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PhD thesis:

Mesenchymal stem cells for therapeutic application in corneal transplantation

A thesis submitted to the National University of Ireland in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

Oliver Treacy, M.Sc.

Supervisor: Dr. Thomas Ritter (REMEDI)

Institution: Regenerative Medicine Institute (REMEDI), National Centre for Biomedical Engineering Science (NCBES)

Submitted: September 2013
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<td>ACAID</td>
<td>Anterior chamber associated immune deviation</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CCL</td>
<td>C-C motif ligand</td>
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<td>CCR</td>
<td>C-C motif receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CD</td>
<td>Sprague-Dawley rat</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
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<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<td>CFU-Fs</td>
<td>Colony forming unit-fibroblasts</td>
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<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
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<td>CX3CL</td>
<td>C-X-X-X-C motif ligand</td>
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<td>DA</td>
<td>Dark-Agouti rat</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DLNs</td>
<td>Draining lymph nodes</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EtOH</td>
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<td>FACS</td>
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<td>Fetal bovine serum</td>
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<td>Fluorescence minus one</td>
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<td>Foxp3</td>
<td>Forkhead box p3</td>
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<td>FSC</td>
<td>Forward scatter</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Graft versus host disease</td>
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<td>H&amp;E</td>
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<td>HIF</td>
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<td>Human leukocyte antigen-D related</td>
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<td>I.V</td>
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<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
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<tr>
<td>KIR</td>
<td>Killer-cell inhibitory receptor</td>
</tr>
<tr>
<td>LEW</td>
<td>Lewis rat</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSC</td>
<td>Limbal stem cell</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagle’s medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miH</td>
<td>Minor histocompatibility</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1 alpha</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
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<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>M-MuLV</td>
<td>Moloney-murine leukemia virus</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MPA</td>
<td>Mycophenolic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MST</td>
<td>Mean survival time</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium iodide symporter</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PD-1</td>
<td>Programmed death-1</td>
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<td>PD-L1</td>
<td>Programmed death-ligand 1</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>Pfu</td>
<td>Plaque forming unit</td>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>POD</td>
<td>Post-operative day</td>
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<td>qRT-PCR</td>
<td>Quantitative real time-polymerase chain reaction</td>
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<td>rATG</td>
<td>Rabbit antithymocyte globulin</td>
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<td>REMEDI</td>
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<tr>
<td>RPE</td>
<td>Retinal pigment epithelial</td>
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<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RV</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
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<td>SEM</td>
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<td>Signal transducer and activator of transcription</td>
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<td>T cell receptor</td>
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<td>TGF-β</td>
<td>Tissue growth factor-beta</td>
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<td>Th</td>
<td>T helper cell</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TSG-6</td>
<td>Tumor necrosis factor stimulated gene-6</td>
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<td>TSP-1</td>
<td>Thrombospondin-1</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VEGF-R2</td>
<td>VEGF-receptor 2</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WF</td>
<td>Wistar-Furth rat</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Alpha-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>β-Gal</td>
<td>Beta-galactosidase</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
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Summary

Although corneal transplantation success rates are high within the first year, the prognosis is much less favourable 5 years post-transplantation with immune-mediated rejection the primary cause of allograft failure. For this reason, on-going preventive and therapeutic measures are needed. Mesenchymal stem cells (MSCs) are being studied extensively in a wide range of diseases/disorders and more recently in solid organ transplantation, due to their ability to suppress or dampen host immune responses. However, reports on the therapeutic efficacy of MSC therapy have been mixed and the genetic modification of MSCs using viral vectors is one option to improve their therapeutic potential. Consequently, the focus of this research work was twofold. Firstly, to investigate if the genetic modification of MSCs using recombinant adenovirus alters the syngeneic host immune response and secondly, to investigate the therapeutic efficacy of MSCs in the promotion of corneal allograft survival.

The results from the first part of this work showed that Ad-transduction of MSCs does not lead to up-regulation of major histocompatibility complex class I and II and co-stimulatory molecule expression. Moreover, Ad-transduction caused no significant changes in terms of pro-inflammatory cytokine expression, chemokine and chemokine receptor and Toll-like receptor expression. Ad-modification of MSCs also had no effect on their ability to suppress T cell proliferation in vitro. In vivo injection of Ad-transduced MSCs did not change the frequency of various immune cell populations either in the blood or in tissues. Overall, the results indicate that Ad-modification has no major influence on the immunological properties of MSCs and therefore can be considered as a suitable gene vector for therapeutic applications of MSCs.

In the second part of this work the results showed that, in contrast to syngeneic MSC treatment which failed to significantly prolong survival, corneal allograft survival was significantly prolonged in approximately 90% of
allogeneic MSC and 80% of 3rd party MSC treated recipients. Flow cytometric analysis showed lower percentages of infiltrating natural killer (NK) T cells in corneas of both allogeneic and 3rd party MSC treated animals, coupled with a significantly higher frequency of splenic regulatory T cells, compared to controls. Thus, allogeneic and 3rd party MSC treatment prolongs corneal allograft survival by suppressing peripheral immune responses and promoting an intragraft immunoregulatory milieu.
Chapter One
General Introduction
1. General Introduction

1.1 Mesenchymal stem cells: origins

The term mesenchymal stem cells (MSCs) was first coined by Caplan (1991) and has its origins in the pioneering work of Tavassoli and Crosby (1968) and Friedenstein and colleagues (Friedenstein et al., 1970, Friedenstein et al., 1974, Friedenstein, 1976) in the 1960s and 1970s and the latter’s discovery of a minor subpopulation of bone marrow (BM)-resident cells with osteogenic potential. These seminal studies also provided a second important discovery. They showed that single cells from BM cell suspensions could give rise to colonies (colony forming unit-fibroblasts, CFU-Fs) (Friedenstein et al., 1970). In addition to their ability to differentiate along the osteogenic lineage and form CFU-Fs, these so-called MSCs could also adhere to tissue culture plastic and their progeny displayed a fibroblast-like morphology, thus suggesting they originated from the hematopoiesis-supporting BM stroma (Bianco et al., 2008). While these studies were widely acknowledged and disseminated at the time, it was only with the publication of similar work in 1999 by Pittenger and colleagues that the concept of a BM-derived non-hematopoietic stem cell gained global recognition (Pittenger et al., 1999). They showed in this study that human MSCs (hMSCs) could proliferate extensively in vitro, be induced to differentiate into adipogenic, osteogenic and chondrogenic lineages and, when expanded to colonies, individual cells could retain their multilineage potential. These characteristics are still employed today as preliminary methods for identifying a multipotent MSC population, albeit heterogeneous in composition. Furthermore, in addition to isolating MSCs from BM, numerous studies have also reported the isolation of populations with similar properties to MSCs from adipose tissue, muscle and umbilical cord, although this list is not exhaustive (Qu-Petersen et al., 2002, Zuk et al., 2002, Erices et al., 2000).

Another routinely used method for identifying an MSC population from BM aspirates is the flow cytometry-based analysis of surface marker expression. In a widely cited position paper published in 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy
(ISCT) proposed a minimal set of criteria for defining multipotent MSCs (Dominici et al., 2006). In addition to the criteria outlined above, i.e. that MSCs must be able to adhere to plastic under standard culture conditions and have proven tri-lineage differentiation capacity, they also proposed a set of surface markers that putative MSCs must express and lack expression of. These include the expression of CD105, CD73 and CD90 and lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. While this set of markers is continually being updated and revised, they are still the markers routinely assayed for. Despite ongoing efforts to identify one or more MSC-specific surface markers, the discovery of which would greatly enhance the field through the development of more rigorous, reproducible and comparable assays, such a discovery remains elusive at present. Moreover, if specific markers were to be identified in the future, it would enable the sorting of a homogenous population of ‘true’ MSCs directly from the BM and circumvent the need to seed heterogeneous BM cells (and thus eliminate the risk of contamination by hematopoietic progenitors) on tissue culture plastic, and subsequent selection based on adherence to this plastic.

While not listed as one of the minimum criteria by which to define MSCs according to the ISCT, it is generally accepted and routinely demonstrated, at least by those working in the field of immunology, that MSCs have the capacity to inhibit T cell proliferation when directly co-cultured together. However, this ability to suppress T cell proliferation is by no means the only way in which MSCs can interact with components/cells of the immune system. This concept will be explored in more detail in the following section.
1.2 Immune recognition of allo-antigen and potential effects of MSCs

While earlier studies focused on the tissue regenerative capacity of MSCs due to their presumed plasticity, attention has shifted in recent times to the ability of MSCs to modulate immune responses. To date, a large body of in vitro work, and to a lesser degree in vivo, has been published on the immunosuppressive capabilities of MSCs upon interaction with multiple immune cell types. Before discussing how MSCs can modulate the recipient’s immune response, it is important to examine how alloantigen (for example, in the form of an allogeneic organ or tissue transplant) comes to be recognised by the cells of the immune system in the first place. While it is well accepted that innate immunity plays a key role in initiation of an adaptive immune response and, as a result, graft rejection in many cases, activation of an innate immune response is largely non-specific and only rarely sufficient to reject an allograft on its own (Wood and Goto, 2012, Griffin et al., 2013a). Therefore, the focus here will primarily be on the adaptive immune response to alloantigen. Moreover, as for many applications (for example organ transplantation), recipient cell deficits or genetic factors will dictate that the cell source must be an allogeneic donor i.e. the ‘off-the-shelf’ cell therapy model (Griffin et al., 2010, Brooke et al., 2007), the interaction particularly of allo-MSCs with the recipient immune system will be the primary focus of the following sections.

1.2.1 Allorecognition and the T cell compartment

Provided a transplant recipient does not have pre-formed donor-specific antibodies, T cell mediated allorecognition is the first step of the adaptive immune response (Wood & Goto, 2012). This allorecognition can occur by three distinct pathways (Figure 1.1). Direct allorecognition involves the priming of recipient CD4+ and CD8+ T cells by intragraft (donor) antigen presenting cells (APCs) mediated through donor major histocompatibility complex (MHC) recipient T cell receptor (TCR) interactions. CD8+ cells then
receive T cell help from the CD4+ population in order to exert their function. *Indirect allorecognition* occurs when peptides derived from allogeneic (source could be cells, tissues or organs) MHC or minor histocompatibility (miH) antigens are taken up, processed and presented by recipient APCs via self-MHC class II molecules to recipient MHC class II restricted CD4+ T cells. The *semi-direct pathway of allorecognition* proposes a mechanism by which recipient APCs can capture intact MHC:peptide complexes from donor APCs either by direct cell to cell contact or, alternatively, through the release of small microvesicles (or exosomes) containing allo-MHC molecules which can fuse with the cell surface of recipient APCs (Montecalvo et al., 2008). These newly generated 'chimeric' recipient APCs can then participate in both direct pathway CD4+ and CD8+ T cell priming and indirect responses (as described above). As T cell priming is mediated, in this model, by the same APC, it is possible for linked help to occur (Afzali et al., 2007, Montecalvo et al., 2008).
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Taking these pathways into account, it is worth examining what effects MSCs may have on both alloantigen presentation and on the outcome of T cell alloantigen recognition (Griffin et al., 2013a) (Figure 1.2). It has been demonstrated extensively in vitro that MSCs can inhibit both alloantigen and mitogen-induced T cell proliferation regardless of whether both cell types are MHC-matched or mismatched, presumably due to the low levels of MHC class I and class II expressed by MSCs (Di Nicola et al., 2002, English et al., 2007, Comoli et al., 2008). It has also been proposed recently that MSCs require pre-activation with the pro-inflammatory cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α and/or interleukin (IL)-1β in order to exert these effects both in vitro and in vivo (Duijvestein et al., 2011, Li et al., 2012,

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**Fig. 1.1** Pathways of donor antigen recognition by T-, B-, and NK cells of the recipient following transplantation of allogeneic organs or tissues. Abbreviations: CD4 – CD4+ T cell; CD8 – CD8+ T cell; NK cell – natural killer cell; dAPC – donor antigen presenting cell; rAPC – recipient antigen presenting cell; dMHC-I – donor major histocompatibility complex class I; dMHC-II – donor major histocompatibility complex class II; rMHC-I – recipient major histocompatibility complex class I; rMHC-II – recipient major histocompatibility complex class II; BCR – B cell receptor; KIR – killer-cell inhibitory receptor. (Adapted from Griffin et al., 2013a).
Ren et al., 2008a). A number of studies have also reported on the effects of MSCs on CD4+ T cell differentiation. Some groups have shown that MSCs can promote Th2-like cytokine responses at the expense of Th1 responses (Batten et al., 2006, Lu et al., 2009), while others have shown that coculturing CD4+ T cells with MSCs leads to an increase in Th1 cells in correlation with a decrease in Th2 cells (Kong et al., 2009). There is, however, general agreement that MSCs can increase the frequency of regulatory T cells (Tregs) within cocultures provided a proportion of these cells are Tregs to begin with, as well as inhibit the differentiation of naïve CD4+ T cells into Th17 cells (Ghannam et al., 2010, Rafei et al., 2009). Ghannam et al (2010) were also able to show that MSCs could suppress production of IFN-γ, TNF-α, IL-17 and IL-22 by mature Th17 cells. Recently, Melief and colleagues could show that human MSCs promote the generation of CD4+CD25hiFoxP3+ Tregs in human peripheral blood mononuclear cell (PBMC) populations and that these populations were functionally suppressive. The authors attributed these effects to constitutive secretion of TGF-β1 by the MSCs and MSC-mediated skewing of monocytes towards IL-10/CCL18 producing, CD206+CD163+ anti-inflammatory M2 macrophages (Melief et al., 2013). The ability of MSCs to promote and sustain Tregs is of particular importance in a transplantation setting, as Tregs play a key role in maintaining tolerance to self-antigens as well as aiding in the prevention of autoimmune disease development (English et al., 2010). At least some of these effects seem to be operational in vivo as, in a mouse model of semi-allogeneic heart transplantation, Casiraghi and colleagues (2008) could show that MSC-induced Tregs were donor-specific. Furthermore, MSC infusion has been shown to significantly prolong the survival of MHC-mismatched skin grafts in baboons (Lazarus et al., 2005) and lower the incidence of graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell (HSC) transplantation in humans (Nemeth et al., 2009).
In addition to the reported effects MSCs have on T cell populations, multiple groups have attempted to elucidate the mechanisms that may be involved in MSC-mediated T cell modulation. A number of potential candidate molecules have been suggested as having key roles to play in MSC-mediated inhibition of T cell proliferation and/or activation such as IL-10 (Batten et al., 2006), transforming growth factor beta (TGF-β) (Groh et al., 2005), matrix metalloproteinases (MMPs: primarily MMP-2 and -9) (Ding et al., 2009), tumor necrosis factor stimulated gene (TSG)-6 (Lee et al., 2009), prostaglandin E2 (PGE2) (Aggarwal and Pittenger, 2005), nitric oxide (NO) (Sato et al., 2007) and indoleamine 2,3-dioxygenase (IDO) (Meisel et al., 2004). It is still unclear as to whether cell to cell contact or release of soluble factors is responsible for these effects. However, in all likelihood, both

**Fig. 1.2 Pathways of donor antigen recognition by T-, B-, and NK cells of the recipient following allogeneic MSC therapy.** For the different pathways, potential modifying factors known to be linked with the immunosuppressive and/or anti-inflammatory properties of MSCs are described in ‘Evidence’ boxes. Other cell types and symbols are as shown in Figure 1.1 (Adapted from Griffin et al., 2013a).
mechanisms have a role to play and may act in synergy. Evidence for this comes from studies showing that pre-treatment of MSCs with a combination of pro-inflammatory cytokines (namely, IFN-γ, TNF-α and/or IL-1β) leads to both an upregulation of inducible nitric oxide synthase (iNOS – the gene encoding NO) and thus an increase in the production of NO, which has antiproliferative effects through inhibiting Stat5 phosphorylation and an increase in chemokine production (Ren et al., 2008a, Shi et al., 2012). Subsequently, these chemokines drive the migration of T cells (via upregulation of CXCR3) into close proximity to the MSCs in cocultures, where the increased levels of NO suppress the T cells (Li et al., 2012). Similar to mouse MSCs, hMSCs also increase chemokine production upon stimulation with pro-inflammatory cytokines. They differ, however, in that they seem to utilize IDO instead of NO, thereby inhibiting T cell proliferation by depleting the essential amino acid tryptophan (required for T cell proliferation) from the local environment (Ren et al., 2009, Shi et al., 2012).

CD8+ T cells or cytotoxic T lymphocytes (CTLs) are MHC class I restricted cells which, when activated, can migrate to the site of transplantation and bind to allogeneic class I molecules on target cells. Once bound, these CTLs initiate target cell killing by inducing apoptosis mediated by the release of cytotoxic molecules such as perforin and granzyme B, coupled with an upregulation of Fas ligand and release of soluble mediators. While CD8+ T cells are not believed to be the key mediator cell type in terms of whether a graft rejects or not (this distinction is reserved for CD4+ T cells), Anglicheau et al (2008) have shown that kidney transplant recipients undergoing rejection have elevated mRNA levels of both perforin and granzyme B in their urine.

Similar to the effects MSCs have on CD4+ T cell proliferation, they have also been shown to be capable of inhibiting CD8+ T cell proliferation. MSCs exogenously loaded with relevant MHC class I peptides were shown to be resistant to CD8+ T cell-mediated lysis in vitro, an effect that coincided with a reduction in IFN-γ and TNF-α production by the T cells themselves (Rasmusson et al., 2007). However, while it has been reported that MSCs themselves may, under specific conditions, evade cytotoxic T cell-mediated
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killing, a number of groups have shown that, in the context of a mixed lymphocyte reaction (MLR), MSCs are only effective at suppressing cytotoxic T cell-mediated lysis when added to a MLR at the beginning of the reaction and ineffective at suppressing cytotoxicity once activation has occurred (Angoulvant et al., 2004, Rasmusson et al., 2003). Moreover, what role MSCs play in suppressing cytotoxic T cell effector functions in vivo remains to be elucidated.

MSC interactions with dendritic cells (DCs)

Given that both MSCs and some dendritic cell (DC) precursors originate in the bone marrow, it may be no surprise that MSCs can influence DC development. Studies have shown that MSCs can inhibit differentiation of DC precursors into DCs, as well as maturation of monocytes into DCs (Jiang et al., 2005, Nauta et al., 2006, Melief et al., 2013). Furthermore, when DCs were cocultured with MSCs in the presence of either lipopolysaccharide (LPS) or TNF-α, they failed to upregulate maturation markers such as MHC class II, CD40, CD80 or CD86 (English et al., 2008, Zhang et al., 2009). Such immature DCs are said to have a tolerogenic phenotype and also secrete higher amounts of anti-inflammatory (e.g. IL-10) and lower amounts of pro-inflammatory cytokines (e.g. IL-12) when compared to mature DCs (Zhang et al., 2009, English et al., 2010). In the absence of conclusive data, to date, on the significance of in vivo MSC-mediated modulation of DCs, one study has shown that MSCs can influence the migratory capacity of DCs to attenuate the development of murine lethal acute GvHD (Li et al., 2008).

Overall, our current understanding of how MSCs influence T cell activation and differentiation suggest the following potential scenarios: (a) direct presentation of allo-antigen by allo-MSCs, while possible, is not likely to lead to functional in vivo anti-donor T cell responses (Griffin et al., 2013a). A more likely outcome of such interactions may be the expansion of Tregs or generation of tolerogenic DCs which may, in turn, prevent T cell-mediated rejection of the allo-MSCs (Griffin et al., 2013a, English et al., 2010). (b) The indirect pathway of allo-antigen recognition (allo-recognition) in the context of
allo-MSC administration has not been investigated in detail as yet. However, it seems likely that such a scenario could occur in vivo especially as MSCs have been shown to upregulate MHC proteins following exposure to pro-inflammatory stimuli (Badillo et al., 2007). As outlined above, however, indirect presentation of MSC-derived alloantigen could lead to tolerogenic outcomes (English et al., 2008, Nauta et al., 2006) (c) While it has yet to be shown what role, if any, the semi-direct pathway of allorecognition plays in graft rejection it appears at least feasible that MSCs could participate in such a model. Recent work from a number of groups has shown that MSCs can secrete exosomes containing cell-specific mRNA, miRNA, lipids and proteins under certain conditions (Collino et al., 2010, Gatti et al., 2011, Kim et al., 2012), as well as participate in bi-directional exchange of membrane components (Strassburg et al., 2012). It would, therefore, seem that MSCs contain the necessary cellular ‘machinery’ to participate in modulating the semi-direct pathway of allorecognition. Future investigation into the feasibility of this concept certainly seems warranted.

1.2.2 Allorecognition and the B cell compartment

While T cell-mediated rejection may be the primary barrier to the successful transplantation of an allogeneic organ or tissue, B cells and B cell-related products can also contribute significantly to tissue/graft destruction. Aside from their role as antibody secreting cells (following differentiation into plasma cells), B cells themselves can act as APCs as they express functional MHC molecules and relevant co-stimulatory molecules such as CD40 (Tarlinton et al., 2008, Stegall et al., 2009). As APCs, B cells can recognize alloantigen through their B cell receptor, internalize it, process it into peptides and present it in the context of self-MHC to T cells, which can then provide help to initiate effector functions and antibody class switching (Afzali et al., 2007). Alloantibody-mediated damage can begin soon after transplantation but also contributes to chronic graft rejection (Terasaki and Cai, 2008) through mechanisms involving complement fixation and antibody-dependent cellular cytotoxicity. In the latter, cells such as macrophages and
natural killer (NK) cells bind to the Fc region of antibodies via specialist cell surface receptors, leading to activation of these cells and subsequent target cell lysis (Wood and Goto, 2012, Griffin et al., 2013a).

Although reports have been varied, for the most part, MSCs have been shown to be capable of inhibiting many B cell functions such as proliferation, differentiation (Tabera et al., 2008) and immunoglobulin production (Comoli et al., 2008) irrespective of the source of stimulation. Some studies suggest, however, that MSCs may actually stimulate in vitro activated B cells isolated from healthy individuals depending on the level of stimulation (Griffin et al., 2010, Rasmusson et al., 2007). These seemingly contradictory findings may be due to the different sources from which the MSCs originated (e.g. bone marrow, adipose tissue, etc.) or how the MSCs were stimulated in culture (non-specific polyclonal stimulation or antigen-dependent). An alternatively cleaved form of CCL2 (Rafei et al., 2008), IFN-γ and the interaction of programmed death (PD)-1 with programmed death-ligand 1 (PD-L1) (Schena et al., 2010) are among the mediators that have been identified as possible for MSC-inhibition of B cell functions. At present, in vivo data is less abundant but one recent study by Ge and colleagues (2009) showed in an in vivo mouse heart transplant model that MSC treatment could reduce both intragraft levels of IgG and circulatory IgM. However, we (Schu et al., 2012) have shown recently that significantly higher levels of serum IgG1 and IgG2 alloantibodies are present in rats treated intravenously (i.v) with allogeneic (allo)-MSCs compared to syngeneic (syn)-MSC treated rats. Therefore, if the ‘drug of choice’ is allo-MSCs, the possible generation of alloantibodies should be taken into account when considering the best course of treatment for the specific clinical indication.

### 1.2.3 Allorecognition and the NK cell compartment

In addition to classical pathways of allorecognition (as discussed at the beginning of this section), the innate immune system is also capable of distinguishing between self and non-self (Petersson et al., 1997). NK cells have the capacity to both directly lyse target cells (similar to cytotoxic T
lymphocytes) and release pro-inflammatory mediators (such as IFN-γ and TNF-α) (Wood and Goto, 2012). Moreover, their unique design means that their functions are tightly controlled by activatory and inhibitory receptors (such as killer cell immunoglobulin-like receptors (KIRs)), allowing them to detect and respond to non-self (Lanier, 2008, Li, 2010). Self-MHC class I molecules serve as ligands for KIRs and lack of engagement of these receptors leads to target cell lysis, the so called ‘missing self’ hypothesis (summarized in Afzali et al., 2007).

As NK cells are known to significantly contribute to the outcome of organ transplantation and MSCs are being used ever more frequently in an attempt to prolong allograft survival, it is important to understand what effect(s) MSCs may have on NK cells (and vice versa). At present, however, and particularly in vivo, reports on MSC-NK cell interactions have been infrequent. In vitro studies have revealed that MSCs can inhibit cytokine-mediated NK cell proliferation, surface receptor expression and expression of the key NK cell activation marker NKG2D (Spaggiari et al., 2008) and that PGE2 and IDO are crucial mediators in this process (Spaggiari et al., 2008). However, it is worth noting that some investigators have found that, despite the fact MSCs do express variable levels of MHC class I molecules, they are susceptible to lysis by activated NK cells (Hoogduijn et al., 2012, Poggi et al., 2005).

While it is well established that MSCs can modulate multiple components of the immune system either directly or indirectly and through mechanisms involving cell to cell contact and/or release of soluble factors, an in-depth understanding of potential mechanisms is lacking. It is because of these pleiotropic effects that MSCs are being extensively investigated in the field of organ transplantation.
1.3 Genetic modification of MSCs

Given the ability of MSCs to rapidly proliferate \textit{in vitro} (without loss of phenotype) and their innate immunosuppressive and anti-inflammatory properties, coupled with their low immunogenicity \textit{in vivo}, it is perhaps not surprising that genetic engineering of MSCs has received considerable attention over the past decade as a means to improve their therapeutic potential. Genetic modification of MSCs has improved their ability to migrate to sites of injury or infection, as well as enhance their survival and engraftment potential at these sites (Hodgkinson et al., 2010). Furthermore, as the primary mechanism by which MSCs exert their beneficial effects is through the release of paracrine factors, genetic modification has the potential to greatly increase these paracrine effects (Gnecchi et al., 2005, Gnecchi et al., 2008, Hodgkinson et al., 2010).

To date, the majority of pre-clinical and clinical studies of gene therapy have employed virus-based transfer of genetic material. These viral vectors generally have broad tropism, high transgene expression and high transduction efficiency, though this can vary depending on the target cell (Griffin et al., 2010). Adenoviruses (Ad) and retroviruses (RV; including lentiviruses) are the most commonly utilized carriers for transferring genetic information in human clinical trials (Edelstein et al., 2007). Importantly, McMahon and colleagues (2006) were able to show that MSCs derived from different species were amenable to transduction by both adenoviral and retroviral vectors (McMahon et al., 2006). The main advantage of using RV-vectors is that they permit long-term expression of the therapeutic gene due to the integration of the vector into the cellular DNA of the target cell. However, the main disadvantage is the potential risk of insertional mutagenesis. Indeed, Nienhuis and co-workers (2006) reported such an adverse event following RV-mediated gene transfer into hematopoietic stem cells (Nienhuis et al., 2006). In contrast, Ad vectors do not integrate into target cell DNA but have been shown to induce undesired immune responses after direct injection. Given this knowledge, transgene expression in rapidly proliferating MSCs will only be transient (Griffin et al., 2010).
However, this should not necessarily be considered a negative outcome as short-term expression may be sufficient (and even preferable) for treatment of diseases in which transient paracrine effects of MSCs could potentially enhance endogenous repair responses and tissue healing (Griffin et al., 2010, Hodgkinson et al., 2010). Furthermore, and of particular importance to the rationale for the work described in Chapter 3 of this thesis, due to the anti-inflammatory properties of MSCs, therapy with Ad-transduced MSCs should be less immunogenic than with direct administration of Ad-vectors as unbound Ad will be removed before cell/tissue transplantation.

In this regard, it is worth considering how genetic modification of MSCs with adenoviral vectors might improve MSC treatment in the context of allogeneic corneal transplantation. What would be the candidate molecules or transgenes one might choose? To date, most gene therapy strategies to prolong corneal allograft survival have focused on two main areas; anti-angiogenesis and modulation of the immune response directed at the allograft and so will be the focus here.

**Anti-angiogenesis**

The risk for corneal allograft rejection is increased in recipients with vascularised corneal beds. Furthermore, corneal vascularity is accompanied with the growth of lymphatic vessels and both contribute significantly to the damage and/or destruction of the previously immune-privileged cornea due to the infiltration of harmful inflammatory cells such as activated APCs and CD4+/CD8+ T cells. Cytokines and chemokines produced by these cells leads to an amplified immune response, culminating in allograft rejection (Qazi and Hamrah, 2013). Therefore, methods aimed at inhibiting the growth of blood and lymphatic vessels are necessary to preserve graft clarity and function. Strategies to address this issue usually involve treating the donor cornea *ex vivo* or direct treatment of the host graft bed *in vivo*. To date, there is little published data relating to the inhibition of angiogenesis using adenoviral vectors as applied to the cornea. However, numerous studies have reported successful inhibition of angiogenesis in cancer models using
adenovirus-mediated overexpression of, for example, the prototypic, naturally occurring angiogenesis inhibitors angiostatin (Raikwar et al., 2005, Frau et al., 2007) and endostatin (Feldman et al., 2000, Sauter et al., 2000). Angiostatin is one of the most potent inhibitors of angiogenesis and has been shown to inhibit endothelial cell migration and proliferation most likely through binding of a cell surface ATP synthase (Persano et al., 2007). Endostatin, on the other hand, can inhibit endothelial cell functions by several means, including attenuation of VEGF receptor signalling and its binding to α5β1 integrins (Persano et al., 2007). Ad-mediated overexpression of either angiostatin or endostatin on MSCs might be an option to reduce or inhibit blood vessel formation in corneal allografts.

**Immunomodulation**

Corneal graft rejection is predominantly caused by an aggressive immune response targeted at donor antigens present on the allograft. While cellular players such as activated APCs (primarily DCs) and molecular players including pro-inflammatory cytokines play important roles in both orchestrating and sustaining this targeted immune response, allo-activated CD4+ T cells have been shown to play a central role in the allograft rejection process (Pleyer et al., 1995, He et al., 1991). In order to achieve full activation, T cells require two signals. The first involves the engagement of the TCR with either MHC class I or II molecules on APCs. The second requires the interaction of the T cell costimulatory molecule CD28 with B7 antigens (B7.1/CD80 and B7.2/CD86) on APCs (Jun and Larkin, 2003). Strategies aimed at interfering with this pathway, in particular, have yielded promising results with regard to inhibition of graft rejection. Gong and colleagues reported significant prolongation of corneal allograft survival by preventing T cell activation following intraperitoneal injection of adenoviral vectors expressing cytotoxic T-lymphocyte antigen 4 (CTLA4-Ig) protein when administered on the day after transplantation (Gong et al., 2006). CTLA4-Ig is a fusion protein that competitively inhibits the binding of CD80/86 with CD28 (Qazi and Hamrah, 2013). Another candidate molecule
that has received considerable attention is Inducible T cell Costimulator (ICOS or CD278). This is an inducible costimulatory receptor expressed by both activated and memory T cells (Hutloff et al., 1999). It has been shown that blocking of the interaction of ICOS with its corresponding receptor can abrogate disease symptoms in murine experimental autoimmune encephalomyelitis (EAE) (Rottman et al., 2001). However, when investigated in the context or corneal transplantation, Fabian and co-workers observed no prolongation of allograft survival after either local or systemic adenovirus-mediated overexpression of soluble ICOSIg (Fabian et al., 2007). Recently, the PD-1/PD-L1 pathway has been investigated due to its role in the negative regulation of immune responses (Ritter et al., 2013). To this end, our group has shown that ex vivo lentivirus-mediated overexpression of PD-L1 on cultured donor corneas prior to transplantation was able to significantly prolong allograft survival in a rat model of corneal transplantation (Nosov et al., 2012).

Another approach for promoting graft survival is the modulation of pro-inflammatory cytokines (either directly or indirectly through overexpression of anti-inflammatory cytokines) known to be involved in mediating graft rejection. Indeed, adenovirus-mediated gene transfer of both IL-10 and the IL-12 inhibitory subunit IL-12p40 have been shown to significantly prolong allograft survival in ovine models of corneal transplantation (Klebe et al., 2001, Klebe et al., 2005). Another candidate gene with potential therapeutic benefit is TGF-β. TGF-β is a key anti-inflammatory cytokine with pleiotropic effects and has been shown previously to be able to suppress immune cell activity in the aqueous humor (anterior chamber) of the eye (Cousins et al., 1990). A study by Dekaris and colleagues analysed the levels of TGF-β in the aqueous humor of patients undergoing corneal transplantation following a previous episode of either non-inflammatory (e.g. keratoconus or stromal dystrophy) or inflammatory corneal disease, including patients that had a previous failed graft. Remarkably, in all cases, they found that patients with a concentration of ≥1,500 pg/ml of TGF-β in their aqueous humor accepted their grafts. In contrast, patients with a concentration lower than this rejected their grafts (Dekaris et al., 2001). Based on these findings, one potential
option to increase corneal allograft survival may be to locally apply Ad- 
transduced MSCs overexpressing TGF-β, assuming therapeutic levels (i.e. 
≥1,500 pg/ml) of the transgene can be achieved. However, despite these 
promising or potentially promising outcomes, the immunogenicity of 
adenviral vectors remains a significant barrier to effective immune 
suppression. It is here that the hypooimmunogenic characteristics of MSCs 
might be best exploited to shield the vector from possible immune-mediated 
attack and thus act as carriers for the delivery of therapeutic genes, such as 
those discussed above.

Other strategies utilising Ad vectors that could potentially further improve 
MSC treatment might include efforts to increase the mobilization and 
engraftment potential of MSCs. In the setting of corneal transplantation, this 
might be enhanced migration to or engraftment in the transplanted cornea 
itself, but also the draining cervical lymph nodes as they have been shown to 
play a crucial role in determining the fate of the graft (Plskova et al., 2002). 
To this end, chemokines and their corresponding receptors are the key 
molecular targets. CCR7 has been shown to be upregulated on APCs 
present within the inflamed cornea and interaction with the CCR7 ligand 
CCL21 (which is itself significantly upregulated) can facilitate the migration of 
these cells to the draining lymph nodes (Jin et al., 2007). Therefore, Ad- 
mediated overexpression of CCR7 on systemically delivered MSCs might be 
one strategy to enhance migration of the cells to this site. Another possible 
target might be the CXCL12/CXCR4 axis. CXCL12 or stromal cell-derived 
factor-1 (SDF-1) is known to be important for MSC chemotaxis and organ- 
specific homing to injured or inflamed tissue and the binding of CXCL12 to its 
receptor CXCR4 is critical to this process (Marquez-Curtis and Janowska- 
Wieczorek, 2013). However, while MSCs highly express CXCR4 in the bone 
marrow, expression is markedly reduced during the process of in vitro 
expansion (Honczarenko et al., 2006). This impairs the ability of MSCs to 
then respond to homing signals emanating from sites of injury/inflammation. 
Due to the importance of this chemokine/receptor signalling axis for MSC 
migration, a number of groups have already reported that overexpression of 
CXCR4 on MSCs using adenoviral, retroviral and lentiviral vectors can
increase homing and engraftment of MSCs in different animal models of injury (Maijenburg et al., 2012, Zhang et al., 2008, Cheng et al., 2008). However, this has yet to be explored in the context of corneal transplantation but would certainly warrant further investigation.

With the development of more advanced gene therapy vectors, the possibility of transducing cells and tissues with more than one transgene has become a reality. Such technology has the potential to greatly increase the therapeutic efficacy of Ad-transduced MSCs. By utilising a bi-cistronic expression construct incorporated into the Ad-vector it is possible to transfer two (or potentially more) genes of interest to MSCs prior to infusion. For example, in the context of corneal transplantation, one could combine a gene that could increase the migratory capacity of MSCs (e.g. CCR7 or CXCR4) to the draining cervical lymph nodes with the overexpression of a therapeutic molecule (such as IDO in human MSCs or iNOS in rodent MSCs) known to inhibit T cell proliferation, which could greatly enhance the therapeutic efficacy of MSC treatment compared to their un-modified counterparts. Furthermore, using MSCs isolated from GFP/RFP transgenic rodent strains would enable visual confirmation of successful migration. While not involving viral vectors, there is at least one recent report demonstrating the clear benefit of this dual-expression approach. Sarkar and co-workers could initially show that by modifying the cell surface of human MSCs with a construct containing sialyl Lewis X (SLeX), a molecule that mediates leukocyte cell rolling in inflamed tissue, MSCs could home to inflamed tissue with higher efficiency compared to native MSCs (Sarkar et al., 2010). This work was further enhanced by a subsequent study including the overexpression of IL-10 in addition to cell surface modification with SLeX. Using an ear inflammation model, these researchers could show that systemic administration of these modified MSCs led to a superior anti-inflammatory effect in vivo that was dependent on rapid migration to the inflamed ear (Levy et al., 2013, Bernardo and Fibbe, 2013).
Other areas which have been targeted extensively include delivery of therapeutic products to different cancer models and improving functionality following acute myocardial infarction (Griffin et al., 2010).

**Acute myocardial infarction (AMI)**

A substantial body of work has investigated the ability of genetically modified MSCs to improve functionality following AMI, with most of these studies focusing on local application (intra-myocardial injection) of transduced or untransduced MSCs. The main aims of these studies were to improve survival of the MSCs, enhance their migratory and engraftment potential and promote angiogenesis and tissue repair by increased production of soluble factors. For example, Mangi and colleagues (2003) were able to show that MSCs engineered to over-express the anti-apoptotic protein Akt using retroviral vectors led to major improvements in overall cardiac function, by reducing infarct size and improving left ventricular function (Mangi et al., 2003). Additional work revealed that these RV.Akt-transduced MSCs mediated their therapeutic effects by increasing production of reparatory and anti-inflammatory factors, as opposed to differentiation into cardiomyocytes which had initially been considered (Gnecchi et al., 2005, Noiseux et al., 2006). More recently, it was shown that MSCs transduced with an Ad-vector encoding both vascular endothelial growth factor (VEGF) and the stem cell homing/retention factor SDF-1 could enhance angiogenesis and improve cardiac function in a rat model of AMI. The authors demonstrated that dual over-expression had increased benefits over MSCs transduced with either gene alone (Tang et al., 2010).

**Cancer**

To date, multiple studies have reported the use of MSCs as delivery agents for various molecules with the ability to inhibit tumor growth. MSCs engineered to over-express either IFN-α or -β were found to have therapeutic
benefit, by accumulating cells in S phase and promoting apoptosis, when used to treat several different cancer models including melanoma (Studeny et al., 2002, Ren et al., 2008b), prostate cancer (Ren et al., 2008c) and glioma (Lee et al., 2009). Other examples of where genetically modified MSCs have been applied successfully to treat cancers include MSCs engineered to over-express the TNF-related apoptosis-inducing ligand (TRAIL) via lentiviral (Loebinger et al., 2009) and adenoviral (Mohr et al., 2008) vectors. TRAIL is a transmembrane protein that causes selective apoptosis of tumor cells while leaving normal cells unaffected (Wiley et al., 1995). Loebinger and colleagues could demonstrate that TRAIL-expressing MSCs induced apoptosis in several cancer cell lines in in vitro coculture experiments. Moreover, they found that TRAIL-expressing MSCs significantly reduced tumor growth and cleared lung metastases in approximately 40% of treated mice compared to 0% of controls (Loebinger et al., 2009).

While the application of genetically modified MSCs in cancer therapy has seen some clear progress, there are disadvantages that must be taken into account when considering the use of MSCs, particularly when applied to models of breast cancer. MSCs have been shown to promote the growth and metastasis of breast cancer cells via production of IL-6 and CCL5, respectively (Sasser et al., 2007, Karnoub et al., 2007). Furthermore, MSCs could protect breast cancer cells by inducing the expansion of Tregs in a TGF-β-dependent manner (Patel et al., 2010). More recently, Chaturvedi and co-workers (2013) reported that hypoxia inducible factor (HIF)-dependent breast cancer-mesenchymal stem cell bidirectional signalling was responsible for promoting metastasis (Chaturvedi et al., 2013). In contrast, however, Dwyer and colleagues reported a significant reduction in tumor growth following administration of recombinant adenovirus-transduced human MSCs engineered to overexpress human sodium iodide symporter (NIS) in a mouse model of breast cancer (Dwyer et al., 2011).
1.4 Corneal transplantation (penetrating keratoplasty)

1.4.1 Immunologic privilege of the eye

The eye is one of only a select few tissues in the body that enjoy immune privileged status. This concept, as applied to the cornea, was first reported 60 years ago by Billingham and Boswell (1953). Inflammatory events in these immune privileged tissues are governed by a different set of rules to those at work in other tissues and organs. Multiple mechanisms operate to confer immune privilege to the eye and have been reviewed extensively (Streilein, 2003, Niederkorn, 2003, Stein-Streilein, 2008, Niederkorn and Larkin, 2010). The cornea is a delicate structure and is under constant threat from injury and/or infection which may lead to inflammation and tissue damage. Such events could result in a debilitating loss of vision. In order to prevent this from happening, the eye is equipped with several features designed to suppress or circumvent inflammatory responses. These include:

1. **Corneal avascularity:** The normal, healthy cornea is an avascular structure and lacks the presence of lymphatic vessels, thereby shielding itself from immune-mediated attack (Niederkorn, 1999). Avascularity is maintained by constitutive expression of soluble VEGF by epithelial cells (Ambati et al., 2006), while lymphangiogenesis is suppressed by secretion of soluble VEGF-receptor 2 (VEGF-R2) by keratocytes and epithelial cells (Albuquerque et al., 2009).

2. **Secretion of immunosuppressive factors:** The eye and the aqueous humor in particular, have been shown to produce several immunomodulatory factors that contribute significantly to maintaining this immune privileged status. These include anti-inflammatory cytokines (e.g. TGF-β2), complement inhibitors (Goslings et al., 1998), neuropeptides, alpha-melanocyte stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP) (Stein-Streilein and Taylor, 2007, Taylor, 2007).
3. **Cell-based immunomodulation:** Retinal pigment epithelial (RPE) cells distributed along the borders of the eye can inhibit primed T cells by production of thrombospondin-1 (TSP-1) (Zamiri et al., 2005). Furthermore, stromal cells from a number of sources (e.g. retina and iris) can convert T cells into cytotoxic T lymphocyte antigen (CTLA)-4 and Foxp3-expressing Tregs (Sugita et al., 2006a, Sugita et al., 2006b).

4. **Fas ligand (FasL):** Both membrane-bound FasL (CD95L) and its soluble form (sFasL) have been shown to induce apoptosis of infiltrating activated T cells and neutrophils (Griffith et al., 1995, Stuart et al., 2005).

5. **Anterior chamber associated immune deviation (ACAID):** ACAID is a physiological phenomenon which describes the induction of peripheral tolerance to alloantigen under non-inflammatory conditions. Briefly, upon encountering alloantigen in the anterior chamber, F4/80+ APCs pick up and transport this antigen to the spleen. There, the alloantigen-loaded APCs interact with T cells, NKT cells and B cells resulting in the induction of antigen-specific Tregs, capable of inhibiting both local and systemic Th1- and Th2-type immune responses (Skelsey et al., 2003, Niederkorn, 2007a, Niederkorn, 2007b, Niederkorn, 2009).

### 1.4.2 MSCs and corneal injury/transplantation

Corneal damage can result from a variety of different clinical disorders including chemical or thermal injury leading to severe inflammation, aniridia, or Stevens-Johnson syndrome. In addition to inflammation, these disorders are often accompanied with stromal opacity, neovascularization or epithelial defects, which ultimately may lead to total and permanent loss of vision. Current treatment strategies involve administration of anti-inflammatory drugs in the acute phase followed by limbal stem cell (LSC) infusion once
inflammation has subsided. However, due to limited availability of sufficient numbers of LSCs, coupled with high immunorejection rates, novel therapeutics are needed for treatment of damaged corneas.

Due to the potent anti-inflammatory effects of MSCs and their ability to be expanded to clinically useful numbers \textit{in vitro}, several groups have investigated the use of MSCs to promote corneal wound healing following chemical injury. In a rabbit model of corneal damage caused by alkali burn, systemically infused rabbit MSCs were shown to engraft in injured corneas and promote wound healing by proliferating, differentiating and synergizing with hematopoietic stem cells (Ye et al., 2006). Ma and colleagues (2006) tested the ability of hMSCs seeded on human amniotic membrane to promote wound healing in a rat model of corneal damage. They found that, rather than differentiating into corneal epithelial cells, the hMSCs could successfully reconstruct the corneal surface by inhibiting inflammation and inflammation-related angiogenesis (Ma et al., 2006). Similarly, it was shown that topically applied rat MSCs could suppress corneal inflammation and reduce the infiltration of CD4+ T cells in chemically burned rat corneas. These effects correlated with decreased expression of the pro-inflammatory cytokines IL-2 and IFN-\(\gamma\) and increased expression of the anti-inflammatory mediators IL-10 and TGF-\(\beta1\) (Oh et al., 2008). Most recently, rat MSCs were again shown to attenuate inflammation and promote corneal wound healing in alkaline burned rat corneas. MSCs also reduced the level of cornea neovascularization. In this study, the authors reported that these effects were related to reduced infiltration of CD68+ macrophages and down-regulation of macrophage inflammatory protein-1 alpha (MIP-1\(\alpha\)), TNF-\(\alpha\) and VEGF (Yao et al., 2012).

As outlined in the studies above, substantial evidence suggests that MSCs can inhibit corneal inflammation, reduce corneal neovascularization and suppress the infiltration of damaging immune cells into the cornea after chemical injury. However, studies reporting the effects of MSCs on corneal allograft transplantation have been less frequent. In one of the first reports, using a pig-to-rat corneal xenotransplantation model, Oh and co-workers showed that topical application of rat MSCs failed to prolong xenograft
survival (Oh et al., 2009). However, more recently, intravenously injected hMSCs were able to prolong allograft survival in a mouse corneal transplantation model. In this study, the authors demonstrated that hMSCs can inhibit the early surgery-induced inflammatory response by increasing expression of the anti-inflammatory multifunctional protein TSG-6 (Oh et al., 2012).

As studies investigating the effects of MSCs on corneal allograft survival are infrequent, more research in this area is certainly warranted.
1.5 Aims and hypotheses of the PhD project

Although corneal transplantation success rates are high within the first year, the prognosis is much less favourable 5 years post-transplantation with immune-mediated rejection the primary cause of allograft failure. While effective treatments, such as the use of topical corticosteroids, can benefit many patients, they are much less effective in individuals deemed to be “high-risk”. That is, patients that have suffered previous graft failure or those with significant corneal neovascularization at the time of transplantation. For this reason, on-going preventive and therapeutic measures are needed to improve the prognosis following corneal transplantation. MSCs are being studied extensively in a wide range of diseases/disorders, and more recently in solid organ transplantation, due to their ability to suppress or dampen host immune responses. However, to date, reports on the therapeutic efficacy of MSC therapy have been mixed and the genetic modification of MSCs using viral vectors is one option to improve their therapeutic potential. Consequently, the focus of this research work was twofold. Firstly, to investigate if the genetic modification of MSCs using recombinant adenovirus alters the syngeneic host immune response and secondly, to investigate the therapeutic efficacy of MSCs in the promotion of corneal allograft survival.

1.5.1 Adenoviral transduction of MSCs: in vitro and in vivo immune response after cell transplantation

MSCs are thought to be hypo-immunogenic due to their low expression of MHC class I and lack of expression of MHC class II molecules, coupled with the absence of expression of T cell activating costimulatory molecules such as CD80, CD86 and CD40 (Di Nicola et al., 2002, Rasmusson, 2006, Nauta and Fibbe, 2007, DelaRosa and Lombardo, 2010). Despite these characteristics, the use of MSC therapy has yielded mixed results. Genetic modification of MSCs using recombinant adenovirus could be a promising approach to enhance the therapeutic efficacy of these cells. The aim of the first part of this project was therefore to investigate what effect(s) adenoviral
transduction of MSCs would have on their expression of immunologically relevant molecules such as chemokines, chemokine receptors, toll-like receptors (TLRs) and pro-inflammatory cytokines. This study also included a comprehensive flow cytometry based analysis of the proportion of immune cell populations in peripheral blood and tissues (i.e. lungs and spleen) following systemic administration of either untransduced MSCs or MSCs transduced with green fluorescent protein-expressing adenovirus (Ad.GFP) in rats. Based on previous reports on the genetic modification of MSCs using recombinant adenovirus (Rooney et al., 2008, McMahon et al., 2006, Shujia et al., 2008) and baculovirus (Chuang et al., 2009), it was hypothesised that adenoviral transduction would lead neither to significant changes in the immune profile of MSCs nor aggressive immune responses to transduced cells in vivo.

1.5.2 The role of MSC treatment in promoting corneal allograft survival

The goal of the second part of this study was to comprehensively investigate the potential therapeutic effects of MSCs on corneal allograft survival. For this purpose, the therapeutic benefit using MSCs from three immunologically distinct sources were investigated. If no therapeutic benefit was observed with unmodified MSCs (i.e. if MSCs failed to prolong allograft survival), the cells would be genetically modified to overexpress therapeutic genes. However, based on recently published reports showing the ability of unmodified MSCs to repair chemically damaged corneas (Ye et al., 2006, Ma et al., 2006, Ye et al., 2008, Oh et al., 2008), it was hypothesised that MSCs, without the need for genetic modification, could significantly prolong corneal allograft survival in a fully MHC-mismatched rat model.
Chapter Two
Materials and Methods
2 Materials and Methods

2.1 Cell culture and genetic modification

2.1.1 Rat MSC isolation, expansion and differentiation

Bone marrow cells were extracted from male Sprague-Dawley (CD), Lewis (LEW), Dark-Agouti (DA) or Wistar Furth (WF) rats (8-12 weeks old; all Harlan Laboratories, UK). Animals were euthanized by CO₂ inhalation and femurs and tibias were flushed with a mixture of alpha modified Eagle’s medium and Ham’s F12 nutrient mixture (αMEM-F12; both Sigma-Aldrich, Dublin, Ireland) to isolate the bone marrow cells. The cells were washed once with Dulbecco’s Phosphate Buffered Saline (DPBS [Invitrogen, Dun Laoghaire, Ireland]) and centrifuged at 400 x g for 5 mins. The centrifuged cells were then transferred to T-175 flasks at a density of 9x10⁵ cells/cm² and rat MSC medium (consisting of αMEM-F12; 10% fetal bovine serum [FBS; Sigma-Aldrich] with penicillin/streptomycin supplements [Invitrogen]) was added to a final volume of 30 ml per flask. Cells were maintained at 37°C, 5% CO₂ and 90% humidity. 3 days after the initial isolation and seeding, medium and non-adherent cells were removed and replaced with fresh rat MSC medium. Medium was changed every 3-4 days until confluency was nearly reached. At the end of culture, adherent cells were detached using 0.25% trypsin/1mM EDTA (Sigma-Aldrich). MSCs between passage 4 and passage 8 (P4-P8) were used for subsequent transduction and transplantation experiments.

In order to investigate the osteogenic and adipogenic capacity of rat MSCs, cells were plated in 6 well plates at various densities; 2x10⁵ cells/well (adipogenic) and 30,000 cells/well (osteogenic) were treated with differentiation-specific induction medias the following day. Differentiation induction medias were prepared as shown in Table 2.1. Lipid accumulation (adipogenesis) and quantification of mineral deposition (osteogenesis) were performed as previously described (Duffy et al., 2010) using Oil Red O and Alizarin Red assays, respectively. The bi-lineage differentiation capacities of different MSC preparations were determined by Georgina Shaw (REMEDI).
The cell surface profile of different MSC preparations were characterised by flow cytometry. Flow cytometric analysis showed that rat MSCs express the cell surface markers CD29, CD73, CD90, and CD44H and lowly express or are negative for CD45RA and CD71.

<table>
<thead>
<tr>
<th>Adipogenesis Induction Medium</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>DMEM (high glucose)</td>
<td>-</td>
</tr>
<tr>
<td>Dexamethasone 1mM</td>
<td>1µM</td>
</tr>
<tr>
<td>Insulin 1mg/ml</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Indomethacin 100mM</td>
<td>200µM</td>
</tr>
<tr>
<td>500mM MIX</td>
<td>500µM</td>
</tr>
<tr>
<td>Antibiotic/Antimycotic solution</td>
<td>100U/mL penicillin</td>
</tr>
<tr>
<td></td>
<td>100µg/mL streptomycin</td>
</tr>
<tr>
<td>FBS</td>
<td>10%</td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Osteogenesis Induction Medium</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>αMEM</td>
<td>-</td>
</tr>
<tr>
<td>Dexamethasone 1mM</td>
<td>100nM</td>
</tr>
<tr>
<td>Ascorbic acid 2-P 10mM</td>
<td>50µM</td>
</tr>
<tr>
<td>β glycerophosphate 1M</td>
<td>10mM</td>
</tr>
<tr>
<td>FBS</td>
<td>10%</td>
</tr>
<tr>
<td>Antibiotic/Antimycotic 100x solution</td>
<td>100U/mL penicillin</td>
</tr>
<tr>
<td></td>
<td>100µg/mL streptomycin</td>
</tr>
<tr>
<td></td>
<td>0.25µg/ml amphotericin B</td>
</tr>
</tbody>
</table>

Table 2.1. Adipogenesis and osteogenesis induction media.

2.1.2 Genetic modification of rat MSCs

First generation E1/E3-deleted serotype 5 adenovirus encoding green fluorescent protein (Ad.GFP) (or β-galactosidase (Ad.β-Gal) which was used in certain experiments where GFP fluorescence would potentially interfere with experimental read-outs) under the control of the cytomegalovirus (CMV) immediate early promoter was generated and purified as described elsewhere (Ritter et al., 1999). Briefly, Human Embryonic Kidney 293T cells (293T) were grown in high glucose DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin in 15 cm plates and Ad.GFP was added at a multiplicity of infection (MOI) of 5-10. 36-48 hr later the cells were
harvested. The virus was released from the cells by freeze/thawing the cells 5 times. The virus was purified over a CsCl-gradient and banded virus was recovered and spin-dialyzed over sepharose CL6B (Pharmacia, Freiburg, Germany). Viruses were then aliquoted after the addition of 10% glycerol and stored at -80°C. To titer the final preparation, an aliquot of virus was serially diluted and assayed for its ability to form plaques on 293T cell monolayers. The plaque-forming capacity of different virus preparations was approximately $1 \times 10^{10}$ plaque forming units (pfu)/ml. Ad.GFP transduction of P4-P8 CD-MSCs was performed as described previously (Rooney et al., 2008). Briefly, $1 \times 10^5$ MSCs/well were seeded into 6 well plates in a total volume of 2 ml of MSC medium per well for 24 h. Medium was then removed and Ad.GFP (or Ad.β-Gal) was added at an MOI of 100 to the cells followed by spin centrifugation at 2,000 x g for 90 mins at 37°C. Medium was removed and fresh MSC medium was added to the cells. Light and fluorescent microscopy was performed 24 h later to confirm successful transduction of the MSCs. MSCs were then detached by incubating with 0.25% trypsin/1 mM EDTA for 5 mins at 37°C and centrifuged at 400 x g for 5 mins. Cells were then washed three times with DPBS and pellets were resuspended in FACS buffer. The percentage of GFP+ MSCs was then measured by flow cytometry using a FACS Aria (BD Biosciences). For certain experiments, where indicated, MSCs were transfected with pNFκB-d2EGFP (a reporter plasmid that permits expression of GFP under control of the NFκB transcription factor binding sites [Clontech, Saint-Germain-en-Laye, France]) using TurboFect™ in vitro Transfection Reagent according to the manufacturer’s instructions (Fermentas, York, UK). Also, in these experiments, transduction of pNFkB-d2EGFP-transfected cells with Ad.β-Gal (MOI 100) was carried out so as not to interfere with GFP expression of transfected cells.

2.1.3 Pre-treatment of MSCs with IFN-γ

LEW-MSCs were expanded as described in Section 2.1.1 until sufficient numbers were reached (this was estimated based on previous observations on the number of cells retrieved from a T175 flask at <90% confluency). To
generate IFN-γ-pre-stimulated MSCs, fresh rat MSC medium supplemented with 500U/ml recombinant rat IFN-γ was added to the cells (PeproTech, Rocky Hill, NJ). Cells were then returned to incubators and cultured for a further 72 h in the presence of IFN-γ.

2.1.4 Isolation of dendritic cells (DCs) from rat spleens

Spleens were collected from CD rats, transferred to petri-dishes and homogenised using a syringe plunger. The released cells were collected in 10 ml of PBS/EDTA solution and filtered using a 40 μm Nylon cell strainer. This step was repeated four times, each time with 10 ml DPBS/EDTA solution, giving a total cell suspension of 50 ml. The cells were collected in a 50 ml tube and centrifuged at 300 x g for 8 mins at 4°C. After washing with DPBS, cells were re-suspended in 20 ml PBS/EDTA solution. The cells were then divided into four 50 ml tubes with each tube containing an equal volume of 5 ml. The 5 ml cell suspensions were then underlaid with 2 ml of Nycodenz solution (Progen Biotechnik, Heidelberg, Germany). In order to generate the density gradient, the tubes were centrifuged at 300 x g for 15 mins at 20°C. After centrifugation, the DC population was contained within a visible white ring. The DCs were then collected and washed twice with 15 ml of PBS/EDTA solution and centrifuged at 500 x g for 5 mins at 4°C. The DCs were then re-suspended in 1 ml of RPMI solution and counted using a haemocytometer. Finally, approximately 1x10^6 DCs/well were seeded in 6 well plates and incubated at 37°C, 5% CO₂.

2.1.5 RAW 264.7 cell culture

RAW 264.7 is a murine monocyte/macrophage cell line (American Type Culture Collection (ATCC), Middlesex, UK). RAW cells can be readily transduced with Ad-vectors and were used as a positive control for detection of IL-1β in cell culture supernatants. The cells were seeded in T-175 flasks in 30 ml of culture medium containing DMEM supplemented with 10% FCS, 2 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1 mM sodium
pyruvate (all Sigma-Aldrich) and incubated at 37°C, 5% CO₂. Cells were passaged every 2/3 days until approximately 80% confluent.

2.2 RNA isolation, cDNA synthesis and qRT-PCR

2.2.1 RNA isolation

Total RNA from rat MSCs and tissue samples was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. Briefly, whole cells were lysed by re-suspending in 1 ml of TRIzol and passing through a pipette tip several times to ensure complete lysis. Samples were then stored at -20°C or -80°C depending on whether short-term or long-term storage was required. Samples were removed from the freezer, thawed and incubated for 5 mins at room temperature to allow dissociation of nucleoprotein complexes. 0.1 ml of chloroform (Sigma-Aldrich) was then added to each sample and the tubes were shaken by hand for 15 seconds and incubated at room temperature for 3 mins. Following incubation, the samples were centrifuged at 11,900 x g for 15 min at 4°C. After this step, three distinct phases formed with the RNA remaining in the uppermost aqueous phase. Thus, the aqueous phase was removed and transferred to a fresh 1.5 ml eppendorf tube. In order to precipitate the RNA from the aqueous phase, 0.5 ml of 2-propanol (Sigma-Aldrich) was added to each sample followed by repeated re-suspension using a pipette. Furthermore, 1 µl of glycogen (Fermentas, York, UK) was added to each sample at this stage to enhance recovery of the RNA. Samples were then incubated room temperature for 10 mins and centrifuged at 11,900 x g for 10 mins at 4°C. A gel-like RNA pellet formed following this step. Supernatants were then carefully removed and the RNA pellet was washed with 1 ml of 75% ethanol (EtOH) (Sigma-Aldrich). Samples were mixed by vortexing briefly and centrifuged at 7,500 x g for 5 mins at 4°C. EtOH was then removed and the RNA pellets were air-dried for 15 mins at room temperature. Finally, the RNA pellet was dissolved in 11 µl of nuclease-free water.
2.2.2 First strand cDNA synthesis

The following components were then added (in the indicated order) to the 11µl containing the RNA, in order to synthesise cDNA: 1 µl of random hexamer primers (0.2 µg/µl), 4 µl of 5X reaction buffer, 1 µl of Ribolock Ribonuclease Inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix, and 1 µl of RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µl) (all Fermentas), giving a final volume of 20 µl. The reaction mixture was then mixed gently and centrifuged briefly. Next, the mixture was incubated at 25°C for 5 mins followed by 42°C for 60 mins. The reaction was stopped by heating at 70°C for 5 mins. The newly synthesised cDNA was then either used directly or stored at -20°C until required.

2.2.3 Quantitative Real-Time polymerase chain reaction (qRT-PCR)

mRNA gene expression analysis was performed using TaqMan RT-PCR. The full list of primer and probe sequences for the genes analysed in this study can be seen in Table 2.2. Generally, cDNA was diluted 1:10 for analysis. The chemokine and chemokine receptor primer and probe sequences were a gift from Dr. Francesca Odoardi of the Institute for Multiple Sclerosis Research, Department of Neuroimmunology, Gemeinnützige Hertie-Stiftung and University Medical Centre Göttingen, Germany. All other primer and probe sequences (unless indicated otherwise) were designed by Dr. Mikhail Nosov and synthesized by Metabion International AG (Germany). mRNA expression levels of all genes were normalized to the mRNA expression of the house-keeping gene β-actin.

The experiment was carried out in MicroAmp Fast optical 96 well reaction plates. Generally, the reaction mix per well consisted of 5 µl of Maxima Probe qPCR Master Mix (2x), 3 µl of 1:10 diluted cDNA, and 2 µl of the appropriate primer mix. Each reaction was done in duplicate/triplicate.

The TaqMan RT-PCR was run with the STEP ONE Plus Real-time PCR System (Applied Biosciences). The real-time PCR program used can be
seen in Figure 2.1. The data was collected at the end of each cycle in stage 2.

<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>β-Actin</td>
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<td>GATATCCCTGACCTCTCTTCTC</td>
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</tr>
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<td>CCR2</td>
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</tr>
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</table>
Table 2.2. Primer and probe sequences used for gene expression analysis.
Figure 2.1. RT-PCR program used for gene expression studies.
2.3 In vitro assays

2.3.1 MSC stimulation with DC-conditioned medium

To isolate dendritic cells from the bone marrow, bone marrow was flushed from both the femur and tibia of male DA rats. The cells were collected in a petri-dish in 10 ml of DPBS and transferred to a 50 ml tube. The cells were then centrifuged at 400 x g for 5 mins. Following centrifugation, the cell pellet was resuspended in Ammonium-Chloride-Potassium (ACK) buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (all Sigma-Aldrich), distilled H₂O) to lyse the red blood cells. The bone marrow cells were then washed in complete culture medium containing RPMI-1640 (Gibco, Grand Island, NY) supplemented with heat inactivated 10% FBS, 2 mM L-glutamine, 0.1 M non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM β-mercaptoethanol (β-ME, Gibco). Next, cells were seeded in a 6 well plate at a concentration of 4.5x10⁶ cells/3ml of complete culture medium per well. Culture medium was supplemented with 5 ng/ml of rat granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml of rat IL-4. Cells were then incubated at 37°C at 5% CO₂. 3 days after initial seeding, half of the medium from each well was removed and the cells were resuspended in fresh complete culture medium supplemented with rat GM-CSF and IL-4 and added back into the culture. On the 5th day of culture all medium was removed and replaced with fresh complete culture medium, again supplemented with GM-CSF and IL-4. Finally, on the 7th day of culture half the medium was removed and replaced with fresh medium supplemented with GM-CSF and IL-4. On the 9th day of culture, the bone marrow-derived dendritic cells (BMDCs) were stimulated in a 6 well plate with 1 μg/ml of LPS in a total volume of 3 ml of complete medium per well. 24 h later the supernatant was harvested, filter sterilised (0.2 μm) and added to either untransduced or Ad.GFP transduced MSCs in 6 well plates in a 1:1 ratio with MSC medium in a final volume of 1.5 ml per well. Before adding the BMDC-conditioned medium to the MSCs, samples were taken and screened for a panel of pro-inflammatory cytokines. It was found that the LPS-stimulated BMDCs produced higher levels of IL-1α, IL-1β, IFN-γ, TNFα and
IL-6 compared to unstimulated BMDCs (data not shown). Untransduced and Ad.GFP transduced MSCs were incubated for 24 h with the BMDC-conditioned medium. Cells were then detached from the wells using 0.25% trypsin/1mM EDTA (Sigma-Aldrich), collected and total RNA was extracted with Trizol-LS reagent (Invitrogen) according to manufacturer’s recommendations (see Section 2.2.1).

2.3.2 MSC stimulation with TLR ligands

2x10^5 MSCs/well were seeded in 6 well plates containing 3 ml of serum-containing medium. Cells were then incubated at 37°C, 5% CO₂. 24 h later, medium was removed and the appropriate TLR ligand dilutions, in serum-free medium, were added (Pam3CSK4 for TLR-2 stimulation (1 μg/ml, Invivogen, Toulouse, France) and ODN2395 for TLR-9 stimulation (5 μM, Invivogen)) to the wells. 24 h later, cells were detached from the wells using 0.25% trypsin/1mM EDTA (Sigma-Aldrich), collected and total RNA was extracted with Trizol-LS reagent (Invitrogen) according to the manufacturer’s recommendations (see Section 2.2.1).

2.3.3 Quantification of IL-1β by ELISA

The mouse monocytic cell line RAW 264.7 (ATCC) was used as a positive control for secretion and detection of IL-1β after Ad-transduction. Cell culture supernatants were collected from Ad.GFP transduced/untransduced MSCs and from Ad.GFP transduced, LPS stimulated and untransduced RAW 264.7 cells 48 hr post-transduction/stimulation. The concentration of IL-1β in the media was measured using a rat IL-1β Tissue Culture ELISA Ready-SET-Go!® (eBioscience) detection kit. Briefly, The ELISA plate was coated with 100 μl/well of capture antibody diluted in 1X PBS. The plate was then sealed and incubated overnight at 4°C. Wells were aspirated and washed 3 times with 200 μl/well of wash buffer. The plate was then blotted on absorbent paper to remove any residual buffer. Wells were blocked with 200 μl/well of 1X assay diluent and incubate at room temperature for 1 hr. The plate was
washed and 100 µl/well of samples or standards diluted in assay diluent were added to the wells and the plate was incubated overnight at 4°C. Following incubation, wells were aspirated and washed 3 times. 100 µl/well of detection antibody was diluted in 1X assay diluent and added to each well. The plate was then sealed and incubated at room temperature for 1 hr. Wells were aspirated and washed 3 times. 100 µl/well of Avidin-HRP* diluted in 1X assay diluent was added to each well, the plate was sealed and incubated at room temperature for 30 mins. Prior to aspiration, the wells were soaked in wash buffer for 1 min. This was repeated 5 more times. 100 µl/well of substrate solution was added to each well and the plate was incubated at room temperature for 15 mins. 50 µl of stop solution was added to each well. Finally, the plate was read at 450 nm on a plate reader to determine the concentration of IL-1β from each of the test samples.

10X PBS – 800g NaCl, 116g Na₂HPO₄, 20g KH₂PO₄ and 20g KCl dissolved in 10L distilled H₂O

Washing buffer – v/v 0.05% Tween (Sigma) in 1X PBS

Assay diluent – w/v 1% bovine serum albumin (BSA) in 1X PBS

Substrate solution – 1:1 mixture of Colour Reagent A (H₂O₂) and Colour Reagent B (Tetramethylbenzidine)

Stop solution – 1M H₂SO₄

2.3.4 Western Blot analysis of CX3CR1

Ad.GFP-transduced and untransduced MSCs were harvested and solubilised in protein lysis buffer containing protease inhibitors. 30 µg of total protein for each sample was separated by 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane. CX3CR1 was detected using a rabbit polyclonal IgG antibody (1:2000 dilution; eBioscience). Cellular α-tubulin levels were detected using a mouse anti-α-tubulin antibody (1:1000 dilution; eBioscience). Membranes were then incubated with HRP-labelled secondary antibodies (1:1000 dilution; eBioscience) and detection was performed using
SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Cheshire, UK).

2.3.5 T cell proliferation assay

Mesenteric lymph nodes were removed from CD rats and processed by disruption of the tissue with a syringe plunger into a single cell suspension of mixed lymphocytes. The cells were then washed with 0.1 % BSA/PBS and stained in pre-warmed (37°C) 10 µM carboxyfluorescein diacetate succinimidyl ester (CFSE)/PBS staining solution (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, Carlsbad, CA) at a concentration of 2x10⁷ cells/ml. The cells were incubated at 37°C for 6 mins in the dark. The reaction was stopped by adding 5 volumes of ice-cold medium containing 10 % FBS. The cells were then washed 3 times with final culture medium to remove all traces of unbound CFSE. Successful CFSE staining was visually confirmed by fluorescent microscopy and the cells were counted using a haemocytometer. 2x10⁵ CFSE-stained T cells were added to wells of a 96 well plate. Untransduced or Ad.GFP transduced MSCs were then added into the wells containing the T cells at a ratio of 1:250 MSCs to T cells. T cells were polyclonally stimulated to induce proliferation by adding an equal amount of anti-rCD3/anti-rCD28-labelled beads in MLC medium containing 2 % heat-inactivated rat serum, 10 % FBS and 55 µM β-mercaptoethanol in RPMI 1640 (Sigma Aldrich). Polyclonally stimulated T cells in the absence of MSCs served as positive controls. The co-cultures were then incubated at 37°C, 5% CO₂. After 4 days, the T cells were collected and CFSE fluorescence was analysed using a FACS Canto (BD Biosciences). Wells were analysed in triplicate.
2.4 *In vivo* administration of MSCs and rat corneal transplantation

2.4.1 Ethics statement

All procedures performed on animals throughout the duration of these studies were approved by the Animal Ethics Committee of the National University of Ireland, Galway and conducted under licence from the Department of Health, Ireland. Additionally, animal care and management followed the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science, Galway, Ireland.

2.4.2 Intravenous/sub-conjunctival injection of MSCs/Ad.GFP transduced MSCs

MSCs were isolated and expanded from male CD, LEW, DA or WF rats as described in Section 2.1.1 and, where appropriate, CD-MSCs were transduced with Ad.GFP as outlined in Section 2.1.2. Rats were briefly anesthetized (2 L/min O₂ and 4.5% isofluorane), transferred to a face mask with a reduced flow rate (0.8 L/min O₂ and 1.5 % isofluorane) and injected i.v. through the lateral tail vein with MSCs (or Ad.GFP MSCs as required). 1 x 10⁶ MSCs (per injection/per rat) were delivered slowly in 1 ml of PBS using a 25G needle. For sub-conjunctival injection of MSCs, rats were anesthetized as described above and 1x10⁶ LEW-MSCs were delivered in 100 µl of PBS using a 27G needle. Each rat was then observed for approximately 20 mins for any sign of bleeding before being returned to the housing room.

2.4.3 Blood sampling

Rats were briefly anesthetized as outlined in Section 2.4.2. Once the lateral tail vein was visualised, a 25G needle was inserted. Once in the lumen of the vein (as determined by the presence of blood in the needle hub),
approximately 600 µl of blood was withdrawn from the needle hub by a P200 pipette and transferred to a 1.5 ml eppendorf tube with or without sodium heparin (if retrieval of leukocytes was required). After removal of the needle tip, pressure was applied to the puncture site until blood had stopped flowing.

2.4.4 Rat corneal transplantation

A well-established, fully allogeneic major histocompatibility complex MHC class I/II disparate cornea transplant model was used for these studies. Male Lewis (LEW, RT-1<sup>i</sup>) rats served as recipients of male Dark Agouti (DA, RT-1<sup>av</sup>) grafts leading to approximately 80% rejection in untreated animals. All animals were 8-14 weeks old, obtained from Harlan Laboratories UK and housed with food and water ad lib.

2.4.4.1 Surgical procedure

Note: The corneal transplantation procedure was performed by a fully trained and licensed ophthalmic surgeon and not by the author himself. Briefly, recipient rats were placed into an anaesthesia box pre-filled with a mixture of medical oxygen and isofluorane (5% anaesthetic in 2 L/min medical oxygen) until fully anaesthetized. Deep anaesthesia was confirmed by abolishment of limb withdrawal and eye reflexes. Donor animals were humanely killed by CO₂ asphyxiation and corneas were excised using a 3 mm trephine and a small angled scissors. Before the donor cornea was completely detached from the eyeball the first suture was introduced. Recipient animals were transferred to a heated operating table which maintained body temperature at 37°C. Pupil dilating drops and Tetracaine drops were administered. The recipient graft bed was prepared by marking the central cornea with a 2.5 mm trephine followed by excision of the corneal tissue using a small angled scissors. The donor cornea was then sutured onto the recipient eye with 8-10 interrupted sutures. Throughout the surgery, the recipient eye was kept moist at all times with sterile saline. Finally, Atropine drops and antibiotic ointment containing chloramphenicol were applied to the ocular surface. The eyelids
remained open throughout the subsequent observation period. Following surgery, transplanted animals were placed in clean cages lined with tissue paper towels and monitored until fully recovered.

2.4.4.2 Post-operative monitoring of transplanted animals

Post-operative observations included monitoring graft transparency and level of neovascularization, as well as development of corneal stromal edema. These checks were performed every 2-3 days using an operating microscope at 25X magnification and a slit-lamp microscope (for estimation of edema levels). Animals with surgical complications were excluded from the study and euthanized by CO₂ inhalation.

Graft transparency, as the primary indicator of rejection, was evaluated every second or third day and graded as follows: 0 – completely transparent cornea; 0.5 – slight corneal opacity, iris structure easily visible; 1.0 – low opacity with visible iris details; 1.5 – modest corneal opacity, iris vessels still visible; 2.0 – moderate opacity, only some iris details visible; 2.5 – high corneal opacity, only pupil margin visible; 3.0 – complete corneal opacity, anterior chamber not visible. Neovascularization was also evaluated and calculated based on the number of quaternary segments of donor corneas in which vessels were present and scored between 0 and 4, with 4 indicating blood vessels present in all 4 segments of donor corneas. Grafts were considered rejected if they had an opacity score above 2.5 on two consecutive observations, in combination with edema and correlating changes in transplant geometry (degree of convex contour, shrinking and surface roughness of graft).
## 2.4.5 Surgical materials

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<td>Iris forceps</td>
<td>15915</td>
<td>World Precision Instruments</td>
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<td>Geuder AG, Germany</td>
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### Ophthalmic surgery drugs

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<td>Isoflurane</td>
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<td>Medical oxygen</td>
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<td>BOC Gases, Ireland</td>
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2.5 Histology and histochemistry

2.5.1 Fixation and embedding of tissue samples

For histological analysis rat eyes were enucleated either on the day of rejection post-operative day (POD) 17 or at the end of the observation period (POD31) for all groups. Eyes were dissected from euthanized animals and fixed in 4% paraformaldehyde (PFA) diluted in PBS for 24 h at 4°C. The eyes were then transferred to 70% EtOH and stored at 4°C until further processed. For processing, tissues were embedded in histological cassettes and processed using an automatic tissue processor (Leica Microsystems, Ashbourne, Ireland) set on a pre-programmed overnight routine program. The eyes were then embedded in molten paraffin wax and left on a cooling plate for approximately 3 h to solidify. Paraffin-embedded tissue samples were then stored at room temperature until sectioned.

2.5.2 Haematoxylin and Eosin (H&E) staining

Eyes embedded in paraffin wax were cut in 5 µm sections using a microtome (Leica Microsystems) and positioned on glass slides. They were transferred to a slide oven and incubated overnight at 56°C to melt away the peripheral wax. The tissues were then deparaffinized twice by placing in xylene for 10 mins, followed by hydration through graded alcohols (75%, 90% and 100% EtOH – 5 mins in each). Next, slides were incubated for 40 seconds in Harris haematoxylin (Sigma-Aldrich), washed in running water for 2 min, then stained in eosin (Sigma-Aldrich) for 7 mins, washed again in running water for 2 min and dehydrated through graded alcohols (100%, 95% and 75% EtOH - 5 mins in each). Eye tissue sections were cleared twice in xylene for 10 mins and mounted in DPX mounting medium (Sigma-Aldrich). Slides were then ready to be visualized microscopically.
2.6 Immune cell isolation from peripheral blood and tissues

Single cell suspensions from individual transplanted corneas were prepared as described previously (Nosov et al., 2012). Briefly, excised corneal buttons were digested with 5% w/v Collagenase D (Sigma-Aldrich) in RPMI containing 25 mM HEPES (Lonza, Basel, Switzerland) plus 1% fetal calf serum (Sigma-Aldrich) at 37°C for 90 min at 900 rpm mixing frequency. Following incubation, the digested corneal tissue was pipetted through a 100 µM cell strainer and disrupted by grinding with a syringe plunger. Cell suspensions were transferred into 15 ml tubes, spun at 400 x g for 5 mins and washed with PBS. Lung tissue was cut into smaller sections using a scalpel blade (in order to aid digestion) and digested as described above for corneal tissue with the exception that lung tissue was incubated at 37°C for 2 hr without mixing. Ipsi-lateral cervical and submandibular draining lymph nodes (DLNs) and spleens were homogenised with a syringe plunger and passed through a 100 µM cell strainer. Following homogenization, splenocytes were resuspended in 5 ml of ACK red blood cell lysis buffer (composed of 0.15 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA (all Sigma-Aldrich) dissolved in distilled water) and incubated for 5 min on ice. Cell suspensions were transferred into 15 ml tubes, spun at 400 x g for 5 min and washed again with PBS. PBMCs were isolated from whole blood according to the same protocol as described above for the spleen. Cell suspensions from individual corneas, DLNs, lungs, spleens and PBMCs were used for subsequent multicolor flow cytometric analysis.

2.7 Flow cytometry

The complete list of monoclonal antibodies (mAbs) used for the characterisation of rat MSCs and immune cells isolated from blood, transplanted corneas, ipsilateral draining lymph nodes, lungs and spleens are shown in Table 2.3 (and isotype controls in Table 2.4). For staining, cells were washed with FACS buffer (PBS containing 2 % FCS and 0.1 % NaN₃,
all Sigma Aldrich). mAbs were diluted in 50 µl FACS buffer, added to the cells and incubated for 30-40 mins at 4°C in the dark. To remove any remaining unbound antibody, cells were washed at least twice with PBS and resuspended in FACS buffer. Cells were then analysed using a FACS Canto flow cytometer (BD Biosciences, Oxford, UK). For some experiments instead of using isotype controls, ‘fluorescence minus one’ (FMO) negative controls were used for construction of gates. Data was analysed and compensated using FlowJo software (Tree Star, Inc.). Results are presented as percent of cell population or, in the case of cells from corneal allografts, as absolute cell number. Fluorescent (CaliBRITE-PerCP beads, BD bioscience, Oxford, UK) or unlabelled beads were added to FACS samples for detection of the absolute number of intragraft cells and was calculated based on the number of fluorescent beads and cells counted per sample. A predetermined number of beads had been added to each sample prior to analysis on the flow cytometer.

2.7.1 Detection of Foxp3 expression by intracellular staining

To analyse Foxp3 expression, cells were first stained for relevant surface markers as described above. The cells were then fixed and permeabilised using a commercial kit (eBioscience, San Diego, CA) according to manufacturer’s recommendations. Briefly, cells were added to individual wells in a 96 well plate and washed with FACS buffer. Supernatants were removed and 200 µl of fixation/permeabilization solution was added to each sample. The plate was then incubated at 4°C for at least 2 hr in the dark. The plate was centrifuged at 400 x g for 3 mins and supernatants were removed. 200 µl of 1X permeabilization buffer was then added to each sample followed by centrifugation and decanting of supernatant. This wash step was repeated. mAbs to Foxp3 were then diluted in 50 µl of 1X permeabilization buffer and added to each sample. Samples were incubated for 30-40 mins at 4°C in the dark. Cells were then washed at least twice with 1X permeabilization buffer and resuspended in FACS buffer before analysing on the flow cytometer.
2.7.2 Detection of allo-antibodies in serum of transplanted animals

Serum alloantibody levels were detected as previously described (Schu et al., 2012). 17 days after corneal transplantation, rats were euthanized and blood was collected from all four study groups by cardiac puncture and stored immediately on ice. Serum was isolated by centrifuging whole blood samples for 15 mins at 2000 × g. Following centrifugation, serum was removed and transferred to fresh 1.5 ml tubes. In order to detect alloantibodies, freshly isolated DA splenocytes were washed with FACS buffer and incubated with anti-rat CD32 (to reduce unspecific binding) for approximately 5 mins. The serum was then diluted 1:2 with FACS buffer and was added to the cells. The serum/DA splenocyte mixtures were then incubated for 45 min at 4°C. The cells were washed twice with FACS buffer and stained with mAbs against rat Igs (anti-rat IgG1-FITC, anti-rat IgG2-FITC, or anti-rat IgM-PE – all Antibodies Online, Aachen, Germany). For anti-IgM staining, anti-CD45RA was added later to allow exclusion of B cells from analysis. Splenocytes were incubated again for 45 min at 4°C, washed and resuspended in FACS buffer for analysis using a FACS Canto.

<table>
<thead>
<tr>
<th>Antibody description</th>
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<th>Clone</th>
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Chapter 2

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Table 2.3. Monoclonal antibodies used for characterisation of MSCs and immune cell populations.

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Table 2.4. Isotype controls used for characterisation of MSCs and immune cell populations.

2.8 Statistics

Statistical analysis was performed with GraphPad Prism software (La Jolla, USA) using non-parametric, unpaired, two-tailed Mann–Whitney or parametric, unpaired, two-tailed Students t-test for analysis. Graphs show mean ± SD/SEM. Survival data were compared using the log-rank (Mantel-Cox) test. Individual experiments were performed in duplicate or triplicate to ensure reproducibility. Differences were considered significant if p ≤ 0.05.
## 2.9 General consumables

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Chapter Three

Adenoviral transduction of MSCs: *in vitro* and *in vivo* immune responses after cell transplantation
Chapter 3

Adenoviral transduction of MSCs: *in vitro* and *in vivo* immune responses after cell transplantation

3.1 Introduction

MSCs are a heterogenous population of adult non-hematopoietic cells which possess a number of attributes that make them attractive targets for regenerative medicine applications. MSCs are predominantly isolated from bone marrow but can also be obtained from other readily accessible tissues such as adipose tissue and umbilical cord blood. They have multi-lineage potential and the capacity to extensively proliferate *in vitro* (Bianco et al., 2001, Pittenger et al., 1999, Barry and Murphy, 2004). To date, a number of studies have shown that MSCs can migrate and successfully engraft in damaged organs and tissues (Ip et al., 2007, Morigi et al., 2004) with the interaction of chemokine receptors and their ligands pivotal to this process (Chamberlain et al., 2008). However, therapeutic success of MSC therapy has been limited and the genetic modification of MSCs is one option to improve their therapeutic potential (Kumar et al., 2008, Huang et al., 2010, Griffin et al., 2010).

The majority of pre-clinical and clinical gene therapy applications reported thus far have utilized virus-based transfer of genetic material due to high transduction efficiency, cell tropism and levels of transgene expression. However, adverse immune reactions directed against the gene therapy vehicle itself, the transgene product and, in some cases, the transduced cells have raised serious concern (Edelstein et al., 2007). Moreover, repeated administration is difficult to achieve as neutralising antibodies may be generated against the viral vectors following the initial injection (Pleyer and Ritter, 2003). *Ex-vivo* genetic modification of cells or tissues using recombinant adenovirus (Ad) could be one option to reduce undesired immune responses as unbound Ad will be removed by repeated washing before cell/tissue transplantation. Another issue that must be addressed relates to possible TLR triggering by viral capsid proteins or dsDNA which may stimulate innate immune mechanisms and render transduced cells more
susceptible to immune-mediated rejection \textit{in vivo} (Cerullo et al., 2007). Indeed, it has been shown previously that differentiated cells e.g. cardiomyocytes and pancreatic islet cells up-regulate various TLRs and chemokine receptors when transduced \textit{ex-vivo} with an Ad-vector (Zhang et al., 2003, Chen et al., 2003). Therefore, both the target cell to be transduced and the viral vector to be used must be carefully considered when designing an appropriate therapeutic intervention.

As mentioned previously, MSCs are, under certain circumstances, considered to be hypo-immunogenic due to their low expression of MHC class I molecules and lack of expression of MHC class II molecules. Furthermore, numerous studies have shown that MSCs do not express co-stimulatory molecules necessary for full T cell activation, namely, CD80, CD86 and CD40 and they can also secrete anti-inflammatory molecules, for example, TGF-β, PGE2, IDO, NO and IL-10. Recently, Chuang and colleagues showed that transduction of MSCs using recombinant baculovirus only led to a mild up-regulation of immune response parameters and did not significantly impair their \textit{in vivo} persistence (Chuang et al., 2009). Moreover, while recombinant Ad has been used for the genetic modification of MSCs (Rooney et al., 2008, McMahon et al., 2006, Shujia et al., 2008, Tang et al., 2010), the immune profile of adenovirally transduced MSCs is not known.

The aim of this study was therefore to investigate if Ad-transduction of MSCs alters their expression of immunologically relevant molecules such as MHC class I and II, co-stimulatory molecules, pro-inflammatory cytokines, chemokine/chemokine receptors and toll-like receptors which, consequently, could lead to an increased risk of recognition by the host immune system. Finally, Ad-transduced MSCs were injected systemically into syngeneic rats and the host immune response to the transduced cells was evaluated.
3.2 Results

3.2.1 Characterisation, differentiation and Ad-transduction of rat MSCs

MSCs were isolated from the bone marrow of CD rats, cultured and subsequently characterized for their expression of relevant cell surface markers. Flow cytometric analysis showed that rat MSCs express the cell surface markers CD29, CD73, CD90, and CD44H and lowly express or are negative for CD45RA and CD71 (Figure 3.1A). Bi-lineage differentiation assays were also performed to assess the adipogenic and osteogenic capacity of the cells. The results showed that CD-MSCs can differentiate along adipogenic and osteogenic lineages as measured by Oil Red O quantification and calcium concentration (Figure 3.1B and 3.1C, respectively). CD-MSCs between passage 4 and passage 8 (P4-P8) were used for subsequent in vitro and in vivo experiments.

MSCs were transduced with recombinant adenoviruses (rAd) encoding GFP at a MOI of 100 by spin centrifugation. While a higher MOI could have been used, preliminary tests showed that this led to higher cytotoxicity (unpublished observations) among transduced cells which could potentially compromise the viability of Ad.GFP transduced MSCs in vivo. The mean transduction efficiency in this study was determined by fluorescence activated cell sorting (FACS) analysis and found to be 66% (n=3, representative gating shown in Figure 3.2A). As a result of limited transduction efficiencies, Ad.GFP MSCs were FACS sorted into GFP positive (+) and GFP negative (-) fractions (n=3, figure 3.2A). From these two distinct populations, RT-PCR analysis was performed to analyse the mRNA expression levels of representative chemokines, chemokine receptors and TLRs. Consistently, non-significant differences in expression levels between the two cell populations were observed (figure 3.2B) and, as a result, a transduction efficiency of 66% was deemed adequate for subsequent experiments.
Fig. 3.1 Cell surface marker expression and differentiation of MSCs. (A) CD-MSCs are CD29, CD73, CD90 and CD44H positive, and CD45RA, CD71 low or negative. Shown are FACS histograms of MSCs stained with antibodies against indicated surface markers or appropriate isotype controls in triplicates. MSCs were able to differentiate into (B) adipocytes and (C) osteocytes following incubation with specific induction medias. Results shown are representative data of 3 separate isolations.
Fig. 3.2 mRNA expression levels of TLRs, chemokines and chemokine receptor following FACS sorting into Ad.GFP+ and Ad.GFP- fractions. (A) Representative dot plot and gating strategy of untransduced and Ad.GFP transduced MSCs (B) RT-PCR analysis of mRNA expression levels of representative TLRs, chemokines and the chemokine receptor CXCR4 after MSCs were sorted into Ad.GFP+ and Ad.GFP- fractions. Shown is one representative experiment from two performed. 2E-ΔCT=2-ΔΔCT = number of copies of gene of interest relative to the number of copies of the internal control gene, β-actin.
3.2.2 MSC immune-phenotyping and pro-inflammatory cytokine expression after Ad-transduction

As one of the key features of MSCs is their low or lack of expression of MHC molecules and co-stimulatory molecules, it was important to investigate if Ad-transduction of MSCs alters the expression of these molecules. Therefore, MSCs were transduced using rAd encoding for the reporter gene β-Galactosidase (Ad.β-Gal) at a MOI of 100. 24 hr after transduction, MSCs were collected and analysed for cell surface marker expression by flow cytometry. The results showed that Ad-transduction caused neither an increase in MHC class I/II expression nor in CD80 and CD86 expression compared to expression levels of untransduced MSCs (n=3) (Figure 3.3A).

The next step was to elucidate whether Ad-transduction of MSCs would lead to higher expression levels of pro-inflammatory cytokines, namely IL-1β, IFN-γ and IL-6, which could indirectly enhance an immune response targeted towards the gene-modified MSCs. The results showed that mRNA expression levels of IL-1β and IL-6 were only slightly increased in Ad-transduced MSCs compared to untransduced MSCs. IFN-γ mRNA expression levels were comparable between untransduced and Ad.GFP transduced MSCs (Figure 3.3B). As IL-1β, in particular, plays a critical role in the early inflammatory response to viral infection, it was important to investigate IL-1β expression at the protein level. Cell culture supernatants of untransduced or Ad.GFP transduced MSCs were harvested and IL-1β levels were assayed by specific ELISA. A mouse monocytic cell line (RAW 264.7) which can be readily transduced with Ad-vectors served as a positive control. IL-1β protein expression levels of both untransduced and Ad.GFP transduced MSCs fell below the detection limits of the kit (sensitivity = ≥2.4 pg/ml, data not shown) thereby confirming that IL-1β expression remains unchanged after Ad-transduction at both the mRNA and protein level. Conversely, both control and Ad-transduced RAW cells were found to secrete high levels of IL-1β (≥2.5 μg/ml) following stimulation with LPS (data not shown). Taken together, this data indicates that Ad-transduction of MSCs does not lead to an increase in expression of immunologically relevant
surface markers or pro-inflammatory cytokines compared to their untransduced counterparts.
Fig. 3.3 Ad-transduction does not alter MHC I/II, co-stimulatory molecule or pro-inflammatory cytokine expression by MSCs. (A) Untransduced and Ad-transduced MSCs were stained for MHC class I/II, CD80 and CD86 with specific antibodies and analysed by flow cytometry. (B) RT-PCR analysis showing mRNA expression levels of IL-1β, IFN-γ and IL-6 by untransduced and Ad.GFP transduced MSCs. Data shown are means ±SD of three independent experiments. 2E-ΔCT=2^(-ΔCT) = number of copies of gene of interest relative to the number of copies of the internal control gene, β-actin.
3.2.3 Chemokine/chemokine receptor mRNA expression by MSCs is not significantly altered after Ad-transduction

Enhanced chemokine and/or chemokine receptor production by MSCs following Ad-transduction may have a detrimental effect on the therapeutic effects of the cells in vivo, as this may induce the migration of greater numbers of potentially harmful immune cells into close proximity to the MSCs and thus rendering the cells more susceptible to immune-mediated lysis. For this reason, it was decided to investigate if Ad-transduction of MSCs altered their mRNA expression profile of a panel of chemokines and chemokine receptors. Rat DCs (rDCs), which are known to express multiple chemokine/chemokine receptors, served as a positive control. As shown in Figure 3.4A, genetic modification of MSCs with Ad.GFP did not lead to a significant up- or down-regulation of chemokine or chemokine receptor mRNA expression. However, the results did show that in Ad-transduced MSCs, mRNA levels of CX3CR1 (the receptor for fractalkine) were clearly reduced compared to untransduced MSCs. Western blot analysis of CX3CR1 protein expression, however, did not show any difference between untransduced and Ad-transduced MSCs (Fig. 3.4B). In summary these data indicate that Ad-transduction of MSCs does not induce major changes in their chemokine/chemokine receptor expression profile compared to untransduced MSCs.

As in an organ transplant setting or disease model the infused MSCs could potentially encounter recipient DCs in an activated state due to an ongoing inflammatory response, it was important to investigate what affect inflammatory stimuli may have on chemokine receptor expression by both untransduced and Ad.GFP transduced MSCs. To mimic this, an in vitro assay was performed in which supernatant from BMDCs stimulated with LPS for 24 hr was removed and added to either the untransduced or Ad.GFP transduced MSCs. RT-PCR analysis of chemokine receptors revealed there was no significant changes in mRNA expression levels of CCR2, CCR5, CXCR3, CXCR4 or CX3CR1 (containing members of the three main sub-
classes of chemokine receptors) even after stimulation with potent inflammatory stimuli (Figure 3.5).
Fig. 3.4 Chemokine/chemokine receptor mRNA expression profile of Ad.GFP-transduced MSCs is comparable to untransduced MSCs. (A) mRNA expression levels of chemokines/chemokine receptors from untransduced and Ad.GFP-transduced MSCs and DCs. Expression levels were normalized to the expression level of the housekeeping gene β-actin. Data shown are means ±SD of four independent experiments. (B) Western blot analysis of CX3CR1 protein expression by untransduced and Ad.GFP-transduced MSCs. Tubulin expression served as control. $2E\Delta CT=2^{\Delta\Delta CT}$ = number of copies of gene of interest relative to the number of copies of the internal control gene, β-actin.
Fig. 3.5 Chemokine receptor expression by Ad-transduced MSCs remains unchanged following stimulation with BMDC-conditioned medium. RT-PCR analysis showing mRNA expression levels of a selection of chemokine receptors from untransduced and Ad.GFP-transduced MSCs stimulated with/without BMDC-conditioned medium. Data shown are means ±SD of two separate isolations. $2E-\Delta CT=2^{-\Delta CT} =$ number of copies of gene of interest relative to the number of copies of the internal control gene, $\beta$-actin.
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3.2.4 Ad-transduction does not cause an increase in TLR activation/expression in MSCs compared to untransduced counterparts

TLR triggering via specific ligands, pathogens or viruses has been shown to induce the production of pro-inflammatory cytokines (Cerullo et al., 2007, Pevsner-Fischer et al., 2006). Therefore, it was important to investigate what effect(s) Ad-transduction would have on TLR expression by MSCs. MSCs were transduced with Ad.GFP (or untransduced) and 24 hr later were harvested for extraction of RNA and subsequent RT-PCR analysis of TLR gene expression. Rat DCs, known to express high levels of TLRs, served as a positive control (Basner-Tschakarjan et al., 2006). The results showed that genetic modification of MSCs with Ad.GFP only led to mild differences in TLR mRNA expression levels between the Ad-transduced and untransduced MSCs (Figure 3.6A). Another interesting finding was that TLR2 and TLR9, both of which play a key role in Ad-induced inflammation (Appledorn et al., 2008), were expressed approximately 100 and 570 times, respectively, less in Ad-transduced MSCs compared to DCs (Figure 3.6A).

As TLR2 and TLR9 are critical for adenovirus-induced inflammation, the next step was to elucidate if these TLRs are functionally active in MSCs. To do this, MSCs were stimulated for 24 hr with the TLR1/2 specific ligand Pam3CSK4 and the TLR9 specific ligand ODN2395. The results showed that both TLR 2 and TLR9 mRNA expression was significantly up-regulated following stimulation with their specific ligands (Figure 3.6B), thus indicating that these signalling pathways are operational in MSCs following TLR triggering. However, as shown in Figure 3.6A, neither TLR2 nor TLR9 mRNA was up-regulated after transduction with Ad.GFP.

TLR activation induces nuclear translocation of NFκB from the cytosol, leading to NFκB-dependent gene expression. To test if TLR-mediated NFκB activation occurs in MSCs after Ad-transduction, MSCs were firstly transfected with a plasmid which expresses GFP under the control of the NFκB transcription factor binding sites (pNFκB-d2GFP). 24 hr later MSCs were either stimulated with Pam3CSK4 or transduced with Ad.β-Gal (to avoid
interference with GFP expression induced from the plasmid), and analysed by flow cytometry for NFκB-mediated GFP expression. The results showed that stimulation with Pam3CSK4 resulted in increased GFP expression compared to untransduced MSCs (Figure 3.6C and D), thus indicating activation of the NFκB-pathway. Transduction of MSCs with Ad.β-Gal, however, did not lead to a significant increase in GFP expression compared to untransduced MSCs (Figure 3.6C and D). These results suggest that Ad-transduction of MSCs does not lead to pro-inflammatory signal transduction pathway activation.
Fig. 3.6 Ad-transduction does not lead to an increase in TLR expression by MSCs. (A) RT-PCR analysis showing mRNA expression levels of TLRs 1-10 from untransduced and Ad.GFP-transduced MSCs. rDCs served as a positive control. Expression levels were normalized to the housekeeping gene β-actin. Data shown are means ±SD of four independent experiments. (B) RT-PCR analysis showing mRNA expression levels of TLR 2 and 9 by MSCs after stimulation with Pam3CSK4 and ODN2395. (C) FACS analysis of GFP-expressing MSCs following transfection with pNFκB-d2EGFP, and subsequent transduction with Ad.β-Gal (black line) or stimulation with Pam3CSK4 (grey line), respectively, compared to untreated MSCs (filled histogram). 2E-ΔCT=2^\(-\Delta\text{CT}\) = number of copies of gene of interest relative to the number of copies of the internal control gene, β-actin. *: p<0.05 compared to control, unpaired Student’s two-tailed t-test.
3.2.5 Ad-transduction does not affect the ability of MSCs to inhibit T cell proliferation in vitro

One of the hallmarks of MSCs is their ability to inhibit T cell proliferation in vitro. It was necessary, therefore, to investigate whether or not Ad-transduction interferes with this ability. To test this, polyclonally activated T cells (stimulated with anti-CD3/anti-CD28 coated beads) were labelled with CFSE and co-cultured together with either untransduced or Ad.GFP-transduced MSCs. 4 days later T cells were collected and analysed by flow cytometry. The results showed that Ad.GFP-transduced MSCs could inhibit T cell proliferation equally as efficiently as untransduced MSCs (p<0.01, Figure 3.7A and 3.7B).

To summarise, the results from this study so far suggest that negligible differences exist between untransduced and Ad-transduced MSCs in terms of TLR, chemokine/chemokine receptor and pro-inflammatory cytokine mRNA expression. Furthermore, both untransduced and Ad-transduced MSCs could inhibit T cell proliferation in vitro with comparable efficiency.
Fig. 3.7 MSC-mediated inhibition of T cell proliferation is not altered by Ad-transduction. (A) Histograms showing percent proliferation of CFSE-labelled T cells stimulated with anti-CD3/anti-CD28 beads in the absence or presence of untransduced or Ad.GFP transduced MSCs (ratio of T cells to MSCs; 250:1). (B) Percentage of cells divided greater than three generations after polyclonal stimulation in the absence or presence of untransduced or Ad.GFP transduced MSCs. CFSE fluorescence was analysed on day 4 after co-culture. Shown is a representative experiment with means of 4 replicates ± SD **: p<0.01; unpaired, Student's two-tailed t-test.
3.2.6 Systemic administration of Ad-transduced MSCs does not lead to increased frequencies of inflammatory cells in vivo

To investigate if Ad-transduction of MSCs might induce an inflammatory response in vivo, MSCs were transduced ex-vivo with Ad.GFP (MOI 100) and injected i.v into syngeneic CD rats (2x10^6 cells/animal). Rats i.v injected with untransduced MSCs (2x10^6 cells/animal) served as the control. Blood was taken from treated animals on days 3, 7 and 14 (the day of sacrifice) and the frequencies of different immune cell populations (antigen presenting cells, CD11b/c+MHCII+; activated T cells, CD4+CD25+; cytotoxic T cells, CD3+CD8+CD161-; activated natural killer cells, CD3-CD8+CD161++ and natural killer T cells CD3+CD8+CD161+, CD3+CD8+CD161++) were analysed by flow cytometry. Furthermore, spleens and lungs from treated animals were also collected on the day of sacrifice and analysed for the presence of the same immune cell populations as for blood. Additionally, cell populations from the lungs and spleens of injected animals were analysed for HIS36 expression, as evidence of the presence of tissue macrophages, on the day of sacrifice. The results showed there were no significant differences in the proportion of circulating activated T cells or APCs at days 3, 7 and 14 or in the lungs and spleens at day 14 after injection of either untransduced or Ad.GFP transduced MSCs (Figure 3.8A and 3.8B). Furthermore, no significant differences were observed in the proportion of cytotoxic T cells, activated NK cells or NKT cells in either the blood or the lungs and spleens of Ad.GFP MSC treated animals compared to animals treated with untransduced MSCs (Figure 3.10A-D and see Figure 3.9A-C for gating strategy). Finally, the proportion of HIS36+ tissue macrophages in the lungs and spleens of Ad.GFP MSC injected animals was almost identical to that of animals receiving untransduced MSCs (Figure 3.8C).

Taken altogether, the in vitro and in vivo data presented here indicate that Ad-modification of MSCs does not significantly alter their immunosuppressive profile which, subsequently, may have important implications for the use of Ad-transduced MSCs within a therapeutic setting.
Fig. 3.8 Ad-transduced MSCs do not elicit a systemic immune response *in vivo*. The proportions of (A) CD4+CD25+ and (B) CD11b/c+MHCII+ cells in the blood at days 3, 7 and 14, and also in the lung and spleen at day 14 post-injection of rats receiving either 2x10⁶ Ad.GFP transduced MSCs (n=4) or 2x10⁶ untransduced MSCs (n=3) as measured by flow cytometry. (C) The proportion of HIS36+ cells in the lung and spleen at day 14 post-injection as measured by flow cytometry. Statistical analysis was performed using the non-parametric, two-tailed, Mann-Whitney test. For all tested parameters, p values were >0.05.
Fig. 3.9 Gating strategy used for the analysis of cell distribution of CD3/CD8/CD161 stained cells in the blood, lungs and spleens of rats receiving either untransduced or Ad.GFP transduced MSCs. Representative dot plots and gating strategy of (A) blood, (B) lungs and (C) spleens from animals receiving intravenous injections of either $2 \times 10^6$ Ad.GFP transduced MSCs ($n=4$) or $2 \times 10^6$ untransduced MSCs ($n=3$).
Fig. 3.10 Ad-transduced MSCs do not elicit a systemic immune response in vivo. The proportions of (A) CD3+CD8+CD161+, (B) CD3+CD8+CD161++, (C) CD3+CD8+CD161- and (D) CD3-CD8+CD161++ cells in the blood at days 3, 7 and 14, and also in the lung and spleen at day 14 post-injection of rats receiving either 2x10^6 Ad.GFP transduced MSCs (n=4) or 2x10^6 untransduced MSCs (n=3) as measured by flow cytometry. Statistical analysis was performed using the non-parametric, two-tailed, Mann-Whitney test. For all tested parameters, p values were >0.05.
3.3 Discussion

Collectively, the data generated in the first part of this study demonstrates that adenoviral transduction of MSCs does not adversely affect the immunological properties of MSCs in vitro or in vivo and therefore can be considered as a suitable gene vector for therapeutic applications of MSCs. This work has relevance as future pre-clinical and clinical studies may well employ the use of gene-modified MSCs using adenoviral vectors to further enhance the therapeutic properties of the cells.

The low immunogenicity of MSCs is primarily attributed to their very low expression of MHC class I and lack of expression of MHC class II and co-stimulatory molecules on their cell surface under non-stimulatory conditions. As MHC class I and II molecules are crucial for the successful presentation of endogenously processed and phagocytosed antigens to CD8+ and CD4+ T cells, respectively, it was important to investigate if Ad-modification alters the cell surface expression of these molecules. Interestingly, the results shown here demonstrated that Ad.GFP transduction of MSCs caused neither an increase in MHC class I/II expression nor an increase in expression levels of the co-stimulatory molecules CD80 and CD86 compared to untransduced MSCs (Figure 3.3A). In contrast to this, antigen presenting cells such as DCs have been shown to significantly upregulate expression of both MHC and co-stimulatory molecules following adenoviral transduction (Tan et al., 2005). However, in certain situations, such Ad-mediated upregulation of MHC and co-stimulatory molecules on DCs may actually be desirable, for example in the context of vaccine adjuvant development and/or enhancement, this may allow for a more efficient generation of T-cell mediated immune responses in vivo (Brandao et al., 2003). A recent study by Chuang and colleagues (2009) investigated immune responses after transduction of MSCs with baculovirus and, although observing a moderate upregulation of MHC class I expression, similar to the observations made in this study, they reported no adverse stimulatory effects following gene-modified cell transplantation (Chuang et al., 2009). In line with the previous observations with regard to MHC class I and II expression after Ad-transduction, the potential of MSCs to suppress T
cell proliferation was also unaffected by Ad-modification (Figure 3.7A and 3.7B). Moreover, there was no significant change to the mRNA expression levels of key pro-inflammatory cytokines (IL-1β, IFN-γ, IL-6) by MSCs after Ad-transduction (Figure 3.3B). Overall, the data presented here suggests that transduction of MSCs with Ad-vectors neither impairs their immunosuppressive properties nor increases their immunogenicity.

A number of studies have shown that interaction of viral vectors with a broad range of different cell types leads to an immediate (and highly upregulated) production of chemokines. Such a response may render the virally transduced cells more susceptible to immune recognition by the host organism. It has been shown previously that multiple chemokines and chemokine receptors were up-regulated in Ad-transduced murine cardiomyocytes compared to their untransduced counterparts. Moreover, Ad-transduction induced a broader panel of chemokines and receptors than that induced by either ischemia injury or alloantigen stimulation (Chen et al., 2003). Similarly, Zhang and colleagues reported significantly higher expression of a number of chemokines (CCL2, 3, 5, 8 and 12, CXCL2, 9 and 10) and chemokine receptors (CCR2 and 5 and CXCR5) following Ad-transduction of pancreatic islet cells compared to untransduced cells (Zhang et al., 2003). The results shown here demonstrate that, at least in terms of mRNA expression, there is little variation between untransduced and Ad.GFP transduced MSCs with regard to the panel of chemokines/chemokine receptors that were analysed (Figure 3.4A). The only notable exception to this observation was in the case of CX3CR1, which showed a greater than 10-fold decrease in mRNA expression in Ad-transduced MSCs compared to untransduced MSCs (Figure 3.4A). However, Western blot analysis showed comparable amounts of CX3CR1 protein produced by both transduced and untransduced MSCs (Figure 3.4B).

Pathogen-mediated stimulation of TLRs or stimulation of TLRs with specific ligands has been shown to result in the increased expression of TLR mRNA and induce the production of pro-inflammatory cytokines, thereby leading to alterations in immunomodulatory responses (Cerullo et al., 2007, Pevsner-
Fischer et al., 2007). It was therefore important to investigate if similar observations could be made after Ad-transduction of MSCs. Particularly, the expression of TLR2 and TLR9 was monitored as these TLRs are thought to be involved in Ad-mediated inflammatory responses (Cerullo et al., 2007). The results showed that unmodified MSCs express low levels of TLR mRNAs to begin with and expression levels remained unchanged after transduction with Ad.GFP (Figure 3.6A and 3.6B). The next step was to determine if, despite low TLR mRNA expression by both untransduced and Ad-transduced MSCs, signalling via TLR receptors occurs after Ad-transduction. As TLR-signalling is predominantly mediated by NF-κB, MSCs were engineered to express a plasmid with NF-κB binding sites followed by a GFP-expression cassette. If TLR triggering could induce NF-κB stimulation of GFP, expression could subsequently be detected by flow cytometry. The results showed that, while transfected MSCs subsequently stimulated with the TLR1/2 specific ligand Pam3CSK4 expressed substantial levels of GFP, similarly transfected MSCs did not respond following Ad-transduction (Figure 3.6C and 3.6D).

The in vitro data presented in the first part of this study indicates genetic modification of MSCs using adenoviral vectors does not adversely alter their immunosuppressive profile. However, minimal changes in the immune expression profile of MSCs following Ad-transduction in vitro does not guarantee a similarly mild response in vivo. Indeed, Ad-transduced MSCs could potentially provoke a robust immune response in vivo which could greatly impact on their therapeutic potential. To investigate this in more detail, 2x10^6 untransduced or Ad.GFP transduced MSCs were injected intravenously into syngeneic rats with the aim of specifically monitoring the effect of Ad-modification only. The frequencies of various immune cell populations in the blood and specific organs were then analysed at different time points in response to the untransduced or Ad.GFP transduced MSCs. A previous report by Gao and co-workers showed that after systemic administration of MSCs into normal, non-injured animals, the cells were found in the lungs, kidneys, spleens and bones for as long as 48 hr post-
injection (Gao et al., 2001). Indeed, the in vivo distribution data of intravenously injected CFSE-labeled syngeneic and allogeneic MSCs shown in Figure 4.10 are in line with these findings. Based on the finding that the majority of MSCs enter and become trapped in the lung after systemic injection, it was decided to analyse if an immune response may have occurred in this organ but also in the spleen (due to its role in immunomodulation) and in the blood of treated animals, in the potential event of immune cell migration. The results showed that at days 3, 7 and 14 post-injection there was no significant differences in either the frequency or the activation status of CD11b/c+MHCII+ (APCs), CD4+CD25+ (activated T cells), CD3+CD8+CD161+ (NKT cells), CD3+CD8+CD161++ (activated NKT cells), CD3+CD8+CD161- (CD8+ T cells) and CD3-CD8+CD161++ (NK cells) in the blood of Ad.GFP transduced MSC compared to untransduced MSC-treated rats. Furthermore, similar results were observed when lungs and spleens of treated animals from both groups were analysed at day 14. The results shown here suggest that Ad-transduced MSCs do not induce a detectable immune response after systemic administration when compared to untransduced MSCs, which may even allow repeated application of Ad-transduced MSCs if required.

In summary, the results from the first part of this study indicate that transduction of undifferentiated MSCs with Ad-vectors does not lead to adverse expression of immunologically-relevant molecules in vitro or provoke a heightened immune response in vivo and therefore can be considered as a suitable gene vector for therapeutic applications of MSCs.
Chapter Four

The role of MSC treatment in promoting corneal allograft survival
Chapter 4

4 The role of MSC treatment in promoting corneal allograft survival

4.1 Introduction

Cornea transplantation (keratoplasty) is the most commonly performed transplant procedure with more than 100,000 procedures per year worldwide. Due to the immune privileged status of the eye, success rates are high within the first year following transplantation (approximately 90%) (Ritter and Pleyer, 2009). With regard to long term observations, the 5-year prognosis for penetrating keratoplasty is similar to that of cardiac, renal and liver allograft transplantation and estimated to be approximately 50% (Streilein, 1999, George and Larkin, 2004, Coster and Williams, 2005, Williams and Coster, 2007, Niederkorn, 2011) with immunologic rejection the leading cause of allograft failure. Treatment of patients with topically applied corticosteroids is currently the gold standard in corneal transplantation. While this treatment is effective for many patients, it is much less effective in patients deemed to be “high-risk”. In these individuals, corneal immune privilege has been lost as a result of preceding corneal inflammation, neovascularization, trauma (surgery-induced or otherwise), infections or those with a history of previously failed grafts (Coster and Williams, 2005). Corneal graft rejection can also be prevented by systemic administration of pharmacological agents. Although Cyclosporine A and corticosteroids have reduced the rejection rate of corneal allografts, a considerable percentage of grafts will still undergo rejection. Furthermore, even if the allograft is accepted for an extended period of time, it has become apparent that true tolerance is not achieved as grafts can undergo immune-mediated rejection years after transplantation (Streilein, 1999). Additionally, the prolonged use of these systemic immunosuppressants can produce significant side effects that may limit their use in a non-life-threatening indication. Therefore, continued preventive and therapeutic efforts are required to improve the prognosis after keratoplasty.
MSCs have been shown to possess potent immunosuppressive properties, predominantly mediated through paracrine effects on processes such as inflammation, tissue repair and angiogenesis (Griffin et al., 2013a, Griffin et al., 2010, Ankrum and Karp, 2010, Caplan, 2009, Dazzi et al., 2012, Prockop and Oh, 2012). In the advent of a large body of research, it is now clear that MSCs can inhibit allogeneic T cell proliferation induced either in a MLC or by non-specific mitogens and these effects seem to be independent of their MHC haplotype (Di Nicola et al., 2002). Moreover, MSCs can influence the maturation and surface marker expression profile of DCs (Beyth et al., 2005) and modulate B cell functions by impairing the production of IgM, IgG and IgA antibodies (Corcione et al., 2006). Additionally, MSCs can inhibit CD4+ Th17 cells in a CCL2-dependent manner (Rafei et al., 2009). More recently, MSCs have been shown to attenuate septic complications by reprogramming host macrophages to increase production of Interleukin IL-10 (Nemeth et al., 2009). Given that MSCs appear capable of modulating multiple immune cell types, it is perhaps not surprising that they are being investigated for the treatment of inflammatory diseases and prolongation of graft survival following organ transplantation (for a comprehensive review see English et al., 2010). In fact, injection of MSCs has been shown to prevent rejection of both allogeneic islet cells and hearts in a mouse model of cardiac transplantation (Ding et al., 2009, Ge et al., 2009). Furthermore, treatment of experimental corneal injuries in rats and limbal stem cell deficiencies in rabbits with MSCs has also been reported (Oh et al., 2008, Reinshagen et al., 2009), however, reports on the therapeutic use of MSCs with regard to corneal transplantation have been limited. One recent study did report the therapeutic benefit of human MSC (hMSCs) infusion in a fully allogeneic mouse model of cornea transplantation (Oh et al., 2012). In this paper, the authors demonstrated that hMSCs can suppress the early surgery-induced inflammatory response leading to the prolongation of corneal allograft survival, an effect mediated, at least in part, by the increased production of TSG-6.
For these reasons, the focus of this study was to comprehensively investigate the potential therapeutic effects of MSCs on corneal allograft survival. For this purpose, the therapeutic benefit using MSCs from three immunologically distinct sources were investigated, with a particular focus on the period corresponding to the average time-point of immune rejection.
4.2 Results

4.2.1 Allogeneic and 3rd party MSC treatment leads to the prolongation of corneal allograft survival in rats, while treatment with syngeneic MSCs has no therapeutic benefit

The first route of administration chosen to test whether syngeneic (syn) MSCs could prolong corneal allograft survival was the sub-conjunctival route, as it was hypothesised that local delivery of the MSCs would be necessary in order for the cells to have some therapeutic benefit. Therefore, the therapeutic strategy involved, firstly, injecting the cells on the day before cornea transplantation (d-1) with a second injection 7 days after transplantation (d+7). Recipient (LEW) rats each received 1x10^6 syn-MSCs in 100 µl of PBS delivered subconjunctivally at d-1 followed by a second injection (same dose) at d+7 (n=5). Untreated corneal allograft recipients served as control group. However, as shown in Figure 4.1, 80% of corneal allografts were rejected by POD 16. Due to these unexpected results, the therapeutic strategy was changed to deliver the MSCs i.v. So, the syn-MSCs were injected i.v through the tail vein of recipient (LEW) rats at d-1 before transplantation with a second injection at d+7. However, as before, i.v injections of syn-MSCs were not associated with prolonged rejection-free survival (Mean survival time (MST): 20.3±6.4, n=11). As previous reports have shown that MSCs are arguably most effective in other transplant models when administered before transplantation (Casiraghi et al., 2008, Casiraghi et al., 2012), the strategy was again amended and it was decided to change the timing of injections. Therefore, 7 days prior to allogeneic corneal transplantation (d-7), recipient (LEW) rats received 1x10^6 syn, allogeneic (allo) or 3rd party (W-F, allogeneic to both recipient and donor) MSCs i.v with a second injection (same dose) on the day of transplantation (d0). Corneal allograft survival was significantly prolonged (p<0.0001, Mantel-Cox test) in approximately 90% of allo-MSC treated rats (MST >30d, n=10, 17d, n=1) and 80% of 3rd party MSC treated rats (MST >30 days, n=4, 20d, n=1) compared to untreated animals (n=7 of 9 transplanted animals -
MST 16.6±1.5d). In contrast, and similar to the results shown in Figure 4.1, injections of syn-MSCs were not associated with prolonged rejection-free survival (MST: 17.1±1.9 days, n=9) (Figure 4.2). Additionally, 100% of untreated syngeneic control grafts were accepted (n=8) as observed until at least day 30. Finally, as neither local nor systemic administration of syn-MSCs had any significant effect on the outcome of corneal allograft survival and injecting the cells pre-, peri-, or post-transplantation also failed to significantly prolong graft survival, it was decided to pre-treat the syn-MSCs with IFN-γ prior to injecting. The decision to do this was based on previous reports showing MSCs have increased therapeutic efficacy when activated or 'licensed' with pro-inflammatory cytokines prior to administration (Ren et al., 2008, Ren et al., 2009, Ren et al., 2010, Duijvestein et al., 2011, Francois et al., 2012, Li et al., 2012). As can be seen in Figure 4.2, however, IFN-γ pre-treatment of syn-MSCs had little effect on the cells’ ability to prolong corneal allograft survival, with survival rates similar to that of the untreated group.

Interestingly, graft opacity, as an indicator of cellular infiltration and endothelial dysfunction, was present in both allo- and 3rd party MSC treated allograft recipients but was significantly reduced in peak severity (p=0.0093 and p=0.0139, respectively) compared to syn-MSC treated allografts (Figure 4.3A). Furthermore, when corneal allografts from allo- and 3rd party MSC treated recipients were observed at day 17/18, which corresponds to the average time-point of rejection for both the untreated control and syn-MSC treated groups, while slight corneal opacification was evident it was not accompanied with changes in the transplant geometry e.g. surface roughness and shrinking. This was in stark contrast to allografts from both the untreated allogeneic control and syn-MSC treated animals. Levels of neovascularization were also investigated and found to be lower in all three MSC treated groups compared to untreated rats at a number of time-points. We also found that 3rd party MSC treated allograft recipients had significantly less neovascularization at day 18 when compared to syn-MSC treated animals (p=0.0276, Figure 4.3B).
Corneal stromal edema development was estimated throughout the observation period using slit-lamp imaging (Figure 4.4) and confirmed by measuring cornea thickness on histological sections at POD 17 and POD 31 (Figure 4.5A). Following analysis of the results, it was evident that there was less edema in grafts from animals that received both allo- and 3rd party MSCs compared to untreated and syn-MSC treated animals at POD 17. A similar outcome was seen at POD 31 albeit with significantly higher levels of edema present in corneal grafts from syn-MSC treated animals only (Figure 4.5A). Histological analysis of H&E stained corneal sections at POD 17 revealed a marked increase in the amount of infiltrating cells in corneas from both untreated allogeneic controls and syn-MSC treated animals compared to allo- and 3rd party MSC treated animals, thus correlating with the level of opacity observed at this time-point (Figure 4.5B). It is also worth noting that there appears to be substantial disruption to the epithelial cell layer in corneas treated with syn-MSCs only (Figure 4.5B).
Fig. 4.1 Syngeneic MSC therapy fails to prolong corneal allograft survival, regardless of route of administration. Kaplan-Meier survival curve analysis of syngeneic (n=8) and allogeneic (n=9) transplants, allograft recipients which received two sub-conjunctival injections of $1 \times 10^6$ syngeneic MSCs (n=5) per 100µl/PBS or two i.v injections of $1 \times 10^6$ syngeneic MSCs (n=11) per ml/PBS.
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Fig. 4.2 Allogeneic and 3rd party MSC therapy promotes corneal allograft survival. Kaplan-Meier survival curve analysis of syngeneic (n=8) and allogeneic (n=9) transplants, allograft recipients which received two i.v injections of either 1x10^6 syngeneic MSCs (n=9), allogeneic MSCs (n=11), 3rd party MSCs (n=5) or syngeneic MSCs that had been pre-treated with 500U/ml of IFN-γ for 72 h prior to injection (n=5), per ml/PBS. ***, p<0.0001, log-rank (Mantel-Cox) test.
Fig. 4.3 Reduced corneal opacity and delayed onset of neovascularization after MSC therapy. (A) Opacity and (B) neovascularization scores up to day 30 post-transplantation of the untreated allogeneic controls, syn-, allo- and 3rd party MSC treated allograft recipients (n=9, 9, 11 and 9, respectively). #/$ - 3rd party-vs syn-MSC treated. £ - allo- vs syn-MSC treated*, p<0.05; **, p<0.01, data shown as mean ± SEM, two-tailed, non-parametric, Mann-Whitney test.
Fig. 4.4 Clinical evaluation of corneal allografts. Light microscope and contrast slit-lamp imaging of untreated control, syn-, allo- and 3rd party MSC treated corneal allografts from postoperative day (POD) 7 to POD 31. Arrows indicate the observable reflection of the slit-lamp beam indicating that allografts remained quite clear throughout the observation period, relative to untreated control and syn-MSC treated allograft recipients. Representative data of four animals is shown.
Fig. 4.5 Thickness measurements and histological evaluation of corneal allografts. (A) Cornea thickness measurements at POD 17 and 31 showing reduced edema development in corneas from both allo- and 3rd party MSC treated allograft recipients compared to the syn-MSC treated group (n=2-4 per group). *, p<0.05, data shown as mean ± SD, Student's unpaired, two-tailed t-test. (B) H&E staining of paraffin-embedded corneal sections at POD 17 and 31 from all groups. Representative images of 2-4 rats per group are shown; 20x magnification).
4.2.2 MSC therapy leads to modulation of immune cells and differential expression of pro-/anti-inflammatory genes in corneal allografts

In order to study the effects of MSC therapy on inflammatory cell populations within the allogeneic cornea, allografts were collected from all four experimental groups at a time-point corresponding to the average day of rejection of the untreated allogeneic control group (between day 17 and 18). Inflammatory cells were isolated from explanted corneas by collagenase D digestion and analysed by flow cytometry. The results showed a marked reduction in both the proportion (%) and the total cell number (Figure 4.6A for gating strategy used to gate out fluorescently labelled beads for calculation of total cell number) of intragraft CD4+CD25+, CD11b/c+ and CD45RA+ cells, corresponding to activated CD4+ T cells, APCs, and B cells respectively, in animals receiving any of the three types of MSCs, compared to corneas from untreated rats (Figure 4.6B). There was also a significant reduction in the numbers of intragraft CD11b/c+MHCII+CD86+ cells (activated APCs) in all three MSC treated groups compared to corneas from untreated rats (Figure 4.6B). Arguably, the most striking results were obtained from analysis of the proportion of infiltrating CD3+CD8+CD161+ cells (see Figure 4.7A for gating strategy), corresponding to NKT cells. The proportion of NKT cells was significantly reduced from 11.23 ± 1.78% in untreated and 10.92 ± 3.87% in syn-MSC treated animals to 5.79 ± 2.27% and 5.47 ± 1.87% in allo- and 3rd party MSC treated allograft recipients, respectively, reflecting an overall reduction of approximately 50% (Figure 4.7B). The results also showed a significant increase in the proportion of intragraft CD3+CD8+CD161- cells, corresponding to CD8+ T cells, from animals receiving any of the three types of MSCs, compared to corneas from untreated rats. However, the increased proportion did not correlate with changes in total cell number, as numbers were broadly similar across the four groups (Figure 4.7B). Additionally, the proportion of intragraft CD4+Foxp3+ Tregs was increased following all three MSC treatments, compared to untreated rats (Figure 4.8A).
To gain a better understanding of what differences may be evident at the gene expression level between the different groups, intragraft mRNA expression levels of a number of key pro- and anti-inflammatory molecules were analysed using real-time RT-PCR. While no detectable differences were observed in mRNA expression levels of IFN-γ between the four groups, both IL-6 and IL-1β mRNA expression was significantly reduced in allografts from rats treated with either allo- or 3rd party MSCs compared to untreated rats (Figure 4.8B). mRNA expression levels of Foxp3, IL-10, iNOS and PD-L1 were also investigated. While there were no significant differences in Foxp3 mRNA expression between the four study groups, intragraft IL-10 mRNA expression was significantly reduced in 3rd party MSC treated animals compared to both untreated and allo-MSC treated animals, while iNOS mRNA expression was significantly reduced in grafts from both allo- and 3rd party MSC treated groups compared to untreated animals (Figure 4.8B). The results also revealed that PD-L1 mRNA expression was significantly reduced in all three MSC treated groups compared to untreated rats (Figure 4.8B). This may be as a result of the reduced inflammation observed in corneas from MSC treated animals compared to the untreated animals, as corneal inflammation has been shown previously to increase PD-L1 expression (Shen et al., 2007).
Fig. 4.6 MSC therapy leads to reduced intragraft immune cell infiltration. (A) Gating strategy used to estimate total cell numbers in subpopulations of intragraft cells from all groups on day 17/18 post-transplantation by use of fluorescently labelled (PerCP-Cy5-5) beads. (B) Proportion (%) and total cell number of intragraft CD4+CD25+, CD11b/c+, CD11b/c+MHCII+CD86+ and CD45RA+ cells, corresponding to activated T cells, APCs, activated APCs and B cells, respectively, from untreated (n=4), syngenic (n=5), allogenic (n=5) and 3rd party (n=4) MSC treated animals. *, p<0.05, data shown as mean ± SD, two-tailed, non-parametric, unpaired, Mann-Whitney test.
Fig. 4.7 Allogeneic & 3rd party MSC therapy leads to a reduced proportion of intragraft NKT cells. (A) Gating strategy of CD3+ subpopulations of graft-infiltrating cells from all groups. (B) Frequency (%) and total cell number of intragraft CD3+CD8+CD161+ and CD3+CD8+CD161- cells, corresponding to NKT cells and CD8+ T cells, respectively, from untreated (n=4), syn- (n=5), allo- (n=5) and third party (n=4) MSC treated animals. *, p<0.05, data shown as mean ± SD, two-tailed, non-parametric, unpaired, Mann-Whitney test.
Fig. 4.8 MSC therapy leads to a higher proportion of intragraft Tregs and differential pro-/anti-inflammatory gene expression. (A) Proportion (%) of intragraft CD4+Foxp3+ regulatory T cells (n=2-5 per group). (B) RT-PCR analysis of intragraft mRNA expression (normalized to the housekeeping gene β-actin and shown as fold-change relative to untreated allogeneic controls) of pro-inflammatory (IL-6, IFN-γ and IL-1β) and anti-inflammatory (Foxp3, IL-10, iNOS and PD-L1) molecules at day 17/18 post-transplantation from untreated (n=4), syn- (n=3), allo- (n=3) and third party (n=4) MSC treated animals. *, p<0.05; **, p<0.01; ***, p<0.0001, data shown as mean ± SD, Student's two-tailed t-test.
4.2.3 Intravenous administration of MSCs leads to minimal changes in immune cell distribution and gene expression in the draining lymph nodes (DLNs)

As the MSCs in this study were administered systemically, it was important to investigate what effects the cells would have on immune cell populations within the DLNs. Therefore, the ipsilateral draining (cervical and sub-mandibular) lymph nodes were harvested from transplanted animals at a time-point corresponding to the average day of rejection (day 17/18) and subjected to the same flow cytometric analysis as described previously for the cornea. Perhaps surprisingly, the results showed that there were no significant differences in the frequency of CD4+Foxp3+ Tregs, CD8+ T cells and NKT cells between the four groups, with only mild differences in frequencies of both activated CD4+ T cells and CD11b/c+ APCs (Figure 4.9B). In contrast, animals from all three MSC groups had a reduced proportion of B cells (CD45RA+) compared to untreated animals (Figure 4.9B).

RT-PCR analysis of the DLNs revealed no significant changes in the mRNA expression levels of either IFN-γ or IL-10 between the four groups, with only minimal differences observed for IL-6 (Figure 4.10). Although we saw no differences in the frequency of CD4+Foxp3+ Tregs between the four groups (Figure 4.9B), RT-PCR analysis did reveal a trend towards higher Foxp3 mRNA expression in the draining lymph nodes of both allo- and 3rd party MSC treated rats compared to syn-MSC treated rats (Figure 4.10). Similar to results from the cornea, PD-L1 mRNA expression was reduced in the DLNs from all three MSC treated groups compared to untreated rats. Finally, we found that expression of IDO was significantly higher in the DLNs of rats treated with both allo- and syn-MSCs compared to untreated rats (Figure 4.10).
Fig. 4.9 Frequency of immune cell populations in the DLNs. (A) Gating strategy of CD3+ subpopulations in the DLNs from all groups. (B) Frequency (%) of the different populations of immune cells from untreated (n=4), syn- (n=5), allo- (n=5) or 3rd party (n=4) MSC treated animals on day 17/18 post-transplantation. *, p<0.05, data shown as mean ± SD, non-parametric, two-tailed Mann-Whitney test.
Fig. 4.10 RT-PCR analysis of gene expression in the DLNs. Analysis of mRNA expression (normalized to the housekeeping gene β-actin and shown as fold-change relative to untreated allogeneic controls) in the DLNs of both pro- (IL-6 and IFN-γ) and anti- (IL-10, Foxp3, IDO and PD-L1) inflammatory modulators at day 17/18 post-transplantation from untreated (n=4), syn- (n=3), allo- (n=3) or 3rd party (n=4) MSC treated animals. *, p<0.05; **, p<0.01; ***, p<0.0001, data shown as mean ± SD, Student’s, unpaired, two-tailed t-test.
4.2.4 Allogeneic and 3rd party MSC therapy leads to a higher proportion of Tregs in the spleens of transplanted rats

As shown in Figure 4.11, MSCs can migrate to the spleen after intravenous administration. It was therefore decided to investigate what effect(s) MSCs may have on immune cell populations and gene expression in the spleen of treated corneal allograft recipients.

Flow cytometric analysis showed there was a significantly higher proportion of Foxp3-expressing Tregs in spleens from both allo- and 3rd party MSC treated rats compared to syn-MSC treated rats (Figure 4.12A). As suggested by a recent report (Chauhan et al., 2009), analysis of the intensity of Foxp3 expression by Tregs is as relevant as the frequency of Tregs themselves. We therefore investigated the intensity of expression as follows. Firstly, lymphocytes were gated based on forward scatter (FSC) and side scatter (SSC) parameters followed by subsequent gating based on CD4 and CD25 expression (Figure 4.12B). As ‘true’ Tregs, in addition to being CD4+Foxp3+, highly express CD25, the CD4+ population was further divided into three distinct subsets, namely, CD4+CD25−, CD4+CD25int and CD4+CD25hi using the gating strategy shown in Figure 4.12B and based on previous reports (Baecher-Allan et al., 2001, Clarke et al., 2006). The percentage of Foxp3 expression was then determined for each of the three subsets and for all four groups (Figure 4.12C). The results showed that Foxp3 expression was highest in allo-MSC treated rats compared to the other groups (Figure 4.12C) for each of the three CD4+CD25 subsets. The levels of Foxp3 were then determined from the CD4+CD25hi population from each of the four groups by calculating the mean fluorescence intensity (MFI). The results showed that levels of Foxp3 were significantly higher in allo-MSC treated compared to both 3rd party MSC treated and untreated animals (Figure 4.12D). There was also clearly higher levels of Foxp3 in the CD4+CD25hi population from allo-MSC treated compared to syn-MSC treated animals, despite a lack of significance following statistical analysis.
RT-PCR analysis of gene expression showed a trend towards lower levels of both IL-6 and IFN-γ mRNA expressed by both allo- and 3rd party MSC treated compared to syn-MSC treated rats. Also, levels of IL-1β mRNA were lower in all three MSC treated groups compared to untreated rats (Figure 4.13). There was also a clear reduction in the level of both iNOS and IL-4 mRNA expression in spleens from both allo- and 3rd party MSC treated groups compared to syn-MSC treated and untreated rats. Furthermore, as seen for both the cornea and the draining lymph nodes of transplant recipients, there was also a trend towards lower PD-L1 mRNA expression in the spleens of both allo- and 3rd party MSC treated compared to untreated animals (Figure 4.13).
Fig. 4.11 MSC distribution following intravenous administration. $2\times10^6$ CFSE-labeled syngeneic (n=4) or allogeneic (n=4) MSCs were injected i.v into naïve LEW rats. 24h post-injection blood, lymph nodes, lungs (results show cell number for one lung) spleens and thymi were harvested, digested and analysed by flow cytometry to detect the presence of MSCs. Fluorescent beads were added to FACS samples for calculation of total cell numbers.
Allogeneic and 3rd party MSC therapy leads to a higher frequency of Tregs in the spleens of transplanted rats. (A) Allo- and 3rd party MSC treated corneal allograft recipients have a significantly higher percentage of splenic CD4+Foxp3+ Tregs compared to syn-MSC treated and untreated animals. (B) CD4+CD25-, CD4+CD25int and CD4+CD25hi cells were identified as depicted. (C) Foxp3 expression within each population was assessed by intracellular staining. Shown is the mean Foxp3 expression from each of the groups. (D) Splenic CD4+CD25hi cells from allo-MSC treated allograft recipients have significantly higher amounts of Foxp3, as determined by analysis of mean fluorescence intensity (MFI), compared to the other groups. n=4/5 per group; *, p<0.05, non-parametric, two-tailed, Mann-Whitney test.
Fig. 4.13 RT-PCR analysis of gene expression in the spleen. Analysis of mRNA expression (normalized to the housekeeping gene β-actin and shown as fold-change relative to untreated allogeneic controls) in the spleen of both pro-(IL-6, IFN-γ and IL1β) and anti- (iNOS, PD-L1 and IL-4) inflammatory modulators at day 17/18 post-transplantation from untreated (n=4), syn- (n=3), allo- (n=3) or 3rd party (n=4) MSC treated animals. *, p<0.05; **, p<0.01; ***, p<0.0001, data shown as mean ± SD, Student’s, unpaired, two-tailed t-test.
4.2.5 Intravenously injected allogeneic MSCs elicit an alloantibody response \textit{in vivo}

Next, it was decided to investigate whether intravenous administration of MSCs elicits an alloantibody response. To do this, freshly isolated splenocytes from allogeneic DA rats were incubated with serum harvested at day 17/18 post-transplantation from untreated rats or from rats receiving a corneal allograft coupled with two injections of syn-, allo- or 3\textsuperscript{rd} party MSCs. Serum donor-specific alloantibodies were detected by the binding of these antibodies to the allogeneic DA indicator-splenocytes and subsequent analysis by flow cytometry. The results showed no significant differences between the groups in terms of IgM antibody formation (Figure 4.14A). However, significantly higher levels of both IgG1 (Figure 4.14B) and IgG2 (Figure 4.14C) were detected in the sera of allo-MSC treated rats compared to the other three groups.
Allogeneic MSCs elicit an alloantibody response in vivo.

Splenocytes from allogeneic DA rats were incubated with serum harvested at day 17/18 post-transplantation from untreated, syn-, allo- or 3rd party MSC treated allograft recipients and, subsequently, bound alloantigen-specific antibodies were stained with anti-rat IgM (A), anti-rat IgG1 (B) or anti-rat IgG2 (C) antibodies. B cells were excluded from analysis of IgM by counterstaining with CD45RA. (n= 4/5 animals/group; *, p<0.05; **, p<0.01; ***, p<0.0001, Student’s, unpaired, two-tailed t-test).
4.3 Discussion

The results generated in the second part of this study show that systemic administration of both allogeneic and third party MSCs, but not syngeneic MSCs, significantly prolong corneal allograft survival in the rat. While the finding that local (sub-conjunctival) administration of syn-MSCs failed to prolong corneal allograft survival (Figure 4.1) was unexpected, it was thought that the route of administration may have been the problem as sub-conjunctival injections are quite an invasive procedure and the overall trauma elicited by two sub-conjunctival injections, coupled with the trauma induced by the corneal transplantation procedure itself all in a short period of time may have been responsible for the failure of this strategy. The rationale to inject the MSCs 7 days before transplantation was based on previous published reports showing that MSCs have enhanced therapeutic benefit when administered pre-transplantation in pre-clinical models of cardiac (Casiraghi et al., 2008) and kidney (Casiraghi et al., 2012) transplantation. While a number of groups have investigated the ability of MSCs from different sources (e.g. rabbit, human and mouse) to repair chemically-damaged corneas (Ye et al., 2006, Ma et al., 2006, Ye et al., 2008, Oh et al., 2008), relatively few have reported on the use of MSCs in the context of corneal allograft transplantation (Oh et al., 2012, Jia et al., 2012). To our knowledge, this is the first report showing MSCs derived from a third party strain are capable of prolonging corneal allograft survival. This is a significant finding as, from a translational viewpoint, the potential clinical use of MSCs to treat corneal transplant patients in the future will, most likely, require cells from a source unrelated (and therefore immunologically distinct) to both the donor and recipient. It is important therefore to understand the mechanisms by which MSCs from donor (allogeneic), recipient (syngeneic) and unrelated (third party) sources modulate, however subtle, the recipients' immune response.
The finding that syngeneic MSCs, although clearly able to modulate recipient immune responses as demonstrated by the results from the graft infiltration and cell distribution experiments, are unable to significantly prolong corneal allograft survival was a little surprising but may be due to their ‘activation status’. That is, pre-treatment of MSCs with a combination of pro-inflammatory cytokines (predominantly a mixture of one or more of IFN-γ, TNFα and IL-1β) has been shown to activate or ‘license’ the cells. A number of studies have reported that MSCs activated in this manner produce higher levels of immunosuppressive molecules such as IDO in the case of human MSCs and iNOS in the case of mouse MSCs both in vitro and in vivo which, subsequently, increases their therapeutic potential (Ren et al., 2008, Ren et al., 2009, Ren et al., 2010, Duijvestein et al., 2011, Francois et al., 2012, Li et al., 2012). Although this concept was investigated in this study, IFN-γ-treated syn-MSCs were only slightly better at prolonging corneal allograft survival than untreated syn-MSCs (Figure 4.2). However, it is important to note that different cytokine combinations and/or concentrations were not tested due to time constraints. As it seems likely that both allogeneic and third party MSCs will be targeted by the recipient’s immune system following encounter, this immune response may have the capacity to provide sufficient pro-inflammatory stimuli to activate the MSCs and, thus, increase their therapeutic potential resulting in allograft protection. However, crucially, as syngeneic MSCs are recipient-derived they will not provoke such an immune response and therefore not become activated, resulting in insufficient graft protection and subsequent rejection. Indeed, in vitro results generated in our group have shown an approximately four-fold increase in nitric oxide (NO) production when MSCs are co-cultured with allogeneic T cells compared to when co-cultured with syngeneic T cells (data not shown).

As shown in Figure 4.3B, while MSC treatment delays the onset of neovascularisation, levels eventually do increase over time. Therefore, transduction of MSCs using adenoviral vectors encoding the angiogenesis inhibitors angiotatin or endostatin could be one strategy that might lead to
the inhibition or regression of blood vessels in to the graft, thereby enhancing
the therapeutic effects of MSC treatment.

Previous results generated in our group have shown that both lentivirus-
mediated overexpression of PD-L1 on corneal cells (Nosov et al., 2012) and
i.v administration of donor-derived BMDCs (O’Flynn et al., 2013) can
significantly inhibit NKT cell infiltration into the corneal allograft. Although in
this study a different approach was employed (systemic infusion of MSCs),
this finding was replicated in the case of both allogeneic and third party MSC
treated recipients, perhaps confirming the importance of this cell type in
determining the fate of the allograft.

Analysis of the effects of MSCs on cell populations in the spleen showed that
both allogeneic and third party MSC treated allograft recipients have
significantly higher proportions of splenic CD4+Foxp3+ Tregs compared to
both untreated and syngeneic MSC treated rats. However, as detailed by
Chauhan and colleagues (2009), predicting and/or correlating allograft fate
solely on the basis of Treg frequency may lead to inaccurate conclusions. It
is therefore important to measure the levels of Foxp3 expression in parallel,
with evidence that higher levels of Foxp3 expression are directly associated
with an increased ability of Tregs to prevent graft rejection. To this end, the
results showed significantly higher levels of Foxp3 expression in splenic
Tregs from allogeneic MSC treated rats compared to the other groups
(Figure 4.12D).

Gene expression analysis revealed differential expression of key pro-
inflammatory cytokines within both the allograft itself (e.g. IL-6 and IL-1β) and
also the spleen between the four study groups. However, it should be
pointed out that the focus in this study was restricted to the average time-
point of rejection (POD 17/18) only and, as a result, changes in levels of
gene expression early post-transplantation (POD 3-7) and towards the end of
the observation period (POD 28-31) were limited in the context of the scope
of the study. A recent report, for example, by Oh and colleagues (2012)
showed reduced expression of intragraft IFN-γ and IL-12α at POD 3 and 7 in mice receiving a corneal allograft along with i.v. administration of 1x10^6 human MSCs one day before transplantation and again on the day of transplantation compared to untreated allograft control mice. However, similar to the results shown in this study for both allogeneic and third party MSC treated rats at POD 17/18, these researchers also observed a significant reduction in both IL-6 and IL-1β expression at POD 7 in their hMSC treated group compared to untreated allograft controls. Indeed, a recent study using a fully MHC-mismatched murine model of cardiac transplantation has highlighted the importance of IL-6 in allograft rejection (Zhao et al., 2012).

As has been reviewed recently (Griffin et al., 2013a, Griffin et al., 2010, English et al., 2010), multiple studies have investigated in vivo immune responses elicited by allogeneic MSCs in various levels of detail. Although until quite recently it was suggested that allogeneic MSCs could evade host immune responses, it is now generally accepted that this is not the case and that allogeneic MSCs are not immune privileged and do in fact evoke an immune response when transplanted into an allogeneic host. Crucially, however, this does not necessarily equate with a lack of efficacy or safety. Indeed, as shown by the results in Figure 4.14, significantly higher levels of IgG1 and IgG2 alloantibodies are generated against allogeneic MSCs (compared to serum from rats that received a corneal allograft only) but, crucially, have no observable effects on the cells’ ability to prolong corneal graft survival. In support of these findings, Hegde and colleagues (2002) have shown previously that while alloantibody can contribute to corneal injury in a complement-dependent manner, it is not necessary or sufficient to cause corneal graft rejection. Taking this into account, alloantibodies generated against the corneal allograft itself, combined with alloantibodies generated against the allogeneic MSCs, may not ultimately determine the fate of the corneal allograft. However, the eye is an immune privileged tissue with both the epithelial cell layer of the cornea and the aqueous humor rich in
complement regulatory proteins capable of counter-acting the effects of complement fixing alloantibodies (Bora et al., 1993, Lass et al., 1990, Goslings et al., 1998). This is not the case in other types of organ transplantation where the presence of alloantibodies may have a more detrimental effect on the outcome of graft survival. For this reason, strategies involving repeated administration of allogeneic MSCs should take this into account.

In summary, the results from the second part of this study demonstrate that systemic administration of both allogeneic and third party MSCs can successfully promote corneal allograft survival in a fully allogeneic MHC-mismatched rat model. These effects may be in part explained by the significant reduction of anti-donor immune responses mediated by allogeneic and third party MSC therapy, such as the reduced frequency of intragraft NKT cells and increased proportion of splenic CD4+Foxp3+ Tregs following treatment. While treatment with syngeneic MSCs can partially modulate the recipients’ immune response, the effect is not sufficient to prevent graft rejection. A possible explanation for this may be the ‘in-activated’ status of the syngeneic MSCs and, as a result, their inability to produce the required levels of immunosuppressive molecules needed for graft protection. While the potential efficacy of pre-treating syn-MSCs with IFN-γ was investigated in this study, only one parameter was used. That is, syn-MSCs were treated with IFN-γ at a concentration of 500U/ml for 72 h prior to intravenous administration to recipient rats. It is certainly possible that by adding other pro-inflammatory cytokines, such as TNF-α and/or IL-1β along with IFN-γ, increasing the length of time the MSCs are exposed to the inflammatory mediators and/or increasing the concentration of the cytokines, this treatment may be more efficacious.
Chapter Five

General Discussion
5 General Discussion

Since the first reported clinical trial using \textit{ex vivo} expanded MSCs in 1995 (Lazarus et al., 1995), over 100 clinical trials involving more than 3000 human subjects have reached safety endpoints (McKernan et al., 2010) with a large number of phase 2/3 trials close to completion (Griffin et al., 2013b). Although, critically, no severe adverse events caused by MSC administration have been reported thus far, a number of issues remain to be resolved before MSCs, as a cellular therapy, can be realistically considered on a global scale. Issues relating to critical parameters such as source of MSCs, timing, dose and route of administration will be discussed in detail in the following discussion but, firstly, it is important to consider some of the key safety issues arising from MSC therapy in humans. Issues such as immunogenicity in human patients, ectopic tissue formation \textit{in vivo} and possible malignant transformation are perhaps the most pertinent. Also, the potential risk of MSC treatment in patients with established tumors will be discussed in this context.

Immunogenicity

While numerous studies have reported on the immunogenicity of MSCs (or lack of in some cases) in pre-clinical animal models (discussed in more detail in the following sections), far less has been published on the potential immunogenicity of human MSCs following administration in human patients. However, one recent study by Moll and co-workers investigated whether or not culture-expanded human MSCs can elicit an innate immune attack, termed instant blood-mediated inflammatory reaction (IBMIR), \textit{in vitro} upon exposure to blood and \textit{in vivo} following systemic infusion in patients that received an hematopoietic stem cell transplantation (Moll et al., 2012). They found that MSCs exposed to blood \textit{in vitro}, while expressing hemostatic regulators, expressed higher amounts of cell surface prothrombotic
tissue/stromal factors, which was responsible for initiating the IBMIR after exposure to blood. Furthermore, expression of these factors was further increased following both extended passaging and co-culture with activated lymphocytes. In contrast, however, low passage (≤ P4) MSCs triggered only weak responses in vitro. In vivo, the authors administered commonly used clinically-relevant MSC doses (1-3x10^6/kg) between P1-P4 and found that these were associated with only weak blood responses (Moll et al., 2012). However, as the authors point out, using higher doses and higher passage MSCs may have serious adverse effects.

Another important consideration when using non-HLA matched human MSCs is the development of alloantibodies generated against the cells. While we (Figure 4.14, Schu et al., 2012) and others (Beggs et al., 2006, Isakova et al., 2010) have reported detectable levels of alloantibodies generated following MSC infusion in different animal models, little has been reported to date with regard to alloantibody formation in patients following treatment with human MSCs. In one clinical study involving 12 patients treated with MSCs (doses ranging from 0.8-2x10^6 cells/kg bodyweight) following haematopoietic stem cell transplantation, none were found to have developed anti-MSC antibodies. However, it is important to note that only 5 of the 12 patients received fully HLA-mismatched MSCs as opposed to sibling-derived or haploidentical MScs and all were immunosuppressed (Sundin et al., 2007). Despite these findings, future studies should include analysis of alloantibody formation following human MSC treatment as alloantibodies may have a detrimental effect on the outcome of the therapy, such as in the case of vascularised solid organ transplantation.

**Ectopic tissue formation**

As with any therapy utilising cells with the ability to differentiate into various cell types, sustained engraftment of the transplanted cells has the potential to lead to ectopic tissue formation in the recipient (Bernardo and Fibbe,
As MSCs are a multipotent population of cells with the ability to differentiate into cells of mesodermal origin, ectopic tissue formation in vivo is certainly a possibility. Indeed, in a rat model of glomerulonephritis, MSCs were found to differentiate (at least partially) into adipocytes in the kidneys of treated rats and this was accompanied with glomerular sclerosis, despite having clear beneficial effects in the early stages post-infusion with regard to preserving damaged glomeruli and maintaining kidney function (Kunter et al., 2007). Similarly, using a rat model of myocardial infarction Breitbach and colleagues found that intramyocardial delivery of MSCs led to the ectopic formation of bone (Breitbach et al., 2007). Furthermore, another study showed that in a mouse model of GvHD, local implantation of MSCs propagated from bone marrow, placenta or umbilical cord resulted in ectopic bone formation in syngeneic recipients and in transplant rejection in allogeneic mice (Prigozhina et al., 2008). While these studies clearly highlight the danger for potential ectopic tissue formation following MSC infusion, very little data has been reported to date in human patients. However, in a recent study in which autopsy material from multiple tissues was examined using both macroscopic and histological analyses from 18 patients who had received HLA-mismatched MSCs, no signs of ectopic tissue formation were found (von Bahr et al., 2012).

**Malignant transformation**

Similar to ectopic tissue formation, malignant transformation must be considered a possible risk with any cell therapy. While the risk is high with certain cell types, such as embryonic stem cells for example, it is far less with cells derived from adult tissues such as in the case of MSCs. However, the risk increases the longer MSCs are expanded in culture which is the case for most, but not all, clinical MSC protocols (Prockop et al., 2010). A few reports had originally suggested that long-term in vitro expansion of both bone marrow and adipose tissue-derived human MSCs could lead to the
accumulation of genetic alterations and subsequent malignant transformation (Rubio et al., 2005, Wang et al., 2005, Røsland et al., 2009). However, the reports by Rubio et al and Røsland et al have since been retracted as it was found that the tumor cells in MSC cultures were derived from contaminating tumor cell lines and not from the original MSCs (Bernardo and Fibbe, 2012). In contrast, multiple groups have demonstrated that human MSCs derived from both bone marrow and umbilical cord can undergo long-term in vitro expansion without losing their typical functional/phenotypical characteristics and without acquiring chromosomal aberrations (Bernardo et al., 2007, Tarte et al., 2010, Wang et al., 2013, Tang et al., 2013). Among these studies, similar methods of analyses have been employed to confirm the absence of malignant transformation and include conventional karyotyping, evaluation of morphological changes, proliferation rates, testing the ability of MSCs to enter senescence, as well as assessment of telomerase activity and telomere length. Moreover, a recent report has estimated the likelihood of long-term expanded MSCs to emerge from senescence and malignant transform to be <10⁻⁹ (Prockop et al., 2010). Despite these consistent findings, however, periodic genomic monitoring of MSCs through conventional and/or molecular karyotyping should be considered before these cells are used in clinical applications.

**MSCs in patients with established tumors**

Numerous studies have investigated the role of MSCs as an effective cell therapy treatment for different forms of cancer. However, the findings from these studies are contrasting to say the least, with MSCs described as being capable of both promoting (Karnoub et al., 2007, Suzuki et al., 2011, Shinagawa et al., 2010, Tsukamoto et al., 2012, Ren et al., 2012) and inhibiting (Khakoo et al., 2006, Qiao et al., 2008, Wei et al., 2009, Lee et al., 2012) tumor growth. It has been suggested that MSCs have the ability to infiltrate into tumors and can subsequently form a major part of the tumor
microenvironment. While in this environment, MSCs may be conditioned into
tumor-resident MSCs and acquire an altered phenotype that is distinct from
that of normal tissue MSCs. Ren and colleagues proposed that a key factor
involved in promoting tumor growth by tumor-resident MSCs is their
production of high levels of the monocyte chemoattractant CCL2. Secretion
of this chemokine leads to the recruitment of macrophages with an
immunosuppressive phenotype which results in promotion of tumor growth.
Interestingly, the authors also found that bone marrow-derived MSCs
stimulated with TNF-α acquired a very similar chemokine profile and had
comparable tumor-promoting properties to tumor-resident MSCs, suggesting
an important role for inflammation in driving tumorigenesis (Ren et al., 2012,
Bernardo and Fibbe, 2013). In contrast to this report, however, Lee et al
could show that pre-stimulation of human MSCs with TNF-α had tumor
suppressive effects by causing an upregulation of TRAIL on MSCs and
subsequent induction of apoptosis of tumor cells in a lung xenograft tumor
model (Lee et al., 2012). It should be noted that the study by Lee and co-
workers used NOD-SCID mice in which tumor-promoting and adaptive
immune responses are missing, which may partially account for the
discrepancies between the two studies (Bernardo and Fibbe, 2013).

It has been proposed that timing of MSC delivery may be a key factor in
tipping the balance in favour of either tumor growth promotion or inhibition.
The majority of studies to date reporting MSC-mediated tumor growth
involved injecting the MSCs at the same time (or before the tumor was
established) as the tumor cells (Karnoub et al., 2007, Suzuki et al., 2011,
Shinagawa et al., 2010, Tsukamoto et al., 2012, Ren et al., 2012). It has
been hypothesised that the presence of MSCs during this early growth phase
may contribute to angiogenesis, which is important for tumor initiation (Klopp
et al., 2011). By contrast, the vast majority of studies in which MSCs were
introduced after tumors had been established reported tumor growth
inhibition (Lu et al., 2008, Otsu et al., 2008, Cousin et al., 2009, Kéramidas et
al., 2013). Although the mechanisms through which MSCs may promote or
suppress tumor growth have yet to be fully elucidated, the potential tumor-promoting activity of MSCs should be carefully considered before choosing to administer MSCs in cancer patients (Bernardo and Fibbe, 2013).

In terms of source, MSCs can be derived from the patient themselves (autologous), from the donor (allogeneic, in the case of organ transplantation) or from a third party (allogeneic to both donor and recipient). From a safety perspective, autologous-derived MSCs should pose little problem with regard to risk of rejection or graft versus host reactions and are therefore considered the safest clinical option. However, the use of autologous MSCs may not always be possible for reasons such as genetic factors, disease acuity and recipient progenitor cell deficits (Griffin et al 2013a). Moreover, it has also been reported that autologous MSCs derived from patients with autoimmune disorders are less efficacious than those derived from healthy donors (Weyand and Goronzy, 2004). In these cases, “off-the-shelf” allogeneic MSC therapy would not only be necessary, but could also be a more feasible approach in terms of availability. However, important considerations pertaining to how MSCs from these different sources would interact with and modulate the recipient’s immune system and how these interactions would affect the immunosuppressive properties of MSCs must be taken into account.

Other critical parameters in the context of MSC therapy are selection of the appropriate dose and route of administration of the cells. In clinical trials reported so far, the numbers of MSCs infused has ranged from $0.4 \times 10^6$ to $10 \times 10^6$ per kg of bodyweight (LeBlanc et al., 2008, Macmillan et al., 2009). However, as of yet, no clear correlation between the amount of MSCs received and the clinical outcome has been made. Moreover, with regard to the clinical outcome, no clear pattern has emerged following single, double or repeated MSC administration. Indeed, LeBlanc and colleagues observed that while some patients responded favourably to a second infusion of MSCs after not responding to the first, other patients remained unresponsive even after multiple infusions (LeBlanc et al., 2008).
In terms of where to deliver the cells, intravenous administration has been the route of choice for many human trials and in pre-clinical animal models. Another possibility is to inject MSCs directly into the donor organ before transplantation. This method could potentially aid in the establishment of an immunomodulatory microenvironment (English et al., 2010). A recent report using a murine model of diabetes showed that graft survival could be significantly prolonged when MSCs were introduced under the kidney capsule in conjunction with the donor islet cells (Ding et al., 2009). Other possible routes of administration, including intra-peritoneal, intra-muscular, subcutaneous and sub-conjunctival have been investigated much less frequently but are nonetheless feasible alternatives, depending on the particular indication.

The use of immunosuppressive drugs in patients receiving organ transplants is currently in effect as a standard protocol. Since MSCs are being used therapeutically in pre-clinical studies to modulate host immune responses to allogeneic grafts, understanding how MSC therapy interacts with immunosuppressive drugs is critical. Consequently, in recent years, a number of reports have investigated how commonly used drugs such as rapamycin, tacrolimus, cyclosporine A (CsA) and mycophenolic acid (MPA) can affect MSC function when co-administered. A recent study, for example, in a rat model of skin transplantation, researchers could show that co-administration of CsA and donor-derived MSCs led to prolongation of graft survival (Sbano et al., 2008). Additionally, in a mouse cardiac allograft model, Ge and colleagues demonstrated that co-infusion of MSCs with rapamycin synergized to induce allograft tolerance as shown by the prolongation of survival up to 100 days post-transplantation, followed by subsequent acceptance of skin grafts of donor origin (Ge et al., 2009). Furthermore, this donor antigen-specific tolerance was confirmed by the finding that third-party derived skin grafts were not accepted by the treated recipients. Interestingly, however, in a mixed lymphocyte reaction, rapamycin and tacrolimus-mediated suppression of PBMC proliferation was abrogated...
when human MSCs were added (Ge et al., 2009). Conversely, another report showed that MSCs enhanced MPA-mediated suppression of PBMC proliferation (Hoogduijn et al., 2008). Moreover, a recent study reported, even in the presence of immunosuppression in the form of mycophenolate mofetil (MMF), that pre-transplant infusion of both recipient and donor-derived MSCs actually accelerated the rate of graft rejection in a rat model of kidney transplantation compared to saline treated control recipients (Seifert et al., 2012). An additional interesting aspect of this particular study was that the protocol the authors used was identical to a protocol used to successfully prolong graft survival in a heart transplant model (Popp et al., 2008). Another recently published study investigated what effects the immunosuppressive agent rabbit antithymocyte globulin (rATG) had on human MSCs (Franquesa et al., 2013). The authors showed that binding of rATG to the MSCs reduces the viability and antiproliferative capacity of the cells in a dose-dependent manner and converts them into targets for CD8+ T cells and NKT cell lysis. While little has been reported to date on the role of combined treatment with MSCs and immunosuppressive drugs on corneal transplant survival, at least one report documented interesting findings following co-administration of MSCs and CsA. In a rat corneal allograft model, Jia and co-workers showed that when MSCs were combined with low dose CsA therapy (1 mg/kg), allograft rejection was actually accelerated. In contrast, when the dosage of CsA was increased to 2 mg/kg and combined with MSCs, allograft survival was significantly prolonged compared to rats treated with MSCs alone or 2 mg/kg of CsA alone (Jia et al., 2012). Of interest and what these studies reveal is that, in addition to the importance of choosing the suitable immunosuppressive drug to be co-administered with MSCs, it appears that choosing the correct dose is equally as crucial.

It has been hypothesised that treatment with immunosuppressive drugs would be necessary after organ transplantation in order to create a favourable environment for MSCs to survive long enough to become activated and, thus, achieve their effect (English et al., 2010). However, as
reported here (see Chapter 4) and by others, MSCs have been successfully infused without accompanying immunosuppression in a number of animal models. In a murine semi-allogeneic heart transplant model, pre-transplant infusion of MSCs alone was shown to prolong graft survival (Casiraghi et al., 2008). Another study showed that administration of MSCs alone could prolong the survival of fully allogeneic islet grafts leading to stable, long-term normoglycaemia and that this affect was mediated by MSC-derived MMP-2 and 9 reducing the expression of CD25 on responding T cells (Ding et al., 2009). In contrast, however, Sbano and co-workers reported the accelerated rejection of skin allografts after administration of donor-derived MSCs (Sbano et al., 2008). What these studies show is that what may work for one disease model may have either no or an adverse effect in another model and that a more complete understanding of the system in question is needed to ensure that MSC therapy, with or without a period of immunosuppression, can be tailored to best suit the model under investigation.

While it was previously suggested that MSCs were immune-privileged (at least to some degree), more recent studies suggest that this is probably not the case and non-self MSCs (e.g. allogeneic, third-party) have immunogenic properties. To date, several studies have examined anti-donor immune responses to allo-MSCs with varying levels of stringency (for comprehensive reviews see English et al., 2010, Griffin et al., 2013a, Kean et al., 2013).

When considering the potential immunogenicity of allo-MSCs, a keenly debated topic is that of in vivo persistence of the cells following administration. While a number of groups have investigated this subject, the results have been quite diverse. Some have found that allo-MSCs can persist for 4 weeks or longer. Examples of which include intramuscular injections of allo-MSCs into healthy baboons (Beggs et al., 2006), intrastratial administration of allo-MSCs in a rat model of Parkinson’s disease (Camp et al., 2009) and intradermal injection of allo-MSCs in a mouse skin wound model (Chen et al., 2009). In contrast, others have found that allo-MSCs have reduced survival compared, for example, to autologous MSCs.
Examples include reports by Eliopoulos and colleagues (2005) where mice received subcutaneous implants of allo-MSCs and by Zangi and colleagues (2009) where mouse luciferase-labelled (Luc+) allo-MSCs survived for a significantly shorter period of time compared to Luc+ syn-MSCs. While not involving allo-MSCs, an interesting recent study by Eggenhofer and co-workers investigating the migration and viability of intravenously injected syn-MSCs (DsRed-C57BL/6 MSCs injected into WT C57BL/6 recipient mice) found, as we (Figure 4.10) and others did, that the majority of cells migrate to the lungs after administration. However, when the cells were isolated from lung tissue and put back into culture (to assess viability), the researchers found that MSCs only remained viable when harvested up to the 24 h time-point, after which no viable cells could be detected (Eggenhofer et al., 2012). The wide range of outcomes from such studies as detailed above suggests that the route of administration of the MSCs (even when immune recognition should not be a concern as in the case of syn-MSCs) may have an important role to play in determining whether allo-MSCs are susceptible to early immunological recognition and, subsequently, how long the cells persist in vivo. It should also be taken into account that studies investigating the in vivo persistence of allo-MSCs frequently involve labelling the cells with a fluorescent dye and that subsequent analysis based solely on fluorescence detection may actually be coming from recipient APCs expressing fluorescent peptides derived from dead MSCs.

While a general consensus has yet to be reached with regard to how long allo-MSCs persist in vivo, numerous studies have demonstrated active anti-donor immune responses to the allogeneic cells under a variety of experimental conditions and which could conceivably be elicited regardless of whether the cells are cleared quickly or persist for a prolonged period of time. These include reports in both healthy mice (Eliopoulos et al., 2005, Nauta et al., 2006a, Badillo et al., 2007) and mice receiving bone marrow transplantation (Nauta et al., 2006b, Zangi et al., 2009), as well as in healthy rats (Schu et al., 2012) and models of rat localised brain injury and kidney
transplantation (Camp et al., 2009, Seifert et al., 2012, respectively). Anti-donor immune responses have also been reported in larger animal models including a porcine myocardial infarction model (Poncelet et al., 2007) and in healthy rhesus macaques (Isakova et al., 2010). However, studies have been published showing that other potential outcomes are possible following in vivo administration of allo-MSCs. For example, Beggs and colleagues (2006) reported a reduction in anti-donor T cell responses following intra-muscular injection of allo-MSCs in healthy baboons that had received a previous injection of allo-MSCs intravenously. Moreover, another study showed that, in contrast to allogeneic fibroblasts, allo-MSCs did not evoke an immune response in a mouse wound repair model (Chen et al., 2009). Therefore, in certain situations, allo-MSCs have the ability to suppress allo-antigen-induced cellular immune responses. How allo-MSCs achieve this, while far from fully understood, may be explained by mechanisms such as induction of Tregs, T cell anergy, clonal deletion or Th phenotype deviation (Lechler et al., 2003, Griffin et al., 2013a).

Taken together, the published literature as a whole suggests that antigens derived from allo-MSCs are actively presented to recipient T cells, regardless of the route by which the cells have been delivered (i.e. systemic or local) and most likely by the indirect pathway of antigen presentation (see Figure 1.2) (Griffin et al., 2013a). While a better understanding of the subsequent anti-donor T cell responses is required, as outlined in the studies above, these would seem to include different T cell effector functions with either destructive or suppressive potential following secondary encounter with allo-antigen (Griffin et al., 2013a).

5.1 Critical analysis of the research hypotheses

This research project explored two separate but closely related questions. Firstly, what effect(s) genetic modification using recombinant adenovirus has on the immune-profile of MSCs both in vitro, by analysing expression levels
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of immunologically relevant molecules such as chemokines, chemokine receptors, toll-like receptors and pro-inflammatory cytokines before and after Ad-transduction and in vivo, by investigating if Ad-transduced MSCs invoke an immune response following intravenous administration. This was an important issue as it was hypothesised that MSCs would need to be genetically modified to overexpress therapeutic molecules in order to have enhanced therapeutic benefit when applied to the corneal transplantation model. The second question dealt with whether or not MSCs either un-modified or, if required, genetically modified, could attenuate corneal allograft rejection in a rat model of cornea transplantation.

The first part of this project investigated what effects Ad-transduction of MSCs had on their gene expression levels of immunologically-relevant molecules. While MSCs have been shown to be able to migrate to damaged or diseased tissues upon systemic administration, the mechanisms underlying this migratory capacity remain to be fully elucidated. It is well established that chemokine receptors and their ligands play a pivotal role in leukocyte recruitment to sites of injury and infection and has therefore been hypothesised that these molecules may also have an important role to play in the homing of MSCs. In a therapeutic setting, as any alteration to chemokine/chemokine receptor gene expression upon viral modification could impair the migratory ability of MSCs in vivo, it was important to investigate chemokine/chemokine receptor mRNA expression levels before and after Ad-transduction. A panel of chemokines and chemokine receptors were chosen that included members from each of the main classification groups, namely, CXC, CC and CX3C. The results indicated that, in terms of mRNA expression, little variation exists between Ad.GFP transduced and untransduced MSCs. The other main aim of this part of the project was to determine if Ad-transduction altered MSC-TLR expression levels. As TLRs are known to regulate innate immune responses after Ad-mediated gene transfer and TLR activation on MSCs plays a role in their proliferation and differentiation ability (Pevsner-Fischer et al., 2006), it was equally important
to elucidate whether or not Ad-transduction alters TLR expression on MSCs. Similar to the results obtained on chemokine/chemokine receptor mRNA expression, TLR mRNA expression levels were almost unchanged after Ad.GFP transduction, compared to their untransduced counterparts. Moreover, Ad-transduction had no significant effect on the expression levels of TLR-associated cytokines by MSCs, such as IL-1β and IL-6. As future preclinical and clinical studies may require the use of Ad-transduced MSCs to further enhance their therapeutic potential, it is extremely important to understand how adenoviral transduction affects the expression of such molecules investigated here by MSCs. Collectively, the data shown in this study contributes significantly to the existing body of literature related to TLR and chemokine/chemokine receptor expression by MSCs and, to the best of the author’s knowledge, reports for the first time on how genetic modification with adenovirus can effect expression levels of these immunologically-relevant molecules. However, it is also important to acknowledge that a more comprehensive analysis would allow for the detection of subtle changes, for example, at the level of chemokine/chemokine receptor and TLR protein expression but was beyond the scope of this project.

The second part of this project investigated the effects of MSC therapy on the prolongation of rat corneal allograft survival. MSCs from three different strains of rat, namely, recipient-derived, donor-derived and a third party source were comprehensively examined in parallel. The data generated in the early part of this study indicated that both wild-type allogeneic and third party MSCs were capable of prolonging corneal allograft survival without the need for genetic modification. For this reason, it was deemed unnecessary to continue any further with genetically modifying the MSCs to overexpress therapeutic molecules of interest. However, as shown in detail in Chapter 4 of this thesis, recipient-derived MSCs were unable to replicate this therapeutic effect and, as a result, may be candidates for genetic modification in the future. As outlined in the discussion above, allo-MSCs are not immune-privileged as once thought and can evoke anti-donor immune
responses. While some transplantation experiments suggest that immune recognition of MSC-derived allo-antigens may include suppressive components, others describe a more destructive outcome (English et al., 2010). In these situations, it may be more favourable to genetically modify syn-MSCs to overexpress therapeutically beneficial molecules and thereby circumvent potential problems associated with immune recognition of allo-MSCs. For this reason, future studies investigating the application of genetically modified syn-MSCs in other transplant models are certainly warranted.

An area of particular importance in relation to manipulating syn-MSCs to potentially enhance their immunosuppressive and/or therapeutic potential is the concept of pre-activation or licensing of the cells via pre-treatment with pro-inflammatory cytokines. While the data generated in this project did not show an increased benefit of IFN-γ-treated syn-MSCs over their un-manipulated counterparts (i.e. the average time-point of rejection was similar between the two groups), it is important to point out only one parameter was investigated. That is, syn-MSCs were treated with IFN-γ at a concentration of 500U/ml for 72 h prior to intravenous administration to recipient rats. The constraints of time did not afford the opportunity to modify this pre-treatment protocol but, given that a number of studies have reported that MSC immunosuppressive properties are enhanced by pre-treatment with inflammatory mediators (Polchert et al., 2008, Duijvestein et al., 2011), it would be important to explore this concept in more detail. Potential options to modify this protocol could include adding other pro-inflammatory cytokines, such as TNF-α and/or IL-1β along with IFN-γ, increasing the length of time the MSCs are exposed to the inflammatory mediators and increasing the concentration of the cytokines from 500U/ml to 750 or 1000U/ml.

Collectively, the body of data generated in the second part of this project clearly show that allo-MSCs and, to the best of the author’s knowledge for the first time, third party MSCs, can prolong rat corneal allograft survival. However, the majority of experimental read-outs were observational in nature
and, from a translational viewpoint, more data pertaining to the mechanism of action of both allo- and third party MSCs would be desirable. One way to probe potential mechanism would be to carry out *in vitro* ‘re-call’ assays for T cell responses to donor-derived allo-antigen after MSC administration. Another possibility could be to perform *in vivo* donor antigen re-challenge experiments, where allo-MSC treated corneal allograft recipients are re-challenged with allogeneic cells or tissue derived from the same donor. For example, this could be in the form of skin transplantation, repeat allo-MSC administration or systemic administration of fluorescently-labelled splenocytes (Griffin et al., 2013a). The outcome from such an experiment (i.e. the duration of survival of a secondary transplant from the same donor strain as compared to transplant survival in naïve recipients) would generate important information with regard to the overall nature of the donor-specific immune responses induced by the primary injection of allo-MSCs. Would the secondary grafts be subjected to accelerated rejection or would they survive indefinitely, indicating tolerance? It would also be important to test the specificity of such responses by monitoring survival of secondary grafts from third-party donors (Griffin et al., 2013a).
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Appendices
Appendices

I. Conference presentations

**Oral presentation**

May 2012  “Mesenchymal stem cell therapy prolongs corneal allograft survival in rats”. Association for Research in Vision and Ophthalmology (ARVO) 2012, Ft. Lauderdale, Florida, USA.

**Poster presentations**

September 2011 “Genetic modification of mesenchymal stem cells with recombinant adenovirus does not alter their immunomodulatory properties”. Irish Society for Immunology (ISI) Annual Conference, NUI, Galway.

October 2011 “Genetic modification of mesenchymal stem cells with recombinant adenovirus does not alter their immunomodulatory properties”. European Society for Gene and Cell Therapy (ESGCT) 2011, Brighton, UK.

July 2012  “Mesenchymal stem cell therapy prolongs corneal allograft survival in rats”. MSC Galway 2012, NUI, Galway.

November 2012 “Mesenchymal stem cell therapy prolongs corneal allograft survival in rats”. Irish Cytometry Society Annual Conference, NUI, Galway.

August 2013 “Mesenchymal stem cell therapy prolongs corneal allograft survival in rats”. International Congress of Immunology (ICI) 2013, Milan, Italy, 22-27th August 2013.
II. Publications by the author


