IMMUNOGENICITY OF ALLOGENEIC MESENCHYMAL STEM CELLS

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By

Senthilkumar Alagesan M.Sc

Immunology and Transplant Biology Group,
Regenerative Medicine Institute,
National Centre for Biomedical Engineering Science, Bioscience Building,
National University of Ireland, Galway.

Thesis Supervisor: Professor. **Matthew D. Griffin**

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ABSTRACT

Allogeneic mesenchymal stem cells (allo-MSC) are a promising “off-the-shelf” therapy and are often cited as failing to elicit anti-donor immune responses. Intra-muscular (IM) administration may be the optimal route for allo-MSC therapy in heart disease and limb ischemia.

In the project described in this thesis, anti-donor cellular and humoral immune responses were compared in fully MHC-mismatched mice following single or repeated IM injections of allo-MSC. As positive and negative controls, additional groups of mice received IM injections of allogeneic splenocytes (allo-splenocyte), of syngeneic MSC (syn-MSC) or of vehicle alone. A panel of assay techniques was developed to assess anti-donor immune responses in secondary lymphoid organs including multi-colour flow cytometry panels, dendritic cell (DC)-stimulated mixed lymphocyte reactions (MLRs), cytotoxicity assays and assays of donor-specific IgG antibody.

Analyses of anti-donor T cell responses 1 week following the final IM injections demonstrated donor-specific hypo-responsiveness in recipients of repeated (but not single) injections of allo-MSC. In contrast, repeated allo-splenocyte injection was associated with enhanced anti-donor T cell responses. The donor-specific hypo-responsiveness associated with repeated IM allo-MSC was present in both $CD4^+$ and $CD8^+$ T cell compartments. It was not accompanied by expansion of $CD4^+/FOXP3^+$ regulatory T cells but was associated with increased production of interferon gamma and interleukin 10 in donor antigen-stimulated MLRs as well as with increased numbers of $CD8^+/CD11c^+$ T cells and alterations to the myeloid and natural killer cell/natural kill T cell compartments in the spleen.

Analyses of humoral (B cell) responses 1 week following the final injections revealed that recipients of single and repeated IM injections of allo-MSC consistently developed anti-donor IgG antibodies. The donor-specific IgG antibodies of allo-MSC recipients were of similar titres to those observed in recipients of allo-splencoyte injections but had higher ratio of IgG1 to IgG2a isotype. Nonetheless, anti-donor IgG induced by single or repeated IM injection of allo-MSC supported complement-mediated lysis of donor cells, indicating potential to mediate rejection *in vivo*. In additional experiments involving mice with induced hind limb ischaemia, it was shown that the development

anti-donor IgG following IM injection of allo-MSC could be prevented by a short course of the T cell-specific immunosuppressive drug tacrolimus.

Taken together, the results from this project provide new insights into the immunogenicity of allo-MSC delivered by the IM route on a single or multiple occasions. Specifically, it was found that IM injection of allo-MSC consistently induced donor-specific T cell and B cell (antibody) immune responses – likely via indirect alloantigen presentation. Importantly, repeated IM administration of allo-MSC resulted in the emergence of donor-specific T cell hypo-responsiveness which we propose may reflect a multi-faceted anti-inflammatory response to MSC-delivered donor antigen that is dependent on an initial "priming" exposure. Also of importance to future clinical application, IM injection of allo-MSC proved to be a potent inducer of IgG1-predominant anti-donor antibody with potential to mediate donor cell lysis. The findings highlight the fact that allo-MSC are, in fact, inherently immunogenic when delivered intramuscularly and that harnessing their tolerogenic potential may, paradoxically, require an initial sensitising phase. These complex interactions between allo-MSC and the recipient immune system should be considered and studied in more detail in human participants in clinical trials involving Allo-MSC administration.

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In memory of my brother

Ajay Alagesan

&

Dedicated to

My parents

ABBREVATIONS

ADCC	Antibody-dependent cell mediated cytotoxicity
Allo	Allogeneic
ANOVA	Analysis of variance
APC	Antigen-presenting cells
BD	Becton Dickinson
BM	Bone marrow
BMCs	Bone marrow cells
BSA	Bovine serum albumin
B6	C57BL/6
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
CEM	Complete expansion media
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFU-f	Colony forming-unit fibroblast
CIM	Complete isolation media
CLI	Critical limb ischaemia
CsA	Cyclosporine
DCs	Dendritic cell
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
Epo	Erythropoietin
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FK506	Tacrolimus
FKBP	FK506-binding proteins
G	Gauge
HSC	Hematopoietic stem cell
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
Ig	Immunoglobulin
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescence protein
GvHD	Graft versus host disease
H&E	Hematoxylin and Eosin
HIF1-α	Hypoxia inducible factor 1 alpha
HLI	Hind limb ischaemia
hMSC	Human mesenchymal stem cells
IHC	Immunohistochemistry
IDO	Indoleamine-2,3-dioxygenase
iTreg	Induced T regulatory cell
IFN-γ	Interferon gamma
IL	Interleukin
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin -1β
ISCT	International Society for Cellular Therapy
LPS	Lipolysaccharide
L-NAME	L-NG-nitroarginine methyl ester
LN	Lymph nodes
ml	Milliliters
MAPCs	Multipotent adult progenitor cells
MCP-1	Monocyte chemotactic protein 1

MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMPs	Matrix metallopreteinase
mMSC	Mouse mesenchymal stem cells
MNCs	Mononuclear cells
MPCs	Mesodermal Progenitor Cells
MSCs	Mesenchymal stromal/stem cells
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NK	Natural killer cells
NKT	Natural killer T cells
OVA	Ovalbumin
P	Passage
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E2
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PI	Propidium iodie
REMEDI	Regenerative Medicine Institute
RT	Room temperature
Sca-1	Stem cell antigen-1
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
Syn	Syngeneic
TAC	Tacrolimus
TCR	T-cell receptor
Th1	T-helper 1 cells
Th2	T-helper 2 cells
Th17	T-helper 17 cells
T-cells	T-lymphocytes
TSG-6	TNF-alpha-induced protein 6
TGF-β	Transforming growth factor-beta
Treg	T regulatory cell
TNF-α	Tumor necrosis factor alpha
VEGF	*Vascular endothelial growth factor*
Xeno	Xenogeneic

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CHAPTER – 1

INTRODUCTION: THE IMMUNE MODULATORY AND IMMUNOGENIC PROPERTIES OF MESENCHYMAL STEM CELLS

1.1 BASIC FEATURES AND IMMUNO-BIOLOGY OF MESENCHYMAL STEM CELLS (MSC)

1.1.1 Identification of MSC:

Mesenchymal stem (or stromal) cells (MSC) were first identified and isolated from bone marrow by Friedenstein and his colleagues in 1970. (Friedenstein *et al.,* 1966, Friedenstein *et al.,* 1968 and Friedenstein *et al.,* 1970) These cells were initially referred to simply as stromal cells but are now popularly referred to as MSC (a term that was first coined by Caplan in 1991). Initially, Friedenstein identified MSC as plastic adherent cells that are capable of forming colonies *in vitro* and also capable of differentiating into osteocytes. (Friedenstein *et al.,* 1966 and Friedenstein *et al.,* 1970) Interestingly, the osteogenic potential of bone marrow cells was reported in the 20th century without knowledge of the biological role of stromal cells in the bone marrow compartment (Goujon 1869 and Tavassoli and Crosby, 1968). Only much later, beginning in the in 1960s did Friedenstein first isolate this sub-population of bone marrow cells and identify their basic characteristics as follows:

1) Non-haematopoietic
2) Plastic adherent
3) Fibroblast-like appearance
4) Capable of forming colonies in culture
5) Capable of differentiating into osteocytes.

Later still, in the 1980s, the differentiation capacity of MSC was studied in more detail and found to include multiple lineages - osteocytes, chondrocytes and adipocytes (Piersma *et al.,* 1985 and Caplan *et al.,* 1986). Interestingly, Wakitani *et al.,* 1995 showed that MSC can also be differentiated into cells of a myogenic phenotypes implicating MSC in a wider range of tissue regenerative functions. Subsequently, however, controversy has arisen regarding the true plasticity of MSC as progenitor cells (Horwitz *et al.,* 2005 and Dominici *et al.,* 2006). Thus, while their capacity to generate osteocytes, chondrocytes and adipocytes under specific culture conditions is now well accepted, other stem cell-like features such as broader multi-potency and self-renewal *in vivo* remain in doubt (**Figure**

1-1). (Wagers *et al.*, 2002 and Bianco, 2007). For this reason, some have favoured the term "mesenchymal stromal cell". Nonetheless, the term "mesenchymal stem cell" has gained global familiarity because of the ground-breaking studies that have confirmed their tri-lineage differentiating potential coupled with the capacity to form colonies and expand *in vitro*. (Pittinger *et al.*, 1999, Paunescu *et al.*, 2007, Akiyama *et al.*,2012 and Tan *et al.*, 2013)

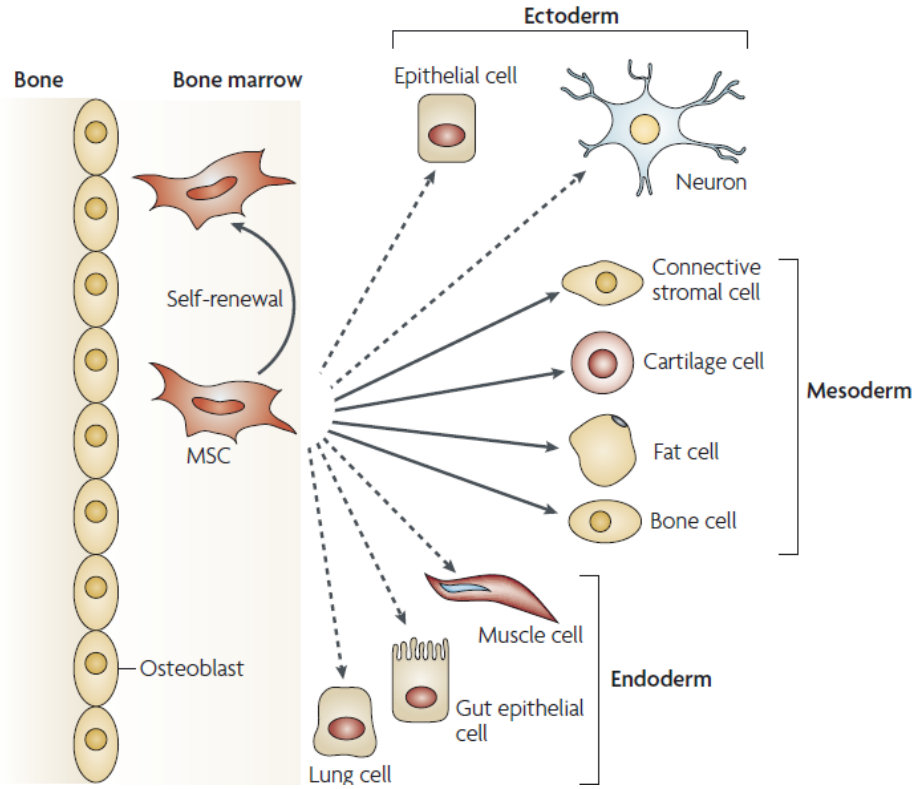


Figure 1-1: The accepted (solid arrows) and putative (dashed arrows) differentiation potential of bone marrow-resident mesenchymal stem cells (MSC (Adopted from Uccelli *et al.*, 2008)

1.1.2 Definition and types of MSC:

Initially, attempts to define MSC were made mainly by exploring colonies from CFU-fibroblast cultures and stromal cells from the bone marrow (Friedenstein *et al.*, 1970 and Sacchetti *et al.*, 2007). Subsequently, isolation of CD146-expressing cells from bone marrow showed that such cells possessed MSC-like properties including clonogenicity and multi-potency suggesting that specific surface markers can be used to define MSC (Sacchetti *et al.*, 2007 and Serafini *et al.*, 2014). Furthermore, as pericytes are also known to express CD146 and MSC-like cells were found to be

present in a range of other tissues besides bone marrow, the concept arose that "*all MSC are pericytes, but not all pericytes are MSC*" (Caplan 2008). Widely-cited studies by da Silva *et al.,* 2008 and Crisan *et al.,* 2008 provided additional experimental evidence that culture-expanded MSC from a range of tissues predominantly derive from pericytic cells. Currently, it is most common for MSC to be defined on the basis of cell surface markers and/or by certain *in vitro* assays. Thus far, however, no unique cell surface marker has been identified which can be used to distinguish MSC from haematopoietic and other cell types present in their tissues of origin. For clinical purposes, The International Society for Cell Therapy has proposed the following criteria to define MSC (Dominici *et al.,* 2006):

1) Plastic adherent in standard culture conditions.
2) More than 95% of cells express CD105, CD73 and CD90 and less than 2% of cells express CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR on the surface.
3) Capable of differentiating into osteocytes, adipocytes and chondrocytes in *in vitro* differentiation assays.

Importantly, these criteria were set to establish the identification of human MSC and do not consistently apply to other species (Peister *et al.,* 2004). Also, when MSC are expanded under standard tissue culture conditions, their cell surface marker patterns may vary, resulting in loss of expression of one or more "specific" MSC markers and upregulation of additional cell surface proteins (Jones *et al.,* 2002). To date, human MSC meeting the ISCT criteria have been successfully isolated and characterised from bone marrow, adipose tissue, umbilical cord, blood, placenta and gingival and synovial tissues (Pittenger *et al.,* 1999, Bieback *et al.,* 2004, Cao & Dong., 2005 and Fraser *et al.,* 2006).

Interestingly, in mice, MSC-like cells have also been isolated from thymus, spleen, lymph node, brain, liver and kidney (among others) suggesting that stromal cells with progenitor capacity may, in fact, be present in most or all postnatal organs (da Silva *et al.,* 2006). Nonetheless, for the present, bone marrow remains the major source of MSC for clinical and experimental purposes. In addition, extensive investigation of the micro-anatomic localisation and physiological properties of MSC in the

bone marrow have revealed that they have unique roles in this tissue which include providing a supportive "niche" for haematopoietic stem cells and serving as progenitors for bone and cartilage growth and repair (**Figure 1-2**). (Prockop, 1997, Mendez-Ferrer *et al.*, 2010 and Frenette *et al.*, 2013).

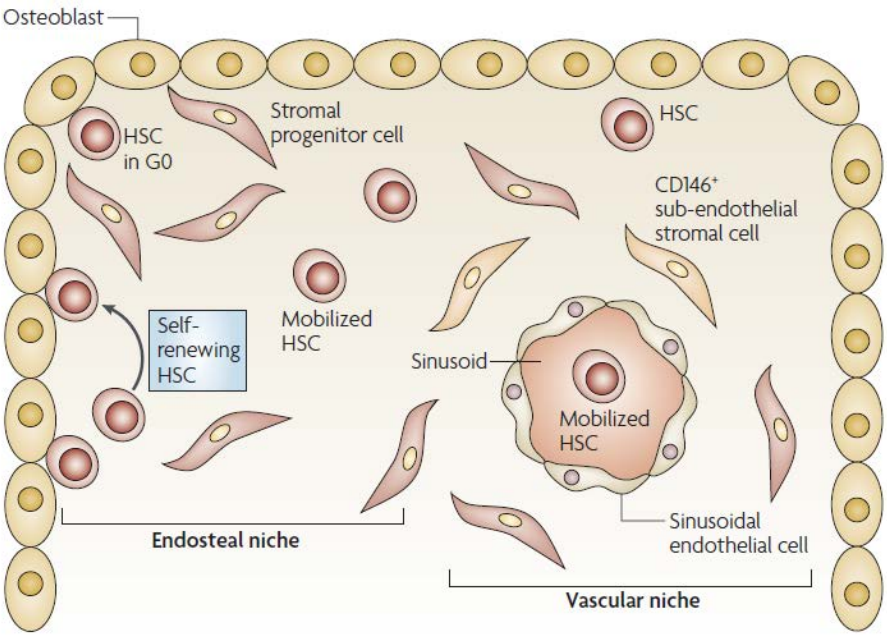


Figure 1-2: Micro-anatomic localisation and biological roles of MSC in the bone marrow (Adopted from Uccelli A *et al.*, 2008)

From a technical perspective, MSC are generally isolated by density gradient centrifugation of cell suspensions from bone marrow or other tissues, followed by culture of the resulting nucleated cells in nutrient medium. The subsequent removal of non-plastic adherent cells allows for outgrowth of fibroblast-like adherent cells which may then be characterized for the MSC-associated phenotype and functions (Pittinger *et al.*, 1999, Lennon *et al.*, 2006 and Akiyama *et al.*, 2012). In other approaches, a subset of mononuclear cells have been isolated based on the expression of more recently identified primary stromal cell markers such as CD271 and Stro-3 – a process sometimes referred to as "prospective isolation" (Hamamoto *et al.*, 2009 and Jones *et al.*, 2010, Kuci *et al.*, 2010). As previously noted, MSC isolated from various tissues of a range of different species have been shown to have species-specific characteristics while maintaining most of the same MSC-like markers and

functions as their human counterparts (Nombela-Arrieta & Ritz , Silberstein , 2011). Overall, there remain significant unanswered questions regarding the definition of MSC and their potential for phenotypic and functional heterogeneity following conventional isolation procedures. Thus, there is a strong need for future research to fully characterize human MSC populations, to further elucidate the differences that exist between MSC from different tissues and species and to better understand MSC multi-potency and self-renewing properties *in vivo* and *in vitro*.

1.1.3 Immunological properties of MSC:

Mesenchymal stem cells have gained a significant amount of clinical attention not only because of their differentiation potential (Barry and Murphy, 2004), but also because of their immune-modulatory properties (Uccelli *et al.,* 2008, Abumaree *et al.,* 2012, De Miguel *et al.,* 2012 and Ma *et al.,* 2014). Furthermore, the capacity of MSC to directly suppress a range of different innate and adaptive immune effectors has led to a widespread belief that they may be administered across the allogeneic barrier without being subject to rejection responses. This concept of low MSC immunogenicity (or "immune privileged" status) has been proposed as a basis for the use of allogeneic MSC (allo-MSC) as therapeutic agent for a broad range of inflammatory diseases (Devine *et al.,* 2000, Majumdar *et al.,* 2003 and Tse *et al.,* 2003) and derives from the following three well-accepted features of culture-expanded MSC:

- Low surface levels of major histocompatibility complex (MHC) proteins.
- Limited or absent surface expression of co-stimulatory ligands for T-cells and other lymphocytes.
- Suppressive activities against both innate and adaptive immune cells.

In the resting state, MSC from human and several other species have been consistently shown to express low surface levels of class I MHC (MHC-I) proteins along with no or minimal surface levels of class II MHC (MHC-II) and co-stimulatory ligands such as CD80 and CD86 (Nicola *et al.,* 2002 , Tse *et al.,* 2003 and Di & Ryan *et al.,* 2005). However, as MSC are often expected to mediate

therapeutic effects in an inflamed environment, the influence of pre-treating MSC with pro-inflammatory cytokines has been the subject of many research studies. In particular, the role of interferon gamma (IFN-γ) to activate MSC has been widely highlighted. It has been known for some time that IFN-γ stimulates MSC surface expression of both MHC-I and MHC-II (Ljunggren and Anderson., 1998), however, as reported by Tse *et al.,* 2003 and Klyushnenkova *et al.,* 2005, this is not accompanied by induction of co-stimulatory ligands. In line with this other pro-inflammatory cytokines such as TNF-α and IL-1β have also been showed to activate/induce the immune-modulatory properties of MSC. (Krampera, 2011, Kwon *et al.,* 2013 and Chen *et al.,* 2015) Furthermore, as discussed in more detail below, MSC exert potent suppressive effects on a wide range of immunological cells through multiple surface-bound and soluble mediators and many of these immune-modulatory mechanisms are further enhanced by MSC exposure to IFN-γ and other inflammatory cytokines – a phenomenon often referred to as "licensing". (Krampera *et al.,* 2006, Sheng *et al.,* 2008, Krampera, 2011, Kwon *et al.,* 2013 and Chen *et al.,* 2015)

Although not the central focus of this thesis, it is important to highlight that MSC also possess non-immunological properties which further enhance their potential for therapeutic application in a variety of diseases. Work by Crisan *et al.* and others proposed that MSC are naturally found in the body as pericytes (as discussed above) in highly vascularised tissues and that their main role in physiological condition is to maintain the blood, tissue and immune cell homeostasis (Bianco *et al.,*2001, Caplan., 2008, Crisan *et al.,*2008 and da Silva Meirelles *et al.,*2008) During an episode of injury or tissue damage, MSC/pericytes may be activated at the site of injury, where they mediate modulatory effects and secrete trophic factors that inhibit apoptosis and fibrosis and stimulate angiogenesis and mitosis of tissue specific progenitors. By this concept, MSC are not only dominant in modulating or suppressing immune cells, they also have a broader role to play in tissue repair and regeneration (Caplan & Dennis., 2006, da Silva Meirelles *et al.,* 2008 and Murphy *et al.,* 2013). In keeping with this, studies of ischemic injury in animal s have reported that local or systemic administration of MSC promotes neo-vascularization through the secretion of angiogenic factors. (Iwase *et al.,* 2005 and Lu *et al.,* 2011)

1.1.4 Immune-modulatory properties of MSC:

Because of the inherent immune-modulatory properties of MSC, many of their putative therapeutic disease targets share the common feature of maladaptive inflammation and/or autoimmune response (Uccelli *et al.,* 2008, Ghannam *et al.,* 2010 and Griffin *et al.,* 2010). The interactions of MSC with a variety of different immune cells and the mechanisms whereby the activation and effector functions of these target cells occur has been the subject of intense research interest for over a decade (Singer & Caplan, 2011). Following some early controversies arising from the results of *in vitro* experiments, it is now well accepted that MSC modulate cellular immune and inflammatory responses by both cell contact-dependent and soluble mechanisms (Krampera *et al.,* 2006, Chavannes et *al.,* 2007, Sato *et al.,* 2007 and Ren *et al.,* 2008). In reality, it now appears likely that MSC-mediated immune-modulation *in vivo* occurs through a combination of contact-dependent and soluble mechanisms as part of a "cross-talk" between MSC and immune cells. (Shi *et al.,* 2012, Kovach *et al.,* 2015 and Vacca *et al.,* 2015) The final mediators of immune-modulation following such cross-talk are generally considered to be soluble mediators including prostaglandin E2, the enzymatic products of idoleamine 2,3-dioxygenase (IDO), TNF-α stimulated gene/protein (TSG-6), nitric oxide (NO) and interleukin 10 (IL-10) (English., 2012). The *in vivo* therapeutic effects of MSC in a disease setting may be further promoted by their ability to specifically migrate to sites of active inflammatory activity, by their production of chemokines that attract immune cells toward them for closer interactions and, as discussed previously, by their susceptibility to "licensing" by pro-inflammatory cytokines (Ren *et al.,* 2008, Lee *et al.,* 2010 and Roddy *et al.,* 2011). Although many of the mechanistic aspects of MSC immune-modulation have been elucidated in highly artificial *in vitro* experimental systems, many of them have also been validated in clinically-relevant animal models (Griffin *et al.,* 2010).

1.1.5 Effects of MSC on immune cells:

A key breakthrough in the translational potential of MSC was the demonstration that culture-expanded MSC potentially suppress T-cell proliferation following mitogenic stimulation (Di Nicola *et al.,* 2002 and Bartholomew *et al.,* 2002). This observation opened the door to many research studies focussed on the diverse effects of MSC on cellular effectors of both the innate and adaptive arms of

the immune system (Uccelli *et al.*, 2008). In the following sections, current knowledge of the effects of MSC on specific immune cell type and some of the key mechanisms involved are described in more detail.

1.1.5.1 Effects of MSC on innate immune cells:

Macrophages: Tissue macrophages are the first responders to almost any type of localised infection or injury being both an early source of pro-inflammatory cytokines and chemokines as well as a direct effector of anti-microbial phagocytosis and killing (Akira S *et al.*, 2006). There is a large body of research to support the importance of MSC/macrophage interactions for MSC-mediated anti-inflammatory effects and this has been studied in a range of animal models of inflammatory disease (Gupta *et al.*, 2007, Chen *et al.*, 2008, Gonzalez *et al.*, 2009, Nemeth *et al.*, 2009, Maggini *et al.*, 2010 and Yagi *et al.*, 2010). Studies of macrophage interactions have been performed with MSC from various tissue sources and from multiple species including human (Kim *et al.*, 2009, Bartosh *et al.*, 2010 and Zhang *et al.*, 2010). Typically, the experimental systems used have involved the stimulation of macrophages with pathogen associated molecular patterns (PAMPS, such as the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) or ligands for TLR2) or the sourcing of macrophages from inflamed tissues of diseased animals. Such studies have confirmed that MSC are capable of mediating potent modulatory effect on the pro-inflammatory responses of macrophages and that the modulation is mediated through secreted factors following a complex cross-talk between the two cell types (Gupta *et al.*, 2007, Nemeth *et al.*, 2009, Bartosh *et al.*, 2010 and Miller *et al.*, 2010). Interestingly, in a zymosan-induced mouse model of colitis, Cho *et al.*, 2011 have illustrated the importance of an initial contact-dependent mechanism involving CD44 for MSC suppression of inflammatory macrophages. Nonetheless, many studies have focussed on identifying the soluble mediators that are produced by MSC and exert a regulatory effect on macrophages. Among those that have been reported are interleukin 1 receptor antagonist (IL1RN), tumour necrosis factor receptor 1 (TNFR1), PGE2 and myristoylated alanine-rich C-kinase substrate (MARCKS). For example, Ortiz *et al.*, 2007 have shown that IL1RN is secreted by MSC and antagonizes IL-1α activity in silica/LPS activated

macrophages resulting in a subsequent block of TNF-α release. Similarly, MARCKS which is involved in immune cell motility is another secreted product of MSC that has been shown to enhance macrophage migration towards MSC in an *in vitro* assay system (Miller *et al.,* 2010). A large body of work has been carried out regarding the involvement of PGE2 in MSC mediated immune-suppression (Najar M *et al.,* 2010 and Hsu WT *et al.,* 2013). In a mouse model of sepsis in which macrophage interactions with intravenously administered (IV) MSC was shown to mediate protective effects, it was found that the inhibition of PGE2 prevented the modulation of macrophages toward an anti-inflammatory phenotype (Nemeth *et al.,* 2009). In acute cerebral ischaemia, Ohtaki *et al.,* 2008 demonstrated that MSC induce the neuroprotective gene Ym1 in microglia/macrophages leading to an increased T helper type 2 (Th2) cytokine profile.

Taken together, a number of *in vivo* studies have documented that local or systemic delivery of MSC results in accumulation/recruitment of macrophages followed by their re-programming toward an anti-inflammatory phenotype with regenerative and reparative effects (Gupta *et al.,* 2007, Xin *et al.,* 2007, Chen *et al.,* 2008, Nemeth *et al.,* 2009, Bartosh *et al.,* 2010 and Zhang *et al.,* 2010).

Dendritic cells: The interactions between MSC and dendritic cells (DC) have also been carefully studied though, perhaps, to a lesser extent than macrophages. Published reports to date have shown that MSC exert immune-modulatory effects on DC that target both their innate and adaptive immune functions. Studies of human MSC/DC interactions have, of necessaity, been limited to *in vitro* experiments (Beyth *et al.,* 2004, Jian *et al.,* 2005, Nauta *et al.,* 2006, Wahnon *et al.,* 2007, Li *et al.,* 2007, Todorova *et al.,* 2009 and Spaggiari *et al.,* 2009). Nevertheless, these studies have convincingly shown that MSC modulate human DC derived from $CD14^+$ monocytes as well as from $CD34^+$ haematopoietic progenitors and that the modulatory effects are dependent upon MSC production of soluble factors (Nauta *et al.,* 2006, Li *et al.,* 2007, Spaggiari *et al.,* 2009, Wehner *et al.,* 2009 and Kronsteiner *et al.,* 2011) and/or contact-dependent mechanisms (Beyth *et al.,* 2004 and Wahnon *et al.,* 2007).

Regarding the specific modulatory effects, it has been shown that human MSC interfere with the differentiation of monocytes to DC and also inhibit the maturation of DC under the influence of pro-

inflammatory stimuli – resulting in reduced potency for primary activation of naïve T-cells. The induction of DC secretion of PGE2 has also been identified as a key mechanism by which MSC modulate immune responses – in this case by altering the effector phenotype of DC-activated T-cells (Spaggiari *et al.,* 2009, Wehner *et al.,* 2009). Mechanistically, modulation of DC maturation and antigen presentation function has been demonstrated to be dependent upon intracellular signalling through STAT3 (Wahnon *et al.,* 2007) and the Notch pathway (Li *et al.,* 2007 and Cahill *et al.,* 2015). Among the phenotypic and functional features of MSC-modulated human DC are reduced surface expression of CD80, CD86, HLA-DR and CD1a, increased production of the anti-inflammatory cytokine IL-10, reduced production of IL-12 and other pro-inflammatory cytokines and increased capacity to induce the survival/expansion of regulatory T-cells (T-reg) (Jiang *et al.,* 2005, Li *et al.,* 2007, Todorova *et al.,* 2009 and Kronsteiner *et al.,* 2011).

Monocytes: Compared to macrophages and DC, there are relatively few published studies focussing on the interaction of MSC with monocytes. This is somewhat surprising given that monocytes are one of the major pro-inflammatory innate immune cells in the bloodstream and that they translocate rapidly to sites of tissue inflammation where they contribute to the local inflammatory environment and serve as precursors for additional macrophages and DC. (Shi *et al.,* 2011 and Yang *et al.,* 2014) Nonetheless, several *in vitro* studies have shown that MSC from bone marrow, adipose tissue and umbilical cord undergo complex interactions with primary monocytes in *in vitro* assays (Cutler *et al.,* 2010, Lee *et al.,* 2010 and Rocher *et al.,* 2012). Monocytes are highly chemotactic and MSC secrete factors such as monocyte chemotactic protein 1 (MCP1/CCL2) which strongly promote monocyte migration and modulation. (Molloy *et al.,* 2009, Boomsma *et al.,* 2012 and Kyurkchiev *et al.,* 2014) It is been documented that MSC-dependent modulation of monocytes is primarily mediated in a paracrine fashion (Groh *et al.,* 2005, Cutler *et al.,* 2010, Lee *et al.,* 2010 and Rocher *et al.,* 2012). Some of the relevant mediators including IL-6, IL-8 and MCP-1 were shown by Lee *et al.,* 2010 to be induced in MSC by the pro-inflammatory cytokine TNF-α. In addition, Cutler *et al.,* 2010 have shown that PGE2 secreted either by MSC or by monocytes mediates the inhibition of T-cell proliferation in *in vitro* assays. Under some conditions, MSC have also been known to secrete high levels of

transforming growth factor beta 1 (TGF-β1) resulting in suppression of T-cell activation by monocytes and in promotion of T-reg (Groh *et al.,* 2005 and Melief *et al.,* 2013). With the limited amount of studies performed to date, it remains unclear how important MSC/monocyte interactions are for the overall therapeutic anti-inflammatory effects of MSC in disease settings.

Neutrophils: Studies specifically addressing the interaction of MSC with neutrophils have been relatively few and have provided for both positive and negative effects. On the one hand, *in vitro* studies by Maqbool *et al.,* 2011 and Hsu *et al.,* 2013 reported that, MSC promote neutrophil viability and enhance the neutrophil phagocytic activity via IL-17 secretion (Maqbool *et al.,* 2011 and Hsu *et al.,* 2013). In other studies focussed on MSC immune modulatory properties, they were shown to mediate significant anti-apoptotic properties on both resting and activated neutrophils without influencing phagocytic and chemotactic functions (Raffaghello *et al.,* 2008). Recently, Brandau et al., 2014 also reported enhanced anti-microbial activity of neutrophils in the presence of MSC. (Brandau *et al.,* 2014). Other studies are more consistent with an inhibitory effect of MSC on neutrophil pro-inflammatory responses, as has been reported for other innate immune cell types (Ghannam *et al.,* 2010, Luu *et al.,* 2013 and Brandau *et al.,* 2014). For example, Luu *et al.,* 2013 reported that MSC cultured with endothelial cells are capable of downregulating the adhesion and subsequent migration of neutrophils (Luu *et al.,* 2013). Overall, the literature to date, while primarily limited to *in vitro* studies, suggests that MSC exert distinct modulatory effects on neutrophils that may blunt some of their pro-inflammatory responses while maintaining or even enhancing anti-microbial functions – a characteristic that could be beneficial in the context of MSC therapies for conditions in which infection risk is a significant concern.

Natural killer (NK) cells: Natural killer cells are best understood for their protective effects against viral infections and transformed/neoplastic cells (Brian CA, 1997, Wright *et al.,* 2010 and Mocsai *et al.,* 2013). The immunological functions of NK cells include direct cytolysis as well as production of inflammatory cytokines (Moretta, 2004). Several studies have investigated the interactions of NK cells with MSC (Sotiropoulou *et al.,* 2006 and Spaggiari *et al.,* 2006 & 2008). Among the observations reported are reduced NK cell expression of the activating receptors NKp30 and NKG2D

and inhibition of IL-2-induced NK cell activation and proliferation (Spaggiari *et al.,* 2006). The mediators of these inhibitory effects were shown to include TGF-β1, IDO and PGE2 (Sotiropoulou *et al.,* 2006 and Spaggiari *et al.,* 2006 & 2008). Clinical interest in understanding MSC interactions with NK cells and their immunological consequences has been increasing and some authors have proposed that there may be specific therapeutic benefits associated with these effects in transplantation and other disease settings (Benichou *et al.,* 2011 and Reinders & Hoogduijn., 2014). Of interest, MSC were shown to inhibit NK cell activation in blood samples taken from renal allograft recipients before and after transplantation (Crop *et al.,* 2009). Overall, however, the *in vivo* relevance of MSC modulation of NK cell function remains unclear.

1.1.5.2 Effects of MSC on T and B lymphocytes:

T-cells and T-cell subsets: In addition to their multiple effects on the innate immune system, MSC also exert well-described, potent suppressive effects on adaptive immune responses – in particular the T-cell compartment. This aspect of MSC immune-modulation was brought to attention by several research groups in a series of landmark studies in the early to med 2000s (Bartholomew *et al.,* 2002, Di Nicola *et al.,* 2002, Tse *et al.,* 2003, Zappia *et al.,* 2005, Krampera *et al.,* 2005 and Aggarwal *et al.,* 2005). In predominantly *in vitro* experiments, MSC were shown to interfere with multiple stages of T-cell activation, differentiation, proliferation and effector function such as cytokine production and cytolysis (Rasmusson *et al.,* 2003, Maccario *et al.,* 2005, Aggarwal *et al.,* 2005, Krampera *et al.,* 2005 and Quaedackers *et al.,* 2009). In regard to the polarisation of $CD4^+$ T-cells to the various T-helper (Th) phenotypes, MSC have been shown, in some but not studies, to re-program IFN-γ-producing Th1 cells to IL-4-producing Th2 cells in both *in vitro* and *in vivo* (Aggarwal *et al.,* 2005 and Zappia *et al.,* 2005). More recently, a suppressive effect of MSC on the pro-inflammatory Th17 differentiation pathway has been reported and has been shown to be mediated by PGE2 (Ghannam *et al.,* 2010 and Duffy *et al.,* 2011). The suppressive effects of MSC on T-cell activation and Th differentiation have now been documented for human, mouse and other experimental animal species in *in vitro* studies and, in many cases, have also been confirmed in disease model settings *in vivo* (Duffy *et al.,* 2011, Tobin *et al.,* 2013, Haddad & Saldanha-Araujo, 2014 and Chinnadurai *et al.,* 2014).

Regulatory T-cells are a subset of T-cells that prevent autoimmunity, counteract transplant rejection and suppress tissue inflammation both by cell contact-dependent mechanisms and by production of anti-inflammatory mediators (Sakaguchi *et al.,* 2008 & 2009, Peterson., 2012, Josefowicz et al., 2012 and Tang & Bluestone.,2013). Importantly, MSC have been reported to induce T-reg *in vitro* and *in vivo* (Aggarwal *et al.,* 2005, Maccario *et al.,* 2005Engela *et al.,* 2012 & 2013, Luz-Crawford *et al.,* 2013 Tang *et al.,* 2015, Liu *et al.,* 2015 and Cahill *et al.,* 2015), although the specific mechanisms reported to be responsible for this effect have varied from one study to the next and may be different across species. Among the candidate factors for T-reg induction are TGF-β1, hepatocyte growth factor (HGF), PGE2, IDO and iNOS. (Chabannes *et al.,* 2007, Jones *et al.,* 2007 Sato *et al.,* 2007 and Selmani *et al.,* 2008). Interestingly, a recent report from Cahill *et al.,* 2015 revealed a role for MSC-expressed jagged-1 in the expansion of regulatory T-cells, implicating Notch signalling in this process (Cahill *et al.,* 2015). Additionally, MSC may also indirectly promote the preferential expansion of T-reg during antigen-specific immune responses through their modulatory effects on DC (Choi *et al.,* 2012)

B-cells: The primary immune function of B-cells is the production of antibodies to eliminate foreign antigens/pathogens. Although less extensively investigated than T-cells, several co-culture studies have shown that MSC interact directly with B-cells and may inhibit their proliferation, activation and immunoglobulin G (IgG) (Corcione *et al.,* 2006, Comoli *et al.,* 2008, Rafei *et al.,* 2008, Asari *et al.,* 2009 and Schena *et al.,* 2010). As for other immune cells, the reported mechanisms underlying these effects include both soluble factors and cell contact-dependent mechanisms (Deng *et al.,* 2005, Glennie *et al.,* 2005, Augello *et al.,* 2005 and Corcione *et al.,* 2006, Franquesa *et al.,*2012 and Fan *et al.,*2016). Some of the mediators that have been identified as mediating these B-cell suppressive effects are IFN-γ, cleaved CCL2 and PD1/PDL1 interaction. (Rafei *et al.,* 2008, Schena *et al.,* 2010). Of interest, the development of regulatory B-cells (B-reg) has also recently been shown to be promoted by MSC. The induced B-reg were associated with high expression of IL-10 (Qin *et al.,* 2015). Importantly, as primary B-cell responses are typically dependent on help from $CD4^+$ T-cells, the previously described modulatory effects of MSC on DC and T-cells are also likely to play a role in

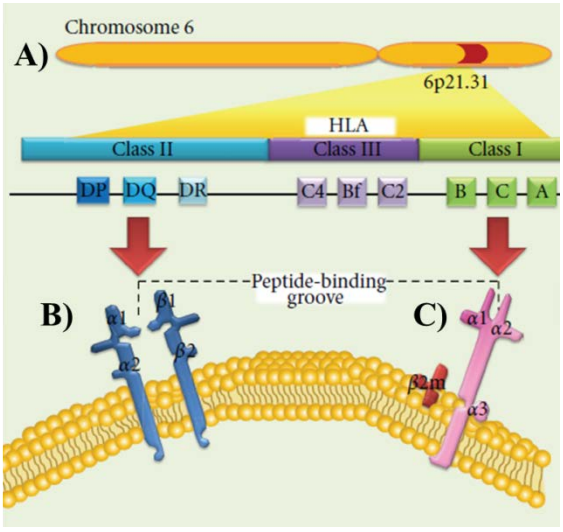
inhibiting *de novo* antibody production *in vivo*. However, under some experimental conditions it has been shown that MSC may also support the survival and proliferation of antibody secreting B-cells from healthy individuals and in disease settings (Rasmusson *et al.,* 2007 and Che *et al.,* 2014, Traggiai *et al.,* 2008, Healy *et al.,* 2015 and Ji *et al.,* 2015).

In vivo studies that aimed to study the immune effects of MSC on B-cells have provided mixed results. For example, Gerdoni *et al.,* 2007, Rafei *et al.,* 2008 and Asari *et al.,* 2009 have shown that MSC can inhibit primary antigen-specific antibody production in experimental animals while Comoli *et al.*,2008 and Rafei *et al.*,2008 have shown that MSC can also suppress the production of pre-existing antibodies by plasma cells. In line with this, two recent studies have reported that MSC administration is associated with suppression of anti-double stranded DNA auto-antibodies as well as reducing total serum antibodies in a murine model of systemic lupus erythematosus (Gu *et al.,* 2012 and Collins *et al.*,2014). Interestingly, recent study by Park *et al.,* 2015 has shown induction of IL-10 secreting regulatory B-cell induction to MSC infusion in murine model of SLE. (Park *et al.,* 2015). In contrast, other groups have reported that MSC have no effect or, in some cases, actively induce antigen-specific antibody production in various disease settings (Poncelet *et al.,* 2007, Schena *et al.,* 2010 and Youd *et al.*,2010). Taken together, the literature to date indicates that influences of MSC on B-cell activation and antibody production in vivo are quite complex, involve both direct and indirect effects and which might have diverse implications for the therapeutic efficacy of MSC – particularly in the case of allo-MSC.

1.2 IMMMUNOGENOCITY OF ALLO-MSC:

1.2.1 Immune responses to allo-antigens:

Before discussing the specific interactions of allo-MSC with the recipient immune cells *in vivo*, it is appropriate to consider how antigens from allogeneic tissue and organ transplants are typically recognised by the host immune system. Major Histocompatability Complex (MHC) proteins play a central role in allo-antigen-mediated sensitization of the host. In humans, the MHC complex genes are located on chromosome 6 **(fig-1.2.1).** In general, organ transplants between MHC-identical individuals are readily accepted while MHC mismatched transplants are rejected acutely or chronically as a result of MHC protein-specific cellular and humoral immunity. The MHC was first discovered in mice by Gorer (1937) & Snell (1948) and for human MHC it was named as the human leukocyte antigen (HLA) system by the World Health Organization (WHO). For this discovery, Snell, Benacerraf and Dausset were awarded the Nobel Prize in Physiology or Medicine in 1980. Following this discovery, Doherty and Zinkernagel were awarded the Nobel Prize for their research demonstrating the specificity of the cell-mediated immune defence.



Adopted from Garica *et al.*,2012, *J.Transplant.*

Figure 1-2.1: Gene locus and structure of HLA molecules: (A) Chromosomal location and basic orientation of the MHC gene cluster in human. **(B)** Basic conformation of Class II MHC protein on the cell surface (B-cells, T-cells, monocytes, macrophages, dendritic cells etc.) **(C)** Basic conformation of Class I MHC protein (all nucleated cells).

The MHC has been divided into three classes designated MHC I, II and III **(fig-1.2.1)** based on structure and function. MHC I and II genes are expressed as cell surface proteins while MHC III genes encode components of the complement system and other immune-related proteins. Class I MHC proteins are composed of a 45-kd transmembrane α chain encoded by genes of the HLA-A/B/C loci on chromosome 6 which is associated non-covalently with a 12-kd protein, β2-microglobulin, encoded by a gene on chromosome 15 **(Fig-1-2.1)**. Class II MHC proteins are heterodimers composed of non-covalently associated α and β polypeptide chains encoded by genes of the HLA-D region. Class III molecules are located between the HLA-B and HLA-D loci and determine the structure of three components of the complement system. Allogeneic immune responses are frequently mediated by T-cells, which recognise peptide antigens presented in the context of MHC by antigen-presenting molecules (APCs). In the transplantation, MHC proteins from the donor act as strong allo-antigens when presented intact by donor APCs or as processed peptides by recipient APCs – with either modality capable of resulting in acute rejection or, in some cases, chronic rejection. In the clinical field of transplantation, HLA typing was initially performed by complement dependent cytotoxic assays using antisera derived from placentas of mothers who had developed immune response to their foetuses during pregnancy. This approach mimicked *in vitro* the pathogenesis of acute antibody-mediated rejection which occurs following transplantation of vascularised organ transplants into recipients with pre-formed anti-donor MHC antibodies (Gloor & Stegall., 2010). More recently, more advanced techniques such as ELISA, flow cytometry and Luminex® bead-based assays have been introduced into the clinical arena and provide substantially greater sensitivity and accuracy (Lung., 2011). Although it is now well accepted that innate immunity has a key role in initiation of adaptive immune responses including transplant rejection, (Chong & Alegre., 2012) innate immune activity is largely non-specific and is only rarely sufficient to mediate allograft rejection in the absence of allo-antigen-specific T- and B-cell responses (Wood and Gotto, 2012). Nonetheless, there is a growing interest in the modifying role of innate immunity on the outcomes of allo-antigen presentation and on the balance between transplant rejection and tolerance that may be of specific relevance to the immunogenicity of allo-MSC therapies (Chong & Alegre., 2012; Wood & Gotto., 2012; Griffin *et al.*, 2013).

1.2.1.1 *Allo-antigen recognition by T-cells:* In the absence of pre-formed donor-specific antibodies, activation of allo-antigen-specific T-cells by professional antigen-presenting cells (APCs) is the first step toward initiation of anti-donor immune response following transplantation (Wood & Gotto., 2012). As summarised in **Figure 1-2.2**, this process of T-cell allo-recognition following transplantation of an organ or tissue can occur by three distinct pathways (Afzali *et al.,* 2008 and Wood & Gotto., 2012). The first, termed Direct allo-recognition, involves the interaction of APCs of donor origin, specifically tissue-resident DC, with $CD4^+$ and $CD8^+$ T-cells of recipient origin. This is considered to occur following DC migration to lymphoid organs such as lymph node and spleen. Responding T cells are activated through their T-cell receptors (TCR) via interactions with donor MHC proteins in the context of additional, co-stimulatory signals provided by the donor DC. Subsequently, $CD8^+$ T-cells receive help from the $CD4^+$ population to expand and mediate their antigen specific cytolytic functions. The second pathway, referred to as Indirect allo-recognition, occurs when donor MHC or non-MHC proteins (often referred to as minor histocompatibility antigens) are internalised, processed into peptides and presented to allo-antigen-specific $CD4^+$ and $CD8^+$ T-cells by APCs of recipient origin via 'self ' MHC molecules. Importantly, this form of allo-antigen recognition may occur at any time post-transplant and is not dependent on the presence of donor DC or other professional APCs in the transplanted tissue (Afzali *et al.*, 2008). Thus, even for transplanted allogeneic cells lacking inherent antigen-presenting function, the potential for indirect allo-recognition exists. Finally, Semi-direct allo-recognition represents a more recently proposed mechanism whereby recipient APCs acquire intact MHC/ peptide complexes from donor cells either by direct cell-to-cell transfer or, alternatively, through the release of extracellular micro-vesicles ("exosomes") containing allo-MHC molecules, which can fuse with the surface of recipient cells (Afzali *et al.*,2008 and Montecalvo *et al.*,2008). These 'chimeric' recipient APCs may subsequently activate allo-specific $CD4^+$ and $CD8^+$ T-cells through both direct and indirect mechanisms with the possibility for linked help to occur. (Afzali *et al.,* 2008 and Montecalvo *et al.*, 2008). As for indirect allo-recognition, the semi-direct pathway does not have an absolute requirement for recipient APCs.

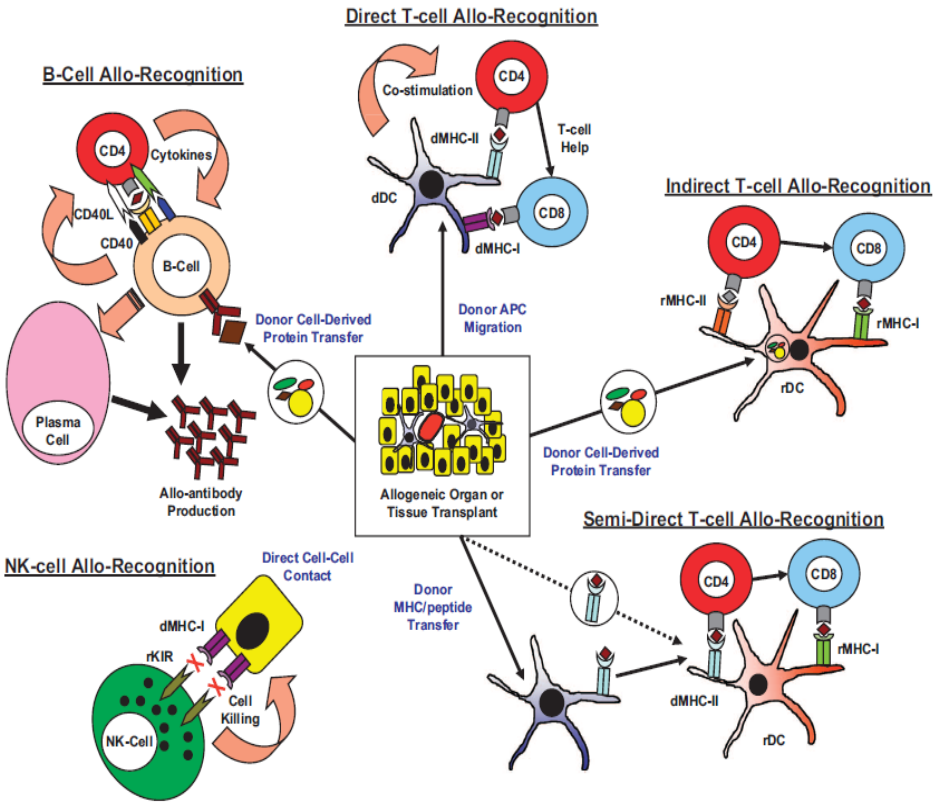


Figure 1.2.2: The major pathways whereby donor antigens are recognised within the T-, B- and NK-cell compartments of the recipient following allogeneic organ or tissue transplantation are illustrated. dDC, donor DC; rDC, recipient DC; dMHC I, donor major histocompatibility complex type I; dMHC II, donor major histocompatibility complex type II; rMHC I, recipient major histocompatibility complex type I; rMHC-II, recipient major histocompatibility complex type II; CD4, $CD4^+$ T cell; CD8, $CD8^+$ T-cell; rKIR, recipient killer-cell immunoglobulin-like receptor. **(Adapted from Griffin *et al.*, 2013)**

1.2.1.2 *Allo-antigen recognition by B-cells:* In addition to T-cell-mediated allograft rejection, B-cells and B-cell related products can also contribute significantly to the destruction of allogeneic organs and tissue transplants (Everly & Terasaki, 2009, Gloor *et al.*, 2009, Nankivell & Alexander, 2010 and Wood and Gotto., 2012). The generation of antibody against allo-antigens (most commonly donor MHC-I and MHC-II proteins) is most likely to be initiated in secondary lymphoid tissues and results from binding of whole-protein antigen to the B-cell receptor in the context of cytokine production and other activation stimuli provided by allo-antigen-specific $CD4^+$ T-cells **(Figure 1.2.2)** (Tarlinton *et al.*, 2008, Stegall *et al.*, 2009 and Wood and Gotto., 2012). Conversely, B-cells can also act as APCs expressing high levels of MHC I and II along with relevant co-stimulatory molecules such as CD40. (Tarlinton *et al.*, 2008, Stegall *et al.*, 2009 and Wood & Gotto., 2012). As APCs, B-cells internalise allo-antigen through the B-cell receptor, process it and present it on MHC to allo-specific T-cells, thus further promoting cellular immune responses against the transplant (Tarlinton *et al.*, 2008 and Wood

& Gotto., 2012). High levels of allo-antibodies mediate acute, severe transplant injury by binding to endothelial or other graft cells and serving as a trigger for complement fixation and/or antibody-dependent cellular cytotoxicity via macrophages and NK cells (Tarlinton *et al.,* 2008, Stegall *et al.,* 2009, Gloor *et al.,* 2009 and Wood & Gotto, 2012). Once fully activated, donor antigen-specific B cells have the capacity to mature into bone marrow-resident plasma cells, which serve as a long-lived source of allo-antibody as well as memory B cells capable of rapidly producing high levels of circulating antibody upon re-exposure to allo-antigen (Stegall *et al.,* 2009). The indefinite persistence of donor specific antibodies following an initial sensitisation event represents one of the most challenging barriers to successful organ and tissue transplantation. Furthermore, the emergence of donor-specific antibodies after transplantation is linked with the development of chronic transplant injury and loss of function (Gloor *et al.,* 2009 and Wood & Gotto., 2012).

1.2.1.3 *Immune recognition of MSC by T and B-cells:* The interaction of MSC with immune cells and the related effects and mechanisms, which are described in earlier sections, may be highly relevant to the *in vivo* immunogenicity of allo-MSC. Summarised in **Figure 1.2.3** are the potential influences of various MSC immune-modulatory properties on different aspects of allo-antigen recognition.

Overall, the current understanding of the specific effects of MSC on T-cell activation and effector function differentiation suggest that:

1) Direct allo-antigen presentation by allo-MSC is unlikely to result in productive anti-donor T-cell responses *in vivo* and may promote responses such as allo-antigen-specific Th2 deviation, T-reg expansion and tolerogenic DC maturation that would serve to prevent aggressive T-cell-mediated allo-MSC rejection (Nauta *et al.,* 2006 and English *et al.,* 2010).
2) Indirect allo-recognition following allo-MSC administration has not been well studied to date but is likely to occur *in vivo* and could be enhanced by MSC death and up-regulation of MHC proteins upon exposure to inflammatory stimuli (Badillo *et al.,* 2007). Conversely, the modulatory effects of MSC on DC functions could also result in tolerogenic outcomes from indirect presentation of MSC-derived alloantigen (Nauta *et al.,* 2006 and English *et al.,* 2008).

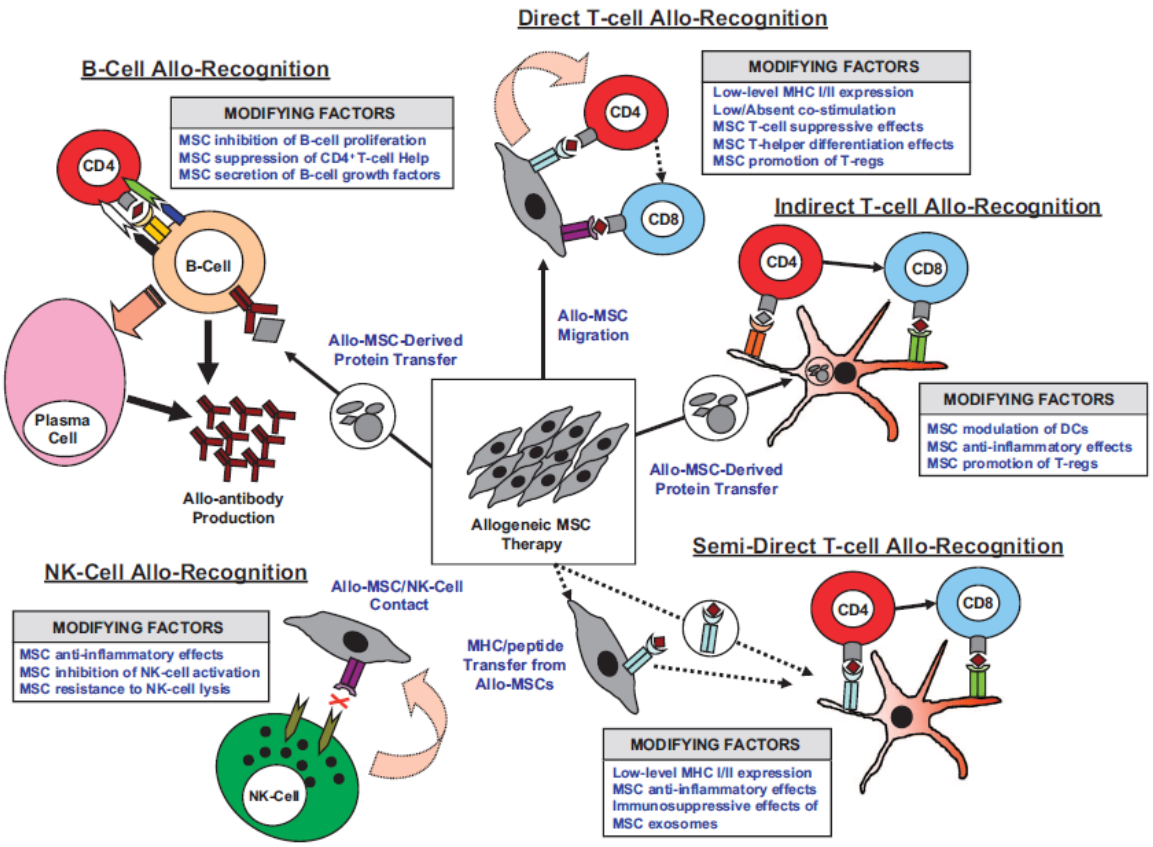


Figure 1.2.3: The major pathways whereby donor antigens may be recognised within the T-, B- and NK-cell compartments of the recipient following allogeneic MSC therapy are illustrated. For each pathway, the potential modifying factors associated with known MSC anti- inflammatory/immunosuppressive properties are indicated in a box. Other cell types and symbols are as indicated for **Figure 1-3 (Adapted from Griffin *et al.*, 2013)**

3) Although it is yet to be evaluated, it also appears feasible that MSC may participate in semi-direct allo-recognition as recent work from several groups has shown that MSC produce high numbers of extracellular micro-vesicles (exosomes) containing cell-specific mRNA, miRNA and proteins(Biancone *et al.*, 2012) and also participate in bidirectional exchange of membrane components.(Strassburg *et al.*,2012).

In contrast to their interactions with T cells, the influence of MSC on B-cells and antibody production is quite poorly understood (Griffin *et al.*, 2010 and Franquesa *et al.*, 2012). As previously discussed, *in vitro* co-culture assays of MSC and B-cells have, for the most part, shown that MSC have the capacity to suppress B-cell proliferation, differentiation and immunoglobulin production (Tabera *et*

al., 2008, Griffin *et al.*, 2010 and Franquesa *et al.*, 2012) under a variety of stimulatory conditions. Other studies, however, indicate that MSC have stimulatory effects on *in vitro*-activated B-cells from healthy subjects depending on the level of stimulation (Rasmusson *et al.*, 2007). Such *in vitro* experiments provide little clear indication of how MSC influence primary and secondary antibody responses *in vivo* either directly or indirectly through effects on T-cell help. Nonetheless, as summarised below, the generation of donor-specific antibodies following experimental allo-MSC administration by a variety of routes in multiple species indicates that MSC-derived antigens are readily accessible to the B-cell compartment and are capable of stimulating an active humoral response.

1.2.1.4 *Anti-donor immune responses to allo-MSC (in vivo evidence)*: Experimental studies attempted to study the immunogenicity of allo-MSC in a number of different species (rat, mouse, pig, monkey, baboon, rabbit) using a variety of delivery routes [intravenous (IV), intraperitoneal (IP), intramuscular (IM), subcutaneous (SC) and local implantation] and cell doses in various disease models have produced mixed results (**Figure 1.2.4**). Thus, the immunogenicity of allo-MSC is highly context-dependent and, potentially, highly variable.

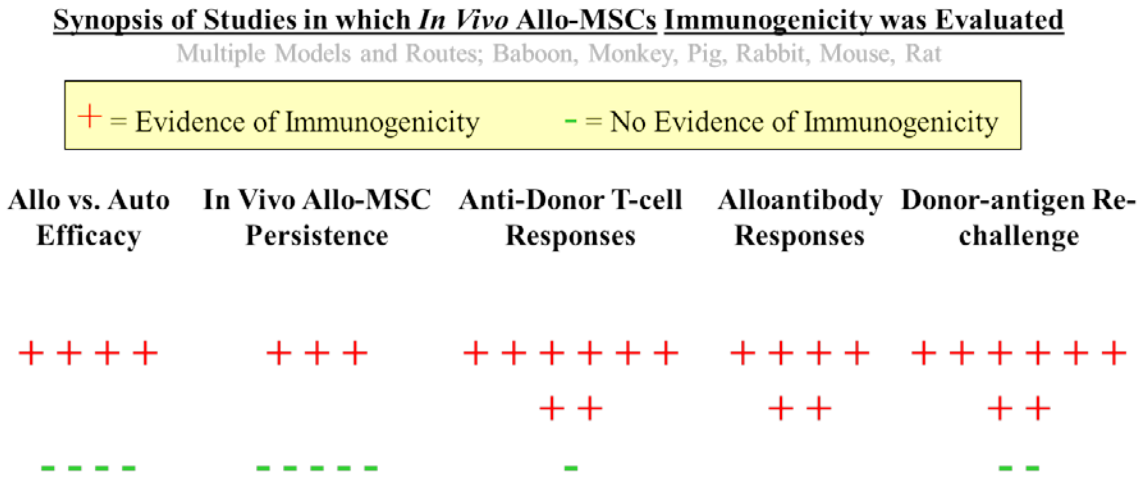


Figure 1.2.4: Summary of the frequency of evidence for (+) or against (-) allo-MSC immunogenicity in various animal model studies based on different types of experimental evidence (**Adapted from Griffin et al, 2010**). As shown, for studies that included specific assays for anti-donor T-cell and alloantibody responses or that included donor-antigen re-challenge experiments following allo-MSC administration, the majority provided evidence of immunogenicity.

Evidence of increased anti-donor T-cell responses following allo-MSC administration to experimental animals and has been reported for a number of different models. This has included studies in healthy mice and rhesus macaques, as well as studies of bone marrow transplantation and myocardial infarction in mouse and pig, respectively (Eliopoulos *et al.*,2005, Nauta *et al.*,2006, Badillo *et al.*,2007, Poncelet *et al.*,2007, Zangi *et al.*,2009, Isakova *et al.*,2010, Ryan *et al.*,2014 and Tano *et al.*,2016).

In one of the earliest and most convincing studies dealing with allo-MSC immunogenicity, Eliopoulos *et al.*,2005 showed that sub-cutaneous implantation of allo-MSC (but not autologous MSC) within a biomaterial scaffold was associated with increased T-cell resulting in cellular infiltrates at the site of the implant. In this study, the implanted MSC were engineered to express murine erythropoietin (EPO) such that "graft survival" could be accurately detected through the effect of EPO production on haematocrit. (**Figure 1.2.5**)

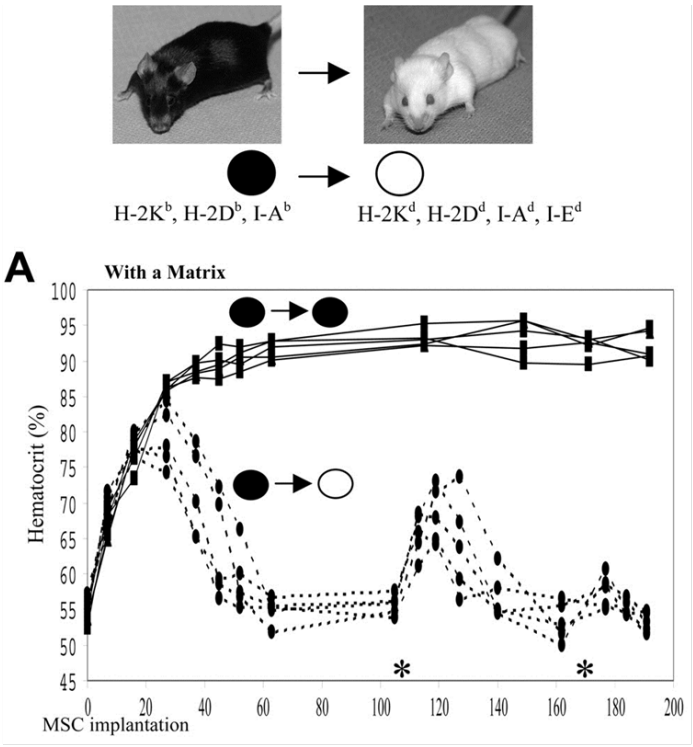


Figure 1.2.5: Allogeneic (black dot/dashed lines) but not autologous (white dot/solid lines) EPO$^+$ MSC-containing subcutaneous implants in mice are rejected in naïve hosts (day 0 – 100) and secondary allo-MSC containing implants are rejected at an accelerated rate (day 100 to 200). In vivo survival of MSC is indicated by increased Hematocrit (%) above baseline of 50-55% **(Adapted from Eliopoulos *et al.*,2005).**

In this way, the authors clearly demonstrated that allo-MSC was immunologically rejected while autologous MSC survived indefinitely. Additionally, re-challenging with a second allo-MSC-containing implant, it was possible to demonstrate donor-specific accelerated rejection following initial allo-MSC-mediated sensitisation.

In an another important and clinically relevant study, Poncelet *et al.*,2007 investigated the immunogenicity of allo-MSC in a pig model of myocardial infarction with a focus on the development of donor-specific antibodies. These authors observed that both intra-cardiac and sub-cutaneous (SC) injection of allo-MSC resulted in the induction of anti-donor IgG antibody along with increased T-cell responses. Of interest, the titres of anti-donor IgG induced by single injections of allo-MSC in this study were lower than those induced by peripheral blood mononuclear cells (PBMCs) or by allogeneic skin grafting. In the case of SC injection, the induced antibodies were not associated with donor-specific complement mediated cytotoxicity *in vitro*. However, following a second round of SC allo-MSC injections, these animals also developed cytotoxic levels of anti-donor IgG (**Figure 1.2.6**). Thus, this study provided clear evidence of the capacity of allo-MSC to induce cytotoxic donor-specific immune sensitisation following one or more localised inoculations.

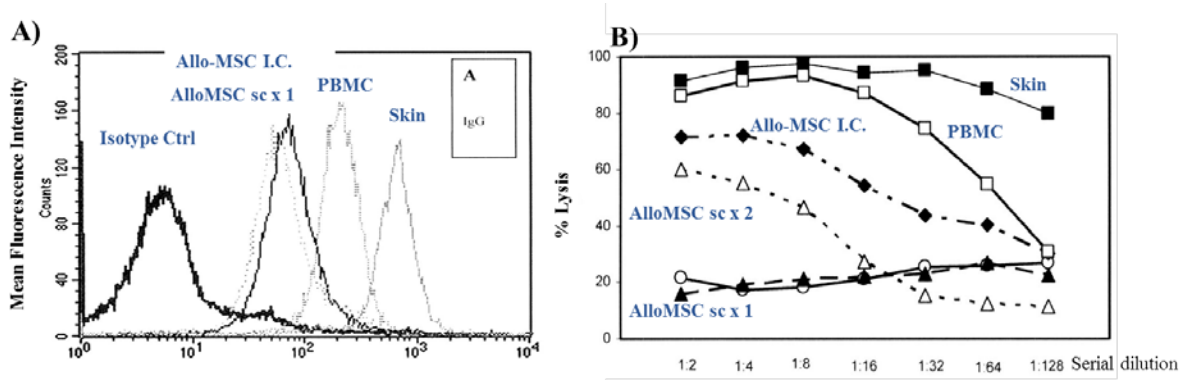


Figure 1.2.6: Donor-specific alloantibodies detected in pig serum by flow cytometry (**A**) and complement dependent cytotoxicity assay (**B**). **A.** Levels of anti-donor IgG compared to Isotype contrl staining of target cells according to the type and site of treatment (I.C. = intra-cardiac; sc = subcutaneous; PBMC = peripheral blood mononuclear cells; Skin = allogeneic skin transplant). (B) Cellular cytotoxicity curves for sera from pigs that had received the various treatments (sc x 2 = two subcutaneous injections of Allo-MSC). **(Adapted from Poncelet *et al.*,2007)**

In addition to these studies, others have also reported accelerated rejection of a secondary transplant or cell injection from the same donor source in multiple species and various routes of allo-MSC delivery. (Nauta *et al.*,2006, Badillo *et al.*,2007, Zangi *et al.*,2009 and Seifret *et al.*,2012).

Overall, therefore, there is quite convincing animal model-based evidence that allo-MSC are capable of initiating both cellular and humoral anti-donor immune responses *in vivo*, although, in some cases, the immunogenicity of allo-MSC appears to be lower than that of other allogeneic cells and tissues. Furthermore, it can be concluded from the evidence to date that immunogenicity of allo-MSC does not always correlate with or predict lack of safety or efficacy in specific disease settings – particularly in the case of acute conditions (e.g. myocardial infarction) where the efficacious effects are expected to occur soon after administration. Also, as discussed in more detail later, some transplantation experiments suggest that MSC-delivered allo-antigens may potentially induce immune regulatory responses that could favour the development of donor-specific tolerance. (English *et al.*,2010). Thus, a better understanding of allo-MSC induced immune responses and their underlying mechanisms and consequences could greatly help to clarify their clinical value for treating specific acute and chronic diseases.

1.3 ALLOGENEIC VS AUTOLOGOUS MSC FOR TRANSLATIONAL APPLICATIONS

1.3.1 *Advantages of allo-MSC for therapeutic applications:*

Pre-clinical studies have provided supportive evidence for the therapeutic benefits of MSC administration in a wide range of diseases. However, the sources of MSC that can be applied to specific clinical therapeutic purposes may be dictated not only by the translation of pre-clinical scientific knowledge but also by practical and logistical considerations. In this regard, autologous MSC (cells prepared from the same person who is to receive treatment) have some limitations in clinical practice. In the first place, it may prove difficult to obtain sufficient numbers of primary MSC from people with medical conditions. For example, isolation of adequate numbers of bone marrow MSC from patients with haematological conditions or of adipose-derived MSC from thinner patients may not be possible. Similarly, in some studies at least, MSC from older individuals have been shown to have less biological activity and regenerative capacity. (Mueller *et al.,* 2001 and Jung *et al.,* 2014). Furthermore, some diseases such as diabetes mellitus, rheumatoid arthritis and systemic lupus erythematosus are known to directly influence MSC and their biological properties in ways that could diminish their clinical efficacy. (Nie *et al.,* 2010, Cianfarani *et al.,* 2013 and Sun *et al.,*2015). Finally, for acute diseases such as myocardial infarction, stroke, sepsis and acute kidney injury, the need for immediate therapy would preclude the use of autologous MSC unless the patient in question had pre-emptively donated tissue for MSC culture expansion and storage.

In settings such as these, allo-MSC, prepared from a healthy donor and cryopreserved as a potential "off the shelf" product would be an ideal option. Furthermore, large-scale expansion and storage of allo-MSC from healthy donors represents a more cost-effective model of production and offers additional theoretical advantages such as selection of optimal donors, use of umbilical cord and placenta for MSC generation and matching of MSC potencies to individual diseases. Based on these considerations, the commercial companies that have emerged in the MSC therapeutics space are predominantly focussed on allo-MSC products. (Griffin *et al.,*2013).

1.3.2 *Relative safety of autologous and allo-MSC equivalent:*

There are now a large number of completed and on-going clinical trials of MSC which include both autologous and allo-MSC-based interventions. (Duijvestein *et al.*,2010, Ciccocioppo *et al.*,2011, Perico *et al.*, 2011, Tan *et al.*, 2012, Reinders *et al.*,2013, Franquesa *et al.*, 2013, and Peng *et al.*, 2013). For many of these studies, particularly those involving early phase trials, the establishment of safety in the context of a specific clinical condition is the primary goal (Griffin *et al.*, 2013). For systemically administered cells, whether autologous or allogeneic, the most immediate safety concern is the potential for acute allergic reactions, pro-thrombotic effects or peripheral artery embolization associated with infusion. Such adverse events may be triggered by culture medium components, cryopreservation reagents, dead or clumped cells or cell-expressed biomolecules that trigger intravascular inflammatory/pro-coagulant responses (Moll *et al.*,2012). In case of allo-MSC, it must also be considered that pre-sensitisation against allogeneic MHC proteins or minor histocompatibility antigens could, theoretically, result in immediate or early immune response to the administered cells. Interestingly, however, clinical studies involving administration of both autologous and allo-MSC by IV and other routes have reported few, if any, severe reactions (Blanc *et al.*, 2008, Hare *et al.*,2009, Duijvestein *et al.*,2010, Vaes *et al.*,2012, Hare *et al.*,2012, Tan *et al.*, 2012, Reinders *et al.*,2013 and Peng *et al.*, 2013). Specifically, Le Blanc *et al.*, 2008 reported no acute adverse response in 55 patients receiving IV infusions of fresh or cryopreserved third-party allo-MSC in steroid-resistant graft-versus-host disease. In another study by Peng *et al.*, 2013, donor-derived allo-MSC were intravenously administered to living donor kidney transplant recipients with no acute adverse events recorded. In the case of autologous MSC, Tan *et al.*, 2012 and Reinders *et al.*, 2013 reported that IV infusion of bone marrow MSC to kidney transplant recipients was well tolerated.

Thus, although there is undoubtedly a need for on-going close monitoring of allo-MSC recipients participating in clinical trials or receiving clinical therapy with allo-MSC products, the reported experiences to date indicate that they have similar safety profile to autologous MSC. Furthermore, completed trials indicate that systemic or localised administration of clinical-grade, culture-expanded MSC is generally safe even among patients with severe conditions such as graft versus host disease.

1.3.3 *In vivo persistence and efficacy of autologous and allo-MSC:*

Pre-clinical studies of various inflammatory disease (skin wound healing, sepsis, kidney ischemia reperfusion injury, allogeneic bone marrow transplant and myocardial infarction) have provided direct comparisons of autologous and allo-MSC. For several of these, the efficacy of allo-MSC was shown to be equivalent to that of autologous MSC (Imanishi *et al.*,2008, Zangi *et al.*,2009, Chen *et al.*,2009, Rafei *et al.*,2009 and Nemeth *et al.*,2009). Others, however, have reported that allo-MSC were less effective than auto-MSC in terms of their therapeutic benefits – in some cases with evidence of immunological rejection (Eliopolous *et al.*,2005, Nauta *et al.,* 2006 and Togel *et al.,* 2009). It is often considered that MSC therapeutic value may be dependent upon the longevity of the delivered cells either at the target site or at another, distant location. Many animal model studies have demonstrated that IV MSC primarily localise to (or are "trapped" in) the lungs for the first 24 hours following administration and that the majority of cells remaining after that point are non-viable. Among the factors reported to be associated with MSC trapping in the lungs and *in vivo* survival following IV administration are cell size, tissue source, passage number and surface markers expression profile (Eggenhofer *et al.,* 2012 and Nystedt *et al.*,2013). To date, it is not clear whether allogeneic source and/or pre-sensitisation to allo-antigens also represent risk factors for early loss of MSC viability or failure of administered cells to be released from the lungs and engraft other at disease-relevant sites.

There is, however, some evidence that engrafted or implanted allo-MSC can elicit a delayed (primary) anti-donor immune responses which results in eventual loss of efficacy. Perhaps the best examples of this comes from the work of Huang *et al*. in a rat model of left ventricular remodelling following acute myocardial infarction (Huang *et al*., 2010). In this study, intra-myocardially injected allo-MSC were shown to have equal early benefits to autologous MSC but subsequently lost efficacy as a result of immunological rejection associated with *in situ* differentiation. In the clinical arena, however, Hare *et al*. have reported similar safety profiles and efficacy signals in two groups of 15 patients with ischemic cardiomyopathy who received intra-myocardial injections of either autologous or allo-MSC (Hare *et al*., 2012). Overall, there is much left to be learned regarding the relative *in vivo* survival and long-term efficacy of autologous and allo-MSC for specific disease indications.

1.4 ALLO-MSCS FOR NON-TRANSPLANT APPLICATIONS

1.4.1 *Factors influencing allo-MSC immunogenicity in inflammatory disease models:*

Clinical trials demonstrating unequivocal therapeutic efficacy of allo-MSC administration compared to current standard-of-care for acute and chronic inflammatory diseases have remained elusive. (Griffin *et al*.,2013 and Zhang *et al.,*2015). Furthermore, the frequency and clinical implications of allo-MSC-induced anti-donor immune responses in human subjects have been infrequently reported. (Le Blanc *et al.*,2008, Hare *et al.*,2012 and Peng *et al.,* 2013) From a pre-clinical perspective, there is convincing evidence that administration of single doses of allo-MSC by IV and other routes can mediate potent anti-inflammatory and immune modulatory effects with distinct beneficial effects in a large number of different animal disease models. To give two relevant examples, allo-MSC have been shown to reduce mortality and organ damage in the mouse caecal ligation model of polymicrobial sepsis (Nemeth *et al.*,2009) and to ameliorate central nervous system inflammation and neurological deficits in experimental autoimmune encephalomyelitis (Zappia *et al.*,2005 and Rafei *et al.*,2009). Of particular relevance to this thesis, there have been a number of reports documenting the potential therapeutic efficacy of MSC in rodent models of limb ischemia as well as some encouraging results from phaseI/II clinical trials. (Al-khaldi *et al.*,2003, Ishikane *et al.*,2008, Mamidi *et al.*,2012, Gupta *et al.*,2013 and Das *et al.*,2013). Importantly, while potential mechanisms of action of allo-MSC in inflammatory diseases have been elucidated through *in vitro* experimentation, the extent to which these have been investigated *in vivo* has varied in the pre-clinical literature. Nonetheless, the existing evidence suggests that different mediators and mechanistic pathways are likely to play a role in different disease settings. Furthermore, the extent to which allo-MSC induced anti-donor immune responses modulate their therapeutic mechanisms *in vivo* under specific disease conditions remains incompletely understood.

As described previously, several studies involving inflammatory disease models in immune competent rodents, pigs and non-human primate have demonstrated the induction of donor-specific cellular and humoral responses to allo-MSCs (Cho *et al.*,2008, Camp *et al.*,2009, Chen *et al.,* 2009, Isakova *et al.*,2010, Huang *et al*., 2010). The reported strength and immunological consequences of

these responses has varied, however, from one model to the next. It is likely that a range of specific factors influence the immunogenicity of allo-MSC administration in the context of a disease model. For example, the nature and site of cell delivery may determine whether a readily detectable anti-donor immune response is induced. While IV, IP, SC and intra-myocardial routes of administration have been well shown to be associated with increased anti-donor T-cell response and/or development of anti-donor antibodies (Badillo *et al.*,2007, Poncelet *et al.*,2007 and Cho *et al.*,2008); intra-cranial, intra-articular and skin wound application of allo-MSC have been reported not to induce such responses (Djouad *et al.*,2003 and Chen *et al.,* 2009). Consistent with this, route of delivery is also likely to impact the *in vivo* persistence/survival of allo-MSC in a given disease model. Interestingly, Camp *et al.*,2009 documented survival of allo-MSC for up to 4 weeks following intra-cranial injection in a rat model of localised brain injury and others observed similarly prolonged persistence of allo-MSC in a mouse skin wound model and in healthy baboons (Beggs *et al.,* 2006 and Chen *et al.,* 2009). Thus, in some locations, allo-MSC clearly may evade immune recognition and/or removal by immunological effectors.

Altogether, experimental evidence suggests that allo-MSC immunogenicity (and possibly therapeutic efficacy) is influenced by: (a) Route of administration. (b) Cell dosage administered. (c) Immunological status of the recipient. (d) Nature of the disease model. (e) Duration of the model. (f) Persistence and differentiation of allo-MSC. For the process of translating allo-MSC administration to clinical practice in specific diseases, we believe that is important for pre-clinical experimentation to be planned and implemented in such a way that these factors are as well matched as possible with the likely human therapeutic scenario.

1.4.2 *Current understanding of anti-donor T-cell and B-cell responses to allo-MSC in inflammatory diseases:*

As already described, the T-cell suppressive properties of MSCs have been well documented *in vitro* for over a decade (Bartholomew *et al.,* 2002, Di Nicola *et al.,* 2002, Tse *et al.,* 2003, Zappia *et al.,* 2005, Aggarwal *et al.,* 2005, Krampera *et al.*,2005 and Duffy *et al.*,2011). However, the extent to which MSC (including allo-MSC) actively prevents or suppress antigen-specific T-cell responses *in*

vivo is less clear. Indeed, some studies have shown that allo-MSCs induce active anti-donor T-cell responses in immune competent experimental animals. (Griffin *et al.*,2010, Schu *et al.*,2012 and Zhang *et al.*,2015). However, the finer details of the T-cell response to allo-MSC are rarely studied. In this regard, some interesting observations were made by Beggs *et al.*,2006. In this report, the authors demonstrated that multiple administrations of allo-MSC resulted in decreased donor-specific T-cell responses suggesting that repeated exposure to MSC-delivered allo-antigens might induce a regulatory or tolerance-like response which could be of therapeutic benefit in auto-immune or inflammatory disorders. Taken together, we believe that the current evidence supports the conclusion that allo-antigens expressed by MSC are actively recognised by the host T-cell compartment when the cells are administered by typical, clinically-relevant routes in the setting of inflammatory disease. It seems likely that presentation of peptide antigens from allo-MSC occurs via the indirect and/or the semi-direct pathway. To better understand the clinical implications of allo-MSC-induced anti-donor cellular immunity, more refined profiling of *in vivo* T-cell responses to allo-MSCs are needed. In this thesis, we specifically addressed the influence of multiple IM administrations of allo-MSC on such responses in immune competent mice.

In addition to anti-donor T-cell responses, the generation of anti-donor antibodies following allo-MSC administration by different routes may have important clinical implications for patients suffering from inflammatory and immune-mediated diseases. Surprisingly, while the field of organ and tissue transplantation has developed a strong focus on studying allo-antibody responses and their clinical implications, the field of regenerative medicine and stem cell therapeutics has not yet fully embraced this area of immunology. The presence of donor-specific and non-donor-specific antibodies against HLA and other allo-antigens in a potential organ transplant recipient is routinely analysed by assaying the serum samples. (Gloor *et al.*,2005, Cai *et al.*,2008 and Everly *et al.*, 2009). Furthermore, the technology for accurately detecting and functionally profiling such antibodies has progressed greatly in the past 10 years. (Hare *et al.*,2012, Arnold *et al.*,2013 and Sicard *et al.*, 2015) Thus, there is great potential for studying allo-MSC-induced allo-antibodies in both experimental animals and human subjects using approaches that are now well established in the field of transplant immunology.

Despite the relative dearth of research, anti-donor antibody responses to allo-MSC have been documented in various animal model settings as we have previously described (Beggs *et al.,* 2006, Badillo *et al.,* 2007, Poncelet *et al.,*2007, Poncelet *et al.,*2008, Griffin *et al.,*2010, Isakova *et al,*.2010, Seifret *et al.,*2012 and Schu *et al.,* 2012). In some of these studies, the functional nature of the allo-antibodies induced by allo-MSC have been tested using complement mediated lysis assays with mixed results (Poncelet *et al.,*2007 and Schu *et al.,* 2012). For example, Poncelet *et al.,*2007 and Schu *et al.,* 2012 observed that allo-MSC-induced IgG are capable of fixing complement and inducing donor target cell lysis *in vitro*. Whether immunosuppressive drugs can prevent the generation of *de novo* anti-donor antibodies following allo-MSC administration may be an important question for future clinical practice. Poncelet *et al.,*2007 have reported that co-administration of tacrolimus with allo-MSC in the pig myocardial infarction model did result in a reduction in anti-donor antibody titers in a small number of animals. In this thesis, we have performed a detailed analysis of the development of anti-donor IgG antibodies and have also investigated the potential value of tacrolimus for its prevention following IM delivery of fully MHC-mismatched allo-MSC in the mouse hind limb ischemia model.

1.5 ALLO-MSC FOR TRANSPLANT APPLICATIONS

1.5.1 *Safety and efficacy of allo-MSC in transplant applications:*

MSCs have recently entered the clinical arena as a novel therapy for recipients of organ transplantation. (Perico *et al.*,2011, Tan *et al.*, 2012, Reinders *et al.*,2013, Franquesa *et al.*,2013 and Peng *et al.*,2013). As is typical for new immunosuppressive/ immunomodulatory agents, clinical studies have been preceded and informed by a decade or so of experimental work in animal models (Griffin *et al.*, 2013). However, in comparison with novel pharmacological products, MSC requires precise considerations to completely understand their safety, efficacy and therapeutic efficacy in clinical setting (Griffin *et al.*, 2013). As previously noted, a growing number of published and anecdotal reports have continued to appear in relation to Phase I and Phase II clinical studies of autologous and allo-MSCs administrated by IV and other routes for diverse medical conditions, and these have consistently described a lack of acute complications from doses as high as 5×10^6 cells/kg (Hare *et al.*,2009, Duijvestein *et al.*,2010, Vaes *et al.*,2012 and Hare *et al.*,2012). Nonetheless, as we have already discussed, the safety implications of MSC entrapment in the lungs following intravenous injection and the degree to which this limits their therapeutic effects at other sites has not been extensively evaluated in humans (Eggenhofer *et al.*, 2012 and Nystedt *et al.*,2013). As for other clinical indications, these recent studies merit close attention by clinicians and researchers involved in the development of clinical trials of MSCs in the setting of organ and tissue transplantation.

1.5.2 *Allo-MSC associated anti-donor immune responses and transplant applications:*

In transplantation field, an important question of special relevance is the degree to which donor-specific or third-party allo-MSCs may induce anti-human leukocyte antigen (HLA) immune responses with potential for acute or chronic harmful effects for organ allografts. The concept of 'immune privilege', which has been frequently evoked in regard to allo-MSCs on the basis of their low expression of HLA proteins and inherent immunosuppressive properties, does not stand up well to scrutiny when relevant *in-vivo* experimental studies are examined (Griffin *et al.*, 2013). For example, most studies in which anti-donor immune responses have been assayed following administration of

allo-MSCs have observed emergence of readily detectable alloantibody (Schu *et al.*, 2012, Seifert *et al.*, 2012 and Griffin *et al.*, 2013). Furthermore, accelerated rejection of subsequent allografts has also been documented in diverse animal models of allo-MSC administration (Schu *et al.*, 2012, Seifert *et al.*, 2012 and Griffin *et al.*, 2013). As an example, Seifert *et al.*, 2012 reported the finding that pre-treatment of rat renal allograft recipients with donor-specific allo-MSCs enhanced the production of anti-donor antibody and worsened graft rejection. An additional potential concern, is the possibility that small numbers of engrafted allo-MSCs may undergo spontaneous differentiation and, in doing so, become more immunogenic (Huang *et al.*, 2010). Counterbalancing these concerns, the capacity of allo-MSC to induce anti-donor immune responses, most likely through indirect antigen presentation pathways (Griffin *et al.*, 2013), may be preventable by co-administration of conventional immunosuppressive medications or may be associated with pro-tolerogenic features, such as expansion of donor-specific regulatory T cells (T-reg) (Casiraghi *et al.*, 2008, Ge *et al.*, 2009 & 2010 and Eggenhofer *et al.*, 2011 & 2013). Nonetheless, the frequency and nature of humoral and cellular anti-donor immune responses following allo-MSC administration to human organ transplant recipients, not to mention the impact of such responses on graft outcomes, is not known precisely so far. Although high-level sensitization of patients receiving allo-MSC for other indications has not been reported from clinical trials to date, this issue has rarely been addressed using the highly sensitive methodologies that are considered state-of-the-art in organ transplant practice.

Importantly, a number of desired clinical efficacies can also be envisioned for allo-MSC in the setting of organ transplantation. For the most part, these can be linked to specific MSC influences on innate and adaptive immune responses associated with adverse transplant out-come (**Table 1-1**). The clinical experiences reported to date for MSC therapy in organ transplant recipients provide encouraging results as regards to safety and efficacy during the first post-transplant year. However, this experience is primarily limited to autologous MSC and the potential for additional immunological or practical benefits from donor-specific and third-party allo-MSC remains largely hypothetical. The potential for auto-MSCs or third-party allo-MSCs to be used to stabilize or reverse chronic inflammation and fibrosis in allografts with deteriorating function appears particularly promising.

Table - 1.5.1: *Potential-specific clinical indications and relevant immune mechanisms association with autologous and allogeneic mesenchymal stem cells in organ transplantation.*

Desired Clinical Efficacy (Compared to Conventional Therapy)	**Likely Administration Timing**	**Possible Cellular Targets**	**Possible Preference for MSC Source**	**Evidence from Animal Studies (Refs)**	**Evidence from Human Trials (Refs)**
Reduced severity of ischemia reperfusion injury resulting in improved early graft function.	Before or during transplant	**Innate Immune:** monocyte/macrophages, dendritic cells, neutrophils, NK cells, other. **Non-immune:** epithelial, endothelial, other.	Autologous or Third-party Allogeneic	Casiraghi *et al.,*2012 and Oh *et al.,*2012	Tan *et al.,*2012 (Auto-MSCs)
Prevention of acute transplant rejection.	Before or during transplant	**Adaptive Immune:** dendritic cells, T-cells, B-cells.	Autologous, Donor-specific Allogeneic or Third-party Allogeneic	Casiraghi *et al.,*2012 & 2008, Eggenhofer *et al.,*2013, Ge *et al.,*2009 & 2010 and Oh *et al.,*2012	Tan *et al.,*2012 (Auto-MSCs) and Peng *et al.*,2013 (Allo-MSCs)
Reversal of acute transplant rejection.	After transplant	**Innate and Adaptive Immune:** dendritic cells, T-cells, B-cells, monocyte/macrophages.	Autologous or Third-party Allogeneic	-	Reinders *et al.,*2013 (Auto-MSCs)
Improved long-term transplant survival with minimization of immunosuppression.	Before, during or after transplant	**Adaptive Immune:** dendritic cells, regulatory T-cells, B-cells.	Autologous, Donor-specific Allogeneic or Third-party Allogeneic	Casiraghi *et al.,*2012 & 2008, Eggenhofer *et al.,*2013,and Ge *et al.,*2009 & 2010	-
Reversal or stabilization of chronic transplant inflammation and fibrosis.	After transplant	**Innate and Adaptive Immune:** monocyte/macrophages, dendritic cells, T-cells, B-cells. **Non-immune:** fibroblast, epithelial, endothelial.	Autologous or Third-party Allogeneic	Franquesa *et al.,*2012	-

1.6 CLINICAL PROMISE OF ALLO-MSC AND UNANSWERED QUESTIONS

As we have outlined in this Introduction to the thesis, interest in the clinical applications of MSC for inflammatory and degenerative diseases as well as for transplantation has consistently increased over the past fifteen years with a strong impetus toward the use of allo-MSC products for both scientific and pragmatic reasons (Bernardo *et al.*,2012 and Wang *et al.*,2012). A search of the ClinicalTrials.gov website on 17/05/16 identified 137 registered clinical trials involving allo-MSC. Furthermore, a number of commercial entities have patented allo-MSC products and have collaborated with clinical centres worldwide to complete early phase and, in some cases, phase 3 clinical trials for several target diseases (Hare *et al.*,2009, Ankrum *et al.*,2010, Mckernan *et al.*, 2010, Patel & Genovesse, 2011, von Bahr *et al.*,2012 and Kurtberg *et al.*,2014). As clinical trial activity as progressed and the number of reported individuals receiving allo-MSC for complex diseases such as GvHD, inflammatory bowel disease, ischemic cardiomyopathy and peripheral vascular disease has increased, there has been a growing confidence in the early safety profile of allo-MSC. Nonetheless, the potential short- and long-term implications of allo-MSC-induced anti-donor immune responses requires more focussed research attention across the range of potential disease applications (Ankrum *et al.*,2010 and Griffin *et al.*, 2013). Among the key under-researched areas in regard to allo-MSC immunogenicity that we identified as being of specific clinical importance and of being directly relevant to our interest area of IM cell injection for inflammatory/ischemic diseases were the following:

1. Is IM injection of a therapeutically relevant dose of allo-MSC consistently immunogenic in healthy and diseased states?
2. What are the potencies and effector phenotypes of anti-donor T-cell and B-cell responses associated with IM allo-MSC and do they differ from those of a more immunogenic cell type?
3. What are the immunological consequences of multiple, compared to single, allo-MSC administrations?
4. Do allo-MSC induced anti-donor T-cells and antibodies carry the potential to directly cause lysis ("rejection") of subsequent MSC- or transplant-based treatments?
5. How are allo-MSC-induced immune responses affected by concomitant immunosuppressive medication?

1.7 AIMS AND HYPOTHESES

Rationale: As outlined in the Introduction, the administration of allo-MSC is viewed as having great promise for the treatment of many acute and chronic diseases and as being a potentially tolerogenic adjuvant therapy for organ and tissue transplantation. However, in contrast to the initial view-point that therapeutically administered allo-MSC are ignored by the host immune system, it has become clear in recent years that MSC-delivered allo-antigen is actively recognised by the host immune system. In some settings, the induction of anti-donor immune responses by allo-MSC could diminish their therapeutic efficacy and, theoretically, could be associated with short- or long-term safety concerns. An added complexity is the fact that the current vision for allo-MSC therapeutics includes multiple different potential administration routes, dosing schedules and concomitant treatments. It is notable in the literature to date, including clinical trials conducted in human subjects, that the immunological principles and techniques that are well established in the field of organ transplantation have not often been rigorously applied to the investigation of the range of anti-donor immune responses and their consequences for allo-MSC recipients. Finally, much of the existing experimental work to document the immune responses to allo-MSC administration has focused on single inocula and has not clearly addressed the nature of secondary or "re-call" immune responses that occur following repeated allo-MSC administration.

Thus, not only are there relatively limited fundamental details regarding allo-MSC immunogenicity in vivo, there is also a strong need to better understand the immunological phenotypes of the anti-donor immune responses that occur in association with specific administration protocols and disease setting.

The **overarching goal** of the project described in this thesis was to characterise the cellular and humoral anti-donor immune responses induced by single and multiple intra-muscular (IM) injections of allo-MSC and to determine their significance to localised allo-MSC therapy for limb ischemia.

The **primary hypotheses** of the project were:

1. Intramuscular injection of fully MHC mis-matched allo-MSC is associated with detectable anti-donor T-cell and B-cell immune responses in the absence of immunosuppressive therapy.
2. Anti-donor immune responses induced by IM injection of allo-MSC differ in magnitude and effector phenotype from those induced by other cell types.
3. Repeated IM injection of allo-MSC is associated with enhanced systemic anti-donor T-cell and B-cell immune response.
4. Intramuscular injection of allo-MSC induces anti-donor immune response in both healthy and diseased states.

The **specific objectives** for the three major experimental sections of the project (**Chapters 2-4**) were:

Chapter 2

1. To develop a clinically-relevant *in vivo* model of single and repeated intra-muscular (IM) injection of allo-MSC and relevant control cells in which to evaluate allo-MSC immunogenicity.
2. To optimise a suite of quantitative or semi-quantitative assays for the analysis of donor-specific T-cell and B-cell responses following IM injection of allo-MSCs.

Chapter 3

1. To compare anti-donor T-cell responses induced by single or multiple IM injections of allo-MSCs and allo-splenocytes.
2. To characterise the local and systemic cellular immune responses associated with IM injections of allo-MSCs.

Chapter 4:

1. To quantify and compare anti-donor antibody responses to single or multiple IM injection of allo-MSCs and allo-splenocytes.
2. To determine the anti-donor antibody response to IM injection of allo-MSC in an animal model of limb ischemia without and with concomitant immunosuppressive therapy.

CHAPTER – 2

DEVELOPMENT OF AN *IN VIVO* MODEL TO DETERMINE THE IMMUNOGENICTY OF INTRAMUSCULAR ALLOGENEIC MESENCHYMAL STEM CELLS

2.1 INTRODUCTION

In general, transplantation of any organ or cells from one individual to a genetically unrelated recipient (allogeneic transplant) will activate the host's innate and adaptive immune system resulting in rejection of the allograft with detrimental effect mediated predominantly by donor antigen-specific T-cell and B-cell immune responses. (Janeway's Immunobiology, 7th Edition Chapter-13) **(Fig. 2.1)**

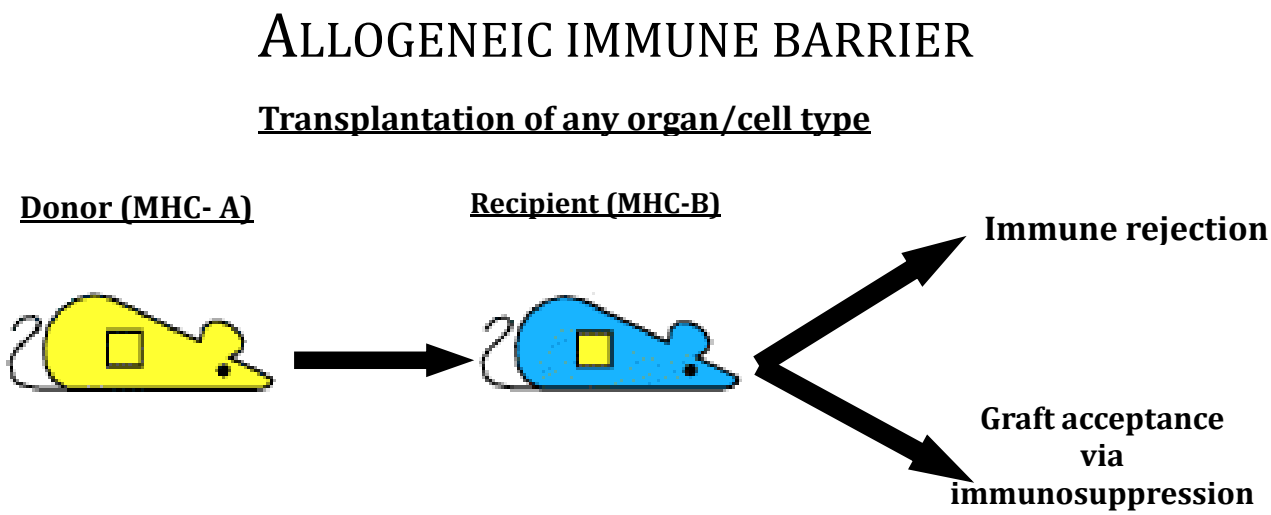


Figure 2.1: Allogeneic immune barrier in living organism (MHC = major histocompatibility complex).

Although innate immune cells are capable of mounting a non-specific immune response to allografts, their functional responses are typically not sufficient to determine the fate of the transplant in the absence of an antigen-specific adaptive immune response (Janeway's Immunobiology, Chapter -13). Nonetheless, the early activation of innate immune cells following transplantation, provides the cytokines and other molecular signals which are necessary for the full activation of donor-specific effector T-cells and antibodies (Janeway's Immunobiology, 7th edition, Chapter -13, Roitt's Immunology, 12th edition Chapter -16). In the case of T-cells, the presentation of donor antigens (alloantigen) along with co-stimulatory signals by professional antigen presenting cells (APCs) represents the first essential step toward clonal selection and activation of effector cells capable of directly mediating graft rejection. Alloantigen presentation may occur via one or more of three pathways: (a) Direct (donor APCs presenting to recipient T cells). (b) Indirect (recipient APCs

presenting peptides derived from donor cells to recipient T cells). (c) Semi-direct (recipient APCs presenting intact MHC/peptide complexes transferred from donor cells to recipient T cells. (Afzali *et al.*, 2007, Griffin MD *et al.*, 2012, Afzali *et al.*, 2013, Benichou *et al.*, 2013 and Nijagal *et al.*, 2013).

On the other hand B-cells respond to allo-antigens directly by interacting with intact proteins from the graft, typically with the "help" from primed $CD4^+$ T-cells in the form of cytokines and co-stimulatory signals. Activation and maturation of donor antigen-specific B cells leads to the development of clonal memory B cells and plasma cells which will secrete high amounts of anti-donor antibodies (alloantibodies), usually of the IgG isotype. (Janeway's Immunobiology, chapter- 13)

The interaction of allogeneic mesenchymal stem cells (allo-MSC) with the host immune system has generated significant interest over the past decade as a result of the growing momentum toward the development of allo-MSC therapy for a wide range of inflammatory diseases. Many published works have concluded that MSCs are hypo-immunogenic and potentially tolerogenic to host immune cells (Batholomew *et al.*, 2002, Spaggiari *et al.*, 2006, English.K *et al.*, 2008, Comoli *et al.*, 2008 and Nemeth *et al.*, 2009). On the basis of their surface phenotype (low MHC expression) and inherent immunosuppressive properties, it has been hypothesized that allo-MSC do not induce any active anti-donor immune response in unrelated recipients following systemic or localised administration (Barthelomov *et al.*, 2002, Spaggiari *et al.*, 2006, English *et al.*, 2008, Comoli *et al.*, 2008, Choi H *et al.*, 2011). However, a number of carefully performed *in vivo* studies have provided evidence that allo-MSCs induce donor-specific T-cell and B-cell response in immunocompetent animals (Eliopoulos *et al.*, 2005, Poncelet *et al.*, 2007, Camp *et al.*, 2009, Salfret *et al.*, 2012 and Li *et al.*, 2012). As the efficacy of therapeutic MSC is often considered to be determined by their longevity in the host, the most immediate practical concern regarding allo-MSC immunogenicity relates to the possibility of their accelerated removal ("rejection") by anti-donor immune responses. Additionally, route of administration of MSC may significantly affect their persistence in the host as well as their primary interface with the host immune system. Thus, studies of allo-MSC immunogenicity should also consider the preferred route of administration for a given disease application.

Mesenchymal stem cells are considered to be a potentially valuable therapeutic option for a wide variety of inflammatory diseases including arthritis, cardiovascular diseases, inflammatory bowel disease, autoimmune conditions, acute kidney injury and critical limb ischaemia (Hoogduijn *et al.,* 2010, English *et al.,* 2008, Griffin *et al.,* 2013 and Ma *et al.,* 2014). Despite an extensive literature demonstrating the immunosuppressive capacity of MSC via modulation of macrophages, neutrophils, dendritic cells (DCs), T-cells, B-cells, natural killer (NK) cells and other innate lymphocytes (Choi H *et al.,* 2001, Bartholomew *et al.,* 2002, Comoli *et al.,* 2008 and English.K *et al.,* 2008), the extent to which allo-MSC elicit anti-donor immune responses in specific *in vivo* settings remains quite unclear. The immunogenicity of allo-MSC has been evaluated in a variety of species ranging from mouse to baboon using different routes of cell delivery and animals healthy animals or those subjected to different disease models. When critically appraised, the majority of these studies in which anti-donor immune responses were carefully evaluated showed that administration of allo-MSC in the absence of concomitant immunosuppression, elicited anti-donor T-cell responses and induction of allo-antibody (Beggs *et al.,* 2006, Poncelet *et al.,* 2007, Isakova *et al.,* 2010, Griffin *et al.,* 2013, Alagesan S *et al.,* 2014 and Hoogduijn *et al.,* 2013&2015). Furthermore, a small number of studies reported that, in *in vivo* re-challenge experiments, donor cells or tissues are strongly rejected after an initial administration of allo-MSC (Eliopolous *et al.,* 2005, Poncelet *et al.,* 2007, Badillo *et al.,* 2007 and Seifert *et al.,* 2012) indicating that allo-MSC are capable of eliciting adverse anti-donor immune responses. It should be emphasised, however, that other experimental studies have demonstrated equal efficacy if syngeneic MSC (syn-MSC) and allo-MSC with failure of allo-MSC to induce any detectable anti-donor immune response (Chen *et al.,* 2009 and Togel *et al.,* 2009). Thus, for the purpose of translating pre-clinical laboratory research into clinical trials of allo-MSC, it is important that the animal model, MSC regimen and immunological analyses selected are closely matched to the target patient population and therapeutic strategy. Also, the potential adverse effects of allo-MSC-induced anti-donor immune responses in a given clinical setting should be carefully considered and studied in a relevant animal model where possible. Among the theoretical concerns related to the immunogenicity of allo-MSC are: (a) Rapid elimination (rejection) of cells shortly after administration. (b) Delayed removal of “engrafted” leading to limited duration of therapeutic efficacy.

(c) Detrimental tissue inflammation due to active immune reaction to engrafted allo-MSC. (d) Limitation of future allogeneic transplant options due to development of persistent anti-MHC antibodies.

Thus, it is of very high importance that immunogenicity of allo-MSC is pre-clinically tested and understood in a particular disease model with a given route of delivery and dose range before it progresses to clinical application. In this regard, it is noteworthy that published studies of allo-MSC immunogenicity have varied greatly in the stringency with which anti-donor immune responses have been analysed. Furthermore, human clinical trials of allo-MSC have not, to date, very clearly examined the development of anti-donor immune response in study subjects (Griffin *et al.*, 2010 and Alagesan S *et al.*, 2014). This chapter, therefore, focusses on the development of an animal model system and panel of assay techniques to specifically analyse anti-donor immune responses to fully MHC-mismatched MSC administered intra-muscularly to the hind limb. The project was initiated in collaboration with the research group of Prof. Timothy O'Brien, REMEDI, National University of Ireland, Galway, which was actively pursuing the clinical translation of IM allo-MSC therapy for critical limb ischaemia (CLI). In planning the experimental strategy for the project, we chose initially to study healthy, adult immunocompetent mice in which clinically relevant doses of fully MHC mismatched allo-MSC were injected into hind limb muscles in similar fashion to the planned administration of human healthy donor bone marrow-derived MSC (BM-MSC) to patients with CLI. We also set out to determine the immunological consequences of multiple IM injections of allo-MSC based on the future clinical potential to treat patients repeatedly with allo-MSC to maximise therapeutic benefit. The specific elements of the anti-donor immune response which we set out to experimentally evaluate were:

1) Effector $CD4^+$ and $CD8^+$ T cell response.
2) Regulatory T-cell response.
3) Effector B cell response (allo-antibody development).
4) Local and systemic innate immune response.

2.1.1 *Effector T-cell response*:

Effector T-cells are the typically the main determinant of the survival or rejection of an allogeneic organ or tissue transplant (Roitt's Immunology, chapter- 16). Anti-donor T-cell responses following a transplantation event can be assayed by quantifying recipient T-cell activation, proliferation and cytokine production *in vitro* in one-way mixed lymphocyte reactions (MLRs) using donor-genotype APCs as the primary stimulator. Necessary controls include responder T-cells from non-transplanted (naïve) animals and stimulators from an allogeneic individual/strain that is genetically distinct from the donor (third party APCs). Distinction of $CD4^+$ ("helper") and $CD8^+$ ("cytotoxic") effector T-cells may be achieved by purification of these subsets in advance or, more commonly, by flow cytometric staining for their specific co-receptors. Also, the T-helper (Th) differentiation phenotype of donor-specific $CD4^+$ T-cells may be determined by analysis of production of specific cytokines during MLRs including interleukin (IL)-2, IL-4, interferon (IFN)-γ, IL-10, transforming growth factor (TGF)-β and IL-17. In a broad sense, Th production of high levels of IL-2, IFN-γ, IL-4 and IL-17 is consistent with potentially destructive T-cell responses while predominant production of IL-10 and TGF-β may signify an anti-inflammatory, pro-tolerogenic phenotype (Janeway's Immunobiology and Roitt's Immunology). Cytokine producing T-cells can be evaluated by immunoassay of MLR culture supernatants or, more precisely, by intra-cellular staining with cytokine-specific antibodies with analysis by multi-colour flow cytometry. $CD8^+$ T-cells which become activated and proliferate in donor APC-stimulated MLRs can be tested for specific cytotoxicity against donor cells by analysis of granzyme B expression and by lysis assays.

2.1.2 *Regulatory T-cell response:*

It has been widely cited that MSC induce and expand $CD4^+/CD25^{++}/FOXP3^+$ regulatory T-cells (T-reg) (Melief *et al.*, 2013, Hu *et al.*, 2013 and Burr *et al.*, 2013). Induction of donor-specific T-reg may result in suppression of anti-donor effector T-cells and of non-specific inflammation, thus enhancing the survival of allo-MSC in the host. For this reason, it is important to characterise the frequency of T-reg following allo-MSC administration in secondary lymphoid organs (spleen and lymph node) as well as following re-exposure to donor antigen in "re-call" MLRs. In mouse, as for

human, enumeration of T-reg is most commonly achieved by flow cytometry with intracellular staining for the T-reg-specific transcription factor FOXP3 along with surface staining for CD4. (Maccario *et al.,* 2005, Di Ianni *et al.,* 2008 and Cahill *et al.,* 2015)

2.1.3 *Effector B-Cell response:*

In general, anti-donor antibody development represents a major hurdle to most types of transplantation and, in the case of allo-MSC administration, the significance of the induction of antibodies against donor MHC or other allo-antigens remains poorly studied. In contrast, a large amount of knowledge has been generated regarding the significance of anti-donor antibody in the field of solid organ transplantation. Highly sensitive assays for quantifying anti-MHC antibodies and determining their specificities, isotypes and potential to support complement-mediated lysis of donor cells have now become state-of-the-art in clinical transplantation and represent essential tools for estimating rejection risk and diagnosing graft complications. In this study, a semi-quantitative, flow-cytometry-based assay for anti-donor IgG in mouse was developed and further modified to detect the major mouse IgG subtypes (IgG1, IgG2a, IgG2b and IgG3) which may, in part, reflect the nature of the underlying donor-specific Th response (Janeway's Immunobiology 7th edition, chapter-13). Additionally, an *in vitro* assay of complement-mediated cytotoxicity assay was developed in order to establish the functional role of anti-donor antibodies induced by IM allo-MSCs.

2.1.4 *Local and Systemic Innate Immune Response*:

The efficacy and longevity of locally administered allo-MSC may be dependent upon their persistence at the site of injection (Zangi *et al.,* 2009, Chen *et al.,* 2009). Cells delivered IM may instantly come in contact with or stimulate the recruitment of innate immune system cells including neutrophils, monocytes, macrophages, DCs, NK cells and NK T-cells). Despite the abundant literature indicating that MSC can modulate or suppress these cell types, the route of administration may determine the susceptibility of MSC to damage or removal innate immune cells (Aggarwal & Pittenger 2005, Ohtaki *et al.,* 2008, Nemeth *et al.,* 2009 and Komoda *et al.,* 2010). In addition, soluble products or subcellular material derived from locally administered MSC and/or innate immune cells "re-

programmed" by contact with tissue-localised MSC may be transferred to secondary sites such as spleen and lymph node. This transfer has the potential to mediate systemic innate immune responses which may amplify the therapeutic benefits of MSC administration (e.g. via production of anti-inflammatory cytokines) or, alternatively, may enhance the generation of anti-donor adaptive immune responses (e.g. via indirect allo-antigen presentation). Furthermore, Li and Lin., 2012 showed that MSCs may be quickly targeted by complement proteins following intravenous (IV) infusion. Hence, it is very important to study the local and systemic innate immune response to *in vivo*-delivered cells in order to better understand their overall immunological impact. Localised immune responses to IM allo-MSC can be determined by histological or immunohistologic analysis of cell infiltrates at the site of injection while systemic innate immune responses can best be analysed by multi-colour flow cytometric profiling of cells from spleen and draining lymph nodes. In this project, such techniques were developed and applied to groups of mice receiving IM allo-MSC or relevant control injections at the time of euthanasia.

2.2 MATERIALS AND METHODS

2.2.1 *Mouse Strains and Ethical Approval*

The aim of this study was to establish an allogeneic mouse model to exclusively test the immunogenicity IM-delivered allo-MSC. For this purpose, two fully MHC mis-matched mouse strains were used. C57BL/6 mice (B6; MHC - H-2^b) served as the donor strain and BALB/c mice (MHC - H-2^d) served as the recipient strain **(fig. 2.2)**. All animal experiments were carried out with approval from the Animal Care Research Ethics Committee (ACREC) of the National University of Ireland, Galway (NUI Galway) and under appropriate individual license and project authorisations from Health Products Regulatory Authority (HPRA) of Ireland. Adult female mice were purchased for all experiments from Charles River Laboratories, United Kingdom.

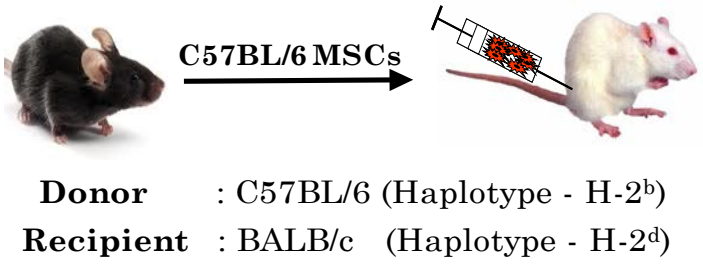


Figure 2.2: Fully MHC-mismatched mouse strains for IM injection of allo-MSCs.

2.2.2 *MSC Isolation and Culture*

Primary B6 MSCs were isolated from bone marrow (BM) according to the method originally described by Peister et al. and modified by our collaborators at NUI Maynooth and characterization of B6 MSC were performed in collaboration with Dr.Clara Sanz Nogues, NUI Galway (Peister *et al.*, 2004, English K *et al.*, 2009 and Kavanagh & Mahon, 2010). Primary BALB/c MSC were isolated from bone marrow according to a similar protocol by our collaborators the Regenerative Medicine Institute (REMEDI) NUI Galway. A brief methodology for the isolation of mouse MSC is as follows: On day 0, femurs and tibiae from 6-8 week old mice were removed and excess tissue cut away. Marrow was flushed from the bones using a 27 gauge (G) needle filled with complete isolation

medium (CIM) (see Appendix 4 for details of media and buffer compositions). An 18G needle was used to break up clumps.

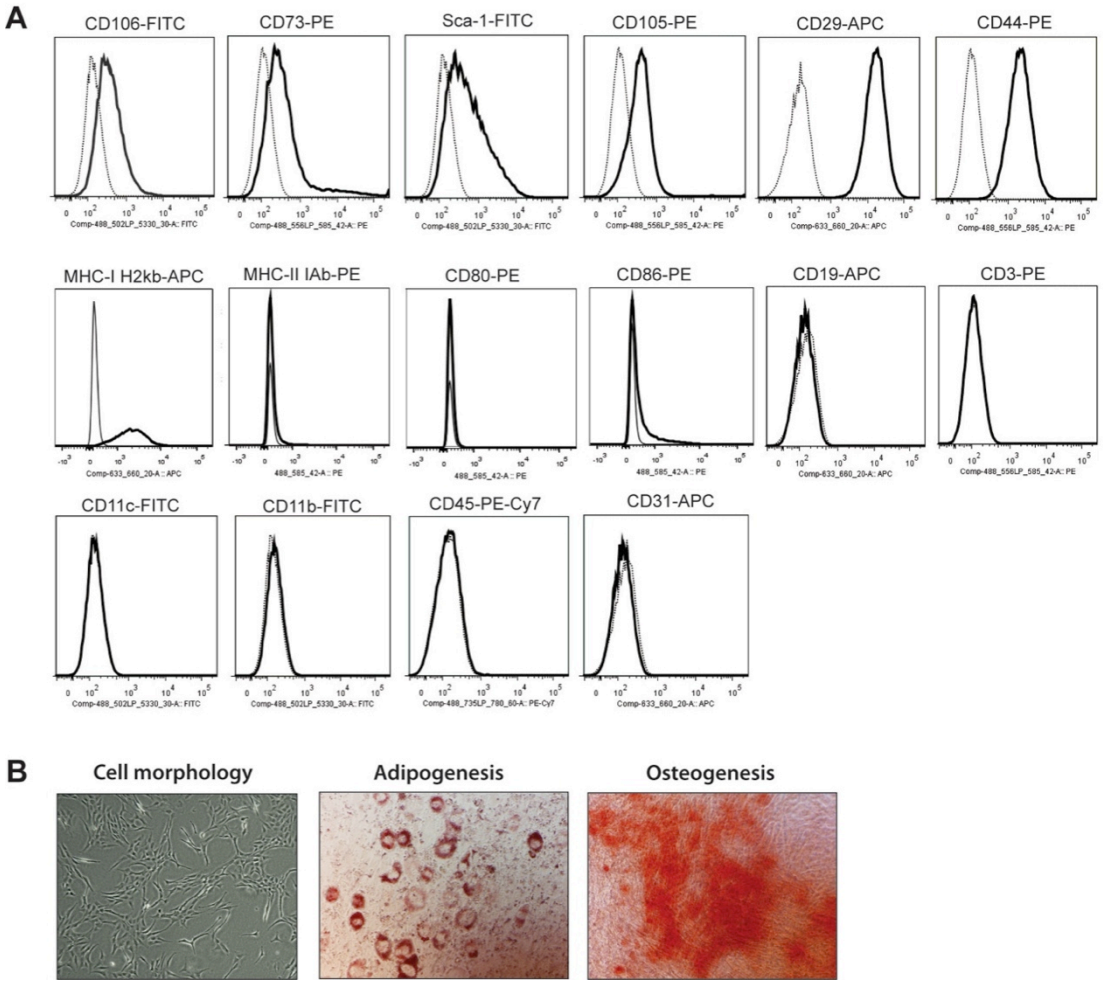


Figure 2.3. ***In vitro*** **characterization of C57BL/6 MSC**. **(A)** Cell surface markers of mMSC characterised by FACS analysis. Isotype control staining (dotted line) was compared with the specific antibody staining profile (heavy line). **(B)** Morphology of C57BL/6 cells, and dipogenic and osteogenic differentiation capacity detected by Oil Red O and Alizarin Red staining, respectively.

Centrifugation was performed at 600 x relative centrifugal force (RCF) for 5 minutes. Passage (P) 0 cells were counted and plated at 20 x 10^6/T25 vented flask in 8 millilitres (ml) CIM. Non-adherent cells were removed on day 1. The attached cells were washed with sterile Dulbecco's phosphate buffered saline (D-PBS) (see Appendix 2 for list of reagents and suppliers). Fresh pre-warmed CIM was added and changed every 3-4 days. Cells were split after 4 weeks using 0.25% trypsin/EDTA for 2 minutes at 37°C. Cells were neutralized with CIM and centrifuged at 600 x RCF for 5 minutes. Passage 1cells were counted and plated at 1 x 10^6/T25 in CIM. Culture medium was exchanged every

3-4 days for 2 weeks. Cells were split and re-plated at 1 x 10^6/T25 or 5 x 10^6/T75 in 8 ml or 15 ml respectively in complete expansion medium (CEM). Passage 2 cells were expanded to P5, cryopreserved in freezing medium and then were subsequently thawed and expanded in culture for use for experiments. Cultured cells were also analysed for phenotypic and morphological characterization. Initially, MSC were analysed for the expression of a panel of cell surface antigens as shown in **fig 2.3A**. Results showed that the cells were strongly positive for CD29 and CD44 and weakly positive for CD106, CD105, CD73, Sca-1 and anti H2-K^b. The cells lacked expression of the endothelial cell marker CD31 and the co-stimulatory molecules CD80 and CD86, as expected. Flow cytometric analysis also showed that there were no contaminating hematopoietic population, as staining for typical hematopoietic markers including CD45, CD11b (granulo-monocytic cells), CD11c (dendritic cells), CD3 (T-cells), CD19 (B-cells) was negative. The cellss were also tested for their differentiation capacity. Before differentiation, bright field microscopy (Olympus CKX41) demonstrated an homogeneous population of adherent cells with fibroblast-like, spindle-shaped morphology.No contaminating hematopoietic cell populations were observed (Figure 2.3B). Finally, we investigated the capacity of C57BL/6 to differentiate into osteocytes and adipocytes (**fig 2.3B**). Cells cultured with osteo-inductive medium underwent a dramatic change in cellular morphology which was accompanied by the presence of calcium-rich deposits, as indicated by Alizarin Red staining. When cultured in adipogenic medium, the cells accumulated intercellular lipid vacuoles which were positively stained by Oil Red O stain. All experiments were carried out with passage (p)6 to p9 MSC grown to 80% confluence in supplemented Iscove's modified Dulbecco's medium (IMDM).

2.2.3 *Basic Experimental Design and Cell Administration*

Cells or equal volumes of vehicle (0.9% NaCl, "saline") were injected into the thigh muscles of mice under general anaesthesia. Culture-expanded MSCs (p6-p9) were lifted from tissue culture flasks by trypsinization and live cells were counted on a haematocytometer by Trypan blue exclusion. The cells were washed and re-suspended in the saline at a concentration of 10^6 cells/200 μl. At the same time, a B6 mouse was euthanized, the spleen was removed by dissection. A red blood cell (RBC)-free single

suspension of splenocytes was prepared by gently pushing the spleen between the two pieces of 150 μM nylon mesh using the end of a sterile syringe plunger into 2ml of saline. Single cells were then pelleted by centrifugation for 5 minutes at 500 x g and were subjected to RBC lysis by re-suspending in 4 ml of 1X ACK lysis buffer for 2 minutes at room temperature (RT) following which an equal volume of saline was added. The cell suspension was centrifuged for 5 minutes at 500 x g and re-suspended in saline then counted on a haemocytometer with Trypan blue exclusion and adjusted to a concentration of 10^6 cells/200 μl.

Groups of BALB/c mice (n=4) received a total of 10^6 B6 (allo)-MSC, B6 (allo)-splenocytes, BALB/c (syn)-MSCs or an equivalent volume of saline (vehicle) by IM injection **(table 2.1 & fig. 2.4)**. Recipient animals were anaesthetized with intra-peritoneal (i.p.) ketamine (75-100 mg/kg) and xylazine (10mg/kg). Intra-muscular injections were carried out at five individual sites [40μl ($2x10^5$ cells) each] in the left thigh muscle. The procedure was carried out by Dr.Xizhe Chen, Senior Research Fellow, REMEDI, NUI Galway. As shown in **fig. 2.4**, two injections were made in the inner part of the thigh muscles and three in the outer part.

Table 2.1 Groups of BALB/c recipients

Type of Infusion (Cell type)	Anticipated anti-donor immune response
Saline	None
Syn – MSC	None
Allo – splenocytes	Strong
Allo – MSC	Unknown

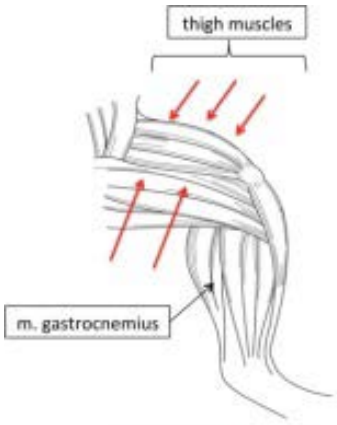


Figure 2.4: IM site of injection

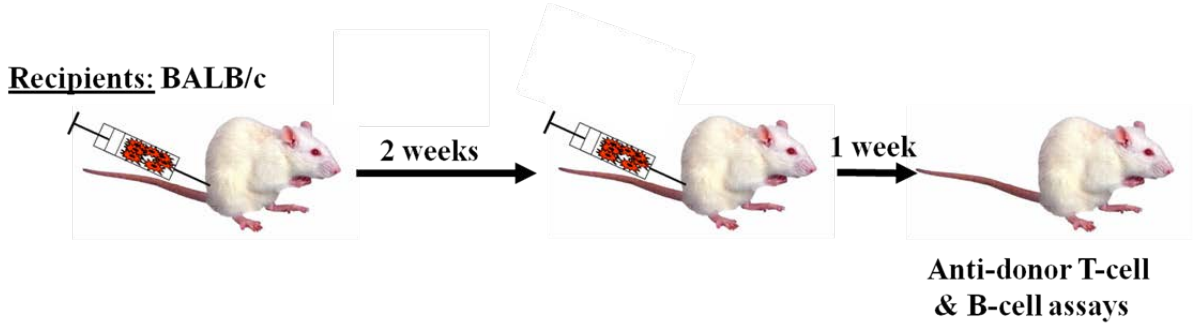


Figure 2.5: Basic design of the *in vivo* experiments.

A typical experimental design is shown in **fig. 2.5**. Eight to 12 week old BALB/c mice received IM cell or vehicle injections at two time-points two weeks apart. After an additional week (21days following the first injections), the animals were humanely euthanized and immune responses were evaluated using the assays described below. Animals were euthanized by terminal exsanguination under deep anesthesia using ketamine (75-100mg/kg i.p) and xylazine (10mg/kg i.p) and cardiac puncture was performed to collect blood samples for anti-donor antibody assays. Secondary lymphoid organs (spleen, ipsilateral and contralateral inguinal lymph nodes) were removed for multi-colour flow cytometric analysis and for anti-donor T-cell assays. Left (injected) and right (non-injected) thigh muscles were dissected for histological analyses. Dissected spleens and lymph nodes were kept on ice in sterile culture medium prior to processing. Blood samples were collected in sterile 1.5ml Eppendorf® tubes and were left at room temperature for a minimum of hour and half to allow clotting to occur following which serum was removed by pipetting and was transferred to cryotubes and stored at -80^{o}C until use. Thigh muscles were transferred into 10% neutral buffered formalin in advance of processed for the histological analysis.

2.2.4 *One-Way Mixed Lymphocyte Reactions (MLR)*

Spleens from the recipient mice were dissected and homogenized into single cell suspension as described in Section 2.2.3. The resulting splenocyte suspensions were labelled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) using the CellTrace CFSE cell proliferation kit® (Invitrogen, Paisley, UK). For CFSE labelling, the splenocytes were counted and re-suspended in pre-warmed 0.1% bovine serum albumin (BSA)/PBS (1 x 10^6 cells /ml). Dimethyl sulfoxide (DMSO) was used to reconstitute the CFSE and this was added to the cells (2 µl/10^6 cells). The cells were incubated in a water bath at 37°C for 10 minutes. Ice-cold 10% serum-supplemented culture medium was then added using twice the volume of BSA/PBS solution and the cells were centrifuged at 400 RCF and washed twice more in culture medium before being re-counted prior to experimentation.

CFSE-labelled splenocytes were co-cultured with magnetic column-purified $CD11c^+$ DCs from B6 spleen. Briefly, RBC-free single cell suspensions were prepared from freshly dissected B6 spleens in MACS buffer as described above and were incubated with anti-mouse CD11c micro-beads (Miltenyi Biotec, Cologne, Germany) for 30 minutes at 4°C. The cells were then washed and re-suspended in MACS buffer and pipetted into a MS magnetic column placed in a MiniMACS cell separation device (Miltenyi Biotec). The column was washed three times with 400 µl MACS buffer following which the column was removed from the magnetic field and the bound ($CD11c^+$) cells were eluted with 400 µl MACS buffer into sterile 15 ml Falcon® tubes then washed and re-suspended in T-cell culture medium and counted.

MLRs were prepared in 96-well, round-bottome tissue culture plates by mixing freshly-prepared CFSE-stained BALB/c splenocytes ("responders") with B6 $CD11c^+$ splenic DCs ("stimulators") at varying ratios in a total volume of 200µl/well. For optimisation experiments, the following DC:T-cell ratios were prepared: 1:10, 1:20, 1:40, 1:80 and 1:160 (1x10^5 responders/well with 10,000, 5000, 2500, 1250, 625 stimulators/well respectively). The plates were placed in a tissue culture incubator at 37°C, 5% CO_2 following which the cells were transferred to 1.5 ml Eppendorf tubes for staining with fluorochrome-labelled antibodies against CD4 and CD8. Briefly, the cells were washed and re-suspended in FACS buffer, incubated with optimised amounts of anti-mouse CD4 and anti-mouse

CD8 for 30 minutes on ice (see Appendix 3 for details of all antibodies used) then again washed and re-suspended in FACS buffer before being analysed by three-colour flow cytometry (FCM). Flow cytometric analysis was performed on a Becton Dickinson (BD) Biosciences FACSCanto® cytometer (San Jose, CA, US) with data analysed by FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR).

2.2.5 *Immune Profiling of Cells From Lymph Node And Spleen*

Single-cell suspensions were prepared from spleen and lymph nodes as described above. For FCM, the cells were suspended in FACS buffer at 10^6 cells/ml and 100 µl aliquots were incubated with various combinations of fluorochrome-labelled antibodies (see table2.2 and Appendix 3) for 30 minutes at 4°C in 96-well, round bottom plates. The stained cells were washed and re-suspended in FACS buffer prior to being analysed using a BD Biosciences FACSCanto® cytometer and FlowJo® v7.0 software (TreeStar® Inc).

Table 2.2 – Combination of antibodies for immune cell analysis in spleen

FITC	PE	PerCP-Cy5.5	PE-Cy7	APC
CD25	CD62L	CD4	CD8a	CD44
CD11b	CD11c	Ly6C	Ly-6G	F4/80
CD8	CD3-Biotin	CD4	CD19	NK1.1

2.2.6 *Intracellular Flow Cytometry*

For analysis of T-cell cytokine production in MLRs, brefeldin A (GolgiPlug® 1 µl/ml, BD Biosciences) was added for the final six hours culture prior to analysis by flow cytometry. Following removal from culture, the cells were suspended in FACS buffer at 5 x 10^6/ml and were first surface-stained by incubation with various combinations of fluorochrome-labelled antibodies for 20 minutes at 4°C (see Appendix 3). Next, the cells were fixed and permeabilised using Cytofix/Cytoperm® reagents (BD Biosciences) according to the manufacturer's instructions and were incubated with fluorochrome-labelled antibodies against cytokines or, in some experiments, against the T-reg-

specific transcription factor FOXP3. The cells were then washed and re-suspended in FACS buffer prior to being analysed using a BD Biosciences FACSCanto® cytometer and FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR). For analysis of FOXP3 expression by freshly-prepared splenocytes, a similar intracellular staining protocol was used but without prior culture in the presence of brefeldin A.

2.2.7 *Flow Cytometric Assay for Detection of Anti-Donor Immunoglobulin G (IgG)*

Blood samples were collected at the time of euthanasia, allowed to clot at room temperature for 4 hours then separated by centrifugation for 10 minutes at 500 x g following which aliquots of serum were frozen at -80˚C. For flow cytometric detection of anti-donor (B6) IgG antibody in serum, freshly prepared, RBC-free B6 splenocytes were suspended at 8 x 10^6 cells/ml in FACS buffer (1x PBS, 2% FCS, 0.05% NaN_3) and were incubated for 30 minutes at 4˚C with either FACS buffer alone (negative control), purified anti-H-$2K^b$ (positive control, clone AF6-88.5, BD Biosciences, Franklin Lakes, NJ) or serum samples diluted 1:100 in FACS buffer. Next, splenocytes were washed twice in FACS buffer then incubated for 30 min at 4˚C with goat anti-mouse IgG Fc $F(ab)_2$-FITC (Beckman Coulter, Brea, CA) at an optimised dilution of 1:400 in addition to anti-mouse T-cell Receptor (TCR)-β- PE (BD Biosciences) to allow discrimination of T-cells and non-T-cells. Finally, stained splenocytes were again washed twice with FACS buffer, re-suspended in 0.5 ml of FACS buffer and analysed immediately on a Becton Dickinson FACSCanto™ flow cytometer. Analysis was carried out using FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR) with FITC fluorescence intensity of TCRβ-negative cells (B-cells) compared between negative control sample and individual serum-incubated samples.

2.2.8 Complement Lysis Assay

Red blood cell-free B6 splenocytes were prepared as described above for use as “target cells”. The splenocytes were re-suspended at 5 × 10^5 /ml in 1X PBS, 2% FCS, 0.05% NaN_3 and were incubated either with serum samples from the recipient mice at different concentrations (final dilution of 1:5, 1:10 and 1:20 in PBS), with PBS alone (negative control) or purified anti-H-$2K^b$ (positive control).

Next, cells were washed to remove unbound serum antibodies and incubated in 20% baby rabbit complement (AbD Serotec, UK) in 1X PBS at a final volume of 100 µl for 1hr at 27°C following which 2 ml of ice cold PBS were added. The cells were pelleted by centrifugation, re-suspended in 50 µl PBS, stained with 0.5 µg propidium iodide/sample and analysed immediately on a Becton Dickinson FACSCanto™ flow cytometer. Analysis was carried out using FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR).

2.2.9 Muscle Tissue Staining with Haematoxylin and Eosin (H&E)

The thigh muscles were dissected from each mouse following euthanasia and were divided into three sagittal portions which were then fixed in 10% formalin, processed in a Leica™ Biosystems™ ASP300 tissue processor and embedded in paraffin wax (Leica™ Biosystems™ EG1150H). Paraffin tissue blocks were then cut into 5-10 µM sections onto polysine microscope adhesion glass slides (Thermo Scientific™, Ireland) using a microtome (Leica™ Biosystems™ RM2235). The sections were washed twice in xylene for ten minutes at RT, followed by two two minute washes in absolute alcohol. The slides were then rehydrated in graded alcohol baths (from 95% to 70% to 50%) for two minutes each and residual alcohol was removed in running distilled water for two minutes. Next, tissue sections were stained with Mayer's haematoxylin (Sigma-Aldrich® Ireland) for six minutes, washed in running water for four minutes then stained with eosin Y (Sigma-Aldrich® Ireland) for two minutes. Stained tissue sections were then hydrated with graded alcohol baths (from 50% to 70% to 95%) for two minutes each. Finally tissue sections were washed with twice in xylene for fifteen minutes and covered with DPX mounting media (Sigma Aldrich® Ireland) cover- slipped and left to dry overnight for microscopic analysis.

2.2.10 *Semi-quantitative Grading of H&E-stained Sections*

Analysis of the H&E-stained tissue sections was carried out by grading multiple sections from each muscle tissue sample in blinded fashion for foci of inflammation (0-4) under bright field microscopy (Olympus™ Ireland) at 10X magnification. The H&E stained sections were photographed using imaging software (Olympus™ CellB™). Fifteen to twenty sections from each animal were graded. The final scores, representing the average of the scores of the each section, were analysed using

Graphpad Prism® 5 (GraphPad Software, Inc., La Jolla, CA, USA). The following *g*rading scale was used to assess muscle histological abnormalities:

Inflammation:

0 = no inflammation

1 = single small focus of inflammation

2 = multiple small foci or single moderate focus of inflammation

3 = multiple moderate foci or single large focus

4 = multiple large foci or widespread inflammation throughout the section

2.2.11 *Statistical Analysis*

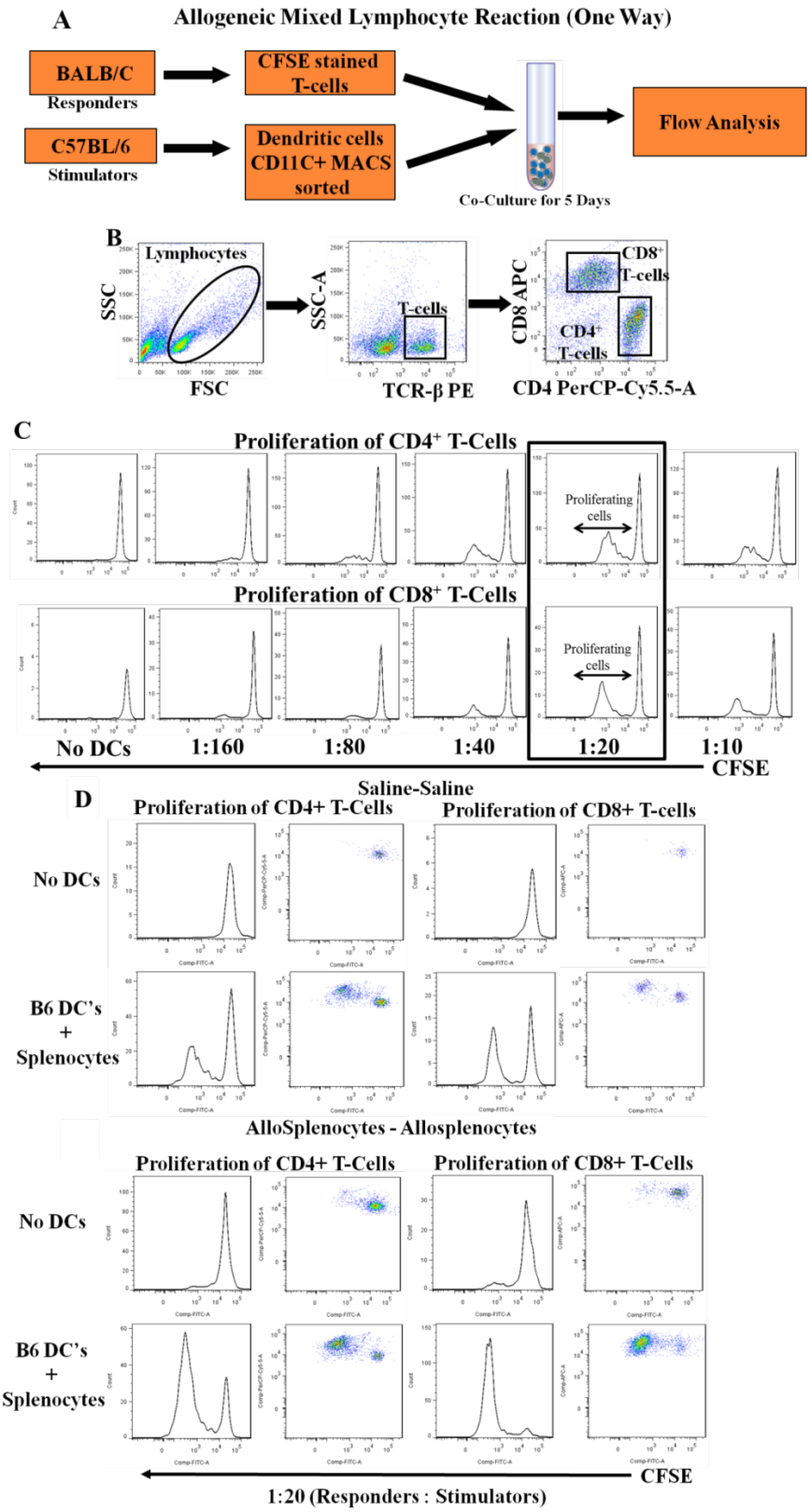
Results were expressed throughout as mean ± standard deviations (SD) and differences among multiple groups were tested statistically by ANOVA with post-hoc testing. Difference between two conditions were statistically analysed by two-tailed Student's t-test. All statistical analyses were performed with GraphPad Prism® 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). Significance was assigned at $p < 0.05$.

2.3 RESULTS

2.3.1 *Optimization of Allogeneic MLRs*

To standardize an *in vitro* one-way allogeneic MLR, prior to performing *in vivo* studies with allo-MSCs, a trial allogeneic MLR assay was performed with CFSE-labelled BALB/c "responders" co-cultured for five days with B6 "stimulators" (DCs) at varying ratios. Proliferation of helper and cytotoxic responder T-cells was analysed by flow cytometry based on CFSE dilution among the $TCR\beta^+/CD4^+$ and $TCR\beta^+/CD8^+$ cell populations **(fig. 2.6A & B)**. Proliferation in the absence of DCs and at the varying DC:T-cell ratios was quantified as the percentage of CFSE-diluted (% divided) among the gated populations **(fig. 2C)**. As shown, there was minimal proliferation in the absence of stimulators, while the highest rate of proliferation in this preliminary experiment occurred at a DC:T-cell ratio of 1:20 and this ratio was selected for subsequent re-call experiments.

Next, the assay was applied to splenocytes from groups of BALB/c mice that were injected repeatedly IM with saline or with B6 allo-splenocytes as detailed in fig – 2.5 and table – 2.1. As shown in **fig. 2.6D**, proliferation of $CD4^+$ and $CD8^+$ T-cell co-cultured with B6 DCs at 1:20 DC:T-cell ratio was greater for responders from mice that had received IM injections of allo-splenocytes than those from mice injected with saline. As an additional demonstration of the sensitivity of this analysis approach to demonstrate differences in donor DC-induced *in vitro* proliferation of T-cells from cell-treated and control animals, individual CFSE histograms were divided into"responding" and "non-responding" $CD4^+$ or $CD8^+$ cells and the percentages of each were calculated. In the example shown in **fig.2.6E & F**, the results of this analysis are shown for groups of BALB/c mice that received two sequential IM injections of either Saline ("Saline-Saline"), B6 splenocytes ("AlloSpleen-AlloSpleen) or B6 MSC (AlloMSC-AlloMSC). As shown, double injection of Allo-splenocytes resulted in increased B6 DC-induced proliferation of $CD4^+$ and $CD8^+$ splenic T-cells compared to the saline control group while double injection of allo-MSC had the opposite effect.


A
Allogeneic Mixed Lymphocyte Reaction (One Way)
BALB/C
Responders
C57BL/6
Stimulators
CFSE stained T-cells
Dendritic cells CD11C+ MACS sorted
Co-Culture for 5 Days
Flow Analysis
B
Lymphocytes
SSC
FSC
SSC-A
T-cells
TCR-β PE
CD8 APC
CD8+ T-cells
CD4+ T-cells
CD4 PerCP-Cy5.5-A
C
Proliferation of CD4+ T-Cells
Proliferating cells
Proliferation of CD8+ T-Cells
Proliferating cells
No DCs
1:160
1:80
1:40
1:20
1:10
CFSE
D
Saline-Saline
Proliferation of CD4+ T-Cells
Proliferation of CD8+ T-cells
No DCs
B6 DC's + Splenocytes
AlloSplenocytes - Allosplenocytes
Proliferation of CD4+ T-Cells
Proliferation of CD8+ T-Cells
No DCs
B6 DC's + Splenocytes
CFSE
1:20 (Responders : Stimulators)

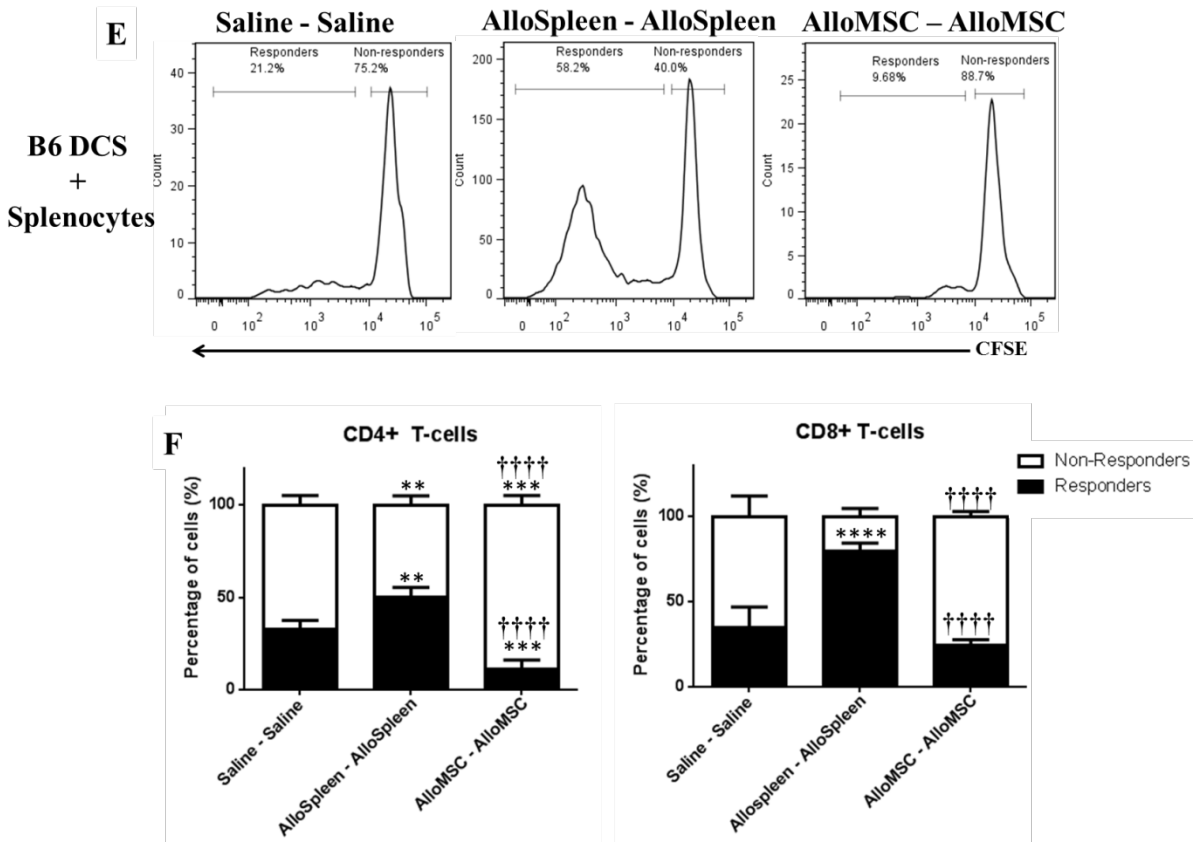


Figure 2.6: One-way Allogeneic Mixed Lymphocyte Reactions: A. CFSE-labelled BALB/c splenocytes were co-cultured with B6 DCs in different ratios for 5 days following which T-cell proliferation was analysed by multi-colour flow cytometry. **B.** Representative dot plots illustrating the gating strategy for $CD4^+$ and $CD8^+$ T-cells. **(C)** Representative histograms illustrating CFSE dilution of $CD4^+$ and $CD8^+$ T-cells from MLR with no DCs or with varying DC:T-cell ratios. **(D)** Representative histograms and dot plots from 5-day MLRs illustrating proliferation of $CD4^+$ and $CD8^+$ T-cells from BALB/c recipients of repeated IM injections of vehicle (saline-saline) or B6 splenocytes (Allosplenocytes-Allosplenocytes). **(E)** Representative CFSE histograms of $CD4^+$ responders and non-responders to B6 DC stimulation in the MLR from treatment groups. **(F)** Proportions of Responder and Non-responder $CD4^+$ T-cells and $CD8^+$ T-cells expressed as mean ± SD percentage (%) of total cells based on CFSE dilution. **Statistics**: One-Way ANOVA, *, **, *** = $p < 0.05, 0.01, 0.001$ compared to saline control group and †, ††, ††† = $p < 0.05, 0.01, 0.001$ compared to appropriate double-injected allo-splecnocyte group.

2.3.2 *Semi-quantitative Anti-Donor IgG assay*

To develop a flow cytometry based semi-quantitative anti-donor antibody assay, B6 splenocytes were used as a target cells and graded amounts of purified anti- BALB/c MHC-I ($H\text{-}2k^b$) were initially used to determine the sensitivity of the assay and to generate a "standard curve" for assignment of quantitative units to results for anti-B6 antibodies within different mouse sera. A fluorescence signal was generated by secondary staining with a fluorochrome-labelled anti-mouse IgG Fc antibody. Because of the varying expression levels of MHC proteins by T-cells and B-cells, the target cells were

also stained for TCRβ to allow for separate fluorescence read-outs from these two target cell types (analogous to T-cell and B-cell flow cytometric cross-matching used in human transplantation practice) (Takakura K *et al.*, 2001). Following trial experiments, it was concluded that fluorescence quantification from $TCR\beta^-$ gated cells (B-cells) provided the most sensitive and reproducible detection of anti-B6 IgG.

Figure 2.7A & B illustrates the basic protocol and the gating strategy for this assay. As shown in **fig. 2.7C**, the assay protocol resulted in variable, readily detected fluorescence shifts within the $TCR\beta^-$ gated cells following target cell incubation with purified anti-H-$2k^b$ at final concentration between 14 and 0.02 ng/ml. When fluorescence intensity of the $TCR\beta^-$ cells was plotted against concentration of anti-H-$2k^b$, a typical sigmoid-shaped standard curve was observed (**fig. 2.7D**).

Furthermore, when target cells were incubated with sera (diluted 1:64) from BALB/c mice that had been injected IM with saline or with B6 allo-splenocytes (as detailed in fig – 2.5 and table – 2.1), a positive fluorescence shift was observed for the sample from the allo-splenocyte recipient only. As shown in fig – 2.7D, plotting of the assay results from the two test sera on the standard curve allowed for attribution of an "anti-B6 IgG concentration" to the positive sample.

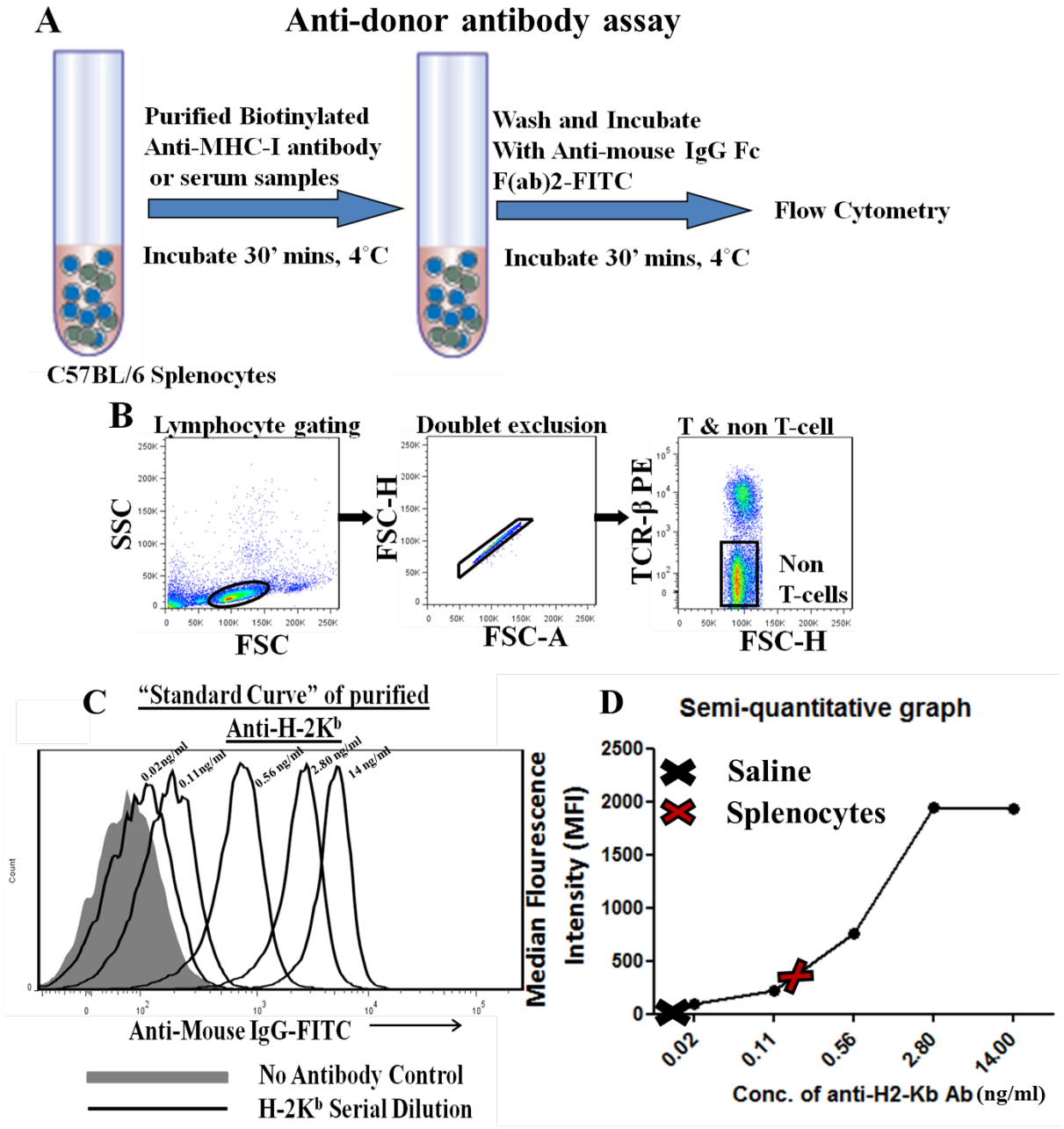


Figure 2.7: Development of a quantitative flow cytometry-based assay of anti-B6 IgG antibody in mouse serum: A. Purified anti- H-2k^b or diluted samples of mouse serum were incubated with B6 splenocytes followed by anti-IgG Fc-FITC and anti-TCRβ-PE then analysed by flow cytometry. **B.** Representative dot plots illustrating the gating strategy used for analysis of fluorescence shift among TCRβ- cells. **C.** Representative histograms illustrating the fluorescence shifts on the FITC channel of B6 TCRβ$^-$ cells incubated with no primary antibody (shaded histogram) or with concentrations of anti-H-2K^b from 0.02 to 14 ng/ml. **D.** Graphical plot of mean fluorescence intensities (MFI) versus anti-H-2K^b concentrations for the histogram shown in C. Fluorescence intensities of target cells incubated with 1:64 diluted sera from a BALB/c recipient of saline (black cross) or from a BALB/c recipient of B6 allo-splenocytes (red cross) are overlaid on the plot. The data shown in C. and D. are representative of results from 6 independent experiments.

2.3.3 *Optimization of Immune Cell Profiling in the Spleen*

On-going systemic innate and adaptive immune responses at the time of euthanasia of MSC-injected and control animals were assessed by analysis of the cellular repertoires of the spleens. Splenic proportions of myeloid and lymphoid lineage immune cells and their subpopulations were analysed by multi-colour flow cytometry using staining panels for characteristic surface and intracellular markers. For myeloid lineage cells, surface staining was carried out with a panel of fluorochrome-labelled antibodies against Ly6G, CD11b, CD11c and F4/80. The major myeloid cell types were defined as follows (see **fig. 2.8A** for representative examples of gating):

1) Monocytes: $Ly6G^-$ $CD11b^+$ $CD11c^-$ $Ly6C^{hi}$ $F4/80^{mid\text{-}low}$ (**fig. 2.8A** –Population IV)
2) Macrophages: $Ly6G^-$ $CD11b^+$ $CD11c^-$ $Ly6C^{lo}$, $F4/80^+$ (**fig. 2.8A** – Population V)
3) Dendritic cells: $Ly6G^-$ $CD11b^-$ $CD11c^+$ (**fig. 2.8A** – Population VII)
4) Neutrophils: $CD11b^+$ $Ly6G^+$ (**fig. 2.8A** – Population VIII)

As shown in **fig. 2.8A**, analysis of neutrophils required a broader initial scatter gate due to the higher level of granularity of these cells, following which a tight cluster of $CD11b^+Ly6G^+$ neutrophils was readily distinguished. Analysis of $Ly6G^-CD11b^+$ cells revealed three distinct clusters based on expression of Ly6C and F4/80. Based on established descriptions from the literature, two of these were designated as monocytes and macrophages as defined above. The third, $Ly6C^-$ $F4/80^-$ population was considered to be indeterminate but likely represents an additional population of splenic macrophages. Finally, $CD11c^+$ cells negative for Ly6G and CD11b were designated as DCs.

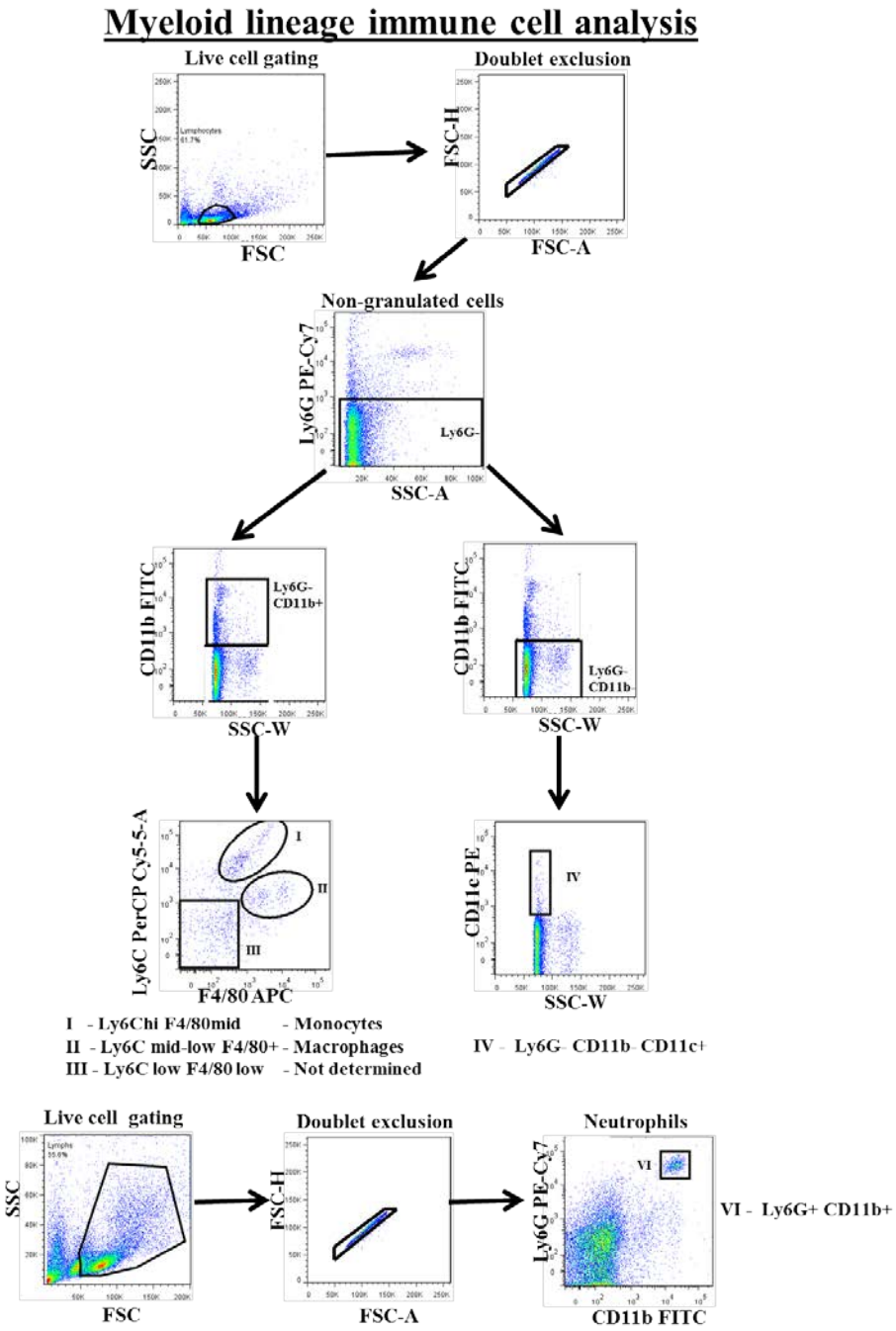


Figure 2.8A: Myeloid lineage immune cell profiling: Representative flow cyometry dot plots illustrating the gating strategies for major myeloid cell populations of spleen. Individual gated populations are numbered I-VI Data is representative of staining patterns from one of four separate *in vivo* studies in which this panel provided consistent results.

As illustrated in **fig. 2.8B**, splenic T-cells and their subpopulations were analysed by multi-antibody staining panels with antibodies against CD4 and CD8 initially used to distinguish the two major subsets ("helper" and "cytotoxic"). Staining for CD62L was then used to distinguish naïve (high CD62L expression) from memory or activated (low CD62L expression) within each subset. Next,

among the $CD62L^{hi}$ and $CD62L^{lo}$ subpopulations, expression of CD44 and CD25 were used to delineate four separate populations as listed below. High expression of CD44 was considered to indicate clonally expanded T-cells with CD25 co-expression indicating recent activation. As expected, the proportions of CD4 and CD8 T-cells with $CD44^{hi} \pm CD25^{+}$ phenotype were higher among the CD62Llo subpopulations (**fig. 2.8B**, lower panels). Identification of regulatory T-cells (T-reg) was achieved by intra-cellular staining for transcription factor Foxp3 as shown in **fig. 2.8B**, right panels.

In total, the following T-cell subsets and subpopulations were identified:

1) T-cells - $CD4^{+}$ and $CD8^{+}$ (T-cell discrimination marker)

2) $CD4^{+}$ subsets - $CD4^{+}$ $CD8^{-}$ $CD62L^{lo}$ (Memory and activation marker phenotype)
   1. $CD4^{+}$ $CD8^{-}$ $CD62L^{lo}$ $CD44^{hi}$ $CD25^{-}$
   2. $CD4^{+}$ $CD8^{-}$ $CD62L^{lo}$ $CD44^{hi}$ $CD25^{+}$
   3. $CD4^{+}$ $CD8^{-}$ $CD62L^{lo}$ $CD44^{mid}$ $CD25^{+}$
   4. $CD4^{+}$ $CD8^{-}$ $CD62L^{lo}$ $CD44^{mid}$ $CD25^{-}$

3) $CD8^{+}$ subsets - $CD8^{+}$ $CD4^{-}$ $CD62L^{lo}$ (Memory and activation marker phenotype)
   1. $CD8^{+}$ $CD4^{-}$ $CD62L^{lo}$ $CD44^{hi}$ $CD25^{-}$
   2. $CD8^{+}$ $CD4^{-}$ $CD62L^{lo}$ $CD44^{hi}$ $CD25^{+}$
   3. $CD8^{+}$ $CD4^{-}$ $CD62L^{lo}$ $CD44^{mid}$ $CD25^{+}$
   4. $CD8^{+}$ $CD4^{-}$ $CD62L^{lo}$ $CD44^{mid}$ $CD25^{-}$

4) T-reg cells - $CD4^{+}$ $CD8^{-}$ $Foxp3^{+}$ (Regulatory T-cell marker)

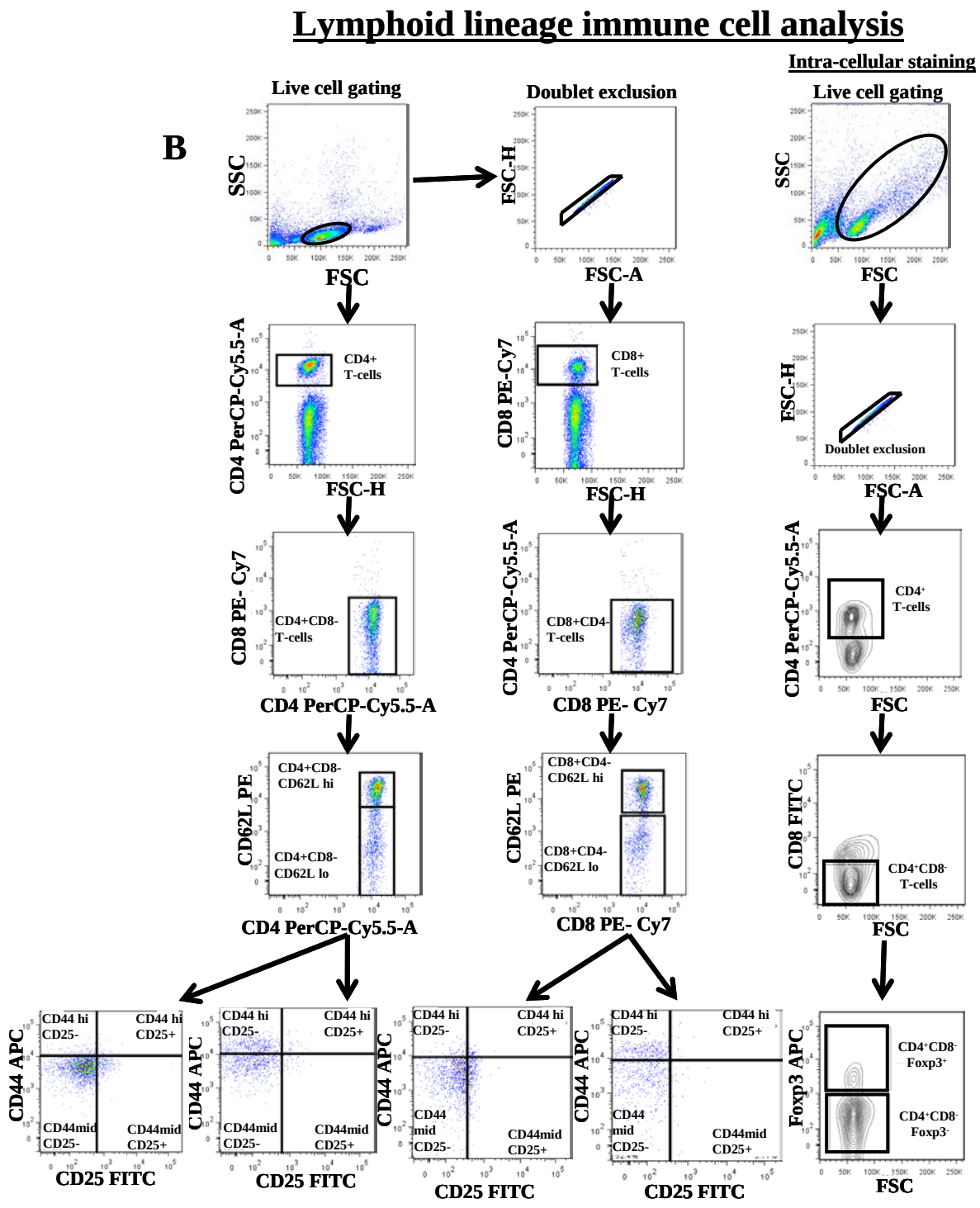


Figure 2.8B: Lymphoid lineage immune cell profiling: Representative flow cyometry dot plots illustrating the gating strategies for major lymphoid cell populations of spleen. Individual gated populations are numbered. Data is representative of staining patterns from one of four separate in vivo studies in which this panel provided consistent results.

2.3.4 *Complement Mediated Lysis Assay*

Complement mediated lysis assays may be used to determine whether antigen-induced antibodies are capable of causing lysis of cells bearing their target antigens via complement fixation. In order to develop an assay system for functionally testing anti-donor antibody induced by B6 allo-MSC, B6 splenocytes were used as a target cells and were incubated with serum samples from injected mice or, as a positive control, with purified anti-H-2K^b as described in the methods section. Following removal of the serum or purified antibody, the "target cells" were incubated with a rabbit complement preparation before being analysed for cell death using propidium iodide (PI) exclusion. The assay system and representative results are summarised in **fig. 2.9A&B**.

Initially, to standardise the assay, the complement preparation was titrated (0 – 50%) on B6 splenocytes pre-incubated with two different dilutions of purified anti-H-2K^b (1:20 and 1:40). As shown in **fig. 2.9C**, pre-incubation with either 1:20 or 1:40 anti-H-2K^b was associated complement dose-dependent increases in cell death with the %PI$^+$ being higher for 1:20 than for 1:40 anti-H-2K^b at in the presence of 10%, 20% or 50% complement. At 50% complement, some increase in non-specific lysis was noted for cells not pre-incubated with anti-H-2K^b. Thus, in subsequent experiments involving serum samples from cell-injected mice, 1:20 dilution of purified anti-H-2K^b was used as a positive control and 20% complement preparation was used for all experimental samples.

To evaluate the assay for our experimental purposes, B6 splenocytes were pre-incubated with serum from two mice – one of which had received IM injection of B6 splenocytes as previously described and one of which had received IM saline injection. For the pre-incubation step, the serum samples were diluted to 1:10, 1:50, 1:100, 1:500 and 1:1000. As shown in **fig. 2.9D**, serum from the allo-splenocytes injected animal but not from the saline injected animal produced a dose-dependent lysis of B6 splenocytes.

It was concluded that the assay provided a sensitive quantitative read-out of the complement fixing capacity of anti-donor antibodies induced in BALB/c mice following IM injection of B6 cells.

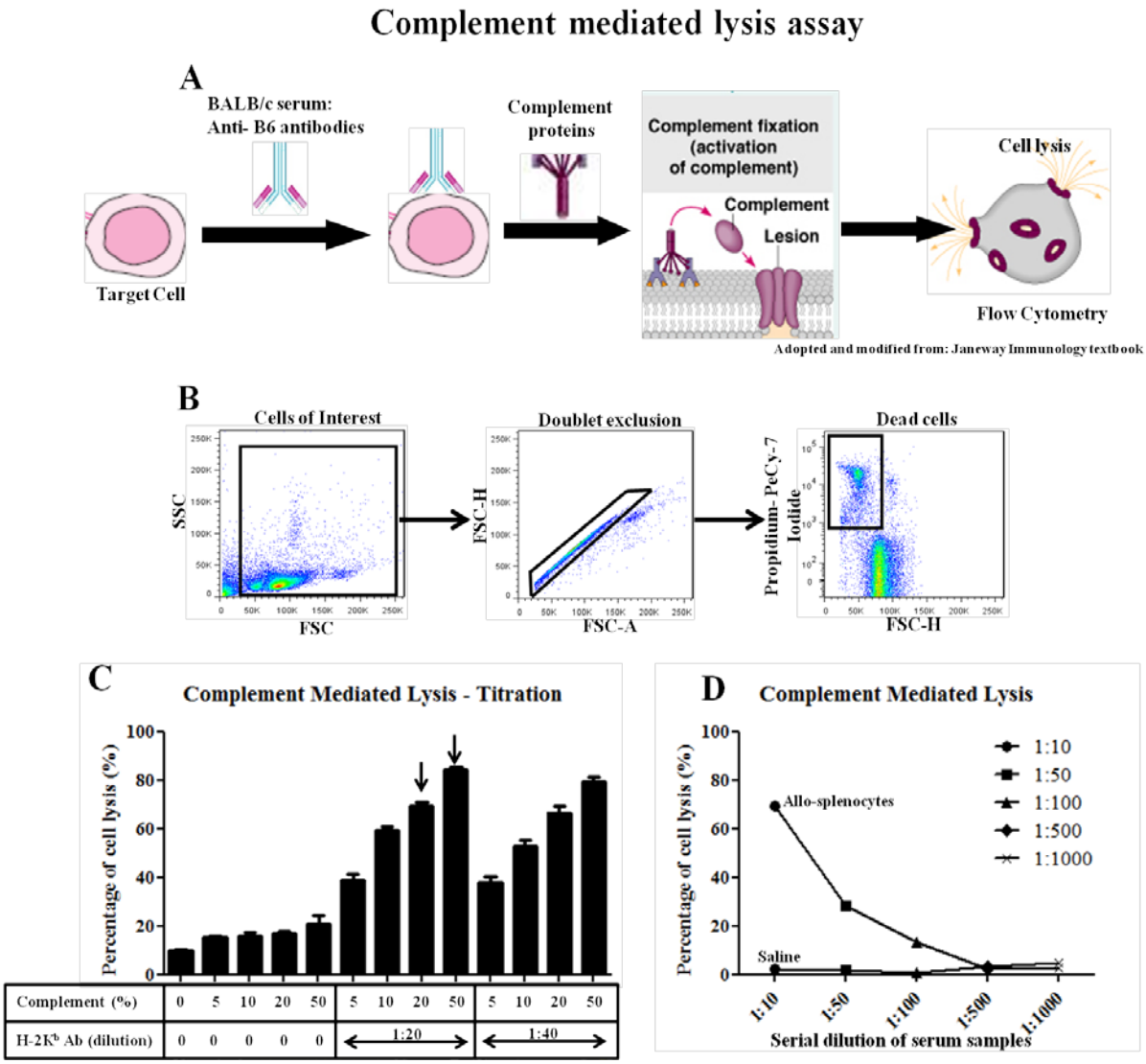


Figure 2.9: Complement mediated lysis assay using B6 splenocytes as targets: A. Schematic illustration of the basic principle of the assay. **B.** Representative dot plots illustrating the gating strategy used to determine the rate of cell death by PI exclusion. **C.** Results for a titration experiment using non-antibody incubated cells and cells incubated with 1:20 or 1:40 purified anti-H-2Kb followed by rabbit complement at 0, 5, 10, 20 or 40%. Arrows indicate optimal lysis conditions. **D.** Lysis curves for B6 splenocytes pre-incubated with serum from mouse recipients of IM B6 splenocytes (allo-splenocytes) or Saline. Cell death percentage is shown for serum dilutions of 1:10 to 1:1000. Data for **C** represent mean ± standard deviation from three independent assays. Data for **D** represent results for samples from one of three separate *in vivo* studies.

2.3.5 *Haematoxylin and Eosin (H&E) staining of Muscle Tissue:*

Haematoxylin and Eosin staining of thigh muscle was performed to investigate the localised cellular infiltrates generated following single or repeated IM injections of allo-MSCs and other cells in BALB/c mice. This staining has been used by others for the purpose of evaluating degenerative and inflammatory processes in skeletal muscle (Cosimo De Beri *et al.*, 2003, Vitali Alexeev *et al.*, 2014). In order to comprehensively screen the injected leg muscles, they were divided into three portions following dissection and, from each portion, 18-34 sections were prepared, stained with H&E and graded from 0 to 4 for inflammatory infiltrates by a researcher who was blinded to the experimental groupings (see Methods section). For each experimental animal, the average grade from all sections was calculated.

Figure 2.10 presents representative examples of H&E stained muscle sections with the results of grading for groups of BALB/c mice that had received IM injections of saline or of allo-splenocytes 21 days prior to euthanasia. As shown, allo-splenocyte injection was associated with significantly greater inflammatory infiltration than saline injection. Based on this validation, H&E staining and blinded, semi-quantitative scoring by this approach was used in subsequent experiments to investigate muscle inflammation following IM injection of allo-MSCs.

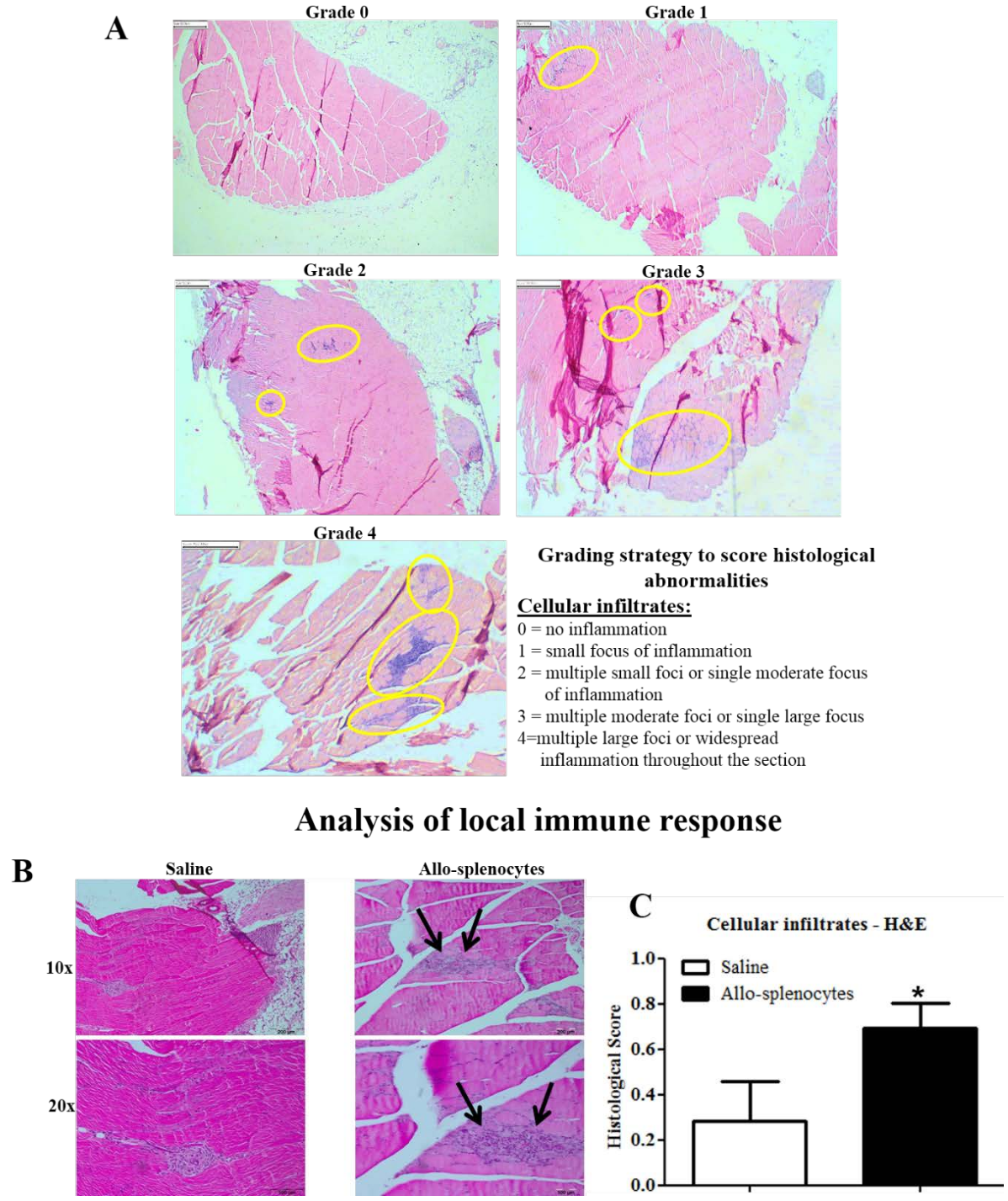


Figure 2.10: Analysis of localised inflammatory cell infiltration by H&E staining of muscle tissue: A. Representative photomicrographs of H&E stained muscle tissue sections from BALB/c mice 21 days following intramuscular injections. Examples are shown at 4x magnification of sections that were graded 0, 1, 2, 3, and 4 for inflammatory cell infiltration based on the grading scheme described above. **B.** Representative photomicrographs of H&E stained muscle tissue sections from BALB/c mice injected IM 21 days previously with Saline (left panels) or with allo (B6)-splenocytes. Examples of 10X and 20X magnification of the same sections are shown. **C.** Graphical representation of the mean ± SD histological scores for saline (n = 10) and allo-splenocytes (n = 10) recipients. * = $P < 0.05$ by unpaired Student's t-test.

2.4 Discussion

In this chapter, we describe the development of an *in vivo* mouse model to study the immunogenicity of IM-delivered allo-MSC in healthy mice using a series of *ex vivo* and *in vitro* assays to quantify and characterise anti-donor cellular and humoral immune responses.

It has been frequently stated that MSC are immune-privileged and fail to stimulate anti-donor immune response (Zappia *et al.*, 2005, OhTaki *et al.*, 2008, Nemeth *et al.*, 2009 and Rafei *et al.*, 2009). However, our own previous reviews of the available literature indicated that studies which employed more rigorous and comprehensive immunological methodologies concluded that allo-MSC do indeed stimulate the activation of donor-specific T-cells and B-cells in a range of different experimental settings (Griffin *et al.*, 2010, Griffin *et al.*, 2013 and Alagesan S *et al.*, 2014). To address this question in a mouse model of hind limb IM injection of allo-MSC, we cultured bone marrow-derived MSC from B6 mice and delivered them into healthy, fully MHC-mismatched BALB/c recipients. Our relevant positive and negative controls included injection of B6 splenocytes (expected to be highly immunogenic), syngeneic MSC from BALB/c bone marrow and vehicle alone. We additionally incorporated the approach of delivering single or repeated injections of cells or vehicle, the experimental results of which are described in the next two Chapters of the thesis. Our rationale in this respect was two-fold: 1. Future therapy of limb ischaemia may include administration of multiple doses of allo-MSC and the immunological impact of repeated injection has, therefore, potential clinical relevance. 2. As is well recognised in the fields of vaccination and transplantation, initial immune sensitisation with a primary antigen exposure may be followed by a more intense (or in some settings reduced) antigen-specific cellular and humoral response on the basis of immunological effector phenotype programming and memory (Nankivell & Alexander., 2010 and Wood *et al.*, 2016). Thus, the immune phenomena associated with repeated administration of allo-MSC may differ significantly from that of a single inoculum.

In the field of transplantation, transfer of solid organs/tissues/cells from a healthy donor to a genetically unrelated individual is consistently associated with either acute rejection or chronic rejection of the graft. (Chapter 13, Janeway's Immunology, Nankivell and Alexander.,2010). In the

case of vascularised, solid organ transplants, rejection may be mediated by donor antigen-specific T-cells (both $CD4^+$ and $CD8^+$), by donor antigen-specific antibodies or by a combination of the two. The dominant ("major") allo-antigens for stimulation of anti-donor immune responses are the class I and class II major histocompatibility complex (MHC) proteins which exhibit a very high level of genetic polymorphism and are also centrally involved in presentation of antigenic peptides to T-cells. In addition, non-MHC proteins containing genetically-encoded amino acid sequence differences between donor and recipient may also serve as a stimulus for allo-immune responses and are typically referred to as "minor" histocompatibility antigens. The use of animal models and *in vitro*/*ex vivo* assays to detect, quantify and functionally characterise donor-specific T-cell and B-cell responses to allo-antigens represents an integral part of transplantation science. However, the extent to which such methods have been applied to study the immunological effects and immunogenicity of allo-MSC, and other stem cell transplants, has been variable compared to the extensive focus on assays of MSC immunosuppressive properties *in vitro* (Mannon *et al.,* 2002, Ara *et al.,* 2005, Poncelet *et al.,* 2007, Griffin *et al.*,2010, Griffin *et al.*,2013 and Valenzuela & Reed., 2013). For the project described in this thesis, the adoption of principles and the development of sensitive assay systems from the field of transplant immunology was an important first step toward better understanding the immunogenicity of IM-delivered allo-MSCs.

The strain combination selected for our experiments can be considered to be inherently highly immunogenic on the basis of genetic sequence differences across all major class I and class II proteins. In the clinical transplant setting, genotyping of class I and class II MHC (HLA) proteins is routinely performed as a strategy to modify overall risk of rejection. (Chapter 13, Janeway Immunology, Fean Villard *et al.,* 2006). The degree of "matching" of donor and recipient HLA genotypes (often referred to as histocompatibility) influences the likelihood of acute rejection as well as the long-term success of the allograft (Neumann *et al.,* 2003). Nonetheless, even full donor/recipient matching for HLA genotyping (as most commonly occurs with living donor transplant between HLA haplo-identical siblings), requires life-long administration of multiple immunosuppressive drugs in order to avoid immune-mediated rejection. In the case of allo-MSC

therapy, the strategy of performing MSC donor genotyping and "matching" of treated patients with optimal donors has been proposed as a means to reduce the potential for anti-donor immune responses (Jacquet *et al.,* 2012 and Wood *et al.,* 2016). However, the overall therapeutic benefit of this approach has yet to be rigorously investigated and it is unclear whether it would justify the large amount of resourcing required. Thus, we believe that it is highly likely that future allo-MSC therapy will typically involve multiple class I and class II MHC mismatches.

In order to characterise anti-donor T-cell responses in naïve and allo-MSC-injected mice, we selected MLR as the technique of choice and utilised non-proliferative, primary donor-phenotype DCs as the optimal stimulator cell type. This approach provided a "one-way" T-cell activation model using a potent APC stimulus that matched the APC type involved in priming and reactivating allo-antigen-specific T-cells *in vivo*. Quantification of proliferation via fluorescence dilution by flow cytometry allowed for the responses to specific immune cell subsets to be analysed individually and for effector read-outs such as cytokine production and expression of cytotoxic proteins to be detected at a single cell level. Although other assay approaches (e.g. ELISPOT) have also been effectively applied to the analysis of antigen-specific T-cell responses, MLRs represent a sensitive, well-established method that has also been applied to human allotransplant recipients (Spencer *et al.*, 1995, Miyahira *et al.,* 1995, Speiser *et al.*, 1996 and Suchin *et al.,* 2001). Discrimination of $CD4^+$ and $CD8^+$ T-cell responses using flow cytometry has also been used as an immune monitoring strategy in human transplant recipients (Altman *et al.,* 1996). It should be emphasised that, unlike other antigen-specific immune responses such as those induced by vaccination, alloantigen-specific T-cell responses are associated with a high proportion of "responders" even in naïve individuals – particularly when the allo-antigen is presented via the "direct" pathway by donor DCs (Warren *et al.,*1994 and Li *et al.,*1999). In keeping with this, we observed significant proliferation of both $CD4^+$ and $CD8^+$ T-cells from saline-injected (B6 antigen naïve) BALB/c mice – providing a clear demonstration of the sensitivity of the MLR system. Furthermore, in our optimisation experiments, the detectable differences between the saline and allo-splenocyte group in terms of proliferation of T-cells represent validation that the system could be used to quantify memory/re-call responses of in vivo-primed allo-

antigen-specific T-cells. By including other splenic cell types from recipient mice (monocytes, macrophages, innate lymphocytes and B-cells) in the MLR culture, we retained the potential to detect modulatory effects of allo-MSC on the innate arm of the immune system. Furthermore, intracellular staining for the transcription factor FOXP3, allowed for the quantification of T-regs which, in many transplant studies, have been shown to be to be closely linked with immune modulation and donor-specific hypo-responsiveness/tolerance (Karim *et al.,* 2004, Long *et al.,* 2007 and Fadi Issa *et al.,* 2010).

Although the role of T-cells in allograft rejection is unequivocal, it is becoming increasingly recognised that donor antigen specific IgG antibodies, typically directed against class I and class II MHC, represent the primary mechanism of injury in many cases of severe acute and chronic allograft rejection (Mason., 1986, Colvin & smith., 2005, Chang & Platt., 2009 and Nankivell & Alexander., 2010). In the field of human organ transplantation, it has been well documented for several decades that high levels of pre-existing antibodies to the ABO blood antigens or donor HLA proteins result in immediate activation of the complement cascade leading to vascular thrombosis and inflammation within minutes to days of transplantation (Mauiyyedi S *et al.,* 2002). Thus, it is now standard practice to match for ABO blood group compatibility and to test for the presence of allo-antibodies in the recipient prior to the transplant surgery (Smith JD *et al.,* 1993, George JF *et al.,* 1995, and Mauiyyedi S *et al.,* 2001). More recently, the role of lower levels of anti-HLA IgG antibodies (either pre-existing or developing de novo post-transplant) in mediating chronic transplant injury through pathogenic interactions with the graft endothelium has been extensively documented and studies experimentally (Colvin & Smith., 2005, Chang & Platt., 2009, Hori *et al.,* 2012, Puttarajappa *et al.,* 2012). These insights have driven important technical advances in the detection and functional characterisation of anti-HLA and donor-specific antibodies (DSA) in human transplant candidates and recipients (Ara *et al.,* 2005 and Konvalinka & Tinckam., 2015). In the setting of non-vascularised allo-transplants, the role of DSA is much less studied and remains unclear. Nonetheless, DSA has recently been shown to exert detrimental effects on graft survival in recipients of pancreatic islet transplants (Brooks *et al.,* 2015). In the case of allo-MSC administration, the development of anti-donor antibody has been

variously documented in a range of animal model studies (Griffin *et al.*, 2013 and Alagesan & Griffin., 2015). However, the significance of this for immediate therapeutic efficacy, for the efficacy of repeated allo-MSC administration or for other long-term outcomes is not currently known. Taking this fact on board, in this study, we developed a sensitive, flow cytometry-based semi-quantitative assay for B6-specific IgG in BALB/c mice. As documented in the preliminary experiments described in this Chapter, this assay confirmed the lack of anti-B6 antibody in saline-injected mice as well as its readily detectable development in animals that had received a single IM injection of B6 splenocytes. Furthermore, using an *in vitro* approach similar to the complement-dependent cytotoxicity (CDC) assay that has been used for decades to "cross-match" human transplant donor/recipient pairs; we optimized a quantitative complement-mediated lysis system for evaluating the potential pathogenic significance of anti-B6 antibody induced by IM allo-MSC (Kovalinka & Tinckam 2015). Importantly, our preliminary results for serum from mice injected IM with a relatively small inoculum of B6 allo-splenocytes confirmed that this route administration is associated with potent induction of complement-fixing IgG DSA. It is of high clinical importance to know whether, antibodies formed against allo-antigens are capable of lysing donor cells following binding as this determines the longevity of the cells *in vivo* (Li & Lin., 2012).

Finally, the localised accumulation and effector functions of donor-specific T-cells and antibodies represent the final link between induction of allo-specific immune responses and damage/destruction of allogeneic organ or tissue transplants. Thus, histological evaluation of tissue samples (biopsies) is routinely used in the field of transplantation in order to determine the severity and nature of immune-mediated complications (Solez *et al.*, 1993 and Nankivell & Alexander., 2010). Infiltration of transplant tissue by innate and adaptive immune cells represents a hallmark of allograft rejection. In a clinical setting, cellular infiltrates in transplant biopsies are categorised and graded by specialised pathologists as a means to diagnose and guide the treatment of acute or chronic rejection (Colvin & Smith., 2005 and Nankivell & Alexander., 2010). Similar techniques and grading systems are also frequently applied to animal models of transplantation. In the context of IM or other localised delivery of allo-MSCs, only a limited number of animal model studies have carefully documented the nature

and extent of cellular immune infiltrates at the site of cell placement (Chuang *et al.*, 2009, Violaine K Harris *et al.*, 2011, Griffin *et al.*, 2013 and Kehoe *et al.*, 2014). Notably, however, Huang *et al.*, 2010 documented evidence of delayed "rejection" of intra-myocardial allo-MSCs in a ray model of post-infarct left ventricular re-modelling that was associated with lymphocytic infiltration of heart muscle and with loss of therapeutic benefit (Huang *et al.*,2010). The effect of repeated local administration of allo-MSC on localised immune response has not been well investigated to date. For this reason, we also developed a histological approach for semi-quantitative scoring of IM inflammatory cell infiltrates and, as described in this Chapter, validated its sensitivity for detecting increased localised inflammation associated with injection of B6 splenocytes into BALB/c mice.

Taken together, the work described in this Chapter centred on the adoption of approaches and techniques from the field of transplant immunology in order to lay the groundwork for experiments to broadly and accurately analyse anti-donor immune response to allo-MSCs delivered IM to the hind-limb.

CHAPTER – 3

CELLULAR IMMUNE RESPONSES TO INTRAMUSCULAR INJECTION OF ALLOGENEIC MSCs

3.1 INTRODUCTION

Recognition of donor (allo)-antigens by recipient T-cells is a key factor in the immunological outcome of transplanted allogeneic organs, tissues and cells (Ingulli, 2010, Chinen & Buckley, 2010 and Griffin *et al.*,2012). Productive allo-recognition by donor-antigen-specific T-cells leads to their activation, expansion and migration to the site of the transplant, leading to acute or chronic "cellular" allograft rejection (Ingulli, 2010). Furthermore, activation of donor-specific T-cells is usually necessary for the *de novo* generation of donor-specific antibodies (DSA) by B-cell clones (Porcheray *et al.,* 2011). Importantly, such productive allo-recognition represents a multi-step process that also involves a key role for cells of the innate immune system which provide essential activating stimuli to APCs (Larosa *et al.,* 2007 and Ingulli, 2010). As already described in Chapter 2, non-immunosuppressed recipients of allogeneic organ or tissue transplants, tend to have a significant number of T-cell clones with inherent responsiveness to allogeneic MHC proteins or allogeneic peptides presented by autologous MHC (Chinen and Buckley, 2010 and Ingulli, 2010). These donor antigen-specific T-cell include both CD4+ ("helper") and CD8+ ("cytotoxic") clones, the majority of which is likely to be in the naïve state (ie. not previously activated) prior to transplantation. Particularly in the absence of pre-formed DSA, the activation of anti-donor T-cells is the primary mechanism of allo-transplant rejection (Wood and Goto, *et al.,* 2012).

3.1.1 **Subtypes of Anti-donor Cellular Immune Responses**

3.1.1.1 Innate Immune Responses: Soluble immune mediators such as chemokines and cytokines play a key role in activating and defining the effector phenotype of T-cell responses to transplant-associated allo-antigens (Larosa *et al.,* 2007). The most significant source of chemokines and cytokines early following transplantation is cells of the innate immune system which become activated as a result of tissue injury associated with the transplant surgery or implantation procedure (Hancock *et al.,* 1996 and Nelson *et al.,* 2001). Among the potential cellular contributors to the innate immune response to organ/tissue transplantation are resident macrophages, DCs and innate lymphocytes as well as monocytes, neutrophils and NK cells which infiltrate from the circulation (Murphy *et al.,* 2011). The subsequent functional programming of donor-specific naïve T-cells and B-

cells is heavily influenced by the intensity and nature of these initial innate immune responses (Larosa *et al.*, 2007). Furthermore, at later time-points, pro-inflammatory events such as infection, ischemia or physical trauma may trigger cellular rejection of previously stable transplants (Riise *et al.*, 1997 and Cainelli & Vento, 2002). In addition, circulating memory T-cells may become rapidly activated following transplantation, either in an allo-antigen-specific or non-specific manner. Examples of mechanisms whereby memory T-cells may be activated in an allo-antigen-non-specific manner include: (a) Bystander activation associated with innate immune cell activation. (b) Homeostatic proliferation following lymphocytes depleting therapies. (c) Heterologous immunity due to cross-reactivity with previously encountered infectious antigens (Wu *et al.*, 2004). In some cases, activated memory T-cells may be responsible for severe rejection that is resistant to conventional immunosuppressive drugs and depleting antibodies (Pearl *et al.*,2005 and Trzonkowski *et al.*, 2006).

3.1.1.2 CD4$^+$ T-Cell Responses: Helper (CD4$^+$) T-cells are central to the orchestration of anti-donor adaptive immune responses to allogeneic transplants and may be programmed, during primary antigen presentation to express different functional responses (referred to as T-helper (Th) differentiation) (Boisgerault *et al.*,2001 and Liu *et al.*,2013). The two best characterised Th differentiation pathways are Th1, which strongly fosters pro-inflammatory, cytotoxic antigen-specific responses and Th2, which promotes antibody-mediated and allergic type immune responses (Liu *et al.*,2013). Both Th1- and Th2-type anti-donor immune responses have the capacity to lead to donor graft/cell destruction, although, in some experimental settings, Th2 polarisation has been associated with an anti-inflammatory, pro-tolerogenic donor-specific response (Le Moine *et al.*, 2002). A more recently recognised Th phenotype, referred to as Th17 as a result of its signature cytokine IL-17A, has also been shown to mediate allograft injury and rejection (Atalar *et al.*, 2009). The Th17 differentiation programme is specifically associated with intense neutrophilic inflammation (Pelletier *et al.*, 2010).

During the course of allograft cellular rejection, the activation of donor-specific CD8$^+$ cytotoxic T-cells, couple with macrophage dependent delayed hypersensitivity reactions and production of donor-specific complement fixing IgG2a represents a highly destructive immunological pathway (Liu *et al.*,1993, Watschinger *et al.*, 1994 and Chen *et al.*,1996). The production of interferon gamma (IFNγ)

by donor-specific Th1-type $CD4^+$ T-cells is a key factor in this form of cellular immunity and, thus, IFNγ is classically viewed as potent mediator of allograft rejection (Wise *et al.,*1999, Wiseman *et al.,*2001 and Benichou *et al.,* 2013). In keeping with this, IFN-γ- and cytotoxic T-cell (CTL)-associated gene expression patterns represent one of the best define molecular signatures of acute rejection in animal models as well as human transplant recipients (Halloran *et al.,* 2001 and Hauser *et al.,* 2005). However, the immunological role of IFN-γ in allotransplant outcomes is more complex. For example, IFN-γ knock-out mice robustly reject allotransplants (Sawitzki *et al.,* 2005 and Sadeghi *et al.,* 2007) and additional experimental data suggest roles for IFN-γ in the induction of T-reg induction and in negative regulation of the Th17 pathway (Feng *et al.,*2008 and Yan *et al.,* 2008). Consistent with this, it has been shown that T-bet deficient mice, which lack Th1 activity, reject allotransplants through strong Th17 activation (Chan *et al.,* 1995).

As mentioned above, Th2 cells may also be involved in allo-antigen-specific cellular immune responses and may mediate graft rejection (Chan *et al.,* 1995, Piccotti *et al.,* 1997 and Barbara *et al.,* 2000). By producing several cytokines, including IL-4, IL-5, IL-9, IL-10 and IL-13, Th2 cells strongly activate antigen-specific B-cells and eosinophils, both of which have high potential for damaging transplant organs and cells. For example, in mouse cardiac allo-graft rejection increased Th2 cytokines were seen in association with eosinophilic infiltration (Martinez *et al.,* 1993) and this observation was confirmed in human renal and liver transplant rejection episodes (Vanbuskirk *et al.,* 1996 and Veldhoen M *et al.,*2008)

3.1.1.3 $CD8^+$ T-Cell Responses: Cytotoxic ($CD8^+$) T-cells typically act as terminal effector cells in the destruction of allotransplants by acute cellular rejection (Ito *et al.,*1995, Walsh *et al.,* 1996, Bergese *et al.,* 1997 and Yang *et al.,* 2007). Anti-donor immune responses mediated by donor-specific $CD8^+$ T-cells are associated with inflammatory features including tissue necrosis and local innate immune cell infiltration (El-Sawy *et al.,* 2004). In kidney transplant rejection activated $CD4^+$ and $CD8^+$ T-cells are seen in the interstitial spaces around the tubules (Robertson *et al.,* 1996 and Cornell *et al.,* 2008). Within the basement membrane of renal tubules undergoing active destruction, however, the infiltrating cells are predominantly clonally expanded $CD8^+$ T-cells with high expression of cytotoxic

and pro-apoptotic proteins including perforin, granzyme A and B and Fas ligand. In line with an important role for CD8+ T-cell-associated cell death mechanisms in allograft rejection, lack of Fas, the binding partner for FasL, has been shown to prolong the survival of allogeneic skin transplants (Surquin *et al.,* 2002).

3.1.2 **Anti-donor T-cell immune responses to allo-MSCs**

Evidence of increased anti-donor T-cell responsiveness following allo-MSC administration has been garnered in a variety of animal models. This has included studies in healthy mice and rhesus macaques, as well as studies of BMT and myocardial infarction in mouse and pig, respectively (Eliopoulos *et al.,* 2005, Nauta *et al.,* 2006, Badillo *et al.,* 2007, Poncelet *et al.,* 2007 and Isakova *et al.,* 2010). These observations indicate that, despite their immuno-modulatory properties, non-manipulated allo-MSCs are capable of eliciting active anti-donor T-cell responses under various experimental conditions. Of interest, little work has been reported to date to characterise the finer details of these responses such as Th phenotype and cytotoxicity. It is clear, that other outcomes are possible following *in vivo* allo-MSC administration. For instance, Beggs *et al.,* 2006 have observed that healthy baboons inoculated first intravenously and then intramuscularly with MSCs from allogeneic donors displayed reduced anti-donor T-cell reactivity following the second inoculum. Furthermore, some groups have reported prolonged or even indefinite survival of allogeneic organ transplants with co-administration of donor-specific MSCs with or without additional pro-tolerogenic immunosuppression. (Casiraghi *et al.,* 2008, Ge *et al.,* 2009, English *et al.,* 2010, Ge *et al.,* 2010 and Casiraghi *et al.,* 2012). Thus, under some circumstances, cellular immunity to allo-antigens may also be suppressed by allo-MSC—a phenomenon that could be explained by classic mechanisms of donor-specific T-cell hypo-responsiveness, such as clonal deletion, T-cell anergy, regulatory T cell induction or Th phenotype deviation. (Game & Lechler, 2002). Taken as a whole, the available literature suggests that allo-antigens associated with non-manipulated MSCs delivered by various systemic and localised delivery routes are actively and productively presented to recipient T cells, most likely by the indirect antigen presentation pathway. Although more precise characterisation of the resulting anti-donor T-cell responses in different models is needed, it would seem that these include a range of

T-cell effector functions with potential for either destructive or suppressive activity upon secondary encounter with allo-antigens.

In this Chapter, results are presented of experiments aimed at quantifying and functionally characterising the donor-specific T-cell responses as well as some of the accompanying innate cell immune responses in immune competent mice following single or repeated IM injections of allo-MSCs along with relevant positive and negative controls.

3.2 MATERIALS AND METHODS

Note: Only those materials and methods not previously described in Chapter 2 are summarised here.

3.2.1 *Mouse Strains*

Donor and recipient mice were as described in Chapter 2. In addition, FVB mice (MHC genotype H-2^q) were used as a source of "third party" APCs in some *in vitro* experiments. FVB mice were purchased from Charles River Laboratories, United Kingdom.

3.2.2 *MACS: Magnetic-Activated Cell Sorting*

Single cell suspensions of RBC-free splenocytes were prepared as described in Chapter 2 and were incubated with anti-mouse CD90.2- or CD11c-coated magnetic microbeads for 30 minutes at 4°C. The cells were then washed and re-suspended in 400 μl of sterile MACS buffer and were loaded onto an MS column placed in a MiniMACS® magnetic separation device (Miltenyi Biotech, Europe) with the flow-through discarded. Next, the columns were removed from the magnetic field and column-bound cells ($CD90.2^+$ or $CD11c^+$) were eluted into a sterile 3 ml polystyrene FACS tube with 400 μl sterile MACS buffer. The eluted cells were washed and re-suspended in T-cell culture medium and were counted prior to use as APCs ($CD11c^+$ DCs) or responders ($CD90.2^+$ T-cells) in MLR experiments.

3.2.3 *Quantification of $CD8^+CD11c^+$ T-cells in Spleen by Flow Cytometry*

Single cell suspensions of RBC-free splenocytes were prepared as described in Chapter 2. The cells were suspended in FACS buffer at 1 x 10^6 cells/ml and were incubated with optimised dilutions anti-mouse CD3-PE, CD8-PE-Cy7 and CD11c-FITC (see Appendix details of antibodies) for 30 minutes at 4°C in round-bottom 96 well plates. Cells were washed and re-suspended in FACS buffer prior to being analysed and using a Becton Dickinson (BD) Biosciences FACSCanto® cytometer (San Jose, CA, US) and FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR). Prior to analysis, SYTOX Red dead cell stain was added to allow for accurate gating on live cells.

3.2.4 *ELISA: Enzyme Linked Immuno-Sorbent Assays*

ELISAs for IFN-γ and IL-10 were performed with R&D System Duo-set® ELISA kits according to the manufacturer's instructions. Briefly, 96-well flat-bottom assay plates were coated with capture antibody, incubated overnight at 4°C then washed, incubated with standards or appropriately diluted samples of culture supernatants for 2 hours at RT. The plates were then washed and incubated with biotinylated detection antibody for 2 hours at RT followed by streptavidin-HRP as a detection system by microplate reader (Wallac 1420 Victor3^{TM} Multilabel Counter plate reader from Perkin Elmer, Waltham, MA, US) set at 450nm with reference reading set at 540nm or 570nm.

3.2.5 *Neutralization of IFN-γ/IL-10 And Blockade of Prostaglandin Synthesis*

To neutralize IFN-γ and IL-10 individually or together in MLRs, CFSE-stained splenocytes from groups of BALB/c mice were mixed with B6 or FVB DCs in 96-well round-bottom tissue culture plates as previously described. Sodium azide-free anti-IFN-γ and/or anti-IL-10 monoclonal antibodies (mAbs) or appropriate isotype control antibodies were added to final concentrations of 10μg/ml each (Catalogue# 504904 & 517904 from Biolegend® and isotype control – catalogue# 400414 from Biolegend®) For control wells, relevant isotype control mAbs were added to the same concentrations. After 5 days of culture at 37°C, T-cell proliferation was quantified by flow cytometric analysis of CFSE dilution as previously described. For prostaglandin synthesis blockade, the non-specific cyclooxygenase (COX) inhibitor indomethacin (Catalogue# I7378, Sigma-Aldrich® Ireland) was added to MLRs a final concentration of 10 μM while an equivalent volume or concentration of vehicle (DMSO, Catalogue# D2650, Sigma-Aldrich® Ireland) was added to control wells.

3.2.6 *$CD8^+$ T-Cell Cytotoxicity Assay and Granzyme B Detection*

To determine the cytotoxicity of activated, donor-specific recipient T-cells, whole splenocytes from groups of BALB/c mice were stimulated with B6 or FVB $CD11c^+$ DCs for 5 days as previously described. The $CD8^+$ T-cells from these MLRs were enriched by magnetic column separation using anti-mouse CD8-coated microbeads according to the previously described protocol. The enriched, DC-activated BALB/c $CD8^+$ T-cells were then cultured for 4 hours at 37°C with B6 MSCs that had

been pre-labelled in a 10 μM solution of calcein (catalogue# C1430 from Invitrogen® Ireland). $CD8^+$ T-cells and calcein-labeled MSCs were plated at 10^6 cells/ml and 10^4 cells/ml respectively giving a T-cell:MSC ratio of 100:1. Culture supernatants were collected to quantify calcein release from MSC in a micro-plate reader (Wallac 1420 Victor3^{TM} Multilabel Counter plate reader from Perkin Elmer, Waltham, MA, US) set at 450nm. For analysis of granzyme B expression by CD8+ T-cells, the cells were removed from the culture plates, washed and re-suspended in FACS buffer then incubated for 30 minutes at 4°C with optimized amounts of anti-mouse CD8-PE-Cy7 and Granzyme B- Pacific blue. After staining, the cells were again washed and re-suspended in FACS buffer and were analysed using a Becton Dickinson (BD) Biosciences FACSCanto® cytometer (San Jose, CA, US) and FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR).

3.2.7 *Statistical Analysis*

Results were expressed throughout as mean ± standard deviations (SD) and differences among multiple groups were tested statistically by ANOVA with post-hoc testing. Difference between two conditions were statistically analysed by two-tailed Student's t-test. All statistical analyses were performed with GraphPad Prism® 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). Significance was assigned at $p < 0.05$.

3.3 RESULTS

3.3.1 *T-cell Re-Call Responses to Single and Repeated IM Injection of Allo-MSCs*

Anti-donor T-cell responses to single or double dose of allogeneic MSCs intervention were tested by one-way MLRs as described in Chapter 2. In the first such experiment, six groups of BALB/c mice (n=4) received two sets of IM injections two weeks apart. The combinations of vehicle (saline), Syn-MSC, allo-splenocyte and allo-MSC injections given to each group are indicated in the legend of **fig. 3.2**. As shown, individual experimental groups received single or repeated ("double") injections of allo-splenocytes and allo-MSCs while control groups received double injections of saline or Syn-MSCs. In all groups, $CD4^+$ and $CD8^+$ T-cell proliferative responses to B6 DCs were quantified 1 week after the second round of injections. As shown in **fig. 3.1B** and **C**, the DC-induced $CD4^+$ T-cell proliferative responses were highest for groups that received single or double injections of allo-splenocytes, while the DC-induced $CD8^+$ T-cell proliferative responses were highest (significantly greater than control groups) for groups that received single injections or double injections allo-splenocytes or single injections of allo-MCSs. In contrast, however, the DC-induced $CD4^+$ and $CD8^+$ T-cell proliferative responses of the group that received double injections of allo-MSCs were significantly lower than all other groups including the saline-injected and Syn-MSC-injected controls.

It was also noted that spontaneous proliferation of $CD4^+$ and $CD8^+$ splenic T-cells in the absence of DC stimulators tended to be higher for groups that had received any cell injections compared to the saline-injected control group. Although these differences did not reach statistical significance, the trend suggested the possibility of some on-going intra-splenic T-cell responses to antigens or non-specific inflammation associated with injection of primary or cultured cells into muscle.

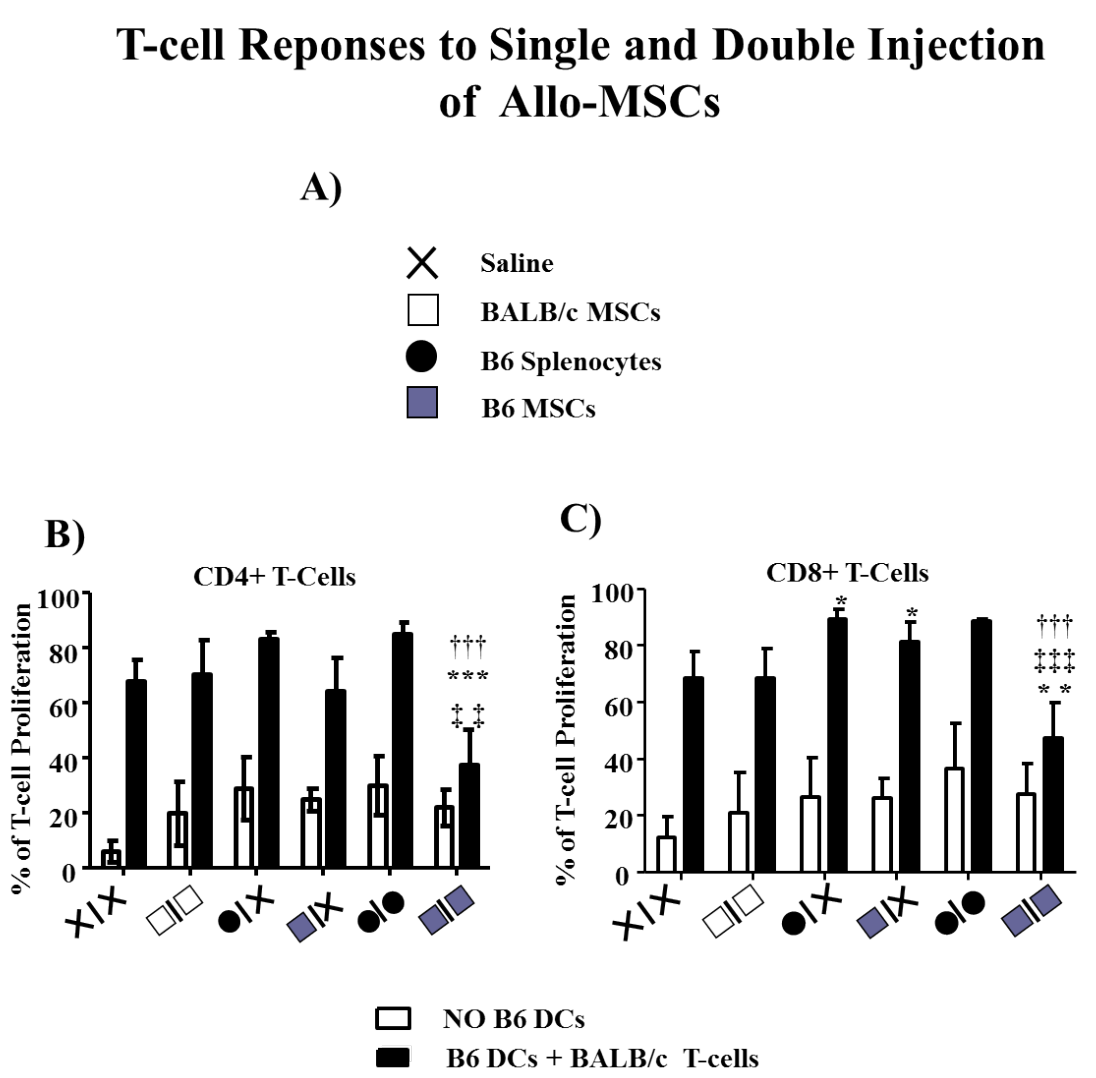


Figure 3.1: T-cell Responses to Single and Double Injection of Allo-MSCs: Proliferative responses of $CD4^+$ and $CD8^+$ splenic BALB/c T-cells from six experimental groups following 5-day cultures without and with B6 DCs (1:20 T-cell:DC ratios). **A.** Symbols indicating different injection types. **B.** Proliferation of $CD4^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **C.** Proliferation of $CD8^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **Statistics**: One-Way ANOVA, *, **, *** = $p < 0.05, 0.01, 0.001$ compared to saline control group †, ††, ††† = $p < 0.05, 0.01, 0.001$ compared to appropriate double-injected allo-splecnocyte group and. ‡‡‡ = $p < 0.001$ compared to single-injected allo-MSC group. (n=4 each group).

Interim Conclusions: (a) There was, as expected, evidence of systemic (splenic) donor-specific sensitisation of $CD4^+$ and $CD8^+$ T-cells following a single or double IM injection of allo-splenocytes MSCs. (b) A single IM injection of allo-MSCs was associated with evidence for donor-specific CD8+ T-cell sensitisation. (c) In striking contrast, a double injection of allo-MSCs resulted in hypo-responsiveness of both $CD4^+$ and $CD8^+$ T-cells upon re-stimulation with donor DCs.

3.3.2 *Donor Antigen Specificity of T-cell Response to Double Injection of Allo-MSCs*

To address whether the T-cell hypo-responsiveness observed following double IM injection was specifically induced by a second IM exposure to allo-MSC-delivered antigens, the next experiment was performed using the same basic design with the following four groups (n=5 each group): 1. Saline double-injected (naïve control). 2. Allo-MSC single injection followed by saline injection. 3. Allo-MSC double injections. 4. Allo-MSC single injection followed by Syn-MSC single injection. Furthermore, in this experiment, the $CD4^+$ and $CD8^+$ splenic T-cell proliferative responses to donor (B6) DCs and to third party (FVB) DCs were quantified in MLRs.

The results for the MLRs from this experiment are summarized in **fig. 3.2A** and **B**. A shown the saline injected control group mounted significant, equal CD4+ and CD8+ T-cell proliferative responses to both B6 and FVB DCs consistent with a baseline level of T-cell allo-reactivity. In comparison, the $CD4^+$ and $CD8^+$ T-cell proliferative responses of the allo-MSC single injected group were significantly greater in both donor (B6) and third party (FVB) DC-stimulated MLRs. Of note, however, the spontaneous levels of splenic T-cell proliferation were also significantly higher for this group compared to the saline injected group. In contrast, T-cell proliferation for the allo-MSC double injected group demonstrated hypo-responsiveness in B6 DC-simulated MLRs with preserved proliferative response to FVB DCs. Strikingly, however, this pattern of donor-specific $CD4^+$ and $CD8^+$ T-cell hypo-responsiveness was not evident for the group that received allo-MSCs followed by syn-MSCs. This group also exhibited a level of spontaneous T-cell proliferation that was significantly higher than that of the saline injected, naïve control group.

Interim Conclusions: (a) Consistent with the first experiment, double, but not single IM injection of allo-MSCs resulted in hypo-responsiveness of $CD4^+$ and $CD8^+$ T-cells in subsequent donor DC-stimulated MLRs. (b) Results for MLRs performed with DCs from a third party allogeneic strain indicated that the observed T-cell hypo-responsiveness was specific for B6 allo-antigens. (c) Results for the group that received allo-MSCs followed by syn-MSCs confirmed that the observed T-cell hypo-responsiveness was dependent on a second exposure to allo-MSCs rather than a non-specific

immunosuppressive effect of a second MSC injection. (d) The previously observed increased rate of spontaneous T-cell proliferation following IM cell injections was confirmed in this experiment.

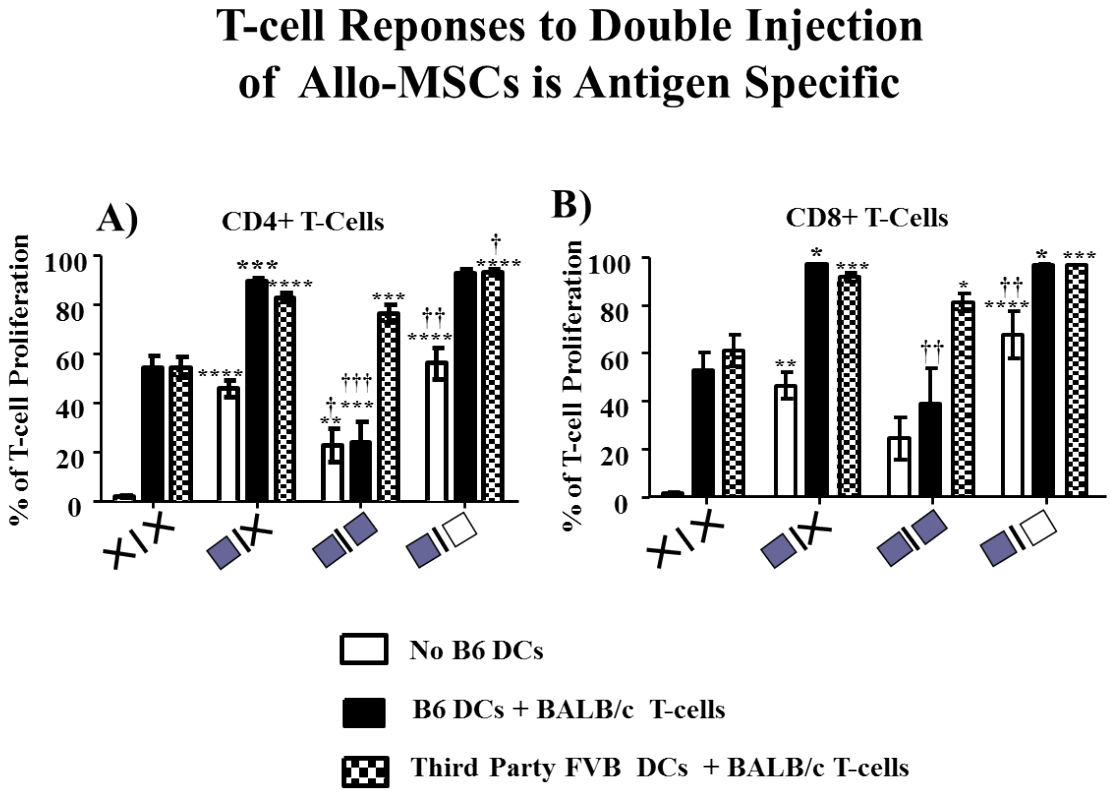


Figure 3.2: T-cell re-call responses to single a double injection of allo-MSCs or two single injection of allo-MSCs followed by injection of syn-MSCs: Proliferative responses of $CD4^+$ and $CD8^+$ splenic BALB/c T-cells from four experimental groups following 5-day cultures without and with B6 or FVB (Third Party) DCs (1:20 T-cell:DC ratios). Symbols used are as for **fig 3.2A**. **(A)** Proliferation of $CD4^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **(B)** Proliferation of $CD8^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **Statistics**: One-Way ANOVA, *, **, **** = $p < 0.05, 0.01, 0.001, 0.0001$ compared to saline control group †, ††, ††† = $p < 0.05, 0.01, 0.001$ compared to appropriate single- or double-injected group. (n=5 each group).

3.3.3 *T-cell Response to Double and Triple Injection of Allo-MSCs*

In the third experiment conducted, we addressed the question of whether the T-cell hypo-responsiveness observed following two sequential injections of allo-MSCs could be further enhanced by administering a third IM injection two weeks after the second. To test this, three groups of BALB/c mice (n=10 each) received sequences of three IM injections at two week intervals according to the

protocol depicted in **fig-3.3A**. The groups compared were as follows: 1. Three IM injections of saline (naïve control). 2. Two IM injections of allo-MSCs followed by a thirds injection of saline. 3. Three injections of allo-MSCs. As with the previous experiments, B6 and FVB DC-stimulated MLRs were performed one week following the final injections.

The MLR proliferation results for $CD4^+$ and $CD8^+$ T-cells are shown in fig 3.3A and B respectively. As shown, the group the allo-MSC "triple injected" group demonstrated lower proliferative responses of $CD4^+$ and $CD8^+$ T-cells to B6 compared to FVB DCs. In contrast, the group that received allo-MSC double injection followed by saline had equal proliferative responses to B6 and FVB DCs with the proliferation to B6 DCs being significantly higher than that of the allo-MSC triple injected group. In keeping with the two previous experiments, both cell injected groups demonstrated higher rates of spontaneous CD4+ and CD8+ T-cell proliferation than the saline control group. Indeed, when this was taken into account, the B6 DC-stimulated $CD4^+$ T-cell proliferation for the allo-MSC triple injected group was no greater than the "background" rate of spontaneous proliferation.

Interim Conclusions: (a) The experiment provided further confirmation that repeated IM injection, in this case triple injection of allo-MSCs, resulted in donor-specific hypo-responsive of $CD4^+$ and $CD8^+$ T-cells as well as a higher rate of spontaneous splenic T-cell proliferation in MLRs. (b) Surprisingly, T-cell hypo-responsiveness was not observed in allo-MSC double injected mice when splenocytes were isolated for MLRs two weeks later than the previous experiments. This result indicated that the donor-specific T-cell hypo-responsiveness associated with multiple IM injections of allo-MSCs was relatively short lived – perhaps suggesting a role for short-lived regulatory cells or soluble mediators.

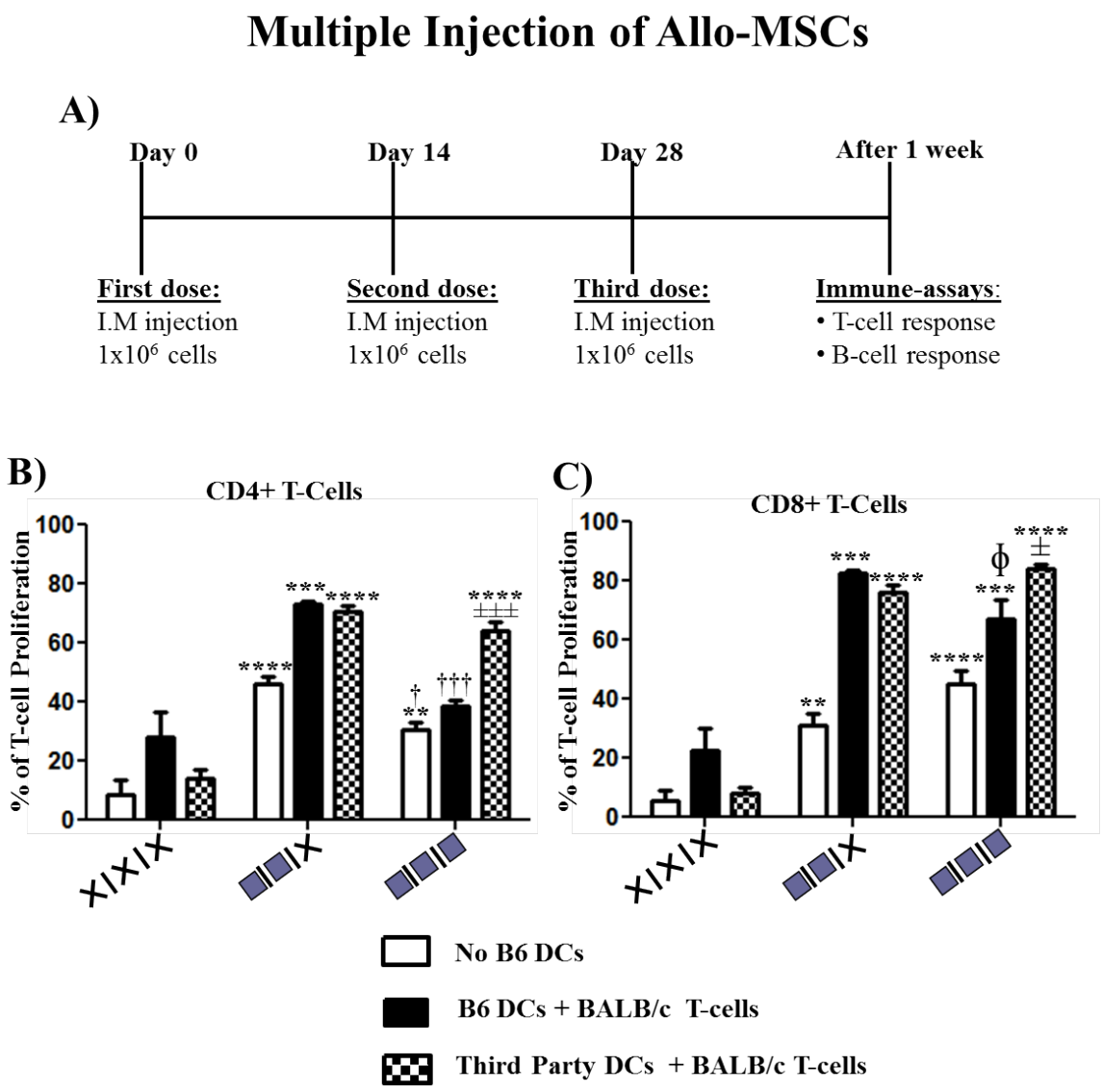


Figure 3.3: T-cell re-call responses to double and triple injection of allo-MSCs: Proliferative responses of $CD4^+$ and $CD8^+$ splenic BALB/c T-cells from three experimental groups following 5-day cultures without and with B6 or FVB DCs (1:20 T-cell:DC ratios). Symbols used are as for **fig 3.2A**. **A.** Experimental design. **B.** Proliferation of $CD4^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **C.** Proliferation of $CD8^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **Statistics**: One Way ANOVA: **, ***, **** = $p < 0.01, 0.001, 0.0001$ compared to equivalent condition for saline control group. †, ††, ††† = $p < 0.05, 0.01, 0.001$ compared to equivalent condition for allo-MSC double-injected group. Student's t-test: ±, ±±, ±±± = $p < 0.05, 0.01, 0.001$ compared to B6 DC-stimulated MLR for the same group. (n=8 each group).

3.3.4 *T-Cell Re-Call Responses in the Absence of Accessory Splenic Cells*

To determine whether the donor-specific T-cell hypo-responsiveness we had observed following multiple IM injections of allo-MSCs was mediated by a non-T-cell population of splenocytes, MLRs were prepared from the experiment described above (fig 3.4A) using CD90 (T-cell)-enriched responders. The results of $CD4^+$ and $CD8^+$ T-cell proliferation from these MLRs are summarized in **fig 3.4A** and **B** respectively. As shown, the pattern of proliferation responses was identical that that observed in MLRs containing total splenocytes (**fig-3.4**).

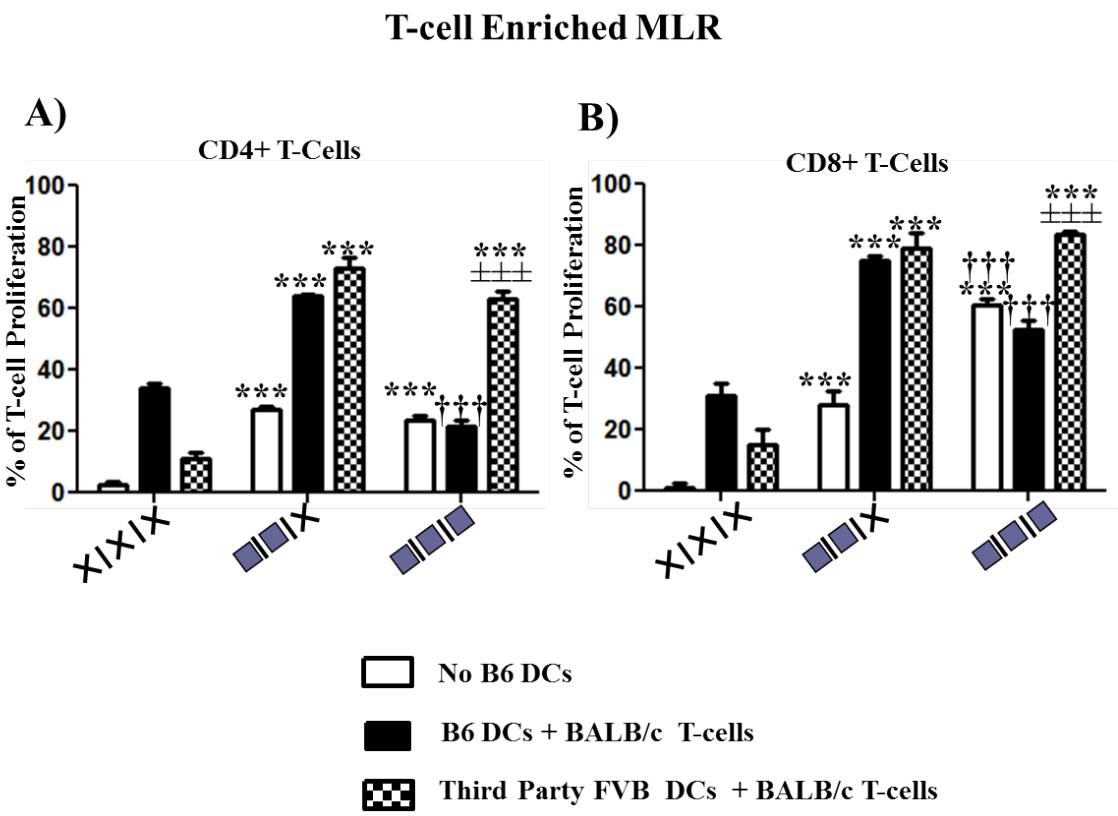


Figure 3.4: T-cell re-call responses to double and triple injection of allo-MSCs (T-cell enriched responders): Proliferative responses of $CD4^+$ and $CD8^+$ splenic BALB/c T-cells from three experimental groups following 5-day cultures without and with B6 or FVB DCs (1:20 T-cell:DC ratios). Symbols used are as for **fig 3.2A**. **A.** Proliferation of $CD4^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **B.** Proliferation of $CD8^+$ T-cells expressed as mean ± SD percentage (%) of proliferation based on CFSE dilution. **Statistics**: One Way ANOVA: *** = $p < 0.001$ compared to equivalent condition for saline control group. ††† = $p < 0.001$ compared to equivalent condition for allo-MSC double-injected group. Student's t-test: ±±± = $p < 0.001$ compared to B6 DC-stimulated MLR for the same group. (n= 8 each group)

Interim Conclusion: The donor-specific T-cell hypo-responsiveness observed following multiple IM injections of allo-MSCs may be mediated by a short-lived regulatory T-cell population.

3.3.5 *Myeloid Immune Cell Responses to Intra-muscular Injection of Allo-MSC:*

With the consistent observation that repeated IM injections of allo-MSC resulted in a period of donor-specific splenic T-cell hypo-responsiveness, it was decided to directly evaluate the splenic myeloid and lymphoid compartments one week following double IM injection of allo-MSCs in comparison to naïve controls (double IM injection of saline) and double IM injection of allo-splenocytes. In this section and the next, the results of myeloid and lymphoid cell profiling are summarized.

It has been well described that MSCs interact with and modulate the functional responses of myeloid cells such as monocytes, macrophages (Tesar *et al.*, 2008, Stout *et al.*, 2009, Nemeth *et al.*,2009 and Geissmann *et al.*,2010), dendritic cells (Zhang *et al.*, 2004, Djouad *et al.*, 2007 and English K *et al.*, 2008) and neutrophils (Raffaghello *et al.*,2008). Splenocytes from three groups of 10 mice each were profiled for proportionate content of several myeloid cell sub-populations identified on the basis of surface expression of the markers CD11b, CD11c, Ly6G, Ly6C and F4/80 (performed and interpreted as described in Chapter 2, Section 2.3.3). The results of this analysis are summarized in **fig. 3.5**. In this analysis, the myeloid cell populations were expressed as proportions of total viable splenocytes.

As shown, double injection of allo-MSCs resulted in significantly lower splenic proportions of neutrophils and $F4/80^{+}$ macrophages compared to double injections of saline or allo-splenocytes. On the other hand, the proportions of $Ly6C^{hi}$ monocytes, $CD11b^{-}/CD11c^{-}$ DCs and $Ly6C^{lo}/F4/80^{lo}$ "other" myeloid cells were significantly higher in the group that received double injection of allo-MSCs.

Interim Conclusions: (a) Repeated IM injection of allo-MSCs is associated with a distinctive myeloid cell response in the spleen that does not occur following injections with allo-splenocytes. (b) The pattern of myeloid cell changes observed following repeated IM injection of allo-MSCs is suggestive of expansion of monocytes and other myeloid cells of unclear functional phenotype with relative reductions in neutrophils and typical macrophages.

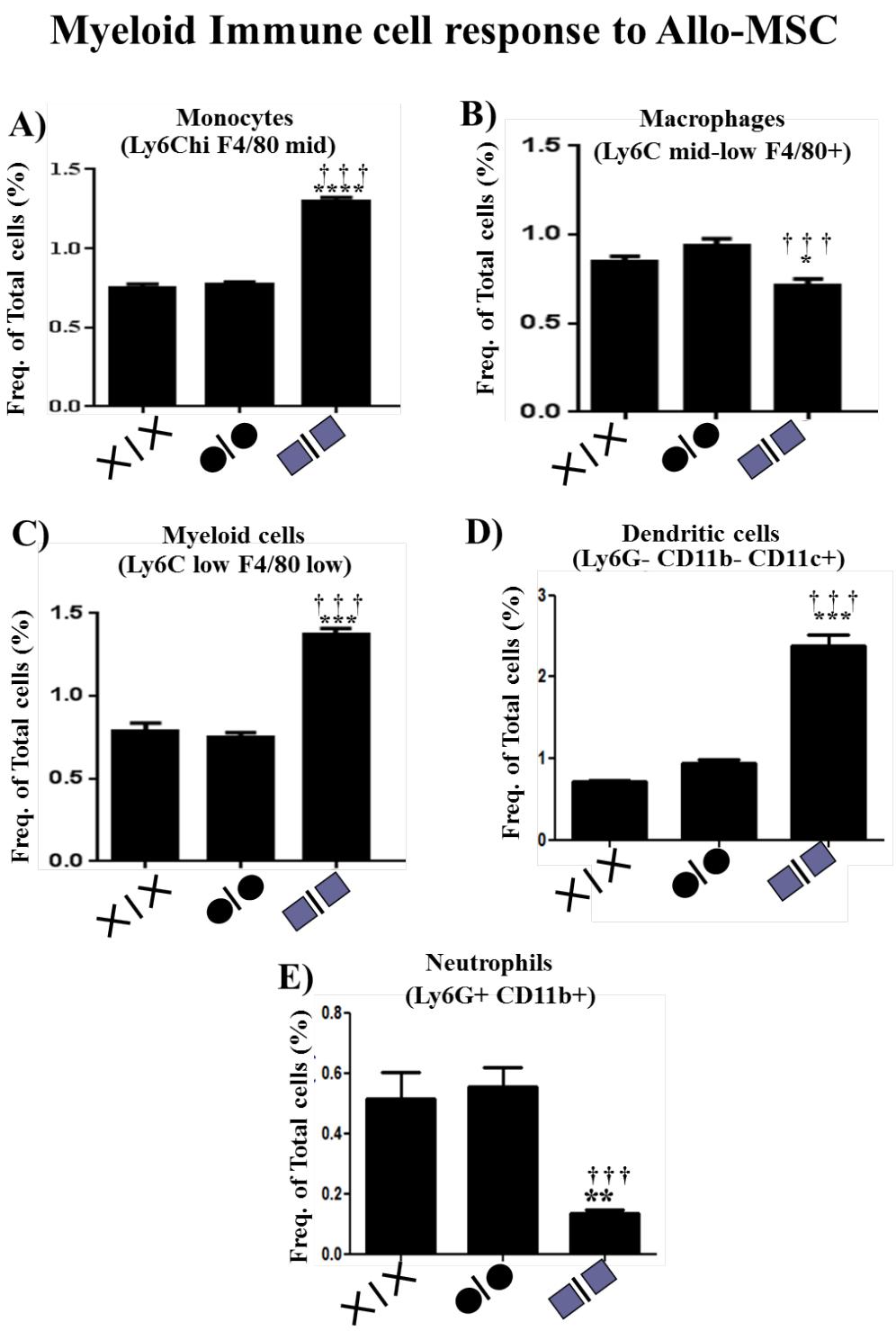


Figure 3.5: Analysis of splenic myeloid cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A-E. Graphs summarizing the results of multi-colour flow cytometry of splenocytes for five individual myeloid cell subpopulations one week following the second of two IM injections are shown for three groups of BALB/c mice (n=10 each). Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**, gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: *, **, *** = $p < 0.05, 0.01, 0.001$ compared to saline control group. ††† = $p < 0.001$ compared to allo-splenocyte double-injected group. (n=8 each group)

3.3.6 *T-Cell Responses to Intra-muscular Injection of Allo-MSC*:

In the same experiment, lymphoid cells including T-cells ($CD4^+$ and $CD8^+$) and regulatory T-cells (T-reg) were analysed by multi-colour flow cytometry as described in Chapter 2, Section 2.3.3. It is well described that MSCs interact both directly and indirectly with T-cells and B-cells and these interactions will contribute to MSC immunomodulatory effects in vivo (Bartholomew *et al.*, 2002 and Di Nicola *et al.*, 2002, Beggs *et al.*,2006, Poncelet *et al.*,2007, Badillo *et al.*,2007 and Isakova *et al.*,2010). Thus, we sought to determine whether there was evidence of ongoing systemic (splenic) T-cell or B-cells response one week following the second of two IM injections of allo-MSCs.

Initially, total $CD4^+$, $CD8^+$ and CD4+/$Foxp3^+$ T-cells were compared across the groups, expressed as the proportions of total viable splenic cells (**fig. 3.6A &B**). As shown, the only difference observed by this analysis was a modest but significant increase in proportionate $CD8^+$ T-cells in the group that received double injection of allo-MSCs. It was notable that there was no evidence of expansion of $FoxP3^+$ T-reg in the spleens of allo-MSC recipients. As further evidence of a lack of involvement of $FoxP3^+$ T-reg in the donor-specific hypo-responsiveness observed following repeated allo-MSC IM injections, the T-reg proportions were also compared at the end point of B6- and FVB-DC-stimulated MLRs of splenocytes isolated from the same experimental groupings. The results, shown in fig. 3.7C, demonstrated similar T-reg frequencies across all groups with, in fact, a small but significant reduction in B6-DC-stimulated MLRs from the allo-MSC double injected group.

Interim Conclusions: (a) Double IM injection of allo-MSCs was associated with a modest proportionate increase in total splenic $CD8^+$, but not $CD4^+$ T-cells. (b) Enumeration of $FoxP3^+$ T-reg in spleens and following donor- and third party allo-specific MLRs provided no evidence for expansion of “classical” T-reg following repeated IM injection of allo-MSCs.

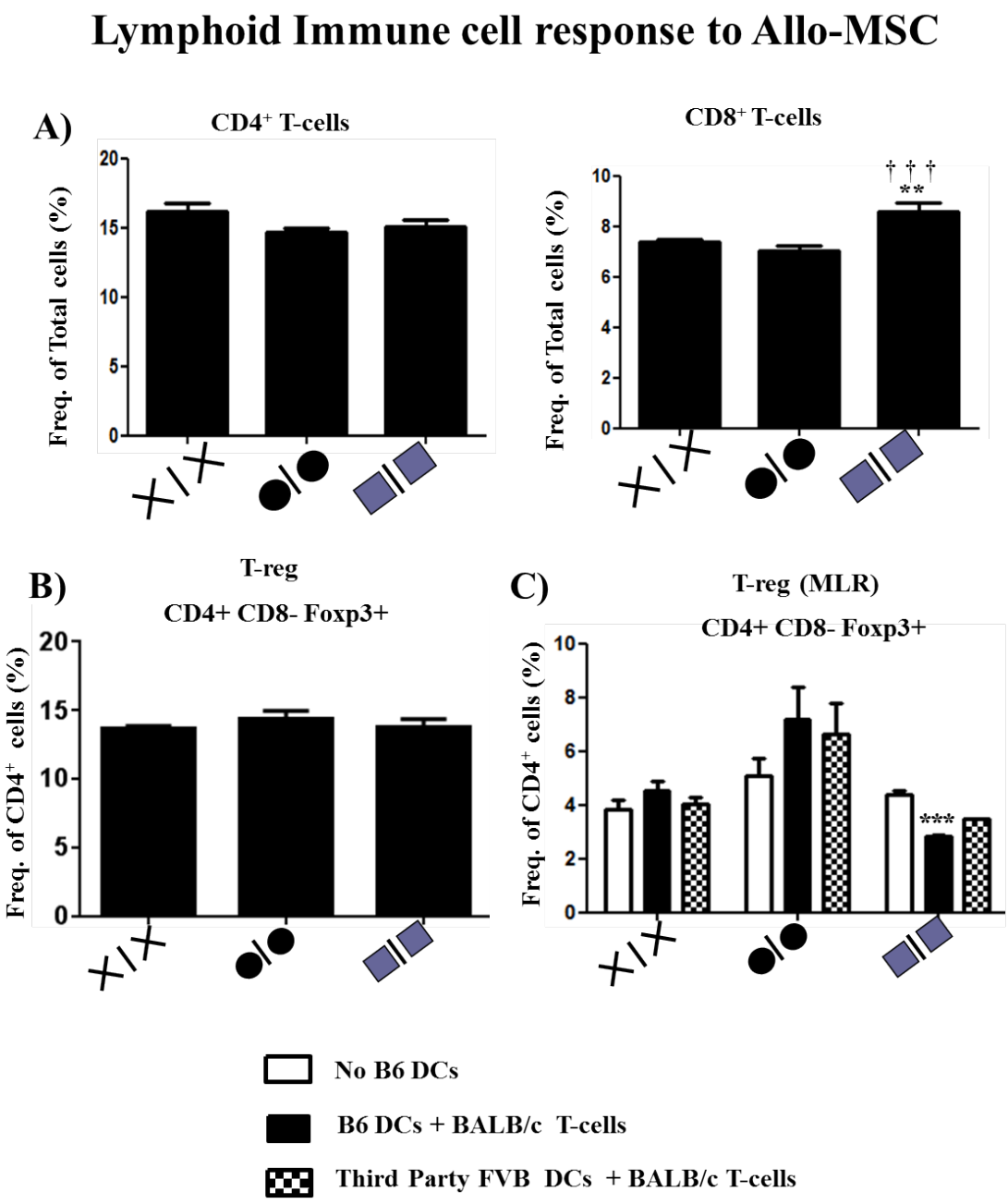


Figure 3.6: Analysis of splenic T-cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A & B. Graphs summarizing the results of multi-colour flow cytometry of splenocytes for $CD4^+$, $CD8^+$ and $CD4^+/FoxP3^+$ T-cells one week following the second of two IM injections are shown for three groups of BALB/c mice (n=10 each). **C.** Graph summarizing the results of staining for FoxP+ T-reg in MLRs from three experimental groups following 5-day cultures without and with B6 or FVB DCs (1:20 T-cell:DC ratios). Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. Symbols used are as for **fig 3.2A**, gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: **, *** = $p < 0.01$, 0.001 compared to saline control group. ††† = $p < 0.001$ compared to allo-splenocyte double-injected group. (n=8 each group)

For $CD4^+$ and $CD8^+$ splenic T-cells, memory and activation status was analyzed by surface staining for CD62L, CD44 and CD25. Initially, each major subset was subdivided into naïve and non-naïve subpopulations based on $CD62L^{hi}$ and $CD62L^{lo}$ state respectively. Following this the naïve and non-naïve subpopulations were further subdivided into four subpopulations: $CD44^{hi}/CD25^-$; $CD44^{hi}/CD25^+$; $CD44^{mid}/CD25^+$; $CD44^{mid}/CD25^+$. The results of these analyses for $CD4^+$ T-cell subpopulation are summarized in **figs. 3.6** ($CD62L^{hi}$ "naïve" $CD4^+$ subpopulations) and **fig. 3.7** ($CD62L^{lo}$ "non-naïve" $CD4^+$ subpopulations). The results for $CD8^+$ T-cell subpopulation are summarized in **fig. 3.9** ($CD62L^{hi}$ "naïve" $CD8^+$ subpopulations) and **fig. 3.10** ($CD62L^{lo}$ "non-naïve" $CD8^+$ subpopulations). For each of these figures, the gating was performed as shown in Chapter 2, **fig. 2.8B**. In all analyses, results were expressed as proportions of total viable splenic cells. As shown in these figures, there were multiple statistically significant differences observed among the three experimental groups suggesting altered splenic proportions of naïve vs. memory and activated vs. non-activated helper and cytotoxic T-cells following IM injections of allo-splenocytes and allo-MSCs. In regard to the results for the allo-MSC groups, the most striking findings were are follows:

$CD4^+$ T-cell Subpopulations: (a) The proportion of $CD62L^{hi}$ cells was lower than the saline and allo-splenocyte injected groups (**fig. 3.7B**), while the proportion of $CD62L^{lo}$ cells was higher than the allo-splenocyte injected group (**fig. 3.8B**). (b) Despite the lower proportion of total $CD62L^{hi}$ cells, the proportions of $CD62L^{hi}/CD44^{hi}$ subpopulations were higher than the saline control (**fig. 38C&**D). (c) In the case of the $CD62L^{lo}$ subpopulations, the $CD44^{hi}/CD25^{lo}$ subpopulations was proportionately expanded while the $CD44^{mid}/CD25^+$ was proportionately lower compared to the saline- and allo-splenocyte injected groups (**fig. 3.8B&**E). *$CD8^+$ T-cell Subpopulations:* (a) The proportion of $CD62L^{lo}$ cells was higher than for the other two groups (**fig. 3.10B**). (b) This difference was accounted for by proportionate increases in the CD44hi subpopulations (**fig. 3.10C&**D).

Interim Conclusion: The differences observed for the allo-MSC-injected group were consistent with skewing toward activated- and memory-phenotype $CD4^+$ and, more strikingly, $CD8^+$ T-cells compared with the saline group and, to a lesser extent, the allo-splenocyte injected group.

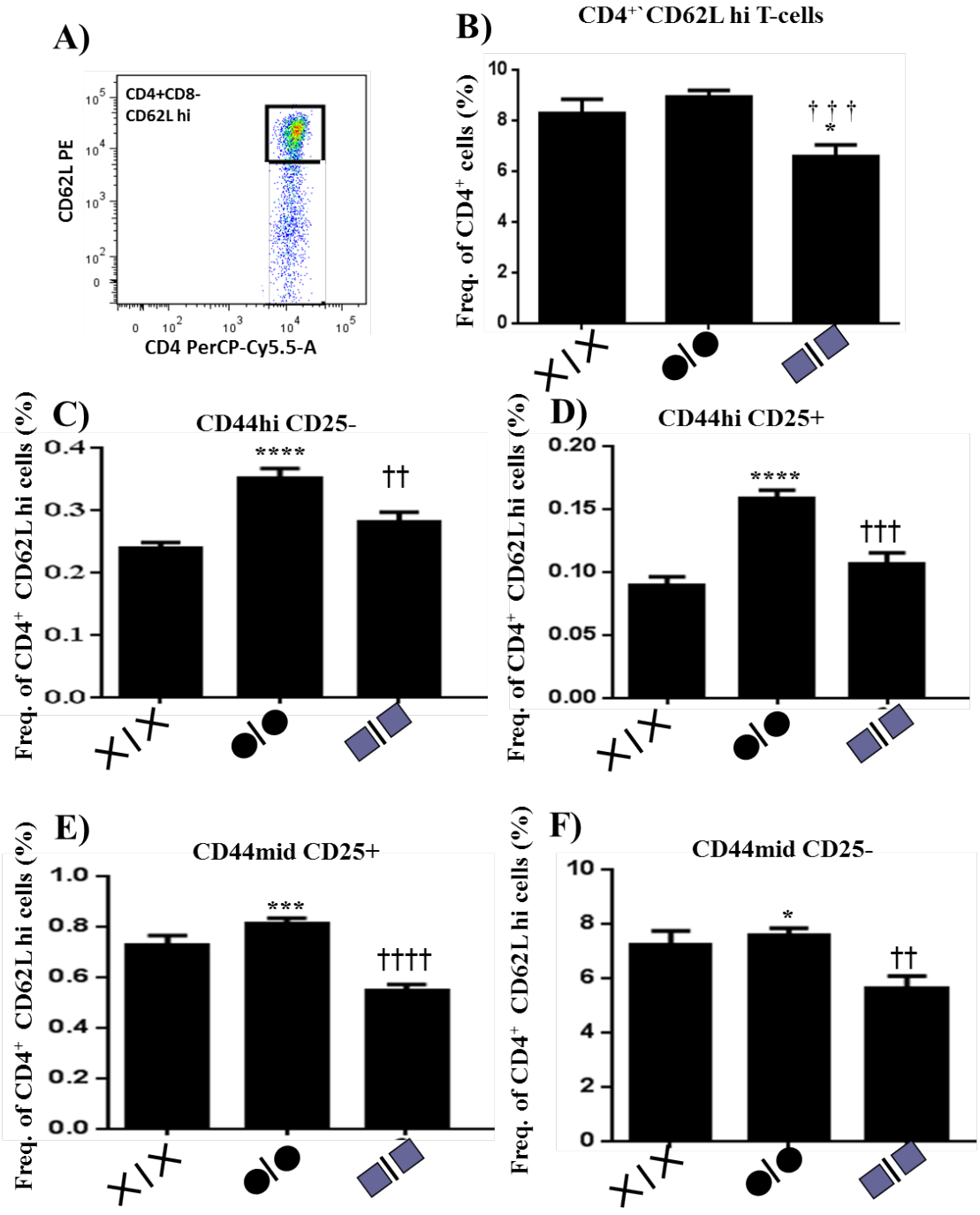


Figure 3.7: Analysis of splenic CD4$^+$/CD62L^{hi} T-cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Dot plot illustrating gating on CD4$^+$/CD62L^{hi} T-cells. **B.** Graph summarizing the frequencies of CD4$^+$/CD62L^{hi} T-cells in spleens from three groups of BALB/c mice (n=10 each). **C-F.** Graphs summarizing the frequencies for four CD4$^+$/CD62L^{hi} subpopulations based on staining for CD44 and CD25. Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. Gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: *, **, ***, **** = $p < 0.05, 0.01, 0.001, 0.0001$ compared to saline control group. †, ††, ††† = $p < 0.05, 0.01, 0.001$ compared to allo-splenocyte double-injected group. (n=8 each group)

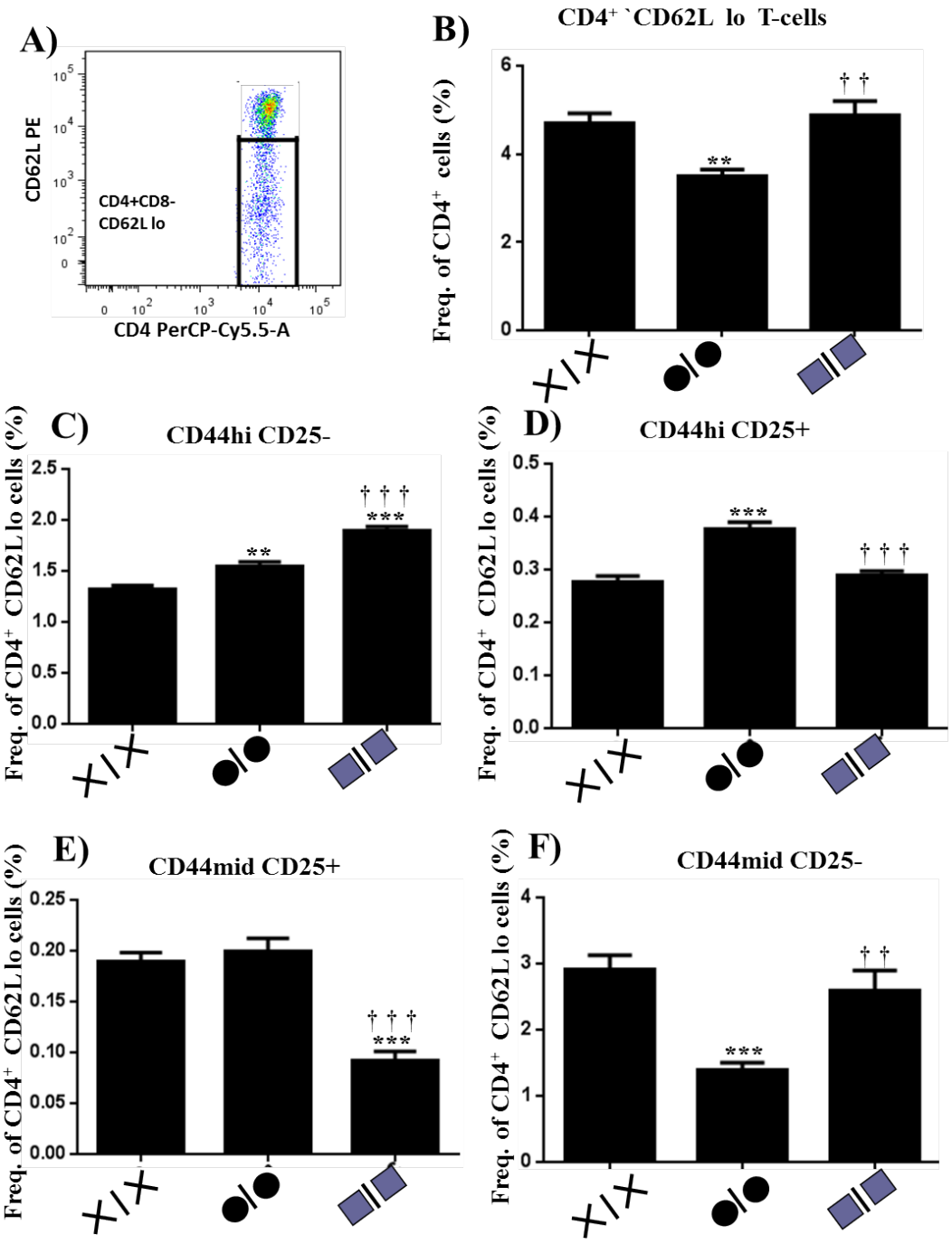


Figure 3.8: Analysis of splenic $CD4^+/CD62L^{lo}$ T-cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Dot plot illustrating gating on $CD4^+/CD62L^{lo}$ T-cells. **B.** Graph summarizing the frequencies of $CD4^+/CD62L^{lo}$ T-cells in spleens from three groups of BALB/c mice (n=10 each). **C-F.** Graphs summarizing the frequencies for four $CD4^+/CD62L^{lo}$ subpopulations based on staining for CD44 and CD25. Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. Gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: **, *** = $p < 0.01, 0.001$ compared to saline control group. ††, ††† = $p < 0.01, 0.001$ compared to allo-splenocyte double-injected group. (n=8 each group)

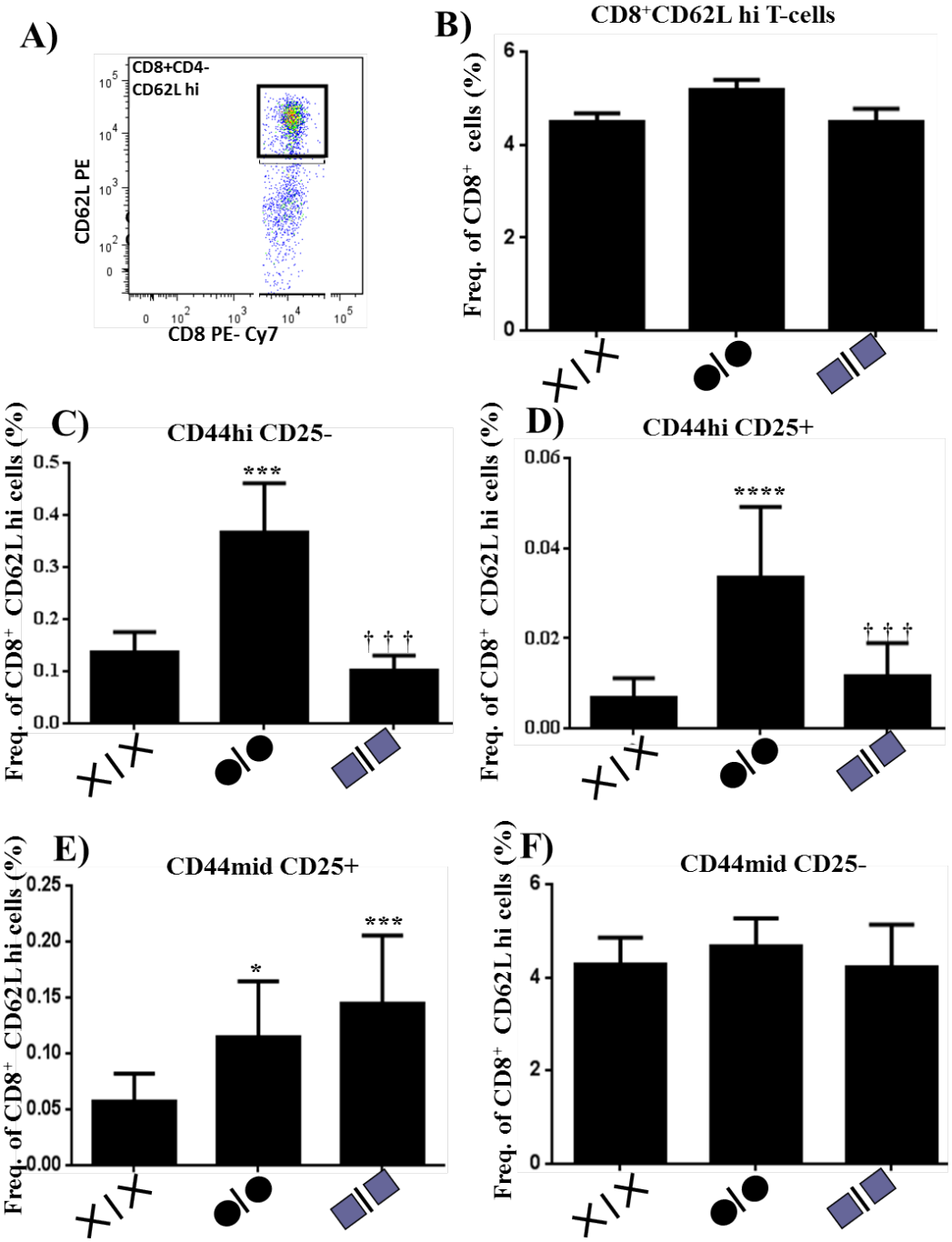


Figure 3.9: Analysis of splenic CD8^{+}/CD62L^{hi} T-cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Dot plot illustrating gating on CD8^{+}/CD62L^{hi} T-cells. **B.** Graph summarizing the frequencies of CD8^{+}/CD62L^{hi} T-cells in spleens from three groups of BALB/c mice (n=10 each). **C-F.** Graphs summarizing the frequencies for four CD8^{+}/CD62L^{hi} subpopulations based on staining for CD44 and CD25. Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. Gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: ***, **** = $p < 0.001, 0.0001$ compared to saline control group. †, ††† = $p < 0.05, 0.001$ compared to allo-splenocyte double-injected group. (n=8 each group)

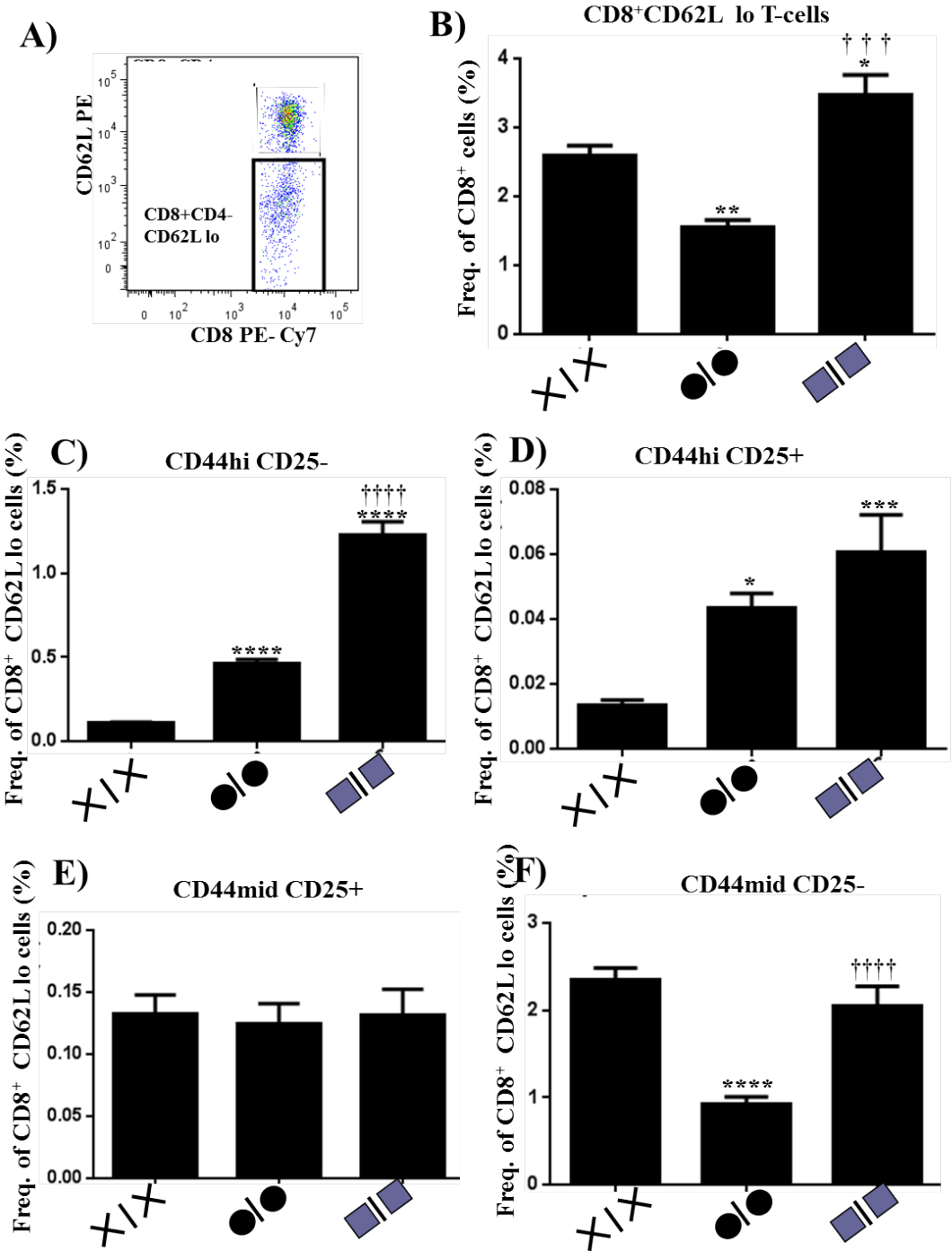


Figure 3.10: Analysis of splenic CD8^{+}/CD62L^{lo} T-cell subpopulations following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Dot plot illustrating gating on CD8^{+}/CD62L^{lo} T-cells. **B.** Graph summarizing the frequencies of CD8^{+}/CD62L^{lo} T-cells in spleens from three groups of BALB/c mice (n=10 each). **C-F.** Graphs summarizing the frequencies for four CD8^{+}/CD62L^{lo} subpopulations based on staining for CD44 and CD25. Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. Gating and subpopulation definitions are as shown in **fig. 2.8B**. **Statistics**: One Way ANOVA: *, **, ***, **** = $p < 0.05, 0.01, 0.001, 0.0001$ compared to saline control group. †††, †††† = $p < 0.001, 0.0001$ compared to allo-splenocyte double-injected group. (n=8 each group)

3.3.7 *Natural Killer and NKT-Cell Responses to Intra-muscular Injection of Allo-MSC:*

A limited number of studies have reported *in vitro* interactions between NK-cells and NKT-cells and MSCs. *In vitro* assays have demonstrated that allo-MSCs may be lysed by NK-cells of the host but have also shown that MSCs may suppress NK-cell secretion of IFN- γ (Rasmusson *et al.*, 2003 and Aggarwal & Pittenger *et al.*, 2005). Mediators reported to be responsible for MSC suppression of NK-cell function include PGE2 and IDO (Poggi *et al.*,2005, Sotiropoulou *et al.*,2006 and Spaggiari *et al.*,2006). Interactions of MSCs with NKT cells are very scarce and only few groups have studied the effects of MSC immune-modulation on NKT cells (Selmani *et al.*, 2008 and Dazzi *et al.*, 2012). For example, Prigione *et al.*, 2009 showed that human MSC can inhibit the expansion of NKT cells *in vitro* assays and related the mechanism of action through PGE_2 blockade experiments. However, the *in vivo* influence of allo-MSCs on NK- and NKT-cells is not well understood, particularly following IM injection.

Thus, in addition to analysing splenic myeloid cell and T-cell subpopulations within the spleens of the three groups of IM injected mice, the proportions of NK-cells ($CD19^-/CD4^-/CD8^-/CD49b^+$) and NKT-cells ($CD19^-/CD49b^+/CD3^+$) were also compared across the groups (**fig. 3.11**).

As shown in **fig. 3.11B&C**, we made the somewhat surprising observation that both NK-cells and NKT-cells were proportionately increased in the spleens of allo-MSC injected compared to saline and allo-splenocytes injected mice. Additionally, NK-cells were proportionately increased in the spleens of allo-splenocyte compared to saline injected mice.

Interim Conclusion: Double IM injection of allo-MSCs is associated with a relative expansion of systemic (splenic) NK- and NKT-cells.

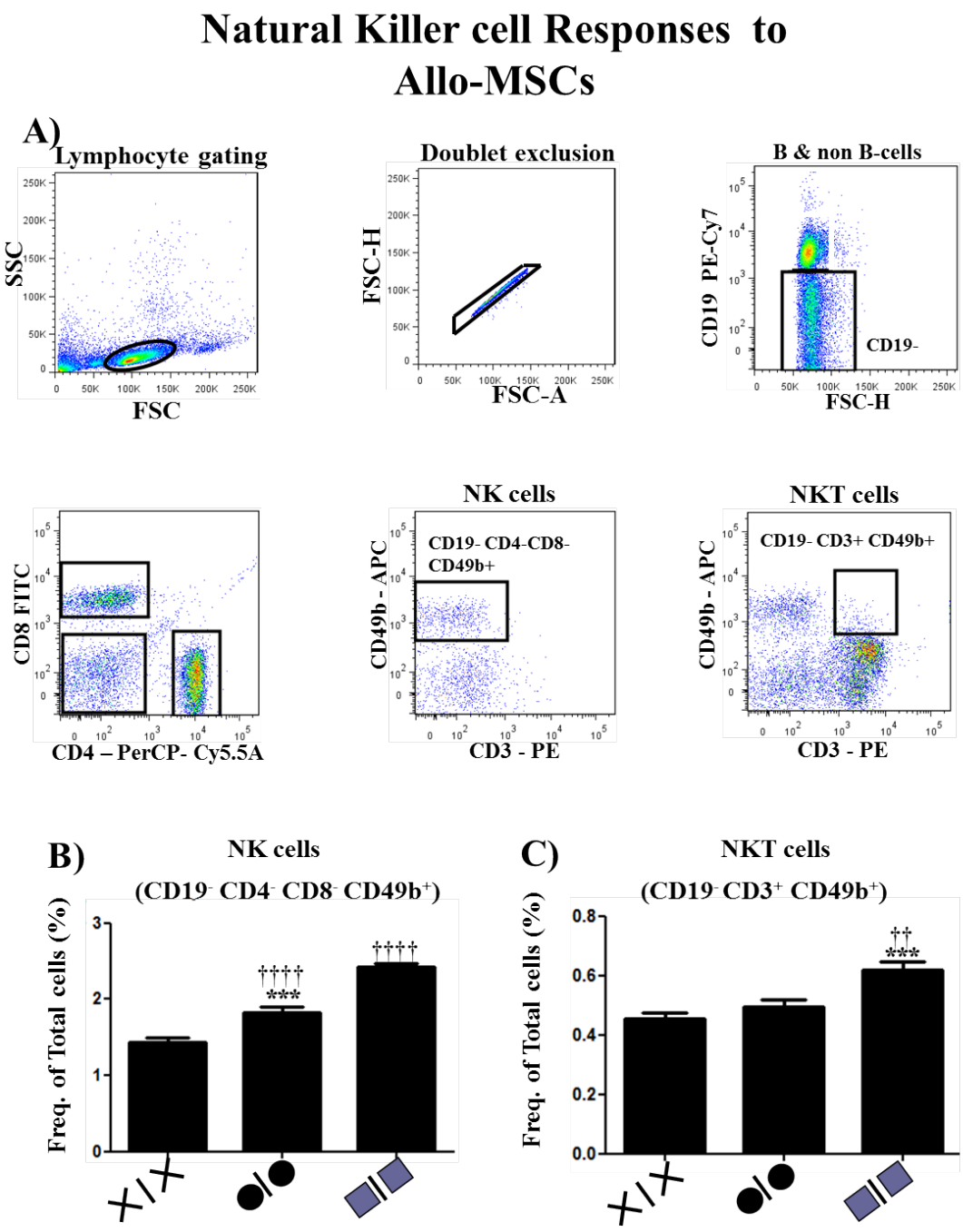


Figure 3.11: Analysis of splenic NK- and NKT-cells following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Representative dot plots illustrating the gating strategies for quantifying splenic NK-cells and NKT-cells in BALB/c mice. **B. & C.** Graphs summarizing the results of multi-colour flow cytometry of splenocytes for NK-cells and NKT-cells one week following the second of two IM injections are shown for three groups of BALB/c mice (n=10 each). Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. **Statistics**: One Way ANOVA: *** = $p < 0.001$ compared to saline control group. ††, †††† = $p < 0.01$, 0.001 compared to allo-splenocyte double-injected group. (n=8 each group)

3.3.8 *Soluble Mediators Associated with T-cell Hypo-responsiveness Following Repeated I.M. Injection of Allo-MSCs*

The results to this point suggested that double injection of allo-MSCs was associated with donor antigen-specific T-cell hypo-responsiveness. As this hypo-responsiveness was observed in MLRs involving T-cell enriched responders, it appeared that the presence of non-T-cell "accessory" cells was not an essential requirement; at least at the time of donor antigen re-call. Results from profiling of splenocyte populations and of analysis of cells at the end of the MLRs cultures also appeared to rule out a specific expansion of $CD4^+/FOXP3^+$ T-reg. It remained unclear, however, whether the observed hypo-responsiveness was associated with a characteristic T-cell differentiation profile. To address this issue, the cytokine profiles of MLR culture supernatants were examined for splenocytes from three groups of BALB/C mice: Recipients of double injections of (a) Saline, (b) Allo-splenocytes and (c) Allo-MSCs. For these analyses, MLRs stimulated with donor-specific (B6) and third-party (FvB) DCs were initiated along with control cultures without DCs. The culture supernatants were analyzed by ELISA for IFN- γ, IL-10, IL-4, IL-2, TFG-β and IL-17. Only IFN- γ and IL-10 were detected in significant amounts and the results for these two cytokines are shown in **fig. 3.12A&B**.

As shown, B6 DC-stimulated MLRs of responder splenocytes from recipients of double injections of allo-MSCs were associated with significantly higher IFN-γ compared to FvB DC-stimulated MLRs and compared to MLRs from the saline and allo-splenocyte groups (**fig.3.13A**). The MLRs from the allo-MSC double injection group also contained higher concentrations of IL-10 than those of the other two groups. However, in this case, the increase in cytokine production occurred equally in unstimulated and B6 DC-stimulated MLRs and to a greater extent in FvB DC-stimulated MLRs (**fig.3.13A**). Thus, the most striking result (which was observed in a total of five individual experiments), was the donor antigen-specific production of large amounts of IFN-γ by splenic T-cell responders from recipients of double IM injections of allo-MSCs. Nonetheless, addition of blocking antibodies to IFN-g, IL-10 singly or together did not result in significant reversal of either the $CD4^+$ or the $CD8^+$ T-cell hypo-responsiveness in this group (**fig. 3.12C&D**).

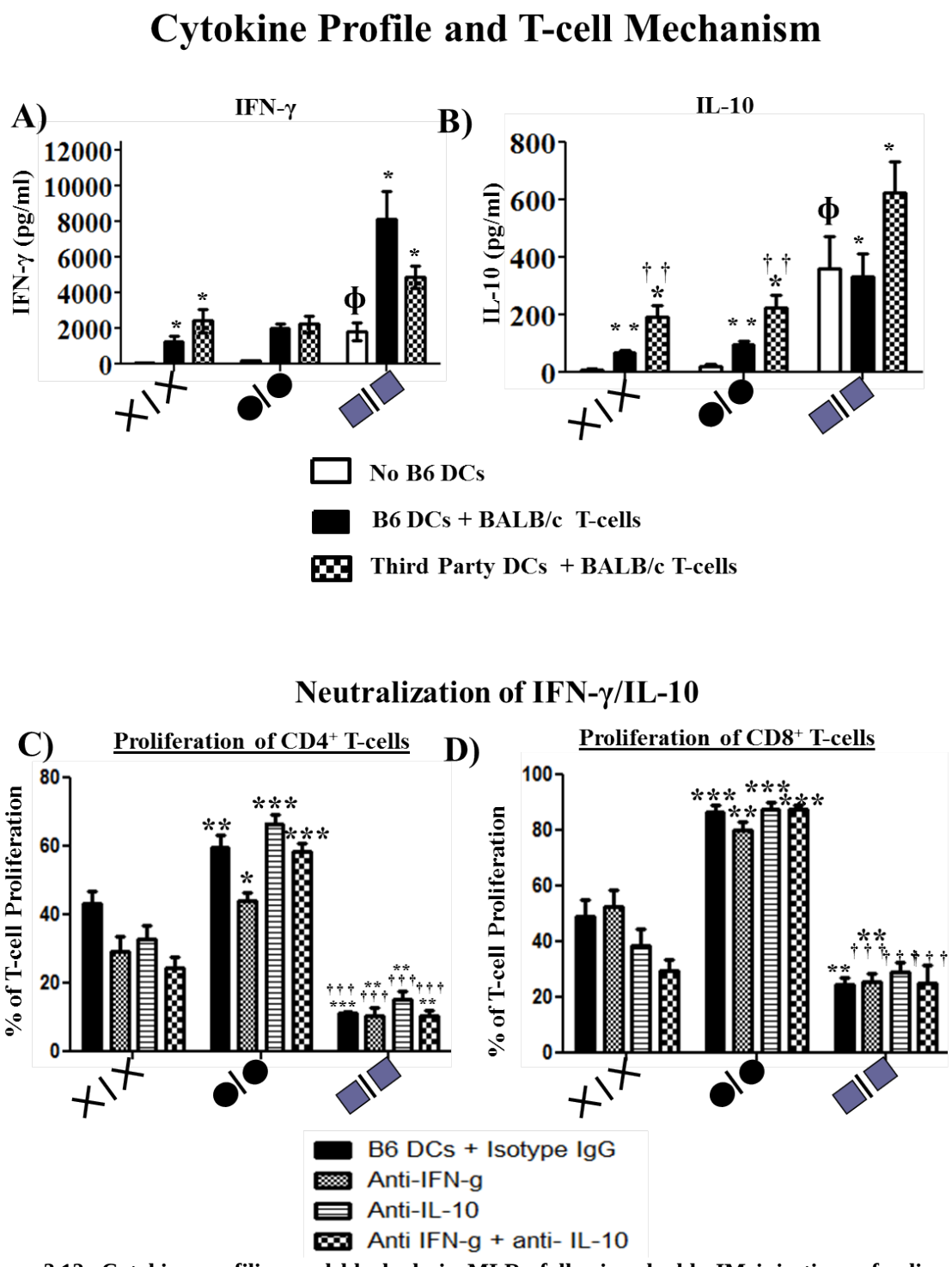


Figure 3.12: Cytokine profiling and blockade in MLRs following double IM injections of saline, allo-splenocytes or allo-MSCs: A&B: Graphs summarizing the results of ELISAs for IFN-γ and IL-10 in day-5 MLR culture supernatants. MLRs were initiated using whole splenocytes with or without donor-specific (B6) and third-party (FvB) DCs one week following the second of two IM injections are shown for three groups of BALB/c mice (n=10 each). **C&D:** Proliferation of T-cells in B6 DC-stimulated MLRs from the same groups in the absence and presence of neutralizing anti-IFN-γ and/or anri-IL-10 antibodies. **Statistics**: One Way ANOVA: *, *** = $p < 0.05$, 0.001 compared to saline control group and appropriate treatment condition. †††† = $p < 0.001$ compared to allo-splenocyte double-injected group and appropriate treatment condition. Also, ϕ = $p<0.05$ compared to no DCs stimulation from saline and allo-splenocytes group. (n=6 each group)

In this same experiment, we also examined the effect of adding the non-specific cyclooxygenase (COX) inhibitor indomethacin to B6 DC-stimulated MLRs in order to determine whether there was a role for prostaglandin E2 (PGE2)-mediated suppression of T-cells as has been reported previously in the setting of MSC administration to animal models (Nemeth *et al.*, 2008). For these analyses, the proliferation of $CD4^+$ and $CD8^+$ T-cells in the MLRs containing indomethacin or an equivalent volume of the relevant vehicle were compared for the three groups of animals.

As shown in **fig. 3.13A&B**, COX inhibition was associated with a significant reduction of both CD4+ and CD8+ T-cell proliferation in MLRs from the allo-splenocyte double injection group, consistent with a positive role for PGE2 in these donor-antigen re-call cultures. In contrast, COX inhibition was associated with a moderate but significant increase in $CD4^+$ T-cell proliferation and no significant effect on CD8+ T-cell proliferation in the MLRs from the allo-MSC double-injected group. Although not conclusive, the results were suggestive of distinctly different roles for (and possibly sources of) PGE2 among splenocyte populations from animals that received repeated IM injections of allo-MSCs and allo-splenocytes.

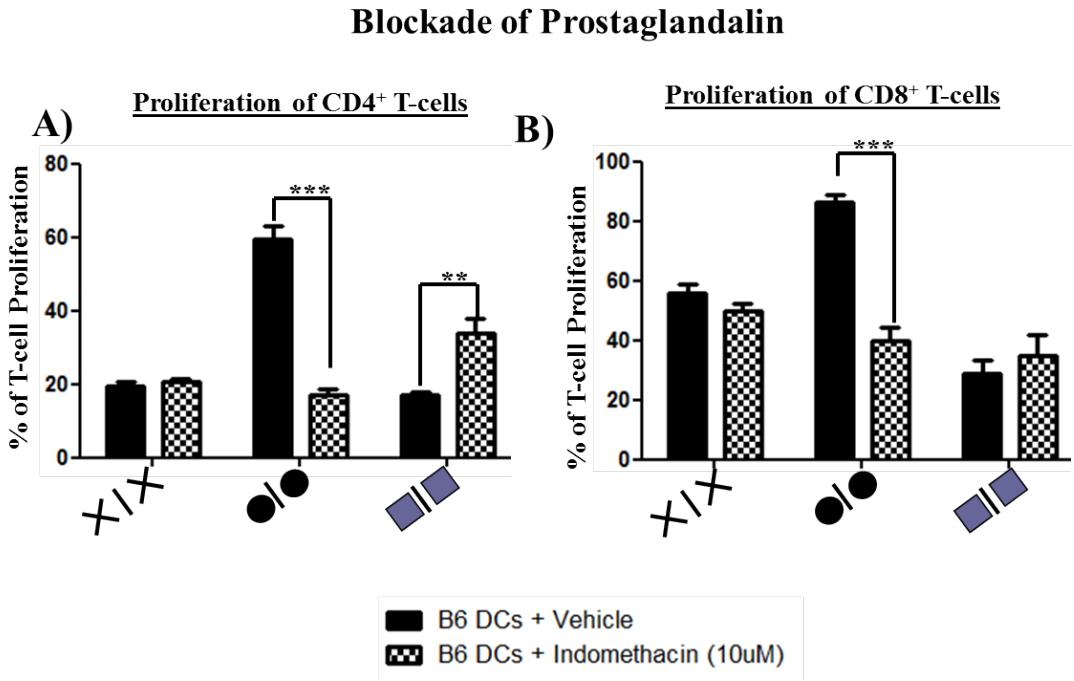


Figure 3.13: Cyclooxygenase blockade in MLRs following double IM injections of saline, allo-splenocytes or allo-MSCs: A&B: Graphs summarizing the proliferation of $CD4^+$ and $CD8^+$ T-cells in B6 DC-stimulated MLRs from three groups of BALB/C mice in the absence and presence of 10μM indomethacin. **Statistics**: Student's t-test, * = $p < 0.05$ compared to vehicle for the corresponding group. (n=6 each group)

Interim Conclusions: (a) The T-cell hypo-responsiveness that is observed following repeated IM injections of allo-MSCs is associated with increased production of IFN-γ by donor antigen-specific T-cells. (b) Splenocytes from recipients of repeated IM injections of allo-MSCs produce increased amounts of the anti-inflammatory cytokine IL-10 but this is not specific for donor antigen re-call. (c) Neither IFN-γ nor IL-10 directly mediates donor-specific T-cell hypo-responsiveness during re-call assays. (d) Production of PGE2 by one or more splenic cell populations may play a minor role in the $CD4^+$ T-cell hypo-responsiveness observed following repeated IM injection of allo-MSCs.

3.3.9 *$CD8^+$ T-cell Cytotoxic Response Following Repeated I.M. Injection of Allo-mscs*

Results to this point had demonstrated donor-specific hypo-responsiveness of splenic $CD8^+$ T-cells in MLRs following double IM injection of allo-MSCs. In addition, flow cytometric profiling of splenic cells indicated expansion of total and activated/memory phenotype $CD8^+$ T-cells in the recipients of allo-MSCs. These results suggested that $CD8^+$ T-cells might have a unique allo-response to repeated IM injections of allo-MSCs. We therefore sought to further characterize the systemic $CD8^+$ T-cell response to allo-MSCs by examining their capacity for donor-specific cellular cytotoxicity as described in the Methods section for this Chapter (section 3.2.6). For these experiments, activated allo-antigen-specific $CD8^+$ T-cells were first generated from total splenocytes of mice that had received double IM injections of allo-splenocytes or allo-MSCs in MLRs stimulated with either B6 (donor-specific) or FvB (third-party) DCs. The targets for the cytotoxicity step were B6 (allo)-MSCs. The sequence of steps for this protocol is summarized in **fig. 3.14** and the results of the assays for splenic $CD8^+$ T-cells from recipients of allo-splenocytes and allo-MSCs are shown in **fig. 3.16**.

As shown, $CD8^+$ T-cells from allo-splenocyte recipients demonstrated increased granzyme B expression and increased lysis of B6 MSC targets following reactivation with B6 DCs (**fig. 3.15A&B**). In contrast, CD8+ T-cells from allo-MSC recipients demonstrated lower granzyme B expression than those from allo-splenocyte recipients under all conditions and did not demonstrate increased cytolysis of B6 MSC targets following re-stimulation with B6 DCs (**fig. 3.15A&B**).

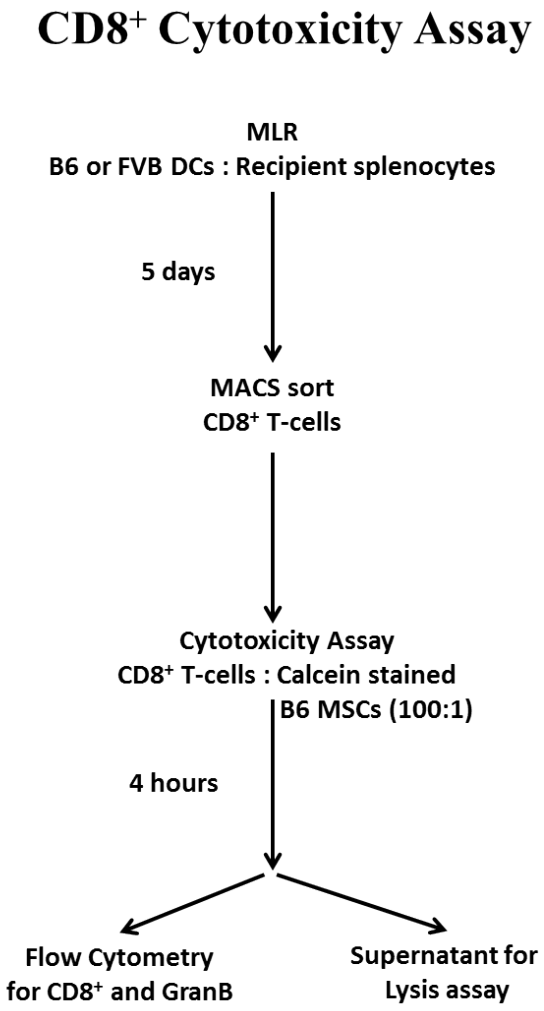


Figure 3.14: Schematic diagram of CD8$^+$ cytotoxicity assay protocol

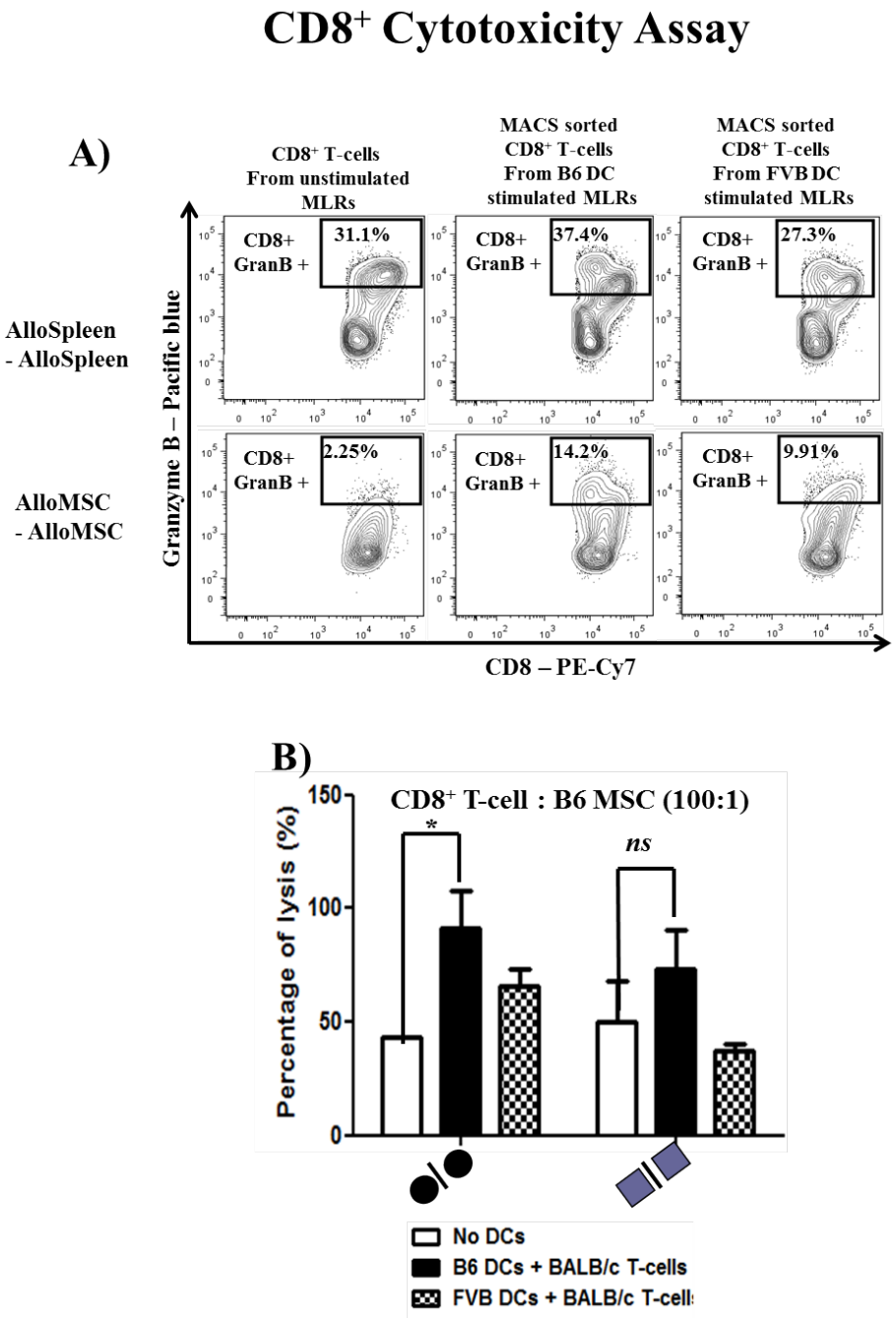


Figure 3.15: Granzyme B expression and cytotoxicity of splenic CD8$^+$ T-cells from recipients of repeated IM injections of allo-splenocytes or allo-MSCs: BALB/C mice received either double IM injections of allo-splenocytes or allo-MSCs. Whole splenocytes from the recipient animals were cultured without DCs (No DCs) or were re-stimulated with B6 DCs or FvB DCs to prime CD8$^+$ T-cells which were then purified and placed in culture for 4 hours with calcein-labelled B6 MSCs. **A.** Representative flow cytometry dot plots demonstrating Granzyme B expression on purified, activated CD8$^+$ T-cells following 4 hour culture with allo-MSCs. Percentages of CD8$^+$ cells that were positive for Granzyme B are indicated in boxes. **B.** Percent lysis of calcein-labelled B6 MSC cell lysis following 4 hour culture with purified, activated CD8$^+$ T-cells from the various conditions. Results shown are mean ± SD for two technical replicates per condition. **Statistics:** Student's t-test. * = $p < 0.05$ compared to relevant result for No DC condition. Ns = non-significant. (n=8 each group)

3.3.10 *Splenic $CD8^+/CD11c^+$ T-cells in Recipients of Repeated IM Injections of Allo-MSCs*

In 1987, Keizer *et al*. reported a subset of $CD8^+$ T-cells expressing CD11c on the cell surface with an apparent regulatory effect on cytotoxic responses. Subsequently, it was shown that $CD8^+/CD11c^+$ T-cells account for less than 3% of total T-cells in healthy mice but expand in an antigen-dependent manner during adaptive immune responses. Additionally, $CD8^+/CD11c^+$ T-cells are reported to suppress $CD4^+$ T-cell activation in a cell contact-dependent manner with IFN-γ as a signature cytokine (Vinay & Kwon *et al.,* 2010 and Segovia *et al.,* 2014). Given this background and the results described above, we evaluated $CD8^+/CD11c^+$ T-cells in spleens of BALB/C recipients of repeated IM injections of saline, allo-splenocytes and allo-MSCs.

Initially, a multi-colour flow cytometry staining panel was designed in order to accurately gate on and quantify this rare population of T-cells. The staining and gating strategy for this analysis is illustrated in **fig. 3.16A**. As shown, forward/side scatter gating and a dead cell exclusion dye were first employed to accurately gate on viable lymphocytes. Next, staining for CD3 and CD8 were used to confine the gating to $CD8^+$ T-cells (thus excluding other potential CD11c+ splenic populations such as DCs, neutrophils, monocytes and NK cells). Finally, $CD3^+/CD8^+/CD11c^+$ cells were gated upon and expressed as a percentage of the total viable splenic cells. For these analyses, a large number of events were acquired for each sample to ensure that the quantified percentages were accurate.

As shown in **fig. 3.16B**, there was a striking and statistically significant proportionate increase in $CD8^+/CD11c^+$ T-cells in spleens of recipients of repeated IM injections of allo-MSCs compared to both saline and allo-splenocyte recipients. Back gating analysis on $CD11c^+$ and $CD11c^-$ cells from $CD3^+/CD8^+$ cell population, as shown in **fig. 3.16C,** demonstrated that $CD11c^+$ CD8 T-cells have similar size (FSC) and granularity (SSC) to the $CD11c^-$ CD8-cells, but showed mildly reduced CD8 surface expression in the allo-splenocyte and allo-MSC injected animals when compared to saline group.

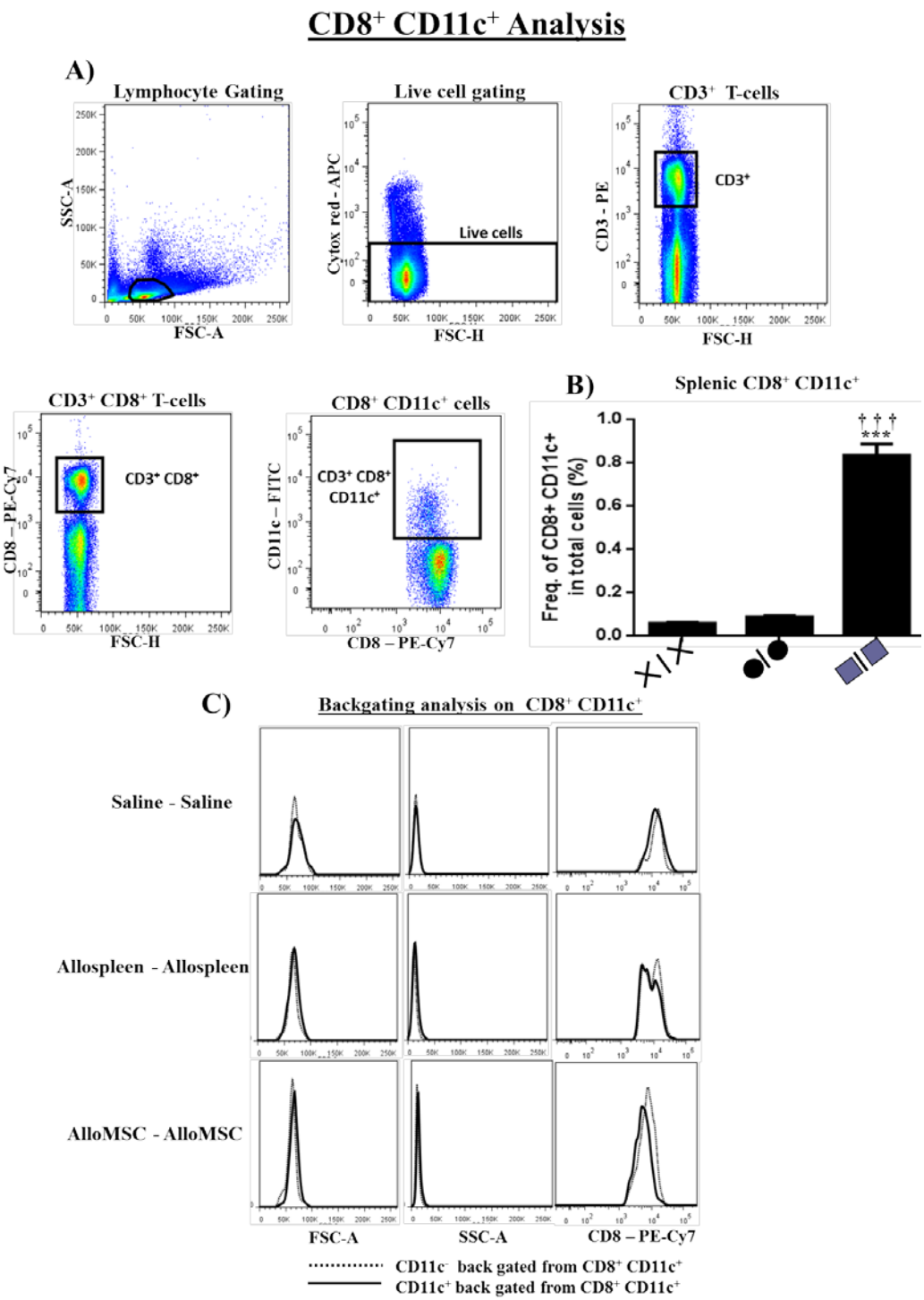


Figure 3.16: Analysis of splenic CD8$^+$/CD11c$^+$ T-cells following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Representative dot plots illustrating the gating strategy for quantifying splenic CD8$^+$/CD11c$^+$ T-cells in BALB/c mice. **B.** Graph summarizing the results of multi-colour flow cytometry of splenocytes for CD8$^+$/CD11c$^+$ T-cells one week following the second of two IM injections are shown for three groups of BALB/c mice (n=10 each). **C.** Representative histograms showing FSC, SSC and CD8$^+$ expression on CD11c$^+$ and CD11c$^-$ cells generated by back-gating from the CD3$^+$/CD8$^+$ population. Results are expressed as mean ± SD frequency among total viable splenocytes. Symbols used are as for **fig 3.2A**. **Statistics**: One Way ANOVA: *** = $p < 0.001$ compared to saline control group. ††† = $p < 0.001$ compared to allo-splenocyte double-injected group. (n=6 each group)

Interim Conclusions: (a) Splenic $CD8^+$ T-cells from recipients of repeated IM injections of allo-MSCs exhibit reduced donor antigen-specific expression of granzyme B and reduced cytolysis of B6 MSCs following *in vitro* reactivation. This result is compatible with clonal deletion or active regulation/re-programming of donor antigen-specific cytotoxic T-cells. (b) Repeated IM of allo-MSCs, but not allo-splenocytes, was associated with relative expansion of $CD8^+/CD11c^+$ T-cells. Considered in the context of existing literature and other findings from the current study, this observation is compatible with induction of a systemic donor antigen-specific regulatory $CD8^+$ T-cell population following repeated IM injection of allo-MSCs.

Limitation: For splenic $CD4^+/FoxP3^+$ and $CD8^+/CD11c^+$ analysis, the results were presented as proportions rather than as absolute number of cells per spleen or per culture well. Although there were no overt differences in the sizes or appearance of the spleens among the experimental groups, this limitation could have been overcome by first recording the total cell number in spleen (in the case of analyses of splenic cell repertoire) and by the use of counting beads or an alternative counting method during the flow cytometric analyses. .

3.3.11 *Local Immune Response to Intra-muscular Delivered Allo-MSCs*

In the final experimental approach for this Chapter, we evaluated the extent of localized inflammatory cell infiltration at the sites of the IM injections in groups of mice that had received repeated inocula of saline, allo-splenocytes or allo-MSCs. The local immune response to allo-MSCs has been previously investigated in healthy animals and in some pre-clinical disease models. In the current study, groups of BALB/C mice received double IM injection of saline, allo-splenocytes or allo-MSCs as previously described and, 1 week later, the injected muscles were dissected and processed and sectioned for H&E staining and blinded, semi-quantitative scoring of inflammatory cell infiltrates as described in Chapter 2. Representative low- and medium-power photomicrographs of the tissues sections from the three groups are shown in **fig. 3.17A.**

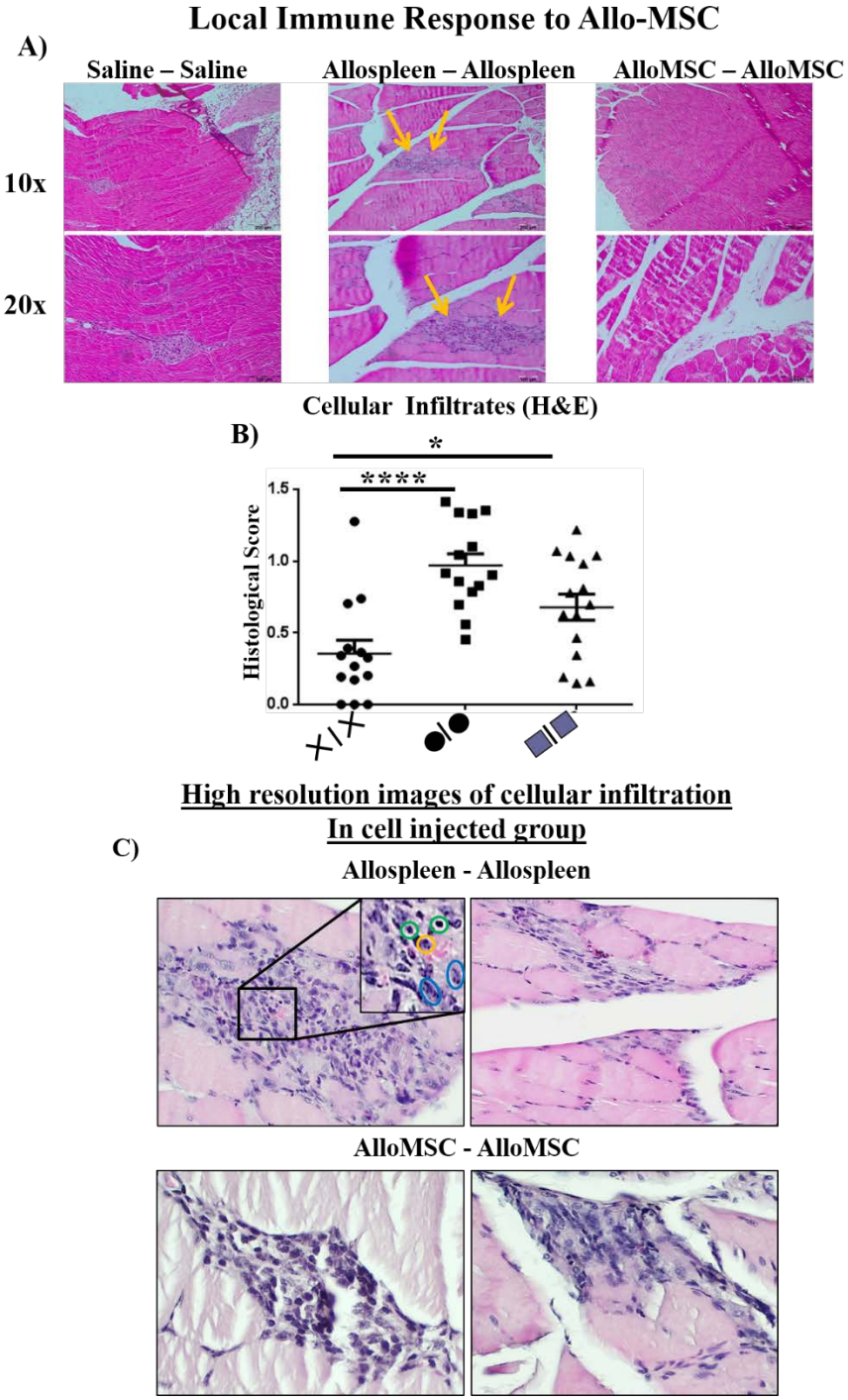


Figure 3.17: Semi-quantitative analysis of muscle tissue inflammatory cell infiltrates following double IM injections of saline, allo-MSCs or allo-splenocytes: A. Representative 10X and 20X photomicrographs of H&E stained muscle tissue sections from each of groups of BALB/c mice. **B.** Graph summarizing the results of semi-quantitative scoring of tissue sections are shown for three groups of BALB/c mice (n=15 each). Each point represents the mean score for multiple sections from an individual animal. The horizontal and vertical bars represent the mean ± SD score for each group. Symbols used are as for fig 3.2A. **C.** Representative 20x/40x photomicrographs of H&E stained muscle tissue sections from allo-splenocytes and allo-MSC groups of BALB/c mice were shown. In the upper right panel, representative cells are circled in red (lymphocytes), orange (neutrophil) and blue (large mononuclear cells – possibly monocyte/macrophages) **Statistics**: One Way ANOVA: *, *** = $p < 0.05$, 0.001 compared to saline control group. (n=10 each group)

The results of the histological scoring for 15 individual animals from each group are summarized on **fig. 3.17B**. As shown, the scores were predictably low in the saline injected animals and were consistently and significantly higher in the allo–splenocyte injected animals. In the allo-MSC injected mice, there was also increased inflammatory cell infiltration that was significantly greater than that of the saline injected group. Although there was a trend toward lower inflammatory cell infiltration scores among the allo-MSC compared to the allo-splenocyte injected group, this did not reach statistical significance. Further, higher magnification of histological sections from both allo-splenocyte and allo-MSC treated groups revealed mixed inflammatory cellular infiltrates in the skeletal muscle tissue. As shown in representative high resolution images in **fig. 3.17C**, infiltrates from the allo-splenocyte-injected group shown the presence of lymphocytes, neutrophil and large mononuclear cells (likely monocytes/macrophages). Infiltrates in muscle sections from the allo-MSC-injected group had similar appearance with a predominance of lymphocytes and mononuclear cells. Many of the lymphocytes had large, irregularly shaped nuclei consistent with localized activation and proliferation of lymphocytes in response to allo-MSC infusion. **(fig.3.17C).**

Interim Conclusion: Repeated IM injection of allo-MSCs is associated with localized inflammatory cell infiltration at the injection sites that is greater than that observed following saline injections and is not significantly in extent to that observed following allo-splenocyte injections.

3.4 DISCUSSION

In this chapter, anti-donor T-cell and innate immune cell responses to single or repeated intra-muscular injections of allo-MSCs were tested and in particular, immune mechanisms associated with repeated IM injections of allo-MSCs were studied in detail with head-to-head comparisons in multiple experiments to injections of allo-splenocytes (which were expected to be strongly immunogenic) and to saline injections (which were expected to have no influence on anto donor immune responses).

Initial results from mice receiving a single IM injection of allo-MSCs showed evidence for increased T-cell proliferation in B6 DC-stimulated "re-call" MLRs that was most prominent for CD8+ T-cells and was comparable to that observed in recipients of single injections of allo-splenocytes. This observation is in keeping with the results of Eliopoulos *et al*. (2005) and Badillo *et al*. (2007) for localised delivery of allo-MSCs in other models. Surprisingly, however, animals that received two sequential IM injections of allo-MSCs demonstrated a clear and consistent hypo-proliferative phenotype for both $CD4^+$ and $CD8^+$ T-cells in similar donor strain DC-driven MLRs – a result that was clearly different to that observed in recipients of double injections of syngeneic (autologous) MSCs and of allo-splenocytes. Thus, our findings were consistent with a distinct, donor-specific immunomodulatory effect of repeated IM inoculation of healthy mice with allo-MSCs that may be indicative of one or more tolerogenic mechanisms. Interestingly, Beggs *et al.*, documented a similar phenomenon in the healthy non-human primates. In this study, baboons received two doses of $5x10^6$ allo-MSCs (I.V followed by I.M) which resulted in decreased anti-donor T-cell response in MLRs along with the persistence of detectable allo-MSCs at the site of injection for up to 28 days. However, the mechanisms associated with anti-donor hypo-responsiveness in the study of Beggs *et al*. were not elucidated and several experiments described in this Chapter were directly toward better understanding the nature and possible mechanistic basis for this observation.

To begin with, we confirmed experimentally that the reduced proliferation of T-cells which occurred following double injection of allo-MSCs was donor antigen-specific (as the responses to third party DCs were not equally suppressed) and also dependent upon the second exposure of allo-MSCs (as it could not be reproduced by administering syngeneic MSCs at the second time-point). Taken together

with the initial results following single IM injections, these findings lend support to some important conclusions. Firstly, we can conclude that allo-MSCs, while expressing low surface levels of MHC proteins and being weak direct stimulators (or even suppressors) of naïve T-cells, still represent a significant source of allo-peptides that are most likely to be initially presented by host APCs via the indirect (or possibly semi-direct) pathway. Secondly, the results indicate that a second exposure to allo-MSC-delivered antigens from the same donor strain produce a strikingly different pattern of T-cell response.

Possible mechanistic explanations for this (which are further developed and discussed in Chapter 5) include a direct role for the injected allo-MSCs in deleting or modulating the phenotype of donor-specific T-cells primed during the first exposure or a step-wise induction of regulatory cell populations by allo-MSCs (English *et al.*,2009, Ge *et al.*,2010 and Kavanagh & Mahon,2011). In the case of the latter mechanism, our results from T-cell enriched MLRs – in which the hypo-responsive phenotype persisted – suggest that any induced regulatory cells represented either a T-cell subset or non-T-cell populations which exerted suppressive effects on donor-specific T-cells *in vivo* that persisted in subsequent *in vitro* experiments involving purified T-cells. Although not conclusive, our finding that "classical" $CD4^+/FOXP3^+$ T-reg were not expanded in number in the spleens or allo-MSC recipients or at the end of the subsequent MLRs suggests that this well-described mechanism of MSC-mediated immunomodulation/tolerance induction was not at play in this setting. Furthermore, the results from our "triple injection" experiment indicate that the T-cell regulatory mechanism(s) induced by sequential IM injections of allo-MSCs is likely to be relatively short-lived – an additional finding that appears to argue against the expansion of a population of donor-specific $CD4^+/FOXP3^+$ T-reg. In this regard, the re-organisation of the myeloid and NK/NKT-cell subpopulations that we documented at the time of euthanasia in the spleens of mice that received double injections of allo-MSCs raises the possibility that a temporary induction of alternatively activated monocyte/macrophages, myeloid-derived suppressor cells or innate lymphocytes in the secondary lymphoid organs played a role in modulating the response of previously primed anti-donor T-cells (Spaggiari *et al.*,2006, Prigione *et al.*,2009, Yen *et al.*,2013, Ko *et al.*,2015 and Lee *et al.*,2015).

Interestingly, the most striking cytokine secretion pattern observed in the MLRs from allo-MSC recipients was very high production of IFN-γ in the absence of high levels of IL-2 or of signature cytokines for the Th2 and Th17 differentiation pathways. The induction of high levels of IFN-γ was specific to B6 DC-stimulated MLRs suggesting that it was produced by donor-antigen-specific T-cell populations. In line with this finding, a role for IFN-γ in MSC-mediated T-cell suppression via B7-H1 (PD-L1) has been reported by Sheng *et al.,* 2008. Furthermore, Konieczny *et al.,*1998 demonstrated, in 1998, that IFN-γ secretion enhances the allo-graft survival by suppressing the expansion of activated T-cells. We also noted increased IL-10 production in these cultures. However, the increased IL-10 was produced even in the absence of DCs and was not further increased by stimulation with B6 DCs, suggesting that it may have been the product of a non-T-cell population. Given the well-documented role for IL-10-producing monocyte/macrophages in several *in vivo* models of MSC anti-inflammatory and immunomodulatory effects, it is tempting to hypothesise that this observation reflects the induction of alternatively activated splenic myeloid cells following the second inoculum of allo-MSCs. However, it was not possible to explore this possibility within the time-frame of this project. It should be emphasised that the observed hypo-responsiveness of $CD4^+$ and $CD8^+$ T-cells to donor APCs was not reversed by neutralisation of IFN-γ and IL-10 either singly or together. Thus, while these cytokines may be interpreted as being part of the "signature" of the altered immunological profile associated with repeated IM injection of allo-MSCs, they are clearly not the final mediators of T-cell suppression. Similarly, blockade of PGE2 production in MLRs by addition of indomethacin resulted in a partial reversal of $CD4^+$ but not $CD8^+$ T-cell hypo-responsiveness. Considered in the context of other experimental observations, these results suggest that the suppression of donor-specific T-cell responses that we describe in this Chapter represents the end result of a complex mechanism that may involve multiple cellular and soluble mediators.

Further experimental data from this Chapter point to specific modulation of anti-donor $CD8^+$ T-cell responses following repeated IM injections of allo-MSCs and, potentially, to a role for a subset of $CD8^+$ T-cells in mediating hypo-responsiveness. As donor-specific, cytotoxic $CD8^+$ T-cells are known to be key mediators of the destruction of MHC-I^+ allogeneic cells following organ and tissue

transplantation (Bueno & Pestana, 2002, Ingulli, 2010 and Harper *et al.*,2015), this may be a significant observation for the use of therapeutic allo-MSCs in general and for their clinical application to transplantation. Specifically, we found that $CD8^+$ T-cells primed by donor B6 DCs from mice that received repeated IM injections of allo-MSC exhibited lower granzyme B expression and less lytic activity against B6 MSC "targets" compared to mice that received allo-splenocytes. Thus, while our profiling of splenic immune cell subtypes demonstrated expansion of total $CD8^+$ T-cells with skewing toward memory/activated sub-populations in the recipients of double injections of allo-MSCs compared to the control groups, the functional experiments we performed *ex vivo* suggested that the induced donor-specific $CD8^+$ T-cells were hypo-proliferative, produced high levels of IFN-γ and had reduced cytolytic capacity. It is of distinct interest, therefore, that we also observed a striking expansion in $CD8^+/CD11c^+$ T-cells in the spleens of the allo-MSC recipients. In addition to the previously mentioned reports of Keizer *et al*. in 1987 and Vinay and Kwon in 2010 regarding the phenotype and potential suppressive functions of IFN-γ-producing, antigen-specific $CD8^+/CD11c^+$ T-cells, Segovia *et al.,* documented their key regulatory role in a mouse allo-transplant model in which autologous "tolerogenic" dendritic cells (ATDCs) were tested as a potential cellular therapeutic. As discussed in greater detail in Chapter 5, we believe that further investigation of the systemic expansion of $CD8^+/CD11c^+$ T-cells following IM injections of allo-MSCs will be potentially of great importance for better understanding the characteristic anti-donor immune responses they induce.

Finally, our results for histological analysis of muscle following repeated IM injections of allo-MSCs confirmed that the second injection was accompanied by readily detectable localized cellular infiltrates, likely composed of multiple immunological cell types. The extent of these infiltrates was not significantly less than that associated with the second injection of allo-splenocytes in our "sensitized" control group. Although time did not permit a more detail immune-phenotyping of these infiltrates, this basic observation also supports our overall conclusion that, far from being immunologically inert, allo-MSCs induce active cellular immune responses that can include localized responses at the site of administration. This finding is in keeping with some previously reported *in vivo* studies. For example, following intra-cranial delivery of allo-MSCs in rhesus monkeys and

subcutaneous implantation of allo-MSC-containing scaffolds in healthy mice, H&E staining of retrieved tissue demonstrated inflammatory cell infiltrates at the delivery sites which included large numbers of neutrophils (Elipoulos *et al.*, 2005 and Isakova *et al.*, 2010). Furthermore, Chen *et* al. (2009) demonstrated that localized application of allo-MSCs in a mouse wound healing model resulted in reduced infiltration of neutrophils and monocytes at the wound site but that this was associated with beneficial effects on wound healing which were comparable to those of autologous MSCs. On the other hand, Camp *et al.* (2009) reported that intra-cerebral allo-MSC injection in the setting of acute cerebral injury stimulated localized cellular infiltration which resulted in loss of therapeutic efficacy. Similarly, Huang *et al.*,2010 observed that intra-myocardial injection of allo-MSCs compared to autologous MSCs was associated with delayed loss of beneficial effects for myocardial remodeling following acute myocardial infarction and that this was accompanied by evidence of localized anti-donor T-cell infiltrates. Thus, it is possible, that allo-MSC-induced local immune infiltrates may reflect an anti-inflammatory, pro-regenerative phenotype or a potentially destructive anti-donor immune response.

Altogether, results from this Chapter convincingly show that repeated IM injection of allo-MSCs is associated with distinct, active cellular immune responses that include a potent, albeit temporary state of donor-specific $CD4^+$ and $CD8^+$ T-cell hypo-responsiveness. Experiments directed toward elucidating the mechanisms underlying this phenomenon point toward a non-classical form of regulation that may involve multiple innate and adaptive cellular populations and is most strikingly characterized by donor-specific IFN-γ-production and systemic expansion of $CD8^+/CD11c^+$ T-cells. How these findings might be further clarified and applied to translation research involving multiple inocula of allo-MSCs is discussed in Chapter 5.

CHAPTER 4

ANTI-DONOR ANTIBODY RESPONSES FOLLOWING INTRAMUSCULAR INJECTION OF ALLOGENEIC MSCs

4.1 INTRODUCTION

4.1.1 Anti-donor antibody responses in allogeneic transplantation:

Humoral (antibody-mediated) immunity plays a vital role in protecting against harmful pathogens but is also an important barrier to successful organ and tissue transplantation. Genetically-based incompatibilities between the donor and recipient of an allogeneic transplant – particularly those involving the ABO blood group antigens and proteins of the MHC complex – may result in the presence of pre-formed antibodies that bind directly to transplanted cells and mediate rapid (hyperacute) rejection. To avoid this complication, ABO blood group matching and "cross-matching" of recipient serum against donor cells or HLA antigens are routinely performed prior to human organ transplantation (Gordon *et al.,* 1986 and Reddy *et al.*,2013). Furthermore, the development of antibodies against HLA proteins of the donor (anti-donor antibodies) after transplantation is now recognised as a major cause of both acute and chronic organ transplant rejection that is not amenable to reversal by conventional immunosuppressive therapies (which predominantly target T-cells) (Covlin & Smith, 2005 and Montgomery *et al.*,2011).

In the case of allogeneic cellular therapies, the significance of anti-donor antibodies for therapeutic safety and efficacy is much less clear. Nonetheless, immune sensitization to donor allo-antigens resulting in anti-donor antibodies is well recognised following blood transfusion and increasingly reported in animal models of allogeneic cellular therapy. (Higgins RM *et al.*,1996, Nauta *et al.,* 2006, Beggs *et al.,* 2006 and Badillo *et al.,* 2007). The effector mechanisms of acute antibody-mediated rejection (AMR) of allografts reflect the primary mechanisms whereby antibodies protect against microbial infection. These include: (a) activation of the complement cascade through the classical pathway resulting in complement-mediated cellular cytotoxicity and induction of inflammation (b) opsonisation resulting in macrophage activation and phagocytosis and (c) antibody dependent cellular cytotoxicity (ADCC) – any or all of which may result in destruction of the graft (Covlin & Smith, 2005 and Puttarajappa *et al.*,2012). Furthermore, low-level deposition of anti-donor antibody on the endothelium of transplanted organs is now recognised to induce aberrant intracellular signalling pathways leading to vascular remodelling and occlusion, often referred to chronic AMR (Colvin,

2007, Hidalgo *et al.*,2010, Farkash & Colvin, 2012 and Djamali *et al.*,2014). Whether these acute and chronic anti-donor antibody-mediated processes could compromise the outcome of an allogeneic cellular therapy such as allo-MSC administration for inflammatory disease is open to question and remains an under-investigated area in the regenerative medicine field (Alagesan & Griffin, 2014). Also, even if allo-MSC-induced anti-donor antibodies are not associated with immediate detrimental effects for treated subjects, it is worth considering that they may create a barrier to future organ transplantation or even to a subsequent allogeneic cellular therapy.

4.1.1.2 Generation of anti-donor antibodies and mechanisms:

Development of *de novo* MHC-specific antibodies is typically dependent on primary $CD4^+$ T-cell activation by MHC-derived peptides presented on class II MHC-I/II by DCs (Steele *et al.*,1996, Pattison & Krensky, 1997, Akalin & Pascal, 2006 and Lionaki *et al.*,2013).

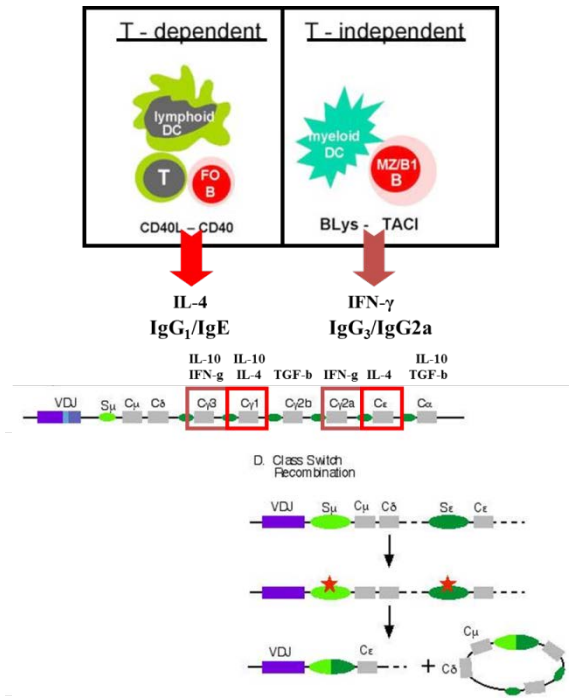


Figure courtesy – Prof. Rhodri Ceredig, REMEDI, NUI Galway.

Figure 4.1: Illustration of the characteristics to T- cell dependent and T-cell independent mechanisms of antibody class switching in the mouse system. The T-cell dependent mechanism involves activation of follicular B cells (FO B cells) by T cells in the presence of lymphoid dendritic cells via CD40 ligand and CD40 interaction in the presence of IL-4, which leads to IgG1/IgE swtiching. T-cell independent mechanism involves activation of marginal zone B cells (MZ/B1 B cells) by myeloid dendritic cells via soluble factor B lymphocyte stimulator (BLys) - Transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) in the presence of IFN- γ induces the isotype switching from IgG3/IgG2a.

Subsequently, when a mature B-cell binds the intact (allogeneic) MHC protein via its surface receptor (BCR), the allo-antigen is internalized, processed and re-presented as MHC/peptide complexes to activated, antigen-specific T-cells. The ensuing T-cell/B-cell cross-talk results in full B-cell activation and production of antigen-specific antibody of the IgM isotype. Further B-cell activation *via* inflammatory cytokines and T-cell-delivered co-stimulatory signals will then result in isotype switching to monomeric IgG antibody, maturation of antibody affinity through somatic hyper-mutation within germinal centres and establishment of long-lived antibody-producing cells (plasma cells) as well as memory B-cells capable of rapid re-activation upon secondary antigen exposure (Raman *et al.*,2003, Kato *et al.*,2004 and Tinckam & Chandraker, 2006). Importantly, antibody class switching may occur through both T- cell dependent and T-cell independent mechanisms based on APC type and the availability of T-cells and co-stimulatory factors at the time of antigen priming (**fig. 4.1**) (Cerutti, 2008 and El Shikh *et al.*,2009). For example, in the murine immune system, B-cell activation in the presence of antigen-specific T-cells and lymphoid DC supports antibody class switching of follicular B-cells from IgM to IgG1 and IgE. As shown in the left panel of **fig. 4.1**, this process is driven by the CD40-CD40L co-stimulatory pathway in the presence of the cytokine IL-4. In contrast, in the absence of T-cells and in the presence of myeloid DC, marginal zone B cells are induced to undergo class switching to IgG3 and IgG2a. During this process, the soluble factor B lymphocyte stimulator (BLyS, also commonly referred to as BAFF) plays a key role through its receptor transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) in the presence of the cytokine IFN-γ (right panel, **fig. 4.1**). As illustrated in lower panel of **fig. 4.1**, differential rearrangement of the individual gene cassettes encoding the isotype-specific Ig Fc segments is regulated by "master" cytokines or cytokine combinations including IL-4, IFNγ, IL-10 and TGFβ1. In this manner, the generation of mature antibodies with varying functional characteristics such as complement fixation, opsonisation and activation of eosinophils can be linked with the innate and cellular adaptive immune responses that are primarily triggered by infectious pathogens. The longevity of antigen-specific B-cell responses that are induced in this manner has been translated into a major health benefit through the field of vaccinology but represents a

formidable barrier to long-term graft success in transplant recipients (Colvin & Smith, 2005 , Siegrist, 2008, Alam *et al.*,2011 and Puttarajappa *et al.*,2012).

The actual clinical impact of anti-donor antibody is dependent on its specificity and amount ("titre") as well as the density and expression pattern of the relevant allo-antigen. Animal models of AMR have been developed to better understand the process in heart, kidney and liver transplantation and to translate insights from pre-clinical to clinical settings. Rejection of an allograft is primarily mediated by pre-formed antibodies (Breinholt *et al.,* 2000 and Meyer *et al.,* 2005), primary B-cell responses (early *de novo*) (DiLillo *et al.,* 2011) and, in some cases, latent B-cell responses (late *de novo*) (Lachmann *et al.,* 2009, Lee *et al.,* 2009). In the clinical setting, acute AMR due to pre-formed anti-donor antibody or to an early memory B-cell response may be prevented or treated by plasmapheresis/immunoadsorption or by high-dose intravenous immunoglobulin (IVIG) to deplete or de-functionalise circulating anti-donor antibodies (Montgomery *et al.*,2000, Glotz *et al.,* 2002, Jordan *et al.*,2003 and Tobian *et al.,* 2008). Other interventions such as anti-CD20 antibody (which results in depletion of $CD20^+$ B-cells), splenectomy, proteasome inhibitors and complement inhibitors have also been used in the clinical setting of acute AMR although their individual efficacies remain unproven (Becker *et al.*,2004, Locke *et al.*,2007, Lefaucheur *et al.*,2009, Stegall *et al.*,2011 and Sadaka *et al.*,2011). Importantly, in transplant recipients without pre-formed anti-donor antibodies or a pre-existing B-cell memory response to donor MHC antigens, the development of *de novo* allo-antibodies resulting in AMR is generally prevented by clinically available immunosuppressive drugs such as cyclosporine, tacrolimus, mycophenolate or sirolimus (rapamycin) which effectively inhibit the primary T-cell activation that is required for antigen-specific B-cell activation (Pascual *et al.*,1998, Coll *et al.*,2004, Knechtle *et al.*,2009, Singh *et al.*,2009 and Djamali *et al.*,2014).

4.1.3 MSC effects on B-cell responses in vitro:

The effects of MSCs on B-cell activation and antibody production have been extensively studied in cultured rodent and human B-cells (Franquesa *et al.*,2012). In general, MSCs have been shown to suppress the proliferation and activation of B-cells with some studies also reporting suppression of antibody production. For example, when pokeweed mitogen-activated B-cells from mice where co-

cultured at 1:1 ratio with MSCs, inhibition of B-cell proliferation was observed and shown to be mediated by PD-1/PD-1L (Augello *et al.,* 2005). Rafei *et al.*,2008 demonstrated that MSCs inhibit the secretion of antibody by ovalbumin-activated B-cells via cleaved CCL2 (Rafei *et al*. 2008). Interestingly, Asari *et al.* showed that MSCs inhibit the *in vitro* induction of plasma cells by LPS treatment. While these authors demonstrated that inhibition of B-cell differentiation is cell-contact independent, Schena *et al.,* 2010 reported that MSC inhibition of B-cell proliferation and differentiation is dependent on cell-cell contact. Thus, the interactions between MSCs and B-cells at different stages of activation and antibody production are likely to be complex and reflect multiple mechanisms and mediators. Experiments with human B-cells co-cultured with MSCs also provide evidence for suppressive effects. For example, Corcione *et al.,*2006 observed that the induction human B-cell proliferation and production of IgM, IgG and IgA by a cocktail of CpG, rCD40L, anti-Ig, IL-2, IL-4 and IL-10 was inhibited by co-culture with MSCs. Of further interest, Comoli *et al.,*2008 reported that addition of MSC-derived culture supernatant inhibited *in vitro* ADCC caused by sera from "sensitised" renal failure patients. This finding suggests that MSC-derived mediators might also inhibit anti-donor antibody effector functions under some conditions, however, contrastingly, few studies suggests that MSCs can also mediate the survival and functioning of B-cells/plasma cells and thus enhancing IgG production and also IgG antibody class switching. (Rasmusson *et al.,*2007, Tabera *et al.,*2008, Traggiai *et al.,*2008, Youd *et al.,*2010 and Healy *et al.,*2015)

4.1.4 MSC effects on B-cell responses in vivo:

As outlined above, studies involving B-cell/MSC co-culture have generally (but not exclusively) indicated that MSCs are capable of inhibiting the proliferation of B-cells and suppressing their antibody-producing functions in simplified *in vitro* systems. Encouragingly, concordant results have been obtained with animal and human cells. (Augello *et al.,*2005, Corcine *et al.,* 2006, Comoli *et al.,* 2008, Rafei *et al.,* 2008, Asari *et al.,* 2009 and Schena *et al.,* 2010). Also, in models of autoimmunity, some effect of MSCs to inhibit pathogenic antibody responses has been observed. For example, Zhou *et al.,* 2008 shown that co-administration of human MSCs with the immunosuppressive drug

cyclophosphamide resulted in reduced levels of circulating anti-DNA antibodies in a mouse model of systemic lupus erythematosis.

However, *in vivo* responses of B-cells to MSCs may be significantly more complex and variable in the absence of immunosuppressive drug therapy – particularly in the case of allo-MSC administration as these cells introduce donor-derived Class I MHC. Several studies have investigated the *in vivo* anti-donor antibody response to MSC administration via various routes (intravenous (IV), intra-arterial (IA), subcutaneous (SC), IM, topical and other routes) (Beggs *et al.,* 2006, Poncelet *et al.*, 2007, Badillo *et al.*, 2007, Isakova *et al.*,2010 and Schu *et al.,* 2012). Such studies have been carried out in healthy and diseased animals. In a limited number of human clinical trials, there also has been analysis of induction of human B cells. Although allo-MSCs have been shown to be safe in human applications (Peng *et al.,* 2013), there are mixed results with induction of anti-HLA antibodies in the allo-MSC human recipients. (Sundin et *al.,* 2007 and Hare *et al.*,2012). Taken together, no adverse anti-HLA antibody effects has been observed in human subjects receiving allo-MSCs, despite, experimental evidence indicating that allo-MSCs can be immunogenic and it would of clinical significance that clinical trials with human allo-MSCs in the future should continue to evaluate anti-donor antibody response in more precise and stringent anti-HLA antibody detection methods. For pre-clinical *in vivo* studies in which the immunogenicity of allo-MSCs was carefully investigated, it has been predominantly found that they do indeed induce anti-donor antibody responses following delivery by various routes (Griffin *et al.,* 2010 and Schu *et al.,* 2012). For example, in a pig model of myocardial infarction, Poncelet *et al.,* 2007 observed that IA and intra-myocardial administration of allo-MSCs resulted in readily detectable anti-donor antibody responses which were capable of complement fixation. Furthermore, they showed an accelerated (memory) antibody response to allo-MSC. Several studies performed in healthy animals have also indicated that anti-donor antibody responses were induced in response to allo-MSC administration. (Beggs *et al.,* 2006, Badillo *et al.,* 2007, Isakova *et al.*,2010 and Schu *et al.,* 2012). Although such studies appear to confirm the potential for induction of anti-donor antibodies following single inocula of allo-MSCs, there has been little experimental work focussed on the question of whether this antibody induction results in

detrimental effects on the recipient. For example, few studies has gone little further to test the physiological impact of allo-MSC induced anti-donor antibody by complement lysis assay and a step further with antigen re-challenge experiments to show the detrimental effects of allo-MSC induced anti-donor antibody. (Cho *et al.*,2004, Poncelet *et al.*, 2007, Huang *et al.*,2010, Seifert *et al.*, 2012 and Schu *et al.*, 2012) Interestingly, it has been shown that allo-MSCs, to a greater extent than autologous MSCs are subject to rapid complement binding following *in vitro* exposure to human serum resulting in a loss of viability (Li & Lin., 2012). This observation suggests that, in an *in vivo* setting, the presence of pre-formed anti-donor antibody could further accelerate the early removal of allo-MSCs.

Altogether, MSCs have been shown to have distinct immune-modulatory effects on B-cells both directly and indirectly through their suppressive effects on T-cells, DCs and monocyte/macrophages. Paradoxically, however, the majority of the *in vivo* work that has been reported indicates that allo-MSCs have clear potential to induce the generation of *de novo* anti-donor antibodies – in some cases of sufficient titre and isotype to mediate complement-mediate lysis of donor-derived target cells. Theoretically, the induction of allo-MSC-induced anti-donor antibodies may be preventable by administration of anti-T-cell immunosuppression although this has not been rigorously tested to date. In this chapter, anti-donor antibody responses to single or double IM injection of allo-MSCs were carefully studied in healthy mice as well as mice with diabetes and with hind-limb ischemia. In addition, we experimentally addressed the potential for a T-cell immunosuppressive drug, tacrolimus, to prevent allo-MSC-induced anti-donor antibody. Finally, we determined whether IM administration of xenogeneic MSC (xeno-MSC) results in the induction of antibodies against xeno-antigens expressed by human MSCs.

4.2 MATERIALS AND METHODS

Note: Only those materials and methods not previously described in Chapter 2 are summarised here.

4.2.1 *Flow Cytometric Assay for IgG Isotyping*

Blood samples were collected at the time of euthanasia, allowed to clot at room temperature for 4 hours then separated by centrifugation for 10 minutes at 500 x g following which aliquots of serum were frozen at -80˚C. For flow cytometric detection of specific isotypes of anti-donor (B6) IgG in serum, freshly prepared, RBC-free splenocytes were suspended at 8 x 10^6 cells/ml in FACS buffer (1x PBS, 2% FCS, 0.05% NaN_3) and were incubated for 30 minutes at 4˚C with FACS buffer alone (negative control) or with serum samples diluted 1:100 in FACS buffer. Next, splenocytes were washed twice in FACS buffer then individual aliquots were incubated for 30 min at 4˚C with one of the following: anti-mouse IgG1-FITC, anti-mouse IgG2a-FITC, anti-mouse IgG2b-FITC or anti-mouse IgG3-APC (BD Pharmingen, UK) at optimised dilutions in addition to anti-mouse TCRβ- PE (BD Biosciences) to allow discrimination of T-cells and non-T-cells. Finally, stained splenocytes were again washed twice with FACS buffer, re-suspended in 0.5 ml of FACS buffer and analysed immediately on a Becton Dickinson FACSCanto™ flow cytometer. Analysis was carried out using FlowJo® software (TreeStar® Inc, Ashland, OR) with FITC fluorescence intensity of TCRβ-negative cells (B-cells) compared between negative control sample and individual serum-incubated samples.

4.2.2 *Anti-donor Antibody Binding Assays to Allo-B6 MSC:*

Serum samples from recipient animals were incubated with freshly-cultured B6 MSCs or pre-treated MSCs (100ng/ml of IFN-γ for 24hours) for 30 minutes at 4˚C with either FACS buffer alone (negative control), purified anti-H-$2K^b$ (positive control, clone AF6-88.5, BD Biosciences, Franklin Lakes, NJ) or serum samples diluted 1:100 in FACS buffer. Next, target MSCs were washed twice in FACS buffer then incubated for 30 min at 4˚C with goat anti-mouse IgG Fc $F(ab)_2$-FITC (Beckman Coulter, Brea, CA) at an optimised dilution of 1:400 in addition to anti-mouse TCRβ- PE (BD Biosciences) to allow discrimination of T-cells and non-T-cells. Finally, stained MSCs were again

washed twice with FACS buffer, re-suspended in 0.5 ml of FACS buffer and analysed immediately on a Becton Dickinson FACSCanto^{TM} flow cytometer. Analysis was carried out using FlowJo® software (TreeStar® Inc, Ashland, OR) with FITC fluorescence intensity of TCRβ-negative cells (B-cells) compared between negative control sample and individual serum-incubated samples.

4.2.3 *Hind Limb Ischemia Model in Healthy Mice*

Note: This work was performed in collaboration with Dr. Clara Sanz Nogues, Dr. Xizhe Chen, Dr. Aaron Liew and Prof. Timothy O' Brien, REMEDI, NUI Galway, Ireland.

Unilateral (left) hind limb ischemia was induced in eight-week-old male BALB/c mice (**Figure 4.1**). Surgical procedures were performed a specialized small animal surgeon, Dr. Xizhe Chen. Animals were anesthetized with 75-100mg/kg ketamine and 10mg/kg xylazine injected intra-peritoneally. The left hind limb was shaved and cleaned with aseptic wipes before surgery. The femoral triangle was exposed by a 1 cm incision made along the axis of the femoral artery across the groin region. The femoral artery was separated from the femoral vein and nerve by blunt dissection distal to the inguinal ligament. The femoral artery was occluded by placement of sutures at the proximal and distal regions above the proximal caudal femoral artery branch and a cut was made between the ligation sites. Finally, the incision was closed with interrupted sutures, following which IM injection of cells/saline in the thigh muscle was performed. The animals received analgesic (0.015-0.03 mg/kg of buprenorphine) prior to surgery and every 12 hours for the next 3 days. Prophylactic antibiotic (0.125mg/mice of Enrofloxacin/Baytril) was also given once post-operatively.

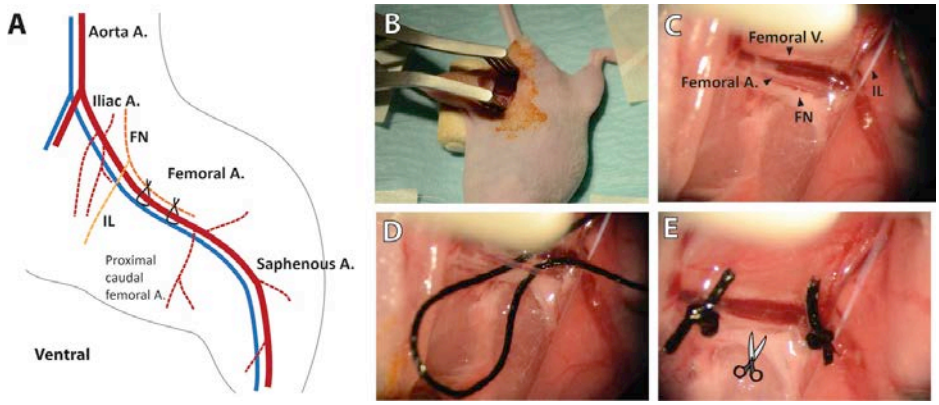


Figure 4.1: Illustrations of the technique of inducing unilateral hind limb ischemia in mice: A. Schematic diagram showing mouse vasculature and site of occlusion. **B.** Midline incision of about 1 cm from the knee towards the medial thigh with retractor used to open the wound. **C.** Components of vasculature including the femoral artery and femoral vein, femoral nerve (FN) and inguinal ligament (IL). **D.** Silk suture underneath the proximal and distal ends of the femoral artery above the proximal caudal femoral artery. **E.** Occlusion of femoral artery at proximal and distal regions using double knots with the segment between the knots transected.

Before surgery, mice were randomly assigned to 5 groups as per **table 3.2** below and were given a code for purposes of blinding the study. All participants in the study reminded blinded until the end of the study including the analysis of the results.

Table 4.1: Experimental groups for HLI experiments.

Group	IM Injection	Donor Strain	Recipient Strain	Immunosuppression	*n*
1	Saline	-	BALB/c	No	8
2	Syn-MSC	BALB/c	BALB/c	No	8
3	Allo-MSC	C57BL/6	BALB/c	No	8
4	Saline -TAC	-	BALB/c	Yes	8
5	Allo-MSC +TAC	C57BL/6	BALB/c	Yes	8
6	Human-MSC	Human	BALB/c	No	5

In mice receiving tacrolimus or vehicle (groups 4 and 5), daily intra-peritoneal injections were made starting from pre-operative day 2 until post-operative day 14. Tacrolimus was administered at a dose of 1 mg/kg/day in accordance with published studies (Song et al. 2012; Herbst et al. 2013). A working solution of tacrolimus (0.2 mg/ml) was prepared each day by mixing stock solution (40 mg/ml) with a vehicle solution containing 10% ethanol and 1% Tween80 in sterile physiological saline (Butcher et al. 1997). Control-treated mice received injections of equal volumes of vehicle alone. Animals were

housed in individually ventilated cages (IVCs) to minimize risk of infections. All animals were monitored daily for adverse effects and were humanely euthanized at day 21 post-surgery with multiple biological samples collected for analysis (including serum for anti-donor antibody assays).

4.2.4 *Hind Limb Ischemia Model in Diabetic Mice*

Eight to 12 week old non-diabetic [C57BL/6, H-2^b (B6)] and diabetic [$C57BKS^{db/db}$, H-2^d (*db/db*)] mice (Leiter & Lee, 2005 and Chodavarapu *et al.*,2013) were purchased from Charles River Laboratories, UK. Hind-limb ischemia was induced in the *db/db* mice as described above. Five IM injections of $2x10^5$ allo-MSCs or PBS (40µl each) were administered into the left thigh (2 medially & 3 posteriorly) following femoral artery ligation. All animals were monitored daily for adverse effects and were humanely euthanized at day 21 post-surgery with multiple biological samples collected for analysis (including serum for anti-donor antibody assays).

4.3 RESULTS

4.3.1 *Anti-donor IgG Following Single and Double IM Injection of Allo-MSCs:*

Anti-donor antibody responses to allo-MSCs were initially studied in 6 groups of healthy BALB/c mice (n=4) that had received two sequential IM injections, 2 weeks apart as described in Chapters 2 and 3. As shown in **fig. 4.2**, the groups consisted of: (a) Double injection of saline, (b) Double injection of BALB/C (Syn)-MSCs. (c) Single injection of allo-splenocytes followed by saline. (d) Single injection of allo-MSCs followed by saline. (e) Double injection of allo-splenocytes. (f) Double injection of allo-MSCs. Serum samples were collected 1 week following the second IM injection and were used in a semi-quantitative, flow cytometry-based assay for anti-B6 IgG (described in detail in Chapter 2). The standard curve that was used to generate a semi-quantitative measure of anti-B6 IgG using purified anti-H-2Kb is illustrated in **fig. 4.2A** and the resulting donor-specific IgG titres for individual mice from the 6 groups are presented in **fig. 4.2B**.

As expected, recipients of repeated saline or Syn-MSC injections had no detectable anti-B6 IgG in serum at the end of the experiment. Also in keeping with expectations, all recipients of allo-splenocytes developed readily detectable anti-donor IgG although, somewhat surprisingly, there was no evidence that the second injection resulted in an increase in antibody titres. Finally, recipients of all-MSC injections also developed significant titres of anti-B6 IgG. In the case of the mice that received a single allo-MSC injection, the observed titres were as high as those of allo-splenocytes recipients. In the case of the recipients of double allo-MSC injections, there was a trend toward lower IgG titre compare to the other sensitised groups but this did not reach statistical and the trend was not borne out in subsequent experiments.

Antibody Reponses to Single and Double Injection of Allo-MSCs

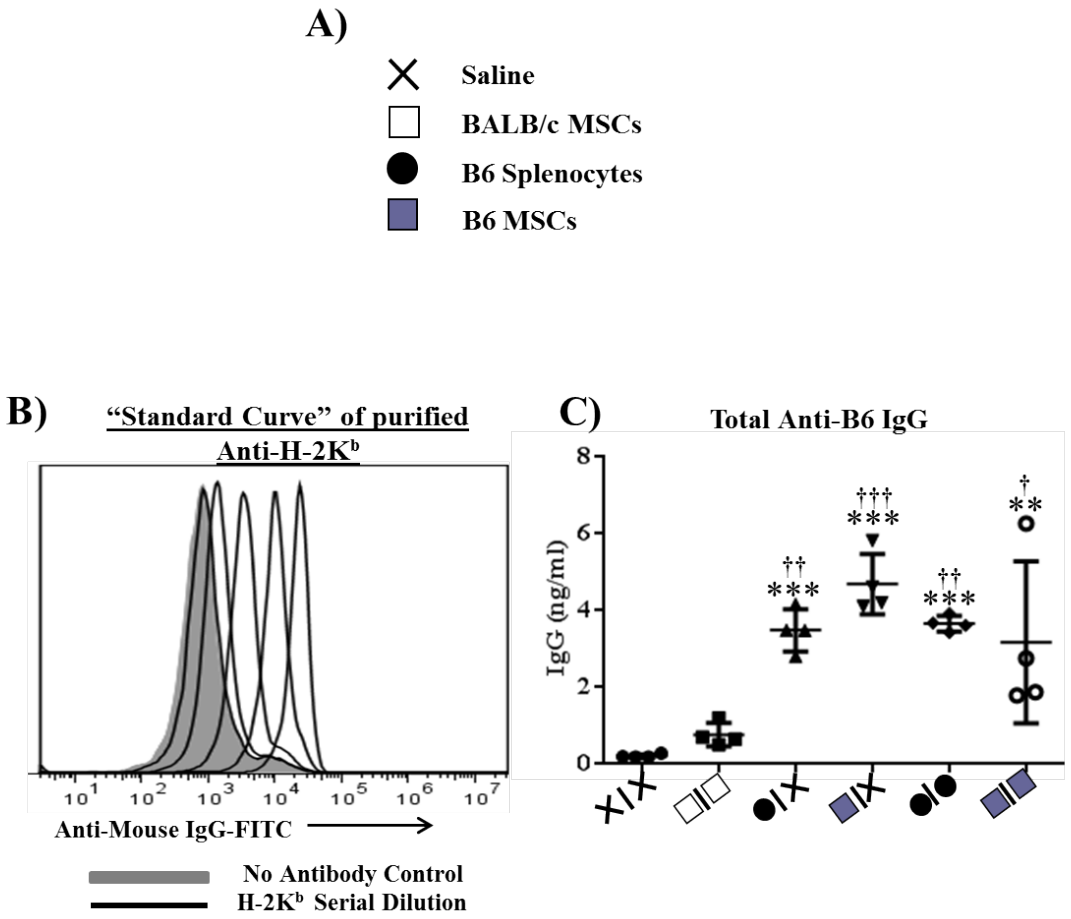


Figure 4.2: Anti-donor antibody responses to single or double IM injections of allo-MSCs and relevant controls: A. Symbols used to denote the injection types used in the experiment. **B.** Histogram overlay illustrating the standard curve generated using five serial dilutions of purified anti-B6 MHC-1 (H-2Kb) antibody. **C.** Graph of anti-B6 IgG titres in sera from the 6 experimental groups. Titres were calculated based on the standard curve and expressed as "ng/ml equivalent". Filled and open symbols represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. **Statistics**: One-Way ANOVA, **, *** = $p < 0.01$, 0.001 compared to saline control group †, ††, ††† = $p < 0.05$, 0.01, 0.001 compared to the Syn-MSC group. (n=4 each group)

Next, to determine the relative contributions of individual IgG isotypes to the overall amount of anti-B6 IgG present in the sera of the different experimental groups, the flow cytometric assay was repeated using FITC-coupled secondary antibodies with specificities for mouse IgG1, IgG2a, IgG2b and IgG3. The results of this analysis are summarised in **Figure 4.3A-D**. In this assay, the results for cell-injected mice were expressed in term of fold-change over the average fluorescence value for the samples from the saline control group. As shown, both single and double injections of allo-splenocytes resulted in comparable increases in detectable anti-B6 IgG1, IgG2a and IgG3. In contrast, recipients of single injections of allo-MSCs developed readily detectable anti-B6 IgG1 and IgG2a with low or absent IgG3. Compared to allo-splenocyte recipients, IgG1 appeared to be the dominant isotype for anti-donor antibody in the single-dose allo-MSC recipients although the small group sizes precluded a definitive result. For the double allo-MSC injection group, the trends were similar but with lower values in 3 of 4 animals.

Interim Conclusions: (a) IM injection of fully MHC-mismatched allo-MSCs into healthy mouse recipients resulted in robust development of anti-donor IgG antibody. (b) Development of anti-donor IgG was not abrogated by a second IM injection of allo-MSC despite the previously described development of donor-specific T-cell hypo-responsiveness. (c) Despite similar titres of total anti-donor IgG, the allo-splenocyte and allo-MSC recipients appeared to develop distinct IgG isotype patterns. Specifically, the anti-donor response of allo-MSC recipients trended toward IgG1-predominance.

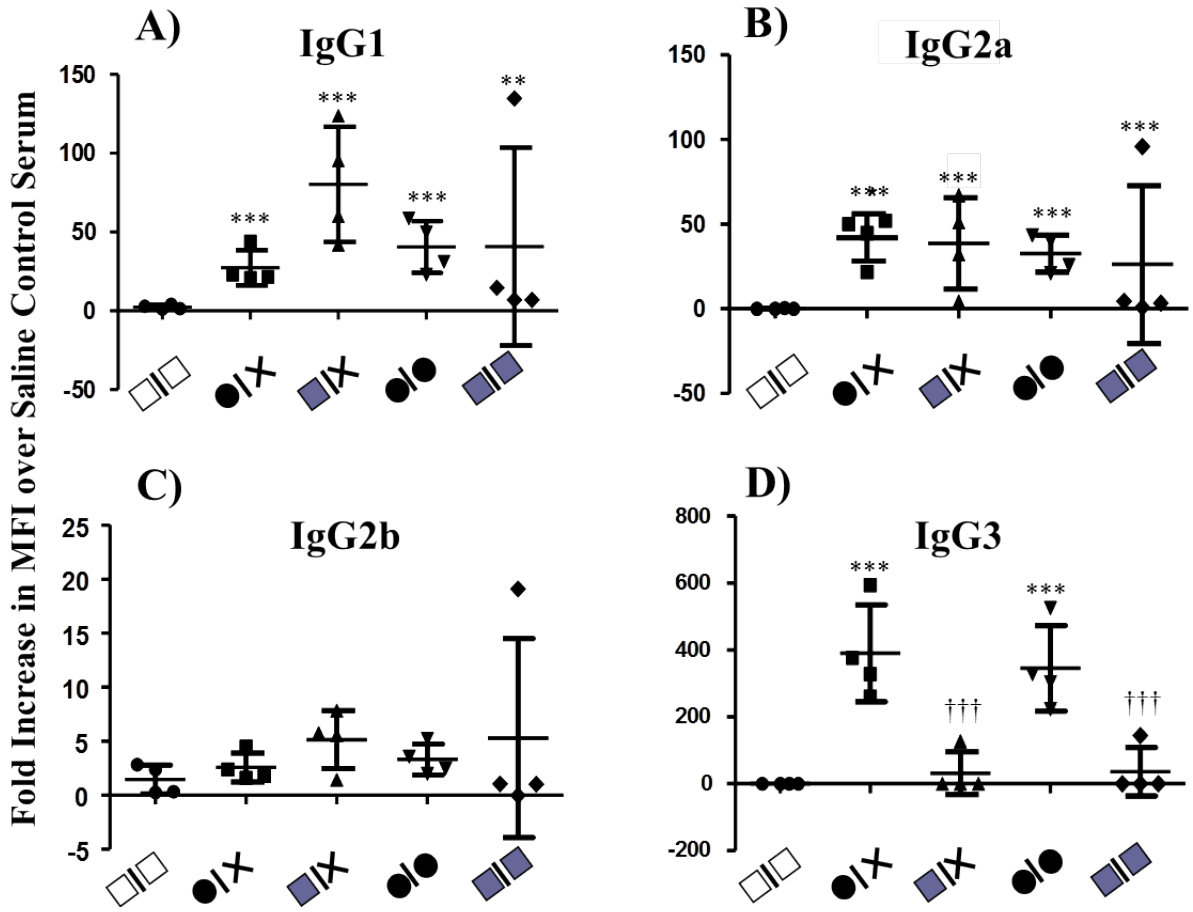


Figure 4.3: Anti-donor IgG isotpye profiles following single or double IM injections of allo-MSCs and relevant controls: Results are shown for semi-quantitative analysis of anti-B6 IgG isotype titres in 5 groups of mice. The groups are indicated by the same symbols as used in Figure 4.2. **A.** IgG1. **B.** IgG2a. **C.** IgG2b. **D.** IgG3. Titres were calculated as fold change over the average fluorescence intensity of the saline control group. Data-points represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. **Statistics**: One-Way ANOVA, **, *** = $p < 0.01$, 0.001 compared to the Syn-MSC group ††† = $p < 0.001$ compared to the relevant allo-splenocyte group. (n=8 each group)

4.3.2 *Anti-donor Antibody IgG isotypes Following Double Injection of Allo-MSCs:*

To further investigate the apparent differences in anti-donor IgG isotype patterns induced by allo-splenocytes and allo-MSCs, sera were analysed from additional groups of BALB/c mice that had received two sequential injections of Saline (n=10), allo-splenocytes (n=10) or allo-MSCs (n=10) using the same methodology as for the preceding section. The results of this analysis are summarised in **fig. 4.4**.

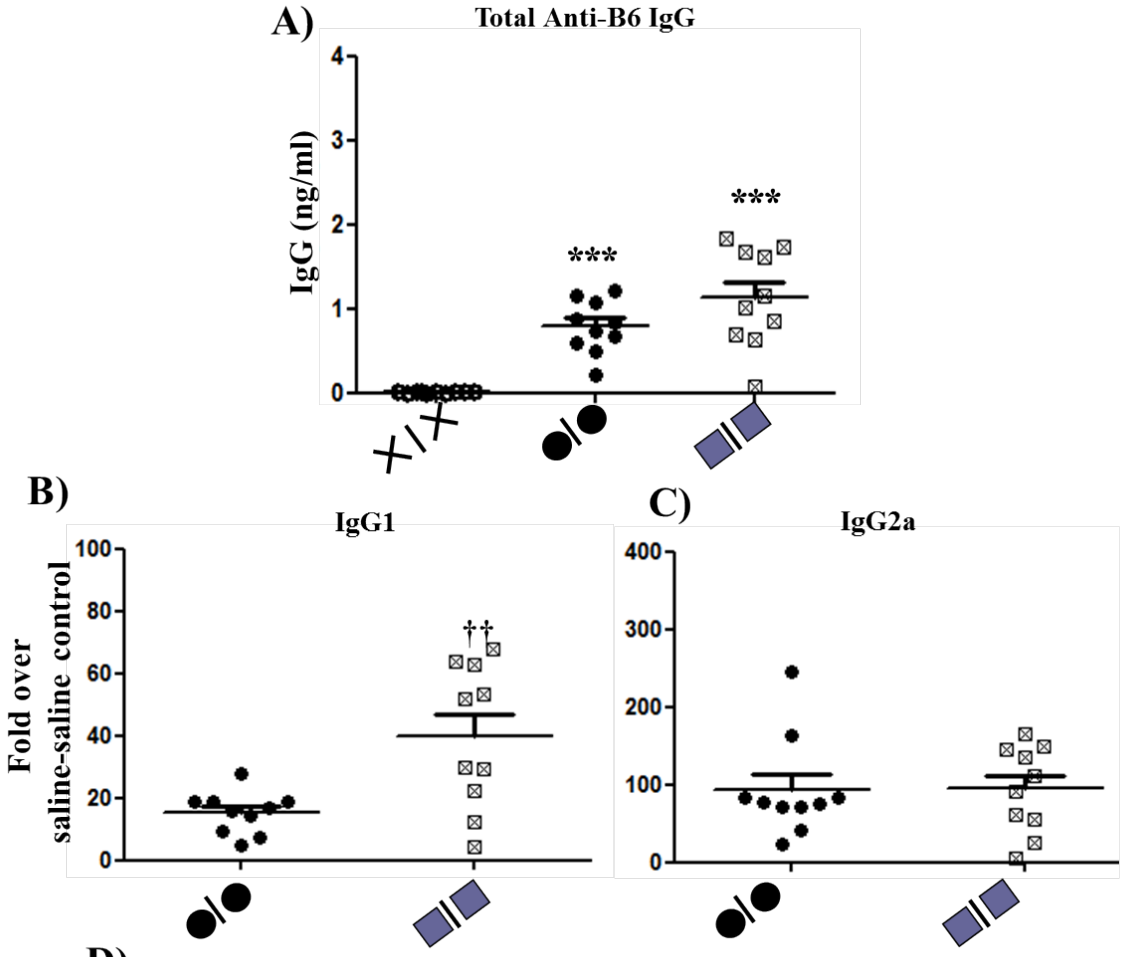
As previously observed, both allo-splenocyte and allo-MSC recipients had readily detectable anti-B6 IgG with no significant difference in titre between the two groups (**fig. 4.4A**).

However, when isotype-specific secondary antibodies were used to stain serum-incubated B6 target cells, it was confirmed that sera from mice that had received double IM injection of allo-MSCs contained higher levels of anti-donor IgG1 than sera from recipients of double IM injection of allo-splenocytes (**fig. 4.4B**). In contrast, the levels of anti-donor IgG2a were similar for the two groups (**fig. 4.4C**).

When the relative levels of the two anti-donor IgG isotypes were expressed as IgG2a/IgG1 ratio for each animal within the two groups, there was a clear difference between allo-splenocyte and allo-MSC recipients (**fig. 4.4D**).

Interim Conclusions: (a) Repeated IM injection of allo-MSCs is associated with readily detectable anti-donor IgG that is of comparable titre to that induced by repeated injection of allo-splenocytes. (b) In contrast to allo-splenocyte recipients, mice that received repeated IM injections of allo-MSCs developed an IgG1 pre-dominant anti-donor antibody response. This observation may be consistent with distinct regulation of IgG class switching by differentiated anti-donor $CD4^+$ T-cells that have been primed by allo-antigens delivered via IM MSCs. (Mountford *et al.*, 1994 and Yadav *et al.*,2001).

Anti-donor antibody response to repeated allo-MSC injections is associated with distinct IgG isotype switching



D)

Groups	Ratio of IgG2a/IgG1 Mean ± SD
AlloSpleen – AlloSpleen	1.08 ± 0.7
AlloMSC - AlloMSC	0.43 ± 0.2

P=0.01

Figure 4.4: Total IgG and IgG isotypes of anti-donor antibody induced by allo-splenoctes and allo-MSC: A. Results are shown for semi-quantitative analysis of anti-B6 total IgG titres in sera from BALB/C recipients of double injections of saline, allo-splenocytes and allo-MSCs (n = 10 for each). The groups are indicated by the same symbols as used in Figure 4.2. **B & C.** Results are shown for semi-quantitative analysis of anti-B6 IgG1 and IgG2a in two groups of cell-injected mice. Titres were calculated as fold change over the average fluorescence intensity of the saline control group. Data-points represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. **D.** Table summarising the mean ± SD IgG2a/IgG1 ratios for the two groups of cell-injected mice. **Statistics**: One-Way ANOVA, *** = $p < 0.001$ compared to the Saline group †† = $p < 0.01$ compared to the allo-splenocyte group.

4.3.3 *Binding of Induced Anti-donor Antibodies to Allo-MSCs:*

To this point, the anti-B6 IgG antibody induced by IM injections of allo-splenocytes and allo-MSCs were detected and quantified by analysing their binding to TCRβ$^-$ B6 splenocytes; which are expected to express high surface levels of the likely targets for anti-donor antibodies - Class I and Class II MHC. We next aimed to determine whether the induced IgG antibodies also bound to allo-MSCs. To test this, serum samples from the *in vivo* study described above, were diluted and incubated with either freshly-cultured B6 MSCs, BALB/C (syngeneic) MSCs or with B6 splenocytes. These samples were then stained with the same FITC-coupled anti-mouse IgG antibody and analysed by flow cytometry as before.

Figure 4.5 shows the results from this assay. As expected, sera from mice that has received IM saline injections did not contain detectable IgG against either type of B6 target cell. Surprisingly, the anti-donor IgG induced by both allo-splenocytes and allo-MSCs provided a higher level of fluorescence when bound to B6 MSCs compared to B6 splenocytes. Furthermore, the level of fluorescence on B6 MSC targets was significantly higher following incubation with sera from allo-MSC compared to allo-splenocyte recipients (**fig 4.5C**). Importantly, neither sera from allo-splenocyte recipients not from allo-MSC recipients contained IgG that bound to BALB/C MSCs, thus ruling out an antibody response against xeno-antigens contained in MSC culture medium (**fig 4.5C**).

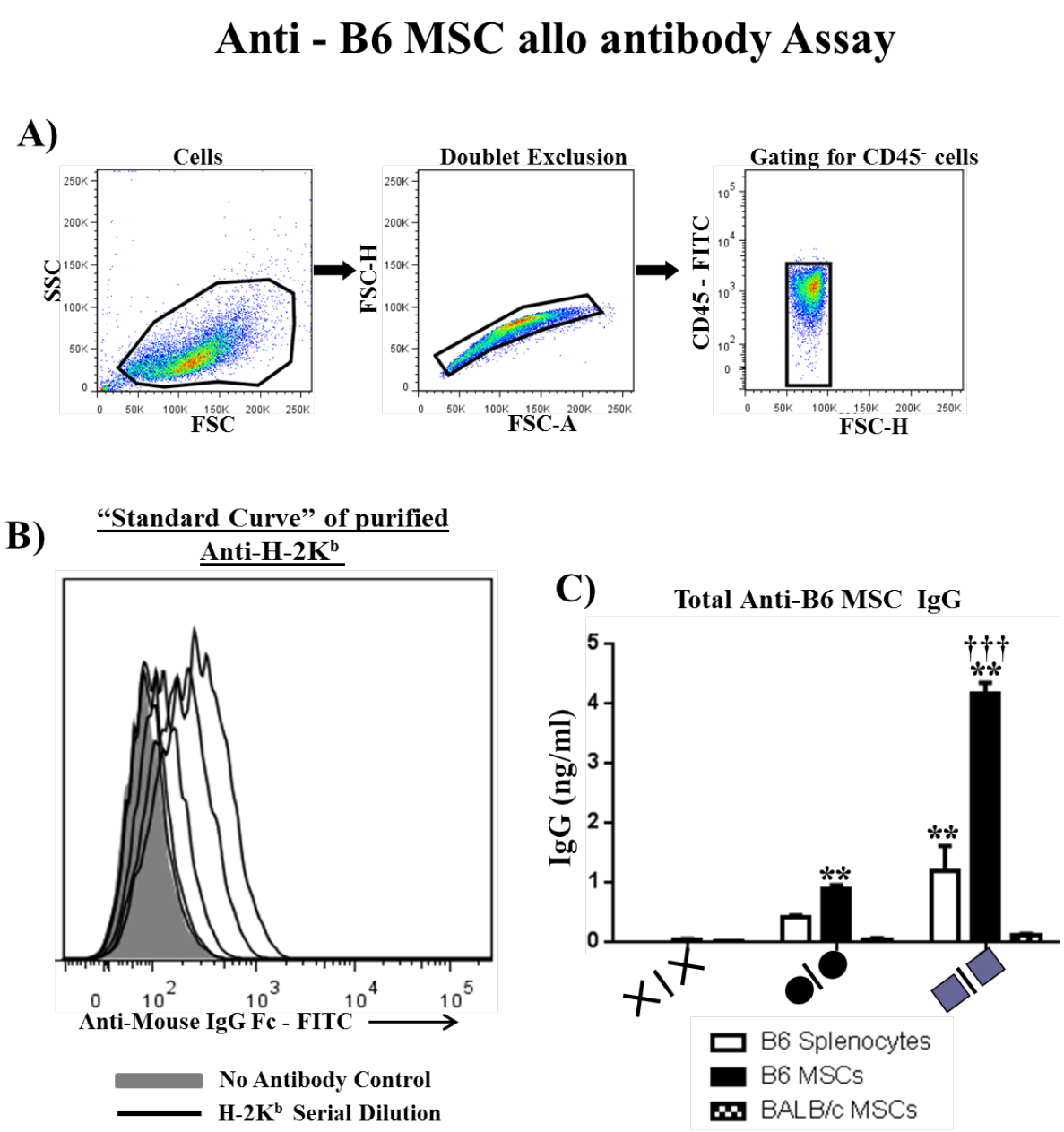


Figure 4.5: Comparative binding of anti-donor IgG to MSCs and splenocytes: A. Representative example of the gating strategy used to detect mouse IgG bound to freshly-cultured MSCs. **B.** Overlay histograms illustrating the standard curve generated by incubation of serial dilutions of purified anti-B6 Class I (H-2Kb) antibody with B6 splenocytes followed by anti-IgG-FITC. **C.** Graph of IgG titres in sera from three groups of mice (n = 10 each) against three different target cell populations: allo-(B6)-splenocytes, allo (B6)-MSCs and syngeneic (BALB/C)-MSCs. The animal groups are indicated by the same symbols as used in Figure 4.2. Titres were calculated based on the standard curve and expressed as "ng/ml equivalent". Data represent the group means ± SD. **Statistics**: One-Way ANOVA, ** = $p < 0.01$ compared to the appropriate results for the Saline group. ††† = $p < 0.001$ compared to the appropriate result for the allo-splenocyte group.

4.3.4 *Binding of Induced Anti-donor Antibodies to Interferon-gamma Stimulated Allo-MSCs:*

The results presented above indicated that double IM injection of allo-MSCs induced donor-specific IgG against target antigens expressed on the surface of the MSCs. In order to determine whether the target antigens were further upregulated on the surface by a pro-inflammatory stimulus, B6 MSCs were pre-treated with IFN-γ (100ng/ml) for 24 hours and the flow cytometry-based assay was performed using the same serum samples as previously. Results for IFN-γ pre-treated SMCs were compared with those for untreated MSCs. Representative example for the flow cytometry analyses and a graphical summary of the results of this experiment are shown in **fig. 4.6**.

As shown, when B6 MSCs were treated with IFN- γ, surface binding of IgG from sera of allo-MSC recipient mice was increased compared to untreated B6 MSCs at serum dilutions of both 1:50 and 1:100. For sera from allo-splenocyte recipients, this increased binding to IFN-γ treated MSCs was only evident at 1:50 dilution (**fig. 4.6A**). In order to formally confirm that the IFN-γ stimulation regimen used in these experiments results in up-regulation of MHC proteins by MSC, the mouse bone marrow stromal cell line B16-14 was cultured for 24 hours in the presence of 100ng/ml of IFN-γ or in control medium and was then analysed by flow cytometry for surface expression of MHC-I and MHC-II. As shown in **fig. 4.6B**, IFN-γ exposure was associated with a substantial increase in surface staining for MHC I as well as a slight increase in MHC-II. When the results for sera from all of the animals in the three groups were analysed **(fig. 4.6C),** it was clear that binding of anti-B6 IgG to MSCs was significantly higher following IFN-g pre-treatment of the MSCs and that the overall levels of bound IgG were greater following incubation with sera from mice that had received double injection of allo-MSCs.

Interim Conclusions: (a) IgG antibodies induced by either allo-splenocytes or allo-MSCs bind strongly to the surface of MSCs in donor-specific manner. (b) Against expectations, allo-MSCs present a high number of surface binding targets for anti-donor IgG antibodies. (c) Anti-donor IgG induced by repeated IM injection of allo-MSC bind to surface targets that are up-regulated by IFN-γ – most likely Class I MHC (which is well reported to be positively regulated on MSCs by IFN-γ).

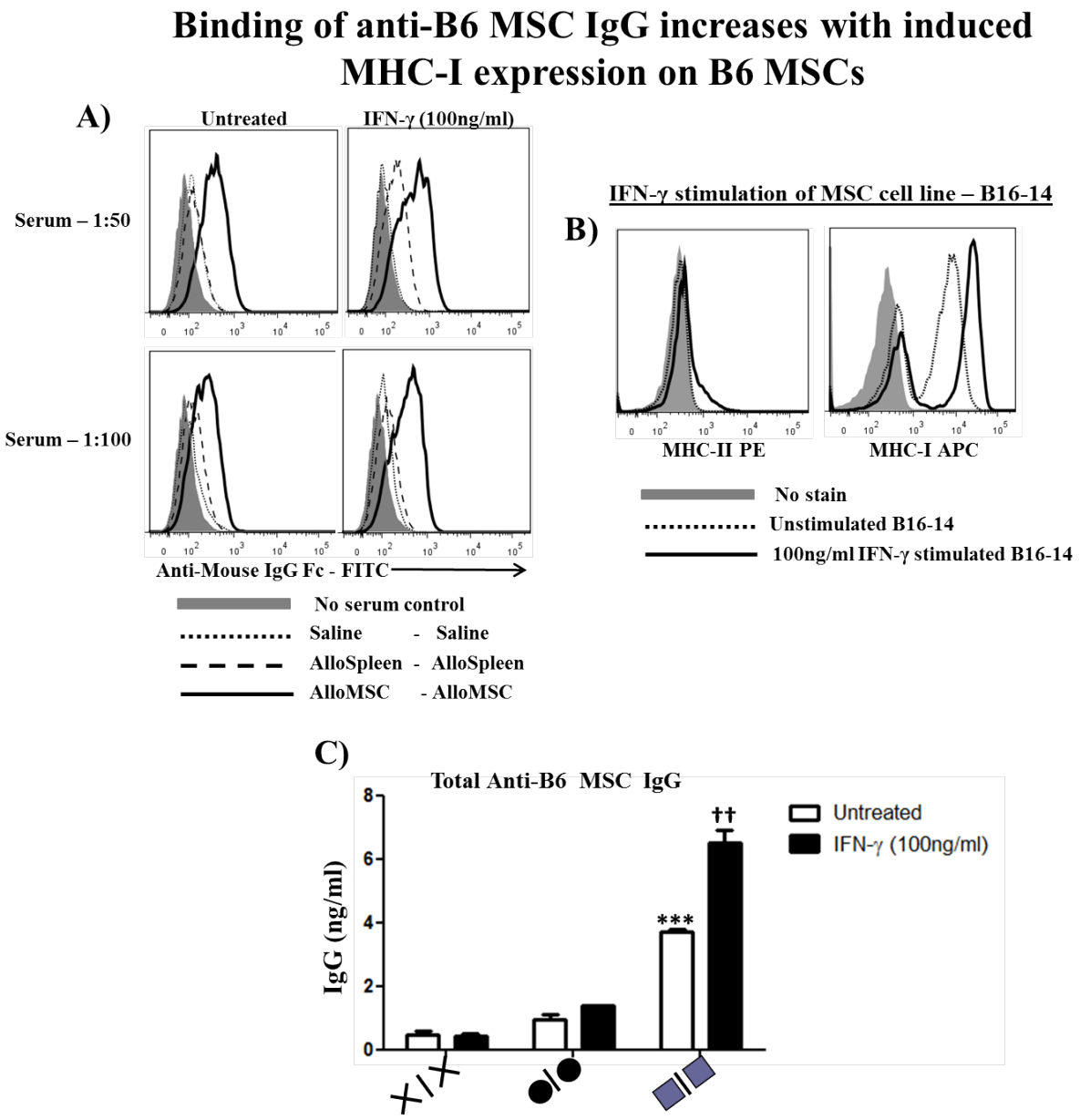


Figure 4.6: Comparative binding of anti-donor IgG to untreated and IFN-γ pre-treated allo-MSCs: A. Representative histogram overlays of the anti-IgG staining levels on untreated (left) and IFN-γ pre-treated (right) B6 MSCs following incubation without serum (no serum control) or with sera from mice that received double injection of saline, allo-splencoytes or allo-MSCs as indicated in the legend. **B**. Representative histograms showing up-regulation of MHC-I and MHC-II upon IFN-γ (100ng/ml) stimulation of the MSC cell line B16-14. C**.** Graph of IgG titres in sera from three groups of mice (n = 10 each) against untreated or IFN-γ pre-treated B6 MSCs. The animal groups are indicated by the same symbols as used in Figure 4.2. Titres were calculated based on a standard curve generated with serial dilutions of purified anti-B6 MHC I and were expressed as "ng/ml equivalent". Data represent the group means ± SD. **Statistics**: One-Way ANOVA, *** = $p < 0.01$ compared to the appropriate results for the saline and allo-splenocyte group. †† = $p < 0.001$ compared to the appropriate result for untreated MSCs.

4.3.5 *Anti-donor antibody Supports Complement-Mediated lysis of donor cells:*

The assays performed to this point confirmed that single or double IM injection of allo-MSCs induces a readily detectable anti-donor antibody response – most likely against donor Class I MHC proteins. We next investigated the capacity of the induced IgG antibodies to mediate donor-specific, complement-mediated cell lysis using the assay system described in Chapter 2. Briefly, frozen serum samples from multiple experiments in which healthy BALB/C mice received single or repeated injections of saline, MSCs or splenocytes were thawed and incubated with B6 "target cells" (freshly-prepared splenocytes) following which the serum was washed off and the cells were incubated at 37^{o}C with or without an optimised dilution of a rabbit complement preparation. Percent target cell lysis was determined by flow cytometry using PI exclusion (**fig. 4.7A**).

An initial dilution experiment confirmed that sera from recipients of double injections of allo-splenocytes and allo-MSCs but not syngeneic MSCs, supported complement-mediated lysis of B6 cells in dose-dependent fashion. For these samples, increased lysis was readily detectable down to a dilution of 1:100 (**fig. 4.7B**). In the subsequent experiment the B6-specific lytic activity of 1:10 diluted stored sera from five groups of mice (n = 4 – 21 per group) was assayed and expressed as fold-change above the mean value associated with sera from mice that were injected with saline alone (**fig. 4.7C**). As expected, sera from recipients of double injections of syngeneic MSCs contained no B6-specific lytic activity. However, for all other groups (single and double injection of allo-splenocytes; single and double injection of allo-MSCs), some or all of the samples supported increased complement-mediated lysis of B6 target cells. It was notable, however, that there was substantial variability in lytic capacity among the sera from recipients of allo-MSCs.

Interim Conclusion: Anti-donor IgG antibodies induced by either single or repeated IM injections of allo-MSCs have potential for donor-specific complement fixation and cell lysis although there is considerable inter-individual variability in this capacity.

Anti-donor antibody response to repeated allo-MSC injections has potential for complement-mediated lysis of donor cells

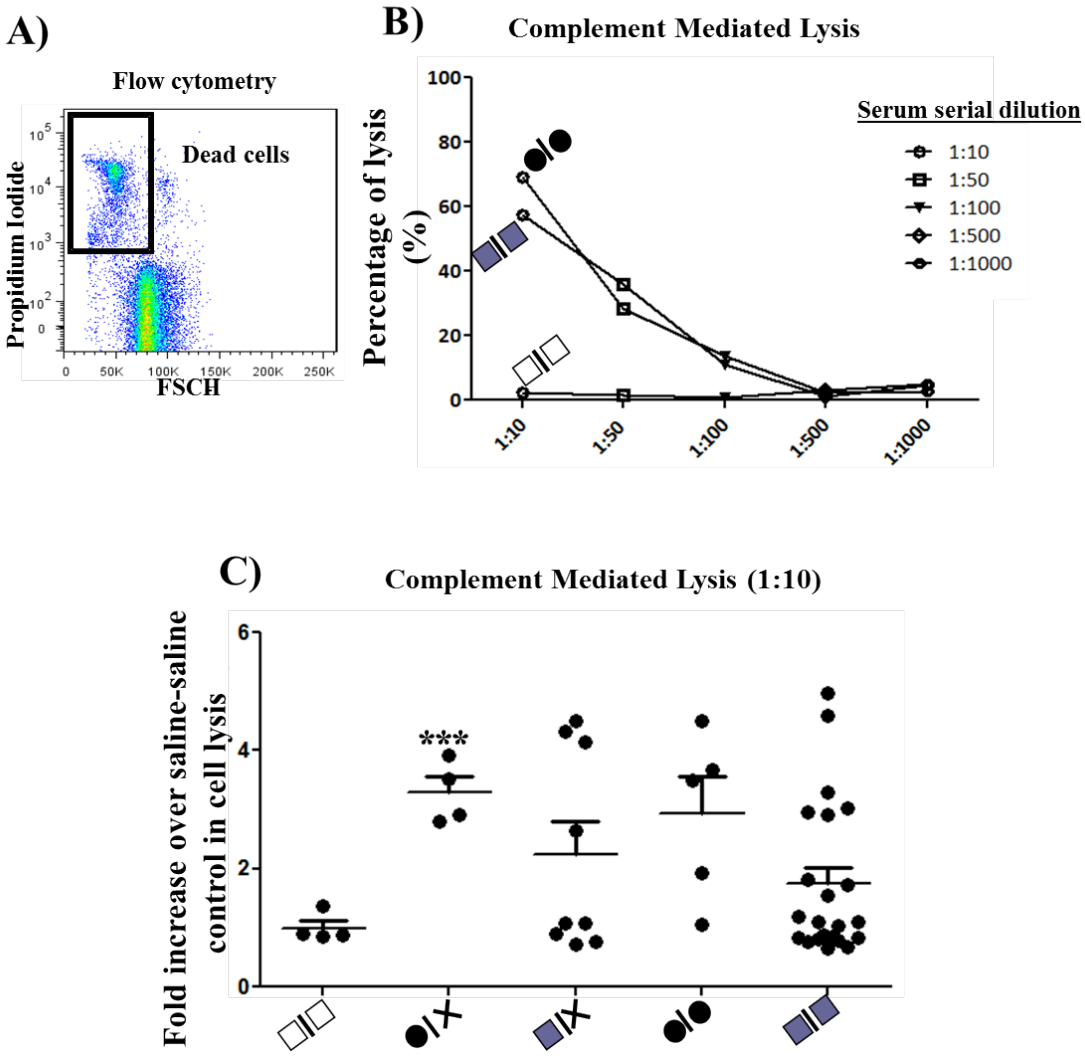


Figure 4.7: Complement-mediated lysis assays of stored sera from mouse recipients of IM injections: A. Representative example of a flow cytometry dot plot showing the positive staining of dead cells by propidium iodide. **B.** Results of complement-mediated lysis assays performed with serial dilutions (1:10 – 1:1000) of sera from mice that had received double injections of saline, allo-splenocytes or allo-MSCs. Results are expressed as mean % Lysis of target B6 splenocytes for 3 technical replicates for each condition. The animal groups are indicated by the same symbols as used in Figure 4.2. **C.** Results of complement-mediated lysis assays performed with 1:10 dilutions of stored sera from groups of mice that had received 1 of 5 different IM cell injection regimens (symbols as for Figure 4.2). Results are expressed as the fold-increase in % Lysis of target B6 splenocytes over the mean result for sera from saline-injected animals. Data-points represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. **Statistics**: One-Way ANOVA, *** = $p < 0.01$ compared to the syngeneic MSC group. (SynMSC-SynMSC = 4, Allospleen-Saline = 4, AlloMSC-saline = 9, Allospleen-Allospleen = 5 and AlloMSC-AlloMSC = 22)

4.3.6 *Anti-donor Antibody Assays Following IM Injections of Allo- and Xeno-MSCs in Mouse Hind-limb Ischemia Models*

In vivo studies described thus far were performed in healthy mice. The results confirmed that either single or double IM injection of allo-MSCs have the potential to induce the production of donor-specific IgG antibodies in recipient animals. In experiments carried out in collaboration with Prof. Timothy O'Brien and members of his laboratory group at REMEDI, we next studied the induction of anti-donor antibody in groups of mice that received single IM injections of allo-MSCs or control IM injections in the context of co-existing diabetes mellitus (DM) and/or surgically created unilateral hind-limb ischemia (HLI). For these studies, which are described in detail in the Methods section of this Chapter, anti-donor antibody assays were performed using the same flow cytometry-based assay system as previously described.

Figure 4.8 summarises the results from the first of these mouse model experiments. In this case, sera were assayed from groups of mice with type 2 (obesity-associated) DM ($C57BKS^{db/db}$ mice) 3 weeks following HLI induction and IM injections of saline, syngeneic (BALB/C)-MSC or allo (B6)-MSC.

As shown in **fig. 4.8C**, anti-B6 IgG was detectable in sera from 3 out of 8 recipients of allo-MSCs compared to 0 of 7 saline recipients and 0 of 8 recipients of syngeneic MSCs.

Allo-antibody Assay in Diabetic CLI Animals

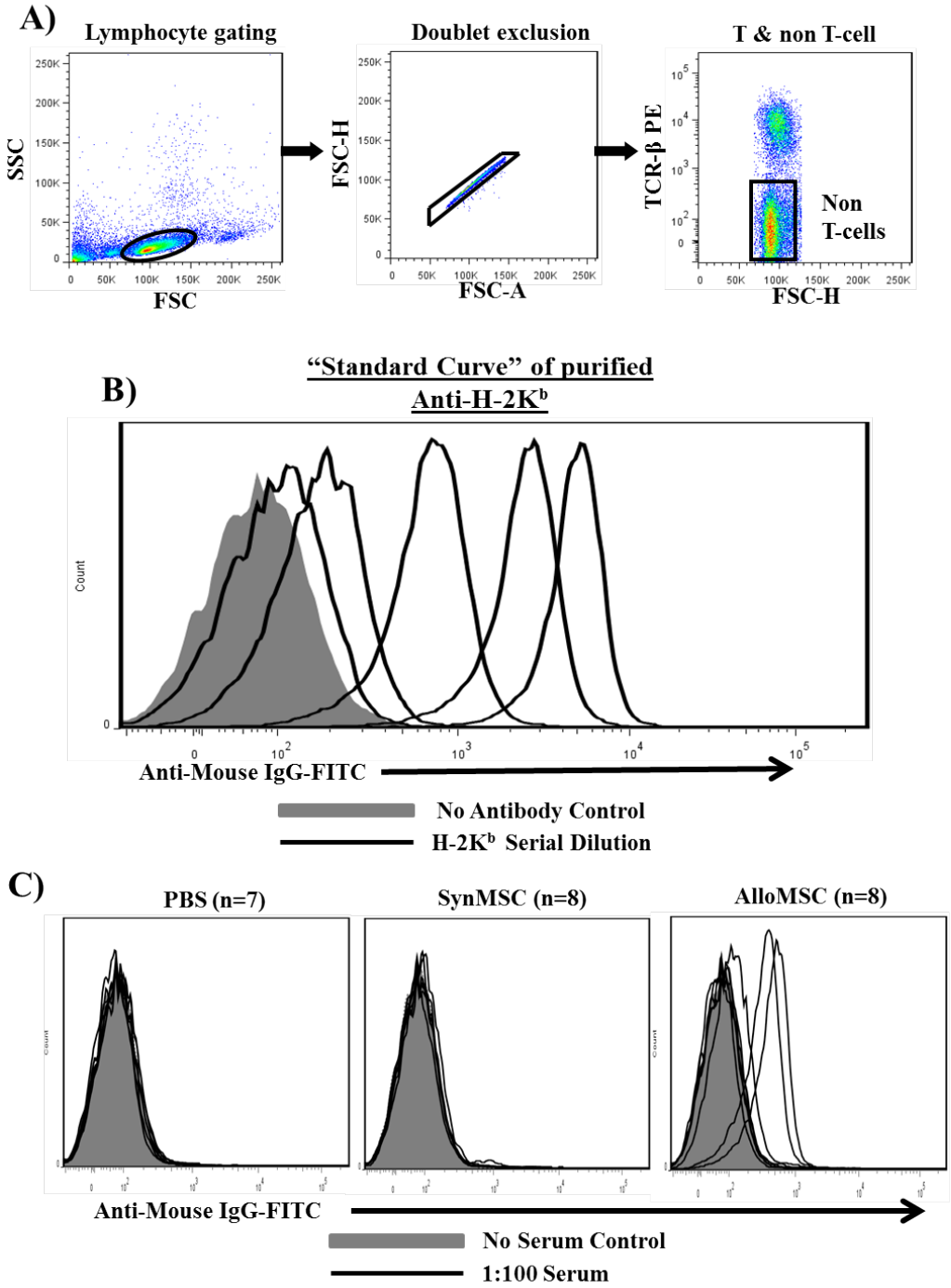


Figure 4.8: Allo-antibody assay in diabetic mice with HLI and injected IM with either saline (PBS), syngeneic (Syn)-MSCs or allo-MSCs. A. Gating strategy used for the analysis of IgG binding to TCRβ$^-$ B6 splenocytes. **B.** Histogram generated from the positive standard dilution using different dilutions using purified MHC-1 antibodies and binding of anti-mouse IgG Fc FITC. **C.** Histogram overlays showing the level of binding of anti-mouse IgG FC FITC to B6 splenocytes incubated with sera from three groups of diabetic mice with HLI. Histograms of staining with individual sera are unshaded while histograms for No Serum control staining are shaded grey.

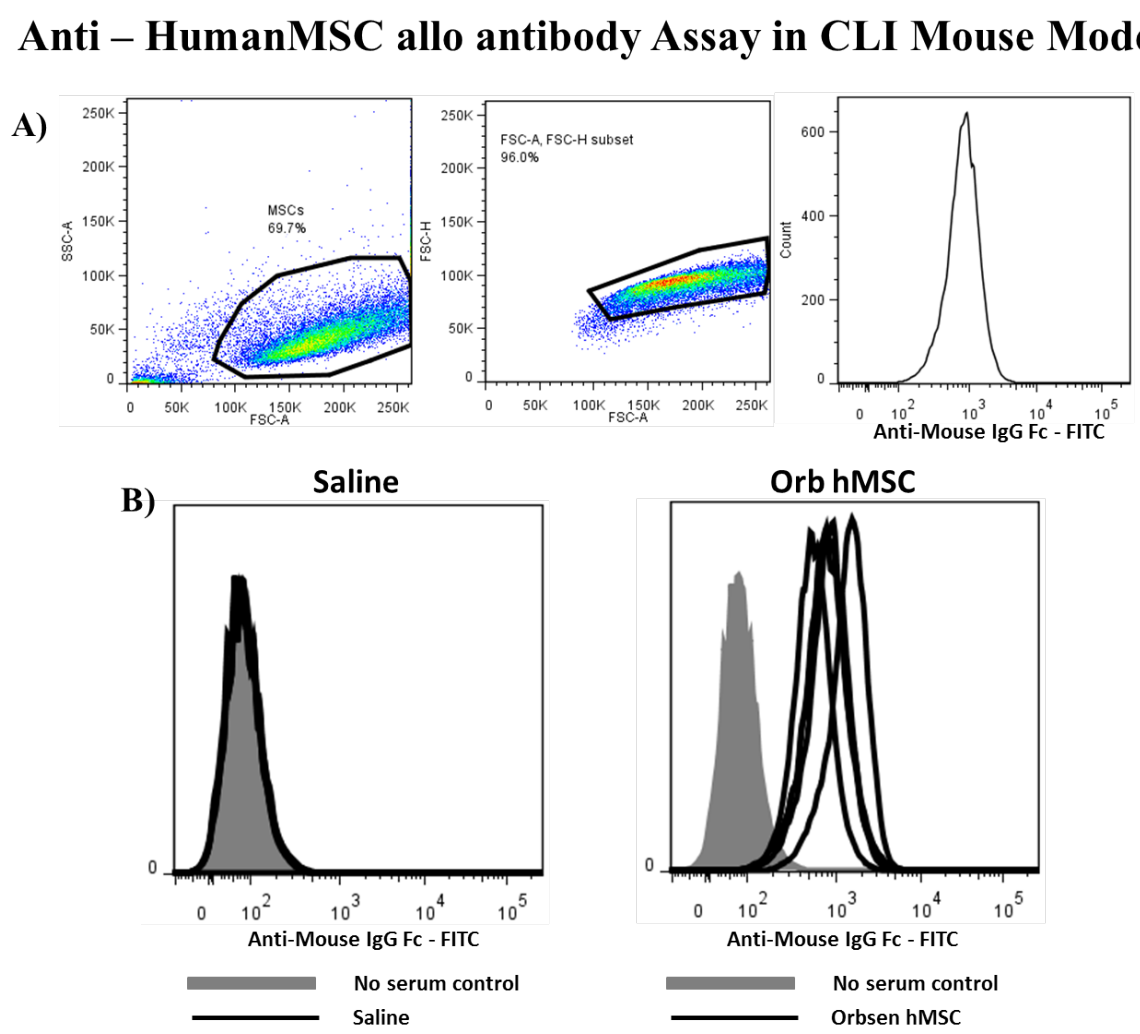


Figure 4.9: Assays for anti-human IgG in sera of non-diabetic mice with HLI and injected IM with either saline or xenogeneic (human)-MSCs. A. Examples of the gating strategy (dot plots) and base-line fluorescence (histogram) for the analysis of mouse IgG binding to human MSCs. **B.** Histogram overlays showing the level of binding of anti-mouse IgG FC FITC to human MSCs (Orbsen hMSC) incubated with sera from two groups of non-diabetic mice with HLI. Histograms of staining with individual sera are unshaded while histograms for No Serum control staining are shaded grey. (n=5 each group)

Next, we performed assays on serum collected 3 weeks following induction of HLI in groups of (non-diabetic) BALB/C mice that had received single IM injections of saline (n = 5) or of human MSCs (n = 5). For these assays, serum samples were incubated with suspensions of the same human MSCs as were used for the IM injections followed by staining with anti-mouse IgG-fc-FITC and flow cytometric analysis with comparison to fluorescence levels of non-serum incubated cells (**fig. 4.9A**).

As shown in **fig. 4.9B**, all of the sera from the human MSC-injected group gave strong positive staining for mouse anti-human IgG while all of the sera from the saline-injected group were negative.

Interim Conclusions: (a) A single IM injection of allo-MSCs resulted in variable development of detectable donor-specific IgG antibody in the setting of DM and HLI. (b) A single IM injection of human MSC resulted in consistent development of donor-specific (anti-human) IgG in the mice with HLI. (c) Allo- (and Xeno-) MSCs retain the potential for inducing readily detectable anti-donor humoral responses in the setting of HLI with and without concomitant DM.

4.3.7 *Effect of Tacrolimus Administration on the Induction of Anti-donor Antibodies Following IM Injection of Allo-MSCs in Non-Diabetic Mice with HLI*

Finally, an experiment was carried out in which BALB/c mice undergoing HLI received single IM injections of saline or allo (B6)-MSCs with and without a 16-day course (day -2 to day +14) of the clinically-approved T-cell-specific immunosuppressive drug tacrolimus. An additional control group received IM injection of syngeneic MSCs at the time of HLI induction. At 21 days following HLI, the animals were euthanized and serum samples were analysed for anti-donor antibodies using the same flow cytometry-based assay as before (**fig. 4.10A**).

The results of the analysis for total anti-donor IgG are summarised in **fig. 4.10B**. As expected, groups of mice that have received IM injections of saline (without or with tacrolimus administration) or of syngeneic MSCs did not have detectable anti-B6 IgG in serum. Consistent with our previous results in healthy BALB/C mice, a single IM injection of allo-MSCs without tacrolimus administration resulted in the induction of readily-detected anti-B6 IgG. However, the group that received allo-MSC injection accompanied by a 16-day course of tacrolimus developed significantly lower levels of anti-donor IgG. When the assay was repeated using secondary antibodies specific for IgG1 and IgG2a, it was found that anti-donor antibodies of both of the major IgG isotypes we suppressed by tacrolimus administration (**fig. 4.10C**).

Interim Conclusion: (a) In non-diabetic mice with HLI, a single injection IM of allo-MSCs is associated with consistent development of anti-donor IgG that includes both IgG1 and IgG2a isotypes. (b) Generation of anti-donor IgG following IM injection of allo-MSCs can be largely (although not completely) suppressed by a short course of the T-cell-specific immunosuppressive drug tacrolimus.

Effect of Tacrolimus on anti-donor antibody response in CLI mouse model

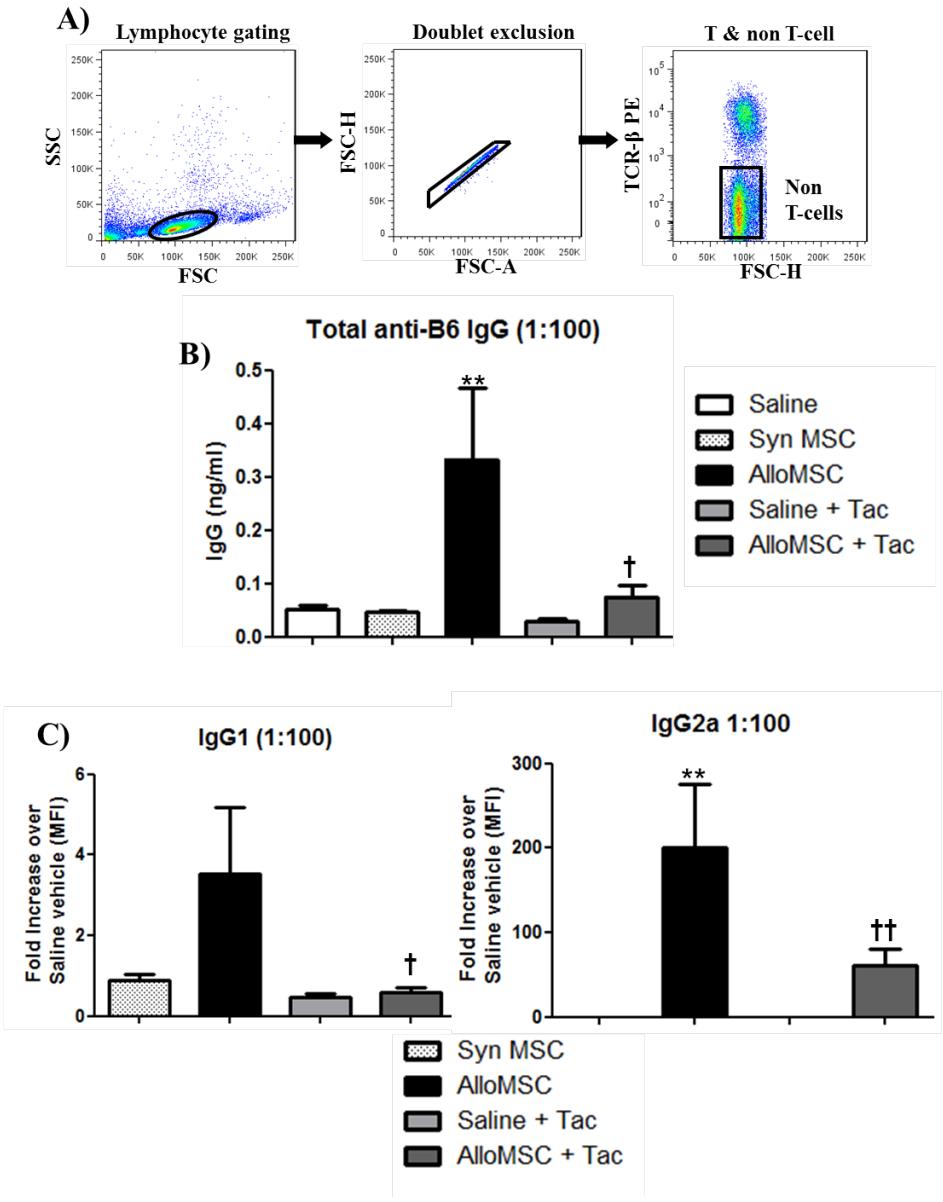


Figure 4.10: Effect of tacrolimus administration on anti-donor antibody response induced by IM injection of allo-MSC at the time of HLI: A. Gating strategy for flow cytometric analysis of the binding of IgG from mouse serum samples to TCRβ$^-$ B6 splenocytes. **B.** Results of semi-quantitative flow cytometry assays for anti-B6 IgG in 1:100 diluted sera from five groups of BALB/C mice (n = 8 each) that had under gone HLI 21 days previously. Titres were calculated based on a standard curve generated with serial dilutions of purified anti-B6 MHC I and were expressed as "ng/ml equivalent". Data represent the group means ± SD. **C.** Results of isotype-specific flow cytometric assays for anti-B6 IgG1 (left) and IgG2a (right) in 1:100 diluted sera from the same five groups of BALB/C mice. Titres were calculated as fold change over the mean fluorescence intensity (MFI) of the saline control group. Data represent the group means ± SD. **Statistics**: One-Way ANOVA, ** = $p < 0.01$ compared to the Saline group; †, †† = $p < 0.05$, < 0.01compared to AlloMSC group.
Abbreviations: SynMSC = syngeneic MSCs; Tac = tacrolimus.

4.3 Discussion

In this chapter, the anti-donor antibody responses to IM injection of allo- or xeno-MSCs were studied in detail in the following settings:

1) Healthy mice (single and double injection of allo-MSCs).
2) Diabetic mice undergoing HLI (single injection of allo-MSCs).
3) Non-diabetic mice undergoing HLI (single injection of allo-MSCs and of xeno (human)-MSCs).

The most striking finding for these experiments was that IM injection of MSCs carrying allogeneic or xenogeneic antigens has high potential to induce anti-donor IgG antibodies in healthy recipients as well as those with concomitant diabetes and/or acute ischemia at the site of MSC injection. Importantly, however, through the use of a semi-quantitative assay system, we observed that the level ("titre") of anti-donor IgG that occurred in individual animals and groups of animals was highly variable and was not clearly boosted upon receipt of a second injection of allo-MSCs. By performing IgG isotype-specific analyses and in vitro assays of donor-specific complement-mediated lysis, we were also able to gain some further insights into the functional roles of the anti-donor antibodies generated in the recipients of allo-MSCs. Finally, through co-administration of immunosuppressive drug tacrolimus, which is commonly used to prevent cellular rejection of organ allografts and to treat autoimmune diseases, we could demonstrate that targeting the T-cell compartment around the time of allo-MSC administration represents an effective approach to suppressing the development of anti-donor IgG antibodies.

As outlined in the Introduction to this Chapter, the direct effects of MSCs on B-cells have been studied extensively *in vitro* with several, though not all, published reports suggesting that MSCs can suppress or modify B-cell activation and proliferation (Asari *et al.,* 2009, Schena *et al.,* 2010). In some of these *in vitro* studies, specific mechanistic pathways were identified whereby MSCs suppress B-cell activation (Augello *et al.,* 2005 and Rafei *et al.,* 2008). Thus, it might be reasonable to propose that allo-MSCs may be resistant to the development of anti-donor antibodies following *in vivo*

administration. Despite this, we find here that IM-delivered allo- (and xeno-) MSCs are quite consistently associated with the generation of anti-donor IgG antibodies. Furthermore, we observed that the IgG titres detected following single or double injections of allo-MSCs were comparable to those induced by IM injection of a highly immunogenic cell type (splenocytes) from the same mouse strain. In this regard, our results are consistent with those reported by other research groups in experiments involving multiple experimental animal species, routes of administration and states of health. (Beggs *et al.*,2006, Poncelet *et al.*,2007, Badillo *et al.*,2007, Cho *et al.*,2008 and Schu *et al.*,2012). Of particular relevance to the results we describe here in mice, it has been previously reported that multiple inocula of allo-MSC via IV, IM and sub-cutaneous routes were associated with readily-detected anti-donor antibody in healthy baboons and pig models (Beggs *et al.,* 2006 and Cho *et al.,* 2008). Significantly, however, the freqency of induction of donor specific anti-HLA antibodies following allo-MSC administration in human subjects participating in clinical trials has been infrequently reported and remains less clear. For example, Sundin *et al,* 2007 and Hare *et al.,* 2012 have reported that a single infusion of allo-MSC leads to induction of anti-HLA antibodies in the human recipients, without any adverse effects. This suggests that single doses of allo-MSC may be immunogenic in a clinical setting in some subjects. Thus, it will be important in the future to also examine the immunological effects of mutiple dosing of allo-MSC in a clinical trials.

Also less well understood at present are the functional characteristics and consequences of allo-MSC-induced anti-donor antibodies. As is now emerging in the field of organ transplantation, the clinical implications of anti-donor IgG antibodies may be strongly influenced not just by antibody level/titre but also by the heavy chain isotype and by the capacity for fixing complement. Indeed, there is now a high level of interest in the clinical application of assays for determining isotypes and complement component binding of anti-HLA antibodies present in serum of transplant recipients. In clinical setting, single-antigen bead assay and flow bead assay are frequently used to quantify anti-HLA antibody isotypes and complement fixing proteins which could be correlated with the graft survival. (Arnold *et al.,* 2013 and Sicard *et al.,* 2015) For example here, kidney transplant recipients who have previously shown presence of anti-donor anti-HLA antibodies were tested for subtypes of anti-donor

antibodies and their functional complement binding activity. Sicard *et al.,* 2015 have used flow bead assay to detect binding of anti-HLA antibodies to C1q and C3d and reported that C3d binding donor-specific antibodies are highly associated with graft rejection. In another study by Arnold *et al.,*2013, single-antigen bead assay have been used to discriminate anti-donor HLA antibody subtypes. In the same study, they have exclusively studied the relevance of non-C' fixing and C' fixing anti-donor IgG (IgG2/IgG4, IgA1/IgA2 and IgG1/IgG3) in the renal transplant recipients and have classified the outcomes of the transplant survival by donor-specific antibodies to non-C' fixing and C' fixing antibody subclasses. In the pre-clinical setting, the capacity of allo-MSC-induced anti-donor antibodies to mediate donor cell lysis has been investigated in a limited number of studies. For example, Cho *et al.,* 2008, in experiments involving multiple dosing of allo-MSCs in healthy pigs, demonstrated that the resulting anti-donor antibody supported complement-mediated lysis of target cells *in vitro* and that the allo-MSC-sensitized animals showed accelerated rejection of a subsequent skin graft from the same donor. Furthermore, Badillo *et al.,*2007 and Schu *et al.,*2012 in rats in baboons reported that single injections of allo-MSCs were associated with the generation of anti-donor antibody and with accelerated clearance of a subsequent inoculum of allo-MSCs. However, the role of antibody and complement-mediated lysis in the "rejection" of the second cell dose was not proven in this study. Our own findings add to this existing evidence that anti-donor IgG antibodies induced by single or double injections of allo-MSCs – in this case by the IM route – have significant potential to fix complement and mediate donor-specific cell lysis. In contrast to the report of Cho *et al.,* however, we did not observe an enhancing effect of a second allo-MSC injection on the anti-donor IgG titre or complement fixing potency. Indeed, in some experiments, there was a non-significant trend toward lower titres of anti-donor IgG among the animals that had received double injections. Furthermore, when complement-mediated lysis of B6 splenocytes was quantified for sera from a relatively large number of double-injected mice, it was increased over that of control sera in only approximately 40% of samples. These observations may reflect a modifying effect of the T-cell hypo-responsiveness that occurred following double IM injection of allo-MSCs upon the quantity and functional properties of allo-MSC-induced anti-donor IgG. They also indicate that there is high potential for significant inter-individual variability in the *in vivo* B-cell responses to allo-MSC

administration – a fact that is consistent with the limited amount of data available from human clinical trials.

Our results have also provided a novel insight into potential qualitative differences between anti-donor IgG induced by allo-MSCs compared to other allogeneic cell types. The flow cytometric assay that we developed to provide a semi-quantitative read-out of total anti-B6 IgG in serum samples from experimental animals was readily adapted to allow for specific detection of the individual mouse IgG isotypes: IgG1, IgG2a, IgG2b and IgG3. As described in the Results section of this Chapter, this approach revealed that, despite comparable titres, recipients of double IM injections of allo-MSCs developed a distinct IgG isotype profile compared to recipients of allo-MSCs. This was characterised by a lack of IgG3 anti-B6 antibody and by a relative predominance of IgG1. As described in the introduction to this Chapter, T-cell dependent and independent activation of B-cells may lead to distinct antibody class and isotype switching depending on the presence and nature of accessory cells and cytokines. Although cytokine analysis of the supernatants from T-cell MLRS described in Chapter 4 did not detect IL-4, there was increased IL-10 present in cultures of splenocytes from mice that received double injection of allo-MSC (both with and without B6 DC stimulation). It is possible, therefore, that the higher titres of IgG1 anti-donor antibodies detected in the serum of recipients of allo-MSC compared to allo-splenocytes resulted from the induction, by MSC, of an IL-10 producing accessory cell. Clearly, additional *in vivo* experiments will be needed to confirm this hypothesis and to better elucidate the mechanisms underlying anti-donor IgG isotype switching following IM administration of allo-MSC. Also requiring further investigation is the question of whether or not allo-MSC injection is associated with additional classes of anti-donor antibody such as IgM, IgE and IgA. Given the roles played of inflammatory milieu and antigen-specific T-helper cell differentiation patterns on the maturation of antigen-specific B-cell responses, we believe that this finding reflects a distinctive influence of MSC-associated modulation of cellular immune responses on the anti-donor humoral response. Such a conclusion would be consistent with other studies in which intravenously administered allo-MSCs anti-donor antibody immune responses were shown to induce a unique pattern of IgG isotypes in recipient animals (Schu *et al.*,2012 and Swart *et al.*, 2013).

Our experiments did not extend to identifying the specific cellular immune response that was responsible for determining the isotype profiles of the anti-donor IgG induced by allo-MSCs and allo-splenocytes. Studies of antibody responses to pathogenic infection in mice have suggested that relative excess of IgG1 over IgG2a antibody is an indication of underlying Th2-type cellular immune response in the recipient animal (Mountford *et al.*,1994). However, assays for Th2-associated cytokines (IL-4, IL-5) in culture supernatants from "re-call" MLRs did not provide evidence for a donor-specific Th2-type T-cell response in splenocytes of allo-MSC recipients (Chapter 3). Alternative explanations for the skewing of the humoral response toward IgG1 isotype include suppressive effects of allo-MSCs or their products on Th1- and Th17-type anti-donor T-cell responses, the influence of induced regulatory T-cell populations, promotion of anti-inflammatory monocytes or myeloid-derived suppressor cells or direct effects upon activated B-cells. (Griffin *et al.*,2013 , Melief *et al.*, 2013, Campioni *et al.*,2013 and Yen *et al.*, 2013) Whether these observations will prove relevant to human subjects receiving allo-MSCs for various disease indications remains to be determined but the field of organ transplantation had already provided the tools to address the issue with high degrees of sensitivity and accuracy.

An additional interesting aspect of the results described in this Chapter is the strong binding of the anti-donor antibodies induced in mice by IM injections of allo-MSCs to freshly prepared B6 MSCs from the mouse strain. Because previous studies have used either donor thymocytes or splenocytes as targets for the anti-donor antibody assays (Poncelet *et al.*,2007, Isakova *et al.*, 2010 and Schu *et al.*, 2012), our result provides the novel insight that a single administration of allo-MSCs may induce anti-donor IgG which will readily bind to subsequent inocula of MSCs from the same donor. Importantly, we ruled out the possibility that the observed binding of IgG to B6 MSCs represented induction of antibodies against FBS components form the culture medium by documenting lack of binding to syngeneic (BALB/C) MSCs cultured under the same conditions. (Horwitz *et al.*,2002 and Sundin *et al.*, 2007) Furthermore, we documented that the cell surface target for anti-donor IgG on MSCs was up-regulated by pre-incubation with IFN-γ. This observation suggests that the primary antibody targets are likely to be MHC as IFN-γ, along with other pro-inflammatory cytokines, is known to

induce increased surface expression of MHC-I expression on MSCs following both *in vitro* and *in vivo* exposure (Chan *et al.,* 2008, Bernardo & Fibbe., 2013 and Griffin *et al.,* 2013). A surprising aspect of our results was that the level of binding of anti-donor IgG to B6 MSCs was significantly higher that the binding level to B6 splenocytes. This was against expectations as MSCs are consistently reported to express low levels of surface MHC-I and absent or very low levels of MHC-II protein in the resting state. (Di Nicola *et al.,* 2002, Tse *et al.,* 2003 and Ryan *et al.,* 2005) We speculate that this finding reflects, at least in part, a potent induction of antibodies against allo-MSC-delivered MHC-I which may be present both on the surface and intra-cellularly in culture-expanded MSCs. (Beggs *et al.,* 2006, Poncelet *et al.,* 2007, Badillo *et al.,* 2007, Isakova *et al.,*2010 and Schu *et al.,*2012) and It is also possible, however, that antibodies were induced against non-MHC allo-antigens expressed on the surface of the administered B6 MSCs – a possibility that merits consideration in the context of human clinical use of allo-MSCs. Although time did not allow us to pursue this question further, experiments involving blocking antibodies against B6 MHC I and II, Western blotting or immune-precipitation of B6 MSC-derived protein lysates with sera from sensitised animals or analysis of antibody binding to recombinant MHC and other proteins could be applied to better identify the target proteins.

Of clinical interest, serum samples from diabetic CLI mice receiving single dose of allogeneic MSCs or CLI mice receiving single dose of allo-MSCs with or without immuno-suppressive drug tacrolimus and or human MSCs (xenogeneic transplantation) were tested for the induction of anti-donor antibodies in a series of mouse *in vivo* studies.

Surprisingly, a single dose of intra-muscularly delivered allogeneic MSCs in a diabetic CLI mice resulted in induction of anti-donor antibodies in 3/8 recipient animals. Although, it's only a small numbered study, the data strongly reflects the frequency of generation of anti-donor antibody in a diseased setting and it supports the findings from previous literatures. (Poncelet *et al.,*2007 and Huang *et al.,* 2010). So far, results from this section confirmed the finding that, injection of allogeneic MSCs are capable of inducing functional anti-donor antibodies both in healthy and diabetic CLI mouse model and emphasize the importance and impact of anti-donor antibodies in allo-MSC therapeutics.

Immunogenicity of allogeneic MSCs are dependent on the immune status of the recipients. (Griffin *et al.,* 2012) When allo-MSCs were tested in an ischemic inflammation model (CLI mouse model), single injection of allo-MSCs resulted in consistent induction of anti-donor antibodies and surprisingly all the allo-MSC recipients (8/8) resulted in high titres of donor specific allo-antibodies. In line with previous findings, intra-muscular injection of allo-MSCs resulted in induction of anti-donor antibodies both in healthy, CLI model and partially in diabetic CLI model and this alarming data can be directly translated to adverse clinical impact of the immunogenicity of allogeneic MSCs. In a clinical setting, this type of anti-donor antibody response would lead to detrimental effects (acute/chronic) and also, affects the future transplant option for the allo-MSC sensitized human subjects. In cell therapy field, human based products must have to be tested in pre-clinical models to ensure that the product is safe for the clinical use, more precisely, several labs have been testing safety of human MSCs for clinical trials in various disease models and in fact, limited clinical trials suggest that allogeneic human MSC application is safe (Peng *et al.,* 2013). Nevertheless, few studies show induction of anti-donor antibodies without any adverse effects (Le Blanc *et al.,* 2008 and Hare *et al.,* 2012). However, the long term effect of this type of response in other disease models is still unclear and if that would affect the efficacy is quite unclear. In the mouse HLI study that was carried out in collaboration with the research group of Prof. Tim O'Brien, the overall aim was to evaluate both anti-donor immune response to allo-MSC and the angiogenic capacity of the allo-MSC in a clinically relevant. In this study, however, no clear evidence of a pro-angiogenic effect of single IM injections of allo-MSC was observed in analyses that included limb functional observations and laser Doppler flow analysis at 7, 14 and 21 days after treatment of the ischemic limbs as well as capillary counting in histological sections of muscle prepared at the study end-point (data not shown as it forms part of the PhD thesis of Dr. Clara Sanz under the supervision of Prof. O'Brien). Importantly, animals that received tacrolimus with or without allo-MSCs did not have worsening of post-ischemic blood flow, limb function or limb muscle capillary density compared groups that did not receive tacrolimus (data not shown). This suggests that administration of a short course of tacrolimus around the time of allo-MSC administration for CLI may represent a safe immunomodulatory strategy if this proves to be clinically indicated. Overall, from a clinical perspective, our experimental data do indicate that

single intra-muscular injections of human MSCs were associated with strong induction of anti-donor antibodies in the recipient HLI mice (5/5) highlighting the importance of safety and monitoring anti-donor antibody response.

Generally in clinics, anti-donor immune responses to donor grafts will be prevented/controlled by appropriate usage of FDA approved immuno-suppressive drugs. In our study, we tested the possibility of controlling the anti-donor antibodies by a limited dose of immuno-suppressive drug tacrolimus. Interestingly, a short course of tacrolimus has resulted in diminished anti-donor antibody responses induced by allo-MSCs in CLI mice. Despite tacrolimus are known to directly suppress activation of T-cells in transplantation settings, this particular data indirectly supports the fact that, recipient B-cells activated by allo-MSC/particles of allo-MSC or in part by primed T-cells were also suppressed by tacrolimus. To this end, it seems very interesting for this project that, anti-donor immune responses to allo-MSCs can possibly be controlled by multiple dosing approach and persistent antibody responses can be easily overcome by co-administration of tacrolimus along with allo-MSCs and promote allo-MSC therapeutic applications. (Beggs *et al.*,2006, Ge *et al.*,2009 and Lutton BV *et al.*, 2010).

Taken together, interestingly, in a diseased setting, where surgically induced local inflammation is active, allo-MSCs or particles of allo-MSCs quite actively reached the B-cell compartment of the recipient host immune system to induce an antibody response indicating the potency of allo-MSCs to induce anti-donor immune responses in naïve and diseased mouse model. MSCs are shown to have induced immuno-suppressive properties, when the recipient immune cells are inflamed or secreting inflammatory cytokines or even inflammatory cytokine pre-treated MSCs (licensed MSCs), (Sheng *et al.*,2008, Krampera *et al.*,2011 and Dazzi *et al.*,2012) but strikingly, our immunogenicity data suggest that despite multiple injection of allo-MSC leads to induced unique T-cell hypo-responsiveness, (discussed earlier in chapter-3) it actively induces anti-donor antibody responses, which can only be prevented by short course of tacrolimus. Experimental data from this chapter is greatly convincing that multiple-dosing of allo-MSCs along with co-administration of immuno-suppressive drugs would be an ideal therapeutic approach for the application of allo-MSCs in inflammation mediated diseases without any adverse anti-donor immune responses.

CHAPTER – 5

FINAL DISCUSSION AND REFLECTION

5.1 Summary of the key findings of the project

The study described in this thesis focussed primarily on investigating the immunogenic effects of single and multiple doses of IM-delivered allo-MSCs in healthy mice. The results from Chapters 3 and 4 suggested, somewhat surprisingly, that single IM doses of allo-MSCs resulted in readily detectable donor-specific T-cell and B-cell responses in a fully MHC-mismatched experimental system that were comparable to those induced by equal numbers of a cell type (splenocytes) that was predicted to be highly immunogenic. Furthermore, in the case of anti-donor antibody responses, additional *in vivo* studies performed in mice with diabetes and/or acute limb ischemia revealed that the immunogenicity of a single IM dose of allo-MSCs is not prevented by the presence of these clinically-relevant co-morbid factors in the recipient animals. *Thus, in the first instance, the project adds to existing evidence that allo-MSCs, rather than being immune privileged as frequently cited, quite effectively deliver allogeneic MHC proteins (and potentially a range of minor histocompatibility antigens) into the host secondary lymphoid compartments where they are effectively presented to T-cells and B-cells*. Among the results we generated for single-dose allo-MSCs compared to single-dose allo-splenocytes during the course of the project, however, the difference in the predominant IgG isotype (IgG1 vs. IgG2a) provide evidence that MSC-delivered allo-antigens are presented to B-cells (and most likely T-cells) in an inflammatory context that is distinct from that associated with other cell types. *Based on this novel finding, we believe that it is important to consider the issue of the immunogenicity of allo-MSCs and its consequences for human clinical applications as being more complex (and interesting) than a simple "presence or absence" question.*

Of greater interest again, when healthy animals were administered a second (or third) dose of allo-MSCs an additional distinctive phenomenon – donor-specific $CD4^+$ and $CD8^+$ T-cell hypo-responsiveness – was consistently observed suggesting that early re-exposure of recipient animals to MSC-delivered allo-antigen is associated with an immunomodulatory effect on the re-call response of previously primed anti-donor T-cells. The persistence of anti-donor antibodies and the subsequent observation that the T-cell hypo-responsiveness to a second IM inoculum may be relatively short-lived, further emphasise the multi-faceted nature of the allo-MSC-associated immune response. These

findings highlight the fact that timing of analysis in *in vivo* experiments of allo-MSC immunogenicity may be of particular importance to the interpretation of the results. This is specifically illustrated by our observation that the T-cell hypo-responsiveness associated with two IM injections of allo-MSC was detectable at 1 week after the second injection but not at 2 weeks **(chapter 3, fig. 3.2 – 3.4)**. In future experiments, approaches to monitoring anti-donor T-cell responses at multiple time-points without the requirement for euthanasia (e.g. *in vivo* imaging of adoptively transferred T-cells, analysis of leukocytes from serial blood samples or biopsies of spleen/lymph node) would be valuable in tracking the course of allo-MSC immunogenicity/immune modulation over time. *Taken together, however, we believe that our results for this project raise the intriguing concept that multiple dosing of allo-MSCs within short time intervals is associated with an initial donor-specific activation of the host immune system which is subsequently converted into a modulatory phase when recipient animals are re-exposed to MSC-delivered allo-antigens. Interestingly, a recent publication by Tano et al.,2016 reports an initial storm of immune responses to single dose of allo-MSC that further resolved into an efficacy phase in a myocardial infarction model. This further strengthens the view that immunogenicity of allo-MSCs is not always inherently detrimental. Thus, immunogenicity and efficacy of allo-MSC must be studied in a distinct fashion for each disease model and, ideally, should also include long-term monitoring/follow-up. Furthermore, we believe that further experimental pursuit of this concept may be of specific value to the optimal use of allo-MSCs in non-transplantation- and transplantation-related clinical applications.*

Figure 5.1 provides a visual illustration of our proposed conceptual model of the immunomodulatory effect of multi-dose IM allo-MSCs with reference to specific elements of the experimental results from the thesis project. As summarised in the left hand portion, the first injection of allo-MSCs results primarily in active donor-specific T-cell and B-cell responses as evidenced by: (a) Increased donor-specific $CD4^+$ and $CD8^+$ proliferation (re-call MLR results). (b) Development of anti-donor IgG with IgG1 pre-dominance (serum alloantibody assays in healthy and diseased models). (c) Potential for anti-donor antibody mediated cell lysis (complement-mediated lysis assays). (d) T-cell dependence of the B-cell responses (tacrolimus suppression of in vivo anti-donor IgG development in CLI).

As summarised in the right hand portion, the second injection of allo-MSCs results in a distinctive modulation of the systemic innate and donor-specific adaptive immune response consisting of: (a) Donor-specific hypo-proliferative responses of $CD4^+$ and $CD8^+$ splenic T-cells with high levels of production of IFN-γ and IL-10 (*ex vivo* re-call MLR results). (b) Reduced $CD8^+$ T-cell cytotoxicity toward donor MSCs (*in vitro* donor-specific CTL assay results). (c) Lack of expansion of $CD4^+$ $FoxP3^+$ "classical" T-reg but striking increase in reportedly suppressive splenic $CD8^+$ $CD11c^+$ T-cells (flow cytometric profiling of splenic T-cells). (d) Proportionate increases in activated/memory-phenotype $CD8^+$ T-cells in spleen (flow cytometric profiling of splenic T-cells). (e) Multiple modulations of the systemic innate immune repertoire (flow cytometric profiling of splenic myeloid cells and NK/NKT-cells). (f) Persistence of complement-fixing, IgGI-predominant anti-donor IgG without a further increase in titre (serum alloantibody and complement-mediated lysis assays). (g) Presence of immune cell infiltrates at the site of injection (histological scoring). (h) Transient nature of the donor-specific T-cell hyporesponsiveness (results of triple-injection IM experiment).

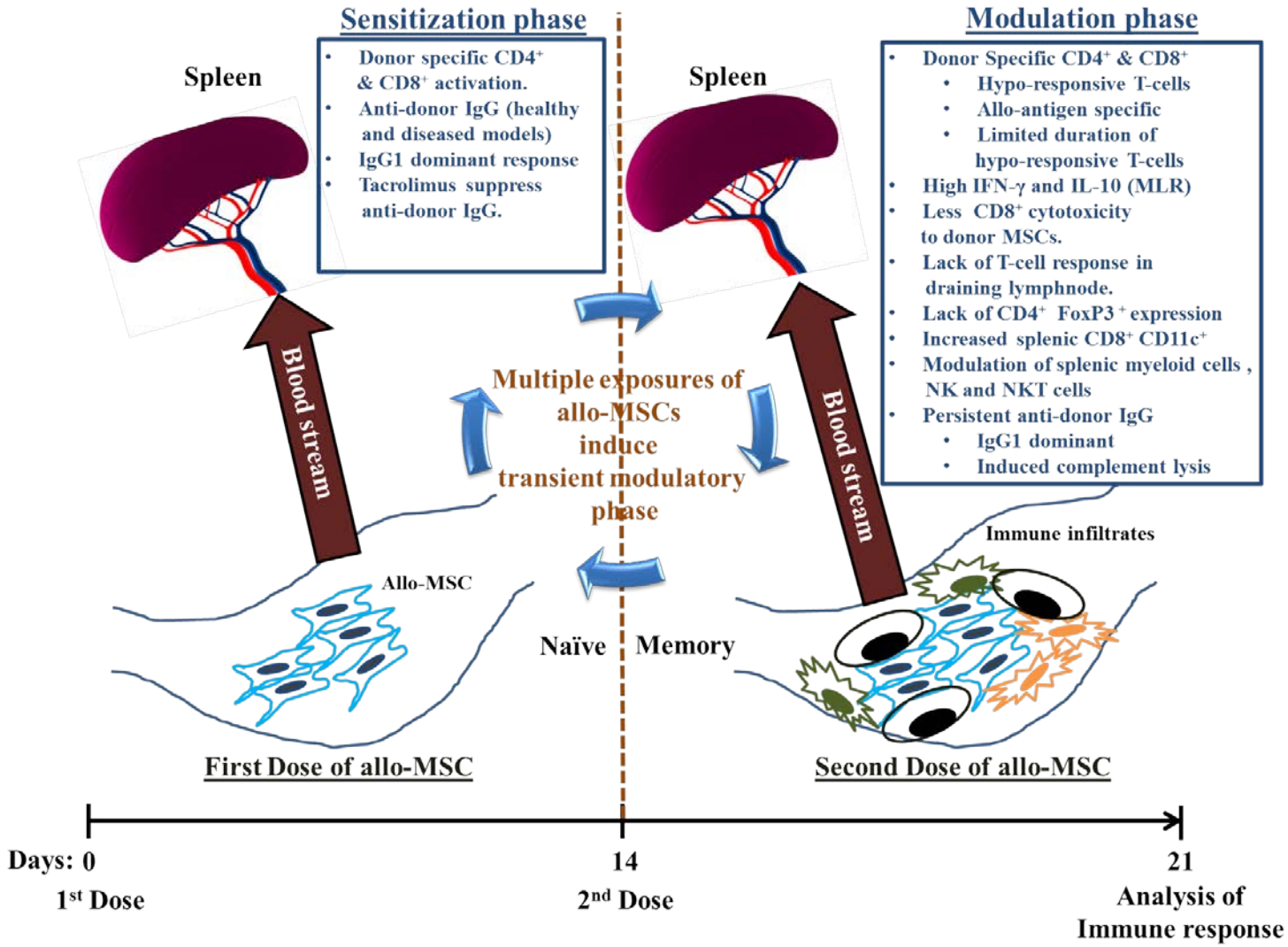


Figure 5-1:

Hypothetical model of allo-MSC multi-dose-associated immune modulation with reference to experimental results from the project.

5.1.1 *Potential Relevance of the Findings to the Field of Transplantation*

Mesenchymal stem cells are considered to be a promising immunotherapy in the field of solid organ transplantation. Among the proposed benefits are the potential for autologous or allo-MSC infusions to facilitate minimisation of long-term immunosuppressive drug doses and the potential to enhance immunological graft acceptance through the promotion of donor-specific tolerance. (Reinders *et al.*, 2013 and Alagesan S *et al.,* 2014) In regard to immunological tolerance, allo-MSCs have been shown, in some experimental setting, to synergise with clinically established immunosuppressants such as sirolimus (rapamycin) and mycophenolate mofetil (MMF). (Poncelet *et al.,* 2008, Popp *et al.,* 2008 and Ge *et al.,* 2009) In addition, the infusion of allo-MSCs in the setting of transplantation procedures has been shown to be feasible in human subjects. For example, Le Blanc *et al.,* 2008 reported that third party allo-MSCs were not associated with severe adverse effects in a clinical trial of graft-versus-host disease. In another study by Peng *et al.,*2013, the authors reported no adverse immune effects of infusion of donor-derived allo-MSCs in kidney transplant patients in a small clinical study. Other clinical studies of solid organ transplantation in which autologous (recipient-derived) MSCs were infused also support the conclusion that this therapeutic approach is generally safe and may facilitate the use of lower doses of maintenance immunosuppressive drugs through anti-inflammatory/immunomodulatory mechanisms. On the other hand most importantly, infusions of MSC alone are shown to be safe in human clinical studies without any adverse effects suggesting that MSC infusion is generally safe in transplant recipients with few exceptions. (Duijvestein *et al.,*2010, Perico *et al.,*2011, Tan *et al.,* 2012, Reinders *et al.,*2013 and Peng *et al.,* 2013)

However, the experimental work that has been published to date raises important questions about the optimal use of allo-MSCs in solid organ transplantation and, in some cases, about the potential for undesired effects. For example, Wu *et al.,* 2003 and Inoue & Popp *et al.,* 2006 in rodent models of allogeneic heart transplantation, observed that allo-MSC infusion alone was not effective in prolonging graft survival, suggesting that the primary immunomodulatory effect of these cells may not be sufficiently potent. Nonetheless, as reported by Popp *et al.,* 2008 the combined administration of donor-derived allo-MSCs with a low dose of MMF did result in long-term graft survival in the

majority of animals. However, this favourable effect was only observed when allo-MSCs were administered on day 4 post-transplant and not when they were administered on day 0 + day 3. While these results provide a context whereby allo-MSCs might be effectively combined with commonly used anti-rejection drugs, they also highlight the complexity of translating such findings into human transplant recipients. Of further relevance, Seifert *et al.,* 2012, reported that allo-MSCs reported a detrimental effect of allo-MSC infusion 4 days prior to kidney transplantation in a rat model in which MMF was administrated for the first post-transplant week. In this study, accelerated graft rejection in allo-MSC recipients was accompanied by enhanced anti-donor humoral immune response suggesting that pre-transplant administration of allo-MSCs into otherwise immunocompetent hosts may be induce or promote the development of harmful anti-donor antibodies. It is of interest, therefore, that our study also provides evidence that single injections of allo-MSCs (in this case IM) do induce an early activation of anti-donor adaptive immune responses – including the development of readily detectable and durable complement fixing anti-donor IgG. Our subsequent finding that an additional exposure to allo-MSCs leads to the development of a period of donor-specific T-cell hypo-responsiveness has an obvious appeal in the context of organ transplantation. However, the initial induction of allo-antibody and its persistence following the second allo-MSC injection raises a significant concern about the overall effectiveness of a "multi-dose" approach to allo-MSC immunotherapy in transplantation – particularly if the first dose is administered prior to the initiation of conventional immunosuppression. Interestingly, Treacy's *et al.*,2014 has shown pre-transplantation of allo-MSCs are associated with induction of high titres of anti-donor antibodies while significantly improving rejection-free survival of the graft in rat corneal transplantation model. This strongly suggest that, immunogenicity of allo-MSCs may be associated with subsequent immuno-modulatory reactions, which might support the survival of the graft and also relates to the relevance of anti-donor antibodies induced by allo-MSCs in vascularised and solid tissue allo-transplants. *Thus, we believe that the results of this project hold high significance to the application of allo-MSC therapy to the field of transplantation and that clinical protocols in this area must pay close attention to the monitoring of both T-cell and B-cell responses to the donor antigens.*

Some important unanswered questions which we were not able to address within the time-frame of the project include: (a) Does co-administration of tacrolimus (or other immunosuppressive drugs) prevent the emergence of donor-specific T-cell hypo-responsiveness following multiple injections of allo-MSCs as it does for anti-donor antibody response? (b) Are the anti-donor IgG antibodies that are induced by IM injection of allo-MSCs sufficiently potent to directly mediate rejection of a subsequent organ or tissue allograft from the same or another donor strain? (c) Is the donor-specific T-cell hypo-responsiveness that we observed sufficiently potent and durable to promote prolonged or indefinite survival of a concomitant organ or tissue allograft from the same donor strain? (d) Do multiple doses of IM allo-MSCs induce a regulatory T-cell population that can mediate "infectious tolerance"?

5.1.*2 Potential Relevance of the Findings to Non-Transplant Applications*

Aside from their application to transplantation, the list of inflammatory and immune-mediated diseases for which allo-MSCs have been shown or proposed to have potential clinical value has been steadily growing over the past 10 years. (Ghannam *et al.*, 2010, Ren *et al.*, 2011, Griffin *et al.*, 2013, and Zhang *et al.*, 2015). In broad terms, the non-transplant disease applications of allo-MSC therapy could be categorised as: (a) Acute tissue injury with inflammatory response (e.g. sepsis, myocardial infarction, acute kidney injury, stroke, acute lung injury, acute limb ischemia). (b) Chronic tissue injury with inflammation and fibrosis (e.g. chronic heart failure, chronic lung disease, chronic kidney disease, liver cirrhosis, critical limb ischemia, diabetic complications, osteoarthritis). (c) Autoimmune and auto-inflammatory diseases (e.g. multiple sclerosis, inflammatory bowel disease, type 1 diabetes, autoimmune hepatitis). As we have described in Chapter 1 and, where relevant, in other Chapters, there is a wealth of published *in vitro* and animal model experimental work to demonstrate the beneficial "paracrine" effects of both autologous and allo-MSCs for resolution of inflammation and promotion of tissue repair and angiogenesis in a variety of disease settings (Oh *et al.*, 2010, Prockop & Youn., 2012, Schlosser *et al.*, 2012, Bianco *et al.*, 2013 and Griffin *et al.*, 2014). In a number of such studies, the therapeutic effects of allo-MSCs have been reported to be equivalent to those of autologous MSCs (Zappia *et al.*,2005, Nemeth *et al.*,2009, Quevedo *et al.*, 2009,Chen *et al.*,2009, and Rafei *et al.*,2009). In addition, several human clinical trials of allo-MSCs for non-transplant-related

diseases have reported good safety profiles with some evidence of efficacy. (Blanc *et al.*, 2008, Hare *et al.*, 2009, Vaes *et al.*, 2012 and Hare *et al.*,2012 and Peng *et al.*, 2013) Nonetheless, as we have already discussed in detail, the issue of allo-MSC immunogenicity and its implications for the short- and long-term safety and efficacy of this therapy in human subjects remains incompletely resolved. Furthermore, the possibility that allo-MSC anti-donor immune responses might, in fact, be harnessed for benefit in some non-transplant-related disease settings has not, to our knowledge, been previously considered.

In regard to safety and efficacy, it is often stated that allo-MSCs do not elicit strong anti-donor immune responses as a result of their low immunogenicity and inherent immunosuppressive functions. (Poncelet *et al.*, 2007 and Ankrum *et al.*, 2014). However, many small animal studies involving allo-MSC administration for inflammatory conditions report short-term rather than long-term outcomes and do not include in-depth immunological analyses or experiments to determine whether the *in vivo* persistence of allo-MSCs is compromised by *do novo* or pre-existing anti-donor immune responses (Kotobuki *et al.*,2008, Polchert *et al.*,2008 and Chen *et al.*,2009). As other animal model studies have demonstrated the capacity for allo-MSCs to induce anti-donor T-cell and B-cell sensitisation following systemic or localised administration (Eliopoulos *et al.*, 2005, Nauta *et al.*, 2006, Poncelet *et al.*, 2007, Badillo *et al.*, 2007 and Huang *et al.*, 2010), further characterisation of allo-MSC *in vivo* immunogenicity is needed. In addition to corroborating the findings from these previous studies, our results reveal that there is a greater complexity to allo-MSC immunogenicity when cells are administered on multiple occasions. The systemic T-cell hypo-responsiveness that we observed along with modulations to the myeloid cell repertoire could be of potential therapeutic benefit in acute diseases in which persistent, pro-inflammatory activation of immune cells plays an important role the progression of tissue injury. (Frangogiannis *et al.*, 2002, Brechot *et al.*, 2008 and Lee *et al.*, 2011). Clearly, additional experiments in animal models such as mouse HLI, will be required to explore this possibility.

Multiple dosing of allo-MSCs could also be applicable to chronic inflammatory and autoimmune disorders such as multiple sclerosis, inflammatory bowel disease and diabetes. Currently, there is very

little published research upon which to predict the immunological consequences of more than a single allo-MSC administration in such diseases. In the clinical arena, GvHD represents the only condition for which patients receiving multiple allo-MSC doses have been well characterised and described in the literature. (Le Blanc *et al.,* 2008) These reports document good safety and possible efficacy of multi-dose allo-MSCs for treatment-resistant GvHD. However, extrapolation to other clinical conditions is difficult given the unique immunobiology of GvHD itself. Nonetheless, it is interesting to consider that multiple-dosing could enhance the immunmodulatory potency of allo-MSC therapy in diseases such as multiple sclerosis in which adaptive and innate cellular immune responses are known to be detrimental. (Dendrou *et al.,* 2015)

In addition to highlighting potential benefits in regard to cellular immunity, our results also sound a note of caution about the persistence of anti-donor antibodies following single and multiple doses of allo-MSCs. As discussed above, this feature of allo-MSC immunogenicity has obvious implications for the long-term success of organ and tissue transplantation. However, it should also be taken into consideration in the context of non-transplant-related disease indications. Specifically, we need to know whether pre-existing anti-donor antibodies could compromise the early survival and efficacy of systemically administered allo-MSCs or whether the emergence of anti-donor antibodies after allo-MSC administration could limit the longevity of their therapeutic benefits. Furthermore, for patient groups who may have a future need for organ transplantation (e.g. chronic heart failure and chronic kidney disease), it will be essential to better document the frequency and persistence of allo-MSC-induced anti-HLA antibodies. Depending on the results of on-going research in this area, our observation that a short course of tacrolimus safely prevents the induction of high-titre anti-donor antibodies following IM injection of allo-MSCs may provide a feasible option for overcoming this immunological hurdle.

Taken together, our results regarding the immunogenicity of IM administered allo-MSCs provide a basis for testing the translational potential of a multiple dosing approach to allo-MSC therapy in both inflammatory and auto-immune diseases. Of equal importance, our findings imply that care must be taken to document the emergence of anti-donor antibody responses in pre-clinical models of allo-

MSC therapy for non-transplant-related diseases. Furthermore, by demonstrating that allo-MSC-induced anti-donor antibodies are suppressible by co-administration of a clinically-established anti-T-cell immunosuppressive drug, we provide a basis for preventing "sensitisation" of patients receiving allo-MSC therapies who may be candidates for organ transplantation in the future. In the context of current clinical trials involving allo-MSC administration for a wide range of inflammatory and degenerative diseases, our work highlights the importance of monitoring anti-donor immune responses in order to firmly establish their long-term safety and to fully harness their efficacy.

5.1.3 *Final Conclusions*

Overall, the findings from this study strongly support the broad conclusion that allo-MSCs are immunogenic in the setting of health as well as in disease states. However, they also reveal the possibility that multiple exposures to allo-MSCs within a short time-frame result in a conversion of the anti-donor cellular immune response to one that is dominated by counter-regulatory and anti-inflammatory effectors – with interesting implications for future clinical translation of allo-MSCs. Our experimental work provides circumstantial evidence for several potential mediators of this "multi-dose allo-MSC immune-modulatory effect" including the presence of donor-antigen-specific IFNγ-producing cells in MLRs, the expansion of $CD8^+/CD11c^+$ T-cells and NK-cells in the spleen and the altered repertoire of myeloid cells. Of equal importance, our work also highlights the propensity for allo-MSCs to induce a characteristic anti-donor IgG antibody response and the need to better understand the frequency and long-term consequences of *de novo* donor-specific antibodies in human subjects participating in clinical trials of allo-MSCs. Clearly, additional research will be needed to validate and expand upon the observations described in this thesis. Of the experimental lines of enquiry that could be pursued, we believe that following could of particular interest:

- Detailed phenotypic characterisation of infiltrating cells involved in the local response to the second IM injection of allo-MSCs
- Isolation and functional analysis of the $CD8^+/CD11c^+$ T-cells that were observed to be expanded in the spleens of mice that had received multiple IM injections of allo-MSC – including, perhaps, adoptive transfer of these cells into naïve recipients.

- Investigation of the tolerogenic potential of the observed donor-specific T-cell hypo-responsiveness through the performance of tissue (e.g. skin, pancreatic islets) or organ transplants (e.g. heart) following single and multiple dose IM allo-MSCs.
- Investigation of the therapeutic potential of the observed T-cell and myeloid cell modulations following multiple IM doses of allo-MSCs in models of acute and chronic tissue inflammation such as critical limb ischaemia, myocardial infarction and inflammatory bowel disease.
- Tracking of the cell types responsible for allo-MSC-derived donor antigen presentation within local and distant lymphoid organs of the recipient animals following single and multiple IM injections of allo-MSCs.
- Investigation of the *in vivo* capacity for anti-donor IgG antibodies to bind to and mediate damage of subsequent doses of allo-MSCs – including transfer of antibody-containing serum from allo-MSC recipients to naïve mice.
- Determination of the impact of a short course of tacrolimus (or other immunosuppressive drugs) on the donor-specific T-cell hypo-responsiveness that occurs following multiple IM doses of allo-MSCs.

APPENDICES

APPENDIX ONE: PUBLICATIONS AND PRESENTATIONS

Publications:

1) Griffin MD, Ryan AE, **Alagesan S**, Lohan P, Treacy O, Ritter T. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunol Cell Biol.* 2013 Jan;91(1):40-51. doi: 10.1038/icb.2012.67. Epub 2012 Dec 4. Review.
2) Duffy MM, McNicholas BA, Monaghan DA, Hanley SA, McMahon JM, Pindjakova J, **Alagesan S**, Fearnhead HO, Griffin MD. "Mesenchymal stem cells and a vitamin D receptor agonist additively suppress T helper 17 cells and the related inflammatory response in the kidney. *Am J Physiol Renal Physiol.* 2014 Dec 15;307(12):F1412-26. doi: 10.1152/ajprenal.00024.2014. Epub 2014 Oct 22
3) Prado-Lopez S, Duffy M, Baustian C, **Alagesan S**, Hanley A, Stocca A, Griffin MD and Ceredig R. The influence of hypoxia on the differentiation capacities and immunosuppressive properties of clonal mouse mesenchymal stromal cell lines. *Immunology and Cell Biology* advance online publication, 29 April 2014; doi:10.1038/icb.2014.30.
4) **Alagesan S**, Griffin MD Alternatively activated macrophages as therapeutic agents for kidney disease: in vivo stability is a key factor. *Kidney Int.* 2014 Apr;85(4):730-3. doi: 10.1038/ki.2013.405.
5) **Alagesan S,** Griffin MD. Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? *Curr Opin Organ Transplant*. 2014 Feb;19(1):65-72. doi:10.1097/MOT.0000000000000043.

Oral presentations:

1) **Senthilkumar Alagesan**, Mikey Creane, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin "Intra-muscularly Delivered Allogeneic Mesenchymal Stem Cells Induce Distinct Anti-donor T-cell and B-cell Response" " VIII Annual Meeting of the Irish Cytometry Society" **NUIG, Galway, Ireland**. November 6th 2012.
2) **Senthilkumar Alagesan**, Mikey Creane, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin "Repeated Intra-muscular Injections of Allogeneic Mesecnhymal Stem Cells Promote Donor Specific T-cell Hypo-responsiveness but also Induce Allo-antibody" "Irish Society for Immunology" **Dublin, Ireland**. September 21st, 2012.
3) **Senthilkumar Alagesan**, Mikey Creane, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin. "Distinctive Donor-specific Systemic CD8+ T-cell and Antibody Responses Following Repeated Intramuscular Administration of Allogeneic Mesenchymal Stem Cells" Stem Cell Galway 2014, **8th UK Mesenchymal Stem Cell Meeting. Venue - Galway, Ireland.** October 29/30 , 2014
4) **Senthilkumar Alagesan**, Mikey Creane, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin. "Distinctive Donor-specific Systemic CD8+ T-cell and Antibody Responses Following Repeated Intramuscular Administration of Allogeneic Mesenchymal Stem Cells" Irish Society for Immunology Annual Meeting**, Venue - The Crowne Plaza, Northwood, Santry, Dublin 9**. 4th & 5th September 2014.

Poster presentations:

1) **Senthilkumar Alagesan**, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin "Re-programming of Toll Like Receptor 2(TLR2)-Activated Macrophages by Mesenchymal Stem Cells" **Poster Presentation for SFI (Science Foundation Ireland)** review on 20.2.2012**.**

2) **Senthilkumar Alagesan,** Mikey Creane, Timothy O'Brien, Rhodri Ceredig, Thomas Ritter and Matthew D. Griffin "Repeated Intra-muscular Injections of Allogeneic Mesecnhymal Stem Cells Promote Donor Specific T-cell Hypo-responsiveness but also Induce Allo-antibody" **"College of Medicine, Nursing & Health sciences Postgraduate Research Day 2013"** NUIG, Galway, Ireland. May 27th, 2013.
3) **Alagesan S**, Creane M, O'Brien T, Ceredig R, Ritter T, Griffin MD. „Repeated intra-muscular injections of allogeneic mesenchymal stem cells (MSC) promote donor-specific T – cell hypo-responsiveness but also induce allo-antibody". **"15th International Congress of Immunology" Milan, Italy.** August 22nd – 27th, 2013
4) Ameya Jagtap, **Senthilkumar Alagesan**, Mikey Creane, Clara Sanz-Noguéz, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin. "Analysis of localized immune response to repeated intra-muscular administration of allogeneic mesenchymal stem cells in mice" REMEDI regenerative medicine **Master degree student's presentation.. Venue – Bioscience building, REMEDI, Galway, Ireland**. August 2014
5) **Senthilkumar Alagesan**, Mikey Creane, Prof. Tim O'Brien, Prof. Rhodri Ceredig, Prof. Thomas Ritter and Prof. Matthew D. Griffin. "Donor-specific systemic CD8+ T-cell and antibody responses to allo-MSCs". Science Foundation Ireland, REMEDI site visit. **Venue – Bioscience building, REMEDI, Galway, Ireland.** . December 8th, 2014
6) Diana Gaspar, Daniela Cigognini, Pramod Kumar, Abhigyan Satyam, **Senthilkumar Alagesan**, Clara Sanz-Noguéz, Matthew Griffin, Timothy O'Brien, Abhay Pandit, Dimitrios Zeugolis. "A Multifactorial Approach Towards Enhanced Extracellular Matrix Deposition And Maintenance Of Mesenchymal Stem Cell Phenotype Using Macromolecular Crowding And Low Oxygen Tension" **27th European Conference on Biomaterials, Krakow, Poland, 30th August – 3rd September 2015**.
7) Clara Sanz-Nogués, **Senthilkumar Alagesan**, Michael Creane, Callum Conroy, Xizhe Chen, Peter Dockery, Matthew D. Griffin and Timothy O'Brien. "Tacrolimus co-administration suppresses anti-donor immune response to allogeneic mesenchymal stem cells but does not influence disease severity in a mouse model of hind limb ischemia" - **4th TERMIS World Congress** - September 8-11, 2015.

APPENDIX TWO: REAGENTS

Reagent Supplier	Catalogue number
0.25% trypsin/EDTA	Sigma-Aldrich T4049
10% neutral buffered formalin	Sigma-Aldrich HT501128
2-mercaptoethanol	Sigma-Aldrich M3148
Alpha-MEM	Gibco-Invitrogen 32561
Ammonium chloride	Sigma-Aldrich A0171
Ammonium persulfate	Sigma-Aldrich A3678
Anti-mouse CD90.2 microbeads	Miltenyi Biotec Inc. 130-049-101
Bovine serum albumin	Sigma-Aldrich A2153
CellTrace CFSE cell proliferation kit® Molecular Probes-	Invitrogen C34554
Chloroform	Sigma-Aldrich 496189
Collagenase I	Sigma-Aldrich C9891
Dimethyl sulfoxide	Sigma-Aldrich D2650
Disodium EDTA	Sigma-Aldrich E5134
DPX mounting medium	Sigma-Aldrich 44581
Dulbecco's modified Eagles medium	Sigma-Aldrich D6429
Dulbecco's PBS	Gibco-Invitrogen 14190
DuoSet® ELISA development systems	R&D Systems (as mentioned in the chapters)
Eosin	Sigma-Aldrich HT110116
Glycerol	Sigma-Aldrich G8773
Hematoxylin	Sigma-Aldrich HHS16
HEPES	Sigma-Aldrich H0887
Hyclone equine serum	Sigma-Aldrich H1270
Hyclone fetal bovine serum (FBS)	Fisher Scientific Ireland SV30143.03
Hydrochloric acid	Sigma-Aldrich H1758
Hydrogen peroxide	Sigma-Aldrich 216763
Indomethacin	Sigma-Aldrich I7378
Iscove's modified Dulbecco's medium	Sigma-Aldrich 13390
Isopropanol	Sigma-Aldrich I9516
L-glutamine	Gibco-Invitrogen 25030-024
L-NAME	Sigma-Aldrich N5751
Non-essential amino-acids	Sigma-Aldrich M7145
NS-398	Sigma-Aldrich N194
Penicillin/streptomycin	Gibco-Invitrogen 15140-122
RNA Later® solution	Applied Biosystems AM7020
RPMI-1640	Sigma-Aldrich R0833
Sodium Chloride 0.9% w/v intravenous infusion	B. Braun Melsungen, Germany 630137
Sodium azide	Sigma-Aldrich A2152
Sodium chloride	Sigma-Aldrich S5886
TMB/E ELISA substrate	Millipore ES001
Trizol®	Invitrogen 15596018
Tween-20®	Sigma-Aldrich P1379
Xylene	Sigma-Aldrich 534056

APPENDIX THREE: ANTIBODY PREPARATIONS

Antibody	Clone	Dilution	Supplier
Anti-mouse CD25 FITC	3C7	1:20	BD 558689
Rat anti-mouse CD8 FITC	53-6.7	1:40	BD 553031
Rat anti-mouse CD62L PE	MEL-14	1:20	eBioscience 12-0621-83
Hamster anti-mouse CD11c FITC	HL3	1:100	BD 553801
Rat anti-mouse CD4 PerCP-Cy5.5	RM4-5	1:40	BD 550954
Rat anti-mouse Ly6C PerCP-Cy5.5	HK1.4	1:20	Biolegend 128012
Rat anti-mouse CD4 PerCP-Cy5.5	RM4-5	1:40	BD 550954
Rat anti-mouse CD8a APC	53-6.7	1:40	BD 553035
Rat anti-mouse Ly-6G PE-Cy7	1A8	1:20	BD 560601
Rat anti-mouse CD19 PE-Cy7	1D3	1:40	552854
Rat anti-mouseCD44 APC	IM7	1:20	BD 559250
Rat anti-mouse F4/80 APC	BM8	1:10	eBioscience 17-4801-82
Anti-mouse CD49b(NK1.1) APC	DX5	1:20	e bioscience 17-5971-82
Rat anti-mouse CD8 APC	53-6.7	1:40	BD 553035
Hamster anti-mouse TCR-β PE	H57-597	1:20	BD 553172
Purified anti H-2k^{b}	AF6-88.5		BD 553567
Anti-IgG Fc F(ab')$_2$ FITC	PN IM1619	1:400	Beckman Coulter
Anti-IgG FITC	RMG1-1	1:200	Biolegend 406606
Anti-IgG2a FITC	RMG2a-62	1:200	Biolegend 407106
Anti-mouse I-Ab FITC	KH74	1:50	Biolegend 115305
Biotin mouse anti-mouse H-2Kb	AF6-88.5	1:50	BD 553568
Purified Rat IgG1, κ antibody	RTK2071	As mentioned	Biolegend 400414

		in chapters	
Purified anti-mouse IFN-γ Antibody	AN-18	As mentioned in chapters	Biolegend 517903
Rat anti-mouse CD45 Pe-Cy7	30-F11	1:40	BD 552848
Purified anti-mouse IL-10 Antibody	JES5-2A5	As mentioned in chapters	Biolegend 504904
Rat anti-mouse IFN-γ PE	XMG1.2	As mentioned in chapters	BD 554412
Rat anti-mouse FoxP3 AF647	MF23	1:10	BD 560401
SA-APC	-	1:100	BD 554067
Anti-mouse CD3 purified	145-2C11	As mentioned in chapters	eBioscience 16-0031-85
Rat anti-mouse IL-10 APC	JES5-16E3	1:20	BD 561059

APPENDIX FOUR: MEDIA/BUFFER FORMULATIONS

Complete isolation media (CIM):
RPMI-1640
Fetal bovine serum (FBS) (10%)
Equine serum (10%)
Penicillin-streptomycin (1%)
L-glutamine (1%)

Complete expansion media (CEM):
Alpha-MEM
FBS (10%)
Equine serum (10%)
Penicillin-streptomycin (1%)
L-glutamine (1%)

MSC culture media:
IMDM
FBS (9%)
Equine serum (9%)
Penicillin-streptomycin (1%)
L-glutamine (1%)

Freezing media:
FBS
DMSO (10%)

T culture media:
DMEM
FBS (10%)
Penicillin-streptomycin (1%)
L-glutamine (2mM)
HEPES (10mM)
Non-essential amino acids (1%)
2-mercaptoethanol (0.1%)

Fibroblast media:
DMEM
F-12 Nutrient mixture (50%)
FBS (10%)
Penicillin-streptomycin (1%)
L-glutamine (1%)
HEPES (1%)
Non-essential amino acids (1%)

Ammonium chloride lysis buffer (10 x):
Ammoniun chloride (1.5 M)
Sodium bicarbonate (100 mM)
Disodium EDTA (10 mM)
Adjusted to pH 7.4
MACS buffer:
D-PBS
Bovine serum albumin (0.5%)
Disodium EDTA (0.2 mM)

FACS sorting buffer:
Calcium- and magnesium-free D-PBS
FBS (1%)
HEPES (25 mM)
Disodium EDTA (2 mM)
FACS buffer:
D-PBS
FBS (2%)
Sodium azide (0.05%)

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