate any issues of immunological rejection when derived in an autologous fashion.

The latter hypothesis was called into question following a paper published in 2011 by Zhao *et al.* The authors initially reprogrammed C57Bl/6 mouse embryonic fibroblasts into iPS cells using retroviral transduction with the 'Yamanaka factors' *Oct4*, *Sox2*, *c-Myc* and *Klf4.* These iPS cells were then injected subcutaneously into syngeneic recipients and immunodeficient SCID mice to form teratomas. Interestingly, when the iPS cells were transplanted into syngeneic recipients, they were unable to form detectable teratomas in the majority of recipients and, where teratomas were found, there was a significant $CD4^+$ T cell infiltrate and tissue necrosis, suggesting that the tissues were in the process of being

immunologically rejected. The authors also reported that teratomas that did not undergo regression likewise infiltrated with $CD4^+$ T cells. Conversely, iPS cells transplanted into immunodeficient mice were found to consistently form teratomas.

As *c-Myc* is a well-characterised oncogene, and given that fibroblasts are routinely reprogrammed into iPS cells without the presence of *c-Myc*, the authors examined whether '*c-Myc*-less' iPS cells undergo the same level of immune rejection. However, no difference in teratoma formation was found between the two reprogramming methods.

One issue that may be associated with immunological rejection is the re-activation of the transgenes used for reprogramming. Indeed, recent studies have shown the presence of *Oct3/4* specific T cells in the periphery of humans (Dhodapkar *et al.* 2010), suggesting that ectopic expression of *Oct3/4* may be sufficient to promote an immune response against transplanted iPS cells. The authors therefore set out to create iPS cell lines using an 'episomal approach'. This method ensured that the iPS cells had lost the episomal vectors and harboured no random integration of the reprogramming vector. Upon transplantation into syngeneic mice, teratoma regression was present in 10% of teratomas formed along with a T cell infiltrate, showing that iPS cells reprogrammed in this manner were less immunogenic than cells reprogrammed using an integrating vector.

Using gene expression analysis, the authors were able to identify a number of genes over-expressed in teratomas formed by iPS cells, in particular the genes *Hormad1* and *Zg16*, the former of which is an identified tumour associated antigen (Chen *et al.* 2005). In order to determine whether these genes played a role in the rejection of iPS cells, the genes identified were ectopically expressed in ES cells, which were subsequently transplanted in a syngeneic

setting. Upon transplantation, 80% of ES cells expressing Zg16 and 50% of ES cells expressing *Hormad1* failed to form teratomas. Within those teratomas that did form, an extensive T cell infiltrate was evident along with tissue necrosis. Upon transplantation into $CD4^{-/-}$ and $CD8^{-/-}$ mice, ES cells expressing the identified genes were able to successfully form teratomas showing that $CD4^{+}$ and $CD8^{+}$ T cells play an important role in the immunological rejection described. The authors suggest that this shows that innate immunity does not have an important role in the rejection of iPS cells and tissues differentiated from them. The mechanism by which the rejection present is likely to result from the up-regulation of at least the two genes *Hormad1* and Zg16 which are developmental antigens meaning that they are only expressed during embryonic development. As a result, the immune system will not have been 'tolerised' to these antigens and, when ectopically expressed on iPS cells, are targeted by the immune system as non-self-antigens.

The Zhao *et al.* paper had a significant impact on the field of regenerative medicine as the work appeared to present a further obstacle to their clinical application. Although the paper looked thoroughly at the mechanisms underpinning the rejection of transplanted iPS cells, there have since been some criticisms as to how well the transplantation conditions in the work would mimic cell replacement therapies.

Firstly, the authors injected undifferentiated iPS cells subcutaneously into the flank of mice, which gave rise to the formation of teratomas. In a clinical setting, replacement tissue would most likely be fully differentiated *in vitro* before transplantation *in vivo* so as to reduce to a minimum the risk of tumorigenesis. It is likely that fully differentiated tissue would have significantly different immunological properties from those of their undifferentiated iPS cell counterparts. Indeed, it has been widely reported that iPS cells have low expression of MHC

class I which is restored by differentiation or treatment with IFNγ (Pick *et al.* 2012). Additionally, ES cells express significantly low levels of MHC class II and co-stimulatory molecules (Drukker *et al.* 2006). It is likely that these immunological properties are also reflected in iPS cells. The low expression of both MHC class I and MHC class II has been shown to be under epigenetic control with various methylation patterns associated with reduced expression of HLA-B in human ES and iPS cell lines (Suarez-Alvarez *et al.* 2010).

Furthermore, it is also likely that the cell type that is differentiated may influence different levels, and perhaps types, of host immune responses. In order to address this, it would be ideal to differentiate one defined tissue type from iPS cells and investigate whether the same level of immune response is observed. Lastly, the authors in the paper reprogrammed mouse embryonic fibroblasts into iPS cells. It would seem prudent however to examine whether the tissue of origin, which is to be reprogrammed, plays a role in the immunogenicity of the resulting iPS cell lines. In particular, the question arises as to whether iPS cells derived from adult dermal fibroblasts, relevant to a clinical scenario, would encounter the same fate as embryonic derived fibroblasts.

Are iPS cells universally rejected?

Eighteen months following the publication of the work by Zhao *et al.*, Araki *et al.* published a paper largely refuting the finding that autologous iPS cells are immunogenic. The Araki work aimed to address the deficiencies identified above. The authors generated multiple ES and iPS cell lines from C57Bl/6 mice using *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* and then selected for genome free integration lines. From the lines generated, seven iPS cell lines and five ES cell lines were used in the transplantation studies and were replicated ten times for all twelve

lines. The authors were unable to detect significant numbers of T cells in either the ES or iPS cell teratomas formed upon subcutaneous injection into syngeneic recipients.

The authors pointed out, as outlined above, that teratoma formation is not necessarily a suitable method for assessing immune responses elicited by transplanted iPS cells, as teratomas are a type of tumour and, therefore, one could argue would naturally poise the immune system to responding against them. To address this, the authors made use of dermal and bone marrow tissues for transplantation experiments, which they obtained from chimaeric mice developed from the same iPS or ES cell lines. A GFP^+ iPS cell clone was used which was aggregated with GFP^- embryos to develop GFP-positive chimaeric mice. Skin was prepared from the chimeras and transplanted onto the flank of syngeneic GFP^- mice and allogeneic recipients. The authors were able to show that all syngeneic mice accepted their grafts whilst grafts onto allogeneic mice were rapidly rejected. The experiments were repeated with other iPS cell clones and the graft survival rates in a syngeneic setting were around 98%. To further investigate the immune response, the authors looked for the presence of $CD3^+$ infiltrating T cells. Very few infiltrating $CD3^+$ cells were found in syngeneic iPS cell grafts compared to the amount in allogeneic transplants.

As an extension to these findings, bone marrow transplantation was mimicked by transplanting marrow derived from the GFP^+ iPS cell chimeras into irradiated C57Bl/6 mice to allow for re-constitution of their immune system. The authors found co-existence of both host and donor-derived bone marrow cells, which suggests that bone marrow cells derived from iPS cells have a low immunogenicity.

The expression of the genes *Zg16* and *Hormad1* have been linked to the immunogenicity of iPS cell teratomas by Zhao *et al.* (2011). However, Araki *et al.* were unable to detect significant up-regulation of either genes in the teratomas formed in their experiments. The authors were also unable to find significant up-regulation of these genes in differentiated tissue of mice derived by tetraploid complementation using iPS cells.

Although this paper aimed to address some of the flaws in the work published by Zhao *et al.* (2011), some questions arise around the methodology used. The authors made use of surgically transplanted tissue but did not use *in vitro* differentiated cells. As this would be the most clinically relevant setting, it would be interesting to observe whether *in vitro* differentiated cells also failed to undergo immunological rejection in their hands. Additionally, the use of chimeric mice may inherently prevent any level of rejection, as during T cell selection in the thymus, any T cells reactive against iPS cell antigens would be negatively selected.

More recently, a third significant study into the immunological properties of iPS cells was published. Guha *et al.* (2013) showed that when transplanting iPS-differentiated embryoid bodies autologously, that no immunological rejection was observed. The authors took the work further by differentiating specific cell-types from iPS cells and transplanted endothelial and hepatic tissue in an autologous manner. Again, the authors observed no immunological rejection, suggesting that the cell type which is transplanted may not influence any subsequent immune response. Finally, the expression of the genes identified by Zhao *et al.* (2011) which were thought to be responsible for the rejection of iPS cell grafts was examined. Guha *et al.* found no evidence of any up-regulation of these genes in their iPS cell lines or grafts.

It is clear that there is currently no clear consensus as to whether autologous iPS cells are rejected or not. A better understanding of the effects and methods of reprogramming and subsequent immune responses is, therefore, required which is one of the central aims of this thesis. However, if it is found that certain iPS cell lines are conducive to initiating an immune response, it is important that the field of regenerative medicine addresses these findings and ways to overcome them. Given the propensity of pluripotent stem cells to become tumorgenic, the use of immunosuppression is contraindicated. An attractive alternative in preventing the rejection of iPS cells would therefore be to manipulate the immune system, to induce immunological tolerance, in order to encourage acceptance of iPS cell-derived tissues.

1.2.2 The immune system and tolerance

An introduction to immunological tolerance

Ray Owen first coined the term 'tolerance' in the context of a physiological state he observed in dizygotic twin cattle (Owen, 1945). Owen observed that when twin cows were injected with red blood cells from one other, no antibody response was mounted; contrary to adult cows that elicited a strong immune response against the red blood cell associated antigens. Immunological tolerance is broadly defined as the process by which the immune system avoids responds to a specific antigen. Tolerance is of two types: 'self-tolerance' whereby the body fails to mount an immune response against self-antigens or 'induced tolerance' whereby tolerance to foreign antigens is artificially created by various mechanisms. It was not until 1953 that Billingham *et al.* showed that injections of allogeneic tissue into neonatal mice significantly dampened the immune response when they were

exposed to these antigens later in life in the form of an allograft. Importantly, the authors showed that the tolerance acquired was antigen specific: inoculated mice were still able to reject third party grafts (Billingham *et al.* 1953). These important findings paved the way for a more thorough understanding of the mechanisms by which the immune system discriminates between antigens and the response, if any, targeted against them.

In the context of organ transplantation, current therapeutic options consist mainly of using drugs to supress the immune system and preventing T cell responses against transplanted tissues. A permanent state of tolerance towards transplanted tissues has long been sought, mainly due to the significant side effects of immunosuppressive medications. By manipulating the tolerance mechanisms *in vivo* it is hoped that a permanent state of tolerance can be induced. Broadly speaking, there are two mechanisms by which tolerance can be induced within the immune system: through central tolerance and through peripheral tolerance.

Central tolerance

Central tolerance refers to events that control the way in which lymphocytes respond to pathogens and are discouraged from attacking healthy tissues. Lederberg proposed this concept in 1959 (Lederberg, 1959) and our understanding of central tolerance has vastly increased in the intervening years.

Central tolerance is inherent to the process of T cell maturation that occurs in the thymus. The first stage is known as positive selection and occurs when thymocytes expressing a newly-rearranged T cell receptor encounter self-major histocompatibility complex (MHC) molecules on cortical epithelial cells affording them a survival signal. Thymocytes, which do

not interact with these self-MHC complexes, undergo apoptosis due to lack of a positive selection signal (Jameson *et al.* 1995). Thymocytes also undergo negative selection, which prevents cells with self-reactive receptors from exiting the thymus, thus protecting the host against autoimmunity (Sprent and Kishimoto, 2002).

Although central tolerance mechanisms are highly efficient, some autoreactive T cells evade these control mechanisms and escape into the periphery. The immune system has therefore evolved mechanisms in the periphery to further regulate tolerance.

Recently, the ability to modulate central tolerance mechanisms has been examined using human ES cell derived thymic epithelial cells (Parent *et al.* 2013). The authors managed to differentiate thymic cells from human ES cells and, upon transplantation into athymic mice, showed that the thymic cells derived from ES cells were able to provide developmental signals for the maturation of thymocytes into T cells. With respect to central tolerance, this approach could induce tolerance to transplanted ES cell derived tissues as autoreactive T cells would be selected out in the reconstituted thymus. This approach could also be examined using iPS cells and inducing tolerance to iPS cell derived tissues.

Peripheral tolerance

Peripheral tolerance is developed once matured T cells exit the thymus into the periphery. As discussed above, even though T cell selection in the thymus is a stringent process, some T cells escape into the periphery, which retain the ability to respond to self-antigens. The immune system has various 'safety' mechanisms in order to restrain the activity of these auto-reactive T cells. These mechanisms include T cell anergy, whereby T cells receive signals from antigen presenting cells to induce functional unresponsiveness. These signals

occur upon T-cell-antigen-presenting cell interaction and are mediated by inhibitory receptors such as CTLA-4 (Fecteau *et al.* 2001). T cell clonal deletion, originally thought to only occur in the thymus, occurs also in the periphery when apoptosis is induced by signals received from antigen presenting cells (Venazi *et al.* 2004).

A more recent discovery has shown the existence of regulatory T cells in the periphery. These regulatory T cells, or T_{regs}, are a subset of T cells, which maintain tolerance to self-antigens. Perhaps the most well characterised form of T_{regs} are $CD4^+CD25^+Foxp3^+$ $T_{regs.}$ The development and survival of these cells is dependent upon continued exposure to antigens and the tolerance maintained in this manner is known as 'infectious tolerance' (Qin *et al.* 1993), a process dependent upon IL-2 and TGF-β (Cobbold *et al.* 2004).

In the context of transplantation, these cells have the ability to suppress effector T cells and have been shown to modulate Th1, Th2 and Th17 responses (Schliesser *et al.* 2012). These type of T cells clearly play an important role in graft rejection and their manipulation could result in tolerance to various exogenous antigens.

Two main classes of T_{regs} exist: naturally occurring T_{reg} cells which express a $CD4^+CD25^+Foxp3^+$ phenotype, develop in the thymus and display a TCR repertoire for self-antigens. The existence of these cells was hypothesised in 1970 by Gershon. Several papers alluded to the existence of these cells with McHugh *et al.* (2002) showing that depleting $CD25^+$ T cells in the periphery resulted in autoimmunity in mice. Additionally, thymectomising mice at day 3 after birth results in the lack of formation of $CD4^+CD25^+$ T_{regs} again resulting in autoimmunity in the form of a fatal wasting disease. The second class, known as induced T_{reg} cells, which are induced from $CD4^+CD25^-$ effector T cells. These

cells can be induced in response to local IL-10 secretion and TGF-β (Roncarolo *et al.* 2006). These cells are secondary suppressor cells and are T cells of altered differentiation rather than a particular T cell lineage. As well as exposure to IL-10 and TGF-β, exposure to $CD103^+$ DC can induce the formation of T_{reg} cells (Coombes *et al.* 2007). Indeed, DC have been shown to expand polyclonal T_{regs} *in vivo* which is mediated by MHC class II interactions between DC and T_{reg} (Zou *et al.* 2010). It is clear, therefore, that DC play a pivotal role in the regulation of peripheral tolerance by mediating T_{reg} induction.

1.2.3. Dendritic cells: a pivotal role in the immune response

Since their characterisation in 1973 (Steinman and Cohn, 1973) there has been much interest in the role DC play within the immune system and how they can be exploited for therapeutic benefit. DC are the main professional antigen presenting cells within the immune system and are often referred to as 'sentinels' of the immune system in that they act as messengers between the adaptive and innate immune responses by controlling the function of T cells, B cells and NK cells. The function of DC falls into three categories:

1. Activation of naïve T cells

DCs have the ability to process and present antigen resulting the activation of either $CD4^+$ or $CD8^+$ naïve T cells. Naïve $CD4^+$ T cells require three major signals for activation by an antigen-presenting cell. The first signal is provided by major histocompatibility complex (MHC) and specific peptide and confer antigen-specificity on the interaction. The second signal consists of the ligation of receptors by co-stimulatory molecules such as CD80, CD86 and CD40 (Haase *et al.* 2003). In order for T cells to become fully activated, pro-

inflammatory cytokines such as IL-6 and IL-12 also must be present. The cytokine milieu influences the resulting phenotype of the activated T cell.

2. Induction of tolerance

DCs have the ability to induce tolerance within the immune system, as outlined earlier in this section. DC induce tolerance within the immune system primarily though T cell anergy and T cell clonal deletion and the promotion of T_{reg} formation (Gong *et al.* 2011). These various mechanisms of inducing tolerance have been exploited therapeutically as discussed later in this section.

3. Memory B-cell response induction

Follicular dendritic cells are found in lymph follicles of B cell areas and are present during germinal center reactions (van Nierop and de Groot, 2002). These specialised dendritic cells, have the ability to present antigen to B cells and are able to induce apoptosis in B cells which do not interact directly with the DC itself. The mechanisms and functions of these DCs are poorly understood, although it is known that these DCs are of a mesenchymal origin, unlike 'conventional' DCs, which are of haematopoietic origin (Maeda *et al.* 2002).

Origin of DCs

DCs arise from haematopoietic precursors, and as discussed later, are heterogeneous in nature. They are found throughout the body located in circulating blood, lymphoid organs and interstitial tissues. Haematopoietic precursor cells form immature DC and stem from both myeloid and lymphoid origins. *In vitro* assays have shown that DCs could be generated from monocytes or myeloid precursor cells (Metcalf, 1997). DCs developed from common lymphoid progenitors have been shown in both mouse and human models (Galy *et al.* 1995).

Subpopulations of DC in the mouse

The variety of DC subsets is extensive in both lymphoid and non-lymphoid organs within the mouse and differs significantly from that of humans.

Two broad categories of DC exist: precursor DC and conventional DC. Precursor DC are able to alter their function in response to stimuli from the extracellular environment (Vremec *et al.* 2000). As discussed above, the ability of DC to respond to signals within their environment allows them to modulate both innate and adaptive immune responses.

In mice there are three subsets of conventional DC which are $CD11c^{hi}$: $CD8\alpha^{+}$ DCs $CD4^{+}CD8\alpha^{-}$ DCs and $CD4^{-}CD8\alpha^{-}$ DC. Around 20-30% of CD8α DCs reside in the spleen and have been suggested to be semi-mature in nature (Morelii and Thomson, 2007). A significant proportion of $CD8\alpha^{+}$ DC also resides in the thymus and are responsible for negative selection of thymocytes. In the mouse, $CD8\alpha^{+}$ DC have the ability to cross-present antigen to MHC class I restricted $CD8^{+}$ T cells. Normally, exogenous antigens are not presented through the MHC class I pathway. The ability of $CD8\alpha^{+}$ DC to cross-present exogenous antigen has been attributed to their ability to phagocytose dying cells (Schulz & Reis E Sousa, 2002). The process of cross-presentation by DC is important in cytotoxic immunity induced by vaccination.

An additional population of DC, known as plasmacytoid DC (pDC), are also present in both mouse and humans. These DC circulate in the blood and often have low expression of CD11c. Upon exposure to viral pathogens, pDC produce large amounts of type I interferons (McKenna *et al.* 2005).

Tolerogenic DCs: phenotype and function

Tolerogenic DC are defined broadly as DC, which have the ability to induce immunological tolerance to defined antigens. There has been much interest in these properties as they may offer a way of inducing a permanent state of immunological tolerance to antigens, which would have broad applications spanning the areas of transplantation and autoimmunity.

Tolerogenic DC are characterised by low expression of co-stimulatory molecules compared with higher expression of inhibitor factors (such as B7-H1) as well as their function of suppressing effector T cell responses.

Within the spectrum of a DC phenotype, DCs that are immature or semi-mature in state have the ability to induce T cell anergy and the development of Treg cells (Hawiger *et al.* 2001). Immature DC are poorly immunogenic due to their low expression of MHC class I and class II along with low expression of co-stimulatory molecules. Transition into a mature phenotype arises from exposure to microbes or danger associated molecular patterns, which are released when cells undergo stress. During this maturation process, the phenotype of the DC changes from one poised towards tolerance, to one with a significantly increased capacity to induce an immune response. Although some mature DCs still have the ability to regulate tolerance (Menges *et al.* 2002), it is widely believed that immature DC have a higher propensity to induce tolerance.

So what constitutes a tolerogenic DC? Morelli and Thomson have set out criteria assembled from the literature, which show some of the minimal properties of a tolerogenic DC:

1. Low constitutive expression of surface MHC molecules and low expression of co-stimulatory molecules such as CD80 and CD86 (although the latter is not an absolute requirement).
2. The ability to acquire and present antigen to antigen-specific T cells.
3. Low production of IL-12p70 and high IL-10 production and expression of indoleamine 2,3-dioxygenase.
4. Resistance to maturation from the presence of 'danger signals' such as TLR agonists or ligation of CD40L (CD154).
5. The ability to generate, select for and expand alloantigen-specific regulatory T cells.
6. The ability to promote apoptotic death of effector T cells.

It would be ideal to identify a set of tolerogenic DC markers, however given the spectrum of the DC phenotype and the impacts that the local cytokine environment has on function, identifying markers has remained a difficult task. A recent study by Chamorro *et al.* (2009) has shown that DC treated with dexamethasone and 1,25-Dihydroxyvitamin D(3) (VD_3) up-regulate TLR2 following ligation of TLR2, TLR3, TLR4 or TLR5. Further evidence for the role of TLR2 in tolerogenic DC showed that TLR2-stimulated splenic DC have the ability to induce $FoxP3^+$ T_{reg} cells via IL-10 secretion and expression of the retinoic acid metabolising enzyme retinaldehyde dehydrogenase type 2. In addition to inducing T_{reg} cells, TLR2 signalling reduced IL-23 secretion and Th17 and Th1 based immune responses (Manicassamy *et al.* 2009).

Conversely, driving DC away from a tolerogenic state and towards an immunogenic phenotype has also been shown to be inhibited by endogenous IL-10 secretion. Chen *et al.*

(2007) showed that IL-10 knockout bmDC had a superior ability to initiate an anti-tumour response *in vivo* compared to wild-type bmDC. This is attributed to IL-10 knockout bmDC exhibiting higher levels of MHC class II and increased secretion of Th1-related cytokines. This shows that IL-10 plays an important role in balancing the functional phenotype of DC.

It is clear, therefore, that identifying tolerogenic DCs by expression of markers alone does not always correlate with the expected phenotype of the DC and, perhaps more importantly, it is the local microenvironment in which the DC is located, which plays an important role in determining its functional phenotype. Although the rationale for identifying and expanding tolerogenic DCs is clear, the generation of the cells can only take place when the environment surrounding the cell is poised towards generating DCs with the ability to induce tolerance. This can be achieved *in vitro* by modulating DCs with various pharmacological agents, as discussed in the next section.

Modulation of DC function by pharmacological agents

As discussed above, there have been various studies, which have teased apart the way in which DCs are able to induce tolerance within the immune system. There has been much interest in either generating DCs with a tolerogenic phenotype *in vitro* or manipulating other DC populations and 'converting' them into DCs with the ability to induce tolerance. The rationale behind generating tolerogenic DCs is that when re-administered back *in vivo*, these cells may induce tolerance to donor-defined antigens for therapeutic purposes. There are several 'steps' in which DC can be targeted pharmacologically to alter their phenotype: differentiation, migration, antigen processing and presentation and maturation. Consequently, with the wide range of pharmacological tools and recombinant cytokines

available, there have been numerous studies into the way in which isolated DCs can adopt tolerogenic potential following exposure to a variety of agents *in vitro.*

One of the most well studied agents is the activated form of vitamin D, VD_3, which is a secosteroid hormone which has, along with its role in bone and calcium metabolism, important immunoregulatory effects (Griffin *et al.* 2003). DCs express the receptor for vitamin D (VDR). There have been a wide range of studies showing that vitamin D_3 analogues modulate DC function by lowering expression of co-stimulatory molecules such as CD40, CD80 and CD86 (Penna *et al.* 2007), increase secretion of IL-10 (Xing *et al.* 2002), decrease production of pro-inflammatory cytokines such as IL-12p70 and reduce responses to alloantigens by dampening T cell responses.

DC have the ability to synthesise VD_3 themselves and the importance of VD_3 in DC function has been shown in VDR-knockout mice which possessed enlarged lymph nodes and a higher frequency of mature DCs (Griffin *et al.* 2001).

The induced tolerogenicity of DCs arising from treatment from VD_3 is further highlighted by their capacity to induce T_{reg} cells. Work by Gregori *et al.* (2001) showed that treatment of adult non-obese diabetic (NOD) mice with VD_3 decreased lipopolysaccharide-induced IL-12 and IFN-γ production along with arresting Th1 cell infiltration and progression of insulitis. They found that this coincided with an increased number of $CD4^+CD25^+$ T_{reg} cells that were able to dampen T cell responses to insulitis-associated antigens. Given that DCs are the main target of VD_3, the authors concluded that the effects seen were DC mediated. Further evidence for the ability of VD_3 to modulate the functional phenotype of DC was demonstrated when Adorini (2004) showed that a short treatment with VD_3 induced

tolerance to fully mismatched mouse islet allografts which correlated with increased frequency of $CD4^+CD25^+$ T_{reg} cells.

Along with VD_3, other pharmacological agents such as rapamycin have been well studied in modulating DC function. *Ex vivo* propagation of mouse bone marrow derived DC in the presence of rapamycin generated DC with characteristics of a tolerogenic DC: low MHC class II expression and reduced expression of co-stimulatory molecules (Hackstein *et al.* 2003). With respect to their function *in vivo,* Battaklia *et al.* (2005) were able to show that DC treated with rapamycin and IL-10 were able to induce T_{reg} cells *in vivo* that mediated antigen specific tolerance in a mouse model of diabetes, proving that the phenotype of treated DC could condition a tolerogenic phenotype *in vivo.*

It is clear, therefore, that pharmacological conditioning of DC with a variety of agents has the ability to induce a more 'tolerogenic' phenotype by imposing on them a maturation-resistant state. This offers hope that DC modulated in this way may have the potential to induce tolerance to defined antigens in a transplantation setting.

1.2.4 Rationale for using DC for tolerance to stem cell derived tissues

Work within our laboratory has shown that DC derived from bone marrow (bmDC) have the ability to induce tolerance to a single minor-histocompatability (mH) antigen difference. Following injection of $5x10^6$ immature bmDC, it was found that this set of conditions had the ability to induce profound transplantation tolerance based on their capacity to polarise responding T cells towards a regulatory phenotype in an antigen specific manner (Yates *et al.* 2007) in the female A1.$RAG1^{-/-}$ mouse, which is detailed later in this chapter.

Given the properties of DC, and their inherent ability to induce tolerance to defined antigens, deriving DC from iPS cells may allow for the induction of tolerance to any erroneously up-regulated developmental antigens, they may express such as *Hormad1* and *Zg16* which may serve as mH antigens. As shown in the figure below, pre-administering DC may allow for tolerance induction prior to administration of any other tissues derived from the same iPS cell line.

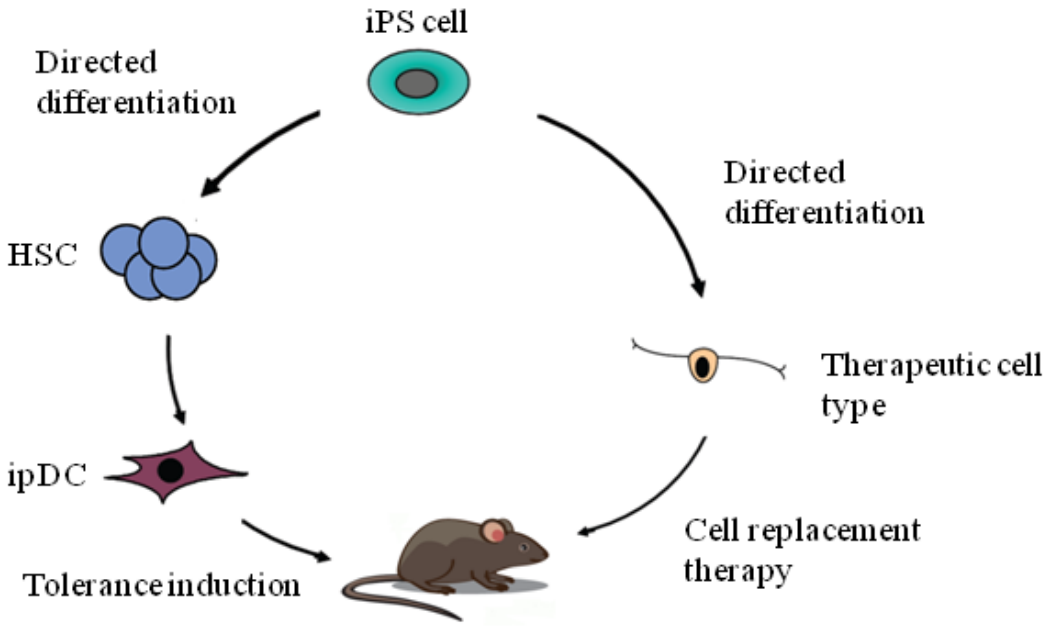

Figure 1 Diagrammatic representation of the possible therapeutic use of iPS derived DC

Indeed, this method has previously been attempted in the context of ES cell transplantation whereby DC were derived from ES cells and administered to mice, which received transplants of a defined mH difference (Fairchild, Personal Communication). However, as discussed below, induction of tolerance with these cells was not achieved under the conditions tested.

Issues arising from attempts to use esDC: low expression of class II and IL-12

Previous efforts from our lab have successfully generated DC from mouse ES cells (esDC) (Fairchild *et al.* 2000, Fairchild *et al.* 2005). Differentiating ES cells into embryoid bodies, followed by culture with IL-3 and GM-CSF, the authors were able to generate DC with similar functional properties to bmDC: the DC were able to process and present antigen and stimulate primary T cells in a mixed leukocyte reaction (MLR). The cells were shown to express DC markers such as CD54, MHC class I, CD45, CD40, CD86 and CD11b. It was also observed that both CD11c and MHC class II, which are typically abundantly present on DC, were expressed only at low levels on esDC. The authors concluded that given the dependence of esDC formation on GM-CSF and their lack of expression of CD8α and CD205, esDCs were most likely of a myeloid origin.

Previous work from our laboratory also explored the potential for genetically modifying esDC: previous efforts to manipulate DC through use of viral vectors have often resulted in altered DC phenotypes through induction of maturation. Therefore, modifying the parent cell line was shown to be a more viable way of genetically altering differentiated DC. Fairchild *et al.* (2003) were able to show that a reporter gene, enhanced green fluorescent protein (eGFP), could be successfully introduced into the parent ES cell line and, upon differentiation, >96% of DC expressed the reporter gene. Most importantly, expression of the gene did not affect their phenotype or immunogenicity *in vivo.* This shows that in principle, genetically manipulating esDC and forcing expression of certain genes involved in tolerance or immunogenicity may have the potential to modulate the immune system towards a tolerogenic or immunogenic state, respectively.

The protocol provides a robust and scalable method for deriving DC in a manner compatible with their downstream therapeutic application. As discussed earlier, many clinical trials have been conducted for using DC for cancer vaccination (Alfaro *et al.* 2011, Nishimoto *et al.* 2011) however, there are no current clinical trials exploiting the tolerance inducing capabilities of DC in a transplantation setting.

As esDC express low levels of MHC class II, secrete IL-10, have the capacity to induce *de novo* polarisation of T_{reg} cells and have the inability to secrete the inflammatory cytokine IL-12, these cells, in principle, have the basis for a tolerogenic phenotype, as defined by Morelli and Thomson.

However, work carried out by a previous D.Phil student in our lab (Lui, *unpublished data)* showed that esDC did not have the capacity to induce tolerance to the male antigen, Dby, across a minor histocompatibility barrier. Further work into elucidating why esDC did not induce tolerance was not carried out, however it was hypothesised that insufficient numbers of esDC could have been administered and also treating them with pharmacological agents, rendering them more 'tolerogenic', may have allowed for acceptance of grafts differing at a single mH antigen.

In addition to work carried out within our own laboratory, there have been other groups that have reported on their successes with esDC both in mouse and human.

Senju *et al.* (2007) reported they were successfully able to modify human ES-derived DC and were able to genetically modify them by forcing expression of programmed death-ligand-1 (PD-L1). These manipulated esDC were shown to induce hyporesponsiveness in

allogeneic T cells upon co-culture. As discussed earlier, these findings suggest that it may be possible to induce a tolerogenic phenotype by genetic manipulation of esDC.

The group was able to take these principles further by deriving esDC which had been genetically modified to present the peptide myelin oligodendrocyte glyocoprotein (MOG) in the context of MHC class II (Hirata *et al.* 2005) whilst simultaneously expressing PD1L1 and TRAIL. When administered *in vivo*, esDC expressing these genes were able to prevent T cell responses against MOG showing that esDC may have the ability to modulate immune response *in vivo* and balance the immune system towards tolerance towards defined antigens.

Given that iPS cells show broadly similar differentiation potential as their ES cell counterparts, it would be interesting to investigate whether iPS cells have the capacity to differentiate into DC and whether their phenotype differs from esDC and bmDC, the latter of which we know have the capability to induce tolerance across a mH barrier. Indeed, if tissues differentiated from iPS cells are rejected autologously, a similar strategy to that tried with esDC could be employed by modulating the immune system with ipDC to induce tolerance to the very developmental antigens responsible for iPS cell rejection that behave as mH antigens in the context of transplantation.

1.3 Experimental models used in this thesis

1.3.1 Differentiation of ES and iPS cells

Initially, the ability for iPS and ES cells to form viable teratomas will be explored in an *in vivo* setting and the level of immunological response against them.

As previous work from the lab has characterised the differentiation of ES cells into DC this protocol will be adapted and modified for the differentiation of DC from iPS cells. The cells derived will then be characterised by comparison with bmDC as work from our laboratory has previously demonstrated that bmDC have the ability to induce tolerance across a mH barrier (Yates, 2007).

1.3.2 Transgenic A1.RAG1$^{-/-}$ mice

The A1.RAG1$^{-/-}$ mouse strain is a well characterised TCR transgenic model in which the mice possess only CD4^{+} T cells that recognise the defined male specific antigen *Dby,* presented in the context of the MHC class II molecule H-2E^{k} (Zelenika *et al.* 1998). Given the defined immunology of this system, it has been shown that when skin grafts from female CBA/Ca mice are transplanted onto the mice, the host A1.RAG1$^{-/-}$ mouse is unable to reject the graft owing to the fact they possess CD4^{+} T cells against one antigen. However, when grafts from male CBA/Ca mice are transplanted onto the transgenic mice, they are infiltrated by host CD4^{+} T cells and macrophages and undergo rapid rejection.

As described earlier, although the level of immunological disparity between iPS cells and 'syngeneic' recipients is likely to be minimal, rejection may still occur due to up-regulation of the developmental antigens *Hormad1* and *Zg16* which may act as mH antigens (Zhao *et*

al. 2011). Although some of the genes, which may be involved, have been identified, the exact epitopes of these antigens, and therefore which ones are initiating the immune response have not yet been determined.

In order to recreate a similar immunological environment to that seen in iPS cell transplantation, I have chosen the A1.RAG1$^{-/-}$ mouse as an experimental model in which to test the capabilities of DC produced in this thesis to polarise antigen-specific CD4^{+} T cells to a regulatory phenotype. Indeed, previous work from our laboratory has shown that it is possible to induce tolerance to the Dby mH antigen by pre-treatment with as few as 2x10^{6} bmDC (Yates *et al.* 2007). The A1.RAG1$^{-/-}$ system also lacks naturally occurring T_{reg} cells. This means that the amount of T_{regs} induced by DC administered can be easily quantified as any T_{regs} present will be induced from existing T cell lineages rather than from expansion of naturally occurring T_{reg} pools.

1.4 Aims of this thesis

The prospect of using iPS cells to regenerate tissues and provide cures for diseases where current treatments are limited is an exciting hypothesis. However, as alluded to earlier, there are several hurdles preventing current translation of 'bench-top' findings into clinical trials.

Therefore, in this thesis I aim to address the following:

1. *What level of immunological activity do transplanted iPS cells attract?*

In this thesis I aim to address the findings of Zhao *et al.* and Araki *et al.* and examine using our iPS cell lines whether, upon transplantation, they have the ability to graft successfully into syngeneic hosts and whether any immunological rejection arises upon transplantation.

2. *Can 'tolerogenic' DC be derived from iPS cells?*

I will also examine whether DC with a tolerogenic capacity, can be differentiated *in vitro* from our iPS cell lines and thoroughly examine their phenotype and suitability for administration *in vivo* to induce tolerance to defined antigens, with the prospect of using them to create immunological tolerance towards iPS cells.

Chapter Two

Materials and Methods

2.0 Experimental mice

Mice were bred in the specific pathogen-free facility at the Sir William Dunn School of Pathology, University of Oxford, UK. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and received local ethical committee approval.

2.1 Cell lines and mouse models

ES cell lines

All ES cell lines used in this thesis are detailed in Table 2.1 below. ES cell lines were derived by Dr. Frances Brook, Mr. Tim Davies and Dr. Paul Fairchild from the inner cell mass of mouse blastocysts as described in Robertson *et al.* (2007) and Gardner and Brook (1997).

iPS cell lines

iPS cell lines were generated as described later in this chapter.

A summary of all ES and iPS cell lines is summarised in Table 2.1 below.

Cell Line	Sex	Strain	MHC Haplotype	Source of derivation
iPS-1	♂	CBA/Ca	H-2E^{k}	Mouse embryonic fibroblasts
iPS-14	♀	CBA/Ca	H-2E^{k}	Mouse embryonic fibroblasts
iPS-32	♂	CBA/K	H-2E^{k} / H-2K^{b}	Mouse embryonic fibroblasts
iMAF-6	♂	CBA/Ca	H-2E^{k}	Mouse adult tail–tip fibroblasts
ESF-116	♂	CBA/Ca	H-2E^{k}	Inner cell mass of embyro
ESF-166	♂	CB/K	H-2E^{k} / H-2K^{b}	Inner cell mass of embyro
ESF-121	♀	CBA/Ca	H-2E^{k}	Inner cell mass of embyro
ESF-75	♂	C57/B6	I-A^{b}	Inner cell mass of embyro

Table 2.1: ES and iPS cell lines used in this thesis. Derivation and cultures of these cell lines is described in section 2.3.

Cell Line		Reference
X63-GMCSF	X63-GMCSF secreting cell line	Stockinger *et al.* (1996)
2G7.1	T cell hybridoma specific for the HEL1-18 peptide in the context of H-2E^{k}	Adorini *et al.* (1991)
Mouse embryonic fibroblasts (MEF)	Mouse fibroblasts derived from E13 gestation embryos	Garfield *et al.* (2010)
Mouse adult fibroblasts	Tail tip fibroblasts derived from 8-10 week mice	Liu *et al.* (2011)

Table 2.2: Cell lines used in this thesis. Derivation and culture of these cell lines is described in sections 2.3 and 2.5.

Mouse strains

Mouse strain	Source	Sex/Age	MHC haplotype
CBA/Ca	Harlan	~6 weeks/♀/♂	H-2E^{k}
C57/B6	Harlan	~6 weeks/♀/♂	I-A^{b}
CB/K	Zelenika *et al.* (1998)	~6 weeks/♀/♂	H-2E^{k} / H-2K^{b}
A1.RAG1$^{-/-}$	Zelenika *et al.* (1998)	~6 weeks/♀	H-2E^{k}
CBA.RAG1$^{-/-}$	Zelenika *et al.* (1998)	~6 weeks/♀	H-2E^{k}

Table 2.3 Mouse strains used in this thesis with corresponding source/reference and MHC haplotype

2.2 Tissue Culture and medium used for cell culture

Tissue Culture

All tissue culture procedures were performed in a sterile tissue culture hood in a Biological Containment Level 2 facility.

R10 medium

R10 medium was made up using 500mL RPMI-1640 (Lonza), 50µM B-mercaptoethanol, 2mM L-glutamine, 10% FCS (Gibco), 2mM Essential Amino Acids and 2mM Sodium Pyruvate.

R10 + GMCSF medium

R10 + GMCSF medium was made up using 500mL RPMI-1640 (Lonza), 50µM β-mercaptoethanol , 2mM L-glutamine, 10% FCS (Gibco), 2mM Essential Amino Acids, 2mM Sodium pyruvate and 4% GM-CSF supernatant (see *GMCSF-X63)* which is equivalent to approximately 50U/mL of rmGMCSF.

ES cell medium

ES cell medium was made up using 500mL DMEM (Lonza), 50µM B-mercaptoethanol, 2mM L-glutamine, 10% FCS (Gibco), 2mM Essential Amino Acids and 2mM Sodium Pyruvate.

SR + LIF medium

SR + LIF medium was made up using 500mL DMEM (Lonza), 50µM B-mercaptoethanol, 2mM L-glutamine, 15% Serum Replacement (Gibco), 2mM Essential Amino Acids, 2mM Sodium Pyruvate and 1000U/mL rmLIF.

DC differentiation medium

DC differentiation medium was made up using 500mL DMEM (Lonza), 50µM B-mercaptoethanol, 2mM L-glutamine, 10% FCS (Gibco), 2mM Essential Amino Acids, 2mM Sodium Pyruvate, 4% GM-CSF supernatant (see *GMCSF-X63)* and 1000U/mL mouse rmIL-3.

Chemotaxis buffer

Chemotaxis buffer was made up by adding 0.5%w/v BSA to R10 (Lonza) with 10mM HEPES.

2.3 Derivation of cell lines

Derivation of mouse embryonic fibroblasts

E13-E14 embryos were harvested from mice and isolated from surrounding uterine and placental tissues under a microscope. Embryos were decapitated and heart and liver tissue removed. Embyronic tissue was then placed in a 5mL volume of Trypsin-EDTA solution in a 100mm tissue culture plastic plate and finely minced with a scalpel blade. The tissue was subject to repeated disaggregation using a 5mL pipette and incubated at 37^{o}C in trypsin-EDTA solution. The remaining cell suspension was passed through a sterile cell strainer and the resulting cell suspension was plated out into T75 tissue culture flasks and the cells expanded up accordingly. When suitable numbers of cells were acquired, they were frozen down in freezing medium and stored in liquid nitrogen until needed.

Derivation of mouse adult fibroblasts

T75 flasks were coated with 0.1% gelatin for 2 hours and washed in PBS. Tails from 6-8 week old mice were removed and rinsed for 1 min in 70% ethanol. The skin was then carefully removed using forceps. The skin was cut into 5mm pieces with dissecting scissors and incubated

in MAF-medium containing 0.1% collagenase IV in a Falcon tube. The tubes were placed on an orbital platform at 37^{o}C for 1 hr. The dissociated cells were centrifuged at 1200rpm for 5 min and plated onto MAF-medium in gelatinised T75 flasks. The cells were allowed to reach confluence before being harvested with TyplExpress and cyropreserved in liquid nitrogen.

Derivation of iPS cell lines from mouse fibroblasts

Fibroblasts were harvested as described above from CBA/Ca or CB/K mice and were cultured in feeder medium. Induction of pluripotency was carried out using the three transcription factors OCT4, SOX2 and KLF4 delivered in separate retroviruses. pMX.hOct4 (17217), hSox2 (17218), hKlf4 (17219) were purchased from Addgene and HiPure maxipreps (Invitrogen) performed according to manufacturer's instructions to amplify plasmid DNA from streaks/liquid cultures. Fibroblasts were plated at 1x10^{5} per well in a 6 well plate and viral supernatant was added to each well. This was repeated 24 hours later and after 48 hours the medium was replaced with SR + LIF medium along with 50µg/mL Vitamin C and 0.5µM valproic acid. The medium was replaced every 2-3 days and the cells were passaged according to their density. iPS cell colonies were identified 24-45 days after transduction at which point they were mechanically isolated and expanded on feeder cells mitotically inactivated with mitomycin-C.

Maintenance of mouse iPS cells

Mouse iPS cells were cultured in T25 flasks containing mitotically inactivated fibroblasts. The cells were maintained in SR + LIF medium and were passaged every three days using TyrpLExpress at a 1:5-1:10 dilutions into a fresh T25 flask containing mitotically inactivated fibroblasts. As well as being split onto MEFs, the cells were also serially passaged onto gelatine coated flasks in order to progressively remove contaminating fibroblasts.

Differentiation of iPS cells into embryoid bodies (EB)

Following two passages on gelatin, the iPS cells were harvested with TryplExpress and plated in ES medium in bacteriological plates at a density of $5x10^6$ cells per plate. The cells were left to form EB for 14 days, the medium being changed when necessary. The EB were removed from culture by sedimentation under unit gravity.

Differentiation of ipDC

Day 14 EB were removed from culture and plated onto tissue culture dishes (Corning) at a density of approximately 15 EB per dish in DC differentiation medium. The cultures were left for 14-25 days depending upon the rate of DC differentiation. The medium was changed every 4-5 days to ensure sustained concentrations of GM-CSF and IL-3. DC were harvested by removing medium and adding 9mL PBS. Lightly adherent cells were removed by gentle pipetting and transferred to a Falcon tube containing 1mL of 0.1% EDTA yielding a final concentration 0.02% EDTA.

2.4 Flow Cytometry

FACS blocking buffer

FACS blocking buffer was made up with 500mL 1x PBS, 10% v/v normal rabbit serum (purchased from Sigma and heat inactivated at 60^oC for 30 min), 0.5% w/v BSA, 0.1% NaN_3 and mouse Serobloc Fc blocking antibody was added at a concentration of 10µg/mL

FACS wash solution

FACS washing solution was made up with 500mL 1x PBS, 1% v/v normal rabbit serum (purchased from Sigma and heat inactivated at 60^oC for 30 min), 0.5% w/v BSA and 0.1% NaN_3

FACS fixative

FACS fixative was made up with 500mL 1x PBS and 1% v/v formaldehyde

FACS surface staining

Cells were pelleted into a 96 well round bottomed plate and blocked with FACS blocking buffer for 20 min on ice. The cells were then washed and incubated in primary antibody at an appropriate concentration for 30 min on ice. The cells were then washed in FACS washing solution and treated with 7-AAD at a concentration of 1µg/mL for 10 min on ice before being washed again. The cells were then fixed in FACS fixative.

FACS intracellular staining

FACS cell permeabilisation buffer was purchased from eBioscience. Briefly, cells were stained with surface markers (if applicable) and washed. The cells were then pelleted and resuspended at 4^{o}C in eBioscience permeabilisation buffer for 2 hr. The cells were then centrifuged at 1200rpm for 5 min, the supernatant removed and stained with the relevant antibodies diluted in 1x permeabilisation buffer for 30 min at 4^{o}C.

Flow Cytometry

Samples were analysed on a FACSCalibur Flow Cytometer.

ImageStream© staining

Cells were first blocked as described above and stained with Live-Dead Aqua (Invitrogen) as per manufacturer's instructions and relevant cell surface markers. The cells were then permeablised overnight and the nucleus was stained with 7-AAD at a concentration of 20µg/mL. The cells were then washed and fixed with FACS fixative.

ImageStream©analysis

Cells stained were analysed using an Amnis ImageStream© Mark II machine.

Antibodies used in FACS staining

The following table (Table 2.3) provides details of the mAb used and their source.

Antigen	Label	Isotype	Supplier	Clone
CD3	PE	Rat IgG2b	BD BioSci	17A2
CD3	Biotin	Rat IgG2a	BD BioSci	17A2
CD4	APC	Rat IgG2a	BD BioSci	RM4-5
CD4	Biotin	Rat IgG2a	BD BioSci	RM4-5
CD8a	PE	Rat IgG2a	BD BioSci	53-6.7
CD8a	Biotin	Rat IgG2a	BD BioSci	53-6.7
CD11b	Biotin	Rat IgG2b	BD BioSci	M1/70
CD11c	APC	Arm Ham IgG1	BD BioSci	HL3
CD25	Biotin	Rat IgM	BD BioSci	7D4
CD40	PE	Rat IgG2a	eBioscience	1C10
CD49b	Biotin	Rat IgM	BD BioSci	DX5

CD54	PE	Arm Ham IgG1	BD BioSci	3 E2
CD80	PE	Arm Ham IgG2	BD BioSci	16-10A1
CD86	PE	Rat IgG2b	Biolegend	PO3.1
F4/80	Biotin	Rat IgG2a	eBioscience	BM8
H-2A/H-2E	FiTC	Rat IgG2a	BD BioSci	2G9
H-2A/H-2E	PE	Rat IgG2b	eBioscience	2G9
H-2K^{k}	PE	Mo IgG2a	BD BioSci	36-7-5
H-2K^{k}	Biotin	Mo IgG2a	BD BioSci	36-7-5
HORMAD1	n/a	n/a	ProteinTech	13917-1-AP
Nanog	AF488	Mo IgG1	BD BioSci	M55-312
NKp46	Biotin	Rat IgG2a	eBioscience	29A1.4
Oct3/4	AF647	Mo IgG1	BD BioSci	40/Oct-3
SIINFEKL-peptide in context H-2K^{b}	PE	Mo IgG1	eBioscience	25-D1.16
SSEA-1	PE	Mo IgM	BD BioSci	MC480
TLR1	PE	Rat IgG2a	eBioSci	eBioTR23
TLR2	FiTC	Mo IgG2a	eBioSci	mT2.7
TLR4	PE	Mo IgG1	eBioSci	UT41
TLR9	FiTC	Rat IgG2a	eBioSci	M9.D6

Secondary reagents

Antigen	Label	Supplier
Streptavidin	APC	BD BioSci
Streptavidin	FiTC	BD BioSci
Streptavidin	PE	BD BioSci

Table 2.4 Antibodies used throughout this thesis along with their fluorochrome conjugation (if any), matched isotype, supplied and clone number.

2.5 Immunohistochemistry

Haematoxylin and eosin staining

Selected tissues were frozen in O.C.T compound by cooling isopropane on dry ice. Slides on which tissue sections of 7µm thickness were mounted were treated with histoclear solution for 5 mins and rehydrated through an alcohol gradient. Slides were immersed in haematoxylin for 3 min, followed by eosin for 30 seconds. Slides were then dehydrated in an alcohol gradient. The slides were dried overnight at room temperature and mounted under a coverslip with DPX.

Immunofluoresence staining

Slides of tissues sections of 6µm thickness were thawed at room temperature for 30 min and then fixed with 4% paraformaldehyde for 10 min. Slides were then washed twice with PBS to remove the PFA. The slides were incubated with goat serum for 30 min to prevent non-specific binding of antibodies. Primary mAb were used at a concentration of X for 1 hour and any unbound mAb removed by washing with PBS. Secondary antibodies, where needed, were also used at a concentration of X for 1 hour and then washed off. Slides were washed thoroughly and mounted

with fluorescent DAPI mounting medium (Vectashield, Cat# H-1200) and allowed to dry overnight at room temperature in the dark.

Visualisation of antibody staining

Staining of sections was visualised using an Olympus confocal microscope with x20 air or x40 oil immersion lenses.

Immunoperoxidase staining

Tissue sections were prepared as previously described. Sections were first allowed to air dry for 15 min before being fixed with 4% PFA for 15 min. The slides were then washed in PBS and blocked with PBS containing 10% Normal Donkey Serum (NDS) and Fcγ-receptor blocking antibody for 2 hr at room temperature. The slides were then washed in PBS and blocked with an avidin/biotin blocking kit (Vector) for 30 min. The slides were then washed in PBS, and the stained with the relevant mAb overnight at 4^{o}C. The following day, the slides were washed with PBS and treated with streptavidin-HRP for 2 hr at room temperature. The slides were washed with PBS three times and the diaminobenzidine (DAB) substrate added (Vector Laboratories, SK-4100). The stain was inspected by eye every 2 min until an appropriate level of staining was visible, and then washed in PBS three times. The slides were then counterstained with haematoxylin and passed through a hydrating then dehydrating gradient. The slides were then mounted with DPX (Sigma-Aldrich) and left to dry overnight.

2.6 Cytokines and pharmacological agents

A summary of cytokines used in this thesis is shown in Table 2.5.

GM-CSF

An X-63 transfectant secreting murine GM-CSF was provided by Dr D. Gray, University of Edinburgh. Cells were grown to confluence in IMDM (Gibco) containing 1µg/mL of the antibiotic G418 to allow for selection. The cells were allowed to condition the medium to produce a source of murine GM-CSF. The supernatant was harvested and tested for activity with murine bone marrow cultures. Briefly, $1x10^5$ cells/well in 200µL of R10 were added to a flat bottomed 96 well plate and the supernatant was titrated and added to each well in triplicate as a two-fold dilution series. Commercial rmGM-CSF (R&D Systems) was used as a standard. After 96 hours, the cultures were pulsed with 0.5µCi/well of ^{3}H-TdR (Amersham Biosciences) and proliferation was assessed 18 hr later by uptake of ^{3}H-TdR, measured using a flatbed scintillation counter (Perkin-Elmer). The aliquots of GM-CSF were stored at -80^{o}C.

Cytokine		Supplier	Catalogue Number
GM-CSF	Granulocyte monocyte cytokine stimulating factor	n/a	n/a
IL-3	Interleukin 3	R&D Systems	403-ML-010
IL-4	Interleukin 4	R&D Systems	404-ML-010
IL-10	Interleukin 10	R&D Systems	410-ML-010
IL-12	Interleukin 12	R&D Systems	412-ML-010
TNF-α	Tumour necrosis factor alpha	R&D Systems	410-MT-010
LIF	Leukaemia inhibitory factor	Millipore	LIF2010

Table 2.5 Source of recombinant cytokines and growth factors and appropriate catalogue number

Lipopolysaccharide (LPS)

LPS, *E. coli* 0127:B8 (Sigma) was diluted to 2mg/mL using RPMI and stored as 2mL aliquots at 4^{o}C. This was then diluted accordingly in cultures to 1µg/mL (or as appropriate).

Rapamycin

Rapamycin (Sigma) was dissolved in PBS at a concentration of 10µg/mL and stored at -80^{o}C.

2.7 Molecular techniques

RNA isolation

RNA was isolated from cell pellets. The SV Total RNA Isolation System (Promega) was used, according the manufacturer's instructions which also included a DNase-1 treatment. RNA concentration was determined by using a Nanodrop spectrophotometer. In integrity of the RNA was determined using the RNA 6000 Nano Assay kit carried out according to manufacturer's instructions and run on an Agilent 2100 Bioanalyser (Agilent Technologies).

First strand synthesis

Reverse transcription of total RNA was performed using the EZ-first strand cDNA synthesis kit (GeneFlow) as per manufacturer's instructions.

PCR

cDNA was transcribed as previously described. cDNA then underwent a PCR reaction with dNTPs, GoTaq Polymerase, $MgCl_2$, primers and DNase-free H_2O.

Preparation of agarose gels

Agarose gels were prepared by dissolving Ultra Pure Agarose (BioRad, UK) in 0.5x TBE and SafeView (NBS Biologicals), diluted according to manufacturer's instructions. Warm agarose was then allowed to set in a gel holder for 30 min at room temperature. Samples were loaded into gel wells with loading dye and electrophoresis carried out at 100mA in 0.5 TBE, SafeView using a Power Pac 300 (BioRad, UK). DNA was visualised and photographed using a UVP GDS 5000 gel documentation system (UVP Inc., USA). For each gel, a 1Kb DNA standard size ladder was run in parallel.

Oligonucleotide primers

Below is a table of all primers used throughout this thesis. All were ordered from Sigma-Aldrich unless otherwise specified and made up to a stock concentration of 100µM in ddH_2O-RNase-DNase free.

Oligo	**Sequence 5`-3`**
Zg16-F	CATCACCGCCTTCCGTAT
Zg16-R	CGTTGAAACTTGTGCCTGA
Hormad1-F (Araki)	CCAGATTACCAACCACCAG
Hormad1-R (Araki)	TGAAAAGGTGTTGGGACT
Hormad1-F (Zhao)	CCAGATTACCAACCACCAG
Hormad1-R (Zhao)	TGAAAAGGTGTTGGGACT
Cyp3a11-F	ATCCCATTGCTAATAGAC
Cyp3a11-R	ATCATCACTGTTGACCCT
Oct3/4-F	CCCCAG GGCCCCATTTTGGTACC
Oct3/4-R	TTATCGTCGACCACTGTGCTGGCG

Sox2-F	GGCACCCCTGGCATGGCTCTTGGCTC
Sox2-R	TTATCGTCGACCACTGTGCTGGCG
Klf4-F	ACGATCGTGGCCCCGGAAAAGGAC
Klf4-R	TTATCGTCGACCACTGTGCTGGCG
CIITA-F	GGACCTGGACTCACTTAGTGA
CIITA-R	ATCCAGCTGCTGCAGGTG
HPRT-F	GTTGGATACAGGCCAGACTTTGTTG
HPRT-R	GAGGGTAGGCTGGCCTATAGGCT

Table 2.6 Summary of primers used in this thesis.

2.8 Immunological techniques

Synthetic peptides

Peptides were synthesised by ThinkPeptides and assessed for purity by the manufacturer. All peptides had a >95% purity level.

Dby male antigen peptide

The 479-493 epitope of the male specific antigen deadbox protein on the Y chromosome (Dby 479-493) has the following sequence: REEALHQFRSGRKPI. The synthetic peptide is recognised by A1.RAG1$^{-/-}$ T cells in an H-2E^{k} restricted manner.

HEL1-18 peptide

The H2E^{k} restricted 1-18 peptide of the hen egg lysozyme has the sequence KVFGRCELAAAMKRHGLD recognised by the T cell hybridoma 2G7.1 when presented in an H2E^{k} restricted manner.

SIINFEKL peptide

The $H2K^b$ restricted peptide of ovalbumin has the sequence SIINFEKL. The peptide is recognised by both OT-1 T cells and SIINFEKL-$H\text{-}2K^b$ mAb.

Processing and presentation of antigen on MHC class II

Dendritic cells were harvested and divided into two equal populations. One population was fixed by treatment with 1% PFA for 5 min. The DC were then pulsed with either the HEL 1-17 peptide or the whole HEL protein. These were co-cultured with the 2G7.1 T cell hybridoma with a 1:1 ratio of T cells:DC ($1x10^5$). DC and the T cell hybridoma were cultured for 18 hours in a 96 well flat bottomed plate. After culture, the plate was centrifuged at 1200rpm for 5 min and the supernatant collected. The amount of IL-2 secreted by the T-cell hybridoma was quantified using an ELISA as a measure of T cell activation.

ELISA

An IL-2/Il-6/IL-10/IL-12p70 ELISA-Ready-Set-Go™ kit was purchased from eBioscience. The ELISA was carried out according to manufacturer's instructions. The ELISA was visualised using a spectrophotometer at $OD_{450nm.}$

Cross presentation of antigen on MHC class I

DC of CB/K origin were plated into a 96 well plate at a density of $2x10^5$/well in triplicate. The DC in each well were then treated either with SIINFEKL peptide (Sigma), whole OVA protein (Sigma) or a scrambled peptide (ProImmune) for 18 hours. The plates were then spun at 1200rpm for 5 min and the cells stained with a mAb specific for the SIINFEKL-$H2K^b$ complex according to FACS staining protocol.

Stimulation of DC by TLR ligands

DC were plated at a density of $2x10^5$ in a round bottom plate in 200µL of R10 medium. A TLR agonist kit was purchased from Invivogen which contained agonists of TLR1-9. The agonists were added at the manufacturer's recommended concentrations to each well in triplicate and incubated overnight at $37^oC/5\%$ CO_2. Subsequently the plate was spun at 1200rpm for 5 min and the supernatants harvested. The supernatant were analysed for IL-6 content using an IL-6 ELISA kit as described.

Mixed leukocyte reaction

DC were harvested from relevant cultures and treated with mitomycin C (10µg/mL) for 30 min at 37^oC followed by three washes in PBS to remove any contaminating mitomycin C. The DC were then serially diluted two fold and were plated in triplicate in a round-bottom 96 well plate with a top concentration of $5x10^4$ DC/well. T cells were purified using a nylon wool column and added into each well at $2x10^5$ cells per well giving a top DC:T cell ratio of 1:5. The co-culture was incubated at $37^oC/5\%CO_2$ for 3 days. During the last 18 hours of culture, the wells were pulsed with 0.5µCi ^{3H}-TdR per well.

Stimulation of ♀ A1.RAG1$^{-/-}$ T cells *in vitro*

DC were harvested from relevant cultures and treated with mitomycin C (10µg/mL) for 30 min at 37^oC followed by three washes in 1x PBS to remove any contaminating mitomycin C. The DC were then serially diluted two fold and were plated out in triplicate in a round-bottom 96 well plate with a top concentration of $5x10^4$ DC/well. T cells were then harvested using a nylon wool column and added into each well at a concentration of $2x10^5$/well giving a top DC:T cell ratio of 1:5. The *Dby* peptide was also added to each well at a final concentration

of 100nM. The co-culture was incubated at 37°C/5%CO_2 for 3 days. During the last 18 hours of culture, the wells were pulsed with 0.5µCi ^{3H}-thymidine per well.

Assessment of CD69 induction in ♀ A1.RAG1$^{-/-}$ T cells *in vivo,* by DC

Male DC were harvested and re-suspended in PBS at a density of $5x10^6$ in 200µL of PBS. The DC were then injected into the tail vein of ♀ A1.RAG1$^{-/-}$ mice using an insulin syringe. Mice injected were also injected with PBS alone or equivalent number of female DC as a control. The spleens from injected mice were then harvested 18 hours later and dispersed into a single cell suspension. Splenocytes were stained for CD4 and CD69 as described in *Flow Cytometry.*

Isolation of splenocytes

Spleens were removed aseptically from mice and pushed through a 70µm cell strainer with a plunger from a 2mL syringe. The resulting homogenate was spun down at 1200rpm for 5 min and then resuspended in 2mL of red blood cell lysis buffer (Sigma) for 3 minutes. Lysis was terminated by adding 10mL of R10 medium.

Isolation of T cells

Nylon wool columns were prepared by placing 2g of teased nylon wool (Robins Scientific, UK) in the barrel of a 20mL syringe that was then autoclaved. On the day recquired, 20mL of RPMI and then 20mL of R10 medium were flushed through the syringe and nylon wool. A butterfly-21 clip (Abbot Labs Ireland) was then attached to the tip of the syringe to control the flow of medium. The wet column was then incubated for 1 hour at 37°C. Spleen homogenate was loaded onto a nylon wool column and left at 37°C for 1 hour. The column was then flushed with 40mL of warm R10 medium to elute non-adherent T-cells.

Culture of bone marrow derived dendritic cells (bmDC)

Bone marrow dendritic cells were derived using a modified protocol as previously described. The hind legs from mice were harvested and soaked in 70% ethanol for 1 min and washed in PBS twice to minimise contamination. The ends of the bones were cut and removed and the bones flushed through with R10 medium loaded into a 20mL syringe equipped with a 20-guage needle. The cells collected were centrifuged at 1200rpm for 5 min. Red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma) for 3 min and then centrifuged for a further 5 min at 1200rpm. The cells were plated in 90mm tissue culture dishes in R10 + GMCSF medium. The medium was replaced on days 3 and 6 of culture. Cells were harvested on day 7 of culture by lightly pipetting PBS over the surface of the dishes.

Induction of Foxp3+ Treg *in vitro*

Female A1.RAG1$^{-/-}$ spleens were harvested from 6 week old mice and homogenised. Red blood cell lysis was performed using red blood cell lysis buffer (Sigma) for three minutes. The remaining splenocytes were then counted using TrypanBlue exclusion. 10^5 DC were added per well in a 24 well plate along with $5x10^5$ splenocytes giving a 5:1 ratio of splenocytes:DC. The cells were suspended in 2mL of R10 along with an appropriate concentration of TGF-β and 100nM of the *Dby* peptide, if appropriate. Cultures were incubated for six days at 37^{o}C/5% CO_2. After six days, the cultures were harvested and stained for CD4, CD25 and Foxp3 as described in *Flow Cytometry*.

Quantification of NO release

DC were plated in a 96 well round bottom plate at a density of $2x10^5$ DC/well and set up in triplicate. The appropriate stimulus was added to each well. The DC were incubated overnight at 37^{o}C/5% CO_2. The following day, the supernatant was harvested and 100µL was

added to a flat bottom plate. 1% w/v N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma) was prepared and added in a 1:1 ratio with 1% w/v sulphanilamide diluted in 5% phosphoric acid to make the Griess reagent. A series of standard NO concentrations were prepared by dissolving $NaNO_2$ in ddH_2O. 100µL of the Griess reagent was added per well of 100µL supernatant. The assay was left to develop for 10 min and measured on a spectrophotometer at $OD_{550nm.}$

2.9 *In vivo* experimental techniques

Grafting of embryoid bodies

Mice were anaesthetised with an intra-peritoneal injection of anaesthetic and an incision was made on the left flank of the mouse. Embryoid bodies were grafted using a modified protocol from Robertson *et al* (2007). Briefly, the left kidney was then gently exposed and 2-5 embryoid bodies were inserted into the kidney capsule using a spatula. The body cavity wall was the stitched along with the skin and the mice were left to recover for several hours. The mice were sacrificed at day 21, unless otherwise stated, and the kidney examined for any growth of teratoma.

Harvesting of teratomas

Mice were exposed to a rising concentration of CO_2 and terminated using Schedule-1 procedure. The left kidney was exposed and any teratoma present was removed with a scalpel and frozen in OCT medium in a plastic mould on isopentane cooled on dry ice.

Administration of non-depleting antibodies

Non-depleting antibodies against CD4 (clone: YTS 177.9.6.1), CD8 (clone: YTS 105.18.10) and CD40L (clone: MR1) were administered to mice intraperitoneally at a concentration of 1mg/mouse on days 0, 2 and 4 to induce tolerance to mismatched tissues.

2.10 Other assays

xCELLigence chemotaxis assay

Experiments were carried out in a 16 well plate (CIM-16) on an xCELLigence RTCA-DP machine. Agonists were made to their desired concentrations in 160µL final volume of chemotaxis assay buffer and loaded into the bottom of the well. The upper chamber was attached and 50µL of chemotaxis buffer was added. The chamber was allowed to equilibrate to a gradient for 30 mins at room temperature. DC were re-suspended in chemotaxis buffer and added to each well at a density $2x10^5$ cells/well. The DC were added to the top chamber of each well and the plate was transferred to the RTCA-DP machine. Measurement of chemotaxis was measured every 5 seconds for 3000 sweeps.

Cell adhesion assay

Experiments were carried out in a 96 well plate on an xCELLigence RTCA-DP machine. $5x10^4$ cells were added in a volume of 50µL per well of the plate, with each condition set out in triplicate. The plate was placed in the machine to record a background reading. Agonists were made up to their desired concentrations in chemotaxis buffer and 10µL added per well. Measurement of adhesion was measured every 2 seconds for 2000 sweeps.

Statistics

All data is presented as mean +/- SD. All statistics were performed using Prism (GraphPad software), applying two-tailed Student's T-test. Values of < 0.05 were considered significant.

Chapter Three:

Differentiation of dendritic cells from mouse iPS cells

3.0 Introduction

In 2006, Takahashi *et al.* showed that stem cells, akin to embryonic stem cells (ES cells), could be derived by reprogramming mouse fibroblasts back to a pluripotent state by the introduction of four transcription factors. These stem cells, induced pluripotent stem cells (iPS cells), may one day be able to act as a novel source of cell types and tissues for cell replacement therapy (CRT). Since this discovery, the field of iPS cell research has expanded exponentially with much interest focusing on the directed differentiation into specific therapeutic cell types.

However, the publication of work carried out by Zhao *et al.* (2011) has raised the question whether, despite being derived in a potentially autologous fashion, iPS cells are targeted by the immune system and recognised as non-self-tissues. Zhao *et al.* suggested that immunological rejection may arise from the erroneous up-regulation of developmental antigens upon reprogramming. These developmental antigens subsequently act as minor histocompatibility antigens (mH) in the context of transplantation. However, several studies since the work from Zhao *et al.* have drawn conflicting conclusions as to whether autologous iPS cells can be immunologically rejected.

Given the possibility that iPS cells may express several developmental antigens, as described by Zhao *et al.* (2011), we sought to investigate whether tolerance could be induced to these antigens using DC derived from iPS cell lines, which at the time, had not been reported in the literature. DC have the unique ability to act as sentinels of the immune system by having the capacity to both initiate immune responses and also to induce tolerance to antigens. If the DC differentiated from iPS cells (ipDC) were found to express the same developmental antigens which the immune system targets on iPS cells, and ipDC could adopt a tolerogenic

phenotype, then their administration might induce tolerance to ectopically expressed antigens on iPS cells prior to their implantation.

Previous efforts by our laboratory have shown that embryonic stem cells (ES cells) can be successfully differentiated into dendritic cells (esDC) (Fairchild *et al.* 2000). The rationale for deriving these esDC was similar to our rationale for deriving ipDC in that esDC also expressed alloantigens which could induce tolerance within the immune system to ES cell-derived transplants. However, ES cell transplantation presents a larger immunological hurdle than iPS cell transplantation as it is likely ES cells would express differences in multiple major histocompatibility complex loci compared with autologous iPS cells which may only differ at minor histocompatibility loci represented by the developmental antigens. It is, therefore, likely that tolerance to iPS cell tissues would be easier to induce than ES cell derived tissues.

Therefore, in this chapter we have examined the ability for iPS cell lines to differentiate into DC and have fully characterised the phenotypes of both the iPS cell lines generated and iPS differentiated DC (ipDC). Following this, we examined the functional phenotype of ipDC and assessed their abilities to process and present antigen and their ability to present antigen to antigen-specific T cells. Given the reported ability for DC to migrate *in vivo* in response to chemokines such as RANTES, MIP1α and CCL19 we sought to investigate whether we could replicate such findings with ipDC *in vitro* using the xCELLigence system. We also examined whether ipDC had the ability to migrate *in vivo* to lymphoid tissues and activate naïve T cells *in situ* in an antigen specific manner. Additionally, the ability for ipDC to be driven towards a tolerogenic state was examined by assessing the effects of various pharmacological agents on ipDC function and phenotype. Finally, the ability for ipDC to induce regulatory T cells (T_{reg}) in antigen specific A1.RAG1$^{-/-}$ T cells was also examined.

3.1 Results

Although several studies have shown the promising use of dendritic cells differentiated from mouse ES cells (Fairchild *et al.* 2000), human ES cells (Silk *et al.* 2011) and human iPS cells (Senju *et al.* 2011), at the outset of this project, no studies had been shown to successfully differentiate DC from mouse iPS cells. As proof of principle we therefore sought to derive iPS cell lines from mouse embryonic fibroblasts (MEF) and induce their differentiation along the DC lineage.

3.1 Derivation of iPS cell lines from mouse embryonic fibroblasts (MEF)

In order to derive iPS cell lines, MEF were chosen as the starting cell population for reprogramming to pluripotency. MEF have been shown to proliferate rapidly *in vitro* and have been widely reported to have the ability undergo reprogramming to iPS cells (Hamilton *et al.* 2009).

3.1.1 Characterisation of iPS cell lines: morphology, karyotype, gender and gene silencing

To date, protocols have been reported by many which somatic cells can be reprogrammed back to a pluripotent state. In order to generate iPS cell lines for this thesis, MEF were derived from E14 CBA/Ca mice as described in Methods 2.3. Following infection with a mixture of retroviruses encoding *Oct3/4, Sox2 and Klf4,* iPS cell colonies were found to appear after approximately 21 days, individual colonies were picked, expanded and characterised in order to determine their level of pluripotency, gender, karyotype and whether the transgenes introduced had been silenced upon reprogramming. As shown below in Figure 3.1, one of the iPS cell lines generated iPS-1, displayed similar morphology to ES cell colonies appearing as densely-packed, refractile colones when viewed under phase contrast.

We used ESF-116 as a comparison throughout these studies, since it has been extensively characterised and has been shown to support DC differentiation (Fairchild *et al.* 2000). iPS-1 also had the ability to differentiate into embryoid bodies (EB) which display similar morphology and size to ES cell-derived EB. The iPS cell line generated also had a normal male karyotype of 40XY with no chromosomal abnormalities, as well as showing silencing of the transgenes *Sox2, Oct3/4* and *Klf4* (data not shown).

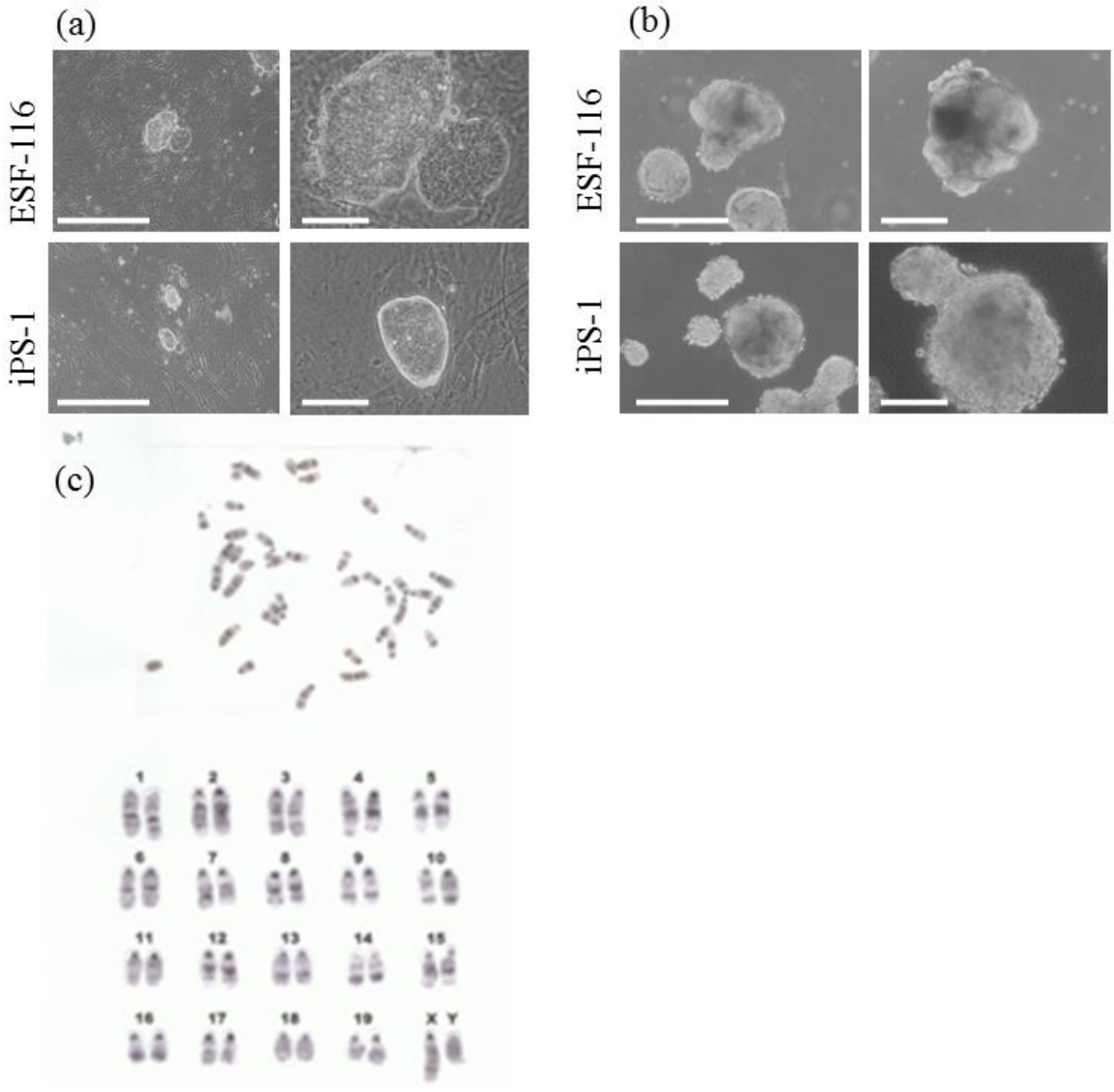

Figure 3.1. iPS cell morphology, karyotype, gender and transgene silencing. iPS-1 cells display similar morphology to their ES cell counterparts (a), have the ability to form embryoid bodies (b) as well as displaying a normal XY male karyotype (c).

3.1.2 Expression of pluripotency markers

The characteristics of pluripotency are reflected in ES cells by the expression of various transcription factors and cell surface markers including Nanog, SSEA-1 and Oct3/4. Subsequently, it has been shown that these pluripotency genes are endogenously expressed in iPS cell lines secondary to their introduction as transgenes (Yu *et al.* 2007). In order to assess whether the iPS cell line we generated displayed similar levels of expression of these pluripotency genes, expression of these markers was assessed by flow cytometry. As shown in Figure 3.2, iPS-1 showed similar levels of expression all three pluripotency markers compared to the ES cell line ESF-116, consistent with the acquisition of pluripotency.

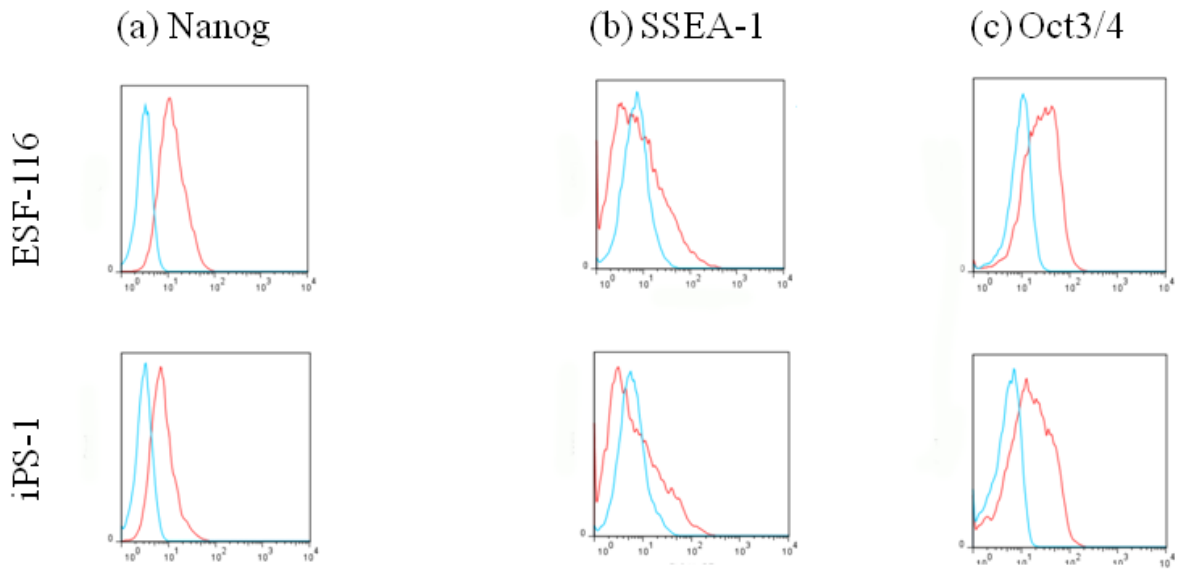

Figure 3.2. Expression of pluripotency markers by ESF-116 and iPS-1 cell lines. Figure 3.2 shows the expression of Nanog, SSEA-1 and Oct3/4 by both ESF-116 and iPS-1 as assessed by flow cytometry. Red line depicts expression of marker of interest, the blue line depicts the level of background staining using isotype-matched control monoclonal antibodies.

Following identification of pluripotency markers in iPS-1, we also sought to examine whether single colonies had similar morphology to ES cell colonies and also expressed the

pluripotency genes *SSEA1, Nanog* and *Oct3/4*. As shown below in Figure 3.3, we were able to show that both iPS-1 and ESF-116 displayed similar morphology and expressed all three pluripotency markers. While Nanog and Oct3/4 were uniformly expressed throughout both ES and iPS cell colonies, SSEA-1 appeared restricted in it expression, being most highly expressed around the periphery.

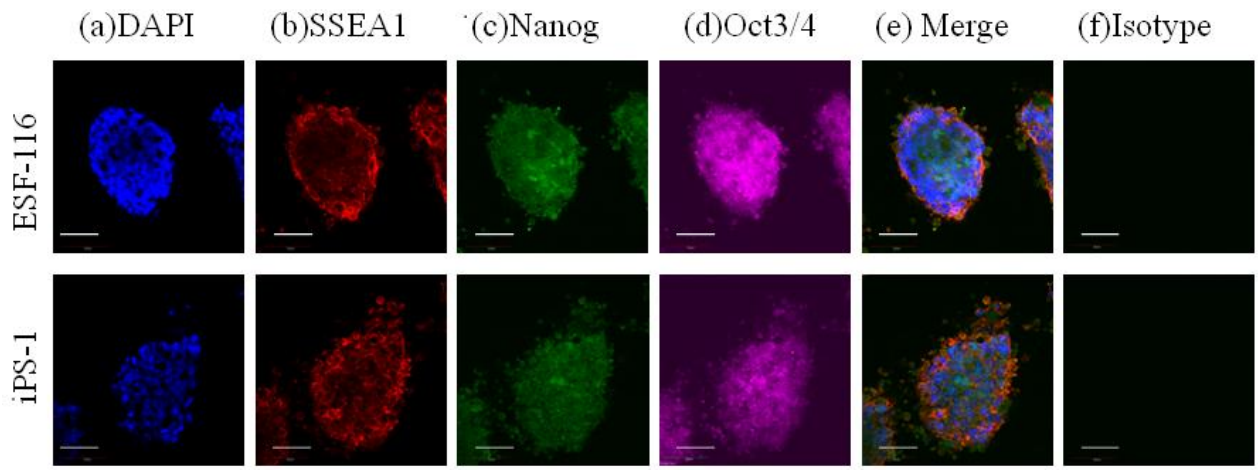

Figure 3.3. Expression of pluripotency markers in single representive iPS and ES cell colonies. iPS and ES cell colonies were grown in feeder-free conditions and stained with antibodies against pluripotency markers. The isotype represents a pooled isotype for all three antibodies. Scale bars represent 100µm

.

We were therefore able to show that the iPS cell line we generated expressed the pluripotency markers characteristic of pluripotent stem cells. In order to examine whether the iPS cell line we generated was truly pluripotent, it is necessary to determine whether the iPS cell line had the capacity to differentiate into tissue from all three germ layers.

3.1.3 Differentiation *in vivo* into lineages from the three embryonic germ layers

Unlike ES cell lines, the differentiation capacity of iPS cells can be heavily influenced by their epigenetic signature and the somatic cell type from which they were reprogrammed. The epigenetic memory, as discussed in *Introduction*, can significantly influence the differentiation pathways along which iPS cells undertake (Cai *et al.* 2013). As a result, the differentiation capacity of the iPS cell line generated was assessed by teratoma formation *in vivo*. Briefly, iPS cells were differentiated into embryoid bodies for 14 days and then transplanted under the kidney capsule of CBA.RAG1$^{-/-}$ mice. The resulting teratomas were harvested 21 days later and 7µm tissue sections prepared. Both iPS-1 and ESF-116 sections were stained with haemotoxylin and eosin and examined under light microscopy. As shown in Figure 3.4, both iPS-1 and ESF-116 had the capacity to generate teratomas in CBA.RAG1$^{-/-}$ mice which displayed cell types of all three germ layers.

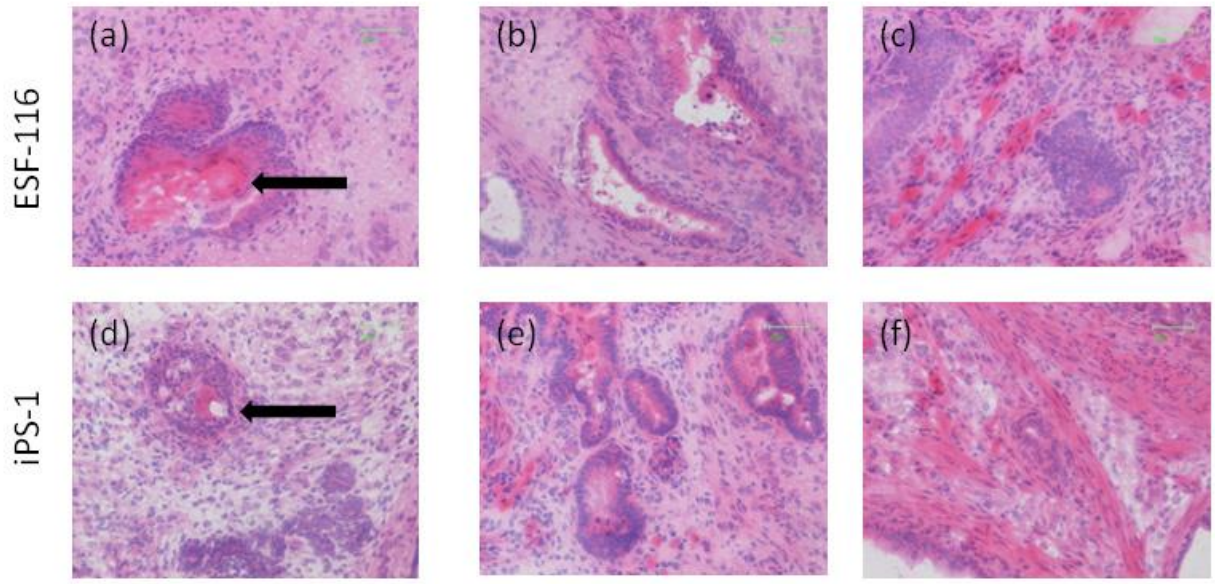

Figure 3.4. Formation of tissue from each of the three embryonic germ layers *in vivo*. The figure above shows haematoxylin and eosin staining of sections from teratomas formed in CBA.RAG1$^{-/-}$ mice by ESF-116 and iPS-1 cell line following transplantation of 14-day embryoid bodies under the kidney capsule. Teratomas were harvested after 21 days.

Photomicrographs show tissues from: cornified epithelial tissue [ectoderm] (a) and (d) gut-like epithelium [endoderm] (b) and (e) and smooth muscle [mesoderm] (c) and (f)

In order to more accurately determine the differentiation capacity of the iPS cell line, tissue sections were also stained with defined lineage markers against endoderm, ectoderm and mesoderm. As shown in Figure 3.5, iPS-1 had the ability to differentiate into tissues expressing the markers smooth muscle actin indicative of mesoderm (Figure 3.5 (b)), the ectoderm marker β-catenin (Figure 3.5 (c)) and GATA-4 (Figure 3.5 (d)). GATA-4 is expressed by endoderm tissue.

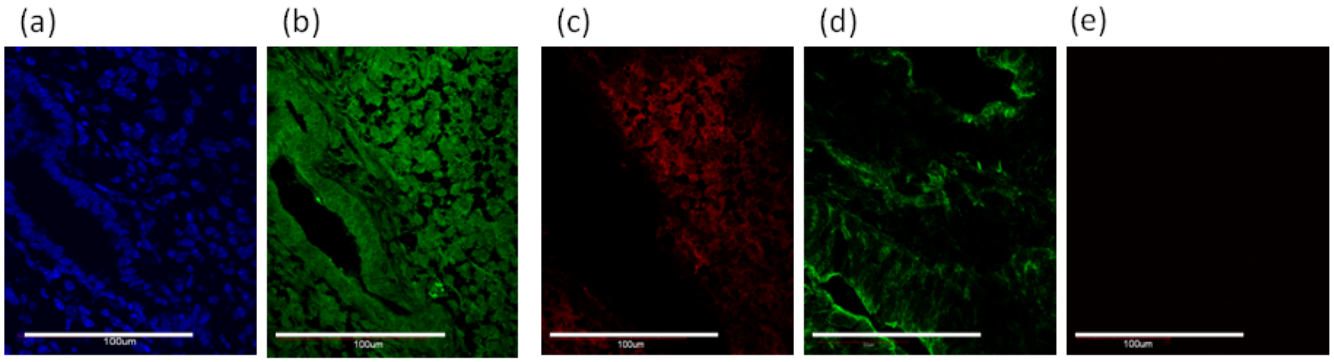

Figure 3.5. Expression of lineage markers by iPS-1 teratoma. iPS-1 were cultured as EB for 14 days and transplanted under the kidney capsule into CBA.RAG1$^{-/-}$ mice. After 21 days, the resulting teratomas were harvested as described in *Methods* and sectioned. Sections were stained with monoclonal antibodies of interest and viewed on a confocal microscope. Panels depict non-overlapping areas of tissue stained with (a) DAPI (b) β-catenin (c) GATA-4 (d) Smooth muscle actin (e) pooled isotype control. Scale bar represents 100μm, except in (d) were scale bar represents 50μm.

3.2 Differentiation of ipDC from iPS cell lines

Previous work in the lab has shown that it is possible to generate DC from ES cell lines (Fairchild *et al.* 2000). As outlined earlier, the rationale for differentiating DC from iPS cells is that these cells may express the same erroneously up-regulated developmental antigens which may be responsible for the possible immunological rejection of autologous iPS cell

derived tissue. These DC may, therefore, have the capacity to induce tolerance to these antigens when administered *in vivo* prior to transplantation of iPS-cell derived tissue. Therefore, we next sought to differentiate DC from the iPS cell lines we had generated and to characterise the DC obtained. Given the down-stream applications of the ipDC in the A1.RAG1$^{-/-}$ TCR-transgenic mouse model, we chose to differentiate ipDC from the male iPS-1 cell line. Firstly, the culture conditions for ipDC were established based upon the previous protocol used for the differentiation of esDC (Fairchild *et al.* 2000). iPS cells were differentiated into embryoid bodies (EB) for 14 days in bacteriological dishes. The EB were then harvested, transferred to tissue culture dishes and cultured in the presence of IL-3 and GM-CSF for 18-25 days. ipDC could then be harvested by gently pipetting to remove lightly adherent cells from culture.

3.2.1 Establishment of protocols for DC differentiation: morphology, timing and yield.

As shown in Figure 3.5, iPS cells were grown on a feeder layer for 3 days and differentiated into embryoid bodies for 14 days. These embryoid bodies were then cultured with GM-CSF and IL-3 which has previously been shown to induce the differentiation of both bmDC (van der Laar, 2012) and esDC (Fairchild *et al.* 2003).

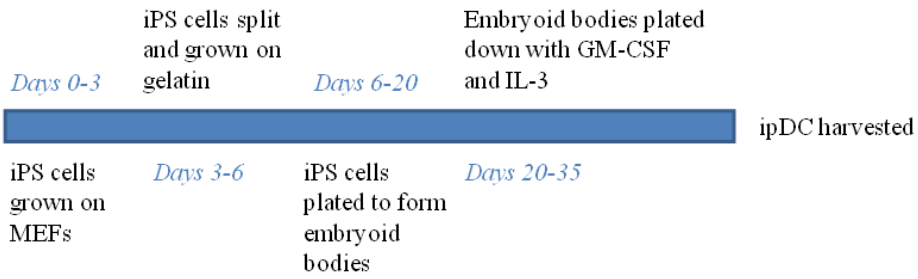

Figure 3.6. Differentiation of ipDC from iPS cell lines. Figure 3.6 depicts the protocol and timeline for differentiation of ipDC from iPS cells and the culture conditions required for differentiation.

Initially, ipDC migrate from embryoid bodies in culture as shown in Figure 3.6 at day 14. These cells are 'early ipDC' which form in relatively small numbers around the edge of the embryoid bodies. Only after a further week do these cells rapidly expand and fill the remaining areas of space within the culture vessel. ipDC also form 'clusters' which are characteristic of bmDC culture as well as exhibiting other features characteristic of bmDC, such as veils of cytoplasm and a large cytoplasmic to nuclear ratio. The complex dendritic cell morphology of ipDC was best observed under scanning EM which revealed many thin dendrites issuing from the surface of each DC (Figure 3.7 (d)).

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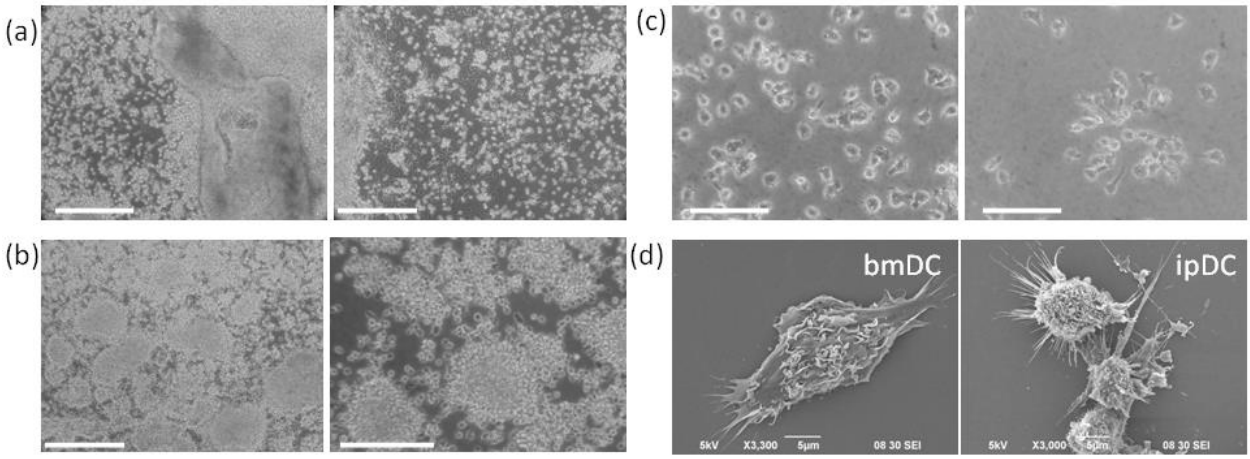


Figure 3.7 Initial characterisation of ipDC. ipDC initially form around the perimeter of EB (a) which then go on to expand and form clusters of DC in the space between EB (b). At high power ipDC have characteristic morphology of DC with pronounced veils of cytoplasm (c). ipDC also have similar structure to bmDC when viewed with scanning electron microscopy (SEM) (d). Parts (a) – (c) show two representative fields of view. Scale bars represent in panels (a) 50µm (b)50µm and 25µm (c) 10µm

After the development of the protocol for ipDC differentiation, we next sought to carefully characterise the cells in order to ascertain the phenotypic and functional characteristics of this novel cell population.

3.2.2 Phenotype of ipDC: myeloid markers, co-stimulatory and inhibitory receptors.

ipDC from confluent cultures were first harvested and analysed for the expression of markers associated with DC. As a control population, we used bmDC since not only are they easily obtainable, but we have previously shown that their administration to A1.RAG1$^{-/-}$ mice will induce tolerance to the male Dby mH-antigenic difference (Yates *et al.* 2007).

In order to analyse their surface phenotype, DC were stained with mAbs specific for a wide range of DC markers. As shown in Figure 3.8, ipDC exhibited similar levels of markers such as CD11b, CD54 and MHC class I compared to bmDC. Expression of CD11b suggests that, as anticipated, ipDC derive from a myeloid origin. Indeed, CD11b^{+}CD11c^{+} DC have been shown to induce tolerance in a model of experimental autoimmune encephalomyelitis (Selles-Moreno *et al.* 2012). ipDC also express co-stimulatory molecules such as CD40, CD80 and CD86 which are necessary for the activation of naïve T cells. Interestingly, ipDC exhibit low levels of the DC marker CD11c. Low expression of CD11c has also been observed in esDC (Fairchild *et al.* 2000). ipDC also express MHC class I and MHC class II, the vehicle for presentation of exogenous antigens to T cells. However, as discussed in the next chapter, considerable variability in the expression of MHC class II has been observed on ipDC throughout this project. Additionally, data shown in the next chapter demonstrates that a proportion of ipDC can undergo maturation following stimulation with LPS and, as discussed later in this chapter, ipDC have the ability to respond to various TLR ligands by secreting IL-6.

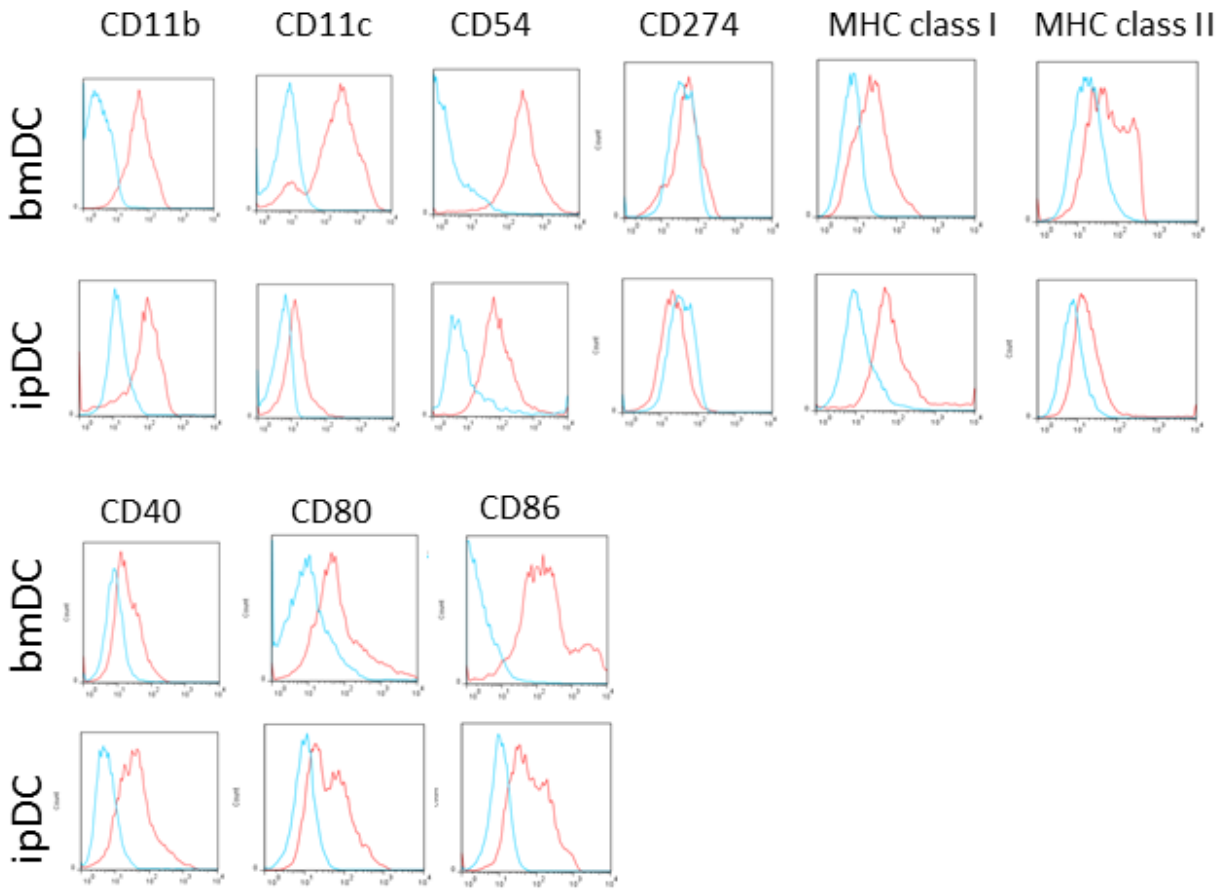


Figure 3.8 A comparison of the expression of myeloid and co-stimulatory molecules by bmDC and ipDC. bmDC and ipDC were grown and harvested as described in section 2.3 and stained with the relevant isotype control and antibody of interest. Red line depicts expression of marker of interest, the blue line depicts the level of background staining using isotype-matched control monoclonal antibodies.

3.2.3 Function and expression of toll-like receptors (TLR) by ipDC

Pathogen associated molecular patterns (PAMPs) enable DC to differentiate between antigen derived from pathogens and those released from dead and dying cells. The recognition of these PAMPS is mediated through toll like receptors (TLR) which are expressed on both the cell surface of DC and intracellular compartments. Indeed, activation of TLR has a multitude of effects on DC including cell migration, maturation and up-regulation of co-stimulatory

molecules (Akira *et al.* 2003). In the mouse, there are broadly 9 types of TLR designated TLR1 through to 9. Table 3.1 shows each TLR and its associated synthetic ligand.

TLR	PAMP
1	PAM3CSK
2	HKLM
3	Poly I:C HMW/LMW
4	LPS
5	FLA-ST
6	FSL1
7	ssRNA40
9	ODN1826

Table 3.1. Recognition of PAMPS by TLR in the mouse.

TLR have been shown to play an important role in the induction of tolerance by DC. Indeed, Albrecht *et al.* (2008) showed that activation of TLR2 and TLR4 lead to the development of tolerogenic-like DC. Given the importance of TLR activation in DC, we sought to investigate the effects of various TLR agonists on ipDC. ipDC were co-cultured with various TLR agonists for a period of 24 hours and their responses to agonists assessed as a function of IL-6 secretion (Dearman *et al.* 2009). As shown in Figure 3.9, ipDC responded to a variety of synthetic TLR ligands in a comparable fashion to their bmDC counterparts, although no detectable responses were found upon ligation of TLR7 in either bmDC or ipDC, and a lower level of TLR3 activation in response to POLYI:C LMW was observed in ipDC compared to bmDC.

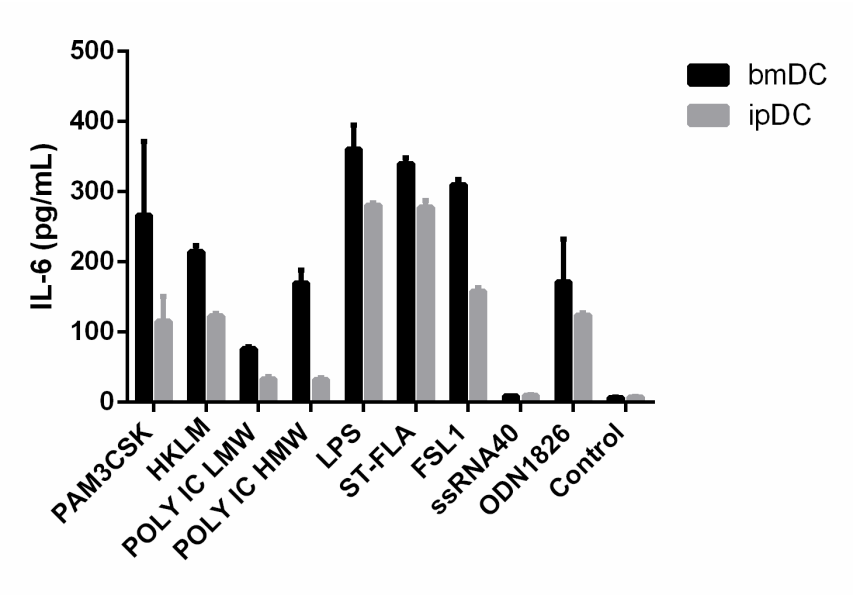


Figure 3.9. Secretion of IL-6 by bmDC and ipDC upon TLR ligation. $3x10^5$ DC were cultured for 24 hours in the presence of various TLR ligands. Supernatants were then harvested and the amount of IL-6 secreted quantified using ELISA. A table showing TLR and corresponding ligand is shown above in Table 3.1.

The expression of TLR was determined by staining ipDC with a variety of mAb. Given the conserved nature of TLR between species, the quality of the available mAbs is limited; therefore we sought to detect the expression of TLR1, TLR2 and TLR4.

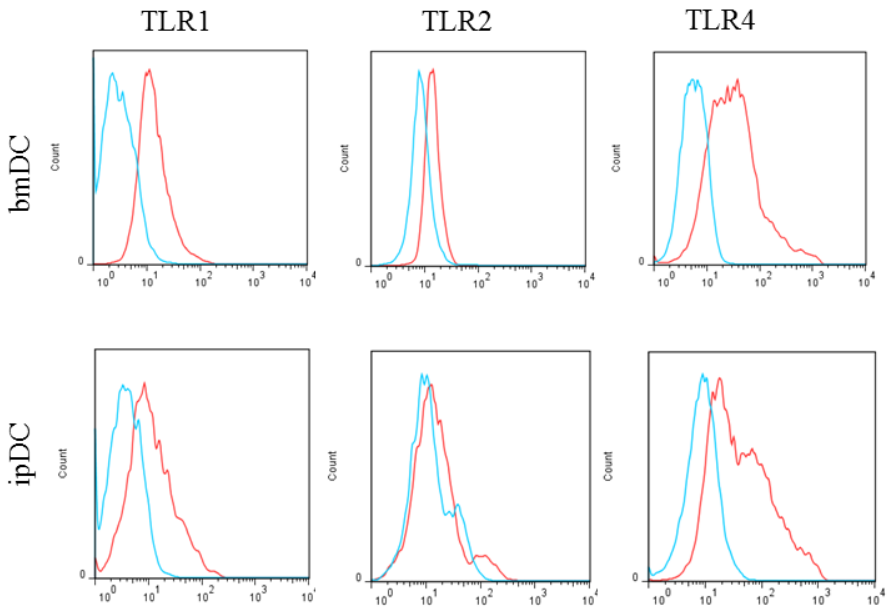


Figure 3.10. The expression of TLR by bmDC and ipDC. bmDC and ipDC were assessed for TLR expression by flow cytometry. Red line depicts expression of marker of interest, the blue line depicts the level of background staining using isotype-matched control monoclonal antibodies.

As shown above in Figure 3.10, ipDC were shown to express similar levels of TLR1 and TLR4 which suggests that, coupled with the data from Figure 3.9, ipDC have the ability to respond to ligation of these receptors. Low levels of TLR2 were expressed by both bmDC and ipDC, however ligation of TLR2 still resulted in IL-6 secretion.

3.2.4. Expression of iNOS by ipDC and secretion of nitric oxide

Nitric oxide (NO) is produced via endogenous L-arginine metabolism and has a wide range of effects *in vivo* including vasodilation, signalling in the central nervous system and immunomodulatory effects. NO plays an important role in intracellular signalling within DC and plays an important role in immune regulation. It has previously been shown that bmDC have the ability to express inducible nitric oxide synthase (iNOS) which is the enzyme responsible for the conversion of L-arginine to NO via the reaction below (Lu *et al.* 1996):

L-arginine + 3/2 NADPH + H^+ + $2O_2$ → Citrulline + NO + 3/2 $NADP^+$

Indeed, the authors showed that bmDC, unlike many populations of DC purified from lymphoid tissues expressed iNOS and secreted NO upon interaction with allogeneic T cells or stimulation with IFNγ or LPS. We sought to see whether ipDC also expressed iNOS and had the ability to secrete NO in both an immature state and upon stimulation with LPS and IFNγ. ipDC were, therefore, plated at 10^5 cells/well and incubated with 1μg/mL of LPS. Supernatants were harvested and NO levels assessed by means of the Griess reaction.

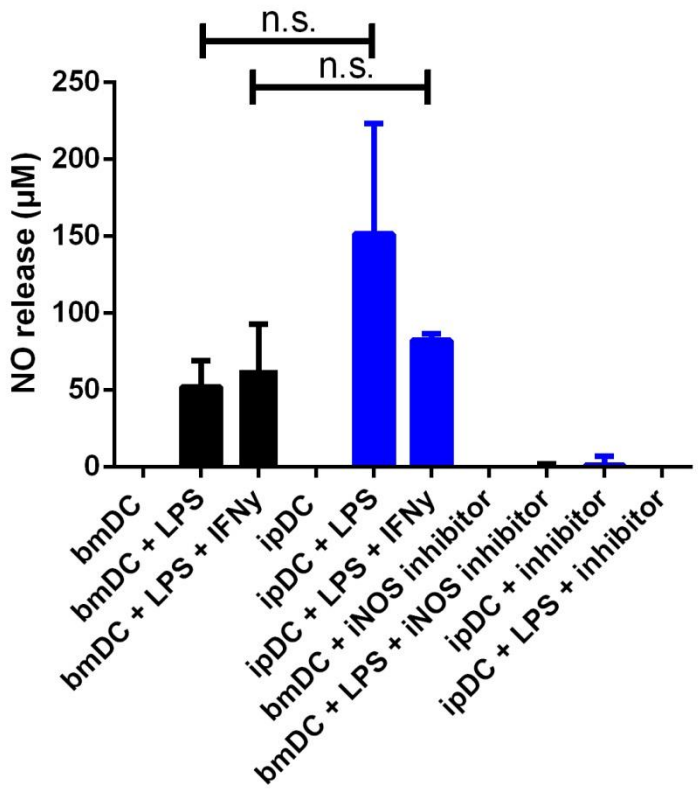


Figure 3.11. Release of NO by bmDC and ipDC. 10^5 DC were cultured overnight after being treated with either 1μg/mL LPS or 1μg/mL LPS and 500U/mL of IFNγ. Inhibition of NO secretion was achieved following the addition of the iNOS inhibitor L-N^G-monomethyl arginine citrate (NMMA). The concentration of NO was quantified by using the Griess reaction as detailed in *Methods.* (P value bmDC+LPS vs. ipDC + LPS = 0.30, P value bmDC + LPS + IFNγ vs. ipDC + LPS + IFNγ = 0.58)

Our results show that ipDC consistently secrete more NO that bmDC, however the results were not of statistical significance. Interestingly, stimulation of bmDC with both LPS and IFNγ resulted in no statistically significant difference compared to treatment with LPS alone. However, stimulation of ipDC with LPS produced significantly higher concentrations of NO than treatment with both LPS and IFNγ suggesting that IFNγ may be acting to suppress NO release in ipDC.

3.2.5 Secretion of IL-10 by ipDC

IL-10 is a cytokine that has been shown to play an important role in DC with a tolerogenic phenotype. Indeed, bmDC derived from BALB/c mice have been shown to express lower levels of co-stimulatory molecules and MHC class II following treatment with IL-10 and TGF-β (Lan *et al.* 2006). Furthermore, these DC were able to secrete IL-10 themselves, secrete lower levels of IL-12p70 and have a superior ability to expand $CD4^+CD25^+Foxp3^+$ T_{reg} cells compared to their untreated counterparts.

To examine whether ipDC may have a similar capacity to adopt a tolerogenic phenotype we sought to determine whether ipDC had the ability to endogenously secrete IL-10.

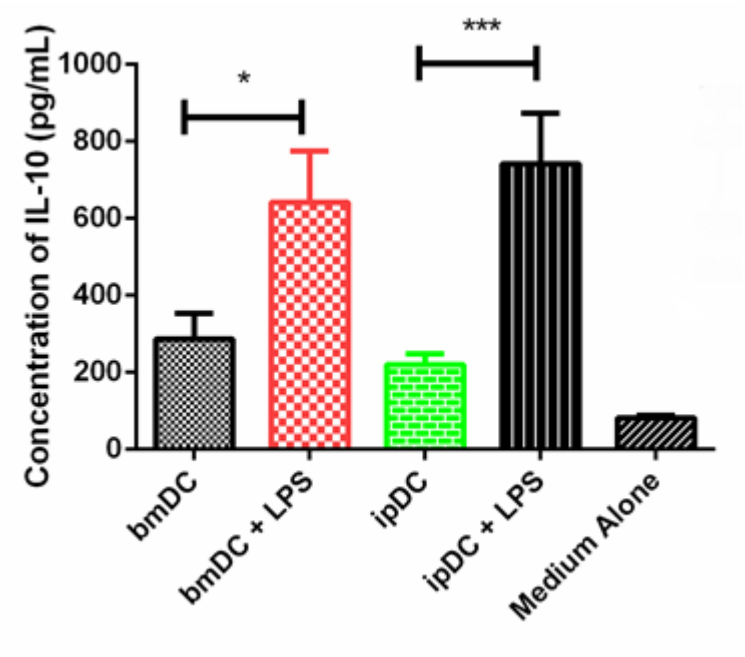


Figure 3.12. Secretion of IL-10 by bmDC and ipDC. $3x10^5$ DC were cultured overnight with or without the presence of 1µg/mL LPS. Supernatant was collected after 24 hours and the amount of IL-10 secreted quantified using ELISA. * depicts $p \leq 0.1$ *** depicts $p = \leq 0.001$.

As shown in Figure 3.12, both bmDC and ipDC had the ability to secrete IL-10 in an immature state. Upon exposure to LPS both bmDC and ipDC significantly up-regulated IL-10 secretion compared to secretion in an immature state. Interestingly, ipDC seemed to secrete slightly more IL-10 than bmDC, although this was not of statistical significance. Nevertheless, similar findings have been reported by others in the laboratory using iPS cell lines derived from other strains of mice (Sachamitr, Personal Communication).

3.3. Functional phenotype of ipDC

Given the low expression of certain DC markers such as CD11c and MHC class II, ipDC may represent more 'primitive' DC and may be more allied to DC of fetal origin and may therefore have different functional characteristics to other conventional DC populations. In order to ascertain whether ipDC had the ability to act as fully functioning DC, we sought to characterise their functional phenotype fully, both *in vitro* and *in vivo.*

3.3.1 Processing and presentation of HEL to the 2G7.1 T cell hybridoma

Processing and presentation of antigen to naive T cells is arguably the defining functional characteristic of a DC. Following the phagocytosis of pathogens, infected cells or apopotic cells, DC process cellular material through internal degradation pathways and present the peptide epitopes of processed antigens on either MHC class I or MHC class II molecules on the cell surface. *In vivo,* following processing of antigen, DC migrate to primary lymphoid tissues and present antigen to naive T cells resulting in clonal expansion of antigen specific T cells.

In order to assess the ability of ipDC to process and present antigen, the 2G7.1 T cell hybridoma was used. This hybridoma has been extensively characterised (Adorini *et al.* 1992), however in brief; the 2G7.1 T cell hybridoma recognises the hen egg lysozyme (HEL) peptide 1-18 in the context of H-2E^{k}. Upon successful recognition of the peptide-MHC complex, the T cell hybridoma will secrete IL-2 which can be used as a read-out of T cell activation and, by extension, successful presentation of HEL. Therefore, to determine whether the ipDC we had differentiated had the capacity to process and present soluble protein antigen, they were incubated with either the whole HEL protein, or the H-2E^{k} restricted HEL1-18 peptide for 24 hours following fixation. The DC were then co-cultured with the 2G7.1 hybridoma for 24 hours and the supernatant assessed for IL-2 content by ELISA.

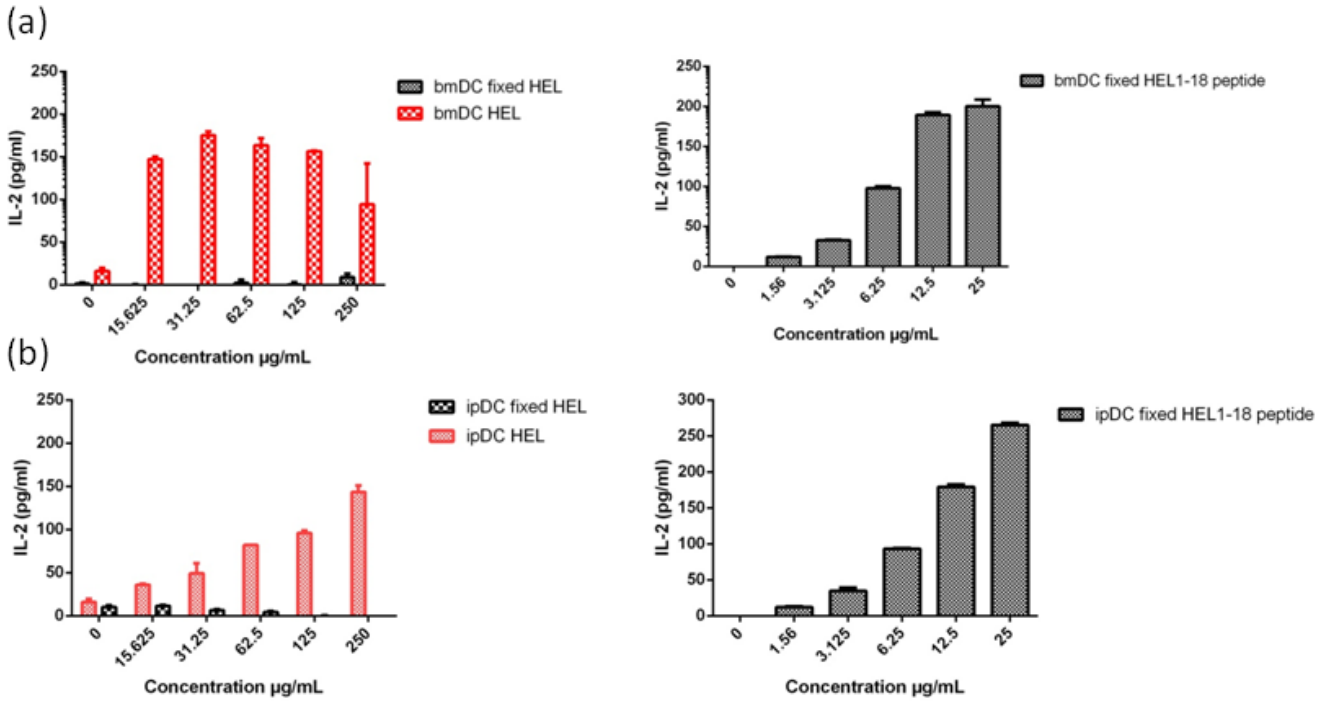


Figure 3.13 Presentation of HEL1-18 on H-2E^k to H-2E^k-restricted T cell hybridoma 2G7.1. Hen egg lysozyme (HEL) was cultured with either bmDC (a) or ipDC (b) or, following fixation, the peptide HEL1-18. They were then cultured with the T cell hybridoma 2G7.1 which recognises HEL1-18 in the context of H-2E^k as described in section 2.5. Activation of 2G7.1 was assessed by IL-2 secretion and quantified using an ELISA.

As shown in Figure 3.13, both bmDC and ipDC had the capacity to endocytose whole HEL, process and present the HEL1-18 peptide on H-2E^k. However when ipDC or bmDC were fixed with PFA before culture with HEL, neither population had the ability to endocytose HEL and process and present the HEL1-18 peptide. Likewise, when fixed with PFA beforehand, both bmDC and ipDC expressed sufficient levels of H-2E^k for the HEL1-18 peptide to bind and stimulate the T cell hybridoma directly. These results suggest that ipDC have a similar capacity to process and present antigen as bmDC.

3.3.2 Lack of capacity for cross-presentation of antigen

Certain populations of DC have the ability to cross-present antigens on MHC class I, as detailed in *Introduction.* Briefly, this involves the uptake of exogenous antigen and presentation on MHC class I to $CD8^+$ T cells. Cross presenting DC play an important role in tolerance induction, as well as playing a pivotal role in mounting immune responses against tumour associated antigens. In the mouse, the two most common markers of cross-presenting DC are CD8α and DEC205 (CD205). In order to predict whether differentiated ipDC may have the ability to cross-present antigen, the expression of these two markers was determined by flow cytometry.

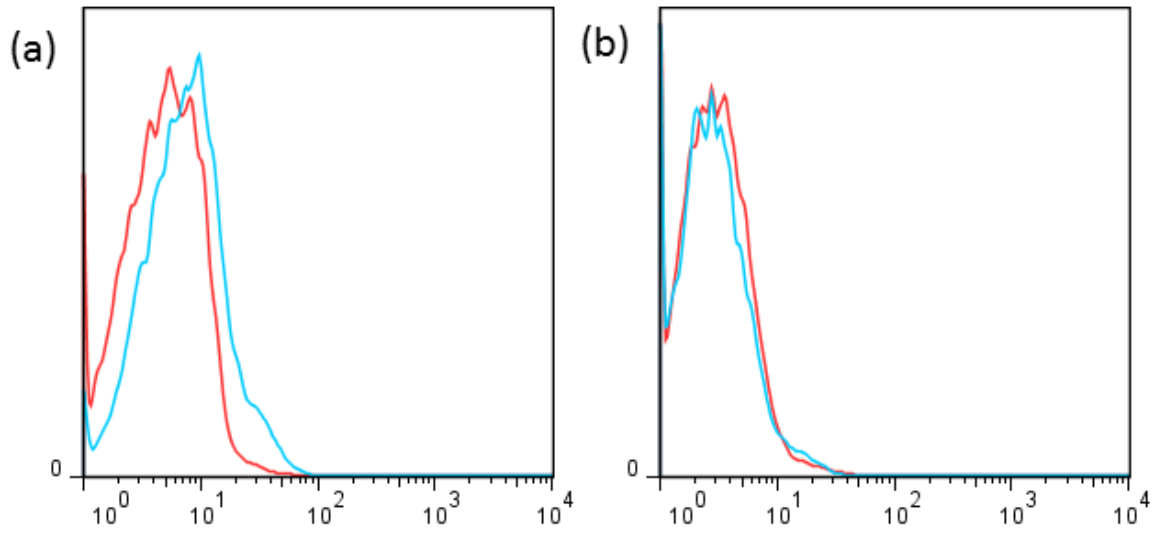
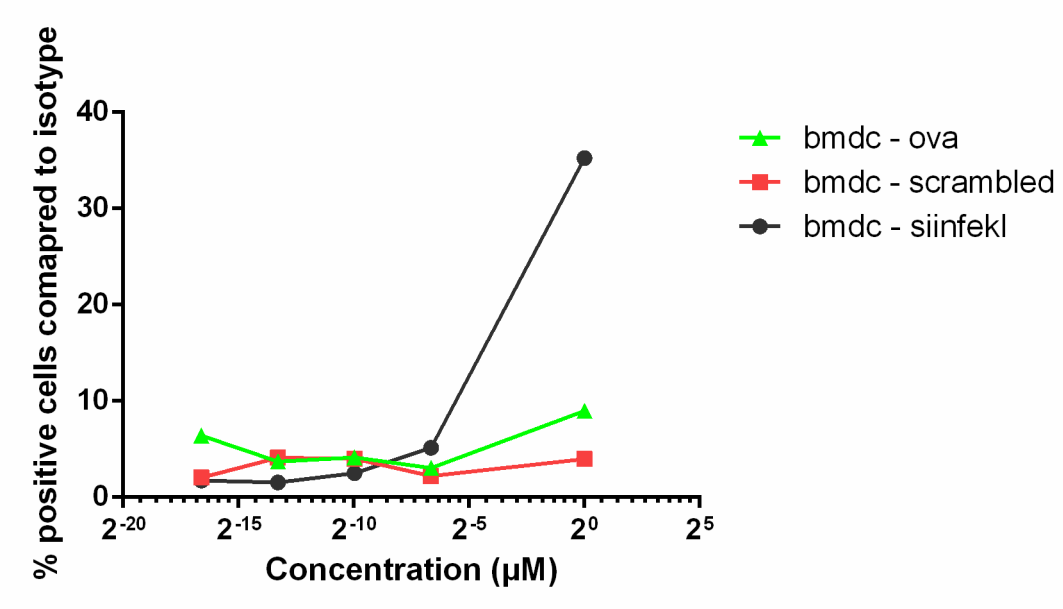


Figure 3.14 Lack of expression of CD8α and CD205 by ipDC. ipDC were analysed for the expression of (a) CD8α and (b) CD205. Red line depicts expression of marker of interest, the blue line depicts the matched isotype control.

As shown in Figure 3.14, ipDC did not express either CD8α or CD205 suggesting that they most likely do not have the ability to cross-present antigen. However, in order to determine definitively whether they have the functional capacity to do so, a novel iPS cell line was derived from the mouse strain CB/K as described in *Methods*. The CB/K strain of mouse expresses a transgene for the MHC class I molecule $H\text{-}2K^b$. From this iPS cell line, ipDC

were differentiated as described earlier. ipDC or bmDC from CB/K mice were cultured with either whole OVA protein or the MHC class I H-2K^b restricted peptide SIINFEKL. DC with the ability to cross-present will uptake OVA and present the SIINFEKL peptide on MHC class I. The presentation of this peptide can then be determined by using a monoclonal antibody specific for the SIINFEKL peptide bound to H-2K^b and quantified using flow cytometry. As shown in Figure 3.14, neither bmDC nor ipDC had the capacity to uptake whole OVA and present SIINFEKL on MHC class I, although when fixed bmDC and ipDC had the capability to present the SIINFEKL peptide on H-2K^b.



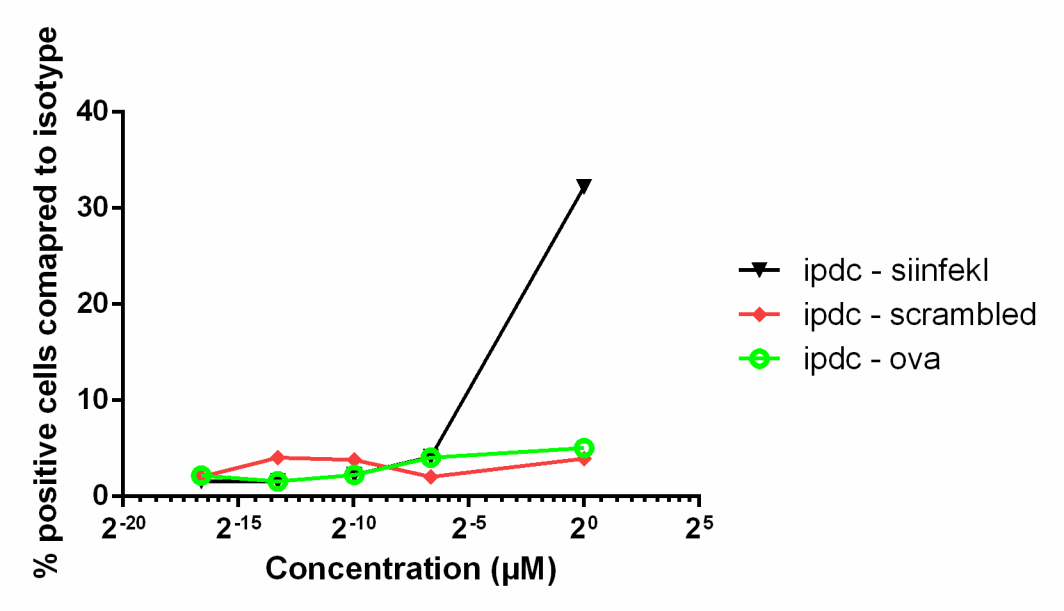


Figure 3.15. Lack of cross-presentation by ipDC. 10^5 DC were cultured overnight with whole OVA, SIINFEKL or the scrambled peptide FILKSINE. The DC were then stained with a mAb specific for SIINFEKL bound to the MHC class I molecule H-$2K^b$. The amount of SIINFEKL presented was then quantified using flow cytometry and expressed as the percentage of positive cells compared to cells stained with an appropriate matched isotype control antibody.

3.3.3 Immunostimulation by ipDC: induction of primary T cell responses in the MLR

The mixed leukocyte reaction (MLR) is an *in vitro* method for assessing the ability of DC to stimulate naïve allogeneic T cells. DC are inactivated with mitomycin C to prevent their proliferation. DC act as the stimulator population and allogeneic T cells act as responder cells by rapidly proliferating. When co-cultured, the amount of T cell proliferation can be quantified by measuring ^{3}H-thymidine incorporation. To assess the ability of ipDC to present antigen to T cells on MHC class II and stimulate T cells via expression of co-stimulatory molecules, ipDC were co-cultured with C57BL/6 T cells. As shown in Figure 3.16, ipDC had the ability to stimulate allogeneic T cells, although T cells exhibited approximately 50% less proliferation than compared with LPS treated bmDC.

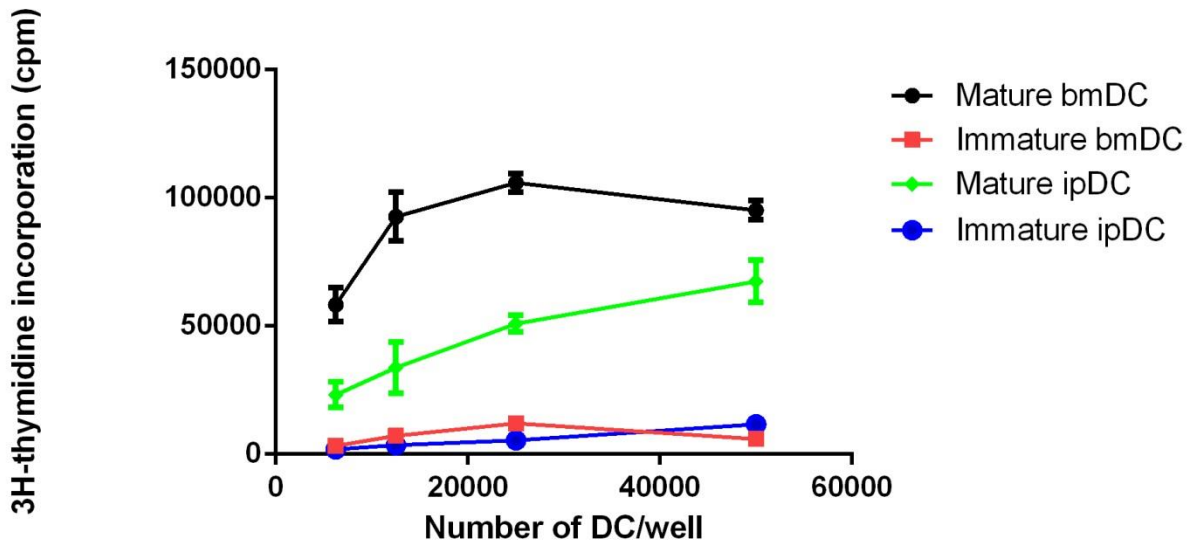


Figure 3.16. Mixed leukocyte reaction with bmDC and ipDC. DC were titrated into cultures of 10^5 C57BL/6 T cells for 72 hours. For the final 18 hours of culture, cultures were pulsed with 0.5µCi/per well of ^{3}H-thymidine to assess proliferation. Data shown is representative of three experiments.

3.3.4 Stimulation of T cells in an antigen specific manner

Given our intention of examining the ability for male ipDC to induce tolerance to the male antigen in the A1.RAG1$^{-/-}$ system *in vivo*, we sought to examine whether ipDC had the ability to stimulate T cells in an antigen specific manner. We therefore cultured ipDC differentiated from a male iPS cell line (ipDC-1) or a control female iPS cell line (ipDC-14) with female A1.RAG1$^{-/-}$ T cells. A1.RAG1$^{-/-}$ T cells possess a T cell receptor (TCR) specific for the male antigen Dby presented in the context of H-2E^k. Proliferation of T cells was measured by ^{3}H-thymidine incorporation. As shown in Figure 3.17 below A1.RAG1$^{-/-}$ T cells only proliferated upon co-culture with male ipDC-1 and did not proliferate when cultured with female ipDC-14 therefore showing that ipDC-1 endogenously express the male antigen Dby and have the ability to present it in the context of H-2E^k in an immunogenic fashion.

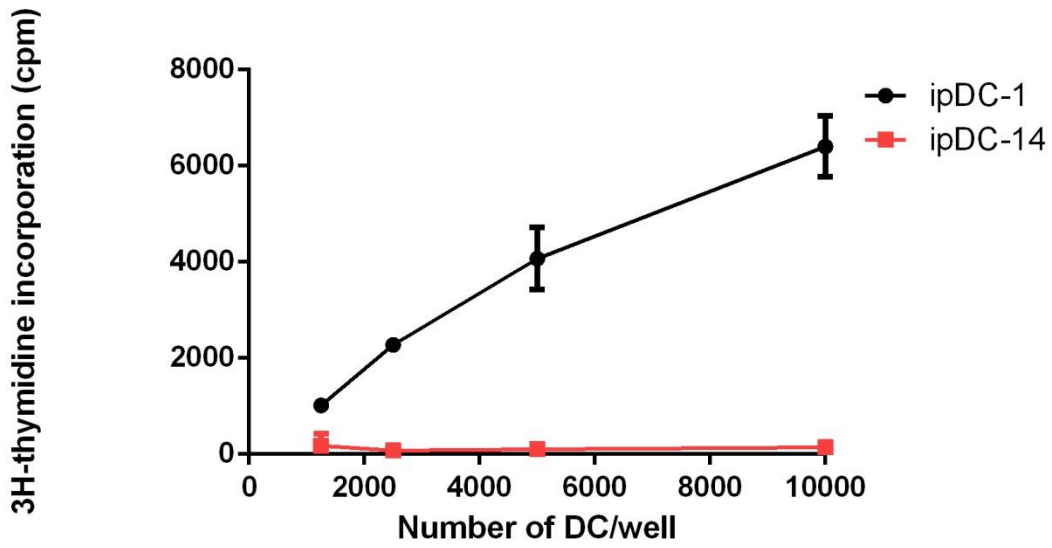


Figure 3.17. Culture of ♂-ipDC-1 and ♀-ipDC-14 with ♀-A1.RAG1$^{-/-}$ T cells. ipDC-1 and ipDC-14 were stimulated with 1µg/mL of LPS, harvested and cultured with 10^5 A1.RAG1$^{-/-}$ T cells for 72 hours. For the last 18 hours of culture, cultures were pulsed with 0.5µCi of ^{3}H-thymidine to assess proliferation.

These results therefore suggest that ipDC have the ability to present endogenous antigen to naïve T cells and stimulate their activation and clonal expansion. Given that ipDC have similar functional characteristics to bmDC, we next sought to examine whether ipDC had the ability to migrate *in vitro* and *in vivo.*

3.3.5 Migration of ipDC *in vitro* in response to chemokine stimulation

DC have the ability to respond to chemokines such as CCL19, CCL9 and CCL7 by expressing the complementary receptor. In order to determine whether DC could migrate *in vitro,* we used the xCELLigence migration assay, in collaboration with David Greave's laboratory, which measures the Cell Index of a cell upon treatment with chemokines. Cell Index (CI) is a dimensionless parameter and measures electrical impedance. When cells are not present or non adherent, CI is zero. When cells adhere to the electrodes, the CI value increases. Changes in cell morphology, adhesion or viability will all cause change in CI.

In order to examine whether ipDC could migrate *in vitro* we incubated either ipDC or control bmDC in the xCELLigence Cell Migration Assay and exposed the cells to a range of concentrations of Chemokine ligand 19 (CCL19) for 6 hours. The migration of cells was measured by their ability to pass through pores of the central membrane in the chamber and adhere to the underside of the insert. Migration was measured as readout of CI. In addition to measuring migration, the expression of chemokine receptor 7 (CCR7) was assessed by flow cytometry on both bmDC and ipDC.

As shown in Figure 3.18, both ipDC and bmDC were able to migrate in response to CCL19 in a dose-dependent manner. However, the way in which ipDC migrated was significantly different from bmDC. Despite expressing similarly low levels of CCR7, as shown in Figure 3.18 (c), ipDC had a slower, continued migration over the period of 6 hours. Conversely, bmDC migrated rapidly within 30 min upon addition of the chemokine and then but failed to maintain their adherence to the underside of the well. These findings correlated with our empirical observations that ipDC are significantly more adherent in culture than their bmDC counterparts.

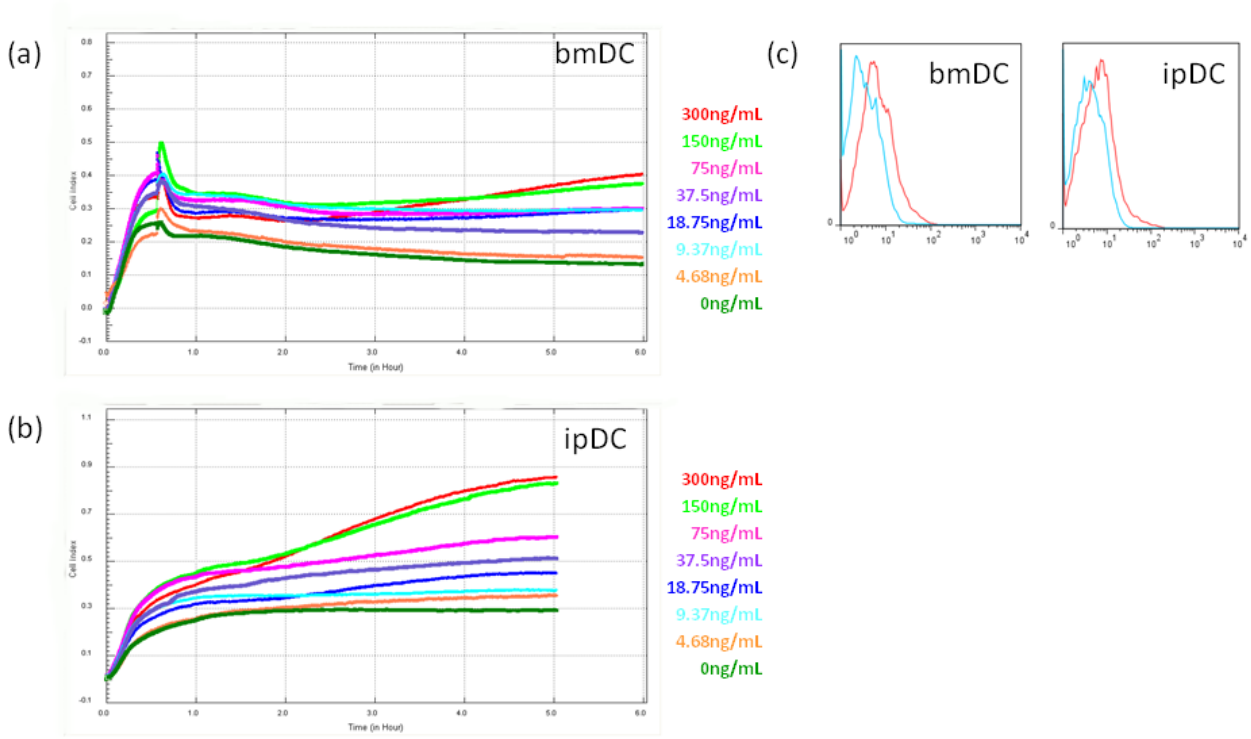


Figure 3.18 Response of bmDC and ipDC to CCL19 and expression of CCR7. 10^5 bmDC (a) and ipDC (b) were cultured in a 12 well xCELLigence plate for 6 hours in the presence of a range of concentrations of CCL19. Migration was assessed by measuring Cell Index. The expression of CCR7 was assessed on bmDC and ipDC using Flow Cytometry (c). *Red line depicts expression of marker of interest, the blue line depicts the matched isotype control.*

3.3.6 Changes in ipDC cell motility by chemokine stimulation

In order to assess the ability for ipDC to change their motility in response to chemokine stimulation, we again used the xCELLigence System. Immature DC were seeded in 96 well plates which were coated with gold electrodes. The principle of the system revolves around the concept that the more cells that adhere to the electrodes, the larger the electrical impedance. The strength of the interaction between cell and electrode is determined by various factors such as cell morphology, viability and degree of adherence. The measure of impedance is, therefore, displayed as Cell Index, a dimensionless parameter. We therefore sought to assess the change in cell motility in response to chemokine stimulation by assessing Cell Index. bmDC and ipDC were cultured with either the chemokine Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), JE, Chemokine Ligand 3

(known as MIP-1α), Complement Component 5a (C5a) or vehicle control for 6 hours. Any response induced by the chemokine was measured as a change in CI. Figure 3.19 represents the change in CI induced on bmDC (a) and ipDC (b) in response to chemokine addition.

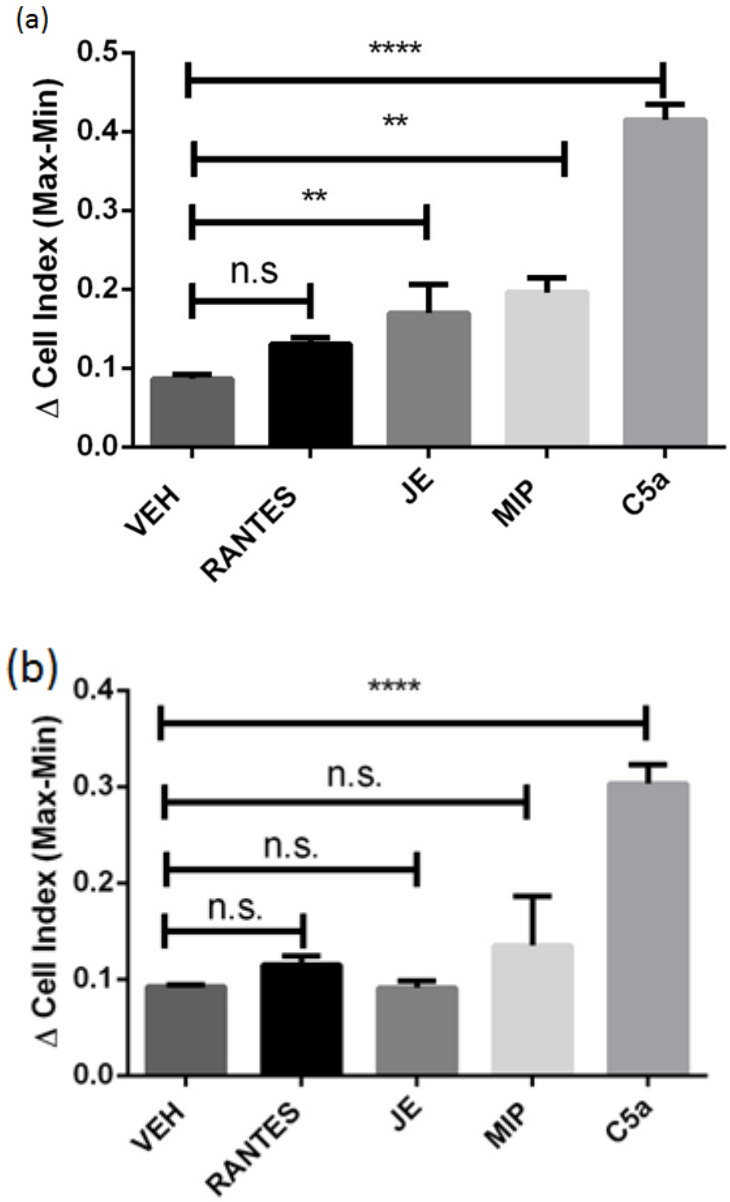


Figure 3.19 Culture of bmDC and ipDC with RANTES, MIP1α and C5a. $5x10^4$ bmDC or ipDC were added to wells of a 96 well plate and placed in the xCELLigence machine to equilibrate for 3 hours. The ligand was then administered and the response measured as CI. RANTES, JE, MIP (MIP-1α) and C5a were used at concentration of 10nM. Figure 3.18 shows the change in maximum versus minimum response level for each chemokine and vehicle control (VEH) in bmDC (a) and ipDC (b). Asterisks indicate level of significance with "*" means $p< 0.05$, "**" means $p< 0.01$, "***" means $p< 0.001$, and "****" means $p<0.0001$. N.S represents no statistical significance.

As shown above, bmDC responded to JE and MIP-1α with the greatest response elicited to C5a. ipDC did not significantly respond to either RANTES, JE or MIP-1α, but ipDC responded significantly to C5a. This may suggest differences in expression of chemokine receptors the two populations which could influence their behaviour *in vivo.* To assess this possibility, we performed *in vivo* experiments in which we examined the ability for ipDC to migrate when injected into the tail vein of mice and activate naïve T cells.

3.3.7 Administration of ipDC to female A1.RAG1$^{-/-}$ mice: assessment of *in vivo* migration and activation

DC have the ability to activate resting naïve T cells and induce proliferation in an antigen specific manner. As shown in section 3.3.4, we were able to demonstrate that ipDC have the capacity to migrate *in vitro in* response to the chemokine CCL19 and they had previously been reported in the literature to respond to the chemokines as well as MIP-2, MIP-5 and RANTES (Foti *et al.* 1999). In order to assess whether ipDC had the capacity to migrate *in vivo* and induce T cell activation, 5x10^6 ipDC were injected into female A1.RAG1$^{-/-}$ mice via the tail vein. At 18 hours post-injection, the spleens of mice were harvested and the level of CD69 expression was determined on CD4$^+$ T cells. CD69 is has been shown to be a marker of early T cell activation (Avgustin *et al.* 2005) allowing activated T cells to be differentiated from resting T cells.

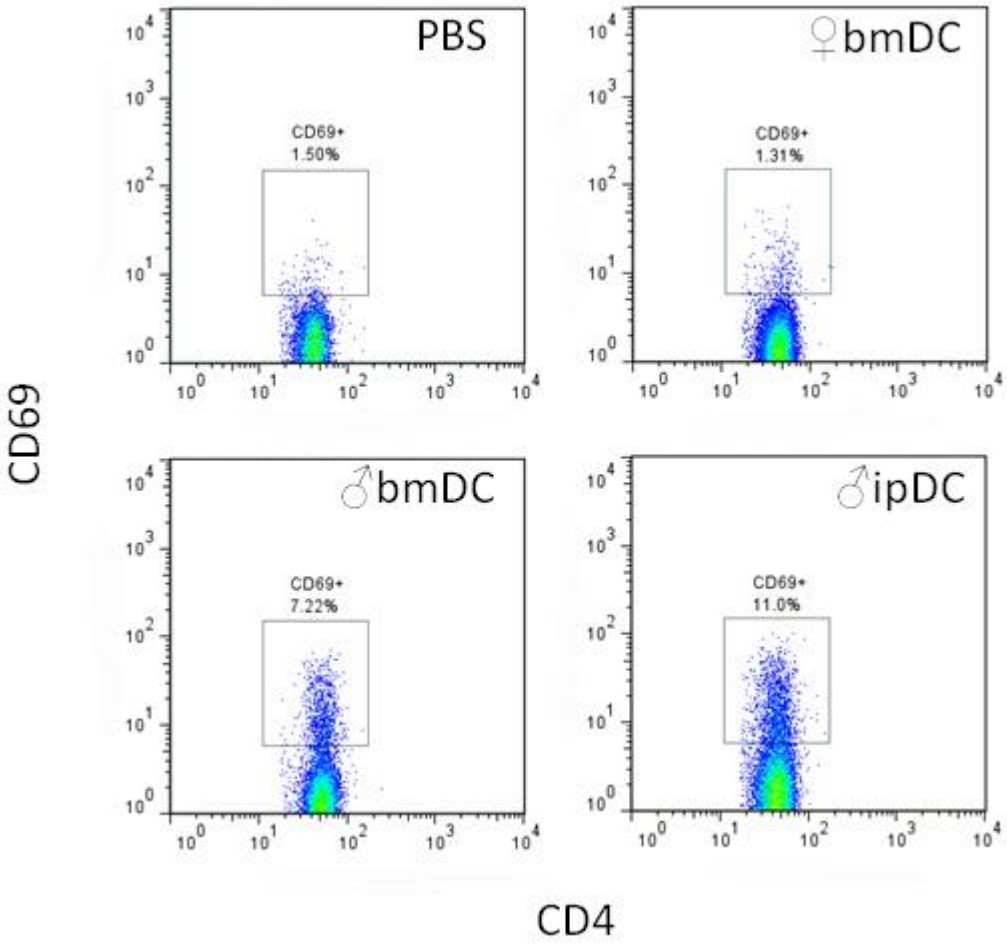


Figure 3.20. Induction of CD69 expression among female A1.RAG1$^{-/-}$ T cells by ipDC. 5x10^{6} DC were injected into the tail vein of female A1.RAG1$^{-/-}$ mice and the spleens from injected mice were harvested 18 hours post injection. Plots are gated on CD4^{+}/7-AAD^{-} cells and the relative amount of CD69 expression shown as % of the total. Plots are representative of n=3.

As shown in Figure 3.20, control male bmDC induced a population of activated T cells compared to female bmDC which did not induce activation of T cells. Likewise, ipDC had the ability to induce a profound T cell response in an antigen specific manner compared to female bmDC or PBS. This suggests that, even though the ipDC may exhibit lower levels of MHC class II in comparison to bmDC, they still have the ability to process and present antigen and productively engage with T cells. It also suggests that they express sufficient levels of the Dby antigen presented in the context of H-2E^{k}, for its recognition by A1.RAG1$^{-/-}$

T cells, and the there is no impediment to their migration *in vivo* to the secondary lymphoid organs.

3.3.8 Modulation of ipDC function by pharmacological agents

Previous work from our laboratory has shown that it is possible to modulate the function of bmDC with various agents such as 1α,25 dihydroxyvitamin D_3 (VD_3), IL-10 and TGF-β in order to drive the phenotype of the DC towards a pro-tolerogenic state. Indeed, when these pharmacologically treated DC were administered to female A1.RAG1$^{-/-}$ mice, they were able to induce tolerance to male skin grafts indefinitely (Yates *et al.* 2007). In order to determine whether we could drive ipDC towards a similar state which may allow for induction of tolerance to initially the male antigen in female A1.RAG1$^{-/-}$ mice, or more broadly towards iPS-cell developmental antigens, we explored the effects of various pharmacological agents on ipDC.

We decided to explore the effects of the mTOR inhibitor rapamycin on ipDC. Rapamycin is an immunosuppressive drug that has historically been used in the prevention of immunological rejection of allografts. In this context, rapamycin functions by preventing T cell activation by inhibiting their response to IL-2 (McAlister *et al.* 2002). Recently however, rapamycin has been shown to drive dendritic cells towards a tolerogenic state. Fischer *et al.* (2009) showed that treating murine bmDC with rapamycin generated immature DC with lower levels of MHC class II, and significantly lower levels of co-stimulatory molecules such as CD86 and which consequently exhibited reduced immunostimulatory capacity reduced among alloreactive T cells. We therefore investigated whether pre-treatment of ipDC with rapamycin followed by stimulation with LPS affected their to stimulate allogeneic T cells.

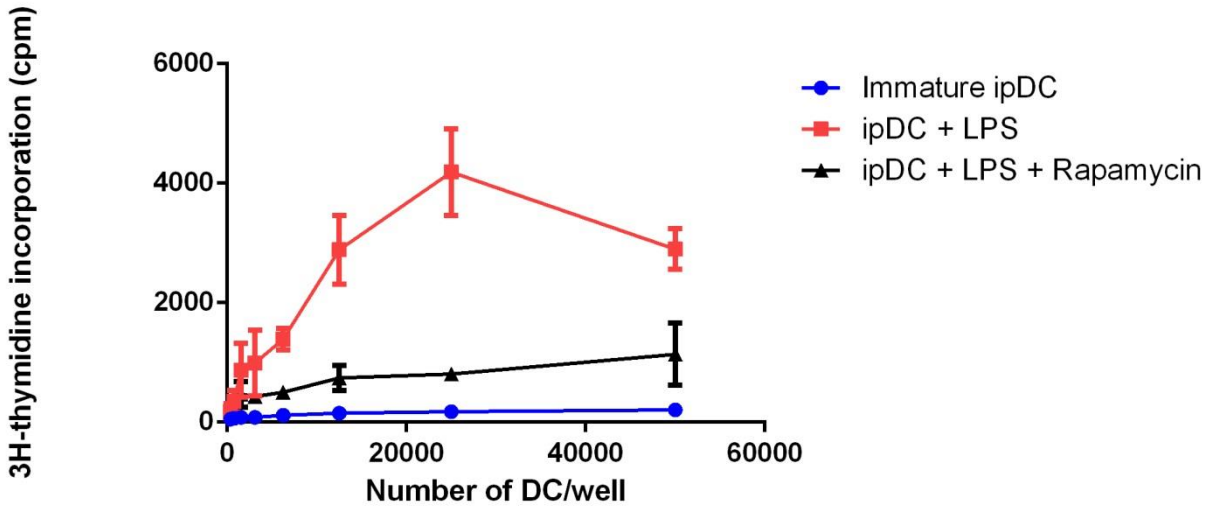


Figure 3.21. Ability of rapamycin treated ipDC to induce hyporesponsiveness in allogeneic T cells. ipDC were treated with 50ng/mL of rapamycin for 24 hours and then treated with 1µg/mL of LPS. ipDC were co-cultured with 10^5 C57BL/6 T cells for 72 hours and were pulsed with ^{3}H-thymidine for the final 18 hours of culture. Proliferation was assessed by ^{3}H-thymidine uptake by T cells.

As shown in Figure 3.21, treating ipDC with rapamycin for 24 hours, followed by exposure to LPS and subsequent co-culture with alloreactive T cells from C57Bl/6 mice resulted in reduced activation of T cells. It is likely that the hyporesponsiveness observed arises from down-regulation of co-stimulatory molecules such as CD86 and CD80 and well as down-regulation of MHC class II. In addition, rapamycin has also been shown to enhance secretion of IL-10 in DC. Therefore, we next sought to establish whether rapamycin treatment affected the secretion of IL-10 in DC.

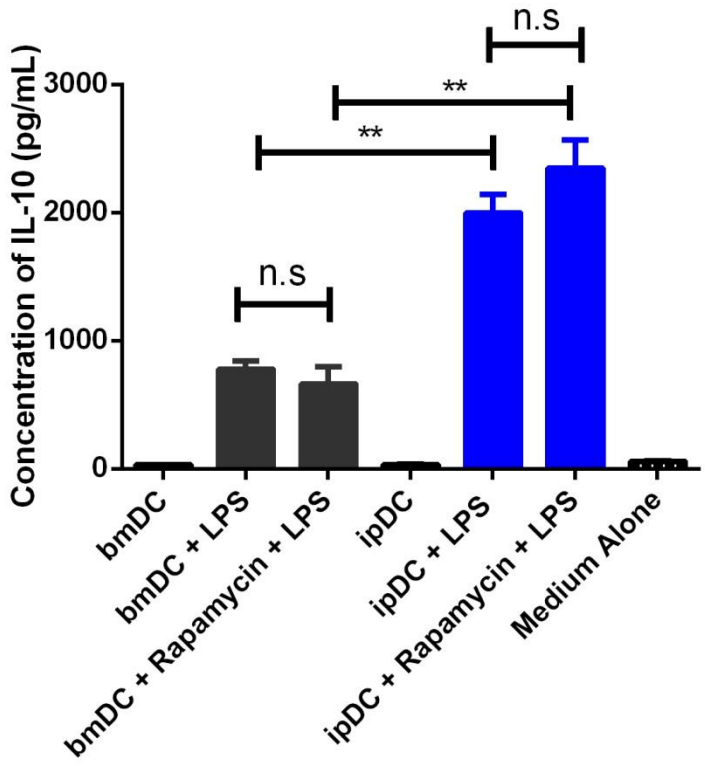


Figure 3.22. Secretion of IL-10 by bmDC and ipDC following treatment with rapamycin. $3x10^5$ DC were cultured for 24 hours either alone, with 1µg/mL of LPS or with concurrent treatment with 50ng/mL rapamycin and 1µg/mL of LPS. The supernatants were then harvested and analysed for IL-10 secretion by ELISA. Asterisks indicate level of significance with "*" means $p< 0.05$, "**" means $p< 0.01$, "***" means $p< 0.001$, and "****" means $p<0.0001$. N.S represents no statistical significance.

bmDC and ipDC were cultured with rapamycin and treated concurrently with LPS overnight. Supernatants were then assessed for IL-10 content. Rapamycin treatment appeared to have no effect on IL-10 secretion by either bmDC or ipDC which suggests that the concentration of rapamycin used may have been sub-optimal. Alternatively, given the unprecedented levels of IL-10 production by ipDC, there may be little scope to increase secretion further with pharmacological agents. Despite these findings, there appeared to be a statistically significant difference in the amount of IL-10 secrete between ipDC and bmDC with ipDC with the latter secreting more IL-10 upon stimulation with LPS.

3.3.9 Induction of T_{reg} cells by ipDC

The existence of suppressor T cells was first suggested by Gershon and Kondo (1970) who showed that T cells could also dampen immune responses as well as elicit them. However it was not until 2003 that Hori *et al.* showed that the development of T_{reg} cells is dependent upon the transcription factor Foxp3. DC have the profound ability to induce $Foxp3^+$ T_{reg} cells which can effectively suppress immunity. Sela *et al.* (2011) described an *in vitro* assay for the induction of Foxp3 T_{reg} cells by DC by culturing spleen cells with $CD11c^{hi}$ splenic DC in the presence of TGF-β and retinoic acid both of which have been shown to enhance the formation of T_{reg} cells (Benson *et al.* 2007). We therefore sought to examine whether ipDC had the capacity to induce Foxp3 T_{reg} cells following co-culture with A1.$RAG1^{-/-}$ T cells in the presence of TGF-β and retinoic acid. A1.$RAG1^{-/-}$ T cells were used, rather than allogeneic T cells, due to the lack of naturally occurring T_{reg} cells in A1.$RAG1^{-/-}$ mice in common with other TCR transenic mice on a $RAG1^{-/-}$ background. Previous work from our laboratory has shown that both bmDC and esDC have the ability to induce $Foxp3^+$ T_{reg} cells *in vitro.* As shown in Figure 3.23 below ipDC have the capacity to induce $Foxp3^+$ T_{reg} cells in an antigen specific manner showing that 25.1% of T cells co-cultured with ipDC adopt a $CD4^+CD25^+Foxp3^+$ phenotype compared to 23.8% when cultured with male bmDC. However, despite these findings, female bmDC also appeared to induce $CD4^+CD25^+Foxp3^+$ (14.4%).

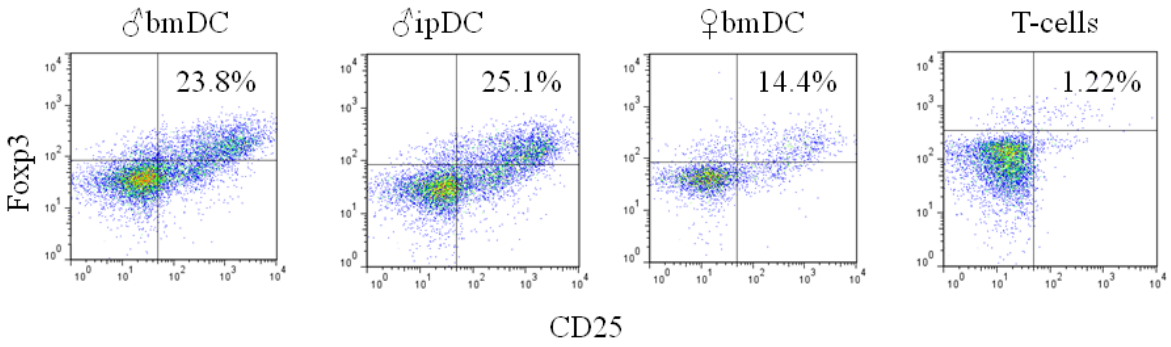


Figure 3.23. Induction of Foxp3 T_{reg} cells by both bmDC and ipDC in an antigen specific manner. $5x10^5$ A1.RAG1$^{-/-}$ T cells were co-cultured with 10^5 DC for 7 days. On day 7, T cells were harvested and stained with CD4, CD25 and Foxp3 as described in *Methods.* 7AAD$^-$ cells were gated and then sub-gated into CD4$^+$ population. Gating is relative to isotype control. Data is representative of three independent experiments.

3.4 Discussion

Over the past decade, the ability of DC to modulate T cell responses has been extensively investigated. Indeed, the prospect they might have the ability to induce a state of tolerance within the immune system towards allografts may hold promise for future clinical applications. Their ability to induce tolerance to mH antigens has already been shown in our laboratory in the A1.RAG1$^{-/-}$ mouse system (Yates *et al.* 2007).

In this chapter, we successfully derived iPS cell lines from mice with two different MHC haplotypes. The line primarily used in this thesis, iPS-1, was characterised extensively and shown to have 'typical' ES-cell-like morphology and possess a normal male karyotype. Furthermore, the cell line was shown to have silenced the transgenes used for reprogramming purposes. Silencing of these genes is of importance since continued expression may impair the differentiation of iPS cell lines towards certain cell types, as well as the possibility that up-regulated antigens from gene expression may act as mH-antigens. Indeed, the existence of

T cells against *Oct3/4* in the periphery has also been demonstrated, suggesting that both silencing of transgenes and silencing of pluripotency genes are critical upon differentiation.

Further to this, we also have shown that the iPS cell lines we generated have the ability to differentiate into DC. These DC were initially characterised with respect to their morphology and expression of markers commonly expressed on DC. ipDC showed similar morphology to bmDC and expression of DC markers.

Additionally, ipDC had the ability to express various TLR and respond to ligation of TLR by IL-6 secretion. Indeed, in the context of tolerance, TLR play an important role. Stimulation of mouse DC with TLR2 or TLR4 agonists has been shown to induce the secretion of TNF-α and, upon re-stimulation with TLR2/TLR4 ligands, the DC were shown to have hyporesponsiveness in terms of TNF-α secretion (Geisel *et al.* 2007). This suggests that priming DC with TLR agonists may render DC more tolerogenic, although the concentration at which the ligand is used dictates whether DC adopt a mature of tolerogenic phenotype. IL-6 has also been shown to modulate immature DC and block maturation *in vivo,* with IL-6 knockout mice exhibiting higher numbers of DC (Park *et al.* 2004). The criteria set out in *Introduction* by Morelli *et al.* for the minimal properties of a tolerogenic DC, suggest that resistance to maturation signals is essential. It would be interesting to examine whether ipDC show hyporesponsiveness to repeat TLR ligation by measuring IL-6 and TNF-α secretion.

As well as responding to TLR ligation, ipDC were shown to secrete NO in response to LPS. Additionally, ipDC were shown to secrete more NO than their bmDC counterparts. This could have several implications on their immunomodulatory properties. Interestingly, Zangi *et al.* (2012) have recently described the generation of immature DC with the ability to 'delete' T cells in an antigen specific manner. $CD4^+$ T cells were shown to be deleted via an MHC independent mechanism whilst $CD8^+$ T cells were shown to be deleted via an MHC

dependent mechanism. The mechanism of $CD8^+$ T cell deletion was shown to occur via the NO system, suggesting that NO plays a pivotal role in the immunomodulatory capacity of this population of DC, known as 'veto DC'. It would be interesting to examine whether ipDC have the capacity to selectively delete $CD8^+$ T cells in an antigen specific manner. Our preliminary data suggest that ipDC also express other markers characteristic of veto DC such as TREM-1 and granzyme A.

ipDC had the ability to process and present antigen to the 2G7.1 T cell hybridoma but were not able to cross-present exogenous antigen on MHC class I. An alternative method for assessing cross-presentation would be to use, as a readout, activation of T cells purified from the TCR transgenic OT-1 mice. These mice contain only $CD8^+$ MHC class I restricted T cells specific for OVA (Clarke *et al.* 2000). Incubation of ipDC and bmDC possessing the $H\text{-}2K^b$ haplotype with OVA will activate $CD8^+$ OT-1 T cells if they have the ability to process and cross-present the SIINFEKL peptide on $H\text{-}2K^b$.

Furthermore, ipDC were able to stimulate naive allogeneic T cells in a MLR showing that not only could ipDC present antigen to resting T cells, but they also possessed the necessary co-stimulatory molecules such as CD40, CD80 and CD86. Given that ipDC induce higher amount of proliferation in T cells upon maturation, this suggests that ipDC up-regulate co-stimulatory molecules upon maturation. The aspects of this maturation process are examined in the next chapter.

We were also able to show that ipDC secreted considerably more IL-10 than their bmDC counterparts upon stimulation with LPS. Given that IL-10 plays a pivotal role in modulating immune responses towards a tolerant state, these findings suggest that ipDC may be naturally pro-tolerogenic. Indeed, ipDC were shown to also induce T_{reg} cells upon co-culture with $A1.RAG1^{-/-}$ T cells suggesting that they have the ability to induce tolerance in an antigen

specific manner. Interestingly, the level of induction of T_{reg} cells, measured by the appearenace in culture of $Foxp3^{+}$ expressing cells, was higher than bmDC. Given the ability of ipDC to secrete higher concentrations of both NO and IL-10, this may make them more tolerogenic than bmDC when administered *in vivo.*

As we were able to show the *in vitro* induction of T_{reg} cells, it would be interesting to examine whether ipDC had the capacity to induce T_{reg} cells *in vivo.* This could be done by injecting ipDC into the tail vein of $A1.RAG1^{-/-}$ and examining whether T_{reg} cells were induced in lymphoid tissues such as the spleen. However, following preliminary experiments along these lines, further investigation into the *in vivo* properties was halted, as it was found that following IV injection of ipDC into mice, the incidence of pulmonary embolism was unacceptably high. ipDC often form clusters of cells which are resistant to dissociation, despite various efforts such as Trypsin treatment and cell straining, were not able to undergo dissociation. These properties are reflected in the findings from the xCelligence assay we performed. In the interest of the safety and welfare of animals, we therefore ceased further investigation of the *in vivo* modulatory properties of ipDC pending resolution of this issue.

In this chapter I have shown that it is possible to derive iPS cell lines from MEF and differentiate them into DC. Furthermore, the ipDC differentiated from these lines display typical DC phenotype including expression of conventional DC markers, the ability to process and present antigen and the ability to stimulate naïve resting T cells in a MLR. In addition, ipDC also have the capacity to retain their functional phenotype *in vivo* with the ability to stimulate naïve $A1.RAG1^{-/-}$ T cells. The ability for ipDC to act in a tolerogenic fashion was also demonstrated as a function of their ability to respond to pharmacological agents and capacity to induce T_{reg} cell *in vitro.*

As examined in the next chapter, the stable phenotype of ipDC will be explored in further detail, with significant variation in the expression of MHC class II noted on ipDC. The impact of these findings on their ability to function as professional antigen presenting cells is examined.

Chapter Four:

A comparison of dendritic cell derived from bone marrow and induced pluripotent stem cells

4.0 Introduction

As detailed in the previous chapter, we have shown that it is possible to differentiate a population of DC from iPS cells. In order to characterise the cells further, and to predict whether they may have the capacity to induce immunological tolerance, we initially compared their phenotype to bone marrow derived dendritic cells (bmDC). bmDC were first produced by Inaba *et al.* (1992) and are now widely used as a convenient source of immature DC capable of maturing in response to TLR agonists or inflammatory stimuli. We have shown that bmDC have the capacity to induce tolerance to the male antigen, Dby, in the A1.RAG1$^{-/-}$ mouse (Yates *et al.* 2007). Given that we wish to investigate whether ipDC possess similar capabilities, bmDC are, therefore, an ideal control population of cells to compare DC differentiated from iPS cells. In this chapter, we have, therefore, carefully analysed the phenotype of ipDC further, and examined similarities and differences between these two populations.

4.1 Results

As the population of cells we have generated are novel and largely uncharacterised, we examined whether the morphology and phenotype of the cells changed over time in culture.

4.1.1 Changes in morphology over time

ipDC were cultured as described previously in Chapter Three. The changes in the morphology were noted from the initial appearance of ipDC at day 7 of culture up until day 28. As shown in Figure 4.1, ipDC adopt a gradually more swollen, vacuolated appearance over time. This is most likely due to continuous uptake of apoptotic material in culture by ipDC. This change in size is likely to have contributed to the complications observed upon administering ipDC *in vivo* as described in Chapter Three. However, in order to acquire a

sufficient number of cells from for experimental purposes, cultures are usually harvested at day 21. We, therefore, encountered a tension between the quality and yield of ipDC.

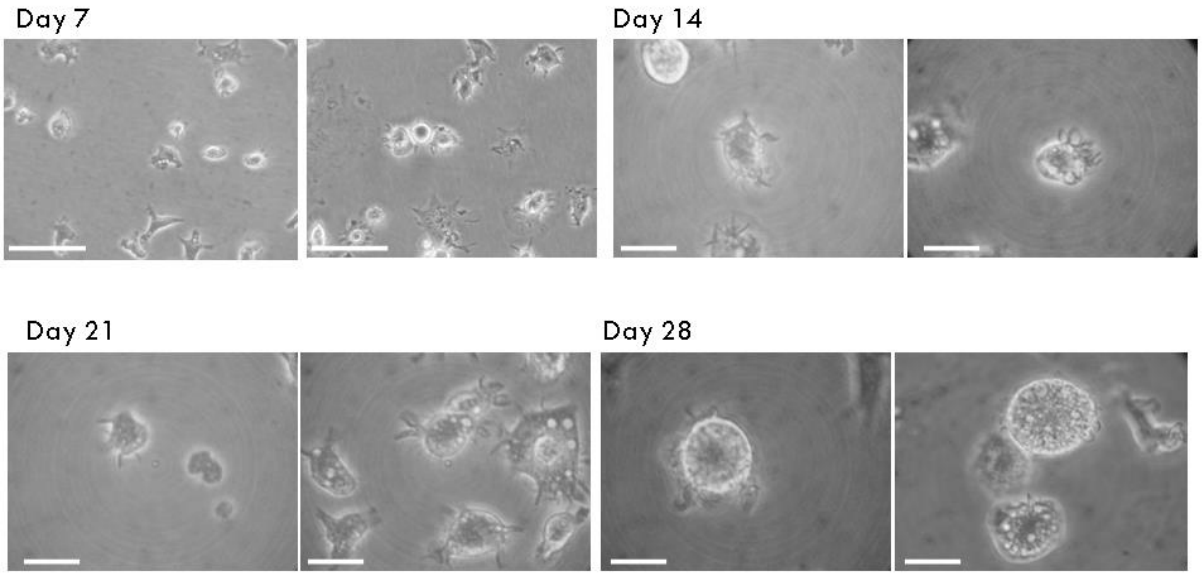


Figure 4.1. Changes in ipDC morphology over time. ipDC were cultured as described in Chapter Three. The same culture of ipDC was photographed over a 28 day period. Figure 4.1 therefore shows the appearance of ipDC at day 7 and their subsequent change in morphology over time. Scale bars represent (Day 7) 50µm (Day 14) 20µm (Day 21 and Day 28) 10µm

4.1.2 Response to LPS to induce maturation

As previously described in Chapter 3, ipDC show some ability to mature in response to LPS and, additionally, activate naïve T cells. However, unlike their bmDC counterparts, proportionally less ipDC adopt a mature phenotype in culture. Consequently, the protocol we derived for inducing maturation is slightly different from that of inducing maturation in bmDC. To induce maturation in bmDC, LPS is conventionally added for a period of 24 hours, during which bmDC undergo rapid maturation. To induce maturation in ipDC, LPS is added for a period of 24 hours and then the medium changed to remove the maturation stimulus. ipDC undergo maturation over the course of the following 72 hours after which, they are harvested. ipDC that adopt a classically mature phenotype including up-regulation of

co-stimulatory molecules are found as non-adherent, free floating cells in culture and, as shown in the previous chapter, have the capability to stimulate naïve T cells in an MLR. The remaining cells that are adherent in culture show only a modest up-regulation of co-stimulatory molecules. As shown in Figure 4.3, free-floating cells adopt a mature phenotype with significant up-regulation of CD40, CD80 and CD86 compared to their immature counterparts. However, the majority of ipDC remain in a semi-mature state showing only partial up-regulation of co-stimulatory molecules as shown in Figure 4.2.

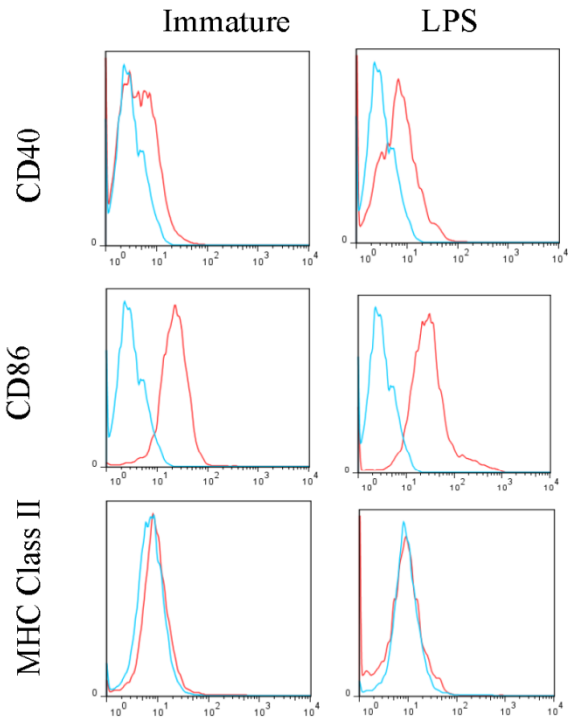


Figure 4.2. Adherent ipDC do not significantly up-regulate co-stimulatory molecules and MHC class II. ipDC were treated with 1µg/mL of LPS for 24 hours. The medium was replaced and the cells left to mature for 72 hours. Free floating cells were removed and adherent cells were removed with vigorous pipetting. Adherent cells were analysed for their expression of CD40, CD86 and MHC class II. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies.

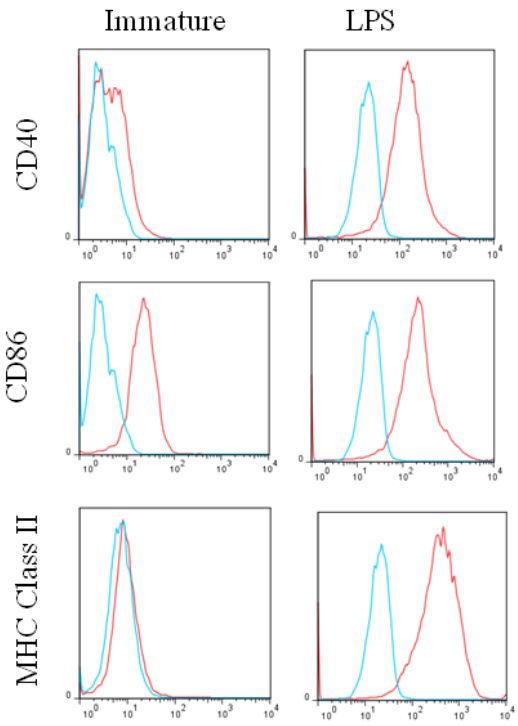


Figure 4.3. Non-adherent ipDC significantly up-regulate co-stimulatory molecules and MHC class II. ipDC were treated with 1µg/mL of LPS for 24 hours and then the medium replaced and left to mature for 72 hours. Free floating cells were removed and analysed for their expression of CD40, CD86 and MHC class II. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies.

4.1.3 Deficiency in IL-12p70 secretion following CD40 cross-linking

IL-12p70 is a cytokine secreted by macrophages, neutrophils and DC upon maturation and is essential in promoting a T helper 1 (Th1) responses (Vieira *et al.* 2000). IL-12p70 is also secreted by DC upon interaction with T cells. It has also been shown that ligation of CD40 causes DC to secrete IL-12p70 *in vitro* but not *in vivo* (Shulz *et al.* 2000). Given that the majority of ipDC appear to show a level of resistance to the 'classical' DC maturation stimulus LPS, we sought to examine whether, upon contact with surface-bound agonistic CD40 *in vitro*, maturation was induced in DC as determined by IL-12p70 secretion. Stimulation via CD40 ligation was induced by coating plates with an agonistic CD40 antibody and cross-linking the antibody as described in *Methods.*

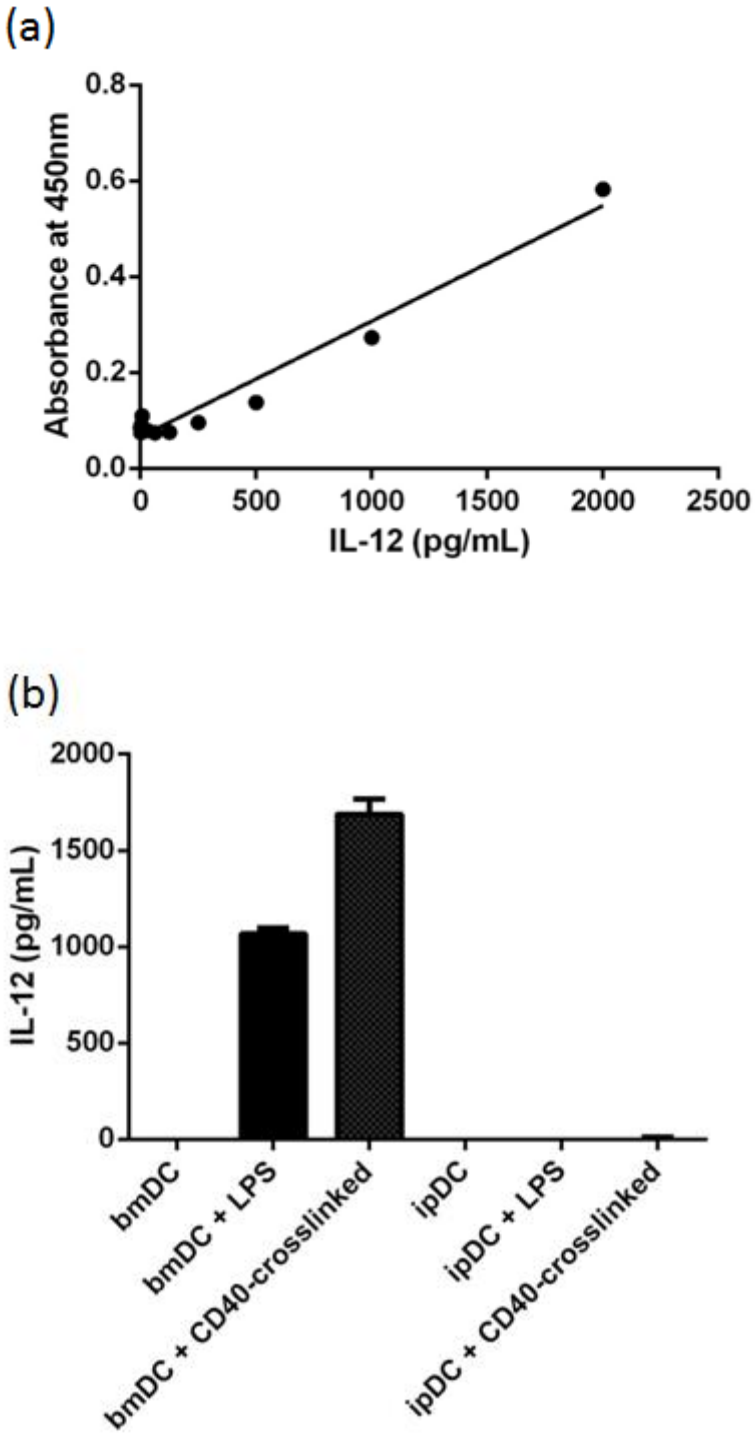


Figure 4.4 Secretion of IL-12p70 induced by LPS and cross-linked CD40 in bmDC and not ipDC. $3x10^5$ DC were cultured in triplicate overnight in wells containing LPS (1µg/mL), surface-bound CD40 antibody or untreated. Supernatant was assessed for IL-12p70 secretion by ELISA. (a) shows the standard curve for the IL-12p70 ELISA used to determine concentration of IL-12p70 (b) shows amount of secretion of IL-12p70 by both bmDC and ipDC. Data is representative of three independent experiments.

As shown in Figure 4.4, although bmDC failed to secrete IL-120p70 in their immature state, they responded to stimulation with both LPS and CD40 cross-linking by secreting IL-12p70. Conversely ipDC were unable to secrete IL-12p70 either in an immature state or following stimulation with either LPS or CD40 ligation. As demonstrated earlier in Chapter Three, ipDC express CD40 so this suggests that ipDC are unable to secrete the inflammatory cytokine IL-12p70 upon stimulation with either LPS or surface-bound agonistic CD40 antibody.

4.1.4 Variability in MHC class II expression: intracellular and cell surface expression and variability

Throughout the course of monitoring ipDC phenotypically, we observed this source of DC often expressed variable levels of MHC class II on the cell surface. Approximately half of all cultures of ipDC expressed very low or undetectable levels of MHC class II compared to control bmDC. This may have several implications on the functions of ipDC. MHC class II is essential for the presentation of peptides to naïve T cells and, therefore, its absence would prevent ipDC from developing cognate interactions with T cells. Given that we wish to induce tolerance to specific antigens, ipDC lacking MHC class II might be anticipated to be incapable of performing such a function. As outlined earlier by Morelli *et al.*, the level of expression of MHC class II can influence the phenotype of DC: high expression of MHC class II can initiate an immunogenic response whilst lower levels of MHC class II can drive the immune system towards a tolerogenic response.

Given that MHC class II has also been shown to be held intracellularly (Liu *et al.* 2011), and that maturation is associated with its translocation to the cell surface, we sought to examine whether ipDC which failed to express MHC class II on the cell surface, retained MHC class II intracellularly.

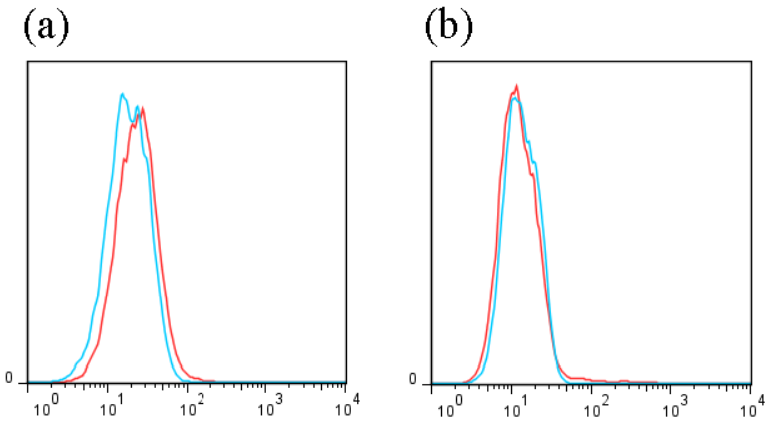


Figure 4.5. Lack of expression of MHC class II in ipDC on both cell surface and intracellular. ipDC which failed to express MHC class II on their cell surface were analysed with mAb against MHC class II for cell surface expression of MHC class II (a) and intracellular expression of MHC class II (b). The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

As shown in Figure 4.5, those ipDC that failed to express MHC class II on either the cell surface also failed to retain it in intracellular compartments, suggesting that lack of MHC class II may be attributed to deficiency in gene expression rather than a failure of translocation.

In order to determine whether these findings are specific to the iPS-1 cell line used throughout this thesis or whether they are common to all iPS cell lines, we compared ipDC differentiated from iPS-1 and iPS-14 and the level of MHC class II expression. As shown below in Figure 4.6, both iPS-1 and iPS-14 (a female MEF-derived iPS cell line derived in parallel with iPS-1) have the capacity to differentiate into ipDC which fail to express MHC class II.

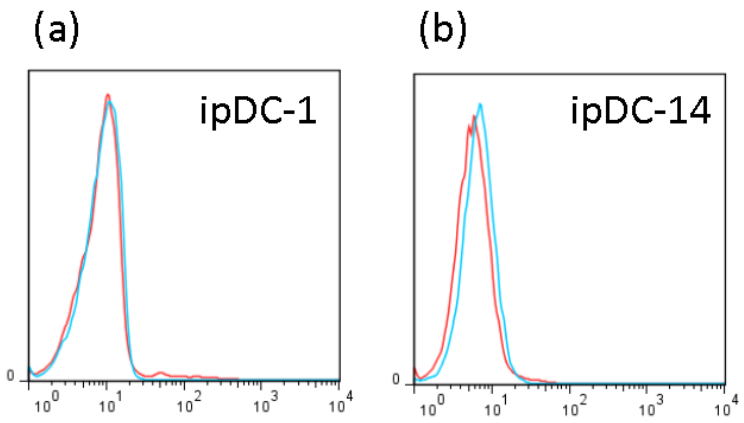


Figure 4.6. Lack of expression of MHC class II on both ipDC-1 and ipDC-14. ipDC which failed to express MHC class II on their cell surface were analysed with mAb against MHC class II for cell surface expression of MHC class II in two lines (a) ipDC-1 and (b) ipDC-14. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

4.1.5 Modulation of MHC class II expression by small molecules

Given that some cultures of ipDC do not express MHC class II on either the cell surface or intracellularly, and show resistance to some maturation stimuli, we sought to examine whether ipDC can up-regulate MHC class II in response to small molecules shown previously to influence MHC class II expression.

The expression of MHC class II has been shown to be modulated by acetylation and methylation of CIITA (Satoh *et al.* 2004) Furthermore, it has been demonstrated that MHC class II can be up-regulated in a cancer cell line (Magner *et al.* 2000). Magner *et al.* showed that culturing the cancer cell line SK-N-MC with the HDAC inhibitors sodium butyrate and trichostatin A resulted in increased levels of MHC class II. The authors rationalised that up-regulation of MHC class II in a cancer cell line may encourage immune intervention.

Given the profound epigenetic regulations involved in MHC class II expression, and the previous findings demonstrated by Magner *et al.*., we explored whether the use of histone

deacetylase inhibitors (HDAC) on ipDC would induce up-regulation of MHC class II expression. We, therefore, cultured both bmDC and a batch of ipDC lacking expression of MHC class II with sodium butyrate (NaBy) and trichostatin A (TSA) at various concentrations for 48 hours. After 48 hours, the cells were harvested and the expression of MHC class II compared to untreated control cells.

As shown below in Figure 4.7, treatment with NaBy or TSA had no observable effect on the surface expression of MHC class II on either bmDC or ipDC.

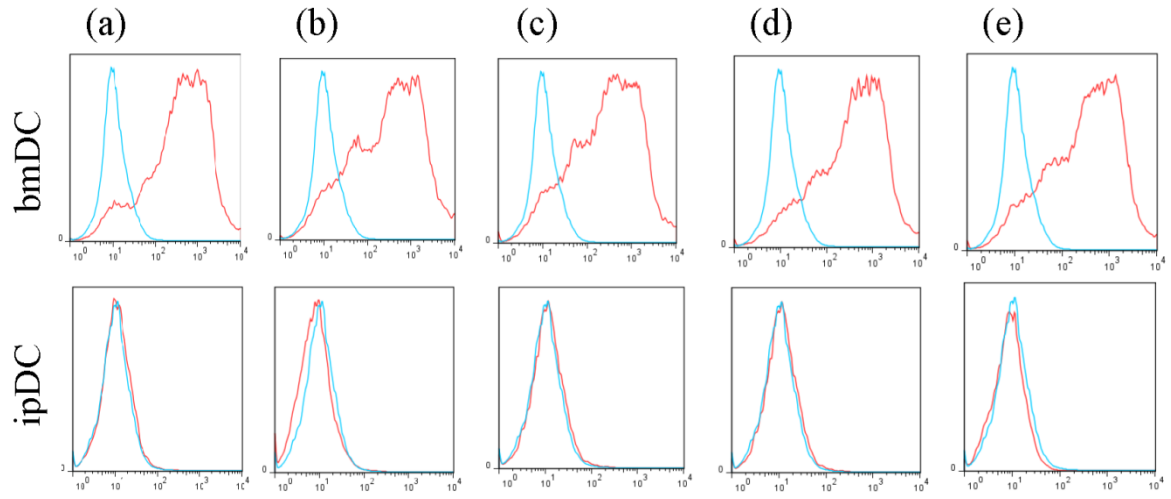


Figure 4.7 Effects of sodium butyrate and trichostatin A on bmDC and ipDC lacking MHC class II expression. 10^5 DC were cultured for 48 hours alone (a) or in the presence of either 1mM of NaBy (b), 2mM NaBy (c), 100nM TSA (d), 200nM TSA (e). DC were then examined for MHC class II expression using FACS. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies.

In the light of these results, we hypothesised that for NaBy or TSA to exert their effects in order to up-regulate of MHC class II, ipDC must first express a detectable level of MHC class II. We, therefore, treated a batch of ipDC expressing MHC class II and bmDC with NaBy and TSA for 48 hours and examined whether MHC class II was up-regulated after this

period. However, as shown in Figure 4.8, even ipDC which expressed detectable levels of MHC class II did not up-regulate MHC class II upon treatment with either TSA or NaBy.

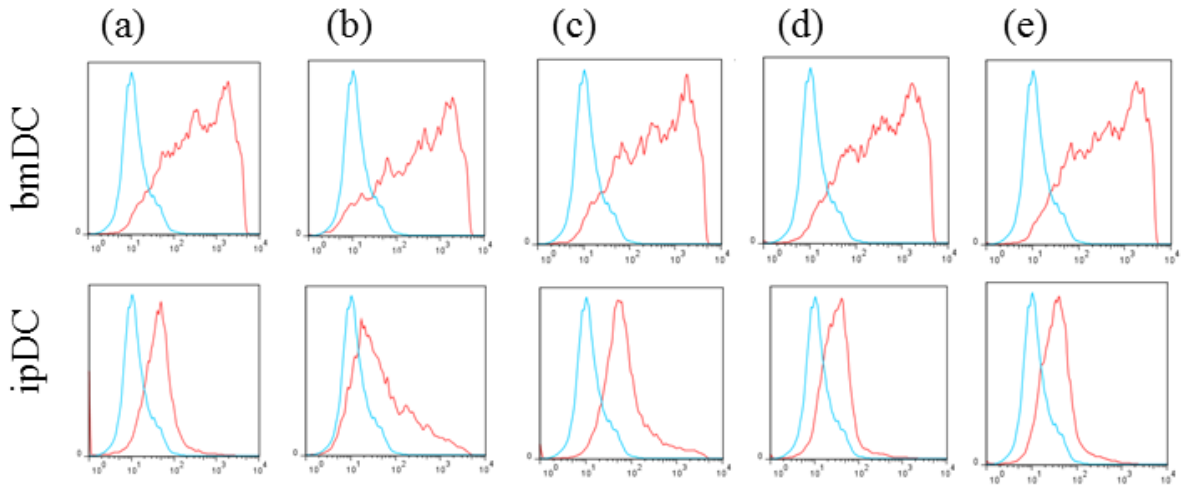


Figure 4.8 Effect of TSA and NaBy on MHC class II-expressing bmDC and ipDC. 10^5 DC were cultured either alone (a) or in the presence of either 1mM of NaBy (b), 2mM NaBy (c), 100nM TSA (d), 200nM TSA (e). DC were then examined for MHC class II expression using FACS. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. As shown in Figure 4.8, neither NaBy or TSA were able to modulate MHC class II expression on either ipDC or bmDC.

These results show that we were unable to translate findings based on the use of a cancer cell line to primary DC, suggesting that the control of MHC class II expression differs between these two distinct cell populations.

4.1.6 Further characterisation of ipDC lacking MHC class II

To confirm that cells with dendritic morphology but lacking MHC Class II expression were indeed DC, we co-stained for MHC I and the DC restricted marker CD11c. As shown in Figure 4.9, ipDC while failing to express MHC class II still maintain expression of MHC class I and CD11c showing that these cells maintain expression of other conventional DC markers.

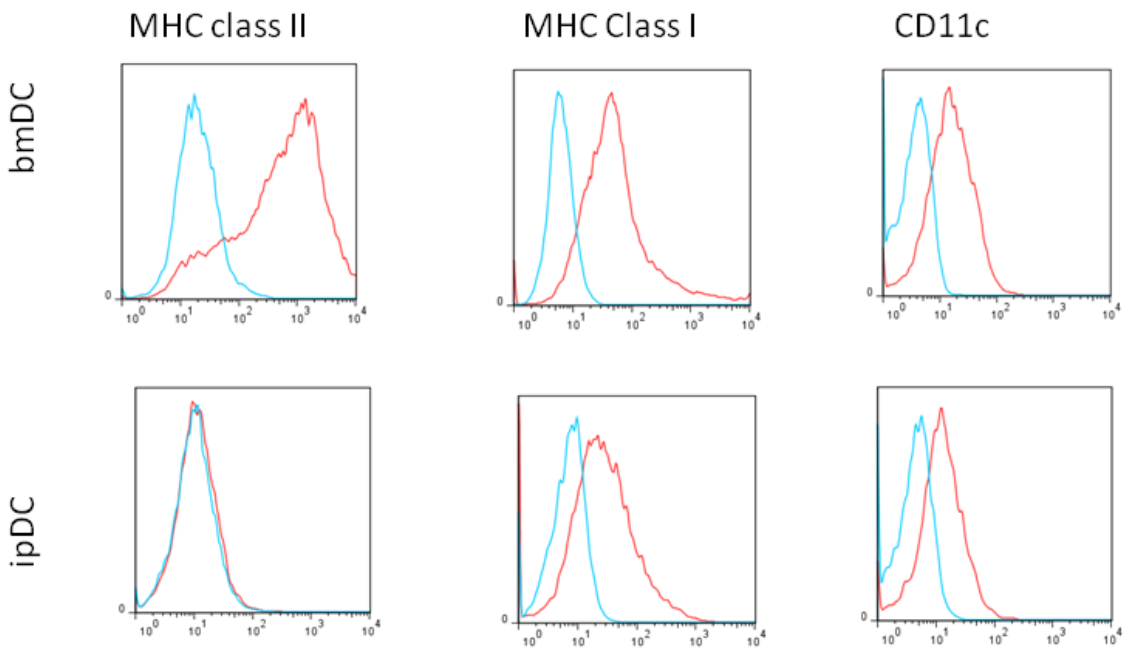


Figure 4.9 Expression of conventional DC markers by ipDC lacking MHC class II. Expression of MHC class I and CD11c is maintained in ipDC which do not express MHC class II on the cell surface showing that ipDC failing to express MHC class II maintain a classic myeloid DC phenotype. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

4.2 Investigation into the regulation of MHC class II expression in ipDC

Following the initial identification of ipDC which failed to express MHC class II, we sought to further examine the molecular mechanisms involved in MHC class II regulation by ipDC: by better understanding the nature of the blockage in MHC class II expression, we hoped to be able to regulate its expression. We, therefore, initially decided to examine the role of transcriptional regulation of MHC class II by investigating expression of the Class II Transactivator gene (CIITA).

4.2.1 Expression of CIITA by bmDC and ipDC

CIITA has been shown to control the expression of MHC class II (Steimle *et al.* 1993). Given that defects in the regulation and expression of CIITA are responsible for the human disease bare-lymphocyte syndrome, a condition whereby lymphocytes fail to express MHC class II, we sought to examine whether ipDC which were found to be as MHC class II^{lo} express CIITA. We also reasoned that the lack of CIITA expression may correlate with the enhanced IL-10 secretion seen in ipDC compared to their bmDC counterparts, as it has been shown previously that DC derived from $CIITA^{-/-}$ mice exhibit enhanced IL-10 secretion (Yee *et al.* 2005).

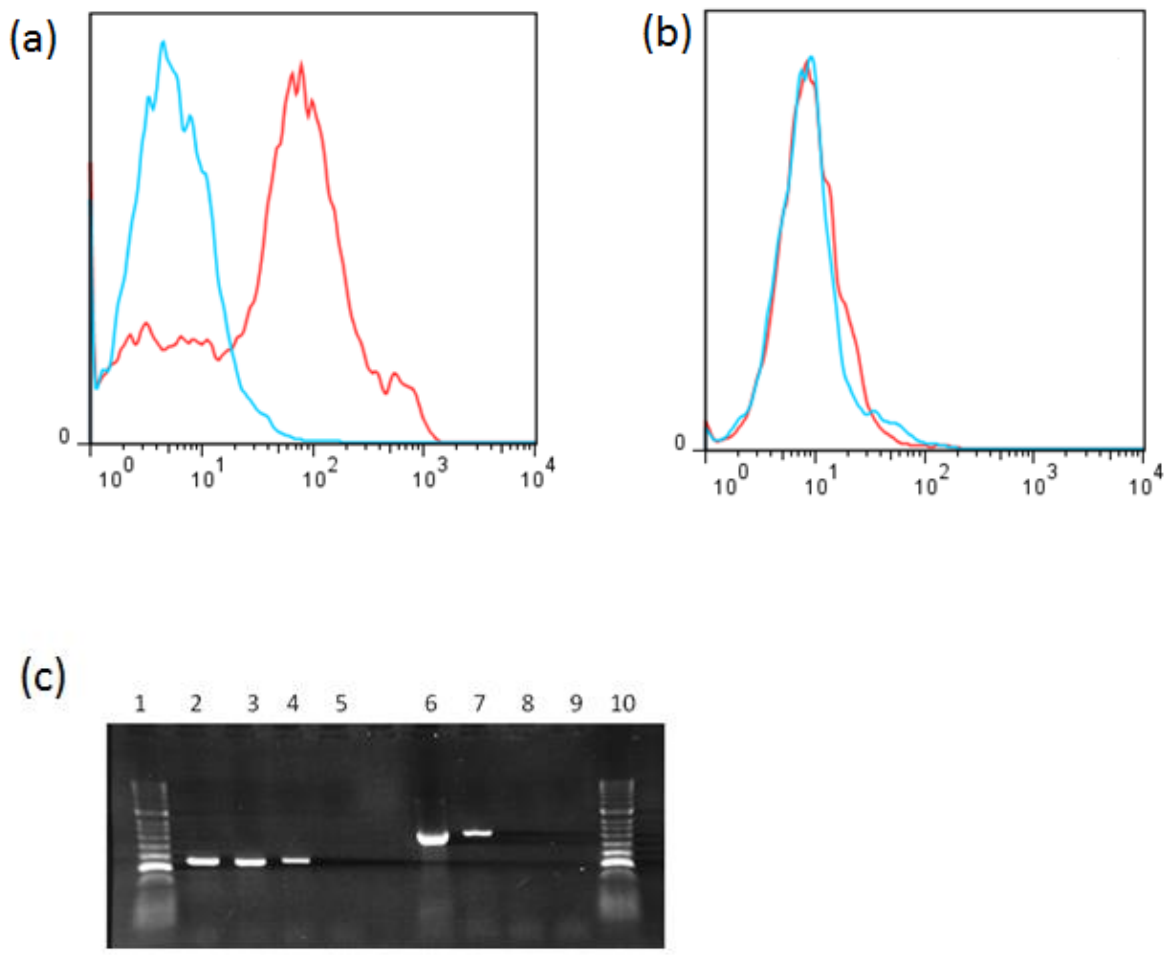


Figure 4.10. (a) Expression of MHC class II by bmDC but not (b) ipDC. (c) Expression of CIITA by ipDC and bmDC. RNA was extracted from bmDC, ipDC and iPS cells. The expression of HPRT is shown in lanes 1-4 by (2) bmDC (3) ipDC (4) iPS cells with (5) H_2O as control. The expression of CIITA was shown in lanes 6 and 7 by (6) bmDC (7) ipDC. iPS cells in lane (8) did not show any expression of CIITA. Lane (9) is H_2O. Lanes (1) and (10) represent 100bp ladders.

As shown in Figure 4.10, bmDC robustly express MHC class II in an immature state however immature ipDC harvested from this culture were shown to be negative for MHC class II by FACS. The RNA from both of these sets of cells was extracted and the expression of CIITA determined by RT-PCR. Figure 4.10 (c) shows that bmDC expressed CIITA along with ipDC, despite the latter failing to express MHC class II on the cell surface. As expected, the parent iPS cell line failed to express CIITA. This suggests that, although ipDC may not

express MHC class II on the cell surface or intracellularly, the genetic control of MHC class II expression is active.

Following this, we sought to examine whether the cell chosen for reprograming effects the phenotype of ipDC differentiated from the parent cell line.

4.3 Generation of iPS cells from adult fibroblasts

It is known that embryonic tissues express low levels of MHC in order to evade recognition by the maternal immune system (Drukker *et al.* 2002). Indeed, this trait is carried through to ES and iPS cells cultured *in vitro.* Suarez-Alvarez *et al.* (2010) showed that human ES and iPS cells in culture express low levels of MHC class I and total absence of MHC class II, the latter of which is thought to be regulated by DNA methylation.

Given the low levels of expression of MHC in embryonic tissues, we hypothesised that deriving iPS cells from adult somatic cells that are not subject to such constraints may influence the expression of MHC class II in ipDC differentiated from them. Consequently, the DC differentiated may display a greater propensity for MHC class II expression compared to those differentiated from embryonic derived fibroblasts.

It is important to address whether the source of the fibroblasts used for reprogramming influences the phenotype of ipDC since and downstream clinical applications would make use of adult human fibroblasts rather than fibroblasts of embryonic origin.

In order to examine this hypothesis, we chose to reprogram tail-tip fibroblasts from adult CBA/Ca mice.

4.3.1 Generation of iMAFs: source of fibroblasts and reprogramming to pluripotency

Tail tip fibroblasts were derived as described in *Methods*, from 8 week old CBA/Ca mice. These fibroblasts were then expanded in culture and reprogrammed using a cocktail of retroviruses containing the reprogramming factors *Sox2, Oct3/4 and Klf4* as described in *Methods.* As demonstrated in Figure 4.11, fibroblasts displayed identical morphology to embryonic derived fibroblasts. Additionally, colonies grown on mitotically inactivated MEF showed similar morphology to iPS cell lines derived in the previous chapter of this thesis using embryonic fibroblasts as the starting material. Furthermore, a representative iMAF cell line generated, iMAF-6, was shown to exhibit a normal male karyotype of 40XY as well as showing silencing of the transgenes used to reprogram cells to pluripotency (data not shown).

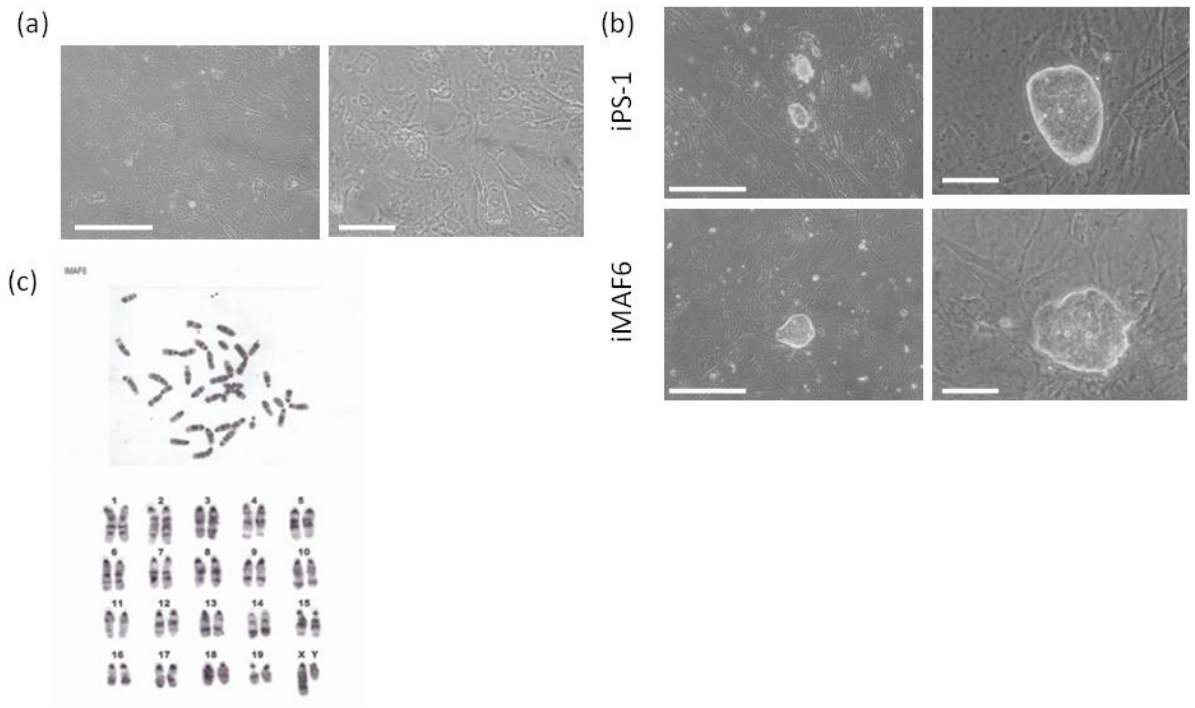


Figure 4.11. iPS cell morphology, karyotype, gender and transgene silencing. (a) Fibroblasts derived from mice adult tail tips display identical morphology to MEF. (b) iMAF6 cells display similar morphology to their iPS cell counterparts (c) as well as displaying a normal XY male karyotype.

4.3.2 Expression of pluripotency markers

As alluded to earlier, the characteristics of pluripotency are reflected in ES cells by the expression of various transcription factors and cell surface markers including Nanog, SSEA-1 and Oct3/4. Subsequently, it has been shown that these pluripotency genes are endogenously expressed in iPS cell lines secondary to their introduction as transgenes (Yu *et al.* 2007). We, therefore, sought to examine whether iMAF-6 had endogenously up-regulated these pluripotency genes. As shown in Figure 4.12, iMAF-6 shows consistently higher levels of expression of these pluripotency markers compared to their ES cell counterparts.

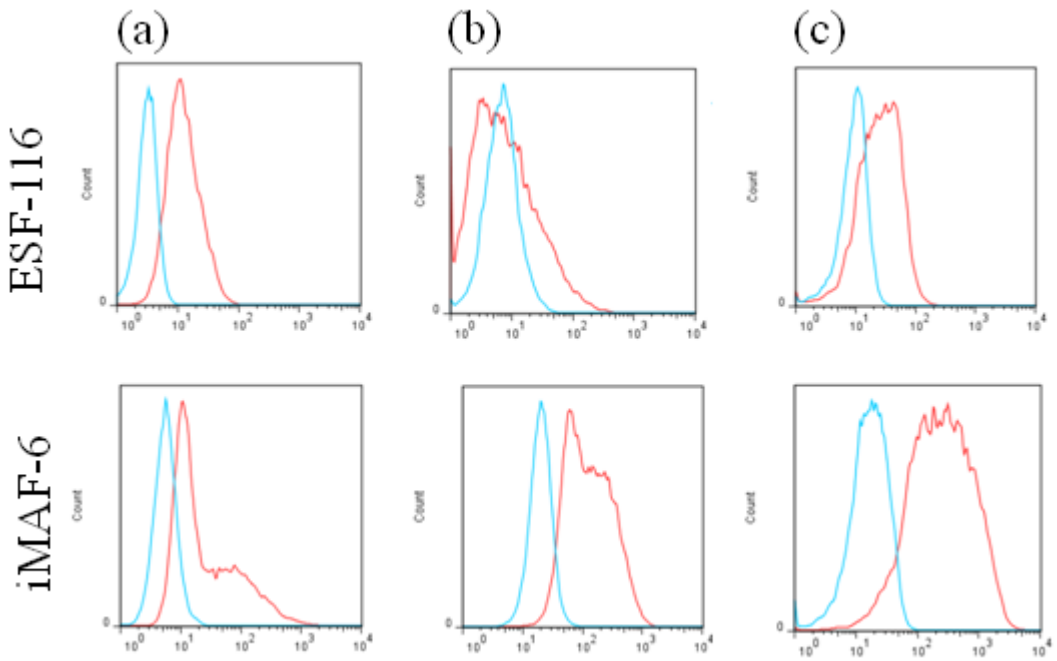


Figure 4.12. Expression of pluripotency markers by ESF-116 and iMAF6 cell lines. Figure 4.12 shows the expression of (a) Nanog, (b) SSEA-1 and (c) Oct3/4 by both ESF-116 and iMAF6 as assessed by flow cytometry. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

Following identification of the expression of pluripotency genes by iMAF6, we sought to examine whether iMAF6 had similar morphology to ES cell colonies and also expressed the

pluripotency genes *SSEA1*, *Nanog* and *Oct3/4*. Similar to the expression of these markers in iPS-1 in Chapter Three, *Nanog* and *Oct3/4* were uniformly expressed throughout both ES and iMAF6 cell colonies, *SSEA-1* appeared restricted in it expression, being most highly expressed around the periphery as shown in Figure 4.13.

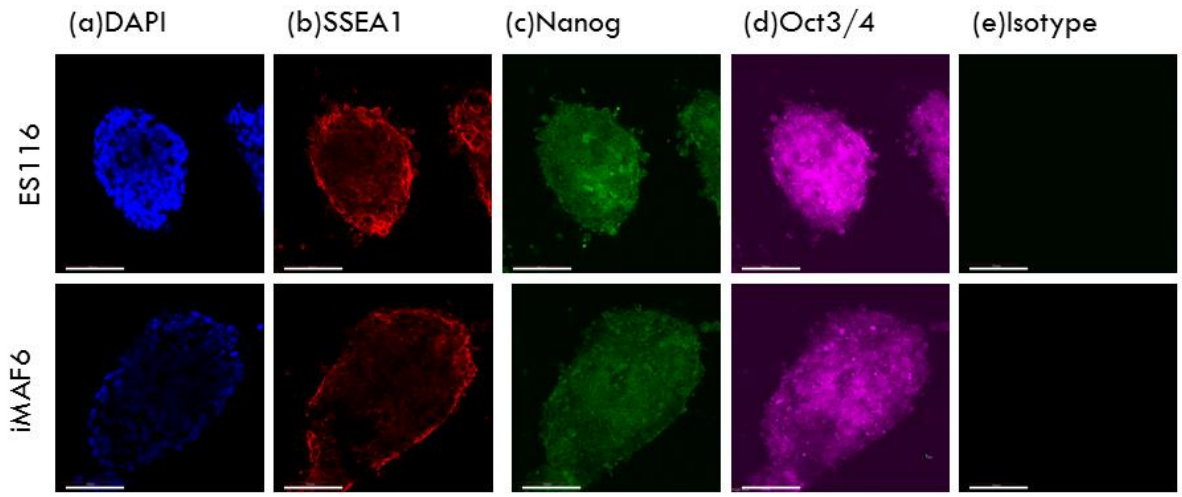


Figure 4.13 Expression of pluripotency markers in a single iMAF and ES cell colony. iMAF and ES cell colonies were grown in feeder-free conditions and stained with antibodies against the pluripotency markers (b) SSEA1, (c) Nanog and (d) Oct3/4. The isotype control represents a pooled isotype for all three antibodies. Scale bar represents 100µm.

We were, therefore, able to show that iMAF-6 expressed transcription factors and surface markers characteristic of pluripotent stem cells.

4.3.3 Differentiation *in vivo* to lineages from three embryonic germ layers

In order to examine the ability for iMAF-6 to differentiate into cell types from all three germ layers, we implanted embryoid bodies into CBA.RAG1$^{-/-}$ mice and harvested teratomas after 21 days. Teratomas were then sectioned and stained with haemotoxylin and eosin and examined by light microscopy. As shown in Figure 4.14, iMAF-6 had the ability to

differentiate into cell types from all three embryonic germ layers, consistent with their pluripotency and suggesting, like their counterparts derived from embryonic fibroblasts, that they may have the capacity to differentiate into DC.

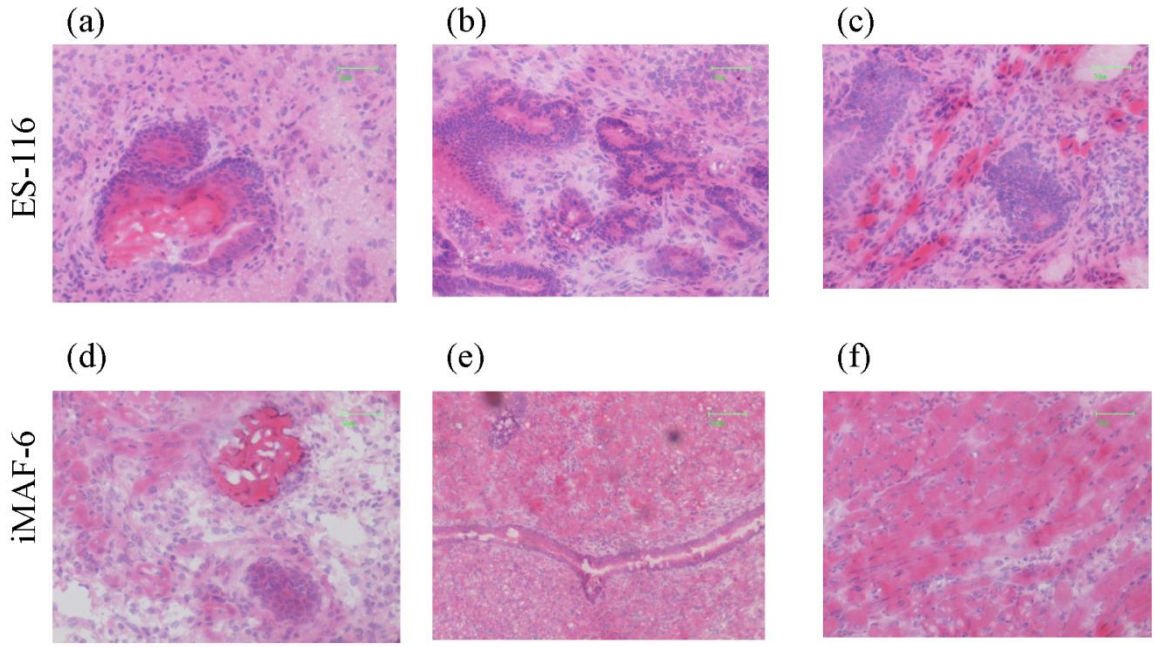


Figure 4.14. Formation of tissue from three embryonic germ layers *in vivo*. Figure above shows haematoxylin and eosin staining of sections from teratomas formed in CBA.RAG1$^{-/-}$ mice by ESF-116 and iMAF6 cell line following transplantation of 14-day embryoid bodies under the kidney capsule. Teratomas were harvested after 21 days. Photomicrographs show examples of: cornified epithelial tissue [ectoderm] (a) (d) putative glandular tissue [endoderm] (b) (e) and smooth muscle [mesoderm] (c) (f).

4.4 Generation of ipDC from iMAF-6 cell line

4.4.1 Differentiation of DC from iMAF6 cell line

Following successful generation of iPS cell lines from MAF and demonstration that these cells had the ability to differentiate into cell types of all three embryonic germ layers, we sought to examine whether these cells had the capacity to differentiate into DC using precisely the same protocol we had developed for iPS-1.

4.4.2 Characterisation of iMAF-DC

To initially characterise this novel population of DC, we sought to examine the expression of various conventional mouse DC markers in the absence of maturation stimuli.

As shown in Figure 4.15, when compared with conventional bmDC (a) iMAF-DC displayed a similar level of expression of classical DC markers such as CD11c, MHC class I and CD54 as bmDC (b). Importantly, initial experiments revealed the expression of MHC class II by iMAF-DC, albeit at far lower levels compared to control bmDC cultures.

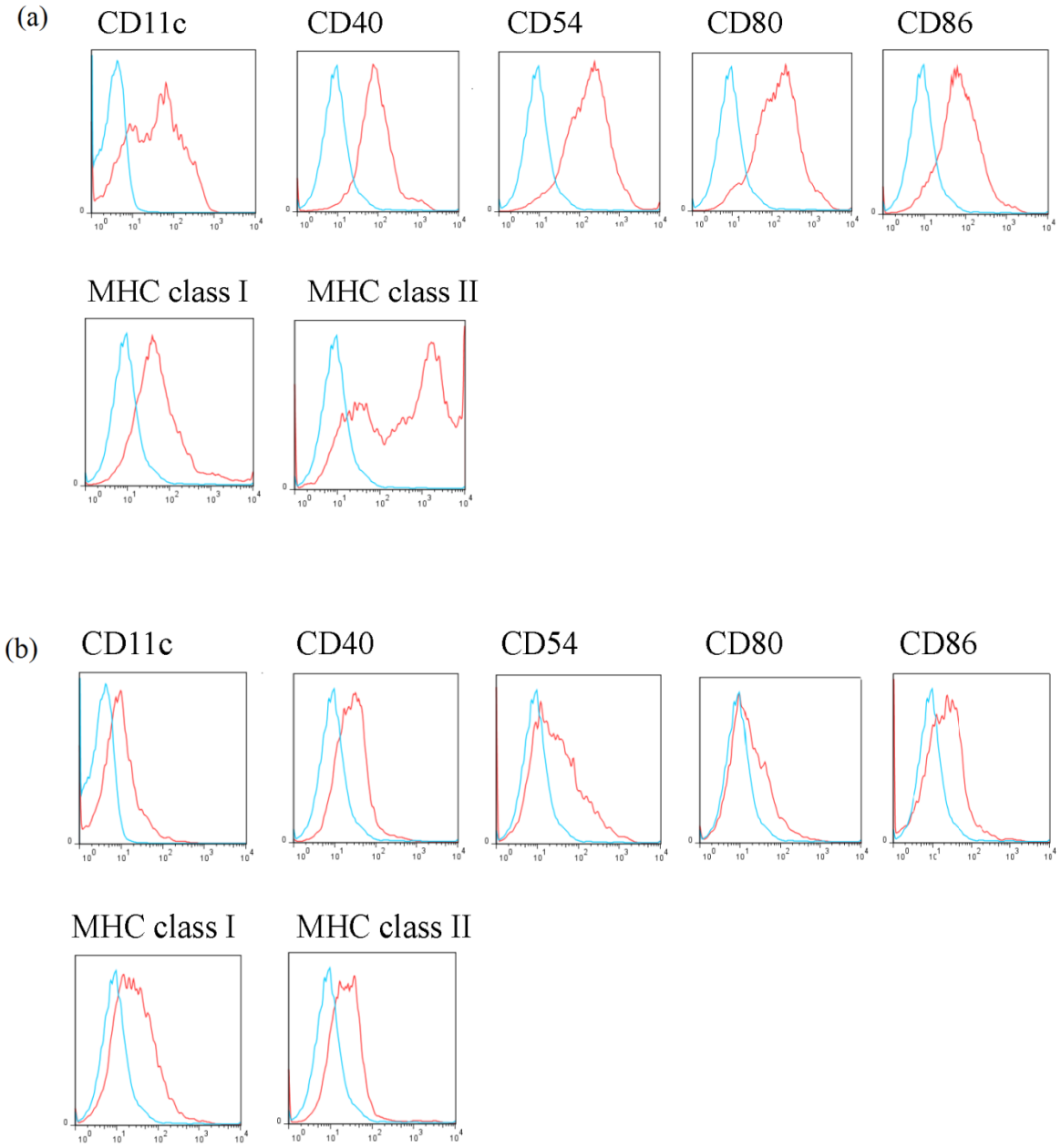


Figure 4.15. Expression of classical DC markers by bmDC (a) and iMAF-DC (b). bmDC (a) and iMAF-DC (b) were compared for their expression of classical DC markers using FACS. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

4.4.3 Response to CD40-cross linking stimulation and IL-12p70 secretion

Given our initial observations of MHC class II expression by iMAF-DC, we next investigated whether the source of fibroblasts from which the parent iPS cell line derived might also influence the ability of DC differentiated from them to secrete IL-12p70 in response to appropriate stimuli.

We, therefore, sought to examine whether iMAF-DC had the ability to respond to cross-linking of surface CD40 by agonistic CD40 antibody and LPS, using ipDC and bmDC for comparison.

As shown in Figure 4.16, bmDC were unable to secrete detectable levels of IL-12p70 in an immature state, however, upon addition of LPS or cross-linking of surface CD40 by agonistic-CD40 antibody, significant IL-12p70 secretion was observed. Consistent with our previous findings, ipDC were unable to secrete IL-12p70 in either an immature state or upon stimulation with LPS or cross-linking agonistic CD40 antibodies. Furthermore, iMAF-DC also failed to secrete IL-12p70 in response to either stimulus. This suggests that both ipDC and iMAF-DC share critical properties by virtue of their common origin that confer a level of maturation resistance that greatly reduced their immunogenicity.

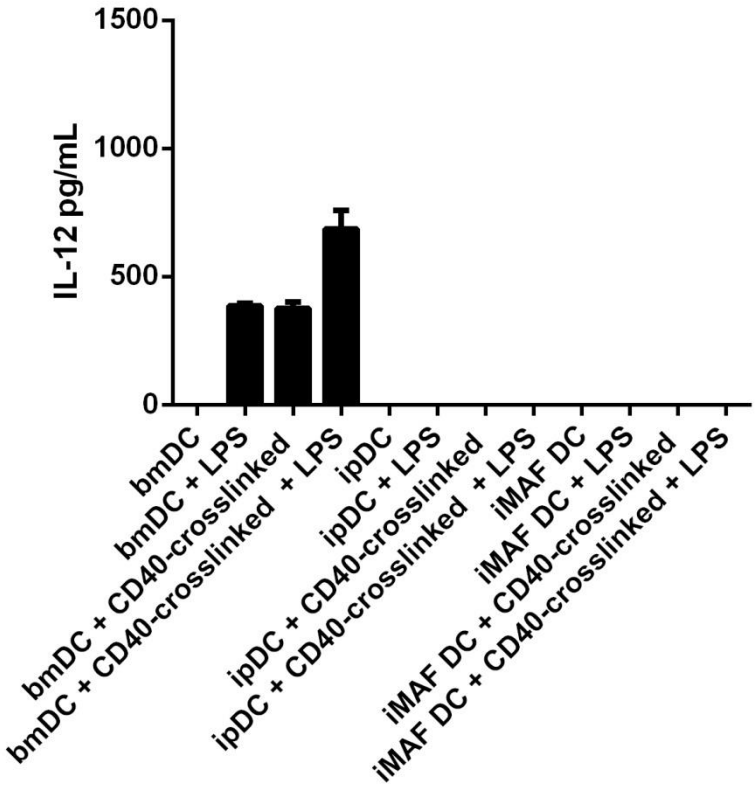


Figure 4.16 Secretion of IL-12p70 by bmDC but not ipDC or iMAF-DC. $3x10^5$ DC were cultured overnight with either 1µg/mL LPS, cross-linked agonistic CD40, both LPS and cross-linked agonistic CD40 or untreated control. Supernatant was then collected after 24 hours and analysed for IL-12p70 content by ELISA. Data is representative of three independent experiments.

4.4.4 Intracellular and surface cell expression of MHC class II in ipDC differentiated from both MEF and MAF derived iPS cells

As described earlier, ipDC differentiated from MEF-derived iPS cell lines exhibit very variable levels of MHC class II expression, from moderately high levels in some cultures to low or undetectable in others. In order to examine whether there are any differences between MEF and MAF derived ipDC, with respect to their expression of MHC class II, we systematically differentiated ipDC from both MEF and MAF derived iPS cells and examined the expression of MHC class II on both the cell surface and held in intracellular compartments by the cells. As shown in Figure 4.17, bmDC consistently expressed robust

levels of MHC class II both on the cell surface and intracellularly. However, in some cultures, both iPS-MEF derived DC (ipDC) and iPS-MAF derived DC (iMAF-DC) expressed low or undetectable levels of MHC class II on the cell surface and intracellularly. Furthermore, permeabilisiation failed to reveal the presence of MHC class II in intracellular compartments that might suggest a deficiency in its translocation to the cell surface.

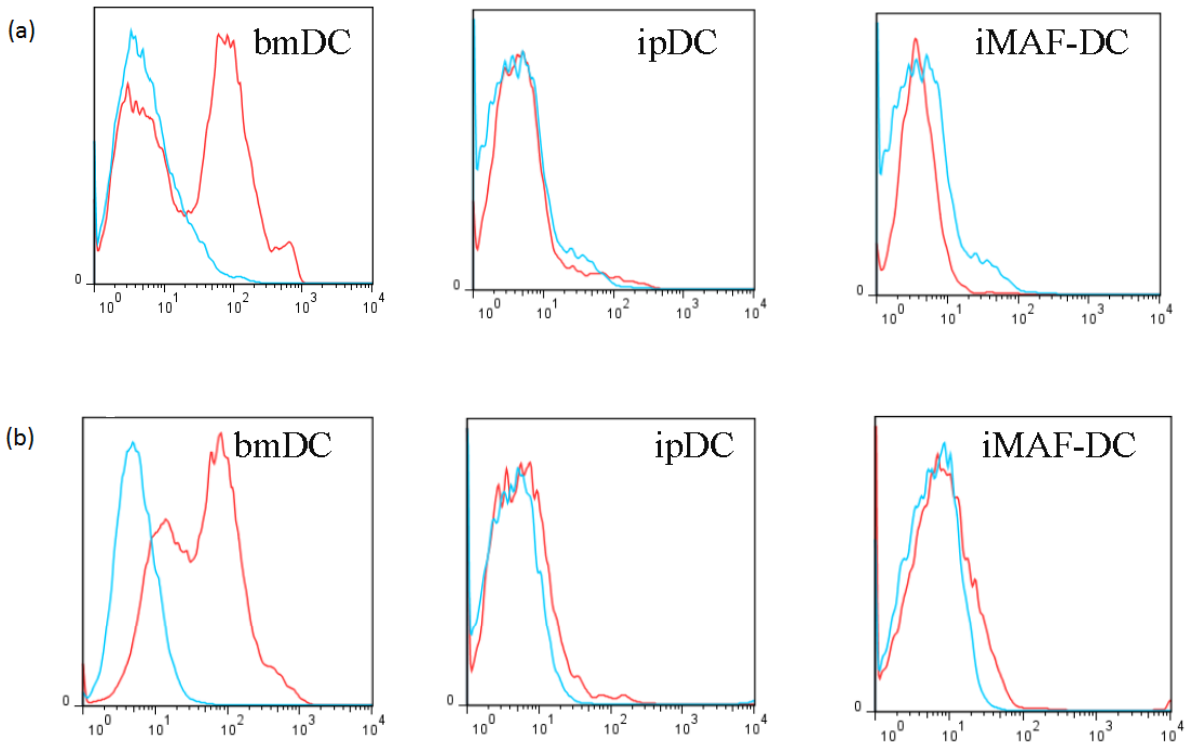


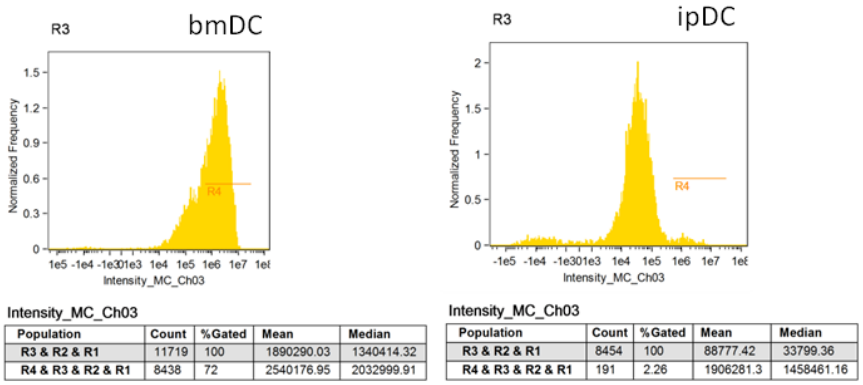
Figure 4.17 Expression of MHC class II in bmDC, iPS-MEF derived DC (ipDC) and iPS-MAF derived DC (iMAF-DC). All three cell types were analysed for the expression of MHC class II both on their (a) cell surface and (b) intracellularly via FACS. The red histogram depicts expression of the marker of interest, the blue histogram depicts the level of background staining using isotype-matched control monoclonal antibodies. Data is representative of three independent experiments.

4.4.5 Visualisation of MHC class II expression using ImageStream

Although our experiments revealed that iMAF-DC suffered the same deficiencies in MHC class II expression evident among ipDC, we consistently observed a subset of MHC class II positive cells in culture. To investigate this population further, we therefore used state of the

art ImageStream analysis. ImageStream was developed in order to combine traditional fluorescence microscopy methods with flow cytometry. This allows large numbers of cells to be analysed at one time and allowing for a wider range of fluorescent probes to be utilised. Additionally, the location of the signal for each antibody can be localised to within the cell allowing users to determine whether the signal is located in the nucleus, intracellular compartments or expressed on the cell surface.

We, therefore, sought to examine the expression of MHC class II in a population of ipDC alongside control bmDC. As shown in Figure 4.18, 97.77% of ipDC failed to express detectable levels of MHC class II on either the cell surface or intracelluarly compared with bmDC which expressed 72%. Additionally, the majority of iMAF-DC also failed to express MHC class II with only 2.49% displaying an MHC class II^+ phenotype. Figure 4.19 represents the majority of ipDC and iMAF-DC which fail to express MHC class II, as well as a small proportion of bmDC. These data suggest that these cells show similar levels of granularity, as demonstrated by the side-scatter profiles (SSC), along with similar morphology as represented by the brightfield view.

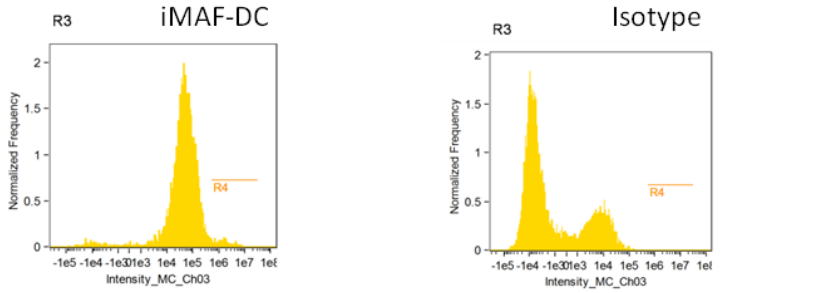


Intensity_MC_Ch03

Population	Count	%Gated	Mean	Median
R3 & R2 & R1	11719	100	1890290.03	1340414.32
R4 & R3 & R2 & R1	8438	72	2540176.95	2032999.91

Intensity_MC_Ch03

Population	Count	%Gated	Mean	Median
R3 & R2 & R1	8454	100	88777.42	33799.36
R4 & R3 & R2 & R1	191	2.26	1906281.3	1458461.16



Intensity_MC_Ch03

Population	Count	%Gated	Mean	Median
R3 & R2 & R1	9382	100	122252.76	49915.54
R4 & R3 & R2 & R1	234	2.49	2206561.23	1572889.58

Intensity_MC_Ch03

Population	Count	%Gated	Mean	Median
R3 & R2 & R1	9246	100	-2707.8	-4436.21
R4 & R3 & R2 & R1	0	0	NaN	NaN

Figure 4.18 Expression of MHC class II on cell surface and intracellularly by bmDC but not ipDC or iMAF-DC. bmDC and ipDC were analysed for their expression of MHC class II on their cell surface and intracelluarly using ImageStream. The nucleus of the cell was visualised used 7-AAD and cell viability was assessed using the marker Live/Dead-Aqua. Data is representative of three independent experiments.

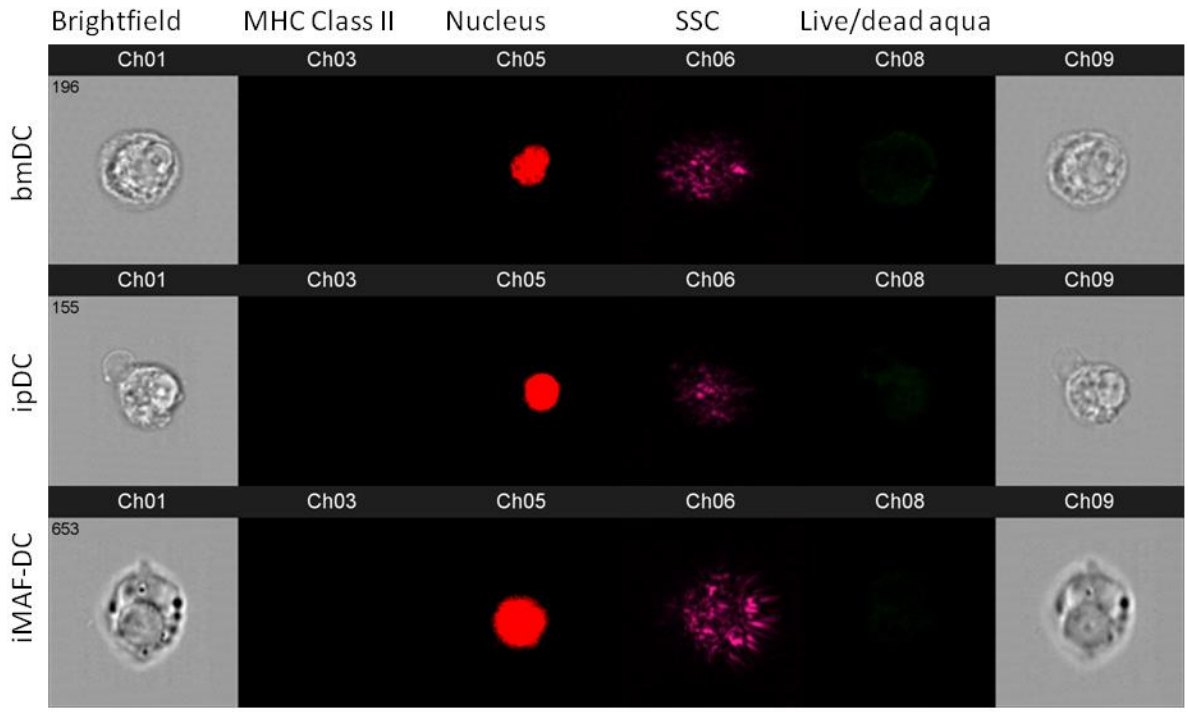


Figure 4.19 Lack of expression of MHC class II on minor population of bmDC and majority of ipDC. bmDC and ipDC were analysed for their expression of MHC class II on their cell surface and intracelluarly using ImageStream. The nucleus of the cell was visualised used 7-AAD and cell viability was assessed using the marker Live/Dead-Aqua. SSC represent the side-scatter of the cell. Data is representative of three independent experiments.

We then sought to examine where MHC class II was expressed in the minor population of ipDC which do express the molecule. As shown in Figure 4.20, whereas the majority of ipDC did not express MHC class II in either intracellular compartments or on the cell surface, we were able to show that in the minor population of MHC class II^+ ipDC, the majority of expression is localised to the cell surface. This suggests that, consistent with earlier findings, cultures of ipDC which express low levels of MHC class II may not have sufficient intracellular MHC class II to up-regulate in response to stimuli such as LPS or small molecule and that such stimuli do not induce *de novo* gene expression.

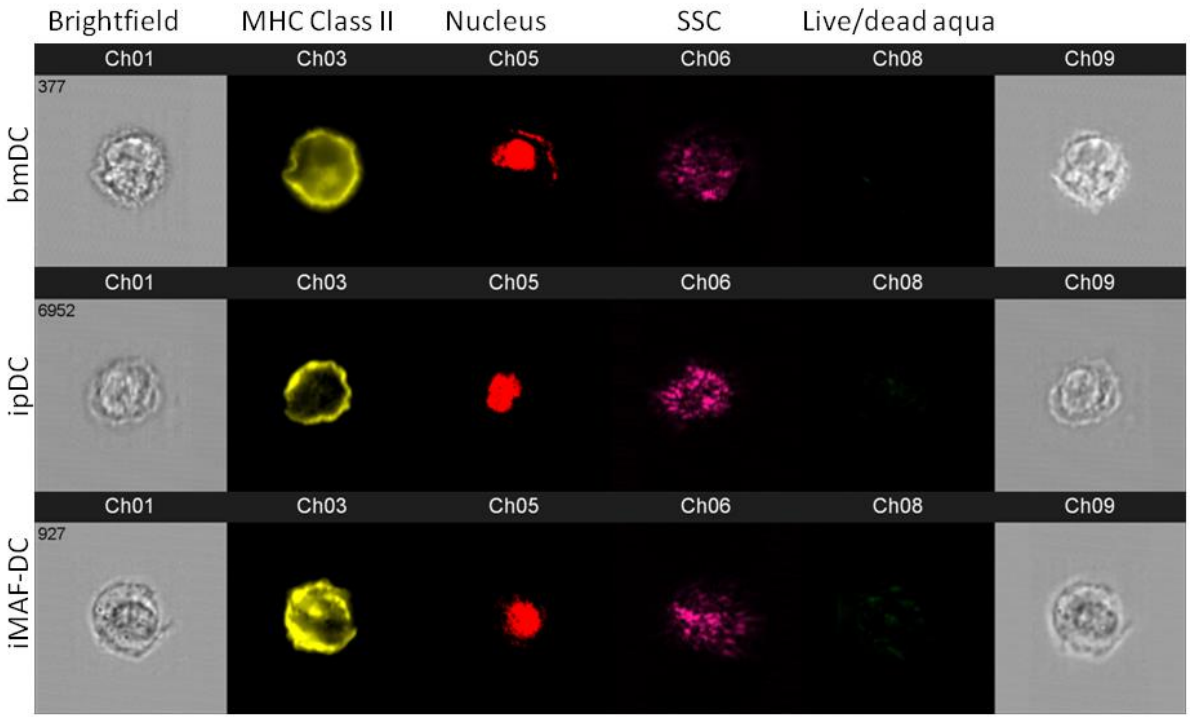


Figure 4.20 Expression of MHC class II by bmDC and minor population of ipDC. bmDC and ipDC were analysed for their expression of MHC class II on their cell surface and intracelluarly using ImageStream. The nucleus of the cell was visualised used 7-AAD and cell viability was assessed using the marker Live/Dead-Aqua. Data is representative of three independent experiments. Data is representative of three independent experiments.

4.4.6 Capacity of fixed MHC class II^{lo} to present the HEL1-18 peptide to 2G7.1 hybridoma

Data presented so far in this chapter show the variability inherent in the differentiation of iPS cells, variability which is particularly evident at the level of MHC class II expression. The fact that some batches of ipDC express undetectable levels of MHC class II suggests that it may be necessary to screen each culture, in advance, to determine its MHC class II 'status', a strategy which is wasteful and therefore costly. We, therefore, sought to determine whether cells defined as MHC class II^{-} by flow cytometry might still undergo productive interactions with T cells, either due to sufficient MHC class II on the cell surface, despite it being below

the level of detection of the cytometer, or because co-culture with T cells might induce the up-regulation of MHC class II.

As described in Chapter 3, ipDC have the ability to process and present HEL to the T cell hybridoma 2G7.1. We sought to examine whether ipDC that were shown not to express MHC class II by FACS nevertheless had sufficient residual MHC class II on their cell surface to present the HEL1-18 peptide to the 2G7.1 hybridoma. In order to examine this hypothesis, we used paraformaldehyde to fix ipDC with undetectable levels of MHC class II expression to prevent any subsequent changes in MHC class II expression. ipDC were then pulsed with the HEL1-18 peptide allowing it to bind directly to any MHC class II on the cell surface. As a control, bmDC were fixed with PFA and incubated with either the HEL1-18 peptide to act as a positive control of presentation, or incubated with whole HEL which would serve as a negative control of presentation.

As shown in Figure 4.21, fixed ipDC which did not express MHC class II by FACS failed to present the HEL1-18 peptide to the 2G7.1 hybridoma. However, fixed bmDC had the ability to present the peptide to 2G7.1, as assessed by IL-2 secretion.

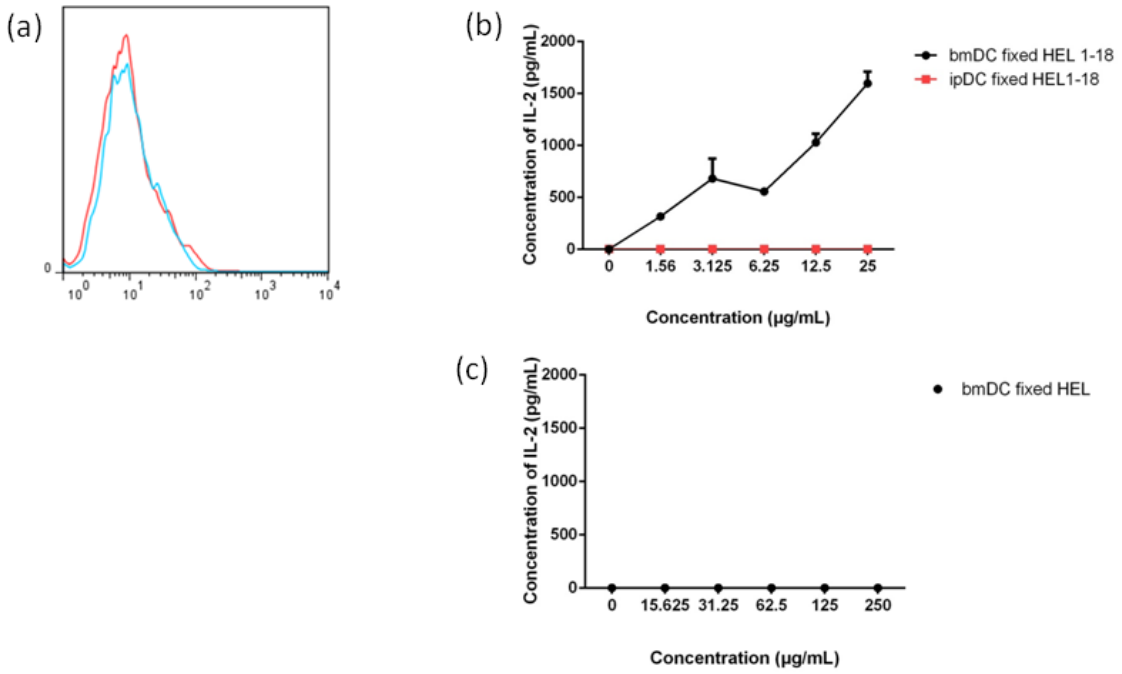


Figure 4.21 Presentation of the HEL1-18 peptide by bmDC and ipDC failing to express MHC class II. ipDC were analysed for the expression of MHC class II by FACS (a). bmDC and ipDC were fixed with PFA and cultured with HEL1-18 peptide (b). Hen egg lysozyme (HEL) was cultured with fixed bmDC (c). They were then cultured with the T cell hybridoma 2G7.1 which recognises HEL1-18 in the context of H-2E^{k} as described in section 2.5. Activation of 2G7.1 was assessed by IL-2 secretion and quantified using an ELISA.

4.4.7 A comparison of the immunogenicity of ipDC and iMAF-DC in the allogeneic MLR

Given that iMAF-DC may have different phenotypic properties to ipDC, we sought to examine whether they showed any difference in their ability to stimulate naïve allogeneic T cells in a MLR. DC were matured with LPS and harvested as described previously. DC were, therefore, titrated into co-culture with 10^5 naïve allogeneic C57Bl/6 T cells for 72 hours. As shown in Figure 4.22, whereas mature bmDC had the ability to stimulate primary proliferative responses among allogeneic T cells, both ipDC and iMAF-DC failed to stimulate allogeneic T cells most likely due to their low MHC class II expression.

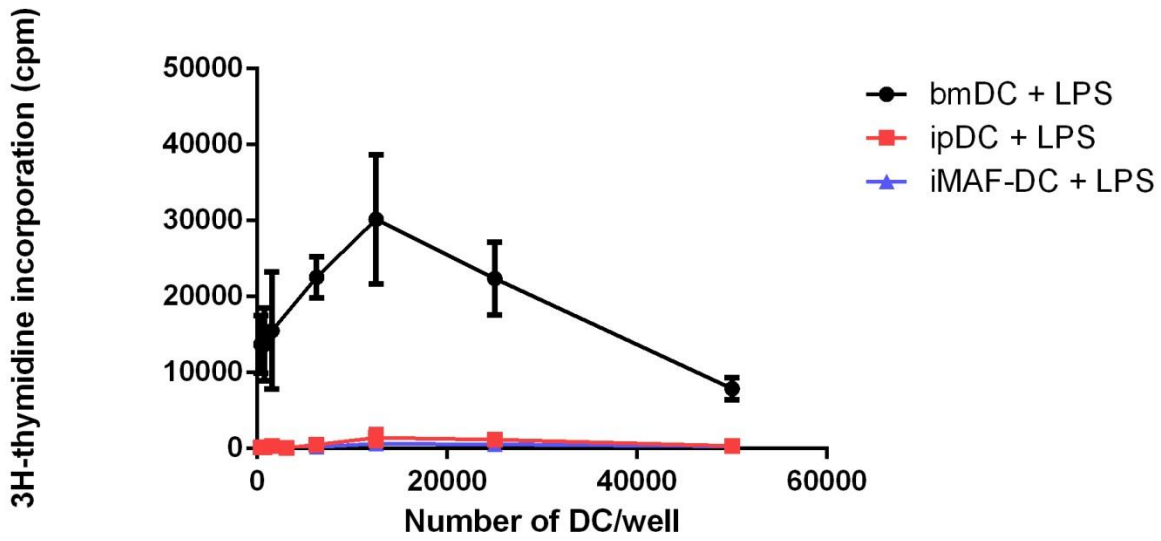


Figure 4.22 Stimulation of allogeneic C57Bl/6 T cells by mature bmDC, ipDC and iMAF-DC. DC were matured with 1µg/mL LPS, harvested and diluted two fold and co-cultured with 10^5 C57Bl/6 T cells for 72 hours. For the last 18 hours of culture, cultures were pulsed with 0.5µCi of ^{3}H-thymidine to assess proliferation. Data shown is representative of three experiments.

4.4.8 Capacity of fixed ipDC not expressing MHC class II to stimulate naïve A1.RAG1$^{-/-}$ T cells

The MLR is a polyclonal response in which 5-10% of naïve allogeneic T cells respond to alloantigen. In order to provide the greatest opportunity for cognate interactions between ipDC and antigen specific T cells, we next made use of the A1.RAG1$^{-/-}$ mouse model. We used TCR transgenic T cells from the A1.RAG1$^{-/-}$ mouse which are uniformly specific for the male antigen Dby presented in the context of H-2E^k.

ipDC which showed no detectable expression of MHC class II via FACS (a) were co-cultured with A1.RAG1$^{-/-}$ T cells, specific for the Dby peptide presented in the context of H-2E^k. Although immature bmDC and ipDC were karyotypically male, they were pulsed with 100nM of Dby peptide to ensure that antigen was not limiting, before being cultured with A1.RAG1$^{-/-}$ T cells for 72 hours.

As shown below in Figure 4.23 (b), pulsed immature bmDC were able to stimulate naïve A1.RAG1$^{-/-}$ T cells to proliferate, however ipDC lacking detectable levels of MHC class II were unable to induce proliferation of T cells within the same time frame. This suggest that, despite interaction with resting naïve cognate T cells, ipDC which fail to express MHC class II at the outset of the co-culture do not up-regulate its expression.

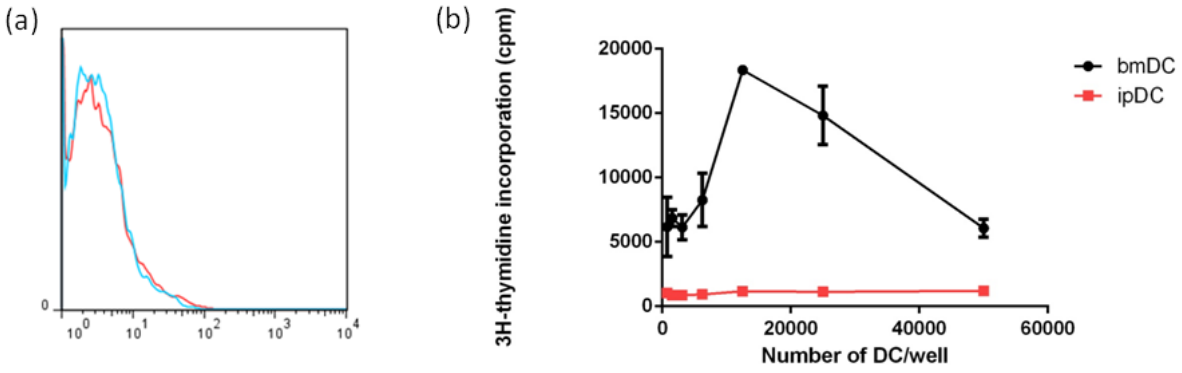


Figure 4.23. Culture of Dby pulsed bmDC and ipDC with A1.RAG1$^{-/-}$ T cells. (a) Immature bmDC and ipDC were analysed for their expression of MHC class II by FACS. (b) These cells were then co-cultured with 10^5 A1.RAG1$^{-/-}$ mice T cells for 72 hours. For the last 18 hours of culture, wells were pulsed with 0.5µCi of ^{3}H-Thymidine to quantify T cell proliferation.

4.5 Discussion

ipDC appear to show some level of 'resistance' to maturation stimuli including LPS and cross-linking agonistic CD40 mAbs. Given that ipDC have previously been shown to express TLR4 (Figure 3.4), we can assume that ipDC have the correct cell surface receptor to recognise LPS. It is also interesting to note that esDC have previously been shown to exhibit a degree of maturation-resistance, similar to ipDC (Fairchild, Personal communication). This suggests that the process, or kinetics, by which TLR4 ligation occurs and the subsequent up-regulation of co-stimulatory molecules differs from that of bmDC.

As outlined in the *Introduction*, Morelli *et al.* documented the minimal necessary criteria to define a tolerogenic DC. These properties include lack of production of IL-12p70 and resistance to CD40 ligation. We have demonstrated that both ipDC and iMAF-DC lack the capacity to secrete IL-12p70 upon stimulation with LPS and agonistic CD40 antibody suggesting that both populations of DC may have the ability to adopt a tolerogenic phenotype.

Morelli *et al.* also suggested that low MHC expression might favour a tolerogenic phenotype, which likewise describes ipDC. However we have consistently found MHC class II expression to be problematic, with only some cultures expressing MHC class II.

We also successfully derived novel iPS cell lines from MAF and compared iPS cells and DC differentiated from them directly with MEF-derived iPS cells. We were unable to show any difference in phenotype between ipDC and iMAF-DC with respect to their expression of conventional DC markers and expression of MHC class II. This may suggest that the cell of origin that is reprogrammed into a pluripotent state may not directly influence the phenotype of the DC differentiated from it. It is, therefore, more plausible that the culture conditions which induce differentiation from a pluripotent state may have more of an effect on DC phenotype than the starting cell population. This comparison between MAF and MEF derived DC is also important for any downstream clinical application, as derivation of iPS cell lines from adult fibroblasts would be a more clinically relevant scenario than derivation from embryonic tissue.

It is most likely that the observation of variable expression of MHC class II in culture is related to other cell types within the culture environment. Given the chaotic and inherently stochastic nature of differentiation and the wide range of cell types observed in culture, it is possible that certain growth factors, secreted by other cells in culture, may down-regulate

MHC class II expression in ipDC and iMAF-DC. Indeed, it has been shown in bmDC that IL-10 inhibits the expression of CIITA during differentiation (Choi *et al.* 2009). Given that we have demonstrated that ipDC secrete significant amounts of IL-10, this may be acting to down-regulate MHC class II expression. Alternatively, other cell types in culture which may secrete IL-10 could be acting indirectly to cause MHC class II down-regulation. To explore this further, it would be interesting to examine whether ipDC which express or lack MHC class II secrete different amounts of IL-10 or to correlate levels of IL-10 in the supernatant of cultures with the ultimate phenotype of ipDC.

Given that the ability for DC is reliant upon presentation of peptide to naïve T cells, it is most likely that ipDC which do not express MHC class II would be unable to induce tolerance *in vivo.* Ideally, only ipDC which express MHC class II should be harvested and used to further examine their tolerogenic phenotype *in vivo,* pending resolution of the aggregation of DC clusters, as discussed in the previous chapter.

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Chapter Five:

An investigation into the immunogenicity of syngeneic iPS cells

5.1 Introduction

It was hoped through the development of iPS cells in 2006 by Takahashi *et al.*, it would now be possible to derive pluripotent stem cells in a completely autologous fashion, thereby overcoming any immunological issues associated with transplantation of differentiated cells. However, the validity of this hypothesis was called into question in 2011 by Zhao *et al.* who showed that transplantation of undifferentiated iPS cells into syngeneic recipients, resulted in their rapid immunological rejection, characterised by the presence of an extensive infiltrate of $CD4^+$ and $CD8^+$ T cells. The rapid rejection observed upon transplantation was linked to the up-regulation of various developmental antigens including *Hormad1, Zg16* and *Cyp3a1*, which occurred upon reprogramming of somatic cells back to a pluripotent state. These antigens are normally only expressed in early embryonic development and are, therefore, down-regulated prior to organogenesis of the thymus and hence, the development of the immune system. Furthermore, their expression in adulthood has been shown to be confined to immune privileged sites, such as the testis. This expression pattern may ensure that T cells specific for such developmental antigens are not purged from the repertoire during negative selection in the thymus and may, therefore, persist into the periphery. Indeed, the authors were able to also demonstrate that ES cells expressed significantly lower levels of these developmental antigens than their iPS cell counterparts and, furthermore, that forced expression of these genes in ES cells resulted in their rapid rejection by syngeneic recipients. These findings therefore provide weight to a working hypothesis that might explain the paradoxical rejection of tissues that were genetically identical to the recipient. Nevertheless, the findings on which this hypothesis is founded has a number of important deficiencies.

Firstly, the strain of mouse from which iPS cells were originally derived and into which they were subsequently transplanted may play an important role in the immunological rejection observed. Zhao *et al.* (2011) used C57Bl/6 mice which are poised towards immunity and

have been shown over many years to be a strain in which tolerance is very difficult to establish. Indeed, had strains been used which were naturally more skewed towards tolerance, such as the CBA/Ca mouse, rejection may not have been observed. Secondly, Zhao *et al.* (2011) chose to derive iPS cells through the re-programming of embryonic fibroblasts. As embryonic fibroblasts (MEF) originate from embryos of mid-stage gestation, these tissues may have a natural tendency to express developmental antigens. If adult fibroblasts, or indeed any other adult tissue, had been chosen as the starting population of cells for reprogramming, the subsequent expression of these developmental antigens following reprogramming may have been different. Lastly, Zhao *et al.* also chose to examine the rejection of undifferentiated iPS cells injected subcutaneously, rather than their differentiated progeny. It is possible that the developmental antigens identified may be highly expressed in their pluripotent state but down-regulated upon differentiation. Therefore, administration of undifferentiated iPS cells may provide a maximal stimulus to the immune system if these antigens are expressed at significantly higher levels than their differentiated counterparts. This is particularly important since, in the context of regenerative medicine, the administration of undifferentiated iPS cells would be considered to carry far too great a risk of tumorigenesis, requiring the administration, instead, of fully-differentiated replacement cell types or tissues.

Given how fundamental the issues of immunogenicity are to the vision of personalised medicine using iPS cells, we sought in this chapter to investigate this controversy that has arisen surrounding the findings of Zhao *et al.* and to determine whether there is any basis to their claims of immunological rejection of syngeneic iPS cell grafts.

5.2 Results

5.2.1 Defining rejection versus acceptance of tissue derived from pluripotent stem cells

In order to be able to interpret the outcome of experiments involving the transplantation of tissues differentiated from iPS cells, it was first necessary to identify which parameters might be usefully employed to define rejection in this unique context. This was particularly important, given that the immune response directed to syngeneic iPS cells might be expected to be far less vigorous than a full allogeneic response, since the disparity would be restricted to a few developmental antigens rather than a full MHC difference.

In order to define these parameters, we made use of ES cells and transplanted them in a syngeneic setting into CBA/Ca recipients thereby setting a gold standard for immunological acceptance. To mimic the scenario in which the immunological disparity is intentionally negligible, we transplanted ES cells from CB/K mice, differing at a single MHC class I locus (H-2K^{b}) into CBA/Ca recipients. These same ES cell lines have been fully characterised previously and used to define the immunogenicity of ES cell-derived tissues, Robertson *et al.* (2007) showing that ES cells differing at a single MHC class I molecule are susceptible to rejection.

We, therefore, implanted EB from the syngeneic male ES cell line ESF-116, or semi-allogeneic male ES cell line ESF-166 into male CBA/Ca recipients. EB were transplanted under the kidney capsule as described in *Methods* for a period of 21 days and resulting teratomas were harvested and their size relative to the size of the kidney was determined, along with the extent of tissue damage evident from histological analysis using H&E staining of tissue sections. Additionally, the presence of an inflammatory infiltrate was assessed using immunohistochemistry.

As shown in Figure 5.1, significant variability in the size of teratomas, relative to the recipient kidney, was encountered, despite EBs being transplanted into syngeneic recipients. This suggests that, in a setting in which the immune system has no influence on the survival or otherwise of EB, numerous factors in the complex *in vivo* environment influence the outcome of teratoma formation and the extent of subsequent tissue growth. Nevertheless, the data presented in Figure 5.1 show that at day 21 post-implantation, syngeneic ES cell teratomas are never smaller than 20% of the size of the recipient kidney.

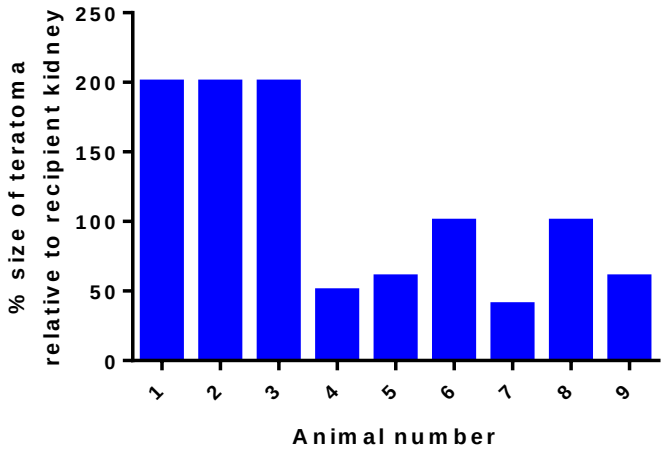

Figure 5.1 Transplantation of syngeneic ES-EB into CBA/Ca recipients. EB were transplanted under the kidney capsule of syngeneic recipients. Any resulting teratomas were harvested 21 days after implantation. Sizes of teratomas are expressed as relative percentage to the size of the kidney.

We next sought to examine whether transplantation of semi-allogeneic ES cells into CBA/Ca recipients displayed similar levels of variability in size of teratoma formation. Indeed, as shown in Figure 5.2, although considerable variation in teratoma size was again observed, the size of teratomas in this setting never exceeded 20% the size of the kidney. Therefore, a size of 20% was chosen as an arbitrary standard that gave a rough indication as to the likelihood

of rejection occurring, which might subsequently be confirmed by histological analysis to determine the presence or otherwise of an inflammatory infiltrate.

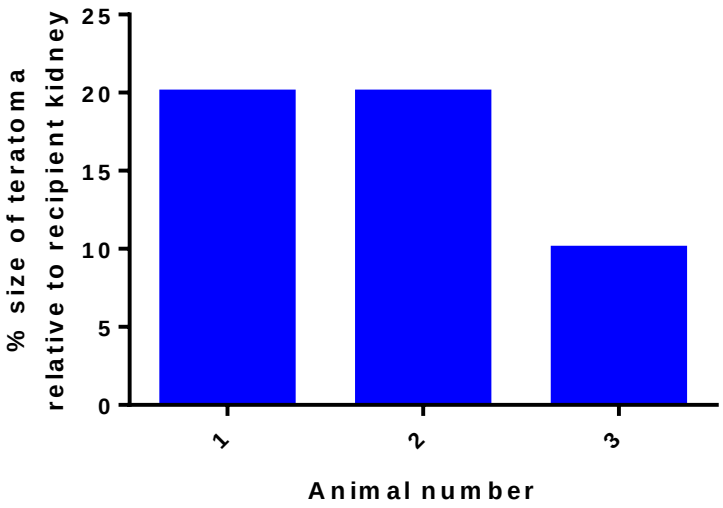

Figure 5.2 Transplantation of single-MHC mismatched ES-EB into CBA/Ca recipients. EB were transplanted under the kidney capsule of recipients. Any resulting teratomas were harvested 21 days after implantation. Sizes of teratomas are expressed as relative percentage to the size of the kidney.

Following the findings outlined above, we performed in-depth histological analysis of teratomas from syngeneic CBA/Ca ES cell grafts. In Figure 5.3 below, panels (a) and (b) show H&E staining from a syngeneic ES cell graft displaying the formation of healthy tissue along with a range of cell types and anatomical structures, characteristic of those formed by pluripotent stem cells. Furthermore, no infiltration of $CD3^+$ T cells could be detected as shown in panels (c) and (d).

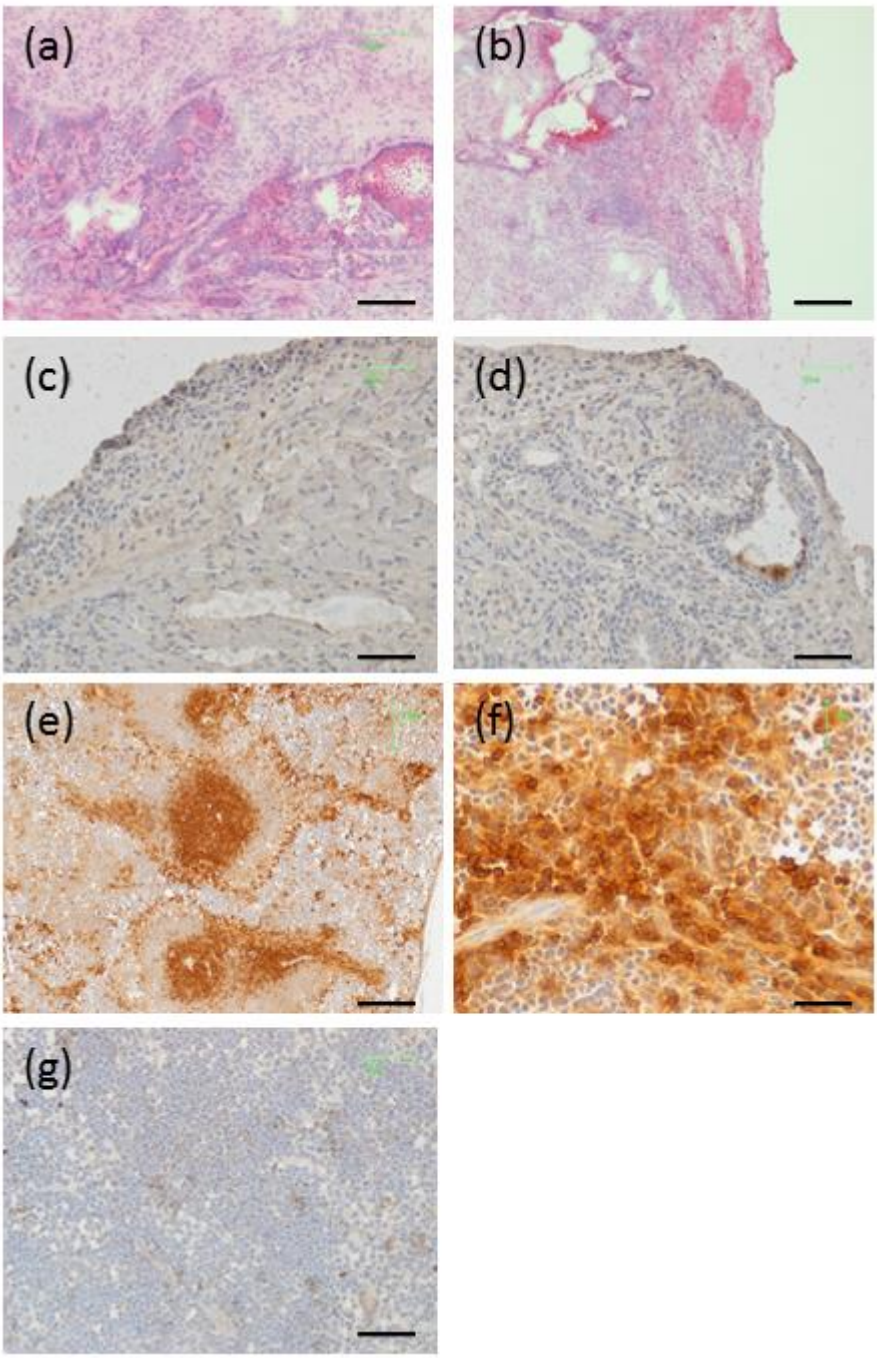

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Figure 5.3 H&E stains of syngeneic ES cell teratoma and lack of CD3 infiltrate. Panels (a) and (b) show H&E stains of syngeneic ES cell teratoma tissue: scale bars are representative of 100µm. Panels (c) and (d) show two representative fields of view of one teratoma, stained with a monoclonal antibody against CD3 demonstrating lack of infiltrate: scale bars are representative of 50µm. Panels (e) and (f) show staining of spleen sections with a monoclonal antibody against CD3 demonstrating extensive staining of follicles in spleen: scale bars are representative of (e) 200µm and (f) 50µm. Panel (g) represents matched isotype control.

Despite the lack of $CD3^+$ T cells in syngeneic grafts, we were able to detect the presence of resident F4/80 macrophages, even in a syngeneic setting which is consistent with previous findings from the laboratory that their presence does not correlate with either acceptance or rejection (Lui *et al.* 2010), as demonstrated in panel (a) and (b) in Figure 5.4. Nevertheless, such macrophages are small in number and display a well-defined morphology, suggestive of a resting, anti-inflammatory phenotype.

NK cells have been shown to be an important effector cell in the rejection of ES cell derived tissues due to the low expression of MHC class I (Rideout *et al.* 2002). We, therefore, used the marker NKp46 as a marker for NK cells since NK1.1, which is more conventionally used for this purpose, is an allotypic marker, not expressed by CBA/Ca mice. As demonstrated in Figure 5.4, despite the consistent presence of small numbers of strongly-stained cells in control sections of spleen (panels (g) and (h)), we were unable to detect the presence of NK cells in syngeneic ES cell recipients, as shown in panels (c) and (d).

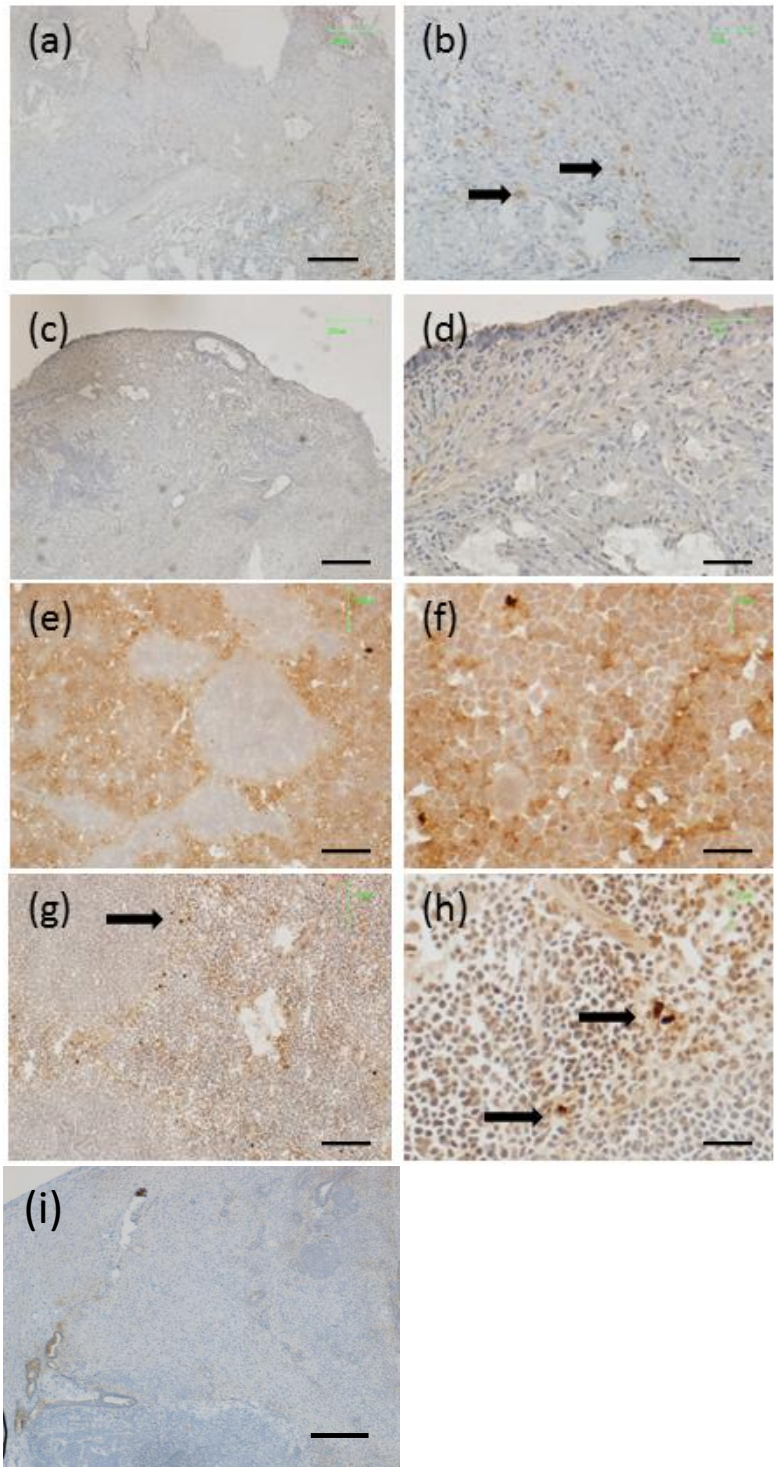

Figure 5.4 Presence of F4/80^{+} macrophages in syngeneic ES cell grafts, but lack of infiltrating NK cells. Panels (a) and (b) show two representative fields of view of one teratoma, stained with the monoclonal antibody F4/80, demonstrating the presence of macrophages: scale bars represent (a) 200µm and (b) 50µm. Panels (c) and (d) show lack of staining with monoclonal antibody NKp46: scale bars represent (c) 200µm and (d) 50µm. Panels (e) and (f) show staining of control spleen sections with F4/80: scale bars represent (e) 200µm (f) 25µm. Panels (g) and (h) show spleen stained with a monoclonal antibody specific for NKp46: scale bars represent (g) 100µm and (h) 25µm. Panel (i) represents matched isotype control: scale bars represent 100µm.

Further to our earlier findings that MHC class I-mismatched ES-EB appear to undergo rejection, we sought to further characterise the events involved by performing histological analysis on sections of teratoma tissue.

As shown in Figure 5.5, panels (a) and (b) demonstrate a significantly more impoverished differentiation of cell and tissue types which has been shown previously by the laboratory to be indicative of ongoing rejection (P. Fairchild, personal communication) and evidence of tissue damage, as highlighted by the arrows.

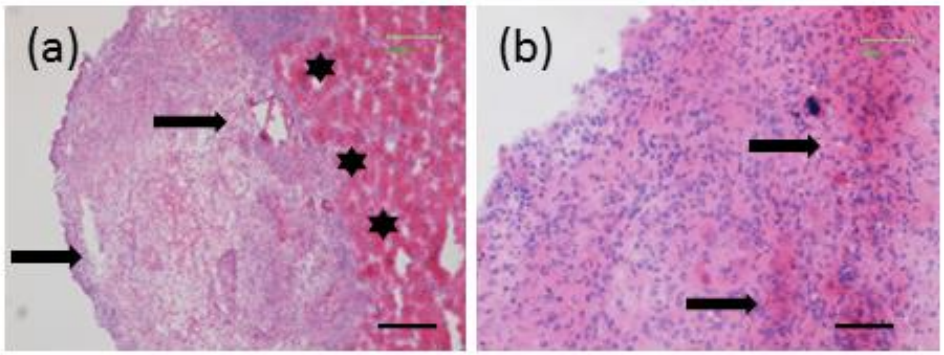

Figure 5.5 H&E staining of MHC class I-mismatched ES-EB teratomas. Arrows indicate areas of putative tissue damage. Asterisks and dotted line highlight the border between the teratoma and the recipient kidney. Scale bars represent (a) 200µm and (b) 100µm.

Further to this, the presence of a $CD4^+$ and $CD8^+$ T cell infiltrate was detected, as shown in Figures 5.6 and 5.7, respectively.

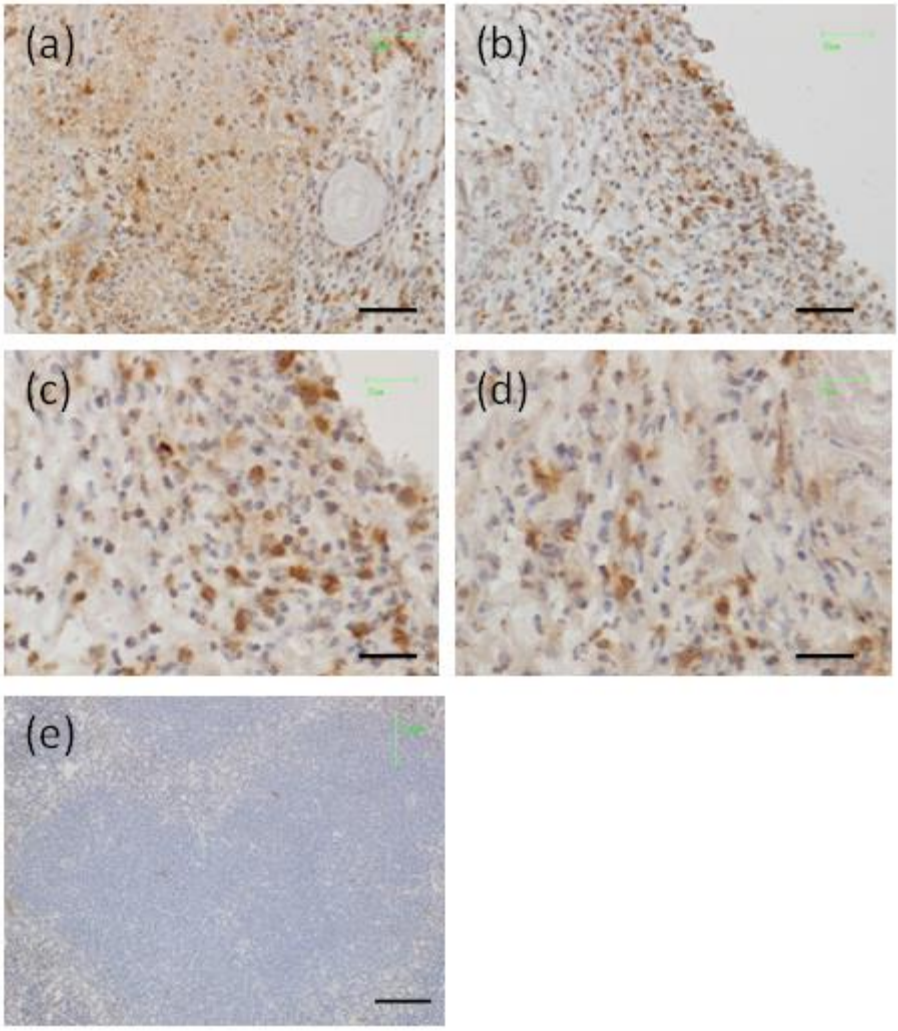

Figure 5.6 Infiltration of CD4$^+$ T cells in MHC class I-mismatched ES teratomas. Panels (a) - (d) show four representative fields of view of one teratoma, stained with a monoclonal antibody specific for CD4: scale bars represent (a-b) 50µm (c-d) 25µm. (e) represents matched isotype control.

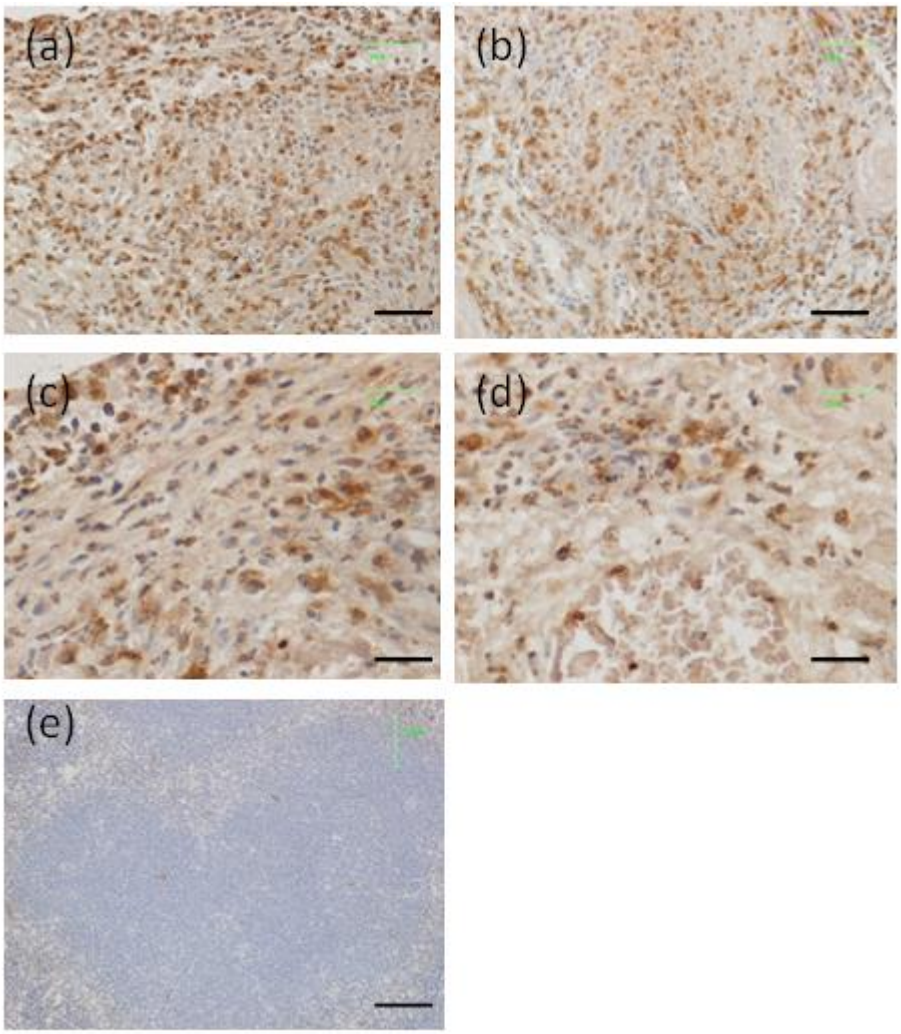

Figure 5.7 Infiltration of CD8$^+$ T cells in MHC class I-mismatched ES teratomas. Panels (a) - (d) show four representative fields of view of one teratoma, stained with a monoclonal antibody specific for CD8: scale bars represent (a-b) 50µm (c-d) 25µm. (e) represents matched isotype control.

Additionally, a significant infiltrate of macrophages was evident, with individual cells having poorly-defined borders consistent with activated pro-inflammatory cells, as demonstrated in Figure 5.8.

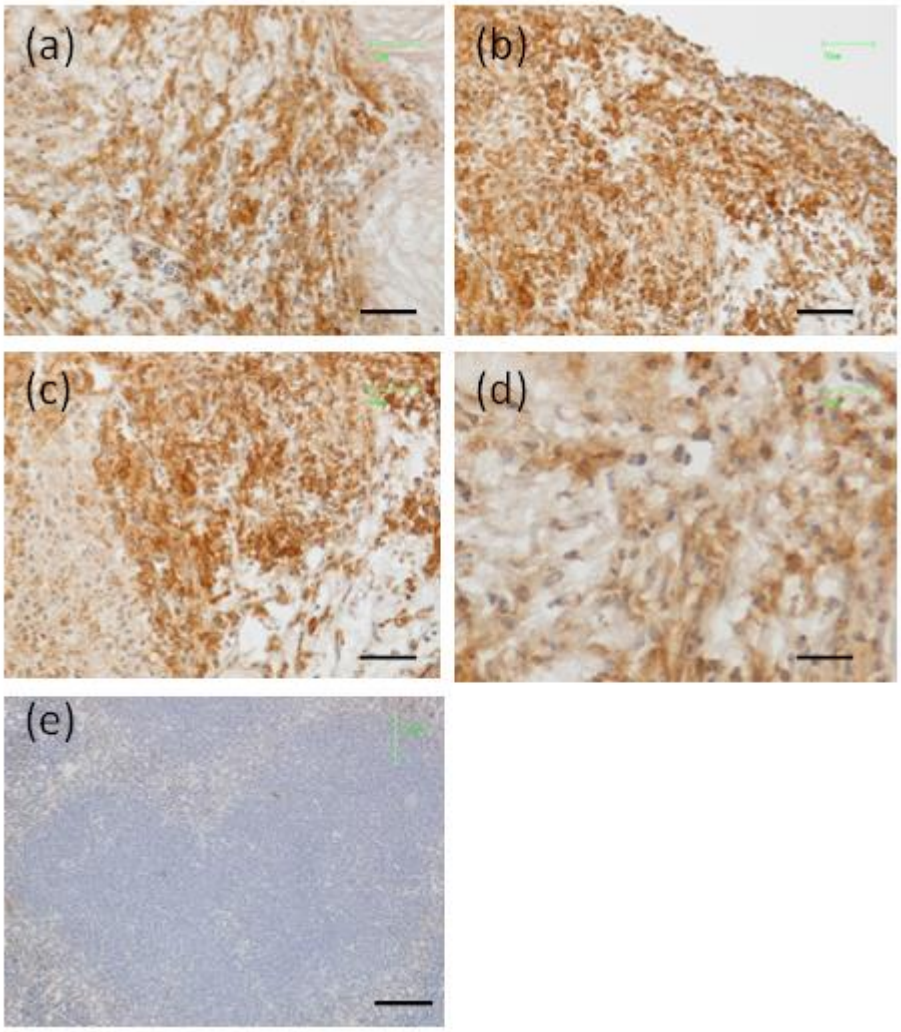

Figure 5.8 Infiltration of F4/80^{+} cells in MHC-class I mismatched ES teratomas. Panels (a) - (d) show four representative fields of view of one teratoma, stained with a monoclonal antibody specific for F4/80 demonstrating the presence of activated macrophages: scale bars represent (a-b) 50µm (c-d) 25µm. (e) represents matched isotype control.

Having carefully defined readouts that would provide a robust indication of acceptance versus rejection of stem cell derived tissues, we next investigated whether iPS cell-derived EB could be recognised as foreign when transplanted into syngeneic and sex-matched recipients.

5.2.2 Is there evidence for the rejection of iPS cells by syngeneic recipients?

Original claims of rejection by Zhao *et al.* (2011) were based on the administration of iPS cells from C57Bl/6 mice to syngeneic recipients but this choice of strain, as discussed earlier, may have contributed to their unexpected observations. C57Bl/6 mice are naturally poised towards immunity and are naturally resistant to protocols of tolerance induction, unlike the CBA/Ca strain which is used preferentially in studies of transplantation tolerance. By choosing a mouse strain in which the tone of the immune system is poised towards immunity rather than tolerance, responses to developmental antigens may have been more readily uncovered, but these may not be physiologically relevant in other strains of mice, and indeed, in a human setting.

To test this hypothesis, we made use of the novel male iPS cell line, iPS-1, derived and characterised fully in Chapter Three. This line was derived from reprogrammed MEF from CBA/Ca mice in which the default status of the immune system is self-tolerance rather than immunity. We, therefore, transplanted EB under the kidney capsule of syngeneic male recipients, a setting with no known immunological disparity.

As shown in Figure 5.9, although one recipient sustained a teratoma approximately three times the size of the host kidney, all others produced teratomas of far more limited size. When applying the criteria of size defined using ES-EB that reject due to a single MHC class I disparity, 13/19 recipients had teratomas less than 20% the relative size of the kidney which might suggest their on-going rejection. Furthermore, 6/19 mice had no visible teratoma tissue at all. These were unlikely to be technical failures since all control syngeneic ES-EB transplanted in parallel, developed teratomas as anticipated. In order to determine whether teratomas of less than 20% the size of the recipient kidney were restricted in their growth due

to an on-going immune response, we assessed rejection as a function of tissue damage and presence of an inflammatory infiltrate.

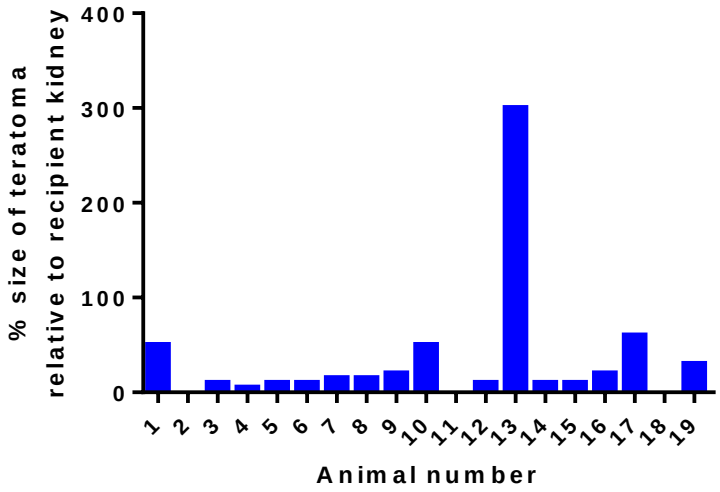

Figure 5.9 Grafting of syngeneic iPS-derived EB. iPS-EB were derived as described in *Methods,* and transplanted under the kidney capsule. Any resulting teratomas were harvested 21 days after implantation. Size of teratomas are expressed relative percentage to size of kidney. Data shown is representative of experiments conducted with transplantation of syngeneic ES cell side-by-side in which successfully formed teratomas.

As shown in panels (a) and (b) in Figure 5.10, H&E staining showed no obvious evidence of tissue damage and sections typically showed a broad range of tissue types and anatomical structures, which appeared healthy.

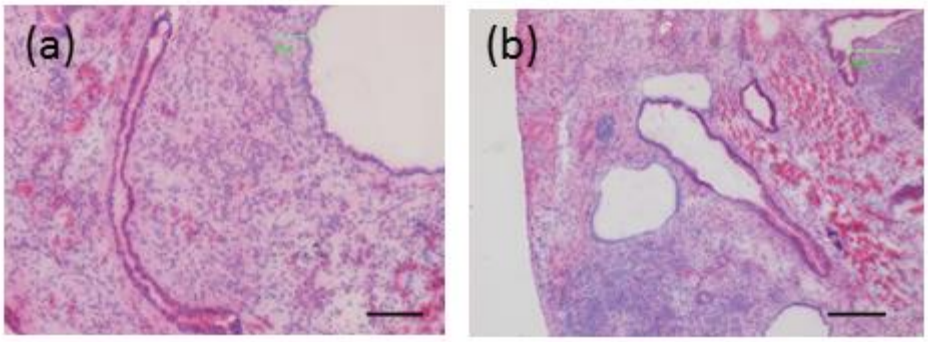

Figure 5.10 H&E staining of syngeneic iPS-EB teratomas H&E staining of syngeneic iPS teratomas reveals no signs of obvious tissue damage (a) and (b). Scale bars represent (a) 75µm and (b) 200µm.

Following this, we sought to examine whether we could detect any inflammatory infiltrate. Staining syngeneic iPS teratomas revealed a very sparse infiltrate of $CD3^+$ T cells which, given their numbers, were not likely to be responsible for limiting tissue growth and differentiation, as demonstrated in panels (a) and (b) in Figure 5.11. Furthermore, analysis of F4/80 staining revealed the presence of sparse populations of macrophages, their size and distribution being more reminiscent of those observed in Figure 5.4 in syngeneic ES-EB teratomas, rather than those found in the vigorous infiltrate characteristic of the rejecting semi-allogeneic ES-EB (Figure 5.8), as demonstrated in panels (c) and (d). In addition, we also examined the presence of NK cells by staining for the marker NKp46. Staining revealed the presence of rare, strongly-stained cells, similar to those observed in spleen sections from immunologically naïve mice, as shown in panels (e) and (f).

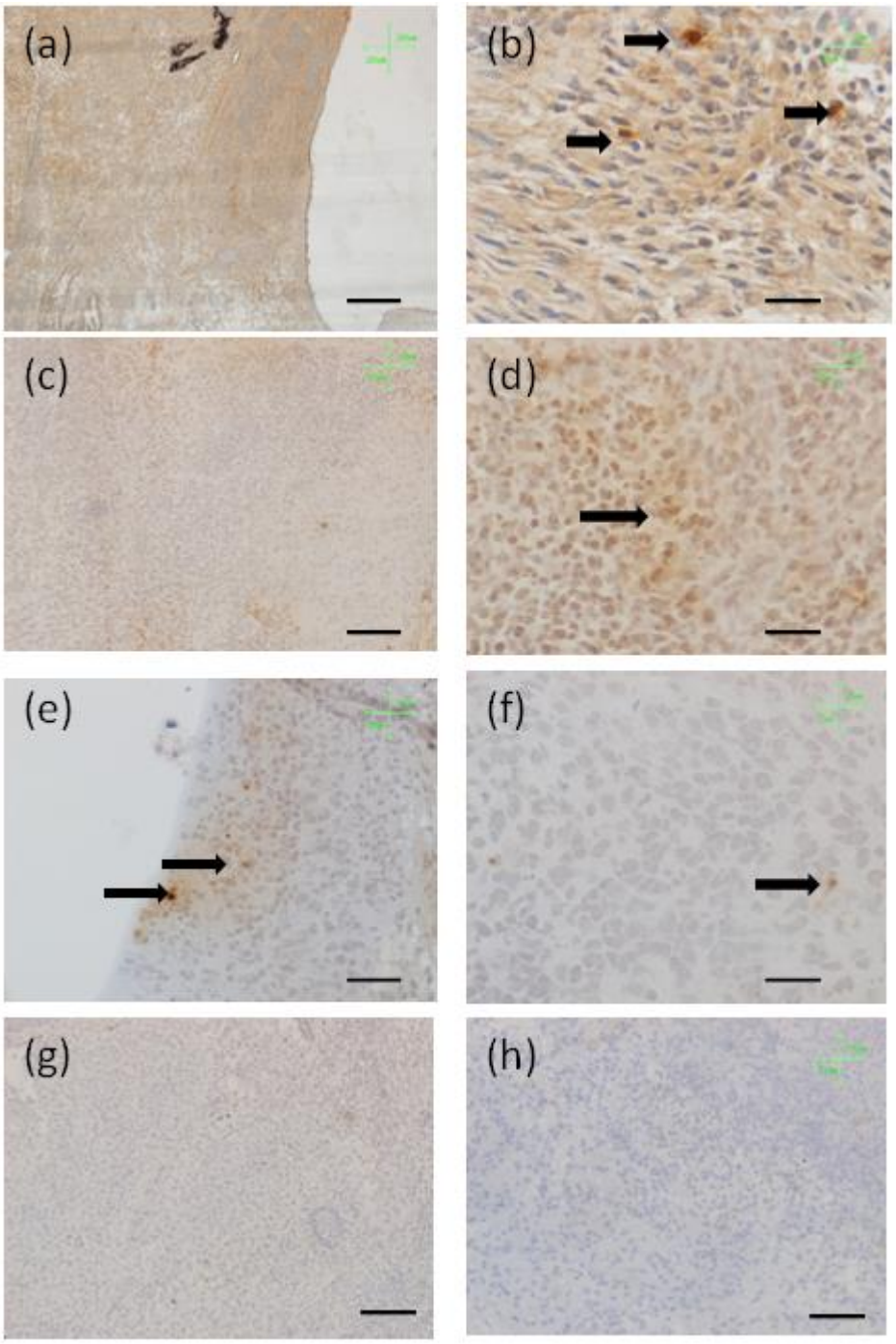

Figure 5.11 Immunohistochemical analysis of syngeneic iPS cell teratomas, for the presence of T cells, macrophages and NK cells. Panels (a) and (b) show sections of syngeneic iPS cell teratoma stained with monoclonal antibody specific for CD3, demonstrating a minor population of $CD3^+$ T cells. Scale bars represent (a) 200µm and (b) 25µm. Panels (c) and (d) show syngeneic iPS cell teratoma stained with the F4/80 monoclonal antibody, demonstrating a minor population of infiltrating macrophages. Scale bars represent (c) 100µm and (d) 25µm. Panels (e) and (f) show syngeneic iPS cell teratoma stained with monoclonal antibody specific for NKp46, demonstrating a minor population of NK cells. Scale bars represent (e) 50µm and (f) 25µm. (g) and (h) represent matched isotype control for (g) CD3, F4/80 (h) NKp46

Despite the presence of NK cells, the numbers detected are unlikely to be responsible for rejection of the tissue although our transplantation model permits intervention at one time point only and, therefore, provides little more than a snapshot of events occurring *in vivo.* It is, therefore, possible that early influx of effector cell types such as $CD3^+$ T cells, macrophages and/or NK cells may have initiated rejection, but the infiltrate may have subsequently dispersed, given the low level of immunological challenge presented by the expression of developmental antigens. Nevertheless, it is uncertain why the inflammatory infiltrate would not have persisted until day 21, as seen in EB transplanted from MHC class I-mismatched ES cell lines. Our data are, therefore, consistent with the findings of Zhao *et al.* (2011) who likewise found very variable outcomes upon sub-cutaneous administrations of undifferentiated iPS cells. Zhao *et al.* (2011) were able to show that in 6/64 mice, teratomas were in a 'regressing' state, 7/64 formed no detectable teratoma at all and 51/64 mice formed detectable teratomas.

Despite the consistent variability observed with transplantation of iPS cell derived EB, our experiments failed to corroborate the findings of Zhao *et al.* (2011) with respect to the presence of an inflammatory infiltrate of $CD3^+$ T cells, reflecting, more closely, the results of Guha *et al.* (2013) who were unable to find any infiltrating $CD3^+$ T cells in syngeneic iPS cell teratomas. Since the size of the teratomas does not appear to correlate with other cardinal signs of rejection, we were unable to show that the limitations in teratoma growth have any immunological basis.

5.2.3 Does the source of fibroblasts from which iPS cells are derived influence the outcome of grafting?

Our results partially replicate the data of Zhao *et al.* (2011) confirming the highly variable survival of iPS-cell derived tissues when transplanted into syngeneic CBA/Ca recipients.

Nevertheless, the absence of an inflammatory infiltrate at day 21 post-transplant appears to undermine the immunological basis of these observations but is subject to the caveat that tissues may attract an early wave of infiltrating cells at a stage when the implanted tissues express the highest level of development antigens; this infiltrate may, however, subsequently wane as antigen expression and hence the magnitude of the stimulus progressively decreases during differentiation. Importantly, our iPS-1 cell line and the iPS cell line used by Zhao *et al.* (2011) were both derived from embryonic fibroblasts which may, therefore, have an intrinsic pre-disposition to express developmental antigens such as *Hormad1,* due to their embryonic origin. We, therefore, sought to investigate whether iPS cell lines derived from adult rather than embryonic fibroblasts behave similarly in our transplantation model. Such issues have important implications since human iPS cell lines for future clinical applications would necessarily be derived from adult rather than embryonic tissues.

We made use of the male CBA/Ca iPS cell line iMAF-6, derived from adult mouse tail-tip fibroblasts and fully characterised in Chapter Four as a source of EB which were grafted under the kidney capsule of male CBA/Ca recipients and harvested 21 days later. As shown in Figure 5.12, of the cohort of 10 recipients used, only 2 retained any detectable tissue although in either case, teratomas were less than 10% of the size of the recipient kidney suggestive of on-going rejection. The remaining 8 recipients had no surviving tissue at the site of implantation. Technical failures were an unlikely explanation as all control mice receiving ES-EB in parallel all developed teratomas, as expected.

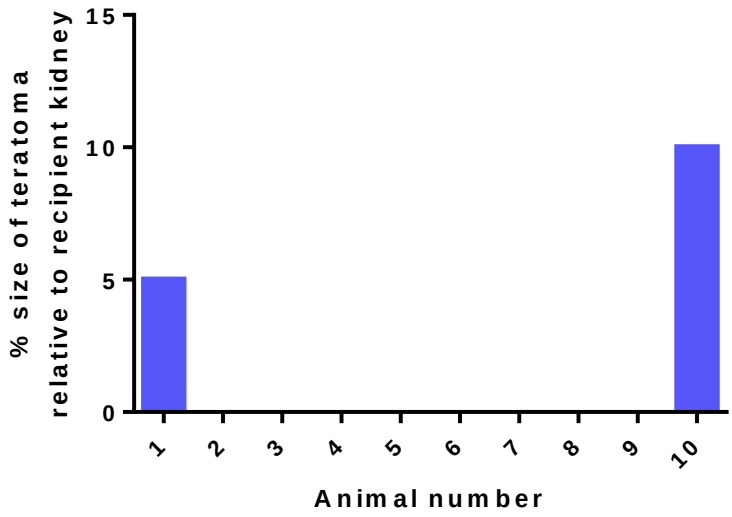

Figure 5.12 Transplantation of iMAF-6 into syngeneic recipients. iMAF6-EB were derived as described in *Methods,* and transplanted under the kidney capsule of male CBA/Ca recipients. Any resulting teratomas were harvested 21 days after implantation. Sizes of teratomas are expressed as relative percentage to the size of the kidney. Data shown are representative of experiments conducted with transplantation of syngeneic ES-EB side-by-side in which teratomas successfully formed.

Our findings suggest that the developmental stage of the cell type of origin from which iPS cells are initially derived has little influence on their subsequent survival in syngeneic recipients. Furthermore, these results confirm that iPS cells behave very differently from their ES cell counterparts when grafted *in vivo,* although our observations call into question the extent to which immunological factors play a role in the failure of engraftment. In order to further investigate whether there is any underlying immunological basis to these findings, we next studied the expression of one of the developmental antigens implicated in the rejection of syngeneic iPS cells by Zhao *et al.,* the gene product encoded by the *Hormad1* gene.

5.2.4 Investigation into the expression of *Hormad1*

Apart from being implicated in role the immunological rejection of syngeneic iPS cells, *Hormad1* has been shown to play an important role in the pairing of homologous chromosomes and formation of synaptonemal complexes during meiosis (Shin *et al.* 2010).

This confines expression primarily to tissues involved in gametogenesis and, in particular, the testis in males. Nevertheless, the fact that *Hormad1* is known to be up-regulated in some tumours and can serve as a tumour associated antigen (Chen *et al.* 2005) suggests that antigen specific T cells persist into the periphery which might also respond to iPS cell derived tissues ectopically expressing the gene. We, therefore, sought to examine the expression of *Hormad1* in iPS and ES cell teratomas, testis and additionally, the thymus using immunohistochemistry.

As shown in Figure 5.13 panels (a) and (b), staining of tissues using a polyclonal rabbit anti-serum specific for *Hormad1* showed strong expression within the testis: the cells around the periphery of seminiferous tubules showed strongly stained nuclei, whereas those towards the centre showed far weaker expression in both the nucleus and cytoplasm. Interestingly, staining of tissue sections from syngeneic iPS cell derived teratomas, (c) and (d), revealed levels of staining above background levels. Areas of diffuse staining were apparent as well as foci of strongly stained cells in which the expression of the target antigen appeared to be confined to the nucleus, a pattern of staining similar to cells in the testis. Indeed, given the recent reports of the capacity of iPS cells to sustain spermatogenesis, the discrete foci of strongly stained cells may represent areas of gametogenesis occurring stochastically within the tissue.

Nevertheless, ES cell derived teratomas, (d) and (e), likewise displayed low levels of diffuse staining and rare intensely stained nuclei suggesting that these observations do not offer a distinction between ES and iPS cells that may explain their ability to form teratomas *in vivo*,

Although ectopic expression of *Hormad1* and other developmental antigens identified by Zhao *et al.* may render tissue susceptible to an immune response following implantation, such an outcome is wholly dependent on the presence of *Hormad1* specific T cells in the periphery

which must, therefore, have evaded the normal processes of central tolerance within the thymus. Many self-antigens confined to immune privileged sites are also expressed centrally in medullary thymic epithelial cells (mTECs) due to the activity of the *Aire* transcription factor which is responsible for promisicous gene expression (Anderson *et al.* 2002). We, therefore, investigated whether, like other autoantigens such as myelin basic protein (Targoni *et al.* 1998), *Hormad1* expression could be detected in the medulla of the thymus.

As shown in Figure 5.13 (g) and (h), preliminary experiments revealed the presence of rare intensely stained cells confined solely to the medulla of the thymus similar to the pattern of expression reported for other known autoantigens (Derbinksi *et al.* 2001). Nevertheless, inspection of negative control slides paradoxically revealed a similar staining pattern.

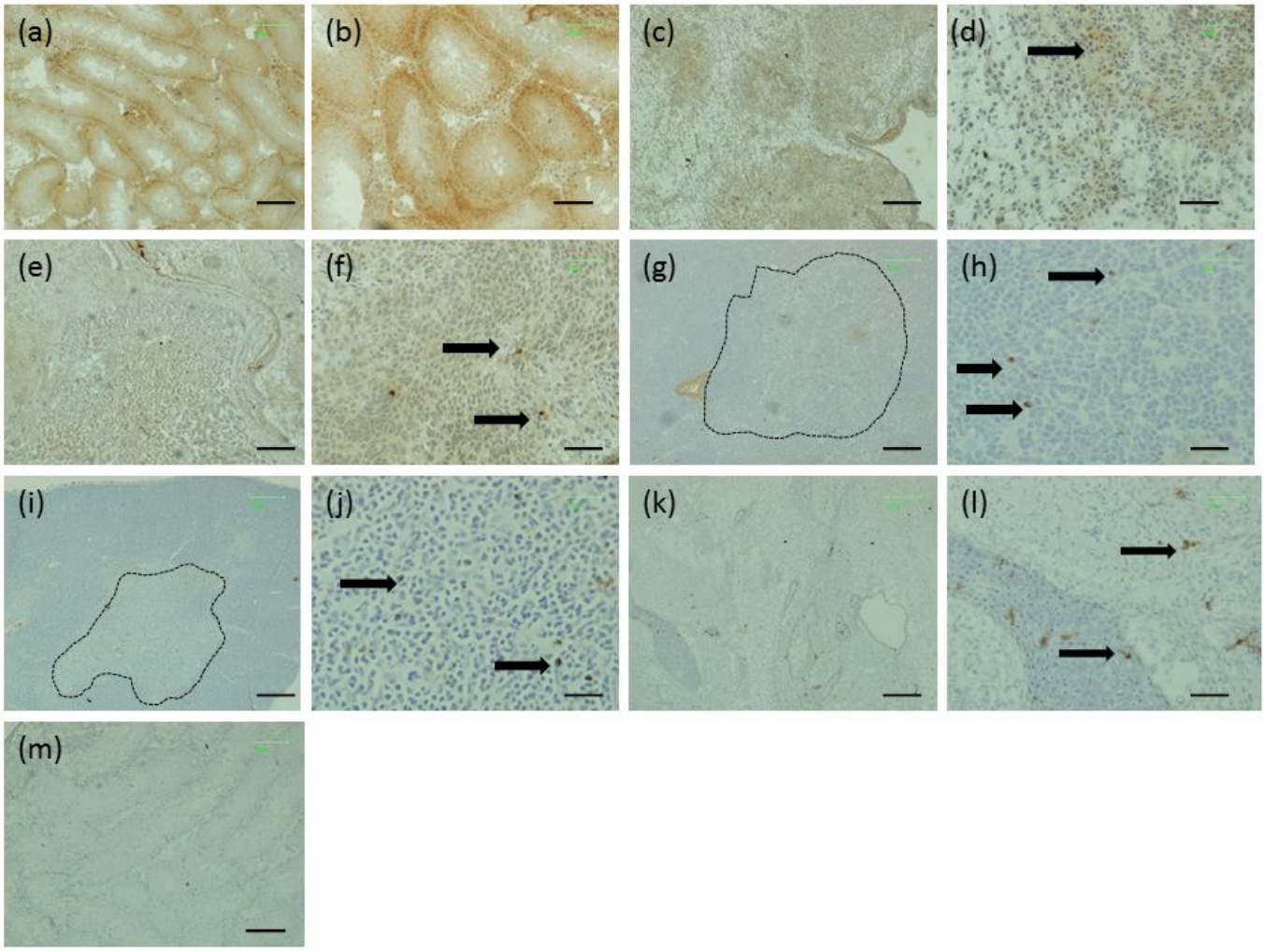

Figure 5.13 Expression pattern of Hormad1 determined by IHC. Panels (a) and (b) represent expression in testis, (c) and (d) iPS cell teratoma, (e) and (f) ES cell teratoma, (g) and (h) thymus, (i) and (j) thymus without primary antibody, (k) and (l) iPS teratoma without primary antibody, (m) testes control. Scale bars represent (a) 200µm (b) 100 µm (c) 100 µm (d) 50µm (e) 100µm (f) 50µm (g) 100µm (h) 25µm (i) 200µm (j) 25µm (k) 200µm (l) 50µm (m) 200µm. Broken lines demark borders of the medulla.

Although the identity of these cells remains unresolved, we have been unable to detect any evidence of *Hormad1* protein expression in the thymus, consistent with a failure of central tolerance that might permit *Hormad1*-specific T cells to escape negative selection. Nevertheless, given this anomaly and the potential of even trace amounts of protein to participate productively in clonal deletion of autoreactive T cells, we used RT-PCR to investigate the expression of *Hormad1* mRNA in the thymus as a more sensitive readout.

We, therefore, pooled thyme from 3 young adult mice and mechanically disrupted them into a single cell suspension before fractionating thymocytes and stromal cells and isolating mRNA as described in *Methods*. As shown in Figure 5.14, testis showed high expression of *Hormad1* mRNA, as expected, however no expression of *Hormad1*mRNA could be detected in either thymocytes or thymic stromal fractions which includes mTEC.

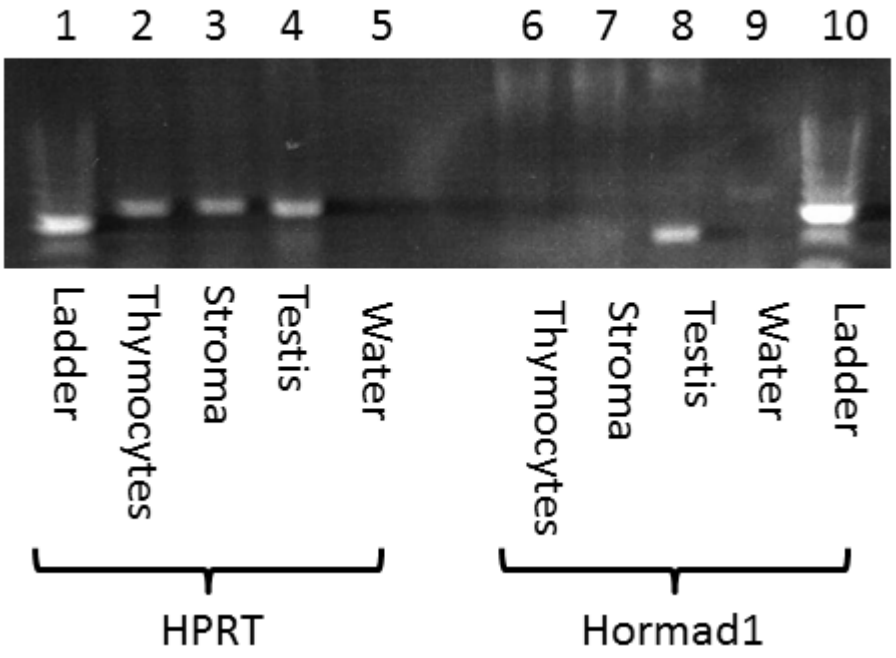

Figure 5.14 Analysis of expression of HPRT and Hormad1 in tissues. Analysis of expression of HPRT and *HORMAD1* was analysed using RT-PCR. (a) Bands represent (1) and (10) 100bp ladder and HPRT expression in (2) thymocytes (3) stroma (4) testis but not (5) water. Hormad1 expression was not found in (6) thymocytes (7) stroma (9) water but found in (8) testis.

These results are, therefore, consistent with the hypothesis that *Hormad1*-specific T cells may escape negative selection due to the absence of antigen expression in the thymus and its confinement to immunologically-privileged sites in the periphery, such as the testis. The question therefore arises as to the extent to which *Hormad1* is expressed in ES and iPS cells and whether its expression is sustained during their respective differentiation *in vitr*o prior to transplantation.

Using testis as a positive control, as shown in Figure 5.15, *Hormad1* expression is maintained equally in undifferentiated iPS and ES cells when assessed using RT-PCR. Importantly, the iMAF6 cell line derived from adult fibroblasts likewise expressed significant *Hormad1* mRNA suggesting that reprogramming of somatic cells to pluripotency is associated with the ectopic up-regulation of developmental antigens such as *Hormad1*. Importantly, as suggested by the results of our immunohistochemistry analysis, both iPS and ES cells continue to express *Hormad1* during their differentiation *in vitro*, although surprisingly ES cells retain expression to a higher degree than iPS cells. Interestingly, bmDC were used as a source of well characterised cells differentiated *in vitro* from adult rather than pluripotent stem cells and showed no detectable expression of *Hormad1*. In contrast, ipDC, differentiated from iPS cells, retained its expression following terminal differentiation, albeit at a low, but detectable level.

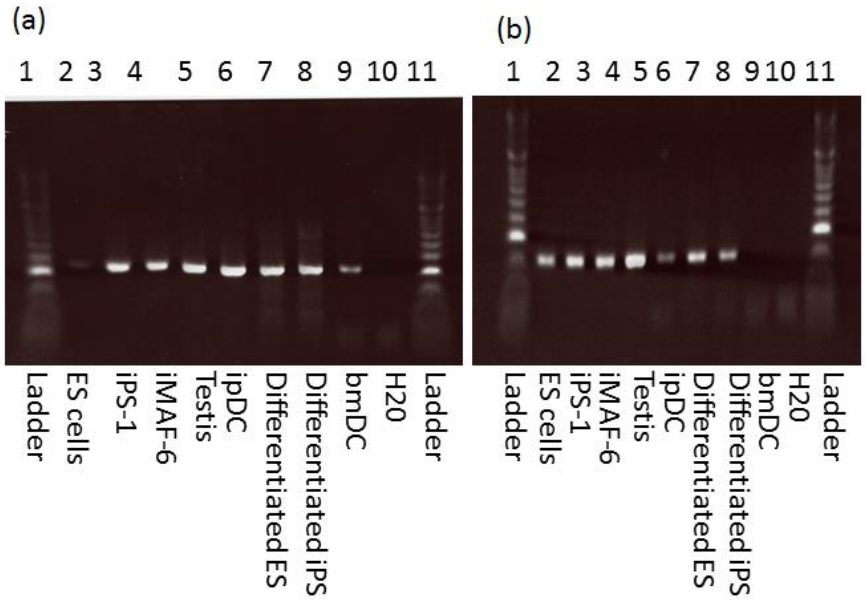

Figure 5.15 Expression of HPRT and Hormad1. Analysis of expression of (a) HPRT and (b) *HORMAD1* was analysed using RT-PCR. (a) Lanes represent (1) and (11) 100bp ladder and other lanes as described. *Note HPRT band in lane 2 (a) is weak and may not easily be visible on printed versions of this thesis.*

These results are not only consistent with the ectopic expression of *Hormad1* by iPS cell derived tissues and cell types, but confirm the basis of our original proposal that ipDC could conceivably used for the induction of tolerance to such ectopically expressed antigens.

In order to determine definitively whether erroneously up-regulated genes were responsible for the lack of formation of iPS-EB teratomas in syngeneic recipients we used two complementary approaches to investigate the extent to which the adaptive immune responses influences the outcome of grafting.

5.2.5 Investigation into the role of the adaptive immune response to the failure of iPS cell engraftment

Many of our findings are consistent with the working hypothesis we developed in response to the original findings by Zhao *et al.* (2011): lack of *Hormad1* expression in the thymus and its confinement to immune privileged sites in the adult, strong expression by iPS cells and iMAF6 cells, even upon subsequent differentiation and identification of protein expression in surviving iPS cell teratomas. Nevertheless, we also found that ES cells express *Hormad1* in both an undifferentiated and differentiated state as determined by both RT-PCR and immunohistochemistry which contrasts with the claims of Zhao *et al*.

Further to these findings it should be remembered that *Hormad1* was only one of the genes implicated in the rejection of iPS cell teratomas with several others, such as *Cyp3A111* and *Zg16* also identified as potential targets. There may, however, also be other genes involved that contribute to rejection of iPS cell teratomas that have yet to be identified. In order to further examine this hypothesis, we devised a two-fold strategy to determine unequivocally the role or otherwise that an adaptive immune response to developmental antigens might play in limiting the survival of syngeneic iPS cell teratomas. We, therefore, firstly grafted tissues into $RAG1^{-/-}$ mice which are devoid of T and B cells and are, therefore, incapable of mounting an adaptive immune response. Secondly, we induced global transplantation tolerance, previously shown to permit the acceptance of skin grafts across a full MHC barrier (Yates *et al.* 2007).

When syngeneic iPS-EB were grafted into CBA.$RAG1^{-/-}$ mice containing no T or B cells, as shown in Figure 5.16, we found significant variation in the outcomes of teratoma formation with only 1/8 mice showing sizable teratoma growth greater than 20% of the size of the kidney.

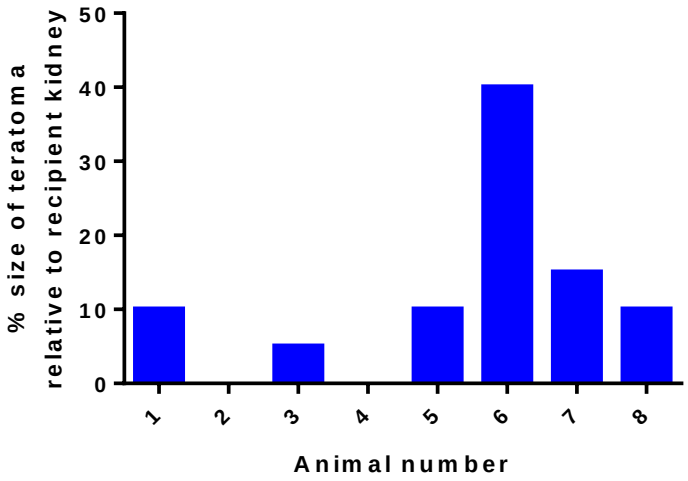

Figure 5.16 Grafting of iPS-1 into CBA.RAG1$^{-/-}$ recipients. iPS-1 EB were grafted into CBA.RAG1$^{-/-}$ recipients and harvested 21 days later and their size expressed relative to that of the recipient kidney.

Likewise, grafting of iMAF-6 into CBA.RAG1$^{-/-}$ mice yielded teratomas in only 1/3 of the recipients, the other two recipients failing to sustain any teratoma growth despite the formation of teratomas in control mice receiving ES-EB, as demonstrated in Figure 5.17.

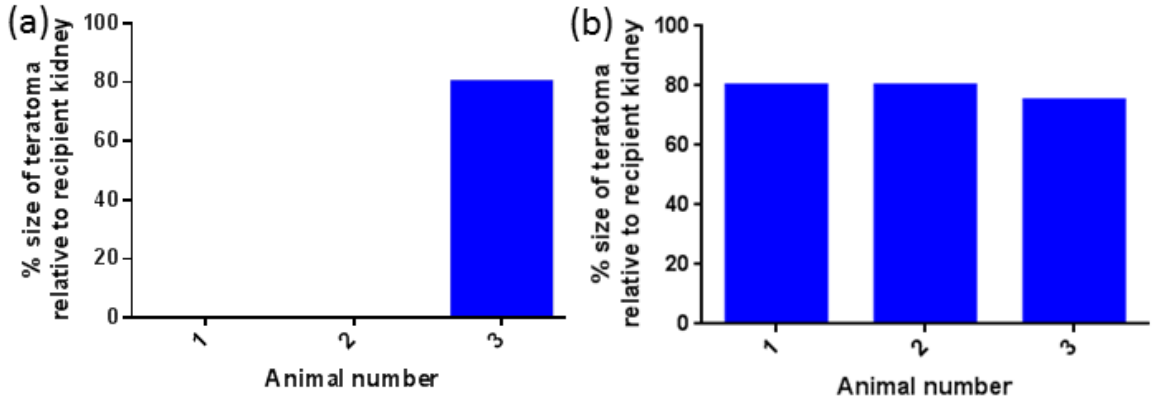

Figure 5.17 Grafting of iMAF-6 into CBA.RAG1$^{-/-}$ recipients. (a) iMAF-6 EB and (b) ES-116 were grafted into CBA.RAG1$^{-/-}$ recipients and harvested 21 days later and their size expressed relative to that of the recipient kidney.

Although in this setting the involvement of a T cell infiltrate is excluded, CBA.RAG1$^{-/-}$ mice are known to have significantly higher than normal numbers of NK cells (Kroemer *et al.*

2008). We therefore investigated the presence of macrophages and NK cells in the one surviving iMAF-6 teratoma, as shown in Figure 5.18. Although resident macrophages could be detected, they were relatively sparse in number with well-defined morphology suggestive of a resting non-inflammatory phenotype. Furthermore, while occasional NK cells could be detected, these were no more prevalent than syngeneic ES cell EB grafts.

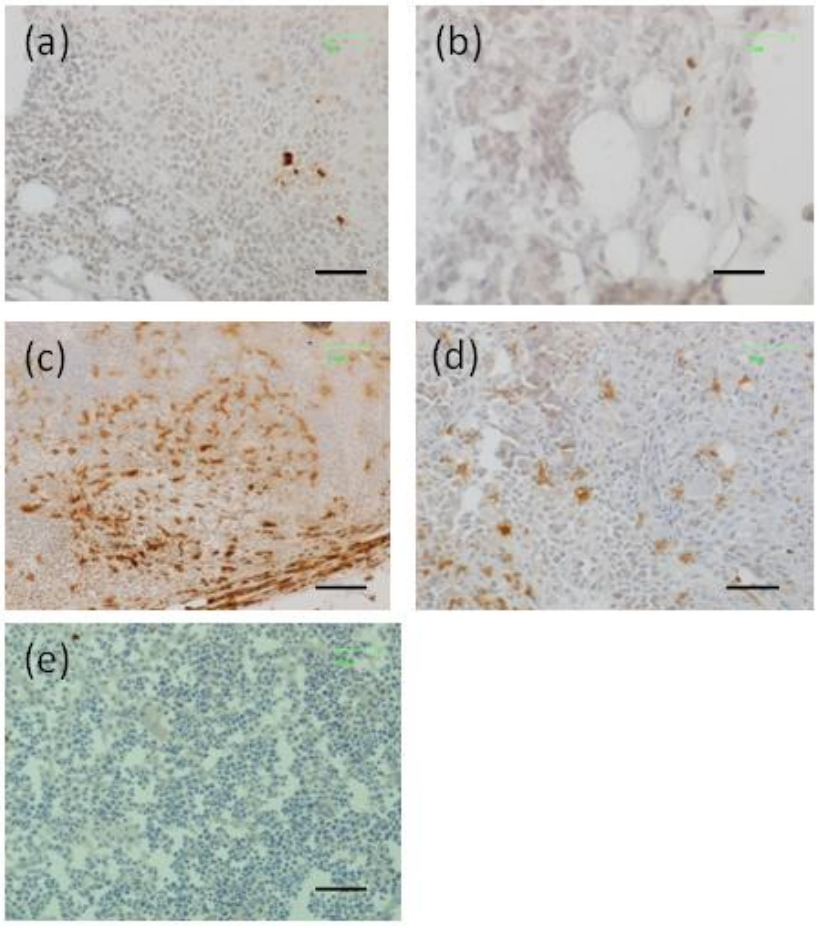

Figure 5.18 Immunohistochemical analysis of syngeneic iMAF-6 cell teratomas, for the presence of macrophages and NK cells. Panels (a) and (b) show sections of iMAF-6 teratoma stained with monoclonal antibody specific for NKp46, demonstrating a minor population of $NKp46^{+}$ cells. Scale bars represent (a) 200µm and (b) 25µm. Panels (c) and (d) show iMAF-6 cell teratoma stained with the F4/80 monoclonal antibody, demonstrating a minor population of infiltrating macrophages. Scale bars represent (c) 100µm and (d) 25µm. Panels (e) represent matched isotype control.

Given, therefore, that CBA.RAG1$^{-/-}$ recipients lack any potential to mount an adaptive immune response, the failure of teratoma formation in a significant proportion of recipients would appear to have little immunological basis.

To unequivocally confirm this conclusion using a complementary approach, we next induced global transplantation tolerance in normal mice prior to implantation of syngeneic iPS cell EB. In order to induce global tolerance, we treated recipients with a short course of non-depleting monoclonal antibodies specific for CD4, CD8 and CD40L which have been routinely used in the past for the induction of tolerance to fully allogeneic skin grafts known to be highly immunogenic (Daley *et al.* 2007). Mice were, therefore, given 1mg of each antibody intraperitoneally one day prior to transplantation and then on days 2 and 4 post transplantation. A control group received doses of PBS on the same days.

In order to demonstrate the effectiveness of this protocol in establishing tolerance in this setting, control CBA/Ca mice received fully allogeneic C57Bl/6 EB differentiated from the ES cell line ESF-75 with either antibody conditioning or PBS. At day 21 post-transplantation we found surviving tissue in all 5 recipients following antibody treatment, but only 1/4 recipients in the PBS control group had any surviving tissue. Nevertheless, our results show significant variation in size with 2/5 teratomas being less than 20% the size of the recipient kidney, as shown in Figure 5.19.

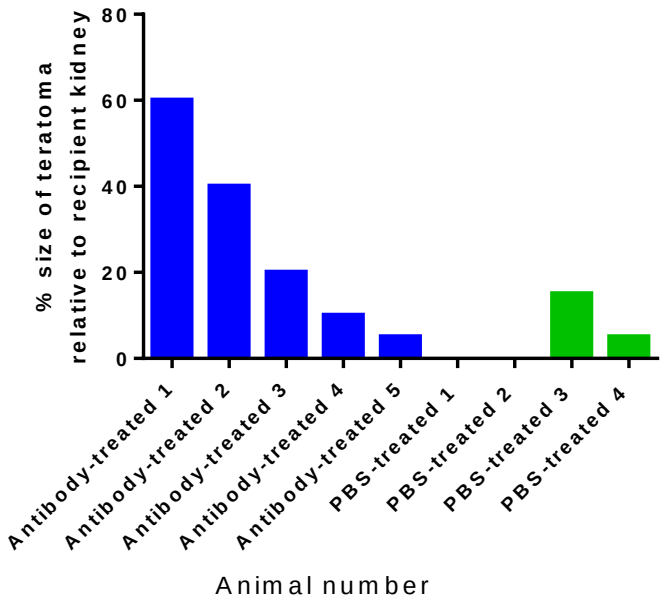

Figure 5.19 Induction of tolerance to allogeneic ES cell grafts. Tolerance to allogeneic ES-EB was induced in antibody treated mice as reflected by teratoma formation in 100% of recipients. Teratoma formation was significantly reduced in PBS-treated control mice.

When iPS-EB were implanted into syngeneic recipients, with or without antibody treatment, we found a very similar level of variation in size, as indicated in Figure 5.20, with some teratomas becoming even larger than ES-cell teratomas.

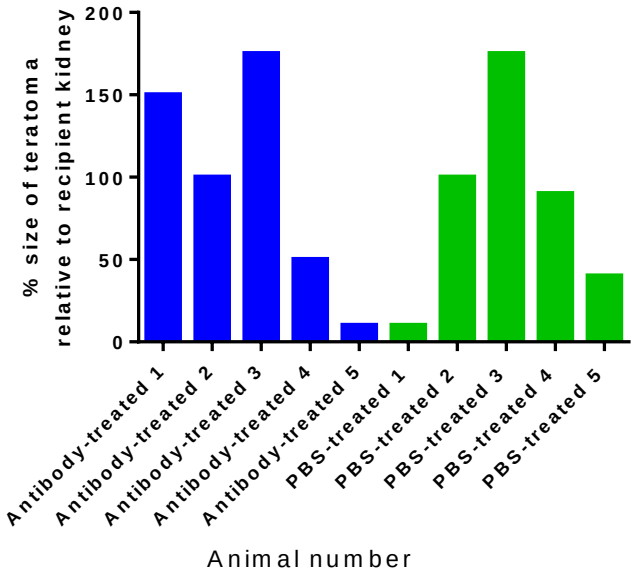

Figure 5.20 Induction of tolerance in recipients of iPS-EB. Tolerance was achieved using a tolerance-inducing antibody regime in mice receiving syngeneic iPS-EB. However no discernible difference in teratoma formation between antibody-treated and PBS-treated control recipients was observed.

To gain further insight into the influence of the immune system on the outcome of grafting, we examined the histology of rejecting ES cell teratomas. We found that the rejecting allogeneic ES cell teratomas displayed extensive infiltration of $CD3^+$ T cells and $F4/80^+$ macrophages, mice that had received antibody treatment at the time of transplantation with allogeneic ES-EB formed teratomas with no detectable level of $CD3^+$ infiltrate, but displayed the presence of $F4/80^+$ macrophages, as demonstrated in Figure 5.21. The presence of a $CD3^+$ infiltrate is, therefore, one of the most diagnostic criteria of rejection under these circumstances and shows the effectiveness of antibody treatment in inducing tolerance across multiple transplantation barriers.

Histological analysis of iPS-EB was also performed, the smallest teratomas being initially chosen given these teratomas were most likely to be undergoing rejection. These teratomas showed only a minimal infiltration of $CD3^+$ T cells as demonstrated in Figure 5.22, in either

PBS control group or antibody-treated mice. These results therefore confirm that even in the absence of an adaptive immune response, the survival of syngeneic iPS cell derived tissue is very variable, suggesting that factors other than rejection contribute to the outcome of grafting. Subsequent histological analysis of larger teratomas yielded very similar conclusions.

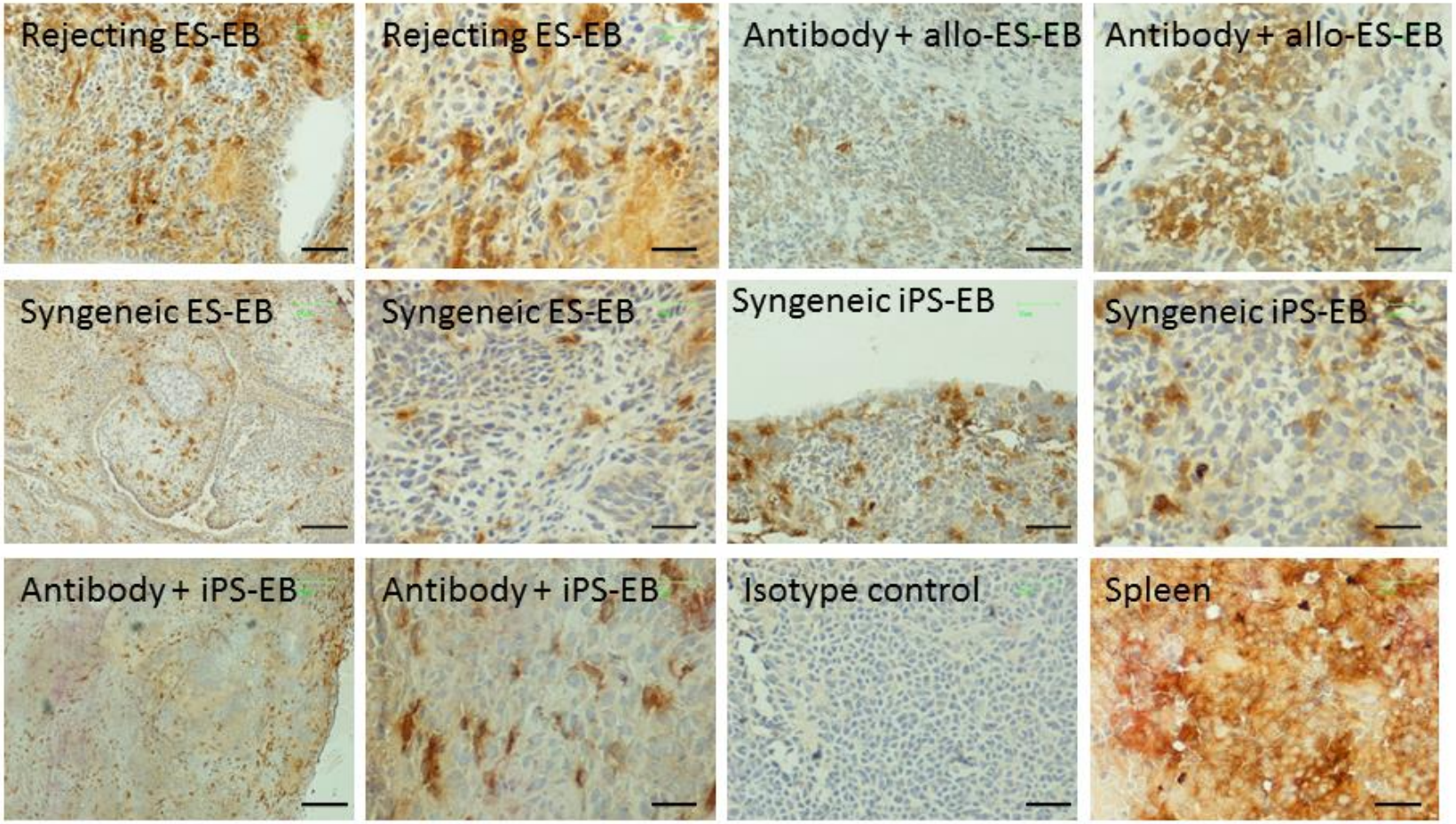

Figure 5.21 Analysis of F4/80$^+$ macrophages in teratomas. 14 day iPS-EB or ES-EB were transplanted into recipient CBA/Ca mice for a period of 21 days and subsequently harvested. Sections of teratoma were analysed for F4/80$^+$ cells and counterstained with haemotoxylin. F4/80$^+$ presence was analysed in rejecting allogeneic ES-EB teratoma, antibody conditioned recipient transplanted with allogeneic ES-EB, syngeneic ES-EB control, control syngeneic iPS-EB, antibody conditioned recipient transplanted with syngeneic iPS-EB, matched isotype control and spleen.

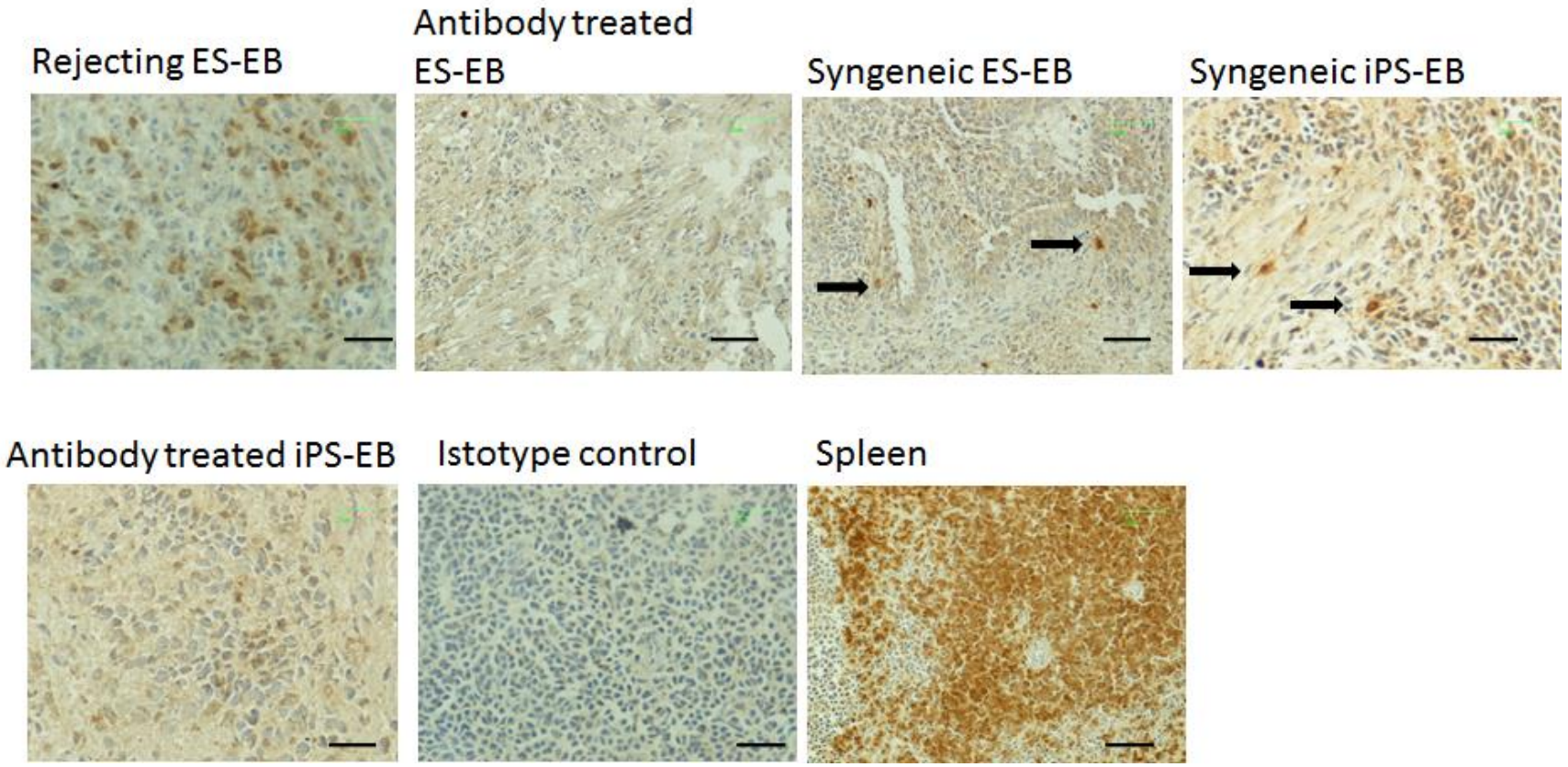

Figure 5.22 Analysis of $CD3^+$ T cell infiltrate in teratomas. 14 day iPS-EB or ES-EB were transplanted into recipient CBA/Ca mice for a period of 21 days and subsequently harvested. Sections of teratoma were analysed for $CD3^+$ cells and counterstained with haemotoxylin. $CD3^+$ presence was analysed in (a) rejecting allogeneic ES-EB, antibody conditioned recipient transplanted with allogeneic ES-EB, syngeneic ES-EB control, control syngeneic iPS-EB control, antibody conditioned recipient receiving syngeneic iPS-EB, matched isotype control and spleen.

5.3 Discussion

In this chapter, we were able to show that the formation of teratomas in syngeneic recipients of iPS-cell EB is a highly variable event. To analyse the mechanisms by contributing to this variability, we examined whether any intervention by the immune system played a role in the formation and/or survival of teratomas. We were unable to identify any significant immunological infiltrate in teratomas of either $CD3^+$ T cells, NK cells or $F4/80^+$ macrophages.

To ultimately determine whether the variability in teratoma formation was influenced by the adaptive immune system, we initially grafted both iPS and iMAF-6 EB into CBA.$RAG1^{-/-}$ mice which are deficient in both T and B cells. We were able to show similar variable levels of teratoma formation, as found with immunocompetent CBA/Ca syngeneic recipients. Furthermore, we induced systemic global tolerance using a defined antibody regime with non-depleting antibodies specific for CD4, CD8 and CD40L. This regime ensured the successful acceptance of allogeneic ES-EB grafts, but made no discernible difference in the extent of teratoma formation of syngeneic iPS-EB compared to the control group. Furthermore, upon histological analysis of syngeneic iPS cell teratomas, only a minor CD3 infiltrate, along with a presence of resting $F4/80^+$ macrophages, was present which suggested that the adaptive immune system does not play a role in the variability observed.

These findings draw parallels with the work published by Zhao *et al.* (2011) showing the considerable variation in teratoma formation by subcutaneous injection of undifferentiated iPS cells syngeneic to the recipients. Despite this finding, we were unable to demonstrate a significant $CD3^+$ infiltrate as shown by Zhao *et al.* It is prudent to point out that several differences exist between our studies. Firstly, Zhao *et al.* injected undifferentiated iPS cells,

whilst our study transplanted differentiated iPS cells in the form of EB under the kidney capsule of mice. Our results also suggest that, whilst the strain of mouse may not account for the variability in teratoma formation, it may explain the differences in CD3 infiltrate observed between our study and Zhao *et al.* As discussed earlier, the CBA/Ca strain is naturally more pre-disposed towards tolerance than the C57Bl/6 strain used by Zhao *et al.* The level of CD3 infiltration between our studies could, therefore, be accounted for by the differences in the immunological responses of the strains used.

Further to our studies, work published by Araki *et al.* (2012) and Guha *et al.* (2013) were unable to replicated the findings of Zhao *et al.* with their studies showing no variability in the capacity of iPS cells to form teratomas in syngeneic recipients, and no presence of a $CD3^+$ infiltrate in the resulting teratomas, consistent with our own findings. It is, therefore, possible that significant variation between iPS cell lines exists with respect to immunogenicity or their ability to engraft into the site of transplantation. The latter possibility is supported by the fact that numerous recipients of syngeneic iPS-EB showed no evidence of viable tissue 21 days later whereas even fully allogeneic ES-EB could be readily detected at the same time point, albeit displaying all the classical signs of rejection. These observations suggest a rather more fundamental failure of engraftment of iPS-EB, possibly due to metabolic issues or the inability to robustly support vasculogenesis. In order to investigate this possibility, we devise a set of experiments involving the construction of chimeric embryoid bodies. Given we have demonstrated that ES-EB have the ability to successfully engraftment in this setting and routinely form viable teratomas, we constructed chimeric embryoid bodies between iPS cells from CB/K mice and ES cells from CBA/Ca mice enabling us to distinguish tissues from either cell type on the basis of $H\text{-}2K^b$ expression. iPS and ES cells can be induced to form chimeric embryoid bodies by introducing

them into hanging drop cultures at a ratio of 1:9. These chimeric embryoid bodies, containing both differentiated ES and iPS cells can be implanted under the kidney capsule of CBA.RAG1$^{-/-}$ recipients ensuring no immunological rejection will occur against either ES or iPS cells. Given that ES-EB consistently provide an environment conducive to vasculogenesis, we were interested to investigate whether iPS-derived tissues could survive when compared to a control group of iPS-EB alone. A preliminary set of experiments using this approach were preformed but due to technical failures, we were unable to reach a definitive conclusion. It is envisaged however that these experiments will be repeated in the near future.

Our studies also confirmed that *Hormad1* was expressed in iPS cells in both differentiated and undifferentiated states. However expression of *Hormad1* was also detected in ES cells, in a differentiated and undifferentiated state, whilst its expression could not be detected in tissue bmDC derived from haematopoietic stem cells through the use of RT-PCR. This lends weight to the observation that *Hormad1* is only expressed during early embryonic development and may indeed fail to be down-regulated upon differentiation. Despite this, *Hormad1* is unlikely to play a role in the lack of teratoma formation observed with iPS cells, given that ES cells display expression of *Hormad1*and have the ability to form teratomas throughout.

We were also able to show that ipDC maintained expression of *Hormad1* upon differentiation from iPS cells. Although the need for ipDC to induce tolerance to *Hormad1* or other developmental antigens for acceptance of iPS differentiated tissues is perhaps unnecessary, our original intention of using them in a tolerogenic regime may have been possible.

Further to our findings that iPS cells express *Hormad1,* we also sought to examine whether expression is also found in the thymus. We were unable to detect expression of *Hormad1* in

thymic stroma using RT-PCR, however some level of staining of *Hormad1* was found using immunohistochemistry. It would be prudent to examine whether differences in *Hormad1* expression in the thymus differ between C57Bl/6 mice and CBA/Ca mice.

The work presented in this chapter provides a valuable contribution to the on-going discussion of the level of immunogenicity of iPS cells and draws parallels between work presented by Zhao *et al.* (2011), Araki *et al.* (2012) and Guha *et al.* (2013). Further examination into the ability of iPS cells and their differentiated counterparts to induce immunogenicity is needed before any definitive conclusions can be drawn.

Chapter Six:

Discussion

6.1 The promise of iPS cells in regenerative medicine

The development of iPS cells by Takahashi *et al.* (2006) transformed the landscape of regenerative medicine by allowing pluripotency to be 'captured' *in vitro* through the reprogramming of any somatic cell. The initial methodology, and the ones evolved since the first description of iPS cell re-programming, offer relatively easy and cost-effective ways of deriving pluripotent stem cells in an autologous fashion. Given the chronic shortage of organ donors and the ageing population in many western countries, novel therapeutics for treating age-related diseases are much needed. iPS cells hold promise that they may be able to aid in the production of specific cell types for cell replacement therapy. In particular, iPS cells overcome not only the ethical concerns associated with ES cells, but particularly immunological barriers, given their ability to be derived in an autologous fashion. Despite these exciting developments, in order for iPS cells to be harnessed for future clinical applications, several hurdles must be addressed.

Directed differentiation and purification of the cell type for transplantation is necessary. Although a vast range of protocols exists for derivation of cells from iPS cells, such as hepatocytes (Espejel *et al.* 2010), cardiomyocytes (Zhang *et al.* 2009), thymic epithelium (Inami *et al.* 2011) and intestinal tissue (Spence *et al.* 2010), purification of cell types is often difficult and further complicated by the ultimate purity of the cell type of interest. Furthermore, the

phenotype of the differentiated cells must be carefully examined, as often, subtle differences exist between populations of cells differentiated *in vitro* and their counterparts *in vivo*, as illustrated by the DC described in this thesis.

It is not only the phenotypic properties of the cells differentiated that present hurdles for clinical therapies: the practical administration of cells and their ability to survive upon transplantation into an area of ischemic or necrotic tissue is another aspect which must be thoroughly researched. For example, administration of dopaminergic neurones, which offers potential for the treatment of Parkinson's disease, may be hampered by the inaccessibility of the striatum of the brain affected by the disease itself, despite the successful generation of dopaminergic neurones from iPS cells (Swistowski *et al.* 2010). Further to this, the functional integration of transplanted cells into the site of administration must also be examined further, although preliminary studies have shown that cell types such as hepatocytes derived from mesenchymal stem cells are successfully able to integrate *in vivo* (Aurich *et al.* 2007). Additionally, animal models of Parkinson's disease have shown some alleviation of symptoms, strongly suggestive of functional integration of differentiated neurones (Kim *et al.* 2002).

In addition to these caveats, the immunogenicity of iPS cells and cells differentiated from the parent cell line remains controversial.

6.2 Immunogenicity of iPS cells

Following the generation of iPS cells in 2006 by Takahashi *et al.* (2006), it was hoped that the issue of the immunogenicity that had threatened the success of cell replacement therapy had been overcome, by facilitating the derivation of pluripotent stem cells in an autologous manner. However, the recent publication of work by Zhao *et al.* (2011) called into question whether, despite being derived in an autologous manner, iPS cells devoid of immunogenicity. The apparent immunological rejection observed upon transplantation of iPS cells into syngeneic mice appeared to be mediated by $CD3^+$ T cells which arose from the expression of ectopically expressed antigens, activated presumably upon reprogramming somatic cells back to a pluripotent state. Several antigens were implicated by Zhao *et al.* (2011), one of which, *Hormad1,* was shown to induce immunological rejection upon forced-expression in ES cells. Given that the antigens described are expressed in developmental stages, the response seen in this scenario is more akin to an autoimmune response than allograft rejection. Given such an immunological barrier might be anticipated to be less significant than a full allo-response, the prospect of inducing tolerance in this setting is likely to be easier than inducing tolerance to full allografts. Furthermore, unlike the treatment of conventional autoimmune disease, that requires the re-establishment of tolerance in a primed immune system, cell replacement therapy offers a window of opportunity for tolerance induction in advance of the administration of cells or tissues. It is, therefore, possible that administration of ipDC, expressing these up-regulated

antigens, prior to transplantation of iPS-derived tissues could induce tolerance to any developmental antigens they may ectopically express. Given our previous findings that bmDC have the ability to induce tolerance to the male antigen, Dby, in the A1.RAG1$^{-/-}$ mouse model but fail to induce tolerance across a full MHC-mismatch barrier, it is likely that ipDC would have similar limitations (Yates *et al.* 2007). However, given that tolerance to only a few antigens, such as those described by Zhao *et al.* (2011), would be needed, we hypothesised that ipDC may have the ability to induce a state of immunological tolerance to these mH antigens.

In an effort to examine whether the findings by Zhao *et al.* were reproducible, we transplanted iPS cell derived EB into syngeneic mice and found they failed to consistently form teratomas consistent with published findings. Our initial experiments suggested that an immunological component may contribute to the lack of teratoma formation given the findings of occasional CD3^{+} T cells and some evidence of tissue damage. In addition to the localised CD3^{+} T cell infiltrate, some evidence of F4/80^{+} macrophages were present in the tissue, although these were also present in non-rejecting ES cell tissue illustrating that macrophages may have either a pro or anti-inflammatory role in a transplantation setting. The significant variability in teratoma formation we encountered when transplanting iPS-EB syngeneically was also reflected in the work by Zhao *et al.* (2011) who showed that in 80% of recipients, a teratoma was present, whilst 9% and 11% had either a regressing teratoma or no detectable teratoma, respectively. The authors, however, did not accurately define what constituted a 'regressing tumour' although they

reported finding a significant $CD3^+$ infiltrate in 'the majority' of teratomas formed in syngeneic recipients. We likewise found an occasional presence of $CD3^+$ T cells in syngeneic teratomas, although we found no qualitative correlation between size of teratoma and level of infiltrate. Additionally, the infiltrate contained only a very minor population of $CD3^+$ T cells compared to fully rejecting allogeneic controls, suggesting that any $CD3^+$ T cells present may not be involved in an active immune response. Given uncertainty surrounding the significance of a minor $CD3^+$ infiltrate, we transplanted iPS-EB into CBA.$RAG^{-/-}$ mice, devoid of endogenous T cells, and found significant variability in teratoma formation suggesting that this variability is not influenced by autoreactive T cells.

However, the methodology of assessing the level of immunological rejection between our studies and that of Zhao *et al.* (2011) carries the caveat that two different methods of iPS cell administration were used. Zhao *et al.* (2011) chose to inject undifferentiated iPS cells and used subcutaneous injection in mice. We chose to differentiate iPS cells into EB and transplant them under the kidney capsule, a protocol that has proven successful in our hands for assessing the immunogenicity of tissues (Robertson *et al.* 2007). EB represent a better, although not ideal, comparison for clinical applications as tissues are in a terminally differentiated state compared to undifferentiated iPS cells which undergo differentiation upon transplantation. It is likely that the site of administration and level of differentiation of iPS cells play an important role in the ability for the immune system to recognise transplanted cells. Ideally, a single cell type should be

transplanted which would allow comparison between terminally differentiated cells and undifferentiated iPS cells with respect to their level of immunogenicity. Since the initial publication of work from Zhao *et al.* (2011), Guha *et al* (2013) showed that 100% of syngeneic recipients of iPS-EB accepted grafts. These grafts showed no apparent $CD3^+$ infiltrate. Additionally, the authors also investigated whether single cell types such as hepatocytes and endothelial cells have a different propensity to induce an immune response. However, upon transplantation of single cell types, the authors were unable to show the presence of any $CD3^+$ T cells in grafts.

Despite the findings by Guha *et al.* (2013), it is also possible that certain cells, other than hepatocytes and endothelial cells, may have a higher propensity to be more immunogenic than others. A full range of cell types should, therefore, be examined in order to establish whether certain cells may express antigens targeting them for immune rejection. In addition to the differentiated cell type, given the significant role of epigenetics in iPS cell differentiation, it is also possible that the cell type chosen to be re-programmed may also influence the immunogenicity of cell types differentiated from the parent iPS cell line.

Given the identification of various antigens which may be responsible for the rejection observed by Zhao *et al.* (2011) in iPS cell transplantation, we sought to examine whether *Hormad1* was initially expressed in the undifferentiated ES and iPS cell lines we derived. We found that our ES

and iPS cell lines expressed *Hormad1*. Furthermore, it appears that tissues differentiated from either ES or iPS cells express *Hormad1*. Furthermore, our findings cast uncertainty on the experiments conducted by Zhao *et al.* which showed that forced expression of *Hormad1* induced the rejection of syngeneic ES cells. Given the ability for ES cell-EB to form teratomas, it is unlikely that *Hormad1* is responsible for any lack of teratoma growth or minor infiltrate observed in iPS-EB transplantation.

In order to further probe the role of *Hormad1*, however, we examined its expression within the thymus, the site of central tolerance induction. We hypothesised that expression of *Hormad1* in the thymus possibly under the control of the AIRE transcription factor expressed by medullary thymic epithelial cells, would induce negative selection of T cells expressing a TCR specific for *Hormad1*, thereby purging them from the peripheral T cell repertoire. Using RT-PCR we were unable to show expression of *Hormad1* in the thymic stroma, which is not consistent with this hypothesis, and indeed, upon staining thymus sections with a polyclonal rabbit antiserum specific for *Hormad1*, we were unable to detect expression of the protein in thymic tissue. It is possible, therefore, that central tolerance to *Hormad1* is indeed compromised given the lack of expression in the thymus.

Further to our findings that *Hormad1* appears to be expressed in both differentiated ES and iPS cells, we also showed that ipDC express *Hormad1* which may permit its presentation for the induction of tolerance.

It is important to also mention that when using the primer sequences for *Hormad1* published by Zhao *et al.* (2011), we were unable to detect expression of *Hormad1* in either testes or any cell line due to extensive primer dimerization. A BLAST search of the primer sequences by Zhao *et al.* (2011) revealed a 100% match against *Hormad1*. As a result, we used primer sequences published by Araki *et al.* (2012) for *Hormad1* which yielded products of the expected base pair size.

Relatively little is known about the role of *Hormad1*, however it has been shown to be immunogenic with expression localised to the immune privileged area of the testes and plays an important role in meiosis by controlling the formation of synaptonemal complexes between sister chromosomes (Chen *et al.* 2005). It has been shown that *Hormad1* mutation results in disruption of recombination and segregation of chromosomes during meiosis (Shin *et al.* 2010). Presumably, if *Hormad1* is expressed only during development, expression post-development may induce an immunogenic response.

It is pertinent to mention that there may be other factors apart from *Hormad1* expression involved in the lack of iPS cell teratoma formation. Given that the iPS cell lines were generated

using a retroviral approach, it is conceivable that the introduction of viral or episomal vectors by the viral reprogramming may be responsible for triggering an immunological response and preventing teratoma formation. Indeed, it has been previously shown that viral vectors have the ability to induce innate immune responses (Zaiss *et al.* 2002). Expression of such viral vectors may, rather than, or in addition to, enhance immune responses alongside expression of *Hormad1*. Given that such viral antigens are not expressed in the thymus, the immune system would not be tolerised towards their expression and would, therefore, target the destruction of such cells expressing these antigens.

6.3 Tolerance induction to iPS cell grafts

In the light of persistent uncertainty surrounding the immunogenicity of syngeneic iPS cells, we sought to induce systemic tolerance to all potential transplantation antigens through the use of non-depleting antibodies specific for CD4, CD8 and CD40L. It has been previously shown that tolerance to mismatched ES cells can be induced using these antibodies which act to induce the polarisation of infiltrating T cells towards a regulatory phenotype. This regime of tolerance induction can induce tolerance not only to multiple mH-antigens but also to fully allogeneic grafts.

Our experiments in this study showed that grafting of fully allogeneic ES cells resulted in rapid rejection of tissue, as highlighted by extensive $CD3^+$ infiltration or lack of teratoma formation.

This was abolished following pre-conditioning with antibody treatment. However, control and antibody treated groups receiving syngeneic iPS-EB showed no differences in their propensity to generate teratomas, and both showed no presence of $CD3^+$ infiltration, providing unequivocal evidence that teratoma formation is not influenced by immunological rejection by infiltrating T cells.

Since the initial publication by Zhao *et al.* (2011), two further studies (Araki *et al.* 2012) (Guha *et al.* 2013) were unable to show any immunological rejection of syngeneic iPS cells, lending weight to our findings that any lack of teratoma formation is not based on any immune induction.

6.4 Future directions of iPS cell grafting

To date, there are no studies that successfully address all the possible variables associated with iPS cell transplantation, further studies must, therefore, be carried out to address these deficiencies which will be conducted by others in the laboratory pursuing this project. These include:

1. A direct comparison between transplantation of undifferentiated iPS cells, 'partially differentiated' EB-tissue and fully differentiated tissues.
2. The differences in the site of administration of transplanted cells by examining whether administration sub-cutaneously or under the kidney capsule biases the immune system into initiating rejection of transplanted tissues.

3. A direct comparison between the protocols of re-programming of somatic cells into iPS cells and whether different methods of reprogramming e.g. use of different viruses or virus-free systems effects the immunogenicity of the iPS cell line generated
4. A direct comparison of immunologically matched and mis-matched ES and iPS cells, side-by-side may aid with better comparison of the level of infiltration, if any, observed in grafted iPS cells. Grafting single MHC mis-matched and fully allogeneic iPS cells as control populations may allow for better analysis of the amount of immunological infiltrate in grafts, along with identically matched ES cell controls.

With respect to our findings, we found that both MEF and MAF derived iPS cell lines had limited capacity to generate teratomas *in vivo*, with no evidence for any immunological component. The variability could be explained by failure of cells differentiated from iPS to integrate into the local microenvironment within the kidney and, perhaps, their limited ability to stimulate vasculogenesis thereby preventing survival of transplanted tissues.

6.5 Derivation of ipDC

Given the findings outlined in Chapter Five we have concluded that there seems to be no apparent immunological component to the failure of teratoma formation demonstrated by iPS-EB. These results suggest that our rationale for deriving ipDC was somewhat ill-founded. Despite their envisaged use of inducing tolerance to mH antigens, such as *Hormad1,* responsible

for iPS cell rejection, their use could be explored numerous other contexts requiring the induction of tolerance to defined protein antigens. Recent developments in the field of enzyme replacement therapy (ERT) have, for example, been hampered by immunological responses causing neutralisation of exogenously administered enzymes (Joseph *et al.* 2008). Although some efforts of immunomodulation with anti-CD3 treatment (Joseph *et al.* 2008) or immunosuppressive medication such as cyclosporine A and azathioprine (Kakkis *et al.* 2003) have managed to induce some level of tolerance to administered enzymes, these regimes are often associated with significant unwanted immunological suppression and side-effects. It is therefore possible that ipDC could be used in this scenario by deriving patient-specific iPS cell lines, differentiating ipDC, pulsing ipDC with the therapeutic enzyme and pre-conditioning individuals to induce a state of tolerance to the enzyme. Indeed, we have recently set up a collaboration to derive patient-specific iPS cells from patients with lysosomal storage disorders and examine whether ipDC differentiated from these iPS cells have the ability to induce enzyme-specific T_{reg} cells.

From the outset of this thesis, DC had yet to be differentiated from mouse iPS cells and the data presented here has constituted their first description. We, therefore, chose to characterise ipDC as fully as possible to assess their phenotype and functional potential, as described in Chapter Three.

From our characterisation studies, we have shown that ipDC have a similar phenotype to bmDC, expressing the myeloid marker CD11b and conventional DC markers such as CD11c, CD54, MHC class I and MHC class II. ipDC appear to show some level of resistance to the classical maturation stimulus LPS with only a small proportion of ipDC adopting a fully mature phenotype. It would be interesting to further probe the ability to mature ipDC using cocktails of inflammatory cytokines which have been reported to induce robust maturation in human ipDC (Silk *et al.* 2012). Such cocktails contain stimuli such as IL-1β, TNF-α and PGE_2 which have been shown to up-regulate co-stimulatory molecules and MHC class II expression.

ipDC also have the ability to process and present antigen as shown by the presentation of HEL to the 2G7.1 hybridoma. Conversely, ipDC do not appear to have the ability to cross-present antigen as determined by the uptake and presentation of OVA on MHC class I, as described in Chapter Three, by using a monoclonal antibody specific for the epitope of HEL bound to H-$2E^k$. An alternative system for assessing cross-presentation of antigen which may be more sensitive would be to use OVA-specific OT-1 T cells as a readout of OVA presentation on MHC class I (Schulz *et al.* 2002).

A proportion of ipDC appear to have the ability to stimulate allogeneic T cells in an MLR as described in Chapter Three. Along with the evidence of the expression of co-stimulatory molecules, this suggests that ipDC have the ability to interact with naïve T cells and stimulate

their proliferation. Further to this, they also have the ability to stimulate antigen specific responses in TCR-transgenic A1.RAG1$^{-/-}$ T cells, specific for an epitope from the male antigen, Dby.

Importantly, ipDC also had the ability to migrate *in vitro* and *in vivo.* When administered *in vivo* to female A1.RAG1$^{-/-}$ mice, ipDC had the ability to migrate to the spleen and activate T cells as demonstrated by CD69 expression. It would be interesting to examine whether ipDC had the ability to induce CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells *in vivo.* Indeed, it has been previously shown that male bmDC administered *in vivo* to A1.RAG1$^{-/-}$ mice had the ability to induce CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells and isolation of these cells showed marked hyporesponsiveness to antigen (Yates *et al.* 2007). It would therefore be prudent to examine, side by side, the ability for bmDC and ipDC to induce regulatory T cells in A1.RAG1$^{-/-}$ mice. Indeed, it is this property, and the ability to adopt pro-tolerogenic phenotypes which are necessary to ensure the ability for ipDC to induce immune tolerance.

As discussed at the beginning of this thesis, some of the essential characteristics of a tolerogenic DC have been outlined by Morelli *et al.* 2000. Indeed, ipDC appear to fulfil some of these criteria such as relatively low CD40 expression, inability to secrete IL-12p70 in response to TLR ligation, generally low expression of MHC class II, high IL-10 secretion and high levels of NO

produiction. All of these attributes suggest that ipDC may have a pro-tolerogenic phenotype. As outlined above, the ultimate test is their ability to induce regulatory T cells *in vivo.*

Ultimately the use of ipDC to induce tolerance within the immune system does come with some limitations. We encountered significant variability in yields of ipDC generated from iPS cells with some cultures containing as many as 2-3x10^6 DC per culture dish but with some failing to generate any ipDC, despite use of the same cell line, passage of cells and *in vitro* culture conditions. These findings, along with the significant variability in MHC class II expression, as described in Chapter Four, represent the chaotic nature of iPS cell differentiation which makes the production of reproducible phenotypes of differentiated cells difficult to attain. Along with the limitations represented by their inherent variability, the issue of cell-aggregation, demonstrated by the thrombo-embolic complications found in a proportion of animals also need to be addressed. It would be interesting to examine whether clumping is a result of cell-cell interactions, up-regulation of cell surface adhesion molecules or whether it can be attributed to mechanical stresses, such as centrifugation of cells. Although we have investigated the use of EDTA, cell dissociation buffer and Trypsin in dissociating cell aggregates, we have so far been unable to resolve this issue.

As examined in Chapter Four, ipDC show significant variability in their ability to express MHC class II. We probed this further by investigating the relationship between the reprogrammed cell

type of origin and ipDC phenotype. We hypothesised that deriving iPS cells from an adult cell type which was not constrained in MHC expression, typical of cells during embryonic development, upon differentiation, give rise to ipDC with a different phenotype compared to cells differentiated from MEF-derived iPS cells. However, we were unable to find any phenotypic differences between ipDC differentiated from MEF or MAF-derived iPS cell lines. In the context of differentiation of DC from iPS cells, this lends weight to the idea that the cell chosen for reprogramming may not influence the phenotype of DC: phenotype of the DC may be more influenced by the differentiation environment, which is reflected by phenotypic differences of ipDC differentiated using other protocols.

Our findings may suggest that ipDC bare a closer resemblance to DC in a primordial state, such as those found in fetal liver. It has been shown that haemtopoeitic progenitor cells derived from fetal liver in mice have the ability to form DC upon differentiation in the presence of PA6 stromal cells, GM-CSF, SCF and Flt3 ligand (Zhang *et al.* 2000). Interestingly, fetal liver DC express low levels of CD11c, similar to our findings with ipDC. Given our findings that ipDC generally have low expression of MHC class II, and low expression of CD11c, it would be interesting to derive fetal liver DC and use them as a control population given they may bare more resemblance to ipDC.

Since the derivation of mouse ipDC by our laboratory, Senju *et al.* (2009) have also successfully derived mouse ipDC using an alternative protocol. The authors differentiated iPS cells in the presence of a feeder layer of mouse OP9 cells with the addition of GM-CSF. The authors did not report any variation in MHC class II expression and showed significantly higher levels of the DC marker CD11c compared to the expression on our populations of ipDC. The authors were also able to mature the ipDC using an inflammatory cocktail of IL-4, anti-CD40 and TNF-α: the authors did not comment on the use of LPS to mature ipDC. The differences in phenotype could be accounted for by the use of the OP9 feeder cell layer. OP9 has the ability to induce lymphohaemotpoeitc cells from ES cells in culture, therefore ipDC differentiated in the presence of OP9 may bias differentiation of ipDC towards a lymphoid phenotype rather than myeloid (Nakano *et al.* 1994). It would, therefore, be interesting to compare our protocol and differentiation of ipDC using OP9 feeder cells directly to establish whether culture with feeder cells induces a more stable phenotype in ipDC differentiation. If such anomalies in the production of ipDC could be better understood and ultimately circumvented, their use in multiple clinical applications requiring the induction of tolerance would be greatly facilitated.

Ultimately, the work presented in this thesis has shown that mouse iPS cells have the ability to differentiate into functional dendritic cells which has, until recently (Senju *et al.* 2009), has not been demonstrated elsewhere. These cells expressed DC markers, albeit at varying levels compared to their bmDC counterparts. ipDC had the ability to process and present antigen,

secrete IL-10 and induce the formation of $Foxp3^{+}$ T_{reg} cells, suggestive of a tolerogenic phenotype. As outlined in Chapter Four, ipDC displayed a varying phenotype with respect to their expression of MHC class II. This variability was explored further but no single factor was attributable to the variation observed.

In addition to the novel differentiation of ipDC from mouse iPS cells, the immunological properties of mouse iPS cells were explored. We were able to show that iPS cells exhibited a significant degree of variability with respect to their ability to form teratomas *in vivo.* Contrary to work presented by Zhao *et al.* (2011), we were unable to find any immunological component responsible for their variability to engraft *in vivo* when compared directly to ES cells. The work shown contributed to the widening body of evidence of the probably lack of immunogenicity of iPS cells.

With respect to future studies, the further characterisation of ipDC and their potential ability to induce tolerance *in vivo* should be further explored. Further characterisation of iPS cell lines needs to be explored with respect to their ability to generate teratomas *in vivo.*

Chapter Seven:

Appendices

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4.17	Expression of MHC class II in bmDC, iPS-MEF derived DC (ipDC) and iPS-MAF derived DC (iMAF-DC
4.18	Expression of MHC class II on cell surface and intracellularly by bmDC but not ipDC or iMAF-DC
4.19	Lack of expression of MHC class II on minor population of bmDC and majority of ipDC

4.20	Expression of MHC class II by bmDC and minor population of ipDC.
4.21	Presentation of the HEL1-18 peptide by bmDC and ipDC failing to express MHC class II
4.22	Stimulation of allogeneic C57Bl/6 T cells by mature bmDC, ipDC and iMAF-DC
4.23	Culture of Dby pulsed bmDC and ipDC with A1.RAG1$^{-/-}$ T cells
5.1	Transplantation of syngeneic ES-EB into CBA/Ca recipients
5.2	Transplantation of single-MHC mismatched ES-EB into CBA/Ca recipients
5.3	H&E stains of syngeneic ES cell teratoma and lack of CD3 infiltrate
5.4	Presence of F4/80^{+} macrophages in syngeneic ES cell grafts, but lack of infiltrating NK cells
5.5	H&E staining of MHC class I-mismatched ES-EB teratomas
5.6	Infiltration of CD4^{+} T cells in MHC class I-mismatched ES teratomas
5.7	Infiltration of CD8^{+} T cells in MHC class I-mismatched ES teratomas
5.8	Infiltration of F4/80^{+} cells in MHC-class I mismatched ES teratomas
5.9	Grafting of syngeneic iPS-derived EB.
5.10	H&E staining of syngeneic iPS-EB teratomas
5.11	Immunohistochemical analysis of syngeneic iPS cell teratomas, for the presence of T cells, macrophages and NK cells
5.12	Transplantation of iMAF-6 into syngeneic recipients
5.13	Expression pattern of Hormad1 determined by IHC
5.14	Analysis of expression of HPRT and Hormad1 in tissues
5.15	Expression of HPRT and Hormad1
5.16	Grafting of iPS-1 into CBA.RAG1$^{-/-}$ recipients
5.17	Grafting of iMAF-6 into CBA.RAG1$^{-/-}$ recipients
5.18	Immunohistochemical analysis of syngeneic iMAF-6 cell teratomas, for the presence of macrophages and NK cells

5.19	Induction of tolerance to allogeneic ES cell grafts
5.20	Induction of tolerance in recipients of iPS-EB
5.21	Analysis of $F4/80^+$ macrophages in teratomas
5.22	Analysis of $CD3^+$ T cell infiltrate in teratomas

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