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Ex vivo generation of bone marrow derived regulatory dendritic cells for promotion of corneal allograft survival.

A PhD Thesis Submitted to the Faculty of Medicine, National University of Ireland, Galway, as partial fulfillment of the requirements for the degree of Doctor of Philosophy

Author
Lisa O’Flynn MSc.

Supervisor: Dr. Thomas Ritter
Institution: Regenerative Medicine Institute (REMEDI)

Submitted: September 2013
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DECLARATION

I declare that all the work in this thesis was performed personally unless otherwise stated. No part of this work has been submitted for consideration as part of any other degree or award.
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Investigations into cell based therapies for application in organ transplantation has grown significantly. Here, I describe the ex vivo generation of donor bone marrow derived dendritic cells (BMDCs) and examine the therapeutic potential of genetic and pharmaceutical modifications of BMDCs for application in allogeneic transplantation. BMDCs were generated via culturing bone marrow precursor cells with rat granulocyte macrophage-colony stimulating factor and interleukin 4 containing medium. Lentiviral gene transfer of BMDCs was examined and although successful was not a feasible approach. The treatment of BMDC with dexamethasone (Dexa) to induce an immature, maturation resistant phenotype was however, comprehensively examined in this study. BMDC and Dexa BMDC phenotype, antigen presenting cell function and their immunomodulatory properties were examined and fully characterised. Both populations displayed significant immunomodulatory properties relative to freshly isolated OX62⁺ DCs, including but not limited to, a significant increase in mRNA expression of programmed death-ligand 1 and indoleamine 2,3-dioxygenase. Both BMDCs and Dexa BMDCs displayed a profound impaired capacity to stimulate allogeneic lymphocytes. Moreover, in a fully MHC I/II mismatched rat corneal transplantation model (Dark Agouti (DA) to Lewis (LEW)), injection of donor (DA) derived BMDC or Dexa BMDCs (1×10⁶ cells/animal, day -7) significantly prolonged corneal allograft survival without the need for additional immunosuppression. With both cell therapies, we observed a significant reduction in the level of allograft cellular infiltration, a significant increase in the ratio of intragraft FoxP3 expressing regulatory cells and evidence of a donor specific allo-antibody response. Taken together, our comprehensive analysis demonstrates a detailed analysis of the significant therapeutic effect of donor derived BMDCs with and without glucocorticoid treatment in corneal allograft survival by modulation of the allo-immune response at the level of both the allograft and the draining lymph nodes. This body of research represents a novel therapeutic approach for the prevention of corneal allograft rejection.
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<td>Adenovirus</td>
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<tr>
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<td>α-rat</td>
<td>anti-rat</td>
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<tr>
<td>amp</td>
<td>ampicillin resistance gene</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ACAID</td>
<td>anterior chamber-associated immune deviation</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMDCs</td>
<td>bone marrow derived dendritic cells</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>DMEM</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>MAA</td>
<td>Maackia amurensis</td>
</tr>
<tr>
<td>MACs.</td>
<td>magnet-activated cell sorting</td>
</tr>
<tr>
<td>mHAg</td>
<td>minor histocompatibility antigens</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage migration inhibitory</td>
</tr>
<tr>
<td>MLC</td>
<td>mixed lymphocyte culture</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction (same as MLC)</td>
</tr>
<tr>
<td>MPLA</td>
<td>monophosphoryl lipid A</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NHP</td>
<td>non-human primates</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NK-T</td>
<td>natural killer-like T cell</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>programmed death-ligand 1</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SNA-1</td>
<td>Sambucus Nigra-1</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SSC</td>
<td>sidewards scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>T reg</td>
<td>regulatory T cell</td>
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<tr>
<td>TCM</td>
<td>T cell media</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>tub</td>
<td>transforming units</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>1-MT</td>
<td>1-methyl-D-tryptophan</td>
</tr>
</tbody>
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Chapter 1

1. Introduction

1.1 Cornea

1.1.1 Anatomy and Function

The cornea is a critical part of the eye which not only acts as a physical barrier along with the lysozyme and IgA containing tear film to provide innate protection against foreign particles or pathogens but it also plays a vital role in the production of an image. The cornea focuses light rays onto the lens which in turn focuses the light onto the retina. As a result the shape, smoothness and clarity of the corneal tissue are essential and any disturbance in the structure of the cornea may result in a loss of function and possibly sight. Cornea transplantation is the main method for visual rehabilitation of patients with decreased or complete loss of corneal clarity due to a wide range of corneal defects and diseases (Tan and Abdulreda 2013).

The cornea consists of five different layers, the epithelium, the stroma and endothelium are the three cellular layers, the Bowman’s zone and Descemet’s membrane the two acellular layers (Fu et al. 2008; Tan et al. 2012) (Figure 1.1). The epithelium consists of six to eight non-keratinized cell layers placed on the Bowman’s membrane. Epithelial cells deep within this layer constantly undergo mitosis and replace the superficial cells (Niederkorn 2012). The Bowman’s zone consists only of irregularly-ordered collagen fibers and is acellular unlike that of organised fibers in the stromal layer. The stromal layer is composed of tightly packed ordered collagen fibers which are populated by scattered keratocytes. Underneath this stromal layer lies another acellular layer the
Descemet’s layer which is a thick basement membrane secreted by the endothelial monolayer cells. The inner layer is the corneal endothelium which forms the barrier between the stroma and the anterior chamber of the eye. This cell layer of polygon-shaped cells with limited proliferation capacity is critical to cornea function. This layer supplies nutrients from the aqueous humor (AH) to the avascular cornea and also acts as a barrier protecting the stroma from swelling by pumping out excess stromal fluid. Loss or damage to this endothelial layer is the main contributor to corneal allograft failure. This can occur due to improper storage, due to surgical trauma or due to post-operative inflammation (George and Larkin 2004; Niederkorn 2012; Tan et al. 2012).

Figure 1.1 Anatomy of the cornea. Adapted from (George and Larkin 2004; Fu et al. 2008).
1.1.2 An immune privileged site

The eye is well-defined as an immune-privileged organ (Stein-Streilein 2008; Taylor 2009; Hori et al. 2010; Niederkorn and Stein-Streilein 2010; Masli and Vega 2011). The preservation and protection of the ocular tissues of the eye described previously is paramount for preserving vision. The eye is therefore governed by different rules than those observed in other organs. Ocular inflammatory processes required to protect and eliminate a threat to the eye may also injure cells of either the corneal endothelium or the retina leading to blindness, as neither of these tissues can regenerate (Niederkorn 2012). Therefore the eye must maintain a state of homeostasis and the anatomical, physiological and immunological adaptations that limit immune-mediated inflammation in the eye create the condition referred to as “immune privilege”, which is believed to be essential for the maintenance of normal vision (Streilein 2003; Niederkorn 2012). This immune privilege is maintained by multiple pathways including the inhibition of the afferent and efferent arms of the immune system. As a result the eye and its immune privileged status have been extensively examined in order to understand the mechanisms involved in the maintenance of such a status (Novak et al. 2003; Streilein 2003; Taylor 2009; Niederkorn 2009; Perez et al. 2013; Forrester et al. 2013).

1.1.2.1 Maintenance of an immune privileged site

A healthy cornea is an avascular tissue; this avascular status is the key to the cornea maintaining its immune-privilege. This is important particularly in transplantation as this avascular status reduces the possibility of antigen presenting cells (APCs) and effector cell trafficking to the cornea through the blood vessels. The central part of the cornea, which is used as donor tissue, contains only a small population of major histocompatibility complex (MHC) class II–expressing APCs (Sosnová et al. 2005).
Bone marrow–derived cells have recently been reported to be present within normal cornea, although most of these cells show an immature phenotype lacking MHC II expression (Hamrah 2003).

The blood-ocular barrier provides further protection to the immune privilege of the eye. In order for cells to access the eye they must first pass through the tight junctions with the iris pigment epithelial layer. The cells of this layer constitutively express the co-stimulatory molecules CD86 which binds to cytotoxic T-lymphocyte antigen (CTLA)-4 on the surface of T cell resulting in an inhibition of T cell proliferation and activation. It also prevents the production of interferon (IFN)-γ and has been reported to induce a regulatory T cell (T reg) phenotype. The inhibition of T cell proliferation and activation was impeded in CD86 or CTLA4 knockout animals, with administration of anti-CD86 antibodies and also with CTLA4 immunoglobulin (Ig) administration (Sugita and Streilein 2003).

Included in the efferent arm of the immune environment of the eye is the AH. The AH consists of immunosuppressive soluble factors capable of modulating immune responses and creating an immunomodulatory milieu. This immunomodulatory environment is created by cytokines, growth factors and neuropeptides. Other factors such as macrophage migration inhibitory factor (MIF), vasoactive intestinal peptide (VIP), transforming growth factor (TGF)-β, CD59, FasL and complement regulatory protein (CRP) which modulates the effect of complement-mediated cytolysis, are also present in the AH to aid in the maintenance of this immune-privilege status of the eye (Bora et al. 1993; Flynn et al. 2008; Hori et al. 2010; Niederkorn 2012).

The normal corneal cells (i.e., epithelial, stromal, and endothelial cells) do not express MHC II and only weak MHC I antigens (Hori, Taniguchi, et al. 2010). Corneal endothelium does however express proteins effective at creating a barrier to effector T
cells or complement activation. The expression of FasL, CRP, and programmed death-ligand 1 (PD-L1) have been detected on the endothelium (Bora et al. 1993; Hori et al. 2006; Shen et al. 2007; Stein-Streilein 2008; Kan-mitchell 2008; Hori, Taniguchi, et al. 2010; Masli and Vega 2011; Nosov et al. 2012). The capacity of complement-fixing alloantibodies to mediate corneal damage is limited by the extensive expression of CRP expressed on corneal cells and in the AH (Niederkorn 2012). Glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR) ligand also contributes to the immune-privileged status of corneal allografts and has been demonstrated to be constitutively expressed on normal corneal endothelium (Hori, Taniguchi, et al. 2010). Also these ligands including FasL, PD-L1 act by interacting with their respective receptors on the surface of T cells resulting in the induction of apoptosis, inhibition of proliferation and IFN-γ production (Hori et al. 2006; Shen, Jin, Freeman, et al. 2007; Masli and Vega 2011).

1.1.2.2 Anterior Chamber-Associated Immune Deviation (ACAID)

In addition to maintaining a local immunosuppressive environment, the eye is also capable of orchestrating systemic immunoregulatory responses against intraocular antigens which has been given the term anterior chamber-associated immune deviation (ACAID) (J W Streilein 2003; Taylor 2009; Stein-Streilein and Lucas 2011; Masli and Vega 2011). The anterior chamber contains AH consisting of biologically relevant concentrations of various immunomodulatory factors as previously described (Bora et al. 1993; Flynn et al. 2008; Hori, Taniguchi, et al. 2010; Niederkorn 2012). These factors modulate and suppress innate and adaptive immunity in order to maintain an immune suppressive microenvironment within the eye. If an alloantigen is introduced to the anterior chamber this can result in an antigen-specific suppression of delayed-type hypersensitivity (DTH). This involves the migration of intraocular APCs to the spleen and formation of two forms of T regs. The first “afferent” set is made up of CD4+ T regs
which prevent the activation and differentiation of antigen specific effector T helper cells (Th1). The second of “efferent” set of cells consists of CD8+ T regs which have been associated with the inhibition of DTH (Masli and Vega 2011). Interestingly it has been demonstrated that the different forms of immune tolerance involved in the induction of ACAID also play a role in the promotion of corneal allograft survival (Yamada et al. 1999; Cunnusamy et al. 2010). However, disturbance of the ACAID due to the trauma of surgery, viral infection or chronic inflammation of the eye are factors which contribute to corneal allograft rejection.

1.1.3 Cornea Transplantation (Penetrating Keratoplasty)

1.1.3.1 Epidemiology and Indications

The first successful penetrating keratoplasty was performed by the Czech ophthalmologist Dr. Eduard Zirm (1887-1944) on 7 December 1905 (Moffatt et al. 2005). In the 108 years since then the procedure of penetrating keratoplasty has evolved and the indications which require keratoplasty intervention have also changed with time. Due to developments in antibiotics and antiviral agents fewer patients with infections, trauma and burns to the cornea require transplantation. Keratoconus (bulging of the cornea (Figure 1.2), corneal dystrophies, corneal scarring and regrafting for failed corneal transplants are now the main indications for penetrating keratoplasty (Moffatt et al. 2005). The cornea is the most commonly transplanted tissue in humans (George and Larkin 2004; Niederkorn 2012) and remains the main method for visual rehabilitation of patients with decreased or complete loss of corneal clarity due to a wide range of corneal diseases (Tan and Abdulreda 2013). Successful engraftment and higher acceptance rate of corneal allografts compared to other solid organs is due in part to its avascular nature and to the inherent immune regulatory properties of the anterior chamber of the eye as previously described (Williams et al. 2006).
Figure 1.2 Keratoconus Cornea. Keratoconus is now one of the main indications for corneal transplantation, figure adapted from http://www.cornea.org.

1.1.3.2 Corneal Allograft Rejection

Although the eye is described as an immune privileged organ, not all corneal allografts succeed in humans or in experimental animals (Yamada et al. 1999). Corneal transplantation itself is a relatively risk free, uncomplicated procedure with 90% survival within the first year post transplantation (Waldock and Cook 2000; Niederkorn 2012), the five year prognosis is similar to that of renal, liver or cardiac allografts and rejection remains the main cause of allograft failure (Waldock and Cook 2000; Niederkorn 2012). It has been reported that graft survival for patients with keratoconus is remarkable (Williams et al. 2006). However, the risk of rejection remains high for
those patients with repeated transplantation in the same eye, corneal neovascularisation, an episode of preoperative inflammation or raised intraocular pressure (Williams et al. 2006). The cornea as previously described, consists of five layers however, only the epithelium, the stroma and the endothelium are targeted in the immune response. The clinical signs for which depend on the layer of the cornea in which rejection is occurring.

The primary initial mechanism described in detail later (section 1.4) which leads to corneal allograft rejection is allo-recognition via the indirect pathway with CD4$^+$ T cells being the cell type required for rejection (Boisgérault et al. 2001). The CD4$^+$ T cells are also capable of recognising donor minor histocompatibility antigens (mHAg) which are processed and presented by recipient APCs on MHC I and MHC II (Sano et al. 1996; Zelenika et al. 1998). It is likely that antigens in the donor cornea are ‘seen’ by the immune system, as it is known that soluble and cell-associated antigens are capable of trafficking within minutes or hours to the eye draining lymph nodes (DLNs) (Vitova et al. 2013).

The procedure of corneal grafting alone can cause the breakdown of the blood ocular barrier and also some degree of corneal vascularisation has been observed in syngeneic grafts possibly due to the presence of sutures. Although the healthy cornea lacks blood vessels the stimulus such as vascular endothelial growth factor (VEGF)-C, that promotes growth of these vessels post transplantation also promotes the growth of lymphatic vessels (Niederkorn 2012). Therefore pre-existing or secondary neovascularisation due to penetrating keratoplasty is the prominent predisposing factors for immunological corneal allograft rejection and is also correlated with a reduced survival rate in both human and murine transplantations (Waldock and Cook 2000; Tan et al. 2012).
T cell extravasation from blood or lymphatic vessels and migration through the anterior chamber into the stroma and/or the endothelium results in the initiation of rejection of these layers. It is coupled with increased levels of the chemokines CCL5, CXCL9, and CXCL10 in allografts which is associated with local activation of infiltrating T cells during ongoing rejection (Tan and Abdulreda 2013). This cell-mediated immunity is believed to play the decisive role in corneal graft rejection (Niederkorn 2012), as the susceptibility of corneal allografts to antibody-mediated rejection is controversial (Hegde et al. 2002; Holán et al. 2005). High numbers of cells expressing natural killer (NK)-markers, that appear to have alloreactivity, have been located in other animal models of corneal transplantation as well as in human samples of AH (Claerhout et al. 2004; Flynn et al. 2008). A recent report indicated that level of Foxp3 expression in T reg in the DLNs is associated with the potential of T reg to prevent corneal allograft rejection (Chauhan and Saban 2009) although it remains unclear whether they are functional within the graft itself (Vitova et al. 2013).

### 1.1.3.3 Potential Therapeutic Approaches in Corneal Transplantation

The majority of cornea transplantations are uncomplicated cases and topical immunosuppression is sufficient to secure graft survival, with no need for systemic immunosuppression (Hori, Taniguchi, et al. 2010). Commonly used immunosuppressive therapies applied locally may be required for several years. However, as previously described the risk of rejection remains high for those patients with repeated transplantation in the same eye, corneal neovascularisation, an episode of preoperative inflammation or raised intraocular pressure (Williams et al. 2006). Therefore, inducing donor-specific immune tolerance would be an ideal solution for improving transplant outcomes by both eliminating rejection and obviating the need for indefinite immunosuppression. Dendritic cell (DC) application in corneal transplantation remains relatively uninvestigated and only recently was it demonstrated that regulatory DCs
suppress the indirect pathway of allosensitisation in corneal transplantation, an important observation for the development of cell therapies for corneal transplantation as the cornea is the most commonly transplanted tissue (Hattori et al. 2012; Khan et al. 2013). At the time of commencing this study the application of ex vivo-generated untreated donor bone marrow derived DCs (BMDCs) or dexamethasone (Dexa) treated BMDCs was not demonstrated in corneal transplantation.

Gene therapeutic approaches explored in preclinical studies into the prevention of corneal allograft rejection have investigated the ex vivo modifications of the corneal allograft directly (Ritter et al. 2013). Although some gene therapeutic approaches have failed to prolong corneal allograft survival (Jessup et al. 2005; Ritter et al. 2007) others have proven successful. These include studies of ex vivo transduction of cornea include equine infectious anemia virus (EIAV) overexpression of indoleamine 2,3-dioxygenase (IDO), Ad expressing nerve growth factor and LV mediated overexpression of PD-L1 in the cornea (Beutelspacher et al. 2006; Gong et al. 2007; Nosov et al. 2012). These modifications resulted in significantly extended survival of corneal allografts following transduction of excised donor cornea ex vivo prior to transplantation.

One of the aims of this thesis study was to examine the over expression of IDO and PD-L1 in BMDCs for the application corneal allograft transplantation. IDO is a monomeric intracellular enzyme that degrades the essential amino acid tryptophan leading directly to an opening of the indole ring of tryptophan. This results in the formation of N-formylkynurenine, which in turn degrades rapidly to L-kynurenine (Higuchi and Hayaishi 1967; Mellor and Munn 2004). It was recently demonstrated by Khan et al. that DCs transduced to express the tryptophan-catabolising enzyme IDO by lentivirus (LV) resulted in inhibition of T cell responses by both depletion of tryptophan and the production of kynurenines (Khan et al. 2013).
PD-L1 is expressed on a variety of cells including APCs (Dong et al. 1999; Keir et al. 2008; Mueller et al. 2010; Amarnath et al. 2010) and interacts with its receptor Programmed death (PD)-1 found on the surface of activated T cells and B cells. In in vitro assays using allogeneic DCs transfected with pseudovirus containing LEW X oligosaccharides with PD-L1 co-cultured with T cells, the analysis of proliferation, cytokines secretion, and activation marker expression of T cells demonstrated that the proliferation and cytokine expression were suppressed after co-cultured with the PD-L1 overexpressing DCs (Wang et al. 2009).

1.2 Current understanding of DC Biology

The birth of DC biology was in 1973 when as a postdoctoral researcher Ralph Steinman and his colleagues first identified a novel cell type in peripheral lymphoid organs of mice (Steinman and Cohn 1973). Their work described how these particular “cells can assume a variety of branching forms and constantly extend and retract many fine cell processes and the term “dendritic” cell would thus be appropriate for the particular cell type” (Steinman and Cohn 1973) (Figure 1.3). It is now well accepted that DCs which are derived from bone marrow hematopoietic stem cells, consist of a family of two major DC phenotypes with distinct immune functions; these are plasmacytoid (pDCs) and conventional DCs (cDCs) (Shortman and Naik 2007). DCs which are of the pDCs phenotype express receptors for viral antigen recognition and produce large amounts of type 1 interferons and now are known to possess functions capable of linking, like cDCs, the innate and adaptive immunity by determining the fate and activation of T cells (Rogers et al. 2013). cDCs, which themselves are further divided into many subtypes, reside in specific tissues in an immature state and are not only potent adaptive immune inducers linking innate and adaptive immunity but they are also essential for the induction and maintenance of T cell tolerance (Steinman et al. 2003; Rogers et al. 2013). All of these functions are dependent on the phenotypical state and the
immunological environment within which the DCs find themselves (Steinman et al. 2003).

**Figure 1.3 Phase-contrast micrographs of dendritic cells isolated from spleen.**
(Steinman and Cohn 1973)

Immature DCs are optimally positioned in peripheral tissues to sense invading pathogens or foreign antigens. Using methods of antigen capture which include phagocytosis, macropinocytosis and receptor mediated endocytosis (Fcy receptor types I (CD64) and II (CD32)), integrins (αvβ3 or αvβ5), C-type lectins (mannose receptor, DEC205), apoptotic cell receptors, and scavenger receptors DCs process antigens into peptides for presentation (Mellman and Steinman 2001; Herrera et al. 2004; Trombetta and Mellman 2005; Platt et al. 2010; Sabado and Bhardwaj 2013). Endocytosis is a major functional characteristic of immature DCs however, their capacity to endocytose does not induce DC activation (Lutz and Schuler 2002). The processed peptides which are loaded onto MHC molecules are transported to the DC surface (Mellman and Steinman 2001). The DCs then travel from the periphery to the lymph nodes (LN) and here DCs mature under inflammatory conditions obtaining the exceptional capacity for T cell stimulation. DCs may also travel to the LN under quiescent states and present antigens in the absence of inflammation resulting in tolerance induction as is the case for the maintenance of peripheral tolerance (Lutz and Schuler 2002; Hubo et al. 2013).
It is evident from the literature that DCs are important modulators of T cell phenotype and function providing the signals required for T cells to become fully activated effector cells or regulatory T cells (Lutz and Schuler 2002; Takahama 2006; Zhu et al. 2010; Hubo et al. 2013). Once DCs mature they develop cellular extensions in order to increase the cellular surface area to improve the interaction with T cells. They also significantly up-regulate their expression of MHC II, co-stimulatory molecules and T cell adhesion molecules (Mellman and Steinman 2001).

The activation of T cells requires multiple signals from the DC; the first signal is mediated by the interaction between the specific T cell receptor (TCR) and the DC MHC II molecule presenting the recognised antigen. In order for complete T cell activation, T cells require a second signal derived from the co-stimulatory molecules CD80 and CD86 (members of the B7 family of costimulatory molecules, B7-1 and B7-2 respectively) expressed on the DC surface (Figure 1.4). Depending on the level of expression of these molecules, the resulting T cell phenotype can differ and the absence of the second signal in the presence of the first can render the T cell anergic (McGrath and Najafian 2012; Hubo et al. 2013). This second signal is a fine balance of positive (CD80/CD86) and negative (PD-L1/PD-L2) co-stimulatory signals delivered by the DC (Sousa 2006). A negative regulator of T cell activation is CTLA4, a member of the immunoglobulin superfamily which interacts with CD80 and CD86. PD-1, a second counter-regulatory molecule with limited structural similarity to both CD28 and CTLA4, has been implicated in peripheral tolerance induction and maintenance based on the development of systemic autoimmunity following deletion of the gene encoding PD-1, or the genes encoding its two ligands PD-L1/ PD-L2 (Mueller 2010) (Figure 1.4). The final signal required for T cell differentiation is provided by cytokines including but not limited to interleukin (IL)-6, IL-12, IFN-α and tumour necrosis factor (TNF)—α expressed by the DC (Banchereau and Steinman 1998; Takahama 2006; Sousa 2006; Zhu et al. 2010; Hubo et al. 2013). It is the integration of all these signals and the maturation status of the DCs which can direct the T cell toward an effector or T reg phenotype (Banchereau and Steinman 1998; Takahama 2006; Zhu et al. 2010).
Cytokines produced during this process influence immune responses generated by differentiating subtypes of CD4\(^+\) T cells such as Th1, Th2, Th17, and T regs. Besides effector T cells, DCs activate naive and memory B cells, NK cells (via IL-12, IL-15, and type I IFNs), and natural killer T (NKT) cells through antigen presentation on CD1 molecules (Chijioke and Münz 2011; Sabado and Bhardwaj 2013). Thus, DCs can qualitatively and quantitatively orchestrate immune responses.

Figure 1.4 Receptors and ligands involved in DC and T cell interactions. Illustration of receptors and ligands expressed by DCs and T cells. Interactions between these molecules either activate or inhibit T cell activation (diagram authors own).
1.3 Effector T cell subsets

T cells can be grouped into four main lineages of specialised Th cells which include Th1, Th2, Th17 and T reg cells which are derived from naïve CD4 T cells. There is further diversity within these four lineages including Th3 cells (TGF-β producing), Tr1 (IL-10 producing), Th9 (IL-9 producing) and T follicular helper (Tfh) cells (Zhu et al. 2010). An acute inflammatory response, characterized by Th1 effector cells, is mainly mediated by DC derived cytokine IL-12. Th1 cells are characterised by their expression of the cytokine IFN-γ, they also produce lymphotoxin, IL-2 and TNF-α (Zhu et al. 2010; Alonso et al. 2011). The Th2 phenotype is associated with allergen mediated immune response and T cells are Th2 polarized by diminished IL-12 secretion and increased OX40L expression or IL-10 producing DCs and also IFNα producing pDCs (Lambrecht et al. 2000; Alonso et al. 2011). Th2 cells themselves do not produce IFN-γ or lymphotoxin and are characterised by their expression of IL-4, IL-5, and IL-13 (Zhu et al. 2010). They have also been identified as producers of TNF-α and IL-9. Expression of IL-1β, IL-6, IL-23, and TGF-β by DCs has been implicated in the polarization of Th17 cells (Alonso et al. 2011). These Th17 cells uniquely produce IL-17A, IL-17F and IL-22 but also IL-21 which is also expressed by several other Th cell phenotypes (Zhu et al. 2010). In contrast to acute inflammatory response, IL-23 induced Th17 effector cells are more related to chronic inflammation and are connected to tumorigenesis (Aggarwal et al. 2003).

The T reg phenotype was initially demonstrated by Nishizuka and Sakakra (Kojima et al. 1976). Work by Hall et al. examined the capacity of separated populations of T cells from rats with long-surviving donor cardiac grafts either effect rejection or transfer unresponsiveness in adoptive allograft assays (Hall and Jelbart 1985; Hall 1985; Hall and Pearce 1990). These studies demonstrated that the state of specific unresponsiveness that follows the induction of graft survival is dependent in part upon active suppression, which is induced or mediated by T lymphocytes (Hall and Jelbart 1985; Hall 1985; Hall
T reg immune regulation has been shown to be orchestrated by the release of immunosuppressive cytokines including TGFβ, IL-10 and IL-35, by modulation of APC and endothelial functions, or by direct suppression of CD8+ and CD4+ T effector cells (Shevach 2009; Ding et al. 2012). The characteristic T reg marker FoxP3 is the major transcription factor which determines the fate and identity of the CD4+ T regs. FoxP3 expression found in natural T regs in the thymus and peripheral T regs which migrate to secondary lymph nodes can also be induced in peripheral naïve CD4+CD25− cells (Mason 2001; Walker et al. 2003). These FoxP3+ cells are highly dependent on IL-2 from an external source for survival (Pandiyan and Lenardo 2008). The human immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) characterised by insulin-dependent diabetes mellitus (IDMM), hypothyroidism, eczema, and immune thrombocytopenias has been shown to have mutations in human FoxP3 (Bennett et al. 2001; Wildin et al. 2001). Other characteristic but not unique expression markers of T regs include GITR, a member of the TNF receptor family involved in the regulation of TCR/CD3-driven T cell activation and programmed cell death, CTLA4 and finally low expression of the IL-7 receptor α-chain (CD127) (Read et al. 2000; Liu et al. 2006; Hori, Taniguchi, et al. 2010).
1.4 Pathways of allo-recognition

Before examining strategies to manipulate transplantation outcomes and promote transplantation survival the pathways of allo-recognition which lead to transplantation, mechanisms of immunological rejection must first be understood.

1.4.1 Direct pathway of allo-recognition

The direct pathway of allo-recognition involves the interaction of donor derived APCs, also referred to as passenger leukocytes, migrating from the donor tissue into secondary lymphoid tissue including the LNs and spleen to stimulate CD4⁺ and CD8⁺ cells of recipient origin (Figure 1.5). Donor MHC alloantigens are recognised by alloreactive T cells which are estimated to be of a relatively high endogenous frequency of between 1:100 and 1:100,000 T cells in humans and even higher (1:10) in mouse (Sagoo et al. 2012). The activation of CD4⁺ Th cells with direct allo-specificity results in the production of pro-inflammatory cytokines which in turn promotes the activation and expansion of CD8⁺ cytotoxic T lymphocytes (CTLs) (Illigens et al. 2002). These CTLs lyse their target cells using Ca²⁺-dependent granule exocytosis pathway (perforin/granzyme system) and/or via the Ca²⁺-independent CD95 (Fas)/CD95 ligand (FasL) pathway (Josien et al. 1998). The direct pathway of allo-recognition is associated with acute rejection as the passenger leukocytes may become activated as a result of the surgical trauma. The activated leukocytes subsequently migrate from the transplant to the recipient lymphoid tissues. The resulting rapid and strong nature of acute rejection is believed to be a result of the high precursor frequency of direct allo-responsive T cells (Illigens et al. 2002; Ford et al. 2009). It has not been fully elucidated as to whether there is in fact similar allo-reactive precursor frequencies between memory and naïve T cell compartments (Ford et al. 2009).
T regs generated with direct allo-specificity suppress the activation of direct CD4$^+$ cells either directly with the production of immunosuppressive cytokines such as IL-10, cell-cell interactions or by down modulation of APC function. The relative contribution of the direct pathway to corneal transplantation is controversial due to the low numbers of passenger leukocytes, many studies have demonstrated that the indirect pathway is the main pathway involved in allograft rejection (Illigens et al. 2002; Huq et al. 2004; Hattori et al. 2012; Khan et al. 2013).

### 1.4.2 Indirect pathway of allo-recognition

The indirect pathway of allo-recognition can occur at any time post transplantation (Figure 1.5). Due to normal cell turn over and surgical trauma significant cell loss may occur and the resulting apoptotic donor cells are phagocytosed by recipients APCs. Allo-peptides from both major and minor histocompatibility determinants are internalised, processed and presented in the context of recipient MHC II molecules. These processed allo-peptides are presented to allo-antigen specific CD4$^+$ effector T cells resulting in the priming and clonal expansion of peptide specific T cells. The frequency of pre-existing recipient allo-reactive T cells with specificity for the indirect pathway is believed to be much lower than that of pre-existing recipient direct pathway, with a range in frequency of 1:100,000–1:1,000,000 T cells (Sagoo et al. 2012). The direct allo-response it is thought to be relatively short-lived and therefore it is the indirect pathway of the anti-donor allo-immune response that poses the major threat to long-term transplant survival (Mirenda 2004). This pathway is correlated with chronic rejection and it is believed that this is the dominant pathway behind the immune response to and the rejection of allogeneic corneal grafts (Illigens et al. 2002; Hattori et al. 2012). It is a pathway which is not dependent on the presence of APCs within the transplanted tissue. T regs with direct specificity can only influence this pathway by
indirect means such as by-stander suppression or secreted cytokines such as TGF-β and IL-10 (Illigens et al. 2002; Huq et al. 2004).

1.4.3 Semi-direct pathway of allo-recognition

It has been shown that DCs are capable of acquiring intact MHC:peptide complexes from donor cells by cell to cell transfer or by means of exosomes (nanovesicles) containing allo-MHC molecules which fuse with the surface of the recipient cell (Herrera et al. 2004) (Figure 1.5). These are incorporated and maintained with sufficient molecular and structural integrity to prime recipient T cell allo-responses to the direct pathway (Herrera et al. 2004; Sagoo et al. 2012). In addition to DCs, macrophage and B cells have also been shown to acquire MHC molecules (Sagoo et al. 2012). This results in an APC which can subsequently activate allo-specific CD4+ and CD8+ T cells through both direct and indirect mechanisms (Herrera et al. 2004; Montecalvo and Shufesky 2008). The semi-direct pathway offers an alternative view on the role of donor APCs in priming the allo-response early post transplantation, where their main effect may also be through the provision of an early and high density source of donor antigen to prime the indirect and semi-direct pathways (Sagoo et al. 2012).

1.4.4 Non classical pathways of allo-recognition

Accumulating evidence now shows that the cells of innate immunity such as NK and NKT cells may also play a key role in allo-recognition. NK cells are highly cytotoxic to target cells that lack self-MHC molecules (missing self-theory) or express polymorphic variation of non-classical MHC molecules (Yu et al. 2006). This NK cell function is
tightly regulated by the pattern of NK receptors both activating, such as NK1.1, and inhibiting, such as killer-cell immunoglobulin-like receptor (KIR) cell surface proteins (Lanier 2005; Orr and Lanier 2010). Thus, NK cells often function as potent effector cells in rejection of allogeneic bone marrow cells and solid organ transplants (Yu et al. 2006) and they have been indicated in corneal transplantation rejection. High numbers of cells expressing NK-markers, that appear to have allo-reactivity, have been located in multiple animal models of corneal transplantation as well as in human samples of AH (Claerhout et al. 2004; Flynn et al. 2008).
Figure 1.5 Illustration of allo-recognition pathways in a corneal allograft model. (a) Direct pathway of allo-recognition: donor derived APCs migrate out of the cornea graft into DLNs and stimulate CD4+ Th cells with direct allo-specificity which in turn provide pro-inflammatory cytokines to stimulate and expand CD8+ CTLs. (b) Indirect pathway of allo-recognition: apoptotic donor material from cornea graft is ingested by host APCs and donor allo-antigens are presented in the context of recipient MHC II molecules to CD4+ Th cells. (c) Semi-direct pathway of allo-recognition: recipient APCs pick up intact donor MHC molecules gaining the possibility to prime T cells with direct and indirect allo-specificity.
1.5 Central and Peripheral Tolerance

Immunological tolerance both central and peripheral were described by Mathis and Benoist as successive barriers, imperfect on their own but powerful in synergy (Mathis and Benoist 2004). Central tolerance is the result of intra-thymic deletion of T cells with high affinity for thymically expressed antigens (Mathis and Benoist 2004). The nuclear factor autoimmune regulator or Aire controls the ectopic expression of tissue restricted antigens within medullary thymic epithelial cells (Takahama 2006). These antigens are also expressed by medullary DCs. T cell precursors which high avidity for the tissue restricted self-peptide-MHC complexes are eliminated (Mason 2001; Mathis and Benoist 2004; Mueller 2010). Negative selection in the thymus is not however, sufficient to eliminate all potentially pathogenic auto-reactive T cells (Mason 2001; Steinman and Nussenzweig 2002). Some of the self-reactive T cells escape negative selection and are kept in check by various methods of peripheral tolerance acting directly on the self-reactive T cell or indirectly via other cells (Li and Boussiotis 2006). Peripheral T cell tolerance includes deletion, the induction of T cell anergy and active immunoregulation (Mathis and Benoist 2004; Li and Boussiotis 2006).

Considerable evidence now suggests regulatory immature DCs incapable of delivering full costimulatory signals in the periphery are capable of expressing tissue restricted antigens, antigens processed from dead or dying cells resulting in the induction of tolerance in naive T cells that bear a TCR with high avidity for self-peptide MHC complexes presented by these immature DCs (Mueller 2010). The DCs uptake apoptotic cells via receptors which include LOX-1, CD36 and the complement receptors (CRs) CR3 and CR4 (Sabado and Bhardwaj 2013). In the absence of maturation signals these immature DCs can tolerise peripheral CD4+ CD8+ (Steinman and Nussenzweig 2002; Steinman et al. 2003) (Figure 1.6). Peripheral tolerance induced by DCs in this immature steady state may therefore, contribute to limiting autoimmunity (Sousa 2006). The expression of receptors such as CTLA4 and PD-1 by auto-reactive T cells also
allows the maintenance and promotion of a state of functional unresponsiveness to peripheral self-peptide MHC presentation (Mueller 2010). The concept that DCs can bring about peripheral tolerance in vivo may lead to the generation of methods for the antigen-specific silencing of immunity, as would be desirable in allergy, autoimmunity and transplantation (Figure 1.6).

**Figure 1.6 DC effector functions.** This figure illustrates immature DCs potential to mature and instruct T cell fates. These T cell fates include induction of immunity, Th1/Th2/CTL effector cell function or tolerance and immune deviation by regulatory T cell function. This illustration also highlights that both immature and DCs of a more mature phenotype have the potential to promote regulation or tolerance induction. Figure adopted from (Sousa 2006).
1.6 Strategies to generate regulatory DCs

Bearing the pathways of allo-recognition in mind and also the concept that DCs can maintain peripheral tolerance, DCs have as a result been extensively investigated as a potential cell therapy. This has generated considerable interest within the field of transplant immunology as an alternative to the current immunosuppressive drug therapies which are the main rejection prophylaxis. Although effective these therapies are often associated with harmful toxic side effects. In particular calcineurin inhibitors which have reduced the loss of allografts due to acute rejection but are themselves associated with severe side effects including increased morbidity and mortality due to cardiovascular complications, infections or malignancies (Rogers et al. 2013). As a result many investigations have aimed to eliminate or reduce administration of these immunosuppressive drug therapies. Accordingly, modified DC therapies for the promotion of allograft survival is a promising alternative but to date there are no tolerising immunosuppressive therapies which are accepted into routine clinical practice. However, by modifying or manipulating the immunoregulatory potential of DCs they may provide a means to accomplish tolerance induction.

In recent years manipulation of DC maturation by alteration of the expression level of MHC II and co-stimulatory molecules, has been demonstrated by treating DCs with various cytokines or pharmaceutical agents (Morelli and Thomson 2007; Unger et al. 2009; Fischer et al. 2009; Boks et al. 2011; Khan et al. 2013) resulting in the generation of immature or regulatory DC phenotypes. This DC phenotype is associated with the adherent or semi-adherent BMDC cell population generated in cell culture (Moreau et al. 2009). BMDCs have been generated using a variety of methods including the culturing of bone marrow cells in the presence of granulocyte- macrophage colony-stimulating factor (GM-CSF) with and without IL-4 (Steinman et al. 1998; Lutz et al. 2000; Pêche et al. 2005; Zhang et al. 2008; Yokota et al. 2009; Ouyang et al. 2010; Pincha et al. 2011; Hattori et al. 2012; Gao et al. 2013), with GM-CSF, IL-4 and the
Fms-like tyrosine kinase 3 ligand (Flt3L) (Stax et al. 2008) or with Flt3 alone (Taieb et al. 2007; Smyth et al. 2013).

In human based assays GM-CSF and IL-4 can be used to differentiate DCs from peripheral blood monocytes (Piemonti et al. 1999; Lutz 2004; Jin et al. 2010). GM-CSF has been demonstrated to preferentially promote cDC at the expense of pDC in a signal transducer and activator of transcription (STAT) 5-dependent manner (Rogers et al. 2013). The Flt3L is known to play an important role in general DC diversification in both humans and mice as well as DC amplification in vitro and in vivo (Rogers et al. 2013; Sabado and Bhardwaj 2013). The DCs generated from such cultures are also phenotypically immature or semi-mature and require further stimuli in order to become fully mature DCs they therefore have the potential to be immunogenic if matured in vivo.

For the induction of an immunoregulatory DC phenotype various methods have been investigated including; the use of pharmaceutical agents such as rapamycin (Taner et al. 2005; Horibe et al. 2008; Fischer et al. 2009; Pothoven et al. 2010; Naranjo-Gómez et al. 2011; Charbonnier and Le Moine 2012), cyclosporin A (Pino-Lagos et al. 2010) and 1,25 dihydroxyvitamin D3 [1,25(OH)2D3] and a related analog, 1,25(OH)2-16-ene-23- yne-26,27-hexafluoro-19-nor-vitamin D3 (D3 analog) (Griffin and Lutz 2001; Xing 2002; Unger et al. 2009; Ferreira et al. 2012). Corticosteroids are among the most efficient and most frequently administered immunosuppressive agents for the prevention of transplant rejection. This is mainly due to the immunosuppressive effects corticosteroids have on T cell activation (Hackstein and Thomson 2004). Corticosteroids such as Dexa have also been shown to induce a maturation-resistant regulatory DC phenotype (Piemonti et al. 1999; Xing et al. 2002; Mirenda 2004; Xia et al. 2005; van Kooten et al. 2009; Naranjo-Gómez et al. 2011; Kooten and Gelderman 2011; Ferreira et al. 2012; García-González et al. 2013; O’Flynn et al. 2013). DC differentiated in the presence of Dexa are at a more immature stage. Dexa has also been demonstrated to
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partially block terminal maturation of already differentiated DC (Piemonti et al. 1999). Gene therapeutic approaches have also been employed to generated immunoregulatory DCs (Yang et al. 2006; Li et al. 2007; Peng et al. 2011; L. Chen et al. 2012; Khan et al. 2013). Immunoregulatory DCs have also been propagated in the presence of cytokines such as IL-10 and TGF-β (Wakkach et al. 2003; Sato et al. 2003; Tiurbe et al. 2009; Dai and Zhu 2009; Hattori et al. 2012).

These strategies result the in accumulation of immature DCs with reduced expression of CD40, CD80, CD86 and low levels of MHC II. They are typically maturation resistant to strong immunogenic stimulus and predominantly produce IL-10 and very low levels of IL-12 (Piemonti et al. 1999; Wakkach et al. 2003; Xia et al. 2005; Pothoven et al. 2010; L. Chen et al. 2012). These cells have been administered in multiple pre-clinical models of transplantation (Table 1.1) including skin grafts (Griffin and Lutz 2001; Xin et al. 2013), allogeneic bone marrow transplantation (Sato et al. 2003), islet transplantation (Pothoven et al. 2010; G. Sun et al. 2012), cardiac transplantation (DePaz et al. 2003; Pêche et al. 2005; Zhang et al. 2008; Heng et al. 2010), renal transplantation (Stax et al. 2008; Ouyang et al. 2010) and also more recently corneal transplantation (Hattori et al. 2012; Khan et al. 2013; O'Flynn et al. 2013).

Administration of these immature, regulatory DCs has been demonstrated with varying degrees of efficacy (Table 1.1) and it has become evident that the role of DCs in the immune response and allograft rejection is complex and depends on a variety of factors, not only their level of maturation but the source (recipient or donor) of the DCs, the nature of the DCs, the environment in which they become activated and the model in which they are administered (Stax et al. 2008; Luo et al. 2008; Alawieh et al. 2009; de Kort et al. 2012).
### Table 1.1 In vivo application of regulatory DCs and the immunological response

<table>
<thead>
<tr>
<th>DC Manipulation/ Source</th>
<th>Species/ DC Culture conditions</th>
<th>Cell dose route</th>
<th>Disease model</th>
<th>In vivo efficacy/survival</th>
<th>Local/ innate immune response</th>
<th>Anti-donor T cell response</th>
<th>Anti-donor Antibody response</th>
<th>Functional Assay of anti donor response</th>
<th>Outcome donor antigen re-challenge</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmaceutical DCs Unmodified Dose</td>
<td>Rat</td>
<td>BMDCs GM-CSF + IL-4</td>
<td>1x10⁶ cells IV</td>
<td>Cornea transplant</td>
<td>Prolongation of allograft survival with both CD4 and CD8 unmodified Control BMDCs</td>
<td>Reduced graft infiltration and re-endothelialization of the corneal stroma</td>
<td>Autologous CD8⁺ and CD4⁺ T cells</td>
<td>T cell response</td>
<td>Increased CD8⁺ T cell response</td>
<td>-</td>
</tr>
<tr>
<td>Pharmaceutical DCs VV D3 Dose</td>
<td>Mouse</td>
<td>BMDCs GM-CSF + IL-4</td>
<td>1x10⁶ cells IV</td>
<td>Cardiac transplant</td>
<td>Prolonged survival of cardiac grafts</td>
<td>All grafts exhibited acute rejection</td>
<td>Decreased frequency of T cells recognizing donor Ag via the direct or indirect pathway</td>
<td>Inhibit the percentage of specific Fas⁺ cells</td>
<td>-</td>
<td>[Weng et al., 2012]</td>
</tr>
<tr>
<td>Pharmaceutical DCs VV D3 Dose</td>
<td>Mouse</td>
<td>BMDCs GM-CSF</td>
<td>1x10⁶ cells IV</td>
<td>Skin and cornea transplant</td>
<td>No prolongation of acute allograft rejection for both models</td>
<td>-</td>
<td>Direct and indirect responses, indirect responses</td>
<td>-</td>
<td>-</td>
<td>[Bollard et al., 2003]</td>
</tr>
<tr>
<td>Pharmaceutical DCs VV D3 &amp; IL-10 Dose</td>
<td>Mouse</td>
<td>BMDCs GM-CSF</td>
<td>1x10⁶ cells IV</td>
<td>Cardiac transplant</td>
<td>Prolongation of graft survival</td>
<td>No evidence of fast rejection</td>
<td>Combined T cell Rhus Hodgkin rejection</td>
<td>Substantial attenuation of donor reactive memory T cells</td>
<td>No evidence of circulating donor specific antibodies</td>
<td>-</td>
</tr>
<tr>
<td>Pharmaceutical DCs</td>
<td>Rat</td>
<td>BMDCs GM-CSF + IL-4</td>
<td>1x10⁶ cells IV</td>
<td>Skin and cornea transplant</td>
<td>Acute graft failure accelerated rejection</td>
<td>Prevalent mononuclear infiltration of a presence of arthritis and uveitis at the absence of complement deposition</td>
<td>-</td>
<td>Allergic reactions were strikingly found in donor DC treated recipients, allergic antibodies detected before first transplant, indicating a transfer of the humoral immune response by donor DCs</td>
<td>-</td>
<td>[Bollard et al., 2003]</td>
</tr>
<tr>
<td>Pharmaceutical V720 Dose</td>
<td>Mouse</td>
<td>BMDCs GM-CSF + IL-4</td>
<td>1x10⁶ cells IV</td>
<td>Cardiac transplant</td>
<td>Significantly prolong functional graft survival</td>
<td>-</td>
<td>Unknown (T) and CD25 response and increased T cell population</td>
<td>-</td>
<td>-</td>
<td>[Weng et al., 2000]</td>
</tr>
<tr>
<td>Pharmaceutical V720 Dose</td>
<td>Rat</td>
<td>BMDCs GM-CSF + IL-4</td>
<td>1x10⁶ cells IV</td>
<td>Skin and cornea transplant</td>
<td>No prolongation of graft survival</td>
<td>Naïve T cells were unresponsive to donor stimulation (non-specific), significant reduction in proliferation of T cells</td>
<td>-</td>
<td>-</td>
<td>[Weng et al., 2000]</td>
<td></td>
</tr>
<tr>
<td>DC Manipulation/Source</td>
<td>Species/DC Culture conditions</td>
<td>Cell dose/route</td>
<td>Disease Model</td>
<td>In vivo efficacy/survival</td>
<td>Local/immune response</td>
<td>Anti-donor T cell response</td>
<td>Anti-donor Antibody response</td>
<td>Functional Assay of anti-donor response</td>
<td>Outcome donor antigen re-challenge</td>
<td>Author (year)</td>
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<tr>
<td>Cytokine GM-CSF &amp; IL-4</td>
<td>Mouse BM/DCs, GM-CSF &amp; IL-4</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Corneal transplant</td>
<td>Prolonged survival</td>
<td>Hyperplastic lesions</td>
<td>Inhibition in infiltration of the immune cells and had no new blood vessels</td>
<td>--</td>
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</tr>
<tr>
<td>Cytokine GM-CSF, IL-10 &amp; TGF-β</td>
<td>Mouse BM/DCs, GM-CSF &amp; IL-4</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Corneal transplant</td>
<td>Prolonged survival</td>
<td>“Therapeutic DCs”</td>
<td>--</td>
<td>Inhibition of this organ, suppression of immunity</td>
<td>Decrease in Th1 cytokine release from DCs</td>
<td>Decrease in frequency of Th1 activity.</td>
<td>[Kaplan et al., 2002]</td>
</tr>
<tr>
<td>Cytokine Low GM-CSF</td>
<td>Mouse BM/DCs, GM-CSF &amp; IL-4</td>
<td>1 x 10⁴ cells i.p.</td>
<td>Skin transplant</td>
<td>Prolongation of allograft survival for all DC sources</td>
<td>--</td>
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<td>[Kaplan et al., 2002]</td>
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<tr>
<td>Adherent BMDCs</td>
<td>Mouse BM/DCs, GM-CSF &amp; IL-4</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Corneal transplant</td>
<td>Increased antigenic response of ALG to donor and syngeneic donor DCs</td>
<td>Decreased infiltrating leukocytes</td>
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<td>[Blau et al., 2001]</td>
</tr>
<tr>
<td>Genetic Lentivirus CTL34 IκBα expression induction</td>
<td>Mouse BM/DCs</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Corneal transplant</td>
<td>Long-term survival of allograft</td>
<td>“Therapeutic induction”</td>
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<td>[Kaplan et al., 2002]</td>
</tr>
<tr>
<td>Genetic Adenovirus IODC-transduced</td>
<td>Mouse BM/DCs</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Skin transplant</td>
<td>Prolonged graft survival; all if allografts were rejected at the end.</td>
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<td>[Kaplan et al., 2002]</td>
</tr>
<tr>
<td>Genetic Adenovirus encoding IL-2</td>
<td>Mouse BM/DCs</td>
<td>1 x 10⁶ cells i.p.</td>
<td>Rat corneal transplant</td>
<td>Prolonged survival of allograft recipient</td>
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<td>--</td>
<td>--</td>
<td>[Blau et al., 2001]</td>
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</tbody>
</table>
1.7 Characterisation of the immunological response to immunoregulatory DCs

The precise mechanisms that underlie the immune regulatory effects of immunoregulatory DC administration still remain unclear. Studies have implicated direct pathway modulation by the immunoregulatory DCs of the recipient T cell leading to deletion or anergy/regulation of reactive T cells (Munn 1998; Steinman and Nussenzweig 2002; Mellor et al. 2004; Munn and Mellor 2013). Several studies have also documented the ability of DC to inhibit mouse or human memory T cell responses which is recognised as an important barrier to the induction of transplantation tolerance (Nasreen et al. 2010; Kenna et al. 2010).

Donor derived DCs have been demonstrated to be able to induce alloantigen specific T cell hyporesponsiveness or anergy in vitro and promote the survival of allografts (O’Connell et al. 2002; Luo et al. 2008; Stax et al. 2008; Alawieh et al. 2010; Pothoven et al. 2010). However, although direct presentation may play a role, this may only be transient. In the case of donor derived BMDCs others suggest that it is in fact the recipient DC processing of donor derived cell therapies by endocytosis or direct transfer of donor MHC molecules that modulates the in-direct and semi-direct pathways and that may be critical for regulation of donor-specific reactivity and prolongation of graft survival (Yu et al. 2006; Luo et al. 2008; Wang et al. 2012).

Production of reactive oxygen species such as inducible nitric oxide (NO) and heme oxygenase-1 (HO-1) are described as potential suppressive mechanisms of DCs cultures (Bogdan 2001; Powell et al. 2003; Rössner et al. 2005; Cobbold et al. 2009; Moreau et al. 2009; van Kooten et al. 2011). In a model of rat cardiac transplantation Moreau et al. demonstrated that an immunosuppressive enzyme HO-1 was up-regulated in their
The promotion and expansion of T reg phenotype has also been implicated as a possible mechanism of immunoregulatory BMDCs (Jonuleit et al. 2000; Dong et al. 2003; Horibe et al. 2008; Fazekasova et al. 2009; Thomson et al. 2009; Unger et al. 2009; Gao et al. 2013). Menges et al. demonstrated the induction of IL-10-producing peptide-specific T cells in vivo and antigen-specific protection from experimental autoimmune encephalomyelitis using peptide-pulsed DCs matured with TNF-α (Menges et al. 2002). BMDC production of enzymes such as IDO (Grohmann 2003; Mellor and Munn 2004; Li et al. 2007; Yu et al. 2008; Curti et al. 2009; Khan et al. 2013), which can locally deplete tryptophan may therefore play an important role in limiting T cell proliferation and effector function within the graft (Cobbold et al. 2010; McGrath and Najafian 2012; Munn and Mellor 2013). IDO expression has been detected constitutively in human regulatory DCs and is believed to contribute to the immunoregulatory capacities of regulatory DCs inhibition of alloantigen-specific T cell responses in vivo and promote and maintain a T reg phenotype (Mellor and Baban 2003; Baban et al. 2011; Sucher et al. 2012).
In order to elucidate the mechanism/s employed by regulatory BMDCs to induce corneal allograft survival the following should be investigated:

- graft quality after administration (cornea: opacity/edema, renal: creatinine levels etc.)
- examination of the immunological cell infiltration at the site of administration
- ex vivo rechallenge assays to examine T cell response to donor antigen following BMDC administration
- measurement of alloantibodies in the serum of BMDCs recipients
- characterisation of the functional nature of the donor-specific T and B cell responses induced by donor derived BMDCs therapy (cytokine, T reg induction, antibody isotype)
- in vivo ‘re-challenge’ experiments in which BMDCs recipients are administered cells or tissue from the same or a different (‘third- party’) genetic background as the allo-BMDCs

The Table 1.1 summarises some the studies investigating the application of BMDC therapies for the promotion of allograft survival. This table also summarises efforts made in these studies to examine the immunological response to regulatory BMDCs. This thesis uses the cornea allograft transplantation model. The clarity of the cornea gives the unique opportunity to directly monitor and examine the rejection process making it an ideal model to investigate the application of a novel therapy.
1.8 Research hypothesis

It is hypothesised that the glucocorticoid treatment of ex vivo generated donor bone marrow derived dendritic cells or their overexpression of immunomodulatory molecules by viral gene transfer will promote graft survival upon injection into corneal transplant recipients.

1.9 Aim of the this research work

This thesis addresses the following specific aims:

- To develop and optimise culture conditions for the ex vivo generation of immunomodulatory BMDCs.
- To characterise the phenotypical and functional effects of glucocorticoid treatment of BMDCs.
- To investigate and establish an efficient method of viral gene transfer of BMDCs.
- To optimise and implement a suitable cell purification approach for the in vivo application of a pure population of transduced BMDCs.
- To develop and implement a strategy for administration of ex vivo-generated immunomodulatory BMDCs in a preclinical model of rat corneal transplantation.
- To evaluate donor derived BMDC therapeutic intervention and detail the local immune environment at the level of both the allograft and the draining lymph nodes as a result of these cell therapies.
- To investigate recipient derived BMDCs administered systemically and locally.
Chapter 1

To our knowledge, the results of this thesis collectively demonstrate for the first time, the efficacy of donor untreated BMDC and Dexa treated BMDC administration in a fully MHC mismatched rodent corneal allograft model and details the level and phenotype of infiltrating immune cell populations and immune microenvironment within the graft and DLNs of corneal allograft accepting recipients.
Chapter 2

2. Materials and Methods

2.1 Introduction

This chapter contains detailed descriptions of the individual techniques and methods used to examine ex vivo-generation of regulatory BMDCs for application in corneal allograft transplantation. The reader is referred to this chapter for detailed description of the individual techniques discussed in later chapters.

2.2 Ex vivo-generation of BMDCs

2.2.1 Isolation and cell culture

BMDCs were generated as previously described for the rat (Taieb et al. 2007; Horibe et al. 2008) with some modifications. Male rats LEW or DA aged 8-14 weeks were sacrificed in a CO₂ atmosphere. After skin disinfection the femur and tibia were surgically removed and kept in ice cold cell culture media. All flesh was removed from the bones and the bones were briefly washed in 70% ethanol. The bones were washed again in petri-dishes filled with 20ml PBS/EDTA (x2) removing all cartilage and flesh. Using a bone cutter the top and bottom end of the bones were removed and using a 25gauge needle the bone marrow (BM) cells were flushed out with serum-free RPMI into another petri dish. The cell suspension was collected and then filtered through a 100nm filter and centrifuged (400 x rcf; 5 mins; 4°C). The BM cell pellet was then re-suspended in ACK buffer (volume x3 times size of pellet ≤10ml) and kept on ice for 5min, the suspension was gently agitated every couple of mins to lyse the red blood cells. The action of the ACK buffer was stopped with ~20ml of serum containing RPMI
or excess of cold PBS. BM cells were counted with Neubauer chamber then seeded in a 6 well plate at a concentration of 4.5x10⁶ cells/ml per well. The culture medium was supplemented with 5ng/ml GM-CSF and 5ng/ml IL-4 and the culture was then incubated at 37°C at 5% CO₂.

On the 3rd day of culture half of the medium from each well was harvested and cells re-suspended in fresh medium supplemented with GM-CSF and IL-4 and added back into the culture. On the 5th day of culture all medium was removed (to remove dead lymphocytes and granulocytes) and replaced with fresh complete medium supplemented with GM-CSF and IL-4. For glucocorticoid treated BMDCs 10⁻⁶M dexamethasone was added to the culture on day 5. On day 7 half the medium and replaced with fresh medium supplemented with GM-CSF, IL-4 or dexamethasone if required. For the preparation of mature BMDCs cultures were subsequently stimulated with lipopolysaccharide (LPS) (1 µg/ml) for 24hrs. Cultures were maintained until day 10 when they were harvested for in vitro assays or in vivo applications.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>PRC2016</td>
<td>Invitrogen, Gibco®, UK</td>
</tr>
<tr>
<td>IL-4</td>
<td>400-04</td>
<td>Peprotech, Rocky Hill, NJ, USA</td>
</tr>
<tr>
<td>Dexa</td>
<td>D8893</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>LPS</td>
<td>L4391</td>
<td>Sigma Aldrich, UK</td>
</tr>
</tbody>
</table>
2.2.1.1 Media, buffers and additives

Complete Medium (CM)
- RPMI 1640, 25 mM HEPES
- 10% FBS (57°C heat inactivated)
- 2 mM L-glutamine
- 100 U/ml Penicillin
- 100 µg/ml Streptomycin
- 1 ml/100ml non-essential amino acids

GM-CSF/IL-4 Reconstitution
Lyophilized GM-CSF/IL-4 (25µg/20 µg respectively) was reconstituted according to the manufacturer’s instructions by adding 250µl/200µl of CM and pipetting gently until fully dissolved. Aliquots of 1µg/10µl were further diluted to a final concentration of 1µg /ml and stored at -20°C.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Phosphate-Buffered Saline (D-PBS)</td>
<td>14190-250</td>
<td>Invitrogen/GIBCO</td>
</tr>
<tr>
<td>RPMI-1640 +25mM HEPES</td>
<td>BE12-115F</td>
<td>Lonza Biologics, UK</td>
</tr>
<tr>
<td>100× non-essential amino acids</td>
<td>M7145</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>100× Pen/Strep</td>
<td>P0781</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G7513</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>F7524-500ml</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>DMSO (molecular biology grade)</td>
<td>41639</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>1× Trypsin/EDTA</td>
<td>T3924</td>
<td>Sigma-Aldrich, UK</td>
</tr>
</tbody>
</table>
2.3 In vitro Assays

2.3.1 DQ Ova Assay

Freshly isolated splenic Ox62+ DCs, day 10 BMDCs and Dexa BMDCs were seeded in a 96 round bottom plate at a concentration of $1 \times 10^5$ cells/250µl in complete medium. DQ ova was added to the DCs at a final concentration of 50µg/ml. Cells were collected at various time points (30min-24hrs) washed and resuspended in fluorescent activated cell sorter (FACS) buffer for analysis by flow cytometry.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ Ova</td>
<td>D-12053</td>
<td>Molecular Probes, Invitrogen, UK</td>
</tr>
</tbody>
</table>

2.3.2 Multi-Analyte Assay

Supernatants from $1 \times 10^6$ unstimulated BMDCs/Dexa BMDCs and LPS (1µg/ml) stimulated cells were harvested on day 10 of culture. IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, GM-CSF and RANTES cytokine determination for these supernatant was quantified using enzyme-linked immunosorbent array (ELISArray Kit) following the manufacturers recommended protocol. Absorbance was read at 450nm and 540nm; wavelength was corrected by subtracting the reading at 540nm from the reading at 450nm. Data was analysed following manufacturers guidelines.
<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi-Analyte ELISArray Kits Rat Inflammatory Cytokines</td>
<td>MER-004A</td>
<td>SABiosciences, Qiagen, Crawley, West Sussex, UK</td>
</tr>
</tbody>
</table>

2.3.3 Enzyme-linked immunosorbent Assay

Supernatants from $1 \times 10^6$ unstimulated BMDCs/Dexa BMDCs and LPS (1µg/ml) stimulated cells were harvested on day 10 of culture. TNF-α and IL-10 cytokine determination for these supernatant was quantified using enzyme-linked immunosorbent assay (ELISA), following the manufacturers recommended protocol. Absorbance was read at 450nm and 540nm; wavelength was corrected by subtracting the reading at 540nm from the reading at 450nm. Standard curves were created by generating a four parameter logistic (4-PL) curve fit.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DuoSet ELISA Development System rat TNF-α</td>
<td>DY510</td>
<td>R&amp;D Systems, UK</td>
</tr>
<tr>
<td>DuoSet ELISA Development System rat IL-10</td>
<td>DY522</td>
<td>R&amp;D Systems, UK</td>
</tr>
<tr>
<td>Substrate Solution</td>
<td>DY999</td>
<td>R&amp;D Systems, UK</td>
</tr>
<tr>
<td>Normal Goat Serum</td>
<td>G9023</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Sulfuric Acid $\text{H}_2\text{SO}_4$</td>
<td>339741</td>
<td>Sigma Aldrich, UK</td>
</tr>
</tbody>
</table>
2.3.4 Griess Assay

100µl of supernatant from cultures of 1x10⁶ untreated BMDCs or Dexa BMDCs with and without LPS (1µg/ml) stimulation and the required standards (NaNO₂ 1st standard 100µM in culture media) were added to the appropriate wells of a 96 flat bottom plate. 50µl of solution A (2.3.5) was added to each well and then 50µl of solution B (2.3.5). Absorbance was read at 550nm.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfanilamide</td>
<td>S9251</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>N-(1-Naphthyl)ethylenediamine dihydrochloride</td>
<td>N9125</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Phosphoric Acid</td>
<td>P5877</td>
<td>Sigma Aldrich, UK</td>
</tr>
</tbody>
</table>

2.3.5 Media, buffers and additives

ELISA Wash Buffer

PBS
0.05% TWEEN®-20
pH 7.2-7.4

ELISA Reagent Diluent

PBS
1% BSA / 2% Goat Serum (57°C heat inactivated) for IL-10 detection
pH 7.2-7.4
0.2µm filter
**ELISA Stop Solution**

2N H₂SO₄ (1ml of H₂SO₄ to 9ml of distilled H₂O).

**Griess Assay Solution A**

- Sulfanilamide (3g)
- Phosphoric Acid (9ml)
- H₂O (291ml)

**Griess Assay Solution B**

- N-(1-Naphthyl) ethylenediamine dihydrochloride (0.9g)
- Phosphoric Acid (9ml)
- H₂O (291ml)

### 2.3.6 RT PCR

#### 2.3.6.1 RNA Isolation

Total RNA from Ox62⁺ DCs, BMDCs, Deda BMDCs, corneas and LNs was isolated using TRIzol reagent according to the manufacturer’s protocol. Briefly cells were lysed with TRIzol reagent by repetitive pipetting of 1 ml of the reagent per 5-10×10⁶ of cells. The homogenized sample was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes and centrifuged to remove cell debris and supernatant was transferred to a new tube. Added 0.2 ml of chloroform per 1 ml of TRIzol reagent and vortexed the samples vigorously for 15 seconds and incubated them at room temperature for 2 to 3 minutes. The sample was centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA located in the aqueous phase was carefully transferred, without disturbing the interphase.
Chapter 2

into fresh tube. 1µl of glycogen was added to each tube to help with precipitation and identification of RNA pellet. The volume of the aqueous phase was noted and RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used for the initial homogenization was added and the samples at R.T. for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed completely and the RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol reagent used for the initial homogenization. Samples were vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. The washing procedure was repeated once and all leftover ethanol removed. The RNA pellet was air dried for 5-10 minutes and then dissolved RNA in 30µl DEPC-treated water by passing solution a few times through a pipette tip. RNA was quantified and the $A_{260}/A_{280}$ ratio for purity was also measured.

2.3.6.2 cDNA Synthesis

cDNA was synthesized using RevertAid™ H Minus Reverse Transcriptase with random primers. Briefly using 1 µg RNA (or closest)/max 10 µl in each cDNA synthesis reaction (2.3.6.2.1). Any samples that contained RNA at less than 100ng/µl was re-precipitated (2.3.6.2.2).

2.3.6.2.1 First Strand Synthesis

All enzymes were vortexed and centrifuged before use.

Template RNA (total RNA quantified – add 1µg)  1µg (max 10µl)
Primer (Oligo DT/Random Hexamer)  1µl
Water, nuclease free  up to 12µl

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Sample was mixed gently and incubated at 65°C (heated both bottom and lid to prevent evaporation) for 5 mins. Sample was placed on ice immediately to prevent strands re-annealing.

The following was added:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>5X Reaction Buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>Ribolock RNase Inhibitor (20U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>10mM dNTPs Mix</td>
<td>2 µl</td>
</tr>
<tr>
<td>Enzyme: RevertAid H Minus M-MuLV RTase (200U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td></td>
<td>8 µl</td>
</tr>
</tbody>
</table>

Added 8µl (1X) (Reaction2) to the 12µl (Reaction 1) = 20µl volume of cDNA.

The following programme was used for synthesis:

42°C for 55min → 72°C 15min → 4°C ∞

180µl of Mol Grade H₂O (1:10) was added to sample however, if RNA concentration was low a lower ratio (1:5) was used. 1µl cDNA/PCR reaction was used (≤10% cDNA in PCR reaction eg 25µl use ≤ 2.5µl cDNA).

### 2.3.6.2.2 Re precipitation

- Added 10% volume 3 M sodium acetate (pH 5.2)
- Added 2 volumes of 200 proof cold ethanol
- Left at -80°C for 1 hour
- Centrifuged for 20 minutes at high speed at 4°C
- Discarded the supernatant and resuspended in 10-15µl of water
- Quantified.
2.3.6.3 RT\textsuperscript{2} Profiler PCR Array and qRT-PCR

The RT\textsuperscript{2} Profiler PCR Array analysis of BMDCs and Dexe BMDCs was performed according to the manufacturer’s protocol (RT\textsuperscript{2} Profiler PCR Array, Qiagen, SA Biosciences, Crawley, UK.).

Two step qRT-PCR based on BMDC and Dexe BMDC RNA was performed on day 10 and on day 18 for grafted cornea and DLNs. For sequences of TaqMan primers and probes for Pdl-1, Il-10, Ido, iNos, Cxcl9, Cxcl10, Cccr2, Tlr2, Tlr4, Tlr7, Tlr9, Ifn-γ, Il-6, Il-1β, Il-2r, Foxp3 and endothelial Nos (eNos) see (Table 2.1).
Table 2.1 RT PCR Primer Sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Probe 5’-FAM Tamra 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hn-r</td>
<td>AACAGTAAAGCAAAAAAGATGACAT</td>
<td>TTTCTGGACAGCTTTTGTCTGG</td>
<td>CGCCAAAGTCTGAGGTAAGACAC</td>
</tr>
<tr>
<td>Il-2r</td>
<td>CACATGCTGTGACCAGGAGAACCT</td>
<td>CCAAGGAAGTGGTAGATTCCTCTTG</td>
<td>CAGGTCACTGCAGGGAGGCCC</td>
</tr>
<tr>
<td>Il-6</td>
<td>TCAAGTCCATCGCCCTCAG</td>
<td>AAGGCAAGTCGCTGGGAAACTCT</td>
<td>AACGACGTCAATGGTCTTCTCCGQA</td>
</tr>
<tr>
<td>b-Actin</td>
<td>GTACAACCTCTCGTGGACTCTC</td>
<td>TTGTCGAGGAGGACGC</td>
<td>CGCCACAGTGGGCTGCACTGATG</td>
</tr>
<tr>
<td>Ccr2</td>
<td>CACCTAGACGCGGCTAGCA</td>
<td>ACTCTGCTGACCAACAAAGGCATAAT</td>
<td>TGACAGAGAGTGTGAATACACAGTCTG</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>TGCCTCAGCGCTTCAACTG</td>
<td>ACCCTGGCTGAATCCTGGCTAC</td>
<td>CATCGTACACTGCAAAGCCGAGATCA</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>GAAAGCCTCTCGATACACGCTC</td>
<td>TGGCTACTGCCTGGCTTTT</td>
<td>CGCTGATCGATTTCTCCCTGGTA</td>
</tr>
<tr>
<td>Tlr2</td>
<td>AGAAGCTAGATACAAAGT</td>
<td>ACAAGGTCAATGCAGTCTTAAA</td>
<td>TTCTGAACTGTCTGAGGAAAT</td>
</tr>
<tr>
<td>Tlr4</td>
<td>CCTGAAGATCTTAAAGAAGAT</td>
<td>CTTGTGCTAATGTCTCAAT</td>
<td>TCCAAATTTTCTCACAACCTCAGT</td>
</tr>
<tr>
<td>Tlr7</td>
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<td>AATTGCAAAAGACTTCTCTAGAAA</td>
<td>CCTCGCAGATTGGCCAACTGTT</td>
</tr>
<tr>
<td>Tlr9</td>
<td>CTGGATCTGGTCTCTTAAAGAA</td>
<td>ACAAGATTTGCGGAGAAAACTGA</td>
<td>ACCTGTACCATGAAATCTGGTA</td>
</tr>
<tr>
<td>Pklr1</td>
<td>TGGAGTATTGGCGCGACTGTC</td>
<td>CTCGCCAATAACTGAATAGAACT</td>
<td>GTGCAGATTCCGAGAAGAAGA</td>
</tr>
<tr>
<td>Ido</td>
<td>CAGGTTGACGGCCTGGCAG</td>
<td>TCAGATCTGGGAGAAGAAG</td>
<td>ACATCCACCATGGCTATTGAGAA</td>
</tr>
<tr>
<td>eNOS</td>
<td>CTGGCATGGATAGAATACGAT</td>
<td>TGCTGCGAAGCTCTCTCCT</td>
<td>ATCCCTAGAACATGGAAAAAGT</td>
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<tr>
<td>NOS</td>
<td>TCCCTACGCTCCCGCTG</td>
<td>CGGCAGCTTGAGTACGCA</td>
<td>AACACAGTAAAGGCACACCTGATG</td>
</tr>
<tr>
<td>Foxp3</td>
<td>NP_001101720.1 (Life Technologies, Carlsbad, CA)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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2.3.6.4 Relative Gene Expression Level Calculations

All samples were normalised to expression of the house-keeping gene β-Actin and relative expression of BMDC and Dexe BMDCs was to Ox62⁺ DC. Relative expression for treated groups cornea and LN analysis was to untreated allogeneic controls. All quantitative real-time PCR was performed according to the standard program on the Applied Biosystems StepOne Plus Real Time PCR System machine.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIzol Reagent</td>
<td>15596-026</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>RT² Profiler PCR Array</td>
<td>PARN-011</td>
<td>Qiagen, SA Biosciences, Crawley, UK</td>
</tr>
<tr>
<td>RevertAid™ H Minus Reverse Transcriptase</td>
<td>EP0451</td>
<td>Fermentas, Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>Maxima Probe/ROX QPCR Master Mix (2X)</td>
<td>KO231</td>
<td>Fermentas, Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>Nanodrop Spectrophotometer</td>
<td></td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>StepOne Plus Real Time PCR System</td>
<td></td>
<td>Applied Biosystems, Life Technologies, CA, USA</td>
</tr>
</tbody>
</table>
2.4 Immunogenicity Assays

2.4.1 Isolation of primary lymphocytes

Male rats LEW or DA aged 8-14 weeks were sacrificed and skin disinfected with IMS. The thorax was opened and blood withdrawn by heart puncture. Submandibular, cervical, brachial, inguinal, popliteal and mesenterial LN were resected and transferred into ice-cold cell culture media. LNs were transferred into a 100 µm cell strainer and placed into a 6 cm Petri dish. Primary lymphocytes were extracted by squeezing the LN tissue through a 100 µm strainer with the piston of a sterile syringe. After isolation, cells were further filtered through a 40 µm cell strainer and collected in a 50 ml Falcon tube. After centrifugation (400 x rcf, 5 min.; 4°C) cell pellets were resuspended in ice-cold D-PBS and pelleted again, counted and stored on ice for further use.

2.4.2 Splenic and Thymic homogenization

Spleens and thymus were harvested and kept in D-PBS on ice until processing. The spleen and thymus were placed in a 100µm filter within a petri-dish containing 10mls of PBS/EDTA. Then using the piston of a sterile syringe the spleen and thymus were gently squeezed to release the cells. The cell suspension was filtered through a 40µm filter. This squeezing and filtering process was continued until the spleen and thymus was transparent and all cells had been released. The cell suspension was centrifuged (400 x rcf, 5 min.; 4°C). The red blood cells were lysed as previously described by the action of ACK buffer which was ceased with x2 volume of serum containing media. The splenocytes and thymocytes were centrifuged as above and washed x2 in D-PBS then stored on ice for further use.
2.4.3 Magnetic assisted cell sorting (MACs)

Isolation of CD4$^+$ T cells or Ox62$^+$ DCs was carried out according to the manufacturer’s instructions. Briefly, freshly isolated rat lymphocytes or splenocytes (2x10$^8$ cells) were resuspended in 80µl of MACs buffer per 10$^7$ total cells and 20µl of CD4 or Ox62 microbeads per 10$^7$ total cells were added. The bead cell suspension was mixed well and incubated for 15min at 4°C. The labelling was stopped with the addition of an excess volume of MACS buffer (1-2ml per 10$^7$ cells) followed by pelleting (300 x rcf; 10min.; 4°C). The supernatant was discarded and cells were resuspended in 500µl MACs buffer per 10$^8$ cells. After placing the magnetic separation column into the magnet and priming with 3ml cold MACS buffer the cell suspension was passed through a 30µm filter and then through the LS column. All unlabelled cells which passed through column were collected in a 15ml Falcon tube. The column was then washed with 3x3ml MACs buffer and total unlabelled negative effluent was collected. The positive cell population was harvested by removing the column from the magnet, adding 5ml of MACs buffer to column and using the supplied plunger the positive fraction of cells was forcefully flushed out into a clean collection Falcon tube. The separation was completed by a last centrifugation step (400 x rcf; 5min.; 4°C). The resulting cells were resuspended in 2-3 ml CM and counted. Typically, a cell number of 8x10$^7$-1x10$^8$ CD4$^+$ cells and 1x10$^7$-3x10$^7$ Ox62$^+$ DCs could be expected from the above given starting material.
<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat Ox62+ Microbeads</td>
<td>130-090-663</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>Rat CD4 Microbeads</td>
<td>130-090-319</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>LS separation columns</td>
<td>130-042-401</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>MACS Multistand</td>
<td>130-042-303</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>MIDIMacs separation magnet</td>
<td>130-042-303</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>Pre-Separation Filters</td>
<td>130-041-407</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
</tbody>
</table>

### 2.4.4 Allogeneic Lymphocyte Assays

Isolation of DA Ox62+ DCs was carried out by MACs as described in (2.4.3). LEW lymphocytes were isolated as described in (2.4.1) stained with CFSE according to the manufacturer’s instructions; lymphocytes were washed and resuspended in appropriate volume of CM (1.5x10^5 cells/125µl) and plated in a 96 well round bottom plate. Gamma irradiated (20Gy) DA Ox62+, harvested day 10 BMDCs and Dexa BMDCs were added to the appropriate wells in a ratio 10:1, lymphocyte:DC. Proliferation and activation of lymphocytes was measured on day 5 by flow cytometric analysis.

### 2.4.5 Suppression Assays

DA Ox62+ cells, BMDCs, Dexa BMDCs and LEW lymphocytes were prepared as described in (2.4.1). Gamma irradiated (20Gy) DA Ox62+ were added to the appropriate wells in a ratio of 10:1, lymphocyte:DC. In addition either day 10 BMDCs or Dexa BMDCs were added in a ratio of 10:1, lymphocyte:BMDC. Proliferation and activation of lymphocytes was measure on day 5 by flow cytometric analysis.
2.4.6 Donor antigen preparation and BMDC antigen pulse

Splenocytes and thymocytes were harvested and processed as described in (2.4.2). Donor cells were resuspended at $1 \times 10^6$ cells/0.5ml complete medium and irradiated at 120Gy to induce apoptosis. On day 8 BMDC were harvested counted and resuspended at $1.5 \times 10^6$ cells in 1.5mls in 6 well dish, irradiated allogeneic splenocytes were added to each well in a ratio of 2:1 for a final volume of 3mls of complete medium supplemented with GM-CSF and IL-4.

2.4.7 Donor antigen pulse Assay

Ox62$^+$ DCs and BMDCs were harvested from antigen pulse preparation as described (1.3.6), cells were washed, centrifuged ($400 \times$ rcf; 5min.; 4°C), resuspended in 1ml CM and counted. Cells were resuspended at a concentration of $2 \times 10^5$ cells/250µl. CFSE labelled CD4$^+$T cells were resuspended at $1 \times 10^6$ cells/250µl in CM. 500ml was added to each well to bring final volume/well to 1ml in a 24 well plate. The cell culture was incubated for 72hrs after which the cells were harvested from each well, washed cells x2 with FACS buffer. Proliferation and activation of lymphocytes was measured on day 5 by flow cytometric analysis.
### 2.4.8 General tissue culture consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>15ml screw cap centrifugation tube</td>
<td>62.554.502 PP</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>50ml screw cap centrifugation tube</td>
<td>62.547.254 PP</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>T-175 TC flask</td>
<td>83.1812.002</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>5 ml, 10 ml, 25ml sterile, single-use serological pipettes</td>
<td>-</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>10 µl, 20 µl, 200 µl, 1000 µl aerosol-resistant pipetting tips</td>
<td>-</td>
<td>StarLab, Germany</td>
</tr>
<tr>
<td>40µm cell strainer</td>
<td>734-0002</td>
<td>BD Falcon, UK</td>
</tr>
<tr>
<td>100µm cell strainer</td>
<td>734-0004</td>
<td></td>
</tr>
<tr>
<td>50ml syringes (Luer-lock)</td>
<td>613-3925</td>
<td>BD Falcon, UK</td>
</tr>
<tr>
<td>0.45µm syringe filter</td>
<td>83.1826</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>0.2µm syringe filters</td>
<td>83.1826.001</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>Filtropur V 25, 500ml filter units 0.2µm</td>
<td>734-2073</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>96 MicroWell Plates round bottom wells</td>
<td>83.1837</td>
<td>Sarstedt Ltd, Ireland</td>
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<tr>
<td>96 MicroWell Plates V bottom wells</td>
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<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>6-well plates</td>
<td>83.1839</td>
<td>Sarstedt Ltd, Ireland</td>
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<td>6 cm Petri dishes</td>
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<td>Sarstedt Ltd, Ireland</td>
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<tr>
<td>10 cm Petri dishes</td>
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</tr>
<tr>
<td>1,8 ml sterile cryo-tubes</td>
<td>368632</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
</tbody>
</table>
2.4.8.1 Media, buffers and additives

Cryo-preservation solution (freezing)
- 90% FBS
- 10% DMSO
- 0.2µm sterile filtration
- stored at -20°C

Reconstitution of Dexamethasone
According to the manufacturer’s recommendations 1mg Dexa was dissolved in 1ml of absolute ethanol. It was further diluted in complete medium for a final concentration of 20µg/ml. After sterile filtration (0.2µm) aliquots were stored at -20°C.

Collagenase D digestion solution recipe:
- RPMI + 25 mM HEPES
- 1% fetal calf serum (57°C heat inactivated)
- 2.5µg/ml Collagenase D (from powder)
- 0.2µm sterile filtration
- store at -20°C

MACS buffer recipe:
- PBS
- 0.5% (w/v) BSA
- 0.2µm sterile filtration
- stored at 4°C
2.4.9 Rat Strains

<table>
<thead>
<tr>
<th>Rat Strain</th>
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<tbody>
<tr>
<td>Lewis (LEW), haplo type: RT-1(^1)</td>
<td>Harlan Laboratories, UK</td>
</tr>
<tr>
<td>Dark Agouti (DA) haplo type: RT-1(^1)</td>
<td>Harlan Laboratories, UK</td>
</tr>
<tr>
<td>Sprague Dawley (CD), outbred strain</td>
<td>Charles River Laboratories, UK</td>
</tr>
<tr>
<td>LEW GFP Transgenic, haplo type: RT-1(^1)</td>
<td>Gift from Max Planck Institute, Munich, Germany</td>
</tr>
</tbody>
</table>

2.5 Production of virus

2.5.1 Ad production

2.5.1.1 293HEK Cell Culture for Ad Transduction

A vial of human embryonic kidney (HEK) cells was thawed in a 37°C water bath and the cells were quickly added to 25ml of high glucose Dulbecco’s modified Eagle’s medium (DMEM). The cells were then centrifuged (300 x rcf; 5mins; 4°C) and resuspended in a volume of 5ml of medium. Cells were then cultured in a T75 flask in a final volume of 10ml of medium for 48hrs. When cells were confluent they were harvested and replated in 150mm plates. Once confluent medium was removed and 4mls of medium containing 50µl of crude Ad were added to each plate. The plates were then incubated for 1h at 37°C, shaking the plate every 15minutes after which the final volume in each plate was brought up to 20mls. The plates were incubated for a further 48-72 hrs, until cytopathic effect was visible. The cell suspension was then harvested.
and the plate washed thoroughly. The cell suspension was then centrifuged (450 rcf; 30 min.; 4°C). 50mls of the supernatant were removed and used to resuspend the cell pellet. This cell suspension was further centrifuged (300 x rcf; 5 min.; 4°C). The cell pellet was resuspended in 2ml of media and stored at -80°C.

2.5.1.2 Ad purification

Ad pellets were thawed at 37°C and then the virus was treated three times to a freeze/thaw cycle vortexing the sample after each thawing step. 8.5ml 0.01M TRIS pH 8.1 was added to each tube and centrifuged (400 rcf; 15 min.; 4°C). Then to SW41 ultracentrifuge tubes 2ml of 1.43 CsCl was placed. This was gently overlaid with 2ml 1.34 CsCl to form a gradient. On top of the gradient 8ml of the TRIS viral solution was carefully added to each tube making sure that none of the cell pellet from the vial solution was added to the gradient. All tubes were balanced and place into the TH641 rotor buckets and centrifuged (30,000 rpm; no brake, Acc 7 Decel 1; 4 hours; 4°C). After centrifugation the white viral band was carefully collected and transferred into one SW55 ultracentrifugation tube. Tubes were balanced and centrifuged (30,000 rpm; no brake, Acc 8 Decel 1; 20 hours; 4°C) in a AH650 rotor. The viral band was collected in cryovials after passing through PD-10 columns following manufacturer’s instructions. Collected fractions were then analysed for the presence of ssDNA. From each fraction 10µl was collected and lysed using 1µl of 0.5% SDS solution. The samples containing the most virus were combined and 10% glycerol was added to the virus, aliquoted and stored at -80°C.
2.5.1.3 AdGFP transduction efficiency

A vial of HEK cells was thawed in a 37°C water bath and cells were quickly added to 25ml of medium DMEM. The cells were then centrifuged cells (300 x rcf; 5mins; 4°C) and resuspend in a volume of 5ml of medium. Cells were then cultured in a T75 flask in a final volume of 10ml of medium for 48hrs. When cells were confluent they were harvested and replated in a 12 well plate. For AdGFP serial dilutions were prepared

- $10^2 = 10\mu l$ of virus + 990$\mu l$ 2% DMEM
- $10^3 = 300\mu l$ of dilution + 270$\mu l$ 2% DMEM
- Continue up to $10^{10}$

Medium was removed from 11 of the 12 wells plated and 1ml viral preparation was added. The cells in the remaining well were harvested and counted. After 48 hours transduced cells were harvested and transduction efficiency was calculated by analysing the percentage of cells transduced measured by flow cytometry.

2.5.2 Ad transduction of BMDCs

2.5.2.1 Spin Centrifugation Adenovirus Transduction

From stock AdGFP different MOI preparation were made using serum free medium. BMDCs were plated 1x$10^5$ cells/well in a 6 well plate containing 2-3mls of medium for 24hrs before transduction. BMDCs were transduced by adding 250$\mu l$ of each MOI to the appropriate wells. The plate was then centrifuged (2000 rcf; 90 min.; 37°C) and after centrifugation medium was aspirated off and replaced with serum containing medium and incubated for 48hrs at 37°C with 5% CO$_2$. Transduction efficiency was calculated by analysing the percentage of cells transduced measured by flow cytometric analysis.
2.5.3 LV production

2.5.3.1 Transfection of 293FT cells for LV production

On d0, 4.5x10⁶ cells 293FT were plated in a 150mm dish containing 10ml of DMEM with L-glutamine supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin, 100μg/ml streptomycin) per dish and incubated at 37°C in 5% CO₂. After 24-36hrs, cultures which were 75-85% confluent were ready for LV transfection. 293T cells were cultured up to passage 20 and mainly used for lentiviral vector generation. The LV packaging and production plasmids were obtained from Prof. Didier Trono (Lausanne, Switzerland) and the vectors used in this study are based on Tat-dependent, second generation self-inactivating (SIN) human immunodeficiency virus-1 (HIV).

Table 2.2 TurboFect transduction reagent

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amount µg</th>
</tr>
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<tbody>
<tr>
<td>pMDL-g/p-RRE</td>
<td>22.5µg</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>11.25µg</td>
</tr>
<tr>
<td>pMD2G</td>
<td>11.25 µg</td>
</tr>
<tr>
<td>Transgene</td>
<td>45µg</td>
</tr>
</tbody>
</table>

Table 2.3 Polyplus JetPEI transfection reagent

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Concentration per 150mm Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMDL g/p RRE (or pPAX2 for 2nd Gen Lenti)</td>
<td>14µg</td>
</tr>
<tr>
<td>pMD2G</td>
<td>6µg</td>
</tr>
<tr>
<td>pRSV Rev</td>
<td>6µg</td>
</tr>
<tr>
<td>Transgene</td>
<td>14µg</td>
</tr>
</tbody>
</table>
Initial LV preparations employed the use of Turbofect transfection reagent however the amount of plasmid DNA required (detailed below) was greater than that required for Polyplus JetPEI reagent and therefore JetPEI was used for all further viral preparations. In a 50ml Falcon tube (Tube A) the concentrations of plasmid shown in Table 1.3 were added to 1.5ml NaCl buffer/150mm plate. In another Falcon tube (Tube B) 1.5ml NaCl buffer and 33µl JetPEI reagent per 150mm plate was added and both tubes were mixed gently by vortex. The contents of Tube B were added to Tube A and immediately mixed by gently vortexing. The plasmid transfection mix was then incubated at room temperature for no greater than 30 minutes. Medium from the 293FT cells was gently removed by tilting the plate at an angle and gently aspirating the medium off. This was replaced with 10mls fresh culture medium by gently adding the fresh medium while tilting the plate to ensure cell layer was not disturbed. Then 3mls of plasmid/transfection mix was added drop wise to each plate and then incubated overnight at 37°C and 5% CO₂.

On d2, the medium was replaced with 10mls fresh culture medium and left for a further 24hrs. Virus containing supernatants were harvested in 50ml Falcon tube and stored at 4°C. The harvested supernatant was replaced with fresh medium and left for a further 24hrs then both supernatants from 24hr and 48hr collection time points were combined and filtered through 0.45µm filter. The viral supernatants were either stored at -80°C or concentrated by ultracentrifugation (27000rpm ; with Accel 9 and Deccel 1; 2.30hrs; 4°C) in Sorvall Tubes (36ml). The supernatant was removed, carefully as so not to disturb the pellet (whitish viral pellet at bottom) and resuspended in 50µl PBS/1% BSA and another 50/100µl total was used to rinse each tube. Virus was stored in a final volume of 400µl PBS/1%BSA in a labelled cryotube at -80°C.
2.5.3.2 LV Titration

293FT cells were seeded $3 \times 10^4 - 5 \times 10^4$ in 500µl/well of 24 well plate. Cells in one well were removed for counting. The following serial dilutions of concentrated virus were prepared:

![Diagram of virus dilutions](Image)

**Figure 2.1 Titration of LV.** Adopted from (Barde et al. 2010).

10µl of each viral dilution was added to the appropriate well, 3 wells received no virus (negative control) and incubated at 37°C for 72 hours. Cells from one well were trypsinised and counted to estimate the cell number at the time of transduction. After
24hrs transduction media was replaced with fresh culture DMEM and left for a further 24hrs. Flow cytometric analysis was used to determine the LV.eGFP virus titer using the following equation:

\[
\text{Titer} = \left[ \frac{(F \times Cn)}{V} \right] \times D_F
\]

- \( F \) = the frequency of GFP+ve cells within the range of 1-20% GFP+ve cells
- \( Cn \) = the total number of target cells
- \( V \) = the volume of inoculum
- \( D_F \) = the virus dilution factor

LV.eGFP virus titers ranged between 1.7x10^7 - 3x10^7 tu/ml.

2.5.4 LV transduction of BMDCs

2.5.4.1 Concentrated LV transduction of BMDCs

BMDCs (d0) were seeded in a 24 well plate at a concentration of 5x10^5 cells in 500µl of complete medium. On d3 cells from one well were harvested and counted. MOI range from 0.125 – 2 was prepared in 250µl of serum free media and added to the appropriate well for a final volume of 750µl and incubated at 37°C for 24hrs. BMDCs LV transduction was also carried out using Polybrene (8µg/ml) and also centrifugation (2000 rcf; 90 min.; 37°C). After 24hrs media was removed and replaced with fresh complete medium. Culture was maintained until d10. On d7 BMDC cell cultures were imaged using an inverted-fluorescent microscope and on d10 flow cytometric analysis was used to determine transduction efficiency.
2.5.4.2 LV containing supernatant transduction of BMDCs

BMDCs (d0) were seeded in a described in (2.2.1) at a concentration of 1.5x10^6 cells/ml in 3mls of complete medium. On d3 cells from one well were harvested and counted. MOI range from 0.125 – 2 was prepared in 250µl of serum free media and added to the appropriate well for a final volume of 750µl and incubated at 37°C. After 24hrs media was removed and replaced with fresh complete medium and culture was maintained until d10. Flow cytometric analysis was used to determine transduction efficiency.

2.5.4.3 Media, buffers and additives

**HEK culture media**

DMEM  
2 mM L-glutamine  
10% FCS  
100 U/ml penicillin  
100µg/ml streptomycin

**Cesium Chloride preparation**

1.34 CsCl  
57g CsCl  
112ml 0.01M TRIS pH8.1  
Filter Sterilized Store at RT

1.43 CsCl  
43g CsCl  
60ml 0.01M TRIS pH8.1  
Filter Sterilized Store at RT
0.01M TRIS Buffer
1.2114g/L
pH8.1

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<td>TRIS</td>
<td>TI503</td>
<td>Sigma Aldrich, UK</td>
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<td>CsCl</td>
<td>289329-25G</td>
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<td>344057</td>
<td>Beckman Coulter, Inc. UK</td>
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<td>52-1308-00 BB</td>
<td>GE Healthcare, UK</td>
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<td>Sorvall Tubes 36ml</td>
<td>3141</td>
<td>Sorvall , Unitech, UK</td>
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<tr>
<td>TurboFect</td>
<td>R0532</td>
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<tr>
<td>Polyplus JetPEI</td>
<td>101-40N</td>
<td>Source Bioscience, UK</td>
</tr>
<tr>
<td>293FT Cell Line</td>
<td>R700-07</td>
<td>Life Technologies, Invitrogen, UK</td>
</tr>
</tbody>
</table>

2.5.5 Cloning eGFP into Destination Vector

2.5.5.1 Primer design for eGFP
Primers designed for use in the Gateway System must contain the following sequence:
Forward 5’ GGGG ACA TTC TAC AAA AAA GCA GCC TTC GAA GGA GAT AGA ACC
Reverse 5’ GGGG ACC ACT TTG TAC AAG AAA GCT GGT TC
After which ATG start codon for forward primer and stop codon (in reverse) for reverse primer.
Figure 2.2 pIRES2-EGFP.

pIRES2-EGFP Vector (PT3267-5 Catalog #6029-1BD Biosciences)

Designed primers:
EGFP- Forward:
5’- GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA GGA GAT AGA ACC ATG GTG AGC AAG GGC GAG GAG CTG TTC A

EGFP- Reverse
5’- GGGG ACC ACT TTG TAC AAG AAA GCT GGG T C TTA CTT GTA CAG CTC GTC CAT GCC GAG A

Primers were manufactured by Metabion, Martinsried, Germany.
2.5.5.2 MultiSite Gateway System: Addition of attB site

Template (eGFP containing plasmid) 10-50ng
Forward Primer (10μM) 5μl
Reverse Primer (10μM) 5μl
dNTPs 5μl
10X Buffer (+Mg²) 5μl
EXT DNA polymerase 1μl
+H₂O Final volume 50μl

The following programme was used for synthesis:
94°C for 5min → 94°C 30sec→ 55°C 45sec → 72°C 1min → 72°C 10min → 4°C ∞.

Sample was mixed with loading dye and run on 1% agarose gel with DNA ladder.

10X TAE Buffer
48.4g TRIS
3.722g EDTA
27.216g Sodium Acetate

1% Agarose Gel
1.5g Agarose
150ml TAE Buffer
4.5μl Sybersafe DNA dye

Gel Extraction

The band containing PCR product of interest was cut from the gel and added to a 1.5ml eppendorf tube and weighed. Using the Quigen Gel Extraction Kit the DNA was extracted following the manufacturer’s guidelines and the concentration was measured.
2.5.5.3 BP Recombination Reaction

Require equimolar concentrations of attB PCR products and donor vector equivalent of 50 femtomoles (fmoles).

Equation used to convert fmoles to nanograms:

\[ \text{ng} = x \text{fmoles} (N) (660 \text{fg/fmoles})(1\text{ng/10}^6\text{fg}) \]

\[ x = \text{fmoles} \]
\[ N = \text{size of DNA bp} \]

\[ \text{eGFP} = 50\text{fmoles} (750\text{bp}) (660\text{fg/fmoles})(1\text{ng/10}^6\text{fg}) \]
\[ = 24.75\text{ng} \]

The BP recombination was carried out according to the manufacturers’ recommendations.

Transformation

Using Top 10 cells 1µl of the produced BP clonase reaction was added and incubated on ice for 30min. The mix was heat shocked for 30sec at 42°C without shaking and immediately transferred to ice. 250µl of Super Optimal broth with Catabolite repression (S.O.C) media was aseptically added to the tube. The mix was then shaken at 200rpm at 37°C for 1hr after which the mix was spread on kanamycin plates. Once BP Clonase step was successful colonies were selected and each colony added to 10µl of nuclease free water. 5µl of mix was added to LB broth and ampicillin and cultured overnight on a shaker at 200rpm at 37°C. The remaining colony prep was heated to 92°C 5-8min and PCR mix was prepared as previously described using the appropriate primers. The PCR product was run on gel and confirmation of successful cloned product was observed (eGFP 750bp).
**Kanamycin Plates**

35g/l agar dissolved in H₂O and autoclaves.
1ml of 50mg/ml kanamycin added to cooled agar.
Plates poured and cooled.
Store at 4°C.

2.5.5.4 eGFP DONR Plasmid

![Diagram of pLenti6/UbC/V5-DEST](image)

Figure 2.3 pLenti6/UbC/V5-DEST.
A mini prep of the overnight culture prepared after the BP clonase was carried out following the manufacturer’s recommendations. Then a LR Recombination was performed and to a microcentrifuge tube the following was added:

- 50-150ng of entry clone (from BP clonase reaction)
- 150ng/µl Destination Vector
- TE Buffer final volumes of 8µl
- 2µl of LR clonase was added

The sample was mixed by pipetting and incubated at R.T. for 1hr. 1µl of proteinase K was added and the sample was incubated for a further 10mins at 37°C.

**Transformation**

The LR Destination reaction was then transformed into One Shot Stbl3 competent E.coli and competent bacteria were spread on ampicillin containing agar plates. Colonies were selected and each colony added to 10µl of nuclease free water. 5µl of mix was added to LB broth and ampicillin and cultured overnight on a shaker at 200rpm at 37°C. The remaining colony prep was heated to 92°C 5-8min and PCR mix was prepared as previously described using the appropriate primers. The PCR product was run on gel and confirmation of successful cloned product was observed (eGFP 750bp).
<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier Code</th>
<th>Manufacturer Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MultiSite Gateway System</td>
<td>12537102</td>
<td>Life Technologies, Invitrogen, UK</td>
</tr>
<tr>
<td>LR Clonase II Plus enzyme</td>
<td>2538120</td>
<td>Life Technologies, Invitrogen, UK</td>
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<tr>
<td>Gateway BP Clonase II Enzyme mix</td>
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<td>Life Technologies, Invitrogen, UK</td>
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<tr>
<td>Gateway pDONR Vectors</td>
<td>11798-014</td>
<td>Life Technologies, Invitrogen, UK</td>
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<tr>
<td>pLenti6/UbC/V5-DEST™ Gateway® Vector</td>
<td>V49910</td>
<td>Life Technologies, Invitrogen, UK</td>
</tr>
<tr>
<td>PureLink® HiPure Plasmid Miniprep Kit</td>
<td>K2100-02</td>
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2.6 Flow cytometry

2.6.1 General cell surface staining procedure

The following monoclonal antibodies (mAbs) were used for the characterisation of BMDCs: CD11b/c APC, CD80-PE, CD86-PE, the macrophage marker HIS36-PE and MHC II-PE, appropriate APC or PE conjugated Ig isotype controls were used. For glycome analysis of BMDC the following FITC-labelled lectins were used WGA (wheat germ agglutinin), MAA (Maackia amurensis) and SNA-I (Sambucus nigra) (1.5µg/ml, gift from Dr. Jared Gerlach and Professor Lokesh Joshi) prepared in lectin staining buffer (PBS containing 1% FBS, 1mM CaCl2 and 2mM MgCl2). Controls were used by incubating BMDCs with lectins in FBS only buffer. Cells were stained on ice for 30mins in lectin staining buffer and washed and resuspended in FACS buffer (PBS containing 2% FCS and 0.01% NaN3) for analysis using a FACS Canto.

For the analysis of lymphocytes isolated from transplanted corneas, ipsilateral submandibular and cervical LNs the following mAbs were used: CD3-FITC, CD8-PE, CD161-AF647, CD4-APC, CD11b/c-APC, CD86-PE, CD45RA-PE, CD25-FITC, FoxP3-PE and MHC II-FITC. For staining, cells were washed with FACS buffer. mAbs were diluted in 50µl FACS buffer, added to the cells and incubated for 30 min at 4°C. Finally, unbound antibody was removed by washing twice with FACS buffer. A commercial kit was used to detect the transcription factor foxp3. After samples were stained with appropriate cell surface stain the permeabilization and staining of foxp3 was performed using the manufacturer’s protocol as a guideline. Cells were resuspended in FACS buffer for analysis. Compensation parameters were established on the FACS Canto using appropriately single stained cells and FMO controls. Data was analysed using FlowJo software. Results are presented as percentage of cell population or as absolute cell number in graft. Unlabelled beads (CaliBRITE unlabelled beads) were
added to FACS samples for detection of absolute number of cells. Absolute number of
graft-infiltrated cells was calculated as a function from number of FACS counted beads
and cells and number of beads which were added into the probe.

The staining was performed according to the manufacturer’s protocol. Freshly isolated
lymphocytes or day 10 BMDCs were washed twice with cold FACS buffer. Cells were
resuspended at a concentration of \(1 \times 10^6\) cells/200µl and aliquoted into 96 well V
bottomed plate. The plate was the centrifuged (400 x rcf; 3min.; 4°C) and 50µl of FACS
buffer containing the appropriate amount of primary antibody was added. Samples were
then incubated in the dark at 4°C for 30mins after which the cells were washed twice
with 150µl of FACS buffer, centrifuging (400 x rcf; 3min.; 4°C) and discarding
supernatant between each wash. Samples were then filtered using a 40µm nylon mesh
when required. Flow cytometry experiments were designed and analysed using isotype
controls and hierarchical ‘fluorescence minus one’ (FMO) negative controls. The gating
strategy was: morphological gate (SSC-A versus FSC-A) appropriate to cells type being
analysed and single cell gates to eliminate doublets (SSC-A versus SSC-H then FSC-A
versus FSC-H).

<table>
<thead>
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<th>Manufacturer Supplier</th>
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<td>340486</td>
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<tr>
<td>Antibody</td>
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**Isotype Controls**

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<td>400130</td>
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### Isotype Controls

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<td>PE</td>
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<td>401208</td>
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<td>MM-30</td>
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### FACS Equipment

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<tr>
<td>FACSCanto</td>
<td>BD Bioscience, UK</td>
</tr>
<tr>
<td>Analysis Software</td>
<td>Tree Star, Inc., USA</td>
</tr>
</tbody>
</table>

#### Intracellular staining procedure for FoxP3 detection

A commercial kit from eBioscience was used to detect the transcription factor foxp3. After samples were stained with appropriate cell surface stains the permeabilization and staining of foxp3 was performed using the manufacturer’s protocol as a guideline. Briefly samples were washed twice with FACS and centrifuged (400 x rcf; 3min.; 4°C) discarding the supernatant. Fresh 1x Fixation/Permeabilization buffer (using the provided diluent) was prepared and 200µl were added to each sample resuspending samples well. Samples were incubated at 4°C in the dark for 2hrs. Cells were then washed twice with 1x Permeabilization buffer by adding 150µl to each sample and centrifuged (400 x rcf; 3min.; 4°C). Pelleted cells were then resuspended in 50µl 1x Permeabilization buffer containing 0.1µg anti-foxp3 antibody and incubated at 4°C in
the dark for 30 minutes. The samples were then centrifuged (400 x rcf; 3min.; 4°C) and washed twice with 150µl of Permeabilization buffer. Cells were resuspended in FACS buffer and filtered through 40µm nylon mesh. Further fixation of samples was not necessary since the Fixation/Permeabilization working solution already contained paraformaldehyde.

<table>
<thead>
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<th>Manufacturer Supplier</th>
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</thead>
<tbody>
<tr>
<td>Anti-Mouse/Rat FoxP3 Staining Set Complete set</td>
<td>72-5775</td>
<td>eBioscience, Inc., UK</td>
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<tr>
<td>Fixation/Permeabilization Concentrate</td>
<td>00-5123</td>
<td>eBioscience, Inc., UK</td>
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<td>Fixation/Permeabilization Diluent</td>
<td>00-5223</td>
<td>eBioscience, Inc., UK</td>
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<td>Permeabilization Buffer (10x)</td>
<td>00-8333</td>
<td>eBioscience, Inc., UK</td>
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<td>Anti-Mouse/Rat Foxp3 PE (FJK-16s)</td>
<td>12-5773-80</td>
<td>eBioscience, Inc., UK</td>
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<tr>
<td>Rat IgG2ak Isotype Control PE</td>
<td>12-4321</td>
<td>eBioscience, Inc., UK</td>
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</tbody>
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2.6.3 Fluorescent cell labeling technique for proliferation measurement

Carboxyfluorescein succinimidyl ester (CFSE) a green fluorescent cell tracer dye was prepared as per manufacturer’s instructions. 18µl DMSO were added to the tube for a final concentration of 50mM. Freshly purified CD4+ responder cells were resuspended at 5x10^7 cells/2.5mls in pre-warmed PBS+0.1% BSA. An appropriate volume of CFSE for a final 10µM concentration was added to the cells (suitable for 2-5x10^7 cells/ml) and mixed thoroughly. Samples were then incubated at 37°C in the dark for 6mins. After 6mins the staining was stopped by the addition of 5 times the volume of stain with ice-cold TCM (additional medium was also added ~10ml to wash the cells). Samples were then centrifuged (400 x rcf; 5min.; 4°C) and this step was repeated twice. Cells were
then counted and resuspended in appropriate volume of TCM to have a concentration of 1x10⁶ cells/0.5ml. The remaining unstained cells were also resuspended in the same way for a 1x10⁶ cells/0.5ml concentration.

**CFSE staining buffer:**

- D-PBS
- 0.1% FBS (57°C heat inactivated)
- 0.2µm sterile filtration
- Store at 4°C

<table>
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<th>Material</th>
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</thead>
<tbody>
<tr>
<td>CFSE</td>
<td>C34554</td>
<td>Life Technologies, Invitrogen, UK</td>
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### 2.6.4 Detection of alloantibodies in rat serum

Blood was withdrawn by cardiac puncture using an 18G needle and transferred into a sterile 15ml screw cap tube. The blood was left at room temperature to allow for coagulation to occur. Clotted blood was then centrifuged (3450 x rcf; 10 min.; 4°C) and the resulting serum fraction was harvested with a sterile pipette and transferred into 1.5ml micro reaction tube. The serum was stored at -20°C for later use. Blood from untreated controls, treated groups and naïve controls, was withdrawn from the tail vein using a 25G needle and transferred into a sterile 1.5ml Eppendorf tube containing 100µl PBS and heparin (2U/ml). The blood was then centrifuged (500 x rcf; 10 min.; 4°C) and the resulting serum fraction was harvested with a sterile pipette and transferred into 1.5ml micro reaction tube. The serum was stored at -20°C for later use. Alloantibody analysis was performed as reported previously (Schu et al. 2011). Recipient serum was diluted (1:2 in FACS buffer) and incubated with 1×10⁶ DA splenocytes for 45 minutes.
on ice in a total volume of 50µl per test. Samples were washed twice with FACS buffer and pelleted (400×rcf for 5 min. at 4°C). In the fashion of a secondary FACS stain, samples were then labelled with either anti-rat IgM-PE, IgG1-FITC or IgG2a-FITC, also with the inclusion of a negative control using DA serum. In the case of anti-IgM-PE staining, anti-CD45RA-FITC was added to later allow exclusion of B cells from analysis. Splenocytes were incubated further for 45 min on ice washed and resuspended in FACS buffer for analysis using a FACS Canto.

<table>
<thead>
<tr>
<th>Antibody</th>
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<td>PE</td>
<td>G53238</td>
<td>Antibodies-online, Germany</td>
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<tr>
<td>α-rat IgG1</td>
<td>FITC</td>
<td>MRG1-58</td>
<td>Antibodies-online, Germany</td>
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<td>α-rat IgG2a</td>
<td>FITC</td>
<td>MRG2a-83</td>
<td>Antibodies-online, Germany</td>
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</tbody>
</table>

2.6.5 Measurement of graft infiltrating lymphocytes draining LN analysis and spleen

**Corneal Tissue Digestion:** The corneal graft was excised using a 3mm trephine and vannas scissors ensuring that the graft was free of iris pigments. The graft was then stored in sterile PBS on ice. The excised graft was then transferred to a 1.5ml Eppendorf tube containing 2.5µg/ml Collagenase D and placed into a tube shaker/heater (900 rpm; 90 min.; 37°C). Digestion was stopped with excess cold PBS and all liquid and tissue poured into a 100µm cell strainer and placed into a 6cm Petri dish. The corneal graft was further disintegrated by mashing with the head of a syringe plunger. The cell suspension was collected in a 15ml Falcon tube and the cell strainer and Petri dish were
thoroughly rinsed and added to the cell suspension. The sample was centrifuged (400 x rcf; 3min.; 4°C) and resuspended in 1.2mls for counting.

**DLN and Spleen Homogenization:** The ipsi-lateral submandibular, cervical and deep cervical lymph nodes were dissected and stored in PBS on ice until homogenization. The LNs and spleen were homogenized as described above for corneal graft without the use of collagenase. The spleen was treated with ACK buffer as previously described. The LNs were resuspended in 5mls of PBS and the spleen in 20mls and stored on ice. Cells were counted as previously described using trypan blue exclusion.

Lymphocytes and splenocytes were resuspended 1x10⁶ cells in 200µl FACS buffer and aliquoted into 96 well V bottomed plate. The cells were then stained following cell surface and intracellular cell staining protocols described (2.6). All cells samples were fixed with 1% Formalin and kept at 4°C cells until analysis.

**2.6.6 Fluorescence activated cell sorting**

Lenti-GFP transduced BMDCs were harvested according to the method described previously and the cells were resuspended in 1ml FACS buffer without sodium azide at 4°C cells were washed once with FACS buffer and passed through a 30µm filter. After centrifugation (400×rcf for 5 min. at 4°C), the pellet was resuspended in sort buffer. The cell density was adjusted to 3x10⁶ cell/ml. The cell sorter was prepared for an aseptic sort according to the manufacturer’s instruction. The sorting procedure was performed using a 100µm nozzle, a 16-16-0 purity mask and commercial sheath buffer. Positively sorted cell were collected in sterile cold CM.
Sort Buffer recipe:
PBS+ 2mM EDTA
1% FBS (57°C heat inactivated)
100 U/ml Penicillin
100 µg/ml Streptomycin
25mM HEPES
0.2µm sterile filtration

<table>
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<th>Material</th>
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<td>SYTOX Blue Dead Cell Stain</td>
<td>S34857</td>
<td>Molecular Probes, Invitrogen, UK</td>
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<td>Cell Sorter FACSaria-II</td>
<td></td>
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<tr>
<td>Sheath Buffer FACSFLOW™</td>
<td>342003</td>
<td>BD Biosciences, UK</td>
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2.6.7 In vivo cell trace

Spleens and thymi were harvested from LEW (syngeneic) GFP transgenic, DA (donor) and CD (3rd party) rats, organs were homogenised and treated with ACK as previously described. Cells from donor and 3rd party origins were stained using CellTrace Far Red DDAO-SE (as per manufacturers’ recommendations, Molecular Probes, Invitrogen, UK), washed and resuspended at 20x10^6 cell/ml PBS. Far Red labelled donor or 3rd party cells were mixed with equal numbers of syngeneic GFP cells. Naïve (ungrafted control), BMDC and Dexa BMDC treated grafted animals received an i.v. injection of a total 20x10^6 cells/ml PBS of Far Red donor/3rd party and syngeneic GFP cell mix. Blood from naive control and treated groups was withdrawn from the tail vein using a 25G needle.
and transferred into a sterile 1.5ml Eppendorf tube containing 100µl PBS and heparin (2U/ml) at various time points. The blood was then treated with ACK buffer, washed, centrifuged (400 x rcf; 5 min.; 4°C) and resuspended in FACS buffer. Fluorescent beads (CaliBRITE-PerCP beads) were added to FACS samples for detection of absolute number of Far Red and GFP labelled cells. Absolute number of circulating fluorescently labelled cells was calculated as a function from number of FACS counted beads and cells and number of beads which were added into the probe. On day 4 post injection right lung, spleen, submandibular and cervical LNs were harvested and homogenised for localisation of fluorescently labelled cells within these tissues. Aliquots of homogenised tissues were resuspended in FACS buffer and fluorescent beads were added for cell enumeration as previously described and samples were analysed on FACS Canto.

Spleens were harvested from LEW GFP Transgenic, DA and CD rats and kept on ice. Spleens were harvested as previously described. DA (allogeneic) cells and CD (allogeneic 3rd Party) cells were stained with Far Red following manufacturer’s protocols and as previously described for CFSE. After staining, cells were washed thoroughly and resuspend in a volume of 10x10^6 cells/500µl. Stained DA or CD cells were mixed with equal amount of LEW transgenic cells (10x10^6 LEW + 10x10^6 DA/CD / ml) and these cells were injected I.V. Following injection, bloods are taken from the tail vein. Animals were placed into an anaesthesia box connected to an isoflurane vaporizer and pre-filled with a mixture of oxygen and isoflurane, 1.5-2% anaesthetic in 2 l / min medical and oxygen flow rate was maintained until the rats were fully anaesthetized. Subsequently, animals were transferred onto a heated operating table and the snout was placed into a breathing tube connected to the anaesthetic machine. The volume percentage of isoflurane was set between 1.5 - 2.5% depending on the size of the animal while the flow rate of oxygen was maintained at 2l/min. Using a 25 ½ G needle 600µl of blood was drawn at the following time points: 12/15hrs, 24hrs, 36hrs, 60hrs and 104hrs post injection and collect in a 1.5ml Eppendorf tube containing 100µl heparin. Samples were centrifuged (500 x rcf; 5min.; 4°C) to collect serum. The blood was the treated with ACK buffer (1.5ml; 5mins) and centrifuged (500 x rcf; 5min.; 4°C)
and this ACK buffer treatment was repeated. The sample was then resuspend in 1ml of FACS buffer, 200µl of the sample was taken and 300µl FACS buffer and 50/100µl beads.

On day 4, lung, spleen and draining submandibular, cervical and deep cervical LNs were harvested. These were processed as previously described. Briefly, harvested lung was digested with 2.5µg/ml Collagenase D for 2 h. The action of the collagenase was stopped with excess cold PBS and all liquid and tissue was transferred to a 100µm filter and the tissue was homogenised with head of a syringe plunger. The spleen and LNs were homogenised using the same technique. Both the lung and spleen were treated with ACK buffer to lyse red blood cells. The spleen and lung cell suspensions were resuspended in 5mls of FACS buffer and the LNs in 1ml. For analysis 500µl of each sample were collected and 50µl of PE labelled beads were added for cell counting.

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<td>Life Technologies, Invitrogen,</td>
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<td>Red DDAO-SE</td>
<td></td>
<td>UK</td>
</tr>
</tbody>
</table>
2.7 Rat cornea transplantation

2.7.1 Surgical procedure

**General anaesthesia:** Induction: Animals were placed into an anaesthesia box connected to an isoflurane vaporizer and pre-filled with a mixture of oxygen and isoflurane, 4% anaesthetic with 2 l/min medical oxygen flow rate was maintained until rats were fully anaesthetized.

**Surgical anaesthesia:** Subsequently, animals were transferred onto a heated operating table and the snout was placed into a breathing tube connected to the anaesthetic machine. The volume percentage of isoflurane was set between 1.5 - 2.5% depending on the size of the animal while the flow rate of oxygen was maintained at 2l/min. Deep anaesthesia was considered to be achieved when limb withdrawal and eye reflexes were abolished. Depth of anaesthesia was monitored by the breathing pattern of the animals.

**Recovery from anaesthesia:** The administration of isoflurane was stopped and rats were allowed to breathe oxygen until they started to hyperventilate. Animals were then carefully placed into a clean cage lined with paper tissue towels. The recovery cage was placed onto a heating pad. Animals were allowed to remain in the recovery cage until fully awake, which was determined by observing normal inquisitive behavior.

**Pre-operative care:** Before the surgical procedure, all animals were screened for ocular abnormalities. Rats were anaesthetised and the eyes were inspected under a surgical microscope. At 16X magnification the eyes were screened for large abnormalities such as corneal scratches and vascularisation, aspherical or asymmetric orbit, iris deformities and blood vessels from the iris extending into the anterior chamber. Furthermore, the central cornea was also inspected in detail at 40X magnification. A drop of saline solution (Alcon BSS) was applied, which caused an additional lensing effect, thus
facilitating a closer inspection of the cornea for possible pigmentation and other abnormalities trans-cornea capillaries.

**Pre-operative injections:** Seven days before surgery animals were anaesthetised according to standard procedure and placed on a heating pad. The cell suspension was mixed and collected with 18G needle and a sterile single use syringe. Infusion of the total volume of 1ml was performed by tail vein injection using a 25 ½ G needle and a sterile single use syringe. For subconjunctival injections the eye was further anaesthetised locally using Tetracaine drops and the 50µl cell suspension was injected using a 25 ½ G needle and a sterile single use syringe.

All procedures described in the following paragraph were performed by a fully trained and licensed ophthalmic surgeon and not by the author. All procedures performed were conducted under animal licence no. B100/3852 and were approved by the Animals Care Research Ethics Committee of the National University of Ireland, Galway and conducted under licence from the Department of Health, Ireland. In addition, animal care and management followed the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science, Galway, Ireland.

A well established, fully allogeneic MHC I/II disparate cornea transplant model was applied for these studies. Male LEW (LEW, RT-1<sup>l</sup>) rats served as recipients of male Dark Agouti (DA, RT-1<sup>av1</sup>) grafts. DA and LEW rats were obtained from Harlan Laboratories UK. The outbred strain Sprague Dawley (CD) rats used for 3<sup>rd</sup> party cell source were obtained from Charles River UK and the LEW GFP transgenic rats were a gift from Naoto Kawakami, Max Planck Institute of Biochemistry, Munich Germany. All animals were 8-14 weeks old and housed with food and water ad lib.

**Surgery:** Donor animals were sacrificed by CO<sub>2</sub> asphyxiation. Both eyes were excised and placed in sterile saline solution (Alcon BSS). Donor corneas were marked with a 3mm trephine and cut out using a small angled scissors. Before the corneal tissue was completely detached from the eyeball the first suture was introduced. Fully
anaesthetised recipient animals were placed on a heated operating table which maintained body temperature at 37°C. Pupil dilating drops were reapplied if necessary and Tetracaine drops were administered until complete abolition of eye reflexes had occurred. Further eye movement was prevented by fixing the eye lobe with two 6-0 braided sutures. The graft bed was prepared by marking the central cornea with a 2.5mm trephine followed by excision of marked tissue. The donor cornea was placed on the recipient eye and sutured into place with 3-4 cardinal stitches. The wound was then completely closed by another 8-9 interrupted sutures. The suture knots were towed away from the donor tissue and knots were trimmed as short as possible. During the surgical procedure the eye was continuously moistened with sterile saline solution. No attempt was made to reconstruct the anterior chamber and wound closure was deemed successful when the eyeball started to re-inflate. The surgery was completed by another dose of Atropine drops and by covering the eye with antibiotic ointment containing chloramphenicol. The eyelids remained open. The approximate length of the procedure was 30-45 minutes. Animals that suffered iris damage during the surgical procedure were not allowed to recover from anaesthesia and were killed by being placed in a CO₂ atmosphere.

2.7.2 Post-operative follow up

Post-surgery animals were anaesthetised and inspected under an operating microscope every second day and the status of the healing process was assessed and corneal opacification and progression of neovascularisation was recorded (see appendix) and images taken. Slit lamp observations were made and extent of graft oedema recoded and images taken. Post-surgical animals that displayed any complications such as active bleeding into the anterior chamber, ocular infection, wound gapping (dehiscence), ruptured sutures or excessive epithelial shedding were excluded from the study and were euthanized.
### Table 2.4 Corneal Opacity Scale.

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<thead>
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<th>Score</th>
<th>Description</th>
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<tr>
<td>0</td>
<td>completely transparent cornea</td>
</tr>
<tr>
<td>0.5</td>
<td>slight corneal opacity, iris structure easily visible</td>
</tr>
<tr>
<td>1</td>
<td>low opacity with visible iris details</td>
</tr>
<tr>
<td>1.5</td>
<td>modest corneal opacity, iris vessels still visible</td>
</tr>
<tr>
<td>2</td>
<td>moderate opacity, only some iris details visible</td>
</tr>
<tr>
<td>2.5</td>
<td>high corneal opacity, only pupil margin visible</td>
</tr>
<tr>
<td>3</td>
<td>complete corneal opacity, anterior chamber not visible.</td>
</tr>
</tbody>
</table>

Graft rejection was defined as an opacity score of 2.5 for three or more consecutive days or an opacity score of 3, in combination with oedema and changes of transplant geometry (degree of convex contour, shrinking and surface roughness of graft) (Matoba et al. 1986; Nosov et al. 2012). Animals with surgical complications were excluded.
Neovascularisation

Neovascularisation was scored on the basis of the number of quadrants of the donor graft which were observed to be infiltrated with vessels (Figure 2.4 Neovascularisation Scoring).

![Figure 2.4 Neovascularisation Scoring.](image)

2.7.3 Histology and Histochemistry

For histological analysis, rat eyes were enucleated at day 18 after transplantation for all groups and at the end of the observation period for graft survival on day 30. Briefly, the eyes were embedded in paraffin wax and 5 µm thick sections were cut, dried overnight at 56°C and then deparaffinized twice in xylene for 10 min, followed by hydration through graded alcohols. Slides were incubated for 40 s in Harris hematoxylin, washed in tap water for 2 min, then stained in eosin for 7 min, washed again in water for 2 min and dehydrated through graded alcohols. Next, sections were cleared twice for 10 min in xylene and mounted in DPX. Microscopic examination was carried out using an Olympus BX51 Upright Fluorescent Microscope with Improvision Optigrid System and Improvision Volocity Software.
### Chapter 2

<table>
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<td><strong>Cold Plate for Modular Tissue Embedding System</strong></td>
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#### 2.7.4 Surgical materials

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<td>Dumont #7 curved student forceps</td>
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<td>Colibri forceps w/ needle holder</td>
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**Ophthalmic Surgery Pharmaceuticals**

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</table>

### 2.8 Statistical Analysis

All individual experiments were carried out in a minimum of duplicate/triplicate to ensure experimental reproducibility. Statistical analysis was performed using GraphPad Prism software (La Jolla, USA). Data is presented as mean ± SEM/SD. Statistical tests including, Student’s t tests (paired/unpaired) one tailed/two tailed were used for the analysis of in vitro data. Non-parametric Mann–Whitney tests were used for analysis of non-parametric data. Kaplan- Meier survival analysis was used for analysis of graft survival and log-rank (Mantel Cox) test applied. Significance is denoted as follows *p ≤ 0.05, **p≤ 0.01, ***≤ 0.001.
3. Ex vivo-generation and modification of BMDCs

3.1 Introduction

In recent years manipulation of DC phenotype, by altering the expression level of MHC II and costimulatory molecules has been investigated. Treating DCs with various cytokines including IL-10 and TGFβ1, by addition of pharmaceutical agents such as rapamycin or glucocorticoids to DC cultures or by gene transfer has resulted in the generation of immature or regulatory DC phenotypes (Dong et al. 2003; Morelli and Thomson 2007; Unger et al. 2009; Fischer et al. 2009; Boks et al. 2011; Hattori et al. 2012; Khan et al. 2013; Luo et al. 2013). Modified ex vivo-generated BMDCs, often referred to as tolerogenic-DCs, are poor stimulators of T cells and actively inhibited T cell proliferation. However, it is now recognized that tolerance induction by DCs is not always related to the absence of stimulatory properties (Sousa 2006).

One of the primary aims of this study was to investigate pharmaceutical modification of ex vivo-generated BMDCs. BMDCs which have been modified by the addition of pharmaceutical agents such as rapamycin or the glucocorticoid steroid Dexa have been used directly in models of transplantation to promote survival and tolerance with varying degrees of success (Taner et al. 2005; Stax et al. 2008; Horibe et al. 2008; Pothoven et al. 2010; de Kort et al. 2012). For this study Dexa was chosen as the pharmaceutical agent to produce immunomodulatory BMDCs over rapamycin as a comparative study of the two pharmaceutical agents reported that Dexa treated BMDCs are not only phenotypically immature but they exhibit a durable immaturity and produce high levels of IL-10 (Naranjo-Gómez et al. 2011). Dexa is known to be an anti-inflammatory and immunosuppressive agent, which has been widely used to treat several autoimmune disorders and prevent graft rejection (van Kooten et al. 2009). In
addition a study by Mirenda et al. found that Dexa had a low toxicity whilst maintaining
the aspired phenotypical and functional effects on DCs, an important consideration
bearing in mind the in vivo application of these cells (Mirenda 2004; García-González et
al. 2013).

In addition to investigating pharmaceutical treatment, genetic modifications of BMDCs
were also explored as an alternative approach to the generation of regulatory BMDCs.
The aim of this study was to examine BMDCs overexpression of the
immunomodulatory molecules PD-L1 and IDO. PD-L1 is an important molecule in the
regulation of auto-reactive T and B cell responses in peripheral tissues and/or plays a
role in regulating inflammatory responses in the target organs (Keir et al. 2008; Riella et
al. 2011). Studies using DCs transfected with PD-L1 have demonstrated that
administration of Ad transduced DC expressing PD-L1 improved kidney function and
survival of transplants. Moreover proliferation, cytokine secretion and activation marker
expression of CD8+ T cells were suppressed (Peng et al. 2011). However the application
of BMDCs overexpression of PD-L1 has not been demonstrated in the cornea
transplantation model.

IDO is a monomeric intracellular enzyme that degrades the essential amino acid
tryptophan leading to N’-formyl-L-kynurenine production which can be further
converted to catabolites including L-kynurenine and L-kynurenic acid (Higuchi and
Hayaishi 1967; Dai and Zhu 2009). Catabolism of tryptophan by IDO and the resulting
production of catabolites such as L-kynurenine has been demonstrated to keep T cells in
an anergic, unproliferative state by arresting activated T lymphocytes in the G1 phase
which can lead to apoptosis (Munn 1998; Mellor et al. 2004; Dai and Zhu 2009; Munn
and Mellor 2013). This results in the suppression of the immune response and
generation and promotion of tolerogenic environment (Mellor and Munn 2004). In a
cardiac transplant model, Ad-IDO gene transfer into the donor heart resulted in
prolongation of survival when compared with control vector or vehicle alone (Li et al.
Ad-IDO transduced donor-specific BMDCs have also been shown to induce skin allograft tolerance in an antigen-dependent manner (Yu et al. 2008). Beutelspacher et al. were the first to examine a role for IDO expression in the maintenance of cornea immune privilege and its contribution to allograft survival (Beutelspacher et al. 2006). Their results not only demonstrated that IDO expression augmented corneal immune privilege but also that overexpression of IDO in donor cornea was found to significantly prolong corneal allograft survival (Beutelspacher et al. 2006).

This chapter examines the pharmaceutical and gene therapeutic approaches employed to modify BMDCs ex vivo. It details the phenotypic and functional analysis of ex vivo-generated BMDCs, including the examination of significant increased expression of important immunoregulatory molecules by ex vivo-generated donor BMDCs and glucocorticoid treated BMDCs.
3.2 Results

3.2.1 Phenotypical and Functional Characterisation of BMDCs

3.2.1.1 Cell surface characterisation of ex vivo-generated BMDCs

Bone marrow cells were differentiated in the presence of rat GM-CSF and rat IL-4 (5ng/ml respectively) as previously described (Grauer et al. 2002; Stax et al. 2008). By day 4 cell aggregates were evident (Figure 3.1) and by day 10 these cell aggregates had grown in size and were easily collected by gently pipetting (Appendix Table 8.1). The phenotype of BMDCs was analysed by flow cytometry on day 10, gating on the CD11b/c+ population. The percentage and MFI of the classical DC markers expressed by BMDCs were examined; these included MHC II and the co-stimulatory molecules CD80 and CD86 (Figure 3.1 and Figure 3.2). Expression of the macrophage marker HIS36 (CD163 or ED2) was also examined to monitor the presence of contaminating macrophage with only 1% of cells in BMDCs cultures staining positive for HIS36. This was indicative of a very pure DC population (Taieb et al. 2007; Horibe et al. 2008) (Figure 3.1 and Figure 3.2). Due to BMDC haematopoietic stem cell origins the percentage and the MFI levels of multiple haematopoietic cell surface markers expressed by the BMDCs were also examined on d10 of culture, these included the receptor-linked protein tyrosine phosphatase CD45 (lymphocyte common antigen), CD73 (ecto-5'-nucleotidase), CD44H (hyaluronic acid receptor), CD172 (signal-regulatory protein), CD71 (transferrin receptor), CD90 and CD106 (vascular cell adhesion molecules) (Figure 3.1 and Figure 3.2).
Figure 3.1 Phenotypic characterisation of ex vivo-generated BMDCs. BMDCs were differentiated with GM-CSF and IL-4 (5ng/ml respectively) for 10 days. (a) Brightfield microscopy images of BMDC culture on d10. (b) Mean percentage and (c) MFI of BMDC phenotypic markers including MHC II, CD80, CD86 and HIS36 expression within the CD11b/c+ population was analysed. Analysis also included CD45, CD73, CD172, CD44H, CD71, CD90 and CD106 (n=3-4).
Figure 3.2 Phenotypic characterisation of ex vivo-generated BMDCs. Representative histograms of 3 independent experiments (isotype controls: grey line and BMDCs: black line) of the MFI of BMDC phenotypic markers including MHC II, CD80, CD86 and HIS36 expression within the CD11b/c+ population. Also analysed was expression of CD45, CD73, CD172, CD44H, CD71, CD90 and CD106.
3.2.1.2 Evaluation of functional properties of ex vivo-generated BMDCs.

To examine ex vivo-generated BMDC functional capacities their ability to phagocytose and process antigen was evaluated. To do this, DQ OVA, a self-quenched conjugate of ovalbumin that exhibits a bright green fluorescence on proteolytic degradation, which can be measured by flow cytometry, was employed. This DQ OVA assay demonstrated that, similarly to that of fresh magnet-activated cell sorting (MACs) sorted OX62+ DCs (OX62 recognises the integrin αE2 subunit) (positive control), ex vivo-generated BMDCs have the capacity to phagocytose and process the soluble OVA antigen (Figure 3.3). Ex vivo-generated BMDCs were also examined for their capacity to present allo-antigen and induce proliferation in T cell population (Figure 3.4). For this LEW and DA rat strains were the chosen as they have been previously described as a high-responder allogeneic combination (Nosov et al. 2012; de Kort et al. 2012). Results revealed that co-culture of LEW CD4+ T cells with DA allo-antigen pulsed BMDCs resulted a reduced level of proliferation (10.96 ± 0.8316%) compared CD4+ T cells co-cultured with allo-antigen pulsed OX62+ DCs (30.50 ± 0.7211) (Figure 3.4).
Figure 3.3 Examination of ex vivo-generated BMDCs capacity to process allo-antigen. Time point analysis of BMDC antigen uptake and processing was analysed using DQ OVA FITC (50µg/ml, Ox62+ DCs were used as a control cell population). DQ OVA assay, MFI at 30mins (dark green line), 4hrs (green line), 24hrs (bright green line) and unstained control (black line, representative histogram of two independent experiments).
Figure 3.4 Examination of ex vivo-generated BMDCs capacity to process and present allo-antigen. (a) Illustration of experimental design to pulse LEW BMDCs with DA donor derived allo-antigen and co-culture with CFSE labelled LEW T cells. (b) Representative graph of two independent experiments (Unpaired two-tailed student’s t test n=3, mean ± SEM *p≤0.05, and ***p≤0.001).

Phenotype analysis demonstrated that the BMDCs displayed a semi-mature phenotype consisting of partial maturation including the up-regulation of MHC and co-stimulatory molecules however, in the absence of an inflammatory stimulus the BMDCs maturation is arrested at a semi-mature stage. These BMDCs were also functionally active APCs capable of processing and presenting antigen. However, ex vivo-generated BMDCs have a reduced capacity to promote proliferation compared to freshly isolated Ox62+ DCs.
3.2.1.3 Changes in maturation of LPS stimulated ex vivo-generated BMDCs.

To investigate the changes in phenotype of ex vivo-generated BMDCs in the presence of an inflammatory stimulus, the semi-mature BMDC cultures were further matured by addition of LPS (1µg/ml) to the BMDC culture on d9. The BMDCs were then analysed for expression of MHC II, CD80, CD86 and HIS36 (Figure 3.5 and Figure 3.6). The flow cytometric results indicated that ex vivo-generated BMDCs display a semi-mature phenotype, expressing moderate amounts of costimulatory molecules CD80 and CD86 and upon receiving a strong inflammatory stimulus these semi-mature BMDCs significantly increased their expression of the co-stimulatory molecules CD80 and CD86 displaying a fully matured phenotype.
Figure 3.5 Phenotypic characterisation of unstimulated versus LPS stimulated ex vivo-generated BMDCs. BMDCs were differentiated with GM-CSF and IL-4 (5ng/ml respectively) for 10 days. On d9 cultures were stimulated with LPS (1µg/ml, 24hrs). (a) Mean percentage and MFI of MHC II, CD80, CD86 and HIS36, expression within the CD11b/c+ population was analysed (Unpaired two-tailed student’s t test n=3, mean ± SEM *p≤0.05, and ** p≤0.01).
Figure 3.6 Changes in phenotype of ex vivo-generated BMDCs after LPS stimulation. Representative histograms of 3 independent experiments (isotype controls: grey line, unstimulated BMDCs: black line and LPS stimulated BMDCs: black dashed line) of MFI of MHC II, CD80, CD86 and HIS36 expression within the CD11b/c+ population of unstimulated BMDC cultures and cultures stimulated with LPS (1µg/ml, 24hrs).
3.2.2 Glucocorticoid treatment of BMDCs

3.2.2.1 Cell surface characterisation of ex vivo-generated BMDCs and Dexa BMDCs.

BMDCs generated as previously described (2.2) are cells that display a semi-mature phenotype in a quiescent environment and are capable of becoming fully mature BMDCs upon stimulation. To investigate the ex vivo-generation of regulatory BMDCs with a maturation-resistant phenotype the addition of Dexa to the cultures was examined. A final concentration of \(10^{-6}\)M of the glucocorticoid was added to the BMDC culture every other day from day 5. By day 10 cell aggregates were evident in Dexa treated cultures (Figure 3.7) however, they were not as large or as numerous as in untreated BMDC cultures. Evaluation of Dexa BMDC phenotype revealed that treatment of BMDCs with Dexa resulted in a significant reduction in the expression level of the following markers MHC II, CD80 and CD86 (Figure 3.8) compared to untreated BMDCs. Dexa BMDC cultures displayed a significant increase in the level of expression of HIS36. This however, does not reflect an increase in contaminating macrophages as it has been previously shown that monocyte derived DCs cultured with glucocorticoids such as Dexa significantly increase their expression of HIS36 (Sulahian et al. 2000; Buechler et al. 2000; Kowal et al. 2011).
Figure 3.7 Brightfield microscopy images of BMDC and Dexa BMDC cultures on d10.

3.2.2.2 Changes in the level of maturation of ex vivo-generated BMDCs and Dexa BMDCs with an inflammatory stimulus.

To examine the effects of Dexa treatment on BMDCs ability to mature under inflammatory conditions, untreated semi-mature BMDC and Dexa BMDC cultures were stimulated for 24hrs with LPS (1µg/ml) and analysed for expression of MHC II, CD80 and CD86. Results illustrated that LPS stimulated Dexa BMDCs had a significantly lower level of expression of these maturation molecules compared to LPS stimulated BMDC cultures. Not only did the LPS stimulated Dexa BMDCs remain immature but the levels of these maturation markers for LPS stimulated Dexa BMDCs did not significantly change from unstimulated Dexa BMDCs, indicating that this BMDC population had a maturation-resistant phenotype (Figure 3.8).
Figure 3.8 Phenotypic characterisation of immunomodulatory ex vivo-generated BMDCs and Dexa BMDCs. BMDCs were differentiated with GM-CSF and IL-4 (5ng/ml respectively) for 10 days in the absence or presence of Dexa (10^{-6}M added to the culture on day 5). (a) Representative histograms of MFI of MHC II, CD80, and CD86 expression within the CD11b/c^+ population of BMDCs (black lined histogram) and Dexa BMDCs (black dashed lined histogram) (b and c) The percentage and MFI of BMDC and Dexa BMDC cultures was analysed, cultures were stimulated with LPS (1µg/ml, 24hrs) (Unpaired two-tailed student’s t test n=3, mean ± SEM *p≤0.05, **p≤0.01 ***p≤0.001).
3.2.2.3 Cytokine expression and immunomodulatory profile of ex-vivo BMDCs and Dexa BMDCs.

The phenotype of DCs is not only based on the level of maturation markers expressed by the cell population but also significantly on the cytokines expressed. In order to fully characterise the phenotype of ex vivo-generated BMDCs and Dexa BMDCs the supernatants from d10 unstimulated and LPS stimulated (24hrs) cultures were examined first using a Multi-Analyte array (Appendix Figure 8.4). Compared to unstimulated BMDCs there was a trend towards reduced expression of the pro-inflammatory cytokines TNF-α, IL-2 and IFN-γ in Dexa BMDC cultures (Appendix Figure 8.4). After 24hr LPS stimulation there was also a trend towards reduced expression of pro-inflammatory cytokine such as IL-1α and β, TNF-α, and IL -6 (Appendix Figure 8.4) in Dexa cultures compared to untreated BMDCs.

To validate these observations and examine other immunomodulatory molecules additional BMDC and Dexa BMDC supernatants were examined by ELISA. It was observed that BMDCs produced 90.82 ± 21.66pg/ml of TNF-α and Dexa 21.48± 8.27 pg/ml (Figure 3.9). Once stimulated TNF-α production increased to 565.3 ± 196.6pg/ml and 140.2 ± 25.95pg/ml for BMDCs and Dexa BMDC cultures respectively. IL-10 production was also examined with BMDCs expressing 4.396 ± 1.88pg/ml before LPS stimulation and 32.23 ± 10.98 after. Dexa BMDCs however, produced significantly more IL-10 before 19.85 ± 9.978pg/ml stimulation and 83.34±2.36 pg/ml after (Figure 3.9). Dexa BMDC culture supernatants were also assayed for nitrite content (NO$_2^-$) by Griess assay. Supernatants from unstimulated Dexa BMDC cultures contained significantly more nitrite (NO$_2^-$) compared to BMDCs alone, 3.608±0.3475 µM and 1.398 ± 0.3930µM respectively. However, results demonstrated a trend although not significant in increased levels of NO$_2^-$ after LPS stimulation for Dexa culture supernatants compared to untreated BMDCs 3.078±0.6487µM and 1.768 ± 0.3265µM respectively (Figure 3.9).
Figure 3.9 Examination by ELISA of cytokine expression of ex vivo-generated untreated BMDCs and Dexa BMDCs. Supernatants of untreated BMDCs and Dexa BMDC cells were analysed by ELISA for production of TNF-α, IL-10 and by Griess assay to measure NO$_2^-$ production, before and after LPS stimulation (Unpaired two-tailed student’s t test n=3-6, mean ± SEM *p≤0.05 and **p≤0.01).

Characterisation of untreated BMDCs and Dexa BMDCs cytokines, chemokines, chemokine receptors and toll like receptors (TLRs) expression profile was further examined using RT-PCR. Preliminary analysis was carried out using PCR-array (Appendix Figure 8.5, 8.6 and 8.7) which revealed significant trends toward decreased mRNA expression levels of the cytokines Il-$1\beta$ and Tgff-$1\beta1$ and increased expression of Il-$10$ and Tnf-$\alpha$ (normalized to β-actin and fold change relative to BMDCs alone) in Dexa BMDC cultures (Appendix Figure 8.6). There was also an increase in mRNA expression levels of Cxcl9 and Cxcl10 in Dexa BMDC cultures compared to untreated BMDCs (Appendix Figure 8.6). Interestingly, compared to BMDCs stimulated with LPS there was a trend towards a reduced expression of Il-$1\beta$ and Cxcl10 and increase of Il-$10$, Tnf-$\alpha$ and after LPS stimulation of Dexa BMDC cultures (Appendix Figure 8.7).

A select number of genes from the PCR-array analysis and some additional immunomodulatory molecules were analysed using RT-PCR for d10 unstimulated and LPS stimulated (24hrs) BMDC and Dexa BMDC cultures (Figure 3.10). Confirming observations made in the PCR-array, it was observed that Dexa BMDC cultures before
and after LPS stimulation expressed significantly higher amounts of *Il-10* mRNA (normalised to β-actin and fold change relative to Ox62⁺ DCs) compared to BMDC cultures. The same was observed for mRNA expression of inducible nitric oxide synthase (*iNos*) (normalised to β-actin and fold change relative to Ox62⁺ DCs), (Figure 3.10). These results provided further confirmation of the ELISA results which demonstrate an increased concentration of IL-10 and increased NO breakdown in the supernatants of Dexa BMDC cultures. RT-PCR for day 10 unstimulated and LPS stimulated cultures also revealed that unstimulated and stimulated BMDCs express significantly higher levels of *Pdl-I* (normalised to β-actin and fold change relative to Ox62⁺ DCs) compared to that of Dexa BMDCs (Figure 3.10). Interestingly mRNA expression of the immunomodulatory molecule *Ido* was significantly higher after LPS stimulation of Dexa BMDC cultures compared to stimulated BMDCs (Figure 3.10).
Figure 3.10 RT-PCR examination of immunoregulatory molecule expression of untreated BMDCs and Dexa BMDCs. Untreated BMDCs and Dexa BMDC cultures mRNA expression (normalised to β-actin and fold change relative to Ox62+ DCs) of immunomodulatory molecules *Il-10, iNos, Pd-l1 and Ido* were analysed (Unpaired two-tailed student’s t test n=3, mean ± SEM *p≤0.05, **p≤0.01 and ***p≤0.001).

Although no significant results were observed for mRNA expression of *Ccr2*, The CXCR3-binding chemokines *Cxcl9* and *Cxcl10* (normalised to β-actin, fold change relative to Ox62+ DCs) were significantly reduced in cultures treated with Dexa BMDCs compared to BMDCs (Figure 3.11). These chemokines are known to be induced by IFN-γ. CXCL10, also referred to as IFN-γ-inducible protein 10 (IP10), is believed to direct leukocytes trafficking to inflammatory sites and may influence the proliferation and development of alloantigen-reactive T cells (Rosenblum et al. 2010). The mRNA expression levels of a select number of TLRs was evaluated for unstimulated BMDCs and Dexa BMDCs, which included TLRs expressed on the cell surface (TLR-2 and
TLR-4) and those expressed in intracellular endosomal compartments (TLR-7 and TLR-9) (Schreibelt et al. 2010). The results showed there were no significant differences in expression levels between BMDCs and Dexa BMDCs (Figure 3.12).

**Figure 3.11 RT-PCR examination of chemokine expression of untreated BMDCs and Dexa BMDCs.** mRNA expression levels of chemokines Cxcl9, Cxcl10 and the chemokine receptor Ccr2 were examined in BMDC and Dexa BMDC cultures with and without the addition of LPS stimulation (Unpaired two-tailed student’s t test n=3, mean ± SEM *p≤0.05 and ***p≤0.001).
Figure 3.12 RT-PCR examination of TLR expression of untreated BMDCs and Dexa BMDCs. mRNA expression (normalised to β-actin and fold change relative to Ox62+ DCs) levels of the Tlr s 2, 4, 7 and 9 did not significantly differ in cultures with the addition of Dexa compared to untreated BMDCs (Unpaired two-tailed student’s t test n=3, mean ± SEM).
3.2.2.4 Evaluation of functional properties of ex vivo-generated untreated BMDCs and Dexa BMDCs.

The maturation status and the cytokine expression profile do not completely define the phenotype and characterisation of DCs. The full characterisation relies also on DC functional capacity. As previously examined for untreated BMDCs, Dexa BMDCs were also evaluated for their capacity to phagocytose and process antigen using a DQ OVA assay as described (2.3.1). This DQ OVA assay demonstrated that, similarly to that of fresh MACs sorted Ox62$^+$ DCs (positive control) and untreated BMDCs the Dexa BMDCs are capable of phagocytosing and processing the soluble OVA antigen (Figure 3.13). The freshly isolated MACs Ox62$^+$ DCs were not cultured prior to use in the DQ OVA assay this may account for the observed reduced MFI or reduced level of soluble OVA antigen processed compared to the highly pure cultured BMDCs.

![Figure 3.13 Examination of BMDCs and Dexa BMDCs capacity to process antigen.](image)

Time point analysis of BMDC and Dexa BMDC antigen uptake and processing was analysed using DQ OVA FITC (50µg/ml, Ox62$^+$ DCs were used as a control cell population). DQ OVA assay, MFI at 30mins (dark green line), 4hrs (green line), 24hrs (bright green line) and unstained control (black line), (representative histogram of two independent experiments).
Given the observed low to moderate levels of costimulatory molecules and immunomodulatory molecule expressed by BMDCs and Dexa BMDCs, their allo-stimulatory capacity was assessed. For this the LEW and DA rat strains were chosen as this has been described by others as a high-responder allogeneic combination (Nosov et al. 2012; de Kort et al. 2012). When in culture LEW lymphocytes stimulated with allogeneic DA Ox62⁺ DC visibly proliferate and form large cell aggregates and clusters however, lymphocytes stimulated with allogeneic BMDCs or Dexa BMDCs fail to form the same number and size of cell aggregates and the lymphocytes remain quiescent (Figure 3.14). Furthermore, proliferation assays which compared freshly isolated allogeneic DA Ox62⁺ DCs to both allogeneic DA BMDCs and Dexa BMDCs demonstrated their reduced capacity to induce lymphocyte proliferation (Figure 3.15). There was also a trend towards a reduced expression of the T cell activation marker CD25 (Figure 3.15) in Dexa BMDC stimulated cultures and a trend for towards higher FoxP3 expression in BMDC and Dexa BMDC stimulated cultures (Figure 3.15). An immunosuppressive capacity by BMDCs and Dexa BMDCs was also observed. At a ratio of 10:1 (lymphocyte:DC), lymphocyte cultures stimulated with allogeneic DA Ox62⁺ DCs, were suppressed with the addition, at a ratio of 10:1 (lymphocyte:BMDC) of allogeneic DA BMDCs or Dexa BMDCs (Figure 3.15 no significant difference between BMDCs and Dexa BMDCs).
Chapter 3

a. CFSE labelled LEW Lymphocytes
   Irradiated DA Ox62+DCs/
   BMDCs+/−Dexa

   d0
   ↓
   d5 Proliferation is measured by Flow Cytometry

b. DA Ox62+ DCs  DA BMDCs  DA Dexa BMDCs
   Lymphocyte:DC 10:1

10X

c. unstimulated

Lew Tcells:DA Ox62+ DCs 10:1

Lew Tcells:DA BMDCs 10:1

Lew Tcells:DA Dexa BMDCs 10:1
Figure 3.14 Examination of the immunogenicity of ex vivo-generated BMDCs and Dexa BMDCs. (a) Illustration of the experimental design to examine the immunogenicity of BMDCs and Dexa BMDCs. (b) Brightfield microscopy images show a visible reduction in the level proliferation and clustering in DA BMDCs and DA Dexa BMDC cultures compared to DA Ox62+ DCs stimulated cultures. (c) Representative flow plots from 2 independent experiments demonstrating BMDCs and Dexa BMDC have a reduced stimulatory capacity, illustrated by the reduction in percentage proliferating CFSE labelled lymphocytes.
Figure 3.15 Examination of the immunogenicity of BMDCs and Dexamethasone (Dexa) BMDCs. (a) At a ratio of 10:1 (lymphocyte:DC) DA BMDCs and Dexa BMDCs have a significantly reduced stimulatory capacity compared to DA Ox62+ DCs and (b) DA BMDCs and Dexa BMDCs showed an immunosuppressive capacity in lymphocyte cultures stimulated with allogeneic DA Ox62+ DCs which were suppressed with the addition, at a ratio of 10:1 (lymphocyte:BMDC) of DA BMDCs or Dexa BMDCs, illustrated by the reduction in percentage proliferating CFSE labelled lymphocytes. (c) LEW lymphocyte percentage population of activated CD4+CD25+ cells after stimulation with DA Ox62+ DCs, BMDCs or Dexa BMDC (d and e) regulatory CD4+FoxP3+ cells within these co-cultures. (a and b representative of 4 independent experiments, unpaired two-tailed student’s t test n=3, c and d combined analysis of 4 experiments unpaired two-tailed student’s t test n=4, mean ± SEM **p≤0.01 and ***p≤0.001).

Taken together, these results have clearly demonstrated that ex vivo-generated BMDC display a semi-mature phenotype which is significantly altered by glucocorticoid treatment resulting in the induction of a maturation-resistant DC phenotype. The ex vivo-generated BMDCs and Dexa BMDCs are functionally active APCs which have a profound capacity to modulate allogeneic immune responses. The results have demonstrated that not only do untreated BMDCs and Dexa BMDCs differ significantly in their cell surface characteristics and expression of immunomodulatory molecules, it would appear that compared to freshly isolated DCs (Ox62+); ex vivo-generated BMDCs differ significantly in their expression of immunomodulatory molecules without additional modification by glucocorticoid treatment.
3.2.3 Genetic Modification of BMDCs

Genetic modification is another method which has been demonstrated to modulate both phenotype and cytokine production of DCs (Newton et al. 2008). DCs are ideal cells for targeted delivery of immunosuppressive therapeutic molecules due their migratory capabilities and essential role in maintaining tolerance. This makes DC therapies an attractive and promising alternative for the promotion of allograft survival and/or tolerance induction. One of the aims of this study was to examine the application of BMDCs overexpressing the immunomodulatory molecules PD-L1 and IDO for the promotion of corneal allograft survival which until recently had not been explored. The application of such IDO overexpressing donor derived BMDCs has recently been demonstrated by Khan et al. not to be an effective therapy to promote corneal allograft survival (Khan et al. 2013). However, the authors did demonstrate prolongation of corneal allograft survival using donor derived BMDCs which were transduced to express the fusion protein CTLA4-KDEL which inhibits the expression of CD80/86 (Khan et al. 2013). Others have also shown that DCs overexpressing PD-L1 induced T cells suppression and long-term allograft acceptance in a rat model of renal transplantation (Peng et al. 2011).

The following results explore the methods examined during this study to genetically modify rat BMDCs for the generation of regulatory BMDCs for application in corneal allograft survival.
3.2.3.1 Ad transduction of rat BMDCs.

The first gene therapy approach adopted during this study to generate regulatory BMDCs was by Ad transduction of ex vivo-generated BMDCs. To examine this as a potential means of generating regulatory BMDCs the optimal method of Ad transduction was first determined. First generation E1/E3-deleted serotype 5 Ad encoding GFP (AdGFP) under the control of the cytomegalovirus (CMV) immediate early promoter was generated and purified as described in (2.5.1). A spin centrifugation method of transducing BMDCs described in detail in (2.5.2.1) was examined. Using various multiplicities of infection (MOI) 250 to 1000 on d3, BMDC cultures were transduced by AdGFP. Successful gene transfer was measured using flow cytometric analysis by detecting GFP expression. However, by d3 and d7 after Ad gene transfer, GFP expression was not detected in the BMDC cultures. In an effort to optimise the day of transduction, AdGFP transduction on d0, d1, d2 and d7 of BMDC culture was investigated again using flow cytometry to detect GFP expression on d10 of BMDC culture (Figure 3.16). Nevertheless, results demonstrated that AdGFP BMDC transduction was unsuccessful (Figure 3.16). An attempt to transduce BMDCs at later time-points was not undertaken, as mature DCs are less readily transduced because of low expression of the coxsackievirus and adenovirus receptor (CAR) (Rea et al. 1999). This method was repeated using new Ad virus preparations which successfully transduced mesenchymal stem cell cultures (data not shown) however, AdGFP transduction of BMDCs by the same spin centrifugation method was not achieved.
Figure 3.16 Adenovirus transduction of BMDCs. BMDCs were transduced with a MOI of 250, 500 or 1000 on days 0, 1, 2 or 7 of BMDC cell culture. Cells were analysed by flow cytometry on day 10 of the BMDC culture (representative plots of three independent experiments).
Alternative methods of Ad transduction which included the use of Polybrene® and LnCl₃ which allow transduction independently of the CAR (data not shown), were also investigated. Again successful transduction of BMDCs was not achieved. Although it has been reported that several vector systems, including Ad vectors, retroviral vectors, Hemagglutinating Virus of Japan (HVJ)-related vector and electroporation are able to transduce genes into mouse and human DC, some have also successfully transduced rat derived DCs with Ad (Ouyang et al. 2010; Schlickeiser et al. 2011; Peng et al. 2011). However, others have also reported the difficulties in using Ad to transduce rat derived DCs (Satoh et al. 2005). Due to the unsuccessful Ad transduction of rat BMDCs an alternative method of transduction using LV was investigated.

3.2.3.2 LV transduction of rat BMDCs

Recombinant LVs were generated by a standard protocol as described in materials and methods (2.5.3) and flow cytometric analysis was used to determine LV.eGFP virus titer. BMDC cultures were transduced with LV on d3 as described in (2.5.4) and the BMDC culture was maintained until d10. Transduction of BMDC on day 3–4 of differentiation was based on the fact that LV transductions of BM cells at earlier stages were demonstrated as being more restrictive (Toscano et al. 2010). On d4 after LV transduction the BMDCs contained clear clusters of GFP expressing cells (Figure 3.17). Using flow cytometric analysis on d10 transduction efficiency of BMDCs by LV.eGFP was assessed. Results indicated successful gene transfer but illustrated no significant difference between the transduction efficiencies obtained with a MOI 0.5, 1 or 2 (Figure 3.18 and Figure 3.19).

In order to increase efficiency of the LV production and transduction protocol alternative methods of LV transduction and additional MOIs were investigated (Appendix Figure 8.8). Surprisingly the most efficient method of transducing BMDCs
was using unconcentrated LV containing supernatant diluted 1:1 with culture medium (Figure 3.20). Applying this method of LV transduction resulted in $4.5 \times 10^6$ BMDCs/3mls transduced with an efficiency of $9.180 \pm 2.892\%$ with 1.5ml of unconcentrated LV supernatant (total of $4.05 \times 10^5$cells transduced). To obtain the same number of cells transduced with concentrated LV preparation with a MOI 2 would require a concentrated LV preparation of $3 \times 10^8$tu/ml which is 10 times the maximum titer obtained during this study.
Figure 3.17 Fluorescent light microscope images of LV eGFP transduced BMDCs. BMDC cultures were transduced with LV eGFP on day 3 with MOI of 0.5, 1 or 2. On d10 cells were examined by fluorescence microscopy at magnifications of 4x and 10x.
Figure 3.18 Flow cytometric analysis of LV eGFP transduction of BMDCs. Representative plots from 3 independent experiments examining LV eGFP transduction of BMDCs on d3 of culture with a MOI of 0.5, 1 or 2.

Figure 3.19 LV eGFP transduction of BMDCs. No significant difference was observed between LV MOI 0.5, MOI 1 or MOI 2 (Unpaired two-tailed student’s t test n=3 mean ± SEM).
Figure 3.20 LV eGFP transduction of BMDC using unconcentrated LV containing supernatant. (a) Representative flow plots from 4 independent experiments examining the transduction of BMDCs on d3 with unconcentrated LV supernatant mixed 1:1 with culture media. (b) Percentage population transduced LV eGFP (Unpaired two-tailed student’s t test n=4 mean ± SEM ***p≤0.001).
3.2.3.3 Selection of transduced BMDCs Population.

For application in vivo it was necessary to establish a method of selection or purifying the transduced BMDC population. A method which employs antibiotic selection of the transduced population was investigated. The antibiotic resistance gene for blasticidin was encoded for on the pLenti6 Destination vector containing our gene/s of interest. Blasticidin is a nucleoside antibiotic that is produced by the bacterium Streptomyces griseochromogenes. It is a potent translational inhibitor in both prokaryotic and eukaryotic cells, resistance to which is conferred by the product of the \textit{bsd} gene from Aspergillus terreus. Blasticidin causes rapid cell death at low antibiotic concentrations with mammalian cells being sensitive to concentrations as low as 2 to 10 µg/ml cell death occurs rapidly.

BMDC sensitivity to blasticidin treatment was examined with the addition of 0-5µg/ml of the antibiotic to cell culture medium on d3, d5 and d7. On d10 cell viability was analysed using trypan blue exclusion (Figure 3.21). Even at the highest concentration 28.5% of the cells remained viable. When blasticidin treatment was repeated on cells transduced with LV.eGFP which also contained blasticidin resistance gene, it was evident that although the transduced eGFP expressing cells were viable many untransduced cells remained contaminating the culture (Figure 3.22).
Figure 3.21 Viability of BMDC culture after addition of blasticidin. BMDC cultures were treated with blasticidin d3, d5 and d7. On d10 cell viability was analysed using trypan blue exclusion. Graph representative of triplicate samples from one experiment.
Figure 3.22 Flow cytometric analysis of LV eGFP transduced BMDC culture after addition of blasticidin. Survival of LV eGFP expressing BMDCs was examined by flow cytometry on d10 of culture after treatment with 0-10µg/ml of the antibiotic blasticidin to cell culture medium on d3, d5 and d7 (representative plots of three sample replicates in one experiment).
An alternative, Fluorescent Activated Cell Sorting (FACS), was also explored as a method of selecting and purifying the transduced BMDCs population. The first sorted BMDC cultures transduced with LV.eGFP supernatants contained <2% eGFP+ve BMDCs (Figure 3.23) of which only 1x10^4 cells were sorted with a purity of 83.4%. The sort was repeated with a starting population of 9x10^6 cells but with a transduction efficiency of 4.22% resulting in a total of 3.8x10^4 eGFP+ve BMDCs sorted with a purity of 85.5% (Figure 3.23). Based on these results and the average transduction efficiency of 9% from optimal LV.eGFP supernatants the schematic (Figure 3.24) illustrates the total cell numbers and LV supernatants required for one in vivo group.
Figure 3.23 Gating Strategy for FACS of LV eGFP transduced BMDCs. A representative image of the gating strategies used in sorts to examine the potential application of FACS to purify transduced BMDCs. Gating approach: (a) gates on morphological BMDC, to exclude non-viable cells, (b) single cell gate to eliminate cell aggregates, (c) gate on untransduced BMDCs to identify transduced population as in illustrated in (d). Arrows indicate gating hierarchy. Purity obtained sort #1 83.4% (Appendix Figure 8.9) and (e) sort #2 85.5%.
Figure 3.24 Schematic of LV production and transduction of BMDCs for application in vivo. A comparison of concentrated LV preparations and unconcentrated supernatants and the quantities required in order to obtain the numbers of FACS transduced cells required for administration in in vivo experiments.
3.2.3.4 Maturation of Transduced BMDCs Population

In addition to requiring a pure transduced BMDC population for application in vivo it was also necessary to monitor the maturation status of the ex vivo-generated BMDCs after LV transduction sorting. Similarly to other reports (He et al. 2005; Veron et al. 2009) the level of maturation marker expression, MHC II, CD86 and CD80, within the total BMDC population before and after LV transduction did not significantly increase (Figure 3.25). This observation is of critical importance when considering LV transduced BMDCs for application in vivo as the immunostimulatory or immunoregulatory properties of BMDCs depends not only on their phenotype and source but on their maturation status.
Chapter 3

a. 

b. 

Untransduced BMDCs

Transduced BMDCs

MFI

Untransduced BMDCs

Transduced BMDCs

Gated on LV.eGFP+ve BMDCs

% Population

Gated CD11b+/c+
Figure 3.25 Expression of maturation markers on BMDCs before and after LV transduction and LV eGFP positive BMDCs. (a) BMDCs (black line) and BMDC cultures after transduction with LV eGFP (green line) had similar levels of expression of MHC II, CD86 and CD80 expression (representative of n=4). Examination of the (b) MFI and (c) percentage population expressing MHC II, CD80, CD86 and HIS36 before and after LV eGFP transduction (Unpaired two-tailed student’s t test n=3-4 mean ± SEM).
3.2.3.5 Summary of gene therapy approach and justification of change of direction.

A variety of gene therapeutic approaches were investigated to transduce rat BMDCs. Despite preparative scale transductions the Ad approach to genetically modifying BMDCs was not successful and so an alternative using a LV method of transduction was explored. Although transduction was successful the number of eGFP positive cells was too low for functional assays or in vivo applications with whole LV preparations being required for transduction of one BMDC culture. This was not a feasible method due to the cost and time required to produce the required quantities of virus. To address this unconcentrated LV preparations were examined and successful transductions of multiple BMDC cultures were achieved from a single LV preparation. However, again transduction efficiencies were low and there was also another limiting factor in the purification of the transduced population after LV transduction. The purity of the transduced population required for in vitro and in vivo applications was a key factor to insure that the recipient would not be pre-sensitised to donor antigen. FACS was explored when antibiotic selection was not successful in this study. In order to use FACS as a method of purification the genes of interest PD-L1 and IDO would be required to be tagged with GFP in order to identify and sort the transduced BMDCs population. Experiments to clone PD-L1 and IDO into a pLenti6 Destination vector containing both the gene of interest and a GFP tag were commenced and proved difficult to achieve in the time remaining in the study (Appendix 8.7.6).

In parallel to the genetically modified BMDC study the glucocorticoid treatment of BMDCs was investigated. The limitation of the gene therapeutic approach, including scalability, time and cost meant that this approach was no longer feasible. The positive results obtained in the study of glucocorticoid treatment of BMDCs meant that this was the more compelling application to investigate in vivo.
3.3 Discussion

3.3.1 Immunoregulatory BMDC Phenotype and Function.

This study began with the successful optimisation of cell culture for the generation of BMDCs from fresh rat bone marrow. Although fresh rat bone marrow was employed throughout the study cryopreserved marrow was also investigated. The phenotype of BMDCs which were cryopreserved at d0 and then differentiated in the presence of rat GM-CSF, rat IL-4 (5ng/ml respectively) revealed no phenotypical difference to fresh marrow BMDCs (Appendix 8.2). The BMDC phenotype of the adherent and semi-adherent BMDC cell population in culture was fully examined. Results revealed that these cells had a semi-mature phenotype (Figure 3.1), a phenotype associated with this population (Lutz and Schuler 2002; Moreau et al. 2009). These cells remained in this semi-mature status unless they were introduced to an inflammatory stimulus which resulted in their maturation (Figure 3.6)(Hattori et al. 2012; Gao et al. 2013). If the semi-mature BMDCs were administered in vivo there was the potential that these cells could become immunogenic and increase the rate of rejection rather than be immunoregulatory and promote allograft survival. This study therefore aimed to investigate methods to promote a maturation-resistant or immunoregulatory BMDC phenotype.

To date studies have employed a variety of different methods to generate regulatory or maturation-resistant DCs including gene transfer, treatment with cytokines such as IL-10 and TGF-β1, rapamycin, the vitamin D₃ metabolite 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) alone or in combination with glucocorticoid steroids (Dong et al. 2003; Morelli and Thomson 2007; Unger et al. 2009; Anon 2011; Boks et al. 2011; Hattori et al. 2012; L. Chen et al. 2012; Khan et al. 2013). For this this study ex vivo-generated
BMDCs treatment with the glucocorticoid Dexa and genetic modification by Ad and LV transduction were examined.

BMDCs treated with Dexa were examined as prototypic therapeutic BMDCs over rapamycin treated BMDCs, as a comparative study of the two pharmaceutical agents reported that Dexa treated BMDCs are not only phenotypically immature but they exhibit a durable immaturity and produce high levels of IL-10 (Figure 3.8 and Figure 3.9) (Naranjo-Gómez et al. 2011). As previously stated Dexa has also been shown to have low toxicity on DCs (Mirenda 2004).

Dexa treatment not only resulted in a maturation-resistant phenotype but the Dexa BMDCs were capable of modulating an allogeneic lymphocyte response (Figure 3.15) with an efficacy comparable with previously described immature, tolerogenic DCs generated by alternative methods (Morelli and Thomson 2007; Unger et al. 2009; Anon 2011; Boks et al. 2011; Hattori et al. 2012). Dexa treatment also ensures BMDC remain in a continued immature state when introduced to an inflammatory environment as these cells did not altered their maturation status when stimulated with a strong inflammatory stimulus such as LPS (Figure 3.8). Additionally, as illustrated in the results of the allogeneic lymphocyte assays BMDCs without additional glucocorticoid treatment, also appear to have a reduced capacity to stimulate allogeneic lymphocytes (Figure 3.15). This was a significant observation made during this study and with further investigation of the immunomodulatory properties of Dexa BMDCs, it became clear that unmodified BMDCs themselves had significant immunomodulatory properties. Similar to Dexa BMDCs, untreated BMDCs may utilise specific molecular mechanisms allowing them to inhibit T cell proliferation and/or modify T cell differentiation independently of their maturation status (Lutz et al. 2000; Lutz and Schuler 2002; Mellor et al. 2004; Berger et al. 2009).
3.3.2 Immunoregulatory Mechanisms.

The molecular basis for BMDCs and Dexa BMDCs profound immunomodulatory properties is partially explained by their differential expression of \( Pd-L1 \), NO, \( Ido \) and IL-10 (Figure 3.9 and Figure 3.10). These molecules are significantly increased for both the untreated BMDCs and Dexa BMDCs in unstimulated and stimulatory conditions relative to ex vivo Ox62\(^+\) DCs. These results give an indication of the potential mechanisms exploited by these cells which results in the observed in vitro inhibition of allogeneic lymphocyte proliferation.

Along with expression of co-stimulatory molecules, DCs also express negative co-stimulatory molecules that are capable of inhibiting T cell activation, an example of which is the well characterized interaction between the T cell receptor PD-1 with its ligand PD-L1 expressed by DCs (Amarnath et al. 2010). During this study a significant increase in mRNA expression levels of this immunomodulatory molecule within untreated BMDCs and Dexa BMDC cultures relative to ex vivo Ox62\(^+\) DC was observed (Figure 3.10). Recently however, it has been suggested that PD-L1 is not essential to inhibit lymphocyte proliferation by myeloid derived suppressor cells. NO, which this study has also shown to be expressed by both BMDCs and Dexa BMDCs, was found to be the key modulator of proliferation inhibition (Rössner et al. 2005).

iNOS expression and the resulting NO production has been demonstrated to mediate suppressive effects on lymphocyte proliferation and both of these molecules were found to be significantly expressed in BMDC cultures (Bogdan 2001; Powell et al. 2003; Rössner et al. 2005; Cobbold et al. 2009). Ex vivo-generated BMDCs unlike, freshly isolated DCs have been demonstrated to secrete NO (Powell et al. 2003). The results observed in this study support this finding as freshly isolated donor Ox62\(^+\) DCs resulted
in LEW lymphocyte proliferation as one would have predicted but significantly the donor untreated BMDCs inhibited proliferation (Figure 3.9 and Figure 3.10).

Studies have also indicated that the NO produced due to iNOS expression inhibits IDO activity by directly interacting with it and promoting its degradation through the proteasome pathway (Samelson-Jones and Yeh 2006). This may explain the differences observed in untreated BMDC cultures where iNOS is significantly expressed and IDO expression is significantly reduced compared to Dexa BMDCs. Catabolism of essential amino acids, such as tryptophan by IDO and arginine by iNOS results in a localized, immune-privileged microenvironment in which naïve T cells that would normally become activated proliferating T cells, are instead kept in an anergic, unproliferative T cell state (Munn 1998; Mellor et al. 2004; Munn and Mellor 2013). The results in this chapter have clearly shown evidence that not only maturation-resistant, immature Dexa BMDCs have significantly reduced immunogenicity but also the ex vivo-generated semi-mature BMDCs.

3.3.3 Gene Transfer to BMDCs

As well as investigating methods to induce maturation-resistant BMDC phenotype this study aimed to investigate the propagation of BMDCs overexpressing immunomodulatory molecules by gene transfer. One of the important decisions in the use of viral vectors to transduce DCs with genes encoding immunoregulatory molecules is the nature of the viral vectors employed. It has been reported that several vector systems, including Ad vectors are able to transduce genes into mouse and human DC (Dyall 2001; Satoh et al. 2005; Schlickeiser et al. 2011). Although Ad vectors have been used successfully by others to transduce rat BMDCs (Ouyang et al. 2010; Peng et al. 2011), during this study transduction of rat BMDCs was not achieved using Ad (Figure
This has also been observed by Satoh et al. who reported LV to be the most effective method of gene transfer in rat DCs (Satoh et al. 2005). Ad vectors for DC modification is hindered by the lack of expression of the primary Ad receptor, CAR on DCs (Pereboev et al. 2004). Nevertheless, others have reported that with specific immunomodulatory constructs, Ad have transduced rat DCs and this has resulted in DCs which exert a profoundly suppressive effect on T cell proliferation (Newton et al. 2008; Peng et al. 2011).

Although successful at transduction of DCs, Ad application has also been reported to lead to the subsequent maturation and cytokine production by DCs (Toscano et al. 2010). Also due to the potential induction of host immunity to immunodominant viral epitopes Ad application is limited (Toscano et al. 2010). Ad are known also to activate TLR-9-dependent and independent pathways of DNA sensing and TLR-2 activation and can result in antibody and T cell response against transgene products especially If nonspecific promoters are used (Nayak and Herzog 2010). Alternatives to Ad transduction have been investigated and LV transduced DCs represent an alternative in immunotherapy. Advantages to using LV include LV stable gene delivery into most nondoning primary cells (Barde et al. 2010), LV vectors genetic material is integrated into the chromosome of the target cell making them an ideal tool to introduce long-term expression of a gene. LV also have a relatively large capacity allowing delivery of most cDNAs and they do not transfer sequences that encode for proteins derived from the packaging virus (Barde et al. 2010). LV are highly efficient at transduction of APCs (Nayak and Herzog 2010) and during this study rat BMDC culture were successfully transduced using LV (Figure 3.19 and Figure 3.20).

The focus of the literature has been on LV transduced DC as immuno-stimulants for possible uses in cancer therapy (He et al. 2005; Lebson et al. 2011; Song et al. 2012), however in vivo models have also examined the therapeutic effects of BMDCs modified by LV vectors genetically engineered to express VIP in models of experimental
autoimmune encephalomyelitis (EAE) (Toscano et al. 2010). Henry et al. expressed the immunosuppressive cytokine IL-10 to control experimental asthma (Henry et al. 2008). In such models the maturation status of the DCs may not negatively impact the therapeutic benefit however, in transplantation the application of mature BMDCs pulsed with donor antigen or a modified donor BMDCs risks pre-sensitising the recipient to the donor antigen. However, it has been argued that maturation of BMDCs may in some setting prove advantageous as the mature phenotype may in fact improve the efficiency with which the BMDCs traffic to appropriate sites for tolerance induction (Khan et al. 2013).

DC morphology and phenotype alterations including changes in expression of surface markers have been reported by others after Ad (Rea et al. 1999; Miller et al. 2002; Lyakh 2002; Newton et al. 2008; Khan et al. 2013). These changes include the loss of CD14 and increased expression of CD80, CD86 or other cell surface molecules including MHC I and II molecules (Rea et al. 1999; Miller et al. 2002; Lyakh 2002; Newton et al. 2008; Khan et al. 2013). Studies have now begun to work on methods to inhibit maturation of DCs due to transduction with Ad. One study examined DCs maturation after transduction with Ad expressing a kinase-defective dominant negative form of IKK2 (IKK2dn) (Ouyang et al. 2010). The form of IKK2 kinase inhibits NF-κB activation and impairs DC maturation by reducing CD80 and CD86 expression under alloantigen stimulation (Ouyang et al. 2010).

This study, in line with findings from previous studies (He et al. 2005; Veron et al. 2009; Richter et al. 2013), has shown that BMDC maturation was not observed after transduction with LV (Figure 3.25). Interestingly a LV construct expressing the fusion protein CTLA4-KDEL has been shown to prevent CD80 and CD86 expression by retaining the costimulatory molecules within the endoplasmic reticulum of the transduced DCs. The same group demonstrated that CTLA4-KDEL-expressing DCs
targeted to the indirect pathway were capable of promoting long-term survival of corneal allografts (Khan et al. 2013).

It is also accepted that DCs have a restricted life span and so studies have now used LV systems that allow transcriptional targeting of DCs in vivo to achieve stable and longer lasting expression of antigen (Dresch et al. 2008). Pincha et al. have demonstrated ex vivo genetic programming of mouse bone marrow precursors with LVs encoding GM-CSF and IL-4, which induced autonomous differentiation of long-lived DCs referred to as self-differentiated myeloid-derived antigen-presenting-cells reactive against tumors (SMART-DCs) (Pincha et al. 2011).

Genetic modification of BMDCs for the generation of immunomodulatory BMDCs for application in vivo was not a feasible method during this study. For a variety of reasons including the costs and time required to produce the necessary quantities of virus in order to obtain high transduction efficiencies of BMDC cultures. There were also other limiting factors including the purification of the transduced BMDC population after LV transduction. The in vivo application of the genetically modified BMDCs for the promotion of corneal transplantation survival required BMDCs with an immature phenotype as injection of BMDCs pulsed with donor antigen with a mature phenotype or administration of donor derived BMDCs risked pre-sensitising the recipient to the donor antigen. There also remained the disadvantages with a gene therapy approach for the modification of BMDCs for in vivo application in the potential adaptive immune response including CTL and antibody responses to the therapeutic gene products that differ from the endogenous protein (Nayak and Herzog 2010). Therefore after extensive analysis of our findings, it was decided that the therapeutic approach which merited further investigation for the prolongation of corneal allograft survival was the glucocorticoid treatment of BMDCs.
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3.3.4 Chapter Summary

Our interpretation of the work analysed and presented in this section can be summarised as follows:

- Functionally active BMDCs can be generated ex vivo from rat bone marrow cultured with GM-CSF and IL-4.
- Dexa can be added to BMDC cultures to induce an immature, maturation-resistant BMDC phenotype.
- Dexa BMDCs exhibit immunoregulatory phenotype but significantly so do untreated BMDCs.
- This immunoregulatory phenotype includes but is not limited to PD-L1, IDO and IL-10 expression.
- Both untreated BMDCs and Dexa BMDCs display significant immunomodulatory effects and inhibit allogeneic T cell proliferation.
- Ad is unable to transduce rat BMDCs cells.
- LV is capable of transducing rat BMDCs and does not lead to a significant increase in the expression of maturation markers MHC II, CD80 and CD86.
4. Administration of ex vivo-generated immunomodulatory BMDCs in corneal allograft transplantation.

4.1 Introduction

In the 108 years that have passed since Dr. Eduard Konrad Zirm performed the first successful corneal transplantation many of the risk factors which are likely to lead corneal allograft failure have been identified (Moffatt et al. 2005). Although the eye is an immune privileged organ due to the immunoregulatory milieu found within the organ itself, the 5 year prognosis is equivalent to that of vascularized renal or cardiac allografts (Waldock and Cook 2000). In particular patients with corneal vascularization, previous graft failure and glaucoma are at an increased risk of accelerated allograft failure due to immune-mediated rejection. Many studies have identified CD4 cells as the key effector immune cell in corneal allograft rejection (He et al. 1991; Yamada et al. 1999; Tan and Abdulreda 2013).

It is evident from the literature that DCs are important modulators of T cell phenotype and function with an inherent capacity to direct antigen-specific immune responses. Once DCs mature they modify their phenotype in order to increase the cellular surface area to improve their interaction with T cells. They also significantly up-regulate their expression of MHC II, co-stimulatory molecules and T cell adhesion molecules (Mellman and Steinman 2001). In the absence of maturation signals and expression of immunomodulatory molecules such as IDO, NO or HO immature DCs can tolerise peripheral CD4+ CD8+ (Steinman and Nussenzweig 2002; Steinman et al. 2003; Mellor and Baban 2003; Moreau et al. 2009; Baban et al. 2011; Sucher et al. 2012) (Figure 1.6).
Due to DCs inherent effector function to instruct distinct T cell fates and their capacity to interact with numerous other cell types of the immune system including B cells, NK cells and NKT cells, they make the ideal target cell population for modification (Chijioke and Münz 2011; Sabado and Bhardwaj 2013). Depending on the DC source and culture conditions these cells can be modified to promote a tolerogenic, regulatory response in the T cells with which they interact. Subsequently this interaction can lead to deletion or anergy/regulation of reactive T cells (Munn 1998; Steinman and Nussenzweig 2002; Mellor et al. 2004; Munn and Mellor 2013) and the recipient’s immune system is orchestrated to accept the allograft by prevention of cellular mediated graft rejection and promotion an immunoregulatory environment.

This chapter investigates the intravenous injection (i.v.) administration of immunoregulatory donor derived untreated BMDCs and Dexa BMDCs as potential cell based therapies for the promotion of corneal allograft survival without the requirement for additional immunosuppressive drug therapies. This chapter aims to extensively characterise the immune microenvironment of the corneal allograft and the graft draining lymph nodes after treatment with these BMDC cell therapies. This chapter also aims to examine the therapeutic effect of local administration by subconjunctival injection of immunoregulatory BMDCs on the survival of corneal allografts.
4.2 Results

4.2.1 Transplantation Model

A MHC I/II mismatched rat corneal transplantation model using the high responder LEW-DA combination was adopted for this study. Rapid rejection is not typical for allogeneic keratoplasty in humans, corneal allografts enjoy high rates of spontaneous acceptance compared with other types of transplantation (40–50%) (Niederkorn 2003; Williams et al. 2006). The LEW-DA strain combination is popular due the fact transplant recipients reject their grafts fast and uniformly. As a result rejection times can be narrowly predicted experimental plans are therefore easier to establish.

4.2.2 Route of Administration

During this study two possible routes of administration were examined the first being a systemic application via i.v. injection and the second a local application via subconjunctival injection described later in the chapter (4.2.7). The in vivo localisation of i.v. injected Far Red labelled fluorescently labelled donor Dexa BMDCs (1x10^6/ml PBS) but also donor antigen pulsed syngenic Dexa BMDCs (1x10^6/ml PBS) were investigated (Figure 4.1 and Figure 4.2). Donor Dexa BMDCs and donor antigen pulsed syngenic Dexa BMDCs were labelled with Far Red dye and a system using fluorescently labelled beads was employed to enumerate the cells found in the tissues analysed by flow cytometry. Analysis of homogenised tissues revealed that Far Red labelled cells antigen pulsed syngenic Dexa BMDCs and donor derived Dexa BMDCs could only be located in the blood for Dexa BMDCs and in the lungs for both Dexa
BMDCs and donor antigen pulsed Dexa BMDCs 24hrs post injection and there were no fluorescently labelled cells in the spleen, bone marrow, thymus and LNs (submandibular, cervical and para-thymic) (Figure 4.1 and Figure 4.2).
Figure 4.1 Localisation of I.V. injected fluorescently labelled donor Deka BMDCs and donor antigen pulsed Deka BMDCs. Far Red labelled donor Deka BMDC (1x10^6 cells/ml PBS) were injected i.v. and 24hrs post injection tissues were harvested, homogenised and analysed by flow cytometry for the presence of Far Red labelled cells. Far Red labelled cells were located in the blood and in the lung for both donor Deka BMDCs and donor antigen pulsed Deka BMDCs. No cells were observed in any of the other tissue examined including spleen, bone marrow, thymus and LNs (naïve n=1, donor Deka BMDCs n=2 and donor antigen pulsed Deka BMDCs n=2).
Figure 4.2 Enumeration of I.V. injected fluorescently labelled donor Dexa BMDCs and donor antigen pulsed Dexa BMDCs. (a and b) Far Red labelled cells located in the blood and both lungs for donor Dexa BMDCs and donor antigen pulsed Dexa BMDCs respectively (naïve n=1, donor Dexa BMDCs n=2 and donor antigen pulsed Dexa BMDCs n=2).
4.2.3 Ex vivo – rechallenge

Evidenced of detectable active donor-reactive immunity has been correlated with poor allograft outcome and can be associated with the development of acute and chronic graft rejection (Sagoo et al. 2012). Therefore the aim of this experiment was to investigate the adaptive immunological consequences of injecting donor derived Dexa BMDCs using untreated donor BMDCs as a control. To do this a comparison of recipient T cell responder frequencies against donor stimulation to that of a third party (3rdParty) stimulation can be used as a method to detect donor- specific responsiveness and, therefore, determine the presence of donor-reactive immune responses after administration of donor derived BMDCs. It is important to note that this experiment was in naïve animals and not corneal allograft recipients.

On d7 post injection having received untreated donor BMDCs (BMDCs Alone) or donor Dexa BMDCs, lymphocytes isolated form recipients were labelled with CFSE and rechallenged with either donor (DA) or control third party (CD) Ox62+ DCs (αCD3αCD28 beads positive control) (Figure 4.3). The time point d7 was analysed as the pre-clinical corneal allograft transplantation model for this study planned to administer the BMDC cell therapy 7 days prior to transplantation.

Lymphocyte proliferation and activation was measured after 4 days in culture. Results demonstrated no significant increase in proliferation on direct restimulation with donor Ox62+ APCs (proliferation – unstimulated background) for lymphocytes and splenocytes for donor Dexa BMDCs groups compared to naïve controls. Significantly again the untreated BMDCs demonstrated similar effects in vivo as the Dexa BMDCs and donor untreated BMDC demonstrated no significant increase in proliferation (Figure 4.3). This may indicate a level of donor-hyporesponsiveness to both types of
donorBMDC cell injections. The percentage proliferation was not significantly increased even though the donor antigen had been previously “seen” by the recipient.

Analysis of lymphocyte activation for both CD4$^+$ and CD8$^+$ did however, demonstrate a significant increase in the percentage of cells expressing the activation marker CD25 on rechallenge with donor Ox62$^+$ having received either donor BMDCs or Dexa BMDCs. It was therefore examined to see if this increased expression of CD25$^+$ was accompanied by an increase in FoxP3 expression and induction of T reg phenotype. Although not significant there was a trend towards an increased expression of FoxP3 in the CD4$^+$ population in recipients of donor Dexa BMDCs and importantly also for donor untreated BMDCs on rechallenge with DA Ox62$^+$ APCs (Figure 4.4). These results provide evidence that donor derived BMDCs and Dexa BMDCs could potentially induce donor specific hyporesponsiveness and T reg phenotype in vivo which may result in the promotion of allograft survival.
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Figure 4.3 Recipient rechallenge with donor antigen after I.V injection of donor BMDCs and D exe BMDCs. (a) Experimental design. Recipients were injected with 1x10^6 donor BMDCs or Dexe BMDCs I.V., d7 lymph nodes (mesenteric, cervical and submandibular) and spleen (b. and c. respectively) were harvested and cells were restimulated with donor Ox62^+ APC (responder : APC 10:1). After 4 days in culture, (b) lymphocytes and (c) splenocytes were analysed for proliferation (CFSE) and activation of (d) CD4^+ and (e) CD8^+ lymphocyte populations (Mann Whitney two tailed test n=4-8 per group, mean ± SEM *p<0.05, **p<0.01), no significant difference was detected in the spleen.
Figure 4.4 Recipient lymphocytes restimulation with donor antigen after injection of donor BMDCs or Dexa BMDCs show a trend in increased FoxP3 expression. (a)Lymphocytes and (b) splenocytes were analysed for expression of Foxp3 on restimulation with donor and third party antigen (Mann Whitney two tailed test n=4-8 per group, mean ± SEM *p<0.05, **p<0.01).
4.2.4 I.V. administration of donor BMDCs and Dexa BMDCs promotes corneal allograft survival.

The systemic application of donor DA Dexa BMDCs to promote corneal allograft survival again using untreated donor DA BMDCs as a control was initially examined (Figure 4.5). Seven days prior to corneal transplantation recipient LEW rats received an i.v. injection of 1x10^6 cells/ml PBS of donor DA BMDCs or Dexa BMDCs and the clinical and immunological outcomes of these cell injections on corneal allograft survival were evaluated. Untreated allogeneic control groups as previously mentioned rejected uniformly with MST ± s.d. of 18 ± 1.57 days. In contrast, significant prolongation of allograft survival was observed in Dexa BMDC treated groups which had a MST ≥ 30 days (Figure 4.6). Interestingly, a significant prolongation of allograft survival in groups treated with BMDCs was observed. As an additional control 7 days prior to corneal transplantation recipient LEW rats received an i.v. injection of 1x10^6 cells/ml donor BMDCs pretreated with LPS (1µg/ml) (MST ± s.d. of 12 ± 10.62 n=5 days Appendix Figure 8.11).
Figure 4.5 Pre-clinical model of donor untreated BMDCs and Dexa BMDCs administration for prolongation of corneal allograft survival.
Figure 4.6 Prolongation of corneal allograft survival with donor derived BMDCs or Dexa BMDC administration. (a) Graft survival curves of allogeneic transplantation (Tx) control (n=26), syngeneic Tx controls (n=8), donor BMDCs (1x10^6 cells/ml PBS i.v. n=30) and donor Dexa BMDCs (1x10^6 cells/ml PBS i.v. n=34), (Kaplan-Meier survival analysis, n numbers include rats used for experiments in addition to the illustrated survival and opacity analysis above). (b and c) Opacity scores and neovascularisation scores day 4-day 30 of control groups with and without spontaneously accepted graphs, donor BMDCs and Dexa BMDCs treated groups. For (a) a log rank (Mantel-Cox) test was applied p≤0.0001 for donor BMDC and Dexa BMDC treatment.
With both BMDC and Dexa BMDC treatments a significant reduction was observed in corneal allograft opacity compared to untreated recipients, indicating a reduction in cell infiltration and endothelial dysfunction (Figure 4.6). Corneal neovascularisation was not affected by BMDC or Dexa BMDC injection (Figure 4.6). Clinical evaluation of the corneal allografts by light and slit lamp microscopy gave further evidence of the reduction in corneal opacity (Figure 4.7). Furthermore, compared to untreated controls, a significant reduction in infiltration of inflammatory cells was observed by H&E staining on day 18 (average day of rejection) and on day 30 in BMDC and Dexa BMDC treated recipients (Figure 4.8). By day 30, BMDC and Dexa BMDC treated groups showed evidence of a reduction in corneal thickness compared to that of untreated controls (Figure 4.9). These results indicate that single i.v. administration of donor derived BMDCs or Dexa BMDCs, without the use of additional immunosuppressive therapies, is sufficient to promote corneal allograft survival.
Figure 4.7 Evaluation of prolongation of corneal allograft survival with donor derived BMDCs or Dexa BMDC administration by light microscopy. (a) Every other day post transplantation, corneal allograft opacity was evaluated by light microscopy, slit lamp and contrast slit lamp images for all groups, arrows indicate slit lamp reflection in iris visible only in donor BMDCs and Dexa BMDCs treated groups. (b) Enlarged light microscopy, slit lamp and contrast slit lamp images for all groups on day 18 post transplantation.
Figure 4.8 Evaluation of prolongation of corneal allograft survival with donor derived BMDCs or Dexa BMDC administration by histology. H&E stained section of the cornea also illustrate a reduction in corneal allograft cell infiltration which was evident for both BMDC and Dexa BMDC treated groups at day 18 and day 30 points (n=2-5 per group).
Figure 4.9 Evaluation of corneal edema of untreated compared to donor derived BMDCs or Dexa BMDC treated groups. H&E stained section of the cornea were also analysed for presence of corneal edema by measuring the thickness of the corneal graft at day 18 and day 30 and a significant reduction in corneal thickness was evident for Dexa BMDC treated groups by day 30 (Unpaired two tailed student’s test n=2-5 per group, mean ± SEM *p<0.05).
4.2.5 Investigation into the mechanism of donor BMDC and Dexa BMDCs mediated promotion of corneal allograft survival.

To further characterise and reveal a mechanism of donor BMDCs and Dexa BMDCs capacity to promote survival of corneal allografts, the phenotype of the cell populations infiltrating the allograft was quantified and examined. The DLNs and the spleen (Appendix Figure 8.10) were also examined. Corneal transplants were collected from each experimental group at the time point corresponding to the average day of rejection for untreated allogeneic controls (18 ± 1.57 days). Explanted grafts were digested with collagenase D and the single cell suspension was stained and analysed by flow cytometry as described previously (Maenz et al. 2011). A certain amount of T cell activation background due to normal immunological processes in non-sterile animals would be expected, and therefore the main focus of the results is on the DLN rather than the spleen (Appendix Figure 8.10) and the blood. Also the DLNs are known to play an important part in determining the fate of a cornea transplant (Plsková et al. 2002; Yamagami and Amano 2003).

4.2.5.1 Quantification of corneal graft infiltrating lymphocytes

In this study lymphocytes were isolated from transplanted corneal allografts from different treatment groups by enzymatic digestion as previously described and quantified using a haemocytometer and trypan blue exclusion of dead cells. As one would expect with a significant reduction on the level of opacity in treated groups, there was also a significant reduction in the absolute number of cells isolated from the corneal allografts for BMDC and Dexa BMDC treated groups (Figure 4.10).
Figure 4.10 Quantification of corneal allograft infiltrating lymphocytes after donor BMDCs and Dexa BMDCs treatment. Compared to untreated allogeneic controls both cell treatments resulted in a significant reduction in the number of cells found within the corneal allograft on day 18 the average day of rejection (Mann Whitney one-tailed t-test, n=4 per group, mean ± SEM *p<0.05).
4.2.5.2 Phenotype analysis of allograft immunomodulatory milieu after donor untreated BMDCs and Dexa BMDCs administration.

In order to examine the resulting phenotype of the cells infiltrating the corneal allograft after untreated BMDC and Dexa BMDC treatment the donor cornea was excised and prepared as previously described (2.6.5) and the isolated cells were stained for flow cytometric analysis. This analysis revealed that the absolute numbers of CD11b/c⁺, monocyte/ macrophage/ dendritic APCs, were reduced in BMDC and Dexa BMDC groups. Within the CD11b/c⁺ population, both treatments resulted in a significant increase in the frequency (percentage population) of CD11b/c⁺ MHC II⁺CD86⁺ dendritic cells present in the graft (Figure 4.11). There was a significant reduction in the total cell number of B cells (CD45RA) in the cornea for both treated groups (Figure 4.11). A trend in reduced frequency and total cell number of activated NKT (CD3⁻CD8⁺CD161⁺⁺) and activated NK cells (CD3⁻CD8⁻CD161⁺⁺) for both treated groups was observed (Figure 4.12). A significant reduction in the frequency of activated T cells (CD4⁺CD25⁺) was also observed in both BMDCs and Dexa BMDCs treated groups, a trend which was also observed in the total CD4⁺CD25⁺ cell numbers (Figure 4.12). There was a significant increase in the percentage of intragraft regulatory CD4⁺FoxP3⁺ cells within the Dexa BMDC treated group and an overall significant increase in the ratio of FoxP3⁺ regulatory T cells to CD4⁺CD25⁺ activated T cells in both treated groups (Figure 4.13) compared to untreated controls.

The ipsilateral DLNs (submandibular, superficial and deep cervical) and also the spleen were collected from grafted animals and subject to the same flow cytometric analysis as described for the corneal graft (Figure 4.14 and Appendix Figure 8.10). Results obtained indicated there was a trend towards a reduction in CD4⁺CD25⁺ T cells in the draining LNs and an increased ratio of regulatory CD4⁺FoxP3⁺ cells (Figure 4.15). The results obtained from the flow cytometry analysis of the spleen from BMDC and Dexa BMDC
treated animals compared to controls revealed a significant increase in the percentage population of CD11bc\textsuperscript{+}MHC II\textsuperscript{+}CD86\textsuperscript{hi} cells in BMDC treated groups with a trend in the same direction for Dexa BMDC groups. There was no significant difference observed in the percentage population of CD4\textsuperscript{+}FoxP3\textsuperscript{−} cells although a trend towards high percentage population was observed for the treated groups compared to the control (Figure 4.15).
Figure 4.11 Both BMDC and Dexa BMDC administration result in a reduction in percentage and absolute number of graft infiltrating cells. (a) Gating strategy for corneal cell infiltrating analysis. (b) The percentage cell population and total cell number of corneal infiltrating populations of APCs (CD11b/c⁺), DCs (CD11b/c⁻ MHC II⁺ CD86hi) and B cells (CD45RA) were evaluated (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).
Figure 4.12 Both BMDC and Dexa BMDC administration result in a significant reduction in percentage of activated T cells. The percentage cell population, total cell number of corneal infiltrating populations of activated NKT (CD3⁺CD8⁻CD161⁺⁺), NK (CD3⁻CD8⁺CD161⁺⁺) and of activated T cells (CD4⁺CD25⁺) were analysed (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).
Figure 4.13 Both BMDC and Dexa BMDC administration result in an increased ratio of intragraft FoxP3+ expressing cells. The percentage cell and total cell number of and regulatory T cells (CD4⁺FoxP3⁺) was measured. The intragraft ratio of regulatory CD4⁺FoxP3⁺ T cells to activated CD4⁺CD25⁺ T cells was also analysed (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).

A number of pro- and anti-inflammatory cytokines were identified for real time RT-PCR analysis, results of which revealed a significant reduction in the levels of the pro-inflammatory cytokines \textit{Il-6} and \textit{Il-1β} mRNA expression for both the BMDCs and Dexa BMDC groups within the corneal graft (Figure 4.16). \textit{Ifn-γ} mRNA expression was also significantly reduced in Dexa BMDC groups (Figure 4.16). Potential mechanisms involved in prolongation of graft survival in donor BMDC and Dexa BMDC treated groups were investigated further by examining expression of the immunomodulatory molecules IDO, PD-L1 and IL-10 within the corneal graft (Figure 4.16). Intriguingly, there was a profound increase in the level of \textit{Ido} mRNA expression in the corneal graft for both BMDC and Dexa BMDC treated groups (Figure 4.16). \textit{Pd-l1} mRNA expression was significantly reduced in corneal grafts of treated groups, which may be due to the low levels of pro-inflammatory cytokines, such as \textit{Ifn-γ}, expressed within the cornea of these groups as corneal inflammation is known to increase PD-L1 expression (Shen, Jin...
and Freeman 2007). There was no detectable change in the level of II-10 mRNA and although there was significant increase in the ratio of CD4⁺FoxP3⁺ cells within the corneal graft, RT-PCR analysis of Foxp3 mRNA expression did not show any significant increase within the graft.

On investigating the mRNA expression of immunomodulatory molecules in the DLN, a profound increase in the level of Ido mRNA expression for both BMDC and Dexa BMDC treated groups (Figure 4.17) was observed. A significant increase in Foxp3 mRNA was also detected in the DLNs of BMDC and Dexa BMDC treated groups (Figure 4.17).

Consequently, unlike in an untreated control corneal allograft which is highly infiltrated by harmful activated immune cell populations and within which there is significant expression of pro-inflammatory cytokines, the administration of donor BMDCs and Dexa BMDCs promotes an immunoregulatory microenvironment within the corneal allograft itself and the DLNs resulting in prolongation of graft survival.
Figure 4.14 Evaluation of the immune cell populations of the DLNs of corneal allograft recipients after donor BMDCs and Dexa BMDCs treatment. (a) The percentage cell population and total cell number per $1 \times 10^6$ cells from DLN APCs (CD11b/c$^+$), DCs (CD11b/c$^+$MHC II$^+$CD86$^{hi}$) and B cells (CD45RA) were evaluated. (b) The percentage cell population total cell number per $1 \times 10^6$ cells from DLN populations of activated NKT (CD3$^+$CD8$^+$CD161$^{++}$), NK (CD3$^+$CD8$^+$CD161$^{++}$) and activated T cells (CD4$^+$CD25$^+$) were also analysed (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).
Figure 4.15 Both BMDC and Dexa BMDC administration result in an increased ratio of intragraft FoxP3+ expressing cells within the DLNs. The percentage cell and total cell number per 1x10^6 cells of regulatory T cells (CD4+FoxP3+) was measured. The ratio of regulatory CD4+FoxP3+ T cells to activated CD4+CD25+ T cells was also analysed (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).
Figure 4.16 Both BMDC and Dexas BMDC administration results in a significant reduction in corneal allograft mRNA levels of pro-inflammatory cytokines. mRNA analysis of intragraft cytokine expression (normalised to β-actin, fold change relative to untreated allogeneic Tx controls) for pro-inflammatory cytokines Il-6, Ifn-γ and Il-1β and Ido, Pd-11 and Il-10 expression. The mRNA of iNos, eNos, Foxp3 and Il-2 were also measured (Mann-Whitney two-tailed test n=4 per group, mean ± SEM *p≤0.05).
Figure 4.17 Both BMDC and Dextra BMDC administration results in a significant increase in FoxP3 and IDO mRNA expression in the DLNs. mRNA expression within the DLN (normalised to β-actin, fold change relative to untreated allogeneic Tx controls) of the pro-inflammatory cytokines Il-6, Ifn-γ, Il-2, eNos and Il-1β was analysed. The mRNA levels of Foxp3, iNos, Ido, Pd-l1 and Il-10 expression was also examined (Mann Whitney two tailed test n=4 per group, mean ± SEM *p<0.05).
4.2.6 Evaluation of peripheral donor specific unresponsiveness and alloantibody production after donor BMDC and Dexa BMDC administration.

4.2.6.1 Peripheral donor unresponsiveness

To examine the induction of peripheral donor specific unresponsiveness following BMDC and Dexa BMDCs treatment a strategy where long-term surviving grafted animals (day 65 – 80 post transplantation) in BMDC and Dexa BMDC treated groups were rechallenged with donor antigen was devised. I.V. injection of $10 \times 10^6$ Far Red labelled donor splenocytes (or control 3rd party splenocytes) mixed with $10 \times 10^6$ GFP+ve syngeneic splenocytes for a total of $20 \times 10^6$ cells/ml PBS was performed, naïve ungrafted animals were used as a control group (Figure 4.18). The rate of clearance of these labelled cells from the circulation for all groups, 15hrs – 4 days post injection was examined by flow cytometry (Figure 4.19). Both control 3rd party and syngeneic splenocytes were detected in all groups and were cleared from the blood at a similar rate in all groups (Figure 4.19). Unlike naïve groups where donor splenocytes were detected and cleared from the blood at a similar rate to that of 3rd party splenocytes, results from grafted BMDC and Dexa BMDC treated groups injected with syngeneic and donor derived splenocyte cell mix, revealed that only the syngeneic cells were detectable in these groups 15hrs post injection (Figure 4.19). This indicated that donor cells were cleared at a faster rate than that of 3rd party or syngeneic cells in BMDC and Dexa BMDC treated groups compared to that of naïve. Analysis of injected cell distribution within LNs, spleen and lung on day 4 illustrates that while syngeneic splenocytes persist in all groups, by day 4 there is clearance of 3rd party and donor derived cells from all groups (Figure 4.19). It is important to note that during a period of up to 4 days post rechallenge of grafted animals with donor splenocytes the graft itself remained clear and did not reject.
Figure 4.18 Illustration of experimental design for evaluation of tolerance induction (including gating strategy). Animals with surviving grafts were rechallenged with donor splenocytes. A mixture of fluorescently labelled donor (or 3rd party control) and syngeneic splenocytes were injected I.V. and blood (15hrs –d4) and organs (d4) were analysed for the presence of fluorescently labelled cells.
Figure 4.19 Effects of donor BMDC and Dexa BMDC administration on peripheral donor specific unresponsiveness. (a) Day 65-day 80 post transplantation long-term allograft surviving and naïve ungrafted LEW rats were injected i.v. with a total of 20x10⁶ labelled Far Red donor (DA) + GFP syngeneic (LEW) splenocytes or Far Red 3rd party (CD) + GFP syngeneic cells. Blood samples were collected from naïve ungrafted, BMDC and Dexa BMDC grafted treated groups 15hrs-day 4 post injection and analysed for the detection of labelled cell populations. On day 4 post injection of labelled cells LNs (submandibular, cervical and deep cervical), spleen and lung were also harvested (mean ± SEM n=2-4 per group). (c) Clinical evaluation of corneal allograft opacity in long-term allograft graft survivors day 4 post injection of donor derived splenocytes (n=3-4 per group).
4.2.6.2 Serum alloantibody production

To further examine the mechanisms involved in the corneal allograft survival after donor BMDC and Dexa BMDC treatment the humoral response was also studied. The results from donor splenocyte rechallenge and the subsequent rapid clearance of the donor splenocytes from the circulation of treated groups indicated the presence of donor specific alloantibodies. Thus, the serum from BMDC and Dexa BMDC treated groups was analysed for the presence of donor specific alloantibodies on day 4 - 14 post transplantation (Figure 4.20) and then again at the time point corresponding to the average day of rejection for untreated allogeneic controls day 18 (Figure 4.20).

The IgM response for all groups did not significantly differ, however, there was a significant increase in the levels of IgG1 and IgG2 antibodies recognising donor antigen in serum from BMDC treated group compared to untreated controls (Figure 4.20). The Dexa BMDCs response was significantly reduced compared to BMDCs treated group and the IgG1 and IgG2 response of Dexa BMDC treated groups remained similar to that of untreated naïve animals until the later time points i.e. day 14/18 at which time it was significantly higher (Figure 4.20).

Again, although there are detectable levels of donor specific alloantibodies and a clearance of the injected donor splenocytes, crucially, the grafts themselves remained clear and were not rejected suggesting some level of local graft protection.
Figure 4.20 Effects of donor BMDC and Dexa BMDC administration on donor specific alloantibody response. (a) Schematic of experimental design for analysis of anti-donor antibody production. (b) Differential levels of anti-donor antibodies detectable in both BMDC and Dexa BMDC treated groups on day 4 – day 14 post transplantation and (c) day 18 (average time point of rejection), (Mann Whitney two tailed test n=3-4 per group, mean ± SEM *p<0.05, **p<0.01).
4.2.6.3 Glycome characterisation of ex vivo-generated BMDCs and Dexa BMDCs.

In order to fully characterise the phenotype of ex vivo-generated BMDCs before and after treatment with Dexa and to potentially explain the difference observed in the alloantibody response between groups administered untreated BMDCs and Dexa BMDCs the glycocalyx, in particular the glycoprotein profile of the BMDCs was studied. Using the fluorescently tagged glycan-binding proteins or lectins Wheat germ agglutinin (WGA), Maackia amurensis (MAA) and Sambucus Nigra (SNA) which recognise and bind to specific types of glycosylation and glycosylation linkages the glycoprotein profiles of BMDCs and Dexa BMDCs were examined using flow cytometry. It has been previously described that DC maturation leads to changes in glycan expression and that regulatory DCs have a characteristic glycan expression pattern (Bax et al. 2007).

Results from this analysis demonstrated that the Dexa BMDCs had a uniquely different glycosylation pattern to BMDCs alone. Although BMDCs and Dexa BMDCs expressed similar levels of N-acetylglucosamine and α 2,3-sialic acid linkages in quiescent states the α 2,3-sialic was significantly increased in the Dexa BMDC population after LPS stimulation compared to BMDCs. Interestingly the α 2,6-sialic acid linkage was significantly increased in the Dexa BMDC in unstimulated and LPS stimulated cultures compared to BMDCs (Figure 4.21). This α-2,6 sialic linked glycan linkage is characteristic of a tolerogenic, immature DC and expression of which is also known to be reduced on DC maturation (Erbacher et al. 2009). The differential glycome patterns may in part explain the difference observed in antibody production, moreover, the donor alloantibody results may explain the results observed in BMDC and Dexa BMDC peripheral clearance of donor splenocytes.
Figure 4.21 Glycome characterisation of ex vivo-generated BMDCs and Dexa BMDCs. (a) Representative histograms of glycan expression of BMDCs (black line) and Dexa BMDCs (black dashed line). (b) Differences in expression of cell surface glycans; N-acetylglucosamine, α2,3 linked sialic acids and α2,6 sialic linked acids were analysed using plant lectins WGA, MAA and SNA-I (respectively), (Unpaired one-tailed student’s t-test, n=3, mean ± SEM *p≤0.05).
4.2.6.4 I.V. administration of syngeneic Dexa BMDCs donor antigen pulsed.

Therapies using donor derived BMDCs will be difficult to implement using deceased donors for application in transplantation models such as renal or cardiac etc. However, in the case of cornea transplantation, donated corneas can be stored for up to one week without impacting graft survival (Simon et al. 2004), giving time for in vivo propagation of the BMDCs. Nevertheless, i.v. injections of syngeneic Dexa BMDCs pulsed with donor antigen was also investigated as potential cell therapy to prolong corneal allograft survival. Results from these studies did not however, show prolongation of the corneal allograft with administration of this treatment (MST 14 ± 7.16 days n=5) (Figure 4.22). Out of five treated, one recipient had MST day 30 with the remaining 4 rejecting uniformly on day 14.
Figure 4.22 I.V. administration of Syngeneic Dexa BMDCs donor antigen pulsed. (a) Graft survival curves of allogeneic transplantation (Tx) controls (n=26), syngeneic Tx controls (n=8), syngeneic Dexa BMDCs pulsed with donor antigen (1x10^6 cells/ml PBS i.v. n=5, Kaplan-Meier survival analysis). (b and c) Opacity scores and neovascularisation scores day 4-day 30 of control groups and syngeneic Dexa BMDCs pulsed with donor antigen group. For (a)log rank (Mantel-Cox) test was applied.
4.2.7 Subconjunctival administration of Dexa BMDCs.

This thesis study examined two possible routes of administration the second of which was local application via subconjunctival injection. As the DLNs are known to play an important part in determining the fate of a cornea transplant (Yamagami and Amano 2003); a more local application to target these LNs was investigated. Preliminary subconjunctival injection experiments employed the use of a dye, Chicago Sky Blue, of which 50µl was injected into the conjunctiva of LEW rats. After 24hrs it was observed that with local subconjunctival injections the dye drained into the superficial lymph nodes, the deep cervical lymph nodes, with low amount of dye observed in the submandibular lymph node (Figure 4.23). Others have also demonstrated that injections into the subconjunctiva drain to the submandibular LN (Dullforce et al. 2004).
Figure 4.23 Targeting the DLNs of the eye via subconjunctival injections. Administration of 50µl of Chicago Blue dye via the conjunctiva. After 24hrs it was the DLNs (submandibular, superficial and deep cervical) were examined for the presence of the dye (n=2).

The following results describe the localisation of syngenic d10 BMDCs from GFP+ve transgenic rats and donor antigen pulsed GFP+ve BMDCs which were injected locally into the conjunctiva of naïve LEW rats. After d3 the eye and the surrounding conjunctiva were harvested, digested and homogenised and analysed for the presence of GFP+ve cells. Results demonstrated that GFP+ve cells could be located in the eye and the conjunctiva on d3 after injection (Figure 4.24). The DLNs were also analysed for the presence of GFP+ve cells however, GFP+ve BMDCs were only observed in one of the two animals injected for each condition.
Figure 4.24 Local subconjunctival injection of GFP+ve BMDCs in naïve LEW rats. GFP+ve LEW BMDCs 1x10^6 cells/75µl were injected into the subconjunctiva of LEW rats. After d3 the a) eye and conjunctiva and (b) DLNs were homogenized followed by flow cytometric analysis demonstrated localisation of GFP+ve cells in these tissues after subconjunctival injections (n=1 naïve, n=2 GFP+ve BMDCs and n=2 donor antigen pulsed GFP+ve BMDCs).
Next the local subconjunctival injections of fluorescently labelled donor Dexa BMDCs and donor antigen pulsed syngenic Dexa BMDCs (2\times10^5 cells/50\mu l PBS) were examined. Donor Dexa BMDCs and donor antigen pulsed syngenic Dexa BMDCs were labelled with Far Red dye and a system using fluorescently labelled beads as previously described was employed to enumerate the cells found in the tissues analysed by flow cytometry (Figure 4.25). Similarly as was observed for the injections of syngeneic BMDCs, Far Red labelled donor antigen pulsed syngenic Dexa BMDCs and donor derived Dexa BMDCs were observed in the eye and conjunctiva however, there were no BMDCs observed in the submandibular or cervical lymph nodes for both Dexa BMDCs and donor pulsed Dexa BMDCs.
Figure 4.25 Localisation of subconjunctivally injected fluorescently labelled donor Dtexa BMDCs and donor antigen pulsed Dtexa BMDCs. (a) Far Red labelled cells (2x10^5 cells/50µl PBS) injected subconjunctivally 24hrs post injection tissues were harvested, homogenised and analysed by flow cytometry for the presence of Far Red labelled cells. BMDCs were found in the eye and conjunctiva but no cells could be identified in the DLNs. (b and c) Enumeration of Far Red labelled cells located in the eye and conjunctiva for donor Dtexa BMDCs and donor antigen pulsed Dtexa BMDCs respectively (naïve n=1, donor Dtexa BMDCs n=2 and donor antigen pulsed Dtexa BMDCs n=2).
Next the capacity of subconjunctivally injected Dexa BMDCs and donor antigen pulsed syngenic Dexa BMDCs to modulate allogeneic immune responses in vivo was examined DA (donor)/LEW (recipient) corneal transplantation model. Initial experiments examined the administration of syngeneic Dexa BMDCs pulsed with donor antigen and donor Dexa BMDCs applied locally by subconjunctival injections (Figure 4.26). Seven days prior (d-7) to corneal transplantation recipient LEW rats received a subconjunctival injection of $2.5 \times 10^5$ BMDCs in 50 µl PBS and clinical outcomes of these cell therapies on corneal allograft survival were evaluated.

Untreated allogeneic control groups rejected uniformly with a MST ± s.d. of 18 ± 1.57 days. Groups treated with syngeneic BMDCs pulsed with donor antigen rejected their grafts with a MST similar to untreated controls (18 ± 6.57 days n=5). However, although not significant (Mantel-Cox test) in groups treated with donor Dexa BMDCs subconjunctivally had a MST of 30 ± 8.01 n=7. Of the 7 animals treated n=4 had a MST of 30 ± 0 days and n=3 had a MST 17 ± 2.89 days (Figure 4.26). Significance may be observed with increased numbers and the results and variation in survival from this group may reflect the technical difficulties with the route of administration rather that the cell therapy itself being ineffective.

The level of neovascularisation was the same for all groups for the period of the study however; there were differences in the level of opacity. Groups treated with donor Dexa BMDCs demonstrated a significantly higher level of opacity between days 6 to 12 post transplantation, however this level of opacity never increased beyond an average of 1.9 (Figure 4.26). Interestingly, the pattern of opacity for syngeneic Dexa BMDCs pulsed with donor antigen was similar to that of untreated allogeneic controls for the same time period. Nevertheless, form day 12 onwards these animals rapidly rejected their transplanted corneas which was coupled with rapid increased opacity levels similar to that observed for untreated allogeneic controls.
Figure 4.26 Administration of donor Dexa BMDCs and syngeneic Dexa BMDCs pulsed with donor alloantigen for prolongation of corneal allograft survival. (a) Graft survival curves of allogeneic transplantation (Tx) controls (n=26), syngeneic Tx controls (n=8), donor Dexa BMDCs (2.5x10^5 cells/50µl PBS n=7) and syngeneic Dexa BMDCs pulsed with donor antigen (2.5x10^5 cells/50µl PBS n=5) and, (Kaplan-Meier survival analysis). (b and c) Opacity scores and neovascularisation scores day 4-day 30 of control groups, syngeneic Dexa BMDCs pulsed with donor antigen and Dexa BMDCs treated groups.(d) Average opacity score for the period day 6-12 post transplantation (a)log rank (Mantel-Cox) test was applied, (d) Mann Whitney two tailed, n=5-26 mean ± SEM, ***p<0.001).
4.3 Discussion

4.3.1 Therapeutic Efficacy of Regulatory BMDCs.

There is a substantial body of work which demonstrates with mixed efficacy, that the application of donor derived DC therapy alone or with additional immunosuppressive therapies prolongs allograft survival and promotes transplantation tolerance (Stax et al. 2008; Alawieh et al. 2010; Pothoven et al. 2010; Hattori et al. 2012; de Kort et al. 2012; Gao et al. 2013; Khan et al. 2013). This study aimed to examine the therapeutic effects of administration of ex vivo-generated regulatory-BMDC on the promotion of corneal allograft survival. For this, a corneal allograft transplantation model using the high-responder allogeneic strain combination of LEW recipients to DA donors was selected (Nosov et al. 2012; de Kort et al. 2012).

Treatment of allograft recipients with donor derived Dexa BMDCs significantly prolonged corneal allograft survival and interestingly, a significant prolongation of allograft survival in groups treated with untreated donor derived BMDCs was also achieved (Figure 4.6). One contributing factor for the promotion of allograft survival is the maturation status of BMDCs at the time of injection, which is a key determinant of transplantation outcomes. Therefore, it is important to note that the BMDCs injected in allograft recipients were in a semi-mature phenotypic state and not a fully (LPS-treated) matured BMDCs as indicated by low expression of co-stimulatory molecules. Recipients of LPS matured donor BMDCs subsequently reject their allograft (Appendix Figure 8.11) an observation made during this study and by others (Hattori et al. 2012). It is believed that injection of a semi-mature DC into a pro-inflammatory environment would risk the induction of donor antigen sensitisation rather than acceptance (Lutz et al. 2000) however, in this study the untreated BMDCs were injected into a quiescent immune environment 7 days prior to transplantation. This may explain why untreated
donor BMDCs, which in the previous chapter were demonstrated to be immunoregulatory and express significant immunomodulatory molecules (Figure 3.10 and Figure 3.14), were able to promote transplant survival rather than inducing rejection. The treatment of BMDCs with the glucocorticoid Dexa resulted in a maturation-resistant phenotype as was observed in the unchanged levels of expression of maturation markers and a significant reduced level of pro-inflammatory cytokines unlike LPS stimulated BMDCs (Figure 3.8 and Figure 3.9). As the results from the previous chapter and also this chapter including the ex vivo re-challenge experiments (Figure 4.3) suggest, as well as the Dexa BMDCs having potential immunoregulatory capabilities the untreated BMDCs also display a significant immunoregulatory capacity. Both cell therapies immunoregulatory capacity is sufficient to promote corneal graft survival.

It is interesting to note that prolongation of corneal allograft survival was not achieved with a systemic injection of syngeneic donor antigen pulsed Dexa BMDCs (Figure 4.22). The efficacy of donor BMDCs and Dexa BMDCs in prolonging corneal allograft survival may in part be due to the direct immunomodulation of donor specific T cells by induction of cell anergy and hyporesponsiveness as was observed in the in vitro assays (Figure 4.3). However, recent articles suggest that maybe in fact the recipient DC processing of donor derived cell therapies and immunomodulation of the in-direct and semi-direct pathways by the recipient DCs that play a significant role in the induction of allograft survival (Wang et al. 2012). The injected donor derived cells whether unmodified or treated with Dexa would therefore serve as donor antigen transporting cells that are processed into alloantigen by the recipient (Divito et al. 2010; Kheradmand et al. 2012; Wang et al. 2012). Groups which received syngeneic alloantigen pulsed BMDCs were unable to prolong allograft survival as it may be that there is insufficient quantities of donor antigen available to the recipient to promote corneal allograft survival. Unlike in the case of donor derived BMDC and Dexa BMDC treated groups where significantly more donor antigen is available to the recipient. Prolongation of survival was not observed with LPS stimulated donor BMDCs so the maturation status
of the injected BMDCs as well as the quantity of donor antigen available is an important factor.

It is well accepted that the immune response may be customised to the organ in which the response is initiated, as well as being specialized for the region in which it has to function. To date, there has not been a detailed analysis in the cornea transplant model on the allograft infiltrating cell populations after treatment with donor derived BMDCs or Dexa BMDCs. The present chapter demonstrates clearly that the application of donor BMDCs leads to an induction of an intragraft immunoregulatory environment which favours graft acceptance. Although immuno-histochemistry has been the method of choice for studying graft infiltrating lymphocytes, immuno-histochemistry method is difficult to establish and laborious. For this study however, a method using flow cytometric analysis of graft infiltrating cells and secondary lymphoid organs was employed (Maenz et al. 2011) to describe the local immune cell populations within the allograft and of the DLNs after BMDC and Dexa BMDC treatment.

4.3.2 Immunological Response to Regulatory BMDC Therapies.

Studies have demonstrated that cell-mediated immunity plays a decisive role in corneal graft rejection (Niederkorn 2012; Tan and Abdulreda 2013). Prevention of corneal allograft rejection was achieved by treating graft recipients with anti-CD4 antibodies, a result further supported by evidence that CD4-deficient mice are severely impaired in their ability to reject corneal allografts (He et al. 1991; Yamada et al. 1999). In contrast the ability of corneal allograft recipients to reject their graft remained unimpaired in mice deficient in CD8$^+$ T cells which may indicate that CD4$^+$ cells are required for corneal allograft rejection (He et al. 1991; Yamada et al. 1999; Tan and Abdulreda 2013). Flow cytometric analysis of the transplanted corneal tissue during this study
revealed a significant reduction in the percentage population of intragraft activated CD4\(^+\)CD25\(^+\) T cells which was accompanied by a profound reduction in the total number of CD4\(^+\)CD25\(^+\) T cells and a significantly increased ratio of CD4\(^+\)Foxp3\(^+\) cells to activated CD4\(^+\)CD25\(^+\) T cells in both BMDC treated groups (Figure 4.12 and Figure 4.13). It is likely that these CD4\(^+\)FoxP3\(^+\) cells, along with the significantly increased expression of Ido (Figure 4.16), which is known to promote and maintain a regulatory T cell phenotype (Baban et al. 2011; Sucher et al. 2012), play a key role in inducing and promoting survival of the corneal allografts.

Results also revealed not only an increase in the ratio of CD4\(^+\)FoxP3\(^+\) in the DLNs but also a significant increase in the level of Foxp3 mRNA expression for both BMDC and Dexa treated groups (Figure 4.15 and Figure 4.17). It has been reported that an increase in Foxp3 mRNA expression in the DLNs of corneal allograft accepting mice was an important indicator of graft acceptance, with the same group also identifying that regulatory T cells isolated from the DLNs of allograft acceptors, when adoptively transferred into grafted mice were able to prevent allograft rejection (Chauhan and Saban 2009).

A profound reduction in the numbers of NK and NKT cells after BMDC and Dexa treatment was also detected (Figure 4.12). Evidence now shows that the cells of innate immunity such as NK and NKT cells play a key role during corneal allograft rejection (Nosov et al. 2012). High numbers of cells expressing NK-markers, that appear to have alloreactivity, have been located in other animal models of corneal transplantation as well as in human samples of aqueous humor (Claerhout et al. 2004; Flynn et al. 2008). This study also demonstrated a trend in a reduction in the numbers of NK and NKT cells in the DLNs of treated animals. Prolongation of graft survival has been established in models where treatment significantly impacts the level of NK and NKT cell infiltration, as was observed in work by Nosov et al. where corneal allografts
engineered to overexpress PD-L1 resulted in a reduction in graft infiltrating NK and NKT cell populations (Nosov et al. 2012).

There was also a significant reduction in the total cell number of B cells (CD45RA) in the cornea for both treated groups (Figure 4.11). Numbers of B cells were also reduced in the DLNs (Figure 4.14). Others have suggested that B cells do not appear to play an key role in corneal allograft rejection however they have been linked to play a role in the induction and maintenance of ACAID (Yamada et al. 2001; Skelsey et al. 2003; Tan and Abdulreda 2013). These B cells present antigen to CD8+T cells promoting their differentiation into suppressor cells which inhibit the expression of DTH (Skelsey et al. 2003).

The observation of coincidental increases of CD11b/c+MHC II+CD86+ and Ido expression within the allografts of both treatment groups is strengthened by recent studies which demonstrated that IDO expressing DCs are required for promotion of graft survival in various transplantation models (Cook et al. 2008; G. Chen et al. 2012; Sucher et al. 2012; X. Sun et al. 2012). Anti-inflammatory cytokines, together with CTLA4, constitutively expressed on the FoxP3+ regulatory T cell surface interacts with CD80/86 molecules expressed on DCs to induce within the DC, production of multiple different enzymes that consume essential amino acids (Cobbold et al. 2010; McGrath and Najafian 2012). Similar to what has been described for other transplantation models, the immunomodulatory microenvironment created within the corneal allograft of BMDC and Dexa BMDC treated groups, allows FoxP3+ cell interaction with DCs, inducing expression of IDO that can locally deplete tryptophan and may therefore play an important role in limiting T cell proliferation and effector function within the graft (Cobbold et al. 2010; McGrath and Najafian 2012; Munn and Mellor 2013). The observed increased ratio of CD4+FoxP3+ cells in the DLNs and also the significant increase in the level of Foxp3 and also Ido mRNA expression for both BMDC and Dexa
treated groups is an important indicator of graft acceptance (Chauhan and Saban 2009; Hattori et al. 2012).

In this study despite prolonging corneal allograft survival, donor BMDC and Dexa BMDC treatment was not effective at inducing peripheral donor specific unresponsiveness (Figure 4.19). Although previous work has described cells, with similar phenotypic and functional characteristics as demonstrated during this study, as “tolerogenic” DCs this study did not observe tolerance induction (Moreau et al. 2009; Hattori et al. 2012). Recent reports have described sensitisation of the recipient to donor antigen with pre-treatment of donor derived Dexa BMDCs (de Kort et al. 2012; Smyth et al. 2013). The allo-antibody response was however, significantly reduced in animals with Dexa BMDC treatment (Figure 4.20), which may be due to the immunomodulatory phenotype and glycome profile of the BMDCs after Dexa treatment (Figure 4.21). This may be an advantage to the use of Dexa BMDCs over BMDCs as treatment of BMDCs with Dexa may allow repeat administration of the cell therapy if required. Unlike the aforementioned reports which demonstrate accelerated rejection of the allografts (de Kort et al. 2012; Smyth et al. 2013), in our corneal allograft model, the allografts remained protected and were not rejected (Figure 4.19).

Although cell-mediated immunity is believed to play the dominant role in corneal graft rejection, the role of antibody-mediated rejection is controversial (Hegde et al. 2002; Holán et al. 2005; Niederkorn 2012). Corneal cells themselves are known to express extensive levels of cell membrane bound CRPs which are also found in a soluble form in the AH (Bora et al. 1993). Therefore, the level of alloantibodies produced may not be sufficient to override the CRPs expressed by and which protect the cornea from a humoral immune response. The immunomodulatory environment generated by treatment with donor BMDCs or donor Dexa BMDCs may protect the corneal allograft from cell mediated immunity which in turn may promote the prevention of rejection of the cornea by complement-fixing alloantibodies.
In one murine corneal allograft study untreated recipient mice having received and accepted an allogeneic corneal allograft, were further challenged with donor skin allografts. The recipients rejected the orthotopic skin grafts, but the corneal grafts themselves remained clear and healthy (Yamada et al. 1999). Furthermore, the same donor challenge was repeated in CD4 knock-out mice which had also accepted their corneal allografts. These mice not only rejected the donor skin allografts, but they also proceeded to reject their long-standing corneal grafts. This indicated that although CD4$^+$ cells can mediate corneal allograft rejection a sub population of CD4$^+$ cells, possibly T regs, are required to maintain graft survival (Yamada et al. 1999). The results from this chapter suggest that a form of regulatory, local protection develops within the corneal allografts of BMDC and Dexa BMDCs treated groups including the increased ratio of CD4$^+$Foxp3$^+$ cells and mRNA expression of *Ido* within the graft itself but significantly also in the DLNs.

### 4.3.3 Alternative Therapeutic Approaches.

As it has been established that the DLNs play an important part in determining the outcome of a cornea transplant (Plsková et al. 2002; Yamagami and Amano 2003); local administration of BMDC cells to target the DLNs was also investigated. Preliminary experiments employing dye and fluorescently labelled BMDCs which were injected into the conjunctiva indicated that this could be explored as feasible approach (Figure 4.23). This approach was technically challenging due to the nature of the area being injected and the volume required for low viscosity in the cell suspension. Transplanted groups which on day -7 received a subconjunctival injection of syngeneic Dexa BMDCs pulsed with donor alloantigen did not show increased survival of their allografts (Figure 4.26). However, although not significant groups treated subconjunctivally with donor Dexa BMDCs had a MST of 30 days (Figure 4.26). Four of the animals treated had a MST of 30 days and three a MST of 17 days. With increased numbers for this treatment
significance may have been achieved. As was observed in the preliminary experiments not all of the injected cells make it to the DLNs all of the time due to the challenges with this type of injection. It may be that due to the nature of the injection, not all animals received the optimum cell dose required to promote survival and large group numbers or multiple injections are required to observe a significant effects of this cell phenotype injected locally into the conjunctiva.

4.3.4 A Novel Therapeutic Application.

The eye is well-defined as an immune-privileged organ (Stein-Streilein 2008; Taylor 2009; Hori, Vega, et al. 2010; Niederkorn and Stein-Streilein 2010; Masli and Vega 2011) and thus there may be differences in the immune responses to donor antigen in a cornea allograft model after i.v. administration of donor derived BMDCs and Dexa BMDCs compared to other transplantation models. In conclusion, the results from this chapter have clearly demonstrated the significant observation that not only ex vivo-generated donor Dexa BMDC therapy has the sufficient immunomodulatory properties to significantly prolong corneal allograft survival but so too does an unmodified donor derived BMDC therapy. Interestingly, the local application of donor derived Dexa BMDCs appears also to modulate corneal allograft survival but further investigation is required. Systemic administration of unmodified donor derived BMDC and Dexa BMDCs induces a local immunoregulatory microenvironment capable of suppressing the induction of allo-immunity in the DLNs resulting in protection of the corneal allograft. These results demonstrate a novel therapeutic application for donor derived BMDCs with and without glucocorticoid treatment for the prevention of corneal allograft rejection.
4.3.5 Chapter Summary

Our interpretation of the work analysed and presented in this section can be summarised as follows:

- Ex vivo-generated donor Dexa BMDCs have sufficient immunomodulatory properties to significantly prolong corneal allograft survival.
- Donor-derived untreated BMDCs also prolong corneal allograft survival.
- Administration of syngeneic Dexa BMDCs pulsed with donor and LPS mature donor BMDCs fail to promote survival demonstrating the importance of availability of donor antigen and the maturation status of BMDC therapies.
- Both cell therapies fail to induce peripheral donor specific unresponsiveness but did induce a local immunoregulatory milieu within the allograft and DLNs resulting in protection of the corneal allograft.
- The potential in the local administration of BMDCs via subconjunctival injections of donor derived BMDCs for promotion of corneal allograft survival.
- Donor derived BMDCs with and without glucocorticoid treatment are novel therapeutic applications for the prevention of corneal allograft rejection.
5. Discussion

The discussions of the preceding chapters focus mainly on the aspects of BMDC biology and therapeutic properties which relate to the results obtained during this project. In the following chapter some of the themes and issues raised during this study are critically analysed and further developed. Suggestions are also made on how these issues could be addressed with future work. This chapter also discusses the potential development of BMDC therapies from a pre-clinical to clinical application.

5.1 Defining Phenotype and Function.

One of the key themes which this study explores is the relationship between BMDC phenotype and function. DCs are not only defined by their phenotype but also by their ability to deliver distinctive immunogenic or regulatory signals to T cells. The view that DCs exist in two basic states; a mature state inducing immunity to foreign antigens and an immature state inducing tolerance to self, has been challenged and to accommodate this the terminology has evolved to include terms like semi-mature, maturation-resistant etc. (Sousa 2006). Defining a BMDC population by both its phenotypic maturation status and its effector function is of particular importance in the context of immunoregulation.

As this study and others have clearly demonstrated the classic immature and maturation-resistant BMDCs have regulatory properties but so do BMDCs with a more mature phenotype (Scheinecker et al. 2002; Gao et al. 2013). This study demonstrated ex vivo-generation of BMDCs from GM-CSF and IL-4 treated bone marrow cultures. The resulting BMDCs which express high levels of MHC II and moderate expression of
CD80/CD86 produced low levels of pro-inflammatory cytokines such as TNF-α (Figure 3.8 and Figure 3.9). Sato et al. have also described a similar phenotype of expanded DC populations with high levels of MHC class II, but low levels of co-stimulatory molecules (Sato et al. 2003). The marked difference between the phenotype of these BMDCs and fully matured (LPS stimulation) BMDCs mean the term semi-mature proposed by Lutz and Schuler to describe regulatory DCs is fitting (Lutz and Schuler 2002). Due to the correlation with T cell priming and expression of maturation molecules it is assumed that these phenotypically semi-mature BMDCs are also functionally mature however, this is not always the case as clearly demonstrated during this body of research (Figure 3.15). A semi-mature phenotype combined with a higher expression of inhibitory co-stimulators and the production of IDO, IL-10 or NO facilitates a regulatory function of semi-mature BMDCs rather than an immunogenic one.

This semi-mature phenotype may be more reflective of ex vivo-generated human DCs from peripheral blood monocytes (Mo-DCs) (Jonuleit et al. 2000). In the Jonuleit et al. study human immature Mo-DCs are used which have reached a developmental stage that is similar to the semi-mature DCs derived from murine BM. Interestingly they report that the immature allogeneic Mo-DCs do not induce anergy, rather induce a regulatory T cell phenotype in vitro, similarly to the in vitro results observed during this study (Figure 3.15) (Jonuleit et al. 2000).

### 5.2 In vivo Application of Regulatory BMDCs.

This study investigated donor derived BMDCs for their potential to prolong corneal allograft survival as there was growing evidence that donor derived DCs were able to induce alloantigen specific T cell hyporesponsiveness or anergy in vitro and promote the
survival of allografts (O’Connell et al. 2002; Luo et al. 2008; Stax et al. 2008; Alawieh et al. 2010; Pothoven et al. 2010). At the time of commencing this study donor derived BMDCs had not been investigated for their therapeutic application in corneal transplantation. However, recently there have been studies which have also demonstrated the efficacy of donor derived BMDCs in corneal transplantation (Hattori et al. 2012; Gao et al. 2013).

Unlike this body of research work which administered donor untreated BMDCs and Dexa BMDCs, the study by Hattori et al. examined the administration of immature regulatory DCs generated by treatment of donor derived DCs cultures with IL-10 and TGF-β. Their results demonstrate that IL-10 and TGF-β treated donor DCs prolong corneal allograft survival by suppressing the indirect pathway of allo-sensitisation as well as the direct pathway. This correlated with a decreased IFN-γ+ T cell frequency and a twofold increase in FoxP3 levels in the DLNs (Hattori et al. 2012). The authors conclude that donor-derived, “tolerogenic” DCs significantly suppress the indirect pathway however, tolerance to the donor antigen is not demonstrated within this study. Although graft survival was prolonged nevertheless, the authors do not examine the in vivo immune response upon rechallenge with donor antigen or examine the effect on the humoral immune response to the donor-derived DCs. The second study by Gao et al. uses a model of high risk corneal transplantation by inducing neovascularisation by creating an alkaline burn on the cornea of the transplant recipient. They administered immature BMDCs which were harvested from a GM-CSF and IL-4 treated bone marrow culture on day 6 without further modification. The authors observed prolongation of allograft survival and also reported that this strongly correlated with the significant increase in FoxP3 expression within the spleen (Gao et al. 2013). The Gao et al. study also failed to examine the in vivo cellular and humoral immune responses to donor-derived BMDCs. It would have also been interesting if the authors had examined the level of FoxP3 expression within the DLNs of grafted animals.
A recent publication by Khan et al. also investigates the application of donor derived BMDCs in murine corneal transplantation however, the authors used genetically modified BMDCs. Using CTLA4-KDEL-expressing BMDCs Khan et al. demonstrated that these genetically modified BMDCs could induce anergy in alloreactive T cell populations and generate CD4^+CD25^+ and CD4^+CD25^- T regs both in vitro and in vivo. However, the T cell unresponsiveness induced by BMDCs transduced to overexpress IDO although suppressive lacked donor specificity. The authors suggest that the effect of IDO can vary, with low levels inducing antigen specific anergy and regulation, and higher levels resulting in non-specific hyporesponsiveness and death (Khan et al. 2013). Interestingly, long-term graft survival was only observed in animals treated with the CTLA4-KDEL-expressing BMDCs (Khan et al. 2013).

One of the primary focuses of this research work was to explore the application of genetically modified BMDCs for application in rat corneal allograft survival however, the difficulties as previously described arising from this experimental approach were underestimated. Even though there may be safety concerns with regard to viral gene therapy vectors and their clinical application, a recent phase I trial using genetically modified DCs to express low co-stimulatory molecules has supported the safe application of genetically modified tolerogenic DCs for the treatment of T1D (Giannoukakis et al. 2011). Nevertheless, alternatives to gene modification to generate immunoregulatory BMDCs were investigated. This included the administration of syngeneic donor antigen pulsed regulatory BMDCs in corneal transplantation.
5.3 Examining Alternative Sources of BMDCs.

The systemic or local administration of recipient derived Dexa BMDCs pulsed with alloantigen did not prolong corneal allograft survival in this study although others have seen efficacy using alloantigen pulsed BMDCs in various models of transplantation (Horibe et al. 2008; Ikeguchi et al. 2008; Kuo et al. 2009; Ouyang et al. 2010; Wang et al. 2012). A study by Menges et al. using peptide-pulsed DCs matured with TNF-α demonstrated the induction of IL-10-producing peptide-specific T cells in vivo and antigen specific protection from EAE. The same was not observed when immature DCs or DCs matured with LPS and CD40L were injected (Menges et al. 2002). Interestingly Dexa treated F1 derived DCs have also been shown to induce indirect allo-specific tolerance, however, additional immunosuppressive therapy was required in the form of a single dose of CTLA4-Ig to prevent sensitisation caused by representation of donor DC-derived alloantigen (Mirenda 2004). During the experiments described in this thesis with recipient-derived BMDCs, no additional immunosuppressive therapies were administered so the cell therapies may have failed due to this recipient sensitisation (Figure 4.22).

Recent reports by Smyth et al. and Kort et al. have highlighted the risks in recipient sensitisation with pre-treatment of donor “tolerogenic” BMDCs resulting in allograft rejection in models of islet and skin transplantation respectively (de Kort et al. 2012; Smyth et al. 2013). Such results may merit further investigations into the development of therapies with recipient derived immunoregulatory BMDCs. An alternative approach to induce tolerance is by targeting recipient DCs directly in vivo with donor antigen, in a quiescent state. Delivering antigen specifically to conventional DCs via DEC-205 or 33D1 leads to effective presentation by MHC class I or II molecules, respectively, which is followed by induction or expansion of T regs and/or T cell deletion (Beriou et al. 2012; Smyth et al. 2013). Tanriver et al. demonstrated in a skin transplant model that
delivering alloantigens to cDCs via 33D1 leads to indefinite transplant survival, but only in the presence of anti–CD8- depleting Ab (Tanriver et al. 2010).

It has also been reported that recipient or autologous derived BMDCs were more efficient than donor derived BMDCs in a rat model of cardiac transplantation (Pêche et al. 2005). Although the effect was not antigen specific, the results demonstrated a significant prolongation of allograft survival and decreased anti-donor humoral and cellular responses (Pêche et al. 2005). It is important to note that in this study 7x10^6 cells were administered via penile vein injection 1 day prior to surgery. Throughout this study the immunoregulatory BMDC cell populations were infused systemically or locally 7 days prior to surgery into an immunologically quiescent recipient. Earlier or later infusion of regulatory DCs have been reported to diminish or abolish their regulatory effect (Lutz et al. 2000; Wang et al. 2012). The best kinetics of a single DC injection, including injection at day 28, 14, 7, 3 or 1 prior to transplantation of the allograft demonstrated was obtained when the DCs were injected 7 days before transplantation survival (Lutz et al. 2000; Wang et al. 2012; Ezzelarab and Zahorchak 2013). However, it is argued that such protocols would not be applicable in the clinic and DCs injected only 1 day prior the graft represent a more realistic clinical approach (Pêche et al. 2005). Although cornea transplanted tissue can be stored for up to 7 days with no adverse effects on the graft, it may be worth investigating a shorter the time between cell administration and transplantation and examining the effects of increasing the dose administered or treating with multiple injections in terms of the local administration.
5.4 Mechanism of Action.

In order fully understand the mechanism/s of action employed by the donor derived BMDCs or Dexa BMDCs it is important to understand where the cells localise after administration. This project examined the localisation of the cells after systemic and local injections. Having demonstrated that local injection of syngeneic BMDCs could be found in DLNs 24hrs after administration interestingly, the same was not observed with allogeneic BMDCs which were found only in the eye and conjunctiva (Figure 4.25). However, systemically administered allogeneic BMDCs were found only in the lung and blood during this study 24hrs after administration (Figure 4.1). Interestingly vitamin D3 DC, IL-10 DC and Dexa DCs have been reported to migrate significantly less when compared to TGFβ DC, rapamycin DC and mature DCs (Boks et al. 2011). Yu et al. demonstrated that the presence of NK cells allogeneic DCs did not survive and were unable migrate to the host spleen and lymphoid tissue where they directly stimulated the activation and effector function of alloreactive T cells. However, it remains unclear whether all kinds of donor APCs are sensitive to NK-mediated killing or different cells like regulatory BMDCs would survive (Yu et al. 2006).

Considering that only a small percentage of i.v. administered untreated BMDCs or Dexa BMDCs may migrate to lymphoid organs once injected, as they are short-lived or attacked by NK or CTLs the injected BMDCs may use an alternative mechanism to induce donor-specific immune-suppression and prolongation of allograft survival (Divito et al. 2010). Wang et al. suggest that injected donor DCs transfer donor antigen to recipient quiescent APCs expressing low levels of costimulatory molecules. This could represent a mechanism for restraining the anti-donor response, since a single donor immunoregulatory BMDCs could transfer donor antigen to several recipient APCs (Wang et al. 2012). Several studies have examined methods of DC antigen transfer and these include via trogocytosis, nanotubes, cross-dressing, apoptotic cell-vesicles or exosomes (Xia et al. 2005; Woltman et al. 2006; Horibe et al. 2008; Fedoric
colleagues also demonstrate that cDCs of the recipient, instead of the injected
immunosuppressive DCs, are critical for donor-specific immune-suppression and
prolongation of allograft survival (Wang et al. 2012).

The administration of semi-immature or immature DCs has been shown to modulate the
direct and indirect T cell responses by promoting deletion, anergy, immune-deviation
and/or regulation (Sato et al. 2003; Woltman et al. 2006; Fazekasova et al. 2009;
fully examine untreated BMDCs and Dexa BMDCs immunomodulation of both the
direct and indirect pathways of allorecognition further ex vivo rechallenge experiments
would be required. During this study in vitro hyporesponsiveness to donor BMDCs and
Dexa BMDCs was observed along with a trend towards an induced T reg phenotype in
BMDC and Dexa BMDC treated animals (Figure 3.15). The ex vivo rechallenge
experiments also demonstrated T cell hyporesponsiveness to direct donor antigen
stimulation in animals treated with donor BMDCs or Dexa BMDCs (Figure 4.3 and
Figure 4.4). However, in order to confidently conclude that this is in fact the case this
experiment would need to be repeated and modifications made. The initial experiments
were carried out in naïve animals; therefore it would be interesting to examine the T cell
response in grafted recipients which have been untreated or treated with donor BMDCs
and Dexa BMDCs. The inclusion of a LPS or cytokine stimulated mature BMDC
population as a control cell group would also be required. Also an additional in vitro
analysis examining the response to donor antigen presented by recipient APCs would
also be required to address the impact of the therapy on the indirect pathway of allo-
recognition. The time point of analysis of day 4 post restimulation with the alloantigen
may have to be expanded to see a significant difference between a naïve animal having
not received a BMDCs injection and one that has seen the donor antigen. Others have
looked at several time-points from day 3 to 7 and found a maximal response only at day
5 (Khan et al. 2013).
Interestingly, significantly increased expression of *Ido* mRNA was found in both accepted allografts and the DLNs of BMDC and Dexa BMDC treated animals (Figure 4.17). This may be an additional explanation for the regulatory capacity of ex vivo-generated BMDCs. IDO catalyses the depletion of the essential amino acid tryptophan and enhances the production of immunoregulatory kynurenine metabolites that inhibit T cell proliferation and promote T cell apoptosis (Grohmann 2003). Constitutive IDO expression has also been detected in human regulatory DCs and is believed to contribute to the immunoregulatory capacities of regulatory DCs inhibition of alloantigen-specific T cell responses in vivo and promote and maintain a regulatory T cell phenotype (Mellor and Baban 2003; Baban et al. 2011; Sucher et al. 2012). Future investigations of untreated BMDC and Dexa BMDC therapy would need to elucidate the role of IDO in the both the in vitro inhibition of alloantigen-specific T cell responses and also the in vivo induction and maintenance of corneal allograft survival. Experiments administering the IDO inhibitor 1-methyl-D-tryptophan (1-MT) in in vitro cell culture assays and in vivo at the time of BMDC and Dexa BMDC injection are required. Also experiments with 1-MT administration at the time of transplantation would be required to elucidate IDO role in the maintenance of allograft survival due BMDC and Dexa BMDC cell therapies.

NO produced due to iNOS activity is recognised as an inhibitor of IDO activity by directly interacting with it and promoting its degradation (Samelson-Jones and Yeh 2006). This may explain the observed difference in the level of *Ido* mRNA expression between untreated BMDC cultures and Dexa BMDCs cultures as *iNos* was significantly expressed in untreated BMDCs (Figure 3.10). Interestingly, Pêche et al. showed that recipient derived BMDC treatment was able to induce significant prolongation of cardiac allograft survival the effect was partially mediated by the production of NO (Pêche et al. 2005). To clarify the role of NO in BMDC and Dexa BMDC ability to modulate and suppress allogeneic T cell proliferation studies using the a selective inhibitor of iNOS, L-NMMA (NG-Monomethyl-L-arginine, monoacetate salt) could be carried out. Also to examine the role of NO in the promotion of corneal allograft
survival by donor derived BMDCs and Dexa BMDC therapies in vivo, administration of the iNOS inhibitor aminoguanidine would contribute to elucidating NO as a potential mechanism.

The results of this study demonstrated a significant increase in the ratio of FoxP3 expressing regulatory cells in the allograft and DLNs of treated animals but also observed significant increase in mRNA expression levels in the DLNs (Figure 4.14 and Figure 4.15). Further studies to elucidate whether the Foxp3 expressing T cells are functionally active could include in vitro examination of their capacity to suppress donor antigen specific responses. In vivo analysis of their function could be carried similarly to the adoptive transfer experiments described by Chauhan et al. where they injected $1 \times 10^5$ FoxP3$^+$ cells (isolated from graft accepting mice) i.v. to allograft recipients at 18h post corneal allograft surgery. Their results demonstrated that these adoptively transferred Tregs were functional and highly effective at preventing corneal allograft rejection (Chauhan and Saban 2009). Goto et al. have recently demonstrated that in their cardiac model of transplantation using a delayed administration of anti-CD3 therapy until day 3 after transplantation had a significant impact on long-term graft acceptance and that intragraft, rather than the draining lymphoid organ Foxp3$^+$ T cells play an important role for long-term graft acceptance (Goto et al. 2013). They suggest that regulatory T cells present in surviving allografts might be more efficient at protecting the allograft from damage.

Sagoo et al. posed an interesting question about Tregs found in accepted allografts and that was whether the Tregs are generated elsewhere (DLNs) and then migrate to the graft site from the periphery or whether they are induced within an allograft tissue itself (Sagoo et al. 2012). This group studied the early trafficking of adoptively transferred human Tregs (3 days) in a humanised mouse xenograft model of skin transplantation. Similar numbers of both allo-specific and polyclonal Tregs were recruited to skin allografts, although a higher proportion of allo-specific Tregs were found to be in
contact with skin resident alloantigen bearing donor cells. The authors speculated that donor-specific T reg mediated suppression occurs primarily at sites of alloantigen expression and effector target tissue, and possibly acts through early interaction and modulation of APC function and effector cell recruitment. As the donor cornea is avascular and does not contain T cells (Sosnová et al. 2005), the presence of T regs in accepted grafts requires trafficking of such cells into the stroma. Similarly to the observations made during this project T regs have been identified in accepted corneal grafts (Hori, Taniguchi, et al. 2010). As naive T cells do not normally patrol the tissues, it can be assumed that alloantigen specific T cells require activation prior to entry to the cornea and if this was halted in the DLNs this trafficking to the graft would not occur (Vitova et al. 2013). In the case of corneal allografts T regs clearly play a major, if not a critical role, as it has been shown by others that graft survival in their absence is not possible (Hori, Taniguchi, et al. 2010).

The data collated during the course of this research work supports the recent evidence of peripheral donor sensitisation with pre-treatment with donor derived BMDCs (de Kort et al. 2012; Smyth et al. 2013) however, the results also detail the unique observation that within the immunomodulatory environment promoted within the eye, the corneal allograft remains protected. This protection of the graft was maintained despite the presence of donor specific alloantibodies. Other studies using donor derived BMDC exosomes have reported the presence of alloantibodies in cardiac transplantation models, these were at levels significantly less compared to untreated rejecting animals (Pêche et al. 2003; Pêche et al. 2006). Having identified the alloantibodies as anti-donor class II alloantibodies the authors of these studies demonstrated that sera harvested 5 days after transplantation from exosome-treated rats delayed acute cardiac allograft rejection, whereas sera from untreated rejecting rats did not have any effect. The results suggest that that anti-donor MHC II alloantibodies could play a role in exosome induced inhibition of allograft rejection (Pêche et al. 2003). This concept could be worth pursuing in the context of our findings in the model of corneal transplantation.
The role of alloantibodies in corneal allograft rejection is however, controversial with cell-mediated rejection believed to play the dominant role (Hegde et al. 2002; Holán et al. 2005; Niederkorn 2012). The determination of the exact epitopes recognised by the alloantibodies is a challenging task and was not attempted during this project. However Pêche et al used an elegant system, which could be employed in future studies, alloantibody staining was performed on both CD45RA\(^+\) (B cells) and CD45RA\(^-\) cell populations enabling the study of alloantibody staining of B cells (MHC I\(^+\) and MHC II\(^+\)) and non-B cells including resting T cells which are MHC I\(^+\) (Pêche et al. 2006). Alternatively if future work was conducted in murine models the proportion of antibodies directed against MHC class I and II could be examined by using target cells from MHC I or II knock-out animals respectively.

5.5 Steps from Pre-Clinical to Clinical Application

Preclinical studies with the aim to develop and characterise tolerogenic monocyte-derived DC for a clinical application have begun (Moreau, Varey, Bériou, et al. 2012). Some pre-clinical studies have already examined regulatory DCs in non-human primates (NHP). One such study using regulatory DCs generated from CD14\(^+\) blood monocytes of allogeneic donor rhesus macaques demonstrated allograft survival of subsequent renal transplants from the same donor monkey (Ezzelarab and Zahorchak 2013). Ezzelarab and Zahorchak administered regulatory DC in combination with CTLA4-Ig and rapamycin and demonstrated inhibition of acute allograft rejection in a clinically relevant NHP model (Ezzelarab and Zahorchak 2013). Studies from rodent organ allograft survival have also demonstrated long-term survival with administration of regulatory DCs alone as previously described but also in combination with the co-stimulation blocking agents CTLA4-Ig (Mirenda 2004) or anti-CD154 mAb (Wang et al. 2003).
Before scaling up for clinical trials it is critical that there is a standardisation of ex vivo-generated high quality, viable BMDCs as there may also be fundamental differences in their functionality in vivo if generated by different methods. In an effort to standardise and develop reproducible experimental conditions Richter et al. generated conditionally immortalised DCs with classical DC properties and a stable phenotype long term. The bone marrow cells from an “immorto-mouse” with a tetracycline regulated expression of SV40 large T-antigen were treated with Dexa and doxycycline to induce immortalization and when the cells are de-induced, the DCs display similar morphology as BMDCs (Richter et al. 2013).

Two potential methods of generating expanding human DCs for clinical application include the use of CD34+ hematopoietic precursors and in vivo expansion of circulating DCs (Sabado and Bhardwaj 2013). DCs can be propagated from CD34+ precursors that are mobilized from the bone marrow by treatment of patients with G-CSF before harvesting by leukapheresis (Banchereau and Palucka 2001). Alternatively administration of growth factors such as Flt3L induces the in vivo expansion of circulating DCs (Marroquin and Westwood 2002). These methods are applicable if the treatment is the application of recipient derived regulatory DCs alone or pulsed with donor alloantigen. However, administration of DCs pulsed with donor alloantigen will require evaluation of different sources of donor Ag, including donor cell lysate, apoptotic cells and exosomes (Ezzelarab and Zahorchak 2013). Moreover therapies using donor derived BMDCs will be difficult to implement using deceased donors for application in transplantation models such as renal or cardiac, as typically deceased donors are not identified until shortly before transplantation and DC propagation takes approximately 7 days. As a result the autologous regulatory DCs pulsed with donor antigen could not be administered until the day of transplantation surgery and/or later (Ezzelarab and Zahorchak 2013).

However, in the case of cornea transplantation, donated corneas can be stored for up to one week without impacting graft survival (Simon et al. 2004), giving time for in vivo
propagation of the donor BMDCs. Nevertheless, groups have begun investigations into shortening the time required to propagate regulatory DCs. Garcia-Gonzalez et al. have developed a 5 day protocol to generate regulatory DCs using Dexa plus monophosphoryl lipid A (MPLA, a clinical grade analog for LPS) which confers the DCs with a stable phenotype and migratory capacity to lymphoid chemokines (García-González et al. 2013).

Interestingly, Hutchinson et al. demonstrated the technical feasibility of using donor-derived transplant acceptance–inducing cells in renal transplantation (Hutchinson et al. 2009). The case report describes the treatment and outcome of a kidney transplant of a male renal transplant recipient who received an infusion of donor derived transplant acceptance–inducing cells from the spleen 5 days post transplantation as a form of adjunct to sirolimus and tacrolimus immunosuppressive therapy (Hutchinson et al. 2009). The Hutchinson study highlights that initial clinical applications of donor-derived cells will be combined with immunosuppressive drug therapies. The choice of immunosuppressive treatment to combine with regulatory DC therapies is critical as certain drug therapies may interfere with the of immunomodulatory effects of the DCs (Moreau et al. 2012). As the lifespan of donor-derived BMDCs has been reported to be short in vivo (Divito et al. 2010) the effect of immunosuppressants on recipient NK or CTLs thus could increase the regulatory DCs probability of interacting directly with donor-reactive T cells (Wang et al. 2012).

Clinical investigations of administering recipient regulatory DC has already begun. One recent clinical safety study examined autologous Mo-DCs treated ex vivo with antisense phosphorothioate-modified oligonucleotides targeting the primary transcripts of the CD40, CD80, and CD86 costimulatory molecules therapy for type 1 diabetes suppression (Giannoukakis et al. 2011). This study demonstrated that treatment of diabetic patients with unmanipulated DCs (two patients) or immunoregulatory DCs (eight patients) proved to be well tolerated, without any detectable adverse events or
toxicities (Moreau et al. 2012; Smyth et al. 2013). An efficacy trial aims to investigate locally injected DexaD3-DCs injected into the knees of patients with rheumatoid arthritis (Isaacs, J. D. 2011). This trial represents one of the first efficacy trials to determine whether recipient pharmaceutically-modified DCs can alter the course of disease locally in a systemic disorder.

Specific markers of regulatory DCs to use as indicators of regulatory efficacy will be required (García-González et al. 2013). Many molecules like the immunoglobulin like transcript inhibitory receptor family members, ILT3 and ILT4 and some molecules which were identified on the ex vivo-generated BMDCs and Dexa BMDCs used in this study and others, such as PD-L1 (Keir et al. 2008; Wölfle et al. 2011), have been shown to be upregulated in DCs with regulatory properties and have been associated with a capacity to induce anergy in T cells (Brenk et al. 2009). Additional studies using labeled regulatory BMDCs will be required to provide critical information regarding the migration pattern, fate and longevity of the administered regulatory DCs.

It is also important to identify biomarkers of the long-term surviving tolerant allografts. A study recently compiled a comprehensive review of over 20 observational clinical studies which have examined the association of detected human T regs with liver, heart, lung, and kidney allograft outcome. The analysis shows that elevated intragraft T regs detected by relative increase in FoxP3+ cells or quantitative mRNA expression could, in general, be positively correlated with improved graft function or outcome (Shan et al. 2011). Others have examined the gene profile of tolerant renal transplant patients and demonstrated increased expression of \textit{FOXP3}, \textit{GATA3 TGF\textbeta}1 however, interestingly this profile did significantly overlap with biomarkers identified in liver transplant recipients highlighting the possible differences between different transplantation models (Chandrasekharan et al. 2013). Cobbold et al. explored biomarkers of tolerance in three different murine skin graft models by examining the graft, spleen and DLN. Although the syngeneic grafts and allografts that were accepted had a similar gene expression
profile, which differed from the gene expression of a rejecting allograft there, was no
difference observed in the spleen or DLN. Cobbold et al. suggest that if a biomarker
profile of tolerance was to exist, the location most likely to reveal this is in the allograft
itself (Cobbold et al. 2011). Further studies are required to include more powerful
prospective cohorts with bigger sample size and longer follow-up times. Also Shan et al
suggest that there is a need for more precise quantitative studies to analyze the threshold
of T regs counts to effectively control rejection and induce transplant tolerance (Shan et
al. 2011). The difficulty with corneal transplantation is that a biopsy is not feasibly
obtained from an accepted graft so alternative methods will have to be examined to
identify bio-markers of long-term tolerant allografts. One alternative could include
examination of the tear film of the eye for bimolecular signatures of acceptance or
rejection.

The overarching aim of this research work was to further transplantation immunologys
transition from the administration of harmful immunosuppressive drug therapies
towards development of more controlled immunoregulatory cellular therapies that
effectively promote long-term graft survival. Although some aspects were unable to be
explored to their full potential (due to time constraints) such as the local administration
of immunomodulatory BMDCs in corneal allograft transplantation the results achieved
give sufficient justification for future investigations.

5.6 Conclusion.

Of particular interest, this research work highlights the fact that unmodified BMDCs,
like the glucocorticoid BMDC treatment protocol, prolongs allograft survival without
the need for additional immunosuppressive drug therapies. In addition to the knowledge
that current literature provides us with, this research work developed a more
comprehensive approach to characterisation of the immune microenvironment of the corneal allograft and the graft DLNs after treatment with unmodified donor BMDC and glucocorticoid treated BMDCs.

This work also highlighted the risks in recipient sensitisation with pre-treatment of donor derived BMDCs. Uniquely this work also detailed the observation that within the immunomodulatory environment promoted within eye the corneal allograft remains protected. This work not only represents a novel therapeutic strategy with significant potential for translation into a clinical application but also gives an insight into the induced mechanism of corneal allograft survival after donor BMDC administration. This thesis also provides the first detailed description of how the application of BMDC therapies, in the absence of additional immunosuppressive drug therapy, can effectively prolong the survival of a corneal allograft by the induction and maintenance of an immunoregulatory environment within the allograft and DLNs. This research may therefore lead to an improved treatment protocol with reduced toxicity and an increase in long-term survival of corneal allografts for cornea transplant patients.
6. Publications and Presentations

6.1 Peer-reviewed Original Research Publications

Promotion of corneal graft survival by donor bone marrow derived dendritic cells is mediated by creating an intragraft immunoregulatory milieu.


To investigate the role of lentivirus-mediated overexpression of programmed death-ligand 1 (PD-L1) on rat corneal allograft survival.


Adenoviral transduction of mesenchymal stem cells: In vitro responses and in vivo immune responses after cell transplantation.


Immunogenicity of allogeneic mesenchymal stem cells.


Mesenchymal stem cell therapy promotes corneal allograft survival in rats by local and systemic immunomodulation.

Publications and Presentations

Chondrogenic differentiation of mesenchymal stem cells alters cellular immunogenicity in vitro and in vivo.

Colon cancer cell NF-κB regulates tumor-associated macrophage polarization and promotes peritoneal metastasis.

#Equal Contribution

6.2 Conference Oral Presentations


6.3 **Conference Poster Presentations**


7. Bibliography


Bibliography


Bibliography


Bibliography


8. Appendix

8.1 Harvesting BMDCs

Table 8.1 Method of harvesting ex vivo-generated BMDCs.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Viable Cell #</th>
<th>Dead Cell #</th>
<th>% Viable</th>
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<tbody>
<tr>
<td>Cell Scraper</td>
<td>5.2x10^6/ml</td>
<td>1.2x10^6/ml</td>
<td>46</td>
</tr>
<tr>
<td>Pipetting Only</td>
<td>1.2x10^6/ml</td>
<td>1.6x10^6/ml</td>
<td>75</td>
</tr>
<tr>
<td>5mM EDTA/PBS + Pipette</td>
<td>1.6x10^6/ml</td>
<td>2.6x10^6/ml</td>
<td>61</td>
</tr>
<tr>
<td>2mM EDTA/PBS + Pipette</td>
<td>1.4x10^6/ml</td>
<td>1.86x10^6/ml</td>
<td>75</td>
</tr>
<tr>
<td>1mM EDTA/PBS + Pipette</td>
<td>1.6x10^6/ml</td>
<td>2.14x10^6/ml</td>
<td>74.7</td>
</tr>
<tr>
<td>Accutase</td>
<td>1.34x10^6/ml</td>
<td>1.54x10^6/ml</td>
<td>87</td>
</tr>
</tbody>
</table>

BMDCs were harvested using the methods listed (3 replicates for each condition) and the percentage viability was calculated. Although Accutase had the highest percentage viability pipetting alone was the method chosen as reagent was not required and it gave reasonably high percentage cell viability.
8.2 Phenotype of Cryopreserved BMDCs

Figure 8.1 Phenotype of Cryopreserved BMDCs. (a) BMDCs from fresh bone marrow were stimulated with pro-inflammatory cytokine containing media from media harvested from LPS stimulated RAW (murine monocyte/macrophage cell line) (n=5). Maturation markers from BMDCs were cultured from cryopreserved bone marrow cells (n=2) or freshly culture bone marrow (n=5) were examined. Each culture was treated with pro-inflammatory cytokine containing media from media harvested from LPS stimulated RAW and no significant difference was observed between the phenotype of the BMDCs from the cryopreserved culture and the fresh bone marrow.
8.3 RAW 264.7 Culture

RAW 264.7 (Cat number 91062702) were seeded at 2-4x10^4 cells/cm²; in DMEM supplemented with 2mM Glutamine and 10% FBS and incubated at 5% CO₂; 37°C. RAW cells are semi-adherent, i.e. some cells grow in suspension, some loosely attach to the surface and others flattened out and attached to the flask. Cells were stimulated with 1μg/ml LPS for 24hrs after which time cells morphology had changed.

**Figure 8.2 RAW 264.7 cells.** (a) The unstimulated appeared round with smooth surface, whereas (b) the LPS stimulated displayed an irregular shape with numerous pseudopodia or lamellapodia and ruffles on the cell membrane besides becoming larger, flatter, and longer.
8.4 Phenotype of MACS Ox62$^+$ DCs

Figure 8.3 Phenotype of MACS Ox62$^+$ DCs. Histograms of MHC II and CD80 expression of Ox62+ MACs purified DCs (n=2, isotype grey line, LEW black line, CD black dashed line).
8.5 Multi-Analyte Assay

Figure 8.4 Multi-Analyte Assay of unstimulated and LPS stimulated untreated BMDCs and Daxa BMDCs. Unstimulated BMDC and Daxa BMDCs supernatant n=2 each (mix of 3 different supernatant preparations per sample) LPS stimulated BMDCs and Daxa BMDCs n=1 each.
8.6 RT2 Profiler PCR Array

Figure 8.5 PCR Array for unstimulated and LPS stimulated BMDCs. Unstimulated BMDC n=1 and LPS stimulated BMDC culture n=1 (normalized to β-actin and fold change relative to BMDCs alone).
Figure 8.6 PCR Array Comparison of untreated BMDCs and Dexa BMDCs. Untreated BMDCs and Dexa BMDC n=1 (normalized to β-actin and fold change relative to BMDCs alone).
Figure 8.7 PCR Array Comparison of LPS stimulated BMDCs and LPS stimulated Dexa BMDCs with and without LPS stimulation. Untreated BMDCs and Dexa BMDC n=1 each (normalized to β-actin and fold change relative to LPS stimulated BMDCs alone).
8.7 Virus

8.7.1 Lanthanide ($\text{Ln}^{3+}$) Adenovirus Transduction

$\text{Ln}^{3+}$ was dissolved in deionised $\text{H}_2\text{O}$ and the pH adjusted to 5.5-6, stocks were maintained at 0.4M at 4°C and fresh stocks prepared every 2 weeks. Dilutions of $\text{LnCl}_3$ e.g. 2mM, 0.2mM and 0.02mM were prepared and different MOI of adenovirus prepared. To an Eppendorf tube 500µl of serum free medium, the appropriate volume of $\text{LnCl}_3$ and MOI of adenovirus was added. The Eppendorfs were incubated at 37°C for 30 mins after gently mixing samples by vortex. After incubation 100µl of each preparation was added to appropriate well of BMDCs. BMDCs were plated 1x10^5 cells/well in a 6 well plate containing 2-3mls of medium for 24hrs before transduction. An additional 150µl of serum free medium was added and incubate for 2-4hrs. After incubation the $\text{Ln}^{3+}$ serum free media was removed and replaced with serum containing medium and incubate for 48hrs at 37°C with 5% CO₂. Transduction efficiency was calculated by analysis the percentage of cells transduced measured by flow cytometric analysis.

8.7.2 Lentivirus Production Calcium Phosphate Transfection Method

Grow cells in standard DMEM + 10% FCS + Pen/Strep at 37°C and 5% CO₂ and passaged every few days so they do not become over-confluent. Prior to transfection cells should be at 70-80% confluency in a 10cm dish. Remove media 30mins before transfection and replace with 9ml of fresh medium containing 10µl/ml chloroquine and allow time for cells to settle back down. 2X HBS is thawed at room temperature (37°C
Appendix

is NOT recommended) and CaCl₂/HBS/DNA precipitate is prepared as follows for each 10cm dish in 1.5 ml microfuge tube:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amount µg/10cm dish</th>
</tr>
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<tbody>
<tr>
<td>pMDL-g/p-RRE</td>
<td>42.33µg</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>19.6µg</td>
</tr>
<tr>
<td>pMD2G</td>
<td>21.62 µg</td>
</tr>
<tr>
<td>Transgene</td>
<td>40.5µg</td>
</tr>
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</table>

279µl of 2M CaCl₂ was added to the plasmid mix using enough distilled water to bring the total volume to 1.98 ml. The CaCl₂/DNA mix to 2.25mls 2X HBS slowly drop-wise, mixing gently during the addition and incubated at R.T. 20-30mins. This mix was then added directly to cells by dropping slowly and evenly into medium, trying to cover as much of the plate as possible. This was not mixed but carrying the dish to the incubator mixes the solutions. Cells were incubated at 37°C/5% CO₂ for 24hrs. The media was removed and replaced with fresh warm complete medium and incubated for a further 24hrs. Supernatants were harvested filtered and stored appropriately.

2X HBS

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>8g NaCl</td>
<td></td>
</tr>
<tr>
<td>0.14 g Na₂HPO₄</td>
<td>(1.5 mM)</td>
</tr>
<tr>
<td>6.5 g HEPES</td>
<td></td>
</tr>
<tr>
<td>pH to 7.0</td>
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</tr>
</tbody>
</table>

Bring up to 500 ml with distilled water
Re-pH
Filter sterilise
Aliquot and store at –20°C.
Appendix

2M CaCl₂

- 29.4g CaCl₂ dihydrate
- Final volume of 100 ml distilled water
- Filter sterilise
- Store at
- 4°C (DO NOT FREEZE).

8.7.3 3rd Generation Lentivirus Packaging Plasmids

**pMD2.G** is a fusogenic envelope G glycoprotein of the vesicular stomatitis virus (VSV-G) plasmid (Addgene cat. # 12259).

**pRSV-Rev** is a helper plasmid with rev sequence (Addgene cat. # 12253).

**pMDL g/p RRE** LV packaging plasmid contains gag, coding for the virion main structural proteins; pol, responsible for the retrovirus-specific enzymes; and RRE, a binding site for the Rev protein which facilitates export of the RNA from the nucleus. (Addgene cat. # 12251).

The 3rd generation packaging system offers maximal biosafety but is more cumbersome, as it involves the transfection of four different plasmids in the producer cells. The forth plasmid contains the gene of interest and for this project the pLenti/V5-Dest vector system was used.

**pLenti/V5-Dest** (Invitrogen cat. # V499-10). Contains the ubiquitin C (UbC) promoter. This human promoter drives constitutive but physiological levels of expression of the gene of interest. It was used as a lentiviral expression vector for GFP, PD-L1 and IDO.
8.7.4 LVeGFP Transduction of BMDCs

Figure 8.8 LV eGFP transduction of BMDCs. Comparison of different methods of LV transduction of BMDCs representative of three repeat samples from one experiment.
8.7.5 FACS of LV eGFP transduced BMDCs.

Figure 8.9 Gating Strategy for FACS #1 of LV eGFP transduced BMDCs. BMDCs Gating approach: (a) gates on morphological BMDC, to exclude non-viable cells, (b) single cell gate to eliminate cell aggregates, (c) gate on untransduced BMDCs to identify transduced population as in illustrated in (d). (e) Purity obtained sort #1 83.4%
8.7.6 Cloning IRESGFP – PDL1/IDO into Lenti Destination Vector

8.7.6.1 Restriction Enzyme Digest

The IRES-eGFP and pTA PD-L1 plasmids were both cut with EcoR1 HF and Sal1 as follows:

- Added 5µg DNA to each tube.
- Added 5µl of 10X NEBuffer 3 +BSA
- Added 2.5µl of appropriate enzyme to appropriate tube.
- Left at 37°C for 1hr.
- Proceeded with ligation.
- RE digest was run on gel and successful digest obtained so carried out a gel extraction on band of interest.

IRES-GFP and pCMV Sport6 IDO plasmids were both cut with Xho1 and Sal1 as follows:

- Add 1µg DNA to each tube.
- Add 2µl of 10X NEBuffer 4 +BSA
- Add 1µl of appropriate enzyme to appropriate tube.
- Leave at 25°C for 6hrs (for Sma1 activity, 50% activity at 37°C and half-life of 15min at 37°C).
- Add in Xho1 enzyme after 6hrs.
- Raise temperature to 37°C (for Xho1 activity) and leave o/n.
- RE digest was run on gel as follows and successful digest so carried out a gel extraction.
Appendix

**Ligation was performed as follows:**

Combined 50ng of vector with a 3-fold molar excess of insert and adjusted volume to 10μl with dH₂O.

**PD-L1 EcoR1 H1 + Sal1**
1:3 Took 50ng of vector and 28ng of PD-L1 insert
1:4.5 Took 50ng of vector and 74.72ng of PD-L1 insert

**IDO Xho1 + Sal1**
1:3 Took 50ng of vector and 37.36ng of IDO insert
1:6 Took 0.6μl (50ng) of vector and 74.72ng of IDO insert

Added 10μl of 2X Quick Ligation Buffer and mixed.

Added 1μl of Quick T4 DNA Ligase and mixed thoroughly.

Centrifuged briefly and incubated at room temperature (25°C) for 5 minutes.
Chilled on ice, then transformed or stored at -20°C.

Do not heat inactivate. Heat inactivation dramatically reduces transformation efficiency.

**Transformation**

Took whole ligase reaction (20 μl) and added it to the Top10 competent bacteria, mix gently.

Left on ice for 30mins and heat shocked for 30sec at 42C.

Added 250ul of SOC media and left shaking at 37C for 1hr.

After centrifuging at 3000rpm for 1min, removed supernatant, added 100ul of fresh soc media and spread on Kanamycin plates left o/n at 37°C.
Mini Prep

Selected colonies from agar plates x2 colonies from each ratio plate. Inoculated 5ml of LB broth+Kanamycin and left o/n shaking at 37°C.

Preformed Mini Prep as follows:

- Collect 1ml from o/n culture and centrifuge at 6800g for 3min at R.T.
- Remove supernatant and resuspend in 300µl of resuspension buffer (B1).
- Add 300µl of lysis buffer (B2) and inverted 4-6 times.
- Add 300µl of precipitation buffer and invert immediately.
- Centrifuge at 18000g R.T. collect supernatant and place in a fresh Eppendorf.
- Add 670µl of isopropanol and resuspend.
- Centrifuge 20min at 20000g at 4°C.
- Wash pellet with 1ml 70% EtOH.
- Centrifuge 10min as above remove supernatant and leave pellet to air dry for 10min.
- Resuspend pellet in 100 µl TE buffer.
- Measure concentration.

Collected 1ml from x2 colonies for each ratio for each PD-L1 and IDO.

RE Digest to confirm insert

- Add 1µg DNA to each tube.
- Add 2µl of 10X Buffer 0 (for EcoR1+ Sal1 - PD-L1) and NEBuffer 3+BSA (for Xho1+Sal1 - IDO)
- Add 1µl of appropriate enzyme to appropriate tube.
- Leave 37°C for 1hr.

Fragment size expected

- PD-L1 = 931bp
- IDO = 1263bp

Results

A band observed for 3 of 4 colonies for IRES-GFP-PD-L1 plasmid however no band observed for IRES-GFP- IDO after multiple tries and modifications to the cloning protocols. IRES-GFP-PD-L1 plasmid stored at -20°C.
8.8 Administration of ex vivo-generated immunomodulatory BMDCs in corneal allograft transplantation

8.8.1 Phenotype analysis of immunomodulatory milieu – Spleen.
Figure 8.10 Evaluation of the immune cell populations of the spleen in corneal allograft recipients after donor BMDCs and Dexa BMDCs treatment. (a) The percentage cell population and total cell number per 1x10⁶ cells from spleen APCs (CD11b/c⁺), DCs (CD11b/c⁺MHC II⁺CD86hi) and B cells (CD45RA) were evaluated. (b) The percentage cell population total cell number per 1x10⁶ cells from splenic populations of activated NKT (CD3⁺CD8⁺CD161++), NK (CD3⁻CD8⁺CD161++) and activated T cells (CD4⁺CD25+) were also analysed. (c) The percentage cell and total cell number per 1x10⁶ cells of regulatory T cells (CD4⁺FoxP3⁺) was measured. The ratio of regulatory CD4⁺FoxP3⁺ T cells to activated CD4⁺CD25⁺ T cells was also analysed (Mann-Whitney two-tailed test n=4 per group, mean ± SEM *p≤0.05).
8.8.2 LPS Stimulated Donor BMDCs

Figure 8.11 I.V. administration of donor LPS stimulated BMDCs. (a) Graft survival curves of allogeneic transplantation (Tx) controls (n=26), syngeneic Tx controls (n=8), and LPS stimulated donor BMDCs (1x10^6 cells/1ml PBS i.v. n=5), (Kaplan-Meier survival analysis). (b and c) Opacity scores and neovascularisation scores day 4-day 30 of control groups, LPS stimulated BMDCs pulsed. (d) Average opacity score for the period day 6-12 post transplantation (*p<0.05, **p<0.01 Mann Whitney n=5-34).
Appendix

8.8.3 I.V. Injections of LPS stimulated Donor BMDCs.

Figure 8.12 Evaluation corneal allograft after I.V. administration of donor derived BMDCs. (n=2-5 per group).

8.8.4 Subconjunctival Injections of Dexa Donor BMDCs
Figure 8.13 Evaluation of prolongation of corneal allograft survival after subconjunctival injections of donor derived BMDCs administration. Groups treated with donor Dexa BMDCs demonstrated a significantly higher level of opacity between days 6 to 12 post transplantation, this level of opacity never increased beyond an average of 1.9 (n=2-7 per group)
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<table>
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**Animal details:**
- **LEWIS** male
- **AF code #:**
- **STUDY CODE:**

**Age:** | **Weight:** | **Time in AF:**
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**Source:**
- | Charles River
- | B&K
- | Harlan

**Cornea donor:**
- **LEWIS (syn) AF code #:**
- **DA (allo) AF code #:**
- ............ (third party) AF code #:

**Anaesthesia:**
- **ISOFLURANE**

**Additional drugs:**
- Atropine 1%
- Tropicamide 1%
- Tetracaine 1%

**Antibiotic treatment:**
- Chloromycetin (chloramphenicol)
- Artificial tears

**Suture:**
- Discontinued-number of stitches: continuous

**Day -01 eye inspection:** passed
- **Yes**
- **No**

If no; specify:

**Day -01 donor inspection:** passed
- **Yes**
- **No**

If no; specify:

**Un-blinding injections:**

**Organ harvest:**
- Cornea for RNA FACS sort
- LNs for RNA FACS serum for allo-AB test

**Other:**

**End of observation period:**
- Day

**Animal sacrificed - date:**

**Final assessment:**
- Success
- **Yes**
- **No**

If no – why:

**Pictures taken:**
- **Yes**
- **No**

**Location**

**File closed:**
- **Yes**
- **No**

**Date, signature:**

266
Follow up:

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- A/C formed: □ yes / □ no
- Infection: □ yes / □ no
- Wound gapping: □ yes / □ no
- Wound edema: □ yes / □ no
  □ mild / □ moderate / □ strong
- Iris mobility: □ full / □ partial
- Anterior synechia: □ yes / □ no
- Pupil shape: □ round / □ irregular
- Rejection: □ yes / □ no
- Other: 

• action taken:
  Atropine drops: □ yes / □ no
  Pictures taken: □ yes / □ no

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<td>name of observer:</td>
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- A/C formed: □ yes / □ no
- Infection: □ yes / □ no
- Wound gapping: □ yes / □ no
- Wound edema: □ yes / □ no
  □ mild / □ moderate / □ strong
- Iris mobility: □ full / □ partial
- Anterior synechia: □ yes / □ no
- Pupil shape: □ round / □ irregular
- Rejection: □ yes / □ no
- Other: 

• action taken:
  Atropine drops: □ yes / □ no
  Pictures taken: □ yes / □ no