# An Investigation of The Effect of Hypoxic Culture on Mesenchymal Stromal Cell Immunomodulation and Biodistribution *in-vivo*

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B.Sc.

A thesis submitted to

The National University of Ireland, Maynooth for the degree of Doctor of Philosophy


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June 2016

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# ABSTRACT

The overarching aim of this work was to investigate the effects of hypoxic culture on mesenchymal stromal cell (MSC) immunomodulation and biodistribution *in-vitro* and *in-vivo*. Thus far, MSC have proved therapeutically beneficial for a number of inflammatory diseases such as acute Graft versus Host Disease (aGvHD). However, despite extensive *in-vitro* characterisations of MSC mechansims of immunomodulation, the exact modes of action *in-vivo* are not well understood. Importantly, large numbers of MSC are required in pre-clinical and clinical studies to further explore their utility in medicine. Despite the availability of MSC from almost all adult tissues, their *ex-vivo* life span is not finite and thus limits their *in-vitro* culture yield. Interestingly, physiological hypoxia can be employed in the laboratory to increase MSC numbers while mirroring a natural micro-environmental niche encountered *in-vivo* and therefore more biologically relevant. However, the effect hypoxia has on MSC immunomodulation has not been fully delineated. Therefore, the key goals of this thesis were to:

(1) Determine what effect, if any, hypoxia exerts on MSC immunomodulatory abilities *in-vitro* and in a humanised mouse model of aGvHD.
(2) Examine the short term homing capacity of hypoxic and normoxic culture expanded MSC in aGvHD.

This study demonstrated that hypoxic culture increases MSC numbers in comparison to normoxic culture. *In-vitro* analysis of the effects of hypoxic MSC on peripheral blood mononuclear cells (PBMC) revealed less potent suppressor capacity than their normoxic counterparts. However, when harnessed in a humanised mouse model of aGvHD, it

was revealed that hypoxic MSC prolonged the survival of aGvHD mice in line with normoxic MSC therapeutic efficacy. Of note, both hypoxic and normoxic MSC displayed similar biodistribution profiles, capable of migrating to aGvHD target organs as assessed by novel 3D Cryo-imaging.

These findings contribute to a wider understanding of the effect of hypoxic culture on MSC immune regulation both *in-vitro* and *in-vivo.* In conclusion, this research provides a clinically and physiologically relevant method of culture expanding MSC for the treatment of inflammatory disease with the aim of reaching more patients in the clinic.

# ABBREVIATIONS

| | |
|---|---|
| APC | Antigen presenting cell |
| ARDS | Acute respiratory distress syndrome |
| ARF | Acute renal failure |
| ATG | Anti-Thymocyte globulin |
| AT-MSC | Adipose tissue derived MSC |
| ATP | Adenosine triphosphate |
| BLI | Bioluminescence |
| BMT | Bone marrow transplant |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CD | Cluster of differentiation |
| cDNA | Complementary Deoxyribonucleic acid |
| CFSE | Carboxyfluorescein succinimidyl ester |
| COX | Cyclooxygenase |
| CXCL9 | Chemokine (C-X-C motif) ligand 9 |
| DC | Dendritic cell |
| dH2O | Distilled Water |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EAE | Experimental autoimmune encephalomyelitis |
| EB/AO | Ethidium bromide/ Acridine orange |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FSC | Forward scatter |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GI | Gastrointestinal |
| GMP | Good manufacturing practice |
| GvHD | Graft versus host disease |
| GvL | Graft versus leukaemia |
| Gy | Gray |
| H&E | Hematoxylin and Eosin |
| HEK | Human embryonic kidney |
| HGF | Hepatocyte growth factor |
| HIF | Hypoxia-inducible factor |
| HLA | Human leukocyte antigen |
| HRP | Horseradish peroxidase |
| HSC | Hematopoietic stem cell |
| HSCT | Hematopoietic stem cell transplantation |
| i.v. | Intravenous |
| ICAM-1 | Intracellular adhesion molecule-1 |
| IDO | Indoleamine 2, 3-dioxygenase |
| IFN | Interferon |
| IL | Interleukin |
| ISCT | International Society for Cellular Therapy |

| | |
|---|---|
| Kg | Kilogram |
| LVEF | Left ventricular ejection fraction |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MEF | Murine embryonic fibroblast |
| MI | Myocardial infarction |
| miHA | Minor histocompatibility antigen |
| MLC | Mixed lymphocyte culture |
| MRI | Magnetic resonance imaging |
| MS | Multiple Sclerosis |
| MSC | Mesenchymal stromal cell |
| NO | Nitric oxide |
| NOD | Non-obese diabetic |
| NSG | NOD-Scid IL-2rγnull |
| NUI | National University of Ireland |
| OD | Optical density |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD-L1 | Programmed death-ligand 1 |
| PEG | Polyethylene glycol |
| Pen/Strep | Penicillin/ Streptomycin |
| PGF | Placental growth factor |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PGE-2 | Prostaglandin E2 |
| QDot | Quantum dot |

| | |
|---|---|
| RBC | Red blood cell lysis |
| RNA | Ribonucleic acid |
| mRNA | Messenger Ribonucleic acid |
| RPM | Revolutions per minute |
| RPMI | Roswell Park Memorial Institute |
| SCID | Severe combined immunodeficiency |
| SSC | Side scatter |
| TGF-β | Transforming growth factor-β |
| TLR | Toll like receptor |
| TNF-α | Tumour necrosis factor- alpha |
| TFG-6 | Tumour necrosis factor-α stimulated gene/protein 6 |
| Treg | Regulatory T cell |
| UC-MSC | Umbilical cord blood MSC |
| VEGF | Vascular endothelial growth factor |

# DECLARATION OF AUTHORSHIP

I certify that the work presented herein is, to the best of my knowledge, original, resulting from research performed by me, except where acknowledged otherwise. This work has not been submitted in whole, or in part, for a degree at this or any other university.

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Laura A. Cahill B.Sc. Date

# ACKNOWLEDGEMENTS

Firstly, I want to thank my supervisor Dr. Karen English. I appreciate all of your support and guidance throughout my Ph.D. Your hard-work and enthusiasm was contagious and motivated me to keep going, especially during the tough times. I am grateful for the inspirational example you have provided as a successful and outstanding woman scientist. I am proud to say I've been your Ph.D. student.

To the girls and guys of the Cellular Immunology Lab both past and present, I want to thank you for your help and wish you all the best for your futures. I know they will be bright!

To my friends Jane, Claire and Grace, your encouragement and constant support helped me through the toughest of times and I doubt I would be here today without you all. I couldn't ask for better friends, probably because no one else would have put up with all my moaning! Your love and advice gave me the strength to get up and keep going, and for that I will forever be grateful.

To Christopher, I bet you're glad I'm done now?! I'm sure I wasn't the easiest of people to live with these past few months but we made it. I am thankful for your love and friendship and for making me laugh when I needed it.

To my family, Dad, Mom, Kimmy and Riona, I love you all and you have always supported my decisions and been there for me when the tough got going. Dad, there are no words to express my gratitude to you, I know I wouldn't have made it to where I am today without your love, support and constant guidance.

# CHAPTER 1

# INTRODUCTION

## 1.1 MESENCHYMAL STROMAL CELLS

Friedenstein and colleagues were the first to describe a population of plastic adherent bone marrow cells which could be differentiated into osteocytes, chondrocytes and adipocytes *in-vitro* (Friedenstein 1966). These cells were termed mesenchymal stem cells (MSC) by Caplan *et al.* in 1991 based on their potential for differentiation and regenerative medicine and became a traditional designation for these stromal cells (Caplan 1991). Although first described in the bone marrow, it has recently been suggested that MSC reside within a perivascular niche (da Silva Meirelles *et al*. 2006; Crisan *et al.* 2008; Caplan & Correa 2011; Bautch 2011; Crisan *et al.* 2012; Paul *et al.* 2012; Lin & Lue 2013) and interestingly, this may explain why MSC can be isolated from most tissues in the body. For example, MSC have been isolated from the bone marrow (Friedenstein 1966; Friedenstein *et al*. 1976; Tuli *et al*. 2003), adipose tissue (Gronthos *et al.* 2001), dental pulp (Perry *et al.* 2008) and umbilical cord blood (Lee et *al.* 2004) amongst others. *In-vitro* differentiation capabilities, as well as ease of isolation and *ex-vivo* expansion, rapidly positioned these cells as key agents in regenerative medicine and has made them the subject of intense research for translational and academic investigators.

Although the initial interest in MSC focused on their potential in regenerative medicine, the discovery of their cytoprotective and tissue reparative mechanisms, facilitated through the secretion of trophic factors, has significantly widened the range of MSC therapeutics in reparative medicine and immunomodulation (Barry *et al.* 2005; Murphy *et al.* 2013). Furthermore, MSC have the capacity to produce soluble factors that support stem cell homeostasis and engraftment (Le Blanc *et al.* 2007; Méndez-Ferrer *et al*. 2010). Moreover, the realisation that administered MSC can home to

damaged tissue (Khaldoyanidi 2008; Ren *et al.* 2012; Sohni & Verfaillie 2013) and exert their reparative effects through the release of soluble factors and influencing neighbouring cells has provided substantial insight into the dynamic features of MSC (Caplan & Dennis 2006; Chen *et al.* 2008; Yew *et al*. 2011). Furthermore, the initial discovery that MSC could potently modulate immune responses prompted a number of studies investigating the mechanisms by which MSC regulate immune responses (Bartholomew *et al.* 2002; Le Blanc *et al.* 2004; Uccelli *et al.* 2008). The array of immunosuppressive mechanisms employed by MSC has now been extensively characterised, identifying both soluble and cell-contact dependant factors as key mediators of MSC immunomodulation (English *et al*. 2007; Duffy *et al*. 2011; English *et al.* 2009; Ren *et al*. 2010;). Despite the extensive understanding of MSC behaviour *in-vitro*, a full understanding of how these cells mediate their therapeutic effects *in-vivo* is required. A wealth of data demonstrates that timing of MSC administration and inflammatory conditions differentially affect MSC fate and thus, a better understanding of how MSC function in different inflammatory scenarios *in-vivo* is needed (Benvenuto *et al.* 2007; Crop *et al.* 2010; Tobin *et al.* 2013; Engela *et al.* 2014; Mancheno-Corvo *et al.* 2014).

Originally, MSC were ascribed as being immune privileged and thus promoted as an "off the shelf" cell therapy. However, this matter now commonly arises as a topic of controversy amongst academics and industrial leaders. Recent reports have suggested that MSC are immune evasive and not immune privileged and may induce immunological memory (Ankrum *et al.* 2014; Zangi *et al.* 2009). However, a number of studies supports the use of autologous (Duijvestein *et al.* 2010; Peng *et al.* 2011; Connick *et al*. 2012) and allogenic MSC as a safe and efficacious cell therapy for a

range of diseases (Le Blanc *et al.* 2004; Liang *et al*. 2009; Forbes *et al.* 2014; Wang *et al.* 2014; Premer *et al.* 2015).

### 1.1.1. THE IDENTITY OF MESENCHYMAL STROMAL CELLS

Given that MSC identity is becoming increasingly ambiguous and is complicated by the lack of specific MSC surface markers, the International Society for Cellular Therapy (ISCT) further addressed this issue by laying forth criterion for defining MSC *in-vitro* (Dominici *et al*. 2006). Thus, it has been proposed that MSC must be plastic adherent, should express CD73, CD90 and CD105 but not CD34, CD45 or human leukocyte antigen-D related (HLA-DR) and be capable of differentiating into osteocytes, adipocytes and chondrocytes. A defining characteristic of MSC is the capacity for lymphocyte modulation and is central to the assessment of their functional properties. Hence this standard criteria suggested by the ISCT is aimed at attaining a universal characterisation of MSC, thus aiding in the exchange of data amongst researchers (Dominici *et al*. 2006). However, since 2006 a number of additional markers have become accepted as identifiers of MSC *in-vitro*, albeit not when used singularly. For example, STRO-1 (Lin *et al*. 2011; Ning *et al.* 2012), Ganglioside GD2 (Martinez *et al*. 2007; Rasini *et al.* 2013), CD200 (Delorme *et al.* 2008), CD271 (Quirici *et al.* 2002; Buhring *et al.* 2007) and stage-specific embryonic antigen-4 (SSEA-4) (Gang *et al*. 2007; Rasini *et al.* 2013). Despite the advances made in furthering our understanding of MSC surface identity, routine use of these markers is not commonly implemented. For this reason, the characteristics of MSC established in this thesis are fibroblast-like morphology upon *in-vitro* culture, expression of a typical set of surface markers, capacity for osteogenic and adipogenic differentiation *in-vitro*

and the capacity to suppress lymphocyte proliferation *in-vitro*. The following thesis will refer to MSC isolated and cultured according to these criteria.

### 1.1.2 MECHANISMS IMPLICATED IN MSC IMMUNOSUPPRESSION

MSC have a broad range of physiological functions. However, the uncovering of their immunosuppressive capabilities on T cell proliferation in 2002 prompted investigators to explore their immunomodulatory attributes (Di Nicola 2002; Bartholomew *et al.* 2002). Since then, a wealth of data has identified how MSC modulate the innate (Spaggiari *et al.* 2009; English *et al.* 2008; Noone *et al.* 2013; Huang *et al.* 2014; Cahill *et al.* 2015) and adaptive immune systems (English *et al.* 2007; Rasmusson *et al.* 2007; Ren *et al*. 2008; Corcione *et al.* 2006; Akiyama *et al.* 2012). The employment of immunosuppressive mechanisms by MSC require that MSC must first be "licensed" or activated by pro-inflammatory cytokines such as IFN-γ and TNF-α (Krampera *et al.* 2006; English *et al.* 2007; Ryan *et al*. 2007; Polchert *et al.* 2008; Sheng *et al.* 2008). Moreover, modulation of the immune system by MSC is reliant on the release of a myriad of soluble factors and cell contact dependant mechanisms. The complete understanding of how MSC regulate immune responses is important for their successful application as a cell based therapy.

#### 1.1.2.1 SOLUBLE FACTORS EMPLOYED IN MESENCHYMAL STROMAL CELL IMMUNOMODULATION

The last decade has seen major advances in identifying mechanisms of MSC immunomodulation. Most of these mechanisms involve the secretion of soluble immunosuppressive factors. Human MSC have been shown to inhibit *in-vitro* T cell

activation and function through soluble factors such as indoleamine 2,3-dioxygenase (IDO) and Prostaglandin E-2 (PGE-2) (Aggarwal & Pittenger 2009). The production of IDO in the modulation of immune responses by MSC has been extensively characterised (Krampera *et al*. 2006; English *et al.* 2007; DelaRosa *et al*. 2009; Ge 2010; Tipnis *et al.* 2010; Tatara *et al*. 2011; François *et al.* 2012). Tryptophan, an essential amino acid required for T cell proliferation is degraded into kynurenine metabolites by the rate limiting enzyme IDO and thus MSC production of IDO regulates T cell proliferation through depletion of local tryptophan (Mellor & Munn 1999). Expression of IDO by MSC is induced by stimulation with IFN-γ (Krampera *et al.* 2006). Furthermore in human MSC, IDO production may also be induced through stimulation with toll-like receptor 3 (TLR) or TLR4 ligands therefore highlighting the effects of differential micro-environmental cues on MSC suppressive functions (Opitz *et al.* 2009). However, a more recent study by Waterman *et al.,* demonstrated that TLR4 was not involved in IDO priming but instead identified TLR3 as an inducer of IDO production by MSC (Waterman *et al.* 2010). Moreover, the use of blocking studies or IDO knockout MSC have elegantly demonstrated the importance of IDO for MSC immunomodulation (Krampera *et al.* 2006; English *et al.* 2007; Ryan *et al*. 2007; Ren *et al.* 2009; Ge 2010; Tipnis *et al.* 2010; Li *et al.* 2012; François *et al*. 2012; Ciccocioppo *et al.* 2015; Donders *et al.* 2015; Spaggiari *et al.* 2015).

In addition to IDO, MSC also utilise the lipid mediator PGE-2 to modulate the immune response. PGE-2 is an eicosanoid derived from fatty acids with immune regulatory roles and its synthesis is regulated by cyclooxygenase (COX) enzymes 1 and 2, COX-1 and COX-2 respectively (Harris *et al.* 2002). PGE-2 possesses multifactorial roles in the immune response and exerts its effects through binding to one or more of its receptors PGE-2 receptor 1 ($EP_1$), PGE-2 receptor 2 ($EP_2$), PGE-2 receptor 3 ($EP_3$), or

PGE-2 receptor 4 ($EP_4$) (Harris *et al.* 2002). Interestingly, MSC constitutively produce PGE-2 and an extensive array of studies have identified the role of MSC derived PGE-2 in modulating the immune system (English *et al*. 2007; Ryan *et al.* 2007; Aggarwal & Pittenger 2009; English *et al*. 2009; Spaggiari *et al.* 2009; Németh *et al.* 2009; Najar, *et al.* 2010; Yañez *et al.* 2010). Studies have elegantly demonstrated that suppression of PGE-2 synthesis (through inhibition of COX-2) increased the percentage of PBMC proliferation in a co-culture with MSC (Sotiropoulou *et al*. 2006; Chen *et al.* 2007; Aggarwal & Pittenger 2009) highlighting the substantial role of PGE-2 in MSC immunomodulation.

It is widely believed that the cascade of events employed by MSC in immune regulation involves the secretion of chemokines. Chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 9 (CXCL9) and have been implicated in MSC immunoregulation (Ren *et al.* 2008). Ultimately, this chemoattraction provides a localised platform for MSC to directly modulate T lymphocytes through the release of both soluble factors and cell contact dependant mechanisms. The immunomodulation employed by MSC has been extensively characterised and is further mediated through the secretion of a number of additional soluble factors namely, tumour necrosis factor-α stimulated gene/protein 6 (TSG-6) (Lee *et al*. 2009; Choi *et al.* 2011; Sala *et al.* 2015), IL-10 (Rasmusson *et al.* 2005; Yang *et al.* 2009; Razmkhah *et al.* 2011), transforming growth factor-β (TGF-β) (Zhao *et al.* 2008; English *et al*. 2009; X.-J. Liu *et al.* 2009; Kong *et al*. 2009; Patel *et al.* 2010; Razmkhah *et al.* 2011; Tasso *et al.* 2012), hepatocyte growth factor (HGF) (Di Nicola 2002; Kang *et al.* 2008; P. Chen *et al.* 2014), human leukocyte antigen G (HLA-G5) (Selmani *et al*. 2008; Ding *et al.* 2015) and IL-6 (Nauta *et al.* 2006; Guangqu *et al.* 2007; English *et al.* 2008; X.-J. Liu *et al.* 2009; Najar *et al.* 2009).

#### 1.1.2.2 CELL-CONTACT DEPENDENT MECHANISMS EMPLOYED IN MESENCHYMAL STROMAL CELL IMMUNOMODULATION

MSC also utilise a number of cell-contact dependent mechanisms of immunomodulation in addition to the release of soluble trophic factors and there is considerable overlap between these two mechanisms. In an elegant study by Akiyama *et. al.*, the infusion of murine MSC in mice induced transient T cell apoptosis and the authors identified the expression of FASL as the key contact dependent mechanism employed by MSC to reduce activated T cells. Moreover, the administration of $FASL^{-/-}$ MSC did not induce T cell apoptosis in recipients and failed to ameliorate systemic sclerosis and experimental colitis mice (Akiyama *et al.* 2012). Importantly, the role of the FAS/FASL cell contact dependent mechanism of immunosuppression has been confirmed in human MSC (Mazar *et al*. 2009; Gu *et al.* 2013). The programmed cell death pathway (PD) has also been implicated in MSC cell-contact dependent modulation of T cell responses. Interestingly, Chinnadurai *et. al.,* demonstrated that increased expression of programmed death-ligand 1 (PD-L1) (also called B7H1) by IFN-γ stimulated human MSC inhibited T cell effector functions through ligands of PD-L1 independent of the MSC soluble factor IDO (Chinnadurai *et al.* 2014). In line with this, Augello *et al.,* (2005) previously demonstrated that murine MSC inhibited the activation of lymphocytes through direct contact of programmed cell death receptor 1 (PD1) with its ligands (PD-L1) (Augello *et al*. 2005). Interestingly, knockdown of this pathway in murine MSC abolished their immunosuppression (Sheng *et al.* 2008). Moreover, the addition of a PD-L1 blocking antibody abolished human umbilical cord-derived MSC (UC-MSC) immunosuppression in a mixed lymphocyte reaction (MLR) (Tipnis *et al.* 2010). In contrast to this however, English *et al.,* demonstrated a redundant role for PD-L1 in murine MSC immunosuppression through the addition of neutralising antibodies to a mixed lymphocyte reaction (MLR) (English *et al.*2007). B

lymphocyte suppression by MSC was also accomplished through the interaction between PD1 and its ligands (Schena *et al*. 2010).

In addition, the upregulation of adhesion molecules on MSC by pro-inflammatory cytokines are critical for murine MSC immunosuppression. An elegant study by Ren *et al.,* (2010) reported that intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are required for the contact dependent interaction of murine MSC with T cells (Ren *et al.* 2010). Moreover, blockade of the adhesion molecules significantly reversed MSC immunosuppression both *in-vitro* and in a model of delayed-type hypersensitivity response (DTH) (Ren *et al.* 2010). However, in human MSC the blockade of these adhesion molecules did not promote the inhibition of T cell proliferation (Najar *et al.* 2010). The Notch/Jagged pathway has also been implicated in MSC mediated modulation of the immune system. For example, a Jagged-2 dependent mechanism employed by MSC induced mature dendritic cells (DC) into a regulatory phenotype (Zhang *et al*. 2009). Moreover, MSC expression of Jagged-1 was blocked by neutralising antibodies and subsequently hindered the suppressive capacities of MSC on T cells (Liotta *et al.* 2008). Furthermore, MSC were also shown to assist the immunosuppressive capacity of Cyclosporine A (CsA), an immunosuppressive drug, on T cells through a Jagged-1 inferred inhibition of NF-κB signalling (Shi *et al.* 2011). More recently, Cahill *et al.,* (2015) revealed that MSC expand Treg populations and that Jagged-1 expression by MSC is responsible for the expansion of Treg *in-vitro* (Cahill *et al.* 2015).

The physiological function of MSC also relies on the collaboration of soluble factors and cell-contact dependent mechanisms. Data suggests that the synergy of both pathways is important for the initial induction of MSC mediated immunosuppression

(Ren *et al.* 2010; Akiyama *et al.* 2012). English *et al.*, 2009 demonstrated the requirement for PGE-2 and TGF-β in the promotion of Treg also depended on cell-contact (English *et al.* 2009). The inhibition of TH17 differentiation was mediated in part by MSC derived PGE-2 following cell-contact dependent COX-2 induction (Ghannam *et al.* 2010; Duffy *et al.* 2011). Moreover, pro-inflammatory stimulation of MSC enhanced ICAM-1 expression, facilitating adhesion to TH17 cells *in-vitro* (Ghannam *et al.* 2010). Furthermore, stimulation of UC-MSC with IFN-γ upregulated PD-L1 and induced IDO; a combination of anti-PD-L1 and anti-IDO inhibitors in a UC-MSC: T cell co-culture showed maximum proliferation of T cells therefore highlighting the involvement of both soluble and cell-contact dependent mechanisms in MSC immunosuppressive activity (Tipnis *et al*. 2010). In line with this, the role of a cell-contact dependent mechanism augmenting MSC immunosuppression on T cells derived from Crohns disease patients was explored. The authors described that although MSC were capable of suppressing T cells in a transwell system, their capacity was weaker. Furthermore, the inhibition of IDO resulted in significantly increased T cell numbers which was confirmed in IDO knockdown MSC (Ciccocioppo *et al.* 2015). Notably, cell contact is required for the complete secretion of soluble HLA-G5 by human MSC. Neutralising antibodies against HLA-G revealed that HLA-G5 first suppressed T cell proliferation and then expanded Tregs. Secretion of HLA-G5 was not observed when MSC and lymphocytes were separated by a semipermeable membrane (Selmani *et al.* 2008).

### 1.1.3 THERAPEUTIC EXPLOITATION OF MSC FOR CLINICAL TRIALS

As previously mentioned, the initial clinical interest in MSC relied on their stem cell-like capacity to regenerate and repair. The identification of a broad range of trophic factors produced by MSC greatly increased the range of applications for which these cells can be applied. In 1995, Lazarus *et al.*, reported the *ex-vivo* expansion and subsequent administration of bone marrow derived human MSC were safe for clinical therapy (Lazarus *et al*. 1995). Leading on from this seminal study, Bartholomew *et al.*, described how bone marrow derived baboon MSC had the capacity to prolong skin graft survival *in-vivo* (Bartholomew *et al.* 2002). Furthermore, ground-breaking work by Le Blanc *et al.*, added significantly to the field of MSC therapeutics for graft versus host disease (GvHD). A 9 year old patient developed severe acute GvHD following administration of a matched, unrelated donor HSC transplant. The infusion of 2 separate doses of haplo-identical MSC was effective in treating GvHD (Le Blanc *et al.* 2004). Since then, the beneficial effects exerted by MSC have been demonstrated in multiple animal models (Zappia *et al.* 2008; Lee *et al.* 2009; Tobin *et al*. 2013).

Early MSC studies have now been propelled from the academic setting to an industrial setting. Mesoblast Ltd., an Australian-based company that acquired Osiris Therapeutics Inc., assessed the efficacy of their MSC cellular therapy product, Prochymal® in several phase II and III clinical trials. Intravenous (i.v) administration of Prochymal® has been investigated in Phase III trials for aGvHD (ClinicalTrials.gov identifier: NCT00562497 and NCT00366145). However, despite demonstrating significant improvements in liver and gastrointestinal aGvHD, the primary end point was not met. Moreover, Prochymal® was being investigated for Crohn's disease (ClinicalTrials.gov identifier: NCT00482092). Prochymal® therapy for the repair of cardiac tissue in patients post myocardial infarction (MI) in a phase I trial demonstrated

a safe profile and demonstrated significant improvement in cardiac function, and Osiris Therapeutics received clearance to progress to a phase II for further evaluation (ClinicalTrials.gov identifier: NCT00877903). In addition, Prochymal® therapy was explored for the protection of pancreatic islets in Type 1 diabetic patients (ClinicalTrials.gov identifier: NCT00690066) albeit, 1-year follow up interim results did not meet primary endpoint despite being well tolerated by patients. Furthermore, Athersys, (a clinical-stage biotechnology company), and Pfizer joined forces and harnessed human bone marrow derived MSC-like cell therapy product (MultiStem®) in a phase II trial for ulcerative colitis. Although a significant improvement with therapy was not demonstrated at midpoint outcomes, the safety profile demonstrated these cells were well tolerated (ClinicalTrials.gov identifier NCT01240915). Furthermore the trial progressed to a second round of dosing at later time points. More recently, Athersys announced that in collaboration with Cell Therapy Catapult, they would conduct a phase I/II clinical trial to evaluate the administration of Multistem® to patients with Acute Respiratory Distress Syndrome (ARDS), an immunological and inflammatory condition (ClinicalTrials.gov identifier NCT02611609). Although the field is rapidly moving it is not without its drawbacks.

The academic model of MSC as a cellular therapy focuses on smaller pilot studies traditionally cultured in planar, 2D plastic flasks. In contrast, industry favours the commercialisation of a bioprocessed, mass produced cell therapy and there is concern pertaining to the population doublings MSC must undergo to meet dosing requirements (Fossett & Khan 2012). Clinical data from Katharina Le Blanc's group (von Bahr *et al.* 2012) followed 31 patients treated with MSC for aGvHD or hemorrhagic cystitis over 5 years. Importantly, in aGvHD patients, the one year survival rate was 75% in patients who received early passaged MSC in contrast to 21%

using later passaged MSC *(*von Bahr *et al.* 2012). The excessive culture of MSC over prolonged periods in standard culture conditions (normoxia; 21% $O_2$) are challenged by the induction of *in-vitro* cell culture-induced senescence and adversely affects their genomic stability, and morphological and functional characteristics (Wagner *et al.* 2008; Katsara *et al.* 2011; Pan *et al.* 2014; Minieri *et al.* 2015**).** Notably, replicative senescence of MSC is linked to a loss of therapeutic potential *in-vitro* and *in-vivo* (Wagner *et al.* 2009; Galipeau 2013; Sepúlveda *et al.* 2014). In fact, these findings suggest that a better method of culturing MSC is required. Importantly, MSC are derived from tissues ranging in physiological oxygen levels but typically much lower than that of 21% $O_2$ (Chow *et al.* 2001; Harrison *et al.* 2002; Bizzarri *et al.* 2006). Hypoxic culture may provide a solution to the challenges facing the traditional culture of MSC; MSC cultured in low oxygen tensions (hypoxia) have been demonstrated to resist this senescence and change in morphology while importantly generating enhanced cell numbers (Fehrer *et al.* 2007; Grayson *et al.* 2007; Jin *et al.* 2010).

## 1.2 HAEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic haematopoietic stem cell transplantation (HSCT) is currently an effective cell therapy for patients suffering with haematological malignancies and inherited blood disorders (Reddy & Ferrara 2003; Baron & Storb 2006). It typically involves the administration of $CD34^+$ stem cells to re-establish a functional haematopoietic compartment in patients whose immune system is compromised as a result of malignant disorders. In a HSCT setting, patients must first undergo a myelosuppressive pre-conditioning regimen to immunosuppress the recipient, thereby enhancing the rate of graft acceptance (Blazar *et al.* 2012). Subsequently, donor $CD34^+$

stem cells are administered to the host to reconstitute a functional immune system and mediate a beneficial Graft versus leukaemia effect (GvL) whereby donor T cells may recognise any residual host leukemic or tumour cells (Horowitz *et al*. 1990; Bleakley & Riddell 2004). However, this treatment may also produce undesirable side effects through which donor T cells cause pathology known as Graft versus Host Disease (GvHD) as a result of MHC mismatches between the host and donor (Lee *et al.* 2007).

The major histocompatibility complex (MHC), known as human leukocyte antigen (HLA) system in humans are highly polymorphic molecules that display endogenous and exogenous antigenic peptides to self-restricted T cells and are essential in the distinction of self from non-self (Doherty & Zinkrnagel 1975). The MHC haplotype is almost exclusive to each individual. Therefore, transplanted donor allogenic cells that express MHC molecules different to that of the host may recognise these MHC molecules as foreign and thus become activated against them (Snell 1948) in order to clear them from the body. The frequency of graft versus host disease (GvHD) in humans is directly related to the degree of mismatch between HLA determinants (Loiseau *et al.* 2007; Lee *et al.* 2007). However, recipients who receive HLA identical grafts may still develop GvHD due to discrepancies between minor histocompatibility antigens (MiHAs) (Glolmy *et al.* 1983; Mutis *et al.* 1999; Dzierzak-Mietla *et al.* 2012). Furthermore, a range of factors have been identified as important predictors of GvHD, including age and dose of HSC (Weisdorf *et al.* 1991; Couriel *et al.* 2004).

## 1.3 PATHOPHYSIOLOGY OF GRAFT VERSUS HOST DISEASE

GvHD is a major complication of HSCT that is fatal in 15% of transplant recipients (Pasquini *et al.* 2010) and develops as a result of an immunological attack

against recipient target tissues. Initially, three elements were identified as necessary for the development of GvHD, conditions now termed Billingham's triad; the recipient must be immunodeficient and thus unable to reject the graft, the donor graft must contain immunocompetent cells and there must be incompatibilities' in transplant antigens (HLA) between the host and donor graft (Billingham 1966). New insights into the immunobiology of GvHD have led to a review of Billingham's original theory and added a fourth requirement for the development of GvHD. Thus, this theory has been expanded to include the prerequisite for effector cells to migrate to target tissues given that it is a relatively organ specific condition (Sackstein 2006). However, despite our understanding of GvHD, it still remains a major cause of treatment failure and a study of HSCT transplant patients from 2003-2007 showed that 70% of patients developed some level of GvHD *(*McSweeney *et al.* 2001).

GvHD develops when immunocompetent donor T cells recognise genetically divergent recipients that are incapable of rejecting donor cells after allogenic HSCT (Welniak *et al.* 2007) and is characterised by the initiation of a cytokine storm, in particular TNF-α, that progresses in 3 phases. Priming of the immune response occurs in phase I. The myelosuppressive preconditioning regimen induces tissue and organ damage. As a result, these tissues respond by releasing a storm of proinflammatory cytokines and chemokines. Moreover, damage to the gastrointestinal (GI) tract causes lipopolysaccharide (LPS) leakage into the periphery, further exacerbating the immune response. Clinical studies have proposed that reduced intensity preconditioning is associated with a reduction in the development of early-onset acute GvHD (aGvHD) and reduced morbidity (Mielcarek *et al.* 2003).

Phase II of GvHD represents the core of this disease. Here, the danger signals generated from phase I activate host antigen presenting cells (APCs), cells which present antigen T cells and are pivotal in inducing adaptive peripheral tolerance. Activated APCs prime donor T cells present in the graft further exacerbating donor cell immune activation through expansion, differentiation and further expression of proinflammatory cytokines (Reddy & Ferrara 2003). The prevailing view is that $CD4^+$ T cells are predominantly activated by non-haematopoietic APCs in the GI tract and produce pro-inflammatory cytokines that mediate robust $CD8^+$ T cell expansion which are further activated upon encounter with host hematopoietic APCs (Markey *et al.* 2014). Studies have also demonstrated the role of co-stimulatory molecules in GvHD. These studies revealed that blockade of CD80/86 protects from GvHD development. However, further treatment was required for complete GvHD prevention (Blazar *et al.* 1996; Saito *et al.* 1996; Koura *et al*. 2013).

The subsequent transition to the effector phase III occurs after the migration of these activated T cells to host GvHD target organs. In mice, donor T cells migrated to lymphoid tissues where activation occurs and subsequently traffic to GvHD target organs (Panoskaltsis-mortari *et al.* 2004). The trafficking of alloreactive donor T cells is possible through the complex combination of a large number of chemotactic signals such as CCL2, CXCL9, CXCL10 and CXCL11 which are overexpressed during GvHD (Serody *et al.* 2000; New *et al.* 2002; Wysocki *et al.* 2005). Phase III is the final stage in the development of GvHD which results in the destruction of host tissue following migration of alloreactive donor T cells to target GvHD organs. This phase is the result of biphasic events involving effector cells and cytokines inducing apoptosis. Cytotoxic activity and recruitment of other immune cells has been implicated in this phase. Chemokines and adhesion molecules facilitate T cell migration to target tissues, pro-

inflammatory cytokines produced by monocytes, macrophages and T cells drive GvHD pathology by inducing apoptosis of target tissue (Antin & Ferrara 1992; Hill & Ferrara 2000). Therefore, strategies to reduce GvHD lethality have been aimed at targeting these effector pathways.

### 1.3.1 CLINICAL FEATURES OF GVHD

GvHD typically presents as acute (aGvHD) or chronic (cGvHD). Classically, aGvHD is defined as occurring within 100 days post transplantation while cGvHD occurs after 100 days (Martin *et al.* 1990; Sullivan *et al.* 1991). However, this definition is substandard given that clinical manifestations of GvHD have been reported to dually display symptoms of acute and chronic GvHD (Filipovich *et al*. 2005; Griffith *et al.* 2008; Brodoefel *et al.* 2010). Thus, a clear definition and differentiation of these diseases is not well resolved. The target organs of aGvHD are predominantly the skin, liver, GI tract and lungs (Serody *et al.* 2000; Schmaltz *et al.* 2003; Tobin *et al.* 2013). Clinically it is characterised by the directed apoptosis of these target organs. The first and most common manifestation appears as lesions in the skin (81%) while aGvHD of the liver and GI tract affect 50% and 54% of patients respectively (Martin *et al.* 1990) (Table 1.1). As a result of epithelial cell necrosis, a painful maculopapular rash develops, displaying a characteristic dispersion initially affecting the palms of the hands and soles of the feet and then progresses to the neck, face and upper trunk (Vogelsang *et al.* 2003). In severe cases, the rash can lead to skin blistering and ulceration and cause intense pain. Damage to the GI tract presents with severe abdominal pain and diarrhoea (Martin *et al.* 1990). Furthermore, as aGvHD of the GI tract progresses, patients may experience nausea, vomiting and bloody diarrhoea which can lead to excessive weight loss (Nevo *et al.* 1999; Ponec *et al.* 1999). Damage to the liver caused by aGvHD is difficult to distinguish from other causes of liver damage following HSCT (e.g. viral

infection, sepsis) (Ferrara *et al.* 2009). However, damage to liver tissue is typically identified as jaundiced patients presenting with hyperbilirubinemia in conjunction with histological patterns of endothelialitis, pericholangitis, bile duct destruction and lymphocytic infiltration (Snover *et al.* 1984; Fujii *et al.* 2001; Ferrara *et al*. 2009).

The progression of aGvHD is characterised within each organ system involved and the extent of damage to each organ by using Glucksbergs criteria for aGvHD staging (Glucksberg *et al*. 1974) (Table 1.2). The diagnosis of GvHD is primarily based on clinical criteria in conjunction with histopathological changes. The assessments described previously are combined to identify an overall clinical stage, progressing from grade I (mild), grade II (moderate), grade III (severe) to grade IV (very severe). The more advanced the staging, the poorer the patient prognosis (Ferrara *et al.* 2009). The estimated 5 year survival rate for patients with grade III aGvHD is 25% which plummets to 5% when patients progress to grade IV aGvHD (Cahn *et al.* 2005). Therefore therapeutic targets for aGvHD are aimed at each grade of the disease pathology in an attempt to increase these survival rates.

### 1.3.2 THERAPEUTIC PREVENTION OF AGVHD

Despite the recent advances in our understanding of the immunobiology of aGvHD, effective treatments for its prevention are lacking. Currently, two basic strategies are employed; pharmacological therapy and partial depletion or elimination of lymphocyte subsets from the graft prior to administration. Steroids in combination with immunosuppressive drugs are used as an initial therapy for aGvHD. The administration of cyclophosphamide post-transplant eliminates rapidly dividing T cells by inhibiting DNA replication and shows promise in recent clinical trials (Luznik & Fuchs 2010;

Ding & Zhou 2012). The calcineurin inhibitors cyclosporine and tacrolimus inhibit calcineurin phosphatase and T cell activation (Halloran 2007) and when combined with methotrexate, have successfully been used for more than 40 years as a prophylactic regimen for GvHD (Storb et al. 2010). Steroid therapy has improved the outcome and enhanced the survival of patients with aGvHD (Van Lint *et al*. 1998; MacMillan, Weisdorf, Wagner, *et al.* 2002). Functionally, pharmacological therapy inhibits the release of inflammatory cytokines (e.g. IFN-γ, TNF-α) and therefore dampens the activation of immune cells (Brattsand & Linden 1996; Maslekar & Anwar 2008) while promoting lymphocyte lysis during interphase (Deeg 2007). A number of types and doses of steroids are used to treat patients with aGvHD and patient responsiveness is usually dependant on the grade and severity of the disease (MacMillan, Weisdorf, Wagner, *et al.* 2002; Lint *et al*. 2013). However, a typical course of treatment for patients diagnosed with aGvHD is the administration of methylprednisolone (2mg/kg) for 7-14 days followed by a gradual reduction in dose depending on patient responsiveness (Messina *et al.* 2008). However, the adverse complications of steroid treatment are well known and include the risk of infection, hyperglycaemia and growth defects (Ruutu *et al*. 1998; Deeg 2007; Reddy *et al*. 2012). Approximately 50% of patients with aGvHD who are treated with steroids in the initial management of the disease will achieve a partial or complete response (Martin *et al.* 1990). There are however patients that do not respond to steroid treatment and thus develop steroid resistant aGvHD resulting in a poor prognosis (Kobbe *et al.* 2001; Deeg 2007). In these patients, a second line of treatment is required.

**Table 1.1 Clinical manifestation and symptoms of aGvHD**

| Target organ | Clinical manifestation | Staging |
|---|---|---|
| Skin | Erythematous, maculopapular rash | 1. <25% rash<br>2. 26%-50% rash<br>3. 51%-75% rash<br>4. >75% rash |
| Liver | Painless jaundice with hyperbilirubinemia | 1. Bili 2-3 mg/dL<br>2. Bili 3.1-6 mg/dL<br>3. Bili 6.1-15 mg/dL<br>4. Bili >15mg/dL |
| G.I tract | Nausea, vomiting, diarrhoea and abdominal pain | 1. Diarrhoea >500 mL/day<br>2. Diarrhoea >1000 mL/day<br>3. Diarrhoea >1500 mL/day<br>4. Diarrhoea >2000 mL/day |

*** Adapted from** (Martin *et al*. 1990; Vogelsang *et al.* 2003)

**Table 1.2 Glucksbergs criteria for aGvHD staging**

| Overall aGvHD grade | Skin | Liver | | Gut |
|---|---|---|---|---|
| I | 1-2 | 0 | | 0 |
| II | 1-3 | 1 | and/or | 1 |
| III | 2-3 | 2-4 | and/or | 2-3 |
| IV | 2-4 | 2-4 | and/or | 2-4 |

***Adapted from** (Vogelsang *et al*. 2003)

#### 1.3.2.1 SECOND LINE TREATMENT OF AGVHD

The progression of aGvHD determines when to begin a second line of treatment therefore the severity and duration of aGvHD manifestations need to be considered. If the manifestations of any aGvHD organ worsen over 3 days of treatment or, if the skin doesn't improve by 5 days it is improbable that a response will be achieved. Therefore, a second line of treatment is considered when organ pathology worsens over 3 days of treatment, if there has been no improvement in condition over 7 days or if there is incomplete response to treatment over 14 days (Deeg 2007). Thus far, there have been a number of second line strategies to treat aGvHD. Monoclonal or polyclonal antibodies have been studied extensively as a secondary line of treatment for GvHD (Doney *et al*. 1985; Carpenter *et al.* 2002).

Anti-thymocyte globulin (ATG) is a potent T cell depleting antibody that mediates its effects on T cells through complement dependent lysis or activation-associated apoptosis (Genestier *et al.* 1998; Michallet *et al.* 2003). ATG has been commonly employed in GvHD prophylaxis and has been successful in reducing the frequency of GvHD in related-donor HSCT patients without increasing the risk of tumour relapse (Doney *et al*. 1985; Kröger *et al*. 2002). Albeit somewhat successful, the administration of ATG for aGvHD still demonstrates adverse side effects in 80%-90% of patients such as hypotension and thrombocytopenia and the long term survival rate for patients on ATG ranges from 5-32% making its use in the clinic dubious (Graziani *et al.* 2002; MacMillan, Weisdorf, Davies, *et al*. 2002). Moreover, treatment with Visilizumab, a humanised anti-CD3 antibody, selectively induces the apoptosis of activated T cells and improves aGvHD (Cole *et al.* 1999; Carpenter *et al.* 2002). However, reactivation of latent Epstein Barr Virus (EBV) in patients with aGvHD led to

post transplant lymphoproliferative disease as a result of Visilizumab therapy (Carpenter *et al*. 2002).

Elevated levels of TNF-α are indicative of more severe aGvHD development, and thus an alternative strategy for treatment is it's blockade (Holler *et al.* 1990; Kitko *et al.* 2008). To date, clinical trials have used two drugs: etanercept, which binds trimeric and membrane bound TNF, or infliximab, a monoclonal antibody that binds the soluble subunit and the membrane bound precursors of TNF-α, blocking the interaction with the receptors and resulting in the lysis of cells that produce TNF-α (Couriel I. 2004; Ehlers 2005). A phase II clinical trial found that etanercept in combination with systemic steroid therapy supported complete resolution of aGvHD symptoms in 70% of patients, with 80% complete responses in GI tract and skin aGvHD (Levine *et al.* 2008). However, the side effects of these drugs have proved problematic in the treatment of some patients (MacMillan,*et al*. 2002; Graziani *et al*. 2002; Carpenter *et al.* 2002; Levine *et al*. 2008). Incomplete efficacy and adverse effects associated with the use of monoclonal and polyclonal antibodies, suggests the need for a more effective therapy for aGvHD (MacMillan,*et al*. 2002; Graziani *et al*. 2002; Carpenter *et al.* 2002; Levine *et al*. 2008).

REGiMMUNE, a biopharmaceutical company, are currently conducting a phase I/II study of their pipeline product RGI-2001, a formulated glycolipid CD1d ligand. Developers suggest that RGI-2001 induces Tregs, maintains normal immune cell function and prolongs the survival of mice with lethal aGvHD (Duramad *et al.* 2011) (ClinicalTrials.gov identifier: NCT01379209). In 2013, Enlivex Therapeutics Ltd. were granted approval for the development of their Allocetra product, ApoCell (ClinicalTrials.gov identifier NCT00524784). Functionally, a blood sample is retrieved from the patient or matched donor, it is then treated to generate an early apoptotic cell

population (mononuclear early apoptotic cells) undergoing apoptosis and then administered to the patient based on the premise that the presence of apoptotic cells favours the induction of tolerance (Griffith *et al.* 2007). A single infusion of ApoCell was administered as a prophylactic treatment in conjunction with immunosuppressant's cyclosporine and methotrexate to 26 patients after HLA-matched myeloablative allogenic HSCT. Engraftment was successful in all patients and the overall survival of patients at day 180 post-transplant was 85%. The overall occurrence of grade II-IV aGvHD was 23%, however in the 2 patients that received a higher dose of ApoCell this incidence was 0% (Mevorach *et al*. 2014). Alternatively, studies are concentrating on cellular therapies as a treatment for inflammatory mediated disease, such as aGvHD.

### 1.3.3 CELL THERAPY FOR AGVHD

#### 1.3.3.1 T REGULATORY CELLS

As previously mentioned, GvHD is caused by the presence of donor T cells in the allograft and targeted destruction of host tissues; theoretically, the deletion of donor T lymphocytes prior to transplantation could solve this problem. However, a beneficial role for donor T cells in recipient haematopoietic reconstitution and the clearance of remaining leukaemic cells (GvL), have highlighted the importance of donor T cells for successful transplantation (Horowitz, 1990). $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory cells (Treg) can induce tolerance by suppressing autoreactive lymphocytes and dually control innate and adaptive immunity (Takahashi *et al*. 2000; Janssens *et al.* 2003; Piccirillo & Shevach 2004; Fehérvari & Sakaguchi 2004). In preclinical models, the adoptive transfer of natural Treg was highly effective at suppressing aGvHD (Cohen *et al*. 2002; Hoffmann *et al*. 2002; Taylor *et al.* 2002). Surprisingly, GVL responses were

maintained. Here, Treg suppressed early expansion of alloreactive murine donor T cells and expression of their IL-2 receptor alpha chain (Edinger *et al.* 2003). Despite the difficulties associated with the isolation and *ex-vivo* expansion of large numbers of Treg, a number of clinical trials have been undertaken to explore the therapeutic value of Treg therapy in GvHD. In a seminal, small scale study, Trzonkowski *et al.,* were the first to demonstrate the ability of Treg to reduce pathology in 2 human GvHD patients (Trzonkowski *et al*. 2009). Building on this, a larger phase I/II clinical trial involving 23 patients harnessed human Treg isolated from umbilical cord blood and expanded *ex-vivo* before transplantation. This therapy reduced the incidence of aGvHD compared with historical controls; however, overall GvHD occurrence was not significantly reduced (Brunstein *et al.* 2011). A third phase I/II clinical trial explored the effect of Treg cell therapy on GvHD development following HLA haploidentical HSCT (Di Ianni *et al*. 2011). This study was the first to demonstrate that adoptive transfer of Treg cells prevented GvHD in the absence of any post-transplantation immunosuppression. However, the survival rate of GvHD patients remained at 50% (Di Ianni *et al.* 2011).

#### 1.3.3.2 MESENCHYMAL STROMAL CELLS

Allogenic MSC therapies have been applied in clinical trials for the prevention or treatment of a number of conditions. The clinical efficacy of MSC for GvHD was first observed by Le Blanc *et al.,* following transplantation of MSC as an allogenic cell therapy for patients with steroid resistant grade IV GvHD (Le Blanc *et al.* 2004). MSC were administered over 2 doses to a 9 year old patient who had been diagnosed with severe steroid-resistant acute GvHD of the gut and liver. The patient showed a complete response following MSC therapy and this study became a cornerstone for

future clinical trials. In a later pilot study by Ringden *et. al.*, 8 patients with steroid resistant GvHD were treated with MSC therapy. Complete aGvHD remission was observed in 6 out of the 8 patients and their overall survival rate was significantly higher than those not treated with MSC (Ringden *et al.* 2006).

A multicentre phase II study comprised of 25 paediatric and 30 adult patients with steroid GvHD demonstrated the efficacy of allogeneic MSC therapy and lessened concerns surrounding HLA disparities between the donor and recipient. Patients were treated with HLA identical and HLA haploidentical sibling donor bone marrow, or third party mismatched bone marrow. 68% of patients showed complete responses and had a significantly reduced level of transplantation related mortality further highlighting the potential of *in-vitro* expanded MSC for aGvHD (Le Blanc *et al*. 2008).

Although considerable progress has been made in the development of MSC for GvHD treatment, contradictory results harnessing MSC therapy has revealed a number of limitations. A phase III trial by Osiris Therapeutics contradicted their previous encouraging results from a phase II study. Their MSC like cell therapy, Prochymal® was proven safe for human administration and beneficial for patients with aGvHD in a phase II study (Kebriaei *et al.* 2009). In a large scale phase III trial Prochymal® did increase response rates in patients with steroid refractory liver GvHD and steroid refractory gastrointestinal disease but did not reach its primary endpoint to significantly increase complete response rates in steroid refractory GvHD patients for at least 28 days (Martin *et al.* 2010). As outlined in section 1.1.3, large scale expansion of MSC by industrial leaders may weaken the physiological function of MSC by altering their secretory phenotype, genomic integrity and inducing cell-culture senescence. These data may provide a rationale for the undesirable results obtained in the phase III trial for aGvHD by Osiris Therapeutics (or undesirable results obtained from clinical trials).

Furthermore, Galipeau highlighted the fact that MSC have a vast inter-donor variability in relation to their physiological and immunoregulatory functions (Phinney *et al.* 1999; Zhukareva *et al.* 2010; Ciuculescu *et al.* 2011; François *et al.* 2012; Moll *et al.* 2012; Galipeau 2013; Ketterl *et al.* 2015; Heo *et al*. 2016). The licensing requirement of MSC by IFN-γ is well understood (section 1.1.2) and responsiveness to IFN-γ is imperative for MSC mediated immunosuppression *in-vivo* (Krampera *et al.* 2006). Accordingly, François *et al.,* demonstrated considerable differences in the extent of IDO responsiveness from IFN-γ stimulated human MSC (François *et al.* 2012). Furthermore, the authors describes the amount of IDO produced by each MSC donor influences their immunosuppressive capabilities (François *et al.* 2012). Given that that phase III Osiris trial used MSC isolated from one single donor, it has been postulated that this donor may have had a low receptivity to IFN-γ. Therefore, a more robust immune plasticity assay to evaluate MSC donor responsiveness to IFN-γ may result in the transfusion of more potent MSC (Krampera *et al.* 2013; Galipeau *et al.* 2016). Importantly, these studies emphasized a large gap in our understanding of how MSC mediate their therapeutic effects *in-vivo* and challenge researchers to critically analyse and investigate the therapeutic mechanisms employed by MSC in GvHD. The development of novel models of aGvHD is essential to this investigation and major emphasis has been placed on the need for robust models to examine the precise mechanisms of MSC immunomodulation.

## 1.4 ANIMAL MODELS OF ACUTE GvHD

Many non-human primates are used to model human disease. However, non-human primate models are often associated with vigorous ethical constraints and can be

very costly. To combat these problems, mouse models of disease offer a more feasible alternative to human observation. Mouse models of aGvHD have provided important insights into the pathophysiology of this disease and have furthered our understanding of the immune response in GvHD pathology. The advantages of a murine model of GvHD lie with the capacity to control environmental conditions, to transplant large numbers of recipients concurrently, image immune reactions and treatment responses at multiple time points and are a relatively cost effective model. The development of aGvHD in these models is in response to alloantigen, as seen in patients and thus mirrors aGvHD seen in the clinic. Most models involve the transplantation of donor lymphocytes into lethally irradiated hosts. The severity of aGvHD development depends on several factors such as the irradiation dose, the amount and type of donor lymphocytes that are transferred with the bone marrow transplant (Schroeder & DiPersio 2011). MHC mismatched mouse models are the most straightforward tools for studying aGvHD. Here, the model involves the transplantation of murine lymphocytes into a murine model (i.e. mouse in mouse). However, current advances in the development of humanised mouse models have facilitated the analysis of the underlying mechanisms of human disease pathogenesis. Similar to human aGvHD, the pathology of aGvHD in murine models typically affects target organs such as the lung, liver, skin and intestinal tract. A key hallmark of murine aGvHD pathology is weight loss as a result of intestinal track damage and reduced food intake. Accordingly, there is also a well-designed scoring system in place for determining the severity of aGvHD in all murine models.

### 1.4.1 Humanised Mouse Models

A major disadvantage to harnessing the murine system is that mouse models do not always truly reflect the pathophysiology of human diseases. The interest in the use

of humanised mouse models of aGvHD is growing and thus the design of these models is continually improving and enabling more complex studies. An understanding of the most well-known humanised models will enable researchers to make the most suitable choice for studying aGvHD.

#### 1.4.1.1 CB17-SCID HUMANISED MOUSE MODEL

A critical development for humanised mouse models of disease was the discovery of the *Prkdc*$^{scid}$ mutation (protein kinase, DNA activated catalytic polypeptide; severe combined immunodeficiency) in a CB17 mouse strain (Bosma *et al.* 1983). These mice have a loss of function mutation of the *Prkdc* gene which encodes the catalytic subunit of a DNA dependant protein kinase that resolves DNA double strand breaks that occur during V(D)J recombination. This mutation results in defective V(D)J rearrangement of lymphocyte antigen receptor genes resulting in the absence of functionally mature T and B cells (Lieber *et al.* 1988).allowing the engraftment of human PBMC. However, engraftment of human cells was exceptionally low and thus was not satisfactory for the functional study of engrafted cells (Mosier *et al*. 1988). Some mice bearing the *scid* mutation developed functional T and B lymphocytes over time, a phenomenon known as "leakiness" (Bosma *et al.* 1988). As a result, the development of functional T and B cells can lead to immune cell activity which resulted in increased numbers of NK cells, ultimately hampering the engraftment of human PBMC (Christianson *et al.* 1996; Greiner *et al*. 1998). The introduction of the *scid* mutation onto the Non obese diabetic (NOD) background, demonstrated 5 to 10 fold higher human PBMC engraftment when compared with other mouse models (Hesselton *et al.* 1995) and quickly positioned this model as the "gold standard" of the humanised mouse model.

#### 1.4.1.2 NOD-SCID HUMANISED MOUSE MODEL

NOD mice have inherited immune defects, including imperfect myeloid lineage production, reduced complement activity and decreased NK activity (Kataoka *et al.* 1983; Baxter & Cooke 1993; Serreze *et al.* 1993). Moreover, levels of engraftment of human PBMC were much higher than that found in the CB17 mouse models and also displayed reduced "leakiness" (Shultz *et al.* 1995; Hesselton *et al.* 1995) resulting in a more stable and robust model. Despite the advancements with the NOD-scid model compared to previous humanised models, some limitations remained. The development of thymic lymphomas severely decreased their lifespan (Shultz *et al.* 1995). Although the engraftment of human cells was more than in the CB17 humanised model (0.01%-0.1%), the levels still remained relatively low (Hesselton *et al.* 1995).

#### 1.4.1.3 NOD-SCID IL-2R γ $^{NULL}$ HUMANISED MOUSE MODEL

The next line of humanised mouse models addressed mutations in the cytokine receptor common γ- chain. The NOD-scid mouse model was the most permissive humanised mouse model to date. A disease known as X linked Severe Combined Immunodeficiency (SCID) occurs in humans due to mutations in the IL-2rγ chain gene (Kovanen & Leonard 2004) and patients are characterised by the lack of or marked reduction in mature T cells and NK cells (Sugamura *et al.* 1996). The introduction of this IL-2rγ mutation onto the NOD-scid background allowed the development of a humanised mouse model, NOD-SCID IL-2R γ $^{NULL}$ (NSG) deficient in T, B and NK cell activity facilitating high engraftment levels without the development of thymic lymphomas (Ishikawa *et al*. 2005; Shultz *et al.* 2005; Pearson *et al.* 2008). In addition to this, the life span of the NSG mouse was markedly longer when compared to the NOD-scid mouse making it ideal for long-term studies. In 2005, the administration of mobilised human peripheral blood $CD34^{+}$ cells to NOD-scid IL-2rγ$^{null}$ humanised mice

resulted in the development of a complete human immune system (Shultz, 2005) making it ideal for the study of functional immune diseases. This model has since been identified as one of the most suitable platforms for studying GvHD given the similarities between this model and the clinic (Ali *et al*. 2012) and has already been engrafted with peripheral blood mononuclear cells (PBMC) for GvHD studies (Hippen *et al.* 2012; Tobin *et al.* 2013). For the purpose of this thesis, the NOD-scid IL2r$\gamma^{null}$ humanised mouse model was used to investigate hypoxic cultured MSC as a therapy for the treatment of aGvHD.

## 1.5 HYPOXIC CULTURE FOR MSC

### 1.5.1 HYPOXIA

In the 17$^{th}$ century a scientist known as John Mayow first discovered the importance of air and the role it plays in the continuation of life. Upon placing lighted candles and an animal simultaneously into a glass jar and inverting it over water, he discovered that as the air was consumed the candle no longer burned and the animal did not long survive thereafter. "[A]nimals and fire draw particles of the same kind from the air...[L]et any animal be enclosed in a glass vessel along with a lamp so that the entrance of air from without is prevented...When this is done we shall soon see the lamp go out and the animal will not long survive the fatal torch." (Partington 1956).

Oxygen tensions vary greatly throughout animal physiology (Wild *et al.* 2005; Saltzman *et al*. 2003; Iii *et al.* 2005; Spencer *et al.* 2014). This variance of oxygen levels has led to a vague distinction between low oxygen and high oxygen concentrations, hypoxia and normoxia respectively. Over the course of evolution oxygen sensing mechanisms have allowed for an adaptive response to fluctuating oxygen levels and has been reviewed intensely (López-maury *et al.* 2008; Costa *et al.*

2014; Stamati *et al.* 2011). A key mediator of the response to hypoxia is the hypoxia inducible factor (HIF) (Semenza & Wang 1992; Wang *et al*. 1995) that promotes changes in gene expression that allow the cell to adapt to a low $O_2$ environment. This thesis will use the term hypoxia to describe $O_2$ concentrations lower than 21% for consistency with conventional terminology.

#### 1.5.1.1 THE HISTORY OF OXYGEN IN CELL CULTURE

Initially, cell culture was performed on cell lines that mostly originated from cancers that were adept at adapting to new, unusual conditions. However, as science advanced, new methodologies for cell culture emerged. Atmospheric oxygen is deemed "normoxic" even though the levels of oxygen cells encounter *in-vivo* is typically much lower. Given that oxygen concentrations *in-vivo* are much lower than the standard 20-21% $O_2$ cells encounter *in-vitro* (Caldwell *et al*. 2001; Saltzman *et al.* 2003; Wild *et al.* 2005), it seems logical that investigations into the effect of low oxygen levels, physiological hypoxia, on MSC culture be ensued. Furthermore, the concept that MSC are likely to be altered by culture conditions must be taken into consideration.

In the 1970s, Packer and colleagues reported the increased lifespan of human fibroblasts when cultured in 10% $O_2$ in comparison to culture in normoxia (Packer, 1977). Since then our understanding of the impact of physiological oxygen on cell culture has significantly advanced (Parrinello *et al.* 2003; Atkuri *et al.* 2007). In a comparative study, Parrinello *et al.*, demonstrated that culture of murine embryonic fibroblasts (MEFs) in 3% $O_2$ grew faster than MEFs cultured in 21% $O_2$ and interestingly showed no sign of senescence (Parrinello *et al.* 2003).

MSC have emerged as promising tools for clinical applications given that they can be readily isolated from a patient and easily expanded *ex-vivo* with maintained differentiation capacity and immunomodulation. MSCs can be found in virtually all tissues, however the oxygen tensions of such tissues vary and are typically characterised as physiologically hypoxic (Saltzman *et al*. 2003; Wild *et al.* 2005; Spencer *et al.* 2014). Recent studies have highlighted the hypoxic conditions that stem cells are exposed to *in-vivo* (Parmar *et al.* 2007; Kubota *et al*. 2008). Thus bone marrow-derived MSC (BM-MSC) are naturally exposed to a hypoxic micro-environment. Furthermore, a study on the analysis of oxygen concentrations in murine adipose tissue have also identified a hypoxic nature to the tissue in which AT-MSC are naturally derived from (Matsumoto *et al.* 2005). Collectively, the above studies have demonstrated the hypoxic milieu that stem cells naturally occur in and highlight the importance of harnessing hypoxia as a method of cell culture.

#### 1.5.1.2 HYPOXIA AND CELL PROLIFERATION KINETICS

The impact of hypoxic culture on MSC proliferation remains controversial. While some studies describe a positive effect of hypoxia on MSC proliferation (Lennon *et al*. 2001; Grayson *et al.* 2007; Nekanti *et al*. 2010; Tsai *et al*. 2011; Hung *et al.* 2012; Valorani *et al*. 2012), others have found that hypoxia inhibits MSC proliferation (Chung *et al*. 2012; Holzwarth *et al*. 2010; Beegle *et al*. 2015). These results vary depending on a number of factors such as the source of MSC, seeding density, the oxygen concentration and the duration of exposure to hypoxia. Regardless of these discrepancies, considerable data supports a positive impact of hypoxic culture on MSC proliferation and suggests that expansion in hypoxic conditions may generate enhanced cell numbers for clinical utility.

Tsai *et al.*, reported an increase in MSC expansion efficiency by hypoxic cultivation whilst maintaining a typical cell surface phenotype. Cell-cycle phase distribution studies revealed a reduction of hypoxic cells in the $G_0/G_1$ phase with a concomitant increase of cells in the $S/G_2m$ phase (Tsai *et al.* 2011). Furthermore, microarray analysis of rat bone-marrow derived MSC cultured in 1% $O_2$ revealed an up-regulation of genes involved in cell proliferation in response to hypoxia such as vascular endothelial growth factor-D (VEGF-D) and placental growth factor (PGF) (Ohnishi *et al.* 2007). In line with this, another group reported the presence of significantly higher amounts of growth factor, basic fibroblast growth factor (b-FGF) and IL-6 in hypoxic conditioned human MSC medium (Chen *et al*. 2014) Overall, a number of molecular and paracrine factors may be responsible for the enhanced proliferation of hypoxic cultivated MSC.

#### 1.5.1.3 HYPOXIA AND CELLULAR DIFFERENTIATION

As previously mentioned, MSC can differentiate into multiple cell type's *in-vitro* thus making them attractive possible agents of regenerative medicine, yet the effect of hypoxia on cellular differentiation kinetics *in-vivo* is poorly understood. While much of the research to date provides evidence that hypoxia maintains MSC in an undifferentiated state, others have recently investigated the idea that hypoxia promotes differentiation (Tsai *et al*. 2011; Hung *et al*. 2012; Valorani *et al.* 2012; Wagegg *et al.* 2012; Binder *et al.* 2014; Ding *et al.* 2014; Prado-Lòpez *et al*. 2014).

Although data on the effects of hypoxia on MSC chondrogenic differentiation are lacking some studies have reported the positive effect of hypoxia and conditioned medium on different sources of MSC. Adipose tissue-derived MSC (AT-MSC)

subjected to hypoxia for 14 days also exhibited an increase in total collagen synthesis (Wang 2005). A range of studies have examined the effect of hypoxia on MSC osteogenic capacity. While numerous studies show the positive effect of hypoxia on this capacity *(*Lennon *et al.* 2001; Valorani *et al.* 2012; Hung *et al.* 2012) a small number of studies report a reduced capacity of MSC osteogenesis (Fehrer *et al.* 2007; Holzwarth *et al.* 2010). In contrast to the role of hypoxia in promoting osteogenesis by MSC hypoxia appears to inhibit adipocyte differentiation. Peroxisome proliferator-activated receptor gamma (PPARγ) is vital for adipocyte differentiation such that its over-expression in non-adipogenic fibroblasts is sufficient to convert them into adipocytes (Spiegelman *et al.* 1997). Culture of 3T3-L1 pre-adipocytes in cobalt chloride ($CoCl_2$), a hypoxia mimetic, significantly reduced PPARγ mRNA expression (Kang *et al.* 2005). Furthermore, studies by Wagegg *et al.,* expanded on these findings in MSC. Human MSC cultured in 2% $O_2$ for two weeks demonstrated enhanced osteogenesis. Interestingly, the culture of MSC in hypoxia suppressed adipogenesis and associated PPARγ gene expression. Moreover, knockdown of HIF1-α, a key mediator of the response to hypoxia, enhanced the adipogenic capacity of MSC cultured in hypoxia and normoxia (Wagegg *et al*. 2012).

#### 1.5.1.4 THE EFFECT OF HYPOXIC PRECONDITIONG ON MSC REGENERATIVE CAPACITY

Recent studies have highlighted the importance of preconditioning MSC in hypoxia to enhance their regenerative and wound healing capacity. In a murine model of bleomycin-induced pulmonary fibrosis, administration of murine MSC exposed to 24 hours of hypoxia resulted in significantly improved pulmonary functions over their normoxic cultured counterparts (Lan *et al.* 2015). In a study comparing the effect of

hypoxia on human MSC wound healing capacity, results revealed significantly higher levels of bFGF and VEGF-A in MSC cultured under hypoxia. Furthermore, the topical administration of conditioned medium from hypoxic MSC to Balb/c nude mouse skin wounds revealed a significantly accelerated wound closure in comparison to those treated with medium from normoxic MSC (Chen et al. 2014). The capacity of murine MSC, exposed to short term hypoxia, to engraft into ischemic tissue and participate in tissue regeneration was analysed in a mouse model of ischemic hind limb. Data revealed enhanced skeletal muscle regeneration in the hypoxic MSC group and increased vascular formation (Leroux *et al.* 2010). Additional studies confirm the benefit of preconditioning MSC with hypoxia for an enhanced regenerative cellular therapy (Chang *et al.* 2013; Jaussaud *et al.* 2013; Yu *et al*. 2013). However, it is important to note that all of the above data used oxygen levels of between 0.5% and 2% $O_2$ for between 24 hours and 7 days and thus can be classified as a preconditioning method for MSC before administration for regenerative purposes. Data on the effects of continual, long term hypoxic culture, as opposed to preconditioning, of MSC are lacking.

#### 1.5.1.5 THE IMPACT OF HYPOXIA ON MSC IMMUNE MEDIATORS

The primary focus of using MSC in regenerative medicine shifted toward elucidating MSC modes of immunosuppression. Their regulation of immune responses by immunosuppressive factors makes them ideal candidates for the treatment of a number of inflammatory disorders harnessing mediators such as IDO and PGE-2. However, the impact of hypoxia on MSC expression of such mediators has yet to be completely elucidated. Few *in-vitro* studies have demonstrated that short term hypoxia does not impair MSC immunosuppressive ability (Gornostaeva *et al*. 2013; Roemeling-

van Rhijn *et al*. 2013; Nold *et al.* 2014; Prado-Lòpez *et al.* 2014; Bobyleva *et al.* 2016) but data on the effect of long term hypoxia on BM-MSC immunosuppressive attributes are lacking. Exposure of AD-MSC to short term 1% $O_2$ did not affect their capacity to upregulate IDO or PD-L1. Furthermore the ability of AD-MSC to suppress $CD4^+$ and $CD8^+$ T lymphocyte proliferation following 3 day co-culture was not hampered by short term hypoxia (Roemeling-van Rhijn *et al.* 2013). The influence of hypoxia on murine MSC was described using a number of different murine MSC lines (OP9.F12, MS5.C2, PA6.G6, ST2.B11 and B16-14.D2) cultured in 5% $O_2$. The authors reported no inhibitory effect of hypoxia on the suppressive capacity of murine MSC (Prado-Lòpez *et al*. 2014). Thus far, studies suggest no negative impact of short term hypoxia on the immunosuppressive capacity of human MSC. However, the effect of continuous physiological hypoxic culture, that of which MSC would chronically encounter *in-vivo* remains to be elucidated.

## 1.6 IMAGING OF MSC THERAPY *IN-VIVO*

MSC are effective therapeutic agents for a number of inflammatory disorders however the mechanisms underlying their actions *in-vivo* are largely unknown. Lack of a single imaging modality that satisfies all requirements of imaging confounds complete elucidation of MSC fate *in-vivo.* In order to extrapolate MSC therapy to the clinic, their biodistribution, engraftment, and proliferation and survival kinetics in preclinical animal models must first be examined. However, these characteristics are not yet understood and thus, reliable imaging techniques to track the outcome of MSC therapy *in-vivo* are required to benefit future clinical trials (Kraitchman & Bulte 2008; McColgan *et al*. 2011). For example, we know that MSC can migrate into damaged

target organs to mediate their therapeutic effects. Furthermore, the local milieu in which they find themselves in regulates how they do this (Barry & Murphy 2004). Confirming whether administered cells have homed to damaged organs and to what extent with *in-vivo* imaging may aid in the development of novel cell strategies and support investigations into the outcomes of clinical trials that employed cellular therapies. Frangioni and Hajjar identified 8 characteristics of an ideal imaging technology for stem cell tracking during clinical trials (Table 1.3). However, most of these characteristics are not yet fulfilled by any single currently applied imaging modality. Therefore in order to obtain the most ideal imaging setting, a combination of imaging techniques are often employed in multimodality techniques ultimately combining the best feature of each individual imaging technology (Josephson *et al.* 2002; Doubrovin *et al.* 2004; Frangioni & Hajjar 2004; Higuchi *et al.* 2009).

### 1.6.1. FLUORESCENT IMAGING

Fluorescent proteins are arguably one of the most popular techniques for molecular imaging of live cells. Fluorescence refers to the property of particular molecules to absorb light at a certain wavelength and subsequently emit detectable light of a longer wavelength (Stepanenko *et al*. 2008). A number of tools exist that permit fluorescent imaging but each has a number of advantages and disadvantages and thus it is important to tailor the choice of imaging modality to the experimental design. Potential challenges to fluorescent imaging include its limited depth of penetration, administration of cells genetically altered to express a fluorescent protein and is limited by natural autofluorescence (Puaux *et al.* 2011). As a whole however, fluorescent imaging is a versatile imaging modality that is relatively inexpensive and does not require the administration of exogenous substrate for visualisation. Interestingly,

preliminary attempts are being made to translate optical fluorescent imaging to the clinic. An elegant study by Hsiung *et. al.,* employed a novel fluorescent confocal micro-endoscope to image topically administered fluorescein labelled heptapeptide against fresh human colonic adenomas (Hsiung *et al*. 2008). Initial results revealed that these heptapeptides bound more strongly to regions containing dysplastic colonocytes compared with adjacent normal tissue. Thus, exciting and important developments in fluorescent imaging will hopefully translate successfully into the clinic and permit thorough analyses of disease by linking *in-vivo* and *in-vitro* assays.

#### 1.6.1.2 CRYOVIZ$^{TM}$ TECHNOLOGY AS A STATE-OF-THE-ART IMAGING MODALITY

CryoViz$^{TM}$ (BioInvision Inc.) is a dynamic and unique imaging modality that is a fully automated, whole mouse or organ section and image system that provides 3-dimensional, tiled, microscopic anatomical and molecular fluorescence images over large volumes (Roy *et al.* 2009). Furthermore, its single cell sensitivity places it at the forefront of preclinical imaging, permitting the imaging and quantification of cellular biodistribution and engraftment. There are 4 major components to the cryo-imaging system: the mouse–sized cryo-microtome, microscope imaging system, the robotic xyz positioner and the computer control system. Molecular fluorescence is aided significantly by anatomical context as well as visualising it in 3D. It offers 3D resolution, high contrast anatomical imaging, fluorescent imaging that permits detection of and quantification of single cell sensitivity. Potential hurdles to Cryo-imaging revolve around its cost. It is a relatively expensive system with maintenance expenses and commercially available QDots can be costly. Moreover, animals must be sacrificed in order to track cell biodistribution which increases animal numbers and thus overall

cost of the experiment. However, the novelty that this system holds far outweighs its disadvantage.

### 1.6.1.3 TOOLS HARNESSED FOR FLUORESCENT IMAGING

#### 1.6.1.3.1 FLUORESCENT PROTEINS

A large body of fluorescent proteins exist that can enable the study of gene expression, protein function and cell tracking (Tsien 2005). However, this extensive development in fluorescent proteins with advanced photophysical properties may make it difficult for a researcher to identify the most suitable protein for a given application. A number of factors are important to consider when choosing the right fluorescent protein. The brightness of a fluorescent protein is one of the most important factors to consider for high quality imaging. Importantly, the fluorescent protein should be bright enough to provide sufficient signal above auto fluorescence. Furthermore, the photostability of fluorescent proteins (the capacity of a fluorescent protein to maintain its integrity when exposed to light) is becoming an increasingly important parameter to consider when choosing a suitable fluorescent protein (Dean & Palmer 2014).

#### 1.6.1.4.1 FLUORESCENT DYES

Cytoplasmic and nuclear dyes and stains are widely exploited in biomedical imaging despite their rapid dilution; their ease of use and wide range of colours available make them attractive contenders for in-vivo tracking studies where cost is an issue. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a cytoplasmic fluorescent, cell permeable dye capable of covalently labelling intracellular cytosolic components that are readily detected (Quah *et al*. 2007) and given its ease of use is

commonly employed for tracking lymphocyte proliferation and tracing MSC *in-vivo* (Herrera *et al.* 2007; Quah *et al.* 2007; LI 2009)**.** However, the main challenges facing the use of fluorescent dyes such as CFSE in imaging are that they are easily photobleached and *in-vivo* fluorescence decays expeditiously due to an overlap of excitation and emission wavelengths with that of auto-fluorescent tissue components (Ushiki *et al.* 2010). Moreover, upon each cellular division of a CFSE labelled cell, the CFSE dye, and thus intensity of cellular fluorescent signal, sequentially halves and therefore represents a major disadvantage of using CFSE in long term *in-vivo* tracking studies (Dittel *et al.* 1999). Importantly, labelled cells are resistant to quenching under a fluorescent microscope however its use is limited by its short retention time within the cell and importantly hoechst 33342 (another fluorescent dye) was reported to inhibit cellular proliferation at high concentrations (Parish 1999). Furthermore, transfer of hoechst 33342 to host cells is also a disadvantage that warrants consideration. Fluorescent lipophilic dyes represent an alternative mechanism of labelling cells for *in-vitro* and *in-vivo* studies through cell membrane staining. One such dye is 1, 1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; $DiIC_{18}(3)$) (DiL) that diffuses across the entire cell membrane. Utilising this dye, Dittel *et., al.* labelled T cells and following adoptive transfer into a murine model of EAE, were capable of detecting the labelled cells in the lymph nodes 24 hours following administration (Dittel *et al.* 1999). Bone marrow fibroblasts could be labelled with DiL in as little as 30 minutes. The dye was not cytotoxic and did not affect *in-vitro* cell proliferation. Furthermore, when DiL labelled allogenic MSC when administered into sheep, signal was detectable 6 weeks after administration (Weir *et al.* 2008). A major hurdle that impedes its use however is if a transplanted cell preparation contains non-viable labelled cells, the debris of the labelled membrane can be absorbed by host cells, furthermore

even if all cells are viable, DiL may still dissociate from the labelled cells membrane and get absorbed by host cells (Kruyt *et al.* 2003).

#### 1.6.1.5.1 FLUORESCENT PROBES

Fluorescent probes can be applied directly to allow visualisation of endogenous structures and tracking of cell migration. For example, quantum dots (QDots) are light-absorbing, light-emitting nanocrystals that possess bright and stable fluorescent light emission and are being extensively applied to biomedical imaging for translational research (Konstantatos & Sargent 2010; Kairdold *et al.* 2013; Kovalenko *et al.* 2015; Vu *et al*. 2015). These nanocrystals are comprised mainly of semiconductor material such as cadmium and selenium and range in size from between 2nm-10nm in diameter (Wang & Chen 2011). They possess superior optical properties such as an intense fluorescent yield, are highly photostable and efficiently combine a narrow emission spectrum with a broad range of absorption (Resch-Genger *et al.* 2008).

The conducting properties of a QDot is a direct result of a quantum effect associated with the size and shape of each crystal (Debbage & Jaschke 2008). Because of this, the emission wavelength of any QDot can be altered by altering its size and has been applied in fluorescent imaging (Kim *et al.* 2004; Wu et al. 2003; Wang & Chen 2011; Auletta *et al. 2014).* Importantly, QDots are commercially available and compared with traditional fluorophores, are much brighter due to their high quantum yield and are more photostable (less susceptible to photo bleaching) (Walling *et al.* 2009). QDots are estimated to be up to 20 times brighter and 100 times more stable than traditional fluorescent reporters (Walling *et al*. 2009). As with the introduction of any material into a cell, cytotoxicity may be an issue. While some studies report

cytotoxicity associated with QDot labelling (Derfus *et al.* 2004; Kirchner *et al*. 2005; Soenen *et al.* 2012), others have not (Jaiswal et al. 2003; Chen & Gerion 2004; Selvan et al. 2005). Importantly, a number of groups have successfully labelled human MSC with QDots (Rosen *et al.* 2007; Muller-Borer *et al*. 2007; Ohyabu *et al*. 2009). The authors concluded QDot labelling represents a non-invasive, non-toxic and viable method for labelling and tracking MSC. No transfer of QDots to neighbouring cells was reported thus adding to its repertoire of advantages.

**Table 1.3** The 8 characteristics of an ideal imaging technology for stem cell tracking during clinical trials.

| | **Characteristics of an ideal imaging technology for stem cell tracking during clinical trials** |
|---|---|
| 1. | Biocompatible, safe, and nontoxic |
| 2. | No genetic modification or perturbation to the stem cell |
| 3. | Single-cell detection at any anatomic location |
| 4. | Quantification of cell number |
| 5. | Minimal or no dilution with cell division |
| 6. | Minimal or no transfer of contrast agent to non-stem cells |
| 7. | Non-invasive imaging in the living subject over months to years |
| 8. | No requirement for injectable contrast agent |

### 1.6.2 BIOLUMINESCENCE IMAGING

Bioluminescence imaging (BLI) is increasingly being harnessed as a powerful modality for modern bioimaging and employs light emitting enzymes. BLI relies on the detection of light emitted by cells that express luciferase, a light generating enzyme and can detect as little as 100-1,000 transduced cells *in-vivo* (Negrin & Contag 2006). The use of BLI imaging possesses several advantages for imaging in small animals. BLI permits the imaging of live, small animals and thus reduces the number of small animals required for a study given that sacrifice at pre-determined time points is not required. Autobioluminescence typically results in very low background emission in animal tissue. Furthermore BLI offers a sensitive mechanism of tracking cell fate *in-vivo.* Assessment of MSC fate and function *in-vivo* has been addressed using BLI in models of acute kidney injury (AKI) (Tögel *et al*. 2008), myocardial ischemia and the infarcted heart (van der Bogt *et al.* 2008; van der Bogt *et al.* 2009), localisation in tumour bearing mice (Wang *et al.* 2010) and diabetic mice (Yaochite *et al.* 2015).

## 1.7 EVIDENCE OF MSC BIODISTRIBUTION IN-VIVO

Cellular therapies provide encouraging approaches for the treatment of injuries and diseases. The increasing applications of MSC in medicine created the demand for long term *in-vivo* tracking of such therapies. Still, data are lacking on the biodistributional signature of MSC *in-vivo* and the mechanisms by which MSC reach target organs. Little data is available on the biodistribution of MSC in human subjects. However mouse models have evolved greatly in the last decade and thus represent a reliable means of evaluating the efficacy of cell therapies (Steindler 2007). Despite the advancements of MSC biodistribution in disease, a number of questions remain

unanswered. What contact do MSC have with other cells upon administration in the bloodstream and what are the results of these interactions? What happens to MSC that do not migrate to the site of insult/damage and what clearance pathways may be responsible for their removal? If surviving MSC are imaged at a site distant to the target tissues yet therapeutic effects are observed, are MSC truly responsible for the beneficial effects? Thus, further investigation into the role of MSC in animal disease models in conjunction with tracking studies will help us understand why we have not obtained answers on MSC biodistribution and therapeutic efficacy.

### 1.7.1 EVIDENCE OF MSC BIODISTRIBUTION IN DISEASE

A potential hurdle for MSC therapy is that MSC do not persist following administration and imaging techniques may aid in understanding MSC fate *in-vivo*. The persistence of human MSC, mouse MSC and rat MSC was limited with the majority of cells not persisting past 48 hours after systemic infusion (Toma *et al.* 2009; Kidd *et al*. 2009; Lee *et al.* 2009). However, an elegant BLI study by Zangi *et. al.*, has shown that syngeneic, luciferase expressing MSC are detectable for the duration of the experiment (40 days). However, in the allogeneic setting, MSC signal was reduced on day 20 with complete elimination of the signal by day 40. Furthermore, mice that were previously injected with allogeneic MSC displayed accelerated rejection of fibroblasts that were from the same donor (Zangi *et al.* 2009). However, it has yet to be shown in the clinic that improved MSC persistence or immune tolerance to MSC leads to an enhanced efficacy of MSC based cell therapy. A study by Yang *et, al.*, demonstrated that the beneficial effects mediated by MSC ensue after their clearance (Yang *et al*. 2012) thus highlighting the need for further imaging studies in conjunction to fully elucidate MSC modes of therapeutic action in animal models of disease.

A number of studies have tracked MSC therapy in pre-clinical models of myocardial infarction (MI). MI occurs when a blocked artery leads to myocardial ischemia and subsequently damages the surrounding cardiac tissue (Thygesen *et al.* 2012). Barbash and colleagues transfused $^{99M}$TC-labelled rat MSC into the left ventricular cavity of MI rats at 2, 10 or 14 days post MI and compared them to placebo MI rats or MI rats treated with MSC administered intravenously. Using gamma camera imaging followed by isolated organ counting, the authors described the overall detection of labelled cells in the lung with less than 1% of cells trafficking to the heart within 4 hours of infusion. Moreover, delivery into the left ventricular cavity resulted in significantly lower lung uptake and better uptake in the heart. Importantly, histological examination 1 week post MSC infusion revealed labelled cells in the infarcted zone or border zone but not in the remote myocardium or in sham MI hearts (Barbash *et al.* 2003). More recently, a study by Kraitchman *et al.*, (2005) used high sensitivity of a combined single-photon emission CT (SPECT)/CT to track allogenic MSC in a canine model of MI. Initial uptake of radiolabelled MSC was seen in the lung with modest uptake in the liver and kidney. Analysis of early redistribution revealed migration to non-target reticuloendothelial organs 24-48 hours after injection. A focal and diffuse uptake of MSC was seen in the myocardium at 24 hours and at later time points of day 4-7 (Kraitchman *et al*. 2005). Furthermore, in a study by Wang *et al.,* (2012) BLI was employed to evaluate the long term survival, efficacy and persistence of MSC therapy for MI. The authors elegantly described the delivery of human MSC into the peri-infarct region of SCID mouse hearts resulted in long term survival, improvement in left ventricular ejection fraction (LVEF), a decrease in fibrosis and an increase in vessel density. Interestingly, human MSC were labelled with an endothelial specific reporter and BLI signal from these labelled MSC revealed human MSC differentiated into

endothelial cells 48 hours post injection. Both constitutive and endothelial specific signals dissipated by day 50. However, the improvement in LVEF persisted for up to 6 months. Injected hMSC did not migrate to peripheral organs in numbers significant enough to be detected. Furthermore, immunofluorescence microscopy verified the study by revealing that a small subset of MSC differentiated into endothelial cells, identified as $GFP^+$ $CD31^+$ cells, and integrated into the blood vessel walls (Wang *et al.* 2012). Collectively, the above studies effectively exploit imaging techniques to describe the dynamic role of MSC for MI.

Acute kidney injury (AKI) is a renal disorder characterised by the rapid loss of the kidney's excretory function which manifests in the clinic as an increase in the by-products of nitrogen metabolism and/or a decrease in urine output (Bellomo *et al.* 2012). Using magnetic resonance imaging (MRI) Lange *et. al.*, administered labelled MSC to rats with ischemic acute renal failure (ARF) via thoracic aortic infusion and demonstrated MSC localised to the kidney cortex. MSC treatment significantly increased renal functions at days 2 and 3 and mice had a better injury score at day 3 post induction of ARF. Histologically, MSC were located in the glomerular capillaries (Lange *et al*. 2005). In line with this, intracarotid administration of MSC to rats with ARF improved renal function and reduced renal injury scores. Fluorescently labelled MSC were detected in the glomeruli and peritubular capillaries within 10 minutes of administration (Tögel *et al.* 2005). More recently, BLI data from the same group demonstrated a disperse distribution of MSC in normal mice following injection in the suprarenal aorta. However, a distinct localised pattern was observed in the kidneys of mice with ischemia-reperfusion induced AKI 24 hours after MSC infusion (Tögel *et al.* 2008). Notwithstanding, MSC involvement in AKI has not been fully demonstrated

and extensive *in-vivo* imaging studies in models of AKI are essential if we are to exploit MSC as a therapy for renal disorders.

Importantly, evaluation of the complex biological process of GvHD has utilised BLI to accelerate animal studies of GvHD. In an elegant study by Beilhack *et al.*, (2005) the authors uncovered the early events of aGvHD with BLI. Syngeneic or allogeneic recipient mice were administered T cell depleted bone marrow cells 3 hours post irradiation. Luciferase positive splenocytes were subsequently transplanted to induce GvHD. Following luciferin administration, sequential imaging displayed striking differences between syngeneic and allogenic recipient animals. The syngeneic animals revealed bone marrow engraftment, most likely from residual stem cells in the splenocyte preparations. In distinct contrast, allogeneic recipients showed early infiltration of cervical lymph nodes and structures in the gut. At 2-4 days post-transplant, proliferation of donor cells was observed in these lymph nodes and gut sites. Furthermore, by day 6, infiltration of the skin was readily evident (Beilhack *et al.* 2005). In addition, Iclozan *et al.*, (2010) utilised BLI to analyse luciferase positive TH1 and TH17 cells expansion *in-vivo* in a mouse model of GvHD. Both T cell subsets migrated to GvHD target organs. However the signal intensity of TH17 cells was significantly higher than that of TH1 cells. Furthermore, the authors demonstrated that RORγt -/- (transcription factor required for TH17 differentiation) T cells induced GvHD and that TH17 subsets alone are not necessary to induce GvHD (Iclozan *et al.* 2010). BLI has therefore provided new insights into the complex biological processes of not only GvHD but numerous disease models. Future objectives include enhancement of techniques that allow simultaneous visualisation of more than one population of cells, and importantly, quantification of cell numbers rather than signal intensities.

### 1.7.2 EVIDENCE OF MSC BIODISTRIBUTION IN GRAFT VERSUS HOST DISEASE

As previously mentioned, GvHD is a potentially life threatening complication of HSCT and extensive research employing MSC as a cellular therapy for GvHD is under investigation. Although MSC have been proven as a safe option for GvHD and great advances have been made, many patients still die thus underscoring the need for further investigation into this lack of efficacy and variability of response. The elucidation of MSC modes of actions in combination with biodistribution studies is essential for the successful implementation of MSC as a cellular therapy for GvHD.

Although imaging techniques have been applied in the field of GvHD (Panoskaltsis-mortari *et al*. 2004; Negrin & Contag 2006; Stelljes *et al.* 2009), biodistribution studies of MSC and their correlation with treatment efficacy in GvHD are lacking. In a comparative study, Christensen *et al.,* (2010) showed that MSC delayed GvHD but did not prevent its development in a major histocompability complex mismatched model of the disease. However, in the sibling transplant mimic model, 30% of the MSC treated mice did not develop GvHD. Evidence of MSC in-vivo was analysed by GFP expression in the hind leg bones, large intestine, and spleen, inguinal and mesenteric lymph nodes by RT-PCR. The expression of $GFP^+$ genomic DNA was low with very few MSC detected in the organs 1 hour after infusion and after 24 hours only 0.2% of MSC were accounted for (Christensen *et al*. 2010). Moreover, an elegant study by Auletta *et al.,* (2014) combined BLI and cryo-imaging to evaluate a distribution pattern for hMSC in a mouse model of GvHD. The authors demonstrated that MSC labelled for BLI migrated initially to the lungs but gradually re-distributed to intra-abdominal organs in alloBMT recipient mice and relieved symptoms of GvHD. In syngeneic mice, transduced MSC migrated to the lungs only. Cryo-imaging

complemented this study by demonstrating that MSC co-localise with murine alloreactive T cells in the spleen during the onset of GvHD (Auletta *et al.* 2014). However, humanised mouse models of GvHD will provide a superior system whereby human haematopoietic cells can engraft thus holding advantage over murine models as they create a platform for studying human T cell mediated GvHD in the lab. In a study by Joo *et al.*,(2011) live animal imaging was employed in conjunction with confocal microscopy in a mouse model of GvHD. BALB/c-nude mice were irradiated and received bone marrow cells from non-irradiated C57BL/6 donor mice. Splenocytes from C57BL/6 mice expressing eGFP were injected to induce GvHD. MSC from C57BL/6 mice expressing RFP were employed to study their biodistribution in this model and were injected 24 hours after recipient mice were irradiated. Analysis revealed that the MSC reached the lungs first, followed by the GI tract, lymph nodes and skin, in that order. At 48 hours post intravenous administration, the eGFP signal (associated with donor splenocytes) was located in the lungs. At the same time point, the RFP signal (associated with the MSC) was also detected in the lungs. At 7 days post infusion, the eGFP signal decreased in the lungs and increased in the GI tract which was mirrored by the RFP signal. 22-37 days following infusion, the signals co-localised to the liver, skin, lymph nodes and the authors suggested the MSC progressively home to the sites of ongoing GvHD to exert direct cell-cell mediated and/or localised paracrine therapeutic effects (Joo *et al.* 2011). More recently, a refined study developed imaging techniques to compare the distribution patterns of hMSC in the context of GvHD when administered either intravenously or intra-arterially (i.a). An intestinal GvHD model was developed by administering bone marrow and lymphocytes from C57BL/6 or B6D2F1 mice into B6D2F1 recipients. Human MSC transduced with a reporter gene for BLI or labelled with [$^{99m}$Tc]-HMPAO for scintigraphy were then

administered either through the tail vein or carotid artery. Interestingly, in allo-BMT recipients i.a administration of MSC resulted in whole body distribution that lasted 2 weeks whereas in syngeneic mice no signal was detected after a week. Both transplant groups revealed an initial entrapment of MSC within the lungs regardless of the route of MSC administration. However, i.v transplanted MSC migrated out of the lungs and to GvHD target organs i.e. intestines in smaller fractions (Wang *et al.* 2015). Collectively, the above studies have attempted to unravel MSC modes of action in GvHD by harnessing tracking and imaging modalities in attempts to generate a biodistribution profile and correlate this to their therapeutic efficacy. While these data are crucial for acting as a stepping stone for future studies, many questions remain. Can biodistribution studies help determine the optimal dose or number of infusions of MSC for GvHD? What route of MSC administration will provide the most efficacious outcome for GvHD? Do MSC have to persist at the site of insult to alleviate symptoms of GvHD, if so for how long? Does long term hypoxic culture of MSC attenuate, maintain or augment their homing capacity in GvHD?

## 1.8 AIMS AND OBJECTIVES

This introduction has highlighted the obstacles associated with normoxic culture of MSC, underscored the role of exploiting hypoxia for MSC culture and explored the biodistribution of MSC *in-vivo*. The hypothesis is that MSC can be used to treat inflammatory disorders following long term *in-vitro* culture expansion in physiological oxygen tensions and will be explored in this thesis. The aim of this work to investigate three distinct areas in MSC therapy which remain to be addressed:

1. The direct effect of long term hypoxic culture on MSC immunosuppressive biology *in-vitro* and *in-vivo.*
2. The efficacy of long term hypoxic cultured MSC in a humanised mouse model of acute GvHD
3. The influence of long term hypoxic culture on the biodistribution of MSC *in-vivo* in comparison to normoxic cultured MSC

Despite the advances made in our understanding of how hypoxia modulates MSC biology, the impact of continual, long term hypoxic culture on MSC remain unclear. The elucidation of this impact will be beneficial for academia and industry with the goal of producing a more cost effective method of expanding MSC and thus reaching more patients in the clinic. The goal of Chapter 3 is to investigate the effect of hypoxia on MSC immunosuppressive mediators' *in-vitro*. Chapter 4 of this thesis will establish the efficacy of hypoxic cultured MSC in a humanised mouse model of aGvHD compared to conventional normoxic MSC. Following verification of the efficacy of hypoxic MSC *in-vivo*, these cells will subsequently be employed in Chapter 5 to examine the biodistribution of MSC in aGvHD following long term hypoxic culture in comparison to normoxic MSC.

Overall, this study is designed to evaluate the effect of hypoxia on the therapeutic efficacy and biodistribution of MSC in aGvHD. This knowledge will contribute to a broader understanding of immune regulation by MSC cultured in physiological oxygen and will benefit the development of future clinical trials utilising hypoxic MSC therapy in aGvHD.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 ETHICAL APPROVAL AND ANIMAL LICENSING

All procedures involving animals or human material were performed by licenced personnel. Ethical approval for all work was granted by Maynooth University research ethics committee. Approval for procedures involving animals was granted by the Department of Health and/or the Health Products Regulatory Agency, formerly the Irish Medicines Board.

## 2.2 ISOLATION AND CULTURE OF CELLS

### 2.2.1 HUMAN MESENCHYMAL STROMAL CELL ISOLATION

Bone marrow aspirates were obtained from consenting healthy donors. Briefly, sample volume was recorded and aspirates were diluted with equal volumes of phosphate buffered saline (PBS) and centrifuged for 10 minutes at 900g. Supernatants were removed and pellets combined. The volume was then adjusted to the initial volume of the aspirate with PBS. Cells were counted using a haemocytometer and primary MSCs were plated at $40x10^6$ per T-175 flask in cDMEM. Following 3 days incubation at either $37^oC$ in normoxia (21% $O_2$ and 5% $CO_2$) or hypoxia (5% $O_2$ and 5% $CO_2$), 15mls cαMEM (Table 2.1) was added. On day 5, the media in the flasks was swirled to dislodge the red blood cells and non-adherent cells were subsequently removed. Fresh cαMEM was added and flasks were returned to their respective incubator. Media changes were carried out every 3-4 days. Following 12-16 days of culture, MSC cultures were passaged.

### 2.2.2 HUMAN MESENCHYMAL STROMAL CELLS SUBCULTURING

MSC were passaged by aspirating media from tissue culture flasks and trypsinising with 0.25% trypsin-1mM EDTA (Invitrogen-Gibco, Paisley, UK) T-175 flasks received 8 mls of trypsin for 5-6 minutes at 37$^{o}$C. After examination using the inverted light microscope to ensure cells had detached, the trypsin was neutralised by adding equal volumes cDMEM (Table 2.1) to each flask. The cell suspension was centrifuged at 300g for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 1ml cDMEM. Cell counts were then performed using ethidium bromide/aquadine orange (EB/AO) (Sigma-Aldrich, Arklow, Ireland), a method to count viable cells and re-seeded at 1x10$^{6}$ per T175 flask in 25ml of cDMEM for up to 5 days until they reached 70-90% confluency. Cells were re-seeded and placed into normoxic or hypoxic culture.

### 2.2.3 HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Whole blood buffy coat packs, which contained red blood cells, white blood cells and platelets, were supplied by the Irish blood transfusion service. Contents of the buffy pack were diluted with 50mls of sterile PBS (1:1) and 15mls of the diluted blood was slowly layered onto 25mls Lymphoprep (StemCell Technologies, Vancouver, Canada). Samples were centrifuged at 2400 rpm for 25 minutes at room temperature with no brake and low acceleration. After centrifugation, the white blood cell layer containing PBMC was transferred into a fresh sterile 50ml falcon tubes with a 3 ml transfer pipette. Each tube was filled to 50ml with sterile PBS. Samples were then centrifuged at 1800rpm for 10 minutes at 4$^{o}$C. The supernatant was carefully discarded and the pellet resuspended in 30ml of sterile PBS and centrifuged at 1500rpm for 5

minutes at 4$^{o}$C. This wash step was repeated. The supernatant was discarded and pellets resuspended in 5mls of 1 X red blood cell (RBC) lysis buffer (BioLegend, San Diego, USA) and left to incubate for 5 minutes at room temperature after which samples were neutralised with 20-25mls cRPMI (Table 2.1). Samples were centrifuged at 1000 rpm for 10 minutes at 4$^{o}$C. Supernatant was discarded and isolated PBMC were resuspended in 20-25 ml of cRPMI and counted using EB/AO.

### 2.2.4 CRYO-PRESERVATION AND RECOVERY OF HUMAN CELLS FROM LIQUID NITROGEN

Cells that were not required for further culture were cryopreserved in liquid nitrogen for long term storage. This was performed by resuspending 1x10$^{6}$ MSC or 5x10$^{7}$ peripheral blood mononuclear cells (PBMC) in MSC or PBMC freezing media respectively (Table 2.1). Cryovials (Thermo Fisher Scientific, Massachusetts, and USA) were placed at -80$^{o}$C overnight in a Mr. Frosty freezing container (Thermo Scientific) and then stored in liquid nitrogen for future use. MSC were recovered quickly by thawing in a heated water bath at 37$^{o}$C. PBMC were thawed from liquid Nitrogen for 30 minutes at -80$^{o}$C followed by a brief thawing in a heated water bath at 37$^{o}$C. Before completely thawed, MSC cells were transferred to 10ml of warmed cDMEM and centrifuged at 300g for 5 minutes. PBMC were added to DNase I (0.4mg/ml) to minimise DNA fragmentation and cell clumping. 10ml of warmed cRPMI was gently added to the cells and centrifuged at 1500 rpm for 5 minutes at room temperature. Supernatant was discarded and cells were resuspended in 1 ml cDMEM or cRPMI for MSC and PBMC respectively (Table 2.1) for counting.

Table 2.1

| Media | Components | Supplier | Cell type |
|---|---|---|---|
| cαMEM | Minimum Essential Medium | Sigma-Aldrich | MSC |
| | FBS (10% v/v) | BioSera | |
| | Pen/Strep(1% v/v) | Sigma-Aldrich | |
| | | | |
| cDMEM | Dulbecco's modified Eagle's Media (DMEM) containing 1000mg/ml glucose | Sigma-Aldrich | MSC |
| | FBS (10% v/v) | BioSera | |
| | Pen/Strep(1% v/v) | Sigma-Aldrich | |
| | | | |
| cRPMI | RPMI 1640 | Sigma-Aldrich | PBMC |
| | Heat inactivated FBS (10% v/v) | BioSera | |
| | Pen/Strep(1% v/v) | Sigma-Aldrich | |
| | L-Glutamine(1% v/v) | Sigma-Aldrich | |
| | β-mercapthoethanol (0.1% v/v) | Invitrogen-Gibco | |
| | | | |
| CHEK DMEM | Dulbecco's modified Eagle's Media (DMEM) containing 4500mg/ml glucose | Sigma-Aldrich | HEK |
| | Heat inactivated FBS (10% v/v) | BioSera | |
| | Pen/Strep(1% v/v) | Sigma-Aldrich | |
| | L-Glutamine(1% v/v) | Sigma-Aldrich | |
| | | | |
| MSC Freezing Media | cDMEM(70% v/v) | Sigma-Aldrich | MSC |
| | FBS (20% v/v) | BioSera | |
| | DMSO (10% v/v) | Sigma-Aldrich | |
| | | | |
| PBMC Freezing Media | FBS (90% v/v) | BioSera | PBMC |
| | DMSO (10% v/v) | Sigma-Aldrich | |

## 2.3 CHARACTERISATION OF MSC DIFFERENTIATION

### 2.3.1 OSTEOGENIC DIFFERENTIATION

MSC were seeded at a density of 5 x $10^4$ cells/well in a 6 well tissue culture plate (Sarstedt, Numbrecht, Germany) in 3 ml of cDMEM and incubated at 37°C. Once MSC had reached 70% confluence, cDMEM was removed and MSC were incubated in either cDMEM (negative control) or osteogenic differentiation media (Table 2.2). Media was changed every 3-4 days for 21 days. On day 21, media was carefully removed and MSC washed 3 times with sterile PBS. Cells were then fixed in 10% (v/v) neutral buffered formalin for 20 minutes at room temperature. Formalin was removed and cells were gently washed twice in PBS. 1 ml of 1% Alizarin Red (Table 2.3) was added to each well and cells were stained for 20 minutes at room temperature. Excess stain was discarded and cells were gently washed twice with $dH_2O$. 1 ml of $dH_2O$ was added to each well and cells were examined under light microscope for the presence of positively stained mineralising cells.

### 2.3.2 ADIPOGENIC DIFFERENTIATION

MSC were seeded at a density of 5 x $10^4$ cells/well in a 6 well tissue culture plate (Sarstedt, Wexford, Ireland) in 3 ml of cDMEM and incubated at 37°C. Once MSC had reached 70% confluence, cDMEM was removed and MSC were incubated in either cDMEM (negative control) or adipogenic differentiation media (Table 2.2). Media was changed every 3-4 days for 21 days. On day 21, media was carefully removed and MSC washed 3 times with sterile PBS. Cells were then fixed in 10% (v/v) neutral buffered formalin for 20 minutes at room temperature. Formalin was removed and cells were gently washed twice in PBS. 1 ml of filtered 0.5% Oil Red O (Table 2.3)

was added to each well and cells were incubated for 20 minutes at room temperature. Excess stain was removed and cells were gently washed twice with PBS. 1 ml of PBS was added to each well and cells were examined under light microscope for positively stained fat globules which indicate the presence of adipogenic differentiation.

Table 2.2. Cell differentiation media

| Media | Components | Supplier |
|---|---|---|
| Osteogenic Differentiation | DMEM;1000mg/ml glucose | Sigma-Aldrich |
| | 1 mM dexamethasone | Sigma-Aldrich |
| | 20 mM β-glycerolphosphate | Sigma-Aldrich |
| | 50 μM L-ascorbic acid-2-phosphate | Sigma-Aldrich |
| | 50 ng/ml L-thyroxine sodium pentahydrate | Sigma-Aldrich |
| | | |
| Adipogenic Differentiation | DMEM;4500mg/ml glucose | Sigma-Aldrich |
| | 5 μg/ml insulin (dissolved in 0.1N acetic acid) | Sigma-Aldrich |
| | 50 μM indomethacin | Sigma-Aldrich |
| | 1 μM dexamethasone | Sigma-Aldrich |
| | 0.5 μM 3-Isobutyl-1-methylxanthine (IBMX) | Sigma-Aldrich |
| | | |
| | | |

Table 2.3. Reagents for staining MSC differentiation

| **Reagent** | **Components** | **Concentration** | **Supplier** |
|---|---|---|---|
| **Osteoblast differentiation** | Alizarin Red S stain | 1% (w/v) | Sigma-Aldrich |
| | $dH_2O$ | 100 ml | |
| | | | |
| **Adipocyte differentiation** | Oil Red O | 0.5% (w/v) | Sigma-Aldrich |
| | Isopropanol | 30ml | Sigma-Aldrich |
| | | | |

## 2.4 FLOW CYTOMETRIC ANALYSIS OF PROTEIN EXPRESSION

### 2.4.1 CELL SURFACE FLOW CYTOMETRY

Analysis of expression of surface bound proteins by flow cytometry was performed by harvesting cells and washing twice in PBS. Cells were resuspended in FACs buffer (PBS with 2% Fetal Bovine Serum (FBS)) to 1 x $10^6$ cells /ml and 100µl of cell suspension was transferred to a V-bottom 96 well plate (Lennox, Dublin, Ireland) and cells were centrifuged for 5 minutes at 4°C and 950 rpm. Supernatant was discarded and flurochrome labelled antibodies (Table 2.4) or isotype controls were added directly to the cells for 15 minutes at 4°C in the dark. Samples were washed twice in 150 µl FACs buffer and centrifugation at 4°C and 950 RPM. The supernatant was discarded and cells were re-suspended in 50 µl of counting beads (Becton Dickinson, New Jersey, USA) and analysed on a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK).

### 2.4.2 INTRA-CELLULAR FLOW CYTOMETRY

The detection of intra-cellular transcription factors or proteins was performed by flow cytometry. To analyse the expression of constitutively expressed transcription factors, cells were harvested, washed twice in PBS and re-suspended in FACs buffer to a concentration of 1x $10^6$ cells /ml. 100 µl of cell suspension was transferred to a V-bottom 96 well plate (Lennox) and surface proteins were labelled exactly as described in section 2.4.1. After cell surface staining, 100 µl of Fix/Perm buffer (eBioscience, San Diego, USA) was added to each well and samples were incubated in the dark at 4°C overnight. 200 µl of Permeabilisation buffer (eBioscience, San Diego, USA) was added to each well and samples were centrifuged at 950 rpm for 5 minutes at 4°C. Supernatant

was discarded and samples were blocked in 3 µl 2% rat serum (Sigma Aldrich) for 20 minutes at 4°C. 1 µl of fluorochrome labelled antibody or isotype control was added directly to the cells and samples were incubated in the dark at 4°C for 1 hour. 150 µl of FACs buffer was then added to each well and samples were centrifuged at 950 rpm for 5 minutes at 4°C. Supernatant was discarded and samples were washed again with 150 µl of FACs buffer. The supernatant was discarded and cells were re-suspended in 50 µl of counting beads (BD) and analysed on a BD Accuri C6 flow cytometer.

For the detection of pro-inflammatory cytokines in lymphocytes *ex vivo*, lymphocytes were isolated from lung, liver and spleen of mice as described in section 2.9.1. Lymphocytes were seeded at 1x $10^5$ cells/well in cRPMI. PBMC were stimulated with 100 ng/ml Phorbol Myristate Acetate (PMA, Sigma) and 1 µg/ml Ionomycin (Sigma) for 4 hours in the presence of 1X Golgi Stop (eBioscience) (for analysis of intracellular proteins). After stimulation samples were transferred to a V-bottom 96 well plate (Lennox) and cells were centrifuged at 950 RPM for 5 minutes at 4°C. Samples were washed twice with FACS buffer before cell surface proteins were stained as previously described in section 2.4.1.

Table 2.4 Antibodies employed in flow cytometry.

| Antibody | Conjugate | Supplier | Clone |
|---|---|---|---|
| CD3 | APC | eBioscience | UCHTI |
| CD4 | FITC | eBioscience | OKT4 |
| CD4 | APC | eBioscience | SK3 |
| CD8 | FITC | eBioscience | RPA-T8 |
| CD25 | APC | eBioscience | BC96 |
| CD34 | PE | eBioscience | 4H11 |
| CD44 | APC | eBioscience | IM7 |
| CD45 | PerCP | eBioscience | HI30 |
| CD45 | APC | eBioscience | 2D1 |
| CD54 | PE | eBioscience | HA58 |
| CD73 | APC | eBioscience | AD2 |
| CD90 | FITC | eBioscience | Ebio5E10 |
| CD105 | APC | eBioscience | SN6 |
| CD106 | PE | eBioscience | STA |
| HLA-ABC | FITC | eBioscience | W6732 |
| HLA-DR | PE | eBioscience | L243 |
| TNF-α | PE | eBioscience | MAb11 |
| FOXP3 | PE | eBioscience | 236A/E7 |
| CXCR4 | APC | eBioscience | 12G5 |
| IDO | PE | eBioscience | eyedio |
| FASL | PE | eBioscience | NOK-1 |
| PDL1 | PE | eBioscience | MIH1 |

## 2.5 ENZYME LINKED IMMUNOSORBENT ASSAY

96 well NUNC Maxisorb plates (Thermo Fisher Scientific) were used for all ELISA experiments. Plates were coated with 100 µl of capture antibody and stored at 4°C overnight in the absence of light. Plates were washed 3 times with wash buffer (PBS with 0.05% (v/v) Tween-20) and dried by a plate microplate washer (BioTek,Vermont, USA). Plates were blocked for 1-2 hours at room temperature using reagent diluent (1% Bovine Serum Albumin (BSA) in PBS). Plates were then washed 3 times again by the plate washer and samples or standards (50µl) were added to each well. Samples were incubated overnight at 4°C in the absence of light. The following morning, samples were aspirated off and plates washed 3 times with wash buffer. 100 µl of detection antibody was added to each well and samples were incubated for 1-2 hours at room temperature. Plates were washed 3 times as before and 100 µl Avidin-HRP was added to each well and left for 20 minutes at room temperature. Samples were washed 3 times as before. 100 µl of substrate solution was added to each well for 20 minutes or until a strong colour change was detected. 50 µl of stop solution (2N H2SO4) was then added to each well and the OD for each sample was determined using an ELx800TM microplate reader with Gen5 analysis software (BioTek,Vermont, USA). Cytokine concentrations for each sample were extrapolated from a standard curve which related the observed OD to a known protein concentration. Data analysis was performed using My Assays analysis software solutions.

## 2.6 MOLECULAR TECHNIQUES

### 2.6.1 RNA ISOLATION

RNA was extracted from MSC using Tri-Reagent (Molecular Research Centre Inc., Cincinnati, USA). cDMEM was removed from cell monolayers and resuspended in 1 ml of Tri-reagent. 1 ml was used to isolate RNA from 2-5 $x10^6$ MSC. 100µl of 1-Bromo-3-Chloropropane (Sigma) was added to samples. After vigorous vortexing for 15 seconds samples were left at room temperature for 5 minutes. Following this samples were then centrifuged at 12,000g for 10 minutes at 4$^oC$. The RNA containing aqueous layer was then transferred to a new sterile tube. RNA was precipitated from this by inverting the tube with 500µl of molecular grade isopropanol (VWR). Samples were left at room temperature for 10 minutes after which they were centrifuged at 4$^oC$ for a further 10 minutes at 12,000g. The RNA pellet was then washed in 75% ETOH and centrifuged at 7,500g for 5 minutes at 4$^oC$. The ETOH was removed and pellets left to air dry and resuspended in 30µl of RNase free $H_2O$. RNA not used immediately for cDNA synthesis was stored at -80$^oC$.

### 2.6.2 CDNA SYNTHESIS FROM RNA

Reverse transcription polymerase chain reaction (RT-PCR) was utilised to amplify complementary DNA. RNA was quantified using a Nanodrop. Only RNA precipitations which yielded an OD 260/280 ratio of 1.8 – 2.0 were used for cDNA synthesis. Genomic DNA was removed from RNA samples by treating 2µg of RNA with amplification grade DNase I (Invitrogen). 1µl of DNase I was added to each sample and samples were incubated for 15 minutes at room temperature. DNase I reaction was then neutralised using 1µl of 25nM EDTA (Invitrogen). Samples were then

heated to 65$^{o}$C for 10 minutes and then placed on ice for a further 2 minutes. 2 µl of 5X All-in-One RT MasterMix (Applied Biological Materials Inc, Huntingdon, England) was added to each tube and sample volume was made to 10 µl per reaction with RNase free $H_2O$. cDNA synthesis was then performed at 45$^{o}$C for 50 minutes and then 70$^{o}$C for 15 minutes. Samples were then stored at 4$^{o}$C until required.

### 2.6.3 REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

cDNA generated as described above was diluted 1 in 2 with nuclease free $H_2O$ and used for RT-PCR using primers specific from gene sequences of interest (Sigma-Aldrich, Ireland). A reaction mix was prepared and 9µl of reaction mix was placed into special optical 96 well plates (Illumina, MSC, Dublin, Ireland) followed by 40-50 cycles of 95$^{o}$C for 30 seconds, 58$^{o}$C for 30 seconds and 72$^{o}$C for 45 seconds. Amplification of one specific product was determined through melt curve analysis where the presence on one single melting peak indicated the absence of primer-dimer association. The relative quantification of target gene expression was determined in relation to the house keeping gene (GAPDH) expression using the delta CT method which is determined by subtracting the GAPDH value from the target CT value for each sample. The fold change in relative gene expression was determined by calculating the $2^{-\text{delta CT}}$ values.

### 2.6.4 AGAROSE GEL ELECTROPHORESIS

Agarose gels were prepared by adding 1.3 g (w/v) agarose (Sigma-Aldrich) to 1 X TAE buffer (buffer made to a final volume of 1 litre $dH_2O$ consisting of 242g TRIS Base, 57.1 ml Acetic acid, 100 ml EDTA) and heating in a microwave until completely

dissolved. The solution was left to cool and 6 µl of Gel Red (Biotium, California, USA) was added and the solution was poured into a gel tray. When solidified, agarose gels were submerged in TAE buffer and subjected to electrophoresis at 100V for 60 minutes. Samples were run simultaneously to a 100 base pair molecular weight (Promega). Nucleic acid products were visualised under UV light and images acquired using a Gel Logic 212 Pro gel (Carestream Health, Rochester, USA) documentation system.

## 2.7 *IN-VITRO* MSC FUNCTIONAL ASSAYS

### 2.7.1 *IN-VITRO* LICENSING OF MSC

MSC were licensed by stimulating with pro-inflammatory cytokines IFN-γ or TNF-α in order to become immunosuppressive. For IFN-γ stimulation MSC were cultured with 50 ng/ml of recombinant human IFN-γ (Peprotech, New Jersey, USA) for 24 or 48 hours. TNF-stimulation was performed by stimulating cells at 20 ng/ml recombinant human TNF-α (Peprotech) for 24 or 48 hours.

### 2.7.2 ANALYSIS OF THE IMMUNOSUPPRESIVE EFFECT OF MSC ON T CELL PROLIFERATION

A Carboxyfluorescein succinimidyl ester (CFSE) assay was performed to analyse levels of T cell proliferation suppressed by MSC. MSC were seeded in 96 well round bottom plates (Nunc) at ratios of 1:5, 1:10, 1:20, and 1:40 MSC: PBMC. The plated MSC were then placed back into their corresponding oxygen tension:

- Normoxia (N) - continual culture in normoxia.
- Normoxia – hypoxia (N-H) –continual culture in normoxia followed by 8 days of hypoxic culture. Therefore N-H were placed back into hypoxia.
- Hypoxia (H) - continual culture in hypoxia.
- Hypoxia – normoxia (H-N) –continual culture in hypoxia followed by 8 days of normoxic culture. Therefore H-N were placed back into normoxia.

24 hours later, 5 x $10^4$ CFSE labelled PBMC were added to the pre-seeded MSC. Briefly PBMC were thawed from liquid Nitrogen for 30 minutes at -80$^o$C as outlined in section 2.2.4. PBMCs were then added to 10mls of cRPMI and centrifuged at 1500 RPM for 5 minutes at room temperature. A working solution of 10µm Carboxyfluorescein succinimidyl ester (CFSE) Invitrogen (Thermo-Fisher) was prepared using warm PBS. After centrifugation, the supernatant was removed and pellet resuspended in 1ml of warm PBS. A final concentration of 10µm CFSE was added and this was left to incubate for 10 minutes in the absence of light at room temperature. While incubating, the pre-plated MSC were washed with PBS and media was replaced with 100µl cRPMI. After 10 minutes the CFSE labelling reaction was stopped with 2ml of cold PBS followed by centrifugation at 1500 RPM for 5 Minutes. The supernatant was removed and resuspended in 5ml cRPMI and cells were subsequently counted. 5x$10^4$ PBMC were added to each well with 1x$10^4$ anti CD3/CD28 activation beads (Life Technologies). Following 4 days of culture in normoxia, PBMC were harvested from the plates and stained for CD3 as described in 2.4.1 and 7AAD in the absence of light for 15 minutes at 4$^o$C. Samples were washed

twice with FACs buffer and resuspended in 50 µl of counting beads (BD) and analysed on a BD Accuri C6 flow cytometer.

## 2.8 LENTIVIRUS PRODUCTION

### 2.8.1 E. coli Transformation

One Shot® Stbl3™ Chemically Competent *E. coli* bacterial cells (Thermo Fisher Scientific, Massachusetts, USA) were placed on ice. All plasmids were vortexed. 1µl of plasmid DNA was added to the chemically competent cells and sample was flicked gently and left on ice for 30 minutes. Following incubation, cells were heat shocked for 45 seconds at 42$^{o}$C then placed on ice for 2 minutes. 960µl of Lennox broth (LB) (Sigma-Aldrich) without any antibiotic was added to each vial and placed on an orbital shaker for 60 minutes at 37 $^{o}$C and 170 rpm. Ampicillin (100µg/ml) was added to nutrient agar (Oxoid, Hampshire, UK) (to select for ampicillin resistant E. coli that successfully incorporated the ampicillin resistance gene from the plasmid) and poured into a sterile petri dish and left to set at room temperature. Following the one hour incubation, cells were taken from the orbital shaker and 50µl of cell suspension was added to the middle of the set ampicillin agar plate and spread around the plate and incubated overnight at 37 $^{o}$C.

### 2.8.2 Culture of transformed E. coli

Agar plates were removed from the incubator following transformation as described in section 2.8.1. Growth of *E. coli* on the ampicillin resistant plate demonstrated successful transformation. 100ml of LB broth was prepared with

ampicillin (100µg/ml) into conical flasks in the late afternoon. A single colony was picked from the plate with a sterile pipette tip and placed into the conical flask. The flask was subsequently covered with tin foil and placed on an orbital shaker at 37 $^{o}$C for 14-16 hours at 180 rpm.

### 2.8.3 Plasmid DNA extraction

Plasmid DNA was extracted from transformed bacterial cells using a HiSpeed® Maxi Kit (Qiagen). Bacterial cells (2.8.2) were pelleted by centrifuging at 4,000 rpm for 30 minutes at 4$^{o}$C. The supernatant was discarded and 10ml of buffer P1 was added directly to the pellet to resuspend all bacterial cells and vortexed. 10ml of buffer P2 was added to the solution to lyse the cells and inverted 4-6 times and incubated at room temperature for 5 minutes. Following this, 10ml of buffer P3 was added to the suspension to neutralise the solution and mixed by vigorous inversion 6 times. The lysate was poured into a QIAfilter cartridge and incubated at room temperature for 10 minutes. A HiSpeed maxi tip was equilibrated with 10ml of buffer QBT. Following the incubation, the lysate was poured into the previously equilibrated cartridge allowed to empty by gravity flow. The tip was washed twice with 60ml of buffer QC. The DNA was eluted from the tip using 15ml buffer QF. The DNA was precipitated by adding 10.5 ml of isopropanol and incubated at room temperature for 5 minutes after mixing. The eluate/isopropanol was then poured into a 30ml syringe and filtered with a QIAprecipitator using constant pressure. The QIAprecipitator was removed from the syringe and 2ml of 70% ethanol (ETOH) was added to the syringe and the QIAprecipitator placed back onto the syringe. The DNA was washed by filtering the ETOH through the QIAprecipitator. Air was then forced through the QIAprecipitator to dry the membrane and repeated. The nozzle of the QIAprecipitator was removed and

dried gently with clean tissue to prevent ETOH carryover. The QIAprecipitator was added to a 5ml syringe and 1ml of buffer TE was added to the syringe and forced through under constant pressure to elute the DNA. The eluate was then replaced into the syringe and filtered again to maximise DNA return. The DNA yield was determined using a Nanodrop 2000 spectrophotometer. DNA was stored at -20$^{o}$C.

### 2.8.4 TRANSFECTION OF HEK 293 CELLS

Cells were seeded at 3 x10$^{6}$ per dish and placed in an incubator overnight. Two hours before transfection, media was removed from 10 cm culture dishes and replaced with 9mlDMEM. 13.2µg/ml reporter (Pfu-Luc2_eGFP) 10µg/ml packaging (PsPAX) and 4µg/ml envelope ( PMDG.2) (Addgene, Massachusetts, USA) were added to 15ml polystyrene tubes (Corning). This was then made up to a volume of 450µl with TE/H20 (Table 2.4). 50µl of 2.5M $CaCl_2$ (Table 2.5) was then added to each tube. The tubes were then vigorously vortexed. 500µl of 2XHBS (Table 2.5) was then added to each tube and vortexed. The solution was then incubated at room temperature for 20 minutes and examined for the presence of calcium precipitates. During the incubation, 100µM chloroquine (Sigma) was added to the HEK 293s and 1ml of the precipitate added to each dish. Dishes were incubated for 14-16 hours after which viral media was removed and replaced with HEK media. 24 hours later this media was collected and stored at 4$^{o}$C (so that additional media can be added to it 24 hours later and all collections concentrated at the same time without need or freeze-thawing). Fresh media was added to the transfected HEK 293s and a further 24 hours later the media was collected and combined with previous collections. This viral supernatant was then filtered with a 0.45µm filter. Supernatants not immediately concentrated were frozen at -80 $^{o}$C.

### 2.8.5 CONCENTRATION OF LENTIVIRAL PARTICLES

All Lentiviral supernatants were combined. For every 100mls of supernatant, 25.5mls 50% PEG (Table 2.6), 10.8mls of a 4M NaCl stock solution (Table 2.6) and 11.6 mls PBS were added. The supernatants were stored at 4$^{o}$C and mixed every 20-30 minutes for a total of 1.5 hours. Supernatants were then centrifuged at 4000 RPM for 30 minutes at 4$^{o}$C. After centrifugation, a white pellet should be visible. Supernatant was carefully decanted and pellets were resuspended in 200-300µl PBS. A smaller aliquot was stored for quantification. Aliquots were stored at -20$^{o}$C.

### 2.8.6 QUANTIFICATION OF LENTIVIRUS

To quantify the amount of viral particles present, a titre was performed on the viral supernatant. HEK 293 cells were seeded at 8 x $10^4$ cells per well of a 24 well plate. 24 hours later control wells were counted to determine how many HEK 293 cells may get transduced. A serial dilution of the virus was then made beginning with 1:10 and from this a further 6 1:10 serial dilutions were made. The media in the wells was removed and each diluted virus sample was added to a corresponding well. 14-16 hours later this viral media was removed and 1ml of fresh media added to each well. Two days later, the cells were harvested, washed and analysed for reporter gene expression by flow cytometry. To calculate the virus titre, the following calculation was employed:

Reporter gene expression of between 5-20% were deemed positive results and used to quantify viral particles

%GFP$^{+}$ cell x number of cells on day on transduction/ 100 x dilution factor x factor to get 1ml of volume = amount of virus in 1 ml.

Table 2.5. Reagents for transfection

| Reagent | Components | Supplier |
|---|---|---|
| **Te/$H_2O$** | 1mM TRIZMA | Sigma-Aldrich |
| | 0.1mM EDTA | Invitrogen-Gibco |
| | 500ml dH2O | |
| | Sterile filtered 0.22µM | |
| | | |
| **2.5M $CaCl_2$** | 36.755mg of Calcium Chloride | Sigma-Aldrich |
| | 100 ml dH20 | |
| | Sterile filtered 0.22µM | |
| | | |
| **2X Hepes buffered saline (HBS)** | 100mM HEPES | Invitrogen-Gibco |
| | 281mM Sodium Chloride | Sigma-Aldrich |
| | 1.5mM Sodium Phosphate Dibasic | Sigma-Aldrich |
| | pH adjusted to 7.1 | |

Table 2.6. Reagents for lentiviral particle concentration

| Reagent | Components | Supplier |
|---|---|---|
| **50% Polyethylene glycol (PEG)** | 250g PEG | Sigma-Aldrich |
| | 500 ml $dH_2O$ | |
| | | |
| **4M NaCl** | 234g NaCl | Sigma-Aldrich |
| | 1 ltr $dH_2O$ | |

### 2.8.7 MSC TRANSDUCTION

Transductions were performed in 6 well plates at $3x10^4$ per well unless otherwise stated. Virus was added to the MSC in a total volume of 800µl cDMEM. Protamine sulfate (50µg/ml) (Sigma-Aldrich) was added to obtain the final desired concentration. Cells were transduced for 24 hours before being replaced with fresh media for a further 24 hours. 24 hours after this, media supplemented with Zeocin (Thermo Fisher Scientific, Massachusetts, USA) was added to the wells for selection.

## 2.9 ACUTE GRAFT VERSUS HOST DISEASE PRE-CLINICAL MODEL

### 2.9.1 HUMANISED MOUSE MODEL OF aGvHD

A humanised mouse model previously developed in the lab (Tobin *et al*. 2013) was harnessed to evaluate the efficacy of hypoxic MSC for aGvHD. NOD.Cg-PrkdcscidIL2tmlWjl/Szj (NOD-Scid IL-2rγnull) (NSG) mice were ear punched, weighed and exposed to a conditioning dose of 2.4 Gy whole body gamma irradiation. 8 x $10^5$ gram-1 freshly isolated PBMC were washed three times with sterile PBS (Section 2.2.3) and administered on day 0 via tail vein injection using a 27 gauge needle and 1 ml syringe in a final volume of 200ul. Animals were closely monitored for the first hour and at regular intervals for signs of ill health. Mice were weighed every 2 days until day 7 and every day thereafter and changes in weight were recorded. 6.4 x $10^4$ gram-1 (Chapter 4) or 1.3 x $10^6$ (Chapter 5) MSC were washed three times with sterile PBS and administered on day 7. Development of acute GvHD was determined by examining features including weight loss, ruffled fur and hunched posture (Section

2.9.2). Animals which displayed greater than 15% weight loss were sacrificed humanely in accordance with the project ethical approval and license conditions.

### 2.9.2 PATHOLOGICAL SCORING SYSTEM FOR GvHD

The scoring system used to assess experimental mice was as follows:

| Score | Activity | General appearance | Behaviour | Clinical signs |
|---|---|---|---|---|
| **0** | Normal | Normal | Normal | No abnormal signs |
| **1** | Spontaneous movement, but reduced | Evidence of poor grooming/ridging | Less mobile but alert | Slight changes in breathing, increased rate only |
| **2** | Moves to stimulus but not spontaneous | Staring coat, shivering, matted/ruffled fur | | |
| **3** | | | Restless but very still, not alert | Marked changes in breathing |
| **4** | Huddled, not moving to stimulus and failure to take food and water | Hunched, badly matted fur | | Marked abdominal breathing |

## 2.10 HISTOLOGICAL PREPERATION AND ANLAYSIS

### 2.10.1 TISSUE PREPARATION

The lungs, liver and small intestine were harvested from experimental mice at day 12 and fixed in 10% (v/v) neutral buffered formalin for 24 hours. Samples were transferred to 70% ethanol for a further 24 hours at 4$^{o}$C. Samples were processed for histology using an automated processor (Shannon Pathcentre) which immersed the tissues in fixatives and sequential dehydration solutions including ethanol (70%, 80%, 95% x 2, 100% x 3) and xylene (x 2) (Sigma-Aldrich). After processing, tissues were embedded in paraffin wax using a Shandon Histocentre 2 (Thermo Fisher Scientific) and left to set at 4°C overnight. A Shandon Finesse 325 microtome (Thermo Fisher Scientific) was used to cut 5 μm sections of each tissue. Sections were placed in cold water before being transferred to a hot water bath (55°C) to remove any folding of the sections. Tissue sections were placed onto microscope slides (Thermo Fisher Scientific) and placed in an oven (60°C) overnight. Samples were then stained with H&E (Section 2.10.2) and blindly scored using the system outlined in section 2.10.3

### 2.10.2 HAEMOTOXYLIN/EOSIN STAINING

Slides were transferred to Xylene (Sigma) for 20 minutes. Samples were then re-hydrated in 3 decreasing concentrations of ethanol for 5 minutes each (100% x 2, 90% and 80%). Samples were then transferred to d$H_2O$ for 5 minutes before being immersed in Haemotoxylin (Sigma; Table 2.7) for 3 minutes. Samples were then washed under $H_2O$ for 2 minutes before being placed in 1% acid alcohol (Table 2.6) for no longer than 20 seconds. Samples were washed again under $H_2O$ before being immersed in Eosin Y (Sigma-Aldrich; Table 2.7) for 2 minutes and back to washing under $H_2O$ again. Slides were dehydrated through immersion in a series of increasing

ethanol concentrations (80%, 90%, 100%) for 5 minutes each. Samples were air dried and mounted with DPX mounting media (BDH) and examined under a light microscope.

### 2.10.3 HISTOLOGICAL SCORING

Following H&E staining, slides were examined blindly. A semi-quantitative scoring system was used to assess disease progression in the lungs, liver and small intestine (Tobin et al. 2013). Pathological scoring was carried out as follows:

**Lung**: 0; normal, 1; scattered areas of mononuclear cells, 2; mild focussed areas of mononuclear cell infiltration, 3; moderate levels of cellular infiltration and damage to lung architecture, 4; extensive mononuclear cell infiltration and extensive damage to lung architecture.

**Liver**: 0; normal, 1; sporadic collections of mononuclear cells in the parenchyma, 2; endothelialitis present around at least one periportal vein and marked increase in mononuclear cell infiltration, 3; endothelialitis present in more than one vessel and further increase in mononuclear cell infiltration, 4; endothelialitis present in virtually all vessels with extensive levels of mononuclear cell infiltration.

**Small intestine**: 0; normal, 1; mild necrotic cells with minor mononuclear cell infiltration, 2; widespread but mild villous blunting, necrosis and increased cell infiltration, 3; widespread and moderate villous blunting, necrosis and further increased cell infiltration, 4; widespread and severe villous blunting, necrotic cells and extensive mononuclear cell infiltration.

Table 2.7 Staining solutions for histological staining of tissue preparations

| **Reagent** | **Components** | **Concentration** | **Supplier** |
|---|---|---|---|
| **Harris Haemotoxylin** | Haemotoxylin | Neat | Sigma-Aldrich |
| | | | |
| **Eosin** | Eosin Y | 1% (w/v) | Sigma-Aldrich |
| | Potassium dichromate | 1.6% (w/v) | Sigma-Aldrich |
| | $dH_2O$ | 100 ml | |
| | | | |
| **Acid Alcohol** | Hydrochloric acid | 1% (w/v) | VWR |
| | 2-propanol | 69.3% (v/v) | Sigma-Aldrich |
| | $dH_2O$ | 29.7% (v/v) | |

### 2.10.4 CELL DEATH DETECTION

In principle, this assay relies on a key hallmark of DNA degradation; apoptosis. DNA "nicks" (occur during apoptosis) can be detected by labelling the free 3′-OH termini with modified fluorescent nucleotides present in this kit. Fixed tissue sections were placed onto charged microscope slides (VWR) and placed in a heated oven overnight at (60°C). The slides were rehydrated as outlined in section 2.10.2. Antigen retrieval buffer (Vector, California, USA) was heated in a microwave until the buffer reached boiling point. Slides were placed into the buffer for 6 minutes during which solutions for *in-situ* cell death detection (Roche) were thawed on ice. Slides were rinsed in PBS for 1 minute. Tissue sections on slides were identified with a wax pen. 10µl *in-situ* cell death detection solution was added directly to tissue sections and incubated for 1 hour at 37°C on a tray lined with damp tissue to mimic a humidified chamber and covered with tin foil. Following incubation, slides were washed in PBS and tissue sections covered with DAPI (100ng/ml) for 20 minutes at room temperature covered in tin foil. Slides were then rinsed in PBS and covered with Vectamount (Vector) mounting media and sealed with a coverslide.

## 2.11 CRYOVIZ™ IMAGING

### 2.11.1 QDOT LABELLING OF MSC

Qtracker® 625 Cell labelling Kit (Thermo Fisher Scientific, Massachusetts, USA) was employed to label MSC for CryoViz imaging. In the absence of light, 5µl Comp A (Qtracker® nanocrystals) and 5µl Comp B (Qtracker® carrier) were placed into a 1.5 ml Eppendorf tube and mixed with a pipette. The mixture was incubated at

room temperature for 5 minutes in the absence of light. 1 ml of cDMEM was added to the Eppendorf tube and vortexed for 130 seconds. $5x10^6$ MSC were then added to the Eppendorf tube and the sample was pipetted up and down. The tubes were then placed in an orbital shaker at 150 rpm for 1 hour at 37$^o$C. After the incubation, the MSC were washed twice with media and then three times with PBS. QDot labelled MSC were then administered to mice at 1.3 $x10^6$ MSC per mouse (equal number of cells per mouse for quantification of cellular distribution post sacrifice of animal). Non QDot labelled MSC were administered to mice as described in section 2.9.1.

### 2.11.2 IMAGING OF WHOLE ORGANS USING CRYOVIZ$^{TM}$ IMAGING SYSTEMS

MSC were labelled with QDots as outlined in section 2.11.1 and administered i.v to recipient mice as outlined in section 2.9.2. Animals were humanely sacrificed and organs for analysis collected 24 hours post MSC administration and fully immersed in cryo-embedding compound (OCT) in a mould (Optimal Cutting Temperature, Tissue-Tek, Terrance, CA). The mould is subsequently placed into a freezing chamber consisting of liquid nitrogen. Once frozen, samples were stored at -80$^o$C. Before running samples on the system (Figure 2.1), the moulds must be removed and placed into the cooled chamber for 2-3 hours after which it is placed on the microtome stage using OCT. The block is then processed through a "face-off" system whereby the mould is cut into until biological animal tissue becomes visible. The desired thickness (40μm) of each slice is then set and the imaging system is then readied. Brightfield and fluorescent images are then alternately captured between each slice.

Following acquisition of 2D images on the imaging workstation, a series of off-line image pre-processing tasks on the image processing and visualization system was

performed. Any non-uniform illumination pattern is compensated using a reference image of a white card. A "next-image" is then employed. In this method, the next section image is taken; it is then attenuated on a tissue- specific basis, and subtracted it from the block-face image to provide a corrected image. The process is repeated in a pair-wise fashion throughout the volume (Roy et al. 2009). Images were then pre-processed and immaterial regions removed. Filters were used to extract features and candidate pixels are identified and classified using a "bagging detection tree" to detect cells. Interactive image segments are then used to segment organs of interest (Steyer et al. 2009; Auletta et al. 2014; Wuttisarnwattana et al. 2015). Stem cell quantification software was then utilised to quantify the numbers of MSC in the organs of interest.

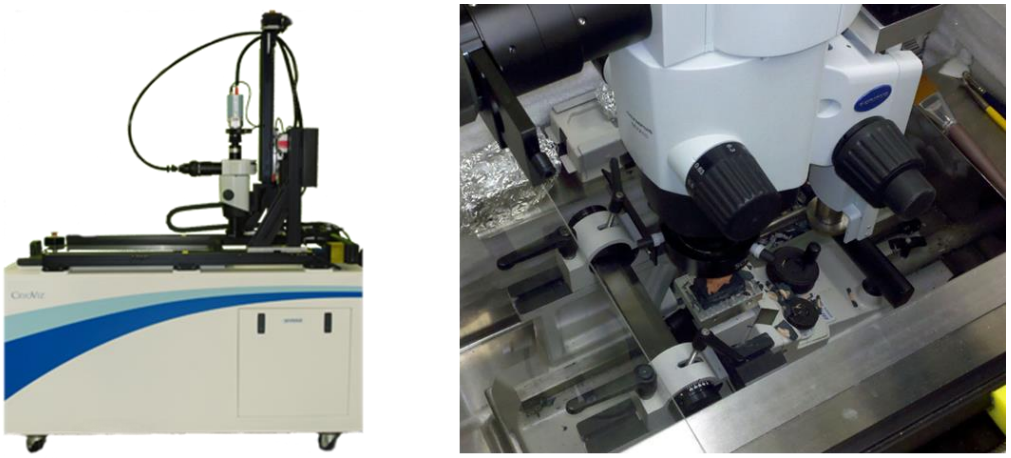


**Figure. 2.1 CryoViz™ (BioInvision Inc.) imaging system.** The CryoViz™ system provides whole body (mouse or organ) cryo-imaging that allows simultaneous fluorescent and brightfield images to be captured and create 3D visualisation and quantification of fluorescent-labelled cells.

# CHAPTER 3

# CHARACTERISATION OF THE EFFECTS OF HYPOXIC CULTURE ON HUMAN MESENCHYMAL STROMAL CELL IMMUNOMODULATION

## 3.1 INTRODUCTION

The multifaceted capabilities of MSC have highlighted the importance of these cells as an attractive source of cell therapy across a wide range of medical fields. Their easy isolation and culture expansion makes them an attractive source for cell therapies. In this chapter MSC were isolated and cultured in 5% $CO_2$ at 37ºC in two different oxygen concentrations; 21% $O_2$ and 5% $O_2$; normoxia and hypoxia respectively. Subsequently, MSC cultured in both conditions were characterised by flow cytometry and differentiation assays. In addition, their capacity to suppress activated lymphocytes and their immune modulatory characteristics were investigated *in-vitro.*

Mesenchymal stromal cells are a heterogeneous population of cells with immense potential for use in cell based therapies owing to their reparative (Quarto et al. 2001; Hofstetter et al. 2002; Morigi 2004; Munoz et al. 2005) and immunosuppressive characteristics (Bartholomew et al. 2002; Le Blanc et al. 2004; Rasmusson et al. 2005; Krampera et al. 2006). MSC can be easily sourced from a number of tissue but their proliferative capabilities are not preserved after prolonged *ex-vivo* expansion which typically is performed at oxygen levels much higher than cells encounter *in-vivo*. Hypoxia can enhance MSC yield (Dos Santos *et al.* 2010) and thus it seems logical to harness hypoxia as a biologically relevant environment for *ex-vivo* MSC expansion. The complex interaction between cells *in-vivo* and functional outcomes of such reciprocities differ than that at normoxia. For example, when cord blood (CB) was co-cultured with MSC at 2%, 5%, 10% or 21% $O_2$, researchers found that 10% $O_2$ produced more efficient $CD34^+$ cell expansion (Andrade *et al*. 2015). Moreover, adipose tissue-derived MSC (AT-MSC) co-cultured with CB-HSC produced more $CD34^+$ cells in 5% $O_2$ than in normoxia (Andreeva *et al*. 2015). In this thesis we chose 5% $O_2$ (termed hypoxia/hypoxic culture). The general consensus is that we use the term

hypoxia for cell culture that is closer to the *in-vivo* oxygen level. However, 5% $O_2$ is actually normoxic for cells *in-vivo* that may be present in venous blood and not thus necessarily hypoxic. Therefore, 21% $O_2$ can be deemed hyperoxic (excess supply of $O_2$). While the concept of using hypoxia for cell culture is gaining momentum, the effects of long term hypoxic culture on MSC immunosuppressive capabilities are not well understood.

This chapter examined in detail the impact of hypoxia on MSC and investigated immune modulation by hypoxic MSC. The MSC isolated from bone marrow aspirates were then cultured in either normoxia or hypoxia until ready for sub-culture. Furthermore, the effect of hypoxic culture on the proliferative capacity of MSC was examined in conjunction with effects on their bi-lineage differentiation capabilities. Importantly, the immunosuppressive capacities of MSC were characterised in hypoxia and subsequent analysis of MSC immunosuppressive mediators (Figure. 3.1) was performed. Results from these different oxygen culture conditions are herein presented which assess MSC parameters when cultured in normoxia or hypoxia.

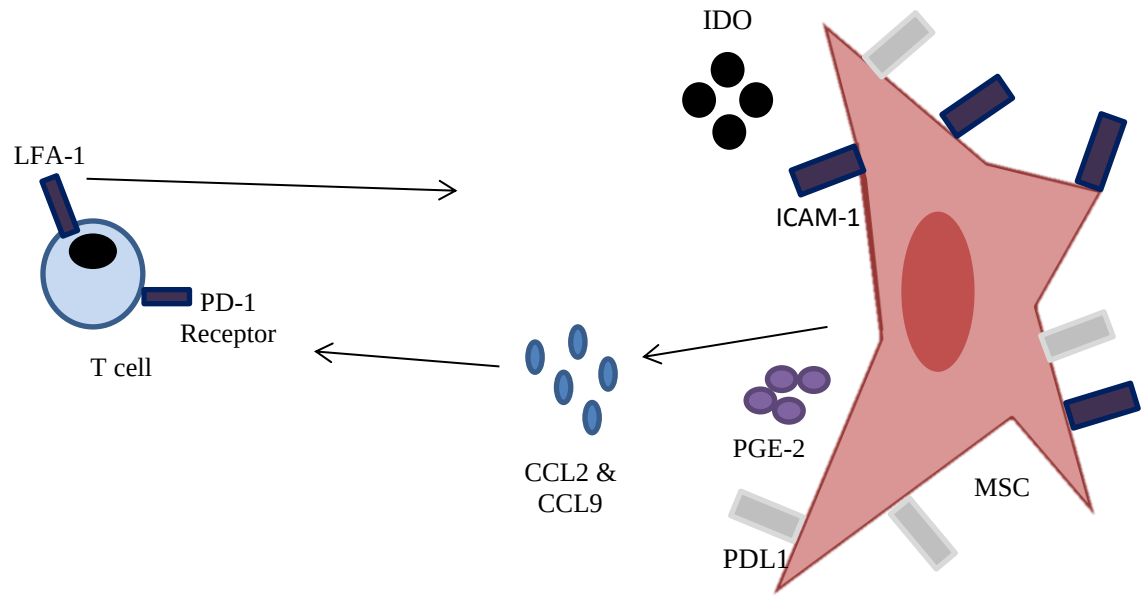



**Figure 3.1 MSC mediators of immunosuppression.** Schematic representation illustrating a select few immunomodulatory effects mediated by MSC on T cells investigated here. MSC employ chemokines to attract T cells into close proximity before anchoring the T cell by adhesion molecules such as ICAM-1 (via its integrin receptor LFA-1), thereby enhancing MSC immunosuppressive potency via the release of soluble mediators IDO and PGE-2 in conjunction with the PD-1/PD-L1 pathway.

## 3.2 BONE MARROW DERIVED MSC ISOLATED FOR THIS STUDY DISPLAY TYPICAL MORPHOLOGY AND GENERATE ENHANCED CELL NUMBERS IN HYPOXIA

Undifferentiated MSC were isolated as described in section 2.2.1. MSC display a characteristic spindle shaped, fibroblastic like appearance (Friedenstein 1966; Pittenger *et al.* 1999). In order to ensure that isolated MSC retained this characteristic appearance, an isolation method based on adherence to tissue culture was selected (Tondreau *et al.* 2004). Human MSC were isolated from bone marrow aspirates of healthy donors. Briefly, bone marrow cells were diluted in PBS and centrifuged as described in 2.2.1. Cells were then seeded at a density of $40x10^6$ per T175 vented flask and fed at interval days thereafter. Cells were examined for any changes in morphology between culture in normoxia and hypoxia; all MSC displayed typical spindle shaped morphology (Figure 3.2 A and B).

The potential for hypoxic preconditioned MSC to generate higher cell numbers would be greatly advantageous to the field. A plethora of studies have examined the effect of hypoxia on MSC expansion. While numerous studies reveal the positive impact of hypoxic culture on MSC proliferation, a small number of studies showing a negative impact of hypoxia on MSC proliferation have also been reported (Fehrer et al. 2007; Grayson et al. 2007; Carrancio et al. 2008; Tsai et al. 2011; Holzwarth et al. 2010; Chung et al. 2012; Nold et al. 2014). Given this discrepancy, the ability of MSC isolated in our hands and cultured in hypoxia, to generate higher cell numbers than normoxic MSC was examined.

The positive effect mediated by hypoxia on MSC cell numbers is presented here. MSC were seeded at $1.4\ x10^5$ per T25 vented flasks in triplicate and cultured in either normoxia or hypoxia over several passages. Cells were then trypsinised, counted and

fold increase calculated (Figure. 3.2 C). Crystal Violet staining of MSC exposed to 5 days of hypoxia also served to show the positive effects of hypoxia on MSC cell numbers (Figure. 3.2 D). MSC consistently generated significantly higher percentages of MSC over all passages (P) tested (P2-P7).

## 3.3. CHARACTERISATION OF MSC EXPRESSION OF SURFACE MARKERS

Given that there is no one specific marker to uniquely identify MSC and given that morphology can be subjective criteria, a panel of surface markers was used to confirm MSC identity. The examination of surface marker expression is routinely used to identify MSC (CD105, CD90 and CD73). Co expression of positive markers and absence of others (HLA-DR, CD34) are used for the identification of MSC (Bobis et al., 2006; Peister et al., 2004). Human MSC were isolated and culture expanded continuously in either normoxia or hypoxia and characterised by flow cytometry. Figure 3.3 demonstrates the manner in which MSC were gated upon and subsequently analysed. MSC were selected based upon a size versus granularity profile (Figure 3.3 A). MSC used in experimentation did not display any non-specific binding to mouse isotype control antibodies (Figure 3.3 B). Gated MSC expressed CD105 and reveals the positive region for MSC surface marker expression. Importantly, culture of MSC in normoxia or hypoxia did not significantly hinder their typical surface marker expression (Figure 3.4). Furthermore MSC did not express hematopoietic markers CD34 or CD45 (Figure 3.4). Importantly, MSC did not express HLA-DR in either normoxic or hypoxic culture (Figure 3.4 A).

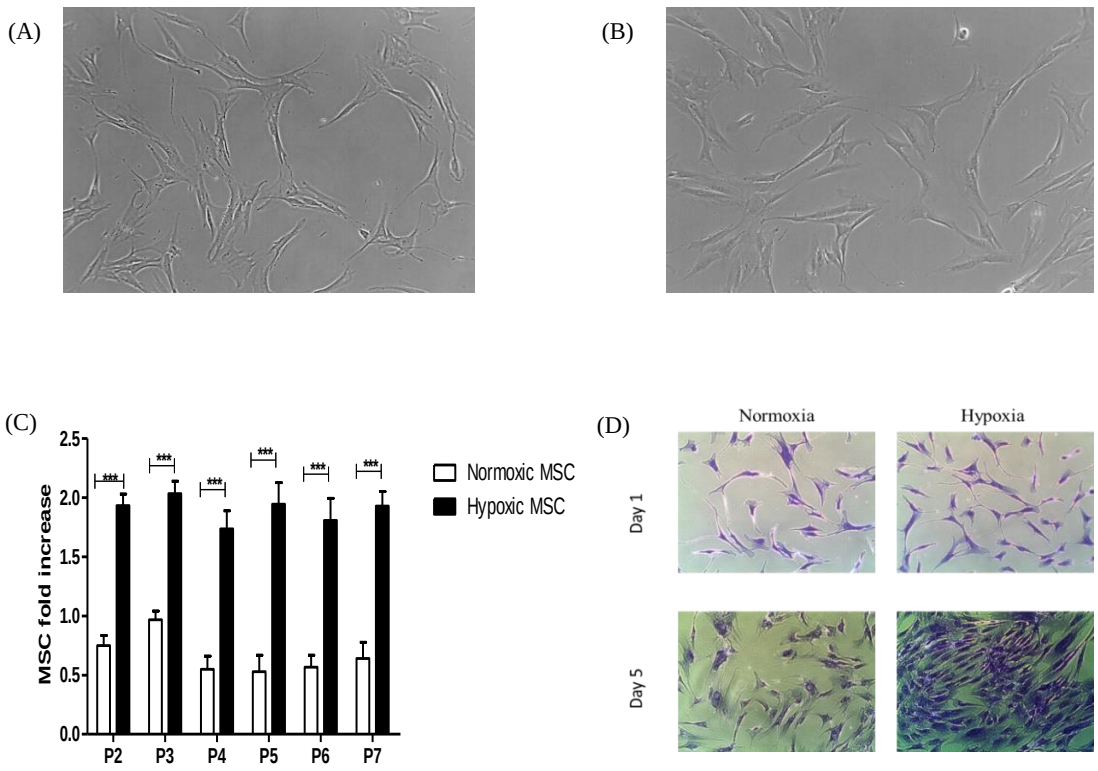



**Figure 3.2. Culture in hypoxia does not alter the typical phenotype of MSC and enhances MSC cell number.** Isolated MSC cultured in both oxygen concentrations had a typical phenotype displaying spindle shaped fibroblastic like cell morphology in normoxia (A) and hypoxia (B). Original magnification 10X, phase contrast, light microscopy**.** Image representative of 3 individual MSC donors. (C) MSC were seeded at 1.4 x$10^5$ per T25 vented flask in triplicate and subcultured. MSC were stained with EB/AO and counted using a haemocytometer. Cells were subsequently replated for a further 4 days. The fold increase was calculated as follows (viable MSC number at day 4 - MSC number seeded on day 0)/ MSC number seeded on day 0. Data are reported as mean ± standard error of the mean (SEM) of 4 individual MSC donors. Hypoxic MSC displayed enhanced cell numbers over all passages. (D) Cells were cultured in 6 well plates and placed into normoxia or hypoxia for 5 days and stained with crystal violet. Images (10X, phase contrast) representative of 3 donors. Statistical analysis was performed using the student's unpaired *t-test* between two means. ***, P<0.001

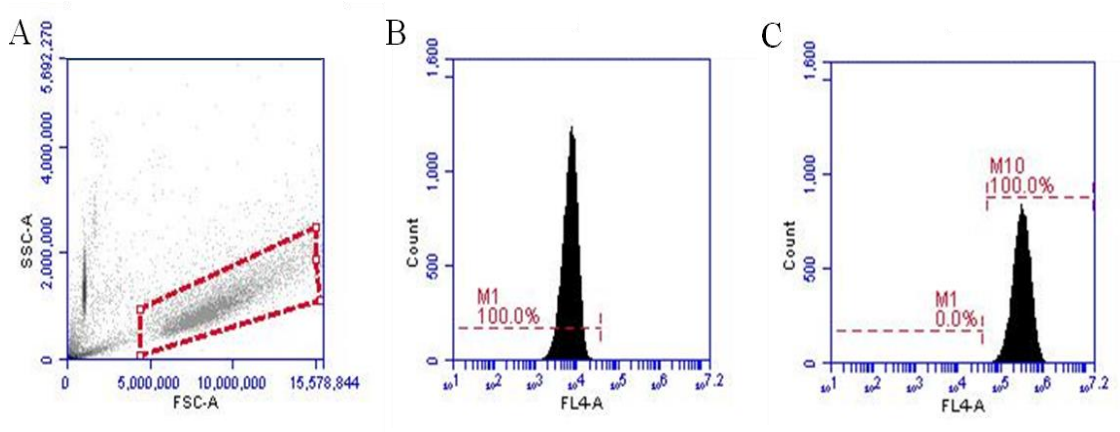



**Figure 3.3 Flow cytometric characterisation of human MSC.** Flow cytometry was performed on MSC over multiple passages. (A) Image shows how MSC were gated on forward scatter (FCS; size) versus side scatter (SSC; granularity) to eliminate cell debris. (B) Human MSC did not display non-specific binding to mouse isotype control antibodies. (C) Histogram showing CD105 (APC/FL4) positive MSC.

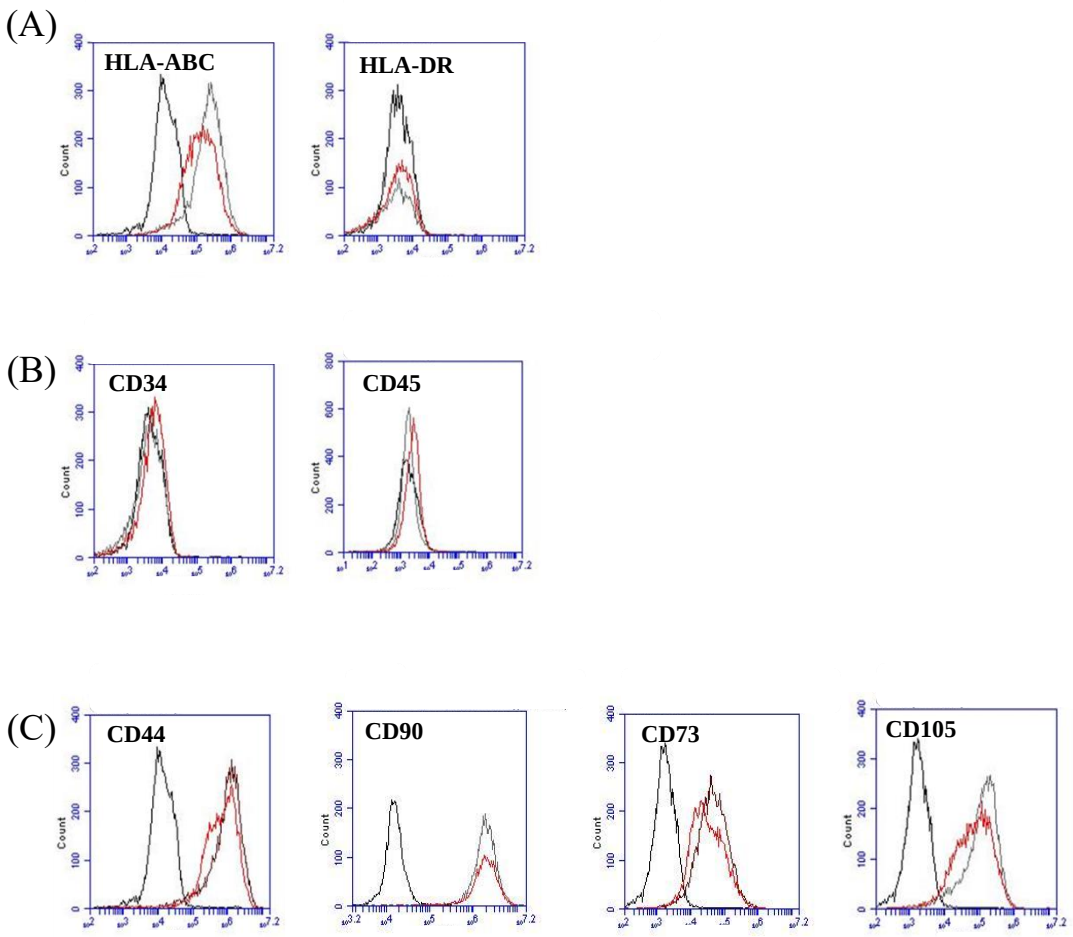



**Figure 3.4 Flow cytometric analysis of surface markers expressed by human MSC.** MSC were characterised according to their surface protein expression after culture in normoxia or hypoxia. Cultures of MSC were labelled with monoclonal antibodies against (A) HLA, (B) hematopoietic markers, (C) cell-cell/cell-matrix interaction markers. Black bars represent the isotype control, the grey and red bars normoxic and hypoxic cultured MSC respectively. Representative from n=3 individual MSC donors.

## 3.4 ISOLATED MSC RETAINED BI-LINEAGE DIFFERENTIATION CAPACITY

In-vitro, MSC have the capacity to differentiate into a number of mesodermal lineages. This property is used to further verify an isolated population of MSC. MSC were seeded into a 6 well plate and fed with control or differentiation media (Table 2.2). Osteogenic differentiation of MSC is detected by the presence of calcium deposits after 21 days of culture in its specific media. To evaluate the level of calcium deposition, MSC were stained with Alizarin Red S (Table 2.3) which in turn identified mineralised MSC. The process of differentiating MSC into adipocytes requires 21 days of culture in controlled media. After 21 days in normoxia or hypoxia, adipogenesis was determined by Oil Red O staining (Table 2.3) to positively identify the formation of lipid vacuoles characteristic to that of adipose cells.

MSC cultured in both oxygen tensions responded differentially to the specific differentiation media. MSC cultured in normoxia generated calcium deposits as indicated by staining with the Alizarin assay (Figure 3.5 C). Furthermore, hypoxic MSC displayed enhanced osteogenesis as assessed (Figure 3.5 D) by staining. Given that the bone marrow is hypoxic in nature it seemed logical that these BM-MSC, when cultured in hypoxia, would display enhanced osteogenesis. In contrast with the above data for hypoxic MSC, when driven towards adipogenic differentiation, they showed a decreased capacity for adipogenesis (Figure 3.6 C) in comparison to normoxic MSC (Figure 3.6 D).

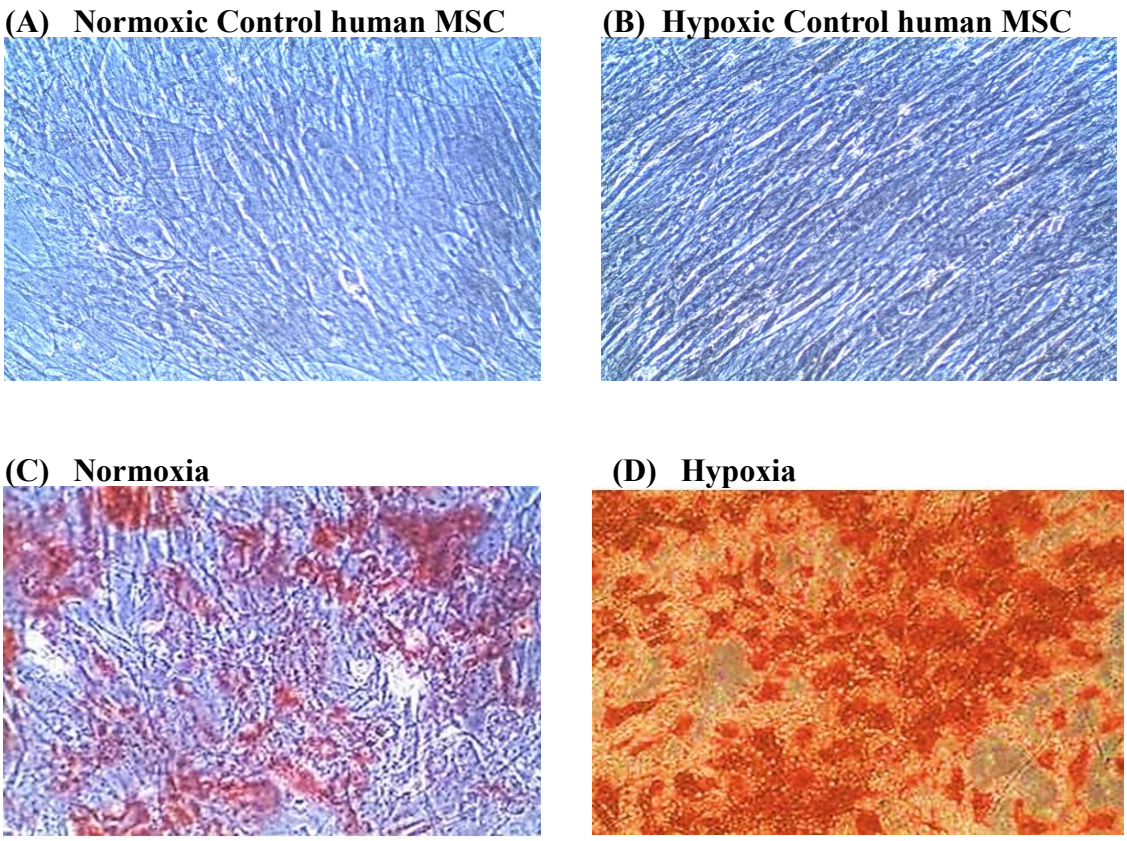



**Figure 3.5 Osteogenic differentiation capacity of MSC *in vitro*.** MSC were seeded at 5 x $10^4$ per well of a 6 well plate and cultured in standard expansion media in normoxia and hypoxia as a control (A and B). MSC were also cultured in osteogenic medium under normoxia and hypoxia for 21 days. Osteogenesis is indicated by mineralised cells staining red with Alizarin Red S staining (A and B; control after Alizarin Red S is washed off) (C and D; Osteogenesis induced in MSC after Alizarin Red S is washed off). Magnification x200. Representative of 3 independent MSC donors.

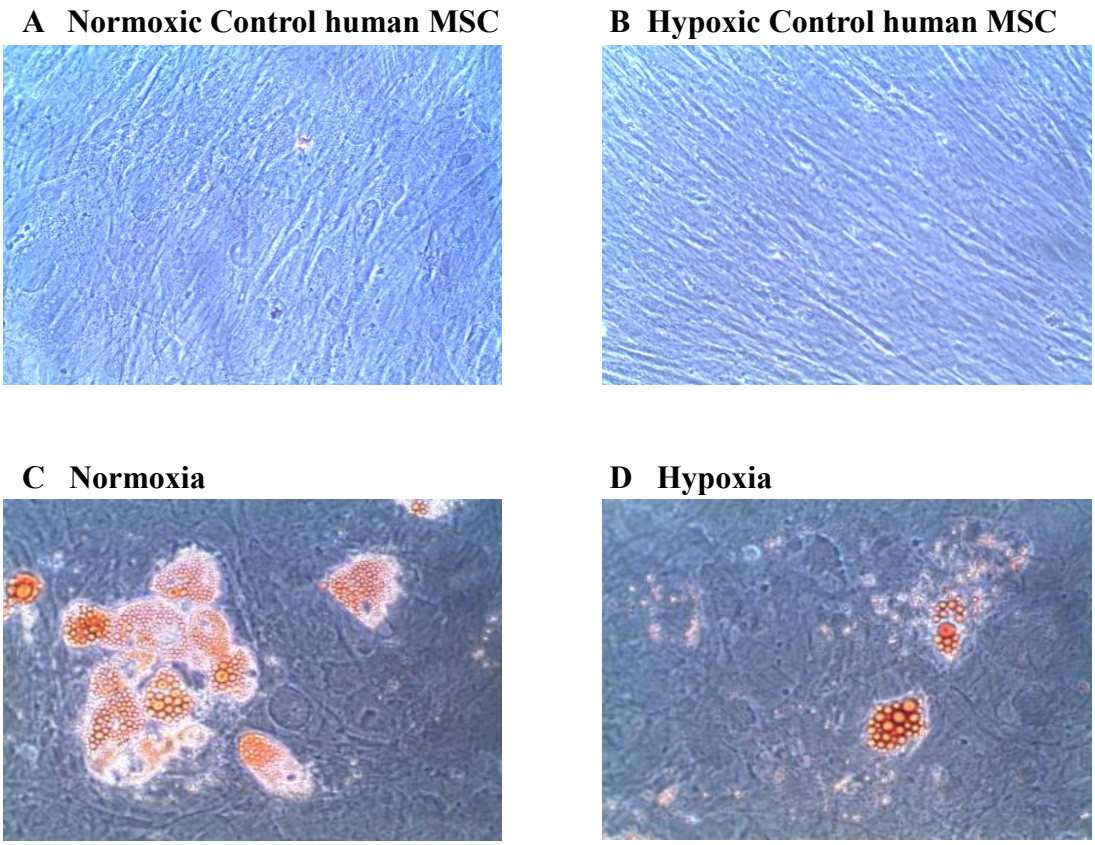



**Figure 3.6 Adipogenic differentiation capacity of hMSC *in vitro*.** MSC were seeded at 5 x $10^4$ per well of a 6 well plate and cultured in standard expansion media in normoxia and hypoxia as a control (A and B) or in adipogenic induction media (C and D) for 21 days. Adipogenesis is indicated by lipid vacuoles stained red with oil red O (A and B; control after Oil Red O is washed off) (C and D; Adipogenesis induced in MSC after Oil Red O is washed off) . Magnification x200. Representative of 3 independent MSC donors.

## 3.5 EFFECTS OF HYPOXIC CONDITIONS ON HUMAN MSC IMMUNOMODULATION OF LYMPHOCYTE PROLIFERATION

An array of studies have proven that MSC have the capacity to suppress lymphocyte proliferation/allogeneic responses (Bartholomew et al. 2002; Krampera et al. 2006; Aggarwal & Pittenger 2009; Tobin et al. 2013). As a result, MSC have entered into clinical trials for a number of inflammatory disorders. It was therefore pivotal that this immunological function was tested *in-vitro* with MSC exposed to multiple passages of hypoxia (as would be present in large scale culture of MSC for clinical trials) to ensure low oxygen culture presented no detrimental impact on this critical function of MSC. MSC continuously exposed to normoxia or hypoxia are termed normoxic or hypoxic MSC respectively.

A carboxyfluorescein succinimidyl ester (CFSE) assay was utilised to ensure MSC cultured in hypoxia over passages retained this important parameter. This model allows for quantitative analysis of peripheral blood mononuclear cells (PBMC) proliferation. In brief, normoxic and hypoxic MSC were seeded in 96 well round bottom plates at diluting ratios for a final ratio MSC: PBMC of $1x10^4$: $5x10^4$. The plated MSC were then placed back into their corresponding oxygen tension. A further 24 hours later, $5 \times 10^4$ CFSE labelled PBMC were added to each well of the pre-plated MSC with anti-CD3/CD28 activation beads and placed into normoxic culture. PBMC controls were either cultured alone unstimulated or stimulated with anti-CD3/CD28 activation beads. Activated PBMC were cultured in the presence of normoxic or hypoxic MSC and harvested on day 4 for analysis by flow cytometry. The number of CFSE diluted $CD3^+$ cells were enumerated using counting beads, after gating on $CD3^+$ T cells. As expected, unstimulated PBMC did not proliferate. Anti-CD3/CD28 stimulated PBMC displayed a strong proliferative response to the activation beads.

Importantly, MSC exposed to at least 20 days of normoxic culture, deemed long term normoxia, significantly suppressed PBMC proliferation at a ratio of 1:5 MSC: PBMC (Figure 3.7). Notably, MSC cultured in hypoxia for at least 20 days, long term hypoxia, failed to significantly suppress anti-CD3/CD28 driven PBMC proliferation in comparison to normoxic MSC (Figure 3.7).

## 3.6 SHORT TERM HYPOXIC HUMAN MSC SUPPRESS ANTI-CD3/CD28 DRIVEN LYMPHOCYTE PROLIFERATION

Given that MSC exposed to long term hypoxic culture lost their capacity to significantly reduce PBMC proliferation in comparison to normoxic MSC, it seemed logical to investigate whether shorter periods of hypoxic exposure resulted in the same adverse outcome. In order to grasp a greater understanding of the effect that long term hypoxia has on MSC, a CFSE assay was performed using normoxic MSC exposed to 8 days of hypoxia (N-H) (Long term normoxic culture of MSC for 20 days to 8 days of hypoxia. See section 2.7.2) as opposed to a minimum of 20 days hypoxic culture. Surprisingly, MSC (N-H) exposed to just 8 days of hypoxic culture did not lose their immunosuppressive capacity at a ratio of 1:5 MSC: PBMC and significantly suppressed anti-CD3/CD28 driven PBMC proliferation (Figure 3.8).

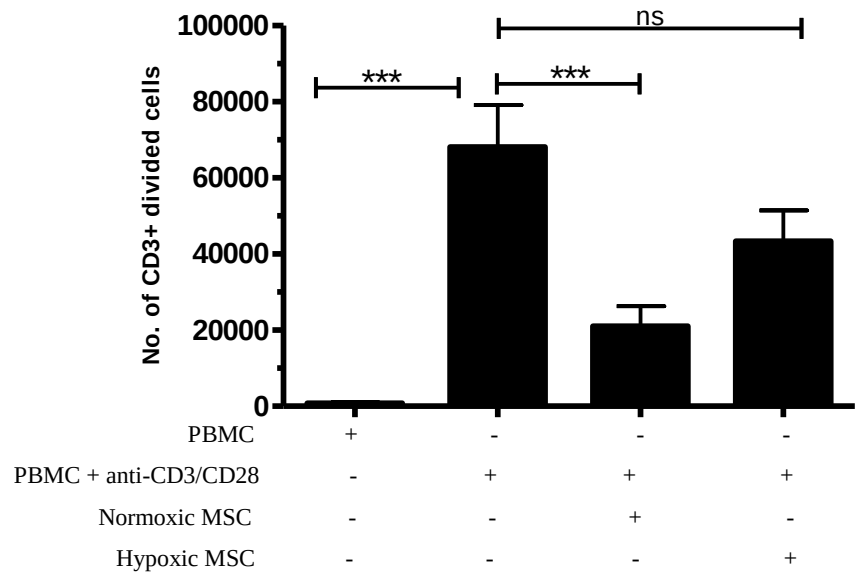



**Figure 3.7 Normoxic MSC but not hypoxic MSC significantly suppress anti-CD3/CD28 driven PBMC proliferation.** The capacity for MSC to inhibit anti-CD3/CD28 driven PBMC was measured using a Carboxyfluorescein succinimidyl ester (CFSE) assay. MSC were seeded in 96 well round bottom plates at 1 x$10^4$ per well and placed back into either normoxia or hypoxia. 5 x$10^4$ CFSE labelled PBMC were added 24 hours later with anti-CD3/CD28 activation beads (1×$10^4$/well). PBMC were cultured alone/unstimulated, or stimulated with anti-CD3/CD28 activation beads as controls and plates placed into normoxia. Activated PBMC were cultured in the presence of long term normoxic or long term hypoxic MSC (20 days) and harvested on day 4. CFSE dilution was analysed in gated $CD3^+7AAD^-$ cells, and the absolute number of $CD3^+$CFSE dividing cells was enumerated using counting beads. Normoxic MSC significantly reduced proliferation of activated PBMC. However MSC cultured in long term hypoxia failed to suppress activated PBMC proliferation. Data reported as mean ± standard error of the mean (SEM) of n=6 (2 MSC donors: 3 PBMC repeated twice performed in duplicate). ***, P<0.001. Statistical analysis was carried out using a student's unpaired *t-test* between two groups.

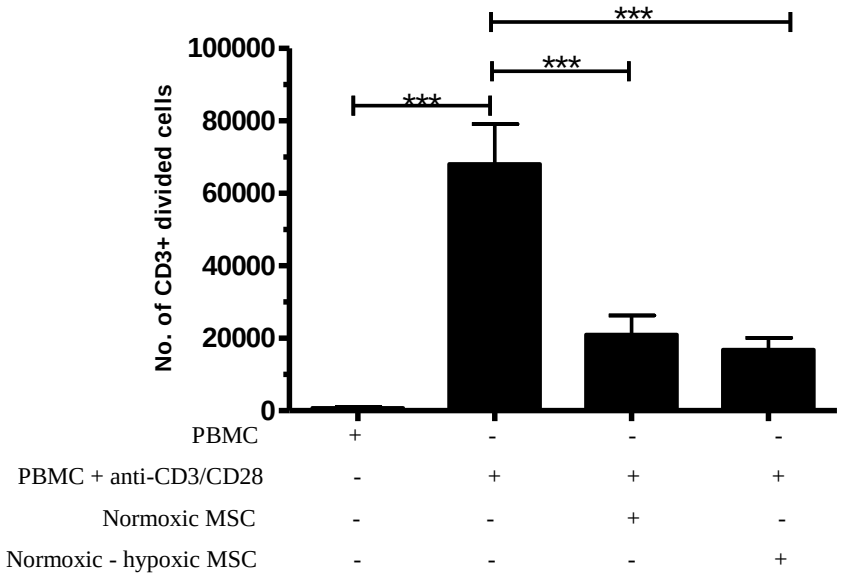



**Figure 3.8 Short term hypoxic MSC retain the capacity to suppress anti-CD3/CD28 driven PBMC proliferation.** Normoxic MSC (20 days) cultured in hypoxia for 8 days (Normoxic-hypoxic) did not lose their capacity to significantly reduce PBMC proliferation. Anti-CD3/CD28 driven PBMC proliferation was significantly decreased in the presence of normoxic and short term hypoxic MSC. Data represented as mean ± standard error of the mean (SEM) of n=6 (2 MSC donors: 3 PBMC repeated twice performed in duplicate) ***, $P<0.001$. Statistical analysis was carried out using a student's unpaired *t-test* between two groups.

## 3.7 LONG TERM HYPOXIC MSC EXPOSED TO NORMOXIA REGAIN IMMUNOSUPPRESSIVE CAPABILITIES

Although long term hypoxia enhanced MSC proliferation capacity, and in turn provided a viable method for generating enhanced MSC numbers, it was surprising to discover that these MSC were less potent immunosuppressors. Consequently, it was important to next determine if these MSC had, as a result of long term hypoxic culture, permanently lost the ability to suppress lymphocyte proliferation. Hypoxic MSC were therefore exposed to 8 days of normoxic culture (H-N) and defined as hypoxic to normoxic MSC, or short term normoxic MSC. These MSC were then harvested for use in a CFSE assay as described above. Interestingly, hypoxic to normoxic MSC regained their previously lost significant immunosuppression. In the presence of these MSC, PBMC proliferation was significantly reduced when compared to the anti-CD3/CD28 driven PBMC proliferation (Figure 3.9).

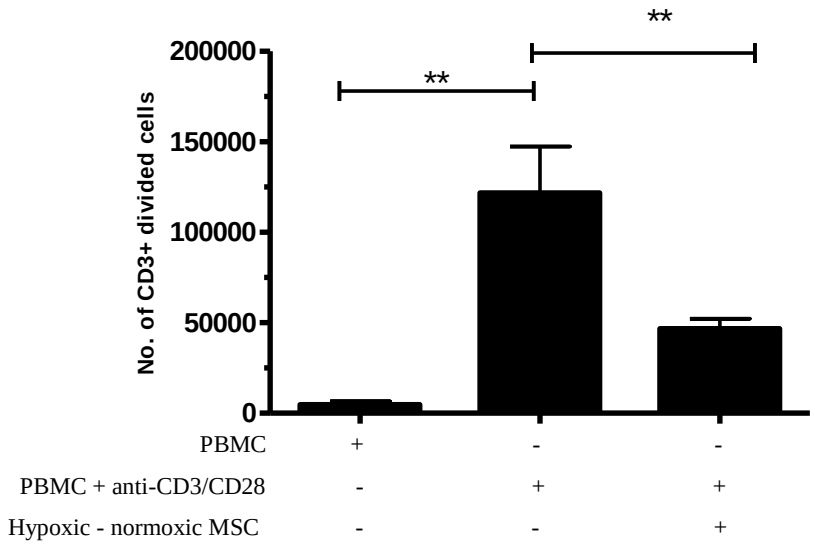



**Figure 3.9 Long term hypoxic MSC retain the capacity to suppress anti-CD3/CD28 driven PBMC proliferation when re-educated by normoxic.** Hypoxic MSC (20 days) which had previously lost their immunosuppressive capacity regained the capacity to significantly reduce anti-CD3/CD28 driven PBMC proliferation upon normoxic culture (8 days). Data represented as mean ± standard error of the mean (SEM) of n=6 (2 MSC donors: 3PBMC donor performed in duplicate). **, $P<0.01$. Statistical analysis was carried out using a student's unpaired *t-test* between two groups.

## 3.8 MSC INHIBITION OF ANTI-CD3/CD28 DRIVEN PROLIFERATION IS DOSE DEPENDENT

MSC suppression on PBMC is dose dependent. Given that hypoxic MSC regain their immunosuppressive capacity following culture in normoxia; it was important to examine a range of different MSC: PBMC ratios across all MSC culture conditions of normoxia, normoxia-hypoxia, hypoxia and hypoxia-normoxia.

All MSC were cultured as previously described, with anti-CD3/CD28 CFSE labelled PBMC at ratios of 1:5, 1:10, 1:20 and 1:40 MSC: PBMC. As observed previously, MSC inhibition of proliferation was significant at a ratio of 1:5 MSC: PBMC (Figures 3.7-3.9). However, normoxic or hypoxic MSC could not significantly reduce T cell proliferation at ratios of 1:10, 1:20 and 1:40 MSC: PBMC (Figure 3.10). Not surprisingly, when short term hypoxic MSC (N-H) were challenged with the same task they too failed to significantly suppress T cell proliferation at ratios of 1:10, 1:20 and 1:40 (Figure 3.11 A). However, in an independent experiment, hypoxic- normoxic MSC (H-N) maintained immunosuppression at 1 MSC: 10 PBMC but this was lost at higher MSC: PBMC ratios (Figure 3.11 B).

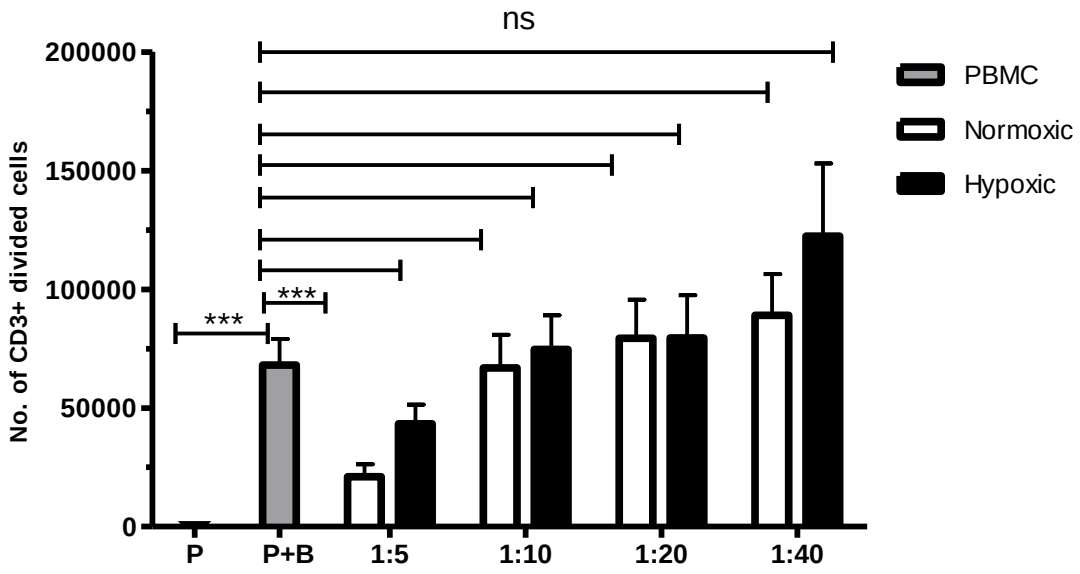



**Figure 3.10 MSC mediated suppression of anti-CD3/CD28 driven PBMC proliferation was dose dependent.** The capacity for MSC to inhibit anti-CD3/CD28 driven PBMC was measured using a carboxyfluorescein succinimidyl ester (CFSE) assay. A CFSE assay was performed as previously described in section 2.7.2, in the presence of increasing concentrations of anti-CD3/CD28 stimulated PBMC (P+B) to MSC per well. PBMC were cultured alone/unstimulated, or stimulated with anti-CD3/CD28 activation beads as controls. CFSE dilution was analysed in gated $CD3^{+}7AAD^{-}$ cells, and the absolute number of $CD3^{+}$CFSE dividing cells was enumerated using counting beads on day 4. Both normoxic and hypoxic MSC (20 days) immunosuppressive capabilities were lost at ratios of 1:10, 1:20, and 1:40 MSC: PBMC. Data represented as mean ± standard error of the mean (SEM) of n=6 (3 PBMC donors and 2 MSC donors performed in duplicate). Statistical analysis was carried out using a student's unpaired *t-test* between two groups. ***, P<0.001

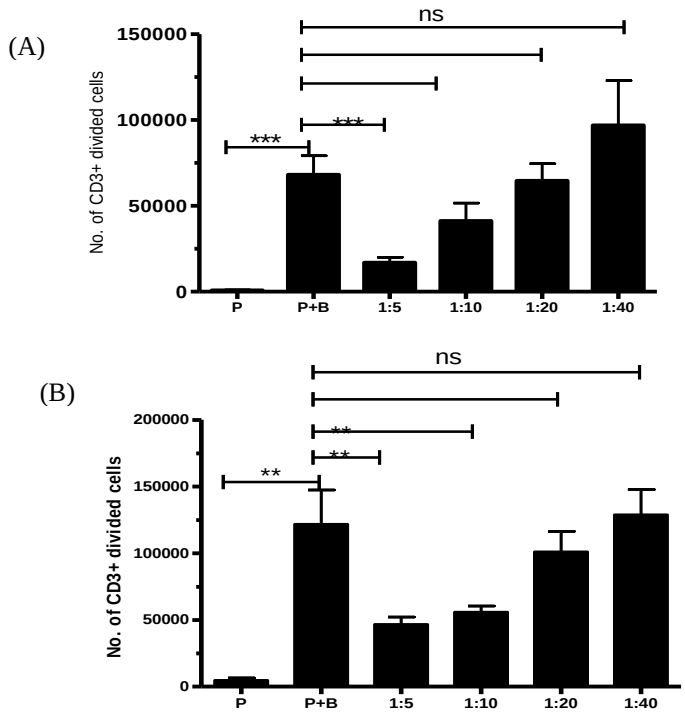



**Figure 3.11 MSC mediated suppression of anti-CD3/CD28 driven PBMC proliferation was dose dependent.** The capacity for normoxic- hypoxic MSC(8 days) (A) and hypoxic- normoxic MSC (8 days) (B) to inhibit anti-CD3/CD28 driven PBMC was measured using a CFSE assay. In brief, PBMC donors were stimulated with anti-CD3/CD28 activation beads and co-cultured with MSC for 4 days at a ratio of 1:5- 1:40 (MSC: PBMC). PBMC were cultured alone/unstimulated, or stimulated with anti-CD3/CD28 activation beads as controls. CFSE dilution was analysed in gated $CD3^{+}7AAD^{-}$ cells, and the absolute number of $CD3^{+}$CFSE dividing cells was enumerated using counting beads on day 4. Normoxic –hypoxic MSC were significantly suppressive at a 1:5 ratio (A). However, in an independent experiment, hypoxic-normoxic MSC displayed significant suppressive properties at both 1:5 and 1:10 ratios (B) Data represented as mean ± standard error of the mean (SEM) of n=6 (2 MSC donors and 3 PBMC donors). (A) and (B) are independent experiments. Statistical analysis was carried out using a student's unpaired *t-test* between two means. **, $P<0.01$; ***, $P<0.001$.

## 3.9 CULTURE IN HYPOXIA HAD NO IMPACT ON IFN-γ INDUCTION OF CCL2 IN HUMAN MSC

A plethora of studies have demonstrated the requirement of cell-contact for MSC Immunomodulation. Chemokines such as CCL2, also known as monocyte chemotactic protein-1 (MCP-1), play an important role in selectively recruiting a number of cells including lymphocytes (Deshmane *et al.* 2009). Once lymphocytes have been recruited to within close proximity of MSC, they can mediate their effects via soluble factors. Akiyama *et al.,* (2012) elegantly demonstrated that murine MSC require cell contact in order to mediate their effects (Akiyama *et al* 2012*).* The authors described the release of CCL2 by murine MSC and consequent recruitment of T lymphocytes for FAS-L mediated apoptosis (Akiyama *et. al,* 2012).

To examine MSC expression of CCL2 by real-time PCR, MSC were seeded into wells of a 6 well plate at 5 x$10^4$ MSC per well and cultured in their respective oxygen tensions. MSC were then stimulated with 50ng/ml IFN-γ for 6 hours. Culture medium was then removed and MSC were lysed with Tri-reagent. RNA isolation was then performed as described in section 2.6.1. RNA was then reverse transcribed into complementary DNA as outlined in section 2.6.2. To examine MSC production of CCL2 by ELISA, MSC were seeded into wells of 6 well plate at 5 x$10^4$ MSC per well and cultured in their respective oxygen tensions. MSC were then stimulated with 50ng/ml IFN-γ for 24 hours. Culture medium was then collected for analysis by ELISA. Real-time PCR analysis of CCL2 expression by MSC revealed that isolated MSC were capable of a strong up regulation of CCL2 in response to IFN-γ (Figure 3.12 A). Furthermore, culture of MSC in long term hypoxia did not hinder their capacity to upregulate CCL2 mRNA or protein (Figure 3.12 B) in response to IFN-γ stimulation.

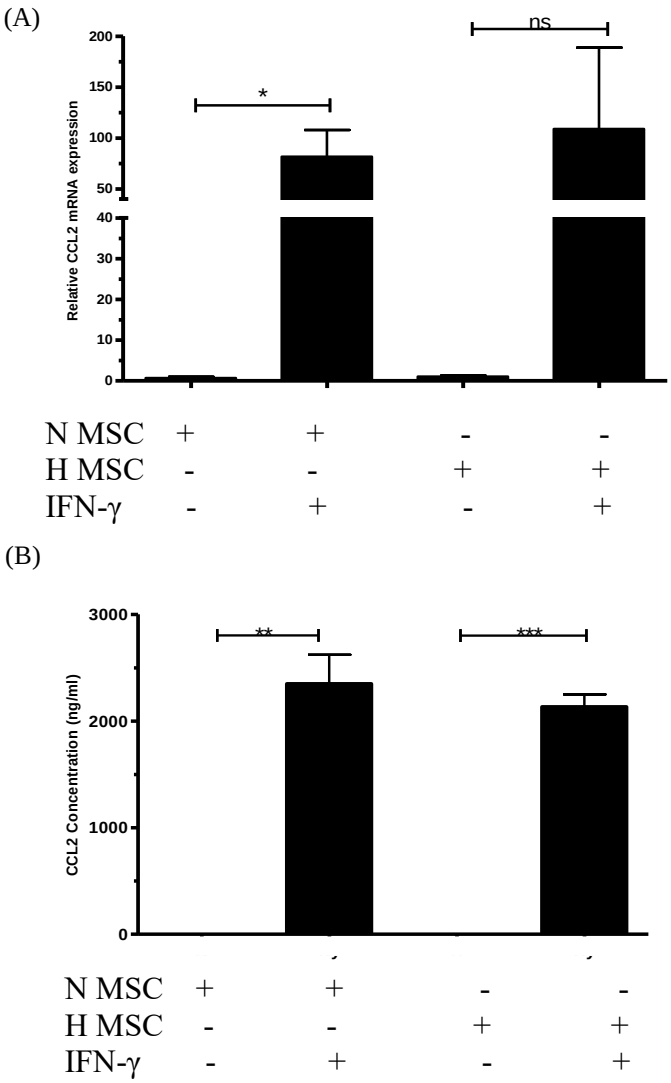



**Figure 3.12 CCL2 expression by MSC is up regulated by IFN-γ.** (A) MSC were seeded into 6 well plates at a density of 5 x $10^4$ MSC per well. mRNA from 6 hours of unstimulated or IFN-γ (50ng/ml) stimulated MSC was isolated and assayed by RT-PCR. Data represented as mean ± standard error of the mean (SEM) of 3 independent MSC donors each performed in duplicate. GAPDH expression was used as a house-keeping control. (B) An ELISA was performed on MSC cultured alone (control) or following IFN-γ (50ng/ml) stimulation for 24 hour. Data represented as mean ± standard error of the mean (SEM) of n=2 independent MSC donors performed in triplicate. Statistical analysis performed using the students' unpaired *t*-test between two groups where *, P<0.05, **, P<0.01 and ***, P<0.001.

## 3.10 CULTURE IN HYPOXIA HAD NO IMPACT ON IFN-γ INDUCTION OF CXCL9 IN HUMAN MSC

The multi-faceted immunoregulatory capacity of MSC also employs the use of lymphocyte chemotactic protein; CXCL9 or monokine induced by gamma interferon (MIG). CXCL9 binds to its receptor CXCR3 on T lymphocytes to induce T lymphocyte migration (Groom & Luster 2011). An elegant study by Ren *et al.,* (2008) showed that cytokine activated MSC expressed CXCL9 and that blockade of CXCR3 inhibited the recruitment of T cells to the MSC lair and prevented the suppression of T cell activation (Ren *et al*. 2008). Real-time PCR analysis of CXCL9 expression by MSC revealed that isolated MSC were capable of a strong up regulation of CXCL9 in response to IFN-γ (Figure 3.13 A). Furthermore, culture of MSC in long term hypoxia did not hinder their capacity to upregulate CXCL9 mRNA or protein (Figure 3.13 B) in response to IFN-γ stimulation.

## 3.11 CULTURE IN HYPOXIA HAD NO IMPACT ON IFN-γ INDUCTION OF ICAM-1 IN HUMAN MSC

The chemoattraction initiated by MSC to recruit lymphocytes allows for localised MSC immunoregulation. Expression of intracellular adhesion molecule 1 (ICAM-1) by human MSC is induced following cytokine stimulation (Majumdar *et al.* 2003) and its ligand, LFA-1 is expressed on lymphocytes (Kürzinger *et al*. 1981; Binnerts *et al*. 1994). Moreover, MSC expression of ICAM-1 is necessary for contact-dependent interaction with T cells (Ren *et al*. 2010). Therefore, the expression of ICAM-1 by normoxic and hypoxic MSC was analysed by RT-PCR and flow cytometry.

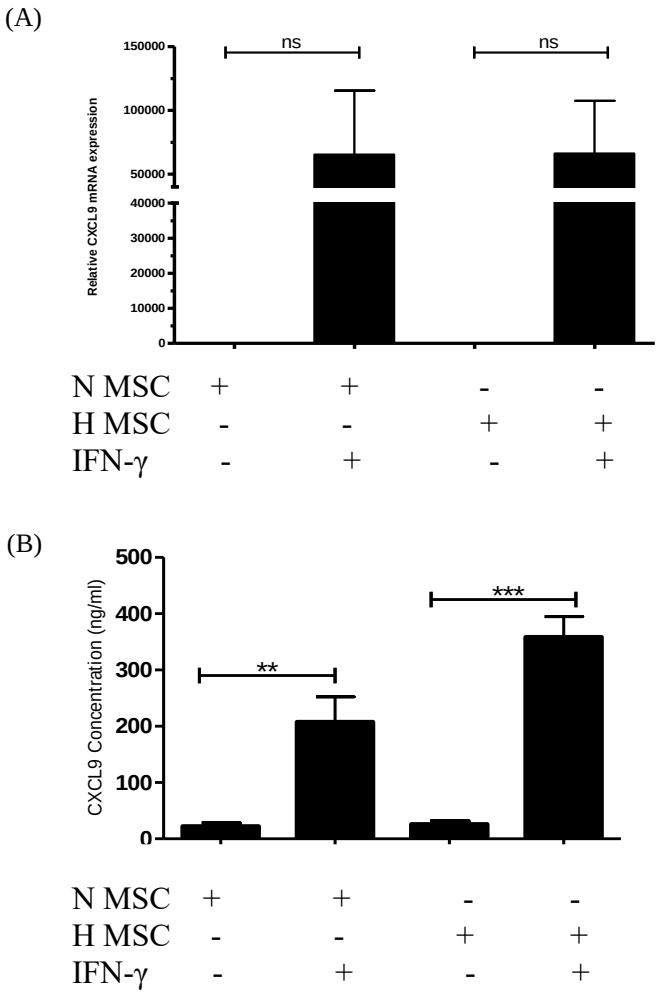



**Figure 3.13 CXCL9 expression by MSC is up regulated by IFN-γ.** (A) MSC (N MSC; long term normoxic, H MSC; long term hypoxic MSC) were seeded into 6 well plates at a density of 5 x$10^4$ MSC per well. mRNA from 6 hours of unstimulated or IFN-γ (50ng/ml) stimulated MSC was isolated and assayed by RT-PCR. n= 3 independent MSC donors performed in duplicate and presented as mean ± standard error of the mean (SEM). (B) An ELISA was performed on MSC cultured alone (control) or following IFN-γ (50ng/ml) stimulation for 24 hours. GAPDH expression was used as a house-keeping control. Data represented as mean ± standard error of the mean (SEM) of 2 individual MSC donors. Statistical analysis was performed using the students' unpaired *t*-test between two groups. **, P<0.01 and ***, P<0.001.

6 hour cultures of MSC were either unstimulated as a control or stimulated with 50ng/ml IFN-γ for 6 hours and subsequently analysed for ICAM-1 mRNA analysis as described previously in section 2.6.1. For surface marker expression, MSC were seeded into wells of a 6 well plate and either unstimulated control or stimulated with 50ng/ml IFN-γ for 24 hours and subsequently analysed via flow cytometry. MSC cultured under normoxia and hypoxia were capable of up regulating ICAM-1 mRNA upon IFN-γ stimulation (Figure 3.14 A). Moreover, this up regulation was equally represented at the cell surface when MSC were stimulated with IFN-γ for 24 hours (Figure 3.14 B). Short term culture of MSC in normoxic or hypoxia for 8 days(N-H and H-N) did not negatively impair capacity to upregulate ICAM-1 in comparison to normoxic or hypoxic MSC. This data shows no negative impact of hypoxic culture of MSC expression of ICAM-1 at the mRNA and protein levels.

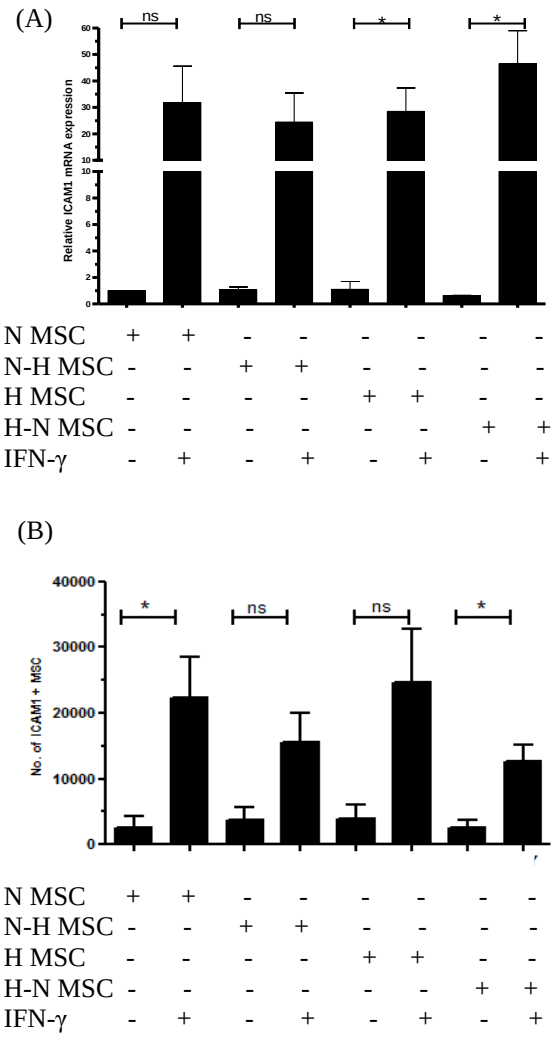



**Figure 3.14 ICAM-1 expression by MSC is up regulated by IFN-γ.** (A) mRNA from 6 hours of unstimulated or IFN-γ (50ng/ml) stimulated MSC was isolated and assayed by RT-PCR. (B) Flow cytometry analysis was performed on MSC cultured alone (control) or following IFN-γ (50ng/ml) stimulation for 24 hour cultures. n=3 independent MSC donors. Statistical analysis performed using the students' unpaired *t*-test using normoxic unstimulated samples as the control. Data represented as mean ± standard error of the mean (SEM) of 3 individual MSC donors. Statistical analysis was performed using the students' unpaired t-*test* between two groups. *, $P<0.05$. N&H MSC; long term N&H(20 days), N-H(+8 days hypoxia), MSC; short term hypoxic MSC H-N(+8 days normoxia) MSC; short term normoxic MSC.

## 3.12 CULTURE IN HYPOXIA HAD NO IMPACT ON IFN-γ INDUCTION OF IDO IN HUMAN MSC

L-Tryptophan is an amino acid required for T cell proliferation. Indoleamine 2, 3-dioxygenase (IDO) is an enzyme that catabolises L-Tryptophan into Kynurenine metabolites and is a pivotal mediator of MSC immunosuppression (Fallarino *et al.* 2002; Jasperson *et al.* 2008). The effects of long term hypoxia on the expression of IDO by human bone marrow derived MSC have yet to be fully elucidated. Therefore, it was important to ensure that long term culture of MSC in hypoxia did not abrogate their ability to up regulate IDO in response to IFN-γ stimulation. To examine MSC expression of IDO by real-time PCR, MSC were seeded into wells of 6 well plates at 5 $x10^4$ MSC per well and cultured in their respective oxygen tensions. MSC were then stimulated with 50ng/ml IFN-γ for 6 hours for RT-PCR analysis and for 6 hours and 24 hours for flow cytometry.

Real-time PCR analysis of IDO expression by MSC revealed that MSC were capable of up regulating IDO in response to a 6 hour stimulation with IFN-γ (Figure 3.15 A). Moreover, IDO was significantly upregulated by hypoxic cultured MSC stimulated with IFN-γ for 6 hours (Figure 3.15 B). Short term culture of MSC in normoxic or hypoxia for 8 days (N-H and H-N) did not negatively impair capacity to upregulate IDO mRNA in comparison to normoxic or hypoxic MSC. Furthermore, 24 hour IFN-γ stimulation increased the number of $IDO^+$ MSC in both normoxic and hypoxic MSC cultures (Figure 3.15 C). Therefore, culture of MSC in long term hypoxia did not hinder their capacity to upregulate IDO mRNA in response to IFN-γ stimulation, suggesting the possible down regulation of a different immunomodulatory mediator produced by hypoxic cultured MSC.

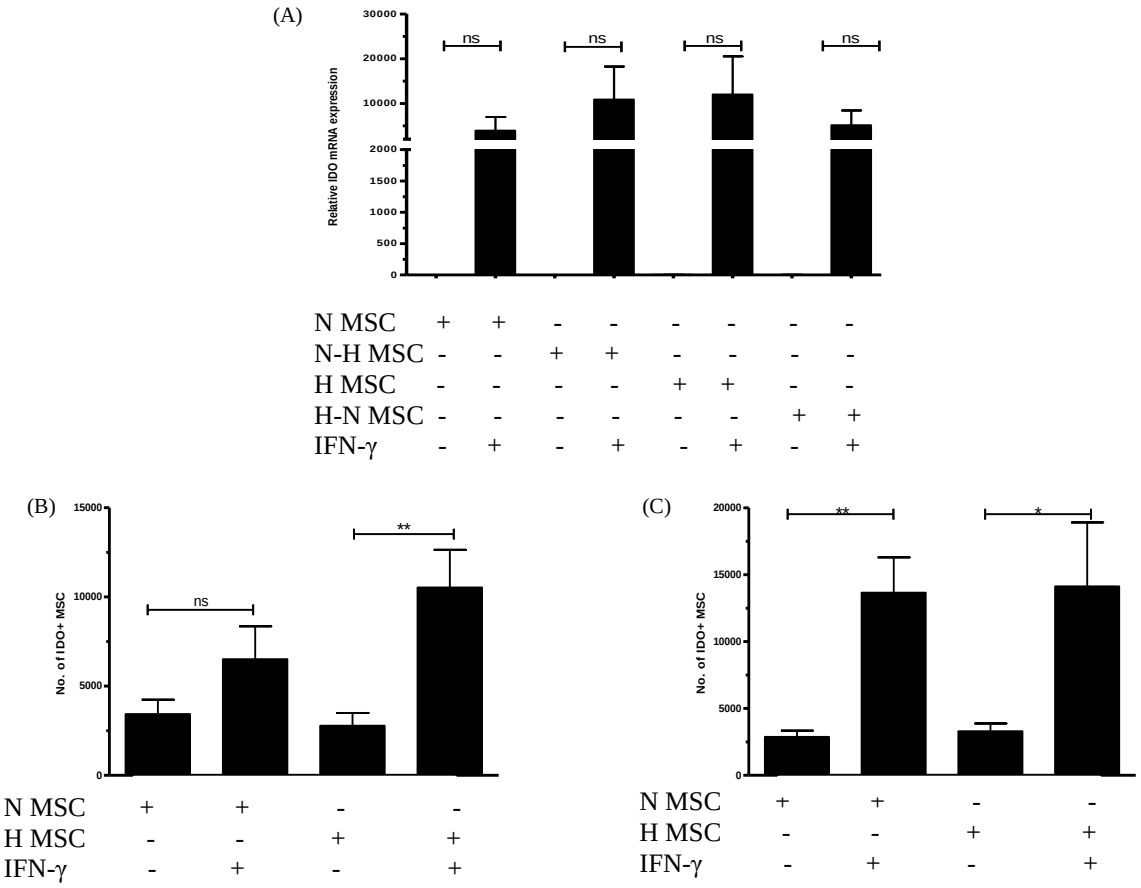



**Figure 3.15 IDO expression by MSC is upregulated by IFN-γ.** (A) Inducible mRNA expression of IDO by MSC was examined by Real Time PCR. Six hour cultures of unstimulated long term MSC (N & H) and IFN-γ (50ng/ml) stimulated long term MSC (Nγ & Hγ) were assayed for IDO mRNA expression. Analysis revealed MSC from all cultures were capable of up regulating IDO in response to IFN-γ stimulation. Flow cytometry analysis was performed on MSC cultured alone (control) or following IFN-γ (50ng/ml) stimulation for 6 hour (B) and (C) 24 hour cultures. Total number of $IDO^+$ MSC was assessed using counting beads. Data reported as mean ± standard error of the mean (SEM) of 3 individual MSC donors. Statistical analysis was performed using the students' unpaired t-*test* between two groups. *, $P<0.05$, **, $P<0.01$.

## 3.13 CULTURE IN HYPOXIA HAD NO IMPACT ON IFN-γ INDUCTION OF PD-L1 IN HUMAN MSC

Programmed death-ligand 1 has emerged as an important inhibitory pathway of T cell activation (Butte *et al*. 2007) and has been implicated in MSC mediated immune suppression (Augello *et al.* 2005). More recently, the PD-L1 pathway has been described as an IDO independent mechanism of suppressing T cell effector functions by MSC (Chinnadurai *et al.* 2014). MSC were therefore stimulated with IFN-γ to examine the effect of hypoxic culture on MSC expression of PD-L1.

6 hour cultures of MSC were either unstimulated as a control or stimulated with 50ng/ml IFN-γ for 6 hours and subsequently analysed for mRNA analysis. For surface marker expression, MSC were seeded into wells of a 6 well plate and either unstimulated control or stimulated with 50ng/ml IFN-γ for 24 and 48 hours and analysed via flow cytometry. All MSC cultured under their respective oxygen tensions were capable of up regulating PD-L1 mRNA when stimulated with IFN-γ (Figure. 3.16 A). Importantly, Short term culture of MSC in normoxic or hypoxia for 8 days (N-H and H-N) did not negatively impair capacity to upregulate PDL-1 in comparison to normoxic or hypoxic MSC. This up regulation was equally represented at the cell surface when MSC were stimulated with IFN-γ for 24 hours (Figure 3.16 B). These data display the importance that IFN-γ plays in up regulating PD-L1 in both normoxic and hypoxic MSC and shows no negative impact of hypoxic culture of MSC expression of PD-L1 at the mRNA and protein levels.

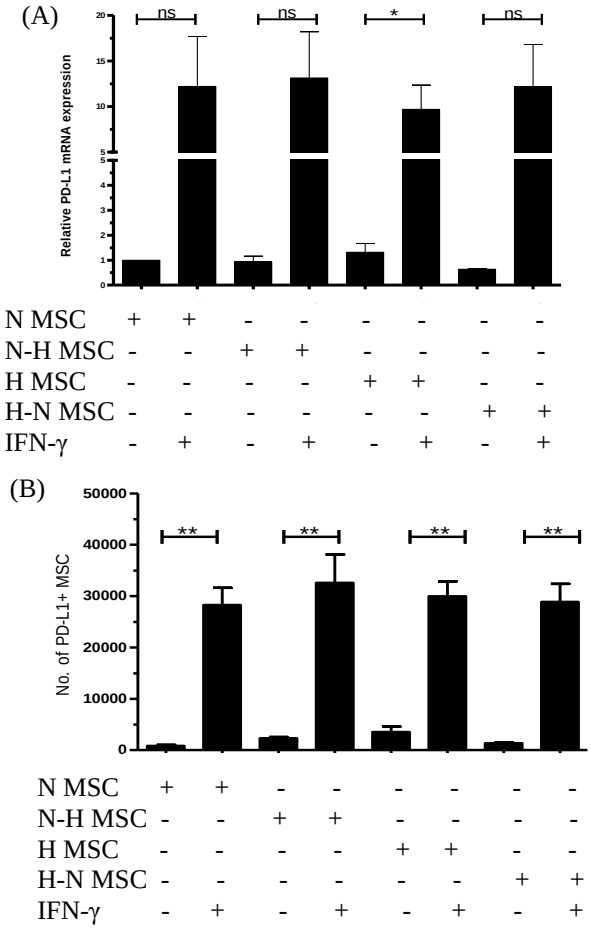



**Figure 3.16 PD-L1 expression by MSC is up regulated by IFN-γ.** (A) Inducible mRNA expression of PD-L1 by MSC was examined by Real Time PCR. Six hour cultures of unstimulated MSC (long term ;N & H and short term; N-H &H-N ) and IFN-γ (50ng/ml) MSC (long term;N & H and short term; N-H &H-N) were assayed for PD-L1 mRNA expression. Analysis revealed MSC from all cultures were capable of up regulating PD-L1 in response to IFN-γ stimulation. Flow cytometry analysis was performed on MSC cultured alone (control) or following IFN-γ (50nηg/ml) stimulation for 24 hours. n= 3 individual MSC donors performed in duplicate and total number of PD-L1$^{+}$ MSC was assessed using counting beads. Data reported as mean ± standard error of the mean (SEM) of 3 individual MSC donors. Statistical analysis was performed using the students' unpaired t-*test* between two groups. *, P<0.05, **, P<0.01.

## 3.14 INVESTIGATION OF THE EFFECT OF LONG TERM HYPOXIC CULTURE ON MSC EXPRESSION OF COX-2 AND PRODUCTION OF PGE-2

### 3.14.1 CULTURE IN HYPOXIA ATTENUATES MSC INDUCIBLE EXPRESSION OF COX-2 AND PGE-2

The expression of the enzyme cyclooxgenase-2 (COX-2) is responsible for the production of the rapid, short acting lipid mediator prostaglandin E-2 (PGE-2) by MSC (Ryan et al. 2007). Furthermore, PGE-2 production by MSC is induced by TNF-α stimulation (English *et al.* 2008; Ren *et al.* 2008; Aggarwal & Pittenger 2009; Hemeda *et al.* 2010). Importantly, a large body of data has highlighted the role of PGE-2 in MSC immunomodulation (English *et al.* 2007; Ryan *et al.* 2007; Aggarwal & Pittenger 2009; English *et al.* 2009; Spaggiari *et al.* 2009; Németh *et al*. 2009; Najar, Raicevic, Boufker, *et al.* 2010; Yañez *et al.* 2010). Studies have shown that TNF-α, a pro-inflammatory cytokine, is required to license MSC so they can exert their immunosuppressive actions (Rasmusson et al. 2005; Aggarwal & Pittenger 2009; Ren *et al*. 2009) and acts synergistically with IFN-γ to enhance its production by MSC (English *et al.* 2007; Ren *et al*. 2008; Spaggiari *et al.* 2015). Therefore, the effect of long term hypoxic culture on MSC expression of COX-2 and production of PGE-2 was examined.

6 hour cultures of normoxic or hypoxic MSC were either unstimulated as a control or stimulated with 20ng/ml TNF-α for 6 hours and subsequently analysed for COX-2 mRNA analysis. For detection of the lipid mediator PGE-2, MSC were seeded into wells of a 6 well plate and either unstimulated control or stimulated with 20ng/ml TNF-α for 24 hours and analysed via ELISA. Normoxic MSC were capable of up regulating COX-2 mRNA when stimulated with TNF-α (Figure. 3.17 A). Intriguingly, this up regulation was not equally represented by TNF-α stimulated hypoxic MSC

(Figure. 3.17 A). Moreover, examination of PGE-2 in TNF-α stimulated MSC supernatants verified the attenuating effect of long term hypoxic culture on MSC production of PGE-2 (Figure 3.17 B).

### 3.14.2 CULTURE IN HYPOXIA IMPAIRS MSC CAPACITY TO UPREGULATE EXPRESSION OF COX-2 DURING CO-CULTURE WITH PBMC

Figure 3.17 highlighted the detrimental effect of hypoxic culture on MSC expression of COX-2 mRNA and PGE-2 production *in-vitro*. Given that hypoxic culture did not negatively impair other MSC mediators investigated, it was hypothesised that hypoxic MSC harvested from a MSC: PBMC co-culture would express reduced COX-2 in comparison to normoxic MSC. Therefore, the levels of COX-2 expression were analysed in normoxic and hypoxic MSC harvested from a MSC PBMC co-culture. In order to investigate sufficient quantities of mRNA, normoxic and hypoxic MSC were seeded into duplicate wells of a 24 well plate at $2.5x10^4$ MSC per well and placed back into normoxia or hypoxia. 24 hours later, $1.25x10^5$ PBMC were added to the MSC with Phorbol Myristate Acetate (PMA) (100ng/ml) and Ionomycin (1µg/ml). On day 4, MSC were harvested and analysed for COX-2 mRNA expression as outlined in section 2.6.

Importantly, COX-2 mRNA was markedly increased in normoxic MSC harvested from a co-culture (Figure 3.18). Interestingly, and in agreement with the hypothesis, hypoxic MSC were less able to upregulate COX-2 mRNA expression (Figure 3.18).

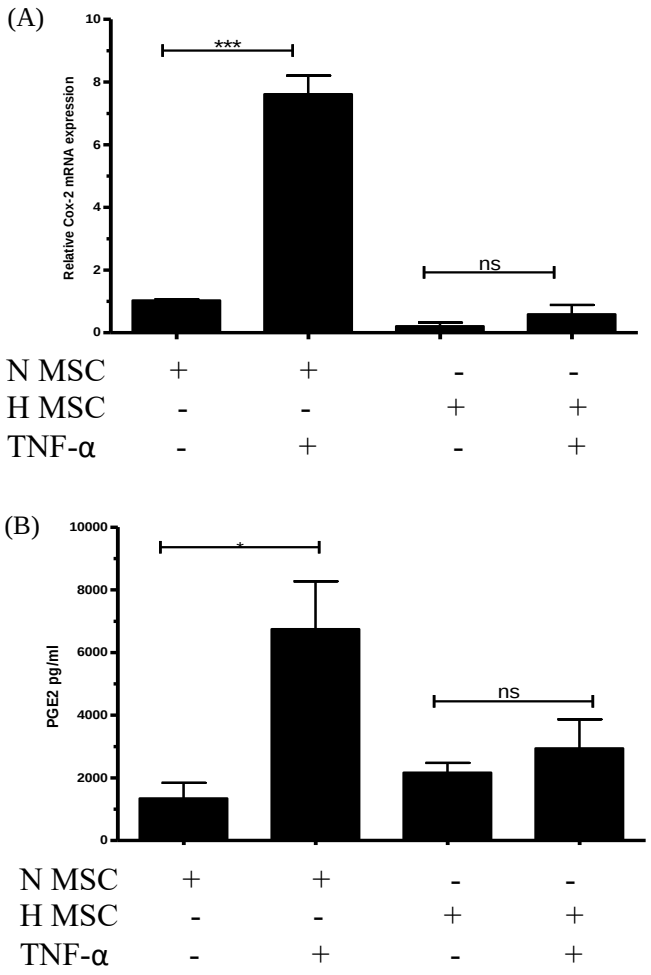



**Figure 3.17 COX-2 and PGE-2 expression by MSC is attenuated by hypoxic culture.** (A) Inducible mRNA expression of COX-2 by MSC was examined by Real Time PCR. Six hour cultures of unstimulated MSC (N & H) and TNF-α (20ng/ml) stimulated MSC (Nα & Hα) were lysed and assayed for COX-2 mRNA expression. Analysis revealed MSC from normoxic cultures were capable of up regulating COX-2 in response to TNF-stimulation. (B) ELISA analysis was performed on MSC supernatant cultured alone (control) or following TNF-α (20ng/ml) stimulation for 24 hours. Supernatant from stimulated MSC were diluted 1:2 in RPMI prior to addition to the wells. Data reported as mean ± standard error of the mean (SEM) of 3 individual MSC donors. Statistical analysis performed using the students' unpaired *t-test* between two groups. *, $P<0.05$, ***, $P<0.001$.

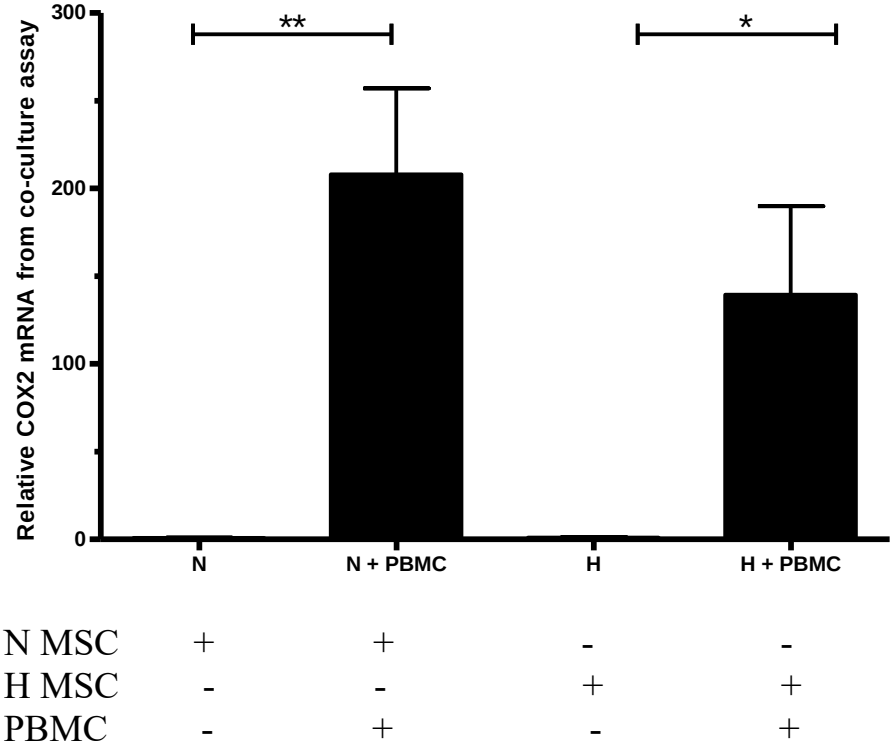



**Figure 3.18 COX-2 expression by MSC is attenuated by hypoxic culture.** Inducible mRNA expression of COX-2 by MSC was examined by Real Time PCR. $2.5x10^4$ MSC were seeded into a 24 well plate and 24 hours later, $1.25x10^5$ PBMC were added with PMA (100ng/ml) and Ionomycin (1µg/ml). MSC were harvested for mRNA analysis as outlined in section 2.6 on day 4 and analysed for COX-2 mRNA expression. Analysis revealed MSC from normoxic cultures were capable of up regulating COX-2 when in a co-culture with PBMC. Moreover, Hypoxic MSC were less potent inducers of COX-2 mRNA in comparison to normoxic MSC in a MSC PBMC co-culture. Data reported as mean ± standard error of the mean (SEM) of 5 MSC donors (1 PBMC donor and 5 individual MSC donors). Statistical analysis performed using the students' unpaired *t-test* between two groups. *, $P<0.05$, **, $P<0.01$. N; normoxic MSC, N+PBMC; normoxic MSC from co-culture with PBMC, H; hypoxic MSC, H +PBMC; hypoxic MSC from co-culture with PBMC.

## 3.15 SUMMARY

The main aims of this chapter were to (1) characterise the effect of hypoxic culture on MSC proliferation, cell surface markers and multi-lineage differentiation capabilities, (2) investigate the impact of hypoxic culture on MSC capacity to reduce T cell proliferation *in-vitro* and (3) to examine the effect, if any, on MSC expression and production of immune mediators. The proliferation of MSC was significantly increased after hypoxic culture in comparison to normoxic culture across a range of passages. Furthermore, hypoxic culture did not impair MSC expression of cell surface markers employed to identify them *in-vitro.* Interestingly, hypoxia enhanced MSC osteogenic capacity and reduced adipogenesis in comparison to normoxic cultured MSC.

The next aim of this study was to examine and compare the capacity of hypoxic cultured MSC to normoxic MSC to suppress T cell proliferation. Not surprisingly, normoxic MSC suppressed T cell proliferation, however, hypoxic MSC were less potent suppressor of T cell proliferation. It was therefore important to investigate if shorter periods of culture in hypoxia generated the same results. Intriguingly, culture of MSC in short term hypoxia (8 days; N-H) did not impair their capacity to reduce T cell proliferation. It was therefore questioned if MSC (that had been cultured in long term hypoxic) could be re-educated by 8 days of normoxic culture (H-N). Surprisingly, MSC (H-N) regained the capacity to suppress T cell proliferation. The next step of investigation was to elucidate the influence of long term hypoxic culture on MSC expression or production of immunomodulatory mediators. Notably, MSC expression of known immunomodulatory mediators, chemokines (CCL2, CXCL9), adhesion molecule ICAM-1, IDO and PD-L1 were not impaired by hypoxic culture. Investigations into inducible COX-2 mRNA and PGE-2 production demonstrated that hypoxic culture impairs MSC capacity to produce comparable levels to normoxic

cultured MSC. This impairment was verified by culturing normoxic and hypoxic MSC with activated PBMC and harvesting mRNA from the MSC.

Investigations into the effect of long term culture in 5% $O_2$ on MSC are severely lacking. This chapter has highlighted the influence of hypoxia on the *in-vitro* nature of MSC. Given that hypoxic MSC were less potent suppressors of T cell proliferation *in-vitro,* it was important to next examine if hypoxic cultured MSC could be successfully employed as an immunosuppressive cell therapy *in-vivo* in a humanised mouse model aGvHD mice in comparison to normoxic MSC.

# CHAPTER 4

# INVESTIGATION OF THE THERAPEUTIC EFFICACY OF HYPOXIC MSC IN A HUMANISED MOUSE MODEL OF AGVHD

## 4.1 INTRODUCTION

HSCT is an effective cellular immunotherapy for a number of hematological malignancies and inherited blood disorders (Reddy & Ferrara 2003; Baron & Storb 2006). The ultimate goal of HSCT is to provide disease free survival, preserve Graft-versus-Leukaemia (GVL) effects and minimise associated effects of immunosuppression and drug toxicities (Deeg 2007). However, its efficacy is restricted by the development of graft-versus-host disease (GvHD). Typical treatment for aGvHD involves a standard course of steroids (MacMillan, Weisdorf, Wagner, *et al*. 2002) which are usually initially effective, but ultimately represent an insufficient therapy for patients with higher grades of GvHD and particularly steroid refractory GvHD patients (MacMillan, Weisdorf, Wagner, *et al.* 2002; Chen *et al.* 2015). Unfortunately there are no effective therapies, however, MSC have emerged as a potential therapy for patients with acute GvHD. The immunosuppressive and immune evasive properties displayed by MSC highlighted these cells as an attractive candidate for the treatment of GvHD (Ryan *et al*. 2005; Le Blanc & Ringdén 2005; Polchert *et al*. 2008). In a seminal study by Le Blanc *et al.* MSC were administered to a patient suffering from steroid refractory GvHD(Le Blanc *et al.* 2004). Remarkably, the patient's symptoms were ameliorated and liver function improved as assessed by bilirubin levels. Long-term benefits were not apparent after post-transplant immunosuppression was discontinued, however a second infusion of MSC resolved GvHD symptoms again (Le Blanc *et al.* 2004). Here, the dynamics of MSC treatment and their exact mechanism of therapy were not delineated and thus intense research is now focused on elucidating the mechanisms of action at play in order to facilitate the routine application of MSC in the clinic for GvHD (Lucchini *et al*. 2010; Martin *et al*. 2010; Prasad *et al.* 2011; von Bahr *et al*. 2012; Kurtzberg *et al*. 2014).

Importantly, MSC require *ex-vivo* expansion to generate sufficient quantities for administration to patients with GvHD. In the clinic, MSC dosing ranges from 1 $x10^6$ - 8 $x10^6$ cells per kg (Le Blanc *et al*. 2004; Fang *et al*. 2007; Martin *et al*. 2010; Prasad *et al*. 2011; Ball *et al*. 2013; Kurtzberg *et al.* 2014) administered as one dose (Fang *et al.* 2007), two doses (Le Blanc *et al.* 2004; Ball *et al.* 2013) or multiple MSC infusions (Martin *et al.* 2010; Prasad *et al.* 2011; Kurtzberg *et al.* 2014) and therefore must undergo long-term *ex-vivo* culture to obtain these numbers. Unpublished data from our lab harnessing a pre-clinical model of aGvHD has demonstrated no benefit of two doses of MSC over one in prolonging the survival of aGvHD mice (Healy, 2015 Thesis).

Culture expansion of MSC is generally performed at 20-21% $O_2$ (normoxia) however, oxygen levels *in-vivo* are much lower than this (Caldwell *et al.* 2001; Saltzman *et al.* 2003; Wild *et al.* 2005; Ivanovic 2009) and normoxia may be regarded considerably hyperoxic (excess supply of oxygen) for cell culture (Masalunga *et al.* 2007; Martinez *et al.*. 2008; Estrada *et al.* 2012; Zhang *et al.* 2016). We and others have reported that lower levels of oxygen (hypoxia) increase MSC proliferation *in-vitro* (Lennon *et al.* 2001; Grayson *et al.*2007; Nekanti *et al.* 2010; Tsai *et al.* 2011; Hung *et al.*. 2012; Valorani *et al.* 2012; Nold *et al.* 2014). The ability to deploy hypoxic cultured MSC to preclinical and clinical scenarios of aGvHD could reduce operating fees associated with long term culture and reach more patients in the clinic. The study performed in chapter 3 identified the importance of hypoxic culture for enhancing MSC proliferation, however, (long term) hypoxic cultured MSC were less potent suppressors of T cell proliferation *in-vitro.* Therefore, the goals of this chapter were:

1. To examine and compare the capacity of hypoxic MSC to normoxic MSC to reduce weight loss, aGvHD pathology and clinical score of aGvHD mice.

2. To investigate the impact, if any, of hypoxic MSC on the engraftment of donor PBMC in a humanised mouse model of aGvHD.

## 4.2 HYPOXIC MSC SIGNIFICANTLY INCREASED SURVIVAL OF AGVHD MICE

A number of studies have examined the capacity of murine MSC (Yañez *et al*. 2006; H. Li *et al*. 2008) and human MSC (Tisato *et al.* 2007; Auletta *et al.*. 2014) to treat GvHD in murine models of the disease. However, it is pivotal to stress that murine and human MSC differ substantially; murine MSC require longer periods of time in culture (Sudres *et al.* 2006) and are more susceptible to undergo transformation in culture (Miura *et al.*. 2006). In addition, murine MSC mediate their immunomodulatory effects via nitric oxide (NO) as opposed to IDO utilised by human MSC (Huang *et al.* 2013). Therefore caution is warranted when interpreting results harnessing murine MSC in murine models of GvHD. Moreover, species differences between mice and humans must be taken into consideration for example, time of onset of GvHD, and choice of strain can impact type and severity of GvHD (Ferrara *et al.* 2009; Schroeder & DiPersio 2011). Whilst murine models have greatly facilitated the advancement of the field, humanised mouse models provide a platform to thoroughly investigate GvHD in the context of human pathology. Here, human donor T cell mediated GvHD can be studied *in-vivo* and importantly, the efficacy of human cellular therapies within the GvHD environment can be delineated and optimised.

This experiment sought to determine whether hypoxic cultured MSC suppressed aGvHD in a humanised mouse model of the disease. Extensive research within our group focussed on developing and optimising a robust and dynamic humanised mouse model of aGvHD by administering human donor PBMC to the NOD-SCID IL-2r$\gamma^{null}$

(NSG) mouse (Figure 4.1). Previous work by Tobin *et al,*. 2013 ascertained the immunosuppressive properties of normoxic cultured human MSC in this humanised mouse model. Human PBMC were isolated from buffy packs and administered to irradiated (2.4Gy) NSG by tail vein injection. Irradiated control groups received sterile PBS. Mice were monitored and the development of aGvHD was defined as previously described (Section 2.9).

As anticipated, control mice receiving sterile PBS only did not develop aGvHD. 40% of mice in the PBMC control group succumbed to aGvHD by day 7 and none survived past day 10 (Fig. 4.2). The administration of normoxic MSC to PBMC mice on day 7 significantly prolonged the survival of aGvHD mice (Fig. 4.2 A) with all mice surviving past day 10 and 60% of mice succumbing to aGvHD by day 15. Importantly, the administration of hypoxic MSC on day 7 also significantly prolonged the survival of aGvHD mice with all mice surviving past day 10 (Fig. 4.2 A) and 80% of Hypoxic MSC treated mice succumbed to aGvHD by day 15. After day 19 however, all MSC treated mice had succumbed to aGvHD (Fig. 4.2 A). Moreover, both normoxic and hypoxic MSC treated mice displayed significantly less weight loss up to day 10 (Fig. 4.2 B) and significantly reduced aGvHD clinical scoring (Fig. 4.2 C).

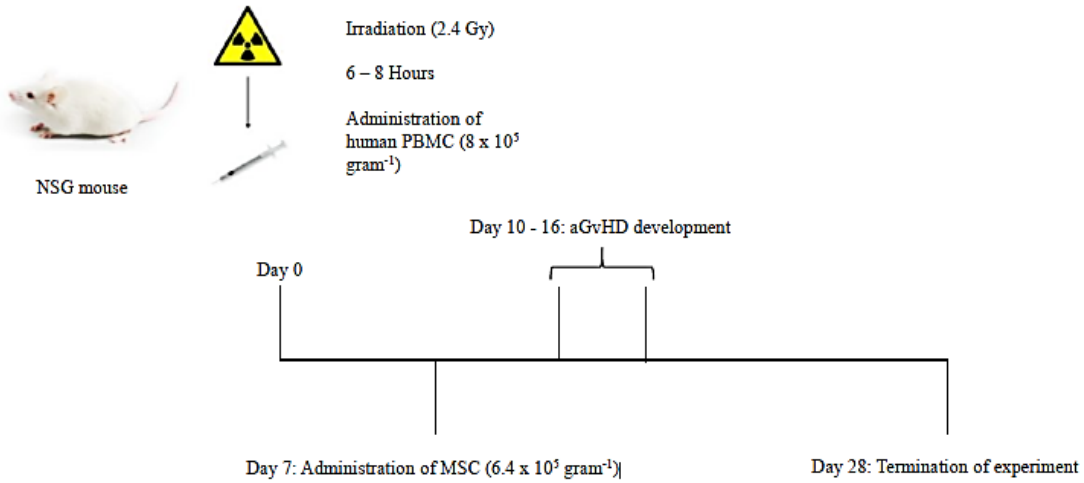



**Figure 4.1 Humanised mouse model of aGvHD experimental design.** (A) NOD-SCID IL-2rγ$^{null}$(NSG) mice were exposed to a 2.4Gy dose of gamma irradiation. 8x10$^5$ PBMC gram$^{-1}$ or sterile PBS was administered to each mouse via tail vein injection in a total volume of 300µl. 6.4x10$^4$ gram$^{-1}$ MSC were administered to mice on day 7 post irradiation. The development of aGvHD was then monitored every second day until day 8 and then everyday thereafter as outlined in section 2.9.3.

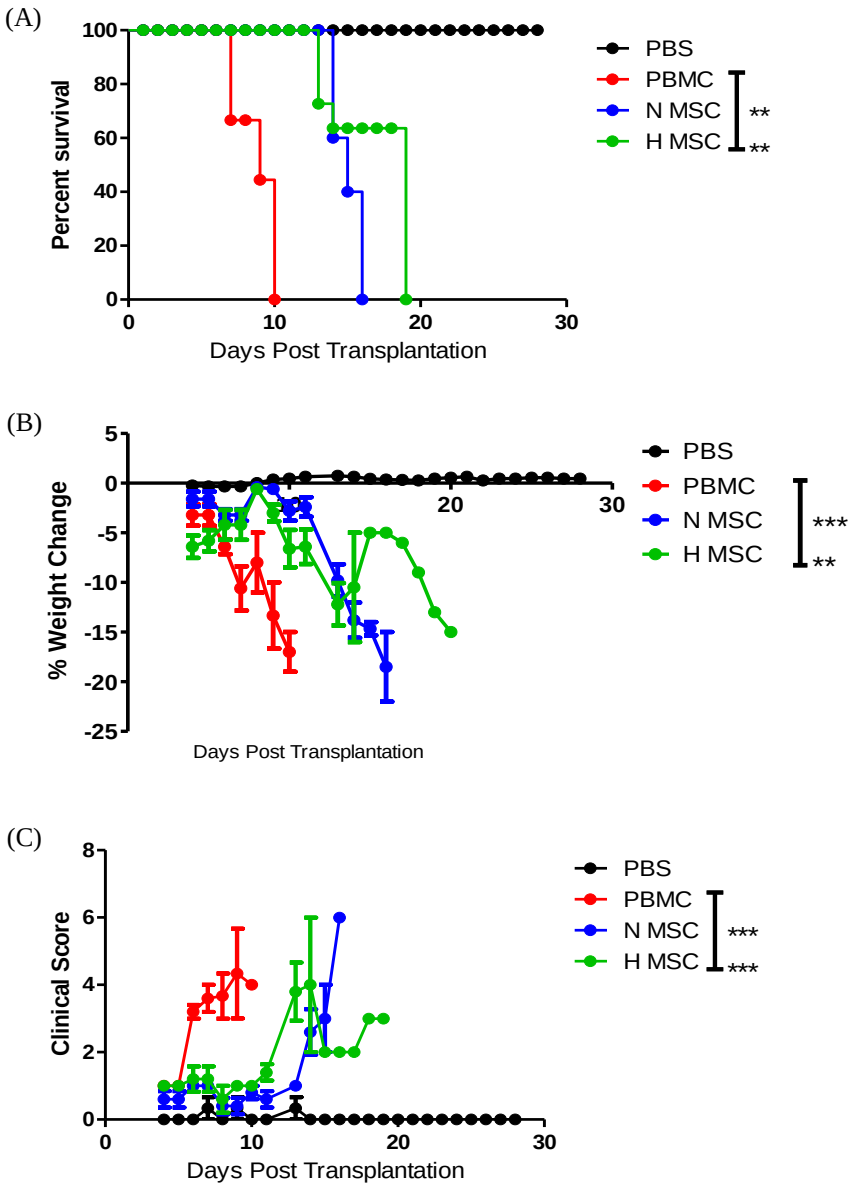
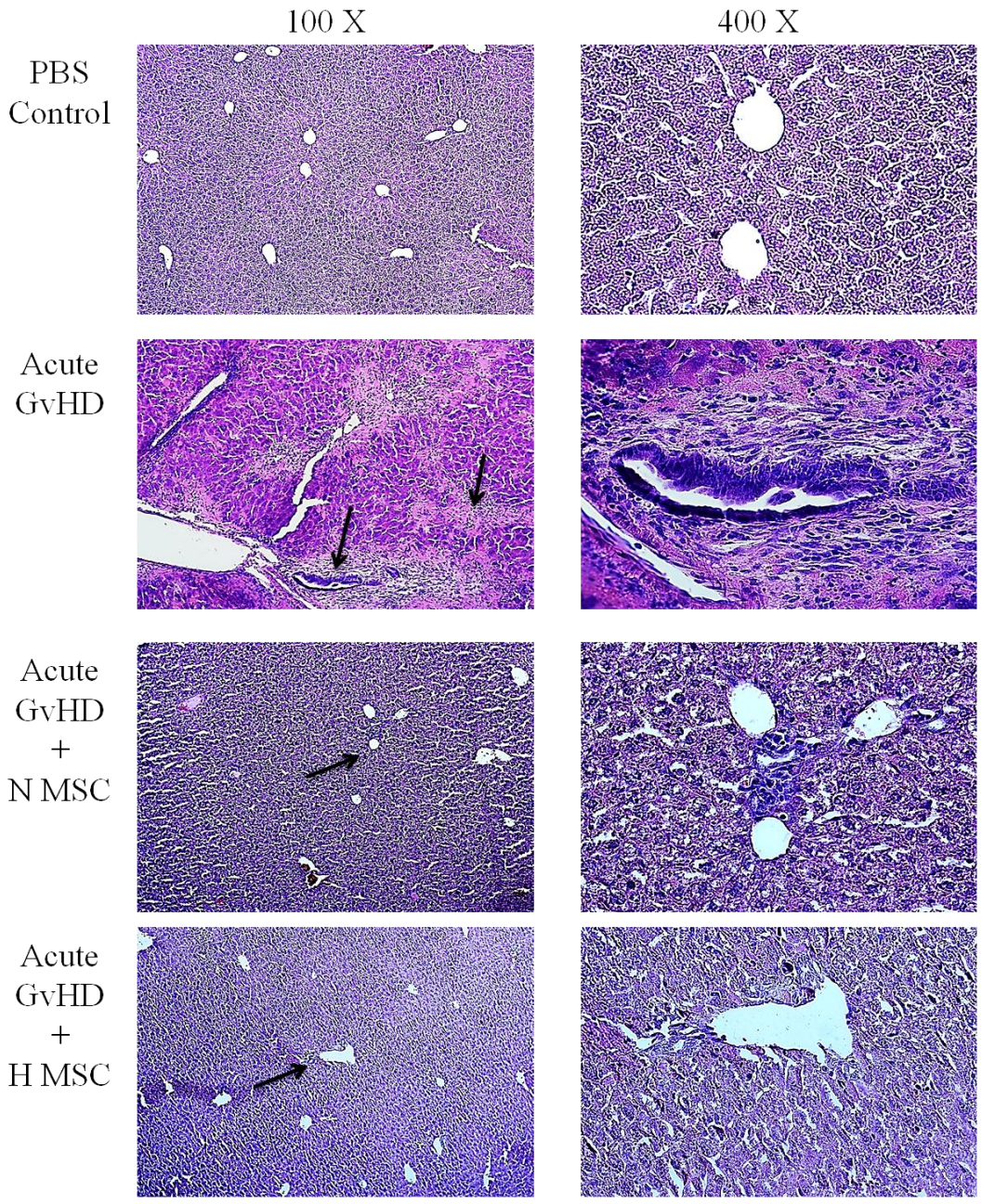



**Figure 4.2. MSC significantly prolong survival and reduce weight loss and aGvHD clinical score.** (A) survival curve, (B) percentage weight change, and (C) aGvHD clinical score. $8x10^5$ PBMC $gram^{-1}$ human donor PBMC were administered to irradiated (2.4Gy) NSG mice on day 0. $6.4x10^4$ $gram^{-1}$ normoxic or hypoxic MSC were administered as a cellular therapy on day 7 post irradiation. Mice were monitored every 2 days until day 7 and then every day for the duration of the experiment. n=3 for PBS mice, n=5 for PBMC mice and n=5 for PBMC + MSC mice. Statistical analysis was performed using a Mantel-Cox test for survival analysis and un-paired student *t*-test for weight change and clinical score where $^{**}, P \leq 0.01$ and $^{***}, P \leq 0.001$.

## 4.3 TRANSPLANTATION OF HYPOXIC MSC REDUCED AGVHD PATHOLOGY

The complex development and augmentation of aGvHD depends on the migration of effector cells to aGvHD organs where damage occurs (Sackstein 2006). MSC therapy to treat aGvHD demonstrated reduced pathology in target organs (Tobin *et al*. 2013). Given that hypoxic MSC therapy comparably prolonged the survival of aGvHD mice to normoxic MSC, the next approach therefore was to ensure hypoxic MSC comparably reduced aGvHD pathology. Tissue sections were stained by H&E and the histological aGvHD score was quantified according to criteria previously established in the lab (Tobin *et al*. 2013) (Section 2.10.2). Significant lymphocyte infiltration was observed around hepatic veins in the livers of the aGvHD group (Figure 4.3 A). Importantly, hypoxic MSC therapy reduced pathology (Figure 4.3 A) and lowered histological score (Figure 4.3 B) in the liver of aGvHD mice. The protective effects observed were similar to those observed in mice treated with normoxic MSC. PBMC administration alone resulted in significant villous blunting and necrotic cells (Figure 4.4 A- 4.4 B) in the small intestine. Akin to the pathology observed in the liver, normoxic MSC significantly reduced aGvHD pathology (Figure 4.4 A) and histological score (Figure 4.4 B) in the small intestine. PBMC delivery resulted in significant mononuclear cell infiltration to the lungs and extensive damage to the lung architecture (Figure 4.5 A). Treatment of mice with either hypoxic or normoxic MSC on day 7 failed to significantly alleviate aGvHD pathology in the lung, demonstrating moderate damage to the lung architecture and reduced cellular infiltration (Figure 4.5 A- 4.5 B). Overall these data suggests that hypoxic MSC therapy was comparable to normoxic MSC therapy in reducing aGvHD pathology in the liver and small intestine of mice with aGvHD.

(A)


100 X
400 X
PBS Control
Acute GvHD
Acute GvHD + N MSC
Acute GvHD + H MSC

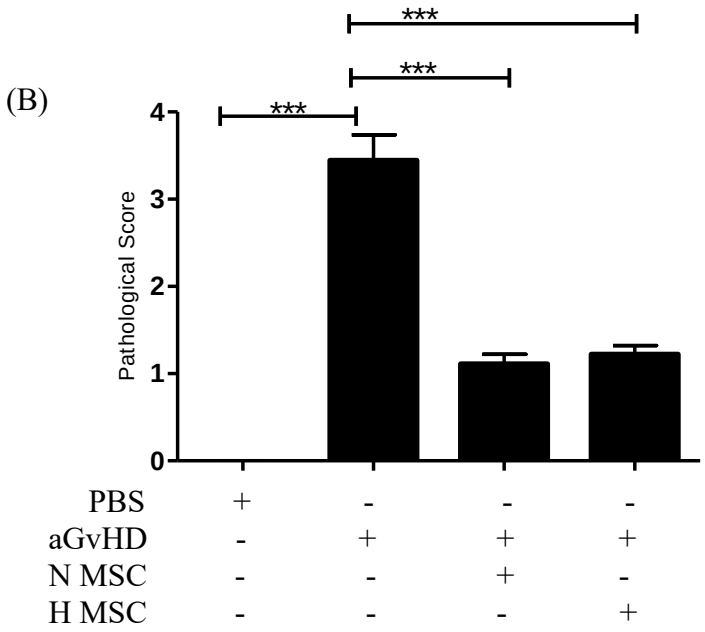
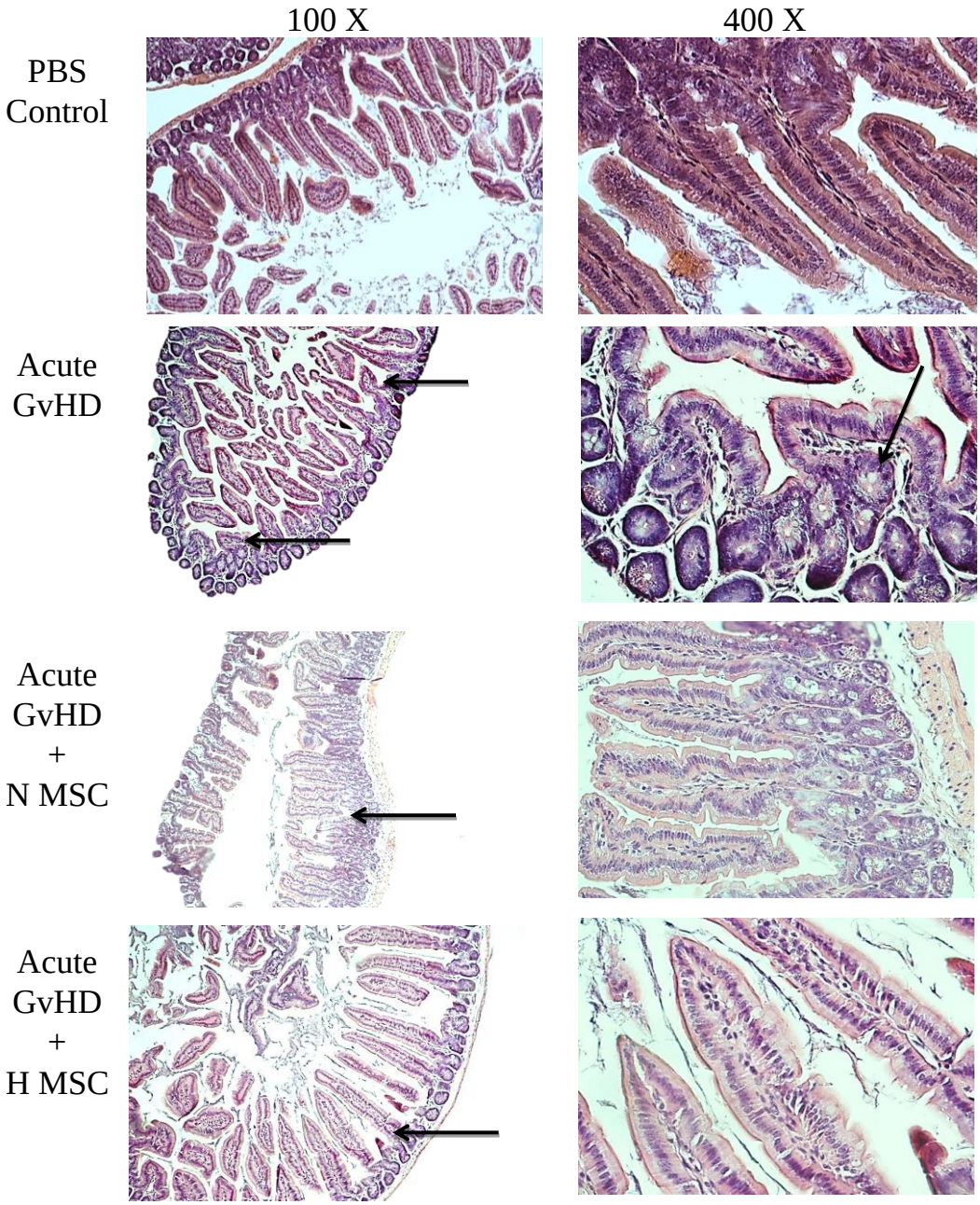



**Figure 4.3. MSC therapy reduced aGvHD pathology of the liver.** NSG mice were administered $8x10^5$ PBMC $gram^{-1}$ human donor PBMC follow a sub-lethal dose of irradiation (2.4Gy) on day 0. Mice were treated with either normoxic or hypoxic cultured MSC ($6.4x10^4$ $gram^{-1}$) as a cellular therapy on day 7. Following aGvHD development, livers were harvested from mice on day 12 and prepared for histological analysis as described in section 2.10 and analysed by H&E staining (A) and a scoring system (B). (A) Black arrows show a few areas of increased mononuclear cell infiltration and endothelialitis around hepatic vessel. Normoxic and hypoxic MSC therapy resulted in reduced aGvHD pathology (B). n= 3 PBS group, n=3 PBMC group, n=6 MSC group (2 independent MSC donors) data reported as the mean ± SEM of multiple fields of view. Statistical analysis was carried out using a student's unpaired *t*-test between two groups. ***, $P \leq 0.001$.

(A)


100 X
400 X
PBS Control
Acute GvHD
Acute GvHD + N MSC
Acute GvHD + H MSC

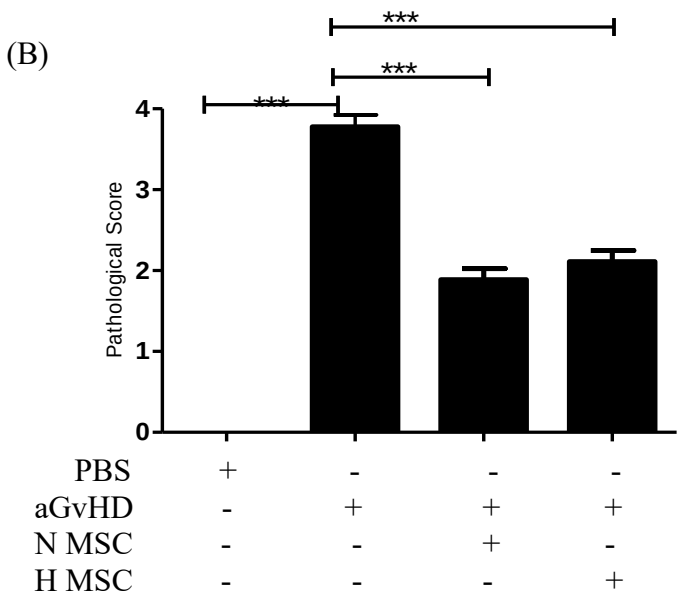
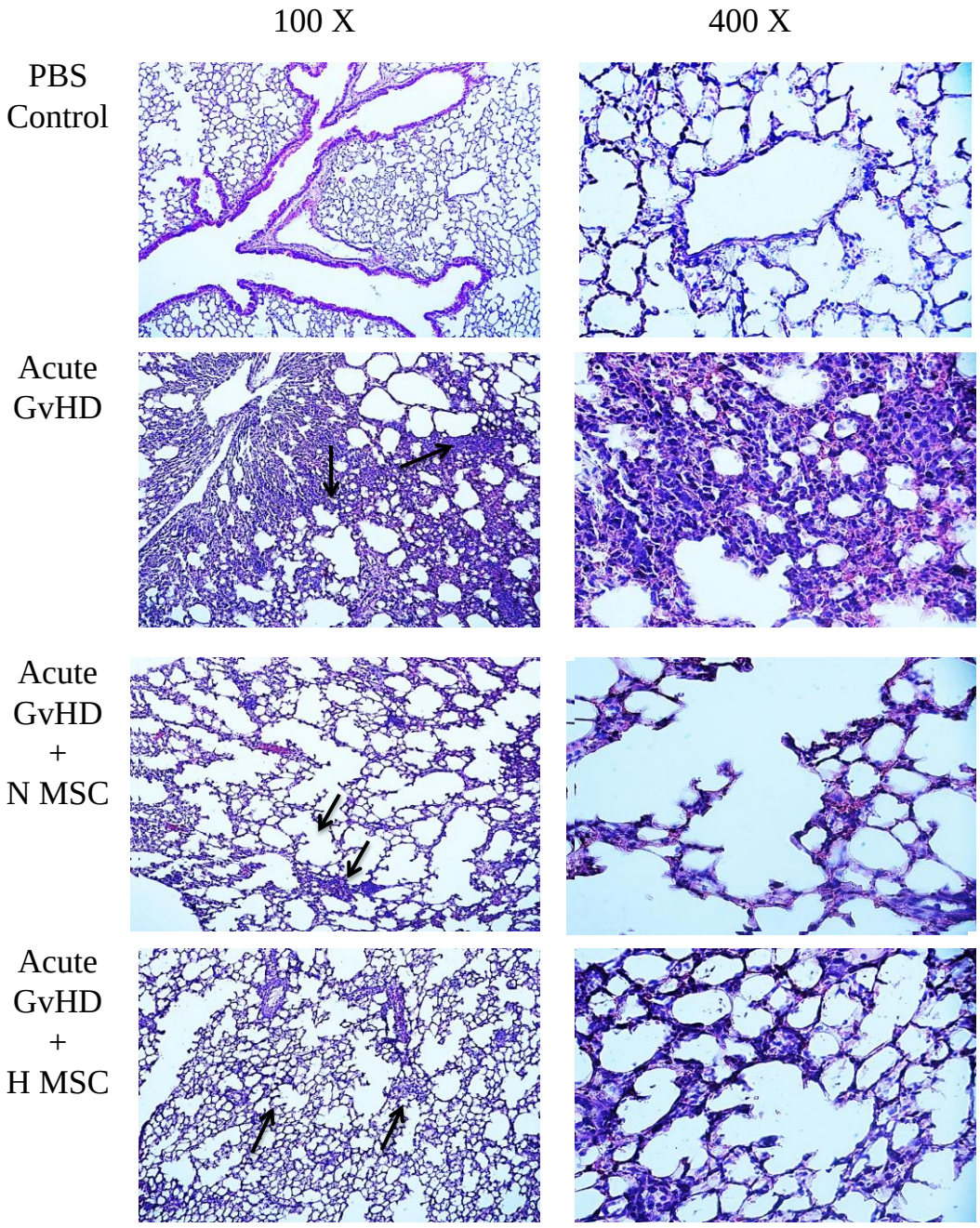



**Figure 4.4. MSC therapy significantly reduced aGvHD pathology of the small intestine.** NSG mice were administered $8x10^5$ PBMC $gram^{-1}$ human donor PBMC follow a sub-lethal dose of irradiation (2.4Gy) on day 0. Mice were treated with either normoxic or hypoxic cultured MSC ($6.4x10^4$ $gram^{-1}$) as a cellular therapy on day 7. Following aGvHD development, the small intestine was harvested from mice on day 12 as prepared for histological analysis as described in section 2.10 and analysed by H&E staining (A) and a scoring system (B). (A) Black arrows show a few areas of villous blunting cell and necrosis. Normoxic and hypoxic MSC therapy resulted in reduced aGvHD pathology (B). n=3 PBS group, n=3 PBMC group, n=6 MSC group (2 independent MSC donors) data reported as the mean ± SEM of multiple fields of view. Statistical analysis was carried out using a student's unpaired *t*-test between two groups. ***, $P \leq 0.001$.

(A)


100 X
400 X
PBS Control
Acute GvHD
Acute GvHD + N MSC
Acute GvHD + H MSC

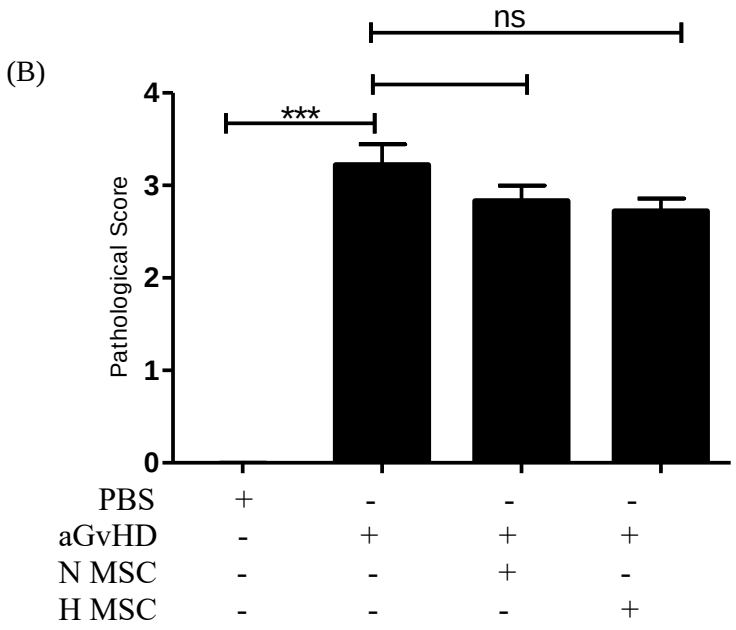



**Figure 4.5. MSC therapy had no effect on aGvHD pathology in the lung.** NSG mice were administered $8x10^5$ PBMC $gram^{-1}$ human donor PBMC follow a sub-lethal dose of irradiation (2.4Gy). Mice were treated with either normoxic or hypoxic cultured MSC ($6.4x10^4$ $gram^{-1}$) as a cellular therapy on day 7. Following aGvHD development, lungs were harvested from mice on day 12 as prepared for histological analysis as described in section 2.10 and analysed by H&E staining (A) and a scoring system (B). (A) Black arrows show a few areas of increased mononuclear cell infiltration and damaged lung architecture. Normoxic and hypoxic MSC therapy resulted in reduced aGvHD pathology (B). n= 3 PBS group, n=3 PBMC group, n=6 MSC group (2 independent MSC donors) data reported as the mean ± SEM of multiple fields of view. Statistical analysis was carried out using a student's unpaired *t*-test between two groups. ***, $P \leq 0.001$.

## 4.4 ADMINISTRATION OF HYPOXIC MSC REDUCED APOPTOSIS IN AGVHD TARGET ORGANS

The intricate pathology of aGvHD is the result of an immunological attack on patient recipient organs by allogeneic donor T cells delivered within the allograft. The cytotoxic effector phase, mediated by a myriad of cell populations (for example $CD4^+$ T cells, $CD8^+$ T cells, monocytes and NK cells) (Podgorny *et al.* 2014), induce apoptotic injury in aGvHD target organs via a number of pathways namely, fas-fas ligand interactions, perforin-granzyme B and the production of pro-inflammatory cytokine TNF-α (Braun *et al.*1996; Schmaltz *et al.* 2001; Remberger & Uzunel 2003; Socei *et al.* 2004; Fowler *et al.* 2004; Maeda *et al.* 2005). This study therefore sought to determine if hypoxic MSC reduced apoptotic death in the liver, small intestine and lungs of aGvHD mice.

aGvHD was induced as described in section 2.9 and tissue was harvested and prepared as outlined in section 2.10.1. Visualisation of apoptosis was analysed by TUNEL (green) staining (section 2.10.4), a dye that identifies molecular level apoptosis by detecting breaks in DNA strands (Kressel & Groscurth 1994). Cells were also stained with DAPI (blue) to identify the apoptotic cells as DAPI binds to nuclear DNA (Chazotte 2011). The administration of PBMC alone resulted in apoptosis in the livers of aGvHD mice in comparison to PBS control (Figure 4.6). Importantly, the delivery of normoxic and hypoxic MSC as a cellular therapy reduced apoptosis in the liver (Figure 4.6). Considerable apoptosis was evident in the small intestines (Figure 4.7) and lungs (Figure 4.8) of aGvHD mice and the administration of normoxic and hypoxic MSC markedly reduced this apoptosis comparably (Figures 4.7- 4.8).

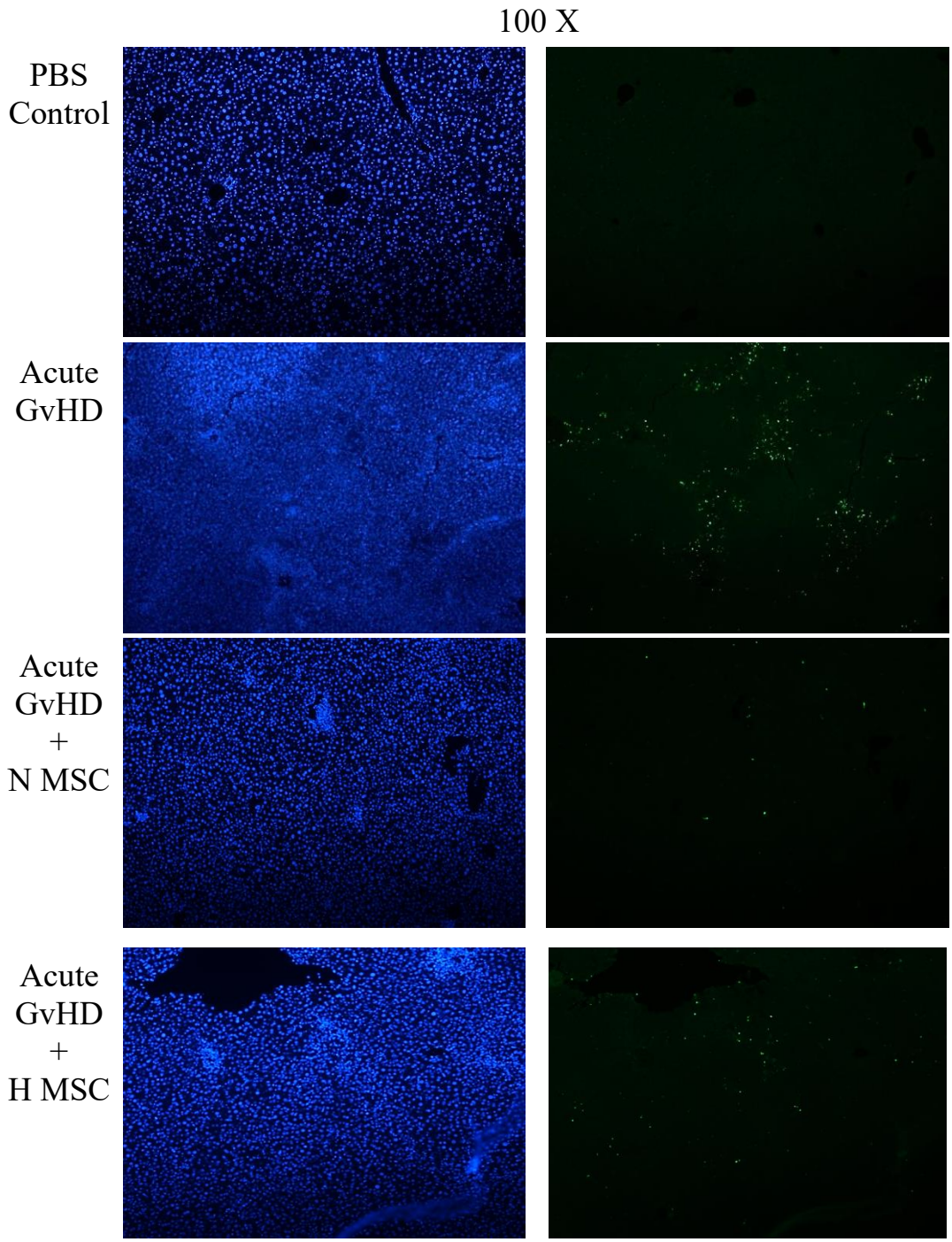



**Figure 4.6. MSC therapy reduced apoptosis in the liver.** The induction of aGvHD in NSG mice (section 2.9) results in significant apoptosis (Left pane: DAPI nuclear counterstain; blue, right pane:TUNEL stain; green) in the liver. Images are representative of multiple fields of view; 10x magnification; fluorescence microscopy. PBS; n=3, PBMC; n=3, MSC; n=6 (2 independent MSC donors).

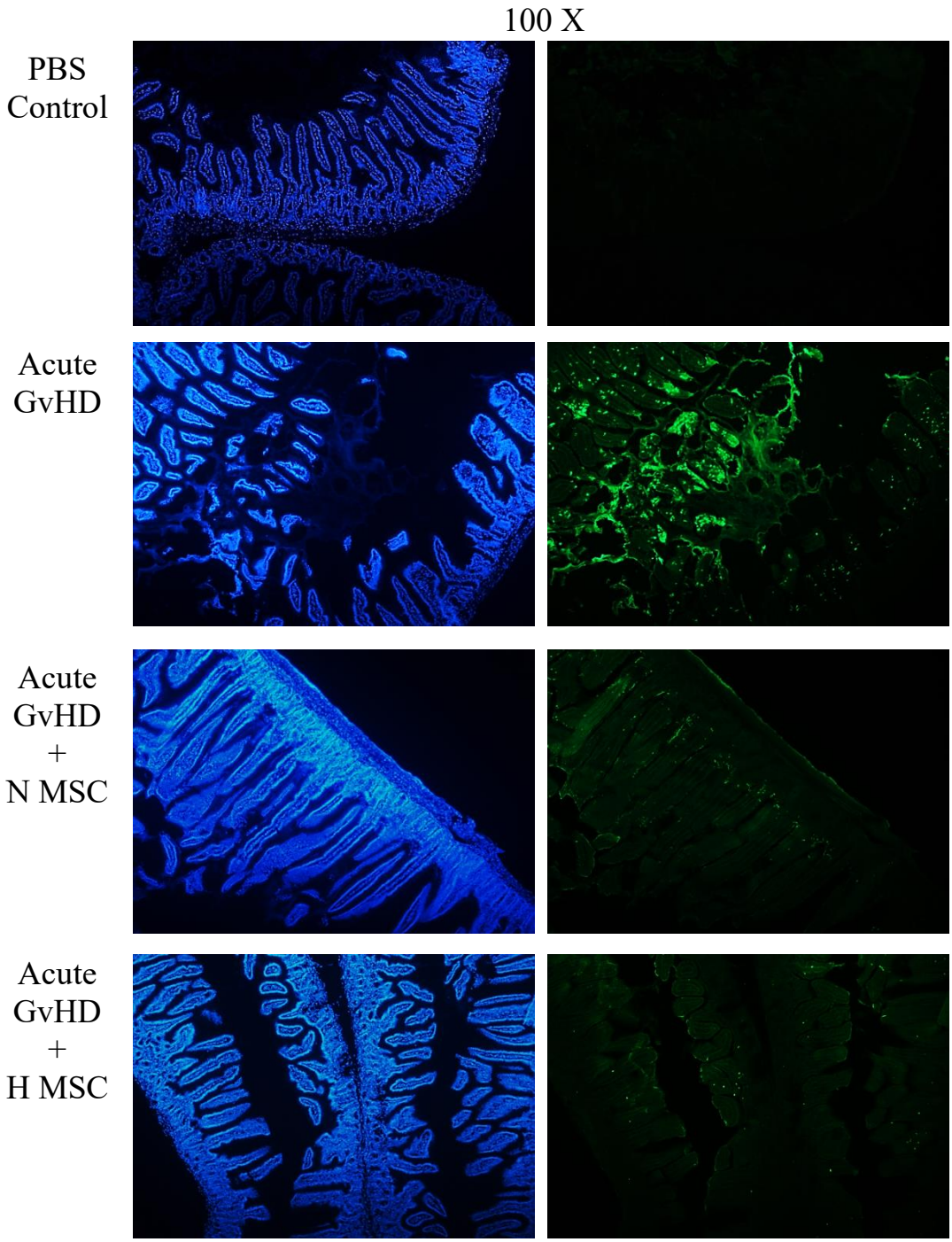



**Figure 4.7. MSC therapy reduced apoptosis in the small intestine.** The induction of aGvHD in NSG mice (section 2.9) results in significant apoptosis (Left pane: DAPI nuclear counterstain; blue, right pane: TUNEL stain; green) in the small intestine. Images are representative of multiple fields of view; 10x magnification; fluorescence microscopy. PBS; n=3, PBMC; n=3, MSC; n=6 (2 independent MSC donors).

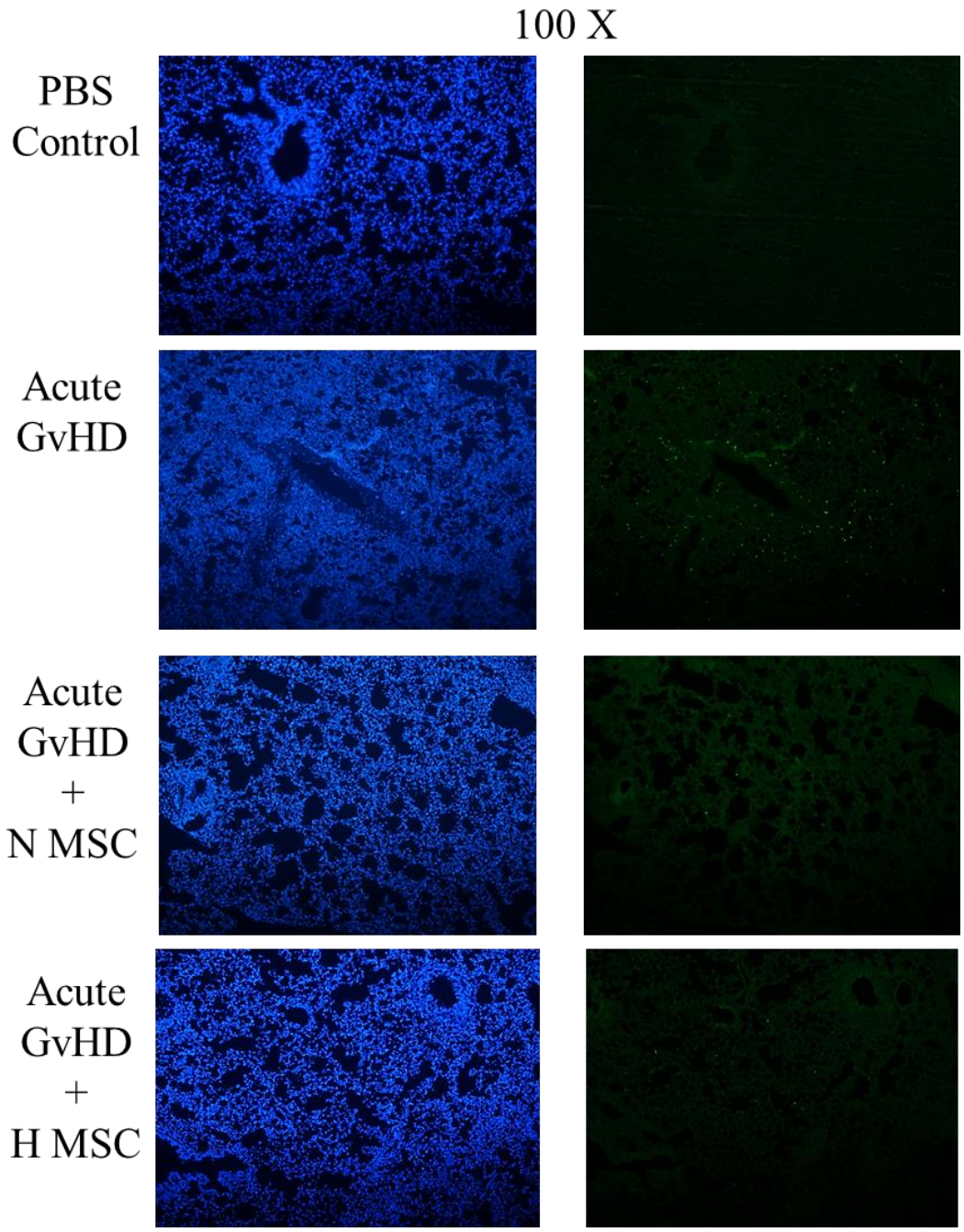



**Figure 4.8. MSC therapy reduced apoptosis in the lung of aGvHD mice.** The induction of aGvHD in NSG mice (section 2.9) results in significant apoptosis (Left pane:DAPI nuclear counterstain; blue, right pane: TUNEL stain; green) in the lung. Images are representative of multiple fields of view; 10x magnification; fluorescence microscopy. PBS; n=3, PBMC; n=3, MSC; n=6 (2 independent MSC donors).

## 4.5 MSC THERAPY SIGNIFICANTLY REDUCED TNF-α PRODUCING T CELLS IN GVHD TARGET ORGANS

TNF-α, a pro-inflammatory cytokine, amplifies donor immune responses to host tissues and its prominence in aGvHD pathogenesis is now well established (Nestel *et al.*1992; Cooke *et al.* 1998; Korngold *et al.*2003; Schmaltz *et al.* 2003). Clinical trials have supported the inhibition of TNF-α as a therapy for aGvHD (Jacobsohn *et al.*2003; Couriel et al. 2004) and previous work in our lab highlighted the capacity of MSC to reduce TNF-α in the serum of aGvHD mice (Tobin *et al.* 2013). It was therefore hypothesised that normoxic and hypoxic MSC could reduce the development of human TNF-α producing $CD4^+$ and $CD8^+$ cells in the livers, spleens and lungs of mice during aGvHD.

Human $CD4^+$ and $CD8^+$ T cells were retrieved from the livers of non-treated MSC and normoxic MSC (N) and hypoxic MSC (H) treated aGvHD mice 12 days post PBMC administration. TNF-α producing T cells were analysed via intra-cellular flow cytometry. As hypothesised, hypoxic MSC significantly reduced the percentage (Figure 4.9 A) and total number (Figure 4.9 B) of $CD4^+$ TNF-α cells in the livers of aGvHD mice. Both normoxic and hypoxic MSC partially reduced the percentage of $CD8^+$ TNF-α cells in the liver, albeit not significantly (Figure 4.9 C). Moreover, normoxic MSC significantly reduced the total number of $CD8^+$ TNF-α cells in the liver in comparison to hypoxic MSC (Figure 4.9 D). Differences between percentage data and number data may be explained by the experimental design; the quantity of PBMC initially administered to each mouse is different (normalised to their individual weight). Thus, subsequent donor expansion *in-vivo* may be different between each mouse resulting in discrepancies between percentage and number data *ex-vivo*. In addition to this, the ability of hypoxic MSC to reduce human $CD4^+$ and $CD8^+$ TNF-α producing T cells

within the spleens of aGvHD mice was also examined. Interestingly, normoxic and hypoxic MSC both significantly reduced the percentage of $CD4^+$ TNF-α producing T cells in the spleens (Figure 4.10 A). Moreover, the administration of hypoxic MSC significantly reduced the total number of $CD4^+$ (Figure 4.10 B) and $CD8^+$ (Figure 4.10 D) TNF-α producing T cells in the spleens of aGvHD mice. However, both normoxic and hypoxic MSC failed to reduce the percentage of $CD8^+$ TNF-α producing T cells in the spleens (Figure 4.10 C). Lastly, the capacity of hypoxic MSC to reduce human $CD4^+$ and $CD8^+$ TNF-α producing T cells within the lungs of aGvHD mice was examined. The administration of both normoxic and hypoxic MSC to aGvHD mice failed to significantly reduce human $CD4^+$ and $CD8^+$ TNF-α producing T cells in the lungs (Figure 4.11). However, the percentage of human $CD4^+$ TNF-α producing T cells was significantly lower than that of the normoxic treated group, although not significantly lower than the PBMC control group (Figure 4.11 A). Collectively, these data suggest that the protection mediated by hypoxic MSC against aGvHD mortality in mice may involve the reduction of TNF-α producing cells up to 12 days post PBMC administration.

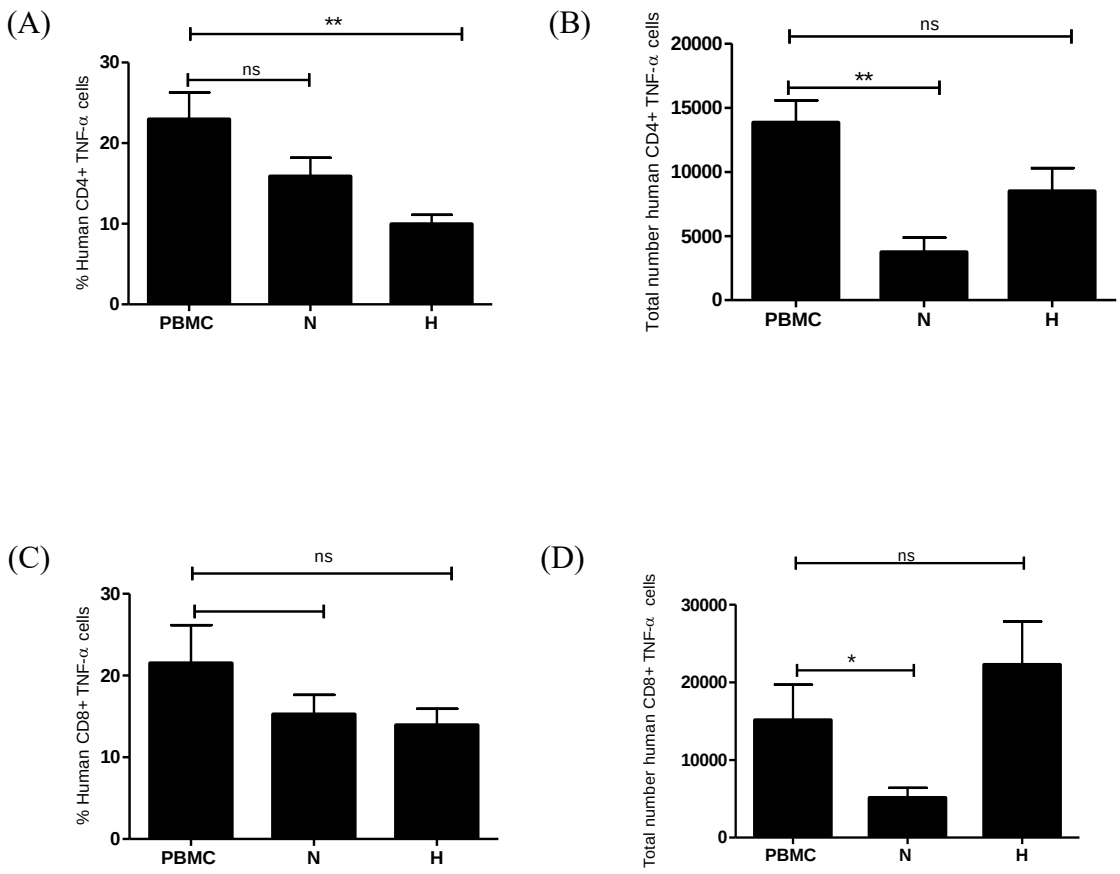



**Figure 4.9. Hypoxic MSC reduce the percentage of human TNF-α producing CD4+ T cells in the liver of aGvHD mice.** aGvHD was induced in NSG mice as described in section 2.9. Total lymphocyte data of the percentage (A) and total number (B) of human $CD4^+$ TNF-α producing cells, the percentage (C) and total number (D) of human $CD8^+$ TNF-α producing cells in the liver of aGvHD mice as analysed 12 days post PBMC administration. The total number of human $CD4^+$ T cells was assessed using counting beads. Statistical analysis was determined using student unpaired *t*- test between two groups *, $P \leq 0.05$; **, $P \leq 0.01$ and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

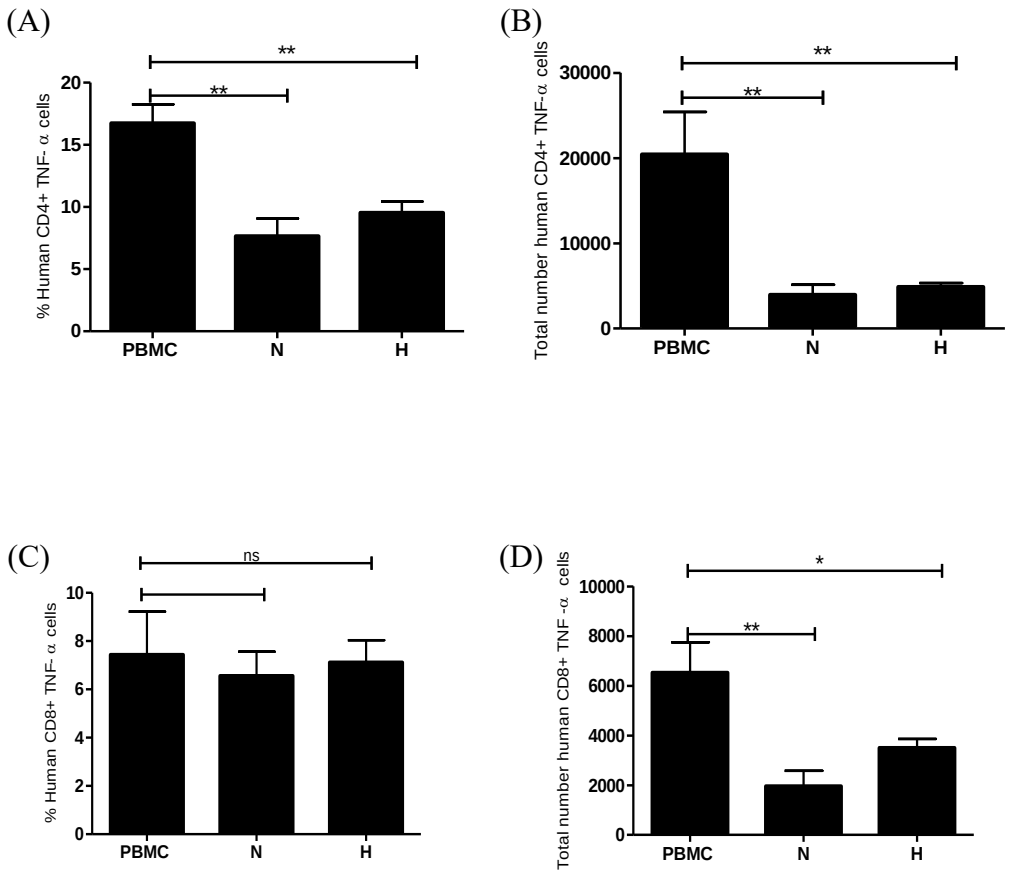



**Figure 4.10. Hypoxic MSC reduce human TNF-α producing CD4$^+$ T cells in the spleen of aGvHD mice.** aGvHD was induced in NSG mice as described in section 2.9. Data of the percentage (A) and total number (B) of human CD4$^+$ TNF-α producing cells, the percentage (C) and total number (D) of human CD8$^+$ TNF-α producing cells in the spleen of aGvHD mice as analysed 12 days post PBMC administration. n=3 per group (1 PBMC and 2 MSC donors). The total number of human PBMC was assessed using counting beads. Statistical analysis was determined using students' unpaired *t*-test between two groups *, P≤0.05; **, P≤0.01 and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

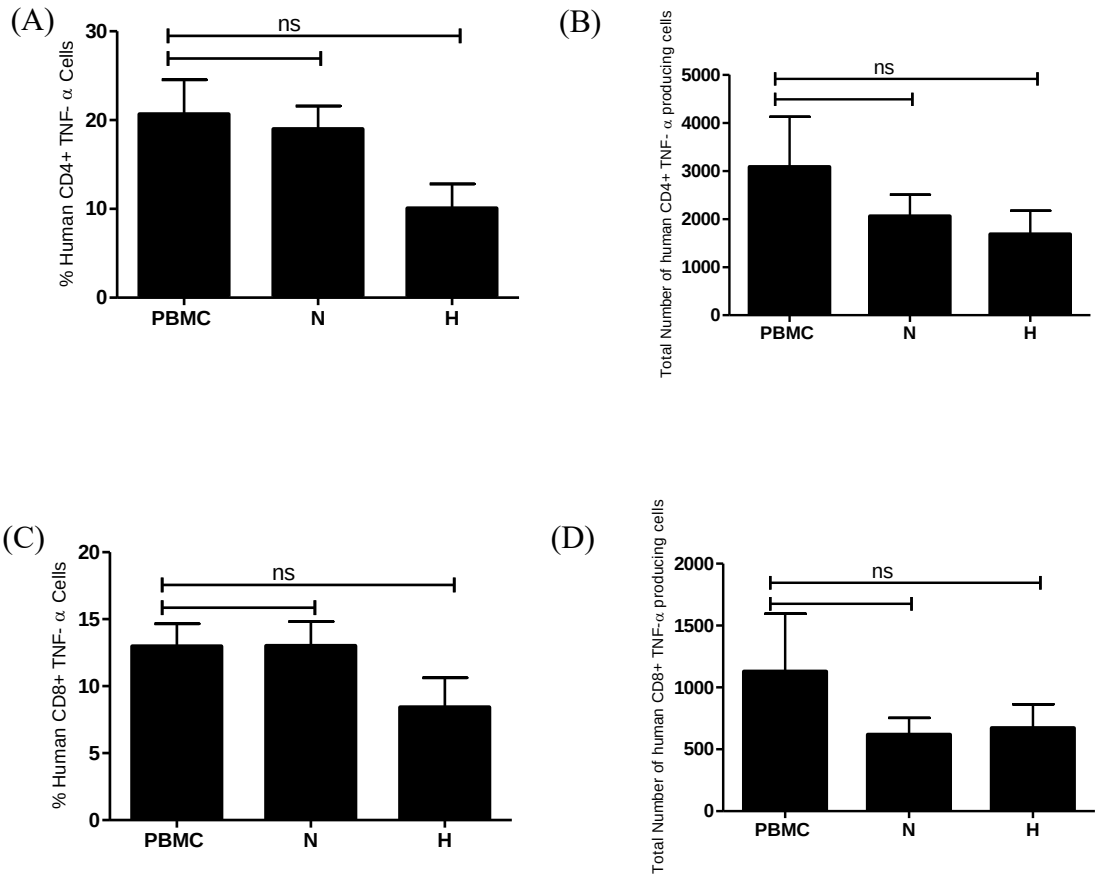



**Figure 4.11. Hypoxic MSC reduce the percentage of human TNF-α producing CD4+ T cells in the lung of aGvHD mice compared to normoxic MSC.** aGvHD was induced in NSG mice as described in section 2.9. Image of the percentage (A) and total number (B) of human $CD4^+$ TNF-α producing cells, the percentage (C) and total number (D) of human $CD8^+$ TNF-α producing cells in the lungs of aGvHD mice as analysed 12 days post PBMC administration. The total number of human PBMC was assessed using counting beads. Statistical analysis was determined using students' unpaired *t*- test between two groups *, P≤0.05; **, P≤0.01 and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

## 4.6 ADMINISTRATION OF HYPOXIC MSC DID NOT REDUCE PBMC ENGRAFTMENT IN THE SPLEEN

Hypoxic MSC significantly enhanced the survival of aGvHD mice, reduced weight loss and lowered pathological score in line with normoxic MSC. Importantly, hypoxic MSC as a cellular therapy for aGvHD significantly reduced the percentage of $CD4^+$ TNF-α producing T cells in the spleens of aGvHD mice and reduced the total number of $CD8^+$ TNF-α producing T cells. However, MSC treated mice began to succumb to this aggressive aGvHD by day 14 post-transplantation. The engraftment of donor HSC is required for the development of a complete functional immune system of HSCT patients after transplantation and therefore this process should not be impeded by cellular therapies. Therefore, this experiment sought to determine whether hypoxic MSC impaired the engraftment of PBMC in the spleens of aGvHD mice. Treatment of aGvHD mice with normoxic (N) or hypoxic (H) MSC did not impede the percent engraftment of human $CD4^+$ (Figure 4.12 A), $CD8^+$ (Figure 4.12 B) and $CD4^+CD8^+$ (Figure 4.12 C) cells in the spleens. Collectively, these data demonstrate that hypoxic MSC do not impair the engraftment of human cells in the spleens of aGvHD mice.

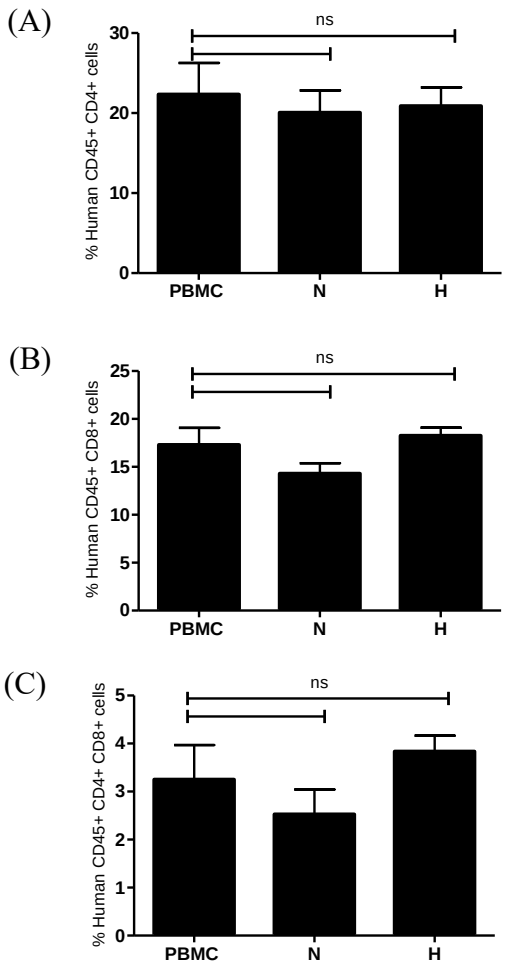



**Figure 4.12. Hypoxic MSC did not impair human lymphocyte engraftment in the spleens of aGvHD mice.** aGvHD was induced in NSG mice as described in section 2.9. (A) Percentage human $CD4^+$ cells recovered from the spleens of aGvHD mice. (B) Percentage human $CD8^+$ human cells recovered from the spleens of aGvHD mice. (C) Percentage human $CD4^+CD8^+$ human cells recovered from the spleens of aGvHD mice. Statistical analysis was determined using students' unpaired *t*- test between two groups and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

## 4.7 ADMINISTRATION OF HYPOXIC MSC REDUCED PBMC ENGRAFTMENT IN THE LIVER OF AGVHD MICE

The trafficking and infiltration of alloreactive effector lymphocytes to aGvHD target organs (i.e. liver and lung) is a pre-requisite for aGvHD development and subsequent destruction of organ tissue (Sackstein 2006). Despite our understanding of T cell migration to secondary lymphoid organs (SLOs) such as the spleen and subsequent aGvHD evolvement, the trafficking of lymphocytes to aGvHD target organs is lesser understood. Thus, the capacity of hypoxic MSC to affect PBMC engraftment in the liver of aGvHD mice as a mechanism of enhancing the survival of aGvHD mice was therefore investigated. As outlined in chapter 2, the livers of aGvHD mice were harvested under sterile conditions and mechanically digested. Human lymphocytes were isolated via density gradient centrifugation and examined for the expression of human CD45, CD4 and CD8 by flow cytometry.

Hypoxic MSC (H) significantly reduced the percentage of $CD45^+$ $CD4^+$ cells in the livers of aGvHD mice in comparison to PBMC alone (Figure 4.13 A). Interestingly, normoxic (N) cultured MSC did not significantly reduce the percentage of $CD45^+$ $CD4^+$ cells in the livers. There were no differences in the percentage of $CD45^+$ $CD8^+$ cells in aGvHD livers (Figure 4.13 B). There was a significant reduction in the $CD45^+$ $CD4^+$ $CD8^+$ cells in the livers of both normoxic and hypoxic MSC treated aGvHD mice (Figure 4.13 C). These results suggest the hypoxic MSC may prevent the infiltration of $CD4^+$ T cells in the livers of aGvHD mice.

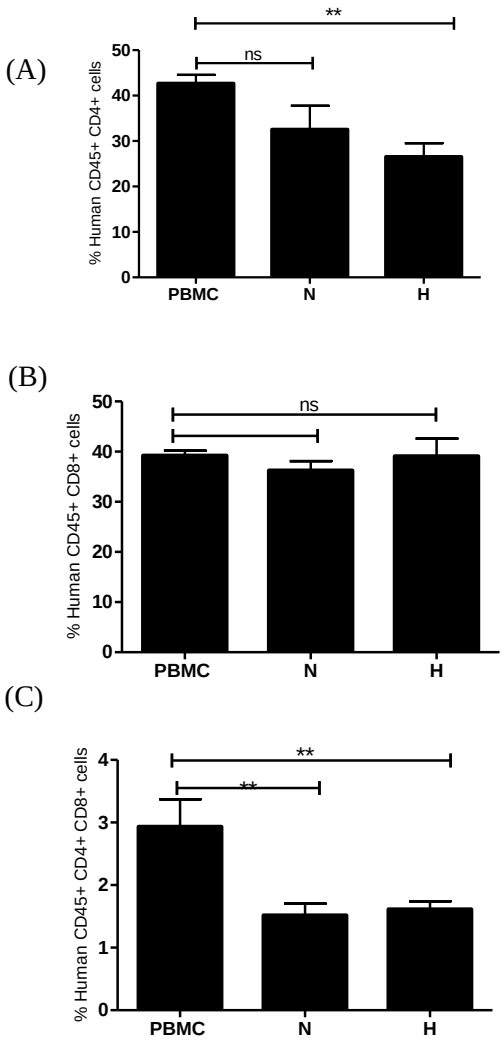



**Figure 4.13. Hypoxic MSC reduced human CD4$^+$ engraftment in the livers of aGvHD mice.** aGvHD was induced in NSG mice as described in section 2.9. (A) Percentage human CD45$^+$CD4$^+$ human cells recovered from the livers of aGvHD mice. (B) Percentage human CD45$^+$CD8$^+$ human cells recovered from the livers of aGvHD mice. (C) Percentage human CD45$^+$CD4$^+$CD8$^+$ human cells recovered from the livers of aGvHD mice. Data representative of one human PBMC donor and 2 MSC donors ± SEM (n=3 for each). Statistical analysis was determined using students' unpaired *t*- test between two groups and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group where **, P≤0.01.

## 4.8 ADMINISTRATION OF HYPOXIC MSC DID NOT REDUCE PBMC ENGRAFTMENT IN THE LUNGS OF AGVHD MICE

aGvHD is a systemic disease induced by the recognition of HLA on patient tissue by alloreactive T cells in the transplanted graft. A target organ of aGvHD is the lung and T cells and their pro-inflammatory cytokines have been implicated in its pathogenesis (Q. Liu et al. 2009). Hypoxic MSC enhanced the survival of aGvHD mice, however no significant improvements in histological score were observed. Nonetheless, it was important to examine the effect of hypoxic MSC on the migration and engraftment of PBMC to the lungs of aGvHD mice.

Human lymphocytes were isolated using density gradient centrifugation from aGvHD mice and MSC treated aGvHD mice. Cells were analysed for the expression of human CD45 in conjunction with CD4, and CD8. The administration of normoxic (N) or hypoxic (H) MSC did not have any significant effect on the percentages of human $CD45^+$ $CD4^+$ cells in the lungs of aGvHD mice (Figure 4.14 A). The engraftment of $CD8^+$ and $CD4^+CD8^+$ human cells was not significantly affected by the administration of normoxic or hypoxic MSC. In fact, there appeared to be a slight increase in the percent of human $CD8^+$ cells in the lungs of hypoxic treated aGvHD mice (Figure 4.14 B) and a slight decrease in $CD4^+CD8^+$ human cells in both MSC therapy groups (Figure 4.14 C). These results suggest that hypoxic MSC therapy for aGvHD does not involve the impairment of human T cells in the lung.

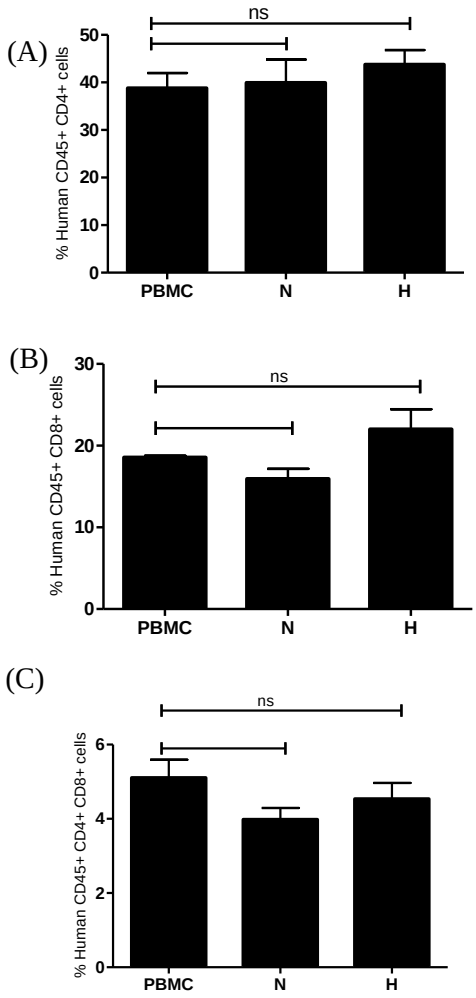



**Figure 4.14. Hypoxic MSC does not affect human CD4$^+$ engraftment in the lung of aGvHD mice.** aGvHD was induced in NSG mice as described in section 2.9. (A) Percentage human CD45$^+$CD4$^+$ human cells recovered from the livers of aGvHD mice. (B) Percentage human CD45$^+$CD8$^+$ human cells recovered from the lung of aGvHD mice. (C) Percentage human CD45$^+$CD4$^+$CD8$^+$ human cells recovered from the lung of aGvHD mice (± SEM). Statistical analysis was determined using students' unpaired *t*-test between two groups and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

## 4.9 MSC THERAPY DID NOT DECREASE REGULATORY T CELL ENGRAFTMENT DURING AGVHD

Regulatory T cells (Tregs) play a key role in the maintenance of self-tolerance (Wing *et al.,* 2010), and strategies to control aGvHD have attempted to exploit their potential to treat aGvHD. Interestingly, the adoptive transfer of Tregs into 28 human patients successfully prevented GvHD in the absence of posttransplantion immunosuppression (Di Ianni *et al.* 2011) and *in-vitro* experiments have demonstrated that MSC promote the generation of functionally suppressive Tregs from human PBMC populations (Sara M. Melief *et al.* 2013). Published data from our lab suggested that MSC do not induce Tregs but rather expand resident Treg populations' *in-vitro* (Tobin *et al.* 2013). Furthermore, this thesis did not detect Tregs in our NSG model. However, we employed a more sensitive mechanism of recovering human cells from murine tissue here. The potential for hypoxic MSC to preserve Tregs during aGvHD was therefore investigated *in-vivo.*

To determine whether MSC modulated Treg expansion *in-vivo,* NSG mice were administered $8x10^5$ PBMC $gram^{-1}$ and treated with normoxic or hypoxic MSC on day 7 post xenograft delivery. Mice were sacrificed on day 12 and human Treg cells were analysed. $CD45^+CD4^+$ $CD25^+$ $FoxP3^+$ cells were defined as Tregs and identified via intra-cellular flow cytometry. Importantly, the detection of Tregs was possible (Figure 4.15). However, the administration of hypoxic MSC (H) or normoxic MSC (N) to aGvHD mice did not increase the percentage of Tregs in the spleens (Figure 4.15 A), livers (Figure 4.15 B) or lungs (Figure 4.15 C). However, there appeared to be a slight, albeit not significant decrease in the percentage of Tregs in the liver. Taken together, these data demonstrates that hypoxic and normoxic MSC do not impair Treg engraftment within inflamed aGvHD target organs.

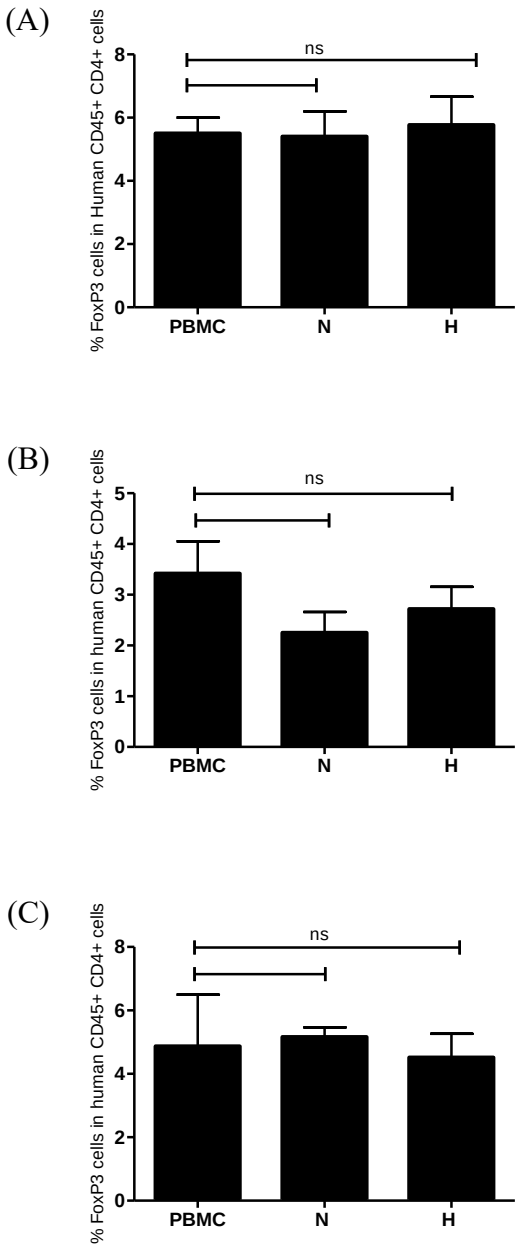



**Figure 4.15. MSC therapy did not impair Treg engraftment during aGvHD.** NSG mice exposed to a 2.4Gy irradiation dose and given $8x10^5$ PBMC $gram^{-1}$ via tail vein injection were analysed on day 12. Treg cells were recovered from the spleen (A), liver (B) and lung (C) of aGvHD mice and normoxic MSC (N) and hypoxic MSC (H) treated aGvHD mice. Statistical analysis was determined using students' unpaired *t*-test between two groups and data reported as the mean ± SEM of n=3 in PBMC group and n=6 in MSC group.

## 4.10 MSC THERAPY DID NOT DECREASE PRO-INFLAMMATORY CYTOKINES TNF-α or IFNγ PRODUCTION BY EX-VIVO CULTURED SPLENOCYTES

A key hallmark of aGvHD is the presence of a "cytokine storm" (Ferrara *et al.* 1999) characterised by the excessive production of pro-inflammatory cytokines such as TNF-α and IFN-γ (Schmaltz *et al.* 2003). Attempts to impede TNF-α production in the clinic are underway and immunoglobulin mediated blockade of the membrane bound precursor of TNF-α is well tolerated ( Couriel *et al.* 2004). Furthermore, IFN-γ is another critical mediator of aGvHD. It was therefore hypothesised that hypoxic MSC may impair IFN-γ and, or TNF-α production from ex-vivo cultured splenocytes from MSC treated aGvHD mice.

Production of pro-inflammatory cytokines were analysed from supernatants of *ex-vivo* cultured splenocytes harvested from aGvHD (PBMC) mice, and normoxic (N) and hypoxic (H) MSC treated aGvHD mice and analysed by ELISA. Contrary to the hypothesis, the administration of normoxic (N) or hypoxic (H) MSC to aGvHD mice did not reduce IFN-γ production by splenocytes harvested from MSC treated aGvHD mice (Figure 4.16 A). Furthermore, the levels of TNF-α from splenocytes of normoxic or hypoxic MSC treated aGvHD were also unaffected by treatment (Figure 4.16 B).

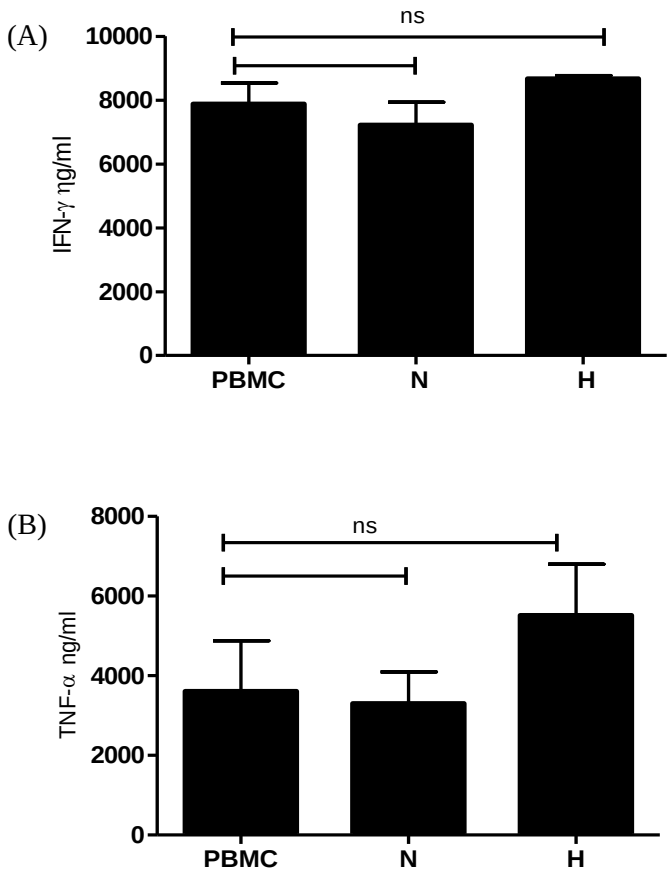



**Figure 4.16. MSC therapy did not reduce IFN-γ or TNF-α production in MSC treated aGvHD splenocytes.** NSG mice which were exposed to irradiation (2.4 Gy) were given $8x10^5$ PBMC $gram^{-1}$. On day 12 post-transplant, spleens were harvested from aGvHD (PBMC) mice, and normoxic (N) and hypoxic (H) MSC treated aGvHD mice and cultured for 3 days in the presence of phorbol myristate acetate (PMA)(100ng/ml) and Ionomycin (ION)(1µg/ml). Supernatants were collected and examined for the expression of pro-inflammatory cytokines via ELISA following dilution (1:10). Data is reported as mean ± SEM of n=3 PBMC and n=6 MSC performed in triplicate two times.

## 4.11 MSC THERAPY DOES NOT AFFECT IL-17 PRODUCTION DURING AGVHD.

Human IL-17 was first described in 1995 and is a characteristic cytokine produced by Th17 cells and has been implicated in aGvHD (Yao *et al*. 1995; Zhao *et al.* 2011). Production of IL-17 was analysed from supernatants of *ex-vivo* cultured splenocytes harvested from aGvHD mice (PBMC), and normoxic (N) and hypoxic (H) MSC treated aGvHD mice and analysed by ELISA. The administration of normoxic (N) MSC to aGvHD mice did not reduce IL-17 production by splenocytes harvested from MSC treated aGvHD mice (Figure 4.17). Normoxic MSC treatment slightly enhanced IL-17 production, however this was not significant. Furthermore, the levels of IL-17 from splenocytes of hypoxic (H) MSC treated aGvHD were also unaffected by treatment (Figure 4.17).

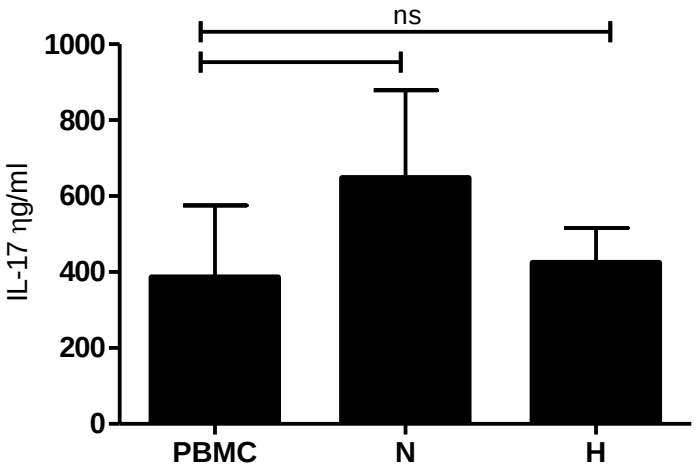



**Figure 4.17. MSC therapy did not reduce IL-17 production in aGvHD splenocytes.** NSG mice which were exposed to irradiation (2.4 Gy) were given $8x10^5$ PBMC $gram^{-1.}$ On day 12 post-transplant, spleens were harvested from aGvHD, and normoxic (N) and hypoxic (H) MSC treated aGvHD mice and cultured for 3 days in the presence of PMA and ION. Supernatants were collected and examined for the expression of IL-17 via ELISA following a 1:5 dilution. Data is reported as mean ± SEM of n=3 PBMC and n=6 MSC performed in triplicate two times.

## 4.12 SUMMARY

The main objectives of this section were to (1) investigate the capacity of hypoxic cultured MSC to prolong the survival of mice with aGvHD, (2) examine and compare the capacity of hypoxic MSC to reduce weight loss and clinical score of aGvHD mice in comparison to normoxic MSC (3) investigate if hypoxic MSC could reduce the percentage of TNF-α producing T cells and proinflammatory aGvHD cytokines, (4) to examine the effect, if any of hypoxic MSC on the engraftment of human PBMC in the humanised mouse model of aGvHD and (5) to analyse the effect of hypoxic MSC on pathology of aGvHD mice. The NOD-SCID IL-2r$\gamma^{null}$ (Pearson *et al.* 2008) (NSG) humanised mouse model of aGvHD was primarily designed by Pearson *et al.,* 2008 and was harnessed in this study to investigate the therapeutic capacity of hypoxic MSC to treat aGvHD.

Through this model, it was demonstrated that hypoxic MSC therapy on day 7 significantly and comparably prolonged the survival of aGvHD mice in comparison to their normoxic counterparts. Furthermore, MSC therapy also reduced weight loss and clinical score during aGvHD. While hypoxic MSC did not impede the engraftment of T cells in the spleen, hypoxic MSC reduced the percentage of $CD4^+$ TNF-α cells in the spleens of aGvHD mice. The administration of hypoxic MSC reduced lymphocyte engraftment in the liver, a target organ of aGvHD but did not affect engraftment in the lung. Importantly, hypoxic MSC were comparable to normoxic MSC in reducing liver and small intestine aGvHD pathology but neither improved lung pathology. Collectively, these data suggest that hypoxic MSC therapy prolongs the survival of aGvHD mice by preventing target organ destruction and reducing the percentage of TNF-α producing T cells. The next step of investigation was to examine whether hypoxic culture

of MSC affected their capacity to engraft in aGvHD target organs and if this had an effect on their capacity to prolong the survival of aGvHD mice

# CHAPTER 5

# IN-VIVO IMAGING OF HYPOXIC AND NORMOXIC EXPANDED MSC BIODISTRIBUTION IN A HUMANISED MOUSE MODEL OF AGVHD

## 5.1 IMAGING OF MSC BIODISTRIBUTION *IN-VIVO*

In line with the identification of the therapeutic properties of MSC in inflammatory and regenerative diseases, the interest in determining their underlying immunomodulatory function broadened to understanding the biodistribution and homing of MSC *in-vivo.* Pioneering studies by Horwitz *et al.,* in children with osteogenesis imperfecta (a bone disorder) demonstrated that infusion of MSC alleviated disease symptoms and accelerated growth velocity during the first 6 months post infusion (Horwitz et al. 1999; Horwitz et al. 2002). Interestingly, bone marrow aspirates in conjunction with bone and skin biopsies revealed engraftment of MSC (by proviral sequences, virus incorporated into the genome) in 5 out of 6 patients in at least one of the tissues examined (Horwitz et al. 2002). This implied that infused MSC migrated to sites in the bone and/or bone marrow and produced measurably therapeutic benefits (Horwitz et al. 2002). Since then, MSC have increasingly been employed as an intravenously applied cellular therapy. The current understanding is that the effectiveness of administered cells for inflammatory disorders typically depends on target organ uptake and the milieu in which they are located (Barry & Murphy 2004). However, the field is challenged by the persistence of unanswered questions. For example, how many cells reach a target organ? Which route of MSC administration is most suited to their desired therapeutic outcome? Therefore, understanding MSC migration and persistence *in-vivo,* more specifically in target organs, is key to the development of future MSC therapeutic strategies for inflammatory disease such as aGvHD. Pre-clinical imaging strategies can identify labelled cells *in-vivo* and further probe the dynamics of MSC migration and efficacy. Although the precise mechanisms controlling MSC migration to sites of injury have yet to be fully elucidated, it is thought that inflammatory cues released at sites of inflammation are involved in recruiting MSC

to the site of injury (Sordi et al. 2005; Ries et al. 2007; Baek et al. 2011). A number of cell labelling methods and imaging techniques exist to aid in these investigations. One such commonly employed imaging modality is fluorescent imaging and integration of a fluorescent protein into the cellular genome (or short term labelling) to facilitate *in-vivo* tracking of cell migration. Lentiviruses are tools commonly employed in the laboratory setting that belongs to the retrovirus category (Kootstra & Verma 2003). The major advantage of using lentiviruses is their ability to stably integrate into the genome of both dividing and non-dividing cells (Cockrell & Kafri 2007). Typically, lentiviral vectors are produced by co-transfection of plasmids in human embryonic kidney (HEK) 293T cells (HEKs) (Naldini *et al.* 1996).

In order to utilise these lentiviral vectors, they must be replicative defective whilst being efficient. This requirement has led to the formation of second and third generation plasmids, ultimately allowing viral vectors to be handled using safe laboratory handling practice. Lentiviruses are divided into generations according to the packaging plasmid or plasmids employed. The first generation lentiviral packaging system contained HIV-1 core proteins, accessory genes and enzymes on the one packaging plasmid (Naldini *et al.*, 1996). However this system is not commonly employed due to advances in viral vector biosafety and therefore, the identification of genes that are not ultimately required for genetic transfer were removed from the first generation system. Second generation lentiviral systems were created by deleting the four dispensable HIV accessory genes Vif, Vpr, Vpu and Nef without any undesirable effects on vector yield (Zufferey et al. 1997) leaving HIV Gag, Pol, Rev and Tat on one packaging plasmid. Thus, second generation systems increased lentivirus biosafety given that any replication competent viruses wouldn't contain any virulence factors. In an effort to further enhance biosafety, the packaging plasmid in a third generation

system is divided into 2 individual packaging plasmids in conjunction with a single envelope plasmid and a transfer vector. Here, this system is composed of Gag and Pol on a single plasmid and Rev on another independent plasmid. Furthermore, this system does not express Tat. Therefore the third generation system is composed of 4 plasmids: a transfer vector, an envelope plasmid and two packaging plasmids in which Tat has been completely eliminated from the packaging assembly and Rev must be expressed on an independent plasmid. Thus, third generation systems, although more difficult to use, offer maximal biosafety.

### 5.1.1. AIMS AND OBJECTIVES

The aim of this work was to investigate the *in-vivo* biodistribution of hypoxic MSC in comparison to normoxic MSC in a humanised mouse model of aGvHD. Therefore, the first step in this study was the generation of a lentiviral vector that permitted fluorescent imaging of transduced MSC by harnessing a dual reporter expressing luciferase and eGFP. In this work, lentiviral vectors harbouring a second generation packaging system and third generation transfer plasmid were produced. This lentivirus was subsequently used to stably transduce MSC *in-vitro*. Transduced MSC were subsequently characterised to ensure maintenance of phenotypical and functional characteristics. Furthermore, a study harnessed normoxic and hypoxic MSC labelled with QDots and administered to aGvHD mice to ensure hypoxic MSC generated a migrational profile similar to normoxic cultured MSC and alleviated clinical scoring of aGvHD mice.

## 5.2 OPTIMISATION OF LENTIVIRAL PARTICLE PRODUCTION

The production of functional lentiviral particles requires a transfer plasmid, a packaging plasmid (or two packaging plasmids) and an envelope plasmid (Kay et al. 2001). Using these components, lentiviral particles can be generated by transfection methods in HEK 293T cells (Lesch et al. 2011; Wright & Zelenaia 2011; Cribbs et al. 2013).

### 5.2.1 PRODUCTION OF LENTIVIRAL PARTICLES WITH MIRUS Transit-293 OR ULTRACENTRIFUGATION WAS UNSUCCESSFUL

Plasmid DNA was amplified and purified from transformed E. coli using plasmid DNA. HEK293T cells (HEKs) were seeded 18-24 hours before transfection in completeDMEM media. 24 hours later, Mirus TransIT-293 was warmed to room temperature and vortexed gently. 200 µl of Opti-MEM was incubated with TransIT-293 and a further 200 µl Opti-MEM was incubated with 1 µg/µl plasmid DNA at a ratio of 3:2:1 (transfer plasmid: packaging: envelope) (Figures Appendix-1-2). The solution was then pipetted to ensure complete mixing and added dropwise to the HEKs in 5ml of complete HEK media. The cells were incubated for 72 hours after which the supernatant was collected and centrifuged at 300g to remove cell debris. Supernatant not required for immediate use was stored at -80$^{o}$C. Viral supernatant was titred on HEKs at a 1:10 dilution or left untransduced as a control (Figure 5.1 A).

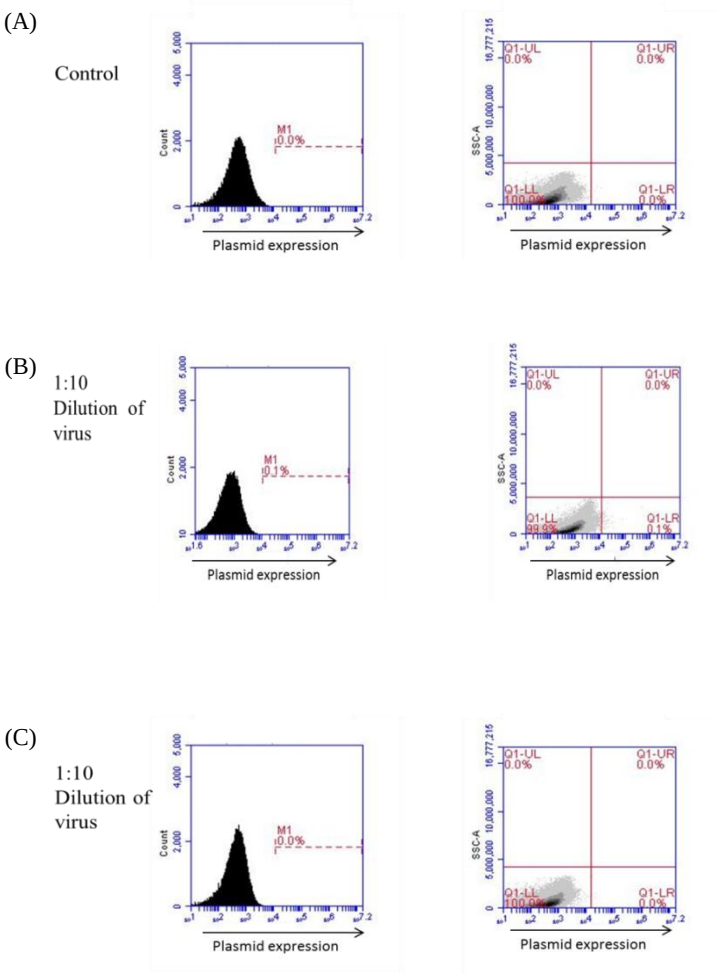



**Figure 5.1. Production of lentiviral particles with TransIT-293 and concentration did not transduce HEKs.** HEKs were seeded at a density of $8.1x10^4$ per well of a 24 well plate 24 hours before incubation with lentivirus. 24 hours later, fresh media was placed onto control cells (A). Additionally, lentivirus in media produced with TransIT-293 alone (B) or ultracentrifuged TransIT-293 lentivirus (C) was serially diluted into the corresponding well of pre-seeded HEKs. 24 hours after this incubation the cell supernatant with virus was discarded and replaced with fresh medium. A further 48 hours after this, the cells were trypsinised, neutralised and analysed by flow cytometry for reporter gene expression (GFP). Analysis of HEKs after incubation with lentivirus demonstrated that lentiviral production with TransIT-293 (A) and ultracentrifuged TransIT-293 lentivirus (B) with DNA plasmids (Figures Appendix1-2) was not successful. n=1

HEKs were then analysed by flow cytometry for GFP protein expression and compared against untransduced HEKs (Figure 5.1 A). Transfection of HEKs with plasmids from Figures A5-A6 by TransIT-293 did not generate sufficient lentiviral particles capable of transducing HEKs (Figure 5.1 B). Concentrated lentiviral stocks increase the titre and removes impurities that may hinder the transduction of target cells (Reiser 2000; Yamada et al. 2003; Ichim & Wells 2011). Furthermore, it is now common practice to conduct a round of ultracentrifugation to transduce different cell types such as T cells and bone marrow cells (Ichim & Wells 2011; Cribbs et al. 2013). Accordingly the next step was to concentrate the viral supernatant by ultracentrifugation and subsequently transduce HEKs. Viral supernatant was produced as previously described in section 2.8.1 and concentrated by ultracentrifugation at 28000 rpm for 3 hours. In order to investigate the transduction capacity of these particles, the concentrated viral supernatant was serially diluted and added to HEKs. This approach did not facilitate successful transduction of HEKs with plasmids from Figures Appendix-1-2 and concentrated by ultracentrifugation (Figure 5.1 C).

### 5.2.2 PRODUCTION OF LENTIVIRAL PARTICLES WITH CALCIUM PHOSPHATE AND CONCENTRATION BY PEG WAS NOT SUCCESSFUL.

Calcium phosphate transfection yields comparable titres to transfection reagents and thus offers a more cost effective transfection method (Cribbs et al. 2013). Moreover, precipitation and concentration of viral proteins can easily be achieved through use of the polymer, polyethylene glycol (PEG) (Kutner et al. 2009). It was therefore investigated if transfection by calcium phosphate with DNA plasmids (Figures A-5 and A-6) and precipitation and concentration with PEG would produce lentivirus

capable of transducing cells. Lentiviral supernatant was produced as previously described in section 2.8.1 and concentrated as outlined in section 2.8.2. Examination of transduced HEKs by flow cytometry revealed that transfection by calcium phosphate with plasmids (Figures Appendix-1 & 2) and subsequent precipitation and concentration by PEG did not yield sufficient quantities of effective viral particles (Figure 5.2).

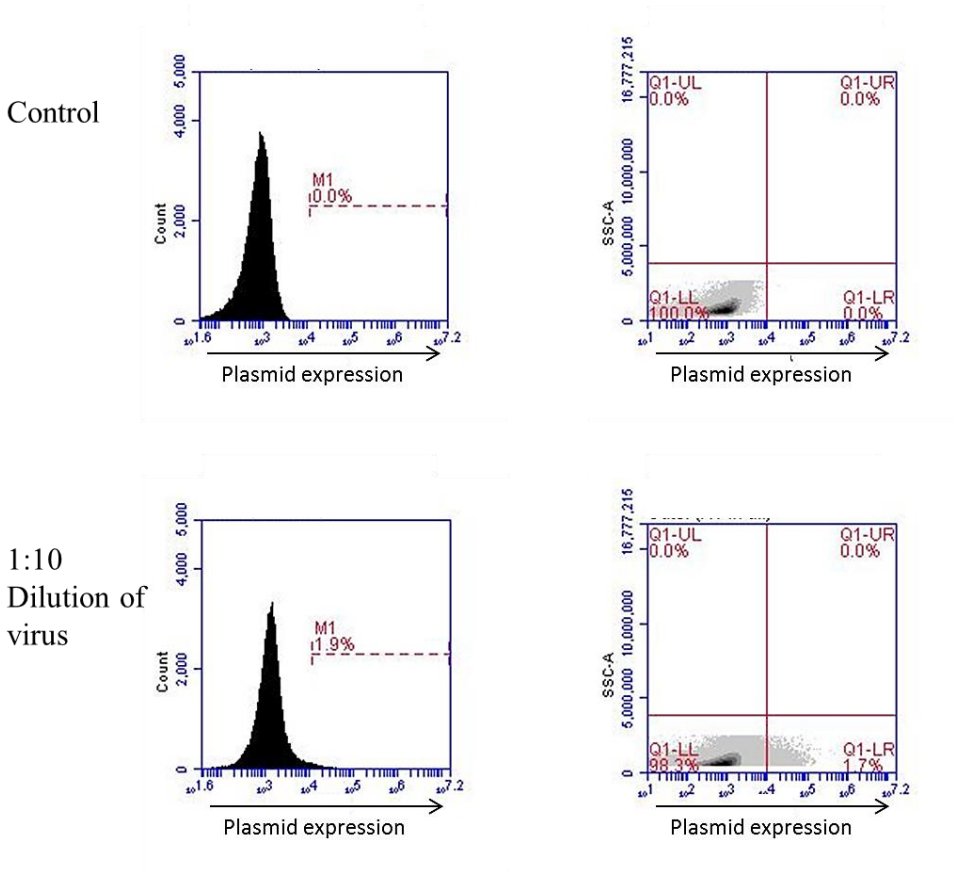



**Figure 5.2. Production of lentiviral particles from transfected HEKs with calcium phosphate and PEG precipitation did not generate sufficient lentiviral particles.** HEKs were seeded at a density of $8.1x10^4$ per well of a 24 well plate 24 hours before incubation with lentivirus. 24 hours later, a serial dilution was prepared of the concentrated lentivirus in media and added to the corresponding well of pre-seeded HEKs. 24 hours after this incubation the cell supernatant with virus was discarded and replaced with fresh medium. A further 48 hours after this the cells were trypsinised, neutralised and analysed for protein expression by flow cytometry for reporter gene expression. Analysis of HEKs after incubation with lentivirus demonstrated that lentiviral production by calcium phosphate and DNA plasmids and subsequent concentration by PEG was not successful. n=1

## 5.3 LUCIFERASE GFP DUAL REPORTER TRANSIENTLY TRANSDUCES HEKs.

Transient transfection allows a cell to express a foreign gene of interest but will not permanently integrate into the genome of the cell therefore permitting rapid testing of functionality. These cells will express the protein of interest for a few days after which the gene is lost through cell proliferation or environmental factors (Kim & Eberwine 2010). Given the lack of expression observed in Figures 5.1-5.2 it was important to probe the functionality of the dual reporter expressing eGFP (Figure Appendix-2). Interestingly, HEKs transiently transfected with the reporter only and observed microscopically 24 hours later, and not 72 hours later via flow cytometry, revealed strong GFP expression (Figure 5.3). This confirmed functionality of the reporter and called into question the suitability of the packaging and envelope plasmids (Figure Appendix-1). Therefore it was important to consider harnessing alternative lentiviral packaging and envelope systems.

## 5.4 LENTIVIRUS PRODUCTION WITH SECOND GENERATION PACKAGING SYSTEM WAS SUCCESSFUL

Seond generation target plasmids must be used in conjunction with a second generation packaging system given that transgene expression from the LTR is dependent on TAT expression (Figure Appendix-3). A third generation target plasmid can be packaged either with a second or third generation system (Figure Appendix-4). It is common practice to use these systems over first generation packging systems due to their enhanced safety feautres as previously outlined. Given that the packaging plasmid in Figure Appendix 1 (A) harboured components only found in first generation

systems and lacked important components, transfection with a second generation system from the Trono lab (Figure Appendix-5) was then tested. Lentiviral supernatant was produced as previously described in section 2.8.1 and titred on HEKs. Examination of transduced HEKs by flow cytometry revealed that transfection by calcium phosphate with plasmids (Figures Appendix-2 and Appendix-5) and subsequent precipitation and concentration by PEG was successful and capable of transducing HEKs (Figure 5.4).

## 5.5 OPTIMISATION OF LENTIVIRAL TRANSDUCTION IN HUMAN MSC

### 5.5.1 Determining Zeocin sensitivy in MSC

Antibiotic selection allows transduced cells expressing a resistance gene to be selected from non transduced populations. The antiobiotic selection in this plasmid was Zeocin. All lentiviral work was preformed in a lentiviral suite and thus MSC were cultured in a normoxic incubator. To investigate the sensitivity of MSC to Zeocin, untransduced MSC were seeded at $5x10^4$ MSC per well of a 6 well plate in duplicate. Media was removed 24 hours later and fresh medium containing Zeocin at varying concentrations was added to the wells. Cell media was replaced with fresh medium containing zeocin or without (control) every 3-4 days thereafter for 14 days. After this, the medium was removed and replaced with fresh cDMEM. Images were taken to examine characteristic morphological changes associated with Zeocin sensitivity and cells were trypsinised, neutralised and counted with EB/AO to investigate sensitivity to Zeocin. The minimum concentration that killed the majority of cells within the 14 days was selected. Zeocin treated MSC displayed typical morphology (Figure 5.5 A). Morphological changes associated with Zeocin sensitity at 200µg/ml were exhibited by

Zeocin treated cells (Figure 5.5 B). Cell count analysis revealed MSC sensitivity to Zeocin at 50 µg/ml (Figure 5.5 C). A concentration of 200 µg/ml was chosen for future experiments.

### 5.5.2 Determining multiplicity of infection

Determining the multiplicity of infection (MOI) is an imporatant parameter to ensure a high percentage of target cells will be transduced and refers to the number of viral particles per cell. Efficient transduction of MSC has been performed with MOI's between 20 and 50 (Lin et al. 2012). In brief, MSC were seeded at 6 $x10^3$ per well of a 96 well plate in duplicate. MSC were allowed to adhere for 24 hours and viral particles were added to each corresponding well at MOIs of 30, 40 and 50 for 24 hours after which media was removed and replaced with fresh cDMEM. 72 hours later, cells were trypsinised, neutralised and total number of GFP expressing MSC was analysed by flow cytometry. Analysis demonstrated no significant difference between transducing MSC at MOI of 30 and 50. Therefore, an MOI of 30 was chosen for future experiements (Figure 5.5 D).

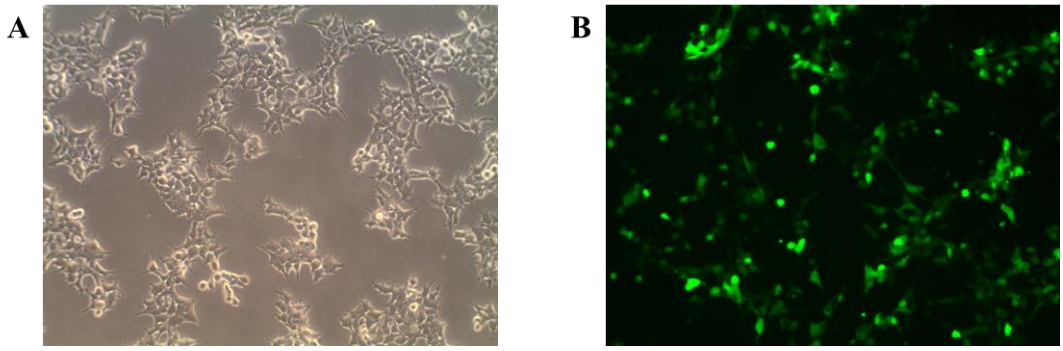



**Figure 5.3. Transient transfection.** A transient transfection assay demonstrated functionality of the dual reporter construct. HEKs were transiently transfected with Mirus- Trans IT and brightfield and fluorescence images were taken (Figure 5.3 A and B). Magnification 100X, phase contrast light microscopy.

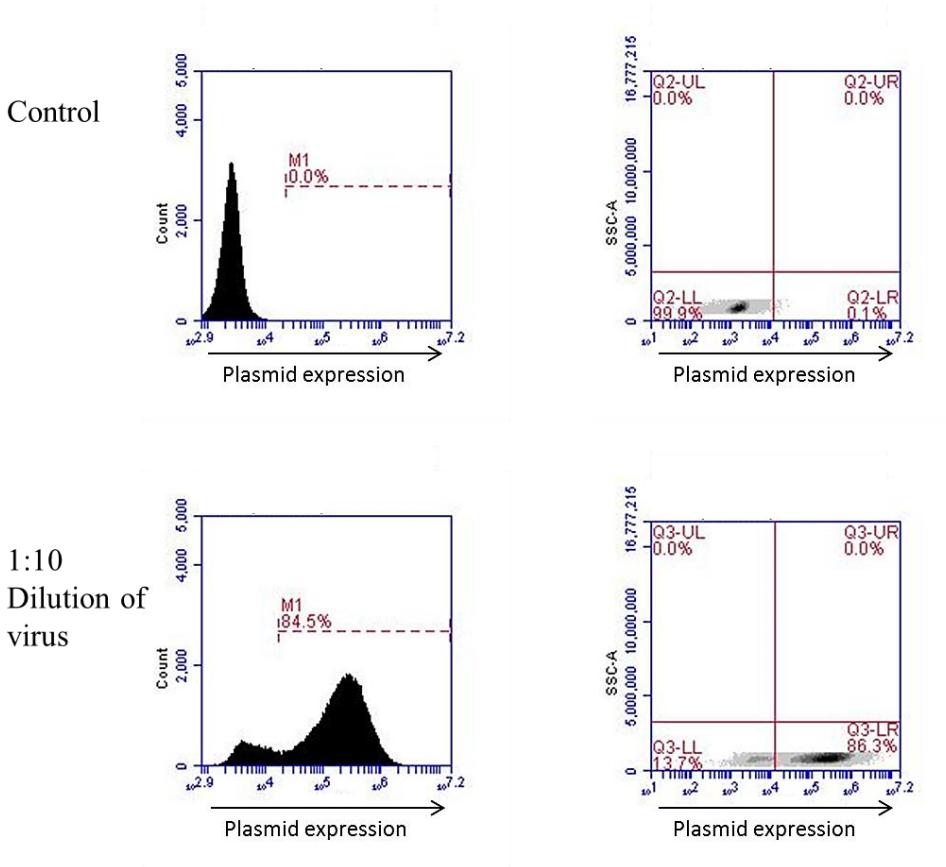



**Figure 5.4. Production of lentivirus with second generation system resulted in expression of reporter plasmid.** HEKs were seeded at a density of $8.1x10^4$ per well of a 24 well plate 24 hours before incubation with lentivirus. 24 hours later, a serial dilution was prepared of the concentrated lentivirus with new second generation system in media and added to the corresponding well of pre-seeded HEKs. 24 hours after this incubation the cell supernatant with virus was discarded and replaced with fresh medium. A further 48 hours after this the cells were trypsinised, neutralised and analysed for protein expression by flow cytometry for reporter gene expression (GFP). Analysis of HEKs after incubation with lentivirus demonstrated that lentiviral production by calcium phosphate and DNA plasmids (Figures Appendix-2 & 5) and subsequent concentration by PEG was capable of transducing HEKs.

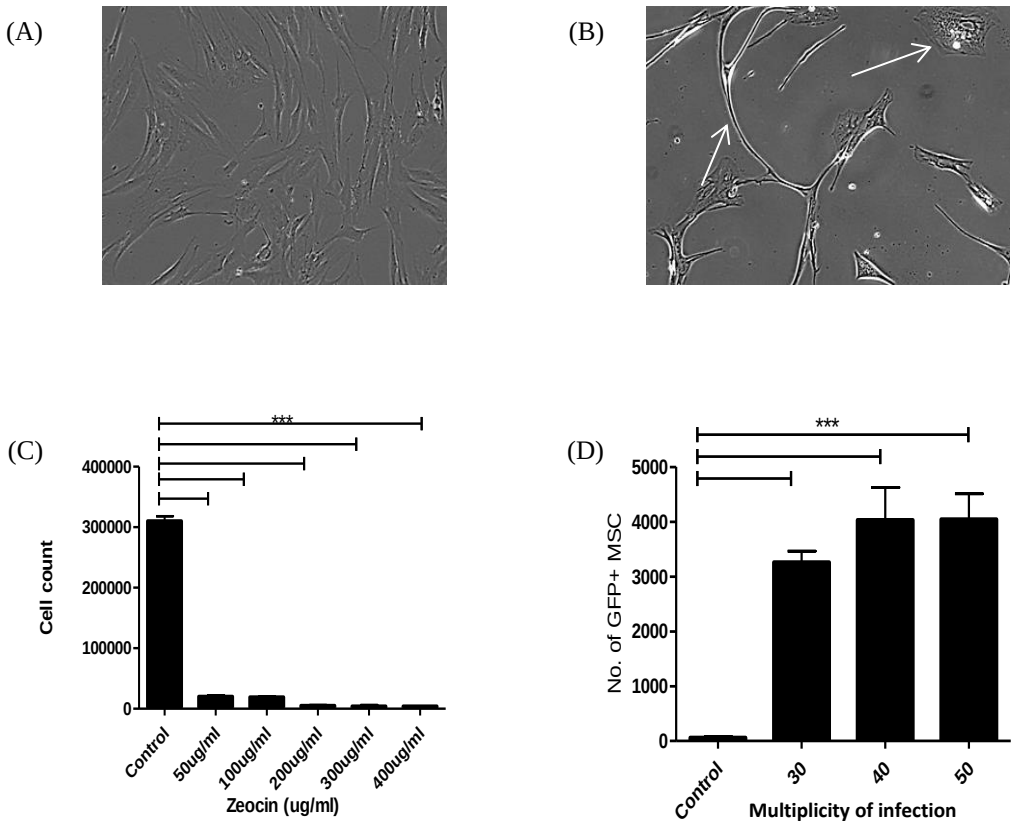



**Figure 5.5 Optimisation of lentiviral transduction in MSC.** Untransduced MSC were seeded at $5x10^4$ MSC per well of a 6 well plate in duplicate and left to adhere overnight. Fresh media containing zeocin or without (control) was added every 3-4 days thereafter for 14 days. In comparison to the control well (A), treatment with 200µg/ml Zeocin resulted in the breakdown of the cytoplasmic membrane and presence of empty vesicles (indicated by white arrows) (B). (C) Cells were trypsinised, neutralised and counted to investigate sensitivity to Zeocin. (D) To determine optimal MOI, MSC were seeded at 6 $x10^3$ per well of a 96 well plate in duplicate and allowed to adhere for 24 hours. Viral particles were added to each corresponding well at an MOI of 30, 40 or 50, or no virus (control). Media was removed and replaced with fresh cDMEM 24 hours later. 72 hours later, cells were trypsinised, neutralised and total number of GFP expressing MSC was analysed by flow cytometry and reported as mean ± SEM of 2 MSC donors. Statistical analysis was carried out using the students' unpaired *t*-test. ***, P<0.001.

## 5.6 TRANSDUCTION DOES NOT ATTENUATE MSC PROLIFERATIVE CAPACITY

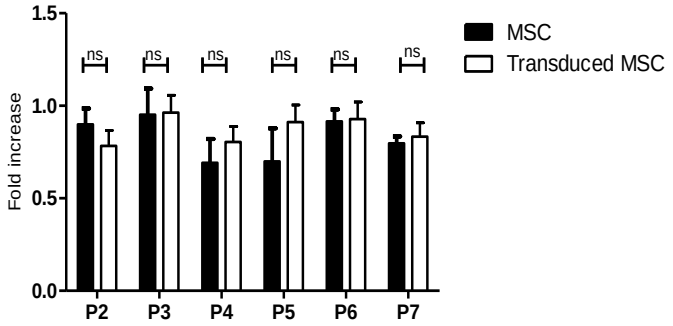
Polycations such as polybrene and protamine sulfate have commonly been employed in tranductions to enhance transduction efficiency (Sambasivarao *et. al.*, 2013). However, a recent report has highlighted the negative impact of polybrene on MSC proliferation during lentiviral transduction (Lin *et al.* 2011). Therefore, the proliferative capacity of MSC transduced in the presence of 50µg/ml protamine sulfate was tested. Transduced and control MSC were seeded in T25 flasks at 1.4 x $10^4$ in triplicate. Every 3-4 days MSC were trypsinised, neutralised and counted with EBAO to measure cell proliferation. Cells were re-seeded at 1.4 x $10^4$ per T25 culture flask. Viral transduction with protamine sulfate (50µg/ml) revealed MSC proliferation was not impaired (Figure 5.6 A). Moreover, MSC displayed typical morphology (Figure 5.6 B) and culture of MSC with protamine sulfate (50µg/ml) did not affect MSC morphology (Figure 5.6 C).

## 5.7 TRANSDUCTION DOES NOT ALTER MSC CELL SURFACE PHENOTYPE.

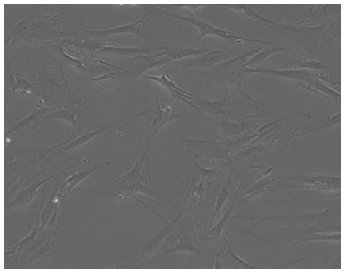
According to the criteria recommended by the ISCT, MSC must posses a panel of cell surface markers in order to be deemed acceptable for laboratory based investigations. Therefore, it was important to ensure that lentiviral transduction did not alter this requirement. Control and transduced MSC were grown to 70-80% confluency. Cell media was removed and cells were detached from the flask with trypsin. Cell media was added 5 minutes later to neutralise the trypsin. Cells were then centrifuged at 300g for 5 minutes at room temperature. Cells were then washed twice with FACS

buffer before plating into v bottom plates. The plates were then centrifuged at 300g for 5 minutes at room temperature. Cells were then stained with a panel of antibodies for 15 minutes at 4$^{o}$C in the absence of light. The cells were then washed twice and centrifuged again before analysis by flow cytometry. Importantly, lentiviral transduction of MSC in did not significantly hinder their typical surface marker expression (Figure 5.7).

(A)




(B)



(C)

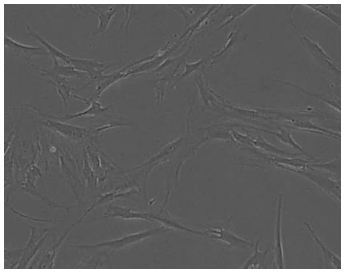


**Figure 5.6 Effect of lentiviral transduction on MSC proliferation.** Transduced MSC were seeded in triplicate into T25 flasks at 1.4 x$10^5$ per flask. Cells were subcultured every 3-4 days. Upon each sub-culture, MSC were trypsinised for 5 minutes followed by subsequent neutralisation with cDMEM. Cells were then centrifuged at 300 RCF for 5 minutes at room temperature. Following resuspension of the cell pellet in 1ml of media, MSC were then stained with EB/AO and counted using a haemocytometer. Cells were subsequently replated and the method repeated until MSC reached passage 7. The fold increase was calculated as follows ((viable MSC number at day 4 - MSC number seeded on day 0)/ MSC number seeded on day 0) and reported as the mean ± SEM of 2 MSC donors. Lentiviral transduction did not impair MSC proliferation as there was no significant difference in cell numbers between transduced and untransduced MSC (A). Furthermore, MSC displayed typical morphology (B) and 50µg/ml protamine sulfate did not affect MSC morphology (C).

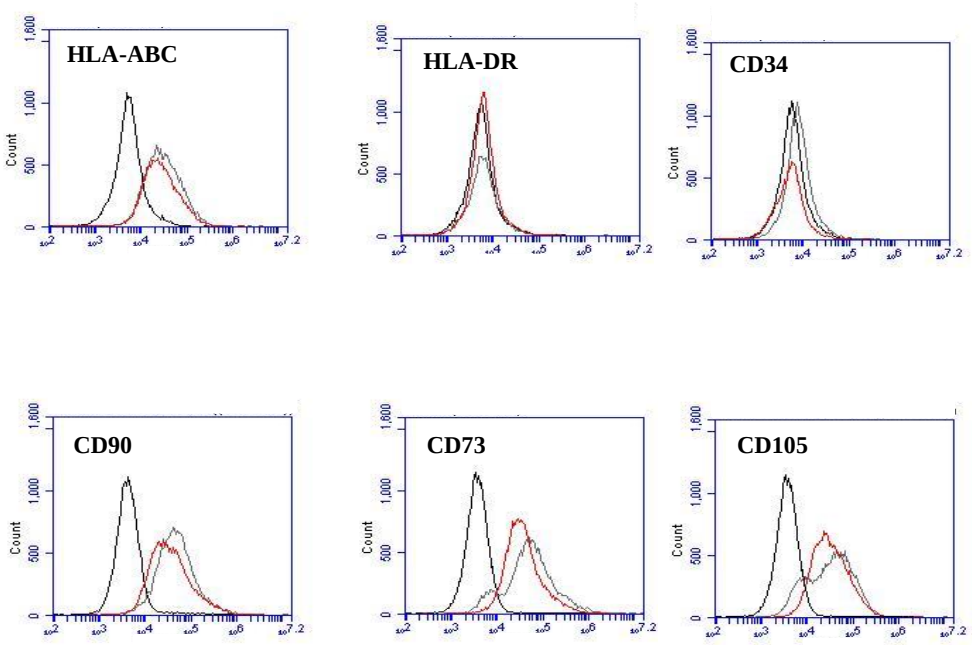



**Figure 5.7 Lentiviral transduction does not alter MSC phenotype.** Transduced and untransduced MSC were analysed for surface marker expression. Cells trypsinised for 5 minutes followed by neutrialisation with cDMEM. Following centrifugation and two washes with PBS cells were then stained with a panel of antibodies for 15 minutes at 4$^{o}$C in the absence of light. The cells were then washed twice and centrifuged again before analysis by flow cytometry. Importantly, lentiviral transduction did not alter MSC surface phenotype. Isotype control; black line, transduced MSC; red line, non transduced MSC; grey line. Data representative of 2 MSC donors.

## 5.8 TRANSDUCED MSC RETAIN OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION POTENTIAL

The capacity of MSC and transduced MSC to differentiate into osteocytes and adipocytes following *in-vitro* induction was determined by calcium deposition and lipid vaculoue formation after a 21 day assay. Non transduced, differentiated MSC displayed osteogenic differentiation and importantly, transduced MSC maintained this osteogenic differentiation capacity (Figure 5.8 I (D)). Furthermore, both non transduced and transduced MSC also demonstrated the capacity to form lipid vacuoles (Figure 5.8 II (D).

## 5.9 TRANSDUCTION WITH SECOND GENERATION LENTIVIRAL VECTOR DOES NOT AFFECT MSC IMMUNOSUPPRESSIVE CAPACITY.

The ability of MSC to modulate immune responses is well established (Ryan et al. 2007; English et al. 2008; Aggarwal & Pittenger 2009). According to ISCT recommendations, validating a population of MSC by the ability to suppress lymphocyte proliferation is pivotal and thus it was important to confirm that lentiviral transduction did not alter this functional capacity of MSC. Anti-CD3/CD28 stimulated PBMCs were co-cultured with non-transduced and transduced MSC (1:5 ratio of MSC: PBMC) in a CFSE suppressor assay as previously described in section 2.7.2. As expected, anti-CD3/CD28 stimulation of PBMC induced a significant increase in PBMC proliferation, whereas the presence of MSC significantly inhibited proliferation (Figure 5.9). Importantly, lentiviral transduction of MSC did not hinder the immunosuppressive function of MSC (Figure 5.9).

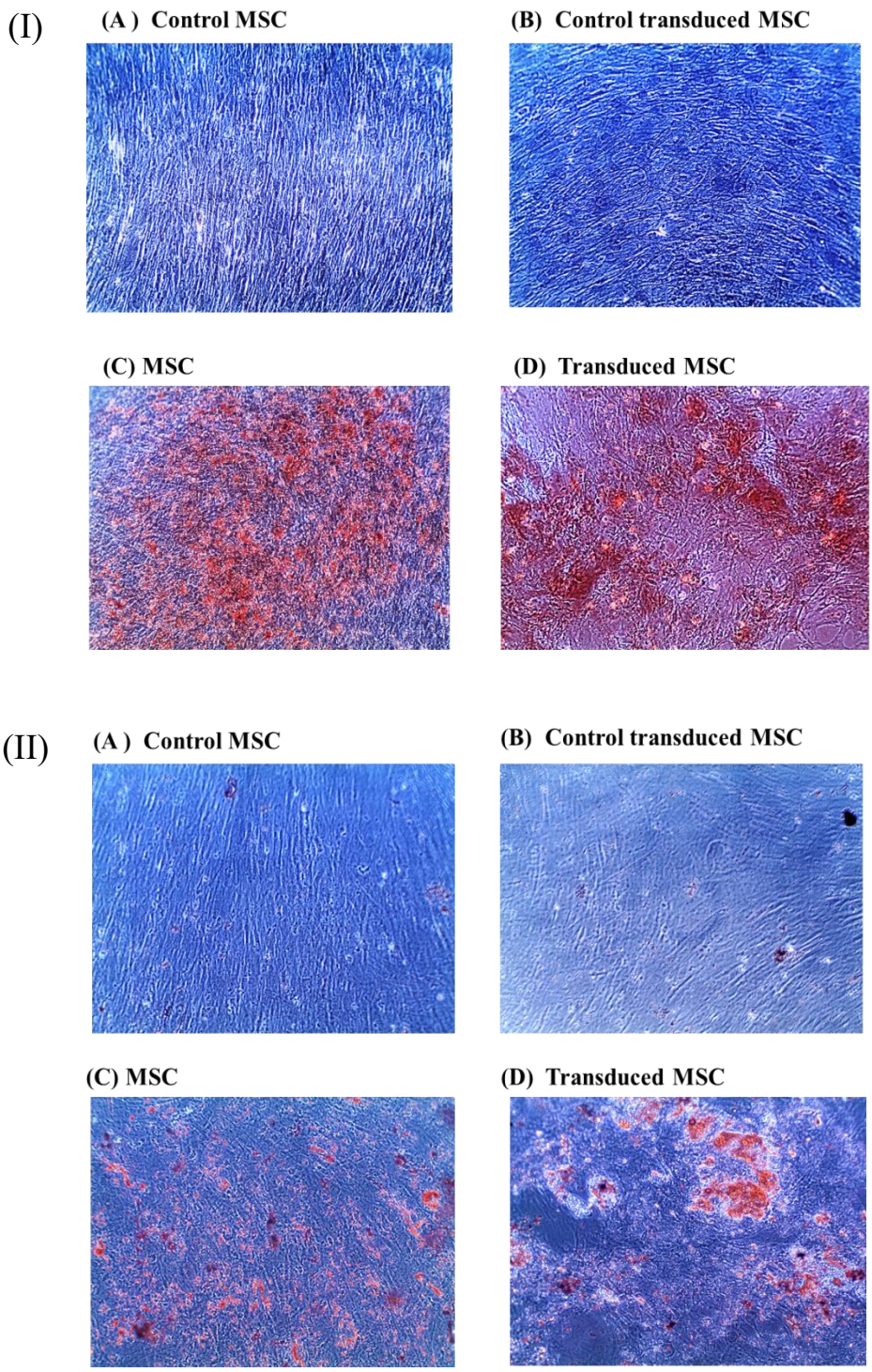



**Figure 5.8. Differentiation capacity of transduced MSC.** Control MSC (A) and control transduced MSC (B) were cultured in cDMEM for 21days. MSC (C) and transduced MSC (D) were differentiated into osteocytes (panel I) and visualised by Alizarin Red S staining and adipocytes (panel II) visualised using Oil Red O staining. Magnification X 100. Data is representative of one human MSC donor.

## 5.10 TRANSDUCED MSC DISPLAY FLUORESCENT AND LUCIFERASE ACTIVITY *IN-VITRO*

The green fluroescent protein (GFP) is produced by the jellyfish *Aequorea Victoria* and is widely used in fluroscent microscopy and gene expression studies (Tsuji 2010). Visualisation of GFP by fluorescent microscopy permits a quick confirmation of successful incorportation of the gene into a target cell. *In-vitro* BLI can also be analysed by measuring the BLI signal emitted from known numbers of cells in culture. Bioluminescence is a versatile and sensitive imaging tool based on the detection of light emission from cells or tissues and depends upon the interaction of the enzyme, luciferase with its specific substrate (Zinn et al. 2008). Furthermore, it represents a non-invasive mechanism of real time imaging (Sato et al. 2004) and provides a tool to monitor tumour growth and metastasis in living animals and track immune cell therapies in tumour bearing mice (Matthias Edinger et al. 2003). Although major advances have been made in the field, GvHD still remains a major complication of HSCT. The complex interactions between MSC and immune cell mediators require additional *in-vivo* studies in order to further elucidate MSC modes of therapeutic action in animal models of GvHD. Therefore, bioluminescence may represent an important tool to investigate the interaction of MSC with immune cell mediators' *in-vivo*.

To measure the luciferase activity *in-vitro,* MSC were plated in duplicate into a 96 well plate and allowed to adhere overnight. The supernatant was then removed and MSC were washed twice with PBS and lysed with 100µl luciferase cell lysis buffer (New England Biolabs). The plate was covered with tinfoil and placed on a shaker for 10 minutes. The plate was then placed at -80$^{o}$C for 2 hours after which it was placed on a shaker to thaw. The lysed cells were then added to a white 96 well plate and 40 µl luciferin buffer (Promega) was added directly to the wells. The plate was then read

immediately on a luminometer. Visualisation of $GFP^+$ MSC confirmed successful transduction (Figure 5.10 A) and importantly cells labelled with the eGFP luciferase reporter also displayed luciferase activity *in-vitro* (Figure 5.10 B).

## 5.11 LENTIVIRALLY TRANSDUCED MSC ARE NOT DETECTABLE USING CRYOVIZ TECHNOLOGY.

Cryo-imaging facilitates the tracking and imaging of fluorescent labelled cells *in-vivo* (Wuttisarnwattana et al. 2015). Given that it is more cost effective than BLI and provides single cell sensitivity it was employed to analyse the biodistribution of hypoxic cultured MSC in comparasion to their normoxic cultured counterparts. However, the GFP labelled cells, although visible microscopically, were not bright enough to be detected by CryoViz$^{TM}$ technology and therefore MSC were labelled with QDots as they have been successfly employed using the CryoViz$^{TM}$ by other research groups (Auletta et al. 2014; Wuttisarnwattana et al. 2015).

MSC were labelled with QDots as decribed in section 2.11.1. In brief, 5µl Comp A (Invitrogen) and 5µl Comp B (Invitrogen) were placed into a 1.5 ml Eppendorf tube, mixed with a pipette and left at room temperature for 5 minutes in the absence of light. 1 ml of cDMEM was added to the eppendorf tube and vortexed for 130 seconds and $5x10^6$ MSC were then added to the eppendorf tube and the sample was pipetted up and down. The tubes were then placed in an orbital shaker for 1 hour set to 37$^o$C. After the incubation, the MSC were washed twice with media and then three times with PBS. Given that QDot labelling efficiency of MSC should be at least 70% (Auletta et al. 2014), QDot labelled MSC were validated before i.v administration (Figure 5.11). MSC were then administered to mice at 1.3 $x10^6$ MSC per mouse as described in section 2.9.2.

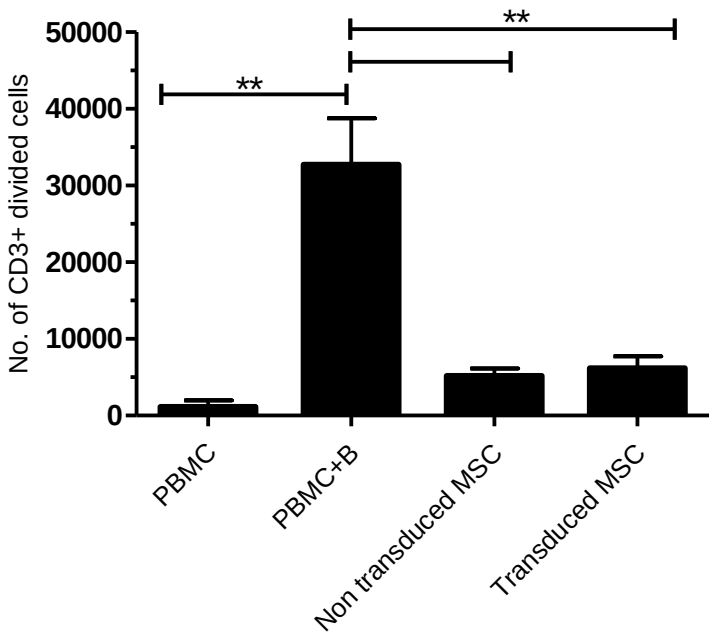
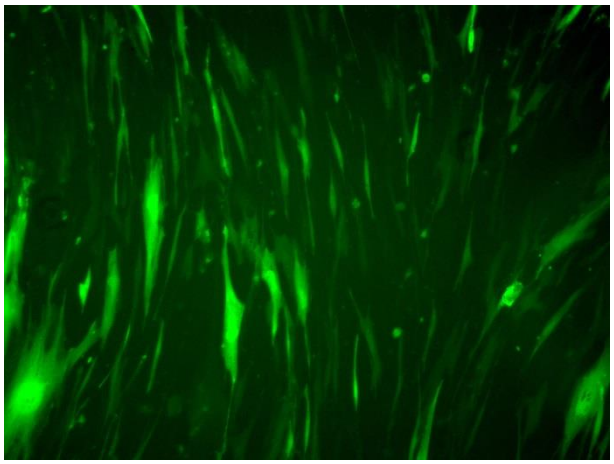



**Figure 5.9. Immune suppression by MSC.** PBMC proliferation was analysed by flow cytometry following a 4 day co-culture with non transduced and transduced MSC at a 1:5 ratio. Anti-CD3/CD28 stimulation significantly promoted PBMC proliferation (PBMC+B). The addition of both non transduced and transduced MSC significantly suppressed anti-CD3/CD28 proliferation and there was no significant difference between non transduced and transduced MSC. Data represented as mean ± SEM from 2 MSC donors. Statistical analysis was carried out using the students' unpaired *t*- test. **, $P \leq 0.01$. SEM; standard error of the mean.

(A)



(B)

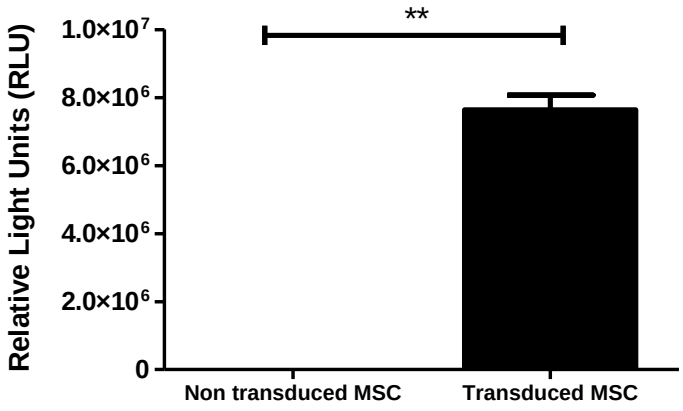



**Figure 5.10. Transduced MSC can be visualised by fluorescent microscopy.** A) MSC were transduced as described in section 2.8.4. MSC positive for eGFP expression could be visualised microscopically; Magnification 10X. B) MSC were analysed by luminometry and light emission displayed as relative light units and reported as mean ± SEM of one MSC donor performed in duplicate. Statistical analysis was carried out using the students' unpaired *t*-test. $^{**}$, $P \leq 0.01$. SEM; standard error of the mean.

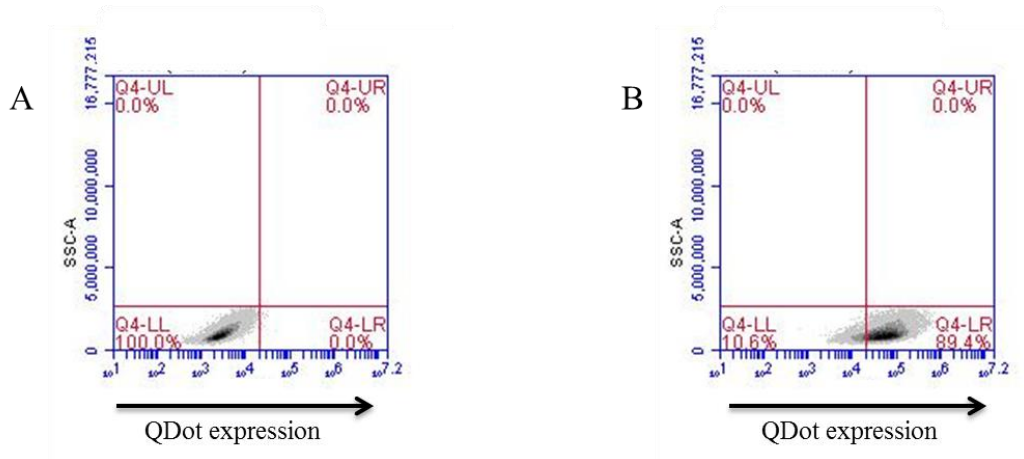



**Figure 5.11. Validation of QDot labelling efficiency.** MSC were fluorescently labelled with red QDots and validated for expression using flow cytometry before administration. Control MSC were unlabelled (A) and 89.4% of MSC were successfully labelled with QDots (B).

## 5.12 BIODISTRIBUTION OF MSC IN A HUMANISED MOUSE MODEL OF AGVHD.

### 5.12.1 Hypoxic MSC enhance survival and reduce weight loss of aGvHD mice

Acute GvHD is a life threatening complication following allogenic HSCT which can occur in 30-50% of patients who receive sibling matched transplants. This experiment was designed to compare and determine the capacity of hypoxic cultured MSC to normoxic cultured MSC to home to aGvHD target organs 24 hours post i.v administration and to increase survival, reduce weigh loss and lower the clinical score in a humanised mouse model of aGvHD.

Normoxic and hypoxic MSC were unlabelled (for survival mice) and QDot labelled (CryoViz$^{TM}$ imaging) as described in section 2.11.1. MSC were then administered to mice as described in section 2.9.2. MSC aGvHD target organs were then harvested 24 hours post MSC administration and prepared for the CryoViz imaging system as outlined in section 2.11.2. The administration of hypoxic MSC significantly increased the survival of mice with aGvHD while all non-treated mice succumbed to GvHD by day 14 (Figure 5.12 A). Human MSC therapy also results in reduced weight loss (Figure 5.12 B) and a lower clinical score (Figure 5.12 C).

### 5.12.2 QDot labelled MSC can be detected *in-vivo* using CryoViz$^{TM}$ technology

Given that MSC transduced with the lentivirus were not detected by the CryoViz, MSC were subsequently labelled with QDots to track the early migration of MSC in aGvHD mice. Single cell resolution identified the presence of MSC in the livers, spleen and lungs of aGvHD mice (Figures 5.13-5.15).

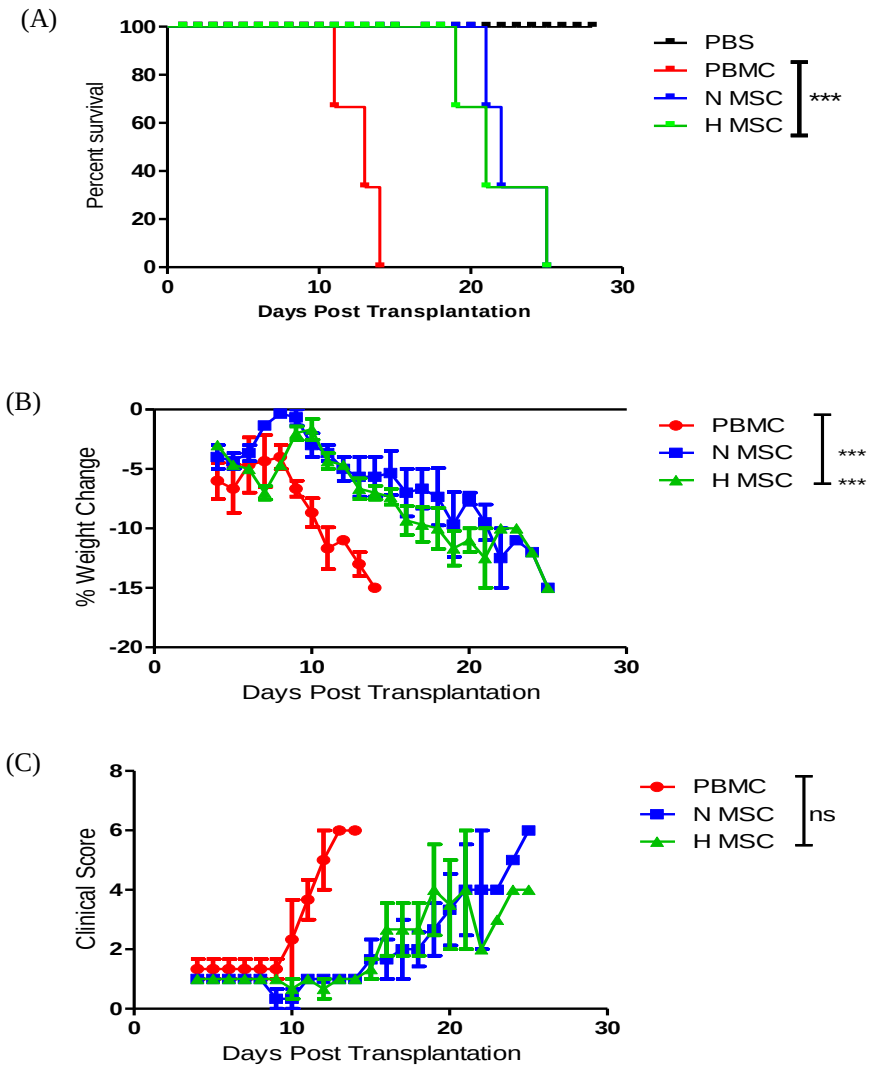



**Figure 5.12. Normoxic and Hypoxic cultured MSC significantly increased survival and reduced weight loss and clinical score in acute GvHD mice.** Graphical representation of (A) Survival, (B) percentage weight loss and (C) clinical score of aGvHD mice and (hypoxic or normoxic) MSC treated mice. 8 x $10^5$ gram-1 PBMC were administered to mice on day 0 post irradiation. 1.3 x$10^6$ MSC per mouse for QDot group were administered on day 7 as a cell therapy. Mice were monitored every second day until day 6 and then every day thereafter. n=3 per group. Statistical analysis was carried out using the survival curve analysis Mantel-Cox log-rank test and the students unpaired t-test for weight change and clinical score, $^{***}$, P≤ 0.001.

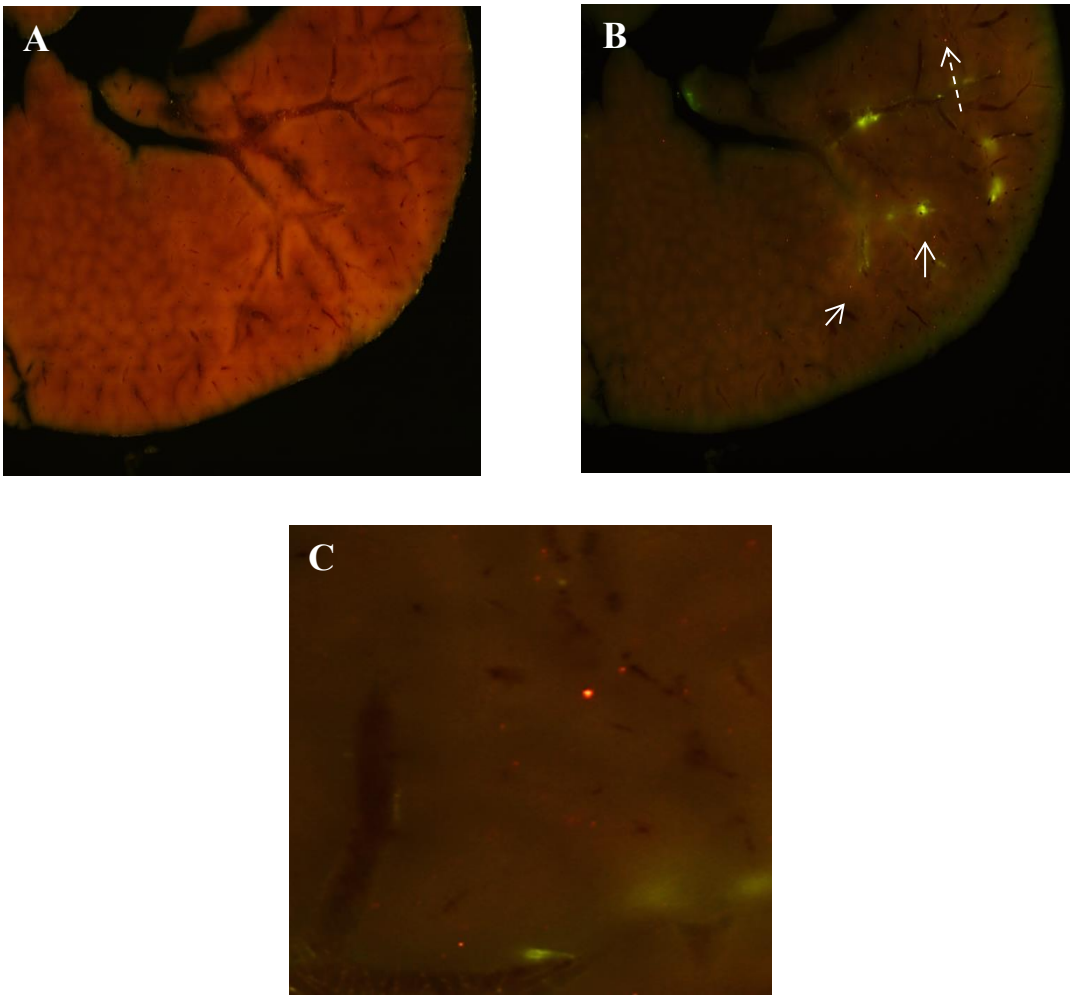



**Figure 5.13. Cryo-imaging of the liver.** Image of a liver following i.v administration of QDot labelled MSC. Cryo-imaging facilitates brightfield contrast (A) as well as fluorescence (B) in the liver. Regions of natural autofluorescence (green) are visible (B & C). Upon zooming into a region of fluorescence (dashed arrow; B) individual cells (labelled with red QDots) can be seen (C). Solid arrows indicate a few other regions of QDot labelled MSC.

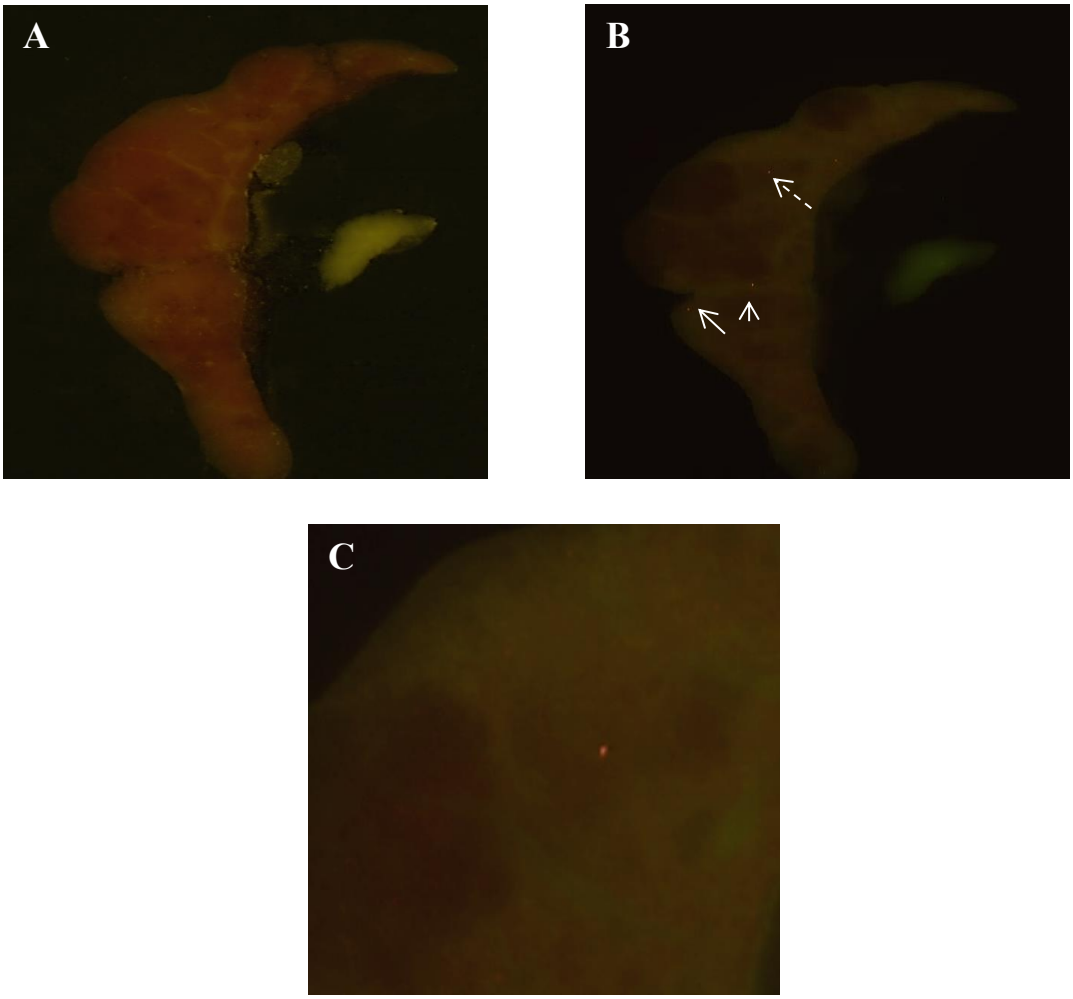



**Figure 5.14. Cryo-imaging of the spleen.** Image of a spleen following i.v administration of QDot labelled MSC. Cryo-imaging facilitates brightfield contrast (A) as well as fluorescence (B) in the spleen. Regions of natural autofluorescence (green) are visible (B & C). Upon zooming into a region of fluorescence (dashed arrow; B) individual cells (labelled with red QDots) can be seen (C). Solid arrows indicate a few other regions of QDot labelled MSC.

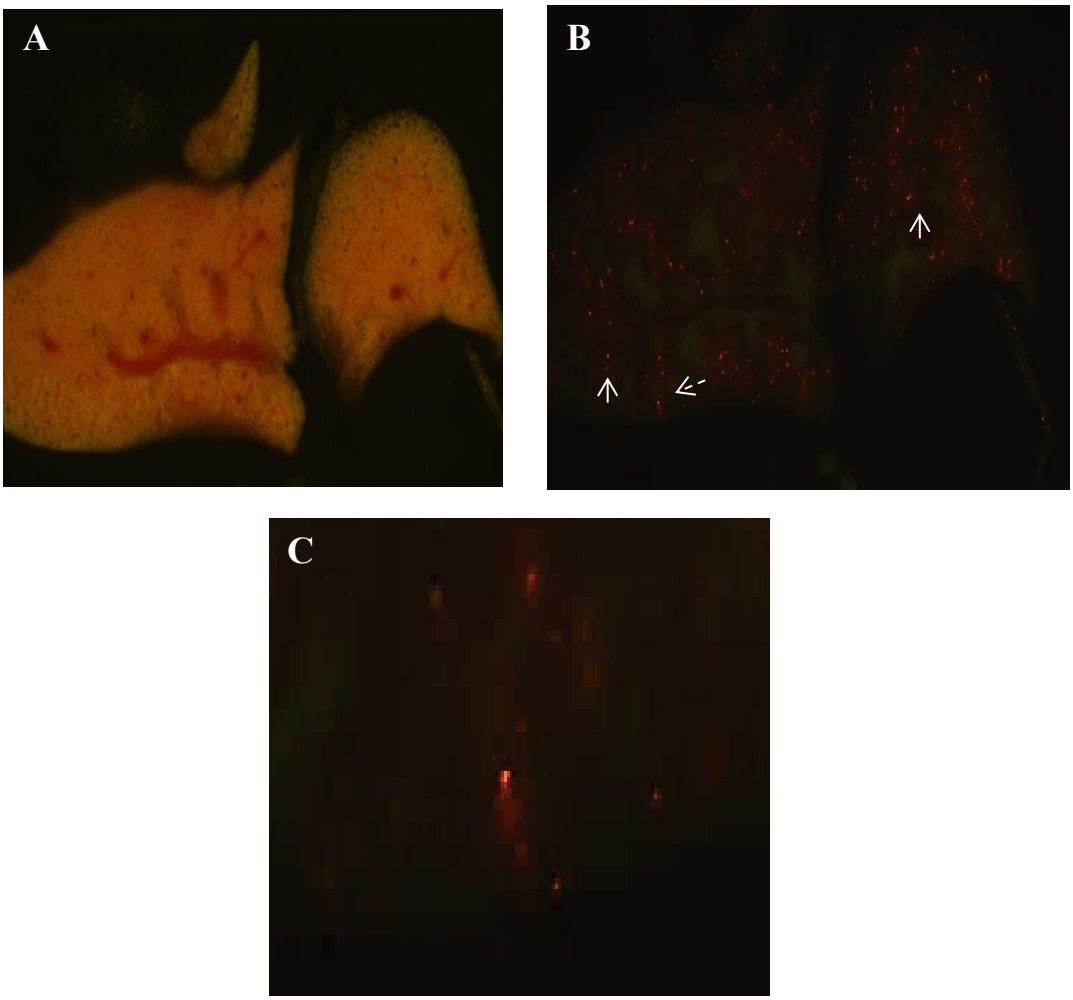



**Figure 5.15. Cryo-imaging of the lung.** Image of a lung following i.v administration of QDot labelled MSC. Cryo-imaging facilitates brightfield contrast (A) as well as fluorescence (B) of the lung. Regions of natural autofluorescence (green) are visible (B & C). Upon zooming into a region of fluorescence (dashed arrow; B) individual cells can be seen (labelled with red QDots) (C). Solid arrows indicate a few other regions of QDot labelled MSC.

### 5.12.3 Hypoxic MSC migrate to aGvHD target organs.

It was next important to determine if hypoxic MSC could comparably migrate to aGvHD target organs and preserve organ integrity. As previously mentioned in sections 1.3.1 and 1.8.3, aGvHD target organs are the skin, liver, GI tract and lungs. The GI tract and skin are intensely auto-fluorescent and thus the capacity of MSC to migrate to the lungs, liver and spleens of mice with aGvHD was analysed by CryoViz$^{TM}$ technology. Moreover, the spleen acts as an important site of donor T cell activation and expansion in HSCT and MSC have been detected in the spleen of aGvHD mice 2 days following bone marrow transplant (Auletta et al. 2014). Cryo-imaging technology was therefore employed to determine a migration profile for normoxic and hypoxic MSC in mice with aGvHD.

MSC were QDot labelled and administered as outlined in section 2.11. Novel CryoViz$^{TM}$ software removes out-of-plane fluorescence and quantifies fluorescently labelled cells at single cell resolution (Steyer et al. 2009). 24 hours after MSC infusion (i.v), target organs were taken and single-cell resolution demonstrated that QDot labelled normoxic and hypoxic MSC migrate to the liver (Figure 5.16) and spleen (Figure 5.17) in similar numbers. However, there were less hypoxic MSC present in the lung at 24 hours post administration (Figure 5.18). Taken together, these novel findings show that the migration capacities of hypoxic and normoxic cultured MSC are comparable.

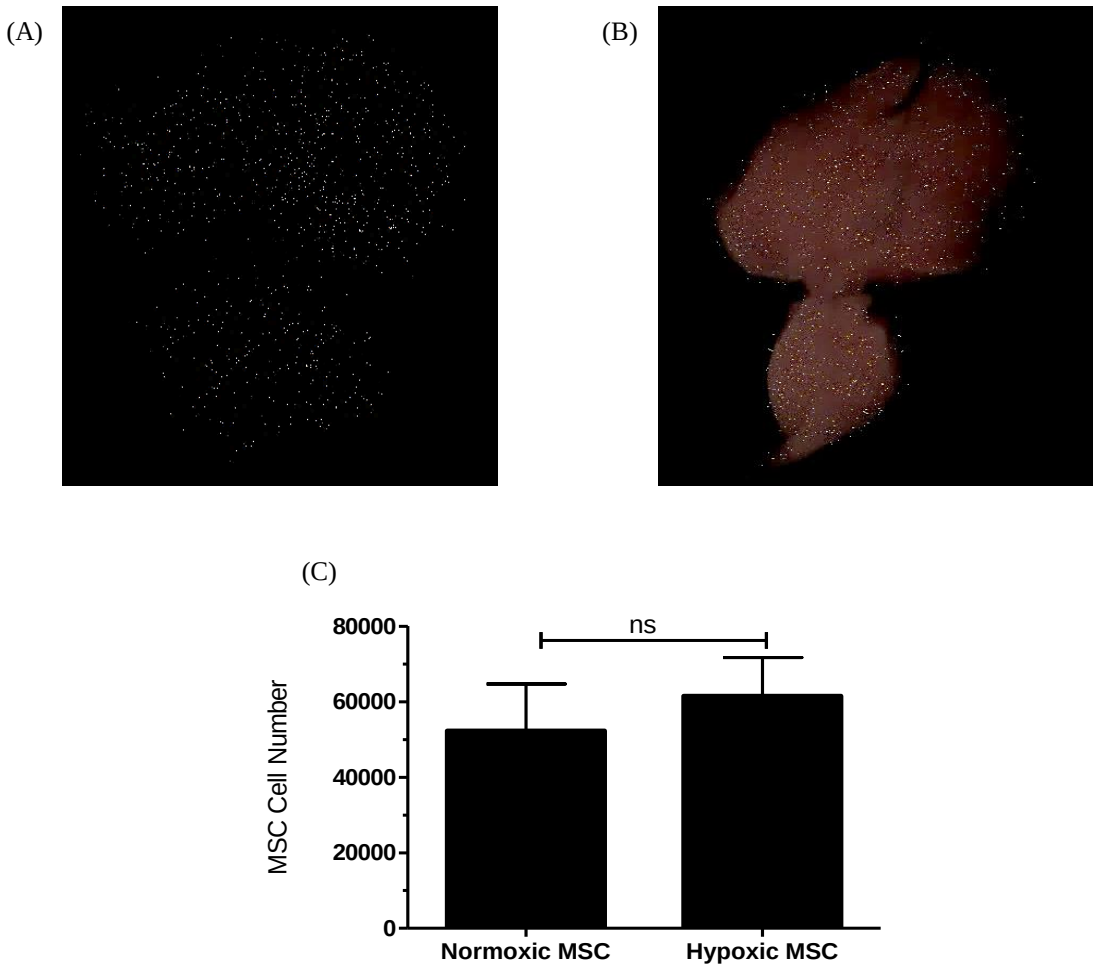



**Figure 5.16. Normoxic and hypoxic cultured MSC migrate to the liver of aGvHD mice.** Livers harvested from three individual mice per MSC group were analysed for MSC migration at 24 hours post i.v administration. (A) Image represents QDot labelled MSC within the liver; not overlaid. (B) Image demonstrating localisation of QDot labelled MSC overlaid onto the liver. (C) Cell detection system quantifies QDot labelled MSC within the livers of aGvHD mice; MSC data reported as mean ± SEM of 3 MSC treated aGvHD livers. There was no significant difference in numbers between normoxic and hypoxic MSC treated livers. Statistical analysis was carried out using a students' unpaired *t*- test. SEM, standard error of the mean.

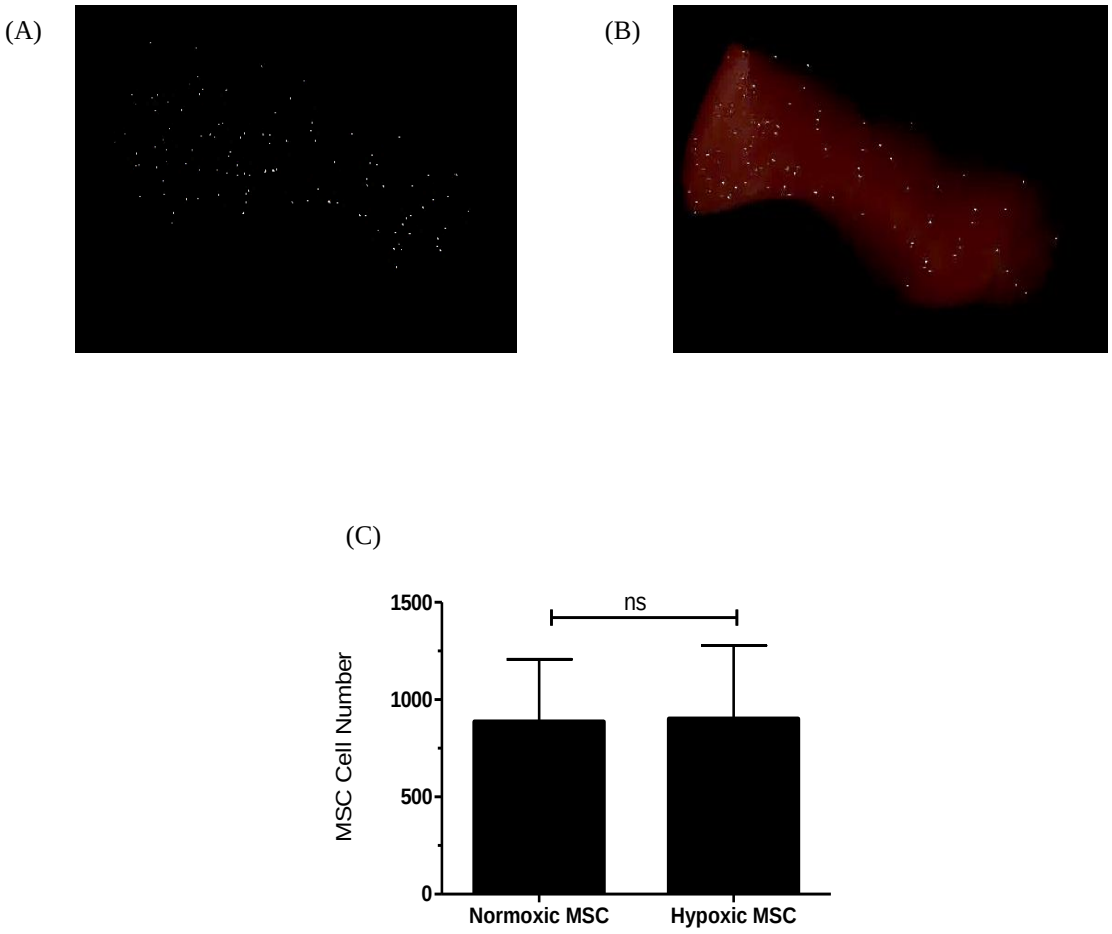
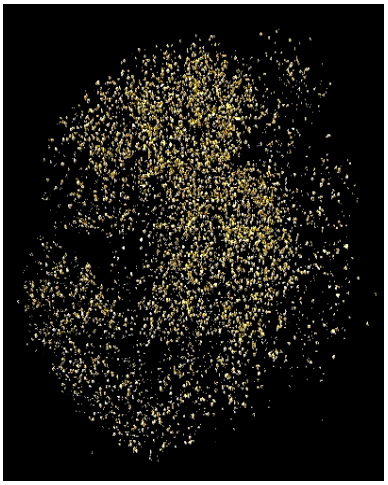



**Figure 5.17. Normoxic and hypoxic cultured MSC migrate to the spleen of aGvHD mice.** Spleens harvested from three individual mice per MSC group were analysed for MSC migration at 24 hours post i.v administration. (A) Image represents QDot labelled MSC within the spleen; not overlaid. (B) Image demonstrating localisation of QDot labelled MSC overlaid onto the spleen. (C) Cell detection system quantifies QDot labelled MSC within the spleens of aGvHD mice; MSC data reported as mean ± SEM of 3 MSC treated aGvHD spleens. There was no significant difference in numbers between normoxic and hypoxic MSC treated livers. Statistical analysis was carried out using a students' unpaired *t*- test. SEM, standard error of the mean.

(A)



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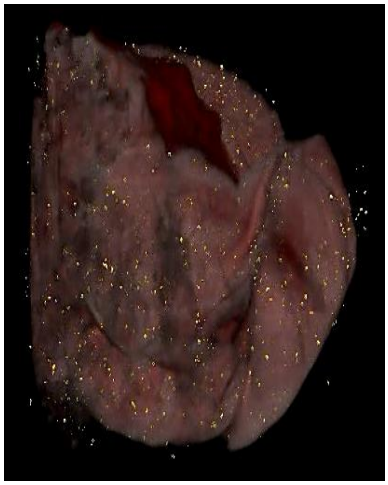

(C)

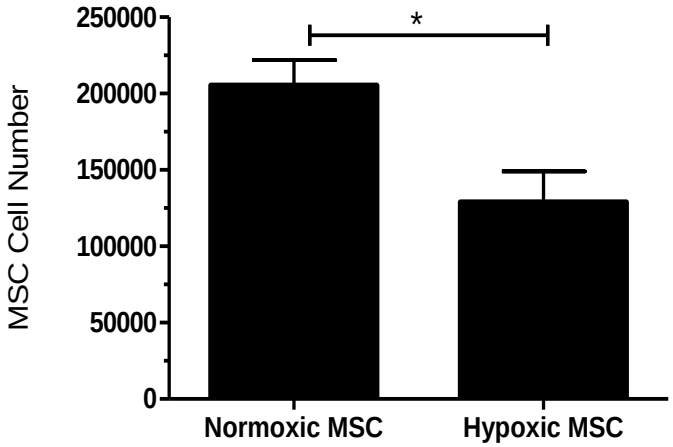


**Figure 5.18. Normoxic MSC are retained in the lungs of aGvHD mice in greater numbers than hypoxic cultured MSC.** Lungs harvested from three individual mice per MSC group were analysed for MSC migration at 24 hours post i.v administration. (A) Image represents QDot labelled MSC within the lung; not overlaid. (B) Image demonstrating localisation of QDot labelled MSC overlaid onto the lung. (C) Cell detection system quantifies QDot labelled MSC within the lungs of aGvHD mice; MSC data reported as mean ± SEM of 3 MSC treated aGvHD lungs. There was a significant difference in numbers between normoxic and hypoxic MSC treated lungs. Statistical analysis was carried out using a students' unpaired *t*- test, *, $P \leq 0.05$. SEM, standard error of the mean.

## 5.13 Summary

The main aims of this chapter were to (1) generate lentiviral particles capable of transducing cells, (2) to characterise MSC transduced with lentivirus and (3) to investigate the capacity for hypoxic cultured MSC to migrate to aGvHD target organs and enhance survival in comparison to normoxic cultured MSC. The generation of lentivirus capable of transducing cells was herein successfully achieved. This chapter presents an optimised method of transducing MSC and demonstrates the possibility of using transduced MSC to track cellular migration *in-vivo*. The subsequent transduction of MSC did not impair their proliferation capacity, cell surface profile, differentiation capacity or capacity to suppress lymphocyte proliferation *in-vitro.* Moreover, transduced MSC displayed light emission *in-vitro* but were not however detectable using the CryoViz$^{TM}$ imaging system. Therefore, the next step was to utilise QDot labelled MSC to examine their biodistribuion in aGvHD harnessing the CryoViz$^{TM}$ imaging system. The admininistration of normoxic and hypoxic cultured MSC to aGvHD significantly prolonged their survival and reduced their weight loss and clinical score (Figure 5.1). Moreover, both normoxic and hypoxic cultured MSC migrated to the livers of aGvHD mice (Figure 5.16). Similarly, normoxic and hypoxic cultured MSC migrated to the spleens of aGvHD mice (Figure 5.17). While the administration of both MSC resulted in their presence in the lung, there were significantly less hypoxic cultured MSC present in the lungs of aGVHD mice 24 hours post administation (Figure 5.18). Collectively, these results display the successfully generation of lentivirus capable of transducing MSC. Furthermore, these findings present for the first time, the migration of QDot labelled, long term hypoxic cultured MSC to aGvHD target organs in a humanised mouse model of aGvHD harnessing CryoViz$^{TM}$ technology.

# CHAPTER 6

# DISCUSSION

The discovery that MSC suppress T cell proliferation *in-vitro* was a milestone for MSC therapeutics and led scientists to investigate the immunomodulatory properties of these cells (Di Nicola 2002; Bartholomew *et al.* 2002). Since then, the prominent therapeutic effects of MSC in inflammatory disorders have been at the front line of cellular therapy development. Interestingly, MSC of various origins possess the capacity to modulate the immune system, however the immunoregulatory activities of MSC first require licensing by inflammatory cytokines (Krampera *et al.* 2006; English *et al*. 2007; Ryan *et al.* 2007; Polchert *et al*. 2008; Sheng *et al*. 2008). In line with this, tissue damage is an inflammatory environment releasing factors that orchestrate the mobilisation of MSC to the injured tissue where activation cues prepare the MSC to modulate inflammatory processes through their physiological functions (Abbott *et al.* 2004; Schenk *et al.* 2007; Belema-Bedada *et al.* 2008; Sasaki *et al.* 2008; Deng *et al.* 2011).

Pre-clinical and clinical studies have demonstrated the efficacy of MSC-based therapy in inflammatory diseases (Le Blanc *et al*. 2008; Gonzalez-Rey *et al.* 2009; Sun *et al*. 2010; Tyndall & van Laar 2010; Tobin *et al.* 2013). Despite these advances, there are numerous unresolved issues which need to be addressed in order to implement MSC as a mainstay cellular therapy for inflammatory conditions. A major hurdle impeding clinical utility is the lack of optimal large scale manufacturing conditions for MSC expansion and culture. Furthermore, the most effective route of MSC administration, dose and dosing schedule remain to be elucidated. Moreover, the lack of understanding of the *in-vivo* persistence and biodistribution of MSC confounds their full potential. Currently, standard practice for expansion of human MSC utilised culture in 21% $O_2$. Given that the *in-vivo* environment is considerably lower than 21% $O_2$, it seems logical to explore the feasibility of utilising more physiological $O_2$ tensions for *in-vitro* cell

expansion. In order to address some of these challenges and develop an optimised MSC therapy for academic and industrial use, it is pivotal to take a step back and harness physiological oxygen tensions to design methods of culturing MSC in a biologically relevant system that will not hinder their physiological functions.

The broad immunosuppressive potential of MSC on the adaptive immune response has been thoroughly investigated and a number of mechanisms identified as key to their therapeutic potential. As a result of such investigations, MSC are now considered to respond to their environment in a multi-faceted manner. For example, T cell chemoattraction is thought to be required for MSC to perform their contact-dependant immunosuppression (Ren *et al.* 2008). Once T cells are in close proximity to MSC, these cells can exert their immunosuppressive effects through short acting soluble factors such as IDO and PGE-2 (Di Nicola 2002; Ren *et al.* 2010) and cell contact-dependant pathways (Akiyama *et al*. 2012; Chinnadurai *et al*. 2014). Moreover, a plethora of immunosuppressive soluble factors released by MSC have been identified (Section 1.1.2.1). The characterisation of mechanisms employed by MSC in modulating the immune responses *in-vitro* and in preclinical models of disease has largely been identified harnessing MSC cultured in normoxia. Culture in hypoxia limits oxidative damage and genetic abnormalities (Estrada *et al*. 2012; Berniakovich & Giorgio 2013). Most clinical trials have not employed hypoxia for cell culture and thus this field is very much in its infancy. However, two trials currently recruiting participants for severe pulmonary emphysema and ischemic limb disease will employ hypoxia to culture expand MSC (ClinicalTrials.gov identifiers NCT01849159 and NCT02336646). Thus, for the full realisation of the potential of hypoxic culture as a solution for MSC-based therapy, the effects of long term hypoxic culture on MSC and the mechanisms by which hypoxic MSC suppress the adaptive immune system must be d



etermined *in-vitro* and *in-vivo.* Hypoxia has proved to be a powerful regulator of MSC proliferation, however little is known about the direct impact of continual hypoxic culture on MSC in terms of immunosuppressive potential. Accordingly, the aim of chapter 3 was committed to determining what effects, if any, hypoxic culture imposed on MSC biology in terms of immunosuppressive capacity *in-vitro*.

Importantly, a faster proliferation rate is pivotal for the efficient use of MSC in large scale studies given that demanding cell numbers are often required for clinical

utility, however large scale expansion remains technically challenging. Therefore, the first line of investigation was to address the effect hypoxia has on MSC proliferation. Importantly, culture of MSC in hypoxia showed higher cell proliferation generating significantly increased MSC numbers between passage 2 and 7. This is an important finding as a method that yields greater cell numbers than the current method of culture may represent a more suitable means of reaching greater numbers of patients in the clinic. This finding is consistent with previous studies which demonstrate enhanced proliferation of MSC under hypoxia (Lennon *et al*. 2001) (Rat BM-MSC; 5% $O_2$), (Grayson *et al.* 2007) (Human BM-MSC; 2% $O_2$), (Carrancio *et al*. 2008) (Human BM-MSC; 5% $O_2$), (X. Li *et al.* 2008) (Rat BM-MSC; 5% and 10% $O_2$), (Dos Santos *et al.* 2010) (Human BM-MSC; 2% $O_2$), (Nekanti *et al.* 2010) (WJ-MSC; 2-3% $O_2$), (Lavrentieva *et al.* 2010) (UC-MSC; 2.5% $O_2$), (Hung *et al.* 2012) (Human BM-MSC; 1% $O_2$), (Berniakovich & Giorgio 2013) (Murine BM-MSC 3% $O_2$), (Boregowda *et al.* 2013) (Murine BM-MSC; 5% $O_2$), (Rylova & Buravkova 2014) (Human AT-MSC; 5% $O_2$), (Feng *et al*. 2014) (Human AT-MSC; 1.5% $O_2$), (Kakudo *et al.* 2015) (Human AT-MSC; 1% $O_2$), (Ali *et al.* 2016) (Human BM-MSC; 5% $O_2$).

However, this result is in contrast to previous reports which suggest hypoxia does not benefit the proliferative capacity of MSC (Holzwarth *et al.* 2010) (Human BM-MSC; 1% $O_2$), (Chung *et al.* 2012) (Canine BM-MSC; 1% and 5% $O_2$), (Ranera *et al.* 2012) (Equine BM-MSC and AT-MSC; 5% $O_2$) (Beegle *et al.* 2015) (Human BM-MSC; 1% and 5% $O_2$), (Kumar & Vaidya 2016) (Rat BM-MSC; 1% $O_2$). Importantly, these inconsistencies may arise dues to variations in the species origin of MSC donor (Carrancio *et al*. 2008; Holzwarth *et al.* 2010; Chung *et al.* 2012; Ranera *et al.* 2012; Kumar & Vaidya 2016), the health of the donor (Holzwarth *et al.* 2010), the level of hypoxia and duration of hypoxic exposure (Dos Santos *et al.* 2010; Feng *et al.* 2014;

Beegle *et al.* 2015). Furthermore, differences in experimental set-up and seeding densities for example, wells of a 12 well plate in comparison to a T-75 culture flask (Carrancio *et al*. 2008; Beegle *et al*. 2015) may contribute to variances.

Friedenstein and others robustly ascertained the differentiation capabilities of a subset of BMSC (Friedenstein *et al,* 1966). However it was demonstrated that these cells were limited in their multipotency to skeletal cell types such as bone (osteoblasts), fat (adipocytes) and cartilage (chondrocytes). The *in-vivo* differentiation capabilities of MSC are less well understood, however the identification of MSC through *in-vitro* functional assays is useful and thus MSC differentiation capacity is one criteria used as a marker to identify MSC *in-vitro* (Dominici *et al.* 2006). The bone marrow is hypoxic in nature (Kofoed *et al*. 1985; Spencer *et al.* 2014) and here, MSC may support hematopoiesis and bone physiology (Majumdar *et al*. 2004; Knight & Hankenson 2013). We found that MSC osteogenic capacity was increased in hypoxic culture and further demonstrates the hypoxic influence on MSC. This result supports previous work by a number of research groups which also harnessed culture in 5% oxygen for rat, human and mouse bone marrow derived MSC (BM-MSC) (Lennon *et al.* 2001; Basciano *et al.* 2011; Hung *et al.* 2012; Binder *et al.* 2014; Prado-Lòpez *et al.* 2014). Furthermore, exposure of human BM-MSC to 2 weeks of hypoxia (2% $O_2$) also displayed enhanced osteogenesis (Wagegg *et al*. 2012). In contrast to these studies, others have demonstrated the inhibition of osteogenesis by human and rat MSC during hypoxic culture (Hung *et al*. 2007) (1% $O_2$), (Fehrer *et al.* 2007) (3% $O_2$), (Potier e*t al.* 2007) ($\leq$ 4% $O_2$), (Holzwarth *et al*. 2010) (1% $O_2$), (Yang *et al*. 2011) (1% $O_2$), (Cicione *et al.* 2013) (1% $O_2$), (Xu *et al*. 2013) (1% $O_2$). The difference in results may in part be due to the level of hypoxic exposure. For example, nearly all of the studies indicating a negative effect of hypoxia on MSC osteogenesis were exposed to 1% $O_2$.

Interestingly, exposure of MSC that had previously been cultured in 1% $O_2$ to 3% $O_2$ restored osteogenic capacity (Holzwarth *et al.* 2010).

*In-vitro* adipogenic differentiation is also used to identify MSC (Dominici *et al.* 2006). In our hands, culture of MSC in hypoxia reduced MSC adipogenic differentiation. This is in line with data from Wagegg *et. al.*, which highlighted that hypoxia (2% $O_2$) suppressed human BM-MSC adipogenesis and promoted osteogenesis (Wagegg et al. 2012). Data from Boyette *et al.*, confirms this finding (Boyette *et al.* 2014) in 5% $O_2$ and a range of studies harnessing hypoxia (1-3% $O_2$) are also in agreement with this data (Fehrer *et al.* 2007; Hung *et al.* 2007; Holzwarth *et al.* 2010; Hung *et al.* 2012; Wagegg *et al.* 2012; Cicione *et al.* 2013).

Given that fatty acid metabolism requires mitochondrial respiration, hypoxia may prevent the use of fatty acids and therefore this result may not be surprising. Interestingly, HIF-1α (key mediator of the hypoxic adaption) has been involved in the inhibition of adipogenesis (Yun *et al.* 2002). Here, the authors elegantly evaluated the role of HIF-1α in murine embryonic fibroblast (MEF) adipogenesis. Treatment of MEFs with hypoxia or hypoxia mimetics suppressed adipogenesis. Furthermore, deletion of the HIF-1α gene from the genome by *cre* allowed MEFs to differentiate into adipocytes in the presence of hypoxia mimetics. Moreover, the authors investigated the effect of hypoxia on adipogenesis differentiation transcription factors such as C/EBPβ, C/EBPδ, and PPARγ2 during differentiation. Intriguingly, under hypoxia the induction of PPARγ2 was completely abolished and C/EBPβ reduced. PPARγ2 expression was also reduced in the presence of hypoxia mimetics (Yun *et al.* 2002). Human MSC express PPARγ transcripts and C/EBPβ (Menssen *et al.* 2011; Amable *et al.* 2014; Kim & Ko 2014; Cohen *et al.* 2015) and PPARγ suppression inhibits MSC adipogenesis (Yu *et al.* 2012). However, other data suggest that hypoxic culture either promotes or

maintains MSC adipogenic capacity (Ren *et al*. 2006; Grayson *et al.* 2007; Basciano *et al.* 2011; Valorani *et al.* 2012). Such variation in the experimental design leads to certain difficulties in the comparative analysis of normoxic versus hypoxic MSC.

The lack of a single surface marker for MSC has constrained MSC identification *in-vivo*, however a number of cell surface proteins can be used to identify MSC *in-vitro* when used in conjunction with each other (Horwitz *et al*. 2005; Dominici *et al.* 2006). Culture of MSC in hypoxia did not alter their cell surface profile. Thus far, a number of data demonstrates hypoxic culture does not impair MSC immunophenotype on BM-MSC (Dos Santos *et al.* 2010; Holzwarth *et al.* 2010; Basciano *et al.* 2011; Wagegg *et al.* 2012; Cicione *et al.* 2013; Nold *et al.* 2014), adipose MSC (AT-MSC) (Valorani *et al*. 2012; Perry *et al*. 2008) or equine BM-MSC (Ranera *et* al. 2012).

MSC interaction with cells of the adaptive immune system is now widely understood and the immunosuppressive properties of MSC are key to their therapeutic utility in inflammatory disorders (Le Blanc *et al.* 2008; Gonzalez-Rey *et al.* 2009; Sun *et al.* 2010; Tyndall & van Laar 2010; Tobin *et al*. 2013). The ability of MSC to suppress lymphocyte proliferation is a hallmark of their identity (Di Nicola 2002; Krampera *et al.* 2006; Aggarwal & Pittenger 2009) and the International Society for Cellular Therapy (ISCT) have called for the functional immunological characterisation of MSC *in-vitro* (Krampera *et al.* 2013). Therefore, the next line of investigation was to investigate the effect of long term hypoxic culture on MSC capacity to reduce PBMC proliferation *in-vitro* using a CFSE co-culture assay. Intriguingly, hypoxia reduced the T-cell suppressive capacity of MSC in comparison to normoxic MSC when MSC are present at a ratio of 1 MSC to 5 PBMC (1:5). This finding is in contrast to the current literature, to our knowledge however, this is the first study to report this finding by long term 5% $O_2$ cultured BM-MSC. Although data concerning the effect of long term

hypoxia on BM-MSC capacity to suppress PBMC proliferation are lacking, few studies suggest hypoxia does not impair the immunosuppressive effects of MSC on PBMC proliferation *in-vitro.* For example, Bobyleva *et al.*, cultured human AT-MSC in 5% $O_2$ for 2-3 passages. Subsequently, these MSC were co-cultured with phytohaemagglutinin (PHA) stimulated PBMC for 72 hours in either normoxia or hypoxia and results indicated that hypoxic AT-MSC retained their immunosuppressive capabilities (Andreeva *et al.* 2015). Moreover, murine MSC primed in normoxia or hypoxia (5% $O_2$) for 6 days before addition to T cells for a further 3 days in either normoxia or hypoxia preserved their immunosuppressive effects (Prado-Lòpez *et al*. 2014). In line with this, human BM-MSC cultured at 5% $O_2$ could also suppress PBMC proliferation in comparison to normoxic MSC in a 5 day CFSE co-culture assay after priming in hypoxic culture for 3 days (Nold *et al*. 2014). Alternatively, Gornostaeva *et al.*, found that co-culture hypoxic (5% $O_2$; cells of 2-3 passages) AT-MSC with PHA stimulated PBMC resulted in a greater inhibition of PBMC proliferation. The authors also analysed CD69 and HLA-DR expression by T cells and found that both normoxic and hypoxic AT-MSC reduced the expression of these activation markers by T cells (Gornostaeva *et al.* 2013). However, PHA stimulated PBMC expressed less CD69 and HLA-DR in hypoxia in comparison to PHA stimulated PBMC in normoxia (Gornostaeva *et al.* 2013). The latter finding is in keeping with the effect of hypoxia on AT-MSC; a 3 day incubation of MSC with PBMC in hypoxia (1% $O_2$) resulted in significantly higher T cell inhibition at a 1 MSC to 5 PBMC (1:5) ratio in comparison to normoxic MSC. Both MSC reduced T cell proliferation at a 1:2.5 ratio. However, the overall proliferation of $CD4^+$ and $CD8^+$ T cells tended to be lower following culture with MSC cultured in hypoxia (Roemeling-van Rhijn *et al.* 2013).

There may be some potential explanations as to the variation between our results and the findings from other studies. Firstly, the main difference is that our system was designed to examine the effect of long term, 5% $O_2$ on BM-MSC capacity to suppress PBMC proliferation in comparison to the standard functional assay (normoxic MSC capacity to supress lymphocytes cultured in normoxia). All the above data cultured normoxic MSC + PBMC in normoxia, and hypoxic MSC + PBMC in hypoxia. The effects of different oxygen tensions on lymphocyte proliferation and activation are a complex variable to add. For example, an interesting study by Atkuri *et. al.,* showed that T cell proliferation in response to stimulation with anti-CD3/CD28 and concanavalin A (ConA) but not PHA was significantly higher in normoxia in comparison to hypoxia (10% $O_2$ and 5% $O_2$) (Atkuri *et al.* 2005). More recently, it was found that blocking aerobic glycolysis abrogated T cell activation (Bottcher *et al.* 2015). Therefore, T cell proliferation may be less profound in hypoxia. Thus in hypoxia, T cell inhibition by MSC could be retained and thereby as suppressive, if not more prominent than normoxic MSC. More recently, the effect of hypoxia on T cell CD69 expression (a marker of early T cell activation) has been described (Atkuri *et al.* 2007). In normoxic cultures CD69 expression on anti-CD3/28 stimulated $CD4^+$ T cells peaks at 12 hours and gradually decreases by 72 hours (Reddy *et al.* 2004; Atkuri *et al*. 2007) and interestingly Atkuri *et al.,* highlighted that culture in hypoxia delayed CD69 expression and does not reach peak levels until 24 hours post stimulation and declines more slowly than in normoxia (Atkuri *et al*. 2007). Therefore, PBMC in co-cultures in normoxia may result in an earlier activation response of these cells and thus proliferation. In turn the MSC may be exposed to an earlier, possibly longer duration of pro-inflammatory cytokine activation (from PBMC) and consequently licensed earlier. It has been demonstrated that VEGF enhances MSC proliferation *in-vitro* (Ball *et al.* 2007; Pons *et*

*al*. 2008; Deuse *et al*. 2009). Culture of activated lymphocytes at 2.5% $O_2$ results in VEGF being detected in supernatants at day 2 of hypoxic culture whereas VEGF wasn't detectable in normoxic cultures until day 4 and the total amount of VEGF was higher in hypoxia (Caldwell *et al.* 2001). It is well understood that MSC require licensing by pro-inflammatory cytokines (i.e. IFN- γ) (Krampera *et al.* 2006; English *et al.* 2007; Dazzi & Marelli-Berg 2008; Polchert *et al.* 2008). The total amount of IFN-γ in the supernatants of activated lymphocytes cultured in hypoxia was lower than in normoxic cultures (Caldwell *et al.* 2001). Others however demonstrated that 16 hours of hypoxia increased IL-4 and IFN-γ production by PBMC although the difference between them was no longer significant at 40 hours (Naldini *et al*. 1997). These select few studies highlight the complexity of oxygen in regulating T cell biology. Therefore, culturing MSC with PBMC in normoxia and MSC with PBMC in hypoxia may generate a confounding system.

A second possible explanation may be the source of MSC utilised. For instance, the two studies that suggest hypoxia enhances MSC suppressive capacity on PBMC proliferation harnessed AT-MSC (Gornostaeva *et al.* 2013; Roemeling-van Rhijn *et al.* 2013) whereas BM-MSC were utilised in our system. It is understood that MSC isolated from different tissues possess different immunomodulatory capabilities and WJ-MSC, AT-MSC, placental-derived MSC (PL-MSC) and umbilical cord blood MSC (UC-MSC) were shown to be more immunomodulatory than BM-MSC (Chang et al. 2006; Ivanova-Todorova et al. 2009; Najar, Raicevic, Boufker, et al. 2010; Sara M Melief et al. 2013; X. Li et al. 2014; Montespan et al. 2014; Rhijn et al. 2014; Bárcia et al. 2015). However, evidence also exists that suggests a more equal immunomodulatory nature between BM-MSC and AT-MSC, WJ-MSC, and UC-MSC (Puissant *et al.* 2005; Yoo *et al.* 2009; Luan *et al.* 2013; Castro-Manrreza *et al.* 2014) or that BM-MSC are

more potent than AT-MSC (Xishan *et al*. 2013). One report using hypoxic cultured BM-MSC highlighted no negative impact on MCS immunosuppressive capabilities although the MSC were not cultured in hypoxia long term (7 days) (Nold *et al.* 2014). Furthermore, experimental design also differed with some studies using a 3 day co-culture assay (Gornostaeva *et al*. 2013; Roemeling-van Rhijn *et al*. 2013; Prado-Lòpez *et al.* 2014; Bobyleva *et al*. 2016) or a 5 day assay (Nold *et al.* 2014) in comparison to a 4 day co-culture assay used in our system.

To further probe the dynamics of hypoxia on MSC immunosuppressive capabilities, it was logical to next investigate if a shorter period of time in hypoxia would preserve this capability. Interestingly, culture of normoxic MSC in short term hypoxia (N-H, 8 days) did not impair their capacity to suppress PBMC proliferation in comparison to normoxic MSC. This finding is in keeping with previous data in which shorter periods of hypoxic exposure allowed MSC to retain this effect on PBMC (Nold *et al.* 2014; Prado-Lòpez *et al*. 2014; Bobyleva *et al*. 2016). This finding raised questions on whether hypoxic MSC, introduced into normoxic culture would still be less potent suppressors of PBMC proliferation. Therefore, the next line of investigation was to examine the effect of long term hypoxic MSC cultured in normoxia (H 20 days-N; 8 days) on PBMC proliferation. Surprisingly, H-N MSC regained the capacity to suppress PBMC proliferation *in-vitro.*

As previously mentioned, the dose of MSC administration in pre-clinical and clinical scenarios of aGvHD is an unresolved issue in the field and varies significantly (Sudres *et al.* 2006; Le Blanc *et al.* 2008; Martin *et al.* 2010; Tobin *et al.* 2013; Z. Y. Li *et al*. 2014) and may provide a rationale for why studies do not meet the primary endpoint. For example, MSC can inhibit or enhance PBMC proliferation depending on extent of baseline PBMC proliferation (and ultimately MSC activation) and the MSC:

PBMC ratio (Bocelli-Tyndall *et al*. 2009; Najar *et al.* 2009). Therefore, it was important to investigate if hypoxic culture of MSC inhibited or enhanced PBMC proliferation at different ratios in comparison to normoxic MSC. We previously discovered that normoxic, but not hypoxic MSC suppressed PBMC proliferation at a ratio of 1 MSC to 5 PBMC (1:5). Normoxic or hypoxic MSC did not significantly suppress PBMC proliferation to ratios of 1:10, 1:20 and 1:40. This is in line with findings that demonstrate only high MSC to PBMC ratios suppress actively proliferating lymphocytes (Bocelli-Tyndall *et al*. 2009; Najar *et al.* 2009; Cuerquis *et al.* 2014). Interestingly, at a 1:40 ratio of hypoxic MSC to PBMC, proliferation of stimulated lymphocytes appeared to be slightly enhanced, albeit not significantly. This latter finding is in keeping with the previous studies which demonstrate that at ratios of MSC: lymphocytes at 1:2 and 1:5, suppression is preserved. However, lymphocyte proliferation (MSC: lymphocytes 1:10 and 1:50) is enhanced above the baseline value at lower MSC to PBMC ratios (Bocelli-Tyndall *et al.* 2009). This trend was also readily apparent in PHA/IL-2 and anti-CD3/CD28 stimulated lymphocytes (Najar *et al.* 2009).

Accordingly, the next step was to explore the capacity of N-H MSC and H-N MSC to inhibit or enhance PBMC proliferation in a dose dependent manner. While N-H MSC suppressed PBMC proliferation at a 1:5 ratio, this effect was not surprisingly lost at higher MSC: PBMC ratios. However, in an independent experiment, H-N MSC maintained the capacity to suppress PBMC proliferation at a 1:10 ratio but was not preserved at higher MSC to PBMC ratios (1:20 and 1:40). A potential explanation for this may be the responsiveness of the PBMC donors resulting in a higher level of PBMC proliferation in this experiment in comparison to the previous one (N-H). It is known that MSC suppression correlates with licensing by pro-inflammatory cytokines produced by activated PBMC and immune cells (Krampera *et al.* 2006; English *et al.*

2007; Dazzi & Marelli-Berg 2008; Polchert *et al.* 2008; Rameshwar 2008; Sheng *et al.* 2008; Hemeda *et al.* 2010; Prasanna *et al.* 2010; Ren *et al.* 2010; Mougiakakos *et al.* 2011; Cuerquis *et al.* 2014) and that MSC suppression of PBMC proliferation occurs on proliferating PBMC (Benvenuto *et al.* 2007; Bocelli-Tyndall *et* al. 2009). This principle of MSC inhibiting or enhancing PBMC proliferation depending on their level of interaction warrants further investigation in pre-clinical and clinical scenarios of inflammatory diseases, as is it not known how many infused MSC reach their target destination and thus, the true ratio of MSC to PBMC *in-vivo* cannot be defined.

Given that hypoxic MSC were less potent suppressors of PBMC proliferation *in-vitro* in comparison to normoxic MSC, the next step was to investigate the levels of known MSC immune mediators expressed by MSC in hypoxia and compare these to normoxic MSC. The chemoattraction of T cells is thought to be a prerequisite for MSC to mediate their immunosuppressive effects within the localised vicinity and thus this avenue was investigated. Data concerning the effects of long term hypoxia on bone marrow derived MSC expression of these chemokines is lacking. Here, hypoxic culture did not impair CCL2 or CXCL9 expression. This is consistent with the few reports that highlight CCL2 or CXCL9 expression by murine BM-MSC was not altered by short term hypoxic culture in comparison to normoxic MSC (Chen *et al.* 2008) or CCL2 by human BM-MSC (Potier *et al.* 2007). However Basciano and colleagues found that culture of MSC in hypoxia (5% $O_2$) for up to 35 days impaired MSC CCL2 production (Basciano *et al.* 2011). Conversely, it was demonstrated that AT-MSC cultured in hypoxia (5% $O_2$) promoted CCL2 production (Bobyleva *et al*. 2016). It is clear from these data that more studies are needed to fully elucidate the impact of hypoxia on MSC expression of chemokines. An elegant study by Ren *et al.*, discovered the role of adhesion molecules in MSC contact-dependent interaction with T cells (Ren *et al.*

2010). To further investigate hypoxic modulation of MSC immunosuppression, the next approach was to analyse ICAM-1 expression by MSC. Hypoxic culture did not impair MSC capacity to upregulate ICAM-1 at the mRNA or cell surface protein level thus suggesting this may not be responsible for the lack of immunosuppression displayed by hypoxic MSC. While Bobyleva *et al.*, found less ICAM-1 expression by hypoxic MSC, there was markedly more soluble ICAM-1 present in conditioned medium, albeit not significantly more in comparison to normoxic MSC (Bobyleva *et al.* 2016).

IDO is a potent pro-inflammatory induced soluble mediator of MSC immunosuppression (Meisel *et al*. 2004; Krampera *et al.* 2006; English *et al.* 2007; François *et al*. 2012). Given that hypoxic MSC were less potent suppressors of PBMC proliferation *in-vitro*, it was hypothesised that hypoxic MSC may have an impaired capacity to up-regulate IDO in response to stimulation with IFN-γ. However, in contrast to the hypothesis, hypoxic MSC displayed comparable levels of IDO up-regulation at the mRNA and protein level. This result is consistent with a previous study by Roemeling-van Rhijn *et al.,* 2013 which examined the effect of hypoxia on AT-MSC IDO mRNA expression and activity (by measurement of l-kynurenine, the breakdown product of tryptophan). AT-MSC maintained the capacity to induce IDO mRNA following pro-inflammatory cytokine stimulation under hypoxia. Furthermore, culture of AT-MSC in hypoxia for 24 or 72 hours did not affect L-kynurenine levels suggesting preserved IDO activity under hypoxic conditions (Roemeling-van Rhijn *et al.* 2013). Pro-inflammatory stimulated MSC employ programmed death-ligand 1 (PD-L1, also called B7-H1) as a contact-dependent mechanism of immunosuppression (Augello *et al*. 2005) through interaction with its receptor PD-1 on lymphocytes. siRNA knockdown of PD-L1 abolished the immunosuppressive capacity of murine

MSC (Sheng *et al.* 2008) and significantly decreased the inhibitory effect of human MSC on T cells (Chinnadurai *et al*. 2014). Initial investigations into the identification of a down-regulated MSC immune mediator by hypoxic culture, as a possible explanation for the observed lack of immunosuppression *in-vitro,* revealed that thus far all selected immune mediators were not affected by hypoxic culture. Therefore, it was hypothesised that PD-L1 expression by MSC may be reduced in long term hypoxia and may provide a rationale for why hypoxic MSC were not as immunosuppressive as normoxic MSC. In agreement with published data, normoxic MSC up-regulated PD-L1 following IFN-γ stimulation. Data concerning the effect of hypoxia on MSC expression of PD-L1 are lacking in the literature. Notably, culture of MSC in hypoxia did not impair MSC capacity to induce PD-L1 mRNA or protein expression following stimulation for 24 hours with IFN-γ. These findings are in line with Roemeling-van Rhijn *et al.,* where short term culture of AT-MSC in hypoxia for up to 72 hours did not impair their capacity to up-regulate PD-L1 mRNA (Roemeling-van Rhijn *et al.* 2013).

All of these findings have suggested hypoxia does not negatively affect MSC mediators of immunosuppression. However, the impact of long term hypoxia on the capacity of BM-MSC to produce PGE-2 has not yet been thoroughly investigated. Accordingly, the next approach was to examine the expression of this well-known mediator of MSC immunosuppression. Stimulation of MSC with pro-inflammatory cytokine TNF-α up-regulates COX-2 expression and subsequently lipid PGE-2 production (English *et al.* 2007). Of note, TNF- α stimulated MSC cultured in long term hypoxia did not significantly up-regulate COX-2 mRNA in comparison to normoxic MSC. The next step was to confirm this finding at the lipid level by competitive ELISA. While the effect of hypoxia on MSC production of PGE-2 is poorly documented, it was hypothesised that hypoxic MSC would produce less PGE-2 in

hypoxia in response to TNF-$\alpha$ stimulation given that COX-2 mRNA was not up-regulated. In line with the hypothesis, hypoxic MSC were less potent up-regulators of PGE-2 production. In order to further verify these results, normoxic and hypoxic MSC were co-cultured with PHA/Ionomycin (ION) stimulated PBMC and examined for expression of COX-2. As expected, expression of COX-2 mRNA was lower in hypoxic MSC in comparison to normoxic MSC following co-culture with activated PBMC.

The hydrolysis of arachidonic acid from phospholipids in cell membranes and conversion to PGE-2 (through a series of enzymatic reactions) is a complex and intricate process. Molecular oxygen is required for the formation of PGE-2 as the COX reaction inserts two molecules of $O_2$ into the arachidonic acid backbone to yield prostaglandin $G_2$ ($PGG_2$). $PGG_2$ is then reduced to prostaglandin $H_2$ ($PGH_2$) + water which can be converted to PGE-2 by prostaglandin E synthase (Smith *et al.* 2011). Therefore it seems reasonable to assume that under conditions of sparse oxygen, the COX-2 reaction may be limited in its production of PGG2 and subsequent intermediates for PGE-2 by BM-MSC. Data concerning the effect of hypoxia on BM-MSC expression and production of PGE-2 are poorly documented. Two recent studies however cultured human UC-MSC (1% $O_2$), and AT-MSC in hypoxia (5% $O_2$) and found increased COX-2 expression or no significant difference in comparison to normoxic MSC (Han *et al.* 2015; Bobyleva *et al.* 2016). In line with our data, hypoxia attenuated PGE-2 production by human placental explants and placental macrophages (Blumenstein *et al.* 2001; Wetzka *et al.* 1997). Interestingly, fatty acid oxidation dependent oxidative phosphorylation and ATP production (required for fatty acid synthesis) was decreased in hypoxia in T cells (Bottcher *et al.* 2015). These data require further exploitation and highlights the need for the investigation into the effect of long term hypoxia on MSC expression and production of COX-2 and PGE-2.

This study has highlighted that the impact of long term hypoxia on BM-MSC immunosuppressive activity *in-vitro.* Few *in-vitro* studies report the preserved capacity of MSC to suppress PBMC proliferation in various degrees and exposures of hypoxia *in-vitro* (Gornostaeva *et al.* 2013; Roemeling-van Rhijn *et al.* 2013; Nold *et al.* 2014; Prado-Lòpez *et al.* 2014; Bobyleva *et al*. 2016). Reports have not investigated the effect of long term hypoxia (5% $O_2$) on bone marrow derived MSC expression and production of COX-2 and PGE-2. We have shown that hypoxic MSC are less potent suppressors of PBMC proliferation *in-vitro* in comparison to the standard functional assay of normoxic MSC + PBMC co-cultured in normoxia. We have also demonstrated that COX-2 mRNA expression and PGE-2 production is reduced in TNF-α stimulated hypoxic MSC. In addition, this thesis has shown that hypoxic MSC expressed less COX-2 mRNA following co-culture with activated PBMC than normoxic MSC. Chapter 3 extensively characterised the effect of hypoxia on MSC mediators of immune suppression *in-vitro* and provided comprehensive data on the effects of hypoxia on MSC immune mediators. However, the challenge facing *in-vitro* studies is that they are merely systems designed to emulate the *in-vivo* setting. Although, it is essential to *ex-vivo* expand MSC for clinical utility, we cannot assume that MSC propagated in a dish supplemented by medium will be comparable to their physiological function *in-vivo.* Nonetheless, these findings represent advancement in our knowledge of how long term hypoxia modulates MSC immunosuppression *in-vitro*, specifically identifying a down regulation in COX-2 mRNA and PGE-2 production by hypoxic MSC following activation.

MSC based cellular immunotherapy for phase I and II trials has rapidly progressed following early result of the promising effects of MSC in a case of steroid refractory GvHD. While *in-vitro* assays and pre-clinical models of disease have

significantly contributed to delineating the mechanisms employed by MSC, the lack of therapeutic efficacy in a phase III trial for GvHD in conjunction with conflicting data from pre-clinical data (Sudres *et al.* 2006; Tisato *et al.* 2007; Badillo *et al.* 2008; H. Li *et al.* 2008; Prigozhina *et al*. 2008; Palmer & Bonnet 2009; Christensen *et al*. 2010; Mielcarek *et al*. 2012; Tobin *et al*. 2013) has highlighted the need for a standardised approach and understanding of MSC therapeutic mechanisms *in-vivo* (Martin *et al.* 2010). This thesis argues that hypoxia may provide a suitable alternative to normoxic culture for obtaining higher MSC yields. However, chapter 3 revealed that hypoxic culture diminished MSC immunosuppressive capacity *in-vitro*. Thus it was important to next examine if these findings could be translated *in-vivo* or was a result of the cell culture phenomena where *ex-vivo* culture alters cell function. Large numbers of MSC are often required for pre-clinical and clinical studies of aGvHD and hypoxic culture has the potential to overcome this issue. However, as chapter 3 has highlighted, a major challenge in implementing MSC therapy in the clinic is the lack of understanding of the influence of hypoxic culture on MSC. Therefore, chapter 4 advocates that the investigation of hypoxic MSC for aGvHD will identify if they may be used for immunotherapies.

The improvement in the development of animal models to study aGvHD has empowered researchers to investigate the pathophysiology of this disease in a relevant system whereby human cells can engraft in an immunocompromised mouse. The NOD-scid IL2rγ$^{null}$ mouse engrafts high levels of human PBMC (King et al. 2008) and therefore the pathogenesis in these models driven by human immune cells mimics a human, clinically relevant system ideal for assessing cellular therapy interventions. As described in chapter 1, the NSG model is the gold standard for aGvHD and has been established and optimised in the English lab (Tobin *et al.* 2013). Tobin *et. al.,*

harnessed this model and used human lymphocytes obtained from freshly drawn blood and optimised the model based on the Pearson protocol (Pearson *et al.* 2008; Tobin *et al.* 2013). This approach was further optimised by using human PBMC isolated from buffy packs and normalising the PBMC dose to the weight of each mouse (Tobin *et al.* 2013; Healy 2015). The progression of this model in the English lab has facilitated the performance of large scale *in-vivo* experiments which were difficult using freshly drawn blood which yielded relatively low numbers of PBMC.

Subsequent investigations into the efficacy of MSC for aGvHD in this model led to the demonstration that MSC delivered on day 7 post irradiation and PBMC administration could prolong the survival of aGvHD mice (Tobin *et al.* 2013; Healy 2015). Building on from findings in chapter 3, this humanised mouse model of aGvHD was harnessed to explore the clinical utility of hypoxic cultured MSC. To mimic the current pre-conditioning regimen, NSG mice were exposed to 2.4 Gy whole body irradiation and administered human PBMC ($8 \times 10^5$ $gram^{-1}$) isolated from buffy packs on day 0. The development of aGvHD was compared between groups of mice which received MSC therapy or mice only receiving PBS. The onset of aGvHD was extremely aggressive as mice started succumbing to the disease by day 7. It is likely that the responsiveness of the PBMC acquired from the human donor is responsible for the aggressive onset of aGvHD. For example, a link between the health and age of individuals and the functional capacity of their immune cells has been reported (Weng 2006; Amar *et al.* 2007; Boynton *et al.* 2007; O'Shea *et al.* 2010; Choi *et al.* 2008). Therefore, it seems plausible that a very healthy, young donor was used in this study. Therefore, it's possible that the PBMC donor was highly responsive resulting in a severe, early onset of aGvHD. Despite this, the administration of normoxic MSC significantly prolonged the survival of aGvHD mice (15 days MST) in agreement with

previous data (Tobin *et al*. 2013). We have furthered this data by demonstrating that hypoxic MSC also significantly prolonged the survival of aGvHD mice (14.5 days MST) in comparison to the PBMC only control (8 days MST).

As previously outlined, MSC must first be activated in order to mediate their immunomodulatory effects. As GvHD develops, the levels of pro-inflammatory cytokines increase and by day 2 to 7, there are sufficient levels of these cytokines present within the aGvHD mouse for the activation of hMSC (Tobin *et al.* 2013). Thus, the administration of MSC during the early phase of aGvHD licenses these cells to orchestrate changes in the microenvironment. GvHD results in the apoptosis of epithelial cells that line the GI tract that leads to abdominal pain, vomiting, bloody diarrhoea and ultimately severe weight loss in patients (Martin *et al*. 1990). Importantly, groups which received normoxic and hypoxic MSC displayed protection from weight loss associated with aGvHD. aGvHD is a systemic disorder affecting a number of organs such as the lungs, liver, GI tract and skin and progression can be identified pre-clinically by one or more signs associated with impaired mobility, appearance or breathing. Mice administered PBMC demonstrated a significant clinical score associated with aGvHD. Notably, aGvHD mice which received normoxic and hypoxic MSC displayed a reduced clinical score. Collectively, these studies have identified that MSC cultured in hypoxia, which had previously demonstrated impaired immunosuppression *in-vitro*, were capable of prolonging the survival and alleviating symptoms of mice with an aggressive form of aGvHD. These were important findings demonstrating that hypoxic MSC may be used as a cellular therapy for aGvHD in pre-clinical and clinical scenarios despite their apparent lack of immunosuppression *in-vitro*.

We wanted to test the capacity of hypoxic MSC to comparably reduce aGvHD pathology. Therefore, the next approach was to examine the histopathology of aGvHD target organs. Importantly, both normoxic and hypoxic MSC therapy significantly reduced the severity of aGvHD pathology in the livers and small intestine of aGvHD mice. This is consistent with reports on MSC in GvHD where therapy significantly improved the histological score of GvHD mice in the small intestine and liver and supports our survival data (Polchert *et al.* 2008; Joo *et al.* 2010; Auletta *et al.* 2014).

Although normoxic or hypoxic MSC therapy appeared to slightly reduce mononuclear infiltration in the lung, this was not significant. Moreover, the lungs of normoxic and hypoxic treated aGvHD mice displayed loss of lung architecture and did not significantly improve lung pathology. This is in line with previous findings from our lab in aGvHD (Tobin *et al.* 2013; Healy 2015). Interestingly in the phase III trial by Osiris Therapeutics for steroid refractory GvHD, patients with aGvHD in the liver and gut showed significant improvement following MSC treatment (Martin *et al.* 2010). Moreover, therapy did not have any improvement on skin pathology or survival of these patients and although pathology of the skin was not examined here in aGvHD mice, these results suggest that beneficial effects mediated by MSC in aGvHD may in fact be organ dependent. Given that MSC are initially entrapped in the lungs before gradual redistribution, analysis of lung pathology at an earlier time point in aGvHD may reveal a different result.

Excessive production of pro-inflammatory cytokines is a hallmark of aGvHD pathogenesis (Antin & Ferrara 1992). For example, TNF-α plays a role in T cell activation, differentiation and alloreactivity in models of GvHD and can result in direct apoptosis in patient target tissues in the final stage of the disease (Speiser *et al.* 1997; Hill *et al.* 1999; Brown & Thiele 2000; Hill *et al.* 2000). Therefore, apoptosis in

aGvHD target organs was examined *ex-vivo.* In terms of liver tissue apoptosis, normoxic and hypoxic MSC both exhibited protective effects. MSC and MSC conditioned medium has been shown to have anti-apoptotic effects on hepatic cells after acute liver injury and fibrosis (Van Poll *et al.* 2008; Nasir *et al.* 2013; Xagorari *et al.* 2013) and recent data suggests hypoxic preconditioning of human AT-MSC does not negatively impair their anti-apoptotic effects in a co-culture with human primary hepatocytes (Qin *et al*. 2015). Following a similar trend hypoxic and normoxic MSC reduced apoptotic damage in the small intestine and lung. These are important findings further highlighting the therapeutic benefits of hypoxic cultured MSC.

A key hallmark of aGvHD pathogenesis is the production of pro-inflammatory cytokines such as IFN-γ, IL-1 and in particular TNF-α (Speiser *et al.* 1997; Hill *et al.* 1999; Ferrara *et al.* 2009). This event, known as a cytokine storm fuels GvHD through the amplification of donor T cells (Henden & Hill 2015). We have previously shown that normoxic MSC treatment reduces TNF-α in the serum of aGvHD mice (Tobin *et al.* 2013) and the number of TNF-α producing T cells in the spleens and lungs of aGvHD mice (Healy 2015). This study sought to further probe the effect of MSC treatment on TNF-α producing T cells in the spleens, livers and lungs of aGvHD mice and in both normoxic and hypoxic MSC treated aGvHD mice. This study investigated the percentage and total number of $CD4^+$ and $CD8^+$ human TNF-α producing cells. Normoxic MSC therapy reduced the percentage of TNF-α producing $CD4^+$ and $CD8^+$ cells in the livers of aGvHD mice, in line with this; hypoxic MSC significantly reduced the percentage of TNF-α producing $CD4^+$ cells in the liver and decreased the percentage of TNF-α producing $CD8^+$ cells. Analysis of the total number of TNF-α producing $CD4^+$ cells in the liver revealed the same trend in the normoxic group, as MSC treatment reduced the total number of TNF-α producing $CD4^+$ cells and $CD8^+$ cells in

the livers of aGvHD mice. Moreover, hypoxic MSC treatment also reduced the total number of TNF-α producing $CD4^+$ cells albeit less significantly than the normoxic group. This may in part be explained by the fact that each mouse received different amounts of PBMC as the dose was normalised to the weight of each mouse and thus, quantifying the total number of lymphocytes *ex-vivo* (after each mouse was transplanted with different amounts initially) may not be suitable in this case. The total number of TNF-α producing $CD8^+$ cells in the liver was slightly increased after hypoxic MSC therapy.

Importantly, hypoxic MSC therapy significantly reduced the percentage and total number of TNF-α producing $CD4^+$ T cells in the spleens of aGvHD mice in line with the normoxic MSC group. MSC therapy failed to significantly reduce the percentage of TNF-α producing $CD8^+$ T cells in the spleens of aGvHD. However, analysis of the total number of TNF-α producing $CD8^+$ T cells in the spleens demonstrated a significant reduction of this subset by hypoxic MSC and more significantly by normoxic MSC therapy. Moreover, investigation of TNF-α producing T cells in the lung revealed that both normoxic and hypoxic MSC therapy decreased the percentage and total number of TNF-α producing $CD4^+$ T cells. Upon examination of the TNF-α producing $CD8^+$ T cells in the lung, it was identified that hypoxic MSC treatment reduced the percentage of this subset. Analysis of the total number of TNF-α producing $CD8^+$ T cells also revealed the capacity of hypoxic MSC to decrease the number of TNF-α producing $CD8^+$ T cells in the lung. These are important findings as we are the first to demonstrate the capacity of long term hypoxic cultured MSC to prolong the survival of aGvHD NSG mice and explore the effect of hypoxic MSC treatment along with normoxic MSC treatment on TNF-α producing $CD8^+$ T cells in the liver, spleen and lungs of aGvHD mice. Furthermore, this study demonstrated that

hypoxic MSC therapy reduced TNF-α producing $CD4^+$ T cells in the liver, spleen and lungs of these aGvHD mice. These results offer a potential mechanism by which hypoxic MSC support the survival of aGvHD mice and is in line with targeting the production of TNF-α in aGvHD, which has provided promising results. For example, antibody mediated blockade of TNF-α production prolonged the survival of aGvHD mice (Korngold *et al.* 2003; King *et al*. 2009).

The engraftment of transplanted cells after HSCT is required for the development of a functional immune system. This thesis has shown that *in-vitro,* normoxic MSC have the capacity to inhibit T cell proliferation while hypoxic MSC are less potent suppressors of T cell proliferation. However, thus far both MSC have demonstrated comparable therapeutic benefits *in-vivo.* Published data demonstrates that MSC therapy does not inhibit the engraftment of administered cells in patients following transplantation (Lee *et al*. 2002; Le Blanc *et al.* 2008; Gonzalo-Daganzo *et al.* 2009; Macmillan *et al.* 2009). Nonetheless, it was important to determine if hypoxic MSC therapy was comparable to normoxic MSC therapy in terms of influence on engraftment of transplanted PBMC in the NSG model. In this system, analysis of MSC therapy was performed on day 12. Consistent with the previous published data, normoxic MSC therapy did not significantly impair lymphocyte engraftment. Importantly, the percentage of human $CD45^+$ $CD4^+$ and $CD8^+$ lymphocytes in the spleen was unaffected by normoxic or hypoxic treatment albeit a slight reduction in the percentage of $CD8^+$ lymphocytes in the spleens of normoxic treated aGvHD mice. Although the presence of CD4 and CD8 co-receptors on T cells is typically exclusive to thymic ontogeny, peripheral $CD4^+CD8^+$ T cells have been described (Munschauer *et al.* 1993; Ortolani *et al.* 1993; Parel & Chizzolini 2004). However, whether the presence of peripheral $CD4^+CD8^+$ T cells is a result of a failure of thymic selection is unknown

(Parel & Chizzolini 2004). Studies linking $CD4^+CD8^+$ T cell function with aGvHD pathophysiology are lacking. One study focussed on skin biopsies from a patient diagnosed with GvHD and demonstrated the capacity of $CD4^+CD8^+$ T cells to produce IL-4 (Eljaafari et al. 2013). Interestingly, the authors reported that the addition of $CD4^+CD8^+$ T cells to a MLR reduced total $CD3^+$ T cells, suggesting an anti-inflammatory effect of these cells in GvHD. However, future work in this field is needed to definitively identify a role of these cells in not only GvHD but in many other pathologies. In this work, the analysis of $CD45^+$ $CD4^+$ $CD8^+$ lymphocytes in the spleens revealed an insignificant increase in these cells in the hypoxic treated group in contrast to the normoxic therapy group.

A pre-requisite for the progression of aGvHD is the trafficking and infiltration of alloreactive effector lymphocytes to aGvHD target organs ultimately resulting in destruction of organ tissue (Sackstein 2006). T cell migration to secondary lymphoid organs ( SLOs) (for example the spleen) and aGvHD development is well established in the literature, however the migration of lymphocytes to the target organs of aGvHD is lesser understood. While engraftment of transplanted cells is essential for the constitution of a functional immune system, migration of lymphocytes to aGvHD organs such as the liver or lung can play a role in further exacerbating the pathogenesis of the disease (Sackstein 2006). Therefore, the next approach was to examine the percent engraftment of human lymphocytes in the livers and lungs of aGvHD mice and MSC treated aGvHD mice. There was a reduction in the percentage of human $CD45^+$ $CD4^+$ lymphocytes in the livers of the normoxic MSC treated group and interestingly, there was a significant reduction in this subset in hypoxic MSC treated groups. Human $CD45^+$ $CD8^+$ lymphocytes were unaffected by MSC treatment while the engraftment of human $CD45^+$ $CD4^+$ $CD8^+$ lymphocytes was reduced in the livers of aGvHD mice. In

the lungs of aGvHD mice, MSC treatment did not affect the engraftment of human $CD45^+$ $CD4^+$ lymphocytes or human $CD45^+$ $CD8^+$ lymphocytes. In contrast to this, the percentage of human $CD45^+$ $CD4^+$ $CD8^+$ lymphocytes in the lungs appeared to be minimally reduced. Collectively, these results suggest that hypoxic MSC therapy can be used without negatively altering the engraftment of human $CD45^+$ lymphocytes in the spleens or lungs of aGvHD mice.

Naturally occurring $CD4^+$ $CD25^+$ forkhead box protein 3 (FOXP3) regulatory T cells (Tregs) are powerful mediators of immunity and are involved in the induction and maintenance of self-tolerance (Piccirillo & Shevach 2004). In murine models of GvHD, the administration of donor derived Tregs in conjunction with allogenic BMT prolonged their survival and reduced aGvHD pathology in an IL-10 dependent mechanism (Hoffmann et al. 2002; M Edinger et al. 2003). In a seminal study by Trzonkowski *et. al.*, aGvHD and cGvHD patients were treated with *ex-vivo* expanded Tregs in conjunction with immunosuppressants. The authors described an interim improvement in aGvHD symtoms and a significant alleviation of cGvHD symptoms (and reduction of immunosuppressants) (Trzonkowski *et al.* 2009) and claimed that adoptive transfer of Tregs may provide a suitable adjuvant therapy in GvHD. More recently however, a study revealed that early infusions of Tregs followed by conventional T cell infusion prevented GvHD in humans in the absence of posttransplantation immunosuppression (Di Ianni *et al.* 2011). Importantly, there is a negative correlation between Treg proportions and aGvHD development in patients (Pabst *et al.* 2007; Wolf *et al.* 2007; Magenau *et al.* 2011; Delia *et al.* 2013; Fujioka *et al.* 2013; Danby *et al.* 2016). Interestingly, MSC are capable of preserving Tregs *in-vivo* (Healy 2015). With this in mind, the next approach in probing the therapeutic efficacy of hypoxic MSC in aGvHD was to ensure MSC did not hamper the engraftment of Tregs in line with normoxic

MSC. Tregs have a potent nature and thus are a small subset of $CD4^+$ T cells typically representing 1-6% of $CD4^+$ T cells in peripheral blood (Baecher-Allan *et al.* 2001; Dieckmann *et al.* 2001; Arram *et al.* 2014; Bahador *et al.* 2014). Normoxic or hypoxic MSC treatment did not increase Tregs during aGvHD compared to untreated aGvHD mice. This was unexpected as previous studies reported MSC therapy increased Tregs in aGvHD mice (Joo *et al.* 2010; Healy 2015). Therefore this study could be further expanded on by examining the percentages of Tregs in aGvHD over multiple time-points and would enlighten the differences between Tregs in aGvHD in this study and others. Importantly however, normoxic and hypoxic MSC treatment did not impair or enhance the engraftment of Tregs in the spleens or lungs of aGvHD mice albeit a slight non-significant reduction in the liver. This has clinical relevance given that as described above, Tregs negatively correlate with GvHD progression and suggests hypoxic MSC are a suitable alternative to normoxic MSC for aGvHD in terms of Treg engraftment. Unfortunately, the advancement of Tregs for aGvHD is limited by their low frequency in the blood. Future studies should focus on the large scale expansion and purification of naturally occurring Tregs for aGvHD and further probe the efficacy of MSC treatment in conjunction with Treg therapy for aGvHD.

As already described TNF-α derived from donor cells play a key role in the progression of GvHD. So far we have provided evidence that hypoxic MSC are capable of prolonging the survival of aGvHD mice and reducing TNF-α producing $CD4^+$ T cells whilst not impairing Treg engraftment. Thus, we attempted to further investigate the immunomodulatory capabilities of hypoxic MSC to modulate pro-inflammatory mediators of aGvHD. In addition to examining the modulation of the percentages and numbers of TNF-α producing cells in this model by hypoxic MSC, cytokine levels of *ex-vivo* cultured splenocytes were also analysed. Many studies have indicated that pro-

inflammatory cytokines such as IFN-γ, TNF-α and IL-17 contribute to GvHD development and progression (Antin & Ferrara 1992; Cooke *et al.* 1998; Elisson *et al.* 1998; Schmaltz *et al.* 2003; Puliaev *et al.* 2004; Carlson *et al.* 2008; Kappel *et al.* 2009; Wang *et al*. 2009; Sun *et al.* 2012). MSC treatment had no significant effect on the levels of TNF-α produced in the splenocyte cultures which was consistent with previous findings (Christensen *et al.* 2010). In fact it was slightly increased in the hypoxic treated group. However, given that this provides global information of TNF-α production from splenocyte cultures it is not possible to determine which cell subset, or combination of subsets, is responsible for the TNF-α production. Alternatively, the analysis of TNF-α production by *ex-vivo* cultured splenocytes on day 12 of an early onset aGvHD, could be too late to examine. Moreover, analysis of IFN-γ in the supernatants of splenocytes from normoxic or hypoxic treated aGvHD mice revealed that hypoxic MSC treatment did not significantly reduce or enhance IFN-γ production by splenocytes in line with normoxic MSC.

The complexity of GvHD is continually being unravelled and recently, increasing evidence indicates the production of pro-inflammatory cytokine IL-17 (TH 17 cells) is involved in GvHD pathogenesis. Importantly, we were able to detect human IL-17 in splenocytes from PBMC only mice. Typically, IL-17 T cells differentiate from naïve T cells and activation of transcription factor retinoic acid-related orphan receptor (RORγt) is essential for IL17-producing T cells (Ivanov *et al.* 2006). The transplantation of murine IL-17$^{-/-}$ CD4$^{+}$ T cells to allogenic BMT recipients significantly delayed GvHD development in comparison to wildtype CD4$^{+}$ T cells (Kappel *et al.* 2009). However, overall mortality was not affected. More recently, data suggests that TH17 cells are not required for aGvHD induction (Iclozan *et al.* 2010). In-*vitro* data suggests that normoxic MSC can inhibit TH17 cell differentiation

(Ghannam *et al.* 2010; Duffy *et al*. 2011; Qu *et al.* 2012). With this in mind, the next approach was to examine cytokine levels of IL-17 in the supernatants of splenocyte cultures of aGvHD mice and normoxic and hypoxic treated aGvHD mice. Normoxic treatment increased IL-17 levels albeit not significantly and importantly, in the hypoxic MSC treated group this increase was much less profound. Despite the lack of data concerning the impact of both normoxic and hypoxic MSC on IL-17 in aGvHD this was a surprising result given the published *in-vitro* data. However, we only examined levels of IL-17 in our splenocyte culture system and thus further investigation could examine IL-17 producing T cells by intra-cellular flow cytometry.

Chapter 4 has comprehensively evaluated the efficacy of hypoxic MSC for aGvHD. This therapy unequivocally demonstrates the ability of hypoxic MSC to prolong the survival of aGvHD mice in comparison to normoxic MSC making hypoxic MSC ideal for the clinic. For the first time we have shown that hypoxic MSC provide safe and comparable efficacy to the routinely used normoxic MSC in reducing pathology in the livers and small intestines of aGvHD mice and reducing apoptotic tissue damage. Moreover, comparable profiles by normoxic and hypoxic MSC were demonstrated for reducing TNF-α producing $CD4^+$ T cells in the spleens, livers and lungs of aGvHD mice and preserving the engraftment of Tregs in these aGvHD target organs and PBMC engraftment in the spleen. This data has attempted to answer the question of whether we can use hypoxic MSC for aGvHD to overcome the limitations associated with culture of MSC in normoxia prior to administration to patients with inflammatory disorders.

For the full realisation of the potential of hypoxic MSC in the clinic, a thorough investigation of their profile *in-vivo* must be established. Assessing the efficacy of

novel cellular therapies by biodistribution studies will further our understanding of the mechanisms mediated by these cells *in-vivo,* thereby aiding the extrapolation of our knowledge to the clinic for aGvHD. While our current understanding of aGvHD pathophysiology and intervention by MSC has significantly widened in the past decade, it is clear from the recent results of a phase III trial for GvHD that we still have a long way to go in optimising MSC therapy for aGvHD. For example, questions on MSC biodistribution in the field of aGvHD remain unanswered; how many MSC reach aGvHD target organs and how long do they stay? Prochymal® therapy was beneficial in the treatment of aGvHD of the gut and liver but not of the skin (Martin *et al.* 2010) which raises the question of why MSC treatment was beneficial for some aGvHD target organs and not others (skin). Biodistribution studies in conjunction with this data may have enlightened researchers on these results; did MSC only home to the GI tract and liver? How long did they stay there for? While quantitative data on cellular homing in patients is difficult, pre-clinical imaging in models of aGvHD can aid significantly in corroborating clinical studies. Therefore the next approach was to examine the biodistribution of hypoxic MSC 24 hours post administration to aGvHD mice and investigate their homing profile in comparison to their normoxic cultured counterparts. Given that hypoxic MSC were comparable to normoxic MSC in-vivo it was hypothesised that hypoxic MSC would comparably migrate to aGvHD target organs. However, it is important to demonstrate this given that there is a paucity of information concerning the effects of long term hypoxic culture on the migrational capacity of bone marrow derived MSC in aGvHD.

As outlined in chapter 1, fluorescent proteins allow *in-vivo* tracking of lentivirally transduced cells. Therefore, in order to test our hypothesis, a dual reporter (fluorescence/bioluminescence) construct/transfer vector was employed to monitor the

long term trafficking of MSC in aGvHD in real time. The transfer vector initially employed was a third generation vector given that it is driven by a CMV promoter and not a natural TAT-dependent HIV-1 promoter. However, our initial packaging system contained Vif, Vpr, Vpu and Nef; components only found in first generation packaging systems which are no longer routinely employed in academic research. Moreover, the Rev protein (exports unspliced, full length vector RNA from the nucleus) was absent from the packaging system (or alternatively may not have been indicated) and this may provide an explanation for why we didn't obtain lentiviral vector particles with these plasmids. Moreover this explains why the transfer vector alone could transiently infect HEKs where most likely we obtained only spliced internal RNA fragments capable of transgene expression but not of packaging of vector particles. Given that TAT was absent from the original packaging (pDelta VPR), transfection may not have been achieved because of this given that transfection was achieved with the alternative PsPAX2 (indicated both TAT and Rev). In order to select for transduced cells, 200µg/ml Zeocin identified as the optimal antibiotic selection concentration. Multiplicity of infection (MOI) is a parameter commonly employed to predict viral infectivity in a cell (Zhang *et al.* 2004) and can specifiy the amount of virions used in an experiment, as indicated by the number of virions used per cell to infect that cell. MSC in all experimentation here were transduced and cultured in normoxia, as all lentiviral work was performed in a separate lentiviral biosafety suite, with an MOI of 30. Importantly transduction did not impair MSC proliferative capacity, cell surface phenotype, differentiation capabilities or immunosuppressive capactiy.

Bioluminescent imaging is a costly technique and whilst initially suitable for our needs unfortunately it was not feasible to investigate the migration of hypoxic MSC in aGvHD with BLI due to budget restrictions. Nonetheless, the English lab acquired a

dynamic CryoViz$^{TM}$ imaging system which reconstructs 3-D bright-field and fluorescent visualisation of whole mouse or isolated tissue. In addition, it provides single cell quantification and therefore presents an advantage over current state-of-the-art imaging systems. Correlation of cell numbers reaching target organs with the therapeutic efficacy will have major implications for the field of MSC based immunotherapy. Despite visualisation of GFP$^{+}$ MSC microscopically, and demonstratable luciferase activity *in-vitro*, it was not possible to detect them using CryoViz$^{TM}$ technololgy. A possible explanation for this is that in order to visualise the fluorescently labelled cell on the CryoViz$^{TM,}$ the excitation range for the fluorophore should be broad enough so that wavelengths in the blue and blue green range can also excite it. The emission can be red. eGFP has an excitation max of 488nm and an emission max of 509nm typically detected with excitation and emission filters of 485/20 and 530/25 respectively.

Quantum Dots (QDots) are nanocrystals that have a broad excitation range and thus are ideal for Cryo-imaging and have been employed in the Cryo-imaging system (Auletta *et al.* 2014). Therefore to overcome the previous problem, MSC were labelled with QDots for analysis of the biodistribution of normoxic and hypoxic MSC in aGvHD. The labelling efficiency of MSC by QDots was validated by flow cytometry for red fluorescent expression prior to administration to aGvHD mice. Similar to findings from chapter 4, normoxic MSC significantly prolonged the survival of aGvHD mice (22.6 days MST) in comparison to untreated aGvHD mice (13.6 days MST). In line with this, hypoxic MSC also significantly prolonged the survival of aGvHD mice (21.6 days MST). Moreover, MSC treatment was efficacious in reducing weight loss and clinical scoring of aGvHD mice. Therefore, the next step was to probe this study further by analysing the migration of QDot labelled normoxic and hypoxic MSC to aGvHD target organs (lungs, livers and spleens) following intravenous administration.

It is well documented that the lung is a barrier for the intra-venous administration of MSC (due to their large size in relation to the small lung microvasculature), resulting in the initial entrapment of cells in the lungs for up to 24 hours, known as the pulmonary first-pass effect (Schrepfer *et al.* 2007; Fischer *et al.* 2009). Published data suggests MSC do not persist *in-vivo* for a long period of time (Kidd *et al.* 2009; Lee *et al.* 2009) and thus a time point of 24 hours post MSC administration was chosen for analysis of biodistribution. *In-vivo* analysis of the early events in a murine model of aGvHD by BLI demonstrated proliferation of $CD4^+$ T cells followed by $CD8^+$ T cells in SLOs following by homing to the intestines, liver and skin (Beilhack *et al.* 2005). Data concerning the *in-vivo* imaging of MSC in an aGvHD setting is severely lacking. Thus we attempted to further our understanding of MSC biodistribution in aGvHD, we took this one step further and for the first time compared and quantified the homing capacities of normoxic and hypoxic MSC in a humanised NSG model of aGvHD. Data from this study revealed that normoxic and hypoxic MSC migrated to aGvHD target organs 24 hours post MSC i.v administration. Both normoxic and hypoxic MSC comparably migrated to the liver of aGvHD mice. There was slightly more MSC in the livers of hypoxic treated MSC mice however this was only slightly increased and not significant. This is an interesting finding because whilst normoxic MSC reduced the percentage of $CD45^+$ $CD4^+$ T cells and TNF-α producing $CD4^+$ T cells, there were significantly less in the aGvHD mice treated with hypoxic MSC (independent data from chapter 4). This study could be further expanded on by whole mouse imaging and investigating if MSC are present in the skin of aGvHD mice however, autofluorescence in the skin and gut limits CryoViz$^{TM}$ imaging given that it relies on fluorescent imaging. This data is in line with findings from a murine model of GvHD by Auletta *et al.*, where QDot labelled MSC injected on day 1 post BMT

migrated to the liver within 24 hours (Auletta *et al.* 2014). Importantly, this data further substantiates our hypothesis that hypoxic MSC represent an efficacious alternative to normoxic MSC.

In aGvHD, donor T cells initially migrate to the spleen and peripheral lymph nodes within hours (Panoskaltsis-mortari *et al.* 2004). In line with published data MSC migrated to the spleens of aGvHD mice (Christensen *et al.* 2010; Auletta *et al.* 2014). In attempts to further explore the capacity of hypoxic MSC to migrate to aGvHD target organs, we analysed the number of QDot labelled MSC in the spleens of MSC treated aGvHD mice. Importantly, hypoxic culture of MSC did not attenuate this capacity. Interestingly, a study demonstrated the co-localisation of MSC with alloreactive T cells in the spleens in a murine model of aGvHD (Auletta *et al.* 2014). In support of these findings, $CD3^+$ lymphocytes were upregulated in lymph nodes and spleens after MSC i.v administration and a shift in the T cells phenotype was observed toward a tolerogenic status and cytokine analysis from splenocytes implied a shift from a Th1 to Th2 polarisation (Li *et al.* 2007).

As previously described, MSC can become entrapped in the lung following i.v infusion (Barbash *et al.* 2003; Kraitchman *et al.* 2005; Fischer *et al.* 2009; Lee *et al.* 2009). In agreement with published data for GvHD, MSC migrated to the lungs of aGvHD mice (Christensen *et al.* 2010; Joo *et al.* 2011; Auletta *et al.* 2014; Wang *et al.* 2015). However, examination of the number of QDot labelled MSC in the lungs of aGvHD mice revealed the presence of significantly less hypoxic MSC in comparison to normoxic MSC. The induction of aGvHD, QDot labelling MSC and subsequent analysis by the Cryo-imaging system can be extremely time consuming and thus one time point was chosen here as we know that at 24 hours MSC can redistribute from the lungs (Fiona Carty, unpublished data). However, it would be important to examine the

numbers of MSC in the lungs of aGvHD mice on day 12 and corroborate this with the numbers of TNF-α producing $CD4^+$ T cells on day 12. Moreover, it may be plausible that hypoxic MSC migrated to the livers earlier than normoxic MSC given that there were slightly more hypoxic MSC in the livers, albeit not significantly. Alternatively, hypoxic MSC may have instead homed to other organs not examined in slightly more numbers than normoxic MSC.

Analysis of radio-labelled DsRed MSC administered to mice as recipients revealed that the majority of the signal was in the lungs one hour after i.v infusion which was reduced after 24 hours; during the first 24 h post administration, living MSC were not found in blood, liver, spleen, kidney, or bone marrow (Eggenhofer *et al.* 2012). Moreover, the authors demonstrated that re-isolation of MSC revealed living donor MSC were present in the lungs as early as 5 minutes after i.v infusion and that 72 hours post i.v infusion, no MSC were detected in the lung implying that they were no longer viable or had redistributed. In a mouse model of ischemia-reperfusion injury to the liver, MSC were administered one hour before the assault (living MSC are present in the lung at this time). Similar to our results in aGvHD, MSC were detected in the lungs 24 hours after reperfusion however, but also in contrast to our data no MSC were detected in the injured liver tissue 24 hours after reperfusion (Eggenhofer *et al.* 2012). The authors concluded that living MSC may not pass the lung and that previous studies have identified phagocytosed MSC. Interestingly however, recent data has shown that macrophages that have phagocytosed MSC adapt an immunoregulatory phenotype (Lu *et al.* 2013; Braza *et al.* 2016). However, MSC have displayed beneficial therapeutic effects in a wide range of pre-clinical models of disease ( Mirotsou *et al.* 2007 ,myocardial survival and repair; Ortiz *et al.* 2007, lung injury; González *et al.* 2009, experimental colitis; Semedo *et al.* 2009, renal fibrosis; Kanazawa *et al.* 2011, hepatic

ischemia reperfusion injury; Tobin *et al.* 2013, aGvHD) despite being cleared or trapped in the lung (Lee *et al.* 2009; Wang *et al.* 2012; Yang *et al*. 2012).

This chapter is the first study to investigate the homing capacity of human bone marrow derived MSC following long term hypoxic culture in a humanised mouse model of aGvHD. These findings present a model for which hypoxic MSC can be extrapolated for aGvHD therapy. We have provided quantitative data of the homing capabilities of hypoxic MSC in aGvHD for the first time. The key findings from this study have demonstrated that 1) 24 hours post i.v infusion, hypoxic and normoxic MSC migrate to aGvHD target organs, 2) normoxic and hypoxic MSC comparably migrate to the spleens of aGvHD mice, 3) hypoxic MSC were present in the livers in slightly increased numbers than normoxic MSC at 24 hours post i.v infusion and 4) despite comparable survival curves, there were significantly less hypoxic MSC in the lungs of aGvHD. These findings are of great relevance as it is typically thought that MSC are short lived but future studies would comprise of analysing hypoxic MSC numbers at a later or multiple time-points in aGvHD and corroborating this to *ex-vivo* analysis of pro-inflammatory cells in aGvHD target organs. Nonetheless, the field of MSC biodistribution as a whole, whether its normoxic or hypoxic MSC, warrants further investigation but importantly we have provided a framework from which future studies can adopt.

This thesis explored the novel concept of utilising hypoxic MSC for inflammatory disorders by harnessing a humanised mouse model of aGvHD. *In-vivo* data refuted *in-vitro* results from chapter 3 thus highlighting the cell culture phenomenon and the disparities associated with *ex-vivo* expansion and *in-vivo* analysis. Moreover, it is not clear whether the culture conditions currently used to propagate MSCs can provide optimal support for their primary plating and subsequent secondary

culture expansion. Notably, (hypoxic culture expanded) MSC therapy *in-vivo* significantly prolonged the survival of aGvHD mice in line with normoxic culture expanded MSC, thus supporting our hypothesis that hypoxic MSC are a suitable and efficacious alternative to normoxic MSC. Moreover, we have comprehensively analysed the early biodistribution of hypoxic MSC in aGvHD and shown that suppression by hypoxic MSC may be associated with migration to aGvHD target organs. An interesting concept to explore would be preconditioning MSC with short term hypoxia (for example 1 day) and investigating the therapeutic efficacy of these MSC in aGvHD. This would highlight the role of hypoxia in not only increasing the availability of these cells (through enhanced numbers) but in the possibility that shorter exposures of hypoxia may actually enhance their therapeutic efficacy thus highlighting the versatility of hypoxic culture for MSC based cellular immunotherapy.

# CHAPTER 7

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# CHAPTER 8

# APPENDIX

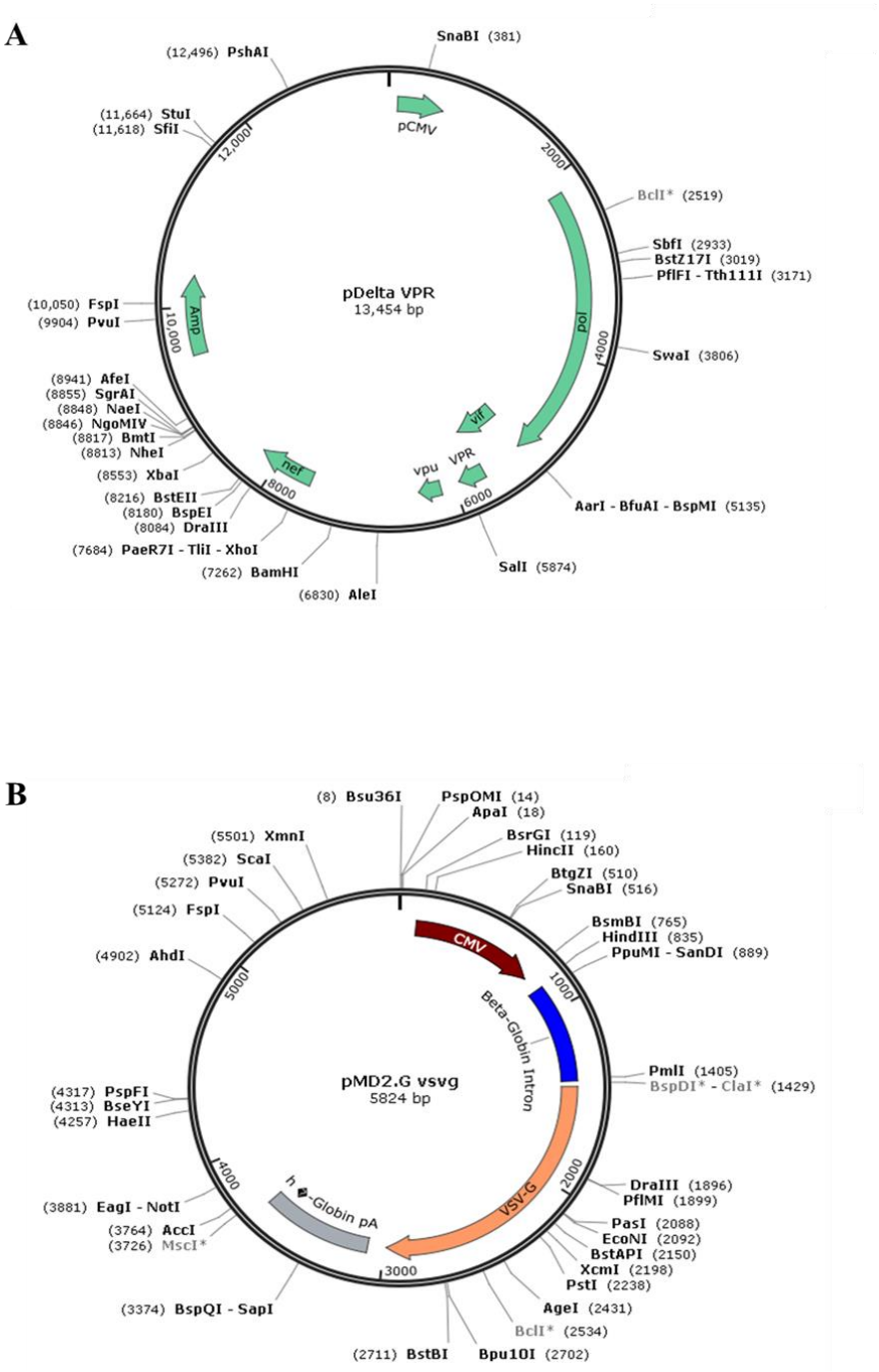


**Figure Appendix 1.** Plasmids used for lentivirus generation; packaging plasmid (A) and envelope plasmid (B).

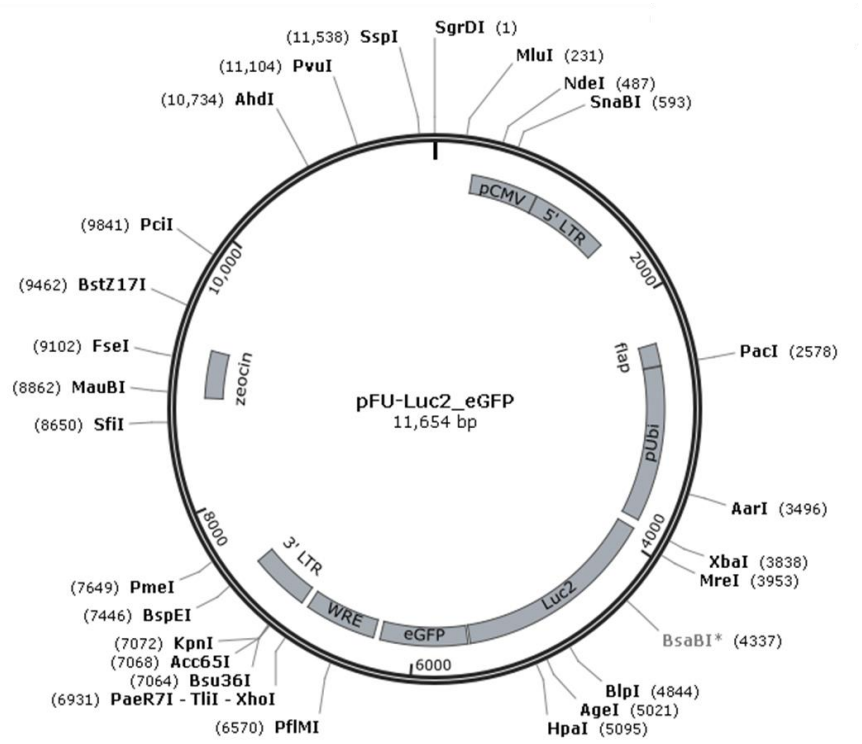


**Figure Appendix 2.** The vector of interest was a dual reporter harbouring luciferase and eGFP that would allow for fluorescent and bioluminescent imaging.

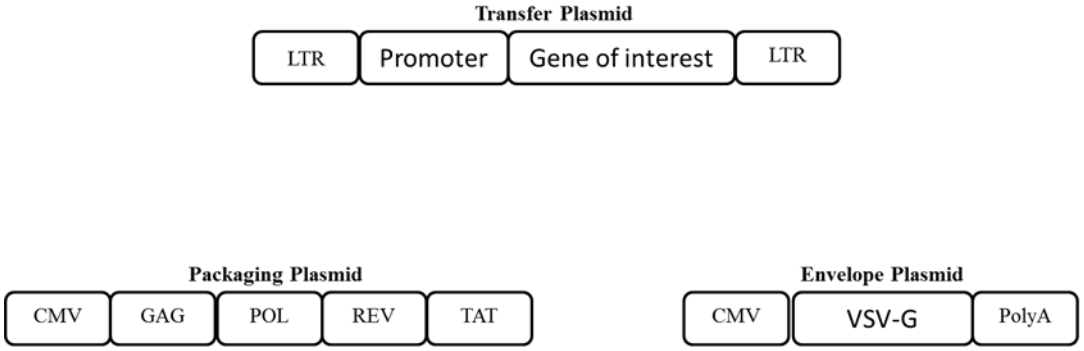


**Figure Appendix 3.** Second generation Lentiviral systems are composed of three individual plasmids; a transfer plasmid, one packaging plasmid containing all the important packaging components and an envelope plasmid.

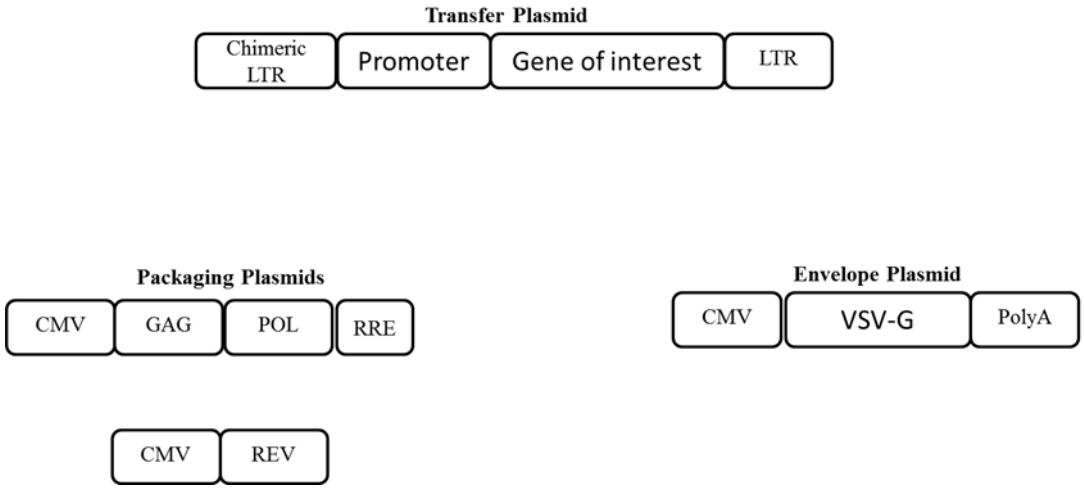


**Figure Appendix 4.** Third generation Lentiviral systems are composed of four individual plasmids; a transfer plasmid, two packaging plasmids in which the Rev protein is expressed independently and an envelope plasmid. Third generation lentiviral systems eliminate the Tat protein and the 5' LTR is modified.

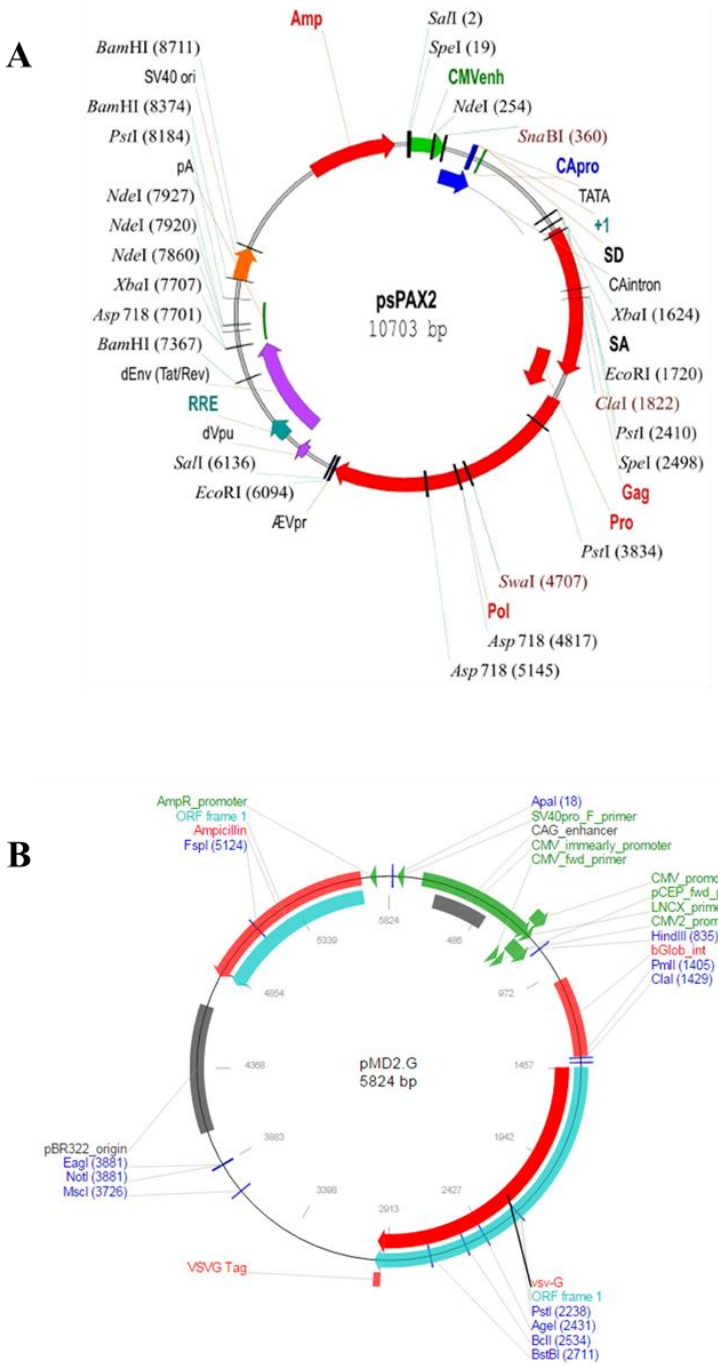


**Figure Appendix 5.** Plasmids used for lentivirus generation from the Trono lab; packaging plasmid (A) and envelope plasmid (B).