

**HARNESSING THE IMMUNOMODULATORY CAPACITY  
OF DENDRITIC CELLS DIFFERENTIATED FROM  
HUMAN INDUCED PLURIPOTENT STEM CELLS AND  
THE THERAPEUTIC POTENTIAL OF DENDRITIC CELL-  
DERIVED EXOSOMES FOR THE TREATMENT OF  
LYSOSOMAL STORAGE DISEASES**

A thesis submitted for the degree of  
Doctor of Philosophy at the University of Oxford  
Trinity Term 2014



**Alison Jane Leishman**

The Sir William Dunn School of Pathology and The King's  
Hall and College of Brasenose

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## **Abstract**

Lysosomal storage diseases (LSDs) are a collection of disorders that feature the pathological accumulation of substrate frequently due to an enzymatic defect within the lysosomes. The most effective treatment regime for LSDs is enzyme replacement therapy. However, this treatment has faced two main challenges which have limited its treatment efficacy and clinical impact. One challenge constitutes the potential immunogenicity of the replaced enzyme, which can lead to the induction of an antibody response that prevents its effective targeting. Therefore, this thesis investigated the potential to derive patient-specific autologous dendritic cells (ipDCs) from fibroblasts which were obtained from a healthy donor and from a patient diagnosed with infantile-onset Pompe disease and were reprogrammed into induced pluripotent stem cells (iPSCs). This study demonstrated the feasibility of differentiating these iPSCs into ipDCs and investigated the potential to modulate their immunogenicity using a variety of agents. Using IL-10, this work was able to show the feasibility of generating patient-specific ipDCs with pro-tolerogenic characteristics which may be exploited for the induction of tolerance towards therapeutic enzymes. Secondly, the delivery of therapeutic enzymes to the central nervous system (CNS), which is frequently involved in disease pathogenesis, is limited by the selective-permeability of the blood brain barrier. As specifically-labelled exosomes have been shown capable of targeting to the CNS for the delivery of therapeutic molecules, this study has shown the possibility of harvesting exosomes from ipDC cultures. The potential of exploiting the endocytic capacity of dendritic cells for the loading of enzyme into exosomes was explored. Furthermore, this study has found that the administration of syngeneic and allogeneic exosomes from mouse bone-marrow derived DCs and ipDCs elicited an antibody-mediated immune response which may limit the clinical application of exosomes further highlighting the need for tolerance induction. Altogether, this study constitutes a first step towards potential improvements in the treatment of LSDs.



**“There is nothing like looking, if you want to find something.  
You certainly usually find something, if you look,  
but it is not always quite the something you were after.”**

*J.R.R. Tolkien, The Hobbit*





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## Abbreviations

|                    |                                                           |
|--------------------|-----------------------------------------------------------|
| 4-MU               | 1,4-Methylumbelliferone                                   |
| 7-AAD              | 7-amino-actinomycin D                                     |
| AAV                | Adeno-associated virus                                    |
| ANOVA              | Analysis of variance                                      |
| AP                 | Activator protein                                         |
| APC                | Antigen presenting cell                                   |
| B6                 | C57BL/6 mice                                              |
| BACE 1             | $\beta$ -site APP cleaving enzyme 1                       |
| BAFF               | B cell activating factor                                  |
| BBB                | Blood brain barrier                                       |
| bmDC               | Bone marrow-derived dendritic cell                        |
| BMP-4              | Bone morphogenic protein-4                                |
| BSA                | Bovine serum albumin                                      |
| CBA                | CBA/Ca mice                                               |
| CCR 7              | Chemokine receptor 7                                      |
| CD                 | Cluster of differentiation                                |
| CD40L              | CD40 ligand                                               |
| cDC                | Conventional DC                                           |
| CFSE               | 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester |
| CNS                | Central nervous system                                    |
| CO <sub>2</sub>    | Carbon dioxide                                            |
| CRIM               | Cross-reactive immunological material                     |
| CRISPR             | Clustered regularly interspaced short palindromic repeats |
| CSF                | Cerebrospinal fluid                                       |
| CTLA-4             | Cytotoxic T-lymphocyte-associated protein-4               |
| DAB                | 3, 3'-Diaminobenzidine                                    |
| DAMPs              | Danger-associated molecular patterns                      |
| DC                 | Dendritic cell                                            |
| ddH <sub>2</sub> O | Double-deioniser water                                    |
| DMEM               | Dulbecco's modified Eagle's medium                        |
| DNJ                | 1-deoxynojirimycin                                        |
| EAE                | Experimental autoimmune encephalomyelitis                 |
| EB                 | Embryoid body                                             |
| EDTA               | Ethylenediaminetetraacetic acid                           |
| ELISA              | Enzyme linked immunosorbent assay                         |
| ER                 | Endoplasmic reticulum                                     |
| ERAD               | ER-associated degradation                                 |
| ERT                | Enzyme replacement therapy                                |
| ESC                | Embryonic stem cell                                       |
| FAS                | First apoptosis signal                                    |
| FCS                | Foetal calf serum                                         |
| FGF                | Fibroblast growth factor                                  |
| Foxp3              | Forkhead box P3                                           |
| GAA                | Acid $\alpha$ -glucosidase                                |
| GAG                | Glycosaminoglycans                                        |
| G-CSF              | Granulocyte-colony stimulating factor                     |
| GM-CSF             | Granulocyte macrophage-colony stimulating factor          |
| gMFI               | geometric mean fluorescent intensity                      |

|                                |                                                                            |
|--------------------------------|----------------------------------------------------------------------------|
| GMP                            | Granulocyte-monocyte precursor                                             |
| GVHD                           | Graft-versus host disease                                                  |
| H <sub>2</sub> SO <sub>4</sub> | Sulphuric acid                                                             |
| HLA                            | Human leukocyte antigen                                                    |
| HRP                            | Horseradish peroxidase                                                     |
| HSC                            | Haematopoietic stem cell                                                   |
| HSCT                           | Haematopoietic stem cell transplant                                        |
| ICOS                           | Inducible costimulatory                                                    |
| IDO                            | Indoleamine 2,3-dioxygenase                                                |
| IFN                            | Interferon                                                                 |
| IL                             | Interleukin                                                                |
| ILT                            | Immunoglobulin-like transcript                                             |
| ipDC                           | Induced pluripotent stem cell-derived dendritic cell                       |
| IPEX                           | Immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome |
| ipMonocytes                    | Induced pluripotent stem cell-derived monocyte-like cells                  |
| ipPo                           | Induced pluripotent stem cells from Pompe disease                          |
| iPSC                           | Induced pluripotent stem cell                                              |
| Jak                            | Janus kinase                                                               |
| Klf4                           | Kruppel-like factor 4                                                      |
| KO                             | Knockout                                                                   |
| Lamp2b                         | Lysosome-associated membrane protein 2b                                    |
| LPS                            | Lipopolysaccharide                                                         |
| LSD                            | Lysosomal storage disease                                                  |
| M6P                            | Mannose-6-phosphate                                                        |
| M6PR                           | Mannose-6-phosphate receptor                                               |
| MAPKK                          | Mitogen-activated protein kinase kinase                                    |
| MEF                            | Mouse embryonic fibroblast                                                 |
| MHC                            | Major histocompatibility complex                                           |
| MHC                            | MHCII compartment                                                          |
| miPSC                          | Mouse induced pluripotent stem cells                                       |
| MLP                            | Multilymphoid progenitor                                                   |
| MLR                            | Mixed leukocyte reaction                                                   |
| MMC                            | Mitomycin C                                                                |
| moDC                           | Monocyte-derived dendritic cell                                            |
| MPS                            | Mucopolysaccharidoses                                                      |
| MSD                            | Multiple sulfatase deficiency                                              |
| mTOR                           | <i>Mammalian target of rapamycin</i>                                       |
| MVB                            | Multi-vesicular body                                                       |
| NFκB                           | Nuclear factor kappa-light-chain-enhancer of activated B cells             |
| NPA                            | Niemann-Pick disease type A                                                |
| NPB                            | Niemann-Pick disease type B                                                |
| NPC                            | Niemann-Pick disease type C                                                |
| ns                             | Not significant                                                            |
| Oct3/4                         | Octamer-binding transcription factor 3/4                                   |
| OD                             | Optical density                                                            |
| OVA                            | Ovalbumin                                                                  |
| PAMPs                          | Pathogen-associated molecular patterns                                     |
| PBMC                           | Peripheral blood mononuclear cells                                         |

|                  |                                                  |
|------------------|--------------------------------------------------|
| PBS              | Phosphate buffered saline                        |
| PD               | Programmed death                                 |
| PD-L             | Programmed death-ligand                          |
| pDC              | Plasmacytoid DC                                  |
| PGE <sub>2</sub> | Prostaglandin E2                                 |
| PI3K             | Phosphatidylinositol-4, 5-bisphosphate 3-kinase  |
| PMA              | Phorbol 12-myristate 13-acetate                  |
| PVDF             | Polyvinylidene fluoride                          |
| RBC              | Red blood cell                                   |
| RNA              | Ribonucleic acid                                 |
| RPMI             | <i>Roswell Park Memorial Institute medium</i>    |
| RVG              | Rabies virus glycoprotein                        |
| SARS             | Severe acute respiratory syndrome                |
| SCF              | Stem cell factor                                 |
| SDS              | Sodium dodecyl sulphate                          |
| siRNA            | Short interfering ribonucleic acid               |
| Sox2             | Sex determining region Y-box-2                   |
| STAT             | Signal Transducer and Activator of Transcription |
| SUMF1            | Sulfatase modifying factor 1                     |
| TAP              | Transporter associated with antigen processing   |
| TBS-T            | Tris-Buffered Saline and Tween 20                |
| TCR              | T cell receptor                                  |
| TGF              | Transforming growth factor                       |
| Th1              | T helper 1                                       |
| Th17             | T helper 17                                      |
| Th2              | T helper 2                                       |
| TLR              | Toll-like receptor                               |
| TNF              | Tumour necrosis factor                           |
| Tr1              | T regulatory type 1                              |
| T <sub>reg</sub> | T regulatory cell                                |
| Tris             | Tris(hydroxymethyl)aminomethane                  |
| Tsg101           | Tumour susceptibility gene 101                   |
| ULA              | Ultra-low attachment                             |
| UPR              | Unfolded Protein Response                        |
| VD3              | 1 $\alpha$ ,25-dihydroxy vitamin D3              |
| VEGF             | Vascular endothelial growth factor               |
| XCR1             | Chemokine (C motif) receptor 1                   |
| XLAAD            | X-linked autoimmunity-allergic dysregulation     |





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## Chapter 1 Introduction





## 1.1 Lysosomal storage diseases

Lysosomal storage diseases (LSDs) are a collection of more than 70 disorders that result in the progressive accumulation of substrate within the lysosomes (Cox and Cachón-González, 2012; Parenti *et al.*, 2015). The overall combined prevalence of LSDs is estimated to be around 1 in 5000 live births (Fuller *et al.*, 2006). Most are the result of a mutation in a single gene which may affect enzyme activity, enzyme folding, enzyme activation, post-translational modifications, transportation mechanisms or endosomal/lysosomal biogenesis amongst others (Ballabio and Gieselmann, 2009; Parenti *et al.*, 2015). Ultimately, many of these mutations lead to the accumulation of substrates which affect numerous metabolic and cellular processes, many of which are not well understood.

LSDs encompass a wide spectrum of disease phenotypes and manifestations. From the accumulation of globotriaosylceramide in Fabry, glucosylceramide in Gaucher, sphingomyelin in Niemann-Pick A (NPA) and B (NPB), heparan and/or dermatan sulphate in mucopolysaccharidosis (MPS), to glycogen in Pompe disease, the range of storage material associated with the LSDs is extensive. Furthermore, clinical manifestations can range from cognitive, skeletal, cardiac and blood cell abnormalities to normal cognitive, skeletal and muscular function. Table 1.1 gives a brief overview over some of the LSDs that will be mentioned in this thesis.

| LSD            | Affected Protein                       | Accumulating substrate/structure                  | Primarily affected tissue                                 | Treatment                                                  | Immune response towards ERT (proportion of patients) |
|----------------|----------------------------------------|---------------------------------------------------|-----------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------|
| Danon          | lysosome-associated membrane protein-2 | autophagic vacuoles, cytoplasmic debris, glycogen | skeletal and cardiomyocytes, CNS                          | Palliative                                                 | -                                                    |
| Fabry          | $\alpha$ -galactosidase A              | glycosphingolipids                                | vascular endothelium, CNS, cardiomyocytes and renal cells | ERT, EET <sup>†</sup> , SRT <sup>†</sup> , GT <sup>†</sup> | Yes (68-89%)                                         |
| Gaucher        | $\beta$ -glucosidases                  | glucosylceramide                                  | macrophages, CNS, liver, spleen, bone                     | ERT, SRT, GT <sup>†</sup>                                  | Yes (15%)                                            |
| MPS I          | $\alpha$ -L-iduronidase                | dermatan sulphate, heparan sulphate               | connective tissue, reticuloendothelial system, bone, CNS  | ERT, BMT                                                   | Yes (91-97%)                                         |
| MPS II         | Iduronate-2-sulphatase                 | dermatan sulphate, heparan sulphate               | connective tissue, reticuloendothelial system, bone, CNS  | ERT, GT <sup>†</sup>                                       | Yes (11-51%)                                         |
| MPS IIIA       | heparan N-sulphatase                   | heparan sulphate                                  | connective tissue, bone, neuronal cells, cardiomyocytes   | ERT <sup>†</sup> , GT <sup>†</sup> , SRT <sup>†</sup>      | -                                                    |
| MPS VI         | arylsulphatase B                       | dermatan sulphate                                 | connective tissue, reticuloendothelial system, bone, CNS  | BMT, ERT                                                   | Yes (97-100%)                                        |
| MSD            | Ca-formylglycine-generating enzyme     | sulphatides, glycolipids, glycosaminoglycans      | CNS, bone, skin, liver, spleen                            | Palliative                                                 | -                                                    |
| Niemann-Pick A | acid sphingomyelinase                  | sphingomyelin                                     | reticuloendothelial system, CNS, liver                    | Palliative, ERT <sup>†</sup>                               | -                                                    |
| Niemann-Pick B | acid sphingomyelinase                  | sphingomyelin                                     | reticuloendothelial system                                | BMT, GT, ERT <sup>†</sup>                                  | -                                                    |
| Niemann-Pick C | NPC1 and NPC2                          | cholesterol and sphingolipids                     | visceral cells, CNS, liver, spleen                        | SRT                                                        | -                                                    |
| Pompe          | acid- $\alpha$ -glucosidase            | glycogen                                          | skeletal and cardiomyocytes, CNS                          | ERT, GT <sup>†</sup> , EET <sup>†</sup>                    | Yes (66-96%)                                         |

<sup>†</sup> Clinical trials, BMT: bone marrow transfer, CNS: central nervous system, EET: enzyme enhancement therapy, ERT: enzyme replacement therapy, GT: gene therapy, MSD: multiple sulfatase deficiency, MPS: mucopolysaccharidosis, SRT: substrate reduction therapy.

**Table 1.1 A brief summary of all LSDs mentioned in this thesis.** Data presented in this table were compiled from numerous reviews (Desnick and Schuchman, 2002, 2012; Futerman and van Meer, 2004; Platt *et al.*, 2012).

The severe substrate accumulation has been described to lead to the enlargement of lysosomes and their rupture and the release of lysosomal enzymes into the cytoplasm which damages cellular structures and processes (Thurberg *et al.*, 2006). This historical causal explanation of the disease pathology is an oversimplification and ignores the more complex aspects and secondary effects of the disease. Not all pathological changes and symptoms can be clearly linked to the physical accumulation of one or several substrates and are often more complex than just lysosomal dysfunction and cellular distortion and rupture.

For instance, the accumulating substrate may have the ability to modulate signalling pathways or membrane fluidity and lipid rafts. The results of these basic changes within the cell may have far-reaching consequences for a vast number of cellular signalling and organ processes (Ballabio and Gieselmann, 2009). In MPS, the accumulation of glycosaminoglycan and its break down products have been shown to activate Toll-like receptor (TLR) 4 due to the structural resemblance to lipopolysaccharides (LPS), leading to the excessive production of pro-inflammatory cytokines and cell death (Johnson *et al.*, 2002; Simonaro *et al.*, 2008). In Hurlers Syndrome, a subtype of MPSI, fibroblast growth factor (FGF)-2 signalling has been found to be impaired due to the lack of heparan sulphate with 6-O sulphation which is a consequence of  $\alpha$ -L-iduronidase deficiency (Holley *et al.*, 2011; Pan *et al.*, 2005; Yayon *et al.*, 1991). This impairment in FGF-2 signalling may explain increased cell death, diminished proliferative response towards FGF-2 and contribute to the neurodegeneration observed in patients (Alzheimer and Werner, 2002; Walton and Wolfe, 2007). There are numerous other signalling pathways that have been found to be

defective in animal models and patients of LSD including insulin, bone morphogenetic protein-4 (BMP-4) and protein kinase C signalling (Ballabio and Gieselmann, 2009; Jatana *et al.*, 2002; Khan *et al.*, 2008; Langeveld *et al.*, 2008; Vainio *et al.*, 2005).

Intracellular signalling via calcium has also been found to be defective and may be a key component in the pathology of LSDs. In Niemann-Pick C (NPC) 1 disease, a reduction of calcium in acidic compartments such as the lysosomes as a result of sphingosine accumulation has been observed (Lloyd-Evans *et al.*, 2008). The depletion of calcium stores, which appears to affect the fusion of late-endosomes with lysosomes, results in secondary accumulation of cholesterol, glycosphingolipid and shingomyelin (Lloyd-Evans *et al.*, 2008). The enhanced release of calcium due to an over-activation of the ryanodine receptor by the accumulating glucosylceramide has been described in Gaucher type 2 mouse models and patients' brain samples (Lloyd-Evans *et al.*, 2003; Pelled *et al.*, 2005). Furthermore, in Sandhoff and NPA disease, defective calcium storage and increased cytosolic calcium concentration was found to be due to a reduced rate of calcium transportation by the sarco/endoplasmic reticulum calcium-ATPase into the ER either due to its inhibition or reduced expression (Ginzburg and Futerman, 2005; Pelled *et al.*, 2003). Disruption of the calcium homeostasis may have far reaching consequences including the induction of apoptosis and over-activation of signalling pathways (Berridge *et al.*, 2003; Lim *et al.*, 2015; Mattson and Chan, 2003).

A defect or increased activation of autophagy has also been reported in different types of LSDs. Autophagy is a major cellular process by which old and damaged organelles and long-lived proteins are degraded and recycled, further supplying

amino acids and energy during periods of nutrient starvation (Mizumura *et al.*, 2014; Mizushima and Komatsu, 2011). Whether increased autophagy is a result of a feedback compensation mechanism is not yet known. In LSDs such as Danon, MPS IIIA, multiple sulfatase deficiency (MSD) and Pompe disease, the secondary accumulation of polyubiquitinated proteins, aberrant mitochondrial fragments and autophagic vacuoles, also referred to as autophagic build-up, have been observed, which are thought to significantly contribute to disease pathogenesis (Endo *et al.*, 2015; Fukuda *et al.*, 2006a; Gabandé-Rodríguez *et al.*, 2014; Lim *et al.*, 2015; Nascimbeni *et al.*, 2012; Nelson *et al.*, 2014; Saftig *et al.*, 2008; Sarkar *et al.*, 2013; Settembre *et al.*, 2008a, 2008b; Tanaka *et al.*, 2000). In some cases, the accumulation of autophagic vesicles has been caused by the defective autophagosome-lysosome fusion while increased autophagy has also been hypothesised to be an attempt to clear leaky lysosomes (Fraldi *et al.*, 2010; Fukuda *et al.*, 2006a; Kiffin *et al.*, 2006; Settembre *et al.*, 2008a, 2008b). The involvement of autophagy in the pathogenesis of LSDs is not surprising as the lysosomes are an important component of the autophagic pathway and both have been found to be regulated by a similar network of genes and transcription factors (Sardiello and Ballabio, 2009; Settembre *et al.*, 2008b, 2011). Impaired autophagy and the accumulation of secondary substrates are thought to play a key role in the pathogenesis of many LSDs, possibly even playing a more important role than primary lysosomal accumulates (Lieberman *et al.*, 2012).

As implied before in the pathogenic activation of TLR4 in MPS, excessive activation of the immune system and inflammation have also been reported in LSDs. Increased inflammation, activation of microglia and macrophage infiltration were detected predominantly in areas of the brain with pathologic lesions in LSD

mouse models of Sandhoff disease, GM1 gangliosidosis and late-onset Tay-Sachs disease (Jeyakumar *et al.*, 2003; Vitner *et al.*, 2010; Wada *et al.*, 2000). This inappropriate activation of the immune system appeared to be linked to the pathological accumulation of glycosphingolipids in the Sandhoff disease mouse model as pharmacological treatment using n-butyl 1-deoxynojirimycin (NB-DNJ), also known as Miglustat, not only reduced glycosphingolipid storage but also inflammation (Jeyakumar *et al.*, 2001, 2003). Similar results were found in the study of NPC1, where the use of non-steroidal anti-inflammatory drugs in combination with standard therapy had the most beneficial impact on the survival of NPC1 mice, their motor function and inflammation of the brain (Smith *et al.*, 2009).

Further cellular processes and mechanisms affected in LSDs include the synthesis of phospholipids, the trafficking of proteins and lipids and overall lipid content (Buccoliero *et al.*, 2004; Hattersley *et al.*, 2013; Labilloy *et al.*, 2014; Mbua *et al.*, 2013; Puri *et al.*, 1999; Vanier, 2015, 2015; te Vrugte *et al.*, 2004). One example is the cholesterol accumulation in NPC1 disease within endosomes and lysosomes which is known to be due to the defective cholesterol transportation mechanism by NPC1 and NPC2 proteins (Puri *et al.*, 1999; Vanier, 2015). Amongst many other downstream effects, this accumulation of cholesterol has been shown to impair neuronal synaptic signalling and insulin signalling pathways (Fletcher *et al.*, 2014; Karten *et al.*, 2006; Vainio *et al.*, 2005; Xu *et al.*, 2010).

Recent research has further pointed in the direction of oxidative stress which may be involved in the initiation of cell death in LSDs (Kiffin *et al.*, 2006; Lim *et al.*, 2015; Villani *et al.*, 2009; Wu *et al.*, 2009). Oxidative stress has been confirmed in the cerebrum of MPSIII<sup>B</sup> mice leading to protein oxidation, lipid peroxidation

and DNA oxidation which will be further discussed in section 1.2 for its relevance in Pompe disease pathogenesis (Villani *et al.*, 2009).

All these examples give a brief illustration of the complexity of disease pathogenesis and molecular interaction in LSDs. This wide spectrum of LSDs has been commonly categorised according to the biochemical properties of the accumulating substrate or the molecular defect. The classification by molecular defect for instance classifies LSDs according to whether the disease is due to a defect in the lysosomal enzyme, post-translational processing of the gene product, enzyme trafficking, lysosomal enzyme protection, transmembrane proteins related to the function of the lysosomal enzyme or soluble proteins likewise related to the lysosomal enzyme (Platt and Walkley, 2004). Another unclassified group exists for those diseases that cannot be categorised into any of the other groups. With this complex diversity in mind, this thesis focuses on one LSD in particular: Pompe disease.

## **1.2 Pompe disease**

Pompe disease, also known as acid maltase deficiency, first described in 1963, is an autosomal recessive disease that results in the absence or insufficient activity of the enzyme acid  $\alpha$ -glucosidase (GAA) (Hers, 1963). GAA is responsible for the breakdown of lysosomal glycogen into glucose. The absence or reduction of GAA activity results in the progressive accumulation of glycogen within the lysosomal compartment of the cell (Hers, 1963; Hirschhorn and Reuser, 2001; Leslie and Tinkle, 1993; Raben *et al.*, 2010). The most severely affected tissues include cardiac, skeletal and smooth muscle (Pena *et al.*, 2015). As mentioned above, early stages of disease pathology are marked by an increase in glycogen



storage within the lysosomes (Hirschhorn and Reuser, 2001; Raben *et al.*, 2010; Thurberg *et al.*, 2006). Progressive accumulation has been reported to cause lysosomes to enlarge, become leaky and release glycogen into the cytoplasm. Damage to myofibrils becomes apparent at early stages followed by further accumulation of glycogen storing lysosomes and increased lysosomal rupture (Griffin, 1984; Raben *et al.*, 2010; Thurberg *et al.*, 2006). Mitochondrial abnormalities and a significantly reduced number of myofibrils within the cells become more apparent during later stages of the disease. Severe cellular damage allows water influx and the swelling of cells. Although for many years it was assumed that lysosomal glycogen accumulation and lysosomal rupture were the major cause of the disease pathology observed in Pompe disease patients, recent research has demonstrated greater complexity of the disease pathology observed in Pompe disease animal models and patients.

More and more studies have shown the accumulation of autophagic vesicles in cells from Pompe mice and juvenile and adult patients with data suggesting that defective autophagosomal-lysosomal fusion and an increase in autophagy might contribute to this pathology (Fukuda *et al.*, 2006a; Lim *et al.*, 2015; Raben *et al.*, 2008; Shea and Raben, 2009; Spanpanato *et al.*, 2013). Here, a difference between early and late-onset Pompe disease pathogenesis was observed. The accumulation of autophagic vesicles was prominent in samples obtained from juvenile and adult patients while infantile patients showed negligible autophagic build-up but severe classical lysosomal pathology very early in life (De Bleecker *et al.*, 1993; Raben *et al.*, 2010). After 6 months of treatment, lysosomal pathology had improved in infantile patients, however the autophagic build-up became increasingly apparent (Raben *et al.*, 2010). These data appeared to indicate two

stages of disease pathogenesis, as the clearance of lysosomal contents in response to treatment appeared to be replaced by autophagic build-up. This autophagic build-up appeared to increase with age culminating in an ultimate loss in vesicle integrity in mice with Pompe disease and led to profound muscle fibre damage (Fukuda *et al.*, 2006b; Shea and Raben, 2009). The effect of autophagic vesicle accumulation is now assumed to have a worse impact on muscle cells and tissue pathology as compared to lysosomal dysfunction and damage (Raben *et al.*, 2012; Shea and Raben, 2009).

Furthermore, the accumulation of undegraded and autofluorescent, highly cross-linked proteins, lipids, metals and carbohydrates, called lipofuscin, have been observed in muscle biopsies of patients (Feeney *et al.*, 2014; Raben *et al.*, 2010; Schoser *et al.*, 2007). These inclusions can result from cellular oxidative damage and mitochondrial stress and were found within lysosomes in regions of autophagic build-up, autophagolysosomes and cytoplasm, the latter possibly a result of lysosomal rupture (Lim *et al.*, 2014). It has been speculated that the accumulation of lipofuscin, due to the reduced lysosomal degradation capacity, may perpetuate disease pathology by further reducing autophagic and lysosomal degradation of aberrant mitochondria and therefore promoting the generation of reactive oxygen species and therefore increased lipofuscin aggregate formation (Lim *et al.*, 2014; Terman *et al.*, 2010). This further illustrates the complexity of the disease pathology originating from a single absent or dysfunctional enzyme.

Furthermore, numerous studies have reported the presence of abnormal mitochondria in muscle biopsies and cells derived from patients. These mitochondria could appear swollen, shortened, fragmented, contain irregular and distorted cristae or appear to contain glycogen particles (Engel and Dale, 1968;

Huang *et al.*, 2011; Lim *et al.*, 2015; Raben *et al.*, 2012; Schoser *et al.*, 2007). Whether the presence of dysfunctional mitochondria is a result of aberrant removal of these dysfunctional mitochondria by autophagy remains to be determined, however, one study showed the accumulation of abnormal mitochondria and oxidative stress as a result of autophagy inhibition in skeletal muscle of wild type mice and therefore indicating a possible link (Lim *et al.*, 2015; Wu *et al.*, 2009). Apart from the morphological appearance of abnormal mitochondria, functional measures have further indicated their involvement in disease pathogenesis. The mitochondrial membrane potential was found to be abnormally low and mitochondrial membrane permeability abnormally high in Pompe disease cells which was further found to lead to the generation of reactive oxygen species and increased cell death (Lim *et al.*, 2015). Furthermore, the mitochondrial oxygen consumption and ATP levels were significantly decreased in Pompe disease cells indicating the possible involvement of dysfunctional mitochondrial bioenergetics in disease pathology (Lim *et al.*, 2015). The cellular distribution of calcium was also found to be abnormal in myotubes of Pompe disease cells. Intracellular calcium and mitochondrial calcium concentrations were significantly higher in Pompe disease cells as compared to wild type cells (Lim *et al.*, 2015).

Another study has indicated a deficit in protein glycosylation in the Golgi of stem cells that had been produced from Pompe disease patients (Raval *et al.*, 2015). However, further studies are required to confirm this in samples from patients and animal models.

All these studies support the awareness of the increasing complexity of Pompe disease pathogenesis as opposed to the previous simplification of the model

based on lysosomal storage accumulation and rupture. These data have led to the proposal of a vicious cycle in which oxidative stress and lipofuscin deposition lead to dysfunctional mitochondria and lysosomes which further impede the clearance of aberrant and old mitochondria leading to further oxidative stress due to reactive oxygen species generation and further pronounced lipofuscin accumulation (Lim *et al.*, 2014, 2015; Terman *et al.*, 2010).

Even though Pompe disease has been mainly described as an LSD affecting muscular tissue, some studies have described the detectable accumulation of glycogen within the central nervous system (CNS) and shown extensive neuropathological alterations (DeRuisseau *et al.*, 2009; Fuller *et al.*, 2013; Sidman *et al.*, 2008). Periodic-acid Schiff (PAS) staining has demonstrated the accumulation of glycogen in Purkinje cells of the cerebellum, cortical neurons and motor neurons of the ventral horn in the spinal cord of patients (Fidziańska *et al.*, 2011; Thurberg *et al.*, 2006). The importance of neuronal glycogen accumulation in disease manifestation was demonstrated by the localized rescue of GAA expression in muscle cells of GAA knockout (KO) mice which retained breathing impairment and reduced phrenic output, indicating a neuronal contribution to respiratory insufficiency in Pompe disease (DeRuisseau *et al.*, 2009).

The current estimates of incidence of Pompe disease are around 1 in 40,000 live births but this can vary between populations from 1 in 14,000 to 1 in 600,000 live births (Ausems *et al.*, 1999; Leslie and Tinkle, 1993; Martiniuk *et al.*, 1998). The disease progression and pathology, similar to all other LSDs, can be highly variable between individuals and may often depend on residual enzyme activity.

A common distinction is made between early-onset and late-onset disease, which may be further divided into classical and non-classical infantile for early-onset Pompe disease and into juvenile and adult for cases of late-onset (Amalfitano *et al.*, 2001; Hirschhorn and Reuser, 2001; van der Ploeg *et al.*, 2010). In severe cases of classical, early-onset Pompe disease, symptoms may include cardiomegaly and hypotonia which manifest themselves within the first few months of life with rapid disease progression culminating in cardiopulmonary insufficiency (Amalfitano *et al.*, 2001; Hahn *et al.*, 2015; Hirschhorn and Reuser, 2001). The non-classical form may occur within the first year of life, and presents itself with progressive muscle weakness, poor muscle tone, slow development of motor activity and cardiomegaly. In contrast to the classical infantile Pompe disease, morbidity and mortality in cases of the non-classical or adult form have been associated to ventilation rather than cardiac insufficiency (Leslie and Tinkle, 1993; Morris *et al.*, 2015). Severe phenotypes are mostly associated with null-mutations or complete absence of enzymatic activity (Polten *et al.*, 1991; Schoser *et al.*, 2008; Wang *et al.*, 2011b). In less severe cases, residual enzymatic function may be detected. In these cases, disease onset may occur between early childhood and late adulthood, with slower but progressive accumulation of glycogen within the lysosomes and autophagic build-up which may manifest itself in less obvious signs and symptoms such as skeletal muscle weakness, sleep apnoea and back pain in the absence of cardiac abnormalities (Hirschhorn and Reuser, 2001; Morris *et al.*, 2015; Raben *et al.*, 2010). Progressive damage to muscular tissue frequently leads to wheelchair and/or ventilation dependency. Respiratory failure constitutes the most common cause of mortality in late-onset Pompe disease (Angelini and Semplicini, 2011; Hirschhorn and Reuser, 2001).

The prevention of high morbidity and mortality amongst individuals with Pompe disease, especially in early-onset cases, depends on early screening and diagnosis (Banugaria *et al.*, 2013; Park *et al.*, 2015). The most common and conclusive diagnostic test for Pompe disease is a biochemical enzyme activity test which assays the presence and level of GAA activity in blood samples, skin fibroblasts or muscle biopsies (Banugaria *et al.*, 2013; Hopkins *et al.*, 2015; Musumeci *et al.*, 2015; Umapathysivam *et al.*, 2001). The ability to identify cases early allows for prompt intervention and initiation of treatment regimens which maximises chances of delaying or preventing the severity of the disease (Banugaria *et al.*, 2013; Musumeci *et al.*, 2015).

### **1.3 The treatment of LSDs**

Due to the broad spectrum of LSD pathogenesis, tissue involvement and genetic origin of disease, a wide range of different treatments have been and are being developed. Some promising approaches are still in the research phase while some treatment regimens have been the clinical standard for decades. A summary of some of the current treatments available is illustrated in Figure 1.1.

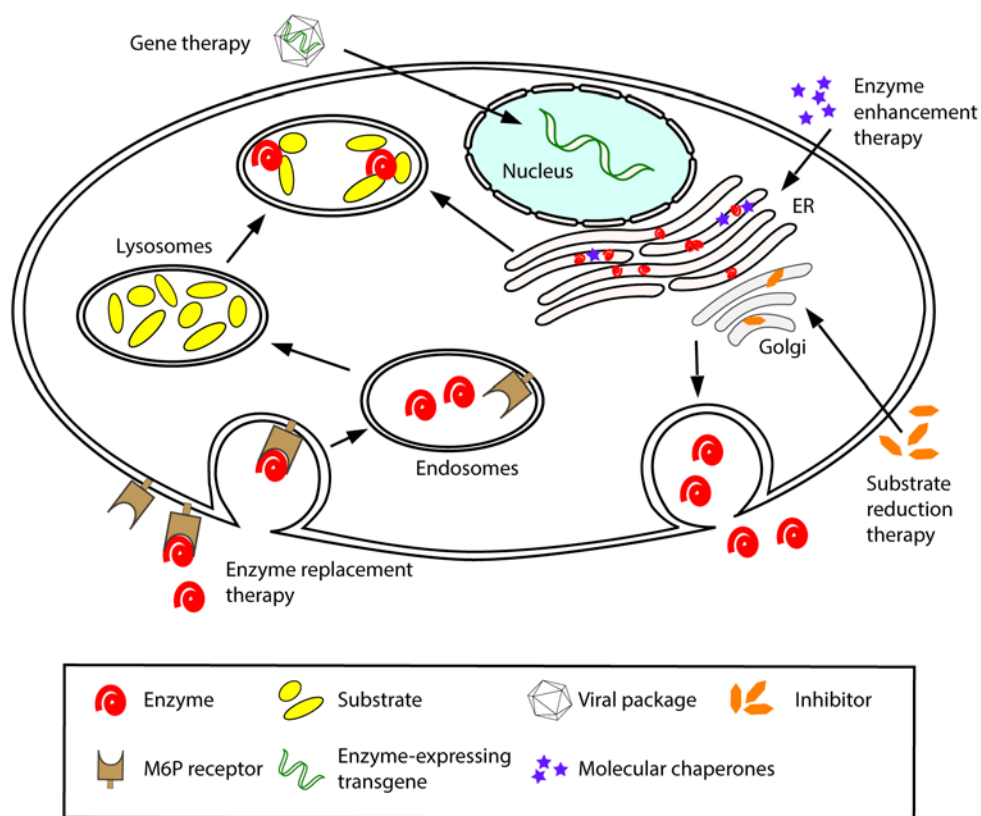


**Figure 1.1 The treatment of LSDs.** During enzyme replacement therapy therapeutic enzyme is administered into the circulation, which enables enzyme uptake into cells primarily by binding to the mannose-6-phosphate (M6P) receptor. Taken up into the endocytic system, the therapeutic enzyme is targeted into the lysosomes where it promotes the breakdown of the accumulated substrate. This pathway of enzyme uptake is also exploited during haematopoietic stem cell transplantation and gene therapy as corrected cells release therapeutic enzyme into the circulation, allowing the cross-correction of other cells. The treatment of LSD with substrate reduction therapy targets the pathway of substrate synthesis by inhibiting its formation. Enzyme enhancement therapy makes use of small molecular chaperones which may support enzyme folding and stability and therefore rescue enzymes from endoplasmic reticulum-associated degradation. This treatment can recover enzyme availability and therefore improve the clearance of accumulating substrate. For gene therapy, transduced cells express transgenes and package them into non-pathogenic viral particles for gene delivery. Once administered, viral particles deliver the transgene to the target cells where their expression allows the production of therapeutic enzyme and improvement of substrate clearance.

### **1.3.1 Haematopoietic stem cell transplantation**

Haematopoietic stem cell transplantation (HSCT) is one approach that has been used for the treatment of MPS I and MPS VI (Turbeville *et al.*, 2011). The transplanted haematopoietic stem cells (HSC) provide a source of enzyme by the generation of enzyme-expressing and secreting blood cells and further release of enzyme from the bone marrow.

HSCT has the advantage of enzyme availability in a wide range of tissues due to cell migration and engraftment, such as differentiation into microglia in the CNS and into Kupffer cells in the liver (Prasad and Kurtzberg, 2010). However, HSCT does not appear to allow the cross-correction of tissues such as the eye and bone (Turbeville *et al.*, 2011). Patient survival after HSCT is low, one study reporting survival to be 78% after 100 days and 66% after 1 and 3 years (Turbeville *et al.*, 2011). The most common causes of death were found to be infections and organ failure. Graft versus host disease (GVHD) was a reported complication which, in this particular study, had occurred in 17% of patients two years after HSCT (Turbeville *et al.*, 2011). In GVHD, donor-derived T cells recognise recipient tissue as foreign and mount an immune response against the host (Sung and Chao, 2013). The dependency on human leukocyte antigen (HLA)-matching is also a limiting factor, greatly complicating the identification of an appropriate donor, since, if not sufficiently matched, HLA disparities increase the likelihood of GVHD and insufficient engraftment. HSCT is, therefore, a last resort therapy due to these potentially severe complications.

However, limiting factors such as GVHD and HLA-matching may be circumvented by the use of autologous HSC. These may be derived from the patient by bone



marrow sampling or HSC-mobilisation through administration of granulocyte-colony stimulating factor and corrected by gene therapy, allowing the expression and production of wild type enzyme by HSC that can then be transplanted back into the patient (Biffi, 2012; Biffi *et al.*, 2013; Harrison *et al.*, 2013; Syres *et al.*, 2009; Visigalli *et al.*, 2010). Animal models and current clinical trials have shown that this approach may be a viable and effective option for the treatment of LSDs, including those with neuronal involvement (Biffi, 2012; Biffi *et al.*, 2013; Harrison *et al.*, 2013; Syres *et al.*, 2009; Visigalli *et al.*, 2010). Apart from the immunological issues it circumvents, this approach may be more effective than allogeneic HSCT as the transfection and integration of the therapeutic gene may lead to the supratherapeutic expression of the enzyme which has been shown to allow improved correction of pathology in a mouse model of MPSI (Visigalli *et al.*, 2010). However, in practice, this method is still hampered by low tissue cross-correction and the poor survival of patients (Grewal *et al.*, 2003; Lund *et al.*, 2014). Furthermore, the genetic manipulation of cells including HSCs may harbour dangers such as insertional mutagenesis and therefore cancer (Condiotti *et al.*, 2014; Donsante *et al.*, 2007; Montini *et al.*, 2009; Nowrouzi *et al.*, 2013; Ranzani *et al.*, 2013; Themis *et al.*, 2005). Recent developments, however, have made available a new method of gene manipulation, called the Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, which enables the highly precise targeting of a particular gene and therefore minimizes the risk of unwanted mutations and gene expression modulations (Hisano *et al.*, 2015; Jiang *et al.*, 2013; Platt *et al.*, 2014; Ran *et al.*, 2013; Xue *et al.*, 2014). Such an approach may find clinical application in the treatment of various LSDs in the future.

### 1.3.2 Substrate reduction therapy

Substrate inhibition/deprivation therapy aims at restoring the balance between synthesis and degradation of the substrate by making use of small molecules with increased bioavailability, broad tissue distribution and potentially improved CNS accessibility as compared to enzyme replacement therapy (ERT), described in section 1.3.6, by inhibiting the production of the accumulating substrate (Jakóbkiewicz-Banecka *et al.*, 2007). A study targeting the muscular isoform of glycogen synthase via ribonucleic acid (RNA) interference was able to reduce glycogen accumulation in primary muscle cells from GAA-knockout mice (Douillard-Guilloux *et al.*, 2008). However, other metabolic pathways are necessary to ensure the clearance of the newly-generated substrate which may accumulate as a result of diverging the metabolic pathway. Miglustat is an iminosugar that was recently approved in Europe for the treatment of neurological manifestations of Niemann-Pick disease type C (NPC) after having been used for the treatment of Gaucher type 1 since 2002 (Brand *et al.*, 2015; Elstein *et al.*, 2004; Kuter *et al.*, 2013; Platt *et al.*, 1994; Wraith *et al.*, 2010). Miglustat reversibly inhibits glucosylceramide synthase, an enzyme involved in the early stages of glycosphingolipid synthesis, which is one of the accumulating substrates in NPC. Treatment allowed the improvement of saccadic eye movement velocity, ability to swallow and stabilization of cognitive manifestations. Therefore, SRT has shown some promising efficacy, however, further research is required, in particular its effect on non-lysosomal disease pathology such as the autophagic build-up.

### 1.3.3 Enzyme enhancement therapy

Enzyme enhancement therapies make use of molecular chaperones which may rescue enzyme folding and prevent their subsequent degradation, correct trafficking and/or improve enzyme stability and therefore activity in some LSDs (Parenti, 2009). As previously mentioned, only a few molecular chaperones have been identified, such as DNJ and NB-DNJ (Miglustat), for Pompe disease. Recent clinical trials have shown the safety of a range of molecular chaperones in patients. The advantage of molecular chaperones for the treatment of LSDs is their non-invasiveness, non-immunogenicity, independence of mannose-6-phosphate receptor (M6PR)-mediated uptake and free diffusability across membranes, which enables drug delivery to the CNS. Studies have also shown the potential of using molecular chaperones in conjunction with ERT (described in section 1.3.6). Co-administration of therapeutic enzyme and NB-DNJ in a Pompe disease mouse model showed improved enzyme activity recovery *in vivo* as compared to ERT alone (Flanagan *et al.*, 2009). This study illustrates that treatment regimes do not have to be mutually exclusive, but may act synergistically on preventing disease pathogenesis and improving disease prognosis. After successful murine *in vivo* studies, this treatment has reached clinical trials for Pompe and Gaucher disease (Khanna *et al.*, 2012; Sun *et al.*, 2012). However, it is important to keep in mind that enzyme enhancement therapy can provide treatment only for patients with the relevant mutations that allow the rescue of activity, which is thought to be around 10-15% of people with Pompe disease (Flanagan *et al.*, 2009; Parenti, 2009). Concerns about further potential problems with enzyme enhancement therapy have been voiced regarding the potential of chaperones to bind close to the active site of the

enzyme and to act as a competitive inhibitor of enzyme activity at certain concentrations (Desnick and Schuchman, 2002). Therefore, more detailed studies are required to fully investigate chaperone function and their optimal therapeutic concentrations.

#### **1.3.4 Proteostasis regulators**

Recently, the potential of general proteostasis regulators that affect processes such as the release of calcium from the ER, unfolded protein response (UPR) mechanisms and heat shock response for the treatment of LSDs has been recognised. Proteostasis regulators diltiazam and verapamil inhibit calcium channels allowing the increase of the calcium concentration within the ER, which is thought to increase the cell's ability to cope with misfolded proteins (Mu *et al.*, 2008). Lacidipine, an inhibitor of calcium mobilisation, was found to decrease cytoplasmic calcium concentrations leading to the activation of endoplasmic reticulum (ER) chaperones, increased expression of UPR associated genes and rescued folding of  $\beta$ -glucosidase in fibroblasts of Gaucher disease patients (Wang *et al.*, 2011a). An increase in cell apoptosis normally induced in response to enhanced UPR was circumvented by Lacidipine and increased  $\beta$ -glucosidase was detected in the ER and lysosomes of treated cells, indicating successful escape from ER-associated degradation (ERAD) mechanisms. A study investigating the effect of proteostasis regulator 1,1'-diheptyl-4,4'-bipyridium on fibroblasts from NPC disease patients showed a similar effect in that treatment caused an increase in ER luminal calcium concentration, increased NPC protein levels, its improved trafficking to late endosomes and lysosomes and reduced lipid storage (Yu *et al.*, 2012). However, despite promising research, this method

of treatment may only be beneficial to patients with relevant missense mutations that lead to protein instability and degradation.

### **1.3.5 Gene therapy**

In order to circumvent regular invasive treatment and potentially decrease the economic burden of treatment, researchers have investigated the potential of gene therapy for the treatment of LSDs. This may make treatment available to rare LSDs and also enable the targeting of the CNS. A study investigating the dual approach of administering recombinant adeno-associated virus (AAV) serotype 9 expressing sulfatase-modifying factor 1 (SUMF1) both systemically and to the cerebral ventricles in a mouse model of MSD has shown the recovery of sulfatase activity, walking speed, spacial memory and the clearance of GAG accumulation (Spampanato *et al.*, 2011). Furthermore, a clinical safety study showed a modest improvement in the ventilation of infantile Pompe disease patients following intradiaphragmatic injection of an AAV-expressed GAA transgene (Smith *et al.*, 2013). Advantages of gene therapy may include the need to transduce only a certain proportion of cells which may allow further cross-correction of other cells due to enzyme secretion and reuptake by other cells. However, the safety of using viral vectors and supra-physiological enzyme concentrations as expressed by transduced cells must be further examined. Integrating vectors may harbour the danger of insertional mutagenesis leading to their transformation (Condiotti *et al.*, 2014; Donsante *et al.*, 2007; Montini *et al.*, 2009; Nowrouzi *et al.*, 2013; Ranzani *et al.*, 2013; Themis *et al.*, 2005). Furthermore, viral vectors may provoke an immune response eliciting the clearance of transduced cells, especially in cases where young patients have to

be administered a second dose of the therapeutic vector which has previously sensitized them. The repeated administration may be required due to a dilution-effect caused by the normal growth and turnover of the liver and therefore the reduced proportion of cells that had been previously transduced with therapeutic vector.

### **1.3.6 Enzyme replacement therapy**

The most commonly used treatment for Pompe disease and some other LSDs such as Fabry, Gaucher and MPS I, II and VI is ERT (Angelini and Semplicini, 2011). This has been made possible by the observation in 1985 of receptor-mediated transport of acid-hydrolases into the lysosomes, identifying M6PR as an important lysosomal targeting component (Sly, 1985). The first LSD to be treated with ERT was type I Gaucher disease in the 1970s.  $\beta$ -glucosidase was purified from placental tissue and administered to patients for treatment (Beutler *et al.*, 1977; Dale and Beutler, 1976). Thereafter, enzymes were produced in recombinant form such as imiglucerase for Gaucher,  $\alpha$ -galactosidase A and B for Fabry, iduronidase for MPSI, idursulfase for MPS II, galsulfase for MPS VI and alglucosidase  $\alpha$  for Pompe disease patients (Bryce A, 2008; Desnick and Schuchman, 2002, 2012). This development has improved patients' life expectancy and quality of life. The treatment of MPS I patients with recombinant  $\alpha$ -L-iduronidase has been shown to improve disease pathology and symptoms considerably, including decreased hepatosplenomegaly, reduced number of apnea and hypopnea episodes, increased motion and growth rate (Dornelles *et al.*, 2014; Jameson *et al.*, 2013; Kakkis *et al.*, 2001; Laraway *et al.*, 2013). The treatment of Gaucher patients has been shown to slow down disease progression

and ameliorate symptoms such as bone pain, thrombocytopenia, anemia and organomegaly (Anderson *et al.*, 2014; Souza *et al.*, 2014; Weinreb *et al.*, 2002). The treatment of Fabry disease with ERT has been shown to be the most effective if started early (Banikazemi *et al.*, 2007; Germain *et al.*, 2013, 2015; Schiffmann *et al.*, 2001; Thurberg *et al.*, 2002; Wilcox *et al.*, 2004). In Fabry, ERT has some beneficial effect on heart pathology however the effectiveness of preventing or reversing nephritic or neuropathology is hampered even though some benefits have been reported (Germain *et al.*, 2013; Rombach *et al.*, 2014; Tøndel *et al.*, 2013; Weidemann *et al.*, 2013). Some of these studies have now questioned the clinical effectiveness of ERT for the treatment of Fabry disease and have called for the development of more effective treatment regimes.

In patients with Pompe disease, ERT was associated with a reduced left ventricular mass, improved skeletal muscle function, reduced glycogen storage and improved histopathology (Amalfitano *et al.*, 2001; Anderson *et al.*, 2014; Park *et al.*, 2015; van der Ploeg *et al.*, 2012). Despite some clinical benefit, the treatment of Pompe disease with ERT has been limited by the low correction of skeletal muscle tissue, even at high therapeutic doses (Angelini and Semplicini, 2011; Chakrapani *et al.*, 2010; Van den Hout *et al.*, 2004; Kishnani *et al.*, 2007; van der Ploeg *et al.*, 2010; Prater *et al.*, 2012, 2013; Schoser *et al.*, 2008). The difficulty of skeletal muscle targeting has been associated to a lower expression of the M6PR in these tissues and therefore current studies are investigating ways of increasing the receptor affinity of GAA or the upregulation of M6PR expression (Farah *et al.*, 2014; Koeberl *et al.*, 2014; Maga *et al.*, 2013; Tiels *et al.*, 2012; Zhou *et al.*, 2011; Zhu *et al.*, 2005, 2009). Furthermore, the reduced responsiveness of type II muscle fibres has also been associated to increased

autophagic build-up in the cells of murine Pompe disease model which appear to sequester therapeutic GAA and prevent its effective targeting to the lysosomes at least partially due to defective autophagosomal-lysosomal fusion (Fukuda *et al.*, 2006a; Moreno-Medinilla *et al.*, 2014; Nascimbeni *et al.*, 2012; Spampinato *et al.*, 2013). Irrespective of these difficulties, improved clinical outcomes could be achieved if treatment regimens had been initiated early on or even before the manifestation of clinical symptoms (Raben *et al.*, 2010).

Therefore, despite being a promising treatment in some respects, the effectiveness of ERT needs to be improved in order to enable a long lasting effect, disease reversal or prevention.

## **1.4 Challenges of LSD treatment**

To date, ERT has been the most successful treatment for the LSDs (Amalfitano *et al.*, 2001; Angelini and Semplicini, 2011). However, as mentioned before, ERT still faces challenges. One challenge is the specific targeting of the replaced enzyme to cells and tissues. Some recombinant enzymes naturally contain specific targeting domains such as mannose-6-phosphate (M6P) residues which allow the uptake by cells expressing the M6PR (Sly, 1985).

### **1.4.1 Challenges to LSD treatment: The blood-brain barrier**

A significant obstacle faced by LSDs with CNS involvement is the selective permeability of the blood-brain barrier (BBB) which has considerable negative impact on the ability to target ERT to the CNS (Abbott, 2013; Raben *et al.*, 2003; Scarpa and Begley, 2013; Sly and Vogler, 2013). Around two thirds of LSDs have



a neurological component and therefore show limited treatment efficacy. The pathway of M6P-dependent cellular uptake, which is commonly targeted in ERT, is limited for the delivery of enzyme to the CNS, as M6PR is down-regulated within the BBB during early development (Urayama *et al.*, 2004).

Intrathecal enzyme administration has been used to overcome the limited permeability of the BBB for the targeting of treatment directly to the CNS. Patients with MPS I and MPS I animal models that had been treated intrathecally showed normalised GAG concentrations in the cerebral spinal fluid (CSF), improved mobility, stability and pulmonary prognosis (Chen *et al.*, 2011a; Munoz-Rojas *et al.*, 2008). However, the frequency of administration and invasiveness of the procedure limits the feasibility of this approach.

Further approaches which may overcome this limitation include gene therapy, enzyme enhancement therapy and substrate reduction therapy as mentioned above. However, as discussed, different approaches harbour different disadvantages, such as treatment invasiveness or applicability to only a limited number of LSD patients.

A recent study showed the improved clearance of GAG in the neocortical and hippocampal neurons of an MPS II mouse model by chemically modifying the recombinant enzyme which caused inactivation of the M6P recognition marker (Grubb *et al.*, 2008). The mechanism of the improved uptake of enzyme due to the inactivation of M6P-dependent enzyme uptake remains to be elucidated.

The possibility of targeting enzymes to receptors which are expressed within the BBB epithelium has been investigated by Osborn *et al.* (2008). The coupling of  $\alpha$ -L-iduronidase to transferrin enabled the delivery of the enzyme into the CNS

via transferrin-receptors in the BBB which lead to a reduction of GAG storage in the cerebellum of MPS I mice (Osborn *et al.*, 2008). Another study looking at improving enzyme-delivery to MPSIIIA mice, was able to significantly improve disease pathology in the brain by enhancing enzyme secretion from transduced liver cells and its transcytosis into the CNS by the addition of a signal peptide from iduronate-2-sulphatase and a BBB-binding domain from apolipoprotein B (Sorrentino *et al.*, 2013). However, the genetic transduction of cells harbours the danger of mutagenesis due to the potential for random integration of the transgene and gene disruption or activation (Magauran and Salgado, 2011; Wingard *et al.*, 2010).

Despite the current attempts to improve enzyme delivery to the CNS, more novel approaches are needed, which are globally applicable with minimal invasiveness and side effects (Brady and Schiffmann, 2004; Parenti *et al.*, 2015; Platt, 2014).

#### **1.4.2 Challenges to LSD treatment: The immunogenicity of the therapeutic enzyme**

Another challenge to LSD treatment using ERT can, in some cases, be presented by the immune system. In patients with null-mutations or mutations causing a significant structural change to the endogenous enzyme, the administration of wild-type recombinant enzyme can trigger an immunological response due to the lack of central tolerance to the wild type enzyme (Abbott *et al.*, 2011; Banati *et al.*, 2011; Banugaria *et al.*, 2011; Berrier *et al.*, 2015; Furusawa *et al.*, 2012; Kishnani *et al.*, 2010; Linthorst *et al.*, 2004; Nayak *et al.*, 2012, 2014).

Patients who lack the endogenous enzyme are defined as cross-reactive immunological material (CRIM) negative, while patients with residual enzyme

levels are categorised as being CRIM positive (Kishnani *et al.*, 2010). CRIM status is determined by Western blotting, where positive refers to the detection of a band specific for the enzyme and negative means the absence of material that cross-reacted with GAA-specific antibodies. Research has shown the higher likelihood of ERT-specific immune responses in CRIM-negative patients which was associated with reduced treatment efficacy, due to the absence of an innate self-tolerance towards the enzyme (Figure 1.2) (Abbott *et al.*, 2011; Banugaria *et al.*, 2011; Berrier *et al.*, 2015; Furusawa *et al.*, 2012; Kishnani *et al.*, 2010; Nayak *et al.*, 2014). However, not all CRIM-positive patients show complete central tolerance towards replaced enzyme. CRIM-positive Pompe disease patients with high antibody titres, so-called high titre CRIM-Positive patients, may have mutations leading to a truncation of the endogenous enzyme which may cause the absence of some immunological epitopes and therefore the immunological recognition of these novel non-cross-reacting polypeptides as foreign (Banugaria *et al.*, 2011; Furusawa *et al.*, 2012; Hunt, 2009; Kishnani *et al.*, 2010; Vedder *et al.*, 2008).

Clinical data suggests that around 66-96% of Pompe disease patients mount an immune response towards ERT (Table 1.1) (Brooks *et al.*, 2003; Desnick and Schuchman, 2012; Rosenberg *et al.*, 2013). Immune responses have also been detected in patients treated for other LSDs. 15% of Gaucher patients are estimated to produce antibodies specific for recombinant enzyme, while an estimated 91-97% respond to MPSI ERT, 11-51% to MPSII ERT, 97-100% to MPSVI ERT and 68-89% to Fabryzyme ERT in Fabry disease (Brooks *et al.*, 2003; Desnick and Schuchman, 2012; Hunt, 2009; Rosenberg *et al.*, 2013). A recently developed method may aid and improve the future determination of drug-

specific antibody titres as this method enables the distinction between free, drug-bound and total antibody titres and may therefore improve our understanding of the prevalence of drug immunogenicity (Bronsema *et al.*, 2015).

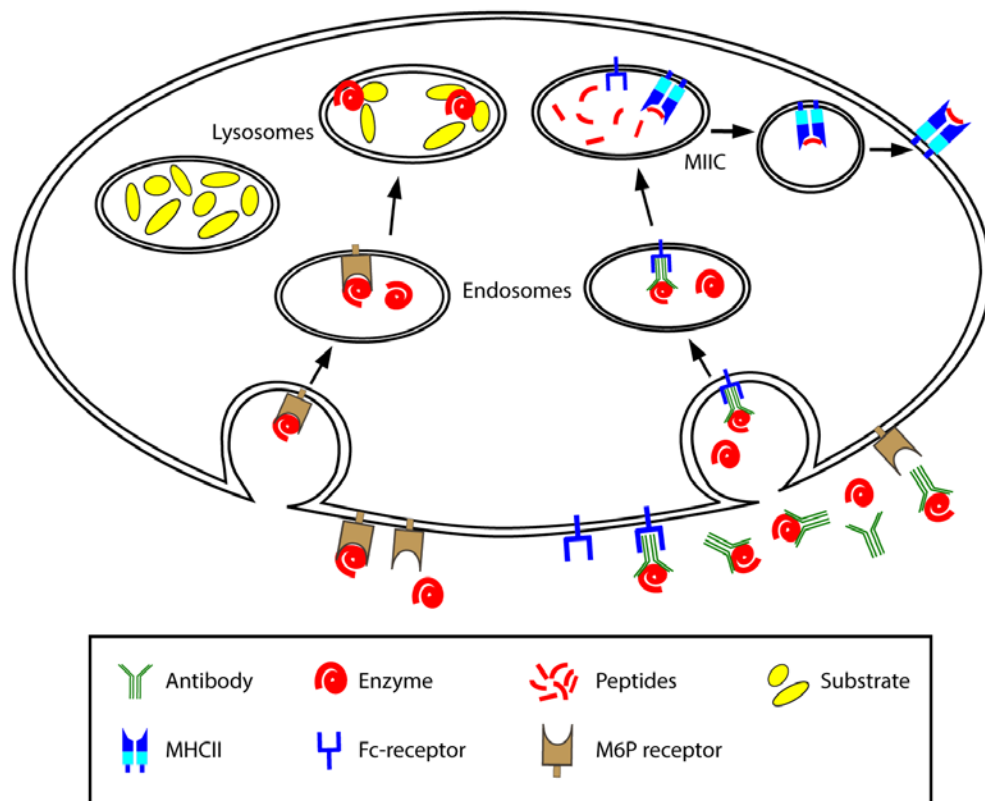


**Figure 1.2 The immunogenicity of therapeutic enzyme.** As illustrated on the left side of the image, in the absence of an immune response, therapeutic enzyme can be taken up via the M6PR into the cell where it supports the degradation of accumulating substrate. However, as illustrated on the right side of the figure, in the presence of enzyme-specific antibodies, enzyme may be opsonized which may prevent the binding of enzyme to M6P receptors and therefore its uptake into the cells. Furthermore, the binding of antibodies to enzyme enhances its uptake into Fc-receptor expressing cells such as antigen-presenting cells. Here, the enzyme may be processed into peptides for antigen-presentation and further stimulation of immune responses.

In the determination of the immunological responses towards ERT, increased interferon (IFN) $\gamma$  expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells has been measured during *in vitro* restimulation of T cells from treated late-onset patients. The strongest T

cell responses were measured in the two patients with the highest anti-GAA antibody titres, as compared to controls (Banati *et al.*, 2011). Research has indicated a link between reduced ERT efficacy and B cell-mediated antibody responses towards the enzyme where initial ERT may show short term improvement followed by a decline coinciding with the development of a high and sustained antibody response (Berrier *et al.*, 2015; Furusawa *et al.*, 2012; Kishnani *et al.*, 2010). CRIM-negative and high titre CRIM-Positive patients, show significantly lower ventilation-free survival, less improvement in motor and cardiac function, higher urinary glucose tetrasaccharide and lower survival rate, indicating that the reduced efficacy of ERT due to antibody responses can also affect CRIM-positive patients (Banugaria *et al.*, 2011; Berrier *et al.*, 2015; Furusawa *et al.*, 2012; van Gelder *et al.*, 2015; Kishnani *et al.*, 2010). These studies have indicated a strong correlation between antibody titres and ERT efficacy, even independently of CRIM status. Antibodies may neutralise administered enzyme by binding close to the active site or altering the distribution within the body. Studies have shown the alteration in enzyme targeting in the presence of high antibody titres from liver to lung and kidneys and intracellularly to endosomes instead of lysosomes (Turner *et al.*, 2000). Opsonisation of enzyme by antibodies may lead to increased enzyme uptake by Fc-receptor-expressing cells, such as macrophages. In a study looking at antibody formation in patients with Fabry disease, a higher  $\alpha$ -galactosidase A activity was detected in leukocytes of patients who had mounted an immune response towards ERT as compared to antibody-negative patients (Linthorst *et al.*, 2004).

These studies have underlined the importance of suppressing an immunological response towards the replaced enzyme in order to achieve an effective clinical

outcome (Abbott *et al.*, 2011; Banugaria *et al.*, 2011, 2012a; Brooks, 1999; Brooks *et al.*, 2003; Cousens *et al.*, 2012; Cresawn *et al.*, 2005; Debiec *et al.*, 2014; Deegan, 2012; Hollak and Linthorst, 2009; Kishnani *et al.*, 2010; Lacan   *et al.*, 2012; Nayak *et al.*, 2012, 2014).

### **1.4.3 Inducing tolerance towards ERT**

Various approaches have been pursued for the induction of tolerance to the administered enzyme. Current research has, for instance, investigated the potential targeting of gene therapy specifically to the liver (Ferla *et al.*, 2014; Franco *et al.*, 2005; Hinderer *et al.*, 2014a; Quiviger *et al.*, 2014; Zhang *et al.*, 2012). This specific targeting may prove advantageous as the high level of blood perfusion of the liver may potentially allow broad tissue distribution of the enzyme and the pro-tolerogenic environment, due to the high level exposure to foreign antigens from the gut, may transfer a state of immunological privilege to the replaced enzyme and transgene. Whether using gene therapy as a tolerizing regime or in creation of an enzyme-producing factory, it was found that antibody production could be prevented if GAA was expressed under liver-specific promoters but not under ubiquitously-active promoters in mice (Franco *et al.*, 2005; Sun *et al.*, 2007, 2010; Zhang *et al.*, 2012). One study found that the combination of liver-specific and ubiquitous transgene expression gave rise to the greatest physiological and biochemical improvement. Furthermore, an immune response to the transgene and GAA expression was not induced unless mice were challenged with exogenous GAA together with an adjuvant (Zhang *et al.*, 2012). T regulatory cells (T<sub>regs</sub>) may play an important role in this tolerizing strategy as their depletion in previously tolerized mice allowed the induction of

GAA-specific antibody titres in response to GAA expressed by the transgene (Sun *et al.*, 2010).

However, as discussed above, gene therapy may harbour considerable dangers for the patient, as viral transgene integration may lead to insertional mutagenesis by disrupting or altering the expression of other genes (Condiotti *et al.*, 2014; Donsante *et al.*, 2007; Montini *et al.*, 2009; Nowrouzi *et al.*, 2013; Ranzani *et al.*, 2013; Themis *et al.*, 2005). Furthermore, more studies are necessary to evaluate the potential non-immunogenicity of this approach, as CD8<sup>+</sup> cytotoxic T cells may cause considerable damage if activated in response to transduced cells.

HSCT as a tolerance induction regime has been investigated in mouse models of Pompe disease by genetically correcting bone marrow cells with human GAA-expressing lentiviral vectors and administering these cells to lethally-irradiated mice prior to ERT initiation (Douillard-Guilloux *et al.*, 2009). This inhibited antibody secretion and restored enzyme activity in bone marrow and peripheral blood cells. This allowed a significant reduction in glycogen storage, however a similar improvement was observed in non-tolerized mice subjected to ERT. A similar study showed the efficacy of HSCT transduced with GAA-expressing lentiviral vector in improving physiological and biochemical phenotype in the absence of ERT (van Til *et al.*, 2010). However, as discussed above, HSCT and gene therapy may harbour considerable pitfalls, such as potential GVHD, severe immune suppression, insertional mutagenesis increasing the likelihood of leukemic transformation and low patient survival (Condiotti *et al.*, 2014; Donsante *et al.*, 2007; Magauran and Salgado, 2011; Montini *et al.*, 2009; Nowrouzi *et al.*, 2013; Ranzani *et al.*, 2013; Themis *et al.*, 2005; Wingard *et al.*, 2010). Whether current scientific developments will improve the clinical applicability by

diminishing these limitations remains to be determined (Biffi, 2012; Biffi *et al.*, 2013; Harrison *et al.*, 2013; Syres *et al.*, 2009; Visigalli *et al.*, 2010).

Apart from gene therapy, clinical trials have shown that the treatment of CRIM-negative infantile Pompe disease patients with methotrexate, rituximab and intravenous immunoglobulin concurrently with ERT can reduce the GAA-specific antibody titre and improve ventilation free survival as compared to CRIM-negative patients receiving ERT only (Banugaria *et al.*, 2013; Markic *et al.*, 2012, 2014; Messinger *et al.*, 2012). This approach allows for the elimination of proliferating B and T cells and all naïve CD20<sup>+</sup> B cells (Chan and Cronstein, 2002; Weiner, 2010; Wessels *et al.*, 2008). The success of the study by Banugaria *et al.* (2013) has been attributed to the early diagnosis of disease and the initiation of immunosuppressive treatment before ERT initiation, preventing the establishment of an entrenched antibody response by long lived plasma cells as a previous study by Banugaria and colleagues was unable to detect any clinical improvement or diminishing of antibody titres in response to cyclophosphamide, intravenous immunoglobulins, rituximab and plasmapheresis initiated after ERT commencement (Banugaria *et al.*, 2012a). However, this approach leaves the patient severely immunocompromised and therefore highly susceptible to infections and malignancy. Furthermore, the use of methotrexate has been associated with gastrointestinal and bone marrow toxicity while rituximab has been linked to infusion reactions, infections, progressive multifocal leukoencephalopathy and hepatitis B reactivation (Banugaria *et al.*, 2012a).

Most of these immunomodulation methods have shown variable efficacy in improving patients' responses to ERT. In addition, considering the potential complications and disadvantages some of these treatment regimens may



harbour, such as depleting a patient's bone marrow cells or introducing viral genes with possible deleterious effects, it has become clear that a more sustainable, reliable and less harmful alternative is needed (Abbott *et al.*, 2011; Banugaria *et al.*, 2011, 2012a; Brooks, 1999; Brooks *et al.*, 2003; Cousens *et al.*, 2012; Cresawn *et al.*, 2005; Debiec *et al.*, 2014; Deegan, 2012; Hollak and Linthorst, 2009; Kishnani *et al.*, 2010; Lacaná *et al.*, 2012; Nayak *et al.*, 2012, 2014).

### **1.5 Dendritic cells in immunomodulating therapies**

One extensively-studied method of tolerance induction makes use of naturally occurring dendritic cells (DCs). These cells orchestrate the immune response by either stimulating a neutralising response or advocating tolerance to defined protein antigens (Cools *et al.*, 2007; Lewis and Reizis, 2012). This dual and opposing capacity of the DC to pivot the immune response in one way or the other has attracted considerable interest from researchers. Considering its central policing role in the immune system, the DC represents the most obvious and potentially effective point of intervention, as any immune response will have had to be initiated by the DC. Hence, harnessing the capacity, unique to DCs, to prime naïve T cells and influence the decision-making process by promoting tolerance rather than immunogenicity, may prove a rational approach to immune intervention.

### **1.5.1 The dendritic cell as the mastermind of immunogenicity and tolerance**

First described in the 1970s, DCs were shown to harbour enhanced stimulatory capacity for the activation and proliferation of antigen naïve T cells in the Mixed Leukocyte Reaction (MLR) and stimulation of T effector cell generation *in vitro* (Nussenzweig and Steinman, 1980; Nussenzweig *et al.*, 1980; Steinman and Cohn, 1973, 1974; Steinman and Witmer, 1978; Steinman *et al.*, 1974, 1975, 1979). The discovery of the DC's potent T cell stimulatory capacity, which was found to be 100 times more potent than that of B cells and macrophages, was the first indication of their importance in immunity.

The origin and differentiation of DCs and their precursors has been difficult to delineate and still requires considerable research (Collin *et al.*, 2013; Doulatov *et al.*, 2010; Merad *et al.*, 2013). Known to originate from HSCs in the bone marrow, the multi-lymphoid progenitor (MLP) and the granulocyte-macrophage progenitors (GMP) are thought to both contribute to DC populations in humans (Collin *et al.*, 2013; Doulatov *et al.*, 2010). The exact development and stages of differentiation and commitment are not clear from these progenitors to the DC subsets found in tissues and organs. Generally, DCs are subdivided into conventional/myeloid DCs (cDCs) that express markers such as CD11c and CD11b and plasmacytoid DCs (pDCs), highly expressing markers such as CD123 (IL-3 receptor) as evidence of their dependence on IL-3 (Collin *et al.*, 2013). The main function of pDCs is thought to involve antiviral immune responses as they express receptors for the recognition of viral antigens and secrete significant quantities of type I IFN on activation (Colonna *et al.*, 2004; Gilliet *et al.*, 2008;

Reizis *et al.*, 2011). pDCs are thought to originate from the MLP in particular, while cDCs can originate from either MLPs or GMPs (Collin *et al.*, 2013).

Other subsets include Langerhans cells, which reside in stratified squamous epithelium, and CD14<sup>+</sup> inflammatory DCs, which are thought to originate from CD14<sup>+</sup> monocytes in the blood and also express cDC markers, such as CD11c. The physiological relevance of the CD14<sup>+</sup> monocyte-derived DCs remains, however, a subject of controversy (Collin *et al.*, 2013).

#### **1.5.1.1 DCs as professional antigen presenting cells**

Since DCs are professional antigen presenting cells (APCs), they continuously sample their local microenvironment, take up and process proteins into peptide fragments, which then bind to products of the major histocompatibility complex (MHC) before being transported to the cell surface for antigen presentation (Guermonprez *et al.*, 2002; Savina and Amigorena, 2007).

The majority of antigen presented via MHC class I (MHCI) molecules is endogenously-derived (Hewitt, 2003; Neefjes *et al.*, 2011; Williams *et al.*, 2002). Ubiquitinated endogenous proteins are cleaved by the proteasome within the cytosol into small peptide fragments that are transported into the ER where they associate with MHCI molecules and are targeted through the Golgi system to the surface of the cell for antigen presentation (Neefjes *et al.*, 2011; Rossi and Young, 2005; Villadangos and Schnorrer, 2007). Peptides presented by DCs via MHCI are recognised by peptide-specific T cell receptors (TCRs) expressed by CD8<sup>+</sup> T cells. The co-receptor CD8, which characterises the MHCI-restricted T cell subset, also binds to a monomorphic region of the MHCI, serving to stabilise the interaction with the TCR (Campanelli *et al.*, 2002; Laugel *et al.*, 2011). This close

binding of CD8<sup>+</sup> T cells with the DC allows for the delivery of secondary activation signals which include the binding to costimulatory molecules CD80 and CD86 to CD28 on naïve T cells (Harris and Ronchese, 1999; Hubo *et al.*, 2013). In response to costimulation, DCs release cytokines such as interleukin (IL)-12 which activate CD8<sup>+</sup> T cells enabling them to mature into cytotoxic T cells that, on degranulation, release granzymes and perforins leading to the induction of apoptosis in the targeted cell (Curtsinger *et al.*, 1999).

The majority of peptides presented via MHC class II (MHCII) are derived from exogenous proteins that have been phagocytosed or endocytosed by DCs (Mantegazza *et al.*, 2013; Neefjes *et al.*, 2011; Roche and Furuta, 2015). While maintained in an immature state, DCs are highly endocytic, continuously sampling the environment for antigen. On entering the endocytic system, protein antigens are processed into smaller peptide fragments by enzymes present within the early endosomes (Mantegazza *et al.*, 2013; Neefjes *et al.*, 2011; Roche and Furuta, 2015). The endosomes merge with ER-derived vesicles carrying inactive MHCII complexes to form the MHCII compartment (MIIC). Here peptide antigens bind to the MHCII peptide-binding groove thereby displacing invariant chain derived peptides and this complex is then translocated to the plasma membrane for antigen presentation (Blum *et al.*, 2013; Mantegazza *et al.*, 2013). Peptide presented in an MHCII-restricted fashion is recognised by CD4<sup>+</sup> T cells. On binding of the TCR and CD4 co-receptor to peptide-bound MHCII, interaction of costimulatory molecules and cytokine release induce CD4<sup>+</sup> T cells to differentiate into effector T cells (Mempel *et al.*, 2004).

The presentation of peptide-MHC complexes constitutes the first and only antigen-specific activation signal to T cells. In the presence of inflammatory

stimuli, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), DCs mature and upregulate their costimulatory molecules of the B7 family such as CD80 and CD86 and increase the expression of peptide-loaded MHC molecules on the cell surface (Kapsenberg, 2003; Newton and Dixit, 2012). The costimulatory molecules interact with CD28 expressed by T cells, which provides the secondary activation signal. This induces the production and release of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-12 and IL-18, sometimes referred to as the third signal, that drive T cells towards full activation and lineage commitment (Curtsinger *et al.*, 1999; Mempel *et al.*, 2004).

There are different subsets of CD4<sup>+</sup> effector T cells including T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 (Th17) (Figure 1.3) (Broere *et al.*, 2011; Romagnani, 2000). The differentiation of these different effector cells is determined by the cytokine environment where IL-12 and IFN $\gamma$ , induced by IL-18, are thought to enhance Th1 commitment, IL-4 promotes Th2 while transforming growth factor (TGF)- $\beta$ , IL-6 and IL-23 are thought to co-operate to enhance Th17 commitment (Broere *et al.*, 2011; Harrington *et al.*, 2005; Swain *et al.*, 1990).

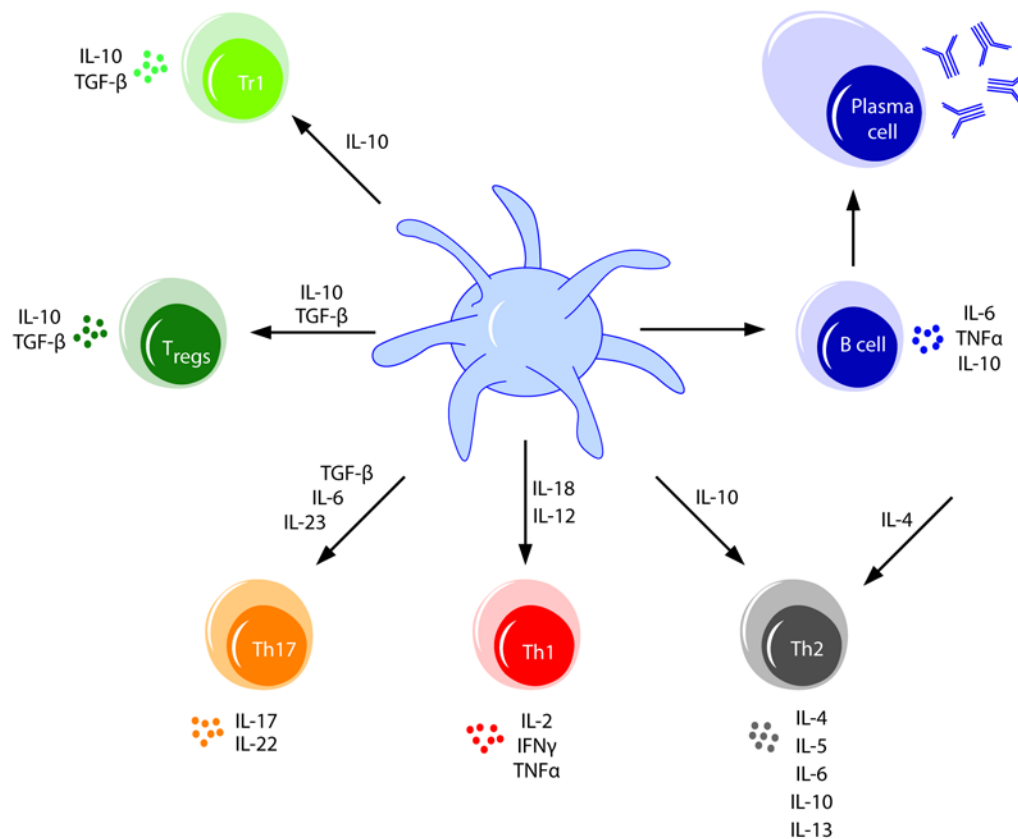


**Figure 1.3 The effect of cytokines on T cell subset differentiation.** DCs and other immune cells secrete a range of cytokines that influence and dictate the differentiation of naïve CD4<sup>+</sup> T cells into a range of T cell subsets. The secretion of IL-10 and TGF- $\beta$  by DCs are particularly thought to be involved in the generation of T<sub>regs</sub> and Tr1 cells. These subsets are particularly involved in the induction of tolerance and the suppression of T effector cell and B cell responses and are thought to also secrete TGF- $\beta$  and IL-10, further enhancing their immunosuppressive capacity. However, TGF- $\beta$ , together with IL-6 and IL-23 has been found to be involved in the generation of the Th17 subset which is known to secrete cytokines such as IL-17 and IL-22. IL-10, on the other hand, in conjunction with IL-4, can mediate Th2 responses which initiate the secretion of numerous cytokines. The secretion of IL-12 and IL-18 has been shown to be involved in the generation of Th1 cells which secrete IL-2, IFN $\gamma$  and tumour necrosis factor (TNF)  $\alpha$ .

These subsets themselves secrete cocktails of cytokines characteristic of their subset and effector function. Th1 cells are well known to secrete IFN $\gamma$  and IL-2 promoting macrophage activation, recruiting further leukocytes to the site of

inflammation and inducing class switching of B cells (Romagnani, 2000). The Th2 subset plays an important role in promoting antibody responses by promoting B cell activation and targeting extracellular pathogens. They release cytokines such as IL-4, IL-5, IL-10 and IL-13 and activate cells such as basophils and eosinophils (Romagnani, 2000). For the induction of B cell-mediated antibody production towards protein antigens, B cells require help from activated T cells. Therefore, DCs have to activate T cells that recognise the same protein antigen as the antigen-specific B cell receptor expressed by the B cell, which is referred to as linked recognition, in order to allow B cell-expressed MHCII and TCR to interact (Dubois *et al.*, 1999; Duddy *et al.*, 2004). Costimulation via CD40-CD40 ligand (CD40L) and the secretion of cytokines further drive the activation of B cells and the development of antibody responses (Elgueta *et al.*, 2009).

### **1.5.2 Antigen cross-presentation**

Apart from the classical pathways of antigen presentation, two further pathways have been recently discovered, the so-called antigen cross-presentation pathways (Bachem *et al.*, 2010; Crozat *et al.*, 2010; Dorner *et al.*, 2009; den Haan *et al.*, 2000; Kovacsovics-Bankowski and Rock, 1995; Sachamitr and Fairchild, 2012). These pathways allow for cytosolic proteins to be presented in an MHCII-restricted fashion and extracellular proteins to be presented via MHCI. Cross-presentation of exogenously acquired antigen via MHCI allows so called cross-priming as tumour antigen and antigen from virus-infected cells can be presented to CD8<sup>+</sup> cytotoxic T cells to allow effective anti-tumour and anti-viral responses (Sachamitr and Fairchild, 2012). Furthermore, self-antigen taken up from the environment can be cross-presented via MHCI following autophagosomal fusion

with the MHC compartment, which may allow for cross-tolerance induction in order to prevent the activation of autoreactive CD8<sup>+</sup> T cells (Schmid *et al.*, 2007).

Despite the identification and characterisation of mouse CD8<sup>+</sup> cross-presenting DCs a decade ago, only recently, have studies been able to identify the population of cells cross-presenting exogenously derived antigen in a MHC-I restricted fashion and their defining markers in humans (Bachem *et al.*, 2010; Crozat *et al.*, 2010; Dorner *et al.*, 2009; den Haan *et al.*, 2000; Kovacsovics-Bankowski and Rock, 1995). These markers include CD141 and chemokine (C motif) receptor 1 (XCR1). These cells may harbour considerable therapeutic potential due to their capacity to cross-prime CD8<sup>+</sup> T cells towards exogenous antigen which may be taken up from tumour cells.

### **1.5.3 Mechanisms of tolerance**

The requirement for mechanisms that prevent the activation of T cells is of considerable importance as the body constitutes a plethora of antigens of self-origin to which immune responses would be detrimental. Clonal deletion of self-reactive T cells occurs early on in life, during T cell maturation in the thymus. The expression of transcription factor autoimmune regulator (AIRE) ensures the expression of many tissue-specific self-antigens on medullary thymic epithelia cells to enable the recognition and elimination of self-antigen-specific T cells by apoptosis or induction of T cells with regulatory function (Blackman *et al.*, 1990; Kappler *et al.*, 1987; Kishimoto and Sprent, 2000; Laan and Peterson, 2013; Peterson *et al.*, 2008). However, as not all antigens are expressed in the thymus, some self-reactive T cells may escape into the periphery. Furthermore, the induction of an immune response towards non-pathogenic environmental



antigens can cause significant immune pathology, requiring another mechanism of tolerance to police the immune system in the periphery of the body (Miller and Morahan, 1992; Mueller, 2010; Saei and Hadjati, 2013). In the periphery, tolerance can be maintained by steady-state DCs. These, but also DCs that had been exposed to anti-inflammatory signals, are capable of suppressing T cell and B cell activation by mechanisms involving the expression of immunosuppressive surface molecules such as programmed-death ligand (PD-L)1, PD-L2, immunoglobulin-like transcript (ILT) 3 and ILT4 (Figure 1.4) (Carter *et al.*, 2002; Manavalan *et al.*, 2003).

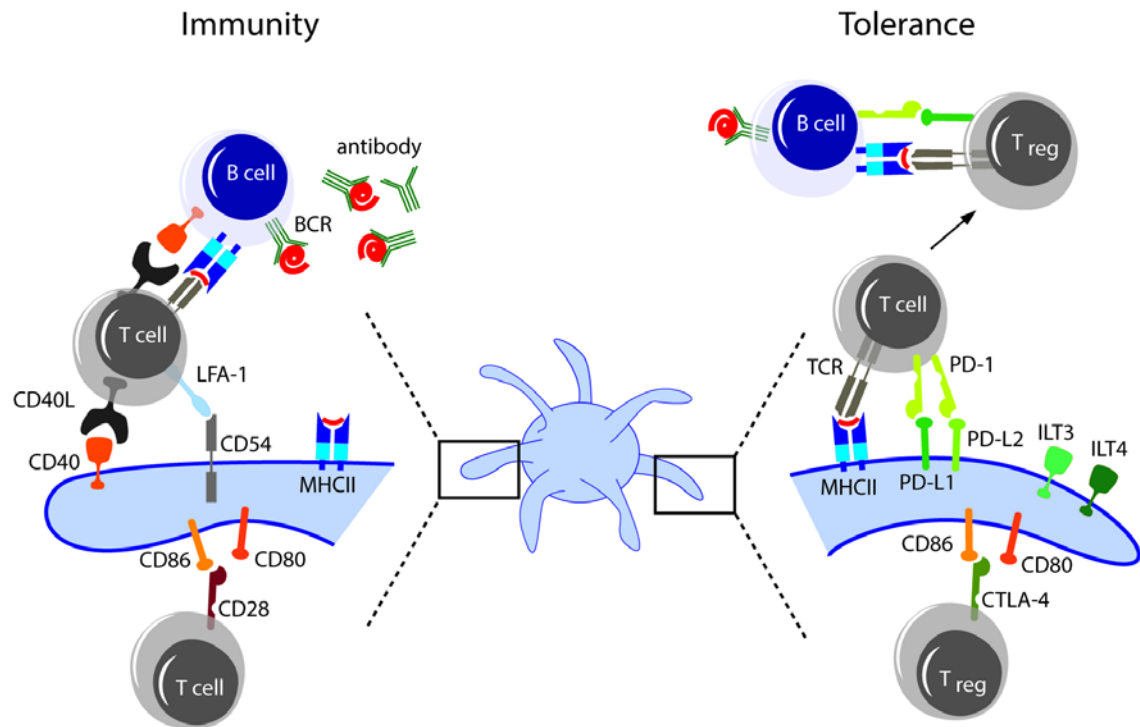


**Figure 1.4 The role of DC surface molecules in immunity and tolerance.** As illustrated on the left-hand side of the figure, the activation of CD4<sup>+</sup> T cells is induced by the presentation of antigen-bound MHCII complexes on the surface of the DC. This occurs in conjunction with costimulatory molecules such as CD80 and CD86 which bind CD28 on T cells and CD40 which interacts with CD40L on T cells. This costimulation induces the secretion of pro-inflammatory cytokines by DCs together with T cell activation and their polarisation into specific subsets (illustrated in figure 1.3). These activated T cells can further activate antigen-specific B cells that have taken up antigen via their B cell receptor (BCR) and present protein-derived peptides via MHCII on their surface. Their activation is initiated by their interaction of their peptide-MHCII complexes with TCRs of the activated T cells and costimulation via CD40 and CD40L. This interaction is further strengthened by close adhesion via CD54 and LFA-1. On the other hand, as illustrated on the right-hand side of the figure, DCs may also display surface molecules such as PD-L1, PD-L2, ILT3 and ILT4 that are conducive of T cell suppression. Antigen-presentation via MHCII and the co-ligation of inhibitory molecules in the absence of strong costimulation may promote the differentiation of naïve T cells into T<sub>regs</sub>. These T<sub>regs</sub> can further act on activated B cells, which express PD-1, via PD-L1 and PD-1 signalling which in conjunction with cytokines, can suppress B cell-mediated immune responses. DCs may themselves be rendered tolerogenic by the interaction of their costimulatory molecules CD80 and CD86 with T<sub>regs</sub> via CTLA-4.

#### 1.5.4 Apoptosis and anergy

One mechanism to prevent unwanted T cell responses involves the requirement of several signals for T cell activation. As mentioned above, the primary signal is provided by TCR and antigen-MHC interaction, while the second signal is provided by costimulatory molecules. If TCR and peptide-MHC interaction occurs in the absence of costimulation, which may occur under steady state conditions in which DCs remain immature, T cells may undergo apoptosis via first apoptosis signal (FAS) signalling, PD-L1 or cytokine withdrawal or enter a state of anergy, where T cells become unresponsive to further stimulation (Sandner *et al.*, 2005; Süss and Shortman, 1996).

Cell surface molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), which competes with CD28 for the binding of CD80 and CD86 on DCs, and the cytokines IL-10 and TGF- $\beta$  have all been shown to contribute to the induction and maintenance of anergic T cells (Akdis and Blaser, 1999; Annunziato *et al.*, 2002). IL-10 may exert its immunosuppressive effect by inhibiting CD80/CD86-mediated CD28 activation, which leads to the promotion of T cell anergy and inhibition of the secretion of Th1 and Th2 associated cytokines (Joss *et al.*, 2000). Anergy may also be induced by immunoinhibitory molecules such as PD-L1 and PD-L2 on DCs, which signal via programmed-death (PD)-1 on T cells, and ILT3 and ILT4 (Butte *et al.*, 2007; Carter *et al.*, 2002; Francisco *et al.*, 2009; Manavalan *et al.*, 2003; Velten *et al.*, 2004).

### 1.5.5 Foxp3<sup>+</sup> T regulatory cells

Another mechanism of tolerance is the induction of T<sub>regs</sub> which are CD4<sup>+</sup> T cells characterised by the expression of the transcription factor forkhead box P3 (Foxp3) (Fontenot *et al.*, 2005; Hori *et al.*, 2003). These Foxp3<sup>+</sup> T<sub>regs</sub> constitute an essential population for immune homeostasis as demonstrated by the autoimmune diseases X-linked autoimmunity-allergic dysregulation (XLAAD) or immunodysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome in humans which are characterised by mutations that abrogate Foxp3 function, resulting in severe autoimmune disease and premature death (Li *et al.*, 2007). T<sub>regs</sub> constitute an important component of central tolerance as they are generated during thymic selection of T cells during T cell development which allows the generation of T cells specific for self-antigen with immunosuppressive capacity (Dzhagalov *et al.*, 2013; Hsieh *et al.*, 2012; Stritesky *et al.*, 2012). These thymic-generated T<sub>regs</sub> are termed 'natural' T<sub>regs</sub>. However, T<sub>regs</sub> can also be generated outside of the thymus in the periphery and are referred to as 'adaptive' or 'induced' T<sub>regs</sub> (Bluestone and Abbas, 2003; Curotto de Lafaille and Lafaille, 2009; Lin *et al.*, 2013). These cells are generated from naïve CD4<sup>+</sup> T cells when DCs provide subthreshold stimulation in the periphery, as is the case in the absence of costimulatory molecules and in the presence of immunoinhibitory molecules (Bilate and Lafaille, 2012; Francisco *et al.*, 2009; Schmitt and Williams, 2013; Semple *et al.*, 2011). Their suppressive function is known to involve cell-to-cell contact including the CTLA-4 binding to costimulatory molecules expressed by the DC and membrane-bound TGF- $\beta$  and PD-L1 binding (Annunziato *et al.*, 2002; Butte *et al.*, 2007; Francisco *et al.*, 2009). Populations of T<sub>regs</sub>, that also secrete IL-10, are thought to play a major role in the induction

of IL-10-dependent anergy in T cells and educate DCs towards a protolerogenic phenotype (Akdis and Blaser, 1999; Alpan *et al.*, 2004; Asseman *et al.*, 1999; Chaudhry *et al.*, 2011; O'Garra *et al.*, 2004; Scheffold *et al.*, 2005; Steinbrink *et al.*, 2002). Apart from the ability of T<sub>regs</sub> to suppress T helper cells, it has also been shown that T<sub>regs</sub> can act directly via cell-to-cell contact on B cells and thereby suppress their activation and the induction of an antibody response (Ikunishi *et al.*, 2009; Lim *et al.*, 2005; Morgan *et al.*, 2003; Seo *et al.*, 2002). This has been shown to be partially dependent on TGF- $\beta$  and CTLA-4-mediated signalling (Lim *et al.*, 2005).

The stability of Foxp3<sup>+</sup> natural and adaptive T<sub>regs</sub> has been the subject of extensive debate as studies have shown that in particular microenvironments, such as during rheumatoid arthritis or experimental autoimmune encephalitis, Foxp3 expression may be lost and former Foxp3<sup>+</sup> T cells may revert to pathogenic T cells (Bailey-Bucktrout *et al.*, 2013; Komatsu *et al.*, 2009, 2014; Zhou *et al.*, 2009).

### **1.5.6 Type 1 regulatory T cells**

Another T cell population that has been shown to retain regulatory function is the so-called T regulatory type 1 (Tr1) subset. These cells are defined as CD4<sup>+</sup> Foxp3<sup>-</sup> cells that secrete high levels of IL-10 but no IL-4, which distinguishes them from the Th2 subset (Groux *et al.*, 1997; Vieira *et al.*, 2004; Wu *et al.*, 2011). The major factor driving Tr1-mediated suppression appears to be IL-10, as antibody-mediated neutralization of this cytokine has been shown to reverse the inhibitory effect of Tr1 cells (Andolfi *et al.*, 2012; Cottrez *et al.*, 2000). For instance, in a recent study, antigen-specific Tr1 cells were purified from splenocytes of diabetic

mice that had been treated with rapamycin and IL-10 for the induction of allogeneic pancreatic islet transplantation tolerance (Battaglia *et al.*, 2006). These Tr1 cells released high levels of IL-10 and were capable of inhibiting T cell proliferation *in vitro*. Tolerance towards the transplant was dependent on IL-10 and CD4<sup>+</sup> T cells could transfer tolerance to mice in an antigen-specific manner as islet transplants from a different donor strain were rejected.

### **1.5.7 Other mechanisms of tolerance**

The local cytokine environment also plays an important role in peripheral tolerance, since cytokines such as TGF- $\beta$  and IL-10 have the capacity to skew towards a more tolerogenic outcome (Taylor *et al.*, 2006). TGF- $\beta$  is known to play an essential role in the induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> from CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells by the TGF- $\beta$ -mediated initiation of Foxp3 expression (Chen *et al.*, 2011b, 2003; Fu *et al.*, 2004; Marie *et al.*, 2005; Pyzik and Piccirillo, 2007). However, the factors that may skew a certain microenvironment towards tolerance may also be involved in promoting certain immunogenic outcomes. IL-10 for example, together with IL-4, plays an important role in Th2 responses (Hernandez *et al.*, 2005; Iwasaki and Kelsall, 1999; Laouini *et al.*, 2003; Swain *et al.*, 1990). TGF- $\beta$ , on the other hand, may be involved in the generation of T<sub>regs</sub>, but can also play a role in B cell class switching or the induction of Th17 cells (Defrance *et al.*, 1992; Fu *et al.*, 2004; Marie *et al.*, 2005; Qin *et al.*, 2009; Stavnezer and Kang, 2009; van Vlasselaer *et al.*, 1992). Furthermore, T cells responsible for driving immune responses also present with suppressive function by inhibiting immune responses of other T helper cell populations sometimes referred to as cross-antagonism. Th2-secreted IL-4 for example is involved in the

inhibition of Th1 development and IFN $\gamma$  secretion, as well as the inhibition of Th17 development (Harrington *et al.*, 2005; Swain *et al.*, 1990). On the other hand, IFN $\gamma$  released by Th1 cells inhibits Th2 proliferation and Th17 development (Gajewski and Fitch, 1988; Harrington *et al.*, 2005). These are only a few examples of the extensive interactions between T cell subsets.

### **1.5.8 The generation of tolerogenic DCs**

As the major players within the immune system, DCs provide an optimal point of intervention. There have been a variety of different attempts to generate stable and functional tolerogenic DCs ranging from genetic manipulation to guided differentiation in a pro-tolerogenic environment (Adorini and Penna, 2009a; Coates *et al.*, 2003; Naranjo-Gómez *et al.*, 2011). Tolerogenic properties have especially been attributed to immature DCs due to their low costimulatory molecule expression and non-immunogenic homeostatic steady state phenotype, even though it is not a capacity exclusive to immature DCs (An *et al.*, 2011; Dhodapkar and Steinman, 2002; Dhodapkar *et al.*, 2001, 2001). However, administration of immature DCs harbours the danger of their subsequent maturation *in vivo* in response to inflammatory signals (Dalod *et al.*, 2014; Hammer and Ma, 2013; Tan and O'Neill, 2005). Therefore generating stably immature or tolerogenic DCs is an important priority for immunotherapy.

Within the last few decades a variety of agents have been identified that enhance tolerogenic properties of DCs. Agents of considerable interest include known immunomodulatory drugs such as rapamycin, the glucocorticoid steroid drug dexamethasone, 1 $\alpha$ ,25-dihydroxy vitamin D3 (VD3), a metabolite of the secosteroid vitamin D3, as well as a variety of recombinant anti-inflammatory

cytokines including IL-10 (Table 1.2) (Bartels *et al.*, 2010; Pothoven *et al.*, 2010; Stallone *et al.*, 2014; Steinbrink *et al.*, 1997; Torres-Aguilar *et al.*, 2010a, 2010b; Taner *et al.*, 2005; Unger *et al.*, 2009; Farias *et al.*, 2013).

Research into human monocyte-derived or mouse bone-marrow derived DCs has shown the potential to generate so-called alternatively-activated or semi-mature DCs by exposure to some of the agents mentioned above. This semi-mature phenotype is characterised by a more immature-like phenotype rather than a fully mature one, with low expression of costimulatory molecules by DCs after maturation, high expression of immunoinhibitory molecules such as PD-L1, PD-L2, ILT3 and ILT4, a low level or lack of secretion of pro-inflammatory cytokines and the release of high levels of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  (Bartels *et al.*, 2010; Fu *et al.*, 2010; Gong *et al.*, 2011; Manavalan *et al.*, 2003; Peng *et al.*; Thomson *et al.*, 2009a; Unger *et al.*, 2009). Functionally, these cells have been shown to suppress T cell activation in co-cultures, induce apoptosis of T effector cells, expand or induce the T<sub>reg</sub> population to a greater extent than their fully-activated counterparts and prolong graft survival in animal models (Naranjo-Gómez *et al.*, 2011; Pothoven *et al.*, 2010; Taner *et al.*, 2005; Turnquist *et al.*, 2007; Unger *et al.*, 2009). However, despite the commonly used simplification of tolerogenic DCs and the vast amount of research conducted in this area, studies vary significantly in their findings of the impact of specific modulatory agents on phenotype and function. This may be due to differences between blood donors, the concentrations used or the timing of treatment.



| Modulator     | DC phenotype                                                                                                   | Impact on co-cultures with T cells                                                                                                                                  | Potential mechanisms                                                                                                                                              | In vivo                                                                                                                 |
|---------------|----------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|
| VD3           | ↓ costimulation molecules<br>↓ MHCII<br>↑ ILT3, ILT4 and PD-L1<br>↓ IL-12<br>↑ IL-6<br>↑/= /↓ IL-10 and TNFα * | ↓ T cell allostimulatory capacity<br>↑ T cell deletion<br>↑ T <sub>reg</sub> induction<br>↑ Tr1 cell induction<br>↓ Th1 and Th17 cells<br>↓ IFNγ-secreting T cells  | T <sub>reg</sub> induction and T effector cell suppression PD-L1 dependent<br>↑ IκBα expression<br>↓ NFκB nuclear translocation<br>↑ IDO expression               | ↓ arthritis severity in RA mouse model<br>↓ EAE severity in rat model<br>↑ skin graft survival in mice (minor mismatch) |
| Dexamethasone | ↓ costimulation molecules<br>↓ MHCII<br>↑ ILT3, PD-L1<br>↓ IL-12, IL-6, TNFα<br>↑/= IL-10*                     | ↓ T cell allostimulatory capacity<br>↑ T cell anergy<br>↑ T <sub>reg</sub> and Tr1 induction<br>↓ Th17 cells<br>↓ IFNγ-secreting T cells                            | T <sub>reg</sub> induction PD-L1 independent<br>↓ NFκB expression<br>↑ IκBα and IDO expression<br>IL-2 and T <sub>reg</sub> independent T cell hyporesponsiveness | ↓ arthritis severity in RA mouse model<br>↑ corneal allotransplant survival in rats                                     |
| rapamycin     | ↓ costimulatory molecules<br>↑ ILT3 and ILT4<br>↓ IL-10<br>↑ IL-12p40                                          | ↓ T cell allostimulatory capacity<br>↑ T <sub>reg</sub> induction<br>↓ IFNγ-secreting T cells<br>↓ IL-2-secreting T cells                                           | = NFκB activity<br>↓ STAT3-phosphorylation<br>↓ AP-1 activity                                                                                                     | ↑ islet and cardiac allograft survival in mice<br>↑ skin allograft survival in rats                                     |
| IL-10         | ↓/= costimulatory molecules*<br>↓ MHCII<br>↑ PD-L1, PD-L2, ILT3 and ILT4<br>↑ IL-10<br>↓ IL-12p70              | ↓ T cell allostimulatory capacity<br>↑ T cell anergy<br>↑ T <sub>reg</sub> and Tr1 induction<br>↓ IFNγ-secreting T cells<br>↑ antigen-specific memory T cell anergy | IL-10-dependent T <sub>reg</sub> induction<br>skin allograft acceptance PD-1/PD-L1 signalling dependent<br>↓ NFκB binding and nuclear translocation               | ↑ skin allograft survival in mice<br>↓ type 1 diabetes in NOD mice<br>↓ DTHR in mouse model                             |

\*Conflicting results found by different studies; AP-1: activator protein-1; EAE: experimental autoimmune encephalomyelitis; DTHR: delayed type hypersensitivity reactions; IDO: indoleamine 2 3-dioxygenase; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; NOD: non-obese diabetic; RA: rheumatoid arthritis; STAT3: signal transducer and activator of transcription 3.

**Table 1.2 The characteristics of modulated DCs.** Data from numerous publications were compiled and are presented in this table (Adorini and Penna, 2009a; Bartels *et al.*, 2010; Bhattacharyya *et al.*, 2004; Chamorro *et al.*, 2009; Dai *et al.*, 2009; Escobar *et al.*, 2014; Farias *et al.*, 2013, 2013; Fazekasova *et al.*, 2009; Fu *et al.*, 2010; Gong *et al.*, 2011; Haase *et al.*, 2010; He *et al.*, 2010; Horibe *et al.*, 2008; Huang *et al.*, 2010; Mu *et al.*, 2008; Müller *et al.*, 2002; Naranjo-Gómez *et al.*, 2011; O'Flynn *et al.*, 2013; Pothoven *et al.*, 2010; Stallone *et al.*, 2014; Stax *et al.*, 2008; Steinbrink *et al.*, 1997; Széles *et al.*, 2009; Taner *et al.*, 2005; Thomson *et al.*, 2009a; Torres-Aguilar *et al.*, 2010a; Turnquist *et al.*, 2010; Unger *et al.*, 2009; Wang *et al.*, 2010; Xia *et al.*, 2005; Ye *et al.*, 2007).

One agent that has been studied extensively for the induction of tolerogenic DCs is rapamycin. Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) signalling pathway which is regulated by signalling pathways sensing the overall metabolic state of the cell via growth factors, nutrient levels and energy levels (Laplante and Sabatini, 2012; Pearce and Everts, 2015; Thomson *et al.*, 2009b). Rapamycin acts by binding immunophilin FK506-binding protein 12. Rapamycin was found not to affect nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) expression, NFkB activity or expression levels of the NFkB-inhibitor Ikb $\alpha$ , which are important for DC survival and maturation, in previously LPS-matured rat DCs or mouse bmDCs (Table 1.2) (Chiang *et al.*, 2004; Wang *et al.*, 2009, 2010). However, decreased signal transducer and activator of transcription (STAT)3, STAT4 and Janus kinase (Jak)2 phosphorylation and activator protein (AP)-1 DNA binding were detected in rapamycin-treated DCs (Chiang *et al.*, 2004; Wang *et al.*, 2010). mTOR signalling or inhibition is thought to play an essential role in the skewing of T cell populations towards a Th1 or Th2 phenotype by enhancing the production of IL-12 or IL-10 respectively. The rapamycin-driven inhibition of mTOR signalling in DCs has shown to increase the expression and secretion of IL-12 and therefore favour the

induction of Th1 cells (Table 1.2) (Ohtani *et al.*, 2008; Thomson *et al.*, 2009b; Wang *et al.*, 2009; Weichhart *et al.*, 2008). Despite the increase in secretion of IL-12 by rapamycin-modulated DCs, these cells have proven to have pro-tolerogenic properties *in vitro* and *in vivo* (Horibe *et al.*, 2008; Macedo *et al.*, 2012; Naranjo-Gómez *et al.*, 2011; Ohtani *et al.*, 2008; Pothoven *et al.*, 2010; Stallone *et al.*, 2014; Turnquist *et al.*, 2007). Rapamycin is a clinically-approved drug and has been commonly used in kidney transplantation to prolong graft acceptance (Dunn and Croom, 2006; Morath *et al.*, 2007; Saunders *et al.*, 2001). It was found that rapamycin-treated DCs had a decreased allostimulatory capacity in the MLR, and were able to increase the induction of T<sub>regs</sub>. Furthermore, alloantigen-pulsed DCs treated with rapamycin were able to prolong heart graft survival in mice (Table 1.2) (Horibe *et al.*, 2008; Macedo *et al.*, 2012; Naranjo-Gómez *et al.*, 2011; Pothoven *et al.*, 2010; Taner *et al.*, 2005; Thomson *et al.*, 2009a; Turnquist *et al.*, 2007).

Another agent frequently used in the modulation of DCs towards a pro-tolerogenic phenotype is VD3, a fat-soluble secosteroid and an active form of vitamin D3 (Adorini and Penna, 2009a, 2009b). VD3 is known to act by binding to the vitamin D3 receptor which heterodimerizes with the retinoid X receptor and binds to vitamin D3 response elements for the induction or inhibition of gene transcription (Peleg *et al.*, 1995; Thompson *et al.*, 1998; Zhang *et al.*, 2011). Studies investigating the pro-tolerogenic effect of VD3 on DCs showed the increase of I $\kappa$ B $\alpha$  expression and decrease RelB translocation and therefore the inhibition of NF $\kappa$ B signalling (Table 1.2) (Adorini and Penna, 2009b; D'Ambrosio *et al.*, 1998; Harant *et al.*, 1998; Song *et al.*, 2013; Yu *et al.*, 1995). VD3-treated DCs have been shown to have significantly reduced allostimulatory capacity in a dose-

dependent manner, which in human moDCs, appeared to be due to T cell deletion while mouse studies indicated T cell anergy (Chamorro *et al.*, 2009; Naranjo-Gómez *et al.*, 2011; Unger *et al.*, 2009; Yates, 2007). The inhibition of T cell proliferation has been found to be antigen-specific and dependent on the expression of PD-L1 (Unger *et al.*, 2009). TGF $\beta$  also appears to play a role in the inhibition of T cell proliferation as VD3 treatment was found to induce an increase in TGF $\beta$  gene transcription while TGF $\beta$  neutralisation reduced the inhibitory effect of VD3-DCs on T cell proliferation (Yates, 2007). Similar to other agents, VD3 inhibited the upregulation of co-stimulatory molecules on DCs and enhanced the upregulation of immunoinhibitory molecules (Table 1.2) (Bartels *et al.*, 2010; Chamorro *et al.*, 2009; Naranjo-Gómez *et al.*, 2011; Piemonti *et al.*, 2000; Stoop *et al.*, 2010; Unger *et al.*, 2009; Yates, 2007). *In vivo* studies further support the pro-tolerogenic properties of VD3 as modulated DCs have been successfully used to prolong skin grafts in mouse models (Griffin *et al.*, 2001; Yates, 2007).

Dexamethasone on the other hand is a steroid drug clinically used as an anti-inflammatory and immunosuppressant (Kunicka *et al.*, 1993; Mazzucconi *et al.*, 2007; Schmelzeisen and Frölich, 1993). Crossing the plasma membrane, dexamethasone binds to cytoplasmic glucocorticoid receptors which bind to nuclear glucocorticoid responsive elements promoting the regulation of genes (Davies *et al.*, 2005; Schaaf and Cidlowski, 2002). This agent has been found to increase the expression of I $\kappa$ B $\alpha$  and therefore inhibit the translocation of NF $\kappa$ B into the nucleus (Table 1.2) (Auphan *et al.*, 1995; Bladh *et al.*, 2005; He *et al.*, 2010; Schaaf and Cidlowski, 2002; Scheinman *et al.*, 1995). One study showed that dexamethasone-treated DCs appeared to have reduced T cell stimulatory capacity in a non-antigen-specific fashion as they were found to suppress T cell

proliferation even in the presence of 3<sup>rd</sup> party DCs (Unger *et al.*, 2009). In human moDCs, this reduced T cell stimulatory capacity appeared to be due to the induction of T cell anergy rather than deletion and occurred independently of PD-L1 expression (Chamorro *et al.*, 2009; Unger *et al.*, 2009). With a low costimulatory molecule expression, low MHCII expression and pro-tolerogenic cytokine profile, numerous studies have indicated the potential use of dexamethasone-treated DCs in immunomodulation therapies (Auphan *et al.*, 1995; Chamorro *et al.*, 2009; Escobar *et al.*, 2014; Gong *et al.*, 2011; He *et al.*, 2010; Mainali *et al.*, 2005; Naranjo-Gómez *et al.*, 2011; Stax *et al.*, 2008; Unger *et al.*, 2009; Xia *et al.*, 2005). Furthermore, the treatment of DCs with dexamethasone or VD3 promoted the increased release of IL-10 while reducing IL-12 secretion and allostimulatory capacity in an MLR (Naranjo-Gómez *et al.*, 2011; Unger *et al.*, 2009; Xia *et al.*, 2005). Here, an important finding was the stability of the tolerogenic phenotype, as restimulation of DCs did not alter their reduced T cell stimulatory capacity.

The naturally-occurring cytokine IL-10 has also been used to successfully modulate DCs. IL-10 is known to act by binding the IL-10 receptor which activates Jak family tyrosine kinases and the Stat3 signalling pathway (Finbloom and Winestock, 1995; Ho *et al.*, 1995; Liu *et al.*, 2013). Furthermore, IL-10 has been shown to inhibit NFκB by inhibiting IκB kinase, which inactivates an inhibitor of NFκB, and by inhibiting the DNA binding activity of NFκB (Bhattacharyya *et al.*, 2004; Liu *et al.*, 2013; Schottelius *et al.*, 1999; Wang *et al.*, 1995). Numerous studies have implicated IL-10 as an effective pro-tolerogenic agent (Müller *et al.*, 2002; Rutella *et al.*, 2006; Thompson and Thomas, 2002; Torres-Aguilar *et al.*, 2010b). Similar to some of the other pharmacological agents, IL-10-treated DCs

display a phenotype conducive to tolerance induction, such as lowered CD80 and CD86 costimulatory molecule expression, increased expression of PD-L1 and PD-L2 immunoinhibitory molecules, decreased T cell stimulatory capacity, increased T<sub>reg</sub> induction potential, decreased IFN $\gamma$  secretion and increased IL-10 secretion (Table 1.2) (Amodio and Gregori, 2012; Boks *et al.*, 2012; Chamorro *et al.*, 2009; Dai *et al.*, 2009; Gregori *et al.*, 2010; Huang *et al.*, 2010; Melillo *et al.*, 2010; Naranjo-Gómez *et al.*, 2011; Steinbrink *et al.*, 1997; Torres-Aguilar *et al.*, 2010a). *In vivo*, IL-10-treated DCs have been found to be capable of suppressing ovalbumin (OVA)-induced asthma in mice which has been partially attributed to de novo induction of T<sub>regs</sub> from T effector cells (Huang *et al.*, 2010, 2013). Interestingly, IL-10 and IL10/TGF- $\beta$ -treated DCs were found to induce anergy in antigen-specific memory T cells and direct them towards a T<sub>reg</sub>-like phenotype which was reversible on IL-2 treatment (Torres-Aguilar *et al.*, 2010a, 2010b). In the study by Dai and colleagues, skin allograft survival was successfully prolonged in mice by the administration of IL-10-treated DCs, which was found to be partially dependent on PD-L1 interaction with its counter-receptor PD-1, as the intravenous infusion of PD-1 specific antibodies abolished graft survival (Dai *et al.*, 2009).

Altogether, these studies have highlighted the availability of numerous promising agents for the generation of pro-tolerogenic DCs. These studies have further highlighted a common mechanism of tolerance promotion in DCs: the inhibition of NF $\kappa$ B activity. Common themes have merged which may be useful in the screening and determination of the pro-tolerogenic capacity of DCs. These include phenotypic markers such as the levels of expression of costimulatory and

inhibitory molecules and functional indicators such as the induction of T<sub>reg</sub> populations and T cell stimulatory capacity.

### **1.5.9 Clinical application of tolDCs**

Various clinical trials are currently investigating the use of DCs for clinical purposes. Most aim to investigate their use as a cell-based vaccine against different types of cancers such as uterine, lung, breast and renal cancer (Coosemans *et al.*, 2010; Cui *et al.*, 2013; Flörcken *et al.*, 2013; Takahashi *et al.*, 2013; Walter *et al.*, 2012). One study, investigating the use of tumour lysate-pulsed, partially HLA-matched DCs for the vaccination against renal cell cancer showed the feasibility and safety of the approach (Flörcken *et al.*, 2013). In this study, around 40% of patients showed Th1-polarised immune responses against renal cancer-associated antigens.

Another clinical trial published in 2011, that aimed to investigate the potential utility of DCs in the induction of tolerance, was able to show that the administration of autologous tolerogenic DCs generated by the knockdown of costimulatory molecules CD40, CD86 and CD80 using antisense oligonucleotides was safe and well tolerated (Giannoukakis *et al.*, 2011). These and other studies have indicated the safety and feasibility of bringing DC immune therapy to the clinic (Dhodapkar *et al.*, 2001).

### **1.5.10 Sources of dendritic cells**

Until recently, the most common source of DCs for research purposes or clinical applications has been monocytes derived from the patient's own peripheral blood

mononuclear cells (PBMCs). These cells are derived from blood obtained from donors or patients and further processed to obtain PBMCs by gradient centrifugation and further purified to obtain populations of CD14<sup>+</sup> monocytes (Cao *et al.*, 2000; Chapuis *et al.*, 1997; Elkord *et al.*, 2005; Motta *et al.*, 2003). These monocytes can be differentiated *in vitro* into myeloid-like monocyte-derived DCs (moDCs) by a standard protocol of a 7-day culture period in the presence of GM-CSF and IL-4 (Holla *et al.*, 2014; Naranjo-Gómez *et al.*, 2011). Studies in mouse models or *in vitro* assays using mouse DCs, conventionally used bone marrow-derived DCs (bmDCs), which are obtained by a 7-day differentiation of bone marrow-derived haematopoietic precursors in GM-CSF-containing culture medium (Boudreau *et al.*, 2008; Inaba *et al.*, 1992). Interestingly, the generation of pure populations of bmDCs and their differentiation efficiency may vary significantly between animal strains (P. Fairchild, personal communication, August 12, 2014). Furthermore, moDCs have been shown to have great donor-to-donor variability in terms of viability, phenotypic profile and capacity for modulation even when derived from healthy individuals (Gogolak *et al.*, 2007; Jensen and Gad; Osugi *et al.*, 2002). This variability may be further exacerbated by disease state and ongoing treatment. Moreover, depending on requirements and the patients to be treated, blood samples may not provide the required number of cells for effective therapy. This may be especially relevant to cases of LSDs with infantile onset, for which the acquisition of large blood samples from young children with compromised health is not likely to be an option. Therefore, a more reliable, reproducible and expandable source of DCs would greatly improve prospects for immunotherapy.



### **1.5.11 Induced pluripotent stem cells**

It is in the context of scalability that pluripotent stem cells have become of considerable interest. The pluripotent capacity of embryonic stem cells (ESCs), which are derived from the inner cell mass of the pre-implantation embryo, the so-called blastocyst, enables the differentiation of any type of cell from the three embryonic germ layers (Park *et al.*, 2004; Reubinoff *et al.*, 2000; Stojkovic *et al.*, 2004; Thomson *et al.*, 1998). In theory, stem cells can grow and expand indefinitely, providing a potentially unlimited source for cells and tissues (Amit *et al.*, 2000; Thomson *et al.*, 1998). Therefore, protocols have been developed for the differentiation of different types of cells from ESCs, including protocols enabling the differentiation of human DCs (Silk *et al.*, 2011; Tseng *et al.*, 2009). However, apart from the ethical controversies surrounding the use of embryo-derived cells, ESCs and their derivatives face the challenge of potential immunogenicity (English and Wood, 2011; Swijnenburg, 2008; Swijnenburg *et al.*, 2005). Despite the possibility of the retention of a degree of immune privilege by ESCs and their differentiated cell products due to the immune privilege of the developing embryo, ESCs may still prove immunogenic as they cannot be derived in an autologous manner and tissue replacement therapy would be targeted to locations external from the tolerogenic environment of the uterus (Ichiryu and Fairchild, 2013; Li *et al.*, 2004; Robertson *et al.*, 2007). Therefore, cells and tissues derived from ESCs for cell and tissue replacement may still be subject to allograft rejection.

Possibly overcoming this limitation, a seminal study published in 2006 by Takahashi and Yamanaka demonstrated that terminally differentiated cells could be reprogrammed back to a pluripotent state allowing the cells to regain the

capacity to differentiate into cell types from each of the three germ layers and to contribute to germline transmission (Takahashi, 2007; Takahashi and Yamanaka, 2006). This was achieved by the transduction of fibroblasts with transgenes expressing factors that are known to be important for proliferation or the pluripotency of ESCs, namely octamer-binding transcription factor 3/4 (Oct3/4), sex determining region Y-box-2 (Sox2), c-Myc, and kruppel-like factor 4 (Klf4). These induced pluripotent stem cells (iPSCs) may revolutionise medicine by serving as a potentially unlimited supply of somatic cells and tissues that may be used for cell replacement therapy.

The method initially used for the generation of these iPSCs made use of retroviral transgene delivery into somatic cells with the inclusion of the transcription factor c-myc (Takahashi and Yamanaka, 2006). This approach leads to multiple random transgene insertions into the genome which may lead to insertional mutagenesis and requires cells to be undergoing cell division at the time of transduction (Laurence and Franklin, 2014). Furthermore, the inclusion of the gene expressing transcription factor c-myc was of concern as its reactivation in iPSC-derived tissue has been associated to tumorigenesis (Okita *et al.*, 2007). Since then, other methods have been developed to overcome the potential problems faced by transgene insertion which include lentiviral reprogramming methods, which also enable the reprogramming of non-proliferative cells, and a *piggyBac* transposon system which allows later removal of the reprogramming genes (Chang *et al.*, 2009; Patel and Yang, 2010; Yusa *et al.*, 2009). Non-integrating methods have also been developed in an attempt to avoid any residual integration site abnormalities using episomal DNA vectors, RNA transfection, non-integrating adenoviruses or recombinant proteins, however all methods face the challenges

of low reprogramming efficiency, the requirement for repeated treatment and therefore higher associated costs (Cho *et al.*, 2010; Laurence and Franklin, 2014; Stadtfeld, 2008; Yakubov *et al.*, 2010; Yu *et al.*, 2009). Another method recently developed makes use of the Sendai virus, a non-integrating RNA virus, which has allowed faster and more efficient reprogramming of somatic cells (Fusaki *et al.*, 2009; Nishimura *et al.*, 2011). This has become a popular approach for the generation of iPSCs as it is fast, non-integrating and viral particles are quickly cleared from the cells (Laurence and Franklin, 2014).

To date, a variety of different sources have been used and shown to be effective. These include embryonic fibroblasts, adult fibroblasts, keratinocytes, B lymphocytes, peripheral blood cells, neuronal cells, pancreatic cells and hepatocytes (Aasen, 2008; Aoi, 2008; Hanna *et al.*, 2008; Kim, 2008; Loh, 2009; Stadtfeld *et al.*, 2008; Takahashi, 2007; Takahashi and Yamanaka, 2006). Further research is required into the most optimal and healthy source of cells for the generation of iPSCs.

The ability to apply the iPSCs generation technique to cells derived from individuals with genetic abnormalities has facilitated the study of genetically-linked diseases, while the ability to derive autologous tissue from individuals may theoretically mitigate the issues of allograft rejection and associated complications. This potential of iPSCs to be derived in an autologous fashion and to be further differentiated into any cell of interest, has drawn our attention to its potential in providing an autologous and potentially unlimited supply of DCs for immunomodulation.

### **1.5.12 Disease-specific iPSCs**

Reprogramming has been successfully achieved for a variety of genetic diseases (Panicker *et al.*, 2012; Park, 2008). Kawagoe and colleagues (2011) have been able to reprogram tail tip fibroblasts from a mouse model of Pompe disease and achieve redifferentiation into skeletal muscle cells without the need to supplement cultures with the missing enzyme. On the other hand, the reprogramming of human fibroblasts from Pompe disease patients appears to have been restricted due to the lack of GAA, as Huang *et al.* (2011) have failed to derive iPSCs from fibroblasts in its absence. In their study, iPSCs production from fibroblasts could only be achieved if the cells had been previously transfected with an inducible lentiviral vector enabling the conditional expression of GAA. Once reprogrammed into iPSCs they were able to differentiate them into mesodermal, ectodermal and endodermal tissue and, more specifically, into cardiac muscle-like cells. Interestingly, a more recent study was able to derive iPSCs from patients with infantile and late-onset Pompe disease without the requirement for GAA-rescue (Higuchi *et al.*, 2014). In light of these studies, the reprogramming of fibroblasts from patients with Pompe disease, their subsequent maintenance *in vitro* and redifferentiation appears feasible and might therefore provide a possible source for large scale production of DCs.

### **1.5.13 The differentiation of iPSCs into DCs**

Protocols previously established in our laboratory have enabled the successful differentiation of DCs from mouse and human embryonic stem cells (ESCs) (Fairchild *et al.*, 2000; Silk *et al.*, 2011; Tseng *et al.*, 2009). Furthermore, we have been able to apply these protocols to mouse and human iPSCs for the generation

of induced pluripotent stem cell-derived DCs (ipDCs) under clinically relevant conditions in the absence of animal products (Leishman and Fairchild, 2014; Silk *et al.*, 2012a). The differentiation makes use of a population of iPSCs which had been expanded by standard techniques. Harvested cells are plated onto ultra-low attachment (ULA) plates to allow for the formation of aggregates which grow into closely packed cellular structures called embryoid bodies (EBs). EBs are reminiscent of the early stages of embryogenesis and have the capacity to form tissues of mesodermal, endodermal and ectodermal lineage (Fuchs *et al.*, 2012). However, differentiation can be guided towards a particular lineage and cell type by the creation of a specific microenvironment which can be achieved by the addition of selected cytokines to the EB culture. The formation of mesoderm lineage and haematopoietic commitment was promoted by and dependent on the presence of factors such as BMP-4 (Goldman *et al.*, 2009; Pick *et al.*, 2007; Sadlon *et al.*, 2004; Tseng *et al.*, 2009). Furthermore, the presence of vascular endothelial growth factor (VEGF) allowed the further expansion of HSCs and their survival (Gerber and Ferrara, 2003; Gerber *et al.*, 2002). Stem cell factor (SCF) was included in the differentiation cytokine environment as SCF has been shown to maintain HSC survival and promote their self-renewal (Audet *et al.*, 2002; Kent *et al.*, 2008; Ogawa *et al.*, 1991). However, Pick *et al.* (2007) investigated the importance of these cytokines in the differentiation of ESCs into HSCs and found no benefit in including SCF in their differentiation cocktail with the exception of its use in combination with FGF which gave rise to an increased number of haematopoietic progenitors and mature haematopoietic cells. In contrast, Tseng *et al.* (2009) found that the presence of VEGF and SCF increased reproducibility in differentiation cultures in the generation of CD14<sup>+</sup> monocytes. The definitive

signal for the generation, proliferation and survival of DCs via mitogen-activated protein kinase kinase (MAPKK), STAT5, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and NF $\kappa$ B signalling arises from granulocyte-macrophage colony stimulating factor (GM-CSF) which is present throughout the differentiation protocol (Chapuis *et al.*, 1997; Conti *et al.*, 2008; van de Laar *et al.*, 2012). Similarly to BMP-4, the absence of GM-CSF from differentiation cultures has detrimental effects on the number of CD34<sup>+</sup>, CD45<sup>+</sup> and CD11c<sup>+</sup> cells generated, underlining the importance of this cytokine in the formation of myeloid-like cells (Tseng *et al.*, 2009). Throughout the differentiation, EBs enlarge and grow cyst-like structures. After around 2 weeks of differentiation, smooth irregularly-shaped cells are released from EBs into the surrounding culture (Leishman and Fairchild, 2014). These cells represent the monocyte-like precursors which significantly increase in number over the following week of culture. During this time, highly adherent “fried egg-shaped” and “spindle-shaped” macrophages appear in differentiation cultures (Leishman and Fairchild, 2014). At this point, IL-4 is added to promote DC differentiation. DC-like cells characterised by their cellular protrusions become more and more prominent and numerous in cultures from day 14 onwards. The final differentiation step is identical to the standard DC differentiation protocol from blood monocytes with a 7-day culture period in the presence of GM-SCF and IL-4.

Using the above described protocol, our laboratory was able to derive ipDCs that had an enhanced capacity to cross-present antigen (Silk *et al.*, 2012a). Despite the early identification of cross-presenting CD8 $\alpha$ <sup>+</sup> DCs in mice, the identification of a homologous subset in humans was achieved only recently, where studies discovered human cross-presenting DCs that were characterised by the

expression of XCR1 and CD141 (Bachem *et al.*, 2010; Crozat *et al.*, 2010; Dorner *et al.*, 2009; den Haan *et al.*, 2000; Schulz and Reis e Sousa, 2002). The derivation of cross-presenting DCs was supported by the surface expression of XCR1 and CD141 and their ability to present exogenous melan A to a CD8<sup>+</sup> T cell clone (Silk *et al.*, 2012a).

In conclusion, our ability to reprogram patient-derived somatic cells such as fibroblast samples, which can be easily obtained, coupled with our ability to maintain, expand and differentiate these cells into ipDCs may enable the large scale production of high quality and autologous cells for potential use in immune modulating therapies.

## **1.6 Treatment barriers to LSDs with neuronal involvement**

Apart from the immunological barriers that limit the effectiveness of ERT, the BBB poses another challenge to the treatment of LSDs with CNS involvement. The BBB, located at the interface between the blood and the CNS, is formed by endothelial cells lining the brain's microvessels and is controlled by the neurovascular unit which includes astrocytes and pericytes (Ballabh *et al.*, 2004; Conti *et al.*, 2008). The BBB is an essential element for neural function due to the need for a controlled and homeostatic environment within the CNS to allow small ionic changes between neurons and their environment (Obermeier *et al.*, 2013). This allows the maintenance of a stable resting membrane potential and generation of action potentials but also limits the access of toxins and enables non-synaptic signalling (Ballabh *et al.*, 2004; Conti *et al.*, 2008; Zlokovic, 2008). The selective permeability of the BBB is controlled by its tight junctions, membrane transporters, enzymes and receptors (Weiss *et al.*, 2009). The

transportation of larger molecules and peptides across the BBB is primarily controlled by absorptive- and receptor-mediated transcytosis (Hervé *et al.*, 2008).

This highly regulated selective permeability of the BBB, even though it may be slightly defective in LSD patients, complicates treatment targeting and the achievement of therapeutic levels of the enzyme within the CNS (Abbott, 2013; Thomas *et al.*, 2006). This challenge is especially faced by ERT due to the limited access of larger proteins to the CNS. Past studies have indicated the requirement of in depth studies of the neuropathological involvement and its impact on disease manifestation in Pompe disease and of extending the targeting of ERT to the CNS due to the possible importance of the neuropathological defects (DeRuisseau *et al.*, 2009; Sidman *et al.*, 2008).

Research has investigated a variety of ways to circumvent this problem. One includes the direct injection of enzymes into the CSF or brain parenchyma (Calias *et al.*, 2012; Jolly *et al.*, 2013). Gene therapy by the intravenous administration of AAV, expressing wild type enzyme, that has the ability to cross the BBB has been promising in the MPS IIIB mouse model where a single administration allowed more than 18 months of pathological improvement within the CNS (Fu *et al.*, 2011). The introduction of genetically modified cells to serve as an enzyme-producing factory is being investigated but may prove to be less of an option due to the high invasiveness of this approach (Kennedy and Abkowitz, 1997; Kim, 2014; Krivit *et al.*, 1999; Lund, 2013; Platt and Lachmann, 2009).

Many lysosomal enzymes naturally incorporate M6P which allows easy delivery to cells expressing M6PR (Sly, 1985). Exploitation of the M6PR pathway for the delivery of enzyme to the CNS is not an option, as M6PR is downregulated during



development within the BBB (Urayama *et al.*, 2004). Other receptors expressed in the BBB at adulthood may, however, be future targets of ERT delivery to the CNS including the transferrin receptor (Osborn *et al.*, 2008).

Further strategies of drug delivery to the BBB currently under investigation make use of naturally occurring or artificially made cargo-vessels such as liposomes, microvesicles and exosomes. A study looking at exosomes as delivery vessels for the targeted delivery into the CNS has been able to generate exosomes expressing rabies virus glycoprotein (RVG) fused to lysosome-associated membrane protein 2b (Lamp2b) for the specific targeting via the nicotinic acetylcholine receptor expressed by the endothelia of the BBB (Alvarez-Erviti *et al.*, 2011). Using this method Alvarez-Erviti and colleagues have been able to target short-interfering RNA (siRNA) into the CNS and knockdown  $\beta$ -site APP cleaving enzyme 1 (BACE1), a protease important in Alzheimer's pathogenesis, in the midbrain, cortex and striatum. Another study, confirmed this methodology by demonstrating the siRNA-mediated knockdown of  $\alpha$ -synuclein RNA in the brains of healthy mice and the reduced RNA and aggregation of  $\alpha$ -synuclein in an  $\alpha$ -synuclein-overexpressing mouse model, which indicated the therapeutic potential of this approach for the treatment of Parkinson's disease (Cooper *et al.*, 2014).

As DCs were used in the study by Alvarez-Erviti and Cooper and colleagues for the generation of exosomes, which is known to be a well-defined and rich source, we hypothesised that ipDCs may likewise serve as a ready source of exosomes and that their endocytic and phagocytic capacity may even serve to load such exosomes with the target enzyme.

## 1.7 Exosomes

Exosomes were first described in 1981 as vesicles that retain enzymatic activity once secreted from cells in culture (Trams *et al.*, 1981). Two years later, two studies revealed that maturing reticulocytes release transferrin receptors that are associated with membrane-bound vesicles (Harding and Stahl, 1983; Pan and Johnstone, 1983; Pan *et al.*, 1985). In 1989, these vesicles, now referred to as exosomes, were shown to be associated with further enzymatic activity (Johnstone *et al.*, 1989).

Over recent years, a more refined definition of exosomes has been developed. Exosomes are defined as vesicles of 40 nm to 100 nm in diameter and are made up of a phospholipid bilayer with a buoyant density of approximately 1.13 to 1.19 g/cm<sup>3</sup> (Théry *et al.*, 2006). The classification of exosomes has been ambiguous and therefore names have frequently been used interchangeably. Classification systems attempt to distinguish exosomes from other membrane-bound vesicles on the basis of their size, density, cellular origin and protein make up (van Dommelen *et al.*, 2011; Keller *et al.*, 2006; Pol *et al.*, 2012; Raposo and Stoorvogel, 2013). Different types of vesicles have been variously referred to as ectosomes, microvesicles, microparticles, nanovesicles, membrane particles and exosomes (Théry *et al.*, 2009).

In contrast to exosomes, microvesicles range in size from 100 nm to 1000 nm in diameter and originate from the plasma membrane of the cell surface by shedding (György *et al.*, 2011a, 2011b; Théry *et al.*, 2009). Exosomes on the other hand, are generated within so-called multi-vesicular bodies (MVBs) which are part of the endosomal system (van Dommelen *et al.*, 2011; Keller *et al.*, 2006; Théry *et*

*al.*, 2006). However, the size range of exosomes may be more dynamic than this definition allows for and difficulties in standard separation methods of exosomes from other vesicles of the same or similar size have been limiting, consequently samples may frequently contain a certain proportion of contaminating vesicles of other origin (van Dommelen *et al.*, 2011; György *et al.*, 2011a; Lane *et al.*, 2015).

MVBs are found in late endosomes, where, by reverse budding, exosomes are generated and collected within its lumen (Figure 1.5) (Keller *et al.*, 2006). These can be further targeted to the lysosomes for degradation or the cell surface for protein recycling or release into the extracellular space. Exosomal surface molecules have the same orientation as cellular surface molecules which arise from 2 invaginations, one occurring at the cell membrane during endocytosis and formation of the early endosome, while the second invagination occurs, as described above, during exosome formation within the MVB where MVB membrane buds into the MVB lumen to create the exosomes (Kowal *et al.*, 2014; Pant *et al.*, 2012; Théry *et al.*, 2002a).

A variety of ubiquitous exosomal markers and cell-origin specific markers have been identified over the last few decades. Ubiquitous markers include Lamp2b, LampP1, Alix, tumour susceptibility gene 101 (Tsg101), CD63, CD81 and CD9 (György *et al.*, 2011a; Simons and Raposo, 2009). Furthermore, parent cell specific markers may also be present on exosomes. Exosomes derived from bmDCs have been shown to carry typical DC markers such as CD11c, costimulatory molecules, MHCII and MHCI (Morelli *et al.*, 2004; Näslund *et al.*, 2013; Vincent-Schneider *et al.*, 2002).

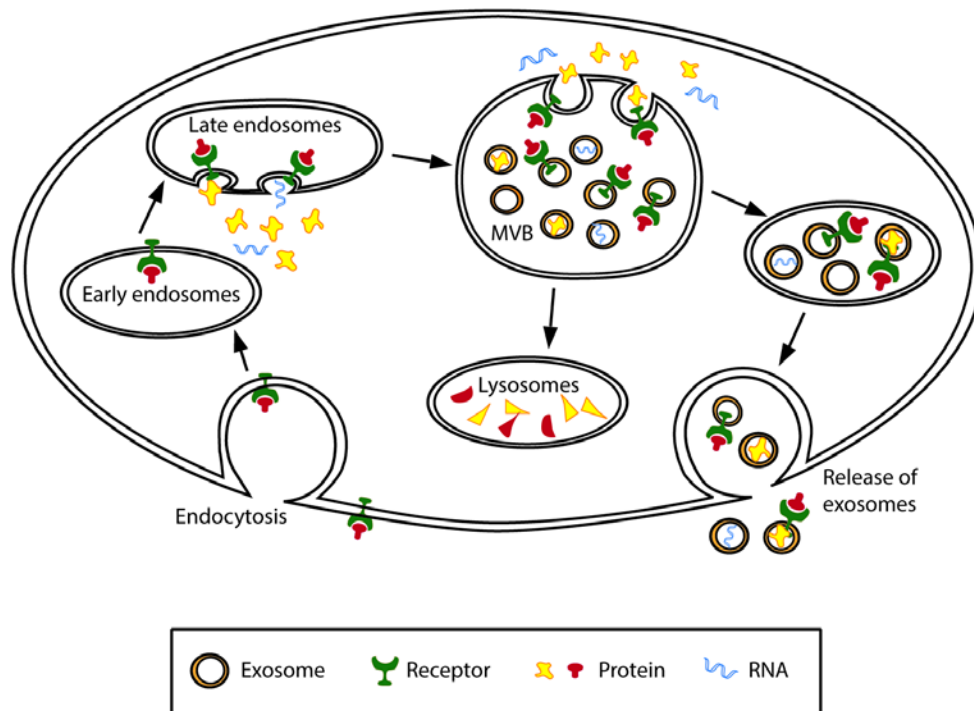


**Figure 1.5 The biogenesis of exosomes.** The biogenesis of exosomes. The biogenesis of exosomes begins with the endocytic internalization of extracellular molecules and membrane bound proteins and lipids by the invagination of the plasma membrane at the cell surface and the formation of a vesicle called the early endosome. At this point proteins and lipids may be recycled back to the plasma membrane or further targeted to the late endosomes. The formation of exosomes occurs within these late endosomes where reverse budding generates so-called MVBs. The reverse budding of MVBs gives rise to intraluminal vesicles which are called exosomes. Molecules found within the MVB may have originated from the cells surface which had been internalized by endocytosis or other intracellular organelles or the cytoplasm. MVBs may be targeted to lysosomes for degradation of lipids, soluble proteins and transmembrane proteins, or to the cell surface for the release of exosomes and other molecules and components into the extracellular space (Keller *et al.*, 2006; Pant *et al.*, 2012).

Exosomes are thought to fulfil a variety of different functions under normal physiological conditions although they are not currently well understood. Identified functions range from intercellular communication such as morphogen

signalling to chemoattraction, cell waste disposal, coagulation, tumorigenesis and immunological regulation (Keller *et al.*, 2006; Pant *et al.*, 2012; Raposo and Stoorvogel, 2013; Simons and Raposo, 2009; Théry *et al.*, 2002a). Apart from the release of exosomes, MVB play an essential role in the degradation of proteins and lipids via the lysosomes. Similar to proteasome-mediated degradation, lysosomal degradation of proteins can be regulated by ubiquitin but, in contrast to the proteasome, appears to play a major role in the degradation of integral membrane proteins that have passed through the Golgi apparatus (Piper and Lehner, 2011; Stringer and Piper, 2011). Integral membrane and intraluminal components that have not been recycled to the plasma membrane or Golgi network are incorporated into intraluminal vesicles which form the MVB (Gruenberg and Maxfield, 1995; Piper and Katzmann, 2007). The lipid composition of these intraluminal vesicles differs from that of the limiting MVB membrane which has been found to be essential to the susceptibility of intraluminal vesicles to lysosomal degradation (Gillooly *et al.*, 2000; Hanson and Cashikar, 2012; Kobayashi *et al.*, 1998, 2002; Trajkovic *et al.*, 2008). The downregulation of proteins and lipids via MVB and lysosomes plays an important role in a cell's maturation and differentiation when significant cellular reorganisation is required (Fujita *et al.*, 2002; Palacios *et al.*, 2005). MVB are further involved in the regulation of receptor-ligand signalling as internalisation of receptors from the cell's surface can terminate its signalling (Bache *et al.*, 2006; Hislop and von Zastrow, 2011; Katzmann *et al.*, 2002). However, as mentioned above, not all intraluminal vesicles are destined for lysosomal degradation. Instead, some intraluminal vesicles are released into the extracellular space at the cell surface as so-called exosomes (Hanson and Cashikar, 2012; Kowal *et*

*al.*, 2014; Pant *et al.*, 2012; Piper and Katzmann, 2007). In recent years, exosomes have aroused considerable attention as they appear to play important roles *in vivo*, but also appear to display useful characteristics that may be exploited for clinical and therapeutic purpose.

### **1.7.1 The potential of exosomes as drug-carrier**

The ability to carry molecules and target them specifically to cells *in vivo* has put exosomes into the spotlight for their potential use in drug delivery (Johnsen *et al.*, 2014; Lai *et al.*, 2013; Lakhal and Wood, 2011). Two studies highlighting this potential made use of RVG linked to exosomal surface protein Lamp2b as a neuronal-specific targeting moiety that targets nicotinic acetylcholine receptors. This enabled the targeted delivery of siRNA loaded exosomes via the BBB into the CNS where it enabled the siRNA-mediated knockdown of BACE1 or  $\alpha$ -synuclein, a therapeutic candidate for Alzheimer's disease and Parkinson's disease respectively (Alvarez-Erviti *et al.*, 2011; Cooper *et al.*, 2014). Therefore, the use of exosomes for the delivery of proteins via the BBB may provide a potential alternative approach to improving the targeting of therapeutic enzyme to the CNS. The specific targeting of protein into exosomes has been explored by one study using membrane anchors, which showed that the introduction of putative myristoyl attachment sites or PIP<sub>2</sub>-binding domain could promote the packaging of proteins into secreted vesicles (Shen *et al.*, 2011). However, the generation of a protein packaging protocol that is independent of protein modifications may be essential to allow the packaging of fully functional enzymes into exosomes.

The advantage of exosomes in drug delivery is assumed to include their natural stability in blood and the provision of a safe environment for the drug, away from nucleases and proteases (van Dommelen *et al.*, 2011). Furthermore, the ability to target defined cell types by expression of specific targeting peptide increases their attractiveness for targeted drug delivery systems. However, for this approach to be effective, exosomes have to be immunologically inert or protolerogenic, as neutralising antibody responses may abrogate any therapeutic effect. Therefore more detailed studies are required to investigate the immunogenicity of autologous exosomes that have been generated *in vitro*. Furthermore, efficient and cost effective methods of loading exosomes with the unmodulated therapeutic drugs are required to allow for large scale production for potential clinical application.

A new avenue for the loading of protein into exosomes may potentially be exploited when deriving exosomes from DCs. This new avenue is based on the ability of DCs to cross-present exogenously acquired antigen via MHC I (Ackerman *et al.*, 2006; Kovacsovics-Bankowski and Rock, 1995; Regnault *et al.*, 1999; Rodriguez *et al.*, 1999). A study by Rodriguez and Colleagues (1999) showed the presence of cytosolic fluorescent horseradish peroxidase (HRP) in DCs following 6 hours of co-incubation. Interestingly, this transportation of exogenous antigen from vesicles into the cytoplasm was unique to DCs and not observed in macrophages or B cells. This capacity is based on their ability to translocate endocytosed and phagocytosed proteins from the phagosomal/endosomal lumen into the cytosol of the cell. This has been shown to involve proteins which are essential to ERAD, as the inhibition of protein translocation from the ER to the cytosol of DCs inhibited the cross-presentation

of exogenous antigen (Ackerman *et al.*, 2006). Further studies showed the importance of transporter associated with antigen processing (TAP) and the proteasome, itself located in the cytosol, in the processing of exogenous antigen for the presentation via MHC I pathway which are known to be essential constituents of the classical MHC I antigen presentation pathway of cytosolic antigen (Ackerman and Cresswell, 2004; Norbury *et al.*, 1997; Rock and Shen, 2005; Sachamitr and Fairchild, 2012). The finding that exogenously acquired antigen may be targeted into the cytosol of the DC indicates a potential link to the biogenesis of exosomes. During the formation of MVB, invaginations of the limiting endosomal membrane into its lumen enable the packaging of cytosolic components into the resulting vesicles (Keller *et al.*, 2006; Kowal *et al.*, 2014; Théry *et al.*, 2002a). Therefore, it is reasonable to suggest that exogenous antigens may find their way into the lumen of exosomes. Hence, the potential of loading exosomes with proteins that have been exogenously supplied to DCs, may prove an attractive option.

Furthermore, recent studies in our laboratory that showed the capacity to derive large proportions of cross-presenting DCs from iPSCs, may open up a strategy for the generation of a population of DCs that could be capable of internalising exogenous proteins, translocating them into the cytosol, packaging them into intraluminal vesicles and subsequently releasing these as exosomes (Leishman and Fairchild, 2014; Silk *et al.*, 2012a). As mentioned above, these exosomes may be tagged to enable tissue-specific targeting and may therefore prove a therapeutic opportunity for the delivery of enzyme during ERT.



### 1.7.2 The immunogenicity of exosomes

Given their potential use in therapeutic contexts, various studies investigated the immunostimulatory and inhibitory capacity of exosomes (Zhang *et al.*, 2014). The injection of exosomes pulsed with the male antigen Dbp into female mice containing a monoclonal population of CD4<sup>+</sup> T cells that are specific to Dbp were capable of inducing antigen-specific T cell responses against this antigen (Théry *et al.*, 2002b). *In vitro*, T cell stimulation could only occur if DCs were present and depended on their expression of costimulatory molecules but was independent of their MHCII expression (Théry *et al.*, 2002b; Vincent-Schneider *et al.*, 2002). This has prompted the authors to assume a mechanism of surface molecule transfer between exosomes and DCs. The lack of independent T cell stimulation by exosomes may be due to the reduced ability to crosslink TCR, which could be improved by the binding of exosomes to latex beads (Vincent-Schneider *et al.*, 2002). There are a variety of hypotheses about the actual role of exosomes within the immune system *in vivo* which are nicely reviewed by Théry *et al.* (2009).

Apart from T cell stimulation, exosomes have also been shown to induce B cell mediated responses. The candidate immunotherapy drug MVA-BN-Pro, which is a virus encoding a number of tumour-associated antigens, was shown to increase the potency of antibody-mediated immune responses against prostate cancer antigen-expressing cells in a mouse model if the expression of one of these tumour antigens was targeted to exosomes (Rountree *et al.*, 2011).

In another study, the derivation of exosomes from a *Neisseria meningitidis* strain that had been genetically modified to express a number of potentially immunostimulatory antigens could induce cross protection against different

serogroups of *N. meningitidis* by inducing antibody responses *in vivo* (Pinto *et al.*, 2011).

The injection of exosomes carrying a glycoprotein of the severe acute respiratory syndrome (SARS) coronavirus or adenoviruses expressing the glycoprotein that is specifically targeted into exosomes was shown to induce neutralising antibody responses and, if both treatments were combined, to reduce SARS glycoprotein-expressing tumour cells *in vivo* (Kuate *et al.*, 2007).

These studies indicate that the potential immunostimulatory capacity of exosomes may be exploited for vaccination purposes but may also hinder other applications of exosomes in which an immune response is undesirable. Therefore, it would be of considerable interest to investigate the potential immunogenicity of unpulsed and autologous exosomes *in vivo*.

### **1.7.3 Tolerogenic potential of exosomes**

Apart from the potential immunostimulatory function of exosomes, research has shown that, possibly depending on the cell of origin, exosomes may harbour intrinsic tolerogenic immunomodulatory function.

Exosomes derived from the blood of patients with squamous cell carcinoma or melanoma or cancer cell lines can inhibit the proliferation of activated CD8<sup>+</sup> T cells and induce their apoptosis (Wieckowski *et al.*, 2009). Furthermore, tumour-derived vesicles were able to expand CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> *in vitro* while bmDC-derived exosomes did not. Another study investigating the immunomodulatory function of exosomes derived from human breast milk, revealed their capacity to inhibit anti-CD3-induced IL-2 and IFN $\gamma$  secretion and

increase the number of T<sub>regs</sub> when incubated with allogeneic PBMC (Admyre *et al.*, 2007).

Research has indicated that exosome immunogenicity may be modulated by the modulatory treatment of the cells of origin. A study published in 2005 showed that exosomes derived from IL-10-treated bmDCs or bmDCs transduced with an IL-10-expressing vector were able to suppress delayed-type hypersensitivity responses after injection into joints, also improving the untreated contralateral joint in the mouse model (Kim *et al.*, 2005). The injection of IL-10-treated DC-derived exosomes was able to prevent the onset of collagen-induced arthritis and, if already established, reduce its severity. Similar results were obtained in a study looking at exosomes derived from the tryptophan catabolising enzyme indoleamine 2,3-dioxygenase (IDO)-expressing DCs, which after injection were able to reverse collagen-induced arthritis (Bianco *et al.*, 2009).

A study looking at the ability to induce cardiac allograft acceptance in mice found that exosomes derived from donor immature DCs or the treatment of mice with rapamycin alone was not sufficient to prolong graft survival, while the combined therapy of exosomes and rapamycin allowed prolonged cardiac allograft survival (Li *et al.*, 2012).

These findings may prove an effective alternative to cell-based therapies, to allow the administration of a cell-like structure with similar properties, but inert to environmental, potentially pro-immunogenic cues.

## **1.8 The aim of this study**

Despite its essential role in protecting us from constant environmental challenges and potentially self-destructive responses, the immune system also poses a considerable barrier towards potentially effective treatments such as tissue and organ transplantation and enzyme replacement therapy.

Our laboratory has been focusing on a simplified model system to look at ways to overcome and prevent the immunological response towards a single foreign antigen while maintaining a healthy and protective immune system. Using iPSCs as a means of differentiating autologous ipDCs in a patient-specific manner, this thesis investigates the possibility of modulating the immunogenicity of ipDCs using a variety of different agents (Figure 1.6).

iPSCs derived from a healthy individual were used in order to investigate different agents and their potential in driving DCs towards a tolerogenic phenotype. Furthermore, this thesis investigates the feasibility of deriving iPSCs from Pompe disease patients, their long term culture, maintenance and capacity to be differentiated into viable and functional ipDCs. Tying in the work of determining modulatory agents with pro-tolerogenic properties using healthy iPSCs, the most effective tolerogenic agent was used to investigate the potential of generating ipDCs from Pompe disease patients with pro-tolerogenic properties, as the effectiveness of ERT has been hampered by immunological responses towards the administered therapeutic enzyme.

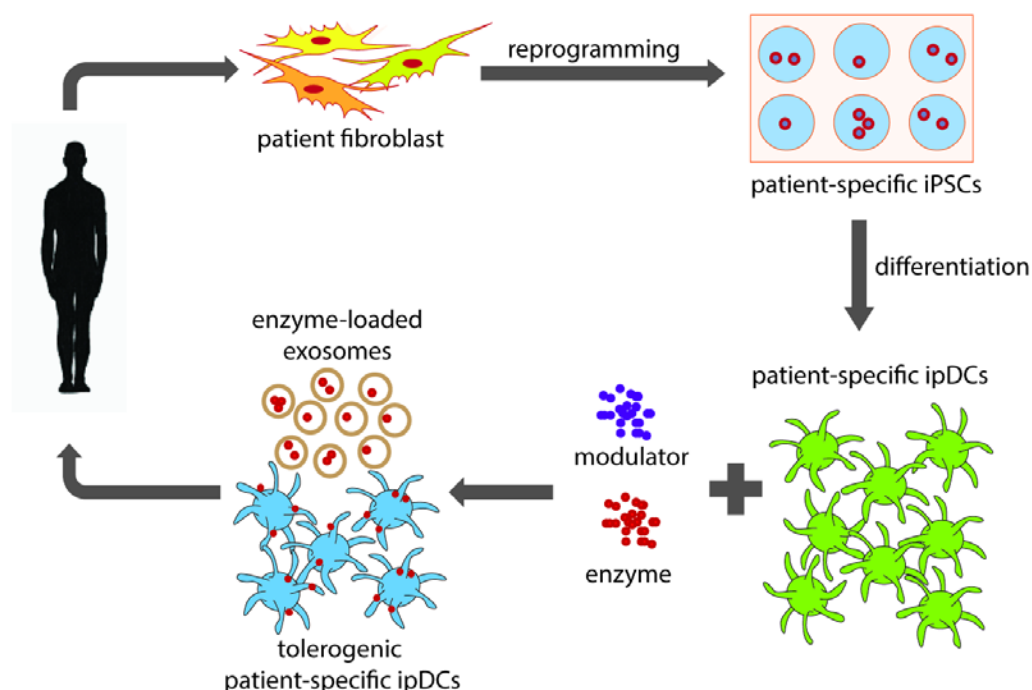


**Figure 1.6 The therapeutic potential of ipDCs and ipDC-derived exosomes.**

The therapeutic potential of ipDCs and ipDC-derived exosomes. Skin fibroblasts obtained from patients' biopsies can be reprogrammed *in vitro* to generate iPSCs. These iPSCs can be cultured and expanded in the laboratory and differentiated to generate patient-specific ipDCs. These ipDCs may be modulated with pro-tolerogenic agents and therapeutic enzymes to generate ipDCs capable of enzyme-specific tolerance induction. At the same time, exosomes may be purified from enzyme-treated tolerogenic ipDC cultures which may carry therapeutic enzymes in their lumen for their specific targeting of enzyme to the CNS. However, this might additionally require the transfection with genes expressing specific exosomal-targeting molecules. The tolerogenic ipDCs and loaded exosomes may be administered to patients in order to induce enzyme-specific immune tolerance and enzyme targeting to the CNS to allow for the improved efficacy of ERT.

On the other hand, DCs were investigated for the potential of loading proteins into exosomes via their endocytic system, as exosomes have been shown to be capable of specifically targeting therapeutic molecules to the CNS via the BBB. Furthermore, research has shown the capacity of endocytosed and phagocytosed protein to be targeted from the phagocytic/endocytic vesicle into

the cytosol where it may be available to exosome loading during MVB formation (Ackerman *et al.*, 2006; Kovacsovics-Bankowski and Rock, 1995; Rodriguez *et al.*, 1999). Given that DC differentiated from human iPSCs have been shown to have the capacity for cross-presentation, we have hypothesised that this route of antigen translocation is especially active providing a cytosolic source of endocytosed enzyme for packaging into exosomes during MVB formation. The BBB has been found to pose a considerable challenge to effective ERT targeting to the CNS and therefore may be exploited for the targeted delivery of lysosomal enzymes through exosome-mediated delivery. Furthermore, the immunogenicity of exosomes was investigated, as any neutralizing effect by antibodies may hamper the successful delivery of exosomes to the CNS. Altogether, this thesis explores different approaches to improving the ERT efficacy for the treatment of LSDs.



## Chapter 2 Materials and Methods





## 2.1 Mice

Mice were bred and maintained under specific pathogen-free conditions in the Sir William Dunn School of Pathology, Oxford, UK. All procedures were approved by the Home Office in accordance to the Home Office Animals (Scientific Procedures) Act 1986 and performed under project licence PPL30/3049.

In this thesis the CBA/Ca mouse strain is referred to as CBA and the C57BL/6 strain is referred to as B6. These two strains were used due to the MHCII mismatch which allowed for allogeneic studies (Table 2.1).

| Mouse Strain | Haplotype |       |       |
|--------------|-----------|-------|-------|
|              | MHCI      | MHCII | MHCII |
|              | H-2K      | I-A   | I-E   |
| CBA/Ca       | k         | k     | k     |
| C57BL/6      | b         | b     | -     |

**Table 2.1 Mouse strains used and their respective MHC I/II haplotypes.**

## 2.2 Human iPSCs

Human iPSC line C15 was kindly provided by Dr Lee Carpenter and Prof Suzanne Watt from the Stem Cell Research Laboratory, National Health Service Blood and Transplant, John Radcliffe Hospital, Oxford, UK (Carpenter *et al.*, 2011).

A skin biopsy from a Pompe disease patient was obtained from Great Ormond Street Hospital, London under Ethical approval (REC reference: 13/LO/0171; IRAS project ID: 100318). The patient was determined to be CRIM positive with a homozygous missense mutation at Gly293Arg (877 G>A) (P. Gissen, personal communication, May 27, 2014). The patient received ERT at the age of 5 months and deceased at the age of 12 months. Whether the patient mounted an immunological response towards the therapeutic enzyme had not been determined (P. Gissen, personal communication, May 27, 2014). The passage of the Pompe disease fibroblasts were at a minimum of 6 passages on receipt. No exact passage number could be determined as passages were recorded at Great Ormond Street Hospital but not at the John Radcliff Hospital in Oxford, where the samples were originally taken. Skin fibroblasts were reprogrammed by Tim Davies using the Sendai virus reprogramming system from the CytoTunes reprogramming kit according to manufacturer's instructions (Life Technologies, A1378002).

## **2.3 Mouse iPSCs**

Mouse iPSC (miPSC) were generated in the lab by Tim Davies using retroviral reprogramming methods as published by Takahashi and Yamanaka (2006) with the exception of the use of 3 times the concentration of Oct4 and the exclusion of c-myc to reduce the risk of tumorigenicity *in vivo*. iPSC cell lines iMEF1 and ipB6/2, which had been extensively characterised in the lab, were further used in this study. Briefly, the iMEF1 iPSC line was derived from mouse embryonic fibroblasts (MEFs) from CBA mice while ipB6/2 was derived from MEFs from B6 mice.

## 2.4 Reprogramming of human Pompe Disease Fibroblasts into iPSCs

Before reprogramming, human fibroblasts were grown in fibroblast culture medium and expanded (Table 2.2). Reprogramming and teratoma assays were performed by Tim Davies in the laboratory.

Two days before reprogramming,  $1.25 \times 10^4$  cells were plated into each well of a 4 well Nunclon Surface plate (Nunc, 176740). The CytoTune®-iPS Sendai Reprogramming Kit (Life Technologies, A1378001) was used according to manufacturer's instructions using CytoTune™ Sendai human Oct3/4 at  $4.7 \times 10^6$  cell infections units/ml (C/U/ml), Sox2 at  $1.5 \times 10^6$  C/U/ml, Klf4 at  $1.5 \times 10^6$  C/U/ml and c-Myc  $1.5 \times 10^6$  C/U/ml in 300ul F12 medium without serum (Table 2.3).

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### Fibroblast culture medium

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Ham's F10 medium (BioWhittaker, BE12-618F)

10% Fetal calf serum (FCS) (Life Technologies, 10828-028)

2 mM L-glutamine (PAA laboratories GmbH, M11-004)

50 U/ml penicillin (PAA laboratories GmbH, P11-010)

0.1 mM MEM non-essential amino acids (PAA laboratories GmbH, M11-003)

50 µg/ml streptomycin (PAA laboratories GmbH, P11-010)

1 mM Sodium Pyruvate (PAA laboratories GmbH, S11-003)

50 µM 2-mercaptoethanol (Sigma, M7522)

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**Table 2.2 Fibroblast culture medium.**

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### **F12 medium**

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Dulbecco's Modified Eagle's Medium/F12+GlutMAX™-I

(Life Technologies, 10565-018)

10% FCS

50 U/ml penicillin

0.1 mM MEM non-essential amino acids

50 µg/ml streptomycin

1 mM Sodium Pyruvate

50 µM 2-mercaptoethanol

---

#### **Table 2.3 F12 medium.**

Cells were rinsed with F12 without serum and 150 µl of the reprogramming mix was added per well. To control wells, 150 µl of F12 without serum was added. After overnight incubation at 37°C, cells were fed with F12 containing 15% serum replacement (SR) (Life Technologies, 10828-028) and 100 ng/ml of basic Fibroblast Growth Factor (bFGF) (Immunotools, 11343625). Subsequently, cells were fed on day 3 and 5 after reprogramming and were passaged using TrypLE Express (Life Technologies, 12604) onto feeder cells on day 6. Cells were grown and maintained in this way, with approximately one passage every week, until day 28, on which stem cell-like colonies appeared. These colonies were mechanically cut using pulled Pasteur pipettes prepared in-house (VWR International Ltd, 612-1702) and a BD Micro-Fine insulin syringe needles

(Becton, Dickinson and Company, 324892). Cut colonies were either replated onto feeder cells or directly plated onto Matrigel (BD Biosciences, 356231)-covered 6 well plates (Corning Incorporated, BC010).

**2.5 Human primary cells**

Peripheral blood mononuclear cells (PBMC) were obtained from leukocyte cones (NHS Blood Transfusion Service). Human primary cells were cultured in R10 medium and supplemented as mentioned in the relevant sections (Table 2.4).

| R10                                  |
|--------------------------------------|
| RPMI 1640 (Lonza, BE12-702F/12)      |
| 10 % heat-inactivated FCS            |
| 2 mM L-glutamine                     |
| 50 U/ml penicillin                   |
| 0.1 mM MEM non-essential amino acids |
| 50 µg/ml streptomycin                |
| 1 mM Sodium Pyruvate                 |
| 50 µM 2-mercaptoethanol              |

**Table 2.4 R10 medium.** RPMI: Roswell Park Memorial Institute medium.

### **2.5.1 Isolation of monocytes and T cells**

Monocytes and T cells were isolated from PBMCs obtained from Buffy Coats or Leukocyte cones obtained from the NHS Blood and Transplant Service at the John Radcliffe Hospital in Oxford.

#### **2.5.1.1 Ficoll gradient centrifugation**

PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare, 17-1440-02) gradient centrifugation. Leukocyte cones were diluted 2-fold with phosphate buffered saline (PBS) (Gibco, 14190) making up a diluted blood sample of around 14 ml which was then carefully layered onto 5 ml of Ficoll. In order to minimize mixing, Ficoll was added first to the tube, the tube was then slightly tilted and the blood sample slowly expelled along the side of the tube layering it on top of the Ficoll. Samples were centrifuged at 900 *g* for 20 minutes at room temperature with the breaks set on low. The white PBMCs interface was harvested using a 1 ml Gilson pipette. PBMCs were repeatedly washed with 50 ml of cold PBS and spun down at 300 *g* for 5 minutes in order to remove contaminating platelets. Cells were washed until supernatants appeared clear.

#### **2.5.1.2 AutoMACS and manual cell sorting**

The AutoMACS separation system (Miltenyi Biotec) was used for the purification of different types of cells from PBMCs.

#### **2.5.1.3 Isolation of Monocytes**

CD14<sup>+</sup> monocytes were isolated from PBMCs using CD14 microbeads (Miltenyi Biotec, 130-050-201) according to manufacturer's instructions. The protocol was altered in that 1 µl of CD14 Microbeads was used for every 2.5x10<sup>6</sup> cells. Briefly, cells were resuspended in Running Buffer (Table 2.5) and labelled with CD14

microbeads for 15 minutes at 4 °C, followed by one washing step in 50 ml of Running Buffer and centrifugation at 300 *g* for 10 minutes.

| Running Buffer           |
|--------------------------|
| 2 mM EDTA (Sigma, E6635) |
| 0.5% BSA (Sigma, A3311)  |
| in PBS                   |

**Table 2.5 Buffer for cell sorting.** BSA: bovine serum albumin; EDTA: Ethylenediaminetetraacetic acid.

Cells were resuspended in 500 µl Running Buffer per 10<sup>8</sup> cells and CD14-labelled cells isolated by positive selection using the “Possel” program on the autoMACS Separator (Miltenyi Biotec) or manually using the QuadroMACS Separator (Miltenyi Biotec, 130-090-976) and LS columns (Miltenyi Biotec, 130-042-401). If the autoMACS was used, Rinsing Buffer and 70% ethanol were made up and attached to the machine according to the manufacturer’s instructions (Table 2.5).

#### **2.5.1.4 Differentiation of monocytes into dendritic cells (moDCs)**

Isolated CD14<sup>+</sup> monocytes were cultured in R10 supplemented with 50 ng/ml GM-CSF (Peprotech, 300-03) and 100 ng/ml IL-4 (Peprotech, 200-04) in 6-well cell bind plates for 7 days in a 37°C humidified incubator at 5 % carbon dioxide (CO<sub>2</sub>). Cells were treated between days 0 and 3 with pharmacological agents and matured on day 5 for 48 hours as described in section 2.10.4/5. Cells were harvested by gently pipetting on day 7.



#### **2.5.1.5 Isolation of naïve CD4<sup>+</sup> T cells and pan T cells**

T cells were isolated from the negative fraction eluted from the CD14<sup>+</sup> monocyte isolation step. Naïve CD4<sup>+</sup> T cells were purified using the naïve CD4<sup>+</sup> T cell selection kit II (Miltenyi Biotec, 130-094-131) using the AutoMACS cell sorter according to the manufacturer's instructions. Pan T cells were isolated from the negative fraction following the monocyte isolation using the Pan T cell Isolation kit II (Miltenyi Biotec, 130-091-156). Briefly, cells were resuspended in Running Buffer after CD14 selection and counted. Cells were resuspended according to the manufacturer's instructions and non-T cells labelled by addition of cocktail of biotinylated monoclonal antibodies for 10 minutes at 4 °C. Thereafter, cells were washed and labelled with anti-biotin microbeads for 15 minutes at 4 °C. Labelling was followed by a washing step in 50 ml Running Buffer and centrifugation at 300 *g* for 10 minutes. Cells were resuspended in 500 µl Running Buffer per 10<sup>8</sup> cells and T cells purified using the "Deplete" program for Pan T cell isolation or "Depletes" program for the isolation of CD4<sup>+</sup> T cells. Naïve CD4<sup>+</sup> T cells were also manually purified using the QuadroMACS Separator and LS columns.

#### **2.5.1.6 Culture of T cells**

T cells were used either fresh on the day of isolation or thawed from frozen stocks one day before use and cultured overnight in R10 supplemented with 10 ng/ml of IL-2 (R&D Systems, 202-IL).

#### **2.5.1.7 Isolation of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> cells**

CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> cells were isolated from DC and T cell co-cultures using the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> regulatory T cell isolation kit II (Miltenyi Biotec, 130-094-775) and the AutoMACS cell sorter according to manufacturer's instructions. Briefly, cells were counted and resuspended in running buffer according to

manufacturer's instructions. First of all, non-CD4<sup>+</sup> and CD127<sup>high</sup> cells were positively selected using biotinylated antibodies and anti-biotin microbeads, which were incubated at 4 °C for 10 minutes and 15 minutes respectively. The unlabelled fraction was collected using the "Depl025" program. The second selection step involved the positive selection of CD25<sup>+</sup> cells by incubating the previously purified cells with CD25 microbeads for 15 minutes at 4 °C. The CD25<sup>+</sup> cells were positively selected using the "Posseld2" program giving rise to a population of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim</sup>- T cells.

## **2.6 Mouse primary cells**

Mice between the age of 6 and 12 weeks were sacrificed according to a schedule 1 method in a CO<sub>2</sub> chamber followed by cervical dislocation to confirm death.

For the differentiation of bmDCs, the growth factor GM-CSF was required. For the derivation of GM-CSF, a cell line called X63-GM-CSF was used.

### **2.6.1 X63 cell culture for the production of GM-CSF**

X63-GM-CSF cells, that had been transfected with a vector expressing murine GM-CSF, were thawed into X6 medium into a T75 flask.

Cells were grown until confluent and plated into two T175 flasks in X63 medium supplemented with G418 Sulfate (Gibco Geneticin, 11811-031) (Table 2.6). Cells were further expanded in presence of 1 mg/ml of G418 sulfate by continuously doubling the number of flasks until four T175 were obtained. The last two passages were cultured in the absence of G418. Cells were maintained in culture until cell death to allow cells to secrete the maximum amount of GM-CSF.

Supernatants were harvested, filter-sterilised and frozen for storage. The new batches of GM-CSF were assayed and compared to previous batches by measuring the proliferation of hematopoietic progenitors in serial dilutions of GM-CSF containing bmDC medium.

| X63 medium                                                  |
|-------------------------------------------------------------|
| Iscove's Modified Dulbeccos Medium (Biowhitaker, BE12-722F) |
| 10 % FCS                                                    |
| 50 U/ml penicillin                                          |
| 50 µg/ml streptomycin                                       |
| 2 mM L-glutamine                                            |
| 50 µM β2-mercaptoethanol                                    |

**Table 2.6 X63 medium.**

**2.6.2 Bone marrow derived dendritic cells**

Femurs and tibia were isolated from mice and cleared of surrounding tissue. Bones were sterilised in 70% ethanol in a bacteriological dish for 1 minute followed by three washes in PBS. A 25 gauge needle (Terumo, NN-2516R), a 20 ml syringe (Terumo, SS+20ES1) and R10 were used to flush the bone marrow from the femur and tibia, which had been lightly cut at each end to allow the needle to be inserted and cells to be expelled. Bone marrow cells were passed through a 70 µm cell strainer (BD Falcon, 352350) to remove debris, and

centrifuged at 300 *g* for 5 minutes. Cells were resuspended in Red Blood Cell Lysing Buffer (RBC) Hybri-Max (Sigma-Aldrich, R7757) for 3 minutes at room temperature, followed by a wash in R10. After another centrifugation step, cells were resuspended in bmDC medium (Table 2.7) and plated into 10 cm cell culture plates (Corning, 430167) (2 per mouse). Medium was replaced on day 3 and day 6 of culture. bmDCs were matured on day 6 by the addition of 10 µg/ml of lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (Sigma, L3880) or left immature and used on day 7.

| bmDC medium                                                                              |
|------------------------------------------------------------------------------------------|
| RPMI 1640 (Lonza, BE12-702F/12)                                                          |
| 10 % heat-inactivated FCS                                                                |
| 4 % supernatant from X6310 cell line (equivalent to ~50 U/mL of recombinant mouse GMCSF) |
| 2 mM L-glutamine                                                                         |
| 50 U/ml penicillin                                                                       |
| 0.1 mM MEM non-essential amino acids                                                     |
| 50 µg/ml streptomycin                                                                    |
| 1 mM Sodium Pyruvate                                                                     |
| 50 µM 2-mercaptoethanol                                                                  |

**Table 2.7 bmDC medium.**

### **2.6.3 Isolation of T cells from the spleen**

For T cell isolation using nylon wool columns, 2 g of nylon wool fibres (Polysciences, 18369) were teased apart and packaged into 20 ml syringes (Terumo, SS+20ES1) for autoclaving. On the day of use, nylon wool-loaded syringes are placed into a clamp stand and filled with R10 medium. The medium was allowed to soak into the wool and drip through the syringe. A 21G butterfly clip (Abbott Labs, 4492) was attached to the syringe and the syringe was topped up with R10 which was eluted until 5 ml remained. The butterfly clip was placed into the top opening of the syringe to stop further elution and the column was placed into a sterile 500 ml Duran bottle with the lid only loosely closed to permit gaseous exchange and placed for one hour into a 37°C humidified incubator at 5 % CO<sub>2</sub>.

Spleens were removed from sacrificed mice by incision into the left flank, cutting away tissue and fat for easy removal. Spleens were teased apart in R10 using sterile forceps, releasing cells into a bacteriological dish containing R10 medium. These cells, together with the spleen fragments, were transferred into a 70 µm cell strainer that had been placed into the neck of a 50 ml falcon tube. The plunger of a 2 ml syringe was used to further homogenise the spleen. Splenocytes were eluted from the cell strainer using R10, centrifuged and resuspended into 5 ml of pre-warmed R10. Splenocytes were loaded onto the R10-equilibrated column and medium was eluted through the butterfly clip so as to allow cells to soak into the nylon wool column. Further R10 was added into the syringe to completely cover the nylon wool with excess medium and replaced in the 500 ml Duran bottle and the incubator for 1-2 hours.

T cells were eluted using 25 ml of warm R10 medium added progressively onto the column by allowing the cells to elute through the butterfly clip into a 50 ml tube. T cells were centrifuged at 300 g for 5 minutes and resuspended in red blood cell lysis buffer for 1-3 minutes at room temperature to allow lysis of red blood cells. T cells were washed twice with R10 after which cells were ready to use.

## **2.7 Culture of mouse iPSCs**

Mouse iPSCs were routinely cultured on mouse embryonic fibroblasts (MEFs), which were referred to as feeder cells.

### **2.7.1 Feeder cells**

MEFs were derived from 13 to 14 day old mouse embryos (Brook 2006), expanded in culture and frozen down for future use by Tim Davies. When required, MEFs were thawed into T75 cell culture flasks in Feeder medium and grown to confluence (Table 2.8).

MEFs were passaged 1:2 every 3-4 days using T75 culture flasks. The day before iPSC passaging, the required amount of MEFs were treated with 10 µg/ml of mitomycin C (MMC) (Sigma, M0503) for 2 hours at 37°C to prevent their proliferation and subsequently referred to as “Feeder cells”. Cells were washed extensively with PBS to prevent any carryover of MMC and passaged into three T25 flasks for the use as Feeder cells for mouse iPSC culture.

| Feeder Medium                                                  |
|----------------------------------------------------------------|
| Dulbecco's Modified Easgle's Medium (DMEM) (Lonza, BE-12-604F) |
| 10% FCS                                                        |
| 1 mM Sodium Pyruvate                                           |
| 0.1 mM MEM non-essential amino acids                           |
| 2 mM L-glutamine                                               |
| 50 mM 2-mercaptoethanol                                        |

**Table 2.8 Feeder medium.**

### **2.7.2 Mouse iPSC thawing**

miPSC were thawed by gently swirling the vial in a 37°C waterbath. Once thawed, cells were plated onto Feeder cells in 7 ml of miPSC medium (Table 2.9). In order to remove dimethyl sulfoxide (used for cell freezing), cultures were fed the following day with fresh miPSC medium.

| miPSC medium                                      |
|---------------------------------------------------|
| DMEM                                              |
| 15% SR                                            |
| 1 mM Sodium Pyruvate                              |
| 0.1 mM MEM non-essential amino acids              |
| 2 mM L-glutamine                                  |
| 50 mM 2-mercaptoethanol                           |
| 1000 U/ml rmLIF (Chemicon International, ESG1107) |

**Table 2.9 miPSC medium.**

### **2.7.3 Mouse iPSC culturing and passaging**

iPSC cultures were fed every 2 to 3 days. At around 80% confluency or once the dome-shaped colonies had increased significantly in size, iPSCs were passaged at 1:10 dilution. For passaging, iPSCs were washed with PBS in order to remove residual serum, and incubated in TrypLE Express (Gibco, 12604) for 5-10 minutes at 37°C. Flasks were agitated to gently dislodge the iPSCs and serum-containing medium at around 5x the volume of TrypLE Express was added to deactivate the trypsin. Cells were centrifuged at 300 *g* for 5 minutes and plated into a fresh flask covered with Feeder cells with fresh miPSC medium.



## **2.8 Differentiation of mouse iPSCs into mouse ipDCs**

For the differentiation of iPSCs into DCs the growth factor GM-CSF is required. GM-CSF was derived from the X63-GM-CSF cell line that secretes GM-CSF into the cell culture supernatants. The derivation of GM-CSF-containing supernatants is described in section 2.6.1.

### **2.8.1 Differentiation set up**

To prepare iPSCs for differentiations and allow the removal of feeder cells, iPSCs were passaged and grown for 2 to 3 passages on 0.1 % gelatin in PBS (Sigma, G1890). Flasks were covered in gelatin for around 30 minutes at 37 °C or left overnight at room temperature before use. Gelatin was removed and passaged cells transferred into the flask as normal.

For the initiation of differentiation, iPSCs were harvested by TrypleE incubation for 5 minutes at 37 °C and washed with serum-containing medium. iPSCs were counted using a haemocytometer. Around  $2.5\text{-}5 \times 10^6$  cells were plated per bacteriological plate in Feeder medium.

Cells were fed with fresh Feeder medium on day 3 and day 8 after differentiation set up by harvesting free-floating embryoid bodies (EBs) from the bacteriological plates and collecting them in a 50 ml falcon tube. EBs were left to settle down in the tube after which old medium was removed and fresh Feeder medium added. In this fresh medium, EBs were plated into new bacteriological plates. After 14 days, large EBs had formed which were harvested with the pipette. Around 15 EBs were plated per 10 cm cell culture plate in mipDC medium (Table 2.10).

| mipDC medium                               |
|--------------------------------------------|
| DMEM                                       |
| 15% FCS or SR                              |
| 1 mM Sodium Pyruvate                       |
| 0.1 mM MEM non-essential amino acids       |
| 2 mM L-glutamine                           |
| 50 $\mu$ M $\beta$ 2-mercaptoethanol       |
| 4% GM-CSF supernatant from X6310 cell line |
| 1000 U/ml IL-3                             |

**Table 2.10 Mouse ipDC medium.**

After a few days, EBs had attached to the surface of the tissue culture plastic. Cells were fed every week or whenever necessary. After 14-21 days, ipDCs were harvested by gentle pipetting.

## 2.9 Culture of human iPSCs

Human iPSC lines C15 and ipPo1 were cultures on Matrigel-covered 6-well plates as follows.

### **2.9.1 Matrigel coating**

Matrigel (BD Biosciences, 356231) was diluted 1 in 30 using ice-cold Knock-out DMEM (Invitrogen, 10829-018), together with cooled tubes and pipettes (Leishman and Fairchild, 2014). 6-well cell bind plates were coated with 1 ml per well of diluted Matrigel and left for 1 hour at 37°C or overnight at 4°C.

### **2.9.2 Human iPSC culture and passaging**

Human iPSC line C15 was cultured in complete mTeSR1 medium (Stemcell Technologies, 05850) on 6-well cell bind plates (Corning Incorporated, BC010) coated with Matrigel. iPSCs were cultured and passaged at 1:12 according to protocols provided by Stem Cell Technologies.

Briefly, iPSC colonies were washed with PBS and incubated in 1 ml per well of 1 mg/ml dispase (Stemcell Technologies, 07913) for 4-5 minutes at 37°C or with 0.02% EDTA in PBS for around 1 minute at room temperature. Once colonies started to round up at the edges, dispase or EDTA was removed and wells were washed with PBS. Fresh mTeSR1 medium containing 10  $\mu$ M Y27632 rho-associated protein kinase inhibitor (Reagents Direct, 53-B85) was added to the iPSC colonies which were gently scraped off the cell culture plastic into the medium using a cell scraper (Corning, 3010). Colonies were further dispersed into smaller fragments by gentle pipetting using a 10 ml pipette. Small clusters were plated onto fresh Matrigel-coated 6 well plates. All cell cultures were maintained in a 37°C humidified incubator at 5 % CO<sub>2</sub>.

## **2.10 Differentiation of human iPSCs into ipDCs**

For the differentiation of iPSCs into ipDCs, iPSCs were expanded and differentiated using the following protocol, also described by Leishman and Fairchild (2014) and illustrated in Chapter 3, Figure 3.2.

### **2.10.1 Differentiation set up**

Following inspection of iPSC cultures, a representative well of average confluency (around 80%) was selected for harvesting using 1 ml of TrypLE Express in order to determine the number of iPSCs present in a representative well to allow an estimation of the total number of iPSCs available for differentiation. After 5 minutes of incubation at 37°C, trypsin was neutralised by the addition of serum-containing medium. Once cells had been broken apart into a single cell suspension by pipetting, cells were counted using a haemocytometer. The number of wells of differentiation to be set up was determined by calculating the total number of iPSCs available and planning to plate  $3 \times 10^6$  cells per well of a 6-well plate of differentiation culture.

For the setup of differentiation cultures, iPSCs were harvested in the same manner as for passaging using dispase or 0.02% EDTA and cell scrapers. However, colonies were scraped into complete mTeSR1 medium supplemented with 50 ng/ml recombinant human bone morphogenetic protein-4 (BMP-4) (R&D Systems, 314-BP/CF), 50 ng/ml recombinant human vascular endothelial growth factor (VEGF) (PeproTech, 100-20), 20 ng/ml recombinant human stem cell factor (SCF) (R&D Systems, 55-SC/CF) and 50 ng/ml recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) (PeproTech, 300-

03). Harvested cell clumps were plated onto 6-well ultra-low attachment (ULA) plates (Corning, 3471) at  $3 \times 10^6$  cells per well in a total volume of 4 ml of supplemented mTeSR1. The day of differentiation set up was defined as day 0.

### 2.10.2 First feeding of differentiations

Differentiations were fed 2 days (designated day 2) after differentiation set up. The medium was gently topped up with 2 ml of complete X-VIVO-15 medium (Table 2.11).

| Complete X-VIVO-15 medium            |
|--------------------------------------|
| X-VIVO-15 medium (Lonza, BE04-418Q)  |
| 1 mM Sodium Pyruvate                 |
| 0.1 mM MEM non-essential amino acids |
| 2 mM L-glutamine                     |
| 50 $\mu$ M 2-mercaptoethanol         |

**Table 2.11 Complete X-VIVO-15 medium.** This medium was supplemented with further growth factors according to the differentiation protocol described in section 2.10.

Complete X-VIVO-15 was supplemented with the growth factors BMP-4, VEGF, SCF and GM-CSF as mentioned above, to produce a total volume of 6 ml.

With this technique, cells were gradually transferred from supplemented mTeSR1 into supplemented X-VIVO-15.

### **2.10.3 Subsequent feeding regime**

Differentiations were fed every 2-3 days by replacing 2-3 ml of old medium with new supplemented complete X-VIVO-15 medium. Throughout the differentiation process, growth factors were successively removed from the X-VIVO-15 supplemented media added to the cells starting with BMP-4 at day 5, followed by VEGF at day 14 and SCF at day 19 of differentiation [19]. Between day 13 and day 17 macrophage-like cells may appear in the cultures. At this point 25 ng/ml of interleukin-4 (IL-4, Peprotech) was added, which was increased stepwise to 100 ng/ml during subsequent feeds. Between days 20-24, monocyte and DC-like cells were harvested by gentle pipetting, preventing the carryover of adherent macrophages in the culture dish. Harvested cells were passed through a 70  $\mu$ m cell strainer (BD Falcon, 352350) to remove any EBs, washed with PBS and plated in X-VIVO-15 supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4 at  $1-1.5 \times 10^6$  monocytes per well of a 6-well cell bind plate. This day was designated day 0 post-harvest.

### **2.10.4 Pharmacological Treatment**

moDCs and ipDCs were treated with rapamycin (Sigma, R0395) on day 3; VD3 (Sigma, D1530) on day 0 and 3; aspirin (Cayman Chemical, 70260) on day 2; dexamethasone (Sigma, D4902) on day 3 and IL-10 (Immunotools, 11340105) on day 3. If not stated otherwise, cells were treated with 10 ng/ml of rapamycin, 100 nM of 1,25VD3, 100  $\mu$ M of dexamethasone and 200pg/ml of IL-10. As dexamethasone was reconstituted in 99% ethanol, controls were treated with the same concentration of ethanol to control for any potential effect or toxicity. This control was referred to as dex-vehicle.

### 2.10.5 DC Maturation

On day 5 after harvest, DCs were matured for 48 hours using a maturation cocktail described in Table 2.12. On day 7, DCs were harvested by gentle pipetting.

| Maturation cocktail                                     |
|---------------------------------------------------------|
| 50 ng/ml of GM-CSF                                      |
| 100 ng/ml IL-4                                          |
| 20 ng/ml IFN $\gamma$ (R&D Systems, 285-IF/CF)          |
| 50 ng/ml TNF $\alpha$ (R&D Systems, 210-TA/CF)          |
| 10 ng/ml of IL-1 $\beta$ (R&D Systems, 201-LB/CF)       |
| 1 $\mu$ g/ml prostaglandin E2 (PGE $_2$ ) (Sigma, 6532) |

**Table 2.12 Maturation cocktail.**

### 2.11 M-FISH

iPSCs were grown on Matrigel-covered 6-well plates until 70% confluency for the M-FISH analysis performed by the Chromosome Dynamics Core at the Wellcome Trust Centre for Human Genetics in Oxford.

### **2.12 HLA tissue typing**

DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, A1120) according to the manufacturer's instructions. Briefly, iPSCs were harvested from cultures and washed with PBS. Spun down cells were resuspended in Nuclei Lysis Solution and mixed. Following nuclear lysis, RNA was eliminated from the sample by the addition of an RNase solution and contaminating protein removed by the addition of a protein precipitation solution and isopropanol. After one wash in 70% ethanol the DNA pellet was air-dried and rehydrated in DNA Rehydration Solution. HLA tissue typing was performed by Proimmune.

### **2.13 Glycogen storage**

iPSCs were grown on 19 mm diameter round coverslips (VWR International, 631-0155) until around 80% confluency. Cells were fixed in 4% paraformaldehyde (Sigma, P6148) for 5-10 minutes at room temperature. Cells were stained using the PAS Periodic Acid Schiff's Stain Kit (CLIN-TECH Limited, 631085) according to manufacturer's instructions. In brief, coverslips were washed in tap water and immersed in Periodic Acid Solution for 5 minutes at room temperature to stain polysaccharides including glycogen. Thereafter, cells were washed with distilled water around 5 times and immersed in Schiff's reagent for a further 15 minutes at room temperature. Cover slips were washed for 5 minutes with numerous changes of tap water. Counter staining was performed using Gill's Hematoxylin Solution for 5 to 10 seconds. Tap water was used to wash away the staining solution after which coverslips were air dried. Once dry, coverslips were mounted onto slides using DPX Mountant for Histology (Sigma-Aldrich, 06522).



## **2.14 Acid $\alpha$ -1,4-glucosidase assay**

The activity of GAA was measured using a standard clinical protocol kindly provided by Derek Burke and Paul Gissen at Great Ormond Street Hospital London.

### **2.14.1 Reagents**

#### **1,4-Methylumbelliferone standard**

17.6 mg of 1,4-Methylumbelliferone (4-MU) (Sigma, M-1381) was dissolved in 1.5 ml of 99% ethanol and made up to 500 ml with double-deionised water (ddH<sub>2</sub>O) to make a 200  $\mu$ M stock solution. This solution was filter-sterilised and further diluted with ddH<sub>2</sub>O to make up a 5  $\mu$ M solution. This working stock was aliquoted as 200  $\mu$ l per tube and stored at -80°C.

#### **50 mmol/L acetate buffer, pH 4.95**

29  $\mu$ l of acetic acid (Sigma-Aldrich, 32,009-9) were added to 8 ml of ddH<sub>2</sub>O to make up a 50 mmol/L buffer. The pH was adjusted to 4.95 using NaOH. Once adjusted, the buffer was made up to a final volume of 10 ml and frozen as aliquots.

#### **100 mmol/L acetate buffer, pH 3.8**

58  $\mu$ l of acetic acid was added to 8 ml of ddH<sub>2</sub>O to make up a 100 mmol/L buffer. The pH was adjusted to 3.8 using NaOH. Once adjusted, the buffer was made up to a final volume of 10 ml and frozen as aliquots.

#### **100 mmol/L acetate buffer, pH 6.5**

58  $\mu$ l of acetic acid was added to 8 ml of ddH<sub>2</sub>O to make up a 50 mmol/L buffer. The pH was adjusted to 6.5 using NaOH. Once adjusted, the buffer was made up to a final volume of 10 ml and frozen as aliquots of 100  $\mu$ l.

#### **4-Methylumbelliferyl $\alpha$ -D-glucoside substrate**

10.15 mg of 4-Methylumbelliferyl (4-MU)  $\alpha$ -D-glucoside (Melford Laboratories Ltd, M1096) was dissolved in 500  $\mu$ l 2-methoxyethanol (Sigma, 284467) with gentle warming. This substrate was made up fresh for every experiment.

#### **0.25M glycine/NaOH, pH10.4**

64 g NaOH was slowly added to 200 ml of ddH<sub>2</sub>O whilst kept cool in iced water to make up an 8 M solution. 75 g of glycine was dissolved in 800 ml of ddH<sub>2</sub>O and adjusted to pH 10.2-10.4 by slowly adding the NaOH solution. Once adjusted, the solution was made up to a final volume of 1 L using ddH<sub>2</sub>O to make up a solution of 1 M. To make up the final working solution 1 part of 1 M glycine/NaOH buffer was diluted in 3 parts of ddH<sub>2</sub>O to make up 0.25 M glycine/NaOH buffer.

#### **2.14.2 Cell preparation**

Cells were harvested by dissociation with 0.02 % EDTA for 5 minutes at 37°C. Cells were washed with cold PBS and centrifuged at 300 *g* for 5 minutes. Cell pellets were stored at -20°C until further processing. Pellets were thawed and placed on ice. Cells were sonicated using a MSE Soniprep 150 at the amplitude of 6 microns for 10 seconds on ice. Whole cell lysates were used for the enzyme assay as specified by a clinical Pompe Disease diagnostic protocol kindly provided by Dr Derek Burke at Great Ormond Street Hospital.

#### **2.14.3 Determination of protein concentration**

The protein concentrations of cell lysates were determined using the Pierce BCA Protein Assay kit (Thermo Scientific, 23225) according to manufacturer's instructions. In short, a standard curve was made up using BSA starting with the

concentration of 2 mg/ml which was diluted to 25 µg/ml. All samples were made up in triplicate. Samples were measured as 10 times or 100 times dilutions. 25 µl was plated per sample into a 96 well flat bottom plate. 200 µl of Working Reagent, made up according to manufacturer's instructions, was added to each well containing sample or standard and incubated for 30 minutes at 37 °C. Absorbance was measured as Optical Density (OD)<sub>562nm</sub> using a BioTek ELx808 spectrophotometer.

#### **2.14.4 Enzyme assay**

Each sample was measured under 4 different conditions. 10 µg of protein was used of each sample for each condition in a maximum volume of 10 µl. If the volume required to achieve the desired protein concentration was lower than 10 µl the sample was topped up with ddH<sub>2</sub>O to a final volume of 10 µl in a 1.5 ml Eppendorf tube. Additionally to the samples, a set of 4 conditions was set up with 10 µl of ddH<sub>2</sub>O as a negative control.

The 4 different conditions were as follows:

1. pH 3.8, pre-treated with acetate buffer pH 4.95: 'inactivated'
2. pH 3.8, untreated active sample
3. pH 6.5, pre-treated with acetate buffer pH 4.95 : 'inactivated'
4. pH 6.5, untreated active sample

To each sample that was to be inactivated 10 µl of acetate buffer pH 4.95 was added, while samples that were to remain active were topped up with 10 µl of ddH<sub>2</sub>O. All samples were kept on ice unless otherwise specified.

The inactivated samples were incubated for 30 minutes at 37°C, followed by cooling on ice. To the pH 3.8 samples 90 µl of acetate buffer pH 3.8 was added while the pH 6.5 samples were treated with 90 µl of acetate buffer of pH 6.5.

Samples were warmed in a waterbath which had been heated to 37°C. At timed intervals 10 µl of 4-MU α-D-glucoside was added per tube and incubated for 30 minutes. At timed intervals 1080 µl of 0.25 M of glycine/NaOH stop reagent was added per sample for termination of the reaction. Tubes were mixed and the fluorescence read using a Spectramax fluorescence spectrometer M5 with excitation at 365 nm and emission at 450 nm.

The enzyme activity was calculated as follows:

$$\frac{\frac{Sample - Blank}{Standard} \times \frac{1000}{V} \times \frac{1}{[Protein]} \times 60}{T}$$

Sample: Fluorescence reading for test sample

Blank: Fluorescence reading for blank

Standard: Fluorescence reading for 4-MU standard

V: Sample volume (µl)

[Protein]: Protein concentration (mg/ml)

T: Assay time (minutes)

## 2.15 Viability

Cell viability was quantified by live and dead cell counts using trypan blue, a haemocytometer (Weber, BS.748) and an Olympus CKX41 microscope.

## **2.16 Yield**

Cell yield was quantified by counting cells as described in section 2.15. The yield of DCs cultured under different conditions was determined as the proportion of cells harvested on the final day relative to the number of precursors plated at the beginning of the final 7 day differentiation period. The yield of ipMonocytes was determined as the number of cells harvested for the final 7 day differentiation period relative to the number of iPSCs plated at the initiation of the differentiation protocol.

## **2.17 Antigen processing assay**

DCs were plated at  $7 \times 10^4$ - $10^5$  cells per well of two 96-well plates (Nunc, 1 63320). The number of cells plated included non-DCs and was not based on the proportion of CD11c-expressing cells. Plated cells were pre-warmed in the incubator or pre-cooled on ice. At different time points 100  $\mu$ g/ml of DQ ovalbumin (Life Technologies, D12053) was added to each well and further incubated either at 37 °C or on ice. Thereafter, cells were washed twice in washing buffer and stained for CD11c as described in section 2.23.

## **2.18 Phagocytosis**

Dendritic cells were harvested by gentle pipetting and counted. For each condition,  $3$ - $6 \times 10^5$  of total cells are plated in 2 ml of R10 per well of a 6 well cell bind plate. For controls, around  $8$ - $7 \times 10^5$  cells of each condition were fixed in 0.5% paraformaldehyde in PBS in a tube and incubated for 30 minutes at room temperature. 3% glycine was added and cells were centrifuged at 300 *g* for 5 minutes and supernatants discarded. Thereafter, cells were washed twice with

10 ml of R10 medium, centrifuged as above and supernatants discarded. Fixed cells were resuspended in 2 ml of R10, counted and plated into the wells of a 6-well cell bind plate at  $3\text{-}6 \times 10^5$ , alongside the live cells.  $4.6 \times 10^6$  FluoSpheres® carboxylate-modified microspheres of 2.0  $\mu\text{m}$  diameter (Molecular Probes, F8827) were added to each well, containing live cells or fixed cells, and incubated for 2-3 hours in the incubator. Thereafter, cells were harvested, washed twice in cold PBS and stained for CD11c as described in section 2.23.

## **2.19 Proliferation assay**

Proliferation was measured either by [ $^3\text{H}$ ]-thymidine (Amsterdam International, TRA120) incorporation into the DNA of proliferating cells or by the dilution of 5- (and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) (eBioscience, 65-0850-85), as it covalently binds to intracellular proteins and is progressively diluted with every cell division.

### **2.19.1 CFSE staining**

T cells were harvested by gentle pipetting and counted. Cells were resuspended as  $2 \times 10^6$  T cells per ml in warm PBS. 1  $\mu\text{l}$  of 10 mM CFSE was added to another tube with 10 ml of warm PBS. Thereafter, the CFSE dilution and suspended T cells were mixed 1 to 1 and incubated in the dark at room temperature for 10 minutes. For the neutralisation of CFSE, 5 ml of cold FCS was added and cells placed onto ice for 5 minutes. T cells were centrifuged and washed twice in 10 ml of R10. Cells were resuspended in R10 and counted.

### 2.19.2 Setting up a Mixed Leukocyte Reaction

A maximum of  $2 \times 10^5$  naïve CD4<sup>+</sup> T cells were plated in a total volume of 100 µl R10 per well of 96-well round bottom plates. A top number of  $5 \times 10^4$  DCs was plated in triplicate in a total volume of 100 µl per well to yield a DC to T cells ratio of 1:4, 1:8, 1:16 and 1:32 in a final volume of 200 µl per well. Co-cultures were incubated for 3-5 days at 37°C. If proliferation was to be measured by [<sup>3</sup>H]-thymidine incorporation, cultures were pulsed for 24 hours before harvest with 0.5 µCi per well of [<sup>3</sup>H]-thymidine (Amsterdam International, TRA120). Recall assays were harvested after 3 days of co-culture. Cells were harvested onto filter matts using a Micro96 harvester (Skatron Instruments). Counts per minute were recorded using a flat-bed scintillation counter (LBK Wallac, Finland). If T cells had been stained with CFSE, wells were centrifuged at 300 g for 5 minutes to remove culture medium and then either stained as described in section 2.23 or fixed in 1% formaldehyde in PBS for a minimum of 30 minutes and analysed by flow cytometry. As illustrated in Figure 2.1, T cells were gated on by size using the FSC and SSC. T cell cultures in the absence of DCs were used to determine the level of CFSE-staining of T cells that had not proliferated. T cells showing staining below that level were defined as having proliferated and gated on in order to determine the percentage of proliferated cells.

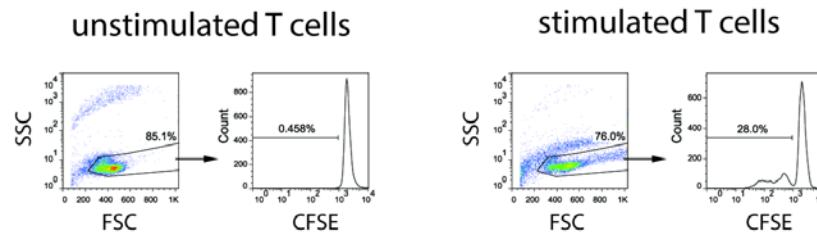


**Figure 2.1 CFSE gating strategy.** T cells were gated on by size using FSC and SSC. The percentage of proliferated cells was determined as the percentage of cells that showed a level of CFSE staining below that detected by T cell control populations which had not been co-cultured together with DCs and therefore remained unstimulated.

## 2.20 Treg and Tr1 induction/expansion assay

DCs were cultured at  $2 \times 10^5$  per well together with  $10^6$  allogeneic T cells in a final volume of 2 ml of R10 in 24-well cell bind plates. Co-cultures were treated with 75 ng/ml IL-2 on day 5. On day 7, cells were harvested and stained for CD4, CD25 and Foxp3, as described in section 2.23, and analysed by flow cytometry as described below.

For the detection of intracellular cytokines, co-cultures were treated on day 7 with 10  $\mu$ g/ml of brefeldin A (Sigma, B7651), 700 ng/ml ionomycin (Sigma, I0634) and 20ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, P8139) and incubated for 5 hours at 37°C and 5% CO<sub>2</sub>. After 5 hours, cells were harvested by pipetting and stained for intracellular cytokines using the fixation/permeabilization buffer and permeabilization buffer obtained from eBioscience (00-5521 and 00-8333) as described in section 2.23.3.



## **2.21 T cell suppression assay**

DCs were co-cultured as described in section 2.20 with unlabelled allogeneic naïve CD4<sup>+</sup> T cells and treated with 75 ng/ml of IL-2 on day 5. On the 7<sup>th</sup> day, cells were harvested and purified for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> cells as described in section 2.5.1.7 and treated with 10 µg/ml of MMC for 30 minutes at 37°C. After several washes in PBS, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> cells were plated in triplicates at  $1.5 \times 10^3$  together with  $2 \times 10^4$  naïve CD4<sup>+</sup> T cells of the same donor per well of a round-bottom 96-well plate. In order to stimulate T cell proliferation, the T<sub>reg</sub> Suppression Inspector (Miltenyi Biotec, 130-092-909) was added at a bead-to-cell ratio of 1:1. Controls included cultures of T cells and DCs alone in presence or absence of stimuli and stimuli alone. After 5 days at 37°C, co-cultures were pulsed with 0.5 µCi per well of [<sup>3</sup>H]-thymidine per well, left for further 16 hours, harvested and analysed using a flat-bed scintillation counter.

## **2.22 ELISA**

The release of cytokines from DCs and T cells was measured by enzyme-linked immunosorbent assay (ELISA).

### **2.22.1 IL-10 cytokine detection**

The presence of IL-10 in supernatants of human cell cultures were measured using the Human IL-10 DuoSet ELISA Development Kit (R&D Systems, DY217B). ELISAs were performed according to the manufacturer's instructions. In brief, supernatants were harvested from cell cultures and stored at -80°C until the assay was performed. 96-well EIA/RIA plates (Costar, 3590) were coated with 100 µl per well of Capture Antibody in washing buffer (Table 2.13) at a

concentration of 2 µg/ml overnight at room temperature. The following day, plates were blocked in Reagent Diluent (1% BSA in PBS, pH 7.2-7.4) and incubated at room temperature for 1 hour. Thereafter, plates were washed 5 times by filling wells with Washing Buffer, incubation of around 2 minutes and removing the liquid by blotting onto absorbent paper towels. Subsequently, samples and standards (starting at 2ng/ml followed by doubling dilutions) were added at a final volume of 100 µl per well and in triplicate. Samples and standards were incubated overnight at 4°C. Following the incubation, plates were washed 5 times as above and 100 µl per well of Detection Antibody was added at 150 ng/ml for 2 hours at room temperature. Detection was followed by 5 further washing steps and addition of 100 µl per well of Streptavidin-horseradish peroxidase (HRP) for 20 minutes at room temperature. Subsequently, plates were extensively washed 8 to 10 times as described above. Finally, 100 µl per well of Substrate Solution (R&D, DY999) was added for 20 minutes at room temperature followed by addition of 50 µl per well of 2N sulphuric acid Stop Solution (H<sub>2</sub>SO<sub>4</sub>) to terminate the colour development. Absorbance was measured using a spectrophotometer at Optical density (OD)<sub>450nm</sub>.

---

**Washing Buffer**

---

0.05% Tween20

(Sigma-Aldrich, P137-9)

in PBS

---

**Table 2.13 Washing Buffer used for ELISA assays.**

### **2.22.2 Human IL-12, IL-6 and IFN $\gamma$**

The presence of IL-12, IL-6 and IFN $\gamma$  in supernatants of human cell cultures was measured using the Human IL-12p70 ELISA Ready-SET-Go (eBioscience, 88-7126), Human IL-6 ELISA Ready-SET-Go (eBioscience, 88-7066) and Human IFN $\gamma$  ELISA Ready-SET-Go (eBioscience, 88-7316) kits according to manufacturer's instructions. ELISA plates (Costar, 9018) were coated with Capture Antibody diluted into 100  $\mu$ l per well of Coating Buffer overnight at 4°C. The following day, Coating Buffer was aspirated and washed 5 times with Washing Buffer by incubating the wells for 2 minutes in Washing Buffer and removing residual liquid by blotting onto absorbent paper. Wells are blocked with 200  $\mu$ l per well of 1x Assay Diluent for 1 hour at room temperature. Plates were washed 5 times followed by addition of samples and standards in triplicate. Samples for IL-6 and IFN $\gamma$  detection were diluted 10x and 20x respectively in 1x Assay Diluent. Top concentrations for standards were 200 pg/ml for IL-6 and 500 pg/ml for IL-12p70 and IFN $\gamma$  standard curves. Samples and standards were incubated overnight at 4°C followed by 5 washes. 100  $\mu$ l per well of Detection Antibody in 1x assay diluent was incubated for 1 hour at room temperature. After 5 further washes 100  $\mu$ l per well of Avidin-HRP was added and incubated for 30 minutes at room temperature, after which plates were washed 8 to 10 times. 100  $\mu$ l per well of Substrate Solution was added and left until samples had adequately developed, after which 50  $\mu$ l per well of Stop Solution (2N H $_2$ SO $_4$ ) was added to terminate the colour development. Absorbance was measured using a spectrophotometer at OD $_{450\text{nm}}$ .

**2.23 Flow cytometry**

Dendritic cells and T cells were harvested by gentle pipetting. iPSCs were harvested by dissociation using 0.02% EDTA for 5 minutes at 37°C. Cells were washed with cold washing buffer (Table 2.14) to remove the dissociation reagent or residual medium. Cells cultured in 96-well plates were centrifuged, supernatant removed and staining performed in the 96-well plate as described below.

| Washing Buffer                   |
|----------------------------------|
| 1% FCS                           |
| 0.01% EDTA                       |
| 0.1% sodium azide (Fulka, 71290) |
| in PBS                           |

**Table 2.14 Flow cytometry Washing Buffer.**

All cells that were harvested from cultures were transferred into 96-well plates at approximately 10<sup>5</sup> per well. Washing steps involved the addition of Washing Buffer (first round 100 µl, second round 150 µl), centrifugation at 300 g for 5 minutes, followed by removal of the supernatant and resuspension of cells in residual medium by gently vortexing.

**2.23.1 Blocking**

Once cells had been plated into a 96-well plate, cells were blocked for 15 minutes in Blocking Buffer on ice (Table 2.15).

| Blocking Buffer                                                                  |
|----------------------------------------------------------------------------------|
| 5% heat-inactivated normal rabbit serum<br>(Jackson ImmunoResearch, 011-000-120) |
| 1 % BSA                                                                          |
| 0.1 % sodium azide                                                               |
| in PBS                                                                           |

**Table 2.15 Flow cytometry Blocking Buffer.**

For mouse DCs an Fc receptor (FcR) blocking antibody (AbD Serotec, BUF041B) was added at 1 in 1000 dilution to inhibit antibody binding to FcR.

### **2.23.2 Cell surface staining**

Cells were washed with Washing Buffer and resuspended in this solution together with one or more purified, biotinylated or fluorescently-labelled monoclonal antibodies (Table 2.16-2.18). In order to control for non-specific antibody binding, control antibodies of the relevant isotype and fluorochrome label were used. Cells were stained at 4°C in the dark for 30 minutes. For the last 10 minutes 250 ng/ml 7-amino-actinomycin D (7-AAD) (Sigma-Aldrich, A9400) was added to all cells other than DCs. This allowed the exclusion of 7-AAD<sup>+</sup> cells during analysis which constitute dying cells. However, work in our laboratory has shown that live phagocytic DCs stain positively for 7-AAD due to the high amount of DNA in their phagocytic vesicles. If relevant, secondary stainings were performed for 20

minutes on ice in Washing Buffer using streptavidin-APC (BD Bioscience, 554067). After staining, cells were washed in Washing Buffer and fixed in 1% formaldehyde (Sigma-Aldrich, F8775) in Washing Buffer. Whenever intracellular staining was to follow surface staining, cells were fixed using Fixation/Permeabilisation Buffer (eBioscience, 88-8118-40).

Data were presented as relative geometric MFI (gMFI) as determined as the gMFI obtained from antigen-specifically stained samples over the gMFI obtained from samples that had been stained with isotype controls.

| Isotype | Label  | Company       | Cat#     | Clone      |
|---------|--------|---------------|----------|------------|
| goatIgG | PE     | R&D           | IC108P   | polyclonal |
| goatIgG | FITC   | R&D           | IC108F   | polyclonal |
| hamIgG1 | APC    | BD Bioscience | 553956   | G235-2356  |
| hamIgG1 | PE     | BD Bioscience | 553972   | A19-3      |
| hamIgG2 | PE     | BD Bioscience | 550085   | B81-3      |
| mlgG1   | APC    | Immunotools   | 21275516 | PPV-06     |
| mlgG1   | FITC   | Immunotools   | 21275513 | PPV-06     |
| mlgG1   | PE     | Serotech      | MCA928   | -          |
| mlgG1   | AF647  | Serotech      | MCA928   | -          |
| mlgG1   | AF488  | BD Bioscience | 557721   | MOPC-21    |
| mlgG1   | AF647  | BD Bioscience | 557732   | MOPC-21    |
| mlgG1   | PE     | BD Bioscience | 550617   | MOPC-31    |
| mlgG2a  | FITC   | eBioscience   | 11-4724  | eBM2a      |
| mlgG2a  | PE     | Immunotools   | 21335024 | PPV-04     |
| mlgG2a  | FITC   | BD Bioscience | 553456   | G155-178   |
| mlgG2a  | PE     | BD Bioscience | 553457   | G155-178   |
| mlgG2b  | PE     | R&D           | IC0041P  | 133303     |
| mlgG2b  | FITC   | Serotech      | MCA691F  | -          |
| mlgG3   | PE     | Serotech      | MCA2063  | -          |
| mlgM    | FITC   | Serotech      | MCA692F  | -          |
| mlgM    | PE     | BD Bioscience | 555584   | G155-228   |
| rlgG2a  | PE     | eBioscience   | 12-4321  | eBR2a      |
| rlgG2a  | FITC   | eBioscience   | 11-4321  | eBR2a      |
| rlgG2a  | APC    | eBioscience   | 17-4321  | eBR2a      |
| rlgG2a  | FITC   | BD Bioscience | 553929   | R35-95     |
| rlgG2a  | APC    | BD Bioscience | 553932   | R35-95     |
| rlgG2a  | Biotin | eBioscience   | 13-4321  | eBR2a      |
| rlgG2a  | PE     | BD Bioscience | 553930   | R35-95     |
| rlgG2b  | FITC   | BD Bioscience | 553988   | A95-1      |

**Table 2.16 Antibody isotype controls for flow cytometry.** Isotype controls with relevant fluorochrome labels were used to account for non-specific antibody binding.

| Antibody<br>Target            | Label       | Isotype      | Company        | Cat#           | Clone        | Dilution       |
|-------------------------------|-------------|--------------|----------------|----------------|--------------|----------------|
| CCR7                          | PE          | mIgG2a       | Biolegend      | 353203         | G043H7       | 1 in 60        |
| CD11b                         | PE          | mIgG2a       | Immunotools    | 21279114       | MEM-174      | 1 in 60        |
| CD11c                         | AF647       | mIgG1        | SeroTec        | MCA2087        | BU15         | 1 in 60        |
| CD11c                         | APC         | mIgG1        | Immunotools    | 21487116       | BU15         | 1 in 60        |
| CD123                         | PE          | mIgG1        | BDPharmingen   | 555644         | 9F5          | 1 in 60        |
| CD127                         | PE          | mIgG1        | R&D            | FAB306P        | 40131        | 1 in 60        |
| CD14                          | PE          | mIgG1        | Immunotools    | 21270144       | MEM-18       | 1 in 60        |
| CD141                         | PE          | mIgG1        | R&D            | FAB3947P       | 501733       | 1 in 60        |
| CD200R                        | FITC        | mIgG1        | SeroTec        | MCA2282        | OX108        | 1 in 60        |
| CD207                         | PE          | mIgG1        | Immunotech     | IM3577         | DCGM4        | 1 in 60        |
| CD209                         | APC         | rlgG2a       | eBioscience    | 17-2099        | eB-h209      | 1 in 60        |
| CD4                           | FITC        | mIgG1        | eBioscience    | 11-0049        | RPA-T4       | 1 in 60        |
| CD4 D25                       | FITC APC    | mIgG1        | eBioscience    | 22-0425        | RPA-T4,BC96  | 1 in 60        |
| CD40                          | FITC        | mIgG2a       | SeroTec        | MCA1590        | LOB7/6       | 1 in 60        |
| CD40                          | PE          | mIgG1        | Immunotools    | 21270404       | HI40a        | 1 in 60        |
| CD54                          | FITC        | mIgG1        | SeroTec        | MCA1615        | 15.2         | 1 in 60        |
| <u>CD68</u>                   | <i>FITC</i> | <i>mIgG1</i> | <i>SeroTec</i> | <i>MCA2375</i> | <i>Ki-M7</i> | <i>1 in 60</i> |
| CD80                          | FITC        | mIgG1        | SeroTec        | MCA2071        | MEM-233      | 1 in 60        |
| CD83                          | PE          | mIgG1        | SeroTec        | MCA1582        | HB15e        | 1 in 60        |
| CD86                          | FITC        | mIgG1        | Immunotools    | 21480863       | BU63         | 1 in 60        |
| CD86                          | FITC        | mIgG1        | SeroTec        | MCA1118        | BU63         | 1 in 60        |
| CD9                           | Biotin      | rlgG2a       | BD Bioscience  | 558749         | KMC8         | 1 in 60        |
| <u>Foxp3</u>                  | APC         | rlgG2a       | eBioscience    | 17-4776        | PCH101       | 1 in 60        |
| <u>Foxp3</u>                  | PE          | rlgG2a       | eBioscience    | 12-4776        | PCH101       | 1 in 60        |
| HLAIIIDPDQDR                  | PE          | mIgG2a       | SeroTec        | MCA477         | WR18         | 1 in 60        |
| <u>IFN<math>\gamma</math></u> | PE          | mIgG2b       | R&D            | IC285D         | 25723        | 1 in 60        |
| <u>IL-10</u>                  | PE          | mIgG2b       | R&D            | IC2171P        | 127107       | 1 in 60        |



| Antibody<br>Target | Label | Isotype | Company     | Cat#     | Clone      | Dilution |
|--------------------|-------|---------|-------------|----------|------------|----------|
| <u>IL-2</u>        | FITC  | rlgG2a  | eBioscience | 11-7029  | MQ1-17H12  | 1 in 60  |
| <u>IL-4</u>        | A488  | mlgG1   | eBioscience | 53-7049  | 8D4-8      | 1 in 60  |
| ILT3               | FITC  | mlgG2a  | R&D         | FAB24251 | 293623     | 1 in 60  |
| ILT4               | FITC  | mlgG2a  | R&D         | FAB2078  | 287219     | 1 in 60  |
| <u>Nanog</u>       | PE    | goatIgG | R&D         | IC1997P  | polyclonal | 1 in 60  |
| <u>Oct3/4</u>      | FITC  | mlgG2b  | R&D         | IC1759F  | 240408     | 1 in 60  |
| PD-L1              | FITC  | mlgG1   | SeroTec     | MCA2627  | MIH2       | 1 in 60  |
| PD-L2              | FITC  | mlgG1   | SeroTec     | MCA2630  | MIH14      | 1 in 60  |
| SSEA-4             | PE    | mlgG3   | R&D         | FAB1435  | MC-813-70  | 1 in 60  |
| TLR2               | FITC  | mlgG2a  | SeroTec     | MCA2152  | TLR2.3     | 1 in 60  |
| <u>TLR3</u>        | PE    | mlgG1   | eBioscience | 12-9039  | TLR3.7     | 1 in 60  |
| TLR4               | FITC  | mlgG2a  | SeroTec     | MCA2061  | HTA125     | 1 in 60  |
| <u>TLR7</u>        | PE    | mlgG2a  | R&D         | IC5875P  | 533707     | 1 in 60  |
| <u>TLR8</u>        | AF488 | mlgG1   | ingenex     | IMG-321  | 44C143     | 1 in 60  |
| <u>TLR9</u>        | PE    | rlgG2a  | eBioscience | 12-9099  | eB72-1665  | 1 in 60  |
| TRA-1-60           | FITC  | mlgM    | Millipore   | FCMAB115 | TRA-1-60   | 1 in 60  |
| XCR1               | FITC  | goatIgG | R&D         | FAB857F  | polyclonal | 1 in 60  |

**Table 2.17 Human antigen-specific antibodies.** Antibodies used for intracellular stainings are underlined.

| Antibody Target | Label  | Isotype | Company       | Cat#     | Clone     | Dilution   |
|-----------------|--------|---------|---------------|----------|-----------|------------|
| CD4             | APC    | rlgG2a  | BD Bioscience | 553051   | RM4-5     | 1.5 in 100 |
| CCR7            | PE     | rlgG2a  | eBioscience   | Dec-71   | 4b12      | 1.5 in 100 |
| CD11b           | FITC   | rlgG2b  | BD Bioscience | 557396   | M1/70     | 1.5 in 100 |
| CD11b           | FITC   | rlgG2b  | Immunotools   | 22159113 | M1/70.15  | 1.5 in 100 |
| CD11c           | APC    | hamIgG1 | BD Bioscience | 550261   | HL3       | 1.5 in 100 |
| CD123           | Biotin | rlgG2a  | BD Bioscience | 555070   | 5B11      | 1.5 in 100 |
| CD40            | PE     | rlgG2a  | eBioscience   | 12-0401  | 1C10      | 1.5 in 100 |
| CD54            | PE     | hamIgG1 | BD Bioscience | 553253   | 3 E2      | 1.5 in 100 |
| CD80            | PE     | hamIgG2 | BD Bioscience | 553769   | 16-10A1   | 1.5 in 100 |
| CD86            | Biotin | rlgG2a  | BD Bioscience | 553690   | GL1       | 1.5 in 100 |
| CD8a            | Biotin | rlgG2a  | R&D Systems   | BAM116   | 53-6.7    | 1.5 in 100 |
| CD9             | Biotin | rlgG2a  | BD Bioscience | 558749   | KMC8      | 1.5 in 100 |
| H-2K[k]         | PE     | mlgG2a  | BD Bioscience | 553593   | 36-7-5    | 1.5 in 100 |
| H-2K[b]         | FITC   | mlgG2a  | BD Bioscience | 553569   | AF6-88.5  | 1.5 in 100 |
| I-A / I-E       | FITC   | rlgG2a  | BD Bioscience | 553623   | 2G9       | 1.5 in 100 |
| I-A[b]          | PE     | mlgG2a  | BD Bioscience | 553552   | AF6-120.1 | 1.5 in 100 |
| ICOSL           | Biotin | rlgG2a  | BD Bioscience | 557788   | HK5.3     | 1.5 in 100 |
| <u>Nanog</u>    | AF488  | mlgG1   | BD Bioscience | 560278   | M55-312   | 1.5 in 100 |
| <u>Oct3/4</u>   | AF647  | mlgG1   | BD Bioscience | 560329   | 40/Oct-3  | 1.5 in 100 |

**Table 2.18 Mouse antigen-specific antibodies.** Antibodies used for intracellular stainings are underlined.

### **2.23.3 Intracellular staining**

Staining for nuclear and cytoplasmic proteins were performed according to the manufacturer's instructions using permeabilization and fixation buffers from a commercial T<sub>reg</sub> staining kit (eBioscience, 00-5521). Molecules detected by intracellular staining are indicated in Table 2.17 and 2.18. These included Foxp3, cytokines and transcription factors Oct3/4 and Nanog. Briefly, cells were blocked and stained for surface markers as described above. Thereafter, cells were fixed in fixation/permeabilization buffer at 4°C for a minimum of 1 hour and for up to 24 hours. Cells were washed in permeabilization buffer followed by intracellular staining with antibodies diluted in permeabilization buffer for 30-60 minutes on ice. Unbound antibodies were removed by further washing in permeabilization buffer. Cells were resuspended in 200 µl of PBS and analysed with a FACS Calibur (BD Biosciences).

### **2.24 Isolation of exosomes**

Cells were cultured in medium containing knock-out serum instead of FCS before harvesting supernatants in order to prevent contamination with serum vesicles. Supernatants were harvested, as described in Figure 2.2, by pipetting and centrifugation at 300 *g* for 5 minutes at 4°C. Supernatants were removed and further spun at 2200 *g* for 10 minutes followed by a filtration step through a 0.22 µm filter unit. Supernatants were removed and spun in polycarbonate ultracentrifuge tubes (Beckman Coulter, 355618) at 120,000 *g* for 70 minutes at 4°C. Supernatants were gently removed by pipetting and pellets resuspended in PBS and stored at -80°C.

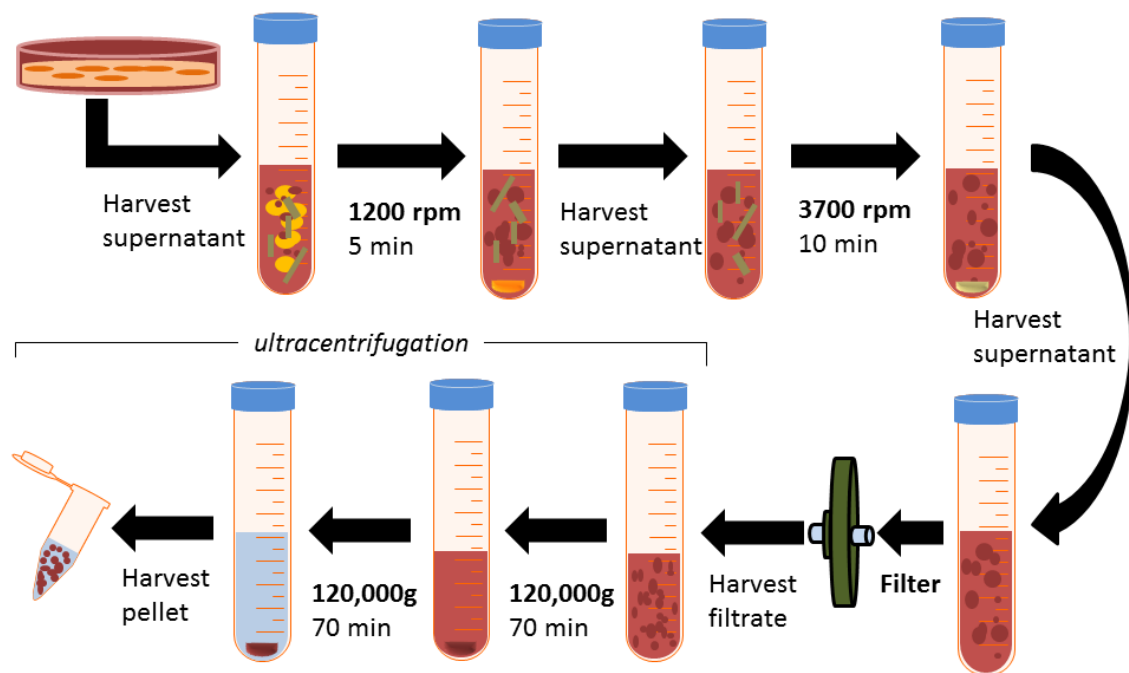


**Figure 2.2 The isolation of exosomes.** Supernatants were harvested from cell cultures by pipetting and centrifugation at 1200 and 3700 rpm in order to remove cells and cellular debris. The supernatants from these centrifugation steps were harvested and filtered through a 0.2 µm filter tip. The eluted medium was ultracentrifuged at 120,000 g for 70 minutes, after which supernatants were discarded and the exosome pellet resuspended in PBS by gentle pipetting. An optional washing step could be included at this point where exosomes would be resuspended in 20 ml of PBS followed by an additional ultracentrifugation step for the final purification of an exosome pellet and resuspension into PBS.

## 2.25 Nanosight

Exosome samples were analysed by Yi Lee at the Department of Physiology, Anatomy and Genetics at the University of Oxford using the NS 500 Nanosight. Nanosight was used to determine the number of particles in a sample and their size range.

## **2.26 Staining of exosomes**

Exosomes were incubated at 250-300 exosomes per 4  $\mu\text{m}$  diameter aldehyde/sulfate Latex bead (Invitrogen, A37304) for 15 minutes at room temperature in a total volume of 100  $\mu\text{l}$ . The number of exosomes was determined by Nanosight as described in section 2.25. Around  $3\text{-}6 \times 10^5$  beads were used for each stain. After 15 minutes, 1 ml of PBS was added to bead-exosome mixtures and incubated on a rotator for 2 hours at room temperature. To block free aldehyde/sulphate groups a final concentration of 100 mM glycine was added and incubated for 30 minutes at room temperature. From this step onwards, exosome-bead complexes were blocked and stained as described in section 2.23.

## **2.27 Western blotting of exosomes**

Western blotting was performed in collaboration with Yi Lee at the Department for Physiology, Anatomy and Genetics at the University of Oxford using the Bio-Rad Mini-PROTEAN Tetra cell according to the manufacturer's instructions.

### **2.27.1 Gel preparation**

Samples were run on a 1.5 mm thick 10% Tris(hydroxymethyl)aminomethane (Tris)-glycine-SDS-polyacrylamide gel. The resolving gel was made up of 10% acrylamide, 0.1% SDS (Table 2.19), 0.1% ammonium persulfate and 1 in 2500 tetramethylethylenediamine in a 375 mM Tris (pH 8.8).

| <b>10% SDS solution</b>                                       |
|---------------------------------------------------------------|
| 10 g of SDS                                                   |
| Made up to a total volume of 100 ml using ddH <sub>2</sub> O. |

**Table 2.19 10% SDS solution.** SDS: Sodium dodecyl sulphate.

## 2.27.2 SDS PAGE

15 µl of exosome sample were mixed with 15 µl of 2x Laemilli sample buffer (Bio-Rad, 161-0737EDU) containing 5% β-mercaptoethanol and incubated at 100 °C for 10 minutes.

Samples were loaded onto the gel and run at 170 V immersed in running buffer (Table 2.20). After around 60 to 70 minutes the dye front had reached the bottom of the tank and the run was terminated.

| <b>Running Buffer</b>                                      |
|------------------------------------------------------------|
| 3 g Tris base                                              |
| 14.8 g Glycine                                             |
| 1 g SDS                                                    |
| Made up to a total volume of 1 L using ddH <sub>2</sub> O. |

**Table 2.20 Running Buffer.**

### 2.27.3 Electrotransfer

Four Whatman 3M filter sheets and one polyvinylidene fluoride (PVDF) membrane (Millipore) were cut per gel. Filter papers and sponges were soaked in transfer buffer (Table 2.21) and the membrane soaked in methanol for a few minutes. These were then sandwiched bottom to top: 1 sponge, 2 sheets of filter paper, gel, PVDF membrane, 2 sheets of filter paper and 1 sponge. This compiled “sandwich” was placed into the rack for electrotransfer at 100 V for 60 to 70 minutes in transfer buffer containing 20% methanol.

| Transfer Buffer                                               |
|---------------------------------------------------------------|
| 3 g Tris base                                                 |
| 14.8 g Glycine                                                |
| 20% methanol                                                  |
| Made up to a total volume of<br>1 L using ddH <sub>2</sub> O. |

**Table 2.21 Transfer buffer.**

### 2.27.4 Antibody staining and imaging

After transfer, membranes were blocked in 5% fat-free dried milk in tris-buffered saline (TBS-T) (Table 2.22) for 1 hours at room temperature with gentle rocking. Antibodies were diluted in 5% fat-free dried milk in TBS-T and incubated either overnight at 4°C or 2 hour at room temperature concurrent rocking (37 oscillations/minute).

| <b>TBS-T buffer</b>                                          |
|--------------------------------------------------------------|
| 8 g of sodium chloride                                       |
| 0.2 g of potassium chloride                                  |
| 450 mg of Tris base                                          |
| in 1L of ddH <sub>2</sub> O                                  |
| 1mL of Tween-20 was added per 1L of TBS<br>to make up TBS-T. |

**Table 2.22 TBS-T buffer**

Primary antibodies used were anti-CD9, anti-PDC6I (Alix) and anti-Tsg101, all used at 1:1000 dilution (all from Abcam, Cambridge UK).

After primary staining, membranes were washed three times in TBS-T every for 10 minutes each with vigorous shaking (45 oscillations/minute). Secondary staining was performed using anti-mouse IgG DyLight-800 for the detection of Alix and anti-rabbit IgG DyLight-800 for the detection of CD9 and Tsg101, all at 1:10,000 dilutions in TBS-T with 0.01% SDS and incubated for 1 hour at room temperature. After staining, membranes were washed three times for 10 minutes each in TBS-T while shaking with vigorous shaking (45 oscillations/minute).

Membranes were imaged with the LI-COR Odyssey CLx infrared imaging system using both the 700 and 800 nm channels under 2 minutes exposure each.



## **2.28 Exosome antigen loading assay**

Immature bmDCs were used on day 7 of culture and ipDCs after harvesting from EB cultures.

Cells were incubated with 100 µg/ml of horseradish peroxidase Type II (HRP) (Sigma-Aldrich, P8250) for 2 hours, after which cells were extensively washed. Removal of HRP was monitored by a colorimetric assay using a Substrate Reagent Pack (R&D Systems, DY999) and 2N H<sub>2</sub>SO<sub>4</sub> as stop solution as described below. Cells were resuspended in DC medium containing SR and cultured overnight or longer. Exosomes were harvested as described in section 2.24.

Supernatants from cultures from which exosomes were isolated were sampled for HRP activity before ultracentrifugation. 1 ml of supernatant from ultracentrifugation and exosome pellets resuspended into 600 µl were sampled for the HRP activity. 100 µl of sample were added in triplicate to a 96-well plate and HRP was detected using 100 µl per well of Substrate Reagent (R&D Systems, DY999). The colour reaction was terminated by the addition of 50 µl per well of 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at OD<sub>450nm</sub> using a spectrophotometer.

## **2.29 Assay for HRP sedimentation**

HRP was added at 1 ng/ml to either 20 ml of PBS or bmDC medium which had been supplemented with SR instead of FCS. These were ultracentrifuged at 120,000 g for 70 minutes followed by the removal of twenty 1 ml fractions.

Fractions were analysed for HRP concentration using the assay described in section 2.28.

### **2.30 Optiprep Gradient Centrifugation**

Gradients were prepared with Optiprep (Sigma-Aldrich, D1556) ranging from approximately 1 g/ml to 1.22 g/ml. The heaviest fraction (fraction 11) was made by mixing 960  $\mu$ l of Optiprep with 240  $\mu$ l of sample and pipetting 1 ml of this into a 11 ml centrifuge tube (Table 2.23). Fraction 11 was overlain by fraction 10, which was repeated for each fraction, until the final layer, fraction 1 was gently pipetted onto fraction 2. The tube was lightly tilted to allow each following fraction to gently flow down the side of the tube as to form a distinct layer without disrupting the transitions. Table 2.23 describes the composition of each fractions of which 1 ml was progressively layered on top of the previous one.

The gradient was centrifuged at 100,000 *g* for 2 hours after which each fraction was carefully removed and separately stored in preweighed Eppendorf tubes.

Following addition of the 1 ml fraction, the samples were weighed again for correct density determination.

For the determination of HRP localisation, 100  $\mu$ l aliquots were taken from each fraction and in triplicate measured for HRP activity using the colorimetric assay described in section 2.28.

For Western blotting, each 1 ml fractions were diluted into 20 ml of PBS for removal of Optiprep, spun down at 120,000 *g* for 70 minutes and resuspended in PBS.

| Fraction | Optiprep (%) | Optiprep (μl) | PBS (μl)   |
|----------|--------------|---------------|------------|
| 1        | 0            | 0             | 1200       |
| 2        | 8            | 96            | 1104       |
| 3        | 16           | 192           | 1008       |
| 4        | 24           | 288           | 912        |
| 5        | 32           | 384           | 816        |
| 6        | 40           | 480           | 720        |
| 7        | 48           | 576           | 624        |
| 8        | 56           | 672           | 528        |
| 9        | 64           | 768           | 432        |
| 10       | 72           | 864           | 336        |
| 11       | 80           | 960           | 240 Sample |

**Table 2.23 Optiprep fractions for gradient centrifugation.** The first fraction to be layered into the tube was fraction 11, followed by fraction 10 through to fraction 1.

### 2.31 The effect of Optiprep on HRP

HRP was incubated at 100 ng/ml in different percentages of Optiprep made up with in PBS. After 2 hours of incubation the colorimetric assay described in section 2.28 was used to determine the concentration of HRP by HRP activity.

## **2.32 EM**

A PHILIPS CM120 Transmission Electron Microscope was used for imaging of samples.

### **2.32.1 Cell sample processing**

Cell pellets were fixed in 2.5% glutaraldehyde (Agar Scientific Ltd, R1020) 4% paraformaldehyde in 100 mM sodium cacodylate buffer (pH 7.0) at 4°C overnight. All solutions and buffers were made up in water.

If uptake of HRP was to be investigated, 3, 3'-Diaminobenzidine (DAB) staining was performed at this point using the 2-Solution DAB kit (Invitrogen, 882014). First of all, the fixation solution was removed by several washes with PBS by gently pipetting out the buffer (so as not to disrupt the cell pellet) and replacing it several times with fresh PBS.

500 µl of DAB substrate reagent was added per cell pellet and incubated for 15 minutes at room temperature. At this point, the colour change in HRP-positive samples could be observed. After staining, samples were repeatedly washed with PBS.

In the following procedure, samples that were not treated with DAB substrate were washed and treated with 100 mM cacodylate buffer (Sigma-Aldrich, 20840), while samples that had been stained with DAB substrate were always washed and treated with PBS. After fixation or DAB staining, samples were incubated in 1 % osmium tetroxide (Elektron Technology UK Ltd, AGR1016) made up in 100 mM cacodylate buffer or PBS for 1-2 hours at room temperature. After postfixation, samples were washed at least 5 times with distilled water to remove

any excess phosphate ions. After washing, samples were incubated in 2% aqueous uranyl acetate mixed 1:1 with 100 mM cacodylate buffer or PBS for approximately 2 hours at 4°C in the dark. Samples were washed twice with distilled water, followed by sample dehydration starting with the incubation of 30% ethanol for 15 minutes, followed by 50% ethanol for 15 minutes on a rotator. Samples were left in 70% ethanol at 4°C overnight. The following day, samples were transferred into 90% ethanol for 15 minutes at room temperature followed by 3 times 30 minute incubations in 100% ethanol. For the last incubation 100% of ultra-dry ethanol was used.

Following dehydration, the samples were slowly embedded into resin without catalyst from the Agar 100 Premix kit (Agar Scientific, R1140) starting with a 2:1 mixture of ethanol with resin for 1 hour at room temperature under continuous rotation, followed by a 1:1 mixture of ethanol and resin for 2 hours, 1:2 mixture of ethanol and resin for 1 hour and 100% resin with benzyldimethylamine catalyst (Agar Scientific, R1140) for 30 minutes followed by a change into fresh 100% resin with catalyst for 4 hour or overnight at room temperature on a rotator.

Following resin infiltration, samples were embedded into moulds and polymerised for 12-24 hours at 60-70°C.

### **2.32.2 Ultrathin sectioning**

Polymerised resin blocks were mounted into chucks and semi-thin sections of around 1-2 µm were cut using a glass knife until the samples were exposed. Sample blocks were then dismantled and trimmed into a trapezoid shape to reduce the amount of resin around the samples and improve sample cutting and

collection. The trimmed sample blocks were mounted into the chuck for sectioning of ultra-thin sections of 90 nm using a diamond knife (Diatome) with water or ultra-thin glass knife with attached water boat. Floating samples were stretched using anhydrous chloroform vapour (Sigma-Aldrich, 288306), collected using a wire loop and placed onto a 200 mesh hexagonal copper grid (Agar Scientific, AGG2450C).

### **2.32.3 Staining of ultra-thin sections**

Samples were stained in 20 µl of lead citrate (Reynolds, 1963) for 5 minutes surrounded by NaOH pellet (Fischer Scientific, S/4920/53) on parafilm. Following the staining, grids were washed three times in 20 µl of warm ddH<sub>2</sub>O. For the last wash, the grids were incubated for 1 minute on the water droplets. Grids were left to dry on filter paper.

### **2.32.4 Grid preparation for exosomes**

200 mesh hexagonal nickel grids (Agar Scientific, AGG2450N) were coated with 0.5% formvar solution in chloroform (Sigma-Aldrich, 09818). Glass slides were dipped into a solution of formvar for 2 seconds, excess liquid removed and the slide left to dry without allowing it to come into contact with any surfaces. Once the film had dried, a razor blade was used to cut the film along the edges of one side of the glass slide. The slide was slowly submerged into a large bowl of clean ddH<sub>2</sub>O at a 45° angle allowing the film to slowly separate from the slide and float on the water.

Nickel grids were gently placed on top of the film (light side facing the film). Nickel grids aligned on the formvar film were picked out of the waterbath by gently lowering parafilm (Starlab, I3080-1075) onto the film starting at one end at an angle. Grids were then stored at room temperature until further use.

Before use, formvar-covered grids were subject to carbon coating using a carbon filament and QT Glow discharge—ve using an Emitech K950 at 20 mA for 20 seconds and grids were subsequently used within 15 minutes of preparation.

### **2.32.5 Mounting of exosome samples onto grids**

Samples were suspended to a final particle concentration of around  $10^9$  particles in 2% paraformaldehyde in PBS. 100  $\mu$ l drops were placed onto parafilm and grids incubated on the drops for 20-30 minutes at room temperature.

### **2.32.6 Immunogold-labelling**

Grids were washed three times in 100  $\mu$ l of PBS drops on parafilm for 5 minutes. Samples were blocked in 100  $\mu$ l drops of blocking buffer (see section 2.23.1) for 15 minutes at room temperature.

Grids were further washed and placed onto 50  $\mu$ l droplets of biotinylated anti-CD9 antibody diluted at 1.5:100 (eBioscience, 13-0098-82) or biotinylated mouse IgG1 isotype control (eBioscience, 13-4714-85) in washing buffer (see section 2.23) and incubated for 30 minutes to 1 hour at room temperature. Grids were washed five times in 100  $\mu$ l droplets of washing buffer to remove excess antibody. Grids were incubated in 50  $\mu$ l droplets of 1:10 diluted secondary 5 nm gold-labelled streptavidin (KPL, 58-30-06) in washing buffer for 20 minutes. Secondary

labelling was followed by five further washes in washing buffer, after which samples were post-fixed in 1% glutaraldehyde for 5 minutes. Grids were washed eight times in 100 µl drops of ddH<sub>2</sub>O for 2 minutes. Immunogold-labelling was followed by contrast staining.

### **2.32.7 Contrast staining**

Grids were incubated in 50 µl of uranyl acetate (pH 7) for 5 minutes at room temperature and protected from light.

## **2.33 Antibody response *in vivo***

The immunogenicity of allogeneic bmDCs and syngeneic and allogeneic exosomes *in vivo* was investigated as follows.

### **2.33.1 Blood sampling**

Animals were tail bled before and after the injection of either PBS, exosomes or DCs. Around 50-100 µl of blood were taken per animal, depending on the number of blood samples to be taken. However, the total volume did not exceed 15 % of the total blood volume of a mouse, as defined by the Animals (Scientific Procedures) Act 1986.

Animals were warmed using a warming lamp until the tail vein was visible. Mouse tails were nicked using a razor blade and blood drops collected into an Eppendorf tube.

Animals were sacrificed at the end of the experiment and bled directly after Schedule 1 killing by cardiac puncture. The heart was held in place using forceps



and a BD Micro-Fine insulin syringe needle was inserted into the beating heart in order to withdraw blood. Blood was transferred into Eppendorf tubes and left for coagulation for 30 minutes at room temperature. After 30 minutes blood samples were centrifuged at 5000 *g* for 10 minutes to separate clotted blood cells from the serum. Serum was harvested by pipetting and stored in an Eppendorf tube at -20°C.

### **2.33.2 Injection**

For the injection of cells and exosomes mice were warmed to encourage vasodilation thereby increasing the visibility of the tail veins. Exosomes and B6 bmDCs were injected into the tail vein at  $5 \times 10^9$  exosomes,  $2 \times 10^6$  bmDCs or 100  $\mu$ l of PBS per animal using a BD Micro-Fine insulin syringe needle which had been bent to an angle of around 45°.

### **2.33.3 The measurement of antibody titres**

bmDCs were obtained as described in section 2.6. These were plated at  $10^5$  cells per well in a 96-well plate for the detection of antibodies present within the serum obtained from mice previously immunized with PBS or bmDCs. For the detection of antibodies generated against injected exosomes,  $3 \times 10^6$  4  $\mu$ m diameter aldehyde/sulfate Latex beads were used per well of a 96-well plate. The beads had been previously coated with the same population of exosomes which had been injected into mice. Three hundred exosomes were incubated per bead as described in section 2.26. Cells and exosome-covered beads were blocked with an Fc-blocking antibody, as described in section 2.23.1, for 30 minutes on ice. Serial dilutions of serum were incubated with the cells and exosome-covered

beads for 40 minutes on ice followed by washing steps as described in section 2.23.2. The Affinity Pure Goat Anti-Mouse IgG (H+L) with minimal cross reactivity to rat, human, bovine, horse and rabbit serum proteins (Jackson ImmunoResearch, 115-095-166) was used for the secondary detection of bound IgG antibody. The secondary staining was performed at 1 in 200 dilution in 30  $\mu$ l per well for 40 minutes on ice. Secondary labelling was followed by multiple washes and fixation as described in section 2.23.2. Cells and bead were analysed by flow cytometry using FlowJo 7.6.5 and GraphPad Prism 6 software.

## **2.34 Statistical analysis**

All data are presented as mean  $\pm$  SEM of independent biological replicated experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) with Sidak's correction for multiple comparisons using GraphPad Prism 6 whenever more than 2 conditions were measured. Paired and unpaired T tests were also performed wherever indicated. Statistical significance was reported as not significant (*ns*) if  $p > 0.05$  and statistically significant if  $*p \leq 0.05$ ;  $**p \leq 0.01$ ;  $***p \leq 0.001$ ;  $****p \leq 0.0001$ . The sample size (*n*) was indicated in all figure legends.



## Chapter 3 The differentiation of human iPSCs into ipDCs and their modulation for the generation of tolerogenic DCs



## Introduction

Up to the current time, ERT has been the most effective treatment regime for a number of LSDs. The replacement of the impaired or missing enzyme has allowed the significant improvement of patient quality of life, life expectancy and disease pathogenesis (Amalfitano *et al.*, 2001; Kakkis, 2004; van der Ploeg *et al.*, 2012). Nevertheless, despite considerable success, the efficacy of ERT has been limited in some cases by the patient's immune response directed towards the replaced enzyme. This has been the case, especially in patients who lack any residual enzyme expression, however it has also been observed in patients with residual enzyme expression (Abbott *et al.*, 2011; Banugaria *et al.*, 2011, 2012a; Furusawa *et al.*, 2012; Kishnani *et al.*, 2010). High antibody titres against recombinant enzyme have been associated with a diminished therapeutic outcome due to enzyme neutralisation, antibody-mediated inhibition and enzyme mistargeting to Fc receptor-expressing cells (Banugaria *et al.*, 2011; Cresawn *et al.*, 2005; Debiec *et al.*, 2014; Desnick and Schuchman, 2012; Linthorst *et al.*, 2004; Rosenberg *et al.*, 2013; Turner *et al.*, 2000). Therefore, it has become clear, that a method of tolerance induction is required in order to improve the efficacy of ERT (Abbott *et al.*, 2011; Banugaria *et al.*, 2011, 2012a, 2012b; Brooks, 1999; Brooks *et al.*, 2003; Cousens *et al.*, 2012; Cresawn *et al.*, 2005; Debiec *et al.*, 2014; Deegan, 2012; Hollak and Linthorst, 2009; Kishnani *et al.*, 2010; Lacaná *et al.*, 2012; Nayak *et al.*, 2012).

Different approaches to addressing the issue of immunogenicity have been investigated, ranging from enzyme-expressing vectors transduced into the livers of mouse models of Pompe disease to drug-based systemic immune inhibition

currently used in the clinic (Banugaria *et al.*, 2013; Franco *et al.*, 2005; Messinger *et al.*, 2012; Zhang *et al.*, 2012).

Most approaches may lead to detrimental side effects, such as drug toxicity, increased susceptibility to infection and cancer, insertional mutagenesis, immune responses towards transduced cells and GvHD in cases of bone marrow transplantation (Banugaria *et al.*, 2012a; Douillard-Guilloux *et al.*, 2009; Magauran and Salgado, 2011; Turbeville *et al.*, 2011; Wingard *et al.*, 2010).

Therefore, we sought to develop a novel approach to the induction of tolerance, merging the well-studied tolerogenic properties of DCs with our recently-established protocols for the differentiation of DCs from human iPSCs to allow the production of DCs in an autologous manner (Silk *et al.*, 2011, 2012a; Tseng *et al.*, 2009). A number of agents have been investigated for the modulatory properties on DCs in pursuit of immune tolerance. These agents include rapamycin, dexamethasone, VD3 and IL-10 which have been shown to downregulate the expression of costimulatory molecules expressed on DCs and reduce their allostimulatory capacity while enhancing their ability to induce T<sub>regs</sub> (Bartels *et al.*, 2010; Chamorro *et al.*, 2009; Gregori *et al.*, 2010; Horibe *et al.*, 2008; Naranjo-Gómez *et al.*, 2011; Pothoven *et al.*, 2010; Stallone *et al.*, 2014; Stoop *et al.*, 2010; Torres-Aguilar *et al.*, 2010a; Unger *et al.*, 2009; Xia *et al.*, 2005; Yates, 2007).

As some LSD patients are of young age or have significantly compromised health, the volume of blood that can be taken to serve as a conventional source of DCs is limited. Therefore, in order to supply the demand for autologous DCs

for tolerance induction, this thesis focuses on the feasibility of deriving patient-specific DCs from pluripotent stem cells.

Derived from the inner cell mass of the blastocyst, ESCs have the potential capacity for unlimited proliferation and differentiation into all three germ layers (Amit *et al.*, 2000; Thomson, 1998). This capacity, called pluripotency, is the reason for the extensive interest in stem cell science as it harbours the potential for the formation of any cells, tissues and organs and therefore presents an exciting new avenue for cell and tissue replacement therapies (Brignier and Gewirtz, 2010; Hirayama *et al.*, 2013; de Lázaro *et al.*, 2014). In collaboration with Geron, our laboratory has been able to develop a differentiation protocol to allow the differentiation of DCs from human ESCs (Silk *et al.*, 2011; Tseng *et al.*, 2009). However, cells differentiated from ESCs cannot be derived in an autologous manner and are likely, therefore, to prove immunogenic (English and Wood, 2011; Fairchild *et al.*, 2004, 2007; Swijnenburg, 2008; Swijnenburg *et al.*, 2005). Consequently, the usage of ESCs and their differentiated tissues are limited by the HLA-matching requirements. Developments within the last decade, might have overcome this limitation, as studies by Takahashi and Yamanaka (2006 and 2007) have shown the possibility of reprogramming fibroblasts taken from skin biopsies into iPSCs thereby reinstating pluripotency. This methodology provides the possibility of deriving any cell type or tissue in a patient-specific manner.

In this chapter, the feasibility of differentiating DCs from human iPSCs, so-called ipDCs, using our previously-established protocol was confirmed (Leishman and Fairchild, 2014; Silk *et al.*, 2011, 2012a; Tseng *et al.*, 2009). Furthermore, the effect of pro-tolerogenic agents rapamycin, VD3, dexamethasone and IL-10 on



ipDC populations was investigated and compared to moDCs. Accordingly, moDCs and ipDCs that had been differentiated from iPSC were treated with rapamycin, VD3, dexamethasone and IL-10 and their immunogenic potential investigated. A variety of readouts were used including cell viability, cell recovery, the expression of costimulatory and immunoinhibitory molecules, T cell stimulatory capacity in the allogeneic MLR, T<sub>reg</sub> induction or expansion, Tr1 induction or expansion and the release of cytokines from DCs and responding T cells. By compiling these various sources of information, this chapter sought to define a protocol that might be applied to ipDC differentiated from Pompe disease patients.

Altogether, this chapter takes a first step towards enabling the derivation of large numbers of patient-specific autologous DCs and determining a range of promising pro-tolerogenic agents with the potential to generate tolerogenic patient-specific ipDCs.

## **Results**

### **3.1 The morphology and pluripotency of iPSC colonies**

In order to investigate, as proof of principle, the feasibility of deriving patient-specific ipDCs, an iPSC line derived from a healthy donor, called C15, kindly provided by Dr Lee Carpenter and Prof Suzanne Watt from the Stem Cell Research Laboratory, National Health Service Blood and Transplant, John Radcliffe Hospital, Oxford, UK, was used. These iPSCs have previously been shown to express markers of pluripotency, have the capacity to differentiate into all three germ layers in a teratoma assay and give rise to haematopoietic and

endothelial cells (Carpenter *et al.*, 2011). Grown on Matrigel, C15 iPSCs displayed a morphology characteristic of iPSCs colonies as illustrated in Figure 3.1. The photomicrographs show the small, tightly packed cells with large nuclei that make up the colonies.

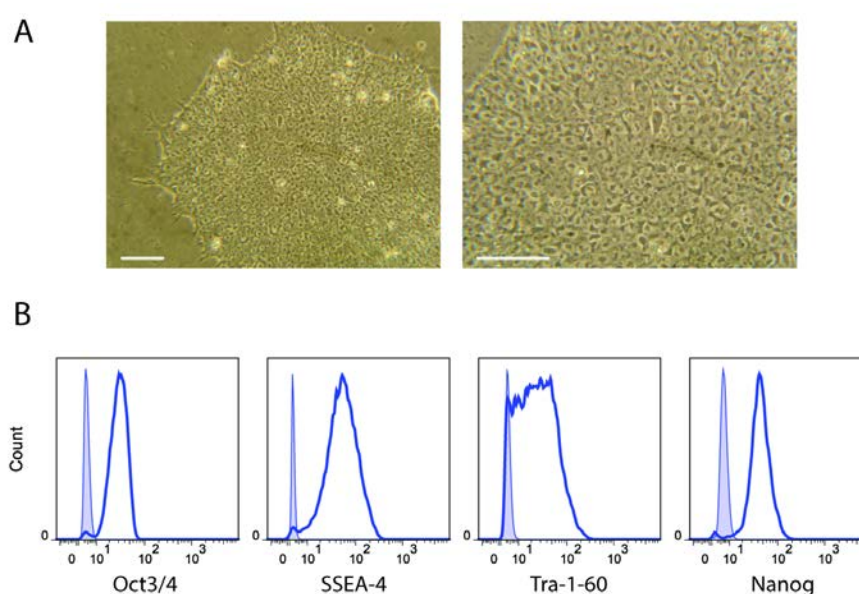


**Figure 3.1 The morphology and expression of pluripotency markers by C15 iPSCs.** (A) Photomicrographs of C15 iPSC colonies grown on a Matrigel-covered 6-well plate at passage 17. Scale bars: 100  $\mu$ m. (B) C15 iPSCs were harvested at passage 19 by dissociation and stained for surface markers and viable cells followed by the permeabilisation and staining for nuclear transcription factors characteristic of pluripotency. After fixation, cells were analysed by flow cytometry. Tinted histograms represent cells stained with relevant isotype controls and the open histograms represent marker-specific stained cells. These data are representative of 3 independent experiments which were further performed on passages 15 and 17.

Pluripotency is known to be maintained by the expression of a range of markers including transcription factors Oct3/4 and Nanog and further characterised by the expression of cell surface markers Tra-1-60 and SSEA-4 (International Stem Cell Initiative *et al.*, 2007; Mitsui *et al.*, 2003; Nichols *et al.*, 1998). In order to investigate the expression of these pluripotency markers by the C15 iPSC starting

population, cells were stained and analysed by flow cytometry. As shown in Figure 3.1B, iPSCs cultured on Matrigel expressed the transcription factors Oct3/4 and Nanog together with the cell surface markers Tra-1-60 and SSEA-4. Having future immunological studies in mind, the HLA-haplotype of the C15 donor was determined to allow the identification of HLA-matched T cell donors for antigen-specific studies. HLA tissue typing for the identification of MHCII loci was performed by Proimmune while the HLA-A locus had been previously identified in our laboratory by Dr Kate Silk using HLA-A2-specific antibodies (Table 3.1)

| Locus    | Allele       | Allele          |
|----------|--------------|-----------------|
| HLA-A    | *02          | -               |
| HLA-DRB1 | *01:02/20    | *11:04/06       |
| HLA-DRB3 | *02:02/10/25 | -               |
| HLA-DQB1 | *05:01/07/11 | *06:03/28/31/41 |
| HLA-DPB1 | *02:01:02    | -               |
| HLA-DQA1 | *01:01/04/05 | *01:03          |

**Table 3.1 HLA-haplotype of the C15 fibroblast donor.** DNA was purified from C15 iPSCs at passage 16 and HLA-tissue typing was performed by Proimmune. Several variants of alleles may be shown indicating that one of the two variants may be possible, however the variant mentioned first is more likely. For examples for HLA-DRB1\*01:02/20 the most likely variant would be HLA-DRB1\*01:02, however HLA-DRB1\*01:20 would also be possible.

Having shown the phenotypic characteristics of C15 iPSCs that are consistent with their pluripotency, it was investigated whether these iPSCs could be expanded and successfully differentiated into ipDCs.

### **3.2 The differentiation of ipDCs from iPSCs**

Once iPSCs had grown close to 80% confluency, cells were harvested and plated into differentiation cultures. Modulation of the cytokine environment during the differentiation process allowed cells to be directed in their differentiation towards the mesoderm lineage for the generation of haematopoietic-like cells (Figure 3.2A). iPSC colonies were removed from Matrigel-covered plates using a cell scraper and transferred onto ULA plates in mTeSR medium containing 50 ng/ml of GM-CSF, 50 ng/ml of VEGF, 20 ng/ml of SCF and 50 ng/ml of BMP-4. These factors were serially diluted out of the culture medium at different times, as illustrated in Figure 3.2A (Leishman and Fairchild, 2014; Silk *et al.*, 2011, 2012a; Tseng *et al.*, 2009). As a key factor for mesoderm and haematopoietic lineage commitment during early embryonic development and HSC survival, BMP-4 was present during early stages of the differentiation process (Goldman *et al.*, 2009; Sadlon *et al.*, 2004). Similarly, VEGF was present during the early phases, as a regulator of HSC survival and repopulation (Gerber and Ferrara, 2003; Gerber *et al.*, 2002). SCF is also an important factor in haematopoiesis further allowing HSC maintenance and self-renewal (Kent *et al.*, 2008; Ogawa *et al.*, 1991). As key player in development of functional DCs from monocytes and haematopoietic cells, GM-CSF was present throughout the differentiation process (Chapuis *et al.*, 1997; Conti *et al.*, 2008; van de Laar *et al.*, 2012).

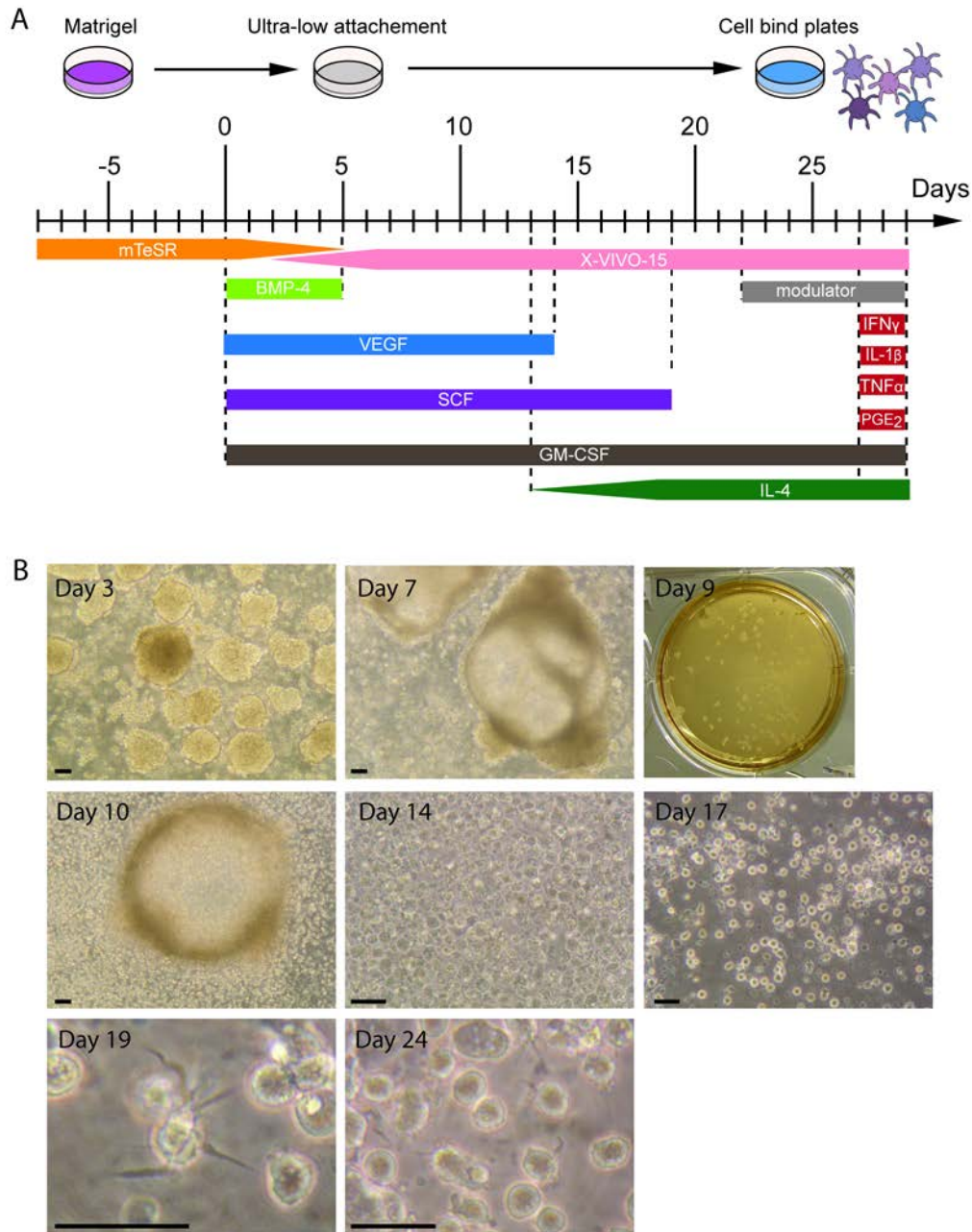


**Figure 3.2 The timeline of the differentiation of iPSCs into ipDCs.** (A) Illustration of the differentiation timeline of iPSCs into ipDCs. iPSCs were grown on Matrigel-covered 6-well plates. For the initiation of differentiation, iPSCs were harvested by scraping and were plated onto ULA plates in a cocktail of cytokines, using mTeSR as a base medium. Differentiation cultures were fed every 2-3 days with the base medium X-VIVO-15 supplemented with cytokines as indicated. (B) Photomicrographs illustrating the morphology and appearance of C15 iPSCs at passage 17 differentiating into ipDCs. By day 3 EBs had become apparent which had grown in size and developed cyst-like structures by day 6. The top right photomicrograph illustrates the size of EBs on day 9 of differentiation. By day 10 and 14, EBs started releasing cells into the surrounding medium, which displayed

monocyte-like appearance as illustrated by the photomicrograph taken on day 17. Cells displaying cellular protrusions and extensions, an appearance reminiscent of DCs, appeared in cultures from around day 17 and increased in number within the next few days. These cells released from EBs including ipDCs and monocyte-like precursors were harvested around day 21-24 for another 7-day differentiation in GM-CSF and IL-4. During this time, ipDCs were either left untreated or modulated using pro-tolerogenic agents and/or matured using a cocktail of pro-inflammatory cytokines. Scale bars: 100  $\mu$ m.

Once cell clusters had been plated, they developed and grew into EBs, reminiscent of early embryonic development (Fuchs *et al.*, 2012). Figure 3.2B illustrates the characteristic stages of differentiation cultures, starting with the early formation of small EBs and their enlargement during the early differentiation process. Around day 10-14, EBs started releasing monocyte-like precursor cells characterised by a large irregular shape and large refractive nucleus. From around day 17 onwards, cells displaying a DC-like morphology with cellular protrusions appeared in the cultures alongside monocyte-like precursors. A few days later, ipDCs with fine and long spikes could be found in cultures. The mixture of monocyte-like precursors and DCs were harvested by pipetting and plated onto cell-bind plates for the final 7-day differentiation, modulation and maturation stages.

### **3.3 The yield of ipDCs**

The yield of monocyte-like precursors (ipMonocytes) from iPSC differentiations was determined as the number of ipMonocytes harvested for the final 7-day differentiation process per plated iPSC (Figure 3.3A). On average, 3 ipMonocytes were obtained from every 4 iPSCs plated, however, as illustrated in Figure 3.3B, the yield of ipMonocytes from plated iPSCs varied considerably between

experiments (mean  $\pm$  SEM,  $71.92 \pm 13.12\%$ ,  $n=18$ ). These data indicated the high variability in differentiation efficiency and DC precursor output, even when generated from one iPSC cell line. These data may indicate the need for further protocol optimization to allow more reliable and reproducible ipMonocyte generation. In future, it would, therefore, be important to investigate the variability between different iPSC lines from different donors. ipMonocytes, harvested from these differentiations, were plated into cell-bind plates for the final 7-day differentiation phase.

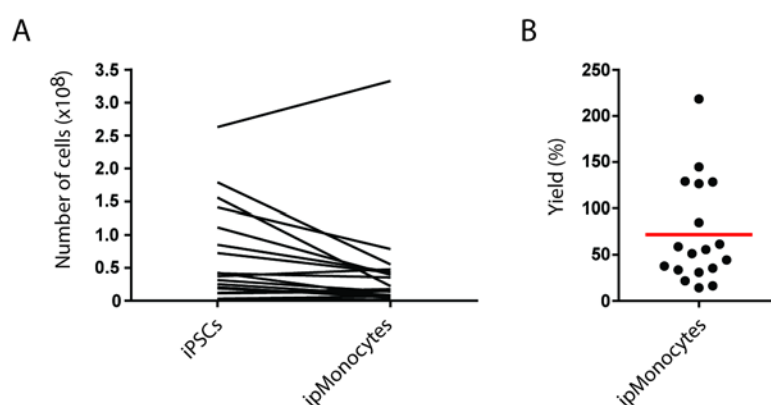


**Figure 3.3 The yield of ipMonocytes from iPSC differentiations.** (A) Data display the number of iPSCs that were plated per differentiation and the number of ipMonocytes generated from these differentiations. Lines represent 14 independent experiments. (B) Data display the yield of ipMonocytes expressed as a percentage of the number of iPSCs that had been plated for the initiation of differentiations. The red line represent the mean of 18 independent experiments which are represented by black dots. Differentiations were set up with iPSCs at passages 15 to 26.

As shown in Figure 3.4A/B, ipDCs displayed a larger variation in size and granularity, measured by FSC and SSC, as compared to moDCs which may be due to the different culture periods, as ipDCs were cultured 4 times longer than moDCs. This was further supported by the observation that during differentiations a vast amount of cell death was observed which created large numbers of

apoptotic cells and cellular fragments in cultures. These cells and debris started to disappear once ipDCs emerged in culture, indicating that the ipDCs may phagocytose and endocytose debris from their local environment which may further account for their enlarged and granular appearance.

High expression of CD11c is thought to be one of the most reliable markers of DCs (Collin *et al.*, 2013). Therefore, in order to determine the yield of DCs from iPSCs and CD14<sup>+</sup> monocytes, the percentage of CD11c<sup>+</sup> cells harvested at the end of the differentiation was analysed. Differentiations of CD14-preselected peripheral blood monocytes gave rise to  $89 \pm 3.93\%$  ( $n=12$ ) and  $89 \pm 4.22\%$  ( $n=13$ ) of CD11c-expressing cells in immature and mature cultures respectively (Figure 3.4). Differentiations from iPSCs gave rise to a significantly smaller proportion of CD11c<sup>+</sup> cells with an average yield of  $43 \pm 7.7\%$  ( $n=12$ ) for both immature and mature ipDCs. However, the yield of CD11c<sup>+</sup> cells is not comparable between the two DC populations as the pre-selection of CD14<sup>+</sup> monocytes for the differentiation of moDCs allowed the differentiation of pure moDC populations while ipMonocytes had not been subject to any purification step and therefore contained contaminating cell types. Whether these cells were haematopoietic cells, macrophages, EB fragments, DC precursors at different developmental stages and therefore with different levels of CD11c expression or other cell types had not been determined, however this may have accounted for the low CD11c<sup>+</sup> cell yield of around 43%.



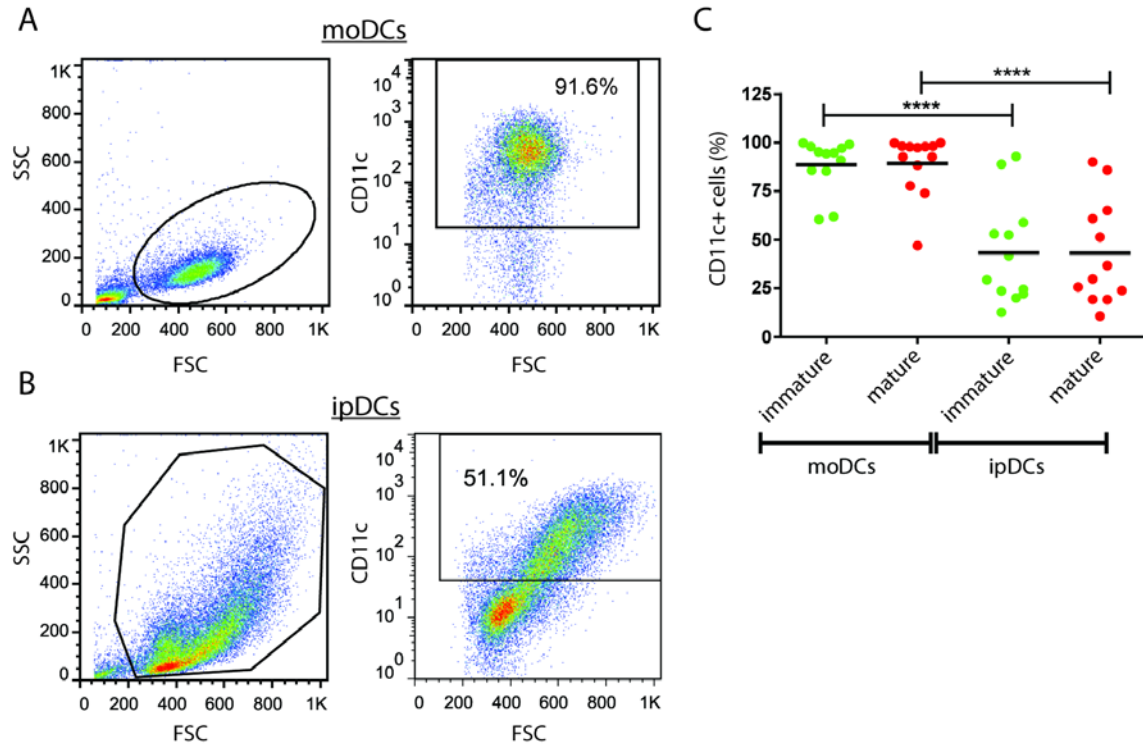


**Figure 3.4 The proportion of CD11c<sup>+</sup> cells obtained from human iPSCs.** (A) CD14<sup>+</sup> monocytes purified from human leucocyte cones or buffy coats were cultured for 7 days and analysed by flow cytometry for size (FSC), granularity (SSC) and CD11c expression. (B) ipMonocytes derived from differentiations of C15 iPSCs at passage 17 were harvested after 21-24 days, cultured for 7 further days and analysed by flow cytometry for size, granularity and CD11c expression. (C) Data display the percentage of CD11c<sup>+</sup> cells generated from differentiations from human blood monocytes and C15 iPSCs at passages 15 to 26 that had been left immature (green) or matured (red) using a cocktail of pro-inflammatory cytokines. Cells were analysed by flow cytometry at the end of differentiations. Cells were gated by size and granularity and then analysed for the percentage of CD11c<sup>+</sup> cells. Black bars represent the mean percentage of CD11c<sup>+</sup> cells, as determined by several independent experiments which are shown as coloured dots. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\*\*\* $p \leq 0.0001$ ). Data on the proportion of CD11c<sup>+</sup> cells were shown again as control groups in Figure 3.17 (page 197) of this chapter.

In order to compare the phenotype of ipDCs harvested from these cultures with conventionally-used moDCs, both DC populations were analysed by flow cytometry.

### **3.4 The characterisation of ipDCs and their resemblance to moDCs**

Having confirmed the presence of DC marker CD11c on DCs derived from iPSCs, ipDCs were further examined for the level of CD11c expression and the presence of other markers, characteristic of DCs.

#### **3.4.1 DC markers**

DCs are characterised by a variety of cell surface and intracellular molecules. As mentioned above, CD11c is one of the most reliable markers of DCs (Collin *et al.*, 2013). In order to investigate the phenotype of ipDCs and exclude cells that are not DCs, cells were gated on the CD11c<sup>+</sup> population for further analysis by flow cytometry. Similar to moDCs, ipDCs were found to consistently express CD11c on their surface in both the immature and mature state, as shown in Figure 3.5. The average level of CD11c expression detected on ipDCs was similar to that displayed by moDCs.

As professional APC, the expression of MHCII molecules clearly separates this population from non-APC populations. During steady state conditions, DCs continuously take up antigens from their environment and present peptides via MHC molecules on their surface (Wilson *et al.*, 2004). On exposure to inflammatory or pathogenic signals, DCs increase the expression of MHCII on their surface and therefore increase their antigen-presentation capacity (Cella *et al.*, 1997; Inaba *et al.*, 2000). This was clearly observed with moDCs and ipDCs which expressed MHCII intracellularly and on their surface which was upregulated in response to inflammatory cytokines on maturation (Figure 3.5).

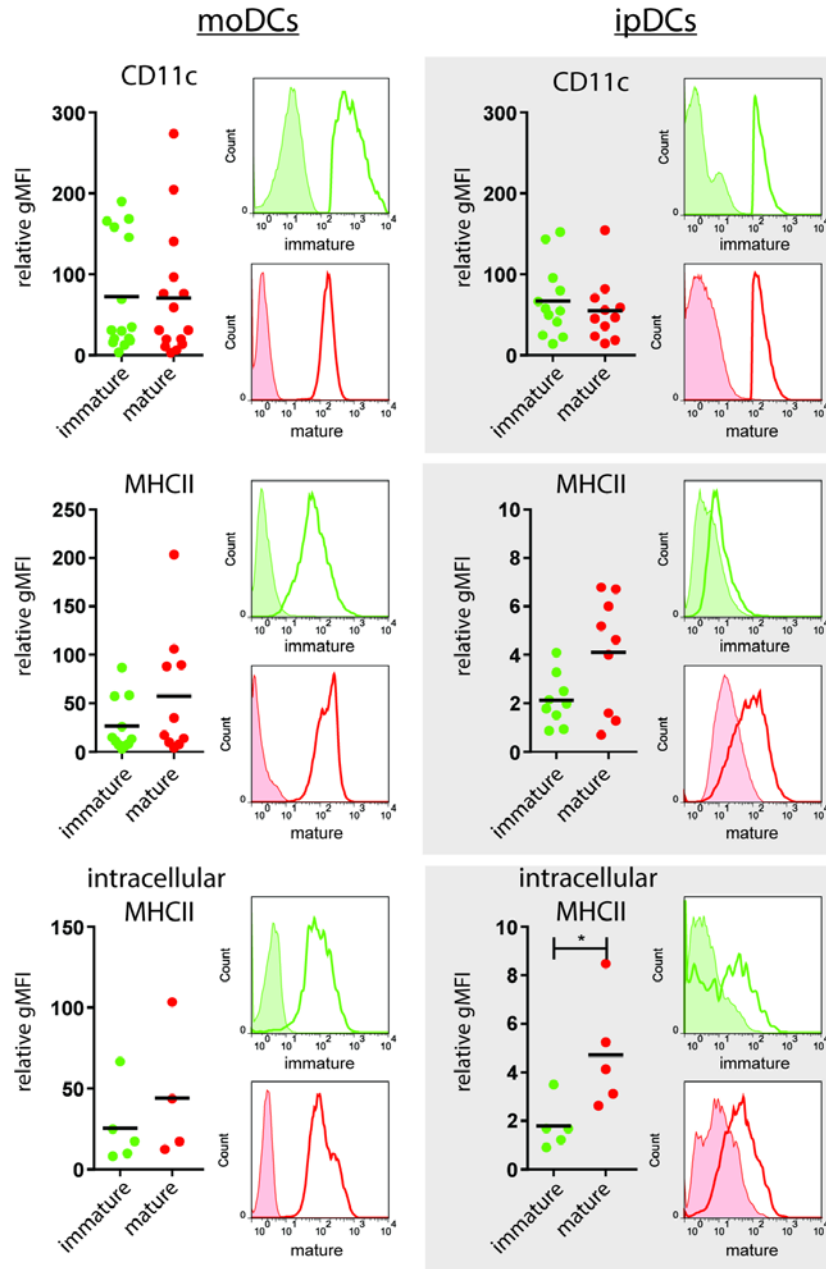


**Figure 3.5 The expression of CD11c, surface MHCII and intracellular MHCII.** Immature (green) and mature (red) ipDCs derived from C15 iPSCs at passages 15 to 26 and moDCs were stained and analysed by flow cytometry. The black lines represent the mean of the relative gMFI obtained from 5 to 15 independent experiments, which are presented as individual coloured dots. The relative gMFI was determined as the gMFI obtained from marker-specific stained samples over the gMFI obtained from samples that had been stained with isotype controls. Histograms illustrate a set of data obtained from an example experiment. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test with the exception for intracellular MHCII, which was analysed using a paired  $t$  test ( $*p \leq 0.05$ ). Data on the expression of CD11c and

MHCII were shown again as control groups in Figure 3.18 (page 198) and Figure 3.19 (page 199) of this chapter.

moDCs and ipDCs were found to express similarly low levels of CD14 and CD11b, which are highly expressed on macrophages but at lower levels on DCs (Figure 3.6) (van Wilgenburg *et al.*, 2013). The level of expression of CD11b and CD14 by ipDCs was similar to control moDCs.

CD123 was expressed at similar levels on moDCs and ipDCs, indicating that ipDCs may phenotypically resemble conventional rather than pDCs.

The expression of CD207, also known as Langerin, which is a marker of Langerhans cells in the epidermis and mucosa, was not detected on moDCs, however on ipDCs weak staining was detected above the background staining detected on controls (Figure 3.7) (Valladeau *et al.*, 2002).

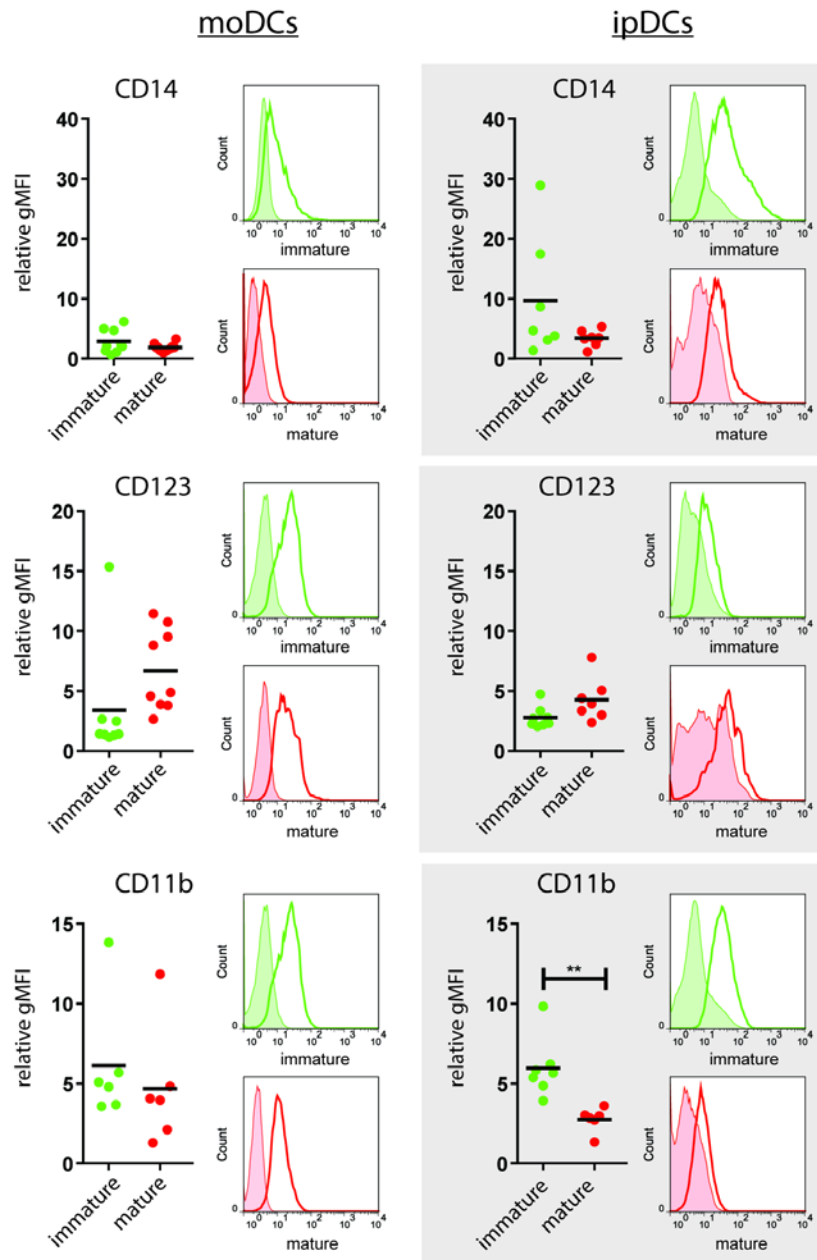


**Figure 3.6 The expression of CD14, CD123 and CD11b.** Immature DCs are displayed in green while mature DCs are displayed in red. ipDCs were differentiated from C15 iPSCs at passages ranging from passage 15 to passage 26. Alongside ipDCs, control moDCs were stained for the expression of CD14, CD123 and CD11b and analysed by flow cytometry. The black lines represent the mean of the relative gMFI as obtained from 3 to 9 independent experiments. Independent experiments are represented by individual coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test ( $**p \leq 0.01$ ). Data on the expression of CD11c and MHCII were shown again as control groups in Figure 3.20 (page 200), Figure 3.21 (page 201) and Figure 3.22 (page 202) of this chapter.

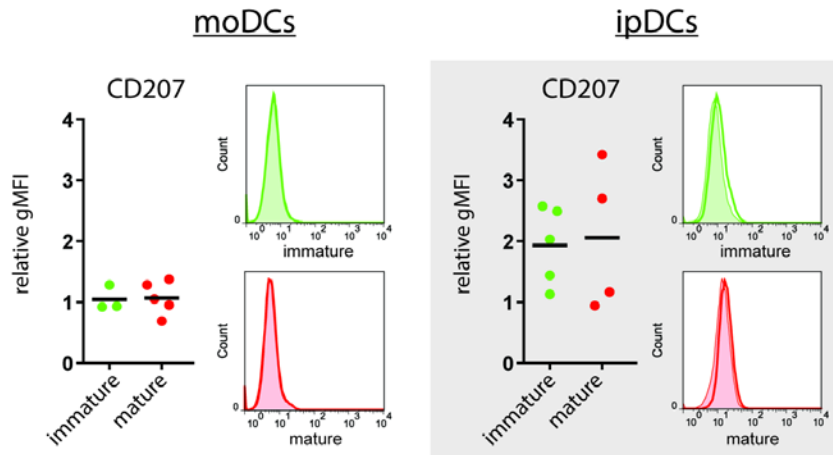


**Figure 3.7 The expression of CD207.** moDCs and ipDCs were left immature (green) or matured (red) and harvested at the end of the differentiation. Differentiations had been set up with C15 iPSCs at passages 20 to 26. Cells were stained for the expression of CD207. The mean of the gMFI is presented as black lines. The mean was determined by 2 to 5 independent experiments which are presented as coloured dots. Histograms illustrate a set of data obtained from an example experiment. Statistical analysis was performed using the paired *t* test. No statistical significance was detected.

The bimodal expression profile of CD1a on moDCs may indicate the presence of two different moDC subsets, CD1a<sup>+</sup> and CD1a<sup>-</sup>, as previously described in the literature (Figure 3.8) (Cernadas *et al.*, 2009; Chang *et al.*, 2000). In contrast, the majority of ipDCs appeared negative for CD1a, indicating the preferential differentiation of the CD1a<sup>-</sup> subset from iPSCs. The expression of CD209, also known as DC-SIGN, is known to play an important role in DC-T cell interaction by binding ICAM-3 on T cells and enabling close binding between these two cells (Geijtenbeek *et al.*, 2000b). Furthermore, it plays an essential role in the transmigration of DCs across the endothelium (Geijtenbeek *et al.*, 2000a). Therefore, CD209 is an important molecule which was found to be expressed on immature and mature moDCs and immature ipDCs. Surprisingly, mature ipDCs lacked any expression of CD209 (Figure 3.8).

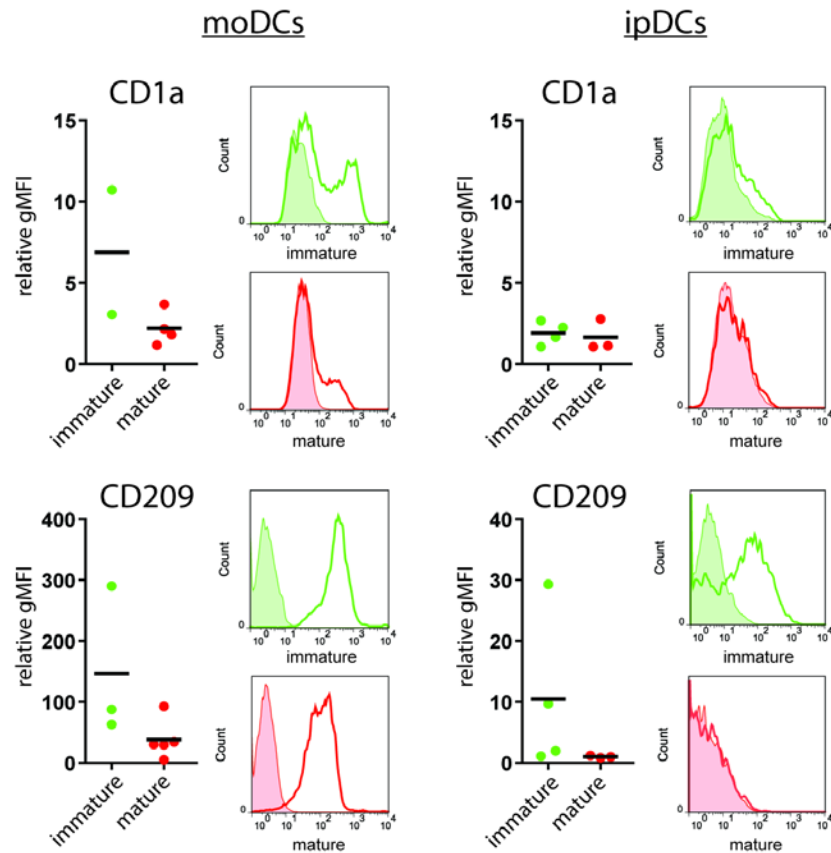


**Figure 3.8 The expression of CD1a and CD209.** Immature and mature moDCs and ipDCs were harvested, stained for the expression of CD1a and CD209 and analysed by flow cytometry. C15 iPSCs were differentiated at passages 22 to 26. The gMFI obtained from 2 to 5 independent experiments are shown as coloured dots. The mean of all gMFI is presented as black lines. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the paired *t* test. No statistical significance was detected.

### 3.4.2 Chemokine receptor for homing to the lymph nodes

For the initiation of immune responses, DCs require the capacity to migrate from peripheral tissues to the draining lymph nodes where they interact with antigen-naïve T cells. Chemokine receptor 7 (CCR7) is a well-characterised lymph node-specific homing receptor that binds the chemokines CCL19 and CCL21 and

induces the migration of cells expressing it along a concentration gradient (Ohl *et al.*, 2004; Sallusto *et al.*, 2000). The expression of CCR7 was detected on average on all DC populations (Figure 3.9).

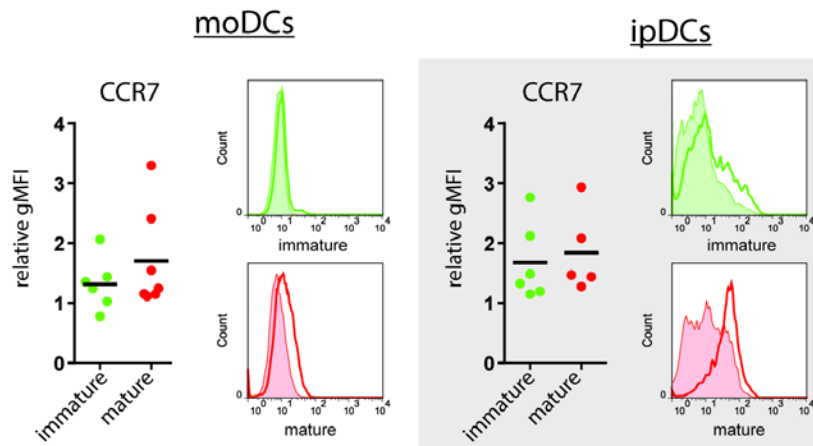


**Figure 3.9 The expression of CCR7.** Immature (green) and mature (red) C15 ipDCs (differentiated from iPSC passages 15 to 26) and moDCs were harvested from cultures, stained for the expression of CCR7 and analysed by flow cytometry. The black lines represent the mean of the relative gMFI obtained from 5 to 7 independent experiments, which are displayed as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the paired *t* test. No statistical significance was detected.

### 3.4.3 Toll-like receptor expression profile

To further characterise ipDCs and their likely responsiveness to environmental stimuli, the TLR expression profile was investigated.

TLR2 and TLR4 were not detected on mature moDCs while low levels were detected on immature moDCs and immature and mature ipDCs (Figure 3.10).

TLR3 was detected on all populations of DCs (Figure 3.10).

TLR7 and TLR8 were detected on moDCs and on ipDCs (Figure 3.11). Immature moDCs showed low levels of TLR9 expression while mature moDCs appeared



negative (Figure 3.11). Furthermore, immature and mature ipDCs both appeared to express TLR9 at levels higher than detected on moDCs.

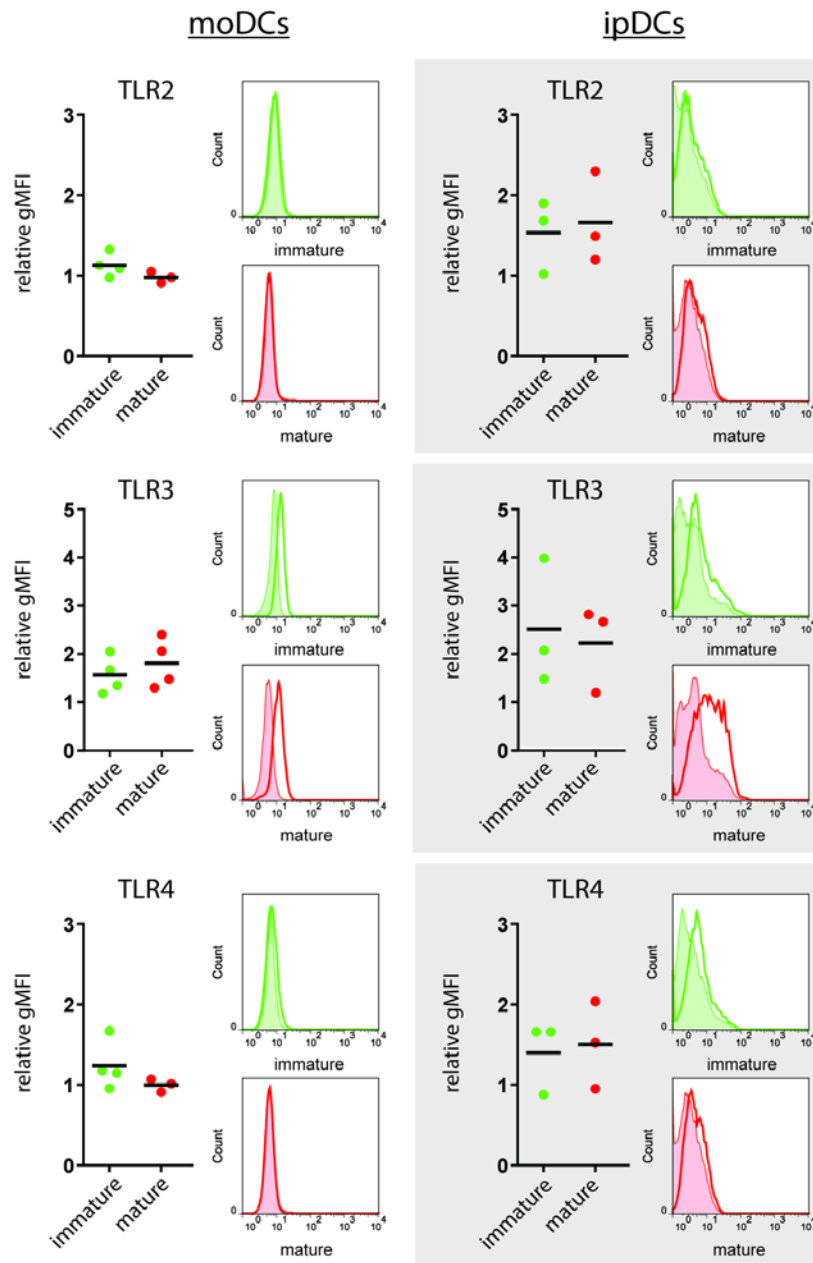


**Figure 3.10 The expression of TLR2, TLR3, and TLR4.** Immature DCs (green) and mature DCs (red) derived from blood monocytes or C15 iPSCs at passages 15 to 22 were stained and analysed by flow cytometry. The black lines represent the mean of the relative gMFI which was obtained from 3 to 5 independent experiments each of which are represented by coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the paired *t* test. No statistical significance was detected.

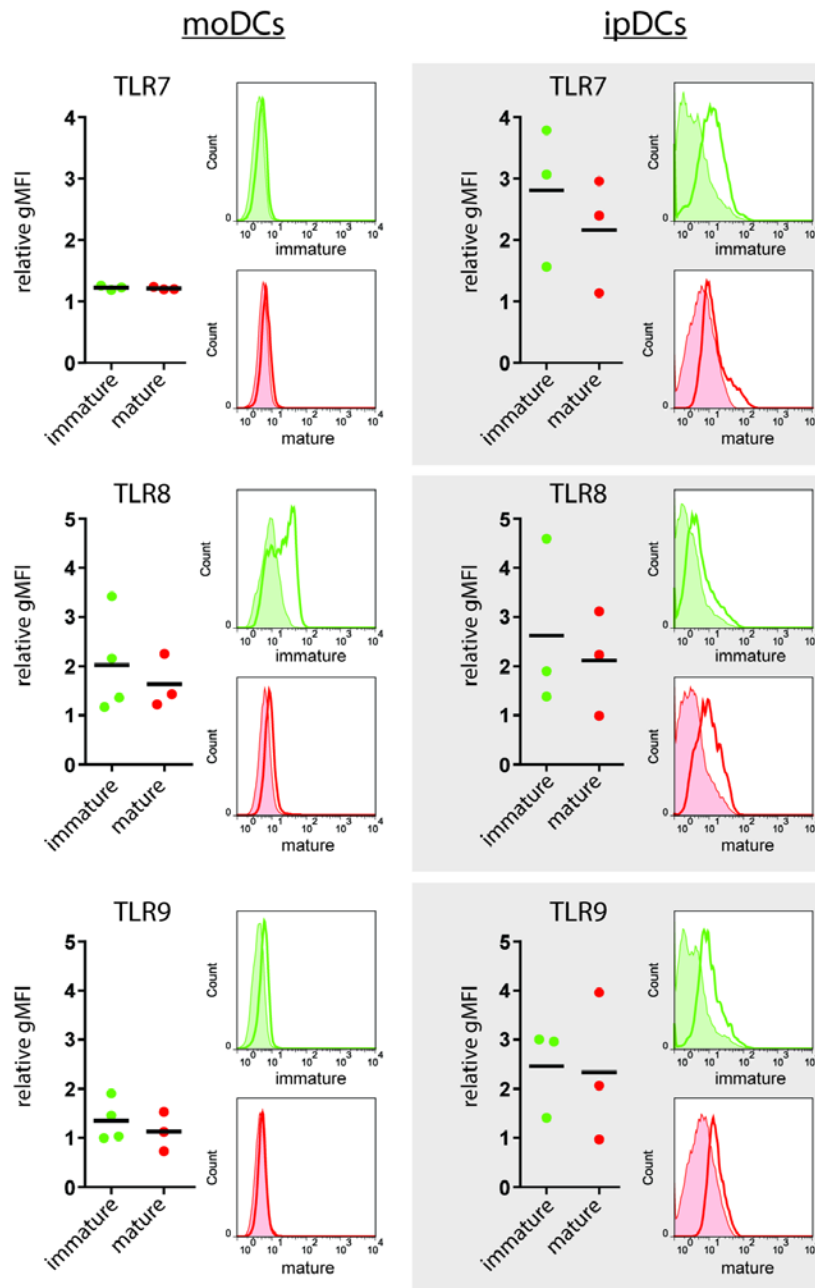


**Figure 3.11 The expression of TLR7, TLR8, and TLR9.** Immature (green) and mature (red) ipDCs and moDCs were harvested from cultures and stained for the expression of TLR7, TLR8 and TLR9 followed by analyses by flow cytometry. ipDCs were derived from C15 iPSCs at passages 15 to 22. The mean of the relative gMFI is presented as black lines which was obtained from 3 to 5 independent experiments. Independent experiments are presented as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the paired *t* test. No statistical significance was detected.

### 3.4.4 Generation of cross-presenting populations of DCs

Previous work in our laboratory has shown that this DC differentiation protocol gives rise to populations of cross-presenting DCs (Silk *et al.*, 2012a). These have been characterised by their expression of CD141 and XCR1 (Bachem *et al.*, 2010; Dorner *et al.*, 2009). The average level of XCR1 expression detected on ipDCs was higher as compared to moDCs and histograms of XCR1 expression by ipDCs indicated the presence of a distinct XCR1<sup>+</sup> subpopulation (Figure 3.12).

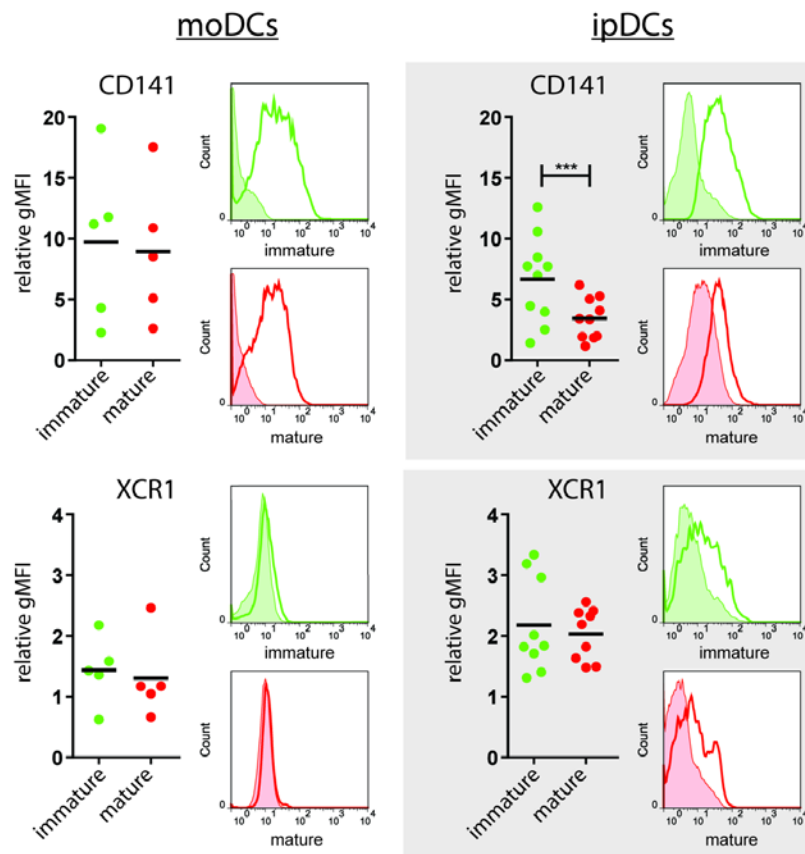


**Figure 3.12 The expression of cross-presentation markers.** Immature (green) and mature (red) ipDCs differentiated from C15 iPSCs at passages 15 to 26 and moDCs were stained for the expression of cross-presentation markers XCR1 and CD141 and analysed by flow cytometry. The black lines represent the mean of the relative gMFI obtained from 5 to 10 independent experiments, which are displayed as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the paired *t* test ( $***p \leq 0.005$ ).

This confirmed that ipDCs provide a better source for cross-presenting cells than blood monocytes (Leishman and Fairchild, 2014; Silk *et al.*, 2012a).

Since phenotypically, ipDC displayed markers characteristic of DCs, their functional properties were next compared to those of moDCs.

### **3.5 The capacity of ipDCs to phagocytose and process antigen**

Next to macrophages and neutrophils, DCs are one of the most phagocytic cells in the human body (Inaba *et al.*, 1993; Savina and Amigorena, 2007; Silva, 2010; Silva and Correia-Neves, 2012; Svensson *et al.*, 1997). Furthermore, their identification as APCs necessitates the capacity for antigen processing and presentation (Savina and Amigorena, 2007; Svensson *et al.*, 1997). Therefore, the capacity for phagocytosis and antigen processing by ipDCs was investigated. As illustrated in Figure 3.13A, moDCs and ipDCs were similar in their capacity for phagocytosis as measured by the uptake of fluorescently-labelled beads of 2  $\mu$ m diameter. Up to 24% of immature CD11c<sup>+</sup> ipDCs had internalized one or more beads over a 3 hour period. Similarly, immature and mature moDCs cultures contained around 20% of CD11c<sup>+</sup> cells with phagocytic capacity while mature CD11c<sup>+</sup> ipDCs contained around 17%. These results could not be attributed to the non-specific association of beads to the cell surface since control cultures of fixed DCs showed considerably low FITC-staining.

Furthermore, for the detection of protein processing, DQ-ovalbumin provided an easy method, as the proteolytic degradation of DQ-ovalbumin generates bright green fluorescence which is detectable by flow cytometry (Garnier *et al.*, 2007;

Wahid *et al.*, 2005). This assay represented the most appropriate readout given the difficulty of obtaining haplotype-matched T cell clones for the detection of antigen-presentation on the cell surface.

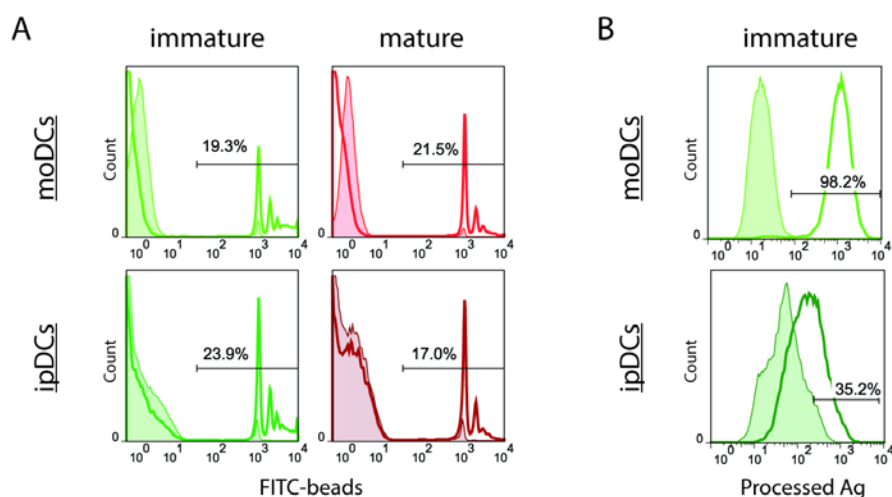


**Figure 3.13 The capacity of ipDC to phagocytose beads and process exogenous protein.** (A) Immature and mature moDCs and C15 ipDCs ( $6 \times 10^5$ ) were incubated with 2  $\mu$ m FITC-labelled beads ( $4.6 \times 10^6$ ) for 3 hours at 37 °C in order to measure their phagocytic capacity (clear, thick lined histograms). The number of cells plated was based on the total number of cells obtained from differentiations rather than the proportion of CD11c-expressing cells. Cells fixed with 0.5% paraformaldehyde were used as controls for non-specific bead adherence and are displayed as tinted histograms. After several washing steps and staining for CD11c, cells were analysed by flow cytometry. Data displayed were gated on CD11c-expressing cells. Data presented are an example of 1 out of 3 independent experiments. ipDCs used in these experiments were derived from iPSCs at passages 13 to 26. (B) Immature moDCs and ipDCs were incubated with 100  $\mu$ g/ml of DQ-ovalbumin for 30 minutes at 37 °C (clear thick lined histograms). The number of cells plated was based on the total number of cells obtained from differentiations rather than the proportion of CD11c-expressing cells. Controls were incubated on ice in order to account for non-specific degradation and fluorescence (tinted histograms). Following several washing steps and CD11c staining, cells were analysed by flow cytometry. Data presented were gated onto CD11c-expressing cells. Data represent a single experiment using moDCs and a representative set of data of 3 independent experiments using C15 ipDCs differentiated from C15 iPSC at passages 21 to 23.

The incubation with DQ-ovalbumin allowed the detection of the product of antigen processing within over 90% of immature CD11c<sup>+</sup> moDCs and 35% of immature CD11c<sup>+</sup> ipDCs (Figure 3.13.B). Controls of moDCs and ipDCs were incubated on ice in order to account for non-specific degradation or fluorescence by slowing down metabolic processes and therefore reduce or inhibit any antigen processing. The lower percentage of antigen-processing immature ipDCs may be explained by the possible presence of DC precursors that have not fully acquired DC-like functional capacity.

Having demonstrated the capacity of ipDCs to phagocytose and process soluble protein antigen, similar to moDCs, the question arose as to whether ipDCs could stimulate naïve allogeneic T cells determined as a function of their proliferation in an MLR.

### **3.6 DCs as stimulators of T cell proliferation**

The central role of DCs in T cell stimulation can be investigated using the MLR as a useful readout of DC function (Naranjo-Gómez *et al.*, 2011; Nussenzweig and Steinman, 1980; Steinman and Witmer, 1978). The MLR is a co-culture of stimulator cells, such as the DCs, together with purified naïve allogeneic T cells. As presented in Figure 3.14, the co-culture of either ipDCs or moDCs with naïve allogeneic CD4<sup>+</sup> T cells induced T cell proliferation in a dose-dependent manner. Interestingly, immature ipDCs displayed considerably lower T cell stimulatory capacity as compared to immature moDCs while mature ipDCs induced higher T cell proliferation as compared to their matured moDC counterparts. This might indicate a more pro-tolerogenic phenotype of immature ipDCs which correlates with the lower level of MHCII expression as compared to moDCs (Figure 3.5).

However, immature and mature moDCs displayed a similar capacity to stimulate T cell proliferation. This was a regular observation, however there were exceptions where immature moDCs stimulated less T cell proliferation than mature moDCs (data not shown). This may indicate a spontaneous *in vitro* maturation of the untreated immature moDC from some donors or a level of resistance to maturation of moDCs in response to pro-inflammatory cytokines. Whether this may have been affected by the health status of the blood donor at the time of donation or whether there was a variant in the cell culture procedure could not be determined. A difference in cell kinetics and cellular interactions between different haplotype-mismatches may also account for the differences observed.

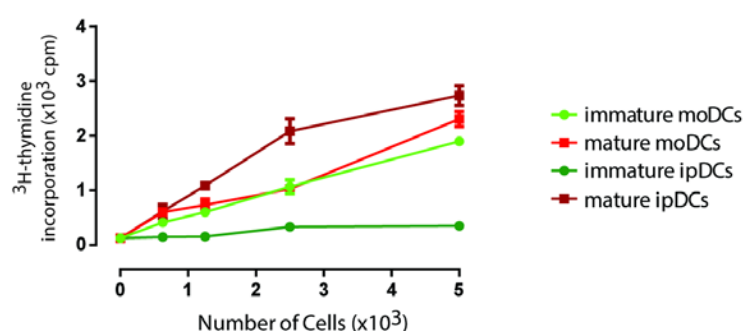


**Figure 3.14 The T cell allostimulatory capacity of ipDCs.** Immature and mature moDCs and ipDCs differentiated from C15 iPSCs at passage 16 were co-cultured at different ratios with  $2 \times 10^4$  allogeneic naïve T cells per well of a 96-well round bottom plate. The number of cells taken from DC differentiations and plated per well are shown on the x-axis. Cultures were pulsed after 48 hours with  $0.5 \mu\text{Ci/well}$  of  $^3\text{H}$ -thymidine and harvested 24 hours later. Counts per minute were recorded using a flat-bed scintillation counter. Data present the mean cpm  $\pm$  SEM of 3 technical replicates.

### 3.7 The capacity of ipDCs to affect $T_{\text{reg}}$ populations

One of the most important cells in the induction and maintenance of immunological tolerance is the  $T_{\text{reg}}$  (Sakaguchi *et al.*, 2008; Wing and Sakaguchi,

2010). T<sub>regs</sub> are generally defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells with immunosuppressive function, preventing T cell activation and proliferation by cell-cell interaction and cytokine release (Corthay, 2009; Roncarolo and Battaglia, 2007). Therefore, the capacity of ipDCs to induce or expand T<sub>regs</sub> *in vitro* was investigated.

Culturing moDCs and ipDCs together with allogeneic naïve CD4<sup>+</sup> T cells gave rise to on average 5% of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> (Figure 3.15A). Whether this proportion of T<sub>regs</sub> was maintained, increased or decreased relative to the starting population was not determined. However these data demonstrated the feasibility of detecting CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> in co-cultures which provides a useful readout for a potential pro-tolerogenic effect of modulated DCs. Furthermore, possibly due to donor-to-donor variation and MHC disparity of responding T cells, the percentage of T<sub>regs</sub> was observed to vary considerably between independent experiments. Traditionally, T<sub>reg</sub> population are described as CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells (Corthay, 2009). However, in this study, CD25 was not used to define T<sub>reg</sub> populations as T<sub>reg</sub>-mediated suppressive function has been shown to be independent of CD25 expression and CD25 is known to be upregulated on all activated CD4<sup>+</sup> T cells, including effector T cells (Fontenot *et al.*, 2005; Passerini *et al.*, 2008). Similarly, Foxp3 expression is also transiently induced in all activated CD4<sup>+</sup> T cells, but can be distinguished from Foxp3-expressed by T<sub>regs</sub> on account of the relatively lower level of expression (Allan *et al.*, 2007; Wang *et al.*, 2007). The inhibitory capacity of these T<sub>regs</sub> was also investigated by the purification of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> cells from co-cultures with mature moDCs, a standard procedure for the purification of T<sub>regs</sub>, and their addition to cultures of naïve CD4<sup>+</sup> T cells of the same T cell donor together with stimulatory beads. As



illustrated in Figure 3.15B, the presence of T<sub>regs</sub> purified from T<sub>reg</sub> induction assays with mature moDCs reduced the proliferation of responder T cells in response to stimulation as compared to the proliferation induced in their absence.

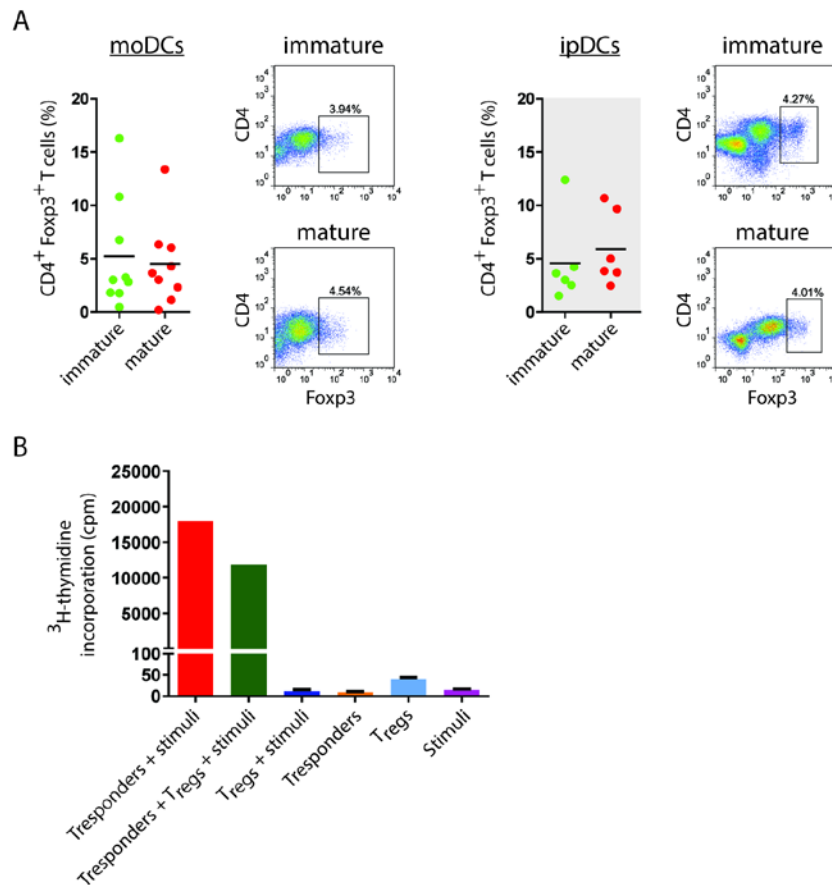


**Figure 3.15 The capacity of ipDCs to maintain a T population.** (A) Allogeneic naïve CD4<sup>+</sup> T cells ( $10^6$ ) were cultured together with  $2 \times 10^5$  moDCs or ipDCs per well of a 24-well plate. ipDCs were differentiated from C15 iPSCs at passages 17 to 26. After 5 days of co-culture, cells were treated with 75 ng/ml of IL-2. Co-cultures were harvested after 7 days and stained for CD4 and Foxp3. Black bars represent the mean percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells from independent experiments which are displayed as individual coloured dots (moDCs:  $n=9$ ; ipDCs:  $n=6$ ). Flow cytometry dot plots illustrate an example set of data obtained in these experiments using moDCs or ipDCs differentiated from passage 17 iPSCs. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected. (B) T cells and mature DCs were co-cultured as described in A. After 7 days, cultures were selectively purified for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> cells which were MMC-treated

and added at  $1.5 \times 10^3$  to  $2 \times 10^4$  naïve CD4<sup>+</sup> T cells (responder T cells) of the same T cell donor and stimulated with beads covered with anti-CD2, CD3 and CD28 antibodies. After 5 days of incubation at 37 °C, co-cultures were pulsed with <sup>3</sup>H-thymidine, harvested after 16 hours and analysed using a flat-bed scintillation counter. Controls for each cell population were included in order to account for background proliferation or radioactivity. Bar charts represent the mean counts per minute (cpm) of 2 or 3 technical replicates  $\pm$  SEM. Data on the average proportion of T<sub>regs</sub> were shown again as control groups in Figure 3.34 (page 220) of this chapter.

The ability to generate ipDCs that phenotypically and functionally resemble moDCs allows the investigation of the immunological effect of a variety of agents with known tolerogenic properties. In particular, the detection of allogeneic T cell proliferation and the induction of T<sub>regs</sub> in response to the co-culture with differently modulated DCs provide useful readouts for the impact of pharmacological agents on the tolerogenic potential of DCs.

### **3.8 Modulating the immunogenicity of ipDCs**

moDCs and ipDCs were generated as described in section 3.2. During the last 7-day differentiation period, DCs were treated with a range of different modulators. These modulators consisted of 10 ng/ml of rapamycin, 100 nM VD3, 100  $\mu$ M dexamethasone or 200 ng/ml of IL-10. As the solvent of dexamethasone was ethanol and the volume added to cultures was relatively large, moDCs and ipDCs were treated with 1% ethanol in order to account for any potential effect of the dex-vehicle. The time points of modulatory treatments were determined by a thorough review of the literature. Two days following maturation by pro-inflammatory cytokines, cells were harvested and examined.

### 3.9 Cell yield and viability

As pharmacological and cytokine-driven modulation of DCs can affect cell adhesion and viability, the number of cells recovered from cultures and the proportion of viable cells was determined. Therefore, C15 iPSCs were differentiated according to the standard protocol. After around 19-24 days, cells released from EBs in differentiation cultures, including ipMonocytes, were harvested from cultures and counted. CD14<sup>+</sup> monocytes derived from PBMCs were thawed on the same day, re-plated and further treated identically to ipMonocytes, which were re-plated for the final 7-day differentiation during which differentiating DCs were treated with dex-vehicle, rapamycin, VD3, dexamethasone or IL-10. On day 5, DCs were treated with a pro-inflammatory cytokine cocktail to induce DC maturation. All modulated DC cultures were matured on day 5. The only cultures that had not been exposed to pro-inflammatory cytokines were referred to as immature. The cell yield was described relative to the number of CD14<sup>+</sup> monocytes or ipMonocytes that had been plated at the beginning of the last 7-day differentiation period in order to determine the effect of maturation and modulatory treatment on survival and cell yield. Most tolerogenic treatments of moDC and ipDC decreased the percentage of cells recovered from cultures (Figure 3.16). The percentage of cells recovered from moDC cultures was generally reduced due to the thawing procedure at the beginning of the 7-day differentiation which is known to cause a high proportion of cell death (Ramachandran *et al.*, 2012; Yokoyama *et al.*, 2001). This reduced viability was not reflected in the data on cell viability as dead cells and apoptotic bodies are actively phagocytosed by viable DCs in the culture and are, therefore,

cleared within the 7 day culture period (Inaba *et al.*, 1993; Savina and Amigorena, 2007).

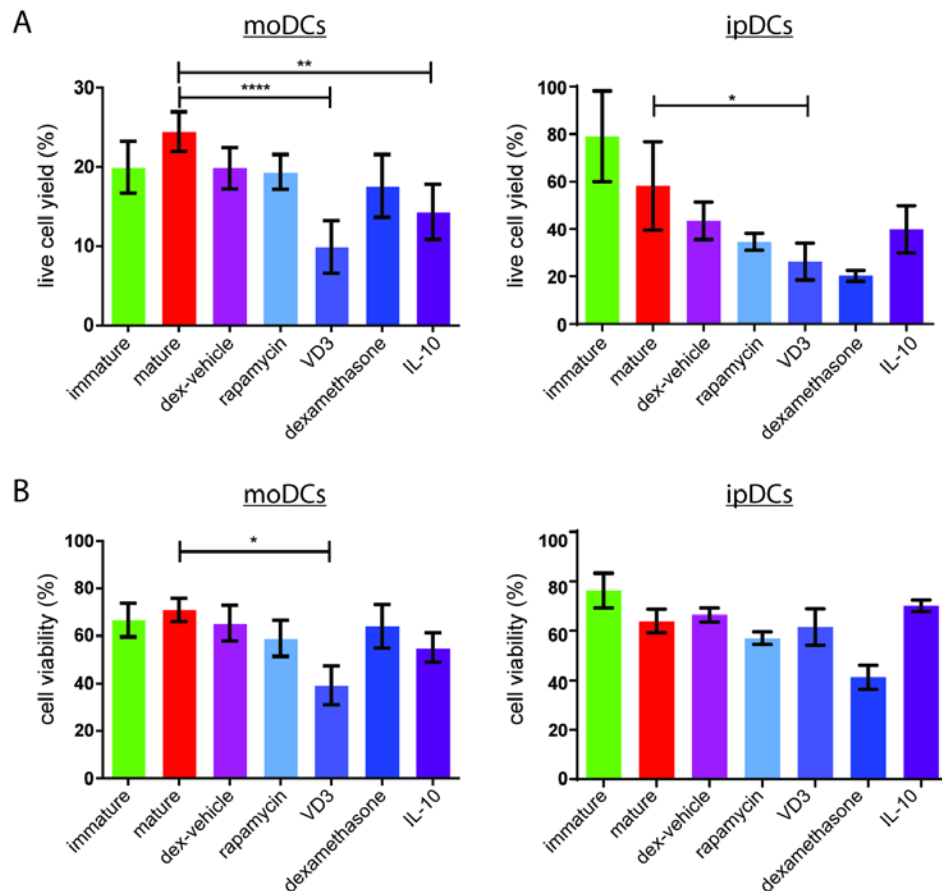


**Figure 3.16 The recovery and viability of modulated moDCs and ipDCs.** moDCs and C15 ipDCs were left untreated, matured or treated with different modulators and matured. (A) Data display the effect of maturation and modulation on recovery of moDCs and ipDCs, as measured by the number of live cells harvested relative to the number of cells that had been plated at the beginning of the 7-day differentiation. ipDC were differentiated from C15 iPSCs at passage 17 to 26 (moDCs:  $n=9$ ; ipDCs:  $n=5$ ). (B) Data display the effect of maturation and pro-tolerogenic manipulation on cell viability as determined as a percentage of live cells per total number of live and dead cells obtained from cultures as measured by trypan blue exclusion. C15 iPSC were differentiated at passages 21 to 26 into ipDCs (moDCs:  $n=8$  in all groups except immature and mature in which  $n=9$ ; ipDCs: all  $n=3$  except immature  $n=5$ ). Bars represent the mean recovery or viability  $\pm$  SED. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\*\* $p \leq 0.001$ ).

A statistically significant reduction in the percentage of cell yield was observed in matured moDC cultures that had been treated with VD3 or IL-10 as compared to untreated matured moDCs (mature:  $24.45 \pm 2.51\%$  ( $n=9$ ), VD3:  $9.92 \pm 3.36\%$  ( $n=9$ ),  $p < 0.0001$  and IL-10:  $14.33 \pm 3.49\%$  ( $n=9$ ),  $p = 0.0034$ ) (Figure 3.16A). Similarly, the VD3-treatment of ipDCs significantly reduced the cell yield as compared to untreated mature ipDC cultures (mature:  $58.21 \pm 18.66\%$  ( $n=6$ ), VD3:  $26.35 \pm 7.77\%$  ( $n=6$ ),  $p = 0.041$ ).

The viability of ipDCs was decreased to around 40% viability in dexamethasone-treated cultures, although this was not statistically significant, while dexamethasone-treated moDCs showed an average viability similar to untreated immature and mature moDCs (Figure 3.16B). The treatment of moDCs with VD3 significantly reduced the cell viability as compared to untreated matured moDCs (mature:  $71.02 \pm 4.94\%$  ( $n=9$ ), VD3:  $39.28 \pm 8.15\%$  ( $n=8$ ),  $p = 0.0175$ ), however this was not observed in ipDC cultures treated with VD3.

Altogether, VD3 appears to have the most striking effect on moDC and ipDC yield and moDC viability. These results correlate with data obtained by a study looking at the generation of clinical grade human tolerogenic DCs, where VD3-treatment significantly reduced the survival and yield of so-called tolDCs (Naranjo-Gómez *et al.*, 2011). When considering the yield of viable ipDC, IL-10 may allow the recovery of the largest proportion of viable cells as compared to the other pro-tolerogenic treatments.

### 3.10 Characterisation of modulated moDCs and ipDCs

In order to investigate whether the modulation of cells has an impact on DC phenotype, iPSCs were grown and differentiated into ipDCs and CD14<sup>+</sup> blood monocytes were differentiated into moDCs. These cells were left immature, matured or matured following modulation using rapamycin, VD3, dexamethasone or IL-10. moDCs and ipDCs were harvested after the final 7-day differentiation stage, stained for different markers and analysed by flow cytometry.

There was no statistically significant difference between the percentage of CD11c<sup>+</sup> cells generated by different treatments of either moDCs or ipDCs (Figure 3.17).

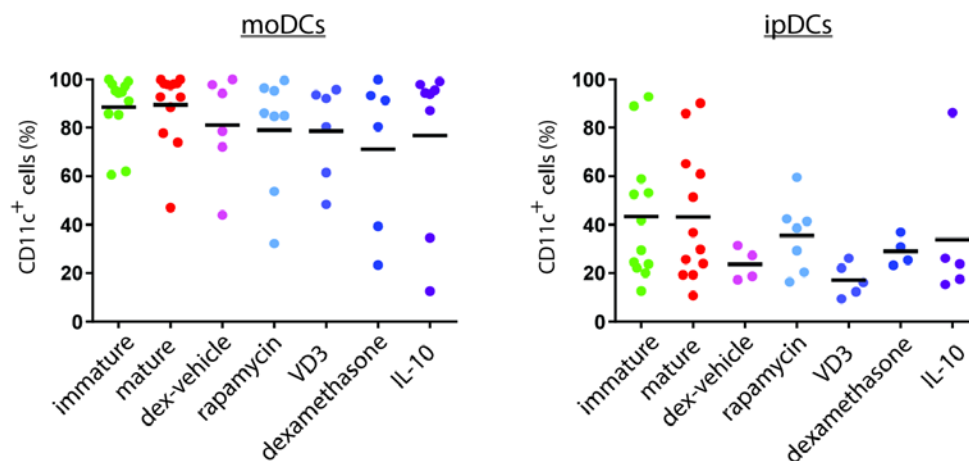


**Figure 3.17 The proportion of CD11c<sup>+</sup> cells obtained from modulated cultures.** The percentage of CD11c<sup>+</sup> cells obtained from moDC and ipDC cultures after the final 7-day differentiation was determined by the staining for CD11c expression and flow cytometric analysis. ipDCs were differentiated from C15 iPSC at passage 15 to 26. Immature and mature moDC and ipDC data were previously presented in Figure 3.4 (page 176) and are shown here as control samples. Black lines represent the mean percentage of CD11c<sup>+</sup> cell yield as determined by independent experiments which are displayed as individual coloured dots ( $n=4-13$ ). Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected ( $ns$ ,  $p > 0.05$ ).

As discussed previously, moDCs generated a purer CD11c<sup>+</sup> population as differentiations had been set up using CD14-preselected monocytes while ipDCs were generated from non-purified differentiations of pluripotent stem cells which may have given rise to cells in addition to DCs. All markers were analysed by gating on CD11c<sup>+</sup> cells in an attempt to exclude any cells that were not committed to the DC lineage.

The average level of CD11c expression was found to be slightly lower on all modulated DCs (Figure 3.18). However, none of these changes were statistically significant, indicating that modulatory treatment did not substantially alter DC differentiation in culture and the maintenance of a DC phenotype.

The expression of MHCII was upregulated by moDCs and ipDCs in response to maturation which, on average, was maintained by modulated ipDCs (Figure 3.19). The treatment of moDCs and ipDCs with modulators did not significantly change the cell surface expression of MHCII.

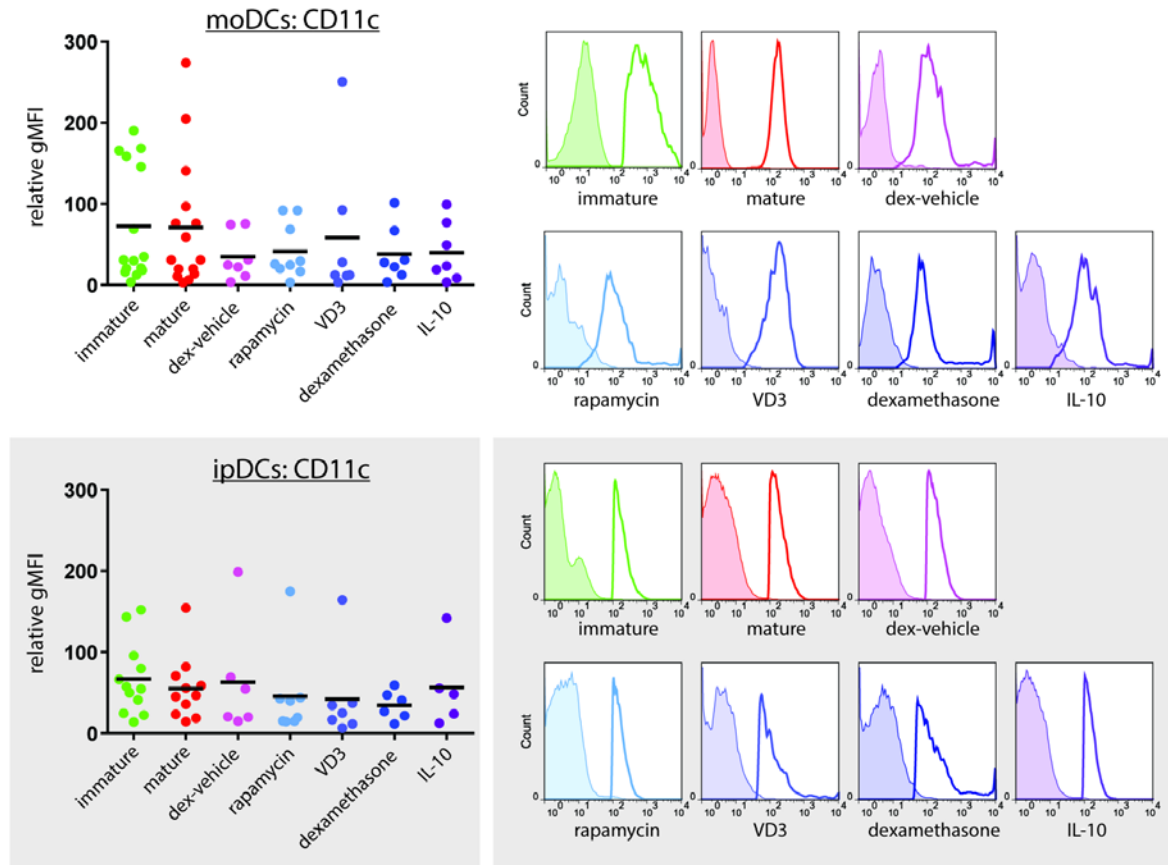


**Figure 3.18 The expression of CD11c by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs at passages 15 to 26 and moDCs were stained for the expression of CD11c and analysed by flow cytometry. Immature and mature moDC and ipDC data were previously presented in Figure 3.5 (page 178) and are shown here as control samples. The black lines in graphs displayed on the left hand side represent the mean of the relative gMFI obtained from 5 to 15 independent experiments which are presented as coloured dots. On the right hand side, histograms of example stainings are presented. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).



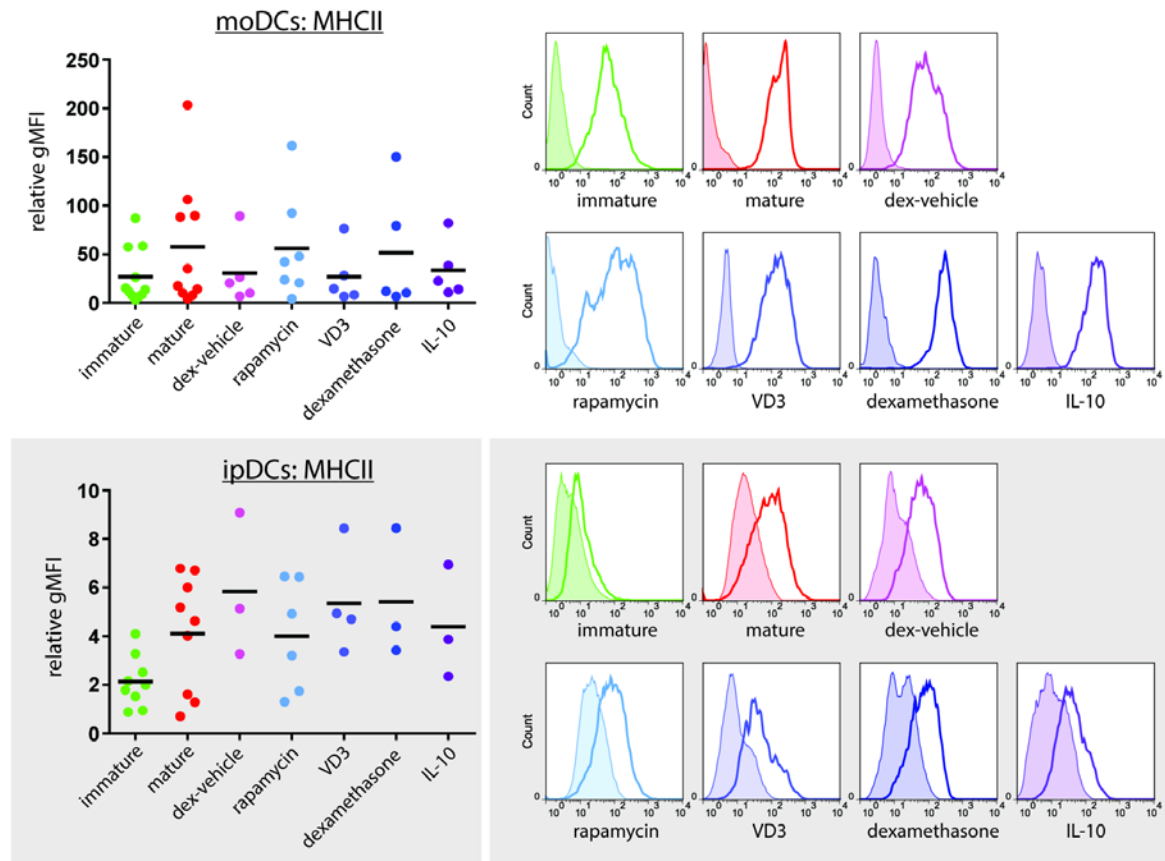


**Figure 3.19 The expression of MHCII by modulated moDCs and ipDCs.**

ipDCs and moDCs were treated with different modulatory agents and stained for the expression of MHCII and analysed by flow cytometry. ipDCs were differentiated from C15 iPSCs at passage 15 to 23. Immature and mature moDC and ipDC data were previously presented in Figure 3.5 (page 178 and are shown here as control samples. The mean of the relative gMFI obtained from 3 to 12 independent experiments is presented by the black lines. Independent experiments are presented as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).

CD14 was expressed at low levels on moDCs and ipDCs, however, VD3 treatment significantly upregulated the average expression of CD14 on moDCs and ipDCs (moDCs mature:  $1.9 \pm 0.20$  relative gMFI ( $n=10$ ), VD3:  $19.2 \pm 7.22$  relative gMFI ( $n=5$ ),  $p < 0.0001$ ; ipDCs mature:  $3.68 \pm 0.47$  relative gMFI ( $n=9$ ), VD3:  $19.14 \pm 9.55$  relative gMFI ( $n=4$ ),  $p = 0.0388$ ) (Figure 3.20).

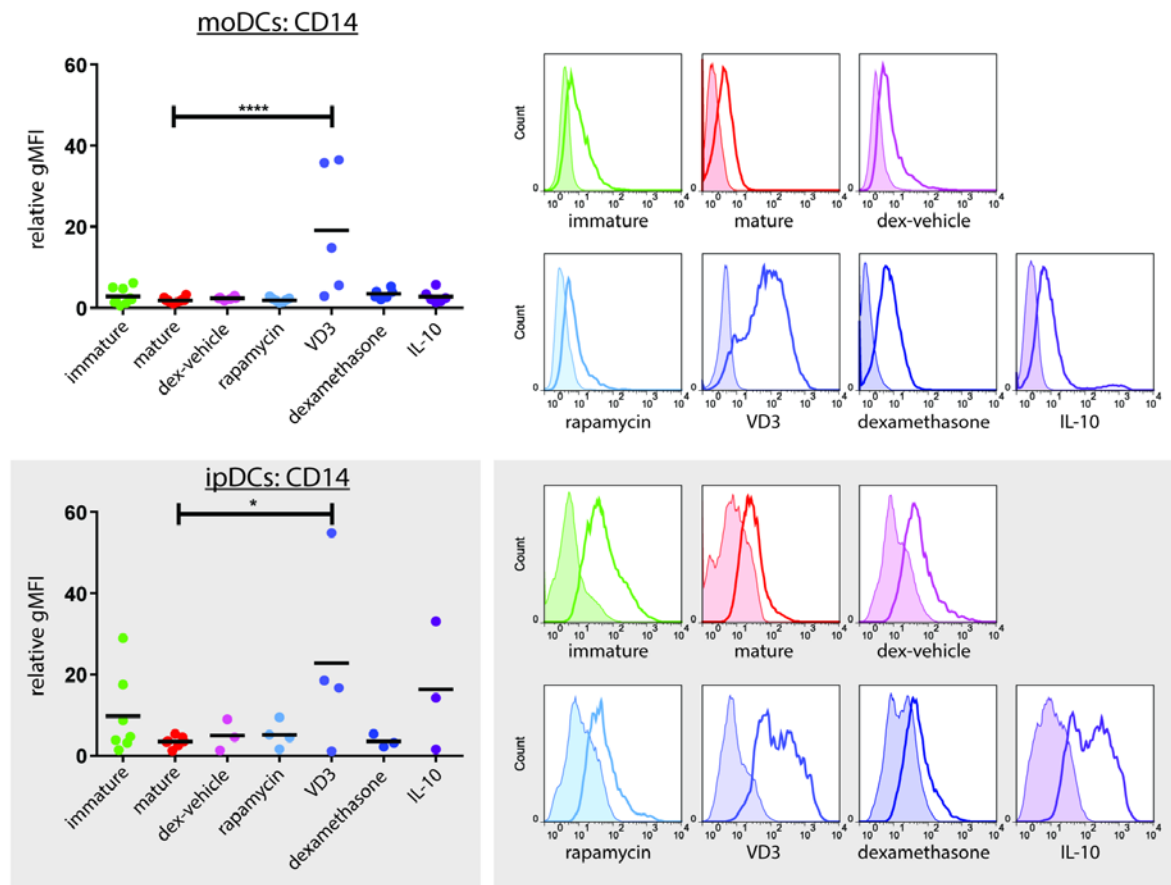


**Figure 3.20 The expression of CD14 by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs at passage 15 to 23 and moDCs were treated with different modulators and matured. Control DCs were left immature, matured or treated with 1% ethanol as a control for the vehicle used to dissolve dexamethasone and matured. DCs were harvested, stained for the expression of CD14 and analysed by flow cytometry. Immature and mature moDC and ipDC data were previously presented in Figure 3.6 (page 180) and are shown here as control samples. The black lines in graphs represent the mean of the relative gMFI obtained from 3 to 9 independent experiments which are presented as coloured dots. On the right hand side, histograms represent example data obtained from an experiment. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test ( $p \leq 0.05$ ,  $****p \leq 0.001$ ).

The average expression of CD123, the IL-3 receptor, was not significantly different between treatments (Figure 3.21). The average expression of CD11b, a myeloid marker known to be expressed on DCs, macrophages and monocytes, was similar on all populations of DCs, with the exception of immature ipDCs

where CD11b expression was significantly higher than on matured ipDCs (moDCs immature:  $5.966 \pm 0.7037$  relative gMFI ( $n=7$ ), mature:  $2.755 \pm 0.3083$  relative gMFI ( $n=6$ ),  $p = 0.0094$ ) (Figure 3.22). This may imply a more immature, developmentally young monocyte-like phenotype of the immature ipDC populations (Eischen *et al.*, 1992; Hudig *et al.*, 2014).

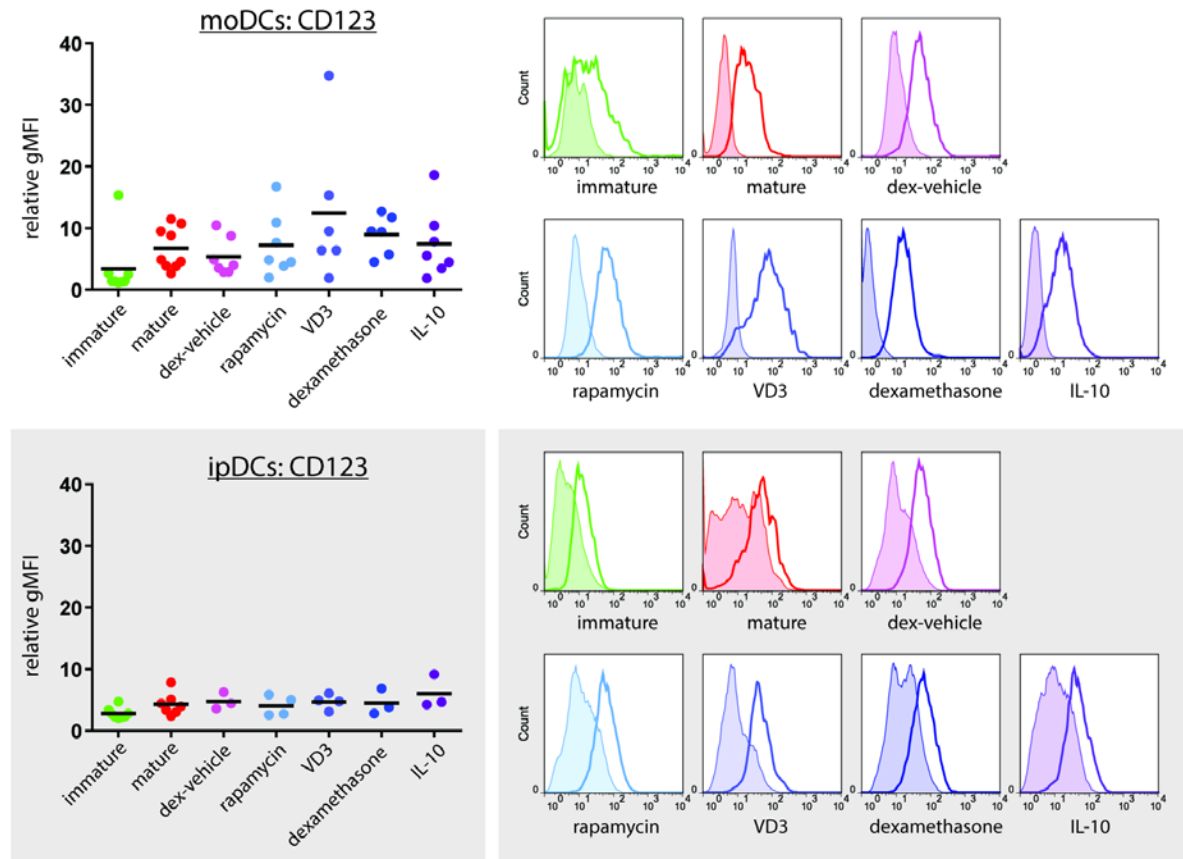


**Figure 3.21 The expression of CD123 by modulated moDCs and ipDCs.** Differentially treated ipDCs and moDCs were stained for the expression of CD123 and analysed by flow cytometry. ipDCs were differentiated from C15 iPSCs at passage 15 to 26. Immature and mature moDC and ipDC data were previously presented in Figure 3.6 (page 180) and are shown here as control samples. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI obtained from 3 to 9 independent experiments which are presented as coloured dots. On the right hand side, histograms of an example experiment are presented. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns.*  $p > 0.05$ ).

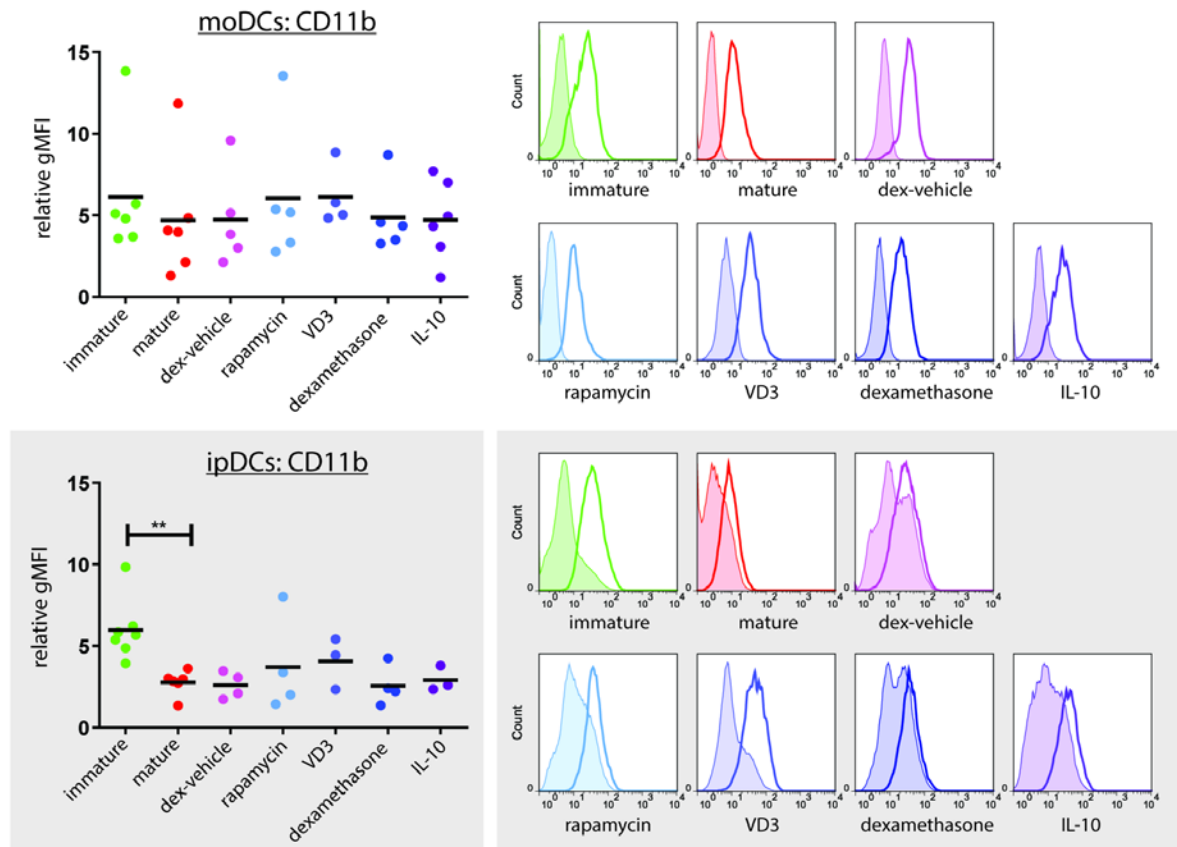


**Figure 3.22 The expression of CD11b by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs at passages 17 to 26 and moDCs were stained for the expression of CD11b and analysed by flow cytometry. Immature and mature moDC and ipDC data were previously presented in Figure 3.6 (page 180) and are shown here as control samples. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI obtained from 3 to 7 independent experiments which are presented as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\* $p \leq 0.01$ ).

The adhesion molecule CD54, also known as ICAM-1, which plays a role in the transmigration of DCs into tissues and the strengthening of cell-to-cell interactions, was expressed by all DCs cultured under different conditions at similar level (Figure 3.23) (Goval *et al.*, 2006; Hamann and Engelhardt, 2006). Research has also indicated the potential use of CD54 as a marker of APCs, further supporting the identity of ipDCs (Sheikh and Jones, 2008).

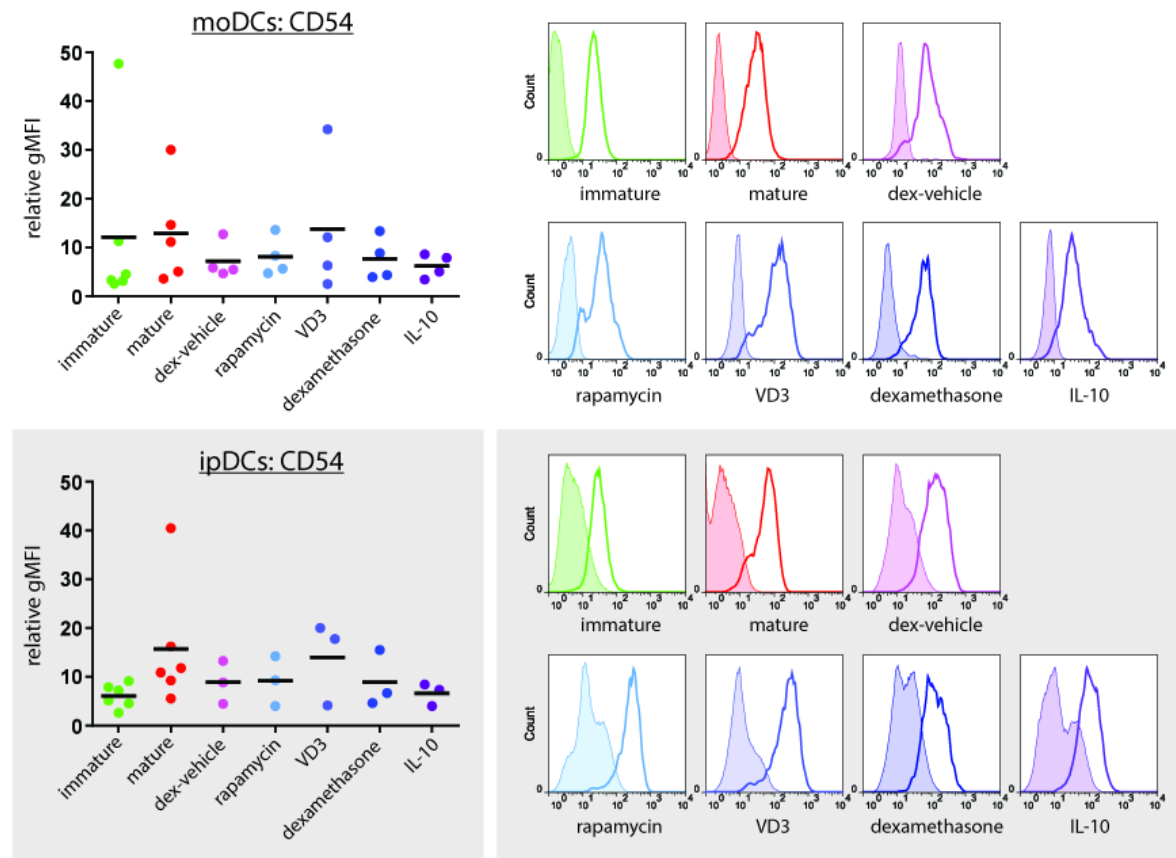


**Figure 3.23 The expression of CD54 by modulated moDCs and ipDCs.** ipDCs, differentiated from C15 iPSCs at passages 15 to 23, and moDCs were stained for the expression of CD54 and analysed by flow cytometry. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI obtained from 3-6 independent experiments which are presented as coloured dots. On the right hand side, histograms of example stainings are presented. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).

As costimulatory molecules play an essential role in the induction of immune responses, the effect of modulation on the expression of costimulatory molecules was investigated (Linsley *et al.*, 1991; Orabona *et al.*, 2004; Svajger *et al.*, 2010).

### **3.11 The expression of costimulatory molecules in response to modulation**

The expression of costimulatory molecules is essential to allow DCs to interact with T cells and stimulate immune responses. Costimulatory molecules CD80 and CD86 are known to interact with CD28 expressed by T cells and provide important survival and expansion signals by the induction of IL-2 secretion (Linsley *et al.*, 1991). Furthermore, costimulation through CD40 on DCs and CD40L on T cells is also important in the induction of T cell responses and is also thought to play a vital role in the mediation of B cell responses, indirectly via T cells and directly via CD40L expression on B cells (Ma and Clark, 2009).

The expression of costimulatory molecule CD40 was detected at similar levels on all ipDC populations (Figure 3.24). The treatment of moDCs with IL-10 or VD3 reduced the average expression of CD40 as detected by flow cytometry, however no statistical significance was achieved. Surprisingly, CD80 was detected at low levels on DCs under all conditions (Figure 3.25).

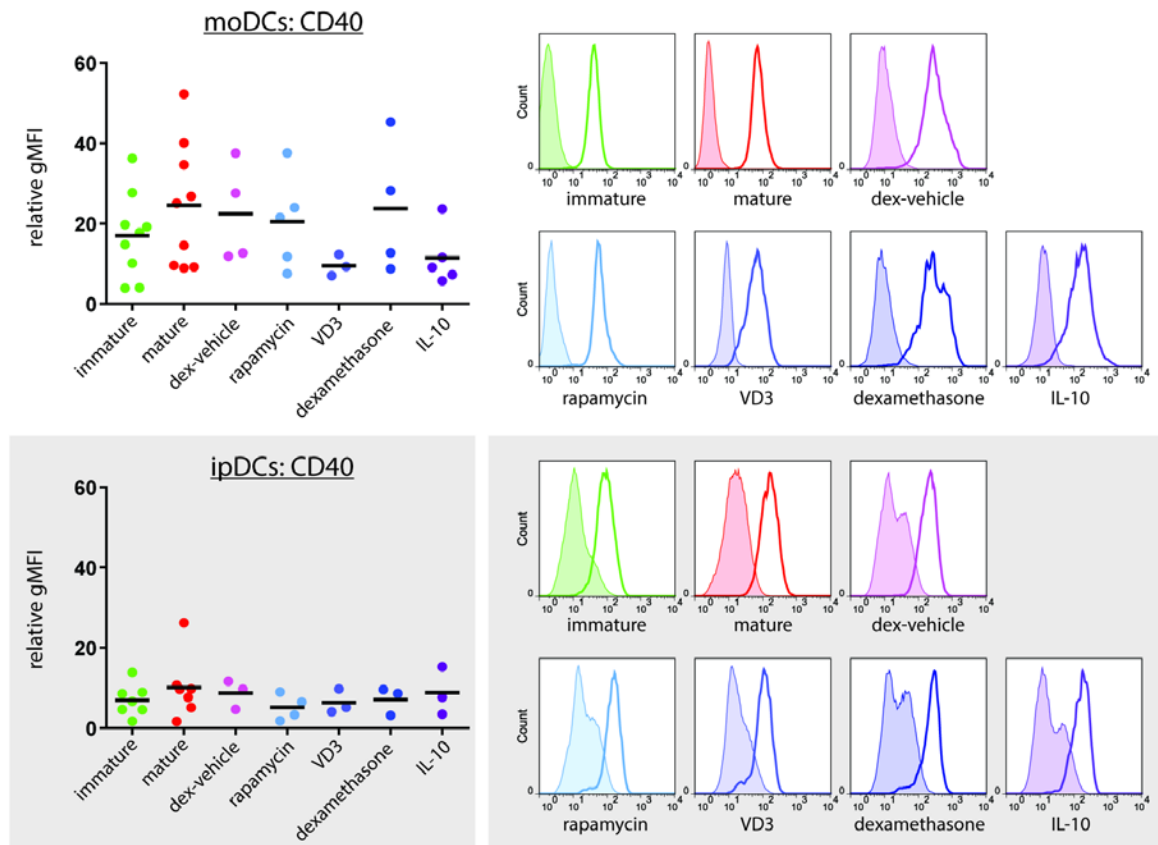


**Figure 3.24 The expression of CD40 by modulated moDCs and ipDCs.** Differentially treated ipDCs and moDCs were harvested, stained for the expression of CD40 and analysed by flow cytometry. ipDCs were differentiated from iPSCs at passages 15 to 23. The black lines in graphs represent the mean of the relative gMFI obtained from 3 to 9 independent experiments. Independent experiments are represented by individual coloured dots. On the right hand side, histograms of example stainings are presented. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).

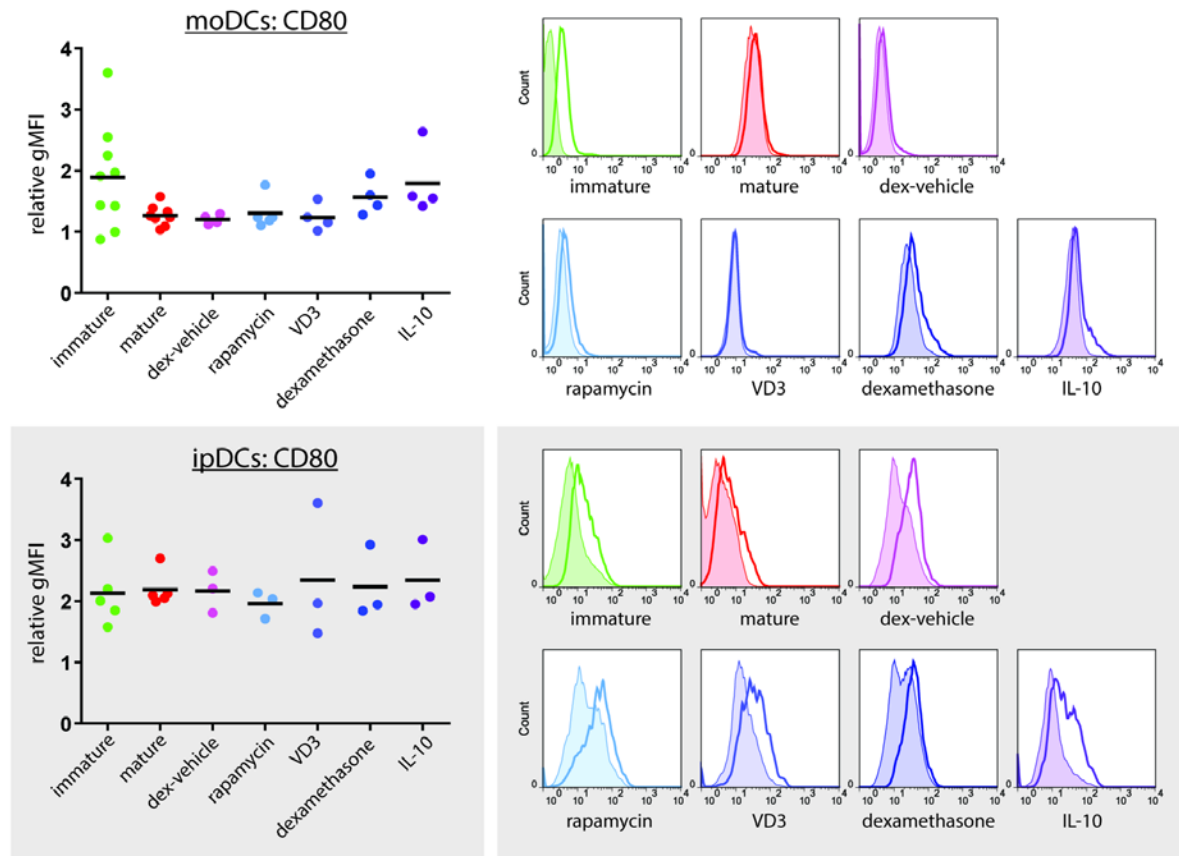


**Figure 3.25 The expression of CD80 by modulated moDCs and ipDCs.** ipDCs and moDCs were stained for the expression of CD80 and analysed by flow cytometry. ipDCs were differentiated from C15 iPSCs at passages 17 to 26. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI which was obtained from 3 to 9 independent experiments. Independent experiments are presented as coloured dots. Histograms illustrate a set of data obtained from an example staining experiment. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).

The average expression of CD86 was upregulated on moDCs and ipDCs in response to the conventional maturation stimulus (Figure 3.26). This upregulation was statistically significant (moDCs immature:  $2.899 \pm 0.469$  relative gMFI ( $n=10$ ), mature:  $37.7 \pm 5.086$  relative gMFI ( $n=9$ ),  $p = 0.0002$ ; ipDCs immature:  $4.81 \pm 0.81$  relative gMFI ( $n=11$ ), mature:  $12.64 \pm 3.58$  relative gMFI ( $n=11$ ),  $p = 0.0455$ ). In response to modulation, however, the average expression of CD86



was found to be moderately, but statistically non-significantly decreased on moDCs and ipDCs of all treatment regimes.

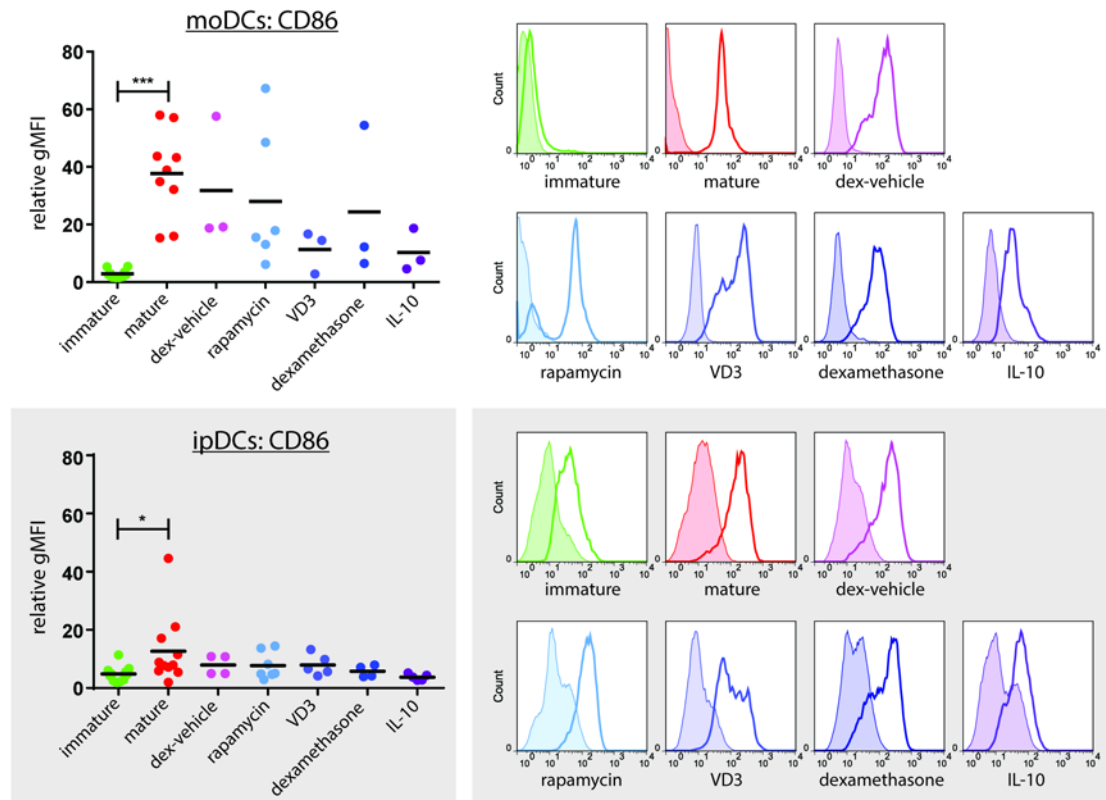


**Figure 3.26 The expression of CD86 by modulated moDCs and ipDCs.** ipDCs and moDCs were stained for the expression of CD86 after exposure to different modulators. ipDCs were differentiated from C15 iPSCs at passages 15 to 23. DCs were harvested and analysed by flow cytometry. The black lines in graphs represent the mean of the relative gMFI. The relative gMFI was obtained from 3 to 10 independent experiments which are presented as coloured dots. On the right hand side, histograms of the most representative staining are presented. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test ( $***p \leq 0.005$ ).

Apart from the reduced expression of costimulatory molecules on the surface of DCs, the increased expression of molecules with immunoinhibitory function may increase the tolerogenic capacity of DCs (Brenk *et al.*, 2009; Freeman *et al.*, 2000; Keir *et al.*, 2008; Manavalan *et al.*, 2003; Zhang *et al.*, 2006).

Consequently, the expression of inhibitory receptors was investigated in response to treatment with modulatory agents.

### **3.12 The expression of immunoinhibitory molecules in response to modulation**

Various immune inhibitory molecules have been explored for their involvement in tolerance induction. The expression profile of four molecules with known immunoinhibitory function was investigated. PD-L1, the ligand for PD-1 expressed by activated T cells, has the potential to aid the induction of T<sub>regs</sub> in the presence of low CD80/CD86 costimulation and has been shown to be essential for the suppression of T effector cells in an animal model of xenogeneic GVHD (Amarnath *et al.*, 2010; Chemnitz *et al.*, 2004). PD-L2, another ligand of PD-1, has been shown to play an important role in the induction of oral tolerance by inhibiting the activation of CD4<sup>+</sup> T cells and the secretion of cytokines such as IFN $\gamma$  and IL-2 (Zhang *et al.*, 2006). While immunoinhibitory molecule PD-L1 is expressed by a broad range of cells, including cells such as macrophages and endothelium, PD-L2 has been found to be limited to APCs (Zhong *et al.*, 2007).

In addition, the expression of members of the ILT family by DCs has been associated with an enhanced immunoinhibitory capacity, by the inhibition of T cell activation and the induction of T cell anergy (Chang *et al.*, 2002; Manavalan *et al.*, 2003; Velten *et al.*, 2004).

In this study, the average expression of PD-L1 was found to be upregulated in response to pro-inflammatory cytokines, which was statistically significant in moDC cultures (immature:  $3.15 \pm 0.54$  relative gMFI ( $n=10$ ), mature:  $12.77 \pm 2.71$  relative gMFI ( $n=10$ ),  $p = 0.0058$ ) (Figure 3.27). No significant difference was

detected in the expression of PD-L1 by ipDCs. In response to modulation, PD-L1 was detected on moDCs at an intermediate level between immature and mature DCs, however no statistically significant difference was found neither in moDCs nor in ipDCs.

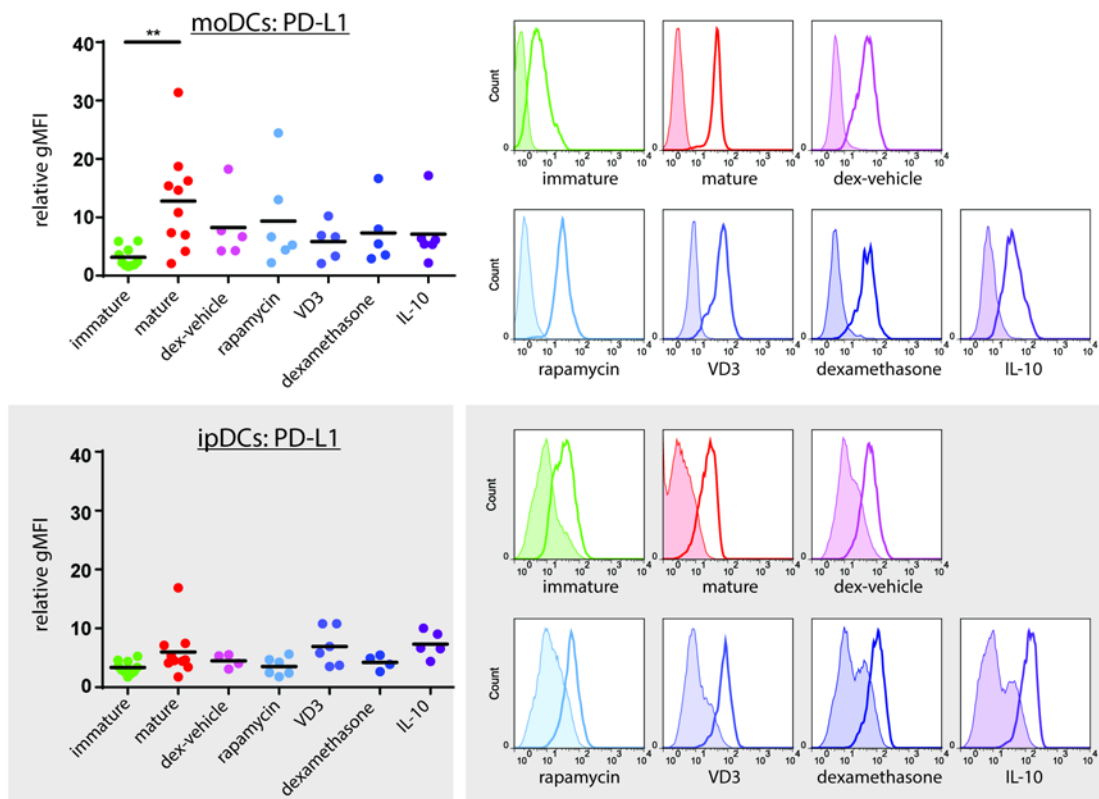


**Figure 3.27 The expression of PD-L1 by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs at passages 15 to 26 and moDCs were treated with different modulators, harvested, stained for the expression of PD-L1 and analysed by flow cytometry. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI obtained from 3 to 11 independent experiments which are presented as coloured dots. On the right hand side, histograms of an example staining are presented. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\* $p \leq 0.01$ ).

Similar to PD-L1, PD-L2, detected on modulated moDCs, was expressed at an intermediate level between immature and mature moDCs, while levels detected on ipDCs were similar between all conditions (Figure 3.28). However, apart from

the statistically-significant upregulation of PD-L2 on moDCs in response to pro-inflammatory cytokines (immature:  $1.4 \pm 0.12$  relative gMFI ( $n=6$ ), mature:  $4.23 \pm 0.77$  relative gMFI ( $n=6$ ),  $p = 0.003$ ), no statistically significant differences were detected.

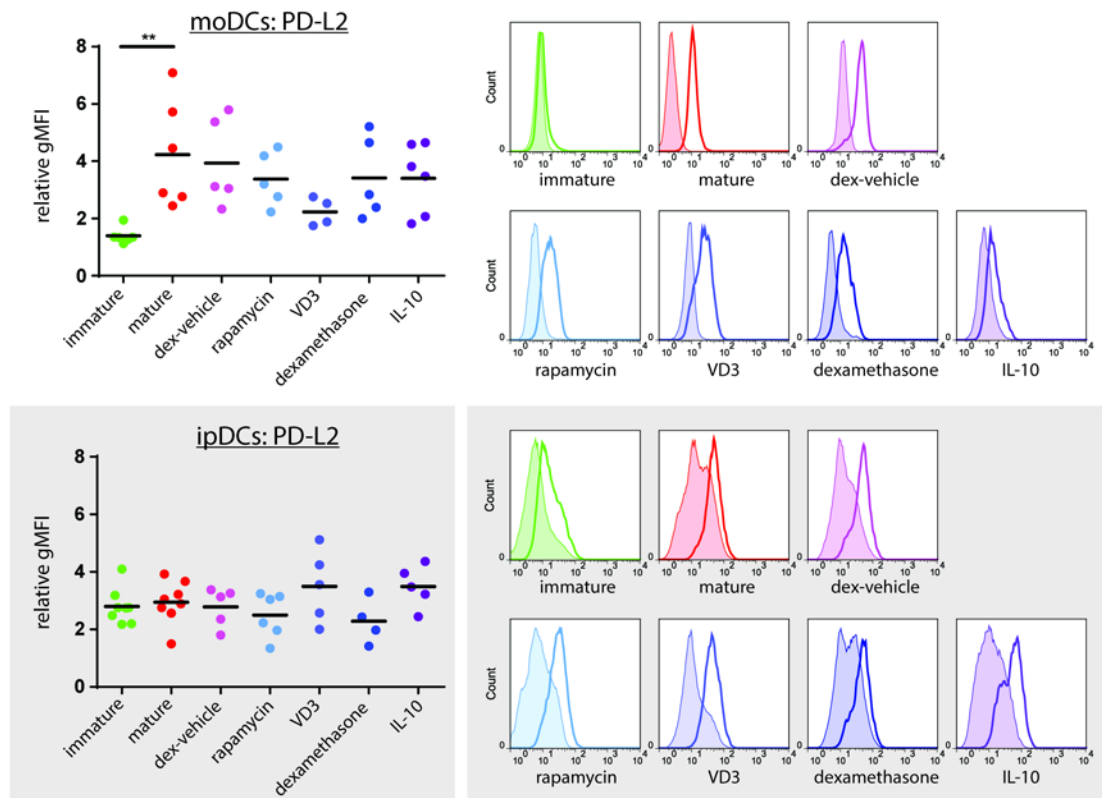


**Figure 3.28 The expression of PD-L2 by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs at passages 17 to 26 and moDCs were stained for the expression of PD-L2 and analysed by flow cytometry. The black lines in graphs represent the mean of the relative gMFI obtained from 3 to 6 independent experiments which are presented as coloured dots. Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\* $p \leq 0.01$ ).

ILT3 and ILT4 are immunoinhibitory molecules expressed on DCs, that are thought to be involved in the induction of T cell anergy and reduced T cell

stimulatory capacity (Chang *et al.*, 2002; Manavalan *et al.*, 2003; Velten *et al.*, 2004).

The expression of ILT3 detected on moDCs was found to be similar between all conditions (Figure 3.29). However, on ipDCs, the expression of ILT3 was significantly upregulated in response to IL-10-mediated modulation (mature:  $3.96 \pm 0.45$  relative gMFI ( $n=7$ ), IL-10:  $7.53 \pm 0.45$  relative gMFI ( $n=5$ ),  $p = 0.0004$ ). However, the bimodal expression profile by VD3-treated moDCs, which had also been observed with dexamethasone and IL-10-treated moDCs in other independent experiments (data not shown), may indicate the presence of a moDC population with enhanced immunoinhibitory capacity.

Similarly, the expression of ILT4 was significantly upregulated on moDCs and ipDCs treated with IL-10 (moDCs mature:  $1.80 \pm 0.18$  relative gMFI ( $n=7$ ), IL-10:  $3.17 \pm 0.51$  relative gMFI ( $n=6$ ),  $p = 0.0098$ ; ipDCs mature:  $2.3 \pm 0.31$  relative gMFI ( $n=8$ ), IL-10:  $4.81 \pm 0.44$  relative gMFI ( $n=5$ ),  $p < 0.0001$ ), while all other conditions showed a similar average expression (Figure 3.30).

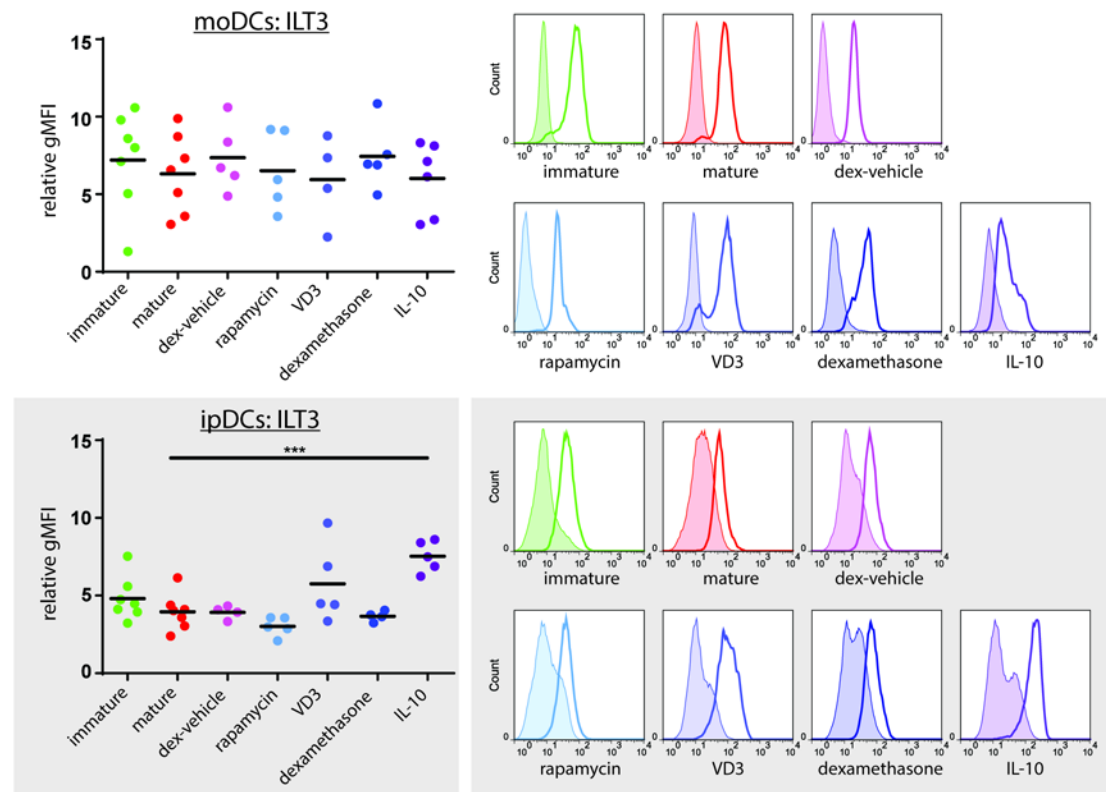


**Figure 3.29 The expression of ILT3 by modulated moDCs and ipDCs.** ipDCs and moDCs were stained for the expression of ILT3 and analysed by flow cytometry. ipDCs were differentiated from C15 iPSCs at passages 17 to 26. The black lines in graphs displayed on the left hand side present the mean of the relative gMFI obtained from 3 to 8 independent experiments which are presented as coloured dots. On the right hand side, histograms represent example stainings obtained from an experiment. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\* $p \leq 0.01$ ).

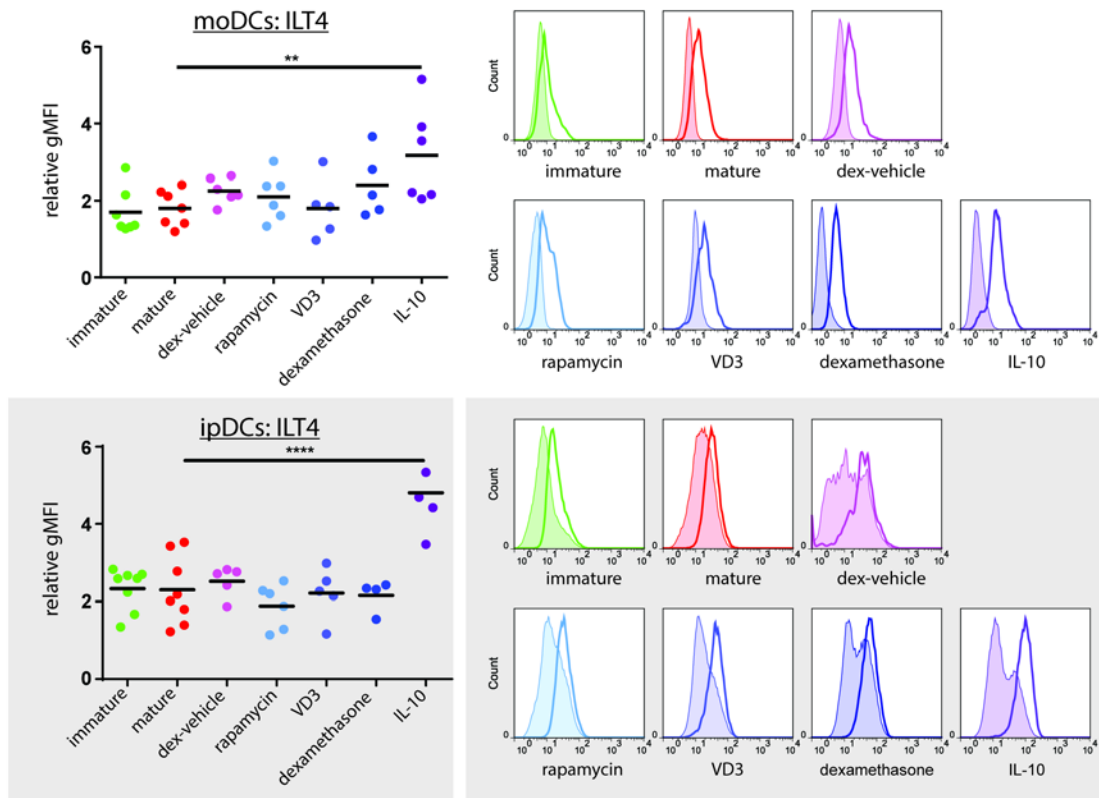


**Figure 3.30 The expression of ILT4 by modulated moDCs and ipDCs.** ipDCs differentiated from C15 iPSCs (passages 17 to 26) and moDCs were modulated with different agents, stained for the expression of ILT4 and analysed using flow cytometry. The black lines in graphs represent the mean of the relative gMFI obtained from 3 to 8 independent experiments which are represented by individual coloured dots. On the right hand side, histograms illustrate data from an example staining. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.001$ ).

Altogether, the expression profile of markers characteristic of DCs on moDCs and ipDCs subjected to different treatment regimes confirms the maintenance of a DC phenotype similar to untreated cells while the expression of costimulatory and immunoinhibitory molecules can be affected by different modulatory treatments.

### 3.13 The modulation of cytokine release by DCs

For the initiation of immune responses and tolerance induction, the release of cytokines by DCs contributes to the local microenvironment, influencing the outcome of the response. DCs secrete a variety of cytokines which are frequently referred to as the third signal in T cell priming. Cytokines may skew T cell populations towards a certain subset, further stimulating an immune response or its inhibition. Therefore, the release of cytokines by modulated moDCs and ipDCs was investigated by harvesting the supernatants from cultures at the end of the 7-day differentiation period. Supernatants were assayed by ELISA and analysed by spectrophotometer at OD<sub>450nm</sub>.

IL-6 is a pro-inflammatory cytokine that can promote the generation of Th17 and Th2 while suppressing the differentiation of naïve CD4<sup>+</sup> T cells into T<sub>regs</sub> (Dodge *et al.*, 2003; Scheller *et al.*, 2011). In this study, moDC and ipDCs were found to increase their IL-6 secretion in response to inflammatory cytokines IL-1 $\beta$ , IFN $\gamma$ , PGE<sub>2</sub> and TNF $\alpha$ , indicating successful DC maturation (Figure 3.31). ipDCs, in contrast to moDCs, were found to significantly increase the secretion of IL-6 in response to maturation (immature: 21.49  $\pm$  10.47 pg/ml ( $n=6$ ), mature: 2811  $\pm$  728.8 pg/ml ( $n=6$ ),  $p = 0.0119$ ). This may support the findings described in Figure 3.14 of an increased immunogenicity of mature ipDCs as compared to mature moDCs, although IL-6 has also been shown to confer pro-tolerogenic properties (Hegde *et al.*, 2004). Immature moDCs released low levels of IL-6 while IL-6 was not detected in cultures of immature ipDCs. Interestingly, the treatment of moDCs with VD3 caused a significant increase in IL-6 secretion as compared to mature moDCs (mature: 1084  $\pm$  265.6 pg/ml ( $n=8$ ), VD3: 2371  $\pm$  415.6 pg/ml ( $n=8$ ),  $p = 0.0013$ ) which was also observed with ipDCs, however no statistical significance



was achieved (Figure 3.31). The treatment with rapamycin, dexamethasone and IL-10 decreased the secretion of IL-6 from ipDCs, however no statistical significance was achieved.

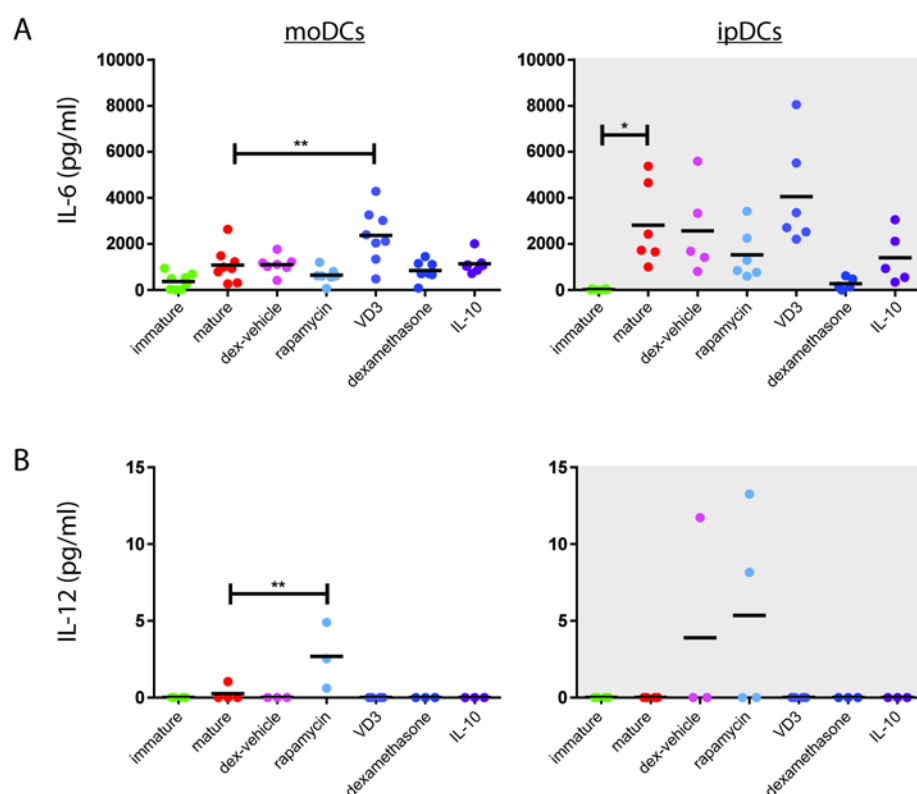


**Figure 3.31 The effect of DC modulation on the secretion of IL-6 and IL-12.** moDCs and ipDCs differentiated from C15 iPSCs at passages 17 to 26 were left untreated, matured or treated with different agents and matured. Cell culture supernatants were harvested on day 7 and stored at -80°C until analysed by ELISA for the presence of (A) IL-6 and (B) IL-12 (moDC IL-6:  $n=8$ ; ipDC IL-6:  $n=6$ ; moDC IL-12:  $n=4$ ; ipDC IL-12:  $n=4$ ). Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test versus matured cells or dex-vehicle treated cells for dexamethasone treated cells ( $*p \leq 0.05$ ,  $**p \leq 0.01$ ).

In contrast, the cytokine IL-12 was only detected in rapamycin-treated moDC and dex-vehicle and rapamycin treated ipDC culture supernatants. As IL-12 plays an important role in the stimulation of Th1 responses and rapamycin-treatment of moDCs significantly upregulated the release of IL-12 (mature:  $0.26 \pm 0.26$  pg/ml ( $n=4$ ), rapamycin:  $2.68 \pm 1.24$  pg/ml ( $n=3$ ),  $p = 0.0042$ ), this treatment regime

may prove not to be suitable for the generation of pro-tolerogenic DCs. These results were not surprising given the recent finding that the induction of IL-12 secretion by moDCs requires the initiation of maturation by TLR3 or/and TLR7/8 ligation while the maturation induced by a range of cytokines, as used in this study, were found not to be sufficient for the induction of IL-12 secretion (Boullart *et al.*, 2008; Zobywalski *et al.*, 2007). Furthermore, PGE<sub>2</sub> was found to inhibit the production of bioactive IL-12p70, which is a heterodimer of IL-12p40 and IL-12p35 subunits (Kaliński *et al.*, 2001). The inclusion of IFN $\gamma$ , TNF $\alpha$  or both together in their maturation cocktail did not alter the inability of moDCs to produce IL-12p70. Therefore, the presence of these cytokines in the maturation cocktail utilised in this study and the exclusion of TLR ligands explains the inability to detect reliable levels of IL-12p70. Therefore, the absence of IL-12 in the majority of cultures correlates with these studies showing the inability of moDCs to secrete IL-12 if stimulated solely with pro-inflammatory cytokines, unless pre-treated with rapamycin (Boullart *et al.*, 2008; Zobywalski *et al.*, 2007).

The release of IL-10 from DCs has been associated with pro-tolerogenic properties *in vivo* (Akbari *et al.*, 2001; Huang *et al.*, 2013). The average release of IL-10 from moDCs and ipDCs were similar under all conditions (Figure 3.32). However, other studies have shown the significant increase in IL-10 secretion by dexamethasone-treated and some by VD3-treated moDCs (Naranjo-Gómez *et al.*, 2011; Unger *et al.*, 2009; Xia *et al.*, 2005). The release of IL-10 from ipDCs was marginally increased in all tolerogenic treatment regimens as compared to their respective controls, however similar to moDCs, no statistical significance was achieved. IL-10-treated DCs were not analysed for the release of IL-10 as

the modulatory treatment would falsify the detection of endogenously produced and released IL-10.

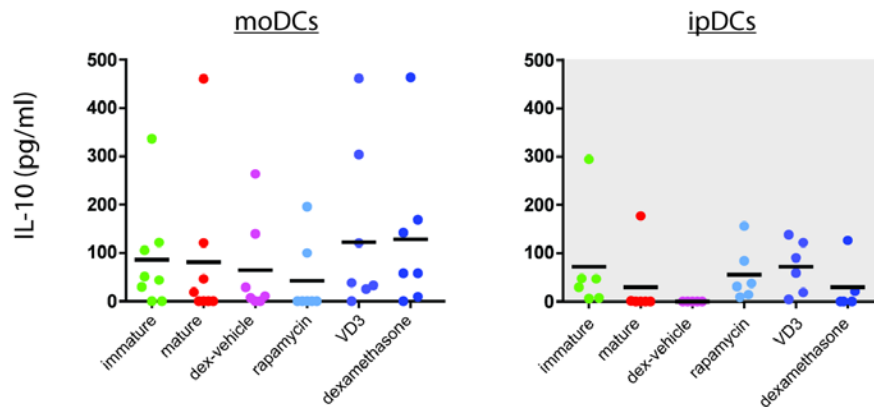


**Figure 3.32 The effect of DC modulation on the secretion of IL-10.** moDCs and ipDCs were left untreated, matured or treated with different agents prior to maturation. ipDCs were differentiated from C15 iPSCs at passages 17 and 26. Cell culture supernatants were harvested on day 7 and stored at  $-80^{\circ}\text{C}$  until analysed by ELISA for the presence of IL-10 (moDC:  $n=8$ ; ipDC:  $n=6$ ). Black lines represent the mean concentration of IL-10 as detected in independent experiments which are represented by coloured dots. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected ( $ns$ ,  $p > 0.05$ ).

The detection of various changes in cytokine secretion by DCs treated with different modulatory agents prompted a further investigation into the functional capacity of these DCs upon their interaction with naïve allogeneic T cells.

### 3.14 The allostimulatory capacity of DCs

In order to investigate the effect of tolerogenic treatments on allostimulatory capacity of DCs, allogeneic naïve  $\text{CD4}^{+}$  T cells were purified from PBMC and stained with CFSE. CFSE passively diffuses into cells, where, upon esterase-mediated cleavage, it becomes fluorescent. This fluorescence enables the detection of T cell proliferation, as with each successive cell division, the amount

of fluorescence retained by a cell is halved, which can be detected by flow cytometry.

These fluorescently-labelled naïve CD4<sup>+</sup> T cells were co-cultured with differentially treated moDCs and ipDCs. After 5 days, cells were harvested and analysed by flow cytometry for the percentage of proliferated cells as an indicator of allostimulatory capacity. Immature moDCs and ipDCs induced significantly less T cell proliferation as compared to their mature counterparts (moDCs immature:  $21.82 \pm 4.682\%$  ( $n=3$ ), mature:  $50.84 \pm 8.872\%$  ( $n=3$ ),  $p = 0.0469$ ; ipDCs immature:  $7.159 \pm 2.107\%$  ( $n=3$ ), mature:  $29.42 \pm 9.14\%$  ( $n=3$ ),  $p = 0.0236$ ).

The average allostimulatory capacity of moDCs was not affected by rapamycin treatment, but was slightly decreased if moDCs had been pre-treated with dexamethasone or IL-10 (Figure 3.33). The functional potential of moDCs treated with VD3 was significantly reduced when compared with the allostimulatory capacity of mature moDCs (moDCs mature:  $50.84 \pm 8.87\%$  ( $n=3$ ), VD3:  $10.62 \pm 5.30\%$  ( $n=3$ ),  $p = 0.0048$ ). In contrast to the data obtained from moDCs, the allostimulatory capacity of ipDCs was significantly reduced after IL-10 treatment but not VD3 treatment (ipDCs mature:  $29.42 \pm 9.14\%$  ( $n=3$ ), IL-10:  $8.357 \pm 3.093\%$  ( $n=3$ ),  $p = 0.0308$ ) (Figure 3.33B).

These data indicate that pro-tolerogenic modulation can affect the T cell allostimulatory properties of moDCs and ipDCs. However, whether reduced T cell proliferation was due to the absence of T cell priming, the induction of antigen-specific T cell anergy or apoptosis was not investigated. In order to gain further insight into the mechanism involved, it was investigated whether the proportion

of T<sub>regs</sub> within co-cultures was altered in response to exposure to modulated DCs, as T<sub>regs</sub> may be responsible for the diminished proliferative response observed (Sakaguchi *et al.*, 2008; Takahashi *et al.*, 1998; Thornton and Shevach, 1998).

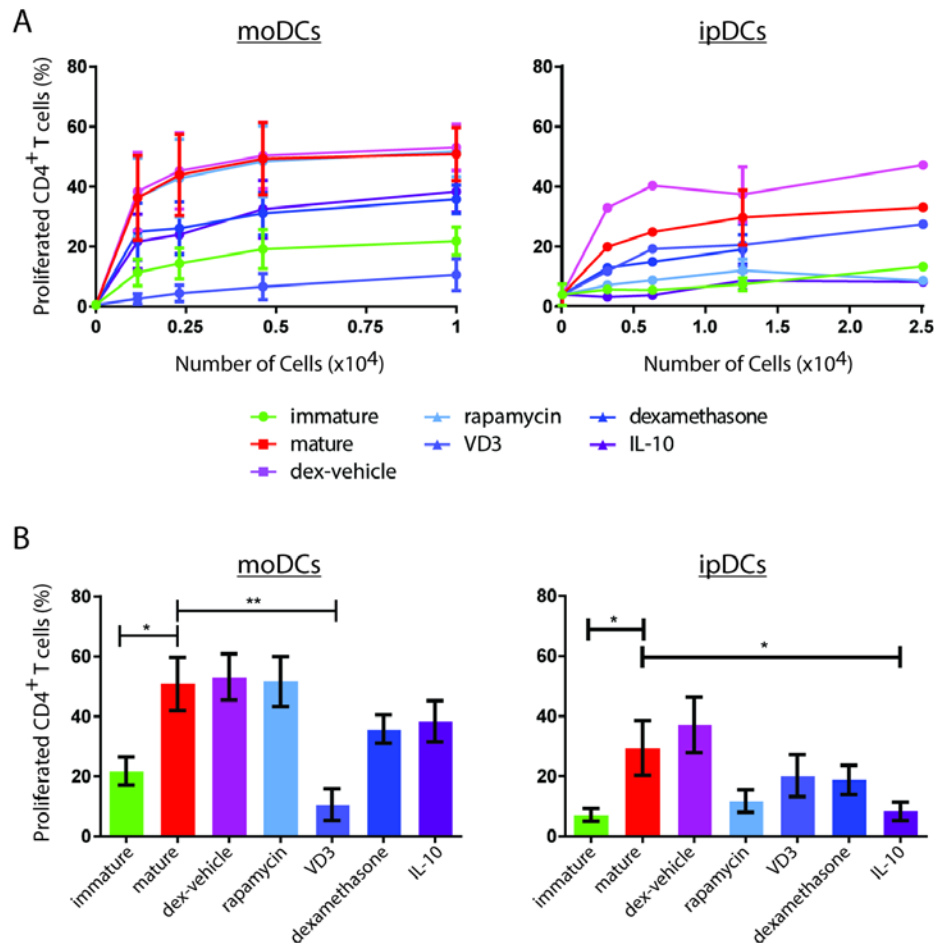


**Figure 3.33 The allostimulatory capacity of modulated moDCs and ipDCs.**

(A) Around  $4 \times 10^4$  naïve CD4<sup>+</sup> T cells were co-cultured with moDCs at different ratios.  $10^5$  naïve CD4<sup>+</sup> T cells were co-cultured with ipDCs at different ratios. The number of cells from DC differentiation cultures that were plated per well are shown on the x-axis. Coloured symbols represent the mean percentage of proliferation as determined by 3 independent experiments for moDC co-cultures  $\pm$  SEM and 2 independent experiments for ipDC co-cultures. At  $1.25 \times 10^4$  ipDCs 3 independent experiments are shown  $\pm$  SEM. (B) Data represent the percentage of proliferated CD4<sup>+</sup> T cells in co-culture with  $10^4$  moDCs together with  $4 \times 10^4$  allogeneic naïve CD4<sup>+</sup> T cells ( $n=3$ ) or  $1.25 \times 10^4$  ipDCs together with  $10^5$  allogeneic naïve CD4<sup>+</sup> T cells ( $n=3$ ). Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ).

### **3.15 The effect of modulated DCs on the T<sub>reg</sub> population**

Since their discovery, it has become clear that T<sub>regs</sub> are instrumental to the establishment or maintenance of peripheral tolerance (Sakaguchi *et al.*, 2008; Wing and Sakaguchi, 2010). To evaluate the effect of modulatory agents on T<sub>reg</sub> polarisation and/or expansion, DCs were co-cultured for 7 days with naïve allogeneic CD4<sup>+</sup> T cells. T<sub>regs</sub> were identified by surface CD4 and the level of Foxp3 expression, as determined by flow cytometry (Figure 3.34A).

Plotting the relative change in the percentage of T<sub>regs</sub> detected in co-cultures of independent experiments did not yield statistically-significant findings (Figure 3.34B).

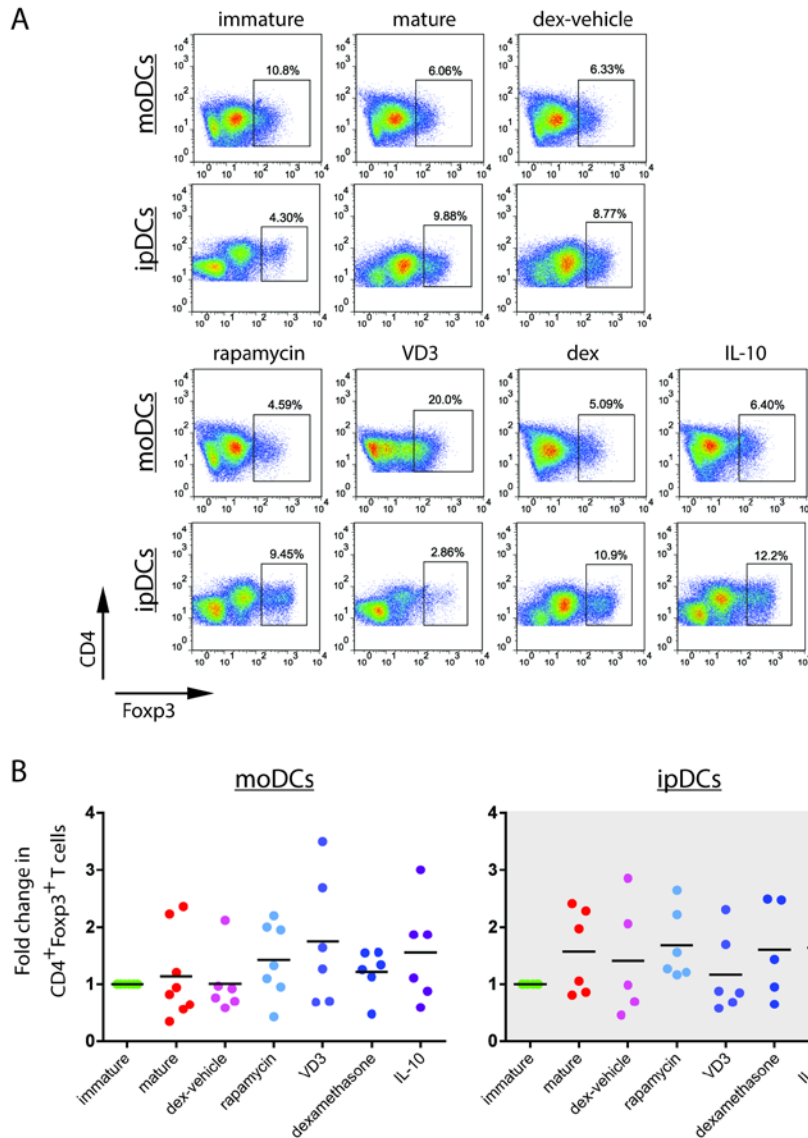


**Figure 3.34 The effect of modulated DCs on T<sub>regs</sub>.** Co-cultures of  $10^5$  allogeneic naïve CD4<sup>+</sup> T cells together with  $2 \times 10^5$  moDCs or ipDCs were treated with 75 ng/ml of IL-2 after 5 days and analysed for CD4<sup>+</sup> Foxp3<sup>+</sup> cells on day 7 by flow cytometry. (A) Data show the appearance and gating of T<sub>reg</sub> populations of example experiments of moDC and a ipDC co-culture with T cells. ipDCs were differentiated from C15 ipDCs at passage 17. (B) Data summarise the fold change in the percentage of T<sub>reg</sub> populations as compared to the percentage of T<sub>regs</sub> obtained in co-cultures with immature moDCs and ipDCs. ipDCs were differentiated from C15 iPSCs at passage 17 to 26. Immature and mature moDC and ipDC data were previously presented in Figure 3.15 and are shown here for comparison. The mean fold change in the proportion of T<sub>regs</sub> from independent experiments is displayed as black lines. Independent experiments are represented by coloured dots ( $n=5-8$ ). Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns*.  $p > 0.05$ ).

### 3.16 The effect of modulated DCs on the IL-10 microenvironment

T<sub>regs</sub> are known to exhibit immunosuppressive capacity by various mechanisms, most importantly via cell-to-cell contact, but also via the release of IL-10 which limits inflammation in locations such as the lungs and gut and inhibits Th17-mediated immune responses (Chaudhry *et al.*, 2011; Rubtsov *et al.*, 2008). Therefore, the supernatants of co-cultures were analysed for the presence of IL-10. In order to allow the detection of an effect exerted by modulated DCs and not the effect of the modulatory agents on T cells, harvested DCs were extensively washed with PBS before being plated into co-cultures with naïve allogeneic CD4<sup>+</sup> T cells. Supernatants were harvested 5 and 7 days after the initiation of co-culture. On day 5, a significantly higher concentration of IL-10 was detected in co-cultures of naïve CD4<sup>+</sup> T cells with moDCs and ipDCs that had been treated with IL-10 (moDCs mature:  $27.81 \pm 27.37$  pg/ml ( $n=4$ ), IL-10:  $1434 \pm 852$  pg/ml ( $n=4$ ),  $p = 0.0288$ ; ipDCs mature:  $35.93 \pm 25.47$  pg/ml ( $n=3$ ), IL-10:  $381.5 \pm 67.09$  pg/ml ( $n=3$ ),  $p < 0.0001$ ) (Figure 3.35). That this was due to the carryover of IL-10 that had been added as a modulator is unlikely as DCs had been extensively washed before being transferred to co-cultures. Furthermore, the presence of IL-10 in supernatants of co-cultures with dexamethasone-treated ipDCs was also significantly higher (dex-vehicle:  $41.36 \pm 22.96$  pg/ml ( $n=3$ ), dexamethasone:  $241 \pm 32.42$  pg/ml ( $n=3$ ),  $p = 0.0088$ ). By day 7, these differences in the concentrations of IL-10 in supernatants of these co-cultures were still detected, however no statistical significance was achieved.



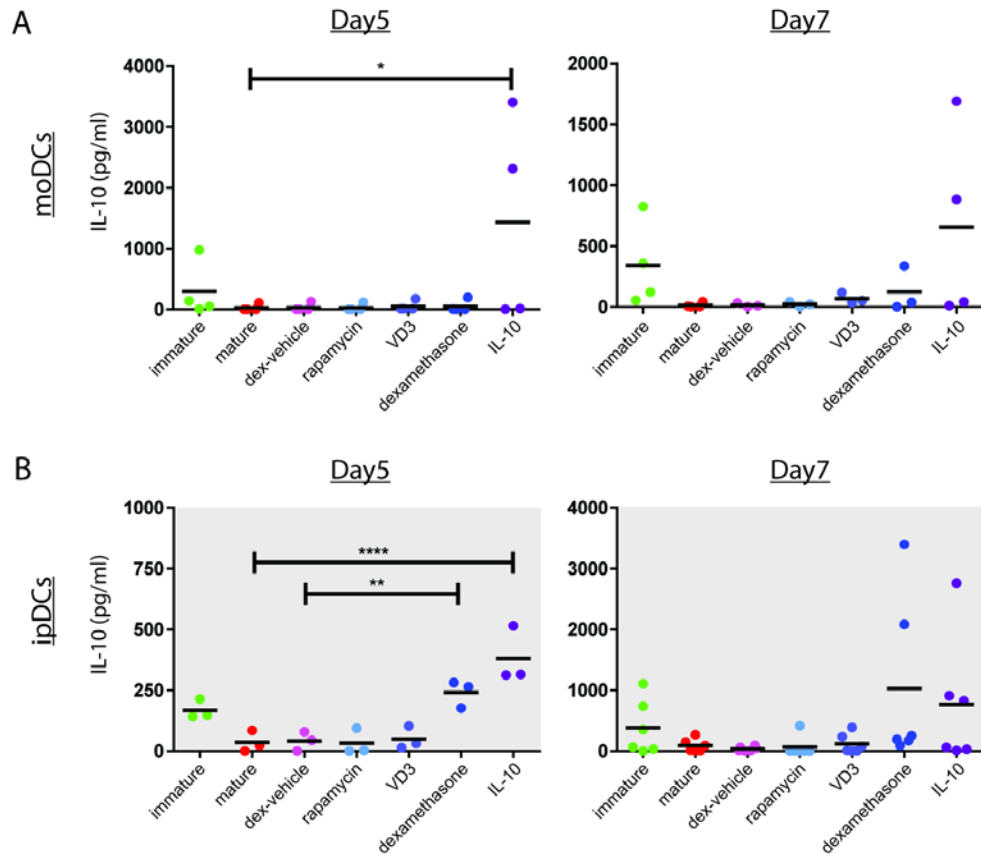


**Figure 3.35 The effect of modulated DCs on the IL-10 microenvironment.**

Co-cultures of  $10^5$  allogeneic naïve  $CD4^+$  T cells together with  $2 \times 10^5$  DCs were treated with 75 ng/ml of IL-2 after 5 days and supernatants harvested on day 7 and analysed by ELISA. ipDCs were differentiated from C15 iPSCs at passages 17 to 26. Black lines represent the mean concentration of IL-10 as determined by independent experiments ( $n=3-6$ ). Independent experiments are presented as individually coloured dots. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test ( $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $****p \leq 0.001$ ).

Nevertheless, similarly to the results obtained on the expression of costimulatory and inhibitory molecules, the presence of IL-10 in co-cultures with moDCs and ipDCs treated with IL-10 is consistent with the acquisition of pro-tolerogenic properties.

### 3.17 The effect of modulated DCs on the Tr1 population

Although they remain poorly understood, another population of T cells with immunosuppressive capacity are the Tr1 cells (Battaglia *et al.*, 2004, 2006; Gregori *et al.*, 2010; Roncarolo *et al.*, 2006). These are defined as CD4<sup>+</sup> Foxp3<sup>-</sup> T cells that produce and secrete large amounts of IL-10 (Battaglia *et al.*, 2004; Roncarolo *et al.*, 2006). They are distinguished from Th2 cells by the absence of IL-4 production. As previous studies have shown the induction or expansion of Tr1 by modulated DCs, in particular following pre-treatment with IL-10, this study examined co-cultures for the presence of Tr1 populations (Battaglia *et al.*, 2006; Gregori *et al.*, 2010). Furthermore, the increase in IL-10 in the microenvironment of co-cultures (Figure 3.35) and upregulation of ILT4 on moDCs and ipDCs treated with IL-10 (Figure 3.30), prompted an interest in the potential effect that IL-10 modulation of DCs may have on Tr1 populations, as ILT4 and IL-10 have been shown to be required for the induction of Tr1 cells (Andolfi *et al.*, 2012; Gregori *et al.*, 2010).

In order to detect the expression of IL-10, T cells were treated with brefeldin A, ionomycin and PMA, in order to activate them and inhibit the release of cytokines, thereby allowing their intracellular detection using flow cytometry. However, no significant changes were detected in the proportion of Tr1 cells found in co-cultures with modulated DCs (Figure 3.36).

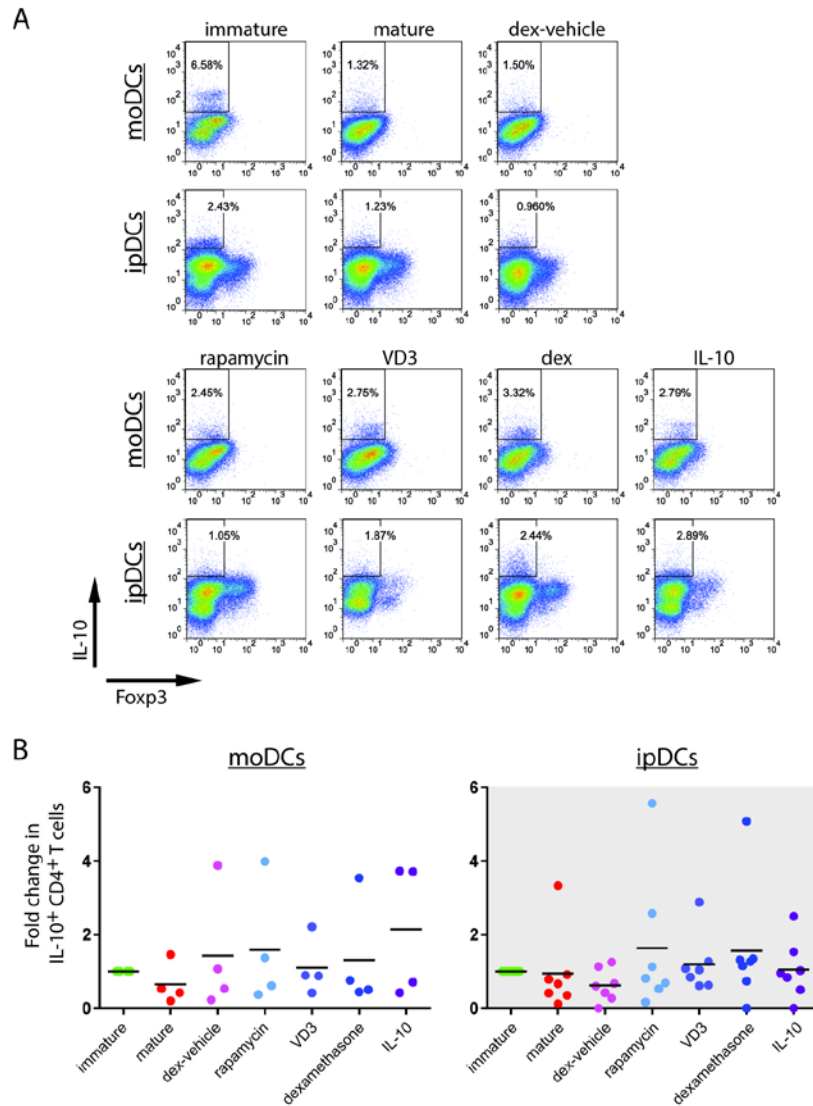


**Figure 3.36 The effect of modulated DCs on the Tr1 population.** Co-cultures were established between  $2 \times 10^5$  allogeneic naïve  $CD4^+$  T cells and  $10^6$  modulated or control DCs. After 7 days of co-culture, cells were stimulated with PMA, ionomycin and brefeldin A, to stimulate the production of cytokines but inhibit their release. After 5 hours, cells were harvested, stained for intracellular IL-10 and Fcγp3 following permeabilization and analysed by flow cytometry. (A) Dot plots present an example of a set of data obtained from one of the Tr1 induction experiments. ipDCs co-cultured with naïve  $CD4^+$  T cells were obtained from C15 iPSC differentiated at passage 17. (B) Graphs summarize the fold change in Tr1 populations as obtained by independent experiments which are displayed as coloured dot (moDCs:  $n=4$ , ipDCs:  $n=7$ ). ipDCs used in these experiments were obtained from C15 iPSCs differentiated at passages 17 to 26. Black lines represent the mean fold change as obtained from independent experiments. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns. p* > 0.05).

### **3.18 The effect of modulated DCs on IFN $\gamma$ and IL-2-producing T cell populations**

As the transcription factor Foxp3 is known to suppress the transcription of genes encoding IFN $\gamma$  and IL-2, thereby rendering T<sub>regs</sub> incapable of IL-2 and IFN $\gamma$  secretion, the detection of IL-2<sup>+</sup> T cells was used as an indicator of the proportion of activated T effector cells while the detection of IFN $\gamma$  indicated the proportion of Th1 cells induced in co-cultures (Bettelli *et al.*, 2005; Ono *et al.*, 2007). A reduction in the proportion of these cells has also been used as an indicator of T cell anergy induction. Furthermore, PD-L1 signalling has been shown to suppress IFN $\gamma$  production in effector cells, providing another useful readout for a pro-tolerogenic phenotype. CD80/CD86 mediated costimulation via CD28 on T cells has been shown to induce the production of IL-2 which acts as a survival and expansion signal to T cells (Linsley *et al.*, 1991). Therefore, the capacity of modulated cells to reduce IL-2-producing T cells was investigated.

As illustrated in Figure 3.37, no statistically-significant difference was found in the proportion of IL-2<sup>+</sup> or IFN $\gamma$ <sup>+</sup> T cells between co-cultures with differently modulated moDCs or ipDCs. Furthermore, rapamycin-treated moDC and ipDCs appeared to induce, on average, a larger proportion of IL-2<sup>+</sup> T cells as compared to matured controls, however none of these results were statistically significant.

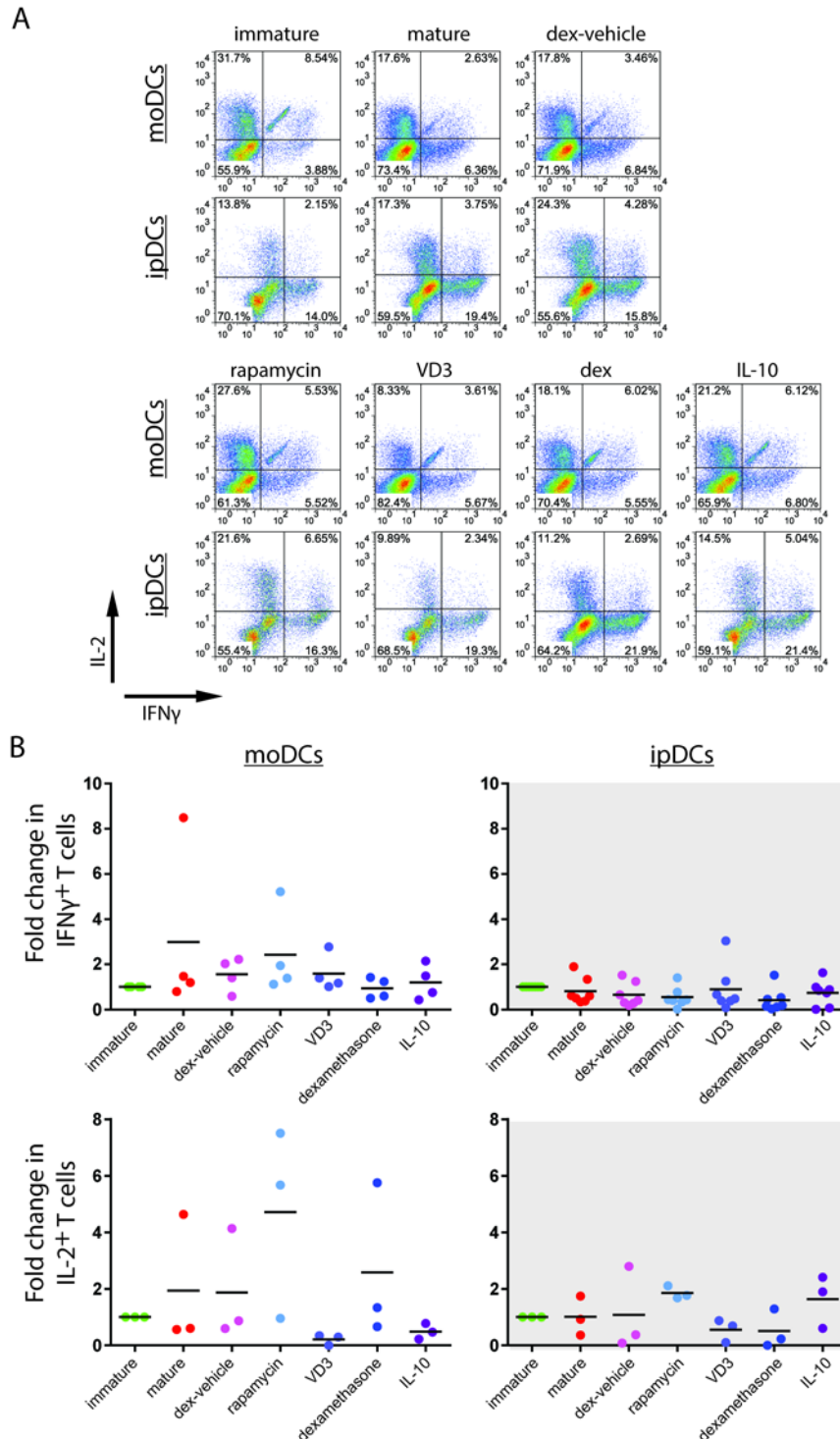


**Figure 3.37 The effect of modulated DCs on IL-2<sup>+</sup> and IFNγ<sup>+</sup> CD4 T cells.** moDCs or ipDCs ( $2 \times 10^5$ ) were co-cultured with  $10^6$  allogeneic naïve CD4<sup>+</sup> T cells per well of a 24-well plate. Cells were treated on day 5 with IL-2 and stimulated with PMA, ionomycin and brefeldin A on day 7. After 5 hours of stimulation, cells were harvested, stained and analysed by flow cytometry. (A) Dot plots represent an example set of data obtained from a co-culture, which therefore not completely match the average fold changes in the percentage of positive T cells. Co-cultured

ipDCs were differentiated from C15 iPSCs at passage 20. (B) Graphs display the average proportion of IFN $\gamma$ <sup>+</sup> and IL-2<sup>+</sup> T cells as black lines which was determined by independent experiments as displayed as coloured dots ( $n=3-7$ ). ipDCs were differentiated from C15 iPSC at passages 17 to 26. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns*.  $p > 0.05$ ).

### **3.19 The effect of modulated DCs on the IFN $\gamma$ microenvironment**

As another indicator of Th1 induction in co-cultures, supernatants harvested on day 5 and day 7 were analysed for the concentration of IFN $\gamma$ . As illustrated in Figure 3.38, no statistically-significant difference was observed in the concentrations of IFN $\gamma$  detected in supernatants on either day 5 or on day 7. However, the average concentration was lower in all co-cultures with modulated DCs as compared to relevant controls.

Similar to past studies, IL-10 treatment of moDCs decreased the average secretion of IFN $\gamma$  from CD4<sup>+</sup> T cells, however, in this study, no statistical significance was achieved (Gregori *et al.*, 2010; Steinbrink *et al.*, 1997).

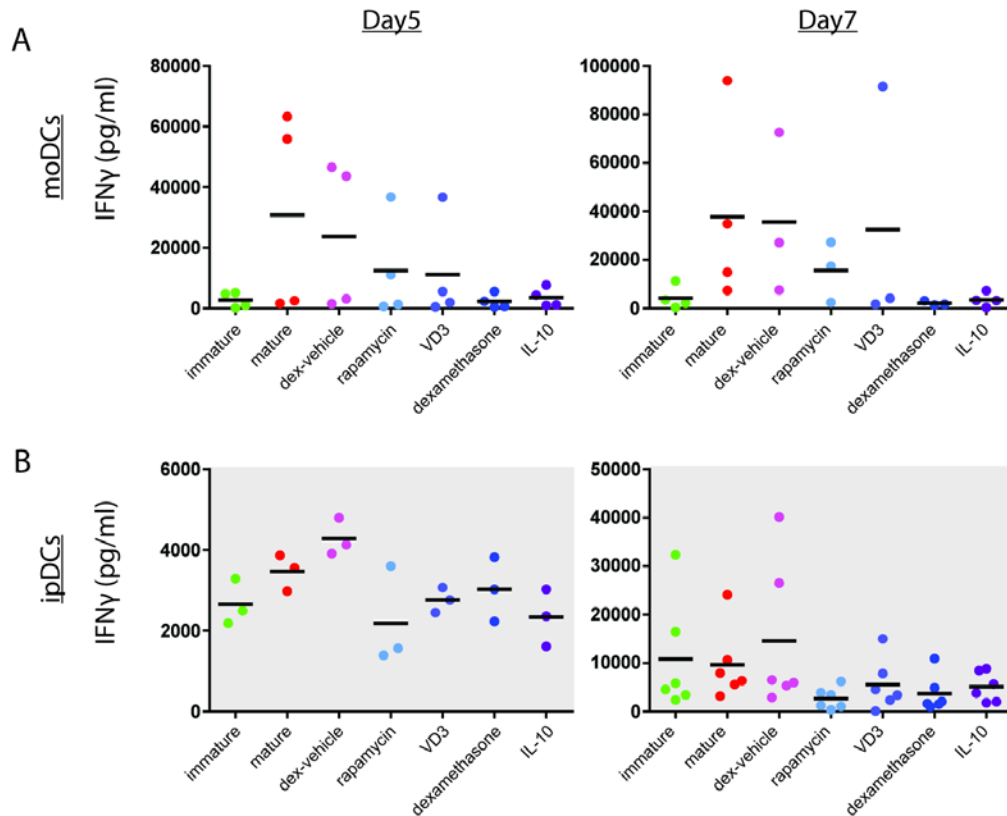


**Figure 3.38 The effect of modulated DCs on the IFN $\gamma$  microenvironment.** DCs derived from CD14<sup>+</sup> monocytes and C15 iPSCs at passages 17 to 26 ( $2 \times 10^5$ ) were co-cultured with  $10^6$  allogeneic naïve CD4<sup>+</sup> T cells per well of a 24-well plate. Cells were treated on day 5 with IL-2 and supernatants were harvested on day 7 for further analyses by ELISA. Black lines represent the mean concentration of IFN $\gamma$  as determined by independent experiments ( $n=3-6$ ). Independent experiments are displayed as coloured dots. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected (*ns*.  $p > 0.05$ ).

## Discussion

This chapter has confirmed the feasibility of deriving cells from iPSCs that resemble moDC in their morphology, surface phenotype and ability to phagocytose, process antigen, maintain T<sub>regs</sub> and induce the proliferation of naïve allogeneic T cells. Therefore, given that iPSCs may be derived in an autologous manner, the possibility of generating large populations of ipDCs in a patient-specific manner appears entirely feasible.

### 3.20 The characterisation of ipDCs and their inherent immaturity

As illustrated in Figure 3.4, the average proportion of CD11c-expressing cells generated varies between differentiations from CD14<sup>+</sup>-selected monocytes and iPSCs. This could be explained by the different potency of the cells where iPSCs are pluripotent and can differentiate into any type of cell of the human body while CD14<sup>+</sup> monocytes have a more restrictive multipotent potential (Kuwana *et al.*, 2003; Seta, 2010). Furthermore, moDCs were derived from purified populations of CD14<sup>+</sup> monocytes which therefore ensured a higher purity of differentiation cultures and resulting DC population. No purification protocol was applied to iPSCs or ipDC differentiations as previous work in our laboratory failed to maintain the viability of cells following CD11c-specific magnetic bead separation (K. Silk, personal communication, December 7, 2010). Therefore a proportion of non-DCs may have been generated and present alongside CD11c<sup>+</sup> cells. This means that data generated in this study need to be interpreted with this in mind. Other cells with an immunological effect may be present in cultures which may have an impact on DC immunogenicity and T cell responses. However, current work in our laboratory is looking at the feasibility of purifying ipMonocytes after



the initial harvest using CD14-specific selection. Furthermore, alternative purification methods making use of negative selection or separation by fluorescence are currently being explored. A few outliers of moDCs that gave rise to a low percentage of CD11c<sup>+</sup> cells may have been due to problems with monocyte purification or the quality of PBMCs from some donors, which is known to be highly variable (Whitney *et al.*, 2003).

Altogether, the phenotype of ipDCs was similar to the phenotype observed on moDCs, with a few exceptions that will be discussed here. All DC-specific markers such as CD11c, MHCII, CD11b, CD14, CD54 and CD123 were detected on moDCs and ipDCs, even though in some cases at different levels.

The expression profile of intracellular MHCII in immature ipDCs indicated the presence of two distinct populations (Figure 3.5). This may be explained by the potential presence of a developmentally immature population of precursor DCs that have yet to upregulate intracellular MHCII. Considering the presence of a single distinct population after exposure to maturation stimuli, it may indicate the presence of ipDCs which have spontaneously acquired a certain degree of maturity.

Furthermore, ipDCs were found to express considerably lower levels of MHCII as compared to moDCs, which correlates with previous findings in our laboratory using mouse ESCs and iPSCs, which have described an inherent naïve and immature phenotype of DCs derived from these stem cells (Figure 3.5) (Hackett, 2013). Therefore, the use of ipDCs rather than moDCs in pro-tolerogenic immune-modulation therapies may be advantageous due to the low level of

MHCII expression and therefore the retention of an extreme immature and pro-tolerogenic phenotype (Dhodapkar *et al.*, 2001; Hawiger, 2001; Li and Shi, 2015).

The expression of chemokine receptors such as CCR7 has been complicated by the low surface expression and sequence conservation between species which has hampered the development of chemokine receptor-specific antibodies (Figure 3.9) (Maecker *et al.*, 2012) (D. Greaves, personal communication, February 28, 2013). This may explain the low level of CCR7 expression detected on moDCs and ipDCs (Figure 3.9). Migration assays previously performed in our laboratory have, however, confirmed the presence of CCR7 and the migration towards CCL19, however, this could significantly vary between different sets of cells and differentiations (P. Sachamitr, personal communication, September 8, 2014). Future attempts to detect this chemokine on the cell surface may make use of fluorescently-labelled cytokine ligands which may improve the sensitivity of detection.

The expression of CD207 on ipDCs, but not moDCs, may indicate the presence of a small proportion of Langerhans-like cells in the ipDC differentiation cultures, which have been differentiated from ESCs and iPSCs in the past (Figure 3.7) (Choi *et al.*, 2009). However, the physiological relevance and reliability of the detection of such a small change is difficult to determine (Valladeau *et al.*, 2002). Statistical analysis had been performed in order to determine whether the gMFI obtained from antigen-specific stainings was significantly different from the gMFI obtained from isotype-controls, however the small sample size and the variation between experiments did not allow sufficient statistical power. However, in order to be objective, any increase in gMFI from antigen-specifically stained samples above background (relative gMFI > 1) was determined to indicate expression. It

remains to be determined whether the level of expression of different markers detected by this method were physiologically relevant and whether their detection may in some cases constitute a false positive due to differently gated populations with different non-specific adhesiveness.

One major difference between DCs derived from CD14<sup>+</sup> monocytes and those derived from iPSCs was the CD1a expression profile (Figure 3.8). Previous publications describing the categorisation of DCs into CD1a<sup>+</sup> and CD1a<sup>-</sup> subsets, indicate the generation of these two subpopulations from CD14<sup>+</sup> monocytes (Cernadas *et al.*, 2009; Chang *et al.*, 2000). In contrast to this, ipDCs appeared to be able to generate the CD1a<sup>-/low</sup> population only (Figure 3.8). This further underlines the differences in the differentiation protocols and raises the question whether alterations in the ipDC differentiation protocol may enable the generation of the CD1a<sup>+</sup> population.

In this study, a striking observation was the absence of CD209 on mature ipDCs, while immature and mature moDCs and immature ipDCs showed CD209 expression (Figure 3.8). Immature ipDC were found to express CD209 at levels more than 10 fold lower as compared to moDCs. CD209 expression is known to be induced in response to GM-CSF and IL-4, however it has also been found to be down-regulated in response to IFN $\gamma$  (Relloso *et al.*, 2002). This explains the reduced expression of CD209, even though not statistically significant, on moDCs exposed to pro-inflammatory cytokines which included IFN $\gamma$  (Figure 3.8). As immature ipDCs already expressed at least 10 times lower levels of CD209, it could be assumed that further downregulation in response to IFN $\gamma$  exposure, may have caused the complete loss of CD209 expression on the surface of mature ipDCs. As CD209 has been shown to be important for the facilitation of DC and

T cell interaction and further downstream T cell activation, these results may be indicative of a pro-tolerogenic phenotype of ipDCs (Geijtenbeek *et al.*, 2000a; Martinez *et al.*, 2005; Zhou *et al.*, 2006). However, whether this low expression may abrogate the effective transmigration of ipDCs *in vivo*, remains to be investigated. Since this observation may have a significant impact on the suitability of ipDCs in immunomodulation therapy, it would be of interest to investigate the expression of CD209 on modulated ipDCs.

The detection of TLR expression was confounded by difficulties in the production of TLR-specific antibodies because of high interspecies conservation which complicated the conclusions about TLR expression profile (Roach *et al.*, 2013). Nevertheless, all TLRs were detected on all DC populations with the exception of TLR2, TLR4 and TLR9 which were not found to be expressed on mature moDCs (Figure 3.10 and Figure 3.11). TLR4 is well known to bind LPS, a component of Gram-negative bacteria, while TLR2 is involved in the recognition of Gram-positive bacteria via peptidoglycans and lipoteichoic acids (Akira and Takeda, 2004). All populations of DCs showed TLR3 expression indicating their capacity to respond to intracellular double-stranded RNA during viral infection (Figure 3.10).

TLR8 and TLR9 were detected on immature and mature ipDCs which, together with the detected expression of TLR7, may indicate the presence of a plasmacytoid-like DC population (Figure 3.11) (Hochrein *et al.*, 2002). Similarly, immature moDCs showed expression of TLR8 which appeared to be down-regulated upon maturation. However, the average expression in 3 independent experiments indicates no significant change. This discrepancy was due to none of the data sets being truly representative of the populations mean.

TLR9 has been shown to induce cross-presentation in response to ligation supporting the finding that ipDCs contain a larger proportion of cross-presenting DCs as compared to moDCs (Datta *et al.*, 2003; Silk *et al.*, 2012a). Expression appeared higher on immature populations while maturation appeared to downregulate these TLRs. TLR8 is thought to recognize guanine-rich oligonucleotides while TLR9 recognises unmethylated cytosine-phosphate guanine dinucleotides which are a common feature of bacterial and viral genomes but are less common within the vertebrate genome (Akira *et al.*, 2006). Our previously-published work supports the expression profile described here with functional assays, which showed the release of cytokines by moDCs and ipDCs in response to TLR3 and TLR8 ligation while ipDCs, but not moDCs, responded to TLR9 ligation (Silk *et al.*, 2012a).

The larger proportion of XCR1-expressing ipDCs and the level of expression has drawn interest towards the ipDC populations especially in terms of their therapeutic potential in cancer immunisation (Leishman and Fairchild, 2014; Sachamitr and Fairchild, 2012; Silk *et al.*, 2012a). This has been confirmed in this study as XCR1 was expressed at relatively higher levels on ipDCs as compared to immature moDCs, while mature moDCs did not show any XCR1 expression (Figure 3.12). Furthermore, the detection of these markers of cross-presentation correlates with the detection of a higher level of TLR9 expression by ipDCs as compared to moDCs, as these TLRs have been shown to promote cross-presentation in mice (Datta *et al.*, 2003; Schulz *et al.*, 2005).

Therefore, one can conclude, that the populations of DCs derived from monocytes and iPSCs share similarities in their phenotypic expression of DC markers but also differ slightly in their DC subtype composition. However, similar

to the expression of CCR7, the reliability of these results is limited due to the low relative gMFIs obtained, small sample sizes and gating strategy (discussed in section 3.23) which complicates the interpretation of results.

The lower expression of MHCII by ipDCs (Figure 3.5) is reminiscent of what had been observed before in our laboratory using mouse ESC or iPSC-derived DCs (Hackett, 2013). DCs derived from mouse iPSCs had been shown to consistently express low levels of MHCII as compared to bmDCs, however, they retained the ability to stimulate T cell proliferation (Hackett, 2013). Data obtained in the past has led to the hypothesis that DCs derived from stem cells have a higher intrinsic tolerogenicity as compared to bmDCs (Hackett, 2013). These data were supported by a number of results in this study and may therefore translate these observations from mouse to human stem cells. One of these observations showed that ipDCs did not significantly upregulate a number of cell surface molecules that were upregulated on moDCs in response to proinflammatory cytokine exposure. These markers include PD-L1 and PD-L2 (Figure 3.27 and Figure 3.28). Furthermore, the lower level of MHCII (Figure 3.5 and Figure 3.19), CD209 (Figure 3.8) and CD40 (Figure 3.24) expression by ipDCs as compared to moDCs may further support a pro-tolerogenic phenotype of these cells, while DC and myeloid markers were expressed at a similar level to moDCs (Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.18, Figure 3.20, Figure 3.22 and Figure 3.23). This was further supported by the lower average base level of T cell proliferation induced by immature and mature ipDCs as compared to moDCs (Figure 3.33). This could be explained by the lower levels of MHCII (Figure 3.5 and Figure 3.19) and CD209 (Figure 3.8) expression, as compared to moDCs, as both MHCII and CD209 are essential for T cell stimulation and interaction (Geijtenbeek *et al.*,

2000b; Martinez *et al.*, 2005; Paul, 2012; Zehn *et al.*, 2012). This inherent immaturity of ipDCs may prove to be advantageous to tolerance induction and therefor potentially be more promising for future clinical application in immune modulation therapies.

### **3.21 The generation of pro-tolerogenic moDCs and ipDCs**

In an attempt to provide a comprehensive overview for the interpretation of the effectiveness of modulatory agents at inducing pro-tolerogenic properties, results are summarised in Table 3.2 and Table 3.3 for the modulation of moDCs and ipDCs respectively.

| moDCs                    |                           | Rapa | VD3   | Dex | IL-10 |
|--------------------------|---------------------------|------|-------|-----|-------|
| moDCs yield              |                           | ns.  | ↓**** | ns. | ↓**   |
| moDC viability           |                           | ns.  | ↓*    | ns. | ns.   |
| Costimulatory molecules  | CD86                      | ns.  | ns.   | ns. | ns.   |
|                          | CD40                      | ns.  | ns.   | ns. | ns.   |
|                          | CD80                      | ns.  | ns.   | ns. | ns.   |
| Inhibitory molecules     | PD-L1                     | ns.  | ns.   | ns. | ns.   |
|                          | PD-L2                     | ns.  | ns.   | ns. | ns.   |
|                          | ILT3                      | ns.  | ns.   | ns. | ns.   |
|                          | ILT4                      | ns.  | ns.   | ns. | ↑**   |
| Cytokine production      | IL-10                     | ns.  | ns.   | ns. | -     |
|                          | IL-6                      | ns.  | ↑**   | ns. | ns.   |
|                          | IL-12                     | ↑**  | ns    | ns. | ns.   |
| Allostimulatory capacity |                           | ns.  | ↓**   | ns. | ns.   |
| T cell populations       | Tregs                     | ns.  | ns.   | ns. | ns.   |
|                          | Tr1                       | ns.  | ns.   | ns. | ns.   |
|                          | IFN $\gamma$ <sup>+</sup> | ns.  | ns.   | ns. | ns.   |
|                          | IL-2 <sup>+</sup>         | ns.  | ns.   | ns. | ns.   |
| Cytokines in co-cultures | IL-10                     | ns.  | ns.   | ns. | ↑*    |
|                          | IFN $\gamma$              | ns.  | ns.   | ns. | ns.   |

**Table 3.2 A summary of the effect of moDC modulation.** Data described in this chapter were translated into scores and summarised to obtain an objective score of the effectiveness of modulatory treatment. Modulation that resulted in a statistically significant change are highlighted with arrows indicating whether the values increased (↑) or decreased (↓) as compared to the mature control population. Results that were not statistically significant were indicated as *ns.* Colour coding highlights whether changes are commonly defined as pro-tolerogenic (green) or pro-immunogenic (red). Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.001$ ).



| ipDCs                    |                           | Rapa | VD3 | Dex | IL-10 |
|--------------------------|---------------------------|------|-----|-----|-------|
| ipDCs yield              |                           | ns.  | ↓*  | ns. | ns.   |
| ipDC viability           |                           | ns.  | ns. | ns. | ns.   |
| Costimulatory molecules  | CD86                      | ns.  | ns. | ns. | ns.   |
|                          | CD40                      | ns.  | ns. | ns. | ns.   |
|                          | CD80                      | ns.  | ns. | ns. | ns.   |
| Inhibitory molecules     | PD-L1                     | ns.  | ns. | ns. | ns.   |
|                          | PD-L2                     | ns.  | ns. | ns. | ns.   |
|                          | ILT3                      | ns.  | ns. | ns. | ↑**   |
|                          | ILT4                      | ns.  | ns. | ns. | ↑**** |
| Cytokine production      | IL-10                     | ns.  | ns. | ns. | -     |
|                          | IL-6                      | ns.  | ns. | ns. | ns.   |
|                          | IL-12                     | ns.  | ns. | ns. | ns.   |
| Allostimulatory capacity |                           | ns.  | ns. | ns. | ↓*    |
| T cell populations       | Tregs                     | ns.  | ns. | ns. | ns.   |
|                          | Tr1                       | ns.  | ns. | ns. | ns.   |
|                          | IFN $\gamma$ <sup>+</sup> | ns.  | ns. | ns. | ns.   |
|                          | IL-2 <sup>+</sup>         | ns.  | ns. | ns. | ns.   |
| Cytokines in co-cultures | IL-10                     | ns.  | ns. | ↑** | ↑**** |
|                          | IFN $\gamma$              | ns.  | ns. | ns. | ns.   |

**Table 3.3 A summary of the effect of ipDC modulation.** Data described in this chapter were translated into scores and summarised to obtain an objective score of the effectiveness of modulatory treatment. Modulation that resulted in a statistically significant change are highlighted with arrows indicating whether the values increased (↑) or decreased (↓) as compared to the mature control population. Results that were not statistically significant were indicated as *ns.* Colour coding highlights whether changes are commonly defined as pro-tolerogenic (green) or pro-immunogenic (red). Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparison test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.001$ ).

The exposure of DCs to pro-inflammatory cytokines for maturation aims at promoting a stable DC phenotype that is terminally matured and therefore refractory to any further activation signals. However, the finding that the exposure to pro-inflammatory cytokines alone may not provide a sufficient stimulus to promote full DC activation, as implied by the inability of cytokine-matured DCs to secrete IL-12, had provoked concern about the potential of subsequent activation of pro-tolerogenic DCs *in vivo*, especially in response to TLR ligands (Boullart *et al.*, 2008; Zobywalski *et al.*, 2007). This was addressed by Naranjo-Gómez and colleagues (2011) and other scientists, who investigated the potential for reactivating tolerogenic DCs that had been previously matured with a pro-inflammatory cytokine cocktail, by exposing these cells to LPS for 24 hours (Chamorro *et al.*, 2009; Taner *et al.*, 2005; Xia *et al.*, 2005; Yates, 2007). They found that moDCs modulated with dexamethasone, VD3 or rapamycin, all maintained their pro-tolerogenic phenotype despite secondary stimulation via TLR4 (Xia *et al.*, 2005). Interestingly, restimulation of dexamethasone-treated moDCs gave rise to a significant increase in the secretion of IL-10, thereby enhancing their tolerogenic phenotype.

No significant change in the expression of MHCII was observed following pro-tolerogenic treatment of moDCs and ipDCs (Table 3.2 and Table 3.3) (Figure 3.19) In contrast to this, Naranjo-Gómez *et al.* (2011) found that VD3-treatment of moDCs significantly down-regulated the cell surface expression of MHCII, while, similar to observations made in this study, rapamycin and dexamethasone-treatment appeared to have no significant effect. Given the role played by MHCII in T cell activation, expression levels may correlate with immunogenicity of DCs. However, no statistical significance was achieved and therefore any functional

effect of the level of MHCII expression on immunogenicity of modulated DCs remains to be determined.

CD80 and CD86 are costimulatory molecules of the B7 family that act by binding CD28 on T cells and are, therefore, essential to T cell activation (Orabona *et al.*, 2004). Considering published data on CD80 expression, a higher average expression on moDCs was expected (Figure 3.25) (Bartels *et al.*, 2010; Chamorro *et al.*, 2009; Harry *et al.*, 2010; Unger *et al.*, 2009). This may have been due to technical difficulties with the antibodies used in this study.

No statistically significant change in the expression of CD40, CD80 or CD86 was observed in response to pro-tolerogenic treatments in this study (Table 3.2 and Table 3.3) (Figure 3.24, Figure 3.25 and Figure 3.26). Other studies, however, showed that rapamycin-treatment of moDCs appeared to exert the least effect on the expression of CD86 while dexamethasone, IL-10 and VD3-treatment were found to promote a significant downregulation of CD86 on the cell surface (Gregori *et al.*, 2010; Naranjo-Gómez *et al.*, 2011). The bimodal expression profile of CD86 on rapamycin-treated moDCs may indicate the generation of two populations with distinct immunostimulatory properties. Further independent experiments need to be performed in an attempt to reach statistical significance.

No statistically significant change was observed in the expression of PD-L1 and PD-L2 following pro-tolerogenic treatment (Table 3.2 and Table 3.3) (Figure 3.27 and Figure 3.28). As PD-L1 plays a role in the induction and expansion of T<sub>reg</sub> populations in the presence of low levels of co-stimulation, a co-staining of ipDCs and moDCs with PD-L1 and CD86 may elucidate the potential presence of DCs

with high PD-L1 but low costimulatory molecule expression and therefore a potentially pro-tolerogenic phenotype (Chemnitz *et al.*, 2004).

The concentrations of cytokines detected were similar between moDC and ipDC cultures, however, the average concentration of IL-6 released by matured ipDC was higher than the concentration measured in matured moDC cultures (Figure 3.31). Here it is important to keep in mind that ipDC cultures were not as pure as moDCs, as shown in Figure 3.4C, and therefore contaminating non-DC cells, such as neutrophils, eosinophils and mast cells, may have contributed to the cytokine profile measured (Ericson *et al.*, 1998; Gagari *et al.*, 1997; Melani *et al.*, 1993). Interestingly, neutrophils were shown to release IL-6 *in vitro* in response to GM-CSF exposure, however, no cells with classic multi-lobed nuclei have been observed (Ericson *et al.*, 1998; Melani *et al.*, 1993).

The modulation of moDCs with rapamycin led to a statistically significant increase in the release of IL-12 (Table 3.2) (Figure 3.31). As IL-12 is associated to pro-inflammatory responses and no other significant changes were observed, it could be assumed that rapamycin may not be an effective pro-tolerogenic modulator. In the case of the treatment of ipDCs with rapamycin, no statistically significant change was observed in any assays performed.

The modulation of DCs using VD3 resulted in a significant decrease in cell yield of moDCs and ipDCs (Figure 3.16, Table 3.2 and 3.3). Furthermore, the viability of moDCs was significantly lower in VD3 treated cultures as compared to untreated mature moDCs (Figure 3.16 and Table 3.2). This was not observed with ipDCs. Whether the detection of reduced viability is due to an impairment in endocytic and phagocytic uptake of dying or apoptotic cells by the modulated

DCs or whether modulatory agents have an impact on DC survival over a longer period, and therefore not allowing for enough time for DCs to clear the cultures from dying cells before measurement, was not determined. The upregulation of CD14 expression on moDC in response to VD3-treatment has also been shown in other studies, which may indicate the immaturity of VD3-treated DCs which retain a more monocyte-like phenotype or the acquisition of a macrophage-like identity (Figure 3.20) (Bartels *et al.*, 2010; Canning *et al.*, 2001). The similar effect of VD3 on the expression of CD14 by moDCs and ipDCs may underline that the DCs derived from the two different sources respond similarly to modulation.

Furthermore, a significant increase in the release of IL-6 by VD3-treated moDCs, but not ipDCs, was detected (Figure 3.31) (Table 3.2 and Table 3.3). Most strikingly, VD3 treatment of moDCs reduced their allostimulatory capacity significantly and therefore reduced their capacity to induce T cell proliferation *in vitro* (Figure 3.33) (Table 3.2). This was not observed with ipDCs, which may be due to biological differences of these two different DC populations or the lower purity of the ipDCs. However, these data indicate that VD3-treatment of moDCs may have a significant effect on their immunological function.

In the case of dexamethasone, modulation had no significant effect on the immunogenicity indicators of moDCs (Table 3.2). However, the treatment of ipDCs with dexamethasone caused a significant increase in the release of IL-10 in co-cultures with T cells (Figure 3.35) (Table 3.3). Whether ipDCs were more susceptible to dexamethasone-mediated modulation as compared to moDCs, due to the significant increase in IL-10 in supernatants was not clear, however previous studies did show the increased release of IL-10 by dexamethasone-treated moDCs (Chamorro *et al.*, 2009; Naranjo-Gómez *et al.*, 2011; Unger *et al.*,

2009; Xia *et al.*, 2005). Other studies have found a significant decrease in the T cell stimulation of moDCs treated with rapamycin, dexamethasone, IL-10 and VD3 (Bartels *et al.*, 2010; Fazekasova *et al.*, 2009; Gregori *et al.*, 2010; Naranjo-Gómez *et al.*, 2011; Steinbrink *et al.*, 1997; Torres-Aguilar *et al.*, 2010b; Unger *et al.*, 2009). Future independent experiments may enable the detection of statistically significant differences in the functional immunostimulatory capacity of dexamethasone-modulated DCs and matured DCs.

IL-10 on the other hand, was found to significantly decrease the yield of moDCs from cultures, but not ipDCs (Figure 3.16) (Table 3.2 and Table 3.3). Furthermore, IL-10 induced a significant upregulation in the expression of ILT4 by moDCs and ipDCs, and ILT3 on ipDCs (Figure 3.29 and Figure 3.30). These results were confirmed by other studies showing the increased expression of ILT3 and ILT4 on moDCs in response to IL-10 treatment, which was maintained even after exposure to IFN $\gamma$  (Gregori *et al.*, 2010; Manavalan *et al.*, 2003). Gene expression studies further confirmed this phenotype, showing the overexpression of the genes encoding ILT3 and ILT4 in response to IL-10 treatment (Velten *et al.*, 2004). In co-culture with T cells, IL-10 modulation of moDCs and ipDCs gave rise to a significant increase in the content of IL-10 in the culture supernatant (Figure 3.35). Previous studies have also demonstrated the ability of IL-10 modulation to increase the secretion of IL-10 from DCs and T cells (Chamorro *et al.*, 2009; Dai *et al.*, 2009; Gregori *et al.*, 2010). Functionally, ipDCs were found to have a significantly reduced allostimulatory capacity when pre-treated with IL-10 (Figure 3.33). The reduced potency of IL-10-treated DCs has been linked to the increased expression of ILT molecules, which is consistent and may explain some of our findings (Gregori *et al.*, 2010; Velten *et al.*, 2004). The effect of IL-10 on DCs and

their immunogenicity is not surprising in light of the published literature, as IL-10 is known to inhibit NF $\kappa$ B signalling and therefore the induction of maturation (Bhattacharyya *et al.*, 2004). DCs modulated with IL-10 are known to be potent inducers of CD4<sup>+</sup> T cell anergy which has been shown to be dependent on ILT4 expression, both of which were observed in this study (Gregori *et al.*, 2010).

The most striking difference between moDCs and ipDCs, however, was the level of MHCII expression, which was approximately 10 times higher on moDCs as compared to the relative gMFI detected on ipDCs (Figure 3.19). This may correlate with previous reports describing the inherent immature and naïve phenotype of DCs derived from mouse iPSCs and ESCs (Hackett, 2013). The relative gMFI of CD40 obtained from ipDC samples was consistently lower as compared to moDCs (Figure 3.24). Interestingly, similar to CD40, the level of CD86 expression was found to be lower on matured ipDCs as compared to matured moDCs (Figure 3.26).

The relative gMFI determined for the expression of PD-L1 and PD-L2 by moDC and ipDCs were at a similar level with the exception of the upregulation of these molecules in response to pro-inflammatory cytokines by moDCs but not by ipDCs. These results are similar to the data presented in Figure 3.26, where a statistically significant upregulation of the expression of CD86 on moDCs, but not on ipDCs, was detected. This may indicate a level of immaturity or failed activation of ipDCs in response to inflammatory cytokines, which may further support the notion that DCs derived from iPSC may be inherently immature or pro-tolerogenic.

Whether this is due to a closer relation of ipDCs to foetal DCs rather than DCs derived from adult haematopoiesis remains to be determined (Aksoy *et al.*, 2007;

Goriely *et al.*, 2001, 2004). Nevertheless, these data may indicate an inherently advantageous phenotype of human ipDCs, as DCs with a lower level of MHCII are commonly associated with tolerance induction (Dhodapkar *et al.*, 2001; Hawiger, 2001; Li and Shi, 2015).

The overall lower percentage of T cell proliferation observed in co-cultures with ipDCs as compared to co-cultures with moDCs may be due to the potential inherent immaturity of cells, also implied by the low level of MHCII (Figure 3.5 and 3.19) and CD209 expression (Figure 3.8), or the lower percentage of DCs present in ipDC cultures as described in Figure 3.4, due to difficulties with further purification. This discrepancy and the possibility of non-DC population to potentially influence the T cell response and overall culture environment further underlines the need for a purification protocol.

In contrast to other studies, this study found no increase in the proportion of T<sub>reg</sub> populations in co-cultures with modulated moDCs or ipDCs (Macedo *et al.*, 2012; Naranjo-Gómez *et al.*, 2011; Silk *et al.*, 2012b; Stallone *et al.*, 2014; Valladeau *et al.*, 2002). This may indicate the requirement for further culture condition optimisation or the requirement for IL-6 neutralisation, as this cytokine has been shown to decrease the proportion of Tregs (Lin *et al.*, 2012). The variation, which has also been observed by other studies, most likely due to donor-to-donor variation among monocyte and T cell donors and the variations between ipDCs derived from different iPSC differentiations, became apparent in the broad spread of data for each treatment regime and therefore requires further in depth studies of the influencing factors (Macedo *et al.*, 2012).



Interestingly, the significant increase in IL-10 in supernatants of co-cultures with IL-10-modulated moDCs and ipDCs and dexamethasone-treated ipDC was not maintained after IL-2 treatment (Figure 3.35). This IL-2 treatment on day 5 may have affected the release of cytokines by T cells and DCs in co-cultures. Previous studies have shown the increase of IL-10 secretion in response to exogenous IL-2 during T cell activation (Cardone *et al.*, 2010; Caudy *et al.*, 2007; Tomova *et al.*, 2006). No such striking increase was observed in co-cultures with moDCs, however ipDCs showed a slight increase in the concentration of IL-10 following exogenous treatment of co-cultures with IL-2, but in comparison to mature untreated ipDCs, this was not statistically significant.

In the case of the detection of Tr1 cells (Figure 3.36), future work needs to attempt to establish an improved protocol for the detection of intracellular IL-10, which may involve the inclusion of monensin in addition to brefeldin A, as another lab has found this approach to improve IL-10 detection within cells (R. Andres, personal communication, February 12, 2015).

In this study, no significant effect was observed on the proportion of IL-2<sup>+</sup> or IFN $\gamma$ <sup>+</sup> T cells in response to co-culturing with differently modulated cells (Figure 3.37). In contrast to this, other studies have shown an average reduction in the proportion of IFN $\gamma$ <sup>+</sup> T cells in co-cultures with dexamethasone-treated and IL-10-treated moDCs (Chamorro *et al.*, 2009; Gregori *et al.*, 2010; Naranjo-Gómez *et al.*, 2011; Unger *et al.*, 2009). However, the concentrations of IFN $\gamma$  detected on day 5 were around 10 times lower in supernatants of ipDC co-cultures as compared to co-cultured with moDCs (Figure 3.38). This may be due to the lower proportion of CD11c<sup>+</sup> DC obtained from differentiation cultures and a higher purity of moDC cultures or the potential immaturity of ipDCs (Figure 3.4). Similar to

other cytokine profiling, it is important to keep in mind the potential presence of non-DCs and non-T cells in the ipDC and T cell co-cultures with the capacity to release IFN $\gamma$ . These might include macrophages, neutrophils and natural killer cells (Darwich *et al.*, 2009; Ellis and Beaman, 2002; Sun and Lanier, 2011).

Altogether, although the significant increase in the release of IL-10 within co-cultures and significant upregulation of ILT4 by IL-10-treated moDCs is a good indicator of a potential increase of pro-tolerogenic properties, no functional change was observed in the allostimulatory capacity of IL-10-treated moDCs (Figure 3.33). Therefore, functionally, VD3 appeared to be the most effective treatment regime, despite the disadvantage of a significantly lower cell yield and viability of moDCs subjected to VD3 modulation. On the other hand, IL-10 appeared to be the most effective treatment for the generation of potentially pro-tolerogenic ipDCs, showing a significantly increased expression of ILT3 (Figure 3.29) and ILT4 (Figure 3.30), increasing the release of IL-10 in co-cultures (Figure 3.35) and significantly reducing their allostimulatory capacity (Figure 3.33).

### **3.22 The indicators of tolerance**

As discussed previously, it is important to keep in mind that all properties that may be indicative of a pro-tolerogenic phenotype are contextual and may not lead to tolerance induction *in vivo*. For example, the secretion of IL-10 at relatively low levels may still prove pro-tolerogenic if CD28 stimulation by costimulatory molecules is low while inducible costimulatory (ICOS) signalling occurs, as T cells may become sensitised to low concentrations of IL-10 by the upregulation of the IL-10 receptor (Tuettenberg *et al.*, 2009). Costimulatory molecules may also play an important role in tolerance induction as their binding to CTLA-4 and

subsequent signalling is required for the activation of IDO in DCs. IDO orchestrates important suppressive effects on T cell proliferation by the catabolism of the essential amino acid tryptophan. Furthermore, CTLA-4-induced inhibition of the expression and production of IL-2 by effector T cells is known to limit the availability of pro-survival and expansion-promoting cytokines (Krummel and Allison, 1995; Linsley *et al.*, 1991; Munn *et al.*, 2004). Therefore, assays and readouts may be taken as an indication of a potential pro-tolerogenic phenotype, as in a context of complex immunological interactions, characteristics that are used here as an indicator of pro-tolerogenicity or pro-immunogenicity may result in a completely different outcome.

Furthermore, the previously assumed correlation between immaturity and tolerogenicity of DCs has been proven to be more complex than initially thought, as mature DCs have been found capable of expanding the T<sub>reg</sub> population while maintaining its suppressive capacity (Brinster and Shevach, 2005; Tarbell *et al.*, 2004; Yamazaki *et al.*, 2003). Therefore, the designation of properties that are reminiscent of matured DCs as pro-immunogenic and the definition of a phenotype characteristic of immature DCs as pro-tolerogenic may be misleading and is an oversimplification of a complex and dynamic phenotype.

Furthermore, the ability to detect changes in T cell allostimulatory capacity or the proportion of T<sub>regs</sub> brings up the question of what level of inhibition or proportion of T<sub>regs</sub> is required for tolerance induction *in vivo*. It is not known what level of pro-tolerogenicity is required in *in vitro* assays for the reliable translation into tolerance *in vivo*, however various animal studies have demonstrated the translatability of modulated DCs with *in vitro* capacity to expand T<sub>reg</sub> populations and inhibit T cell proliferation to a pro-tolerance phenotype *in vivo* (Dai *et al.*,

2009; Huang *et al.*, 2010). Therefore, data presented in this and in the following chapters may be a useful indicator of pro-tolerogenicity of differently modulated DCs but their actual capacity to induce tolerance requires *in vivo* studies.

Therefore, the categorization of changes in phenotypes into pro-tolerogenic versus immunogenic is an oversimplification of a complex system. Certain changes may prove pro-tolerogenic in a particular context while exerting immunogenic effects in another. Furthermore, the bipolar role of different surface molecules and cytokines can complicate the interpretation of the data in this chapter. However, keeping these limitations in mind, the scoring system provides a comprehensive summary of the results obtained in this study.

### **3.23 Technical challenges and future improvements**

As illustrated by Figure 3.3, the differentiation protocol requires further optimization in order to improve the yield of ipDCs and the reproducibility and consistency of differentiations. Past protocol development and optimisations have investigated the use of different mixtures of cytokines including BMP-4, VEGF, SCF and GM-CSF for the differentiation of ESC-derived DCs (Silk *et al.*, 2011; Tseng *et al.*, 2009). However, the inclusion of FGF in the differentiation had not been previously considered, until cell survival became an issue. In a past study, the survival of cells and generation of HSCs from ESC has been shown to be significantly improved if FGF was present in differentiation cultures (Pick *et al.*, 2007). Recent changes in the protocol have led to the alteration of the initial differentiation set up in complete mTeSR1 medium and therefore in the presence of FGF (Leishman and Fairchild, 2014). This has improved the survival of cells during the initiation of differentiations. However, being very heat-labile, FGF

would have been present for only a short time, and therefore, further addition of FGF during at least the first week or two, may significantly improve the generation of HSCs and cell viability.

Furthermore, in order to improve the purity of ipDC populations and minimize any potential interfering factors, a purification protocol needs to be developed. Previous work in our lab has looked at the purification of ipDCs by positive selection for CD11c (K. Silk, personal communication, December 7, 2010). However, using this approach, the viability of the resulting cells was significantly compromised. Therefore, another protocol would be required which may make use of positive selection kits for the purification of CD14<sup>+</sup> cells at the ipMonocytes harvest stage or negative selection kits for myeloid DCs (J. Buchresier, personal communication, September 3, 2014). The ability to purify ipMonocytes using CD14<sup>+</sup> selection beads is currently being investigated in our laboratory.

The characterisation of contaminating non-DCs is required in order to allow a better understanding of potential interfering populations and their potential effect on the results obtained in this study. Similarly, the understanding of the types of cells present in these differentiation cultures and how they may influence the effect of modulated DCs on T cells is essential for the potential application of DCs in a clinical setting. The study of other interfering cells and factors found within the body will therefore form an essential part of the future investigation of the clinical applicability of moDCs and ipDCs.

Furthermore, the use of multi-colour flow cytometry allowing the use of more than 4 concomitant fluorochromes would have enabled a more detailed analysis of

different DC subpopulations and other undefined cell populations obtained from differentiations.

It is important to note that the readouts for pro-tolerogenic potential were limiting. The difficulty of MHC-matching to allow for syngeneic rather than allogeneic co-cultures limits the transferability of these results to potential clinical translation. Obtaining blood donations of particular haplotypes from the clinic is difficult and other sources such as volunteers may be unsafe. The MHC-matching further complicates the variation of results between different donors of monocytes and T cells as different degrees of MHC mismatching may significantly affect the immunological outcome. This variation may be reduced in the studies of ipDCs as the MHC haplotype of ipDCs remains unchanged with only the haplotype of the T cell donors changing which may complicate the comparability of the results obtained from moDC and ipDC populations and co-cultures.

The bimodal expression profile observed on modulated DCs complicates the use of the gMFI, as it may obscure the presence of two populations. The gMFI of a population displaying negative or low expression of a particular marker together with a population expressing high levels portrays the presence of a single population with a medium expression profile. Therefore, data may further imply the need for purifying populations with high immunoinhibitory molecule expression for the generation of a pure population of pro-tolerogenic DCs.

However, the staining methodology and gating strategy for the analysis of flow cytometry data could have been improved by the staining of control samples for the expression of CD11c, which may have circumvented the occurrence of bimodal populations in isotype control stained samples. This would have enabled

the determination of isotype-specific background levels of the CD11c-expressing population in particular. This would have allowed for a more appropriate and accurate control for the determination of the level of marker expression by CD11c-expressing cells.

### **3.24 Conclusion**

Altogether, these data highlight the potential modulatory capacity of IL-10 for the generation of pro-tolerogenic ipDCs and VD3 for the generation of pro-tolerogenic moDCs. Therefore, in order to investigate the transferability of the ipDC differentiation protocol and its pro-tolerogenic modulation to the potential application to LSD patients, the next chapter explores the potential of differentiating ipDCs from Pompe disease patient-derived iPSCs and their susceptibility to IL-10-mediated modulation.

## Chapter 4 The derivation of iPSCs from Pompe disease patients and their differentiation into tolerogenic ipDCs





## Introduction

Having explored a number of agents reported to have a pro-tolerogenic effect on DC function, the previous chapter demonstrated the potential use of IL-10 for the generation of patient-specific pro-tolerogenic ipDCs. However, the feasibility of producing DCs from patient-derived iPSCs and their pro-tolerogenic modulation was demonstrated using a healthy donor. Therefore, as a first step towards investigating the clinical potential of this method for improving the treatment efficacy of LSDs, the feasibility of reprogramming fibroblasts from individuals with compromised health, the subsequent viability of iPSCs in culture and their capacity to generate ipDCs needed to be explored.

The capacity to derive iPSCs from a Pompe disease mouse model and to differentiate these into skeletal muscle cells has been shown (Kawagoe *et al.*, 2011). Furthermore, previous studies have demonstrated the feasibility of reprogramming fibroblasts that had been derived from patients diagnosed with Pompe disease, Gaucher disease, Fabry disease, Hurler syndrome and Niemann-Pick type C (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Kawagoe *et al.*, 2013; Lemonnier *et al.*, 2011; Panicker *et al.*, 2012; Park, 2008; Tolar *et al.*, 2011; Trilck *et al.*, 2013). The capacity of iPSCs derived from Pompe patients to form endoderm, mesoderm and ectoderm tissue on differentiation and their guided differentiation into cardiomyocyte-like cells has been demonstrated (Huang *et al.*, 2011). However, the study by Huang *et al.* (2011) found that the reprogramming of fibroblast from Pompe disease patients was only successful in the presence of GAA delivered in the form of a doxycycline-inducible human GAA-expressing cDNA that had been transfected into the cells using lentiviral vectors. In contrast to this study, other studies showed the feasibility of reprogramming fibroblasts

from LSD patients, including Pompe disease, in the absence of any supplementary enzyme (Higuchi *et al.*, 2014; Kawagoe *et al.*, 2013; Panicker *et al.*, 2012; Raval *et al.*, 2015). When considering the reprogramming protocols used by Huang *et al.* (2011) and Higuchi *et al.* (2014) it appears that the only major difference were the mutations of the fibroblast donors. Therefore, future studies may further explore the viability and permissibility to reprogramming of fibroblasts taken from patients with different mutations. Technical failures in the study by Huang and his colleagues (2011) appear unlikely as healthy control cells were successfully reprogrammed alongside the attempted Pompe disease reprogramming.

As the treatment of LSDs, including Pompe disease, has been limited in a number of cases by the immunogenicity of the therapeutic enzyme and the consequential induction of an antibody-mediated immune response, a tolerance induction regime may greatly improve outcomes (Banugaria *et al.*, 2011; Brooks *et al.*, 2003; Desnick and Schuchman, 2012; Furusawa *et al.*, 2012; Hunt, 2009; Kishnani *et al.*, 2010; Rosenberg *et al.*, 2013; Vedder *et al.*, 2008). Current approaches have shown some successes using mouse models and clinical trials to investigate the potential of different treatment approaches or conditioning regimes such as gene therapy and HSCT (Douillard-Guilloux *et al.*, 2009; Franco *et al.*, 2005; Sun *et al.*, 2007, 2010; van Til *et al.*, 2010; Zhang *et al.*, 2012). The current clinically applied strategy constitutes a harsh immune conditioning regime in which naïve and proliferating B cells and proliferating T cells are eliminated, leaving the patient immune compromised and vulnerable to infections and malignancies (Banugaria *et al.*, 2013; Chan and Cronstein, 2002; Messinger *et al.*, 2012). Therefore, this thesis has been exploring an alternative approach,

which aimed at exploiting DCs for the induction of antigen-specific tolerance, allowing the maintenance of a functioning immune system while specifically tolerating a defined protein antigen.

Having explored this concept using healthy iPSCs, this chapter investigated the possibility of reprogramming fibroblasts, which had been obtained from a patient with Pompe disease, into patient-specific iPSCs and maintaining these cells in culture. Furthermore, their capacity to differentiate and generate ipDCs was investigated. The capacity to modulate these ipDCs with IL-10 for the generation of a population of patient-specific pro-tolerogenic ipDCs was investigated.

## **Results**

### **4.1 The generation of iPSCs from a Pompe disease patient**

Fibroblasts were obtained from a patient with infantile-onset Pompe disease. As described in Table 4.1, the patient had a relatively rare homozygous missense mutation at nucleotide 877 which led to the exchange of the amino acid glycine at position 293 with arginine (Hermans *et al.*, 2004; Joshi *et al.*, 2008; Montalvo *et al.*, 2006; Müller-Felber *et al.*, 2007; Pittis *et al.*, 2008; Sharma *et al.*, 2005). Western blotting performed in the clinic showed residual enzyme expression in the patient's cells who was, therefore, defined as CRIM<sup>+</sup> (P. Giessen, personal communication, December 3, 2014).

At the age of 5 months, the patient received the first dose of therapeutic enzyme, however no clinical response was observed throughout the treatment. The potential of the induction of an antibody-mediated immune response towards the

therapeutic enzyme had not been investigated. However, the patient showed no clinical or pathological improvement and died at the age of 12 months.

---

|                                      |                                                   |
|--------------------------------------|---------------------------------------------------|
| CRIM Status:                         | Positive                                          |
| Mutation:                            | Homozygous missense mutation, Gly293Arg (877 G>A) |
| Treatment received:                  | ERT                                               |
| Age at treatment initiation:         | 5 months                                          |
| Clinical response to treatment:      | No response detected                              |
| Age at death:                        | 12 months                                         |
| Immunological response to treatment: | Not determined                                    |

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**Table 4.1 Clinical details describing the diagnosis and treatment of the Pompe disease patient.**

The fibroblasts were expanded in cultures and reprogrammed in the lab by Tim Davies using the Sendai virus carrying vectors encoding pluripotency factors Oct3/4, Sox2, Klf4 and c-Myc (Fusaki *et al.*, 2009; Macarthur *et al.*, 2012). After 7 days, morphological differences were observed between cells that had been treated with Sendai virus and untreated controls (Figure 4.1). While untreated controls retained the morphological appearance of fibroblasts, reprogrammed cultures gave rise to rounded cells with large nuclei. By day 28, reprogrammed cells formed large ESC-like colonies on feeder cells which were composed of small rounded cells with large nuclei (Figure 4.1). Reprogrammed cells were incubated for approximately 1 hour in mTeSR medium and subsequently harvested by mechanical cutting of colonies away from feeder cells and directly transferring them into Matrigel-covered 6-well plates. Within 1 day, cells had

attached and formed colonies reminiscent of healthy iPSCs (Figure 4.1 and Figure 3.1).

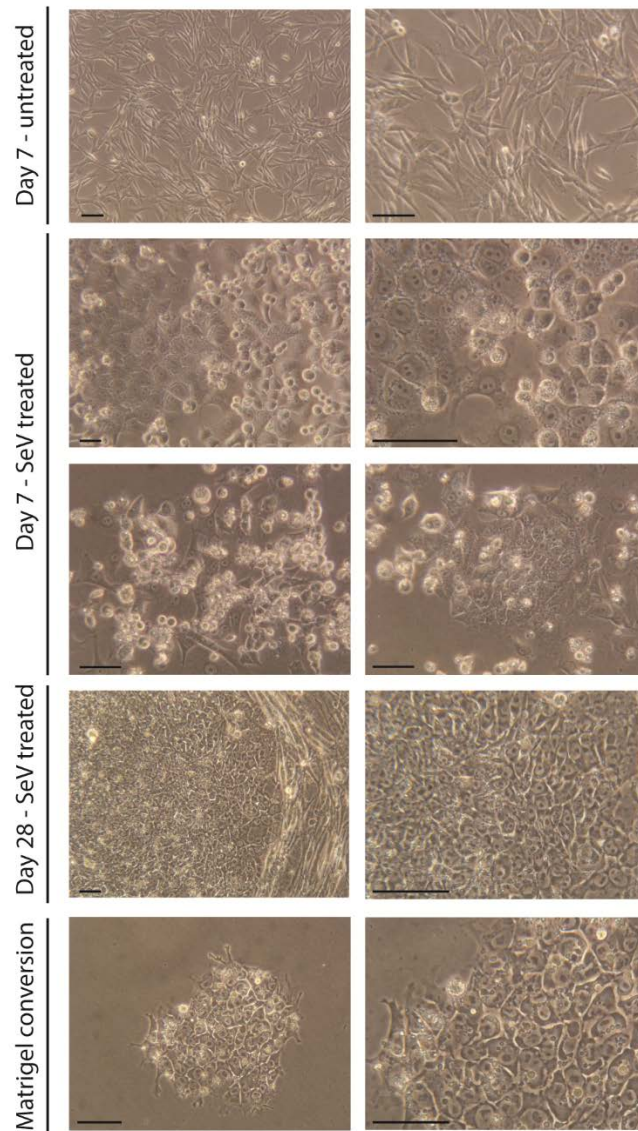


**Figure 4.1 The reprogramming of human Pompe-disease fibroblast.**

Fibroblasts at passage 6 or above were expanded in culture and reprogrammed by Tim Davies using the Cytotune Sendai virus reprogramming kit. Reprogrammed cultures were regularly passaged onto feeder cells every 5-6 days starting 6 days after reprogramming. Photomicrographs illustrate the cell morphology and organisation of reprogrammed and control cultures on day 7 and day 28. Once stem cell-like colonies became apparent, colonies were manually harvested from feeder cell cultures by cutting and transferred onto feeder-free Matrigel-covered plates which was referred to as passage 1. Scale bars: 100  $\mu$ m.

No problems were encountered during the expansion of reprogrammed fibroblasts which were grown continuously until passage 26. As shown in Figure 4.2A, reprogrammed fibroblasts maintained the iPSC-like morphology throughout all passages. Furthermore, reprogrammed cells were shown to be capable of generating teratomas *in vivo* and forming tissues of putative endodermal, ectodermal and mesodermal origin (Figure 4.2.B-E). In order to determine the karyotype of the cells, metaphase spreads were produced from cells at 70% confluence from a 6-well plate and M-FISH was performed by the Chromosome Dynamics Core at the Wellcome Trust Centre for Human Genetics in Oxford. Out of 40 metaphase spreads, one abnormal karyotype was found with a trisomy of chromosome 17 and a monosomy of chromosome 20. This metaphase spread was deemed invalid due to a technical error possibly causing chromosome cross-contamination between burst cells. Therefore, M-FISH confirmed a normal female karyotype of 46 chromosomes (Figure 4.2F). In order to determine whether reprogrammed cells expressed pluripotency markers, cells were harvested, stained and analysed by flow cytometry. Results confirmed the expression of pluripotency markers Oct3/4, SSEA-4 and Nanog (Figure 4.2G) (International Stem Cell Initiative *et al.*, 2007; Mitsui *et al.*, 2003; Nichols *et al.*, 1998; Saunders *et al.*, 2013). Whether Oct3/4 was endogenously expressed or whether residual viral RNA was present was not determined, however the expression of SSEA-4 and Nanog demonstrated the endogenous induction of pluripotency genes. From this point on, reprogrammed Pompe disease fibroblasts were referred to as ipPo. Five ipPo lines were produced named ipPo0, ipPo1, ipPo2, ipPo3 and ipPo4.

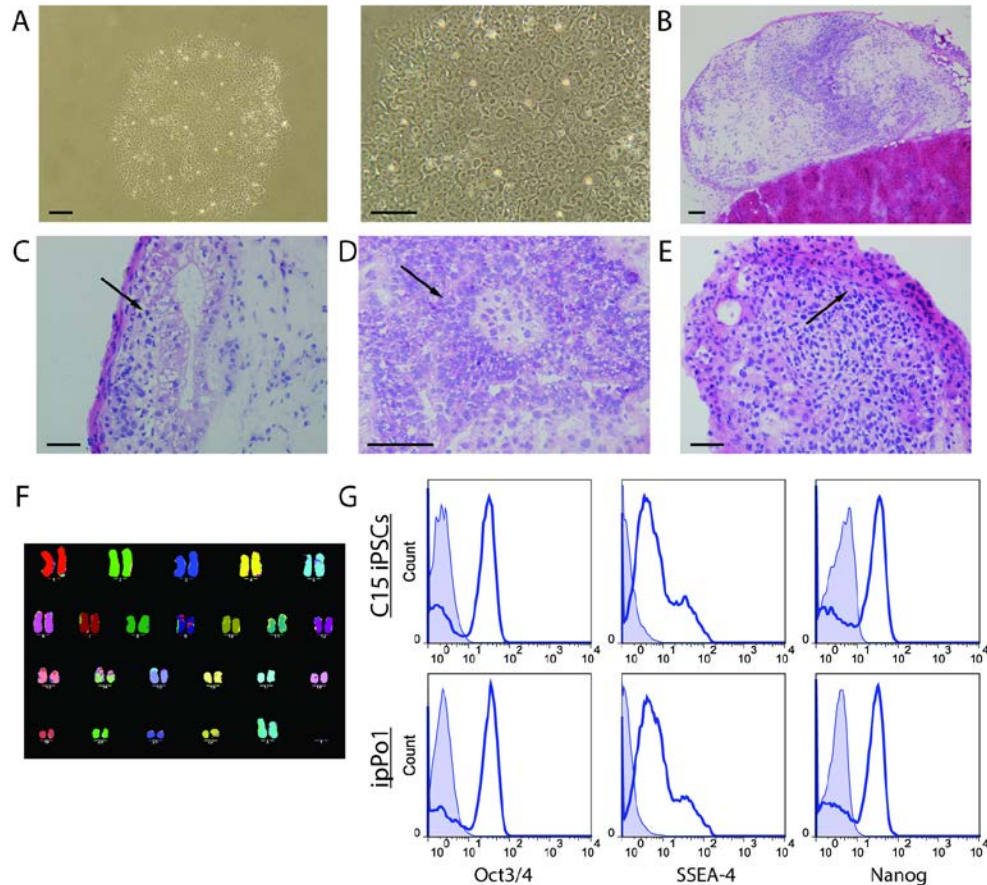


**Figure 4.2 The pluripotency of Pompe disease-specific iPSCs.** (A) Photomicrographs show the stem cell-like morphology of the ipPo1 line at passage 1 which had been reprogrammed from the fibroblasts of a patient with infantile-onset Pompe disease. Scale bars: 100  $\mu$ m. (B-E) ipPo1 were differentiated at passage 20 for 7 days for the formation of EBs, which were transplanted under the kidney capsule of CBA.RAG1<sup>-/-</sup> mice by Tim Davies. After 14 days, the kidneys were excised and embedded in OTC compound for sectioning. Sections 7  $\mu$ m thick were processed and stained with haematoxylin and eosin. (B) Displayed is a teratoma (arrow) beneath the kidney capsule. Photomicrographs show regions within the teratoma which morphologically resemble one of the three germ layers with (C) showing a putative endodermal structure with a glandular appearance, (D) showing putative ectoderm with a neuronal rosette-like structure and (E) showing putative mesoderm with smooth muscle-like appearance. Scale bars: 100  $\mu$ m. (F) M-FISH was performed in order to investigate the karyotype of ipPo1 cells at passage 19. Metaphase spreads were obtained from 40 individual cells and analysed using Cytovision (Leica) software. (G) C15 iPSCs (passage 13) and ipPo1s (passage 11) were harvested by EDTA dissociation, stained for the cell surface expression of SSEA-4 and intracellular expression of transcription factors Nanog and Oct3/4 and analysed by flow cytometry.



DNA was purified from ipPo0 cells and sent for tissue typing in order to determine the HLA-haplotype of the patient for future identification of an HLA-matched T cell donor for antigen-specific studies (Table 4.2).

As all ipPo lines appeared phenotypically normal in culture, the GAA enzyme activity was measured and the accumulation of glycogen investigated in order to confirm the disease phenotype.

| Locus    | Allele                    | Allele                                                                                                                                                    |
|----------|---------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| HLA-A    | *02:11P                   | *24:02/02Q/09N/11N/40N/76/79/83N/135/144/150/153/154/155N/163N/183N/192/193/195/196/202/203/205/209/212/217/221/222N/223-226/230/231/232N/233/237/242-245 |
| HLA-B    | *08:01/19N/109            | *51:01/11N/30/32/48/51/142                                                                                                                                |
| HLA-DRB1 | *03:01/50/68N/82/83/86/89 | *13:01/105/109/112/117/121/143/147/148/153                                                                                                                |
| HLA-DQB1 | *02:01/27                 | *06:03P                                                                                                                                                   |
| HLA-DPB1 | *03:01:01G                | *04:01:01G                                                                                                                                                |
| HLA-DQA1 | *01:03/10                 | *05:01P                                                                                                                                                   |
| HLA-DPA1 | *01:03P                   | -                                                                                                                                                         |

**Table 4.2 HLA-haplotype of Pompe disease patient.** DNA was purified from ipPo0 cells at passage 9 and tissue typed by Proimmune. Wherever more than 2 variants of an allele are given, any of the variants are possible, however the first being more likely than the following ones. The HLA-DQB1\*02:01/27 may therefore be HLA-DQB1\*02:01 or HLA-DQB1\*02:27, however the first option is more likely than the second.

## 4.2 The deficiency of GAA activity and the accumulation of glycogen in ipPo cells

In order to determine the level of GAA activity in ipPo cells as compared to wild type C15 iPSCs, iPSCs were harvested from C15 and ipPo1 iPSC cultures and lysed by sonication. Sonicates were used to measure the activity of GAA at pH 3.8 using 4-Methylumbelliferyl  $\alpha$ -D-glucoside as a substrate. As a control, activity was measured at pH 6.5 which measured the activity of non-lysosomal glucosidases. Pre-treatment of sonicates with acidic pH inactivated the control enzymes ensuring that the activity of non-lysosomal enzymes was eliminated while the activity of acidic glucosidase remained relatively unaffected (although it may increase or decrease slightly depending on the pH for optimal enzymatic activity). As shown in Figure 4.3, sonicates obtained from ipPo1 showed significantly lower glucosidase activity at pH 3.8 as compared to sonicates obtained from healthy C15 iPSCs (C15 iPSCs:  $2.6 \pm 0.21$  nmol/h/mg protein ( $n=3$ ), ipPo1:  $0.4 \pm 0.21$  nmol/h/mg protein ( $n=3$ ),  $p = 0.005$ ). The level of activity detected in ipPo1 samples was below the background levels detected in “water only” control samples and therefore the data indicate the absence of any residual GAA activity. This difference was not observed when glycosidase activity was measured at pH 6.5, confirming that the enzyme deficiency was confined to enzymes functional at lysosomal pH.

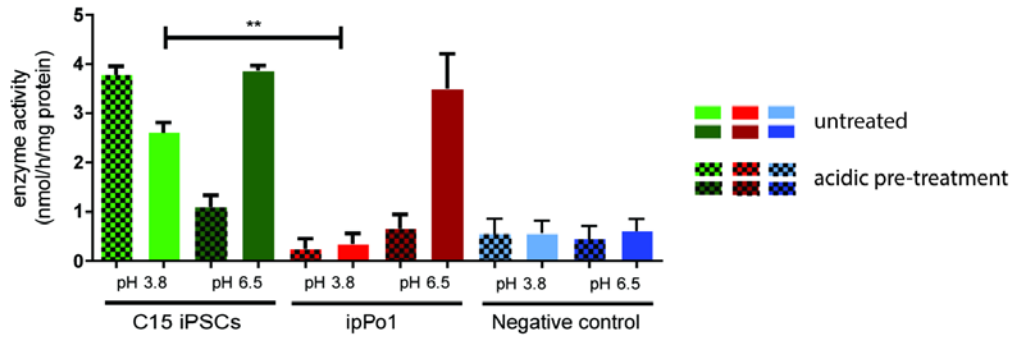


**Figure 4.3 The functional deficiency of GAA in ipPo cells.** ipPo1 cells at passages 17 to 20 and control C15 iPSCs at passages 21 to 24 were harvested by EDTA dissociation, washed and lysed by sonication. Sonicates were assayed for the activity of lysosomal and non-lysosomal glucosidases at pH 3.8 and pH 6.5 respectively. In order to confirm that the activity detected at pH 6.5 measures non-lysosomal enzymes, unlike the activity detected at acidic pH, sonicates were pre-treated with an acidic buffer to inactivate non-lysosomal glucosidases (chequered bars). Furthermore, a control samples of water was included in order to account for any potential enzymatic contamination of reagents. Bars represent the mean activity (nmol/h/mg/protein)  $\pm$  SEM of 3 independent experiments. Statistical analyses was performed using the paired T test (\*\* $p \leq 0.01$ ).

In order to investigate whether ipPo featured the characteristic glycogen accumulation of Pompe disease, ipPo1 cells were cultured on coverslips and stained for carbohydrates including glycogen using periodic-acid Schiff staining. Imaging using light microscopy showed that the majority of ipPo1 colonies resembled the morphology and staining displayed by healthy C15 iPSCs colonies (Figure 4.4A/B). However, larger colonies showed excessive positive staining for carbohydrates in areas of highest cell density, indicating that large ipPo colonies may start accumulating glycogen once they have grown close to confluency or once cells begin to differentiate (Figure 4.4C). However, the strong staining in the centre of the colonies may also be a result of the higher cell number and cell density. Therefore, a more specific method such as a fluorimetric assay will be used in the future to verify the accumulation of glycogen within ipPo1 iPSCs (Pelletier *et al.*, 2013).

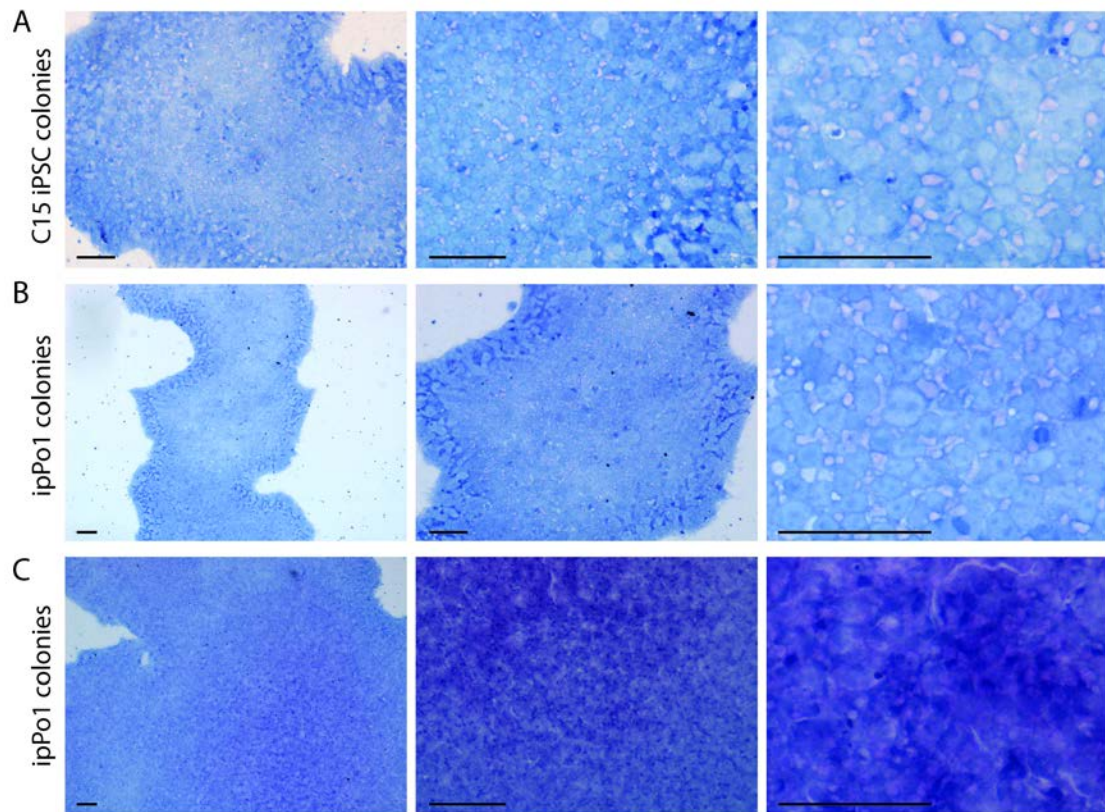


**Figure 4.4 The glycogen accumulation in ipPos.** C15 iPSCs (A) at passage 24 and ipPo1 (B/C) at passage 22 were grown on coverslips until close to 80% confluence. Cultures were fixed in 4% PFA and stained using a periodic-acid Schiff staining kit (purple). Counter staining was performed using hematoxylin (blue). Images are representative of 2 independent experiments. Scale bars: 100  $\mu$ m.

Having confirmed the absence of GAA activity in ipPo1 cells, their capacity to differentiate into ipDCs was investigated, as it may be limited by the disease phenotype. The capacity to differentiate into ipDCs is essential for the potential of DC-mediated immunomodulation and the induction of enzyme-specific tolerance.

### **4.3 The differentiation of ipPos into patient-specific ipDCs**

In order to determine and compare the capacity of ipPo differentiation into ipDCs with that of C15, differentiations were set up simultaneously using C15 iPSCs and ipPo0 according to the differentiation protocol previously described in Figure 3.2. iPSCs were harvested as clusters using a short treatment with EDTA and plated into ULA plates to promote EB formation. One day after the onset of differentiation, C15 iPSCs had formed large EBs, that were visible by the naked eye, while no similar structures were found in differentiations of ipPo0 (Figure 4.5).

Similarly, on day 4, C15 iPSC differentiations contained numerous large EBs while ipPo0 differentiations remained free of any EB-like structures. Sheets and clusters of ipPo0 cells were present, similar to those created during the harvesting process, however no signs of further aggregation were observed (Figure 4.5). C15 differentiations produced viable ipDCs while ipPo0 differentiations did not generate any viable cells. The ipPo0 line was further expanded and a master cell bank produced, however further differentiations and experiments were performed using ipPo1.

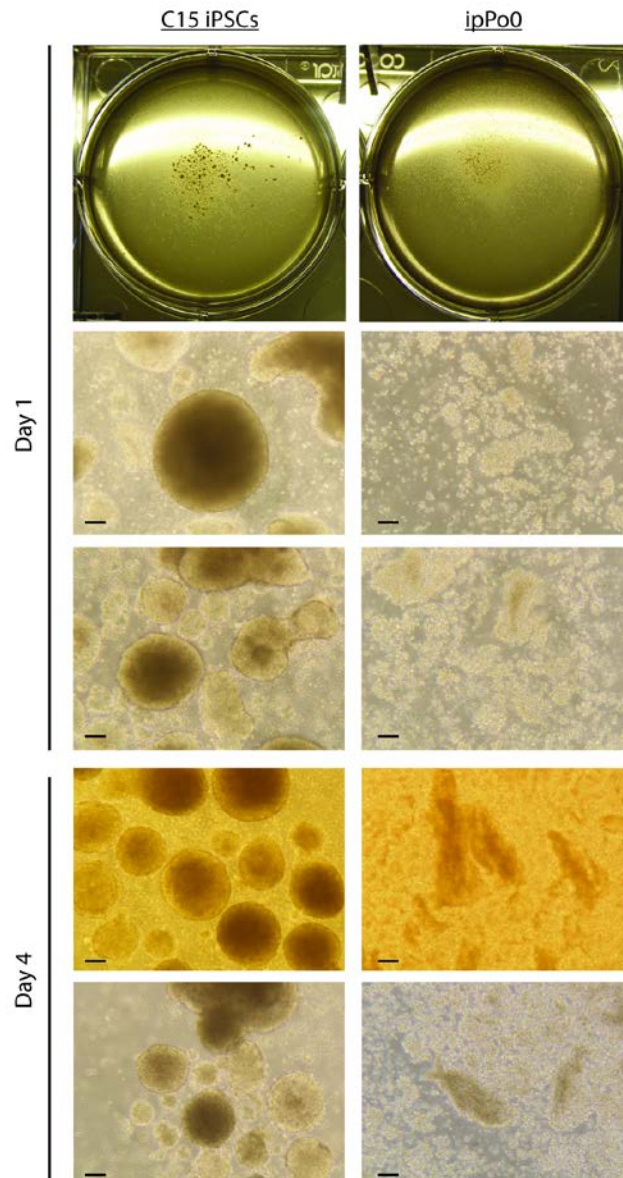


**Figure 4.5 The inability of ipPo cells to form EBs.** ipPo0 and C15 iPSCs were harvested by EDTA dissociation at passage 10 and passage 12 respectively and plated at  $3 \times 10^6$  per well into ULA plates. Photomicrographs were taken on day 1 and 4 of differentiation. Scale bars: 100  $\mu\text{m}$ .

#### 4.3.1 Setting up alternative differentiation protocols

In order to circumvent these difficulties, 4 different methods of initiating differentiations were tested using ipPo1. One approach constituted the standard EDTA dissociation method, while another made use of mechanical cutting of small patches of iPSC colonies, which were subsequently plated into ULA plates for the formation of EBs. Another approach was the differentiation of cells directly from iPSCs *in situ* while remaining attached to Matrigel (“adherent” approach). After 3 days, half of the “adherent” fraction was cut into small patches and plated into ULA plates to allow for EB formation, while the other half was left to continue differentiating while remaining adherent.

After 1 day, EBs had formed in differentiations set up by EDTA dissociation, similar to those obtained in C15 iPSC differentiations (Figure 3.2B), and mechanical cutting (Figure 4.6). Free clusters and ipPo1 patches were also observed in cultures. Cultures with adherent ipPo1s showed cells with large round nuclei, elongated cells around the periphery of colonies and small round differentiating cells lifting off the centre of the colonies. At day 3, EBs obtained from EDTA dissociation cultures and cultures cut manually had grown in size and number. The size and number of EBs obtained from the EDTA dissociation protocol were similar to the appearance of differentiating C15 iPSCs (Figure 3.2B). Cells with a cobblestone-like appearance emerged in adherent cultures (bottom image) and large cell clusters formed on top of cultures. Some wells of the adherent cultures had been harvested on day 3, which, 2 days later, showed ribbons and sheets of cells (Figure 4.7). No EB-like structures were observed in these cultures. By day 5, it became apparent that the EDTA dissociation method



gave rise to more numerous but smaller EBs as compared to mechanical cutting which gave rise to fewer but larger EBs.

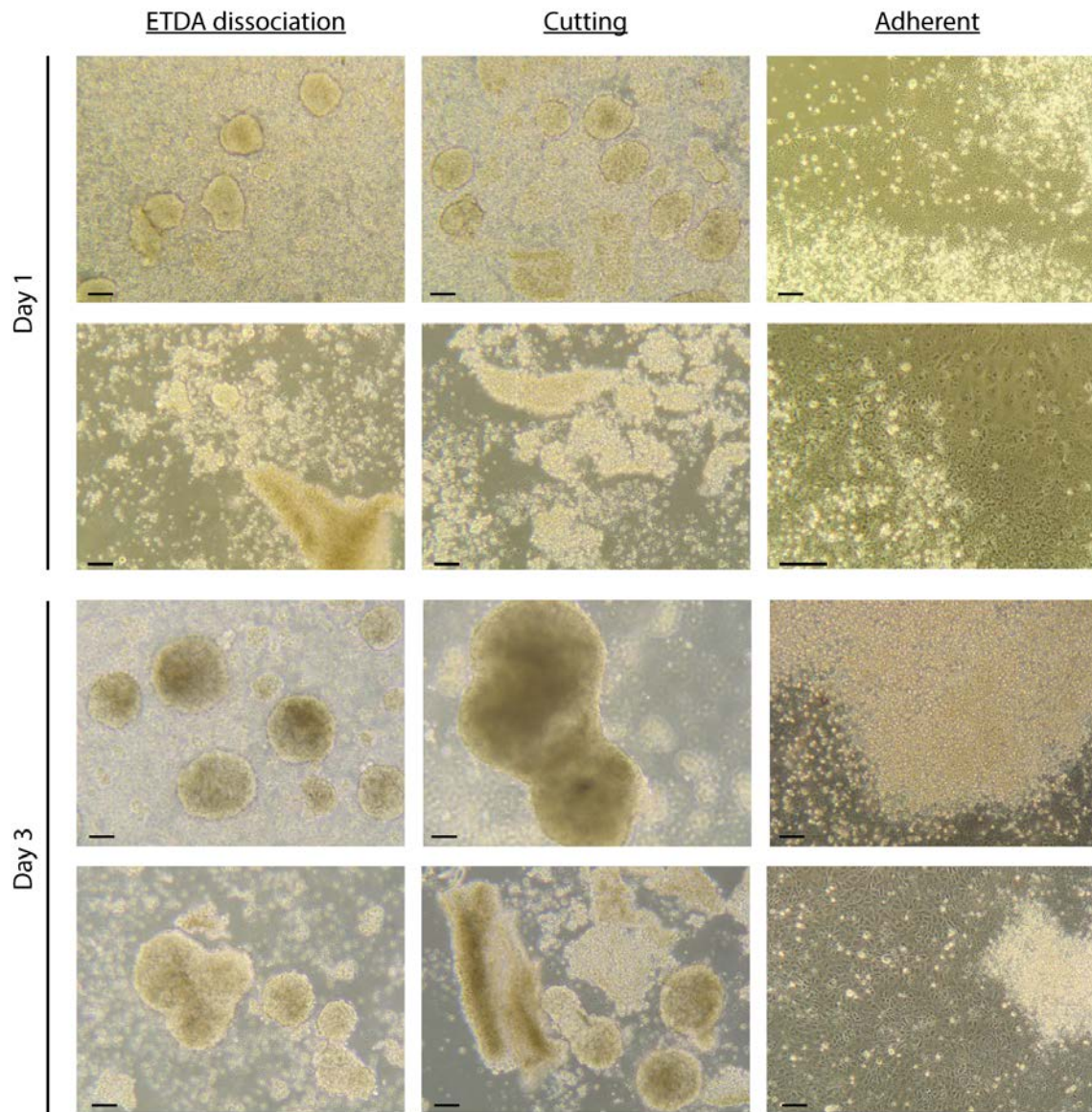


**Figure 4.6 The differentiation of ipPo cells using different methods: Days 1 and 3.** ipPo1s were differentiated by 4 different methods at passage 14. First of all, ipPo1s were harvested by the standard EDTA dissociation method. Secondly, ipPo1 colonies were mechanically cut and cell patches transferred into ULA plates. For the other 2 approaches, the differentiation was initiated in situ with iPSC colonies attached to Matrigel-covered plates. Scale bars: 100  $\mu$ m.



Furthermore, EBs in EDTA dissociation cultures appeared more homogenous in size as compared to cut cultures. The cultures that had been left adherent formed neuronal-like structures and networks suggesting a propensity for ectodermal commitment and were not therefore pursued further.

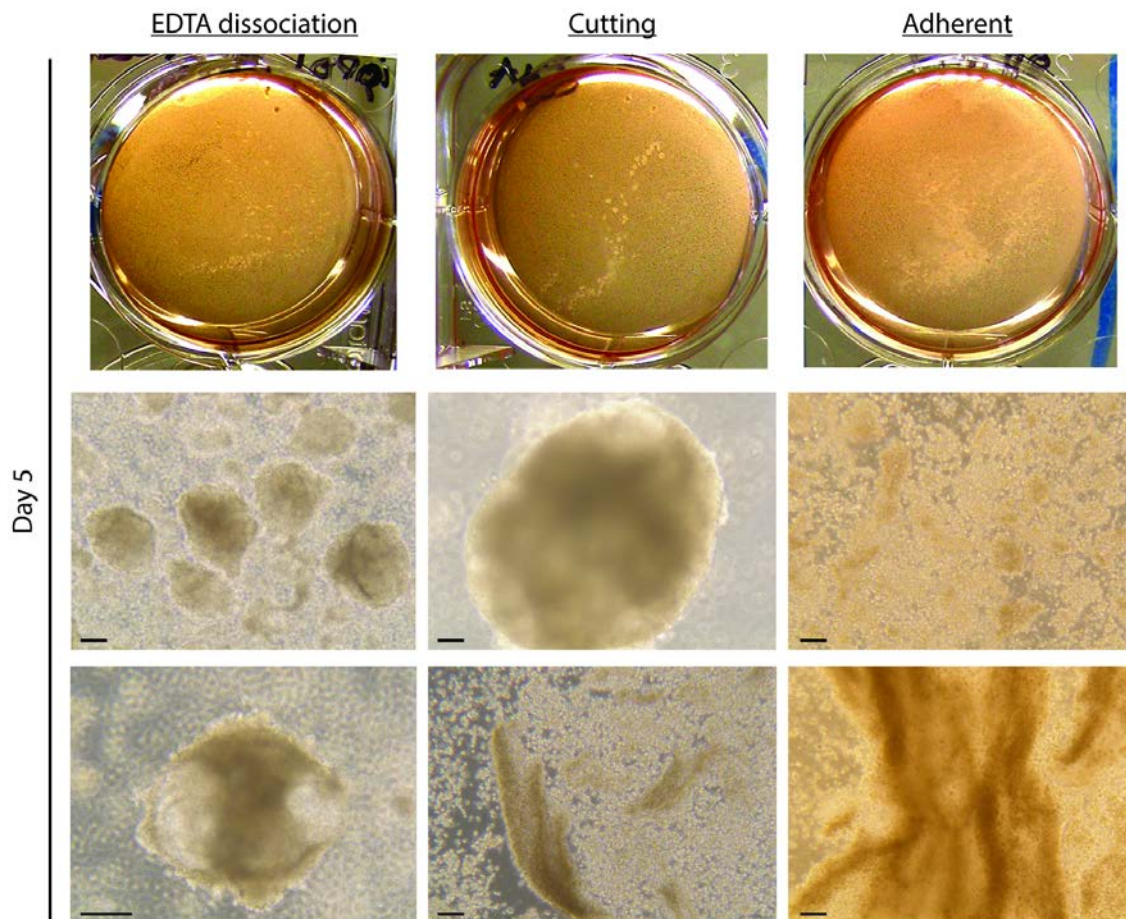


**Figure 4.7 The differentiation of ipPo cells using different methods: Day 5.** At passage 14, ipPo1s were differentiated using 4 different methodologies. Differentiations initiated by EDTA dissociation, cutting and “adherent” culture were fed every 2-3 days according to the standard differentiation protocol. However, after 3 days of “adherent” differentiation, half of the attached cultures were harvested by cutting and scraping, while the other half was left attached for the full course of the differentiation. Scale bars: 100  $\mu$ m.

By day 12, as displayed in Figure 4.8, monocyte-like precursor cells appeared in cultures generated by EDTA dissociation and mechanical cutting, which phenotypically resembled the cells observed in C15 iPSC differentiations (Figure 3.2B). No such cells were observed in adherent cultures that had been harvested on day 3. Due to the appearance of apoptotic and dead cells, the adherent cultures harvested on day 3 were terminated and not further examined. Further observation of the remaining 2 differentiation methods, showed DC-like cells with long protrusions and cytoplasmic veil-like morphology appearing in cultures, which clearly resembled ipDCs generated from healthy C15 iPSCs (Figure 3.2B). Large numbers of DC-like cells were observed early on in EDTA dissociation cultures and therefore harvested as early as day 16 while cut cultures were left for a further 5 days in order to allow the formation of a larger number of DC-like cells.

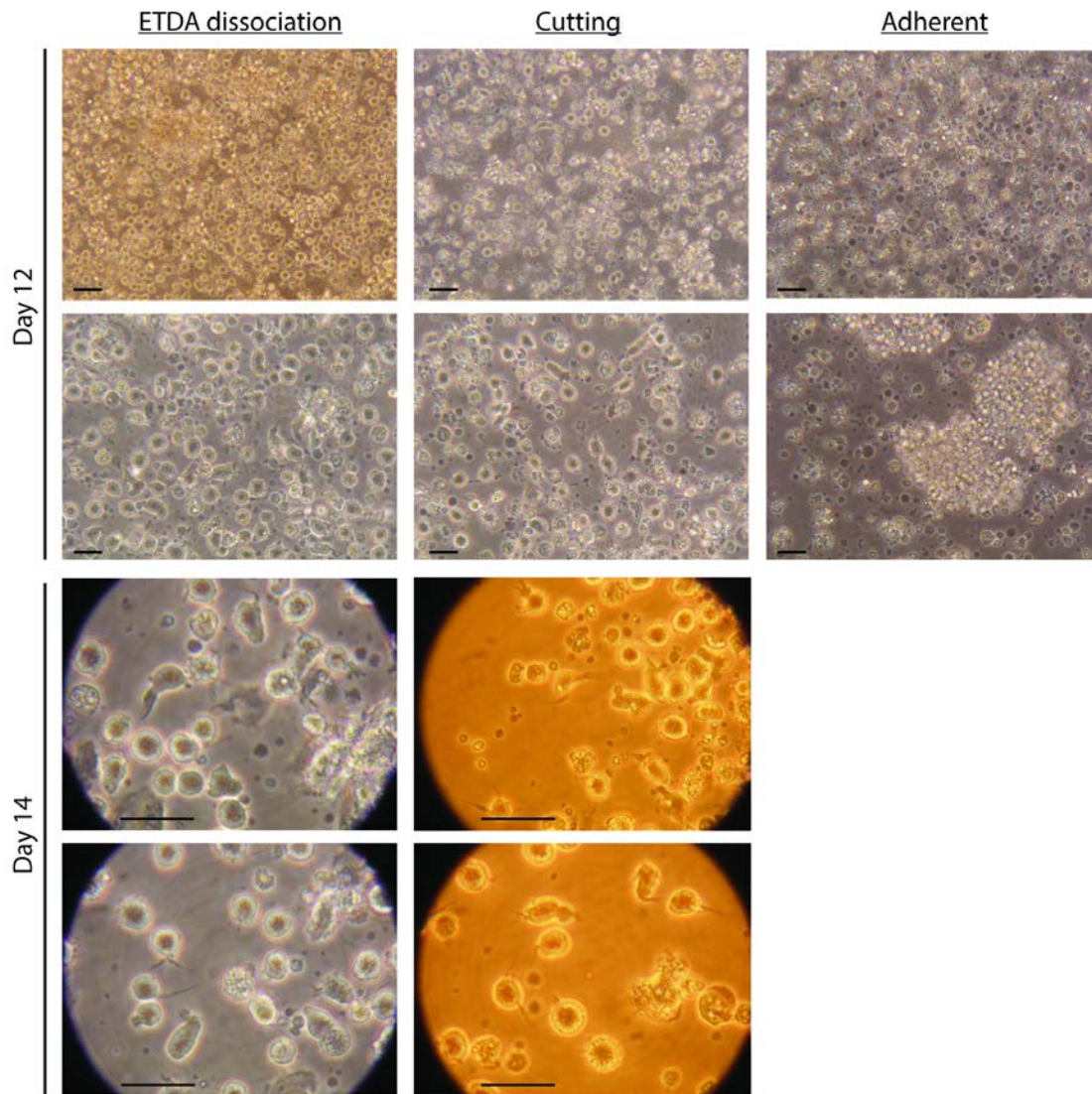


**Figure 4.8 The differentiation of ipPo cells using different methods: Days 12 and 14.** Photomicrographs display the differentiations of ipPo1s (passage 14) which were observed for the appearance of monocyte and DC-like cells and precursors.

In order to investigate whether the cells that had been generated phenotypically resembled DCs, DC and precursor-like cells were harvested and analysed by flow cytometry for the expression of DC markers. CD11c expression was detected on populations generated by both protocols indicating the presence of ipDCs (Figure 4.9A). By gating on CD11c<sup>+</sup> cells, cells were found to express low levels of MHCII, which is characteristic of immature DCs and low levels of CD11b,

CD14 and CD123, indicating a myeloid-like lineage. This expression profile was similar to that observed with C15 iPSC-derived ipDCs (Figure 3.5 and Figure 3.6). The higher expression of CD11c and the larger proportion of CD11c<sup>+</sup> cells at around 45% in the differentiations derived by cutting as compared to the EDTA dissociation may be due to the longer period of differentiation. This may have enabled precursors to become fully committed to the DC lineage allowing the further upregulation of DC markers as compared to dissociation cultures which had been harvested 5 days earlier and gave rise to around 30% CD11c<sup>+</sup> cells (Figure 4.9B). Both methods therefore gave rise to a yield and proportion of CD11c<sup>+</sup> cells within the range of that obtained from C15 iPSC differentiations (Figure 3.3 and Figure 3.4).

However, considering the number of ipMonocytes produced by both differentiation procedures, cut cultures did not produce the number of cells observed in dissociation cultures, despite the extended culture period. EDTA dissociation gave rise to a yield of 94% while cut cultures showed a yield of around 50% (Figure 4.9C). Due to the difference in cell yield and in order to increase the comparability of ipPo1 differentiations to C15 iPSC differentiations, the EDTA dissociation protocol was used for all subsequent differentiations. The reason for the previous failure to differentiate ipPo0 was not investigated, however the residual expression of reprogramming factors from viral RNA may have counteracted the tendency to initiate differentiation or the cells may not have fully adapted to the culture conditions.

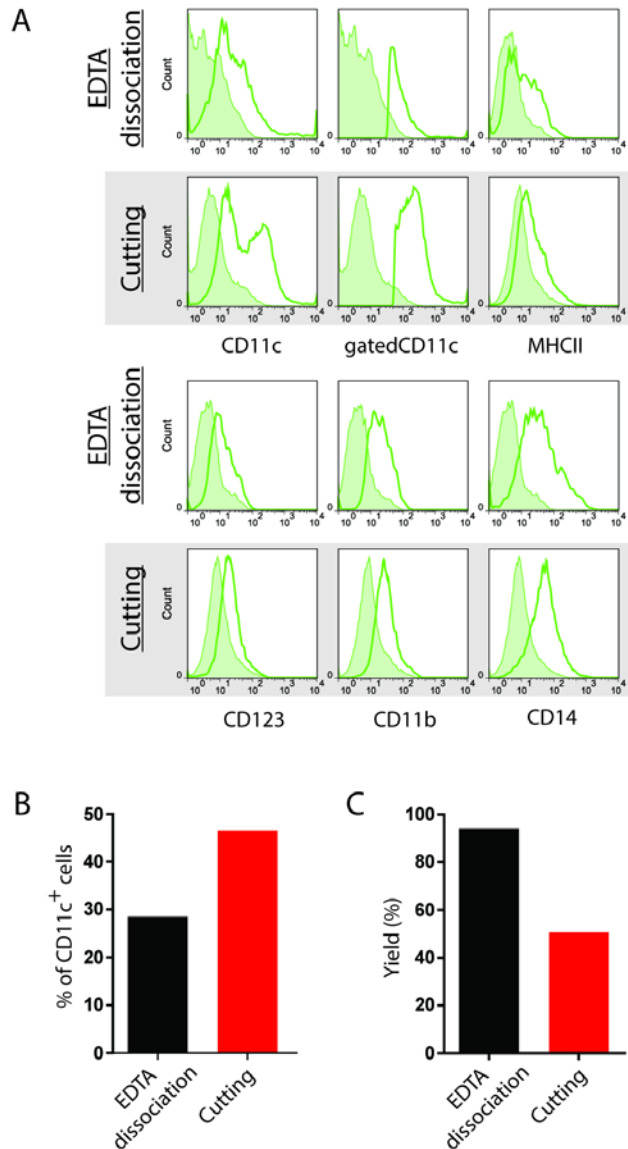


**Figure 4.9 The efficiency of different differentiation protocols in the generation of ipDCs.** DC and precursor-like cells were harvested from differentiations that had been set up by EDTA dissociation or mechanical cutting of ipPo1 at passage 14. (A) These cells were stained for the expression of DC markers and analysed by flow cytometry. (B) The percentage of CD11c<sup>+</sup> cells present in the cell populations harvested at the end of differentiations was determined. (C) The percentage of ipMonocytes recovered from cultures was determined relative to the number of cells initially plated. Data shown were obtained from a single experiment.

This difference in the capacity for guided differentiation between iPSC clones was also observed in other studies, which may be influenced by the parental cell's

unique mutational history (Thatava *et al.*, 2013; Young *et al.*, 2012). Therefore, as ipPo1s have been shown to have a normal karyotype at passage 19 and show the ability to differentiate into ipDCs, the ipPo1 cell line was used in all further studies.

#### 4.4 The characterisation of ipDCs derived from Pompe disease iPSCs

Having devised a protocol for the successful differentiation of ipPo1, the generated ipDCs were characterised by flow cytometry. However, in contrast to the experiment conducted for the determination of an effective differentiation protocol, the yields of ipMonocytes obtained from subsequent differentiations of ipPo1 were lower (although statistically not significant) with an average yield of around  $23.97 \pm 14.23\%$  ( $n=6$ ) as compared to an average yield of  $71.92 \pm 13.12\%$  ( $n=18$ ) from C15 iPSC differentiations (Figure 3.3), possibly suggesting that disease pathology may adversely affect the differentiation of iPSCs (Figure 4.10).

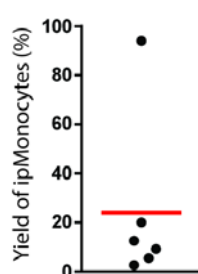


**Figure 4.10 The yield of ipMonocytes and CD11c<sup>+</sup> ipDCs and their viability and recovery from cultures.** The percentage of ipMonocytes harvested from ipPo1 (passages 14 to 22) differentiations was determined relative to the number of iPSCs that were plated for differentiation. Red lines represent the mean percentage of ipMonocytes as determined by independent experiments displayed as individual black dots ( $n=6$ ). Statistical analyses was performed using the unpaired *t* test (*ns.*  $p > 0.05$ ) and compared to ipMonocytes obtained from C15 iPSCs differentiations.



In order to confirm the successful derivation of DCs, cells were further analysed for the expression of DC markers. The expression of CD11c, MHCII and CD54 was detected on mature ipDCs which are markers characteristic of APCs (Figure 4.11) (Sheikh and Jones, 2008). Furthermore, the expression of MHCII was significantly upregulated on the surface of ipPo1 ipDCs following exposure to proinflammatory cytokines (immature:  $2.565 \pm 0.4191$  ( $n=7$ ), mature:  $4.548 \pm 0.7331$  ( $n=5$ ),  $P=0.0306$ ), indicating the successful maturation of these cells (Figure 4.11). Levels of MHCII expression were similar to those of C15 ipDCs and therefore considerably lower than levels of MHCII on moDCs which may further support a pro-tolerogenic phenotype of DCs derived from iPSCs (Figure 4.11, Figure 3.5 and Figure 3.19).

Monocyte and myeloid markers CD14 and CD11b and plasmacytoid marker CD123 were expressed at levels similar to moDCs and C15 ipDCs, indicating the myeloid-like phenotype of generated ipDCs (Figure 4.11, Figure 3.6, Figure 3.20, Figure 3.21 and Figure 3.22). The detection of CCR7 expression on ipPo1 ipDCs similar to levels observed on moDCs and C15 ipDCs indicates the potential capacity for migration towards local lymph nodes in response to the chemokines CCL19 and CCL21 (Figure 4.12 and Figure 3.9) (Ohl *et al.*, 2004).

The relatively low expression of CD1a and CD207 may indicate the absence of the CD1a<sup>+</sup> DC subset and low proportion or absence of ipDCs with a Langerhans cell-like phenotype, although other DCs have also been shown to express CD207 (Figure 4.12) (Idoyaga *et al.*, 2008; Ito *et al.*, 1999; Valladeau *et al.*, 2002). This expression profile is consistent with the data obtained from C15 ipDCs stainings, with low levels of CD207 and the absence of the CD1a<sup>+</sup> DC subset (Figure 3.7 and Figure 3.8). The latter was in contrast to data obtained from moDCs, which

showed the presence of two distinct subsets of DCs, the CD1a<sup>+</sup> and CD1a<sup>-</sup> DCs (Figure 3.8).

Furthermore, the expression of CD209, which is an intercellular adhesion molecules important for antigen-internalisation and contact stabilization between DCs and T cells, was detected on immature and mature ipPo1 ipDCs (Figure 4.12) (Engering *et al.*, 2002; Geijtenbeek *et al.*, 2000b). These results were interesting as they were in contrast with findings in Chapter 3, where mature C15 ipDCs were found not to express CD209 which was explained by its known downregulation in response to IFN $\gamma$  (Figure 3.8), while the levels detected on immature C15 ipDCs were at similar levels as found on immature ipPo1 ipDCs (Relloso *et al.*, 2002; Zhou *et al.*, 2006). Whether C15 and ipPo1 ipDCs respond differently to inflammatory cytokines remains to be determined.



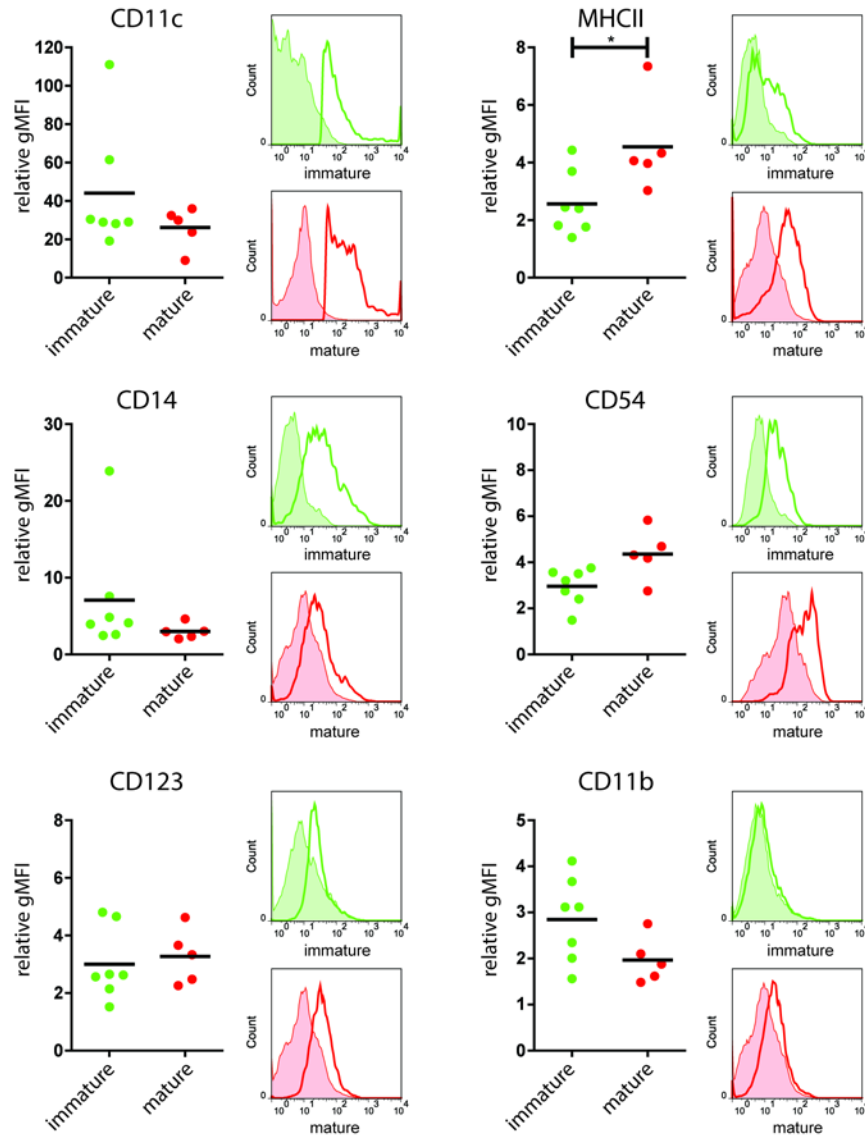


**Figure 4.11 The expression of DC markers by ipPo1 ipDCs.** Immature and mature ipPo1 ipDCs were differentiated from ipPo1 iPSCs at passages 14 to 22 and harvested from differentiation cultures and stained for the expression of CD11c, MHCII, CD54, CD14, CD123 and CD11b as detected by flow cytometry. Black lines represent the mean of the relative gMFI as obtained from independent experiments. The relative gMFI was determined as the gMFI obtained from stained samples over the gMFI obtained from isotype control stained samples. Independent experiments are displayed as individually coloured dots ( $n=5-7$ ). Histograms illustrate a set of data obtained from an example experiments. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test ( $*p \leq 0.05$ ). Data presented in this Figure are shown again as control groups in Figure 4.16 (page 286) and Figure 4.17 (page 287).

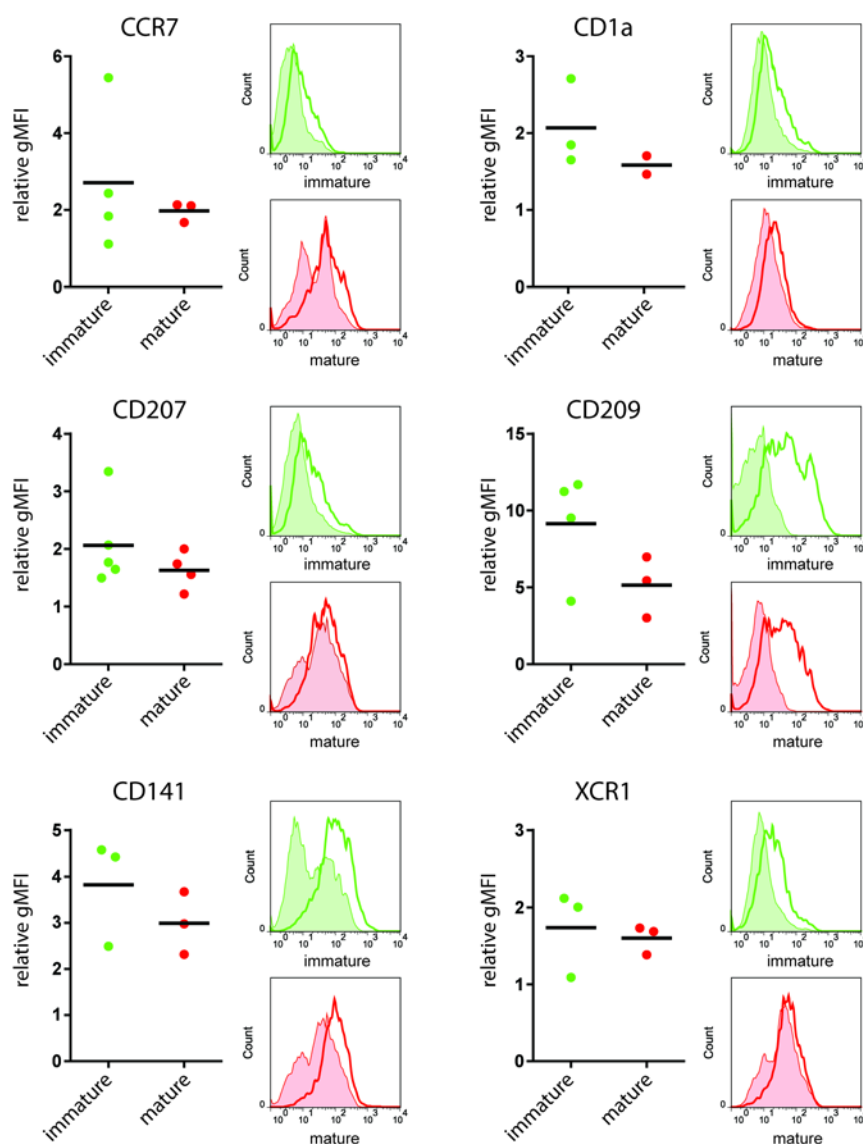


**Figure 4.12 The expression of DC and antigen cross-presentation markers.** Immature and mature ipDCs were derived from ipPo1 differentiations at passages 14 to 22. Harvested cells were stained for different markers and analysed by flow cytometry. Black lines are representative of the mean of the relative gMFI, as determined by independent experiments which are displayed as individually coloured dots ( $n=2-5$ ). Histograms illustrate a set of data obtained from an example experiments. Statistical analysis was performed using the unpaired  $t$  test. No significant difference was detected ( $ns$ ,  $p > 0.05$ ). Data on the expression of CCR7 are shown as control groups in Figure 4.17 (page 287).

Furthermore, the detection of CD141 and XCR1 indicate the presence of ipDCs with the capacity for cross-presentation (Figure 4.12), both of which were

expressed at levels similar to C15 ipDCs (Figure 3.12) (Bachem *et al.*, 2010; Dorner *et al.*, 2009).

Having demonstrated the feasibility of differentiating ipDCs from ipPo1s, the functional capacity of these ipDCs was further investigated.

#### **4.4.1 The functional integrity of ipDCs derived from Pompe disease iPSCs**

Given that Pompe disease specifically affects lysosomal function leading to the accumulation of substrate, the capacity of ipDCs generated from health-compromised individuals to take up protein antigens and process them for antigen presentation to T cells was investigated (Fukuda *et al.*, 2006a, 2006b). These properties are a prerequisite for the induction of antigen-specific tolerance to therapeutic enzymes. Although therapeutic enzyme is generally taken up by receptor-mediated endocytosis, phagocytosis may also provide a useful indication of the functionality of ipDCs (Roche and Furuta, 2015; Savina and Amigorena, 2007; Sly, 1985).

In order to measure their phagocytic capacity, ipPo1 ipDCs were incubated for 3 hours with FITC-labelled beads. As a control for the non-specific binding of beads to the surface of cells, a separate set of ipPo1 ipDCs were fixed and incubated with FITC-labelled beads for 3 hours. As shown in Figure 4.13A, ipPo1 ipDCs displayed phagocytic capacity, showing the phagocytic uptake of beads by around 29.1% of immature and 11.5% of mature CD11c<sup>+</sup> cells. This is similar to the phagocytic capacity of moDCs, of which 19.3% of immature and 21.5% of mature CD11c<sup>+</sup> moDCs were able to internalize latex beads (Figure 3.13). These

data indicate that the accumulation of substrate within the ipPo1 ipDCs may not negatively affect phagocytosis.

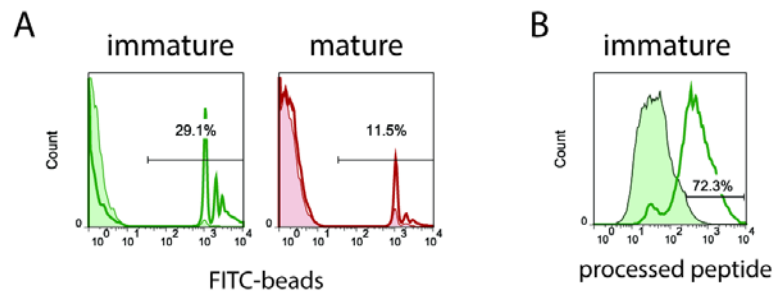


**Figure 4.13 The phagocytic and antigen-processing capacity of ipPo1 ipDCs.** (A) For the analysis of the phagocytic capacity,  $3 \times 10^5$  ipPo1 DCs were incubated with  $2.3 \times 10^6$  2  $\mu$ m diameter FITC-labelled beads for 3 hours at 37°C. Fixed cells were included as controls for non-specific binding of beads. Cells were washed, stained for CD11c and analysed by flow cytometry. The histograms presented were gated on CD11c<sup>+</sup> populations. The data presented constitute a single experiment using ipPo1 ipDCs differentiated from passage 14 ipPo1 iPSCs. (B) The capacity of ipDCs for the processing of antigen was determined by incubating  $7 \times 10^4$  ipPo1 ipDCs together with 100  $\mu$ g/ml of DQ ovalbumin for 30 minutes at 37°C. Controls were left on ice to provide an indication of non-specific protein breakdown and autofluorescence. Cells were washed, stained for CD11c and analysed by flow cytometry. The histograms displayed were gated on CD11c<sup>+</sup> cells. Histogram represents an example set of data from 2 independent experiments using ipPo1 ipDCs differentiated from ipPo1 iPSCs at passage 17.

In the absence of appropriately MHC-restricted T cells to serve as a physiological readout of antigen processing and presentation, ipPo1 ipDCs were incubated with DQ-ovalbumin which, on cleavage by antigen processing, yields a fluorescent product that is detectable by flow cytometry. This assay confirmed the ability of around 72% of CD11c<sup>+</sup> ipDCs differentiated from ipPo1 to process antigen (Figure 4.13B). As previously shown in Chapter 3, around 98% of CD11c<sup>+</sup> moDCs and 35% of CD11c<sup>+</sup> C15 ipDCs had the capacity to process DQ ovalbumin (Figure 3.13). Therefore, from these data, ipPo1 derived ipDCs were

not compromised in their ability to phagocytose beads or process protein antigens.

Having shown the feasibility of generating ipDCs from Pompe disease iPSCs with the capacity to phagocytose and process antigen, the immunological capacity of ipPo1 ipDCs was next investigated.

In order to investigate the capacity of ipPo1 ipDCs to induce T cell proliferation, immature and mature ipPo1 ipDCs were co-cultured with CFSE-stained allogeneic naïve CD4<sup>+</sup> T cells. The allostimulatory capacity of ipPo1 ipDCs was measured by the percentage of T cells that had reduced CFSE staining thereby identifying T cells that had proliferated. As shown in Figure 4.14, in co-cultures with allogeneic naïve CD4<sup>+</sup> T cells, mature ipPo1 ipDCs were able to induce T cell activation and proliferation which increased in a dose-dependent manner.

Similarly, immature ipPo1 ipDCs were capable of inducing T cell proliferation however with lower potency. These data are consistent with the observed allostimulatory capacity of moDCs and healthy C15 ipDCs (Figure 3.33). Furthermore, immature and mature ipPo1 ipDCs gave rise to different proportions of T<sub>regs</sub> in co-cultures as determined by the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, indicating the capacity of ipPo1 ipDCs to maintain, induce or potentially expand T<sub>reg</sub> populations. However, the percentage of T<sub>regs</sub> detected was below the average 5% obtained in co-cultures of T cells together with moDCs and C15 ipDCs (Figure 3.15). Whether this may indicate a lower T<sub>reg</sub> induction potential of ipPo1 ipDCs, remains to be investigated, however donor-related difference between the proportions of T<sub>regs</sub> among peripheral blood T cells in the steady

state have been observed, making it difficult to draw comparisons between experiments.(Finney *et al.*, 2010; Gregg *et al.*, 2005).

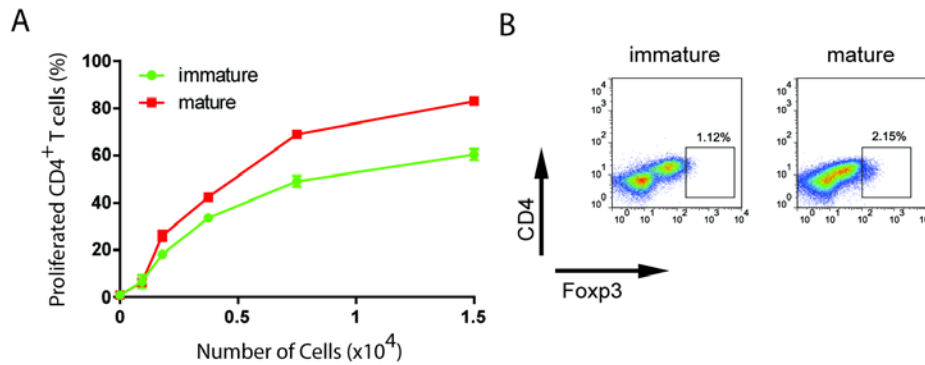


**Figure 4.14 The allostimulatory capacity of ipPo1 ipDCs and the detection of T<sub>reg</sub> populations in co-cultures.** (A) ipPo1 ipDCs were cultured at different ratios (x-axis) with CFSE-labelled 6x10<sup>4</sup> naïve allogeneic CD4<sup>+</sup> T cells per well of a 96-well plate. After 5 days, co-cultures were harvested and analysed by flow cytometry in order to determine the proportion of cells that had divided. Control naïve CD4<sup>+</sup> T cells were used to determine the level of CFSE staining in undivided cells or due to background proliferation. Cells were gated by size using the FSC and SSC and the percentage of proliferated cells was determined by gating cells showing lower CFSE staining than the positive T cell only control. The data are representative of 3 independent experiments using ipPo1 passages 14 to 20 (shown are ipDCs differentiated from passage 20 ipPo1 iPSCs). (B) 10<sup>6</sup> allogeneic naïve CD4<sup>+</sup> T cells were co-cultured with 2x10<sup>5</sup> ipPo1 ipDCs, treated with 75 ng/ml of IL-2 on day 5 and harvested on day 7. Cells were stained for CD4 and Foxp3 and analysed by flow cytometry. Cells were gated by size using FSC and SSC. Data are representative of 2 independent experiments using ipDCs differentiated from ipPo1 iPSCs at passages 17 and 22 (shown is passage 17).

Having shown their capacity to induce T cell proliferation and the presence of T<sub>reg</sub> populations in co-cultures, it was investigated whether ipPo1 ipDCs could be modulated in their ability to exert these functions to favour a pro-tolerogenic outcome.

## **4.5 The characterisation of ipDCs derived from Pompe disease iPSCs and their modulation with IL-10**

Four agents were screened in Chapter 3 for their capacity to modulate ipDC to promote a pro-tolerogenic phenotype. IL-10 was selected as the most promising agent and this chapter therefore explores the effect of IL-10 on ipPo1-derived ipDCs.

### **4.5.1 The yield and viability of ipPo1 ipDCs**

No significant difference was detected in the viability of immature and mature ipDCs derived from ipPo1s (Figure 4.15A), also if compared to C15 iPSCs (Figure 3.16A). C15 ipDCs had an average viability of  $72.8 \pm 7.9\%$  ( $n=4$ ) for immature and  $60.3\%$  ( $n=2$ ) for mature populations (Figure 3.16A). ipPo1 ipDCs showed an average viability of  $69.54 \pm 9.8\%$  ( $n=6$ ) for immature and  $54.91 \pm 9.5\%$  ( $n=5$ ) for mature populations (Figure 4.15A). Similar to moDCs and ipDCs, the treatment of ipPo1 ipDCs with IL-10 and their exposure to pro-inflammatory cytokines did not significantly affect ipDC survival with  $69.5 \pm 9.8\%$  ( $n=6$ ) of viable cells obtained from immature untreated cells,  $54.9 \pm 9.5\%$  ( $n=5$ ,  $p > 0.05$ ) of viable cells derived from matured cultures and  $53.2 \pm 6.6$  ( $n=3$ ,  $p > 0.05$ ) of viable cells derived from IL-10 treated and matured cultures (Figure 4.15A).

No significant difference was observed in the percentage yield of viable ipPo1 and C15 ipDCs, as determined by the number of viable cells harvested relative to the number of ipMonocytes plated for the final 7-day differentiation stage (Figure 4.15B and Figure 3.16B). The average yield for immature C15 ipDCs was  $73.6 \pm 22.4\%$  ( $n=5$ ) and  $59.5 \pm 22.8\%$  ( $n=5$ ) for mature (Figure 3.16B). ipPo1

derived ipDCs gave a yield of  $85.9 \pm 23.61\%$  ( $n=5$ ) from immature and  $56.3 \pm 9.2\%$  ( $n=5$ ) from matured cultures (Figure 4.15B).

The yield of viable cells from differentially-treated cultures was not significantly different in response to IL-10 modulation, giving rise to a recovery of  $47.3 \pm 15.77\%$  ( $n=3$ ) (Figure 4.15B). The yield and percentage of viable cells were similar to those obtained from immature, mature and IL-10-modulated moDCs and C15 ipDCs, with the exception of a statistically significant decrease in the live cell yield of IL-10-modulated moDCs as compared to mature moDCs (Figure 3.16).

Despite the indicted difference in the yield of ipMonocytes (Figure 4.10), the percentage of CD11c<sup>+</sup> cells derived from these ipMonocytes was similar, with an average of  $46 \pm 10.18\%$  ( $n=7$ ) CD11c<sup>+</sup> cells from immature and  $46 \pm 7.3\%$  ( $n=5$ ) from mature ipPo1 ipDC differentiations (Figure 4.15) as compared to an average of  $43 \pm 7.7\%$  ( $n=12$ ) CD11c<sup>+</sup> cells from immature and  $43 \pm 7.8\%$  ( $n=12$ ) from mature C15 ipDC differentiations (Figure 3.4). Cultures treated with IL-10 and pro-inflammatory cytokines gave rise to around  $35.5 \pm 11.7\%$  ( $n=3$ ) CD11c<sup>+</sup> cells (Figure 4.15), showing no statistically significant difference to immature and mature ipPo1 ipDCs. These data are consistent with results obtained from C15 ipDC cultures, which showed an average yield of around 40% of CD11c<sup>+</sup> cells (Figure 3.17).

In order to confirm the DC-like phenotype of ipPo1 ipDCs modulated with IL-10, the expression profile of DC markers was investigated. The average expression of DC markers was found to be similar to either the untreated immature control population or mature control population. The average level of expression of



CD11c, CD11b, CD54 and CCR7 on ipDCs modulated with IL-10 was similar to that expressed by matured ipDCs (Figure 4.16 and Figure 4.17). No significant change in the expression of CD14 and CD123 was observed after IL-10-modulation (Figure 4.16 and Figure 4.17). IL-10-treated ipDCs displayed a cell surface expression of MHCII similar to immature ipDCs, however no statistical significance was achieved on comparison to expression levels on matured ipDCs (Figure 4.16).

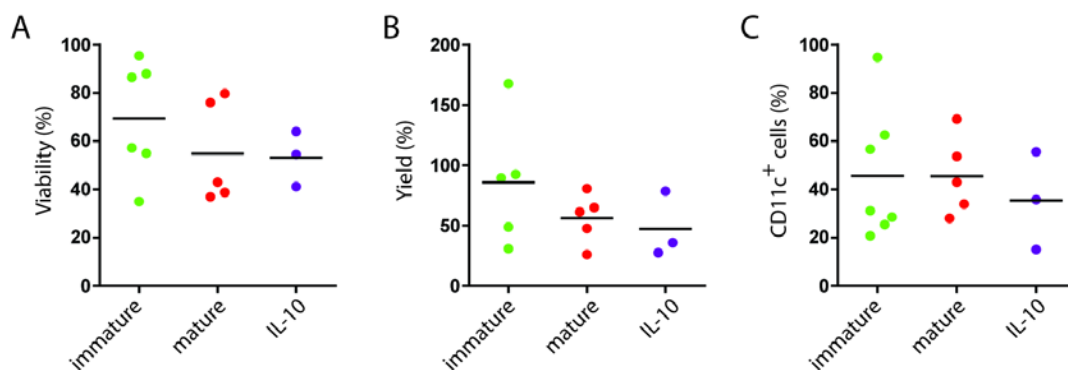


**Figure 4.15 The yield of viable CD11c<sup>+</sup> ipDCs from ipPo1 differentiations.** ipPo1 cells were differentiated at passages 14 to 22 according to the standard protocol. After around 19-24 days, ipMonocytes were harvested from EB cultures and plated for the final 7-day differentiation stage. On day 3 of this period, ipDCs were only matured on day 5, left untreated (immature) or treated with 200 pg/ml of IL-10 and matured on day 5. On day 7 ipDCs were harvested and (A) assessed for the percentage of viable cells by counting the viable and total number of cells obtained using trypan blue exclusion (immature:  $n=6$ , mature:  $n=5$ , IL-10:  $n=3$ ). (B) The percentage yield of ipPo1 ipDCs was determined as the number of ipDCs recovered at the end of the differentiation relative the number of ipMonocytes plated at the beginning of the 7-day differentiation stage (immature:  $n=5$ , mature:  $n=5$ , IL-10:  $n=3$ ). (C) Differentiations were stained for the expression of CD11c after harvest and analysed by flow cytometry for the percentage of CD11c-expressing cells. Black lines represent the mean of the relative gMFI as obtained from independent experiments which are displayed as individual coloured dots (immature:  $n=7$ , mature:  $n=5$ , IL-10:  $n=3$ ). Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test ( $ns. p > 0.05$ ). No significant difference was detected unless indicated.

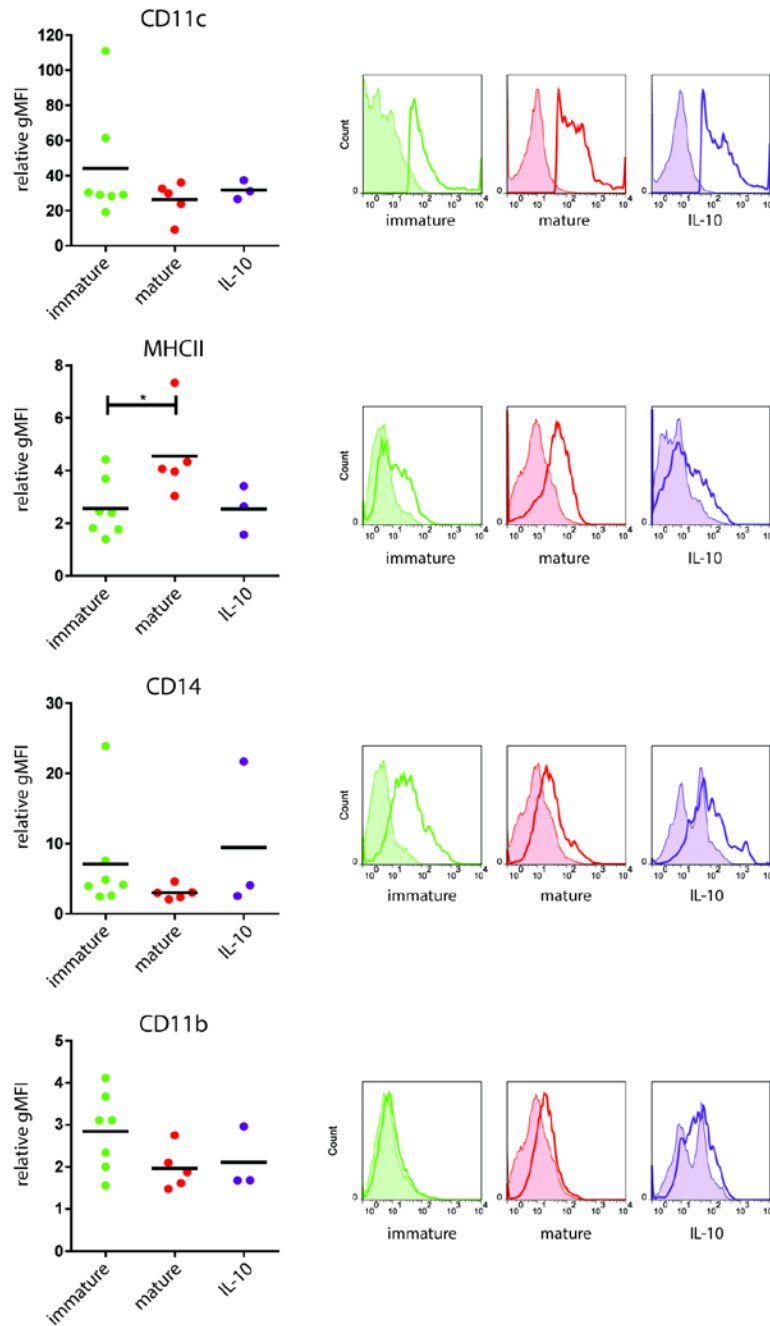


**Figure 4.16 The expression of DC and myeloid markers by modulated ipPo1 ipDCs.** Control and modulated ipDCs were harvested at the end of the differentiation process using ipPo1 iPSCs at passages 14 to 22 and stained for DC markers. Cells were analysed by flow cytometry and plotted using Graphpad Prism. The mean of the relative gMFI is represented by black lines as obtained from independent experiments which are displayed as individually coloured dots ( $n=3-7$ ). Histograms illustrate a set of data obtained from an example experiment. Data on immature and mature ipPo1 ipDCs have been previously presented in Figure 4.11 (page 277). Data are presented here as a control group to IL-10-modulated ipDCs. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test ( $*p \leq 0.05$ ).

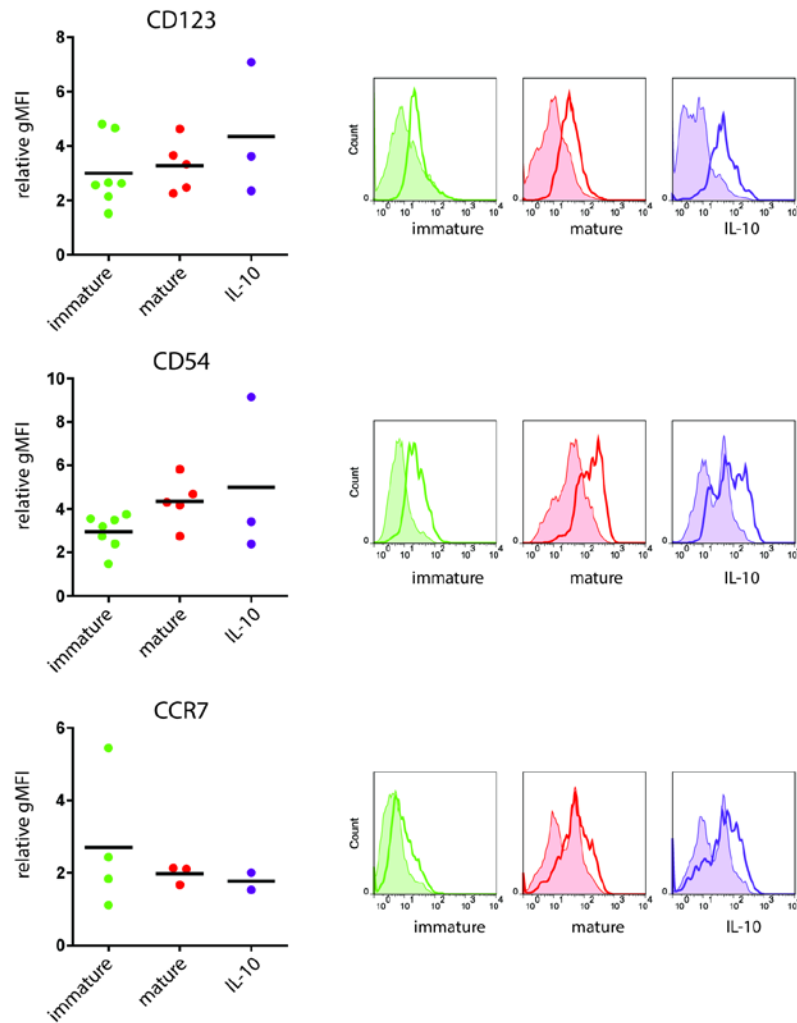


**Figure 4.17 The expression of APC and DC markers by modulated ipDCs.**

ipDCs were harvested from untreated (immature), matured and IL-10-modulated cultures and stained for a variety of markers for analysis by flow cytometry. ipPo1 iPSCs were differentiated at passages 14 to 22. The mean of the relative gMFI is represented by the black lines as determined by independent experiments. Independent experiments are presented as individual coloured dots ( $n=2-7$ ). Histograms illustrate a set of data obtained from an example staining. Data on immature and mature ipPo1 ipDCs have been previously presented in Figure 4.11 (page 277) and Figure 4.12 (page 278). Data are presented here as a control group to IL-10-modulated ipDCs. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test (*ns. p* > 0.05). No significant difference was detected unless indicated.

Having recovered viable ipDCs from ipPo1 differentiations that had been left untreated, matured or treated with IL-10 and matured, the potential for immunomodulation using this treatment was investigated.

#### **4.5.2 The effect of IL-10 on the expression of costimulatory molecules and immunoinhibitory molecules**

Apart from the expression of MHCII, the expression level of costimulatory molecules is fundamental to the capacity of DCs to induce an immune response.

The average expression of the costimulatory molecule CD86 was found to be significantly downregulated on IL-10-treated ipDCs as compared to matured ipDCs (mature:  $5.222 \pm 0.7474$  relative gMFI ( $n=5$ ), IL-10:  $2.248 \pm 0.4768$  relative gMFI ( $n=3$ ),  $p = 0.0305$ ) (Figure 4.18). In contrast to this, no statistically significant difference had been found in the previous chapter between mature and IL-10-modulated C15 ipDCs or moDCs (Figure 3.26). The apparent upregulation of CD86 on ipDCs, however not statistically significant, implies the successful maturation of ipDCs in response to proinflammatory cytokines, which was also supported by the statistically significant upregulation of MHCII (Figure 4.16). The average expression of CD80 and CD40 was similar between all three ipPo1 ipDC groups (Figure 4.18). Consistent with these data, no statistically significant change in the expression of CD80 and CD40 were found when treating C15 ipDCs with IL-10 (Figure 3.24 and Figure 3.25)

Apart from the expression of molecules involved in the stimulation of immune responses, the expression profile of molecules known to be involved in immune regulation and suppression was investigated.

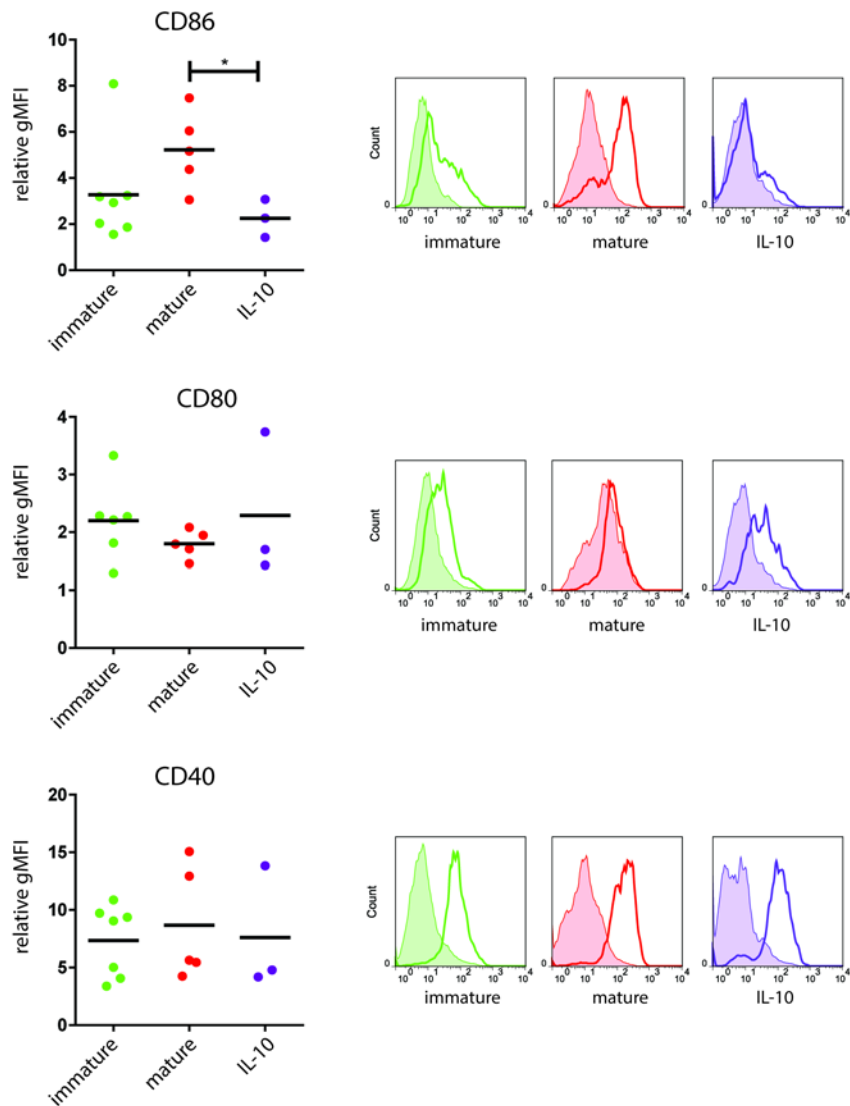


**Figure 4.18 The expression of costimulatory molecules CD86, CD80 and CD40 by modulated ipPo1 ipDCs.** ipPo1s were differentiated at passages 14 to 22 into ipDCs and modulated with IL-10 during the last 7-day differentiation stage. After harvest, cells were stained and analysed for the expression of costimulatory molecules CD86, CD80 and CD40 using flow cytometry. Black lines represent the mean of the relative gMFI as obtained from independent experiments which are displayed as individual coloured dots ( $n=3-7$ ). Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test ( $*p \leq 0.05$ ).

As shown in Figure 4.19 and Figure 4.20, although no statistically significant difference was found in the expression PD-L1, PD-L2, ILT3 and ILT4 on the surface of IL-10-treated ipDCs, the average expression of all markers was

increased as compared to the matured populations. However, whether such changes in levels of expression are immunologically relevant remains to be determined.

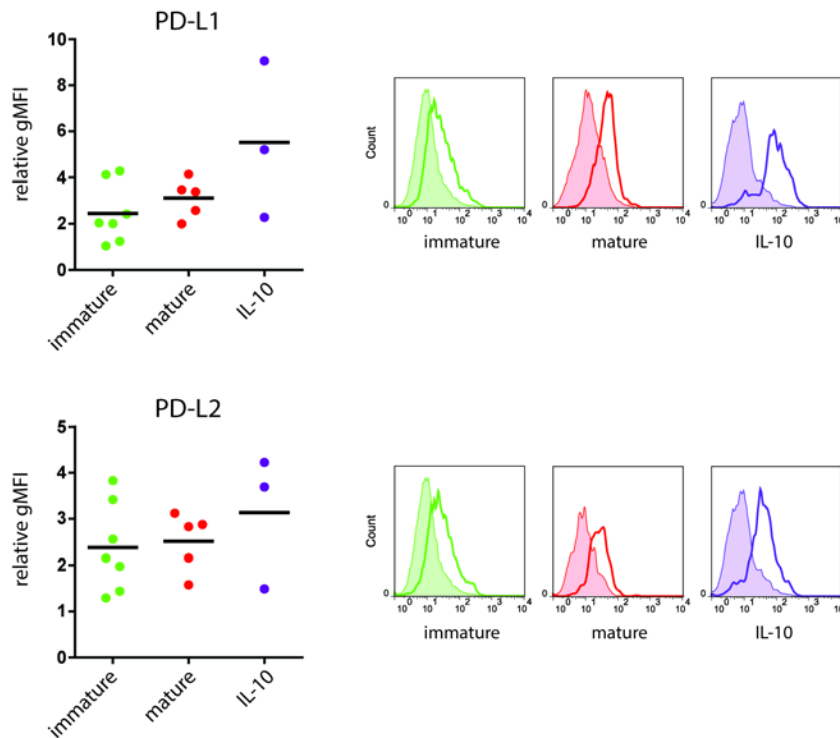


**Figure 4.19 The expression of immunoinhibitory PD-L1 and PD-L2 by modulated ipPo1 ipDCs.** ipDCs were harvested from ipPo1 differentiations using passages 14 to 22 which had been left immature, matured or modulated with IL-10 and matured. ipDCs were stained for the expression of PD-L1 and PD-L2 and analysed by flow cytometry. Black lines represent the mean of the relative gMFI as obtained from independent experiments which are displayed as individual coloured dots ( $n=3-7$ ). Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected unless indicated (*ns. p* > 0.05).

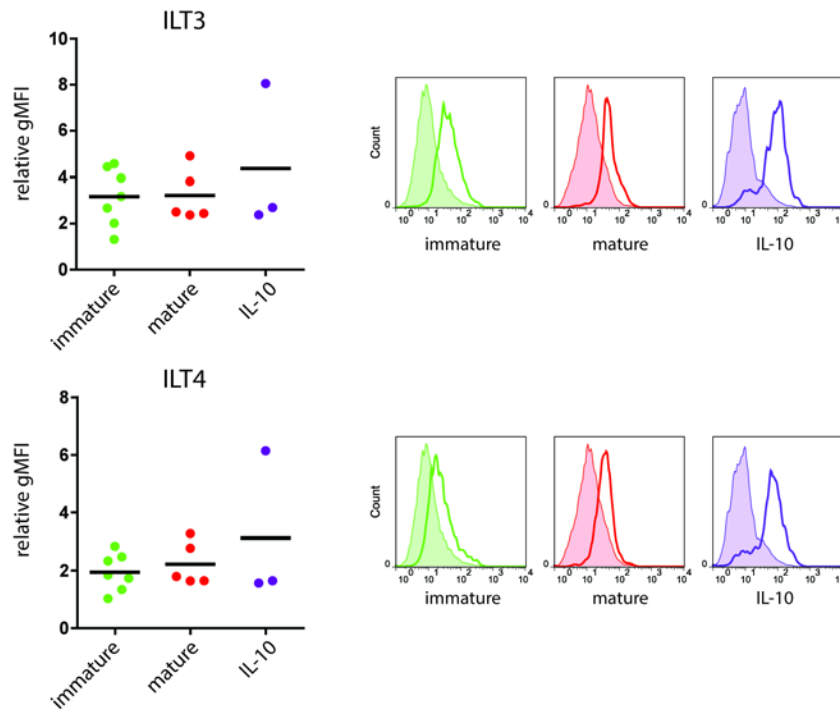


**Figure 4.20 The expression of immunoinhibitory ILT3 and ILT4 by modulated ipPo1 ipDCs.** Immature, mature and IL-10-modulated ipDCs were harvested, stained for the expression of ILT3 and ILT4 and analysed by flow cytometry. ipDCs were differentiated from ipPo1 iPSCs at passages 14 to 22. The mean of the relative gMFI is represented by the black lines. The mean was determined from independent experiments which are displayed as individually coloured dots ( $n=3-7$ ). Histograms illustrate a set of data obtained from an example staining. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected unless indicated (*ns.*  $p > 0.05$ ).

Cytokines secreted by DCs play an important contributing factor in driving immunological responses and the induction of tolerance. They are essential in the creation of a microenvironment which can influence a tolerogenic outcome and can drive the development of specific T cell subsets (Curtsinger *et al.*, 1999; Romagnani, 2000). IL-6 is known to be involved in inflammatory responses and the development of Th17 cells (Dodge *et al.*, 2003; Scheller *et al.*, 2011). As the secretion of cytokines may be an important determinant for the capacity of ipPo1

ipDCs to modulate immune responses, their capacity to secrete IL-6 was investigated.

Accordingly, supernatants were harvested from cultures of DCs at the end of the 7-day differentiation period and analysed by ELISA for the concentration of IL-6. As displayed in Figure 4.21, immature ipPo1 ipDCs secreted on average lower levels of IL-6 as compared to mature ipDCs and those modulated with IL-10, however no statistical significance was achieved.

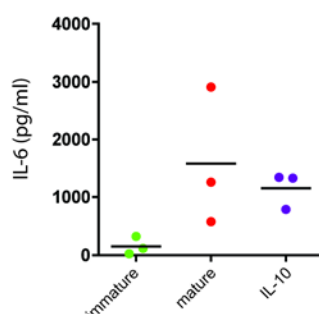


**Figure 4.21 The capacity of ipPo1 ipDCs to secrete IL-6.** Supernatants from ipDC differentiations using ipPo1 iPSCs at passages 17 to 22 were harvested on day 7 of the final differentiation stage and analysed for the presence of IL-6 using ELISA. Black lines represent the mean concentration as detected by 3 independent experiments which are presented as coloured dots. Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparison test. No significant difference was detected unless indicated (*ns.*  $p > 0.05$ ).

In contrast to the release of IL-6 by ipPo1 ipDCs, C15 ipDCs showed a statistically significant upregulation in the release of IL-6 in response to proinflammatory cytokine exposure (Figure 3.31A). This difference may have been due to the lower number of independent experiments performed and therefore statistical significance may be achieved with future repetition of experiments.



#### **4.5.3 The effect of IL-10 on the allostimulatory capacity of ipDCs from Pompe patients**

Having detected a significant reduction in the expression of the costimulatory molecule CD86 on IL-10-treated ipDCs, it was investigated whether IL-10-treatment of ipPo1 ipDCs could reduce their immunostimulatory potential as measured by the allogeneic MLR.

As shown in Figure 4.22, ipPo1 ipDCs that had been modulated using IL-10 showed reduced allostimulatory capacity, inducing less T cell proliferation as compared to mature and immature ipDCs. Furthermore, the increased allostimulatory capacity of proinflammatory-exposed ipDCs indicates the successful maturation of ipPo1 ipDCs. These data are consistent with the results obtained from healthy control C15 ipDC in which proinflammatory cytokine-exposure significantly increased the percentage of proliferated CD4<sup>+</sup> T cells in co-cultures as compared to immature C15 ipDCs (Figure 3.33). Furthermore, IL-10-treatment significantly reduced the allostimulatory capacity of C15 ipDCs, however this could not be determined for ipPo1 ipDCs due to the limited number of independently performed experiments.

As T<sub>regs</sub> can exert suppressive capacity by the inhibition of effector T cell proliferation, the proportion of T<sub>regs</sub> in co-cultures with differently-treated ipDCs was investigated (Cobbold *et al.*, 2006; Fontenot *et al.*, 2005; Hori *et al.*, 2003; Li *et al.*, 2007).

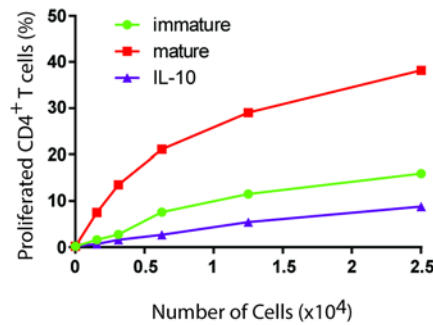


**Figure 4.22 The allostimulatory capacity of IL-10-modulated ipPo1 ipDCs.** After differentiation and modulation of ipPo1 ipDCs, different ratios of ipDCs (x-axis) were co-cultured with  $10^5$  CFSE-labelled naïve  $CD4^+$  T cells per well of a 96-well plate in duplicate. Cells were harvested after 5 days and analysed for the percentage of proliferated cells using flow cytometry. Data constitute a single experiment using ipPo1 iPSCs at passage 17.

#### 4.5.4 The effect of IL-10 on the capacity of ipPo1 ipDCs to induce or expand $T_{regs}$

The reduced allostimulatory capacity of IL-10-treated ipPo1 ipDCs may be due to the induction of a larger proportion of  $T_{regs}$  in co-cultures (Chauhan *et al.*, 2009; McMurchy and Levings, 2012; Sakaguchi *et al.*, 2009). Therefore, the proportion of  $CD4^+Foxp3^+$  T cells was investigated.

As displayed in Figure 4.23, IL-10-modulated ipPo1 ipDCs were able to induce a larger proportion of  $T_{regs}$  or expand this population in co-cultures to a larger extent than mature ipDCs.

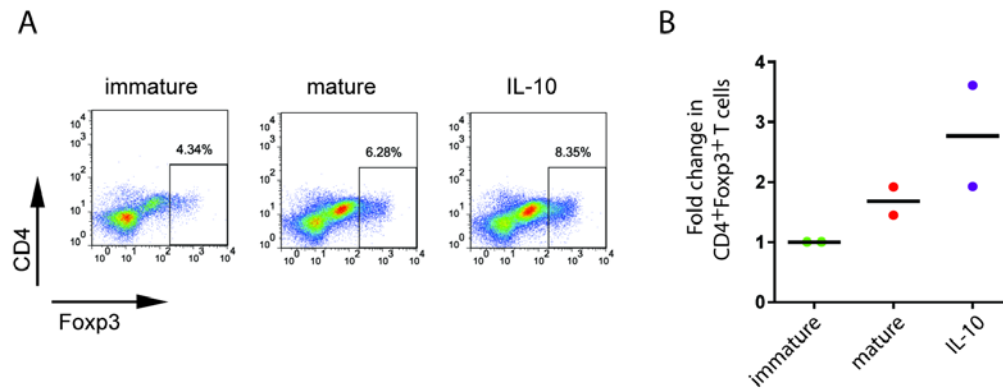


**Figure 4.23 The effect of IL-10-modulated ipPo1 ipDCs on T<sub>reg</sub> populations.**

The potential for the expansion or maintenance of T<sub>regs</sub> was investigated by the co-culture of  $10^6$  naïve CD4<sup>+</sup> T cells with  $2 \times 10^5$  ipDCs per well of a 24-well plate. Co-cultures were treated with 75 ng/ml of IL-2 on day 5 and harvested on day 7. Cells were stained for the expression of CD4 and Foxp3 and analysed by flow cytometry. (A) Data presented show an example of the data obtained from one of the T<sub>reg</sub> induction assays using ipPo1 iPSCs differentiated at passage 22. (B) Data from 2 independent experiments are presented as fold changes in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells relative to the percentage obtained in co-cultures with immature ipPo1 ipDCs. ipDCs used in co-cultures had been differentiated from ipPo1 iPSCs at passages 17 and 22. Black lines represent the mean fold change of independent experiments which are represented by individually coloured dots.

The average increase of the proportion in T<sub>regs</sub> induced or expanded by mature ipDCs as compared to immature ipDCs was 1.683 fold ( $n=2$ ) fold while IL-10-modulated ipDCs increased the proportion of T<sub>regs</sub> 2.766 fold ( $n=2$ ). However, in order to enable statistical evaluation of these data, further experiment are required.

Having detected an average increase in the proportion of T<sub>regs</sub> in co-cultures with IL-10 modulated ipPo1 ipDCs, the cytokine environment was investigated for the presence of IL-10.

#### **4.5.5 The effect of IL-10-modulation of ipDCs on the IL-10 cytokine environment**

IL-10 is a cytokine that is secreted by T<sub>regs</sub>, with known immunoregulatory functions (Corthay, 2009; Hara *et al.*, 2001; Sakaguchi *et al.*, 2009). IL-10 may act by inhibiting the expression of pro-inflammatory cytokines and therefore suppress the development of Th1 effector cells and inhibit the proliferation of CD4<sup>+</sup> T cells (Akdis and Blaser, 1999; Ye *et al.*, 2007).

Therefore, in order to investigate the potential of IL-10-modulated ipPo1 ipDCs to display pro-tolerogenic properties, the presence of IL-10 in co-cultures was investigated. IL-10 that had been added to DC cultures for modulations were unlikely to influence these results as DCs had been extensively washed before their addition to co-cultures. Supernatants were harvested from co-cultures on day 5 and day 7 and the concentration of IL-10 was measured by ELISA. As shown in Figure 4.24, the average concentration of IL-10 was increased in co-cultures with IL-10-modulated ipPo1 ipDCs as compared to co-cultures with immature or mature ipPo1 ipDCs. However, no statistical significance was achieved. Levels of IL-10 detected in these co-cultures were similar to those measured in C15 ipDC co-cultures together with CD4<sup>+</sup> T cells (Figure 3.35).

An increased release of IL-10 in the microenvironment may be indicative of an increased presence of Tr1 cells. Tr1 cells are CD4<sup>+</sup>Foxp3<sup>-</sup> T cells that express and secrete large amounts of IL-10 and have been shown to retain regulatory capacity (Battaglia *et al.*, 2004; Roncarolo *et al.*, 2006). Therefore, the presence of Tr1 cells in co-cultures was investigated.

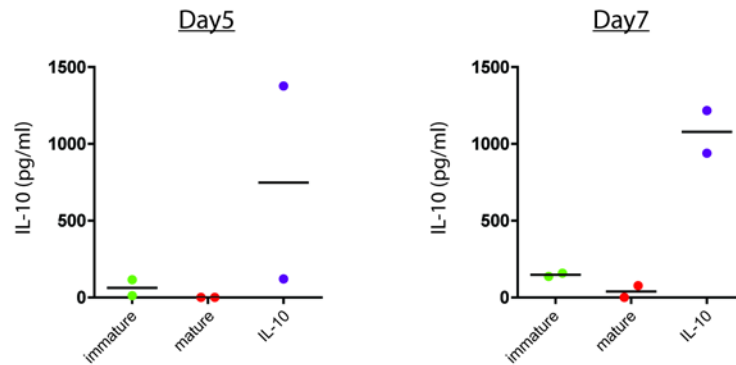


**Figure 4.24 The presence of IL-10 in co-cultures of ipPo1 ipDCs and T cells.** Supernatants were harvested from co-cultures of ipDCs differentiated from ipPo1 iPSCs at passages 17 to 22 and naïve CD4<sup>+</sup> T cells on day 5 and day 7. These were analysed for the presence and concentration of IL-10 by ELISA. Black lines indicate the mean concentration detected as determined by 2 independent experiments. The independent experiments are displayed as coloured dots.

#### 4.5.6 The effect of IL-10 on the capacity of ipPo1 ipDCs to induce or expand Tr1 cells

Tr1 cells, previously thought to be a phenomenon of *in vitro* culture, have been shown to be generated *in vivo* in response to the nasal administration of anti-CD3 antibody and display suppressive capacity in lymph nodes (Wu *et al.*, 2011). Furthermore, IL-10 treated DCs have been previously shown to exert enhanced capacity for the induction of Tr1 populations (Gregori *et al.*, 2010).

Therefore, it was investigated whether the IL-10-modulation of ipPo1 ipDCs had an effect on the Tr1 population. In this study, no difference was detected in the proportion of Tr1 cells obtained from co-cultures (Figure 4.25). These data are consistent with data obtained from C15 ipDC co-cultures where no significant difference was detected between different modulation protocols and only a low proportion of Tr1 cells was detected (Figure 3.36). Whether these results were

affected by technical limitations may be explored in the near future by investigating the use of alternative intracellular IL-10 detection protocols.

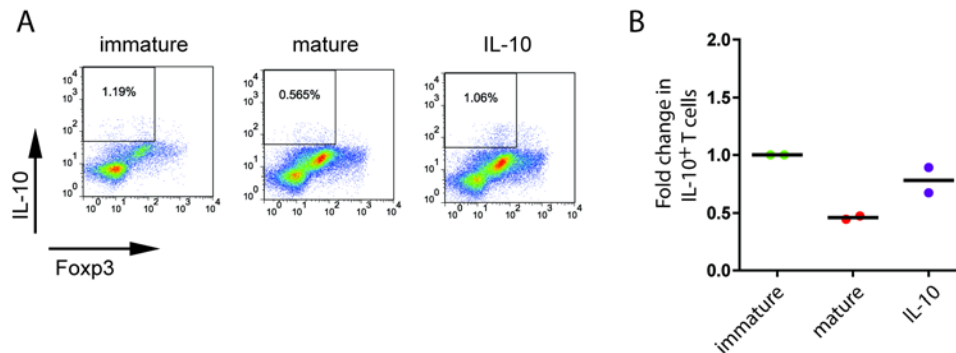


**Figure 4.25 The effect of IL-10-modulated ipPo1 ipDCs on Tr1 populations.**

Immature, mature and IL-10-modulated ipDCs were co-cultured at  $2 \times 10^5$  ipDC (derived from ipPo1 passages 17 and 22) with  $10^6$  allogeneic naïve CD4<sup>+</sup> T cells per well of a 24-well plate. After treatment with 75 ng/ml of IL-2 on day 5, cells were stimulated on day 7 using a cocktail of PMA, ionomycin and brefeldin A. After 5 hours of stimulation and the inhibition of cytokine release, cells were harvested and stained for CD4, intracellular IL-10 and Foxp3 and analysed by flow cytometry. (A) Data presented show the percentage of Tr1 cells induced by immature, IL-10-modulated and mature ipPo1 ipDCs derived from ipPo1 passage 17. Data presented are an example of 2 independent experiments. (B) Data display 2 independent experiments as the fold change in the percentage of CD4<sup>+</sup>IL-10<sup>+</sup>Foxp3<sup>+</sup> T cells relative to the percentage obtained from immature co-cultures. The mean fold change is represented by black lines as obtained from independent experiments which are represented by coloured dots.

#### 4.5.7 The effect of IL-10-modulated ipPo1 ipDCs on IFN $\gamma$ <sup>+</sup> and IL-2<sup>+</sup> T cells

A mechanism of anergy induction by T<sub>regs</sub> is known to involve the suppression of IL-2 secretion by T effector cells (Akdís and Blaser, 1999; Lechler *et al.*, 2001). Furthermore, the expression and secretion of IFN $\gamma$  has been used for the identification of Th1 cells and, together with IL-2, used as an indicator of T cell anergy, as these two cytokines are known to be transcriptionally repressed by T<sub>regs</sub> (Bettelli *et al.*, 2005; Ono *et al.*, 2007; Romagnani, 2000). Pro-tolerogenic

conditions are, therefore, expected to give rise to T cells with reduced IL-2 and IFN $\gamma$  expression.

In order to determine any potential effect of IL-10-modulated ipPo1 ipDCs on T cell activation and Th1 populations, co-cultures were analysed for the presence of IL-2 and IFN $\gamma$ -expressing CD4 $^{+}$  T cells. The co-culture of naïve CD4 $^{+}$  T cells with mature ipPo1 ipDCs increased the proportion of IL-2 $^{+}$  T cells on average 3.66 fold ( $n=2$ ) while ipDCs that had been modulated with IL-10 decreased the proportion of IL2-expressing T cells on average 0.9 fold ( $n=2$ ) as compared to co-cultures with immature ipDCs (Figure 4.26). Further experiments are required for the determination of statistical significance.

On average, the proportion of IFN $\gamma^{+}$  T cells decreased when co-cultured with mature (0.617 fold,  $n=2$ ) or IL-10-treated ipDCs (0.62 fold,  $n=2$ ) as compared to co-cultures with immature ipPo1 ipDCs, however no statistical analysis was performed. This may be explained with the dual properties of IFN $\gamma$ , as this cytokine can also exert immunoregulatory functions by the induction of IDO, which promotes the catabolism of the essential amino acid tryptophan and therefore limits the proliferation of T effector cells (Romani *et al.*, 2008; Williams *et al.*, 2008). These data may further indicate a difference between healthy control C15 ipDCs and ipPo1 ipDCs, as the proportion of IL-2 $^{+}$  and IFN $\gamma^{+}$  T cells was consistently lower in co-cultures with ipPo1 ipDCs as compared to the proportions obtained in co-cultures with C15 ipDCs (Figure 3.37). In order to confirm this phenotype, the secretion of IFN $\gamma$  in co-cultures was investigated.

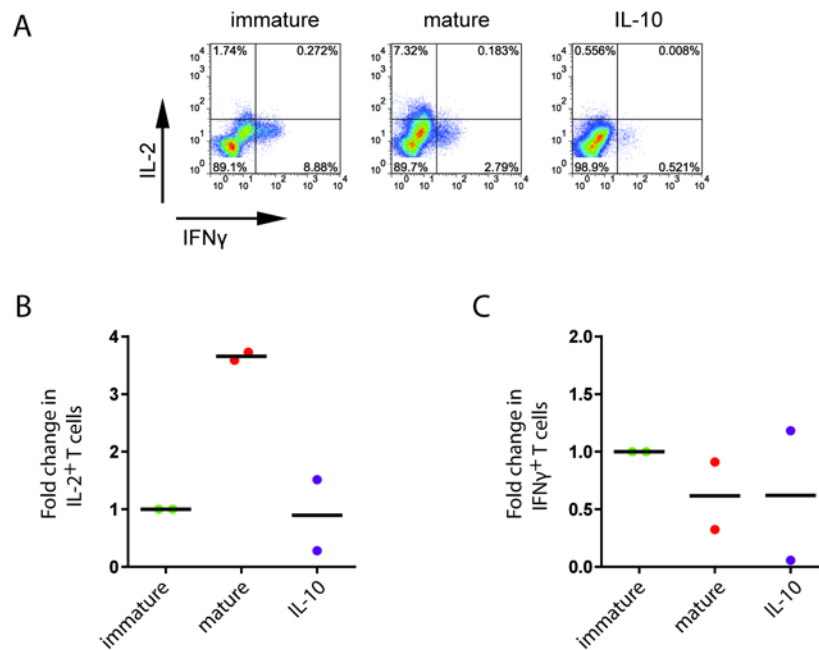


**Figure 4.26 The effect of IL-10-modulated ipPo1 ipDCs on IL-2<sup>+</sup> and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells.** ipDCs were co-cultured at  $2 \times 10^5$  with  $10^6$  allogeneic naïve CD4<sup>+</sup> T cells per well of a 24-well plate. Co-cultures were treated on day 5 with 75 ng/ml of IL-2 and stimulated on day 7 with a mixture of PMA, ionomycin and brefeldin A at 37°C. After 5 hours, cells were harvested, stained for IL-2 and IFN $\gamma$  and analysed by flow cytometry. (A) Data show the percentage of IL-2<sup>+</sup> and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells induced by immature, IL-10-modulated and mature ipPo1 ipDCs differentiated from ipPo1 iPSCs at passage 17. Data presented are derived from one of 2 independent experiments. (B and C) Data display 2 independent experiments using ipPo1 iPSCs at passages 17 and 22 as the fold change in the percentage of IL-2<sup>+</sup> and IFN $\gamma$ <sup>+</sup> T cells, respectively, relative to the percentage obtained from immature co-cultures. The black lines represent the mean fold change as obtained from independent experiments which are represented by coloured dots.

#### 4.5.8 The effect of IL-10-modulation of ipDCs on the IFN $\gamma$ cytokine environment

Supernatants that had been harvested from co-cultures on day 5 and day 7 were analysed by ELISA for the presence and concentration of IFN $\gamma$ . On day 5, the co-culture of immature and IL-10-treated ipDCs with naïve CD4<sup>+</sup> T cells gave rise to similar levels of IFN $\gamma$  in the cultures supernatants (Figure 4.27). Similar to day 5, day 7 showed no striking difference in the levels of IFN $\gamma$  between immature,



mature and IL-10-modulated ipDC co-cultures. Levels of IFN $\gamma$  detected were generally lower in ipPo1 ipDC co-cultures as compared to co-cultures containing C15 ipDCs (Figure 3.39). This further supports findings described in Figure 4.26, where a lower proportion of T cells was found to be producing IFN $\gamma$  as compared to experiments using C15 ipDCs (Figure 3.38).

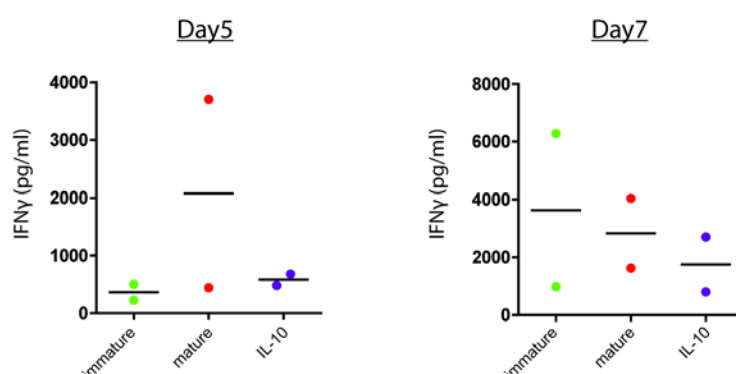


**Figure 4.27 The presence of IFN $\gamma$  in co-cultures of ipPo1 ipDCs and T cells.** Supernatants were obtained from co-cultures of ipPo1 ipDCs differentiated from ipPo1 iPSCs at passages 17 and 22 and allogeneic naïve CD4<sup>+</sup> T cells on day 5 and day 7. Supernatants were analysed by ELISA for the concentration of IFN $\gamma$ . Coloured dots represent 2 independent experiments of which the mean concentration was plotted as a black line.

## Discussion

This chapter has demonstrated the feasibility of deriving ipDCs from iPSCs generated from a health-compromised patient diagnosed with infantile-onset Pompe disease which appeared healthy and viable in cell culture. The relatively normal viability could not be attributed to the CRIM<sup>+</sup> status of the patient as no GAA activity was detected in ipPo1 cells (Figure 4.3).

#### **4.6 The successful generation of iPSC from an infantile Pompe disease patient and their differentiation into ipDCs**

In contrast to reports by Huang *et al.* (2011), this study was able to reprogram Pompe disease patient's fibroblasts without the need to supplement cultures with GAA. This work further supports the findings by other groups that the generation of iPSCs from Pompe disease patients and an appropriate mouse model is feasible, even in the absence of GAA (Higuchi *et al.*, 2014; Kawagoe *et al.*, 2011; Raval *et al.*, 2015). It is not known whether the dependence on GAA supplementation in the study by Huang *et al.* (2011) was due to a complete absence of endogenous GAA and therefore a CRIM<sup>-</sup> status. Similar to what is presented in this thesis, all other studies that were able to reprogram fibroblasts in the absence of GAA supplementation, had derived fibroblasts from patients with infantile-onset, which in all cases implied a severe disease phenotype (Chen *et al.*, 2004; Hermans *et al.*, 1994, 2004; Higuchi *et al.*, 2014; Huang *et al.*, 2011; Huie *et al.*, 1994a; Kawagoe *et al.*, 2011; Kroos *et al.*, 2008; Raval *et al.*, 2015; Wan *et al.*, 2008). However, despite the fact that our patient was known to be CRIM<sup>+</sup>, the enzyme activity assay showed the complete absence of GAA activity in ipPo1 cells (Figure 4.3) and therefore is unlikely to have improved the propensity of fibroblasts to be reprogrammed. In this study, the reprogramming strategy made use of Sendai virus which differed from other studies, some of which made use of the traditional retroviral transgene or lentiviral approach (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Kawagoe *et al.*, 2011; Raval *et al.*, 2015). The study requiring GAA supplementation for the successful iPSC generation used the retroviral approach (Huang *et al.*, 2011). Other studies using the same approach with no obvious alterations to the protocol did not, however,

require GAA supplementation and therefore indicated that the need for GAA supplementation was not due to different reprogramming strategies (Higuchi *et al.*, 2014; Kawagoe *et al.*, 2011).

The capacity to culture iPSCs from Pompe disease patients and their successful differentiation was also observed by other studies which showed the feasibility of generating skeletal muscle and cardiomyocyte-like cells (Huang *et al.*, 2011; Kawagoe *et al.*, 2011; Raval *et al.*, 2015).

Interestingly, the accumulation of glycogen in ipPo1 cells was only found in a few, localised regions of colonies (Figure 4.4). In contrast, other studies have found profound and wide spread glycogen accumulation in human and mouse iPSC colonies of Pompe disease (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Kawagoe *et al.*, 2011). Whether this was due to technical issues with the staining reagents or whether it is a characteristic of the studied Pompe disease patient or iPSC line could not be determined. Therefore, future work needs to further investigate the glycogen accumulation in these cells using a sensitive and more specific method such as a fluorimetric assay (Pelletier *et al.*, 2013).

Despite studying the differentiation of iPSCs derived from a single Pompe disease patient, the differentiation efficiency in terms of ipMonocytes and ipDC yield, similar to C15 iPSCs, was highly variable (Figure 4.10, Figure 3.3 and Figure 3.4). However, important to note is that the yield of ipMonocytes was not determined by considering the total number of iPSCs that were plated for a differentiation but the number of iPSCs plated into those wells that yielded EBs and ultimately ipMonocytes. Wells that failed to produce EBs or ipMonocytes were not included in this calculation. Therefore, the capacity of ipPo1 to generate

ipMonocytes may be lower than implied by these data. However, whether this is due to the disease phenotype cannot be determined as variations in differentiation efficacy between cultures and wells within the same culture had also been observed with C15 iPSC differentiations. Furthermore, periods of unsuccessful ipDC differentiations have occurred with C15 iPSCs which could not be further explained. However, these results underline the importance for further protocol optimization to allow more consistent and reliable differentiation of ipDCs.

The unsuccessful differentiation of the ipPo0 line at passages 7 to 11, as shown in this study, was not just confined to this line but was also shown by the ipPo1 line. The failure to establish viable differentiations may be explained by the possible residual expression of viral RNA that was introduced for reprogramming, which may maintain the expression of pluripotency factors even in the presence of differentiation stimuli. This RNA from the Sendai virus is known to remain within iPSCs for several passages until it is cleared. Studies by the Oxford Stem Cell Facility have found that the elimination of viral RNA generally occurs after around 10 to 15 passages, but can also occur as late as passage 20 (J. Vowles, personal communication, September 24, 2014). This may explain the inability of ipPo cells to differentiate at early passage as virally-expressed pro-pluripotency transcription factors may counteract the induction of gene expression in response to BMP-4, VEGF, SCF and GM-CSF. However, the absence of FGF in differentiation cultures may lead to the expression of pro-apoptotic genes and therefore promote stress-induced cell death (Eiselleova *et al.*, 2009). The presence and clearance of viral RNA was not investigated in this study, however

the capacity of ipPo1 cells to differentiate from passage 14 onwards, may indicate the clearance of residual reprogramming RNA.

In this chapter it has been demonstrated that ipDCs generated from human Pompe disease iPSCs have a DC generation potential and viability *in vitro* similar to that of ipDCs generated from healthy control C15 iPSCs (Figure 4.15, Figure 3.4 and Figure 3.16). Furthermore, the expression of most DC markers on ipPo1 ipDCs was observed at similar levels to those previously observed on C15 ipDCs. The statistically significant upregulation of MHCII on ipPo1 ipDCs in response to pro-inflammatory cytokines indicated the successful maturation of these cells, even though statistical significance was not achieved in healthy C15 ipDCs or moDCs (Figure 4.11 and Figure 3.5). Furthermore, in contrast to C15 ipDCs, the expression of CD209, a cell surface molecule involved in close adhesion between T cells and DCs and their migration, was maintained by ipPo1 ipDCs following exposure to proinflammatory cytokines (Figure 4.12 and Figure 3.8). CD209 is known to be negatively regulated by IFN $\gamma$  which therefore explained the inability to detect its expression on C15 ipDCs following maturation (Figure 3.8) (Relloso *et al.*, 2002; Zhou *et al.*, 2006). However, the maintained expression of CD209 by matured ipPo1 ipDCs may possibly indicate a reduced responsiveness towards IFN $\gamma$  or that the cells may reside in a different developmental stage.

The capacity to phagocytose latex beads and process protein antigen were similar between ipPo1 ipDCs (Figure 4.13) and C15 ipDCs (Figure 3.13), while the average proportion of T<sub>regs</sub> detected in co-cultures was lower in those with ipPo1 ipDCs (Figure 4.14) as compared to C15 ipDCs (Figure 3.15). Whether this may be indicative of differences between cell donors or potential to polarise T cell populations is not yet known. However, the overall allostimulatory capacity of C15

ipDCs and ipPo1 ipDCs was equivalent, showing a similar proportion of proliferated T cells in co-cultures (Figure 4.14 and Figure 3.33).

#### **4.7 The pro-tolerogenic modulation of ipDCs from a Pompe disease patient**

Following the derivation and characterisation of ipPo1 ipDCs, the ability to modulate these cells using IL-10 was investigated. Similar to C15 ipDCs, ipPo1 ipDCs were not affected by IL-10 modulation with respect to their viability, yield or proportion of CD11c<sup>+</sup> cells (Figure 4.15, Figure 3.16 and Figure 3.17). In contrast to C15 ipDCs, however, IL-10-modulated ipPo1 ipDCs showed a significant downregulation of CD86 expression (Figure 4.18 and Figure 3.26) and no significant change in the expression of immunoinhibitory molecules ILT3 and ILT4 (Figure 4.20, Figure 3.29 and Figure 3.30).

Despite this difference, IL-10-modulated ipPo1 ipDCs showed reduced allostimulatory capacity as compared to the untreated matured ipPo1 ipDCs (Figure 4.22) which was also found in co-cultures with C15 ipDCs where IL-10 modulation significantly reduced their allostimulatory capacity (Figure 3.33). No statistical analysis was performed on the allostimulatory capacity of ipPo1 ipDCs due to the limited number of independent experiments. Therefore, future experimental repetition is required in order to determine the actual effect of IL-10 on ipPo1 ipDC allostimulatory capacity.

Similarly, this will be required for the determination of the effect of IL-10-modulated ipPo1 ipDCs on the proportion of T<sub>regs</sub> and IL-10 concentration in supernatants of co-cultures, as a potential trend towards an increased proportion and concentration is indicated by the data (Figure 4.23 and Figure 4.24),

however, further experiments are required in order to enable statistical analysis. Levels of IL-10 concentration in co-cultures were similar between healthy C15 ipDCs and ipPo1 ipDC cultures on days 5 and 7 (Figure 3.35 and Figure 4.24).

Similar to C15 ipDCs, no difference was observed in the induction or expansion potential of Tr1, IL-2<sup>+</sup> or IFN $\gamma$ <sup>+</sup> T cells by IL-10-modulated ipPo1 ipDCs (Figure 4.25, Figure 3.36, Figure 4.26 and Figure 3.37). However, a striking difference was observed in the proportion of IL-2<sup>+</sup> and IFN $\gamma$ <sup>+</sup> T cell subsets. While C15 ipDCs in co-culture with CD4<sup>+</sup> T cells gave rise to 10-30% of IL-2<sup>+</sup> T cells and 15-30% of IFN $\gamma$ <sup>+</sup> T cells (Figure 3.37), co-cultures with ipPo1 ipDCs gave rise to 1-10% of IL-2<sup>+</sup> or IFN $\gamma$ <sup>+</sup> T cells (Figure 4.26). It remains to be determined whether this indicates a difference in the functional capacity of ipPo1 ipDCs to induce Th1 cells or anergy.

Altogether, despite of some differences, ipPo1 ipDCs have been shown to express DC markers at a level similar to C15 ipDCs, to be uncompromised in their capacity for phagocytosis and endocytosis and function similarly in the stimulation of antigen-naïve T cells.

#### **4.8 Technical challenges and future improvements**

As discussed before in chapter 3, there were numerous technical challenges that complicated the interpretation of results in this chapter.

The variation between blood donors from which T cells were obtained and the variation between MHC haplotypes and therefore the degree of mismatch may have contributed to the significant variation in the results. Therefore, any statistical significant changes in response to IL-10-treatment as compared to the

control group may have been lost due to this variation and the low number of independent experimental repetitions. Therefore, it is paramount that these experiments are reproduced a number of times in order to enable statistical significance to be reached.

Furthermore, as mentioned in chapter 3, bimodal expression profiles were observed in a number of stainings and conditions, causing the gMFI to be misleading as it does not reflect the presence of two populations but rather portrays the existence of one single populations with an expression level in between the two extremes. Whether this bimodal profile may indicate the presence of two distinct DC populations with different immunological capacity remains to be determined. Besides, the interpretability of the flow cytometry data has been limited by the fact that the isotype controls had not been gated the same way as the antigen-specific stainings. The latter were displayed and analysed following the gating onto CD11c-expressing cells while control groups had not been gated specifically on the CD11c<sup>+</sup> populations and may, therefore, include non-DCs. Therefore, populations may have appeared more or less positive for a particular marker depending on whether non-CD11c<sup>+</sup> cells showed more or less background stainings. Future work needs to take this problem into account and ensure that the same gating strategy is applied to specifically-stained and control isotype-stained populations.

As further discussed in chapter 3 and chapter 6, the data obtained in this and other chapters is limited due to the lack of DC-purification. Therefore, contaminating non-DCs were present in DC cultures and DC-T cell co-cultures which may have contributed to the cytokine profile detected and affected the T cell proliferation and polarisation response. Due to the ability of numerous cell



types to contribute to an immunological response, a highly effective purification method is needed to enable the study of DCs and T cells in the absence of other cells. However, for its future clinical application, the potential interfering cells and their effect needs to be investigated in order to broaden our understanding of the factors involved and predict the challenges faced by modulated-DCs *in vivo*.

Apart from the purity and the challenges encountered in the flow cytometric analysis of the cells, the detection of the accumulation of glycogen was not reliable. The detection of purple staining may have been non-specific and confined to locations of high cell density. Whether this was due to high concentration of glycogen or non-specific staining is not clear, however in comparison to other studies, the PAS stainings appeared diffuse and not clearly defined (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Kawagoe *et al.*, 2011). Therefore, a new PAS staining reagent should be used and a more sensitive alternative method applied, such as a fluorimetric assay, in order to detect glycogen specifically (Pelletier *et al.*, 2013).

#### **4.9 The clinical potential of ipDCs and current challenges**

Similar to the clinical potential of C15 ipDCs, ipPo1 ipDCs may present an advantageous DC population with enhanced tolerogenic potential due to their low MHCII expression as compared to moDCs (Figure 3.5, Figure 3.19, Figure 4.11 and Figure 4.16). However, whether the pathogenesis of the disease may have further implications for the clinical potential of ipDCs derived from patients' needs to be further addressed.

Having the disease mechanism of Pompe disease in mind, it remains imperative to further investigate the effect of disease pathogenesis on ipDC function. For example, the impaired lysosomal function may affect the capacity for antigen presentation even though antigen-processing appeared normal in this study (Figure 4.13). Furthermore, the effect of autophagic build up, calcium imbalance and mitochondrial dysfunction on ipDC function needs to be investigated as these abnormalities are known to modulate signalling pathways (Berridge *et al.*, 2003; Kiffin *et al.*, 2006; Lloyd-Evans *et al.*, 2008). Future work needs to either exclude the interference of these pathological pathways in the potential pro-tolerogenic capacity of DCs or ensure the prevention of these interferences by pathological correction.

Therefore, the genetic correction of iPSC may be further explored in order to determine whether the enzymatic defect may render ipDCs less effective in a clinical setting. Future studies may investigate the efficacy of iPSC differentiation into ipDCs in the presence of GAA in order to evaluate potential protocol optimisation and to ensure a reproducible and high yield in order to render ipDCs more attractive than moDCs for immunotherapy. Furthermore, differentiation optimisation and the development of a DC purification method would improve the ability to fully and reliably characterise the ipDC populations and generate pure populations for clinical application. Similar to C15 ipDCs, it is not known what other cells may have been present in differentiation cultures and therefore what effect they may have had on T cell and DC interaction and whether they may have affected or contributed to the cytokine profile detected.

Despite the potential enhanced pro-tolerogenicity of ipDCs, the cost and duration required for the generation of iPSCs and ipDCs in a patient-specific manner

would require expensive investments and in an optimal setting at least around 3 months (Leishman and Fairchild, 2014; Silk *et al.*, 2012a). This may prove to be a significant disadvantage to this approach as monocytes may be obtained within a day and differentiated and modulated within a week while other treatment regimes may not just avoid the immunogenicity of the therapeutic enzyme but at the same time improve the treatment efficacy. Nevertheless, iPSC may remain the only feasible source for the generation of a large numbers of DCs from infantile patients for immunomodulation therapy.

#### **4.10 Conclusion**

Altogether, the data presented in this chapter have underlined the similarities between ipDCs derived from healthy iPSCs and Pompe disease iPSCs and the potential use of IL-10 for the generation of ipDCs with pro-tolerogenic characteristics. IL-10 treatment appeared to reduce the T cell allostimulatory capacity of ipPo1 ipDCs (Figure 4.22), expression of costimulatory molecule CD86 (Figure 4.18) and the induction of IL-2-expressing cells (Figure 4.26). On the other hand, IL-10-modulation could be, but not significantly, increasing the expression of immunoinhibitory molecules (Figure 4.19 and Figure 4.20) and the proportion of T<sub>regs</sub> (Figure 4.23) induced by ipPo1 ipDCs as compared to untreated mature controls. Further studies of additional fibroblast donors are required to allow for estimates about the reliability, reproducibility and feasibility of deriving ipDCs from Pompe disease patients and reliably modulating them to generate ipDCs with pro-tolerogenic characteristics. In particular, the potential impact of the disease phenotype on ipDC function requires more in depth exploration.

Chapter 5 The potential of protein loading into  
DC-derived exosomes via the endocytic  
system and the immunogenicity of exosomes  
as an efficacy indicator for the treatment of  
LSDs



## Introduction

The treatment of LSDs with a neuronal component using ERT has been challenging. The selective permeability of the BBB and the low expression of M6PR, on which most ERT relies, has been the limiting factor in efficient delivery of enzyme to the CNS (Rozaklis *et al.*, 2011; Urayama *et al.*, 2004). A variety of approaches have been explored in order to tackle this challenge.

One study investigated the use of plasmid-encoded transferrin-labelled iduronidase that, upon hydrodynamic tail vein injection into NOD-SCID mice, was taken up and expressed in hepatocytes but not in the brain (Osborn *et al.*, 2008). The presence of the transferrin-label improved iduronidase secretion into the blood and was essential for its uptake into the brain. Short term treatment of MPS I mice with transferrin-labelled iduronidase-carrying plasmid resulted in the reduction of GAG storage in the choroid plexus and meninges. However, interestingly, in immune competent MPS I mice enzyme activity peaked around 48 hours after administration and disappeared within the next three weeks, while activity persisted for longer in immune compromised SCID mice indicating the induction of an immune response that hampers the effectiveness of this approach. Other studies have shown the efficacy of intracerebral or intrathecal administration of enzyme or enzyme-encoding genes or the combination of systemic and intracerebral administration in mouse models and patients (Calias *et al.*, 2012; Jolly *et al.*, 2013; Munoz-Rojas *et al.*, 2008; Spampinato *et al.*, 2011). However, these treatments are highly invasive and carry significant risk. Furthermore, the long term efficacy of these treatments needs to be investigated.

As described in previous chapters, this thesis explores another challenge to efficient ERT, the immune response initiated towards replaced enzyme. The focus of our immunological intervention is on the DC. In order to bring two challenges together and overcome them with a related approach, this thesis explores the possibility of exploiting DCs to overcome the challenge posed by the BBB. This approach arose from a publication by Matthew Wood's Laboratory in Oxford, who showed that exosomes derived from mouse bmDCs could be used for targeted delivery of siRNA via the BBB to the CNS (Alvarez-Erviti *et al.*, 2011). Their data showed that labelling exosomes with RVG, which specifically binds to acetyl choline receptors in the BBB, allowed the transcytosis of exosomes from the circulation into the CNS. This resulted in the specific knockdown of the target gene BACE1 in the CNS. Three years later, another study using the same approach, but targeting the expression of  $\alpha$ -synuclein, has confirmed the feasibility of specially targeting RVG-tagged and siRNA loaded exosomes to the CNS (Cooper *et al.*, 2014). The accumulation of aggregates of  $\alpha$ -synuclein is a hallmark of Parkinson's disease indicating the therapeutic potential of siRNA-loaded exosomes. Although their results showed the delivery of siRNA to the CNS, the question arises as to whether the same strategy might be used for the delivery of therapeutic proteins if they could only be packaged into the exosomes. One study has investigated a variety of membrane anchors for the packaging of proteins into exosomes and microvesicles (Shen *et al.*, 2011). This study found that the addition of acyl groups as putative myristoyl attachment sites and the addition of a PIP<sub>2</sub>-binding domain allowed the packaging of a GFP-labelled protein into secreted vesicles. However, the effect of modulations on enzyme activity remains to be determined.

This chapter investigates the possibility of deriving exosomes, which may also include vesicles of other origin, from mouse and human DCs and whether the endocytic and antigen cross-presentation capacity of DCs might be exploited to take up proteins and package them into exosomes for subsequent release. This potential approach may be supported by studies into the mechanism of exogenous antigen cross-presentation onto MHC I as one particular pathway has been shown to allow the translocation of endocytosed and phagocytosed proteins into the cytosol of the cell (Ackerman and Cresswell, 2004; Ackerman *et al.*, 2006; Kovacsovics-Bankowski and Rock, 1995; Norbury *et al.*, 1997; Rodriguez *et al.*, 1999). Once in the cytosol, proteins have been shown to widely diffuse throughout the cell and retain functional integrity from which they might be packaged into exosomes during MVB formation (Rodriguez *et al.*, 1999). Therefore, we used the easily traceable enzyme HRP in order to investigate the potential of targeting protein into exosomes via the endocytic system of DC via their cross-presentation capacity.

Using the endocytic and cross-presentation capacity of ipDCs may allow for the dual usage of patient-derived iPSCs not only for the generation of tolerogenic DCs for immunomodulation, but as a novel source of protein-loaded exosomes for the delivery of enzymes to the CNS.

Such a strategy raises the important question of whether exosomes derived from DCs are immunogenic. Past studies have shown that exosomes derived from bmDCs can induce antigen-specific naïve CD4<sup>+</sup> T cells *in vivo* or *in vitro* in the presence of DCs (Théry *et al.*, 2002b; Vincent-Schneider *et al.*, 2002). Consequently, the potential of exosomes for cancer immunotherapy as an acellular approach has also been investigated (Hsu *et al.*, 2003; Näslund *et al.*,



2013). However, reports have also described the tolerogenicity of exosomes derived from immature DCs or DCs that have been treated with pro-tolerogenic agents or transgenically induced to express pro-tolerogenic molecules (Bianco *et al.*, 2009; Kim *et al.*, 2005; Li *et al.*, 2012; Yang *et al.*, 2011). The study by Alvarez-Erviti *et al.* (2011), briefly investigated the potential of exosomes loaded with siRNA by electroporation to induce an immune response *in vivo*. Assessing the serum concentrations of IL-10, IFN $\gamma$ -Inducible Protein 10, TNF $\alpha$  and IFN $\alpha$  only, the study was not able to detect any significant changes in these cytokines in response to siRNA-loaded RVG-tagged exosomes, however they did detect a significant increase in IL-6 serum concentrations in response to unloaded RVG-labelled exosomes. These studies call for a more detailed investigation of the immunogenicity of exosomes, in particular the potential of an antibody-mediated neutralising response, as this may considerably hamper the therapeutic efficacy as indicated by former studies (Osborn *et al.*, 2008). Therefore, this thesis makes an attempt at pinpointing the potential *in vivo* immunogenicity of exosomes that had been derived from different populations of DCs from syngeneic or allogeneic strains of mice.

## Results

### 5.1 iPSCs as a novel source for DCs

Since most studies have made use of exosomes derived from mouse bmDCs, we additionally investigated the capacity of DCs differentiated from iPSCs to serve as a novel source of exosomes. More interestingly, previous work in our laboratory has implied an inherent immature or tolerogenic phenotype of ipDCs which may prove advantageous for clinical therapies where an immune response may be detrimental to treatment efficacy (Hackett, 2013).

In anticipation of the downstream immunological studies, two iPSC lines were used that had been derived from two different strains of mice to allow for syngeneic and allogeneic strain combinations.

iMEF1 is a line that had been derived from MEFs from CBA mice while ipB6/2 iPSCs were derived from B6 mice. The reprogramming, expansion and teratoma assays were performed in our laboratory by Tim Davies. iPSCs had been further characterised by Simon Hackett and Patty Sachamitr (Hackett, 2013) (P. Sachamitr, personal communication, September 18, 2014) . As seen in Figure 5.1, iPSCs derived from reprogramming displayed a typical stem cell morphology of rounded cells with large nuclei growing in dense colonies on mitotically-inactivated feeder cells (Hackett, 2013). Transcription factor Oct3/4, essential to the pluripotency of cells, and pluripotency marker SSEA-1 were detected at high levels in and on the surface of iPSCs respectively (International Stem Cell Initiative *et al.*, 2007; Nichols *et al.*, 1998). The expression of Nanog was detected at low levels which may be explained by the finding that Nanog expression levels may vary depending on the phase of cell cycle (van der Laan *et al.*, 2014). Other

laboratories have found great variation in their ability to detect Nanog expression which they found was sensitive to fixation methods and strongly dependent on the antibody and antibody isotype (S. Cowley, personal communication, March 25, 2013). However, previous studies in our laboratory have described the iMEF1 and ipB6/2 lines in detail and confirmed their pluripotency by teratoma assays and the formation of all three germ layers (Hackett, 2013) (T. Davies, personal communication, August 14, 2014).

For the differentiation of mouse iPSCs into ipDCs, cells were transferred from feeder cell cultures onto gelatine and cultured for several passages before the setup of differentiation cultures (Figure 5.2A). iPSCs were harvested and plated into suspension cultures on bacteriological dishes for 2 weeks to allow the formation of EBs as illustrated in Figure 5.2B/C. After the 2 week period, EBs were plated onto cell culture plates where they attached and further differentiated (Figure 5.2D-F). After around 2 weeks, EBs started to release DC-precursors and DC-like cells (Figure 5.2G-I). Within the next few days, ipDCs appeared in culture and accumulated over time for harvesting and further processing.

The DC-like morphology was confirmed by comparison of ipDCs with bmDCs derived from CBA or B6 mice, all of which displayed veils of cytoplasm and long cellular protrusions typical of DCs (Figure 5.3).

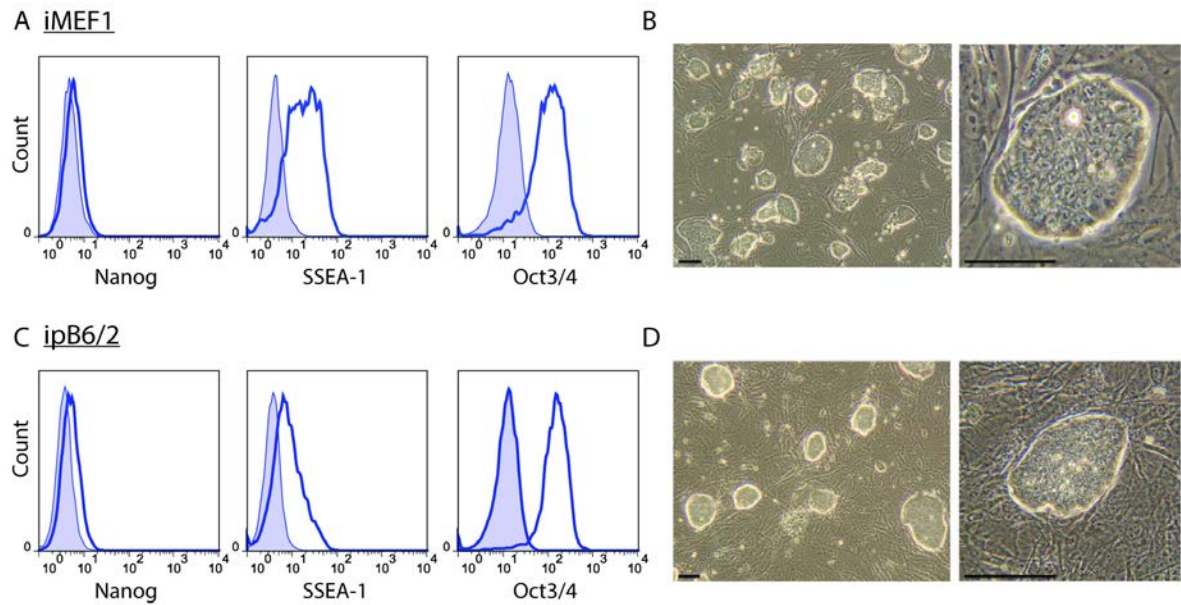


**Figure 5.1 The expression of pluripotency markers and morphology of mouse iPSCs.** Mouse iPSC colonies of the (A/B) iMEF1 line had been derived from CBA MEFs and the (C/D) ipB6/2 line had been derived from B6 MEFs by Tim Davies. (A/C) The expression of pluripotency factors Nanog, SSEA-1 and Oct3/4 was investigated by antigen-specific antibody staining of iMEF1 at passage 12 and ipB6/2 at passage 20 and analysed by flow cytometry. Tinted histograms represent isotype controls of the antigen-specific antibody which is displayed as a clear and thick-lined histogram. Data presented is the most representative of 2 independent experiments. (B/D) Photomicrographs illustrate the typical stem cell morphology of mouse iPSCs grown on Feeder cells. Scale bar: 100µm.

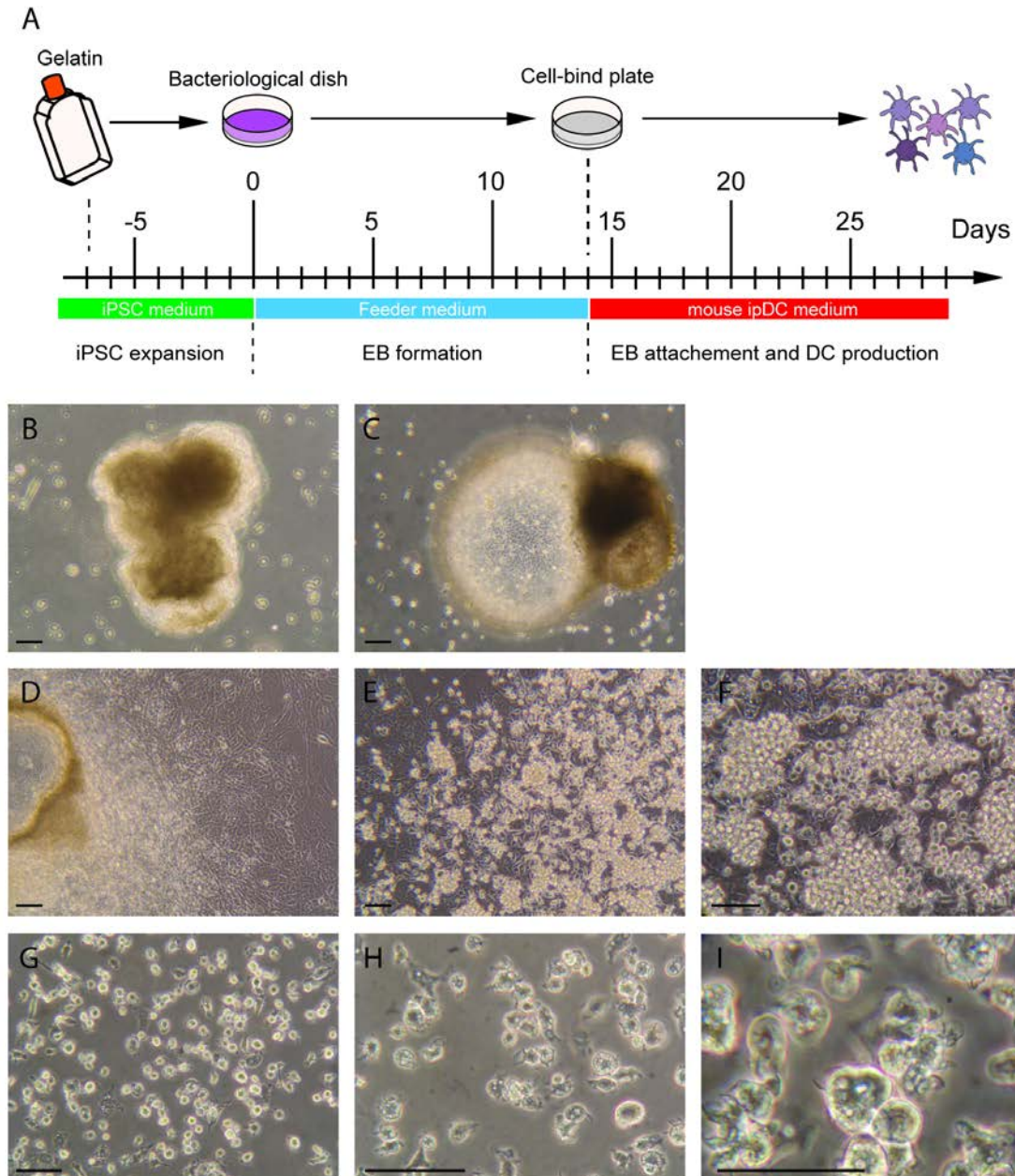


**Figure 5.2 Differentiation timeline.** (A) A timeline of the differentiation process of mouse iPSCs into ipDCs. (B-I) Photomicrographs illustrate the typical appearance and morphology of different stages of iPSC differentiation as illustrated in (A). Prior differentiation, iPSCs were expanded on gelatine-covered flasks to remove Feeder cells. Differentiation was initiated by plating iPSCs into a suspension culture on bacteriological plates. After 2 weeks EBs had formed (B/C) and were plated onto cell culture plastic to allow their attachment (D) and outgrowth (E/F) and subsequent production of ipDCs and precursors after around 2-3 further weeks (G-I). Scale bar: 100  $\mu$ m.

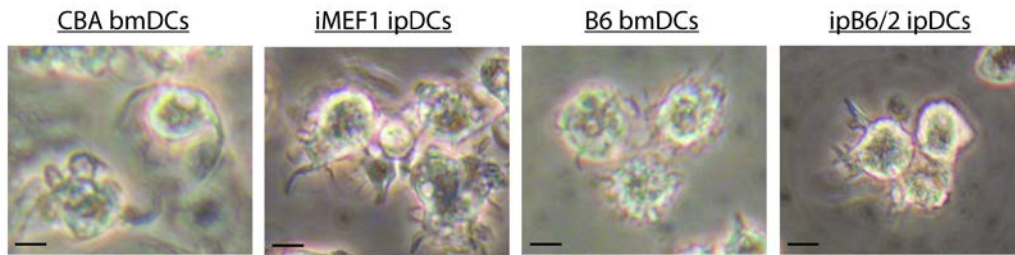


**Figure 5.3 The morphology of mouse bmDCs and ipDCs.** Photomicrographs displaying the characteristic appearance of bmDCs and ipDCs. Mouse ipDCs were differentiated from iPSCs at passages 14 to 26. Scale car: 10  $\mu$ m.

## 5.2 The phenotype of ipDCs

In order to confirm that the cells derived from iPSCs display features consistent with DCs, cells were further analysed for the expression of DC markers by flow cytometry. First of all, ipDCs appeared to express lower levels of CD11c as compared to immature and mature bmDCs as displayed in Figure 5.4 (mean  $\pm$  SEM ;immature CBA bmDC:  $27.05 \pm 6.04$  relative gMFI ( $n=10$ ), iMEF1:  $8.97 \pm 1.8$  relative gMFI ( $n=5$ ),  $p = 0.0386$ ). ipDCs derived from ipB6/2 were found to, on average, not to express CD11c (immature B6 bmDCs:  $27.07 \pm 2.36$  relative gMFI ( $n=4$ ), ipB6/2:  $0.9899 \pm 0.46$  relative gMFI ( $n=3$ ),  $p = 0.045$ ). Studies in our laboratory had previously shown the variability of CD11c expression by ipB6/2 ipDCs, however, functional studies have confirmed their DC-like phenotype (P. Sachamitr, personal communication, August 7, 2014).

CD11b and CD123 were detected on the surface of all DCs, however no expression was detect on ipDCs with the exception of CD123 which was detected at very low levels on iMEF1 ipDCs (Figure 5.4). The expression of CD54 was detected on bmDCs and upregulated on maturation, which was found to be statistically significant in the CBA strain (immature CBA bmDCs:  $37.33 \pm 5.15$

relative gMFI ( $n=10$ ), mature CBA bmDCs:  $111 \pm 15.61$  relative gMFI ( $n=5$ ),  $p < 0.0001$ ). Although ipDCs were not negative for CD54, they were found to express CD54 at considerably lower level as compared to bmDCs which was statistically significant for the CBA strain (immature CBA bmDCs:  $37.33 \pm 5.15$  relative gMFI ( $n=10$ ), iMEF1:  $8.45 \pm 0.9$  relative gMFI ( $n=6$ ),  $p = 0.038$ ).

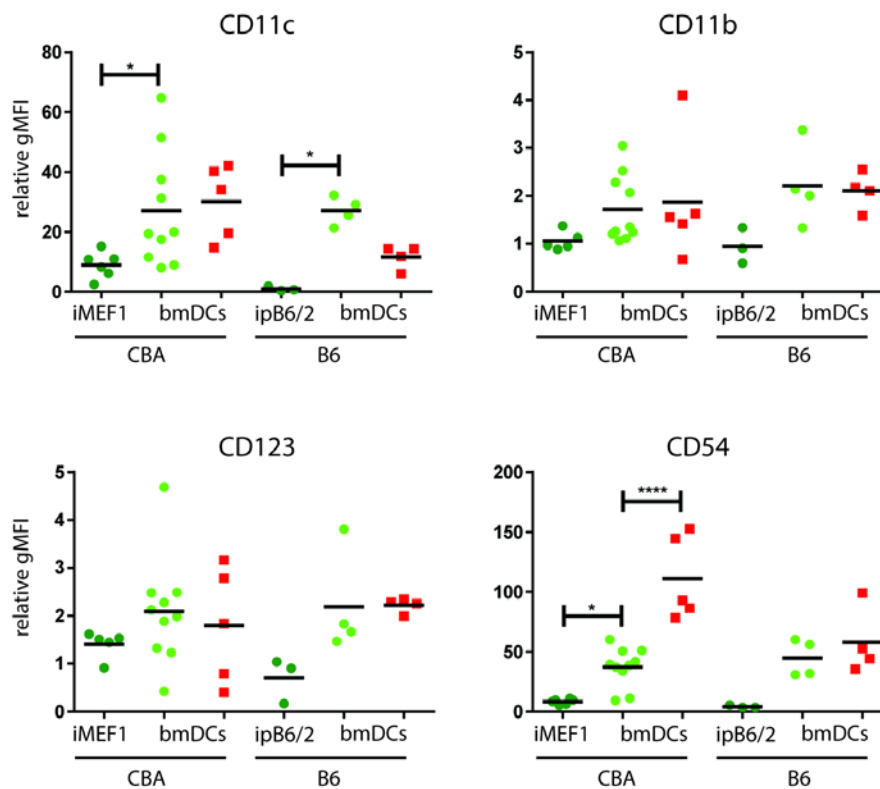


**Figure 5.4 The expression of DC markers by mouse ipDCs.** Mouse ipDCs derived from CBA and B6 iPSCs at passages 14 to 26 and CBA and B6 bmDCs were harvested, stained for the cell surface expression of DC markers CD11c, CD11b, CD123 and CD54 and analysed by flow cytometry. Immature DCs are presented as green dots and mature DCs are presented as red squares. The relative geometric MFI was determined as the geometric MFI obtained from antigen-specific antibody labelled cells divided by the geometric MFI obtained from cells stained with the relative isotype control. Black lines represent the mean of the relative gMFI as determined by independent experiments which are displayed as the dots (immature) and squares (mature) ( $n=3-10$ ). Statistical analyses was performed using the One-Way ANOVA with Sidak's multiple comparisons test (\* $p \leq 0.05$ ; \*\*\*\* $p \leq 0.001$ ).

This is consistent with previous findings in our laboratory that described ipDCs to retain an extremely immature phenotype reminiscent of DCs from the early foetus, even in response to inflammatory signals such as LPS (Aksoy *et al.*, 2007; Hunt *et al.*, 1994; Velilla *et al.*, 2006). Supporting this theory, Figure 5.5 illustrates the finding that ipDCs consistently expressed lower levels of MHCI and MHCII, even lower than the levels detected on immature bmDCs (H-2K<sup>b</sup>: immature B6 bmDCs:  $7.513 \pm 1.033$  relative gMFI ( $n=3$ ), ipB6/2:  $2.235 \pm 0.284$  relative gMFI ( $n=3$ ),  $p = 0.0102$ ). As expected, maturation of bmDCs by LPS exposure significantly upregulated the expression of MHC determinants on their surface (H-2K<sup>k</sup>: immature CBA bmDCs:  $4.842 \pm 0.709$  relative gMFI ( $n=9$ ), mature CBA bmDCs:  $27.27 \pm 3.78$  relative gMFI ( $n=4$ ),  $p < 0.0001$ ) (A/IE: immature CBA bmDCs:  $3.67 \pm 0.814$  relative gMFI ( $n=10$ ), mature CBA bmDCs:  $17.37 \pm 8.72$  relative gMFI ( $n=5$ ),  $p = 0.0078$ ) (H-2K<sup>b</sup>: immature B6 bmDCs:  $7.51 \pm 1.033$  relative gMFI ( $n=3$ ), mature B6 bmDCs:  $11.75 \pm 1.06$  relative gMFI ( $n=3$ ),  $p = 0.0272$ ). This was similar to observations made in Chapter 3, where human iPSC-derived DCs showed more than 10 times lower expression levels of MHCII as compared to that detected on moDCs, indicating an inherent immaturity of iPSC-derived DCs (Figure 3.5).

In this study, matured ipDCs were not characterised, as exosomes derived from mature DCs have been described to be more immunogenic which would preclude their use for enzyme delivery *in vivo* (Li *et al.*, 2012; Näslund *et al.*, 2013; Quah and O'Neill, 2005; Yang *et al.*, 2011; Yin *et al.*, 2013). Furthermore, exosomes administered *in vivo* are thought to be inert towards any further inflammatory signals which would allow exosomes to retain the stimulatory or inhibitory



capacity they inherit from the cell of origin (Näslund *et al.*, 2013; O'Loughlin *et al.*, 2012).

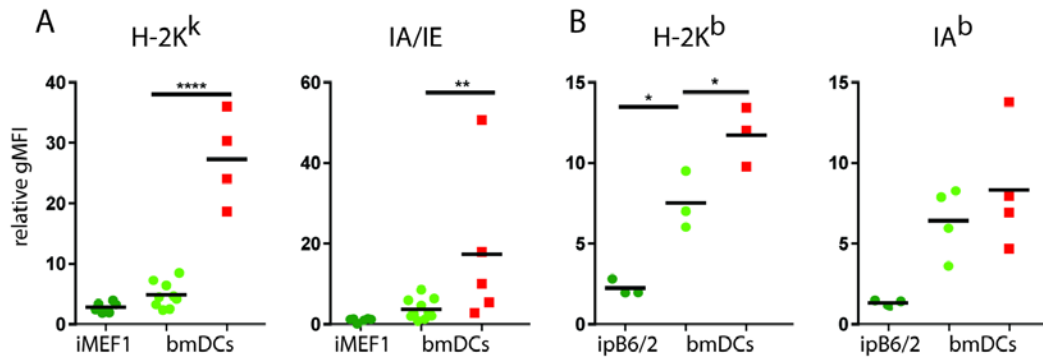


**Figure 5.5 The expression of MHCs by mouse ipDCs.** The cell surface expression of MHCII (IA/IE, IA<sup>b</sup>) and MHCI (H-2K<sup>k</sup>, H-2K<sup>b</sup>) was investigated by flow cytometry. The relative gMFI was determined as the geometric MFI obtained from antigen-specific antibody labelled cells divided by the geometric MFI obtained from cells stained with the relative isotype control. (A) The expression of H2-K<sup>k</sup> and IA/IE, which includes IA<sup>k</sup>, by bmDCs and ipDCs of the CBA mouse strain was determined. (B) The expression of H2K<sup>b</sup> and IA<sup>b</sup> by bmDCs and ipDCs of the B6 mouse strain was determined. ipDCs of both strains were differentiated from iPSCs at passages ranging from passage 14 to 26. Black lines are representative of the mean of the relative gMFI as obtained from 3-10 independent experiments that are displayed as green dots (immature DCs) and red squares (mature DCs). Statistical analyses was performed using the One-Way ANOVA with Sidak's multiple comparisons test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\*\* $p \leq 0.001$ ).

As displayed in Figure 5.6, costimulatory molecules CD86, CD80 and CD40 were found to be expressed on immature and mature DCs. The expression of CD86 and CD80 were found to be statistically significantly increased on CBA bmDCs in response to LPS exposure (CD86: immature CBA bmDCs:  $5.855 \pm 1.397$  relative gMFI ( $n=10$ ), mature CBA bmDCs:  $26.15 \pm 5.601$  relative gMFI ( $n=5$ ),  $p < 0.0001$ ) (CD80: immature CBA bmDCs:  $4.868 \pm 0.6602$  relative gMFI ( $n=10$ ), mature CBA bmDCs:  $21.20 \pm 10.77$  ( $n=5$ ),  $p = 0.0212$ ). Consistent with previous work and data presented above that describe ipDCs as cells with an extreme immature

phenotype, the expression of all costimulatory molecules described here was on average below levels detected on immature bmDCs (Hackett, 2013).

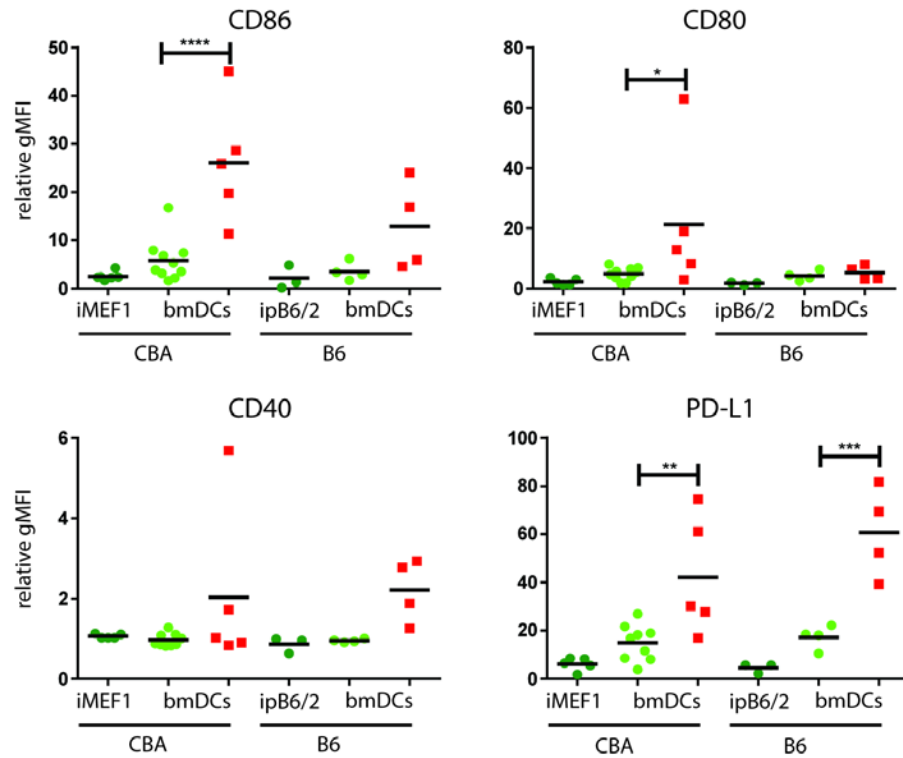


**Figure 5.6 The expression of costimulatory and inhibitory molecules by mouse ipDCs.** The cell surface expression of costimulatory molecules CD86, CD80, CD40 and ICOSL and immunoinhibitory molecule PD-L1 was investigated by flow cytometry. ipDCs were differentiated from CBA and B6 iPSCs at passages 14 to 26. The relative geometric MFI was determined as the geometric MFI obtained from antigen-specific antibody labelled cells divided by the geometric MFI obtained from cells stained with the relative isotype control. The mean of the gMFI as determined by 3-10 independent experiments is represented by the black lines. Independent experiments are displayed as green dots (immature DCs) and red squares (mature DCs). Statistical analyses was performed using the One-Way ANOVA with Sidak's multiple comparisons test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ ; \*\*\*\* $p \leq 0.001$ ).

Interestingly, the immunoinhibitory molecule PD-L1 was also expressed at low levels by ipDCs (Figure 5.6). However, mature bmDCs expressed higher levels of PD-L1 indicating that the difference in phenotype between ipDCs and bmDCs

may not be due to inherent tolerogenic properties but an immature phenotype reminiscent of neonatal DCs (Goriely *et al.*, 2001, 2004). Additionally, the low levels of marker detection on ipDCs may be due to the low purity of samples as the differentiation of iPSCs into DCs yields a variety of cells which include ipDCs as well as non-DCs. Future work needs to look into the generation of a purification protocol to enable the study of more pure populations. Making use of CD11c-specific purification methods may not be an option for mouse ipDCs due to the varied level of CD11c-expression.

Altogether, these data support the investigation of the feasibility and immunogenicity of ipDC-derived exosomes as similar to immature bmDCs, immature ipDCs expressed low levels of costimulatory molecules. More importantly, immature ipDCs expressed lower levels of MHC class I and II, even in some cases lower than the levels detected on immature bmDCs. These characteristics may support the production of exosomes with low inherent immunostimulatory capacity and therefore highlight the importance of investigating the phenotype of ipDC-derived exosomes.

### **5.3 The generation and purification of exosomes**

The main distinction between exosomes and microvesicles, apart from molecular make up and size, is their cellular origin. While microvesicles bud off the membrane at the cell surface, exosomes are generated within the cell in so called MVB. As shown in Figure 5.7, bmDCs displayed structures that strongly resemble MVB. Vesicles of around 100 nm diameter were also observed enclosed within membrane-bound structures within the cells.

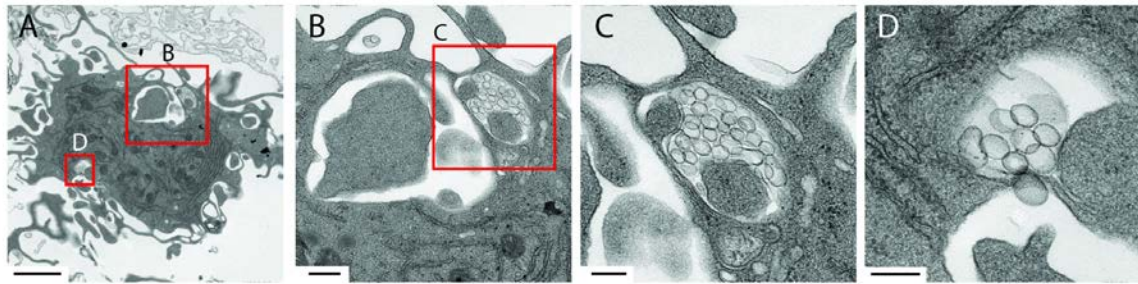


**Figure 5.7 The presence of multivesicular bodies.** Images generated by the EM show intracellular structures reminiscent of MVB present in CBA bmDCs. (A-C) A large membrane-bound structure enclosing many smaller vesicular structures of a homogenous size of around 100 nm diameter was observed. (D) Vesicles of around 100 nm diameter appear to be released from a larger intracellular membranous structure into the surrounding extracellular space. Scale bars: A: 2 $\mu$ m; B,C,D: 200nm.

In order to investigate whether exosomes could be purified from bmDCs and ipDCs, supernatants were harvested from cell cultures, centrifuged at 300 *g* to remove any cell debris, filtered in order to remove particles larger than 200 nm and ultracentrifuged to sediment any particles within the size range of exosomes. As a control, exosomes purified from the neuroblastoma cell line N2a were kindly provided by Yi Lee in the Department of Physiology, Anatomy and Genetics in Oxford, as the N2a cells had been well characterised for the release of large numbers of exosomes positive for the exosome markers CD9 and Alix. The pellets generated from the purification steps were resuspended in PBS and analysed by Nanosight which detects the number of particles within a sample and their respective sizes by Brownian motion. As shown in Figure 5.8, using the centrifugation protocol allowed the purification of vesicles ranging from a size of 50 nm to 400 nm diameter, however the vast majority of particles were around 100 nm diameter. The size distribution of vesicles derived from bmDCs and

ipDCs resembled that obtained from the control N2a cell line. The presence of vesicles larger than 200 nm diameter despite a filtration step through a 200 nm-pore sized filter may be due to a flexibility of vesicles in shape, which may allow larger vesicles to pass through the pores, or potentially some degree of post-purification aggregation.

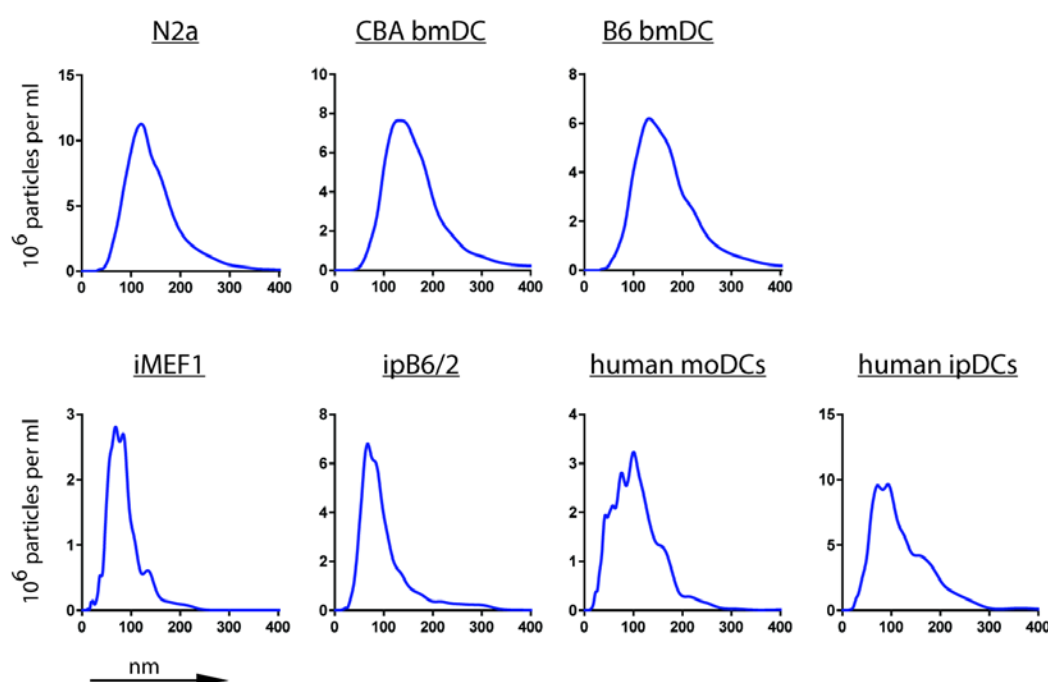


**Figure 5.8 The size distribution of DC-derived vesicles.** A standard ultracentrifugation protocol was used for the purification of exosomes from cell culture supernatants. Exosomes were purified from cultures of N2a cells, mouse bmDCs and ipDCs and human moDCs and ipDCs and analysed by Nanosight in collaboration with Yi Lee at the Department of Physiology, Anatomy and Genetics. Note that the number of particles per ml may vary due to the culture of different numbers of cells. Data shown are representative data of independent experiments (N2a:  $n=4$ ; CBA bmDCs:  $n=11$ ; B6 bmDCs:  $n=6$ ; iMEF1:  $n=10$ , ipB6/2:  $n=4$ ; human moDCs:  $n=5$ ; human ipDCs:  $n=7$ ). Human and mouse ipDCs were differentiated from iPSCs at passages 14 to 26.

The presence of exosome markers Alix and Tsg101 detected in Western blots confirmed the presence of exosomes in the pellets purified from mouse bmDCs, mouse ipDCs, human moDCs and human ipDCs (Figure 5.9A). However, these

markers were not detected in all samples. The variation and heterogeneity of the phenotype of exosomes is not well understood. Whether this variation may be influenced by different stages of cell cycle or the age of a cell remains to be answered. EM confirmed the presence of vesicle-like structures of around 50 to 100 nm diameter that stained positively with anti-CD9 labelled immunogold, confirming the expression of exosome-marker CD9 (Figure 5.9B).

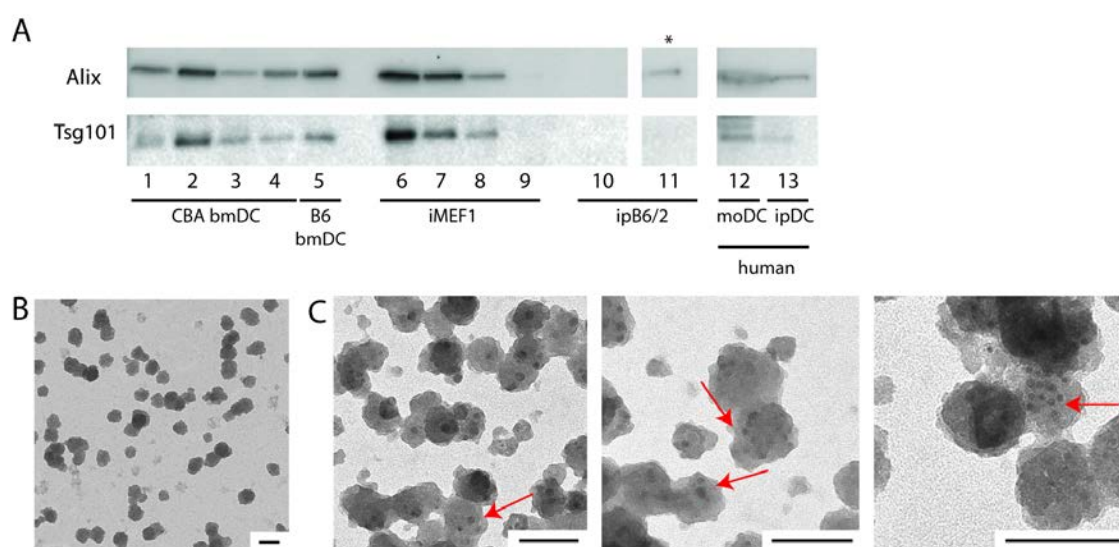


**Figure 5.9 The detection of exosome markers on DC-derived vesicles.** (A) Western blotting performed in collaboration with Yi Lee at the Department of Physiology, Anatomy and Genetics in Oxford, confirmed the presence of Alix (96 kDa) and Tsg101 (43 kDa) in exosome samples. Of all samples (1-13)  $10^9$  vesicles were loaded per well with the exception of the \* labelled sample which was loaded with  $5 \times 10^9$  vesicles. Both ipB6/2 exosome samples were of the same experiment, however loaded at different numbers. Data presented is representative of 2 independent experiments. (B) EM confirmed the presence of vesicles of 50-100 nm diameter. Very few vesicles were non-specifically stained with an immunogold isotype control. (C) Vesicles stained positively for anti-CD9 antibody secondary labelled with immunogold as indicated by small black dots associated to the vesicles (arrows). Scale bars: 100  $\mu$ m.

#### **5.4 The characterisation of exosomes derived from different populations of DCs**

Previous studies have shown the possibility of analysing exosomes with high resolution flow cytometry using the BD Influx flow cytometer or by standard procedures if exosomes were attached to larger beads to enable reliable detection with the flow cytometer (Hoen *et al.*; Näslund *et al.*, 2013; van der Vlist *et al.*, 2012). Therefore, exosomes were analysed by allowing their non-specific binding to aldehyde-sulphate latex beads to allow their detection and analysis of marker expression using a FACS Calibur.

Exosomes were analysed for the expression of a range of DC markers and costimulatory molecules. The exosome populations characterised in this thesis were derived from immature mouse bmDCs and ipDCs of the CBA or B6 strain. Exosomes derived from matured CBA bmDCs were also analysed to represent exosome populations with known immunogenic properties (Segura *et al.*, 2005).

As displayed in Figure 5.10, the exosome marker CD9 was detected on exosomes derived from all different DC populations. However, exosomes derived from the B6 background had consistently lower expression of CD9. Furthermore, exosomes samples derived from different cultures varied in their CD9 expression even if derived from the same cell type and strain.

As exosomes had been derived from DCs, it was of interest to investigate whether they retained the DC marker CD11c which might distinguish them from exosomes derived from other sources. CD11c was detected in a few samples derived from immature and mature CBA bmDCs, however the majority of purified exosomes were negative for CD11c when analysed using this method (Figure 5.10). On

average CD11b was detected on all populations and strains of exosomes derived from bmDCs, while ipDC-derived exosomes appeared low in their CD11b expression or negative. CD54 was detected in the majority of immature and mature CBA bmDC and ipDC-derived exosomes while immature B6 bmDC and ipDC-derived exosomes appeared on average negative for CD54.

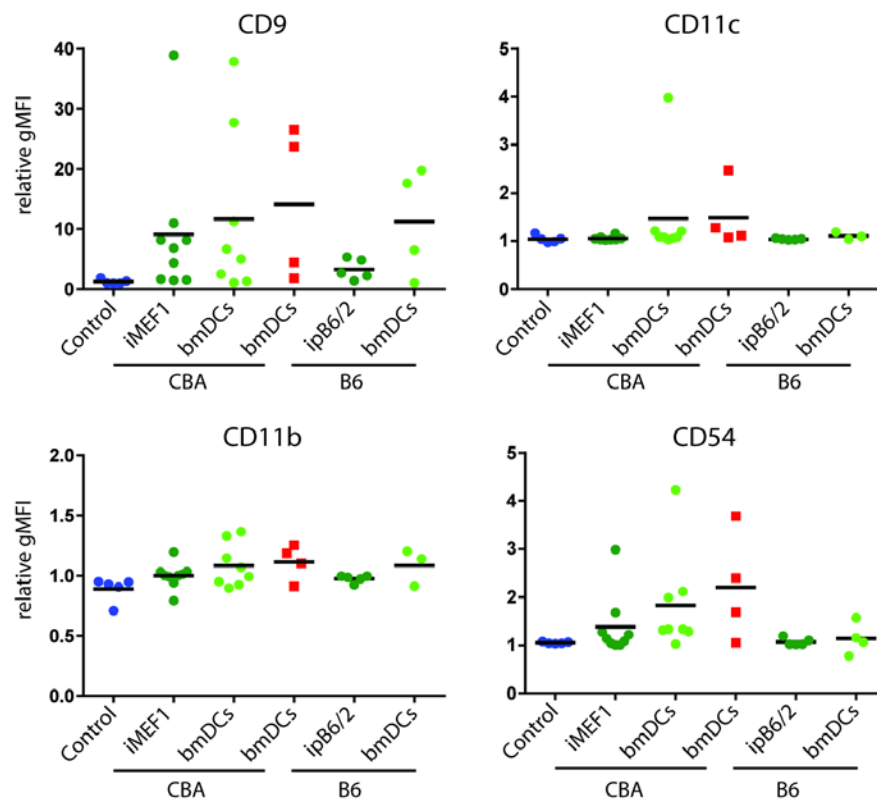


**Figure 5.10 The phenotypic characterisation of exosomes derived from different DC populations: Exosome and DC markers.** The presence of CD9, CD11c, CD11b, and CD54 on exosomes derived from immature (green dots) and mature (red squares) bmDC and ipDCs of different strains. Black lines represent the mean of the gMFI as determined by independent experiments which are presented as dots and squares ( $n=3-9$ ). Controls represent samples containing beads in the absence of exosomes to account for any background staining. No statistical significance was achieved using the One-Way ANOVA with Sidak's multiple comparisons test ( $ns. p > 0.05$ ).



As an indicator of the potential immunogenicity, exosomes were analysed for the presence of MHCII and MHCI on their surface (Figure 5.11). A varied population was observed for all CBA-derived exosomes, some exosomes appearing to display H-2K<sup>k</sup> on their surface while some appeared negative. No reliable staining for H-2K<sup>b</sup> or IA/IE was detected in all exosome samples.

CD80 was detected on mature CBA bmDC-derived exosomes while CD86 was detected on immature and mature CBA bmDC-derived exosomes. PD-L1 was detected on mature CBA bmDC-derived exosomes and one sample of immature CBA bmDC-derived exosomes appeared positive. These results reflect the expression profiles detected on the cells from which the exosome populations originated. CD11c, CD11b and CD54 were either not expressed or expressed at very low levels by ipDCs, which was reflected in the detection profile obtained from exosome analysis (Figure 5.10 and 5.4). Similarly, CD80, CD86 and PD-L1 were expressed at significantly higher levels on mature bmDCs as compared to immature bmDCs or ipDCs (Figure 5.11 and 5.5). These data may indicate a relation between the expression profile of exosomes and their parental cell type.

The variation in the detection of markers on exosomes may be due to an inadequate sensitivity of this method of detection or a great heterogeneity of exosome populations produced by a particular type of cell. Therefore, a more sensitive method will need to be used for future characterisation of bmDC and ipDC-derived exosomes such as Nanosight with fluorescence detectors.

For the clinical application of exosomes, it is however important to ensure the possibility of deriving exosomes from human DC populations. Therefore, human ipDC-derived exosomes were next characterised.

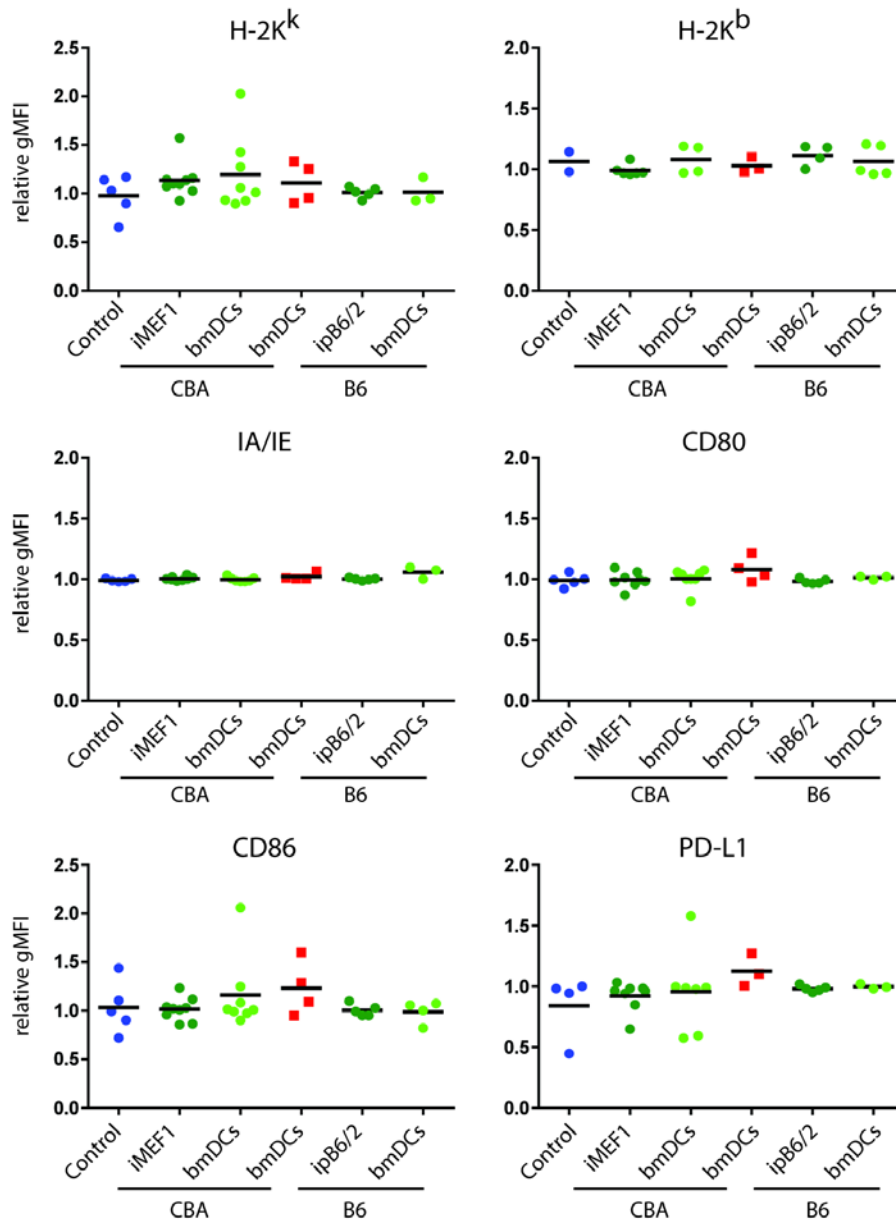


**Figure 5.11 The phenotypic characterisation of exosomes derived from different DC populations: MHCs, costimulatory and inhibitory molecules.**

The presence of H-2K<sup>k</sup>, H-2K<sup>b</sup>, IA/IE, CD80, CD86 and PD-L1 on exosomes derived from different DC populations and strains was investigated. Black lines represent the mean of gMFI as determined by independent experiments ( $n=3-9$ ). Independent experiments are presented as green dots (exosomes derived from immature DCs) and red squares (exosomes derived from mature DCs). Controls represent samples containing beads in the absence of exosomes to account for any background staining. No statistical significance was achieved using the One-Way ANOVA with Sidak's multiple comparisons test ( $ns$ .  $p > 0.05$ ).

## **5.5 Exosomes derived from human ipDCs**

Vesicles ranging in size from 50 nm to 300 nm diameter were purified from supernatants of human moDCs and ipDCs cultures (Figure 5.8). In order to confirm the presence of exosomes and their respective phenotype, human ipDC-derived exosomes were analysed for the expression of DC markers. The presence of exosome marker CD9 was confirmed by flow cytometry as shown in Figure 5.12. Low levels of MHCII were detected on exosomes derived from matured ipDCs, however, no other DC markers were detected on any of the two ipDC exosome populations. This is an interesting finding as MHCII had been previously found to be expressed at very low levels by human ipDCs, especially if compared to levels expressed by moDCs (Figure 3.5). However, these data may further support the increased immunogenicity of exosomes derived from mature DCs as compared to immature DC-derived exosomes on which no MHCII expression was detected (Figure 5.12). Similar to Western blotting of human moDC and ipDC-derived exosomes as displayed in Figure 5.9, western blotting of proteins derived from human ipDC vesicles confirmed the presence of CD9, Flot-1 and Alix. Furthermore, the detection of calnexin may indicate a degree of sample contamination with cellular fragments containing ER membranes.

Having determined the feasibility of purifying exosomes from different populations of DCs, the next step was to investigate the possibility of exploiting the endocytic capacity of DCs and their capacity to translocate proteins into the cytoplasm to load exosomes with proteins such as lysosomal enzymes.

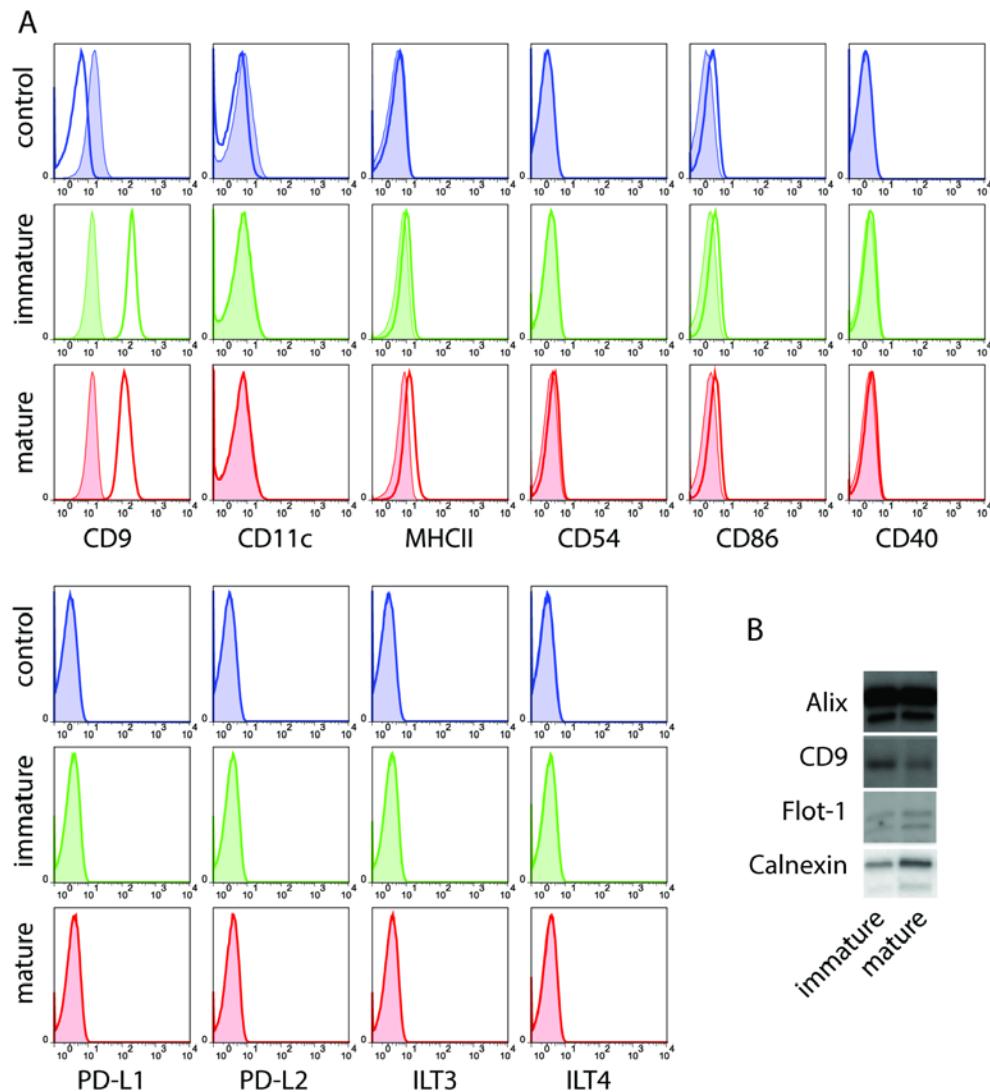


**Figure 5.12 The characterisation of human ipDC-derived exosomes.** (A) Exosomes were harvested in collaboration with Yi Lee at the Department of Physiology, Anatomy and Genetics, Oxford, from supernatants of human C15 ipDC cultures differentiated from C15 iPSCs at passage 23, coated onto beads, stained for specific markers and analysed by flow cytometry. Controls represent beads that had not been coated with exosomes but were treated identically to exosome-coated beads in order to control for unspecific antibody binding to beads. Exosomes were derived from ipDCs which were untreated and therefore immature and ipDCs that had been matured with a cocktail of pro-inflammatory cytokines. This experiment was performed once. (B) The same set of exosomes analysed by flow cytometry was analysed by Western blotting for the detection of exosome markers CD9 and Alix and endosome marker Flot-1 and ER marker calnexin. Western blotting had been performed a total of five times with human ipDC-derived exosomes derived from five independently performed differentiation experiments using ipDCs derived from human C15 iPSCs at passages 17 to 23.

## **5.6 The possibility of exploiting the endocytic capacity of DCs for the loading of proteins into exosomes**

In order to explore the possibility of harnessing the endocytic capacity of DCs to internalise antigens of interest from the environment and their translocation into the cytosol for packaging into exosomes for subsequent release, HRP was used as a model protein that might mimic the use of lysosomal enzymes used in ERT. HRP was chosen as it is easily detectable by a variety of methods and has been previously used to study the translocation of antigen from internalised vesicles into the cytosol of cells (Rodriguez *et al.*, 1999). Furthermore, the possibility of detecting HRP by functional assays such as a colorimetric assays or the detection of reaction product deposits enables the detection of enzyme that has maintained its functional integrity and has escaped antigen processing. Due to the ease of obtaining bmDCs as compared to ipDCs and their capacity to internalise antigen and translocate it from internalised vesicles into the cytoplasm, bmDCs were mainly used to investigate this possibility (Ackerman *et al.*, 2006; Rodriguez *et al.*, 1999). To the best of our knowledge, this potential exosomal cross-loading pathway has not been previously explored for the delivery of proteins into exosomes.

First of all, it was investigated whether the internalisation of HRP could be followed by incubating cells for 2 hours with HRP and subsequently analysing the cells using EM. Analysis by EM showed the presence of large intravesicular structures within cells that had been incubated with 100 µg/ml of HRP as shown in Figure 5.13 (E-K), while samples which had not been incubated with the protein did not display any of these characteristics (A-D).

Furthermore, it was of interest whether HRP retained its enzymatic activity after internalisation by DCs. Therefore, after the 2 hour incubation with HRP, cells were extensively washed and processed for EM. Samples were incubated with DAB staining reagent which in presence of peroxide was converted by HRP into an insoluble dark and electron-dense product.

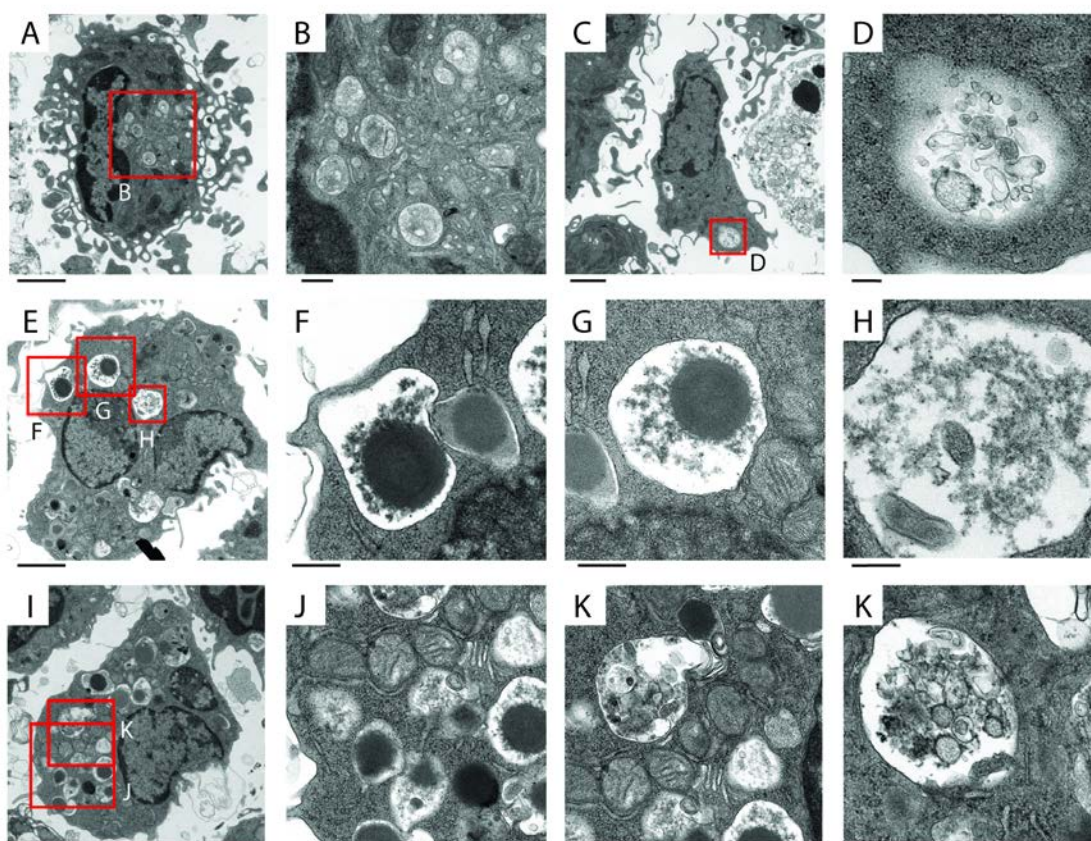


**Figure 5.13 The endocytosis of protein by DCs.** bmDCs were cultured for 2 hours in presence of 100 µg/ml of HRP (E-K) or left untreated as a control (A-D). After extensive washing bmDCs were fixed and processed according to the EM standard protocol described in Chapter 2. Scale bars: A,C,E,I: 2 µm; B,J,K: 500 nm; D,F,G,H,K: 200 nm.

As illustrated in Figure 5.14, DCs that had been incubated with HRP showed electron-dense round particles which appeared to be confined to membrane-enclosed structures within the cell. These structures were not observed in control

cells which had not been incubated with HRP. Furthermore, performing a colorimetric assay on cells after the 2 hour incubation and extensive washing showed that DCs retained HRP activity while T cells that lack endocytic capacity that had been treated with HRP or cells that had remained untreated did not produce any colour reaction (Figure 5.14C). These results suggest that enzyme uptake is dependent on the endocytic capacity of DCs as T cells were not capable of retaining HRP activity.

Further investigation of EM data showed the intracellular localisation of HRP in membrane bound structures which resemble MVB (Figure 5.15). Whether other areas of electron-dense material were located within membrane-bound structures or within the cytoplasm could not be determined. The inability to determine the presence of HRP in the cytoplasm may have been due to the relatively short HRP incubation time until cells were processed for EM. An incubation time of 2 hours was used in this study while Rodriguez *et al.* (1999) used 1 hour time points up to 8 hours which showed a progressive increase in the percentage of cells with cytoplasmic HRP, starting with less than 10% after 2 hours and increasing to nearly 50% of cells after 6 hours.

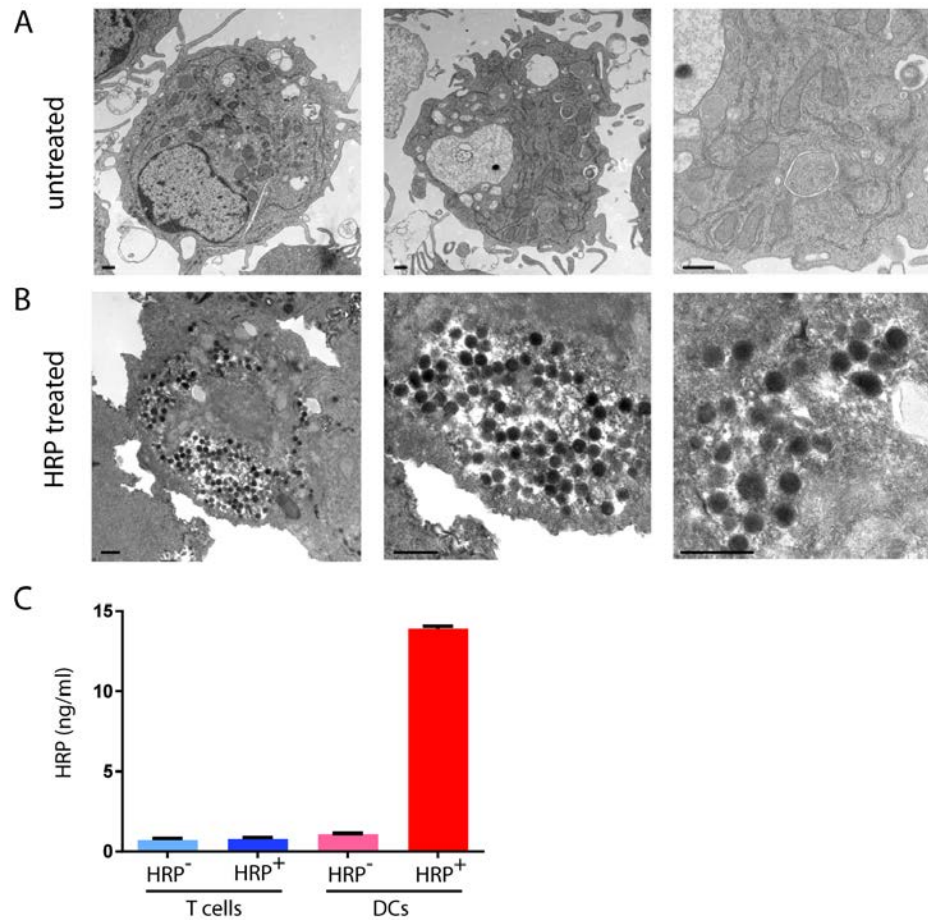


**Figure 5.14 The endocytosis of enzyme by DCs.** (A) Exosomes were purified from bmDCs that had been left untreated (A) or incubated for 2 hours with 100  $\mu\text{g/ml}$  of HRP (B). After extensive washing cells were stained with DAB according to the EM protocol described in Chapter 2 and imaged. Scale bars: 500 nm. (C) DCs and T cells that had been treated with HRP (labelled: HRP<sup>+</sup>) or left untreated (labelled: HRP<sup>-</sup>) were extensively washed and treated with triton-X-100 to permeabilise the cells. Cells were then incubated with HRP colorimetric reagent kit to allow colour development in presence of HRP. Data is shown as mean HRP concentration  $\pm$  SEM of 3 technical replicates.



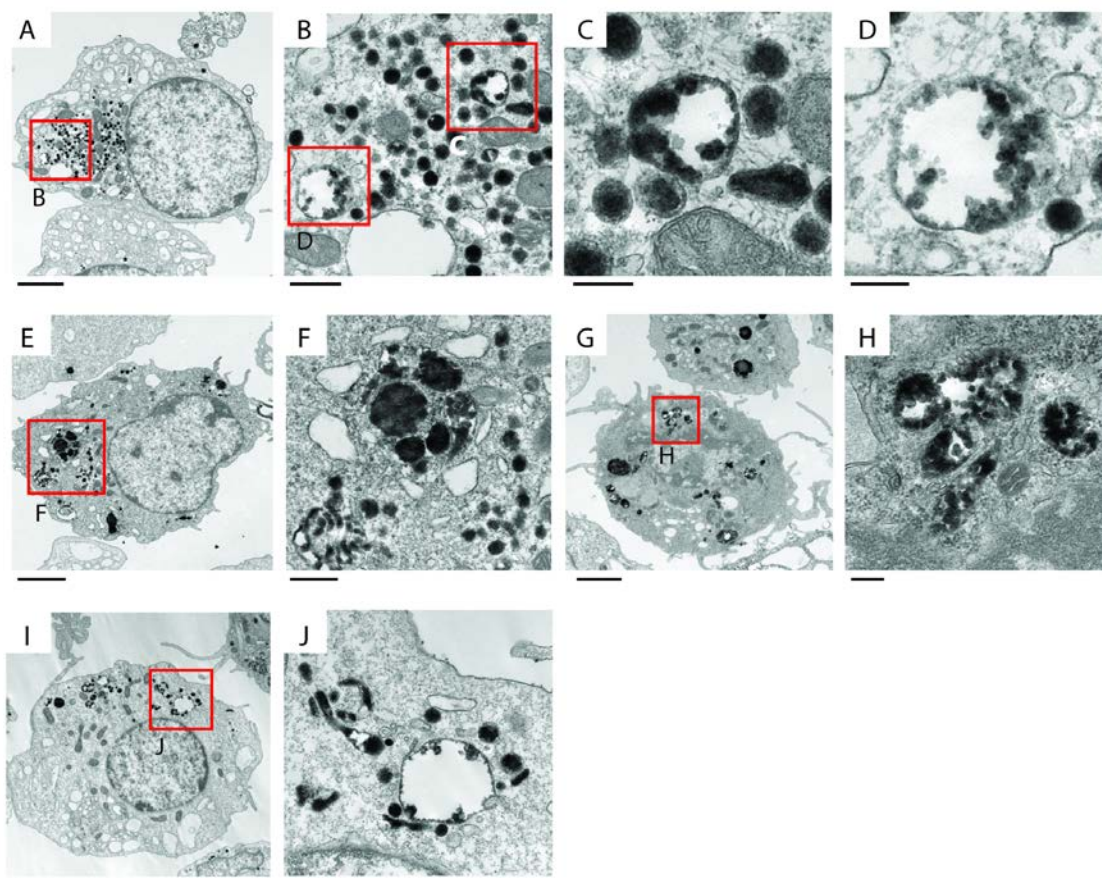


**Figure 5.15 Intracellular localisation of HRP.** DCs had been cultured in presence of 100  $\mu\text{g/ml}$  of HRP for 2 hours, followed by extensive washing and sample staining and processing according to the standard EM protocol. Images C,D,H and J show putative MVB. Scale bars: A, E, G, I: 2 $\mu\text{m}$ ; B, F, J: 500 nm; C, D, H: 200 nm.

These data suggest that the endocytic capacity of DCs allows the internalisation of HRP while maintaining the functional integrity of the enzyme. The co-localisation of HRP in structures resembling MVB may suggest the possibility that HRP could be packaged into exosomes. This would considerably facilitate protein loading into exosomes for clinical application.

## 5.7 The potential of HRP packaging into exosomes

In order to investigate this possibility, exosomes were harvested from supernatants from DC and T cell cultures that had been treated with HRP for 2 hours and, after extensive washing, were left overnight to produce and release exosomes into the supernatant. Untreated cells and HRP-treated T cells, which have low endocytic capacity, were included as controls. Supernatants from ultracentrifuged samples were included to account for carryover of free HRP when harvesting the exosome pellet.

As illustrated in Figure 5.16, HRP activity was detected in exosomes harvested from bmDCs treated with HRP while T cells treated in precisely the same way gave rise to significantly less HRP activity (HRP<sup>+</sup> T cells:  $336.4 \pm 186.6$  pg/ml per  $10^6$  cells ( $n=3$ ), HRP<sup>+</sup> bmDCs:  $1666 \pm 578.8$  pg/ml per  $10^6$  cells ( $n=3$ ),  $p = 0.0025$ ). The background level of HRP activity detected in the supernatants following ultracentrifugation was also significantly lower than the activity detected in exosome fractions purified from HRP-treated bmDCs (HRP<sup>+</sup> bmDC supernatant:  $488.3 \pm 205.5$  pg/ml per  $10^6$  cells ( $n=3$ ), HRP<sup>+</sup> bmDC exosomes:  $1666 \pm 578.8$  pg/ml per  $10^6$  cells ( $n=3$ ),  $p = 0.0064$ ). HRP activity was also recovered in exosome fractions derived from ipDCs, however this was not statistically significant as compared to HRP activity recovered from supernatants of the ultracentrifugation step.

Since such experiments yielded very variable results, it was important to exclude alternative explanations. In particular, the possibility arose that HRP could sediment during ultracentrifugation independently of exosomes.

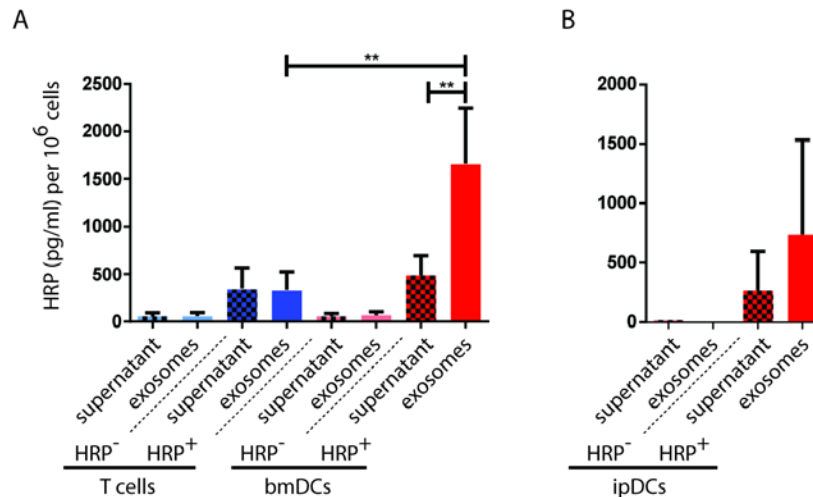


**Figure 5.16 The packaging of HRP into exosomes.** Exosomes were purified from (A) bmDC and T cell cultures and (B) ipDC cultures. Cells had either been left untreated or been subject to a 2 hour incubation with 100  $\mu\text{g/ml}$  of HRP, washed and returned into culture overnight to allow the generation and release of exosomes. These were harvested, purified and analysed for HRP activity by colorimetric assay. Bar chart represents the mean HRP concentration released per  $10^6$  cells  $\pm$ SEM of independent experiments ( $n=3$ ). Statistical analysis was performed using One-Way ANOVA with Sidak's multiple comparisons test ( $*p \leq 0.05$ ,  $**p \leq 0.01$ ). No statistical significance was achieved using ipDCs ( $ns. p > 0.5$ ).

### 5.7.1 The aggregation of HRP

In order to investigate whether free HRP could be concentrated in the absence of exosomes at 120,000  $g$  used for their purification, HRP was centrifuged in either PBS or medium and detected by a colorimetric assay. Following centrifugation, twenty 1 ml fractions were taken starting from the top of the tube with fraction 20 and ending with fraction 1 containing the final spun down pellet.

It was found that HRP showed no evidence of aggregation and sedimentation when centrifuged in PBS and was found at equal concentrations throughout the 20 fractions (Figure 5.17). In contrast, however, HRP became progressively

concentrated when centrifuged in bmDC medium supplemented with SR (Medium: Fraction 1:  $2047 \pm 390.5$  pg/ml ( $n=3$ ), Fraction 20:  $551.6 \pm 130.3$  pg/ml ( $n=3$ ),  $p = 0.0028$ ).

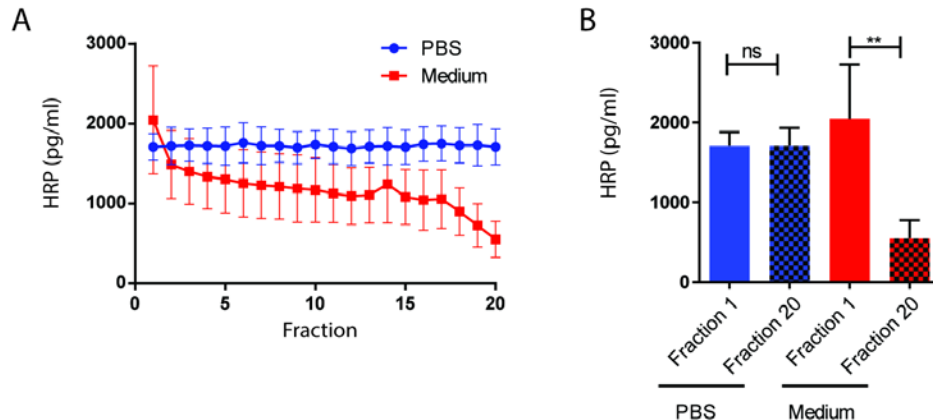


**Figure 5.17 The aggregation and sedimentation of HRP in absence of exosomes.** HRP was added to 20 ml of PBS or bmDC medium supplemented with SR rather than FCS and subject to ultracentrifugation at 120,000  $g$ . (A) Twenty fractions of 1 ml were taken, starting with fraction 1 at the bottom to fraction 20 at the top of the tube, and analysed for HRP concentration by colorimetric assay. Symbols represent the mean HRP concentration  $\pm$  SEM as determined by 3 independent experiments. (B) Fraction 1, which is the fraction that normally contains the exosome pellet, and fraction 20 which is normally taken as a supernatant control sample were plotted as a bar chart showing the mean HRP concentration measured  $\pm$  SEM in PBS and medium by 3 independent experiments. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparisons test (\*\* $p \leq 0.01$ ).

These data suggest that HRP recovered from HRP-treated bmDCs supernatants may not be solely associated with exosomes but may also result from its aggregation with proteins in the medium and consequential pelleting during ultracentrifugation. In order to address the contribution of aggregated HRP to

previous data centrifugation was used as exosomes and free HRP protein differ in their buoyant density.

#### **5.7.2 Gradient centrifugation for the determination of HRP association with exosomes**

A common method of exosome characterisation is by sucrose gradient centrifugation as exosomes are known to possess a buoyant density between 1.13 to 1.19 g/cm<sup>3</sup> (Thery *et al.*, 2009). Recently, Optiprep has become available to replace sucrose gradients as a simpler way to produce gradients (Y. Lee, personal communication, February 2, 2014). This system was used to determine whether HRP activity could be detected in the Optiprep gradient centrifugation fractions in which exosomes are known to localise according to their buoyant density.

Therefore, supernatants from bmDCs cultured with or without HRP were harvested and spun down for the purification of exosomes according to the standard protocol. The resuspended pellets were carefully layered onto the Optiprep gradient and centrifuged at 100,000 *g*. A gradient overlaid with HRP alone was also spun to confirm that the localisation of HRP in the gradient did not coincide with the exosome fraction. After centrifugation, fractions were taken and analysed for HRP activity and density. As shown in Figure 5.18, no HRP was detected in samples purified from untreated or treated bmDCs. HRP activity was however detected in gradients loaded with the enzyme alone which could be detected in fraction 10 and 11 which coincides with a density of 1.2 to 1.3 g/ml. Exosomes may not have been in sufficient numbers to be detected, since Western blotting did not detect the presence of CD9 in any of the fractions, after

a washing step in PBS which enabled the removal of Optiprep. However, Alix was detected in exosomes samples derived from loaded and unloaded-bmDCs before the gradient centrifugation and washing step, indicating the possible loss of exosomes during this procedure.

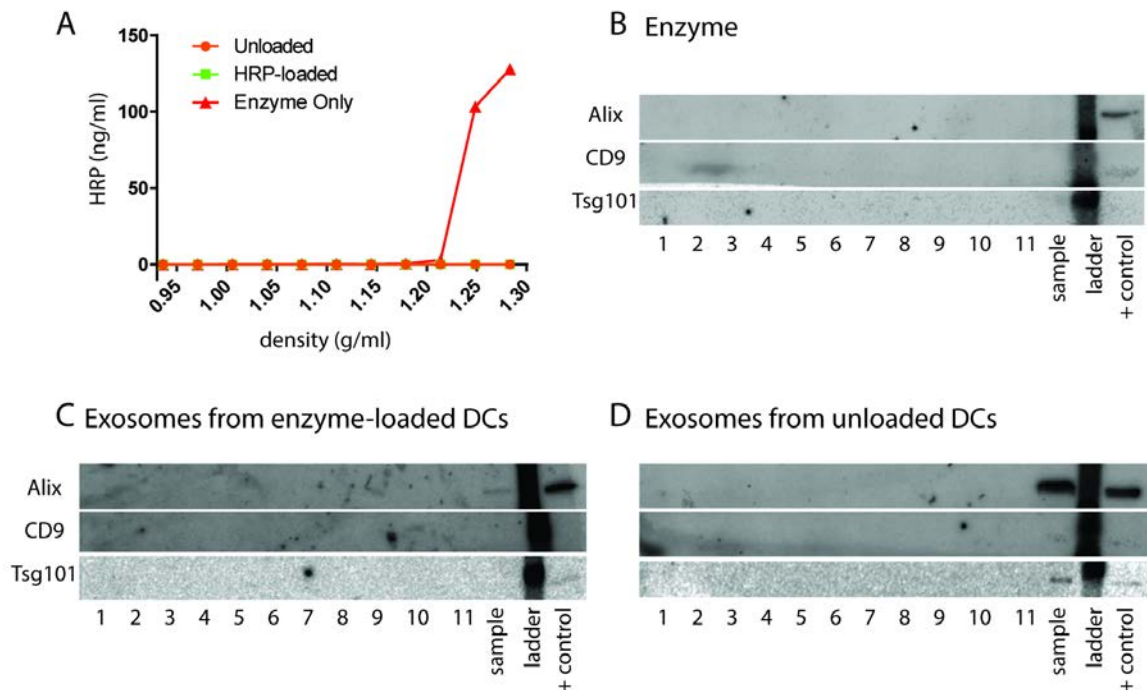


**Figure 5.18 The co-localisation or separation of exosomes and HRP by gradient centrifugation.** (A) Exosomes purified from HRP-treated or untreated bmDC cultures or HRP alone were loaded into the bottom of a tube and different ratios of Optiprep were layered on top for the creation of a density gradient of 11 ml. After ultracentrifugation at 100,000  $g$  for 2 hour eleven 1 ml fractions were taken and analysed for HRP concentration in a colorimetric assay. Data presented is the most representative data of 2 independently performed experiments. (B-D) Fractions were also analysed for the presence of exosome markers CD9, Alix and Tsg101 by Western blotting in collaboration with Yi lee from the Department of Physiology, Anatomy and genetics, Oxford. After Optiprep gradient centrifugation, fractions were washed in 20 ml PBS and exosome fractions collected and resuspended in 100  $\mu$ l PBS. The columns designated 'sample' represent the samples that were obtained from exosome purification or enzyme addition before being loaded onto the Optiprep gradient for centrifugation. As positive control N2a exosomes were included in the Western blot.

To investigate whether Optiprep affected HRP function and therefore the detectability of HRP using the colorimetric assay, the concentration of known amount of enzyme was measured in different ratios of Optiprep in PBS. The data suggest that Optiprep has an inhibitory effect on HRP activity leading to the measurement of a lower HRP concentration, which was reduced to 20% in samples containing 100% Optiprep as compared to HRP concentrations detected in 100% PBS (Figure 5.19). These data suggest that Optiprep has an inhibitory effect on HRP enzymatic activity and may therefore hamper the reliable and sensitive detection of HRP in Optiprep fractions.

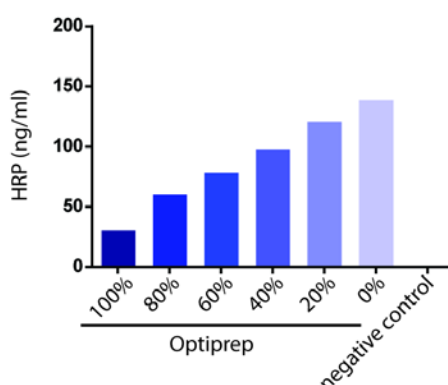


**Figure 5.19 The effect of Optiprep on HRP concentration detected by colorimetric assay.** HRP was added to different percentages from Optiprep made up in PBS. Negative controls constituted PBS in absence of added HRP. Bar charts represent the mean HRP concentration measured by colorimetric assay from 2 independent experiments.

In conclusion, the current data do not allow a definitive answer about the possibility of loading enzyme into exosomes, via the endocytic system and antigen cross-presentation pathway of the DC. Technical limitations have hampered the reliable detection of HRP associated to exosomes and distinction between free HRP and potential exosome-associated HRP. Therefore, future studies are required to investigate any potential association which is suggested

by HRP loading assays. EM may be an appropriate method to allow the detection of HRP by DAB precipitate or immunogold-staining specific against HRP. Nevertheless, the clinical applicability of exosomes, whether unloaded or loaded with protein or siRNA, requires more in depth knowledge of their immunogenicity *in vivo*.

## **5.8 Immunogenicity of exosomes**

To date, the immunogenicity of exosomes derived from bmDCs has been investigated with emphasis on their potential in vaccinations against cancers and infectious agents (Kuate *et al.*, 2007; Pinto *et al.*, 2011; Rountree *et al.*, 2011). The ability of exosomes to induce antigen-specific immune responses has also been explored, however only a few studies have further investigated the immunogenic potential of syngeneic versus allogeneic exosomes *in vivo* (Théry *et al.*, 2002b; Vincent-Schneider *et al.*, 2002). Furthermore, considering the potential tolerogenic and enhanced immaturity of DCs derived from iPSCs, exosomes derived from these ipDCs may provide a novel source for non-immunogenic vesicles.

Therefore, the *in vivo* immunogenicity of exosomes derived from bmDCs and ipDCs from syngeneic CBA and allogeneic B6 mouse strains was investigated.

### **5.8.1 Experimental set up**

In order to establish an experimental system that allowed the detection of an immune response *in vivo* specific against intravenously injected agents such as



exosomes, bmDCs derived from allogeneic B6 mice were injected at two different time points into CBA mice.

In order to investigate the ability to detect an antibody-mediated immune response, samples of serum were derived from blood samples taken from mice before treatment and at specified time points after each injection. These serum samples were incubated with B6 bmDCs in order to detect the presence of alloantibodies, especially against allogeneic MHCII determinants, the binding of which was subsequently detected by incubation with an anti-mouse IgG FITC-labelled secondary antiserum.

As shown in Figure 5.20A/B, a significant increase in antibody titre was detected by day 14 following two B6 bmDC injections, indicating the feasibility of detecting antibody titres using flow cytometry (PBS Day 14:  $1.14 \pm 0.067$  relative gMFI ( $n=3$ ), B6 bmDCs Day 14:  $7.684 \pm 1.191$  relative gMFI ( $n=3$ ),  $p < 0.0001$ ).

The titre detected was between 1 in 100 and 1 in 1,000 for mice injected with allogeneic bmDCs while PBS injected mice showed no antibody response above baseline (Figure 5.20C/D).

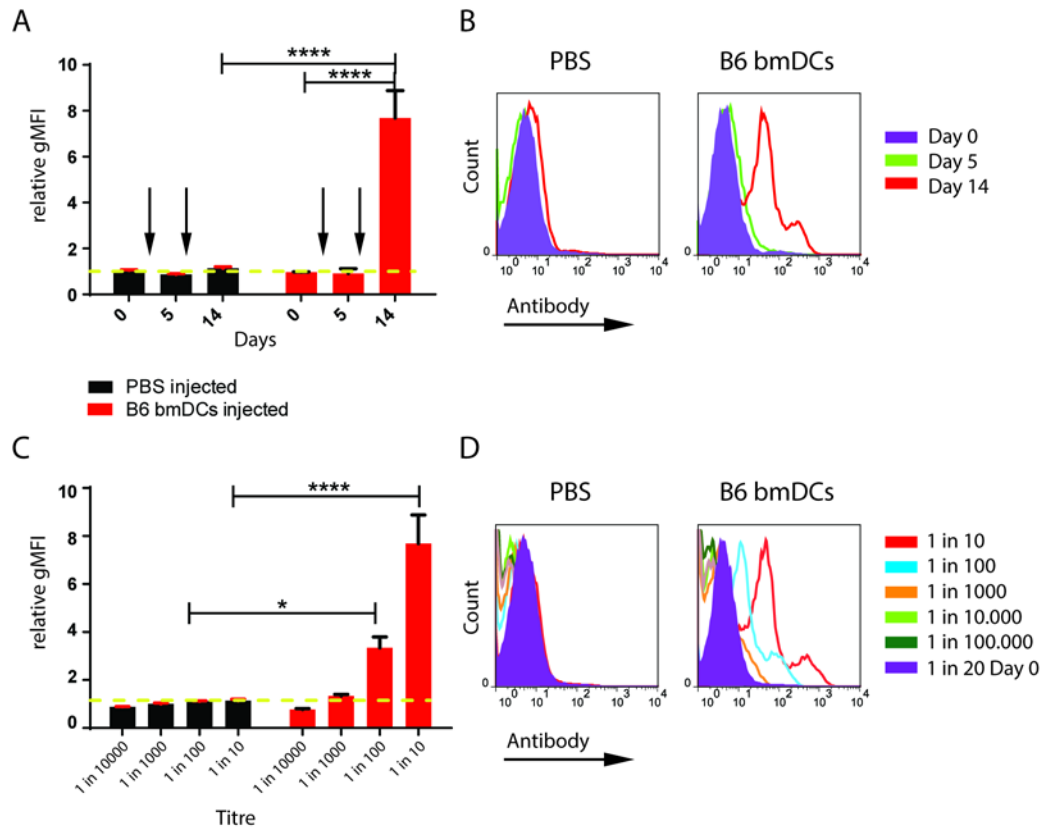


**Figure 5.20 The detection of B6-specific alloantibodies after boost immunization.** CBA mice were injected with 100  $\mu$ l of PBS or  $2 \times 10^6$  B6 bmDCs on day 0 and day 7. Serum obtained from injected mice before injection and on days 5 and 14 was incubated with B6 bmDCs followed by secondary staining with anti-mouse IgG antibodies labelled with FITC. Stained bmDCs were analysed by flow cytometry. The relative gMFI of antibody stainings was determined as the fold increase of gMFI of stained samples as compared to the gMFI obtained from serum samples from naïve mice before injection  $\pm$  SEM. (A/B) The detection of antibodies at 1 in 20 dilutions on different days following injections (indicated by arrows) was determined. (C/D) The antibody titre was determined by measuring the relative gMFI on day 14 at a range of serum dilutions. Each experimental group represents data from three biological replicates. Histograms illustrate example data obtained by flow cytometry. Statistical analysis was performed using the One-Way ANOVA with Sidak's multiple comparisons test (\* $p \leq 0.05$ , \*\*\*\* $p \leq 0.0001$ ).

In order to confirm this antibody-mediated immune response by the detection of a T cell-mediated immune response which is required for the full activation of B cells in response to T cell-dependent antigen, T cells purified from the

splenocytes of immunized mice were labelled with CFSE and co-cultured with allogeneic B6 bmDCs for detection of a recall response indicative of direct allorecognition. After 3 days of co-culture, cells were analysed by flow cytometry and the percentage of proliferated cells determined. Using this direct allorecognition assay, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells derived from CBA mice injected with B6 bmDCs were found to respond to the recall stimulation, showing around 10% of proliferated CD4<sup>+</sup> T cells and up to 30% of proliferated CD8<sup>+</sup> T cells when stimulated with  $2.5 \times 10^3$  allogeneic DCs (Figure 5.21). These data confirm the initiation of a cell-mediated immune response following injection of allogeneic B6 bmDCs.

Having established a reproducible method of detecting antibody responses towards injected cells, this protocol was used to address the question of whether DC-derived exosomes may likewise prove to be immunogenic.

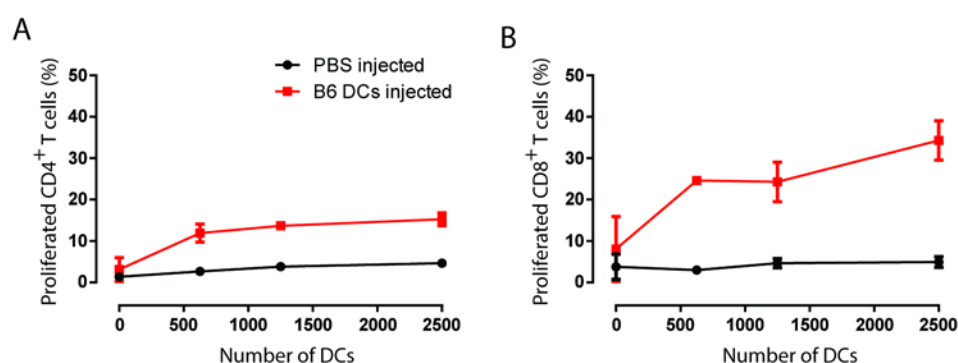


**Figure 5.21 Direct allorecognition recall assay shows T cell priming in bmDCs injected mice.** Splenocytes that had been harvested from immunized mice were purified for T cells and stained with CFSE. Different ratios of allogeneic B6 bmDCs were co-cultured with CFSE-labelled T cells and after 3 days stained for (A) CD4 and (B) CD8 and analysed by flow cytometry. Symbols represent data from 2 independent experiments  $\pm$  SEM.

### 5.8.2 The immunogenicity of exosomes

In order to investigate the potential immunogenicity of exosomes *in vivo*, blood samples were taken from mice before the first injection of exosomes into the tail vein to serve as a baseline. Another boost was administered after 7 days of the first injection and the final blood samples were taken after another 7 days. In order to detect antibodies produced in response to exosome injection, aldehyde-sulphide latex beads were coated with the same exosome population that had been originally injected. Further blocking of beads prevented any non-specific binding of antibodies as measured by beads that had not been labelled with exosomes which did not give rise to any background staining. Comparing antibody detection on day 14 with background levels detected before the injection of exosomes, designated Control Day 0, showed the generation of antibodies specific to injected exosome populations and control allogeneic bmDCs, however only the antibody titres detected from mice injected with CBA bmDC-derived exosomes were statistically significant as compared to titres detected before injection (Control:  $1.349 \pm 0.1544$  relative gMFI ( $n=6$ ), CBA bmDC exosomes:  $14.29 \pm 3.257$  relative gMFI ( $n=3$ ),  $p = 0.0033$ ) (Figure 5.22).

No response was observed in mice that had been injected with PBS alone. Looking at the antibody response towards different exosome populations it was of interest that syngeneic bmDC-derived exosomes stimulated the highest antibody response with a titre around 1 in 10,000 as compared to all other exosome populations which induced a titre of around 1 in 1000 (Figure 5.23).

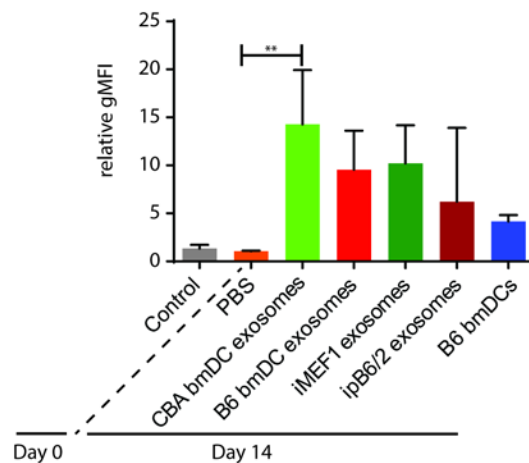


**Figure 5.22 The production of an antibody response towards administered exosomes after 14 days.** Exosomes were injected on day 0 and day 7 into CBA mice. Serum samples were harvested before (labelled control) and 14 days after treatment initiation. Serum (diluted 1 in 10) was incubated with either bmDCs or exosome-coated bead for the detection of bmDC or exosome-specific antibodies. Secondary labelling with a cocktail of anti-mouse IgG antibody allowed the detection of an antibody mediated immune response by flow cytometry. Exosomes injected had been derived from syngeneic CBA bmDCs and ipDCs or allogeneic bmDCs and ipDCs. Positive controls of allogeneic B6 bmDCs and negative controls of PBS were also administered. Bar charts represent the mean relative gMFI of 6 (Control before injection) or 3 biological replicates  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Sidak's test for the correction for multiple comparison (\*\* $p \leq 0.01$ ).

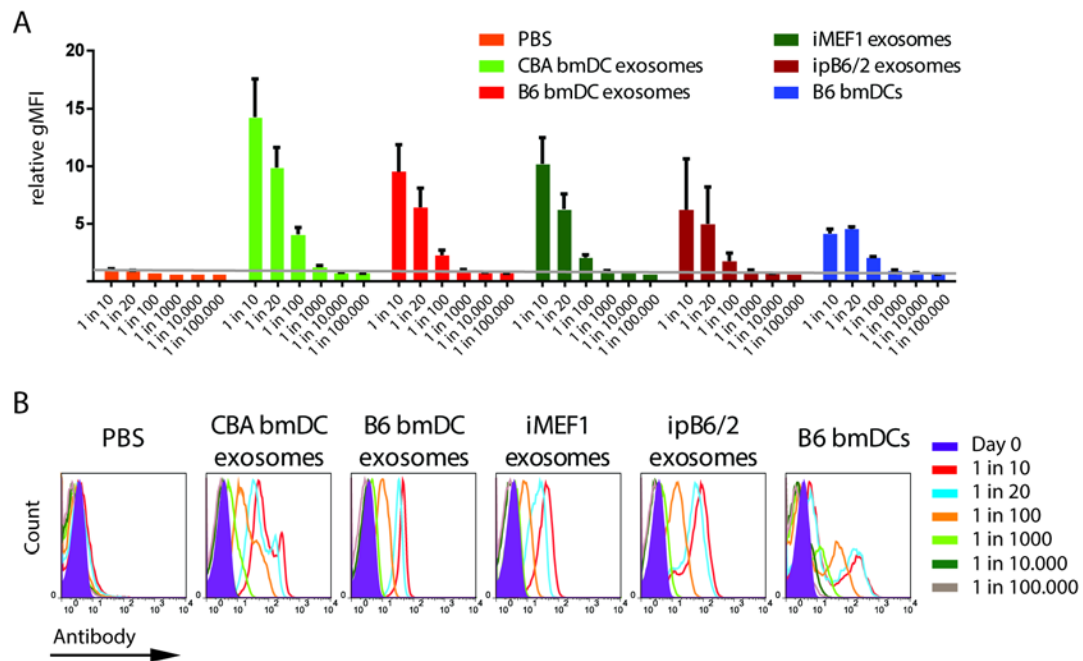


**Figure 5.23 The antibody response towards administered exosomes derived from different DC populations.** Exosomes were injected on day 0 and day 7. Serum samples were harvested before and 14 days after treatment initiation. Serum was incubated with either bmDCs or exosome-coated bead for the detection of bmDC or exosome-specific antibodies. Secondary labelling with a cocktail of anti-mouse IgG antibody allowed the detection of an antibody mediated immune response by flow cytometry. Exosomes injected had been derived from syngeneic CBA bmDCs and ipDCs or allogeneic bmDCs and ipDCs. Positive controls of allogeneic B6 bmDCs and negative controls of PBS were also administered. Bar charts represent the mean relative gMFI of 3 biological replicates  $\pm$  SEM. Histograms illustrate example data obtained by flow cytometry.

Upon reflection, the use of smaller dilution steps may have been of interest which may have allowed the detection of more subtle differences in titre. Furthermore, the readout may compromise comparability as B6 bmDCs are impure cultures with a variety of non-DC cells present which could account for the large number of cells that did not stain for antibodies. Therefore, taking the gMFI of the data may have caused the antibody response to appear smaller than it actually was

as it included cells that may not stain positive for antibodies raised against the bmDC population.

The disparity between the level of antibody response mounted in response to the injection of exosomes and bmDCs may be due to a difference in biodistribution *in vivo*. However, research has found similarities in that DCs and exosomes have been found to traffic and accumulate in organs such as the liver, spleen, kidneys and lungs (Eggert *et al.*, 1999; Wiklander, O.P.B. *et al.*, 2013).

Interestingly, despite the lower proimmunogenic phenotype of ipDCs as illustrated in Figures 5.4-6 and previously demonstrated in our laboratory (Hackett, 2013), ipDC-derived exosomes appear to exhibit an immunostimulatory capacity similar to that observed with bmDC-derived exosomes.

## **Discussion**

In this chapter, the potential capacity to load exosomes with protein via the endocytic and protein translocation system of DCs and the potential immunogenicity of DC-derived exosomes were investigated. As previous work has implied an enhanced immaturity of ipDCs and their potential to be derived at high numbers due to extensive proliferation and therefore expansion, DCs derived from iPSCs were investigated as a potential source of exosomes (Hackett, 2013). Therefore, iPSCs and their differentiation product, ipDCs, were characterised (Figure 5.1 to Figure 5.6). The significantly lower expression or absence of CD11c on ipDCs, as shown in Figure 5.4, was considerably striking. Therefore, more in-detail characterisation of mouse ipDCs is required in order to confirm their identity. However, despite the low level of DC-characteristic markers on ipDCs, previous work has shown their capacity to clearly act like DCs as

stimulators of primary immune responses (Hackett, 2013) (P. Sachamitr, personal communication, August 7, 2014). Future application of this research to human ipDCs will not face this issue as human ipDCs have been shown to consistently express CD11c (Figure 3.4 and Figure 3.5).

Apart from having found that ipDCs appear to be in an enhanced immature state, future work is required to further investigate whether this difference in cell marker expression has wider implications on the functionality of ipDCs. Furthermore, alternative differentiation protocols may be explored in order to determine whether ipDCs with a more DC-like phenotype may be generated and whether these may differ in their immunological behaviour as compared to bmDCs or classical ipDCs. One alternative method that may be explored makes use of OP9 cells with which stem cells are co-cultured in the presence of GM-CSF in order to generate DCs (Senju, 2009; Senju *et al.*, 2003, 2010).

## **5.9 The purification of exosomes from DCs**

Initial purification experiments have confirmed the feasibility of purifying exosomes from mouse bmDCs, mouse ipDCs, human moDCs and human ipDCs (Figure 5.8 and Figure 5.9). The detection of exosome marker CD9 on samples derived from mouse bmDCs, mouse ipDCs and human ipDCs confirmed the presence of exosomal particles (Figure 5.10 and Figure 5.12). However, the presence of other markers was difficult to determine, as the characterisation of exosomes may have been hampered by the relatively low sensitivity of flow cytometry for this purpose. Whether this was due to technical difficulties as other studies have showed clearer stainings using a similar methodology was not determined (Kim *et al.*, 2005; Li *et al.*, 2012; Näslund *et al.*, 2013).



Therefore, future characterisation may make use of Nanosight machines with fluorescence detectors for more reliable and sensitive characterisation of exosomes and exosomes subpopulations. However, studies on exosomes are still hampered by the difficulties in purifying exosomes and eliminating the presence of contaminating vesicles of other origin. What other vesicles were present in purifications in this study and at what proportions was not determined. Insights into this may have aided the interpretation of results especially if exosomes were a minor population in the heterogeneous vesicle purification.

#### **5.10 The possibility of loading exosomes via the endocytic/phagocytic and translocation ability of DCs**

Further in this chapter, the possibility of exploiting the endocytic and cross-presentation capacity of DCs to take up exogenous protein and load it into exosomes was investigated. Data have shown the effective internalisation of protein into the cell and the retention of enzyme activity following 2 hours of uptake (Figure 5.13, Figure 5.14 and Figure 5.15). Whether HRP was localised in the cytosol or MVB could not be determined, however staining experiments indicated that HRP was enclosed in membrane-bound structures (Figure 5.15). If the transportation of HRP into the cytoplasm was confirmed, which would be consistent with previous studies, this may enable the availability of protein for the potential loading into the lumen of exosomes via the formation of MVB (Ackerman *et al.*, 2006; Rodriguez *et al.*, 1999; Zehner *et al.*, 2011).

In this study, enzyme activity could be recovered from cell culture supernatants one day after the treatment of bmDCs or ipDCs with HRP (Figure 5.16). The HRP activity was recovered by means of exosome purification which co-localised and

was concentrated in the purified exosome pellet. However, further investigation has shown that HRP could aggregate if ultracentrifuged in complete medium supplemented with SR but not in PBS possibly due to its combining with proteins to form large complexes (Figure 5.17). Therefore, the question arose, as to whether HRP recovered from supernatants was an artefact due to its aggregation in complete medium or whether the HRP purification was due to its delivery into exosomes following uptake by DCs.

In order to investigate this, gradient centrifugation was performed in order to determine the buoyant density of HRP and whether HRP would co-localise with exosomes in the gradient (Figure 5.18). Despite the scaling up of DC cultures and HRP treatment, no HRP could be detected after Optiprep gradient centrifugation with the exception of gradients that had been directly loaded with HRP alone. Analysis of individual fractions by Western blotting after one ultracentrifugation wash in PBS, revealed that no exosome markers could be detected, despite their detection in the exosome samples prior to loading onto the Optiprep gradients. These data may therefore imply the loss of exosomes during the ultracentrifugation processes and indicate the requirement of larger samples of exosomes in order to reliably detect their presence and that of HRP in Optiprep gradients.

Furthermore, in order to investigate whether Optiprep may affect HRP enzyme function, HRP was incubated in different percentages of Optiprep in PBS and subsequently the enzyme activity was measured by colorimetric assay (Figure 5.19). Here it became clear that Optiprep had an inhibitory effect on the enzymatic activity of HRP or colour production in the colorimetric assay. Due to these methodological limitations further studies are required to unequivocally demonstrate

the association of HRP with exosomes by virtue of the high capacity of DCs for internalisation of proteins from the local microenvironment and their translocation to the cytosol. Nevertheless, data indicated that the release of HRP from DCs into the supernatant may be relying on a DC-specific feature, as no significant level of HRP was detected in supernatants from HRP-treated T cells (Figure 5.16).

Future work may look at the specific sub-cellular localisation of HRP in the cell in order to determine whether HRP was present in the cytosol, within vesicles or within a membrane-bound organelle such as the endosomes or lysosomes. Furthermore, the use of an enzyme more similar to a therapeutic LSD enzyme would provide better insights into the clinical applicability and the enzymes actual intracellular targeting in DCs. Here, purification protocols may further improve the usability of EM as protein contamination from cell culture medium appeared to considerably affect the visibility of exosomes on EM grids.

Therefore, numerous technical studies are required in order to enable a more reliable and more accurate determination of the potential to exploit the endocytic and translocation ability of DCs for the loading of proteins into exosomes.

### **5.11 The immunogenicity of exosomes derived from DCs**

This chapter further established a method by which the immunogenicity of intravenously injected exosomes could be investigated. Data in this chapter have confirmed the immunogenicity of exosomes derived from syngeneic bmDCs and further implied the immunogenicity of exosomes derived from allogeneic bmDCs and syngeneic and allogeneic ipDCs (Figure 5.22 and Figure 5.23). All exosome

populations injected into CBA mice gave rise to antibody titres which were detectable via exosome-labelled beads analysed by flow cytometry. Interestingly, the immunogenicity of syngeneic bmDC-derived exosomes appeared to induce the strongest and only statistically significant antibody response (Figure 5.22 and Figure 5.23). The induction of exosome-specific antibody production in response to syngeneic exosomes may be due to the presence or presentation of foreign proteins taken up from the cell culture such as the proteins contained in FCS or SR. However, this theory requires further investigation. Any immunological advantage of exosomes derived from mouse ipDCs could not be deduced from the results obtained in this study. A more detailed determination of antibody titres may facilitate the identification of any potential differences between the immunogenicity of exosomes of different origin or haplotype.

Furthermore, methodological adaptations and developments may improve the sensitivity of the antibody titre assay. Other approaches may make use of exosomes-covered ELISA plates, enabling the amplification of an antibody signal.

## **5.12 Conclusion**

Despite the requirement of more in-depth studies, this chapter has indicated the immunogenicity of exosomes derived from syngeneic and allogeneic bmDCs and ipDCs. Whether the immunogenicity may be circumvented by the development of alternative culture and purification methods remains to be explored. Nevertheless, the data presented in this chapter further underline the importance of determining reliable protocols for the generation of tolerogenic DCs. Previous studies have shown the immunosuppressive capacity of exosomes derived from

modulated DCs in situations such as collagen-induced arthritis, cardiac allograft transplantation and allergies (Bianco *et al.*, 2009; Kim *et al.*, 2005; Li *et al.*, 2012). Therefore, the ability to generate syngeneic tolerogenic ipDCs may provide two therapeutic strategies: the induction of tolerance towards therapeutic enzyme and the generation of non-immunogenic exosomes for the delivery of therapeutic molecules to the CNS.

## Chapter 6 Discussion



## 6.1 Introduction

The efficacy of ERT for the treatment of a number of LSDs has been limited in some cases due to the induction of an antibody-mediated immune response towards the therapeutic enzyme. In some patients this has prevented the effective targeting of enzymes to the tissues and organs, diminishing the clinical response to treatment. The issues of immunogenicity are currently being addressed through a variety of approaches. These range from immune cell depletion, the use of immunosuppressants and HSCT to gene therapy (Banugaria *et al.*, 2013; Douillard-Guilloux *et al.*, 2009; Franco *et al.*, 2005; Messinger *et al.*, 2012; Zhang *et al.*, 2012). However, all currently explored approaches harbour considerable risks such as increased vulnerability to infection and cancer and insertional mutagenesis, due to the insertion of transgenes into the genome which proved to be a limiting factor in early clinical trials of gene therapy. Accordingly, this study has investigated the potential use of DCs for the induction of tolerance, as DCs act in an antigen-specific manner to promote or inhibit immune responses (Abe *et al.*, 2004; Banchereau and Steinman, 1998). This could potentially allow for the tolerization of patients towards the therapeutic enzyme, while maintaining a functional and protective immune system.

As patients diagnosed with LSDs are frequently of young age and have significantly compromised health, using blood monocytes as the conventional source of DCs may not be feasible, as this would necessitate the sampling of a large volume of blood. Furthermore, the use of blood from other donors for the derivation of DCs would require rigorous HLA-matching, as the correct MHC haplotype is required for the presentation of epitopes from the enzyme produced and presented endogenously and semi-allogeneic DCs would induce significant



alloreactivity *in vivo*. Therefore, this thesis has explored the potential use of patient-specific iPSCs for the derivation of autologous ipDCs.

This strategy made use of a recently developed protocol for the reprogramming of patient-derived fibroblasts into a state where the cells regain pluripotent potential (Takahashi, 2007; Takahashi and Yamanaka, 2006). The acquisition of a pluripotent state theoretically allows the generation of any cells and tissues in an autologous fashion. Since we have previously shown the feasibility of differentiating DCs from mouse and human ESCs and iPSCs, this thesis sought to explore the potential of generating ipDCs from human iPSCs with protolerogenic properties (Fairchild *et al.*, 2000; Silk *et al.*, 2011, 2012a; Tseng *et al.*, 2009). Furthermore, this project sought to tackle another limiting factor in the treatment of LSDs. The BBB poses a selectively permeable barrier, which has prevented the effective targeting of therapeutic enzyme into the CNS (Bryce A, 2008; Fu *et al.*, 2011; Grubb *et al.*, 2008; Hemsley and Hopwood; Urayama *et al.*, 2004). As further explored in section 6.6 and 6.7, the use of DC-derived vesicles for the packaging of proteins and their potential immunogenicity was explored as these so-called exosomes have proven to be a promising approach in mouse models to deliver therapeutic agents into the brain (Alvarez-Erviti *et al.*, 2011; Cooper *et al.*, 2014).

## **6.2 The generation of iPSCs from a patient with Pompe disease**

This study has shown the generation of new iPSC lines (ipPo0-4) from fibroblasts obtained from a patient diagnosed with infantile-onset Pompe disease. Interestingly, during the reprogramming of Pompe disease fibroblasts by Tim Davies, it was observed that healthy human skin fibroblasts, which were

reprogrammed concurrently, produced stem cell-like colonies a few days earlier and at greater numbers than those from the Pompe disease donor (T. Davies, personal communication, March 19, 2015). The frequency of infantile Pompe disease is estimated to be around 1 in 138,000, while late-onset is estimated to be around 1 in 57,000 (Ausems *et al.*, 1999). The Pompe disease fibroblast donor had a guanine to adenosine transitional mutation at nucleotide 877 in exon 5 in both GAA alleles (Table 4.1). This mutation is known to lead to a change in the amino acid, causing the incorporation of arginine instead of glycine (Hermans *et al.*, 2004). This mutation has been found to cause a loss of 98% or more GAA activity in the lysosomes of patients and has been especially associated with infantile-onset Pompe disease and a severe pathological phenotype (Hermans *et al.*, 2004). The mutation is relatively rare with an estimated allele frequency of around 0.017 amongst infantile Pompe disease patients in Italy and 0.06 amongst late-onset patients in Germany, however numerous studies did not have participating patients with this mutation and therefore the allele frequency may be lower than these estimates (Hermans *et al.*, 2004; Joshi *et al.*, 2008; Montalvo *et al.*, 2006; Müller-Felber *et al.*, 2007; Pittis *et al.*, 2008; Sharma *et al.*, 2005).

One study showed that around 70% of pathogenic GAA mutations are due to a unique sequence variation which has been reported only once while only 4% of sequence variations had been reported 8 times or more (Kroos *et al.*, 2012). The mutation found in the fibroblast donor in this study is one of the rarest sequence variation within these reoccurring 4%. The most representable mutations that could have been used would have been c.2481+102\_2646+31del or 525delT, which have been shown to be the second and third most common sequence variation with a severe disease phenotype (Hermans *et al.*, 1994; Kroos *et al.*,

2012). The most commonly found sequence variation, -32-13T>G, may have been less useful due to its relative mild pathology (Hermans *et al.*, 2004; Huie *et al.*, 1994b; Kroos *et al.*, 2012, 1995; Montagnese *et al.*, 2015). However, due to availability and patient consent, the fibroblasts donated by a patient with infantile-onset and a c.877G>A mutation had been used and reprogrammed in this study.

Interestingly, one of three other studies that showed the reprogramming of human Pompe disease fibroblasts, required the conditional lentiviral expression of GAA for the successful generation of iPSC colonies (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Raval *et al.*, 2015). In contrast to our own study in which Sendai virus was used for the reprogramming of human fibroblasts, Huang and his colleagues used the classical retroviral transduction of the Yamanaka reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc (Huang *et al.*, 2011; Takahashi, 2007). However, as a laboratory in Japan was able to reprogram human Pompe disease fibroblasts using the same traditional Yamanaka reprogramming approach and protocol but without GAA-expressing lentiviral transduction, it is unlikely that a difference in the reprogramming methods may explain the requirement of GAA-expression in the Huang *et al.* (2011) study (Higuchi *et al.*, 2014). However, this thesis and the two publications used fibroblasts obtained from Pompe disease patients with different mutations. As mentioned above, the patient used in this study had a transitional point mutation that is associated with a severe pathogenesis and early onset (Hermans *et al.*, 2004). Higuchi and colleagues (2014) reprogrammed fibroblasts obtained from late and early-onset patients where the late-onset patients had the -13 thymidine to guanine point mutation in exon 1, which is known to lead to the splicing out of exon 1, and a deletion of exon 18 (Huie *et al.*, 1994b). However, -13T>G mutations have been associated with a mild

phenotype and a reduction in GAA activity down to 20-40% (Kroos *et al.*, 1995). Pompe disease fibroblasts from patients with a heterozygous and a homozygous deletion in exon 18 have also been reprogrammed in another study, indicating that this mutation, known to produce the GAA precursor but not the active form, may not interfere with iPSC generation (Ausems *et al.*, 1996; Raval *et al.*, 2015). The early-onset patient used in the study by Higuchi *et al.* (2014) also had an exon 18 deletion in one allele but a single base pair deletion at nucleotide 525 on the other. This latter mutation has been associated with severe cases of Pompe disease and the inability to express GAA from this allele (Hermans *et al.*, 1994, 2004). On the other hand, Huang *et al.* (2011) used fibroblasts obtained from patients which all presented with at least one allele with a cytosine to adenosine transversion at position 1935 in exon 14 (Chen *et al.*, 2004). This mutation has been frequently found in infantile-onset Pompe disease in South East Asian countries (Wan *et al.*, 2008). Furthermore, one patient was heterozygous for a cytosine to guanosine transition at position 1062 in exon 6 which has been associated to a severe phenotype (Kroos *et al.*, 2008). That the inability to reprogram these fibroblasts into iPSCs without enzyme supplementation in the study by Huang *et al.* (2011) was not due to technical failure was supported by the concomitant reprogramming of fibroblasts from healthy donors which successfully yielded iPSC colonies. Similar to all other studies discussed here, the fibroblasts used in this thesis for the generation of iPSCs were obtained from a CRIM<sup>+</sup> Pompe disease patient. However, no residual enzyme activity was detected in any of the studies, with the exception of Higuchi's study where enzymatic activity was not investigated (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Raval *et al.*, 2015). Therefore, these studies may indicate a potential challenge

to iPSC generation posed by some genetic mutations such as the 1935C>A transversion. Whether other reprogramming strategies such as the Sendai system may overcome this challenge in the absence of GAA-expression remains to be elucidated. Similar to Higuchi *et al* (2014) and Raval (2015), no difficulties were encountered during the reprogramming procedure.

As mentioned in chapter 1, different sources of cells have been shown to be reprogrammable such as embryonic fibroblasts, adult fibroblasts, pancreatic cells, hepatocytes, peripheral blood cells, neuronal cells, keratinocytes, and B lymphocytes (Aasen, 2008; Aoi, 2008; Hanna *et al.*, 2008; Kim, 2008; Loh, 2009; Stadtfeld *et al.*, 2008; Takahashi, 2007; Takahashi and Yamanaka, 2006). Different sources of cells may harbour different advantages and disadvantages, however in order to access some of these sources highly invasive procedures would be required which may not, therefore, be a regular option. Fibroblasts may be cheap and easy to handle but taking the sample by punch biopsy may be painful, carries a risk of wound infection and scar formation (Nguyen, 2014). Highly proliferative fibroblasts may overgrow reprogrammed cells but at the same time be more readily reprogrammed due to the low promoter methylation of OCT4 and NANOG (Streckfuss-Bömeke *et al.*, 2013). Using blood as a ready source of cells, whether CD34<sup>+</sup> HSC or peripheral mononuclear cells, may also be an option (Loh, 2009; Staerk *et al.*, 2010). However, the harvest of mobilised HSC by G-CSF administration can be associated with complications such as bone pain and nausea, be relatively invasive, expensive, time consuming and only an option for healthy donors (Cashen *et al.*, 2007; Streckfuss-Bömeke *et al.*, 2013). Furthermore, the reprogramming efficiency of peripheral mononuclear cells has been found to be about 50 times lower than that of fibroblasts (Staerk *et al.*, 2010).

Another study however, has been able to generate iPSC from a drop of blood at around 100 to 600 colonies per ml of capillary blood (Tan *et al.*, 2014). This approach allows the easy sampling of cells, even in a non-clinical setting, relatively good reprogramming efficiency and the potential for high throughput protocols. Two further cell sources that are sampled in a non-invasive manner are keratinocytes and exfoliated renal epithelial cells (Aasen, 2008; Aasen and Izpisua Belmonte, 2010; Xue *et al.*, 2013). Keratinocytes have been shown to be easily isolated from plucked hair, stored and cultured. The reprogramming efficiency of these cells has been found to be 100 fold higher and faster than that of fibroblast (Aasen, 2008; Aasen and Izpisua Belmonte, 2010). Similarly, exfoliated renal epithelial cells can be easily obtained and purified from collected urine samples with a reprogramming efficacy around 10 fold higher than fibroblasts (Xue *et al.*, 2013). These new potential sources for iPSC generation indicate that in this fast moving field, accessible, non-invasive, cheap, easily cultured and highly reprogrammable cell populations are available for future standardisation and routine sampling, some of which may one day replace the current dependence on dermal fibroblasts.

### **6.3 The use of iPSCs as a source of DCs**

In this study, we showed the capacity to grow, expand and differentiate ipPo1 cells into viable and functional ipDCs. However, further severe cases of LSDs will need to be tested for their capacity to donate reprogrammable fibroblasts. The disease state was confirmed by an enzymatic assay which showed the significant absence of any glucosidase activity under acidic conditions (Figure 4.3). However, the detection of PAS staining in cell dense areas only of iPSC colonies

may have been affected by the reagents used (Figure 4.4), as previous studies have shown the clear and widely distributed accumulation of glycogen in iPSCs derived from Pompe disease patients' fibroblasts (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Kawagoe *et al.*, 2011). Therefore, the measurement of glycogen deposition in ipPo1 iPSCs has to be repeated using different reagents and possibly a different and more sensitive approach such as a fluorimetric assays (Pelletier *et al.*, 2013). Nevertheless, consistent with the anzyme assay, PAS staining was only observed in ipPo1 colonies and not C15 iPSC colonies.

The differentiation protocol applied in this study appeared to be transferable between iPSCs from different individuals. The yield of ipMonocytes did not differ significantly, even though a lower average yield was observed from ipPo1 differentiations, indicating that even in presence of a severe disease phenotype, the capacity to generate ipDCs remains relatively unaffected (Figure 3.3 and Figure 4.10). ipDCs generated from both Pompe disease and healthy donors showed phenotypic similarities, such as expression of the well-defined DC-markers CD11c, MHCII, CD54 and low levels of CD11b and CD123 (Figure 3.5, Figure 3.6 and Figure 4.11). Costimulatory and immunoinhibitory molecules were found to be expressed on C15 and ipPo1 ipDCs which further endorsed their commitment to the DC lineage (Figure 3.24-30 and Figure 4.18-20). Furthermore, the expression of CCR7, CD141, XCR1 and CD207 was similar between ipDCs derived from Pompe disease patient iPSCs and healthy control C15 iPSCs (Figure 3.7-9, Figure 3.12 and Figure 4.12). Similar to C15 ipDCs, the large majority of ipPo1 ipDCs were CD1a<sup>-</sup>, whereas moDCs contained clearly separated CD1a<sup>+</sup> and CD1a<sup>-</sup> populations (Figure 3.8 and Figure 4.12). This may indicate the generation of a specific DC subset using the iPSCs differentiation

protocol which are low in their IL-12 secretion and may be more potent at the polarisation of naïve T cells towards a Th2 phenotype (Cernadas *et al.*, 2009; Chang *et al.*, 2000; Ito *et al.*, 1999).

The expression of CD209 was lower in ipDCs as compared to moDCs which may indicate a reduced T cell migratory and stimulatory potency of ipDCs as compared to moDCs (Figure 3.8) (Engering *et al.*, 2002; Geijtenbeek *et al.*, 2000a, 2000b). Immature ipPo1 ipDCs showed similarly low levels of CD209, however, these cells remained positive for the expression of CD209 following exposure to proinflammatory cytokines while C15 ipDCs completely down-regulated its expression (Figure 3.8 and Figure 4.12). It is not clear why C15 ipDCs and ipPo1 ipDCs differed in their response towards inflammatory cytokines in terms of their CD209 expression, however, it would be interesting to investigate whether this may correlate to their immunogenic potential, antigen uptake or migratory capacity and what effect modulatory agents have on the expression of CD209 (Engering *et al.*, 2002; Geijtenbeek *et al.*, 2000a, 2000b; Zhou *et al.*, 2006).

This lower level of CD209 expression by ipDCs as compared to moDCs (Figure 3.8 and Figure 4.12), together with the lower level of MHCII expression (Figure 3.5 and 4.11) may indicate an increased immaturity of ipDCs and potentially a lower immunostimulatory capacity as compared to their monocyte-derived counterparts. This may be further supported by the lower average percentage of proliferating cells detected in co-cultures with ipDCs as compared to co-cultures with moDCs (Figure 3.33). This lower immunogenic potential of ipDCs had previously been described in our laboratory using mouse iPSCs and may therefore significantly support the use of iPSC-derived DCs as compared to more



conventionally used moDCs due to a potentially enhanced pro-tolerogenic phenotype (Hackett, 2013).

#### **6.4 The generation of ipDCs with pro-tolerogenic properties**

Apart from an apparent immature and potentially pro-tolerogenic phenotype, it was of interest to determine whether ipDCs, similar to moDCs, may be further modulated in order to confer on them an enhanced tolerogenic potential (Horibe *et al.*, 2008; Pothoven *et al.*, 2010; Steinbrink *et al.*, 1997; Torres-Aguilar *et al.*, 2010a; Turnquist *et al.*, 2007; Unger *et al.*, 2009; Xia *et al.*, 2005). Therefore, in order to further determine the potential of DCs for immunomodulation therapies, modulatory agents with known pro-tolerogenic properties were used in chapter 3 in an attempt to generate ipDCs with pro-tolerogenic features.

Starting with ipDCs from the healthy donor, rapamycin, VD3, dexamethasone and IL-10 were used for the modulation protocol. Data confirmed the successful generation of CD11c<sup>+</sup> moDCs and ipDCs in the presence of these modulators (Figure 3.17 and 3.18). The majority of cell surface molecules investigated for their expression profile following modulation showed no significant change. However, a statistically significant change in the expression of CD14 in response to VD3 modulation was detected which was significantly increased on moDCs and ipDCs (Figure 3.20). This effect of VD3-treatment on moDCs has been observed in a number of other studies which further implies the similarities between moDCs and ipDCs even in response to modulation (Bartels *et al.*, 2010; Brosbøl-Ravnborg *et al.*, 2013; Unger *et al.*, 2009). The failed downregulation of CD14 during monocyte-differentiation may indicate the immaturity of VD3-treated DCs, which may retain a phenotype similar to undifferentiated CD14<sup>+</sup> monocytes,

or the enhanced differentiation of monocytes along the macrophage developmental pathway. This may correlate with the decrease in cell yield observed in this and other studies (Naranjo-Gómez *et al.*, 2011), which was explained by the presence of a larger proportion of flattened and strongly adherent cells in VD3-treated moDCs and ipDCs cultures (Figure 3.16). However, CD68, a marker of macrophages, which had been found to be expressed at low levels in moDCs and ipDCs, was not upregulated in DCs treated with VD3 and did not, therefore, support the theory of enhanced macrophage differentiation (data not shown) (Murray and Wynn, 2011). The possibility that the high adhesiveness of macrophages may have affected the recovery of these cells from cultures which may therefore have been present only at very low numbers during staining remains a possibility. Furthermore, other DC markers did not appear to be significantly altered in response to VD3-treatment.

Moreover, the expression of ILT3 and ILT4 was significantly upregulated on IL-10-modulated ipDCs, whilst IL-10-modulated moDCs only expressed significantly elevated levels of ILT4 but not ILT3 (Figure 3.29 and 3.30). In contrast, other studies reported the significant increase in ILT3 expression on IL-10-modulated moDCs (Chamorro *et al.*, 2009; Gregori *et al.*, 2010). The significant upregulation of ILT4 was further confirmed by Gregori *et al.* (2010), who demonstrated its essential role in the induction of Tr1 cells.

The release of IL-6 was significantly increased in VD3-treated and IL-12 in rapamycin-treated moDC cultures (Figure 3.31). The increase in IL-6 secretion by moDCs treated with VD3 has also been observed by others, which may imply the increased potential of these modulated DCs to induce inflammation and Th17 responses, which conflicts with previous reports about the pro-tolerogenic

potential of VD3-modulated moDCs (Bartels *et al.*, 2010; Chamorro *et al.*, 2009). Despite the increase in the release of IL-6, VD3-treated moDCs were the only modulated population that had a statistically significant inhibitory effect on T cell allostimulatory capacity in co-cultures (Figure 3.33). However, a reduction in T cell allostimulatory capacity was also observed in co-cultures with dexamethasone and IL-10-modulated moDCs, although, this was not statistically significant. In contrast, ipDCs showed a significantly lower T cell allostimulatory capacity if modulated with IL-10, while no significant reduction was observed if ipDCs had been treated with VD3 (Figure 3.33). This may indicate a different susceptibility towards different modulatory agents which may be connected to differences in maturity and subpopulation predominance as observed in this study (Figure 3.5, Figure 3.8, Figure 4.11 and Figure 4.12).

Other studies have confirmed this finding, however, many have also been able to show the statistically significant reduction of T cell allostimulatory capacity of rapamycin, dexamethasone and IL-10-treated moDCs (Bartels *et al.*, 2010; Chamorro *et al.*, 2009; Naranjo-Gómez *et al.*, 2011; Steinbrink *et al.*, 1997; Unger *et al.*, 2009; Xia *et al.*, 2005). An increase in the number of repeated experiments may in future enable the detection of statistical significance for these agents.

The secretion of IL-10 was found to be significantly increased in co-cultures of allogeneic CD4<sup>+</sup> T cells with IL-10-treated moDCs and ipDCs on day 5 (Figure 3.35). In other studies, this was confirmed showing that IL-10-treated moDCs secreted increased concentrations of IL-10, as did T cells (Chamorro *et al.*, 2009; Gregori *et al.*, 2010; Torres-Aguilar *et al.*, 2010a). IL-10 may constitute an important component in the capacity of IL-10-modulated DCs to exert immune inhibition as IL-10 is known to be involved in the suppression of DC activation

and T cell and B cell responses (Akbari *et al.*, 2001; Duddy *et al.*, 2004; Huang *et al.*, 2013). Similarly, a significantly higher concentration of IL-10 was detected in co-cultures on day 5 with dexamethasone-treated ipDCs but not moDCs (Figure 3.35). The increase in IL-10 released by ipDCs treated with dexamethasone was observed by several other studies using moDCs, with one study being able to determine IL-10 to be an important factor in the immunosuppressive capacity of dexamethasone-treated moDCs, as IL-10-neutralizing antibodies partially restored the stimulation of T cell proliferation and upregulation of CD86 (Naranjo-Gómez *et al.*, 2011; Stax *et al.*, 2008; Unger *et al.*, 2009; Xia *et al.*, 2005). However, in this study the release of IL-10 was not significantly increased in dexamethasone-treated moDC cultures, but only in ipDC co-cultures on day 5. The presence of IL-10 in these co-cultures may explain the reduced allostimulatory capacity of dexamethasone-treated ipDCs, although these results were not statistically significant (Figure 3.33 and Figure 3.35). The differences in cytokine profile observed between day 5 and day 7 of co-culture may have been due to the removal of half the medium for the analysis by ELISA, and addition of fresh IL-2-containing R10, which would have diluted and changed the constitution of cytokines in the supernatant. Therefore, IL-2 may have modulated the cytokine secretion by DCs and T cells as previous studies have shown the increase of IL-10 secretion in response to exogenous IL-2 (Cardone *et al.*, 2010; Caudy *et al.*, 2007; Tomova *et al.*, 2006).

In a study comparing the use of dexamethasone and rapamycin with that of VD3 for the generation of tolerogenic DCs, different pro-tolerogenic characteristics were identified for differently modulated DCs. While rapamycin-treated DCs did not show significant downregulation of costimulatory molecule expression or a

significant increase in IL-10 secretion, these cells did display a significantly reduced ability to induce IFN $\gamma$ -secreting T cells and significantly increased capacity to induce T<sub>regs</sub> (Naranjo-Gómez *et al.*, 2011). On the other hand, VD3 and dexamethasone-treated DCs did not display enhanced capacity to induce T<sub>regs</sub> or reduced capacity of induce IFN $\gamma$ -expressing T cells, but showed a significantly reduced expression of costimulatory molecule expression. Furthermore, dexamethasone-treated DCs secreted a significantly increased amount of IL-10 as compared to controls (Naranjo-Gómez *et al.*, 2011). However, all agents displayed a significantly lower capacity to stimulate T cell proliferation, further indicating that a similar pro-tolerogenic phenotype may be achieved via distinct pathways.

In this study, most agents did not significantly affect any of the measurements. The exceptions were the IL-10-mediated modulation which showed significant upregulation of the expression of ILT4 on moDCs and ILT3 and ILT4 on ipDCs (Figure 3.29 and Figure 3.30) and increased production of IL-10 in co-cultures of T cells with moDCs and ipDCs which has also been shown in past studies using moDCs and bmDCs (Figure 3.35) (Chamorro *et al.*, 2009; Dai *et al.*, 2009; Gregori *et al.*, 2010; Manavalan *et al.*, 2003). A significant increase in IL-10 secretion was also observed in co-cultures with dexamethasone-treated ipDCs which has also been shown in the past using moDCs (Figure 3.35) (Naranjo-Gómez *et al.*, 2011; Xia *et al.*, 2005). However, no significant change in the proportion of T<sub>regs</sub> was observed for any of the agents and the significant effect on T cell proliferation was limited to VD3-treated moDCs and IL-10-treated ipDCs (Figure 3.34 and Figure 3.33).

In an attempt to evaluate the effect of modulation on moDC and ipDC phenotype and function, a table was constructed to summarise the results which highlighted VD3 and IL-10 to be the most promising pro-tolerogenic agent for the modulation of moDCs and ipDCs respectively (Table 3.2 and 3.3).

The reasons for the difference in the most effective modulatory agent between moDCs and ipDC are not known. Past studies have shown the effectiveness of all four agents in the pro-tolerogenic modulation of moDCs (Bartels *et al.*, 2010; Chamorro *et al.*, 2009; Farias *et al.*, 2013; Gregori *et al.*, 2010; Naranjo-Gómez *et al.*, 2011; Pothoven *et al.*, 2010; Stax *et al.*, 2008; Turnquist *et al.*, 2007; Unger *et al.*, 2009; Xia *et al.*, 2005). No previous studies have investigated the effect of these agents on ipDCs. However, work performed in our laboratory has investigated the modulation of human ESC-derived DCs using rapamycin (Silk *et al.*, 2012b). This study has shown the capacity of enhancing the pro-tolerogenic phenotype of ESC-derived DCs by demonstrating a decreased T cell stimulatory capacity and enhanced T<sub>reg</sub> induction capacity in response to rapamycin modulation. However, no comparative study of different agents has been published using human ESC-derived DCs.

It was interesting to note that the potentially promising agents, VD3 and IL-10, are modulators that occur naturally in the human body with native immunomodulatory functions. Apart from dietary intake, vitamin D3 is cutaneously generated from 7-dihydrocholesterol in response to the absorption of ultraviolet B radiation. Vitamin D3 is then hydroxylated in the liver to give rise to 25-hydroxy vitamin D3 which is further processed in the kidneys to form VD3 (1 $\alpha$ ,25-dihydroxy vitamin D3) (Aranow, 2011; Cantorna *et al.*, 2004; Holick, 2007). Dendritic cells and macrophages are also known to be capable of

generating VD3 from 25-hydroxy vitamin D3 (Bartels *et al.*, 2010; Hewison *et al.*, 2003; Overbergh *et al.*, 2000). As previously discussed in section 1.3.8, VD3 acts by binding to the vitamin D3 receptor which can heterodimerize with the retinoid X receptor and together bind to vitamin D3 response elements in the genome for the regulation of gene expression (Peleg *et al.*, 1995; Thompson *et al.*, 1998; Zhang *et al.*, 2011). In T cells, VD3 is known to inhibit the expression of IL-2 and IFN $\gamma$  and divert the polarisation of T cells from a Th1 to a Th2 phenotype (Boonstra *et al.*, 2001; Cippitelli and Santoni, 1998; Mahon *et al.*, 2003; Takeuchi *et al.*, 1998). This subversion of the Th1 response is thought to play an important role in the prevention of autoimmune diseases since low levels of VD3 precursors have been found to be possible predictors of autoimmune disease development (Adorini, 2005; Froicu *et al.*, 2003; Littorin *et al.*, 2006; Merlino *et al.*, 2004; Munger *et al.*, 2006). There are a variety of other pathways that VD3 has been known to affect which include the inhibition of the differentiation and activation of B cells and enhanced immunity against various infections, indicating a pro-immunogenic action of VD3 in addition to its immunosuppressive role (Bodnar *et al.*, 2009; Cannell *et al.*, 2006; Chen *et al.*, 2007; Lemire *et al.*, 1984; Rodríguez *et al.*, 2009).

IL-10 on the other hand, is known to act via binding the IL-10 receptor and inducing Stat signalling pathway and inhibiting NF $\kappa$ B signalling (Bhattacharyya *et al.*, 2004; Finbloom and Winestock, 1995; Ho *et al.*, 1995; Liu *et al.*, 2013, 2013; Schottelius *et al.*, 1999; Wang *et al.*, 1995). IL-10 is produced by most leukocytes and acts on a variety of cell types (Bijlga and Martino, 2011; Ouyang *et al.*, 2011). This cytokine is known to have various immune regulatory functions and is, therefore, a molecule of interest for clinical application in tolerogenic

immunotherapies (Asadullah *et al.*, 2003). IL-10 has been shown to inhibit the generation and secretion of a range of proinflammatory cytokines such as IL-2, IL-1 $\beta$ , IL-12, IL-6, IFN $\gamma$  and TNF $\alpha$  (Tang-Feldman *et al.*, 2011). IL-10 knockout mice have further demonstrated the importance of IL-10 in the homeostasis of the immune system as the low levels or ratio of this cytokine or its absence has been associated to hypersensitivity, severe inflammatory conditions and autoimmune diseases (Cai *et al.*, 2012; Rennick *et al.*, 1997; Schmitt *et al.*, 2012; Toptygina *et al.*, 2014; Trifunović *et al.*, 2015). Accordingly, VD3 and IL-10 are known to have *in vivo* pro-tolerogenic effects and may, therefore, represent effective agents for potential clinical use. Whether their physiological occurrence minimizes unwanted side effects as compared to the other immune suppressants is not clear. However, the data obtained in this study provides a good starting point for further investigations into the effectiveness of VD3 and IL-10 for the immune modulation of moDCs and ipDCs respectively.

The table included in Chapter 3 to enable evaluation of the pro-tolerogenic potential of a modulatory agent has to be interpreted with care (Table 3.2 and Table 3.3). A particular significant change may indicate a potential contribution to a pro-tolerogenic phenotype, however, in some cases, it may also lead to enhanced immunogenicity, as these changes and effects are strongly context-dependent and are not easily translated from *in vitro* to *in vivo* studies. Therefore, the fact that many molecules, either expressed on the cell surface or released as cytokines, have the capacity to act either in an immunoinhibitory or immunostimulatory manner, depending on the context of antigen-presentation, considerably complicates the interpretation of data. For example, an increase in IL-10 may indicate increased tolerogenic T cell regulation and DC suppression,



however, it may also indicate Th2 polarisation and B cell class-switching (Itoh and Hirohata, 1995; Joss *et al.*, 2000; Malisan *et al.*, 1996; Velten *et al.*, 2004). On the other hand, IFN $\gamma$  has been attributed immunoregulatory functions in situations such as oral tolerance, while its secretion by Th1 cells confers considerable immunostimulatory capacity (Lee *et al.*, 2000; Schroder *et al.*, 2004). Furthermore, the downregulation of MHCII and costimulatory molecules may be advantageous to tolerance induction, however, their expression is also essential as it enables the induction of antigen-specific T<sub>regs</sub> and the deletion and suppression of antigen-specific T effector cells (Krummel and Allison, 1995; Lamb and Feldmann, 1984; Orabona *et al.*, 2004). Therefore, data presented in this thesis remain an indication of potential tolerogenicity of modulated ipDCs which could only be formally proven in an *in vivo* setting.

Since the modulation of healthy ipDCs identified IL-10 as the most promising modulatory agent, IL-10 was used to investigate the potential generation of pro-tolerogenic ipDCs from the Pompe disease-specific ipPo cell lines.

As shown in Chapter 4, preliminary results have demonstrated characteristics that may be indicative of a pro-tolerogenic phenotype, including the indicative average upregulation of immunoinhibitory molecules (Figure 4.19 and 4.20), the significant downregulation of the costimulatory molecule CD86 (Figure 4.18) and average down regulation of MHCII (Figure 4.16), all of which was shown to culminate in a reduced T cell allostimulatory capacity (Figure 4.22). Furthermore, a modest increase in the proportion of T<sub>regs</sub> and IL-10 secretion was observed (Figure 4.23 and 4.24). The increase in the proportion of T<sub>regs</sub> provides an important readout, as T<sub>regs</sub> are known to be capable of suppressing B cell

responses by cell-to-cell contact and therefore inhibit the induction of antibody responses (Lim *et al.*, 2005).

The decrease in IL-2<sup>+</sup> CD4<sup>+</sup> T cells in co-cultures with IL-10-modulated ipPo1 ipDCs, as compared to co-cultures with mature ipPo1 ipDCs (Figure 4.26), indicated the increased induction of anergy or apoptotic elimination of alloreactive T cells correlating with the reduced T cell allostimulatory capacity observed in the MLR (Figure 4.22). It is interesting to note that ipPo1 ipDCs consistently induce lower proportions of IL-2<sup>+</sup> and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in co-cultures as compared to C15 ipDCs (Figure 3.37 and Figure 4.26). Whether this is due to donor variation, disease pathology or any other factor remains to be investigated, however these data indicate the necessity for more in depth understanding of the influencing factors.

Altogether, ipPo1 ipDCs appeared phenotypically and functionally similar to C15 ipDCs. Although no striking difference has been observed, further in depth studies are required to confirm this. Furthermore, statistical analysis could not be performed on a number of experiments due to the limited number of independently performed replicates. Therefore, experiments need to be reproduced in order to enable this analysis. The comparison of ipPo1 ipDCs with GAA-corrected ipPo1 ipDCs using CRISPR/Cas9 technology may prove useful for the identification of potential differences.

Future work is needed to assess the potential requirement of the correction of the disease-causing gene in LSD-derived iPSCs and their differentiation products. Apart from the feasibility of deriving iPSCs from LSD patients, previous studies have also shown the capacity of correcting genes in iPSCs, including iPSCs from

Fabry disease mouse model (Higuchi *et al.*, 2014; Huang *et al.*, 2011; Mitani, 2014; Panicker *et al.*, 2012; Xue *et al.*, 2014; Yoshimitsu *et al.*, 2015). Whether this is required for the functionality of ipDCs in immunomodulation remains to be determined, but the ability to genetically modulate human iPSCs broadens their clinical applicability. Gene-corrected iPSC-derived cells or their differentiated progeny, similar to transduced liver cells, may serve as an enzyme production factory as a study by Yoshimitsu *et al.* (2015) has shown the increased secretion of enzyme from transduced iPSCs which may, therefore, lead to cross-correction. *In vivo*, this strategy has proven promising with an increased release of Factor VIII and increased survival of mice with haemophilia A following the hepatic injection of gene-corrected iPSC-derived endothelial cells (Xu, 2009). Further protocols have shown the ability to derive hepatocytes from iPSCs which may provide an interesting target for gene correction and transplantation for the generation of an immune-privileged enzyme-producing factory, as the liver provides an immunoprivileged site due to the constant exposure to gut-antigens via the hepatic portal vein (Crispe *et al.*, 2006; Forrester *et al.*, 2008; Mobarra *et al.*, 2014; Tsuruya *et al.*, 2015; Wohlleber and Knolle, 2012; Yanagida *et al.*, 2015). As discussed in the introduction of this thesis, this immune privilege is the reason why previous studies have targeted gene therapy to the liver in order to induce tolerance to the therapeutic enzyme in LSDs (Franco *et al.*, 2005; Hinderer *et al.*, 2014a; Quiviger *et al.*, 2014; Zhang *et al.*, 2012).

A study showing the increased efficacy of HSCT using HSC that had been genetically modified to express supranormal levels of  $\alpha$ -L-iduronidase could completely reverse the disease pathology in MPSI mice and even improve the cross-correction achieved in the CNS (Visigalli *et al.*, 2010). iPSCs may be used

in a similar way, however the generation and production cost and speed would require improvement in order to make this option feasible. The use of gene-corrected HSC in different mouse models of LSDs and in the clinic have shown some successes in the treatment of LSDs such as Metachromatic Leukodystrophy and other diseases and have the advantage of being considerably less time consuming as compared to the usage of iPSC, despite being more invasive (Biffi *et al.*, 2013; Bigger and Wynn, 2014; Cartier *et al.*, 2009, 2012; Harrison *et al.*, 2013). However, genetically manipulated HSCs and iPSCs still face challenges such as the danger of insertional mutagenesis and the potentially resulting malignancy (Hacein-Bey-Abina *et al.*, 2003, 2008). Recent advances in the methodology of genetic manipulation may have overcome these challenges, as the newly developed CRISPR/Cas9 system enables the targeted integration or removal of genes and therefore minimizes unwanted off-target effects (Hisano *et al.*, 2015; Jiang *et al.*, 2013; Platt *et al.*, 2014; Ran *et al.*, 2013; Xue *et al.*, 2014). Therefore, significant progress may be made in the near future in the development of safe and reliable genetically-manipulated cells for clinical use.

Another recent study has investigated the prophylactic use of anti-B cell activating factor (BAFF) antibodies in order to inhibit the activation of B cells and production of neutralizing antibodies towards ERT in a Pompe disease mouse model (Doerfler *et al.*, 2015). This method successfully prevented hypersensitivity reactions during ERT and was able to reduce anti-GAA in response to treatment. However, despite initial success in the animal model, clinical translation of this approach may be feasible but limited by its non-specificity and therefore potential side-effects such as the increased susceptibility to infections. Different groups

are currently investigating various strategies for improving the effectiveness of ERT or new LSD treatment regimes which include the use of autophagy inhibitors in order to reduce the burden of autophagic accumulation, stimulation of lysosomal exocytosis as a mechanism of substrate clearance and agents that increase calcium levels in the cytosol in an attempt to recover some calcium-dependent cellular processes (Li *et al.*, 2013; Medina *et al.*, 2011; Raben *et al.*, 2008; Spampinato *et al.*, 2013; Williams *et al.*, 2014).

Therefore, a variety of approaches are currently investigated in order to determine and define a more effective and less immunogenic treatment regime for the treatment of LSDs.

Nevertheless, this study was able to modulate ipDCs, which had been derived from a healthy individual and a patient with Pompe disease, using IL-10 to favour pro-tolerogenic properties. This provides a promising foundation for the introduction of immunomodulation therapies for health compromised individuals using autologous ipDCs.

## **6.5 The limitations of the study of moDCs and ipDCs and their clinical application**

One of the most striking limitations of this study was the natural and inherent variation between PBMC donors that has complicated the study of the modulation of moDCs, which may likewise prove to be the case with ipDCs derived from different fibroblast donors. Even the use of an iPSC line derived from a single donor has shown variation between differentiation experiments in the efficiency of differentiation and the effect of modulation. Furthermore, the percentage of CD11c<sup>+</sup> ipDCs generated from a single donor and iPSC line could vary from as

low as 10% to as high as 90% (Figure 3.4B). The exact reason for the variations in the differentiation success and yield of CD11c<sup>+</sup> cells is not known, however the elucidation of these variables will prove to be essential to enable the further optimisation of the differentiation protocol. Studies have shown the inter-clonal variation of differentiation potential and efficacy among iPSC lines, as demonstrated by Thatava and colleagues, who showed that individual iPSC lines derived from the same patient demonstrated different capacities for the formation of the primitive gut tube and islet-like cells (Thatava *et al.*, 2013). Another study found that single nucleotide variants identified in every clone of iPSCs were primarily due to the reprogramming of individual cells with a particular mutational history rather than a consequence of reprogramming (Young *et al.*, 2012). This variation may be particularly evident using dermal fibroblasts as this source of cells had been exposed to UV radiation. This further indicates the need for a different source of cells for iPSC generation as skin fibroblasts may not represent an optimal source due to relatively higher UV exposure and therefore potential for DNA damage and mutations (Section 6.2). Furthermore, whether the intra-clonal variation observed in this study was due to limitations of the protocol, different metabolic or proliferative states of the iPSCs or any other factors is currently unknown. However, the identification of these variables and the minimization of their impact would greatly benefit the reproducibility and efficacy of iPSC differentiation.

In terms of the analysis of flow cytometry data, the measurement and comparison of the cell surface expression of different markers by the relative gMFI may in some cases have been misleading, as bimodal expression profiles were occasionally observed and the occurrence of low levels of staining by isotype

controls that span the the y-axis cause inaccuracies in the relative gMFI. In the case of the biomodal expression of markers, profiles may show a population of cells with high marker expression while a distinct second population displays low or no expression at all. In these cases the gMFI does not reflect the fact that two populations may be present, but rather conveys the presence of a single population with a medium level of marker expression. In this study, bimodal expression profiles were observed in several samples, however most are not displayed in the representative plots. In some cases bimodal expression profile was observed in isotype control stainings which may have been avoided by staining and gating CD11c<sup>+</sup> cells to serve as isotype controls. This would have allowed a more reliable isotype control and gMFI determination as, similar to marker-specific stainings, the populations analysed would only include CD11c<sup>+</sup> cells such as DCs and provide the most appropriate control for staining purposes. This may have further limited the ability to detect marker expression at low levels. Most frequently, bimodal expression profiles were observed in moDC populations treated with IL-10. These data may further underline the requirement for a protocol enabling the purification of a specific DC subset, as these two distinct populations may prove to exert different immunomodulatory effects. This requirement for the generation and purification of a well-defined ipDC population is further underlined by the yield of CD11c<sup>+</sup> cells (Figure 3.4 and Figure 3.17), which may indicate the presence of a proportion of non-DC at the end of the differentiation phase.

The impurity of ipDC cultures in particular may have affected the data obtained in this study as other cells such as macrophages, neutrophils and mesenchymal stem cells may exert immunological functions (Figure 3.4) (Murray and Wynn,

2011; Nathan, 2006; Ryan *et al.*, 2005). The presence of mesenchymal stem cells for example may have partially contributed to the reduced immunostimulatory capacity and immaturity of ipDCs as they have been shown to inhibit the maturation of DCs (Aggarwal and Pittenger, 2005; Jiang *et al.*, 2005; Tse *et al.*, 2003; Zhang *et al.*, 2004). Furthermore, the increased secretion of IL-10 in some ipDC co-cultures with T cells may have also been affected or originate from mesenchymal stem cells which are known to secrete IL-10 as a mechanism of T cell suppression (Beyth *et al.*, 2005; Najar *et al.*, 2014; Ryan *et al.*, 2005; Tse *et al.*, 2003; Yan *et al.*, 2014; Zhang *et al.*, 2004). Furthermore, mesenchymal stem cells have been shown to enhance the immunosuppressive capacity of T<sub>regs</sub>, which is partially regulated by increased IL-10 concentrations and the subsequent upregulation of PD-1 on T<sub>regs</sub> (Ryan *et al.*, 2005; Yan *et al.*, 2014). Having detected high levels of IL-6 (Figure 3.31 and Figure 4.21) and IL-10 (Figure 3.35 and Figure 4.24) in some ipDC cultures or co-cultures with T cells and a very low MHCII expression profile (Figure 3.19 and Figure 4.16) of ipDCs, the influence of mesenchymal stem cells on ipDC immunology and the immunological potential of the environmental milieu may have been possible in this study (Beyth *et al.*, 2005; Jiang *et al.*, 2005; Najar *et al.*, 2014; Silva *et al.*, 2003; Zhang *et al.*, 2004). However, no cell types with the distinct features of mesenchymal stem cells have been observed in cell cultures.

Furthermore, the presence of macrophages may have contributed to the cytokine profile measured on culture supernatants and therefore influenced the immunological state of DCs and T cell polarisation. The treatment of DCs with IL-10 may have also affected contaminating macrophages which could further contribute to an increased presence of IL-10, as macrophages have been shown



to increase the secretion of IL-10 in response to IL-10 exposure (Figure 3.35 and Figure 4.24) (Fiorentino *et al.*, 1991; Rey-Giraud *et al.*, 2012; Staples *et al.*, 2007). Furthermore, IL-10 treatment may inhibit the secretion of IL-6 and TNF $\alpha$  from macrophages and therefore affect the cytokine expression profile observed in cultures (Fiorentino *et al.*, 1991; Yilma *et al.*, 2012). On the other hand, the treatment of cultures with VD3 may have affected contaminating macrophages after exposure to maturation signals such as IFN $\gamma$ , which has been shown to inhibit the expression of IFN $\gamma$ -regulated genes and defence mechanisms such as the oxidative burst (Helming *et al.*, 2005; Zhang *et al.*, 2012). Dexamethasone treatment may also affect macrophages in cultures by enhancing their proliferative response to GM-CSF but also by inhibiting the secretion of a set of pro-inflammatory cytokines (Abraham *et al.*, 2006; Lloberas *et al.*, 1998). This change in the cytokine environment may have further downstream effects on the polarisation of T cells during co-cultures and therefore has to be investigated more closely.

The presence of neutrophils in cultures may further affect the immunogenicity of DCs as activated neutrophils have been shown to induce the maturation of DCs and enhance their T cell stimulatory and Th1 polarisation capacity (van Gisbergen *et al.*, 2005).

Nevertheless, future studies are required in order to investigate this potential contamination and ensure, using a DC purification protocol, the reliability and specificity of future results. Furthermore, understanding the potential effect of other cells on DCs and T cells is paramount in order to enable a more accurate indication of the possible effect of modulated DCs *in vivo*.

Apart from the purity of DC cultures following differentiation, the speed, ease and cost effectiveness of the iPSC generation and ipDC differentiation procedure is a clear disadvantage and requires optimisation. The approximate timeframe of 3 months for this process is a protracted time period if patients of young age and short life expectancy are to be treated using the approach investigated in this thesis. Therefore the practicality of this approach may be significantly compromised until an improved and faster protocol is developed. Nevertheless, significant efforts are currently being invested by companies around the world to streamline the derivation and differentiation of iPSC, so new advances are anticipated in the near future (Cramer and MacLaren, 2013; Jung *et al.*, 2012; Madonna, 2012).

The fact that all readouts for the effect of DC modulation on DC-T cell interaction relied on allogeneic co-cultures may limit their interpretation and application to the induction of tolerance towards therapeutic enzymes. The optimal readout would have been antigen-specific T cell activation and the detection of antigen-specific T<sub>reg</sub> populations. However, the HLA-restriction of T cells greatly complicates such assays. The possibility of assaying antigen-specific responses necessitates the availability of a HLA-matched T cell donor in order to allow MHCII and TCR interaction and recognition of presented peptides. The complete HLA-matching would also circumvent the activation of alloantigen-specific T cells and therefore provide a more accurate measure of the potential impact of autologous DC therapy. However, T cell donors of a specific HLA-haplotype are difficult to obtain from clinical sources. Furthermore, as the C15 fibroblasts were obtained commercially and ipPo fibroblasts were derived from a patient who is now deceased, obtaining HLA-matched T cells from these donors by taking blood

samples was not possible. Similarly, as the Pompe disease patient was of Asian origin, the HLA-haplotype may be less frequently represented among clinically obtained blood banks in the UK. Despite, these caveats, this study has indicated the feasibility of generating large numbers of ipDCs and modulating them using IL-10 to generate ipDCs with pro-tolerogenic characteristics.

## **6.6 Exosomes as vehicles for the delivery of therapeutic enzymes and their immunogenicity**

Another challenge to effective ERT is the targeting of therapeutic enzymes to the CNS via the BBB. The CNS is frequently involved in the disease pathogenesis of LSDs (Crumrine, 2001). The microenvironment of the CNS is a highly regulated area which limits the access of larger molecules, such as therapeutic enzymes, into the cerebrospinal fluid. Various approaches have been explored to overcome this challenge, which include HSCT and the intrathecal injection of enzyme or enzyme-expressing transgenes directly into the spinal cord (Banugaria *et al.*, 2013; Douillard-Guilloux *et al.*, 2009; Messinger *et al.*, 2012; Raya, 2009; Sun *et al.*, 2007, 2010; Zhang *et al.*, 2012).

A recently established protocol for the delivery of siRNA into the CNS of mice has made use of small vesicles, so-called exosomes, which are naturally produced by most cells (Alvarez-Erviti *et al.*, 2011; Cooper *et al.*, 2014). These exosomes were derived from mouse bmDCs as they serve as a rich source and had been modified to express RVG to specifically target exosomes across the BBB. Therefore, this thesis further explored the possibility of deriving exosomes from mouse and human ipDCs and their use as vehicles for the delivery of enzymes. Apart from the possibility of improving drug-targeting to the CNS, enzyme-

carrying vesicles may improve enzyme targeting to other cells and tissues of the body by containing specific targeting peptides or antibodies. The therapeutic advantage of specific enzyme targeting was illustrated by a number of recent studies showing significantly improved substrate clearance using therapeutic enzyme and anti-ICAM-1 antibodies that had been coupled to polystyrene bead nanocarriers as compared to ERT with free therapeutic enzyme (Hsu *et al.*, 2011, 2012, 2014).

The incentive behind the investigation of the capacity of ipDCs to generate exosomes was first of all, the possibility of generating large quantities of patient-derived exosomes, and second of all, the potential low immunogenicity due to the seemingly 'naïve' state of ipDCs. This naïve phenotype was confirmed by the characterisation of mouse ipDCs and their comparison to mouse bmDCs, as ipDCs consistently showed a reduced expression of MHCI, MHCII and costimulatory molecules, even below levels observed for immature bmDCs (Figure 5.4-6).

As determined by Nanosight, vesicles ranging in size from 50 to around 400 nm diameter were purified by standard exosome purification methods from mouse and human bmDC, moDCs and ipDCs (Figure 5.8). Western blotting confirmed the presence of exosome markers Alix and Tsg101, which, nevertheless, varied between exosome samples (Figure 5.9). The presence of vesicles larger than 100 nm diameter may imply the presence of non-exosomal vesicles such as shedding microvesicles which originate from the plasma membrane of the cell surface (van Dommelen *et al.*, 2011; György *et al.*, 2011a). The inability to separate exosomes from microvesicles has limited the generation of pure

exosome populations and, therefore, both types of vesicles are expected to be present in samples. However, to simplify the matter, purified vesicles were generally referred to as exosomes. Further characterisation of exosomes showed the expression of the tetraspanin, CD9, and the myeloid marker CD11b (Figure 5.10). Exosomes derived from ipDCs appeared to express low or undetectable levels of MHC or costimulatory molecules (Figure 5.11). However, in contrast to other studies, levels of marker expression by bmDC-derived exosomes was also low and variable. Despite using the same purification and detection method, other studies have shown the detection of higher levels of MHCII, MHCI, CD54, CD80 CD86 and CD11c on exosomes derived from immature and mature mouse bmDCs (Kim *et al.*, 2005; Li *et al.*, 2012; Näslund *et al.*, 2013). Therefore, we questioned the reliability and reproducibility of these methods of exosome characterisation and are currently working towards a proteomics analysis of exosomes from human moDC and ipDC populations in collaboration with Professor Matthew Wood's laboratory. However, the demonstration that exosomes positive for CD9, Alix and Flot-1 could be purified from human ipDC samples, indicated their potential derivation for clinical purposes (Figure 5.12).

As the generation of exosomes in the MVBs within the endosomal system is thought to be closely linked to the endocytic pathway, this study sought to explore the possibility of targeting exogenous proteins into exosomes by virtue of the endocytic capacity of DCs. Furthermore, it has been shown that phagocytosed and endocytosed protein can be targeted for cross-presentation by translocation of protein from the endocytic/phagocytic vesicles into the cytosol for degradation (Ackerman and Cresswell, 2004; Ackerman *et al.*, 2006; Rock and Shen, 2005; Rodriguez *et al.*, 1999). Using HRP, this translocation has also been shown to

maintain the functional integrity of the protein enzyme which has also been achieved using bmDCs (Rodriguez *et al.*, 1999). Therefore, the easily traceable enzyme (HRP) was used which enabled the localisation of functional HRP within the endocytic system and organelles reminiscent of MVBs within bmDCs (Figure 5.14 and Figure 5.15). HRP was further detected in culture supernatants of HRP-treated cells which indicated its subsequent release from cells (Figure 5.16). However this study was not able to reliably determine whether this detected HRP activity was specifically associated with exosomes. This was due to the potential of HRP to aggregate in medium which caused its sedimentation at gravitational forces used for the purification and harvest of exosomes (Figure 5.17). However, the finding that significantly higher concentrations of HRP could be recovered by exosome purification from HRP-treated DCs than from HRP-treated T cells, and HRP could be detected in DCs but not in T cells, indicated the dependence of HRP uptake and release on a cellular property unique to DCs, which may potentially constitute their capacity to translocate endocytosed protein into the cytosol of the cell (Figure 5.14 and Figure 5.16).

Nevertheless, the association of HRP with exosomes had to be further investigated, as the possibility of free HRP aggregation and sedimentations remained. The use of a gradient centrifugation for the determination of the buoyant density associated with exosomes, HRP harvested from cultures and HRP alone, did not achieve conclusive results, possibly due to the loss of a large proportion of exosomes and the inhibitory effect of the gradient matrix on HRP activity (Figure 5.18 and 5.19). The visualization of exosomes from HRP loading experiments using EM was attempted, however the large proportion of protein contaminants from media did not allow any conclusive results to be obtained

(data not shown). Once the protocol is optimized, future studies may investigate this strategy of exosomal loading using human ipDCs, as the differentiation protocol described in this thesis has been shown to generate a larger proportion of cross-presenting DCs and therefore cells with a potential higher capacity to translocate functional protein into the cytosol thereby ensuring the cytosolic provision of protein for uptake into exosomes during MVB formation (Ackerman and Cresswell, 2004; Silk *et al.*, 2012a).

Despite these technical set-backs, results have shown the capacity of DCs to take up enzyme and preserve their activity over a 2 hour period (Figure 5.14 and Figure 5.15). EM analysis of DCs treated with HRP showed electron-dense areas in cellular structures reminiscent of MVB, which may indicate the co-localisation of HRP in areas of exosome generation (Figure 5.14 and Figure 5.15). Furthermore, the detection of HRP in supernatants, above that present after washing steps, was promising and may indicate the release of HRP from cells which was a property unique to DCs (Figure 5.16). However, whether this was due to the release of free HRP from cells due to exocytosis or cell death, was not determined. Therefore, it remains to be confirmed whether HRP detected in supernatants of treated DCs is directly associated with exosomes.

Other approaches to improving enzyme targeting via the BBB into the CNS have been very promising. These include the *in vivo* delivery of transgenes expressing the therapeutic enzyme fused to a signal peptides that allow the specific binding and transcytosis across the BBB into the CNS (Osborn *et al.*, 2008; Sorrentino *et al.*, 2013). However, the potential for transgene integration into the cell's genome remains a considerable danger and therefore an alternative method is desirable (Magauran and Salgado, 2011; Wingard *et al.*, 2010). Intrathecal enzyme

administration has also been an approach to improve the targeting of therapeutic enzyme to the CNS (Giugliani *et al.*, 2014; Hinderer *et al.*, 2014b; Kakkis *et al.*, 2004; Muenzer *et al.*, 2015; Munoz-Rojas *et al.*, 2008). However, recent data have indicated a potential for immunological responses and inflammation in response to intrathecal ERT (Vera *et al.*, 2013).

As previously discussed, HSCT has been another approach in tackling the challenges of the BBB in ERT. Here, HSCT, whether using genetically modified HSC or not, has been found to cross-correct enzyme deficiency in the CNS due to cell engraftment of microglia from the transplant into the CNS (Biffi *et al.*, 2013; Krivit *et al.*, 1995; Visigalli *et al.*, 2010). Epinephrine-induced enhancement of enzyme transportation across the BBB has been shown which appeared to restore developmentally downregulated M6PR-mediated transportation (Urayama *et al.*, 2007). Therefore, a number of approaches are being investigated in order to one day determine an efficient, non-invasive and easy method for enzyme delivery to the CNS.

## **6.7 The immunogenicity of DC-derived exosomes**

Irrespective of issues related to the loading of exosomes, their immunogenicity was investigated following the administration of syngeneic and allogeneic exosomes into mice. Exosomes obtained from autologous CBA bmDCs appeared to induce the highest antibody response as compared to exosomes derived from CBA ipDCs, B6 bmDCs or B6 ipDCs (Figure 5.22 and 5.23). The responses induced by the administration of exosomes was also found to be more potent for the induction of antibody responses as compared to that induced by allogeneic B6 bmDCs. However, the detection of antibodies specific for B6



bmDCs and the analysis of data as gMFI produced a similar problem as discussed above. The bimodal expression profile was hypothesized to be due to the presence of an immature and a mature population of bmDCs. Therefore, on injection, mature bmDCs may induce a potent immune response, while immature bmDCs may have acted in a tolerogenic fashion similar to steady-state conditions. This may have allowed the staining of only mature bmDCs with serum obtained from injected mice. Recently acquired data appear to support this, as co-staining with CD86 confirmed that the bmDC populations staining positively with serum antibodies, expressed higher levels of CD86 as compared to the unstained population. Therefore, a gating strategy to exclude immature or possible non-DCs from the analysis, or cell sorting before injection, may allow the improvement of antibody titre detection. Altogether, these data have further underlined the need for the generation of pro-tolerogenic ipDCs as these may provide a source of non-immunogenic exosomes for the therapeutic delivery of CNS-targeted drugs.

However, the presence of contaminating non-DC populations means that exosomes may be present that had not originated from DCs. These exosomes may further harbour immunomodulation capacity which needs to be considered and in future its effect minimized by DC purification methods. This challenge is especially relevant when studying exosomes derived from ipDCs, as these cultures may harbour larger proportions of non-DCs including macrophages or mesenchymal stem cells.

The potential to derive exosomes from ipDCs, whether from human or mouse, has been shown to be feasible in this study. The potential to use these exosomes for further improvement of the treatment efficacy of LSDs by targeting enzyme

specifically to the CNS, allows the usage of an easily obtainable by-product of ipDC differentiation. As this study has demonstrated the immunogenicity of exosomes derived from *in vitro* cultured syngeneic and allogeneic bmDCs and ipDCs, the derivation of exosomes from modulated ipDCs with pro-tolerogenic characteristics may prove advantageous. Exosomes derived from immature or modulated DCs have previously been shown to confer immunoregulatory properties in settings such as collagen-induced arthritis and animal intestinal and cardiac transplantation models (Kim *et al.*, 2005; Li *et al.*, 2012; Yang *et al.*, 2011). Of particular interest was the study by Kim *et al.* 2005, which reported that exosomes derived from IL-10-modulated mouse bmDCs were able to suppress collagen-induced arthritis at onset and diminish its progress in established cases, as evaluated by footpad swelling. Therefore, the use of exosomes derived from IL-10-modulated ipDCs may prove to harbour immunosuppressive capacity, which may be similar to that exerted by the modulated ipDC. Hence, the immunomodulatory capacity of rapamycin, VD3, dexamethasone and IL-10 may further serve future investigations into the potential immunoinhibitory function of their respective exosomes. As the CNS-specific targeting of exosomes makes use of the RVG peptide, further studies are required in order to investigate the immunogenicity of this targeting moiety. If this targeting strategy was found to be immunogenic, exosomes or ipDCs may provide a useful approach for the induction of tolerance towards this molecule. This may potentially improve exosome-mediated delivery of siRNA, or whole protein, to the CNS, as tolerance induction towards immunogenic exosomes may suppress the induction of neutralizing antibody responses which may prevent the potential mistargeting of exosomes to FcR-expressing cells. Furthermore, in light of these studies,

exosomes may provide an interesting alternative to DCs for the induction of tolerance towards recombinant enzyme in ERT.

## **6.8 Conclusion**

This study has highlighted the need for further optimization of the ipDC differentiation protocol to allow the reliable and consistent production of patient-specific ipDCs. Most importantly, ipDC populations need to be subjected to a highly effective purification method to enable the study and use of pure populations. This would allow the minimization of secondary effects on the immunogenicity and T cell responses exerted by cells other than DCs. Furthermore, differentiation and modulatory protocols should to be applied to iPSCs derived from other donors to allow the evaluation of their reproducibility and potential transferability to patients.

Apart from the investigation of the effect of modulated ipDCs on antigen-specific T cell activation, future work may include the performance of an *in vitro* assay for the evaluation of B cell responses in co-cultures with modulated DCs and T cells. This may achieve a further level of refinement in the determination of the effectiveness of modulated ipDCs in the suppression of an antibody-mediated immune response towards ERT. Furthermore, the application of this immunomodulation regime to animal models of LSDs with known immunoreactivity to therapeutic enzyme is essential for the potential progress of this strategy into the clinic.

The potential of generating non-immunogenic autologous exosomes by developing alternative culture and differentiation methods may improve the

clinical applicability of exosomes. Whether autologous exosomes acquired immunogenic antigens during the culture process remains to be determined. However, the immunogenicity of exosomes may be further circumvented by their purification from modulated DCs with pro-tolerogenic properties. The feasibility of generating exosomes from patients with LSDs is to be determined as a number of accumulating substrates such as sphingolipids also play an essential role in the biogenesis of exosomes. One study for example has shown the enhanced release of cholesterol via exosomes from cells derived from a patient diagnosed with NPC, indicating that exosome biogenesis can be altered in this disease state (Strauss *et al.*, 2010). Therefore, future studies need to determine the effect of particular LSD on exosome biogenesis.

The capacity to load exosomes with protein via the endocytic capacity of DCs should be further explored. The use of EM and immuno-gold labelling or HRP substrate for the localisation of HRP in exosome samples that have been subject to extensive purification, may be used in order to investigate whether HRP may be found in the lumen of these vesicles, associated with the outer membrane or freely in the sample. Other proteins, especially proteins containing M6P-residues, may be investigated for their capacity to be loaded into exosomes via this system. The immunogenicity of exosomes loaded with therapeutic enzyme also requires considerable attention, as exosomes may be mistargeted and neutralised in the presence of an immune response.

The future clinical application of pro-tolerogenic ipDCs or exosomes derived from them demands the development of protocols for the commercial generation of iPSCs, their expansion and differentiation. The number of ipDCs required for effective treatment, the frequency and the route of administration are all essential

variables which will need to be determined. The choice of the route of administration has been shown to have significant effect on the outcome as one study was able to prolong allogeneic heart graft survival if matured dexamethasone-treated DCs were administered intravenously, while subcutaneous injection did not prolong survival (Emmer *et al.*, 2006).

Altogether, this study has provided the first step towards the generation of autologous DCs with pro-tolerogenic properties for the potential improvement of treatment efficacy of LSDs. Furthermore, exosomes derived from these populations of DCs may provide a further means to improve the targeting of therapeutic enzymes across the BBB into the CNS for the improved efficacy of ERT.

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**“Strength does not come from physical capacity.  
It comes from an indomitable will.”**

*Mahatma Gandhi, 1920*