Development of methods for the *in-vitro* assessment of the adipogenesis and immunogenicity of human multipotent mesenchymal stromal cells

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy (PhD)

The University of Leeds Faculty of Biological Sciences

September 2011

The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisors, Professor Eileen Ingham and Dr. Elena Jones, who have provided continued guidance and encouragement throughout my PhD, especially in the preparation of this thesis. Their combined knowledge and mentorship has developed me into the scientist I am today. I am eternally grateful for the time and effort they have invested in me over the last four years, and I am not able to express how thankful I am for the hard work they have put into my tutorage.

Thanks also goes out to the other postdoctoral researchers who have aided my in the various techniques used throughout this thesis; Dr. Chris Brown for his help in the immunological assays, Dr. Sarah Churchman for her help with PCR and Dr. Gareth Howell for his help with imaging, flow cytometry and FACS.

Special thanks goes to the members of the lab who have served as a great source of stress relief, were always available to discuss problems with and who always reminded me that things can only go wrong for so long! Chris Brown, Kirsty Owen, Stacy Wilshaw, Gemma Jones, Stuart Kyle and Dan Thomas, as well as all the other members of who made my journey that little bit easier.

Finally, words cannot begin to describe how thankful I am to my fiancée Claire for her constant support and love throughout this PhD. I do not know how you have coped with me over the years, but I will be forever grateful. I also thank my family, whose love and support have brought me to where I am today, and without them I would never have achieved anything that I have.

The work undertaken here was funded by the Biotechnology and Biological Sciences Research Council at the University of Leeds.

It has been said “Science is built up of facts as a house is built of stones; but an accumulation of facts is no more a science than a heap of stones is a house” ~ Henri Poincaré

ABSTRACT

Adult mesenchymal stromal cells (MSCs) have enormous potential in tissue engineering and regenerative medicine applications. MSC have also been reported to be immune-privileged.

The aim of this study was to develop methods to test two related hypotheses. Firstly, that MSCs will not directly stimulate allogeneic lymphocytes in classical lymphocyte proliferation assays carried out over 5-7 days due to lack of expression of immune-regulatory molecules (CD40, CD80, CD86 and MHC Class-II) but may be capable of the stimulation of allogeneic lymphocytes when cultured over extended time periods that allow the indirect pathway of antigen presentation. Secondly, that following differentiation of MSCs into mature differentiated adipose cells, the cells would become immunogenic or increase their immunogenicity, as determined by the stimulation of allogeneic peripheral blood mononuclear cells in lymphocyte transformation assays.

MSCs from 4 human bone marrow donors were isolated, expanded and characterised and shown to conform to the ISCT definition of MSC through FACs analysis of key antigen expression and trilineage differentiation.

MSCs (from donors 1-3) were differentiated into adipocytes, and current methodology for assaying adipogenesis was evaluated. A new fluorescent microplate assay was developed using DAPI to normalise for cell number and Nile red to stain for intracellular lipids, to generate a ratio of adipogenesis.

Peripheral blood mononuclear cells (PBMCs) from 6 volunteers were isolated, *in-vitro* assay conditions for performing lymphocyte transformation assays (LTAs) were optimised, and the duration of the assays extended to 16-21 days.

It was shown that undifferentiated MSCs stimulated PBMCs in all except 2 out of 24 cases, with the highest stimulation typically post-day 7 with stimulation indices as high as 36 observed. It was shown that MSCs differentiated into adipocytes also stimulated PBMCs with higher stimulation indices observed in differentiated MSCs compared to the undifferentiated counterparts.

It was concluded that MSC, whether undifferentiated or differentiated into adipose cells, are indeed capable of stimulating a proliferative response in allogeneic lymphocytes. Therefore, the use of allogeneic MSCs in the traditional engineering setting may be limited, and a cautious approach should be taken before allogeneic MSCs are used in the regeneration or creation of tissue replacements for clinical use.

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LIST OF ABBREVIATIONS

ADSCs	**Adipose derived stem cell**
AMP	**Adenosine monophosphate**
ANOVA	**Analysis of variance**
APCs	**Antiggen presenting cell**
ATCC	**American type culture collection**
ATP	**Adenosine triphosphate**
BM	**Bone marrow**
cDNA	**Coding DNA**
CFU-Fs	**Colony-forming unit fibroblast assay**
CFSE	**Carboxyfluorescein succinimidyl ester**
Ct	**Cycle Threshold**
CTL	**Cytotoxic T-lymphocyte**
DAPI	**4',6-diamidino-2-phenylindole**
DCs	**Dendritic cells**
DFPCs	**Dental follicle precursor cell**
DMEM	**Dulbecco's modified Eagles medium**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide triphosphate**
DPSCs	**Dental pulp stem cells**
DTH	**Delayed type hypersensitivity**
EBMT	**European group for blood and bone marrow transplantation**

EDTA	**Ethylenediaminetetraacetic acid**
ESCs	**Embryonic stem cell**
FABP4	**Fatty acid binding protein 4**
FACS	**Fluorecent activated cell sorting**
FCS	**Fetal calf serum**
FDA	**Fluoescein diacetate**
FITC	**Fluorescein isothiocyanate**
GAG	**Glycosaminoglycan**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GVHD	**Graft vs host disease**
HBSS	**Hank's balanced salt solution**
HLA	**Human leukocyte antigen**
HSCs	**Hematopoietic stem cell**
HSCT	**Hematopoietic stem cell transfer**
ICAM 1/2	**Inter-Cellular Adhesion Molecule 1/2**
IDO	**Indoleamine-2,3-dioxygenase**
IFN-ɣ	**Interferon gamma**
IMBE	**Institute of mechanical and biological engineering**
iPSCs	**Indiced pluripotent stem cells**
ISCT	**International society for cellular therapy**
ITS	**Insulin-transferrin-selenium**
LFA-1	**Lymphocyte function associated antigen-1**

LIMM	**Leeds institute of molecular medicine**
LTA	**Lymphocyte transformation assay**
MHC	**Major histocompatability complex**
MLR	**Mixed lymphocyte reaction**
MMC	**Mitomycin C**
MSCs	**Multipotent mesenchymal stromal cells**
MSD	**Minimum significant difference**
NF_kB	**Nuclear factor kappa B**
NSCs	**Neural stem cell**
PBMCs	**Peripheral blood mononuclear cells**
PBS	**Phosphate buffered saline**
PDGF	**Platelet derived growth factor**
PDLSCs	**Peridontal ligament stem cells**
PEG	**Polyethylene glycol**
PGA	**Polyglycolic acid**
PGE_2	**Prostaglandin E_2**
PHA	**Phytohaemagglutinin**
PHDFs	**Primary human dermal fibroblasts**
PLA	**Polylactic acid**
PLGA	**Poly (lactic-co-glycolic) acid**
PPAR-ɣ	**peroxisome proliferator-activated receptor gamma**
qRT-PCR	**Quantatative real time polymerase chain reaction**

RNA	**Ribonucleic acid**
RPMI 1640	**Roswell Park Memorial Institute**
SCAP	**Stem cells from apical papilla**
SCNT	**Somatic cell nuclear transfer**
SHED	**Stem cells from human exfoliated deciduous teeth**
SI	**Stimulation index**
SSC	**Side scatter**
TCA	**Trichloroacetic acid**
TCR	**T-cell receptor**
TGF-β 3	**Transforming growth factor beta**
VEGF	**Vascular endothelial growth factor**

Chapter 1: INTRODUCTION

1.1 REGENERATIVE MEDICINE

Regenerative medicine is an emergent multi-disciplinary field of research that aims to repair, replace, or restore normal function to diseased organs or tissues. The approaches taken include research in cell therapy, scaffolds, use of growth factors or any combination of these.

Tissue engineering is one branch of regenerative medicine that aims to combine cells with scaffolds to generate living tissues which have the potential to be used to replace or repair diseased or damaged tissues. The base scaffold is the primary component and can be composed of a variety of materials, ranging from artificial scaffolds made from synthetic materials to fulfil specific criteria in order to replicate that natural tissue micro-environment, to decellularised natural biological scaffolds that may be generated from native tissues from a range of species.

The second component is the cells. Scaffolds can be seeded with pluripotent cells (such as embryonic stem cells), multi-potent cells (such as multipotential mesenchymal stromal cells), or alternatively with precursor cells (such as seeding potential bone scaffolds with osteogenic cells) or even with fully differentiated cells (such as seeding decellularised blood vessels with endothelial cells and smooth muscle cells). Pluri- and multi-potent cells have received a great deal of attention in tissue engineering applications.

1.2 STEM CELLS

The term “stem cell” was originally used to name the progenitor cell which exhibited the capacity to self renew and provide a population of undifferentiated stem cells that have the potential to differentiate into one or more committed descendents, including mature cells of a fully functional tissue (Choumerianou et al., 2007). There are 4 main classes of stem cells; totipotent, pluripotent, multipotent and unipotent.

Totipotent cells in humans are only present at the onset of the developmental process, just after fertilisation when the zygote is formed. This cell has the ability to differentiate into any mature cell type present within the body, including the extra-embryonic tissues (such as the placental support tissue and protective membranes). These totipotent cells do not exist for long before their ability to differentiate into extra-embryonic tissues is lost during cell division

and differentiation (Mitalipov and Wolf, 2009). Totipotent cells are the initial zygote and the early embryonic blastomeres up to about the 4-cell stage, with totipotency gradually lost as the embryo reaches the 8-cell stage and beyond. Following this, cells of the early embryo specialise into two lineages that go on to form the inner cell mass which form the trophectoderm and the fetus and the extra-embryonic cells.

Pluripotent cells are cells that have the potential to form many cell types. The term pluripotency is now generally viewed as describing a stem cell that demonstrates the potential to differentiate into any of the 3 germ layers; the endoderm, ectoderm and the mesoderm. These cells are commonly called embryonic stem cells (ESCs). The endoderm is the innermost layer of the developing embryo and differentiates into cells that form the gastro-intestinal tract, the respiratory tract, endocrine glands and organs and the auditory and urinal system. The ectoderm is the exterior layer, and cells that form this layer are able to differentiate into the nervous system and the epidermis. The final germ layer mentioned is the mesoderm. This is the middle layer, and cells from this are able to differentiate into the mesenchyme (connective tissues), skeletal and cardiac muscle, tissues within the kidneys, red blood cells and also bone and cartilage within the developing embryo. Pluripotent cells are able to differentiate into any cell of a mature animal, but differ from totipotent cells in that they are unable to develop into a fetus or adult organism as they lack the potential to organise into an embryo and they lack the ability to differentiate into extra-embryonic tissue (Mitalipov and Wolf, 2009).

Multipotent cells have the ability to differentiate into cells of a reduced subset of lineages. In humans, examples of multipotent cells are multipotent mesenchymal stromal cells (MSCs) that are able to differentiate into bone, fat and cartilage, and haematopoietic stem cells, that are able to give rise to various cell types within the blood.

Unipotent stem cells differ from the general view of stem cells in that they are only able to differentiate into a single cell type, and are often called precursor cells. What defines them as a stem cell is their ability to self-renew (maintain a population of these cells in the undifferentiated state). Examples of cells of this type are hepatocytes of the liver, and keratinocytes in skin. These cells are more restricted in their potential as they are more differentiated, and because of this, issues such as ease of isolation and expansion affect the use of these cells in the chosen tissue regeneration/engineering strategy.

1.2.1 EMBRYONIC STEM CELLS

Far from being a 'new science', stem cells have been studied for many years. Research has been conducted on murine embryonic stem cells for over 25 years with the first mouse ESC line created in 1981 (Martin, 1981). The first report of deriving embryonic stem cells from a human embryo however occurred much later (Thomson et al., 1998). Embryonic stem cells are derived from the inner cell mass of the 3-5 day old blastocyst, and when grown *in-vitro* have the capacity to proliferate indefinitely, while also retaining their potential to form any tissues of a developing organism (Daley et al., 2003). The inner cell mass is able to generate the initial primitive ectoderm that further develops into the 3 primary germ layers discussed previously; the ectoderm, mesoderm, and endoderm. Further evidence of the ability of embryonic stem cells to differentiate into cells of all three of the primary germ layers was produced when ESCs were injected into severe combined immunodeficient mice, and resulted in teratomas containing differentiated cell types from the 3 primary germ layers (a teratoma is a tumour or encapsulated cell growth containing cell types derived from the 3 primary germ layers) (Choumerianou et al., 2007).

The blastocysts that human ESCs are isolated from are typically obtained from unused embryos generated for use in fertility treatments after *in-vitro* fertilisation (Brignier and Gewirtz, 2010). The stem cells that are obtained from embryonic tissues have a unique feature that sets them apart from other stem cells; they are able to maintain their pluripotent nature in long-term culture. Traditionally the isolated human stem cells were grown on mitotically inactivated murine fibroblast feeder cell layers in serum containing medium in culture conditions replicating those used to isolate and grow mouse embryonic stem cells (Thomson et al., 1998), but it has now come to light that the specific factors needed to maintain murine embryonic stem cells do not support human embryonic stem cells, and there are specific growth factors provided by the feeder cell layer that are required for extended growth of the stem cells without spontaneous differentiation (Yu and Thomson, 2008). This mode of culture introduced risks of unknown components and xeno-protein transmission, which restricted the potential uses of these cells in regenerative medicine. In order to combat this, an alternative to using the fibroblast feeder layer was sought, and some progress has been made into solving this issue. These solutions involve either the use of human feeder cell layers, or feeder free systems which involve growth medium supplemented with specific growth factors (Cho et al., 2011). There have now been several reports on the refinement of methods to allow the culture and maintenance of human embryonic stem cells in xeno-free conditions, but there is little follow-up work on the long term safety of these systems. Chromosomal abnormalities arising from culturing human embryonic stem cells in the absence of the murine embryonic

fibroblast feeder cells have been described and this issue needs to be overcome before clinical grade embryonic stem cells can be obtained (Draper et al., 2004). The use of feeder cell layers that are human in origin eliminates the need for xeno-derived feeder cell layers and derivatives, while maintaining the use of feeder cells to prevent the occurrence of chromosomal abnormalities. Feeder cell layers obtained from adult marrow cells, foreskin fibroblasts, foetal skin fibroblasts, fallopian tube endothelial cells, and uterine endometrium have been used, and these have been shown to allow unlimited proliferation of human embryonic stem cells without spontaneous differentiation (Cho et al., 2011). The preparation of these feeder cells however requires invasive procedures and alternative cell types that are easier to isolate, or do not require an invasive method are needed. Cell types such as umbilical cord stem cells have been suggested as a suitable feeder cell layer, and good initial results have been obtained in one study in which these cells were used (Cho et al., 2011).

There are still many barriers to be overcome before embryonic stem cells can routinely be used in the clinic, from their maintenance and *in-vitro* expansion, to controlling their differentiation when re-implanted *in-vivo*. Until more is understood about how these cells can be maintained in culture and the risk of teratoma formation is reduced, the risks of using these cells clinically outweigh the rewards. However, there are currently a small number of clinical trials involving implanting ESCs in patients. The first of these is being performed by the US company Geron who are conducting a phase I trial on the efficacy of injecting oligodendrocyte progenitor cells (derived from ESCs) directly into the patients' spinal cord 7-14 days after injury. A second study is underway is patients with Stargardt's macular dystrophy (a condition in which retinal pigment epithelial cells in the macula of the eye stop working) by another USA based company called Advanced Cell Technology. In this trial the lab grown retinal pigment epithelial cells derived from human ECSs will be implanted into the eyes of patients with advanced Stargardts macular dystrophy. The retinal pigment epithelial cells normally function to provide nutrients and protection for the light sensitive cells in the retina that allow vision. This company has another clinical trial using the same differentiated ESCs to attempt to improve loss of vision due to age-related macular degeneration (information obtained from the eurostemcell.org clinical trials news). All of these studies have only commenced relatively recently, and as of now, no long term outcomes have been documented.

1.2.2 SCNT AND iPS CELLS

An alternative to embryonic stem cells, while still offering pluripotency, are induced pluripotent stem cells (iPS). These are somatic cells that are subject to nuclear reprogramming to reset their fate to a primordial, embryonic stem-cell-like state. The concept evolved from a

significant breakthrough that made headline news in many countries; the cloning of Dolly the sheep in 1997. When researchers did this they demonstrated that trans-acting material in mammalian oocytes was sufficient to reprogram a differentiated nucleus to a pluripotent state (Yu and Thomson, 2008). The technique of somatic cell nuclear transfer (SCNT) was suggested as a method to create patient specific stem cells. This procedure involves the isolation of a donor cell and the removal of its nucleus. The nucleus of a host oocyte is then removed, and the donor nucleus is inserted. The new hybrid of oocyte and donor nucleus creat a reprogrammed pluripotential cell that is patient matched and able to form a blastocyst containing embryonic stem cells with almost identical DNA to the donor nucleus. SCNT has only recently been demonstrated in non-human primates, and showed that reprogramming of donor cells could be mediated by then unknown transacting factors (Yu and Thomson, 2008). There are various issues, both ethical and scientific regarding the use of SCNT to generate human ESCs, and therefore SCNT has not yet been demonstrated in a human system (Noisa and Parnpai, 2011). The generation of human ESCs using SCNT requires the use of human oocytes, which are difficult (rare) to obtain, and the process itself has very low efficiency. Using SCNT with human material also raises ethical issues about human cloning, in that the blastula created could be used to enable reproductive cloning.

Due to the very low efficiencies associated with SCNT, the generation of pluripotent cells by inducible factors has become a much more desirable method. It was later shown that enforcing the expression of just four transcription factors (Oct4, Sox2, Klf4 and c-Myc) reprogrammed human somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Even four years on from this initial discovery, the most commonly used factors for the generation of human iPS cells remain those four transcription factors (Hussein et al., 2011). There are a variety of methods currently used to drive the expression of these transcription factors, with one of the most common the delivery of transgenes via lenti- or retro-viral constructs (Nelson et al., 2010). Dangers exist with viral transgene delivery such as random genomic integrations and incomplete silencing or unwanted activation of viral transgenes, but retroviral vectors do have inherent sequences that become inactive when the cells enter the pluripotent state which is desired to prevent tumorgenicity (Nelson et al., 2010). The expression of the 4 transgenes initiates a sequence of events that allows a small fraction of the total cell population treated to acquire an imposed pluripotent state that is characterised by a stable epigenetic profile. A more desirable method to generate iPS cells would utilise an excisable system that would allow transient integration or expression of the transgenes within cells until the stable epigenetic profile has been achieved, and then the transgenes could be removed. Methods have been attempted using cell-permeable

recombinant proteins to generate iPS cells, but had a very low efficiency of production (Kim et al., 2009). Potentially the most promising method of generating iPS cells to date is through the use of mRNA transfection of the transgenes into the cells. This technique uses synthetic mRNA that contains modified ribonucleotides attenuating the traditional intrinsic antiviral responses that are typically associated with the introduction of mRNA into human cells (Warren et al., 2010). This technique has been reported to generate iPS cells with a high degree of efficiency. An overview of human embryonic stem cell isolation, SCNT and iPS cell generation is shown in **Figure 1.1**.

Possibly one of the most important hurdles to overcome in order to bring both embryonic stem cells and induced pluripotent stem cells to the clinic is the risk of tumorgenicity. Human ESCs have a great deal in common with cancer cell lines; rapid proliferation rate, lack of contact inhibition, propensity for genomic instability and high expression of oncogenes such as c-Myc and klf4. The risk of tumorgenicity has also been shown to apply to ESCs even with a normal karyotype in mouse models (Ben-David and Benvenisty, 2010). The transgenes expressed in order to form iPS cells are highly expressed in various forms of human cancers, and IPS cells have been shown to more readily acquire chromatic aberrations than ESCs (due to reprogramming stress and due to their somatic cell origin). The self-renewal genes are key factors in the tumorgenesis of ESCs and iPS cells, but are also key factors in their pluripotency. In order to generate effective clinical therapies utilising ESCs and iPS cells the tumorgencity of these cells must be overcome. Progress is being made in this area through the use of terminal differentiation of these cells, complete elimination of any residual pluripotent cells, or interfering with the expression of tumorgenic genes, however much still needs to be done before routine use of these cells can be performed in safe therapies (Ben-David and Benvenisty, 2010).

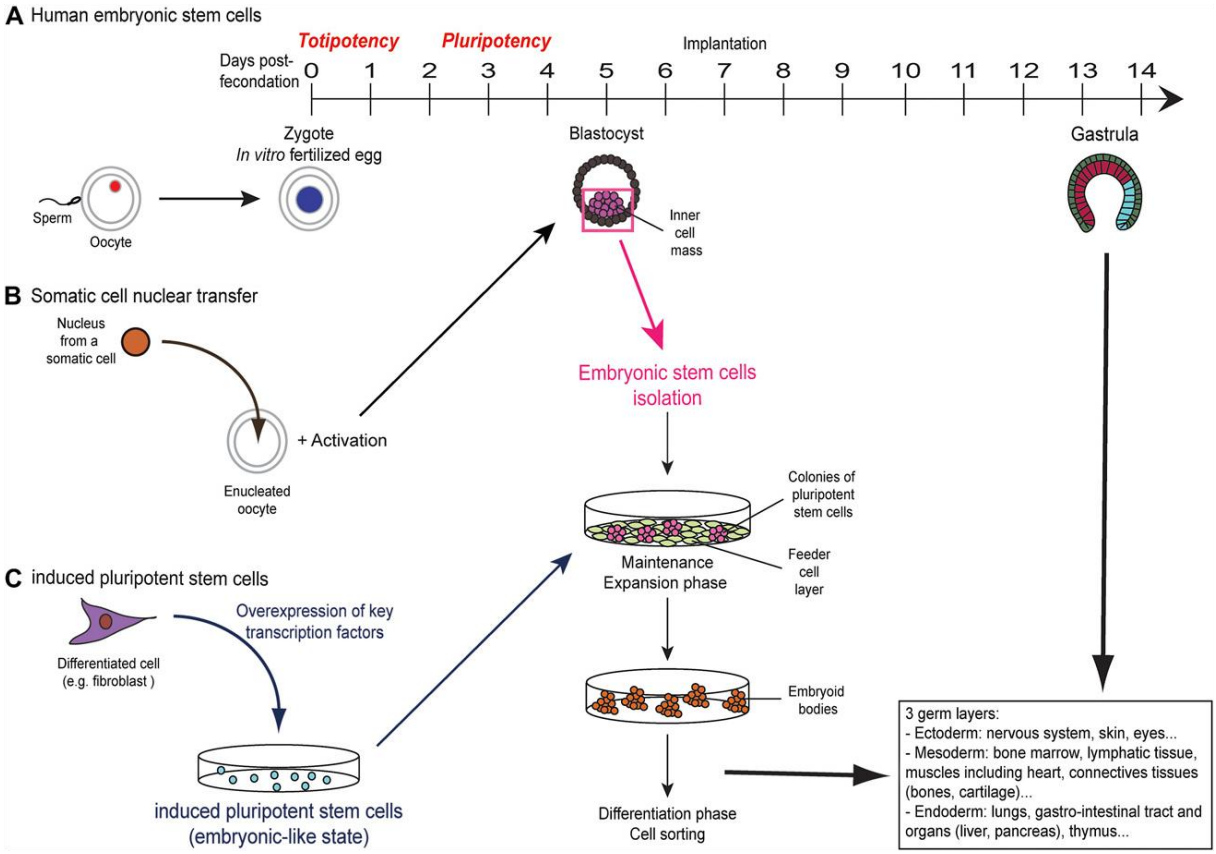


Figure 1.1: An overview of the methods used to obtain and culture pluripotent stem cells. A: Following isolation from the inner cell mass embryonic cells (ESCs) are expanded in culture classically grown on feeder cell layers (used to expand the cells while maintaining their undifferentiated state). When removed from the feeder cell layer and transferred to suspension cultures the ESCs form aggregates of differentiated and undifferentiated cells termed embryoid bodies. Plated embryoid bodies spontaneously display a variety of cellular types from the 3 primary germ layers at various differentiation stages. More specific differentiation can occur with the addition of growth factors or selection for more differentiated cell types. It is difficult to differentiate ESCs into a specific lineage without highly definite culture techniques. **B:** Somatic cell nuclear transfer involves injecting the nucleus from a somatic cell into an enucleated oocyte, followed by activation stimuli to generate an embryo to derive ESCs from. **C:** iPS cells are generated through the overexpression of key transcription factors, shown to be Oct4, Sox2, Klf4 and c-Myc. This can be achieved through viral vectors, treatment of cells with modified transgene mRNA, and plasmid vectors. This image is taken from Brignier and Gewirtz (2010).

1.2.3 ADULT STEM CELLS

The formation of organs and tissues occurs naturally in the developing human embryo, from the embryonic stem cells discussed previously. The final product is a human, composed of several hundred distinct cell types, each with a specific function. The differences between the various cell types within the body is astounding, from the brain and nervous tissue capable of transmitting and receiving electrical stimulus, to cells able to recognise and engulf invading pathogens. As established, the formation of these cells occurs in the developing embryo. In the mature human however the majority of these tissues and organs grow and repair if damaged; they need to be maintained throughout the life of the person. The maintenance of these tissues *in-vivo* is believed to be due to the presence of cell populations called 'adult stem cells', or a pool of cells within an individual that exhibit the characteristics of stem cells (self-renewal, and potency to differentiate into more than 2 different cell types). The presence of these cells in the majority of tissues within the body has been documented, from specific unipotent precursor cells such as the basal epithelial cells of the gastrointestinal mucosa, to bipotent adipofibroblasts of adipose tissue and also multipotent stem cells such as the hematopoietic cells in the bone marrow (Young and Black, 2004). It is currently the multipotent stem cells that hold the greatest promise in regenerative medicine, allowing the derivation of multiple cell lineages from one cell type. There are many types of multipotent adult stem cells that have been discovered including hematopoietic stem cells, neural stem cells, adipose derived stem cells, dental pulp stem cells, and multipotential mesenchymal stromal cells.

1.2.4 HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs) are perhaps the best characterised of all stem cells due to their ease of isolation, developmental potential and therapeutic applications. The existence of these cells was first postulated by a military doctor called Alexander Maximow in 1909, who suggested that there existed hematopoietic stem cells with the morphological appearance of lymphocytes that were able to migrate through the blood system to specific microecological niches where they were able to proliferate and differentiate along lineage specific pathways (Denham et al., 2005). HSCs are located within adult bone marrow and give rise to all of the lymphoid (NK cells, B-cells and T-cells) myeloid types (platelets, granulocytes, macrophages, dendritic cells and erythroid blood cell types). HSCs are divided into two subtypes based on the longevity of the cell; long term HSCs and short term HSCs. The long term HSCs undergo extensive self-renewal, and in dividing form their progeny, the short term HCSs. The short term HSCs undergo more limited self renewal, prior to further differentiating into multipotent myeloid or lymphoid progenitors (Denham et al., 2005). HSCs form between 0.05 and 0.5% of the total cell population of the bone marrow, with the remainder mainly composed of

quiescent cells. Even with this small percentage of HSCs, the average bone marrow still releases 200 billion erythrocytes and 70 billion neutrophillic leukocytes into the blood stream every day (Gunsilius et al., 2001). HSCs can be identified within bone marrow extracts due to their small size, low side staining with vital dyes Hoechst 33342 or rhodamine 1223 and a panel of cell surface marker expression; Sca-1^+, c-kit^+, $CD34^+$,$CD43^+$, $CD45^+$ and lineage specific marker negative (Gunsilius et al., 2001; Denham et al., 2005). Examples of the lineage specific markers used for negative selection are CD13 and CD33 for myeloid cells, CD71 for erythroid cells, or CD4/8 for T-cells. Hematopoietic stem cells have been used for clinical therapies for over three decades, with the main use being HSC transplantation in patients with severe aplastic anemias, fatal leukemias, haematological malignancies, thalassemias and severe combined autoimmune disease (Gunsilius et al., 2001). The use of HSC transplantation is increasing in frequency with over 20,000 transplants performed worldwide in 2004 (Denham et al., 2005), increasing to over 50,000 by 2006 (Gratwohl et al., 2010).

1.2.5 NEURAL STEM CELLS

Neural stem cells (NSCs) are a more recent addition to the adult stem cell field and although they make up the majority of early foetal brain cells, it was not until the early 1990's that the presence of these stem cells in the hippocampus and lateral ventricle of adults was discovered (Galli et al., 2003; Massirer et al., 2010). These stem cells have been shown to have the capacity to self-renew and to differentiate into neurons and glia (astrocytes and oligodendrites). NSCs are believed to differentiate before migration to more specific areas of the brain as neural precursor cells where the newly formed neurons integrate into the local network (Massirer et al., 2010). The general dogma of neural tissue, that it was irreplaceable and could not be regenerated is slowly unravelling, and it is now believed that cell replacement occurs within specific regions of the brain throughout adulthood (Galli et al., 2003). Isolation of these cells for study *in-vitro* was a major milestone that opened up the potential for the study of neurogenesis and the effects of pharmaceutical drug testing (Rietze and Reynolds, 2006). The isolation and culture of these cells involved harvesting cells from the periventricular region of the brain and culturing these cells in serum-free conditions in the presence of epidermal growth factor. This selected for a small percentage of these cells that were epidermal growth factor responsive (0.1%) and able to proliferate *in-vitro* (Rietze and Reynolds, 2006). These cells formed a ball of undifferentiated neural progenitor cells called a neurosphere, which in turn could be dissociated and re-plated to form additional neurospheres, or differentiated to form the 3 major cell types of the central nervous system.

The therapeutic potential of these cells to treat neurological diseases is yet to be realised. NSCs have been isolated from foetal brain tissue, but the use of this tissue as a routine source of these cells is beset with problems such as lack of availability and major ethical hurdles. The availability of a renewable source of NSCs generated from the establishment of NSC lines allows potential therapeutic avenues to be tested, but much more research is needed before these cells can be used clinically.

1.2.6 MULTIPOTENT MESENCHYMAL STROMAL CELLS

Mesenchymal stem cells were first characterised by Friedenstein and Petrakova, who identified a population of cells in adult bone marrow that were adherent, fibroblast-like, and able to repair bone *in-vivo* (Friedenstein et al., 1966; Friedenstein et al., 1968; Owen and Friedenstein, 1988). The cells were first purified using plastic adhesion due to their ability to rapidly and relatively strongly adhere to plastic surfaces. In the 1980s and 1990s further studies were performed that demonstrated the multipotency of these cells and in culture they could give rise to osteoblasts, chondrocytes, and adipocytes. Since then mesenchymal stem cells have also been demonstrated to differentiate into tenocytes and myoblasts (Prockop, 1997; Pittenger et al., 1999; Barry and Murphy, 2004; Alexanian et al., 2008; McGonagle and Jones, 2008). It has been demonstrated more recently that MSCs are not only restricted to differentiating into cells from the mesoderm, but when stimulated with specific growth factors and cultured under specific conditions other cell types can be produced such as a neuronal cell phenotype (Alexanian et al., 2008; Bae et al., 2011). However, functional electro-transduction by these putative neuronal cells was not demonstrated.

Due to the inconsistent terminology used to identify the isolated cells, the International Society For Cellular Therapy (ISCT) proposed the term “multipotent mesenchymal stromal cells” or MSCs (Le Blanc et al., 2005) and also defined a minimal criteria for labelling cells as MSCs (Dominici et al., 2006):

1. Plastic adherent under standard culture conditions.

2. Be positive for the expression of CD73, CD90, and CD105, and negative for the expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR.

3. Be able to differentiate into adipocytes, osteocytes and chondrocytes under specific stimulus in vitro.

Although the first putative methods to isolate MSCs relied on plastic adherence, presently a panel of antibody markers is used, with MSCs having been shown to be CD105, LNGFR, HLA-

DR, CD10, CD13, CD90, STRO-1 and bone morphogenetic protein receptor type IA (BMPRIA) positive and CD14, CD34, CD117 and CD133 negative (Jones et al., 2002; Jones et al., 2006). The ISCT simplified the panel of cell surface markers so that in order for cells to be called MSCs, they must express CD73, CD90 and CD105, but be negative for the expression of CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR. A table of the cell surface markers and their functions is shown in **Table 1.1**.

Mesenchymal stromal cells have been reported to be present in almost all postnatal organs, but there are tissues which contain a higher ratio of MSCs than others. Typically these cells have been isolated from bone marrow taken from the iliac crest or the hip, but MSCs represent only a very small fraction of nucleated cells in the bone marrow, between 0.001-0.01%. However, they can be isolated and expanded with high efficiency (Pittenger et al., 1999). More recently it has been shown that placenta and umbilical cord blood/cord contain higher levels of MSCs than bone marrow, and these tissues are typically considered as biological waste and discarded after childbirth (Spitkovsky and Hescheler, 2008). Adipose tissue has also been shown to contain a much higher frequency of MSCs than bone marrow; with a study carried out finding 2% of nucleated cells in lipoaspirate after a low invasive subcutaneous lipoaspiration procedure were MSCs (Strem et al., 2005). It has been shown that MSCs from alternative areas other than the bone marrow may be more predisposed to differentiate into certain lineages, for example, MSCs isolated from the cartilaginous areas of the knee demonstrated increased levels of chondrogenesis when compared to bone marrow MSCs, but reduced levels of adipogenesis (English et al., 2007).

The nomenclature associated with MSCs isolated from different tissues is currently inconsistent. Dental derived stem cells and adipose derived stem cells can be isolated from dental and adipose tissue respectively, and exhibit many of the same properties and cell surface markers as bone marrow derived MSCs. Here, these two classes of adult stem cells have therefore been grouped under the category of MSCs.

Marker	+/-	Other names	Functions	Reference
CD105	+	Endoglin	A proliferation associated and hypoxia inducible protein that acts as a receptor for transforming growth factor-β (TGF-β) I and III. TGF-β signalling is involved in cell proliferation, differentiation, and apoptosis	(Janeway et al., 2005)
LNGFR (low affinity nerve growth factor receptor)	+	CD271 and p75 neurotrophon receptor ($p75^{TNR}$)	This cell surface molecule acts as one of two receptors for the neurotrophin cell signalling molecules, with the other receptor being Trk receptor tyrpsine kinase. The neurotrophins are a family of 5 proteins; nerve growth factor, brain-derived neurotrophic factor, novel neurotrophin-1, neurotrophon 3, and neurotrophin-4.	(Deponti et al., 2009)
HLA-DR	-		HLA-DR is encoded by the human leukocyte antigen genes in humans. These genes are also known as the major histocompatibility complex receptors, but the nomenclature in humans is HLA. HLA-DR is an MHC class-II receptor that presents peptide to CD4+ T-cells.	(Janeway et al., 2005)
CD10	+	Neprilysin, or neutral endopeptidase (NEP)	A zinc metalloendopeptidase that is involved in the metabolism of a number of peptides of the nervous, cardiovascular, immune, and inflammatory systems, reducing the effect that signalling molecules (such as enkephalins, natriuretic, and chemotactic peptides) from these systems have	(Turner et al., 2001)
CD13	+	Aminopeptidase N	A membrane bound zinc dependent ectopeptidase with broad substrate specificity that is involved in protein modification, activation, and degradation, in addition to the metabolism of bioactive peptides that are involved in leukaemia and tumour metastasis.	(Zhang et al., 2011)
CD90	+	Thy-1	Originally studied in mice, and is a highly glycosylated GPI-anchored surface protein, and a member of the immunoglobin family. Its functions are not fully known, but it has been shown to be involved in mediating the binding of neutrophils and monocytes to activated microvascular endothelial cells. It is usually highly expressed at sights of inflammation or in tumors, and has also been shown to be expressed on fibroblasts, neurons, and a subpopulation of haematopoietic stem cells in humans. It is thought that CD90 is able to provide mechanical support for cell adhesion, and is also able to initiate neutrophil effector functions (the secretion of matrix metalloprotineases such as MMP-9 and chemotactic factors such as CXCL8.	(Schubert et al., 2011)

Table 1.1: A summary of the panel of cell surface markers used to characterise MSCs – continued below.

Marker	+/-	Other names	Functions	Reference
STRO-1	+		Originally discovered in 1991, and was utlilised as a novel marker for the identification of stromal precursor cells within the bone marrow. The antibody developed for this purpose bound a trypsin-insensitive epitope that was present on these stromal cells, which later became known as STRO-1.	(Simmons and Torok-Storb, 1991)
BMPR-IA	+		A receptor for the bone morphogenenetic proteins (BMPs) that are secreted multifunctional proteins that belong to the transforming growth factor-β superfamily. These proteins are involved in a variety of roles within the development, regeneration, and maintenance of tissues in humans. Binding of BMPs to BMPR-IA results in the oligomerisation of the receptor, before phosphorylation occurs in the intracellular domain resulting in the downstream activation of the SMAD signalling cascade affecting the transcription of responsive genes within the nucleus.	(Harth et al., 2010)
CD14	-		A member of the pattern recognition receptors that are able to recognise pathogen-associated molecular pathogens. This receptor exists in a membrane bound form attached to the membrane by a glycosyl-phosphatidyl-inositol anchor, and also in a soluble form after cleavage from the membrane. CD14 binds to the endotoxin lipopolysaccharide (LPS), a polysaccharide found in the outer layer of gram negative bacteria. Binding of LPS to CD14 is catalysed by an acute-phase serum reactant lipopolysaccharide binding protein (LBP).	(Stelter, 2000)
CD34	-		A cell surface marker widely used in the isolation and identification of hematopoietic stem and progenitor cells, and has more recently been used to aid in the identification of other tissue specific stem cells including epidermal precursor cells, and muscle satellite stem cells. The function of CD34 has not been fully determined, but has been demonstrated to have a role in promoting the proliferation and block the differentiation of progenitor cells.	(Nielsen and McNagny, 2008)
CD117	-	Receptor tyrosine kinase (RTK) c-Kit	A transmembrane glycoprotein whose ligand is a mast cell growth factor. This receptor is expressed on hematopoietic cells, and also some non-hematopoietic tissues and tumours. In the bone marrow CD117 is specifically expressed in the progenitor department but its expression is downregulated except in mast cells. Binding of ligand to CD117 results in the activation of a transcription cascade terminating in the production of genes involved in proliferation and survival.	(Edling and Hallberg, 2007)

Table 1.1 continued: A summary of the panel of cell surface markers used to characterise MSCs – continued below.

Marker	+/-	Other names	Functions	Reference
CD133	-	AC133	Little is currently known about the biological significance, except that CD133 is generally expressed on undifferentiated cells such as hematopoietic stem cells. The function remains unclear, except it is used as an important marker to identify various stem cell populations.	(Shmelkov et al., 2005)
CD73	+	5'Nucleotidase	Involved in the salvage of extracellular nucleotides. It is thought that this enzyme plays a major role in tissue homeostasis catalysing the final step in the hydrolysis of extracellular messenger ATP. The expression of this cell surface protein in increased during certain stages of cell maturation and has been hypothesized to be involved in cellular interactions such as cell recognition and cell adhesion.	(Zimmermann, 1992)
CD45	-	Leukocyte common antigen (LCA)	CD45 is expressed on all hematopoietic cells. It acts as a phosphatase that regulates signalling via the B-cell receptor and MHC class II molecules.	(Janeway et al., 2005)
CD19	-		CD19 forms a complex with CD21 and CD81 together they form the B-cell co-receptor complex. In innate immunity the complement fragment C3d binds to antigens, and the C3d-antigen complex binds to the B-cell receptor and the cell surface protein CD21. Following binding, cross linking and clustering of the co-receptor with the antigen receptor results in the phosphorylation of tyrosine residues in the cytoplasmic domain of CD19, by receptor associated kinases. The phosphorylated CD19 is able to bind to the src-family of tyrosine kinases and further kinases resulting in the activation of signalling pathways that activate the B-cell.	(Janeway et al., 2005)

Table 1.1 continued: A summary of the panel of cell surface markers used to characterise MSCs.

1.2.7 DENTAL DERIVED STEM CELLS

There are currently 5 types of dental stem cells that have been identified in humans. These are dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), periodontal ligament stem cells (PDLSCs) and dental follicle progenitor cells (DFPCs). These cells have been shown to be multipotent, capable of expression of bone, cartilage, fat and even neural cell markers (Liu et al., 2006; Petrovic and Stefanovic, 2009; Sloan and Waddington, 2009).

Dental pulp stem cells were first proposed in 1990, but their actual isolation and characterisation did not occur until 2000 in humans (Gronthos et al., 2000). DPSCs occur in low numbers in dental pulp and represent less than 1% of the total cell population. It has been shown that there are two stem cell populations within the dental pulp, with different characteristics. One of the stem cells has been proposed to originate from the neural crest (derived from the ectomesenchyme) and the other from the mesenchyme (Sloan and Waddington, 2009).

Stem cells from the pulp of exfoliated deciduous teeth have been isolated and shown to have an increased proliferation rate and an increased number of cell population doublings when compared to bone marrow derived MSCs. These cells have been reported to be unable to form a complete dentin pulp-like complex, although they are able to differentiate into putative odontoblasts (Sloan and Waddington, 2009). These cells have also been shown to differentiate into adipocytes, chondrocytes, neuronal-like cells and glial cells and have also demonstrated the ability to stimulate the osteogenic differentiation of murine cells in immunocompromised murine models of calvarial defects (Petrovic and Stefanovic, 2009).

The dental papilla is a collection of ectomesenchymal cells found in the developing tooth and as tooth developments nears completion the papilla eventually converts to pulp tissue. It has been shown that stem cells isolated from the apical papilla have a twofold to threefold higher proliferation rate than those from the dental pulp and are more committed to osteogenic and dentinogenic lineages. These cells also express several neural markers and are very weakly adipogenic (Petrovic and Stefanovic, 2009).

The peridontal ligament is a connective tissue that attaches the tooth to the alveolar bone that helps the tooth to remain attached in the mouth through the rigours of chewing food. It has been demonstrated that these cells are able to form a dentin-pulp complex, and periodontal ligament-cementum when transplanted into immunocompromised mice (Liu et al., 2006). These cells have also been demonstrated to be able to differentiate into odontoblasts,

adipocytes, neurons, oligodendrocyte and astrocyte-like cells (Seo et al., 2004; Techawattanawisal et al., 2007).

The final types of stem cells currently found in the tooth are dental follicle progenitor cells and these are found in the dental follicle, a protective tissue that surrounds the developing tooth germ that can be isolated during wisdom tooth extraction. These cells have been shown to differentiate into periodontal ligament fibroblasts, cementoblasts, adipocytes, and neuron-like cells (Petrovic and Stefanovic, 2009).

1.2.8 ADIPOSE DERIVED STEM CELLS

Adipose derived stem cells (ADSCs) are another type of adult stem cell that has received attention, as a stem cell type that can be obtained from an autologous and self-replenishing source and differentiated into a variety of cell types. The stromal vascular fraction of adipose tissue has received the most attention for containing the ADSCs. The stromal vascular fraction of adipose tissue is composed of a heterogenous mixture of cells including fibroblasts, pericytes, endothelial cells, the aforementioned ADSCs (which form up to 2%) and circulating blood cells. Adipose tissue is a promising source of ADSCs because it is available in relatively large volumes through processes such as liposuction (100 ml-1 litre) with minimal morbidity (Yarak and Okamoto, 2010). ADSCs have been differentiated into bone, fat, cartilage, skeletal and cardiac myocytes and endothelial and neuronal lineages (Wilson et al., 2011). ADSCs appear to be very similar to bone marrow mesenchymal stromal cells and umbilical cord stem cells, with no differences in morphology or phenotype. ADSCs however occur at a higher frequency in adipose tissue than bone marrow mesenchymal stromal cells in the medullary stroma and also have an increased proliferative capacity (Yarak and Okamoto, 2010). These stem cells have been used therapeutically in a range of applications, for orthopaedic surgery, neurosurgery, otolaryngology (vocal cord grafting) and in general and vascular surgeries to fill defects and enhance wound healing (Wilson et al., 2011). ADSCs are able to express multiple growth factors and when differentiated into endothelial cells have been shown to express vascular endothelial growth factor (VEGF) and hepatocyte growth factor, which demonstrates the potential of these stem cells to be involved in neoangiogenesis (Fraser et al., 2006).

1.2.9 CLINICAL POTENTIAL OF MSCS

Aside from the fact that MSCs represent a rapidly expandable, easy to access source of multipotent cells that could be used to treat a wide variety of conditions, there is another feature that makes them very desirable for use in therapy; the reported ability of MSCs to reduce inflammation and exhibit immunosuppressive effects. It has been reported that human

MSCs are able to suppress lymphocyte alloreactivity *in-vitro* in mixed lymphocyte reactions (MLRs) through HLA independent mechanisms (Bartholomew et al., 2002; Le Blanc et al., 2003a; Tse et al., 2003). Animal models intravenously administered with MSCs have shown improved outcomes of lung, renal and neural injury, through both paracrine effects and a switch from pro-inflammatory to anti-inflammatory cytokines at the site of injury (Ortiz et al., 2003; Togel et al., 2005; Zappia et al., 2005; Le Blanc and Ringden, 2007).

Bone marrow MSCs have many therapeutic uses that can be split into two main areas, as a cell therapy using systematic infusion or localised injection, or as the stromal cell used in tissue engineered constructs (**Figure 1.2**).

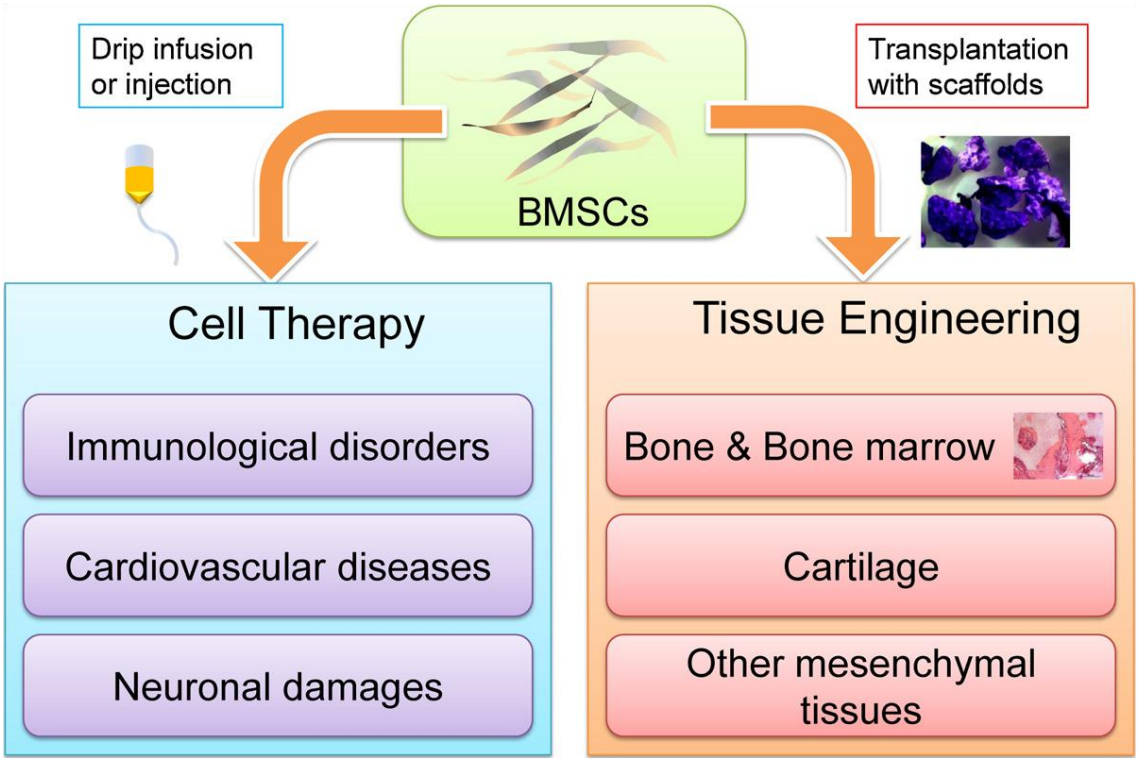


Figure 1.2: An overview of the therapeutic potential of MSCs as systemic cell therapies, or localised tissue engineered replacements. Image taken from (Kagami et al., 2011).

The use of MSCs in tissue engineering has generated a large amount of interest, due to the relative ease and isolation of these cells and their multipotentiality. MSCs have been investigated extensively for attempt the regeneration of bone, cartilage and adipose tissue.

1.2.10 Bone tissue engineering

Bone defects arising from trauma, infection and tumors represent a medical and socioeconomic challenge, with the majority of these defects treated using surgical techniques including autologous, allogeneic or artificial bone grafting. Artificial bone grafts have

limitations including their mechanical properties and biodegradation (artificial bone should exibit the same mechanical properties as natural bone). Autologous bone grafting may result donor site morbidity (Hosseinkhani et al., 2006) and allogeneic bone may suffer with problems relating to the immunogenicity of the grafted bone. Under physiological conditions, one of the roles of MSCs is the supply of osteoblasts for bone synthesis. In order to induce osteogenic differentiation of MSCs *in-vitro*, various supplements are added to the culture medium. The most common of these are β-glycerophosphate, ascorbic acid, and dexamethasone (Jaiswal et al., 1997; Pittenger et al., 1999; Jones et al., 2002). Various 3D supporting matrices have been investigated as scaffolds to aid in the differentiation of bone formation by MSCs. There are numerous reports of utilising MSCs in various scaffolds with and without growth factors and with or without physical stimulation to generate tissue engineered bone *in-vitro*. Bone marrow MSCs have been used to treat bone defects in a small number of clinical trials/studies, with various results obtained. Autologous culture expanded bone marrow MSCs have been implanted along with hydroxyapatite particles to treat intra-oral osseous defects. This resulted in the formation of bone in a proportion of patients, but failed to perform as well in others (Meijer et al., 2008). This study highlighted some of the issues in bone tissue engineering. When the same experiment was performed in proof of concept studies in mice all the test subjects demonstrated bone formation, whereas in the clinic a large degree of variability was observed. Clinical products for the regeneration of bone using MSCs in man are starting to make their way onto the market. Mesoblast offers an allogeneic based MSC product that promises to aid in the union of long bone fracture after trauma or stress. A pilot clinical trial demonstrated increased union and healing in what would have otherwise been permanent non-healing of the fractures without this cell therapy. Interestingly, it has recently been demonstrated in an immunocompromised murine model that implantation of ceramic scaffolds seeded with murine MSCs stimulated the formation of host bone, rather than the MSCs themselves differentiating into osteoblasts (Tortelli et al., 2010).

1.2.11 CARTILAGE TISSUE ENGINEERING

Repair of defects in cartilage tissue, arising from trauma, osteoarthritis or osteochondritis, is another promising avenue for tissue engineered constructs incorporating MSCs. Articular cartilage has very limited regenerative capabilities, and defects rarely heal unless the defect extends to the subchondral bone (Khan et al., 2010). Intervention using autologous osteochondral plugs can result in donor site morbidity and poor integration due to chondrocyte death. The repair of defects using autologous chondrocyte implantion is problematic due to difficulty of cell isolation and expansion in culture. Micro-fracture to release bone marrow cells to heal the defect is also not ideal, as it can often result in the

formation of fibrous rather than hyaline tissue. MSCs are routinely differentiated into cartilage (chondrocytes) as part of their characterisation in the trilineage assay and this is usually performed in 3D cultures. These cultures are usually pellet cultures, in which MSCs are centrifuged to form a pellet of cells that are grown in chondrogenic medium (Pittenger et al., 1999), or in micromasses, whereby a very high density of cells is cultured in a small area in chondrogenic medium (De Bari et al., 2001). The medium (both low and high glucose has been used) is usually supplemented with dexamethasone, ascorbic-2-phosphate, sodium pyruvate, insulin-transferrin-selenium, and transforming growth factor-β 3 (TGF-β 3) (Pittenger et al., 1999; Jones et al., 2002; English et al., 2007; Richardson et al., 2009). Preliminary studies assessing chondrogenesis in MSCs for therapeutic uses have focussed on culturing MSCs in 3D scaffolds for implantation. This provides a framework to fill the defect *in-vivo*, and allows cellular proliferation and differentiation. Natural materials such as collagen, fibrin, agarose and hyaluronan have been used as the scaffold (Khan et al., 2010). Likewise synthetic scaffolds made from polylactic acid (PLA) and polyglycolic acid (PGA), have shown promising results, but further long-term results are awaited (Williams and Gamradt, 2008; Khan et al., 2010).

1.2.12 ADIPOSE TISSUE ENGINEERING

There is a clinical need for engineered adipose tissue. Subcutaneous adipose tissue defects can arise from birth defects, oncologic resection and traumatic injury. Adipose tissue reconstruction is currently treated using methods such as autografting, fat free grafting and implantation of alloplastic materials (Girandon et al., 2011). These methods all suffer from disadvantages such as donor site morbidity and graft resorption. Multipotential mesenchymal stromal cells have been investigated as the cell source seeded onto suitable scaffolds to generate an artificial fat graft. MSCs are routinely differentiated into adipose cells *in-vitro*, and the adipogenesis of these cells is one of their characteristic features that along with osteogenesis and chondrogenesis, is used in the characterisation of these cells. *In-vitro* differentiation of MSCs into adipocytes is usually performed by supplementation of culture medium with isobutylmethylxanthine, indomethacin and hydrocortisone (Jones et al., 2002). Insulin has also been used to differentiate MSCs into adipose cells (Pittenger et al., 1999; Girandon et al., 2011) and the thiazolidinedione rosiglitasone (Fink et al., 2004; Crossno et al., 2006). This class of compounds is used clinically as insulin-sensitising anti-diabetic agents that stimulate the generation of adipocytes via the activation of the nuclear hormone receptor PPAR-ɣ.

The proof of concept studies using small animal models for *in-vivo* analysis have shown promising results using a variety of scaffolds in tandem with bone marrow MSCs for fat grafts.

PLGA hollow fibre scaffolds seeded with bone marrow MSCs encapsulated in alginate/chitin hydrogel capsules demonstrated sustained adipogenesis 4 weeks after implantation in nude mice (Bauer-Kreisel et al., 2010). Polyethylene glycol (PEG) based hydrogels have also been used in *in-vivo* models for fat grafts, again seeded with MSCs and these demonstrated retention of shape and dimension 4 weeks after implantation. MSCs seeded onto the scaffold and adipogenically differentiated before implantation resulted in the formation of adipose tissue *in-vivo* (Bauer-Kreisel et al., 2010). The majority of *in-vivo* studies thus far has been in small animal models and further *in-vivo* studies using longer term studies and in larger animals is needed. ADSCs have been also been suggested to use in soft tissue reconstruction to attempt to restore function and/or cosmesis. Therapies involving the use of synthetic filler materials are troubled with complications such as infection, cost and need for re-operative procedures (Wilson et al., 2011). Non-synthetic options involving fat grafting also have problems, with donor site morbidity/availability and graft reabsorption rates of between 20 and 90%. The use of biomaterials has been proposed as an alternative to free ADSC transfer, using materials such as collagen and hyaluronic acid gel. The use of biomaterials may aid differentiation of the ADSCs, and provides mechanical support to the cells.

1.2.13 AUTOLOGOUS VS ALLOGENEIC MSCS

There are some issues that need to be addressed in the context of tissue engineering using MSCs. One of the main issues that needs to be addressed is the choice of donor cells; autologous cells, or allogeneic? If autologous cells are used the cells have to be harvested from the patient. Due to the low number of MSCs usually obtained in primary harvesting, there is inevitably a time delay as the patient's own cells are expanded to generate sufficient numbers. If a scaffold is to be used, will undifferentiated MSCs be seeded directly before implantation, or will the cells be differentiated on the scaffold before implantation? The latter would result in an even greater delay before the patient receives the tissue engineered construct. If allogeneic cells are used MSCs could be isolated and expanded from donors, seeded onto scaffolds and differentiated in readiness for implantation in the patient. This approach has the advantage of a single invasive procedure for the recipient patient and the availability of the construct 'off the shelf'. Thus the use of allogeneic MSCs is commercially extremely attractive. The use of allogeneic cells could potentially be restricted due to immune rejection of transplanted cells. MSCs are perhaps ideally suited to allogeneic transplantation, as there is a great deal of evidence in the literature suggesting that MSCs are immuno-privileged and are not subject to recognition, or elimination by the immune system (Bartholomew et al., 2002; Tse et al., 2003; Jones et al., 2007; Niemeyer et al., 2007; Oh et al., 2008; Suva et al., 2008).

1.3 IMMUNOLOGICAL RESPONSES TO ALLOGENEIC CELLS AND TISSUES

In order to understand the concept of MSCs being immuno-priviledged, it is nessesary to understand how the immune system recognises alloantigens and therefore how cells are recognised as self, or non-self.

1.3.1 ADAPTIVE VERSUS INNATE IMMUNITY

The immune response in the human body is split into two levels; the innate response, and the adaptive (acquired) response.

Innate Immunity is mainly comprised of reactions of a non-specific nature, such as direct barriers to infection (the skin), complement activation pathway, interferon secretion and killing of virus infected cells by natural killer (NK) cells. One of the most important cell types of the innate response is the phagocyte. These cells, comprised of macrophages and neutrophils, are able to recognise, ingest and subsequently destroy many pathogens without the need of the adaptive response (Janeway et al., 2005). Phagocytes are able to recognise different 'patterns' expressed by microbes (known as pathogen associated molecular patterns). Upon recognition of these molecules on the surface of the invading microbes, the cell engulfs the pathogen and internalises it in a process known as phagocytosis, culminating in the elimination of the engulfed pathogen.

Adaptive immunity is characterised by two main attributes; the specificity for the 'foreign' antigen and immunological memory (the increased more rapid response towards a previously encountered/similar antigen). The cell types associated with adaptive immunity are antigen-presenting cells (APCs), B-cells (bone marrow-derived lymphocytes), and T-cells (thymus-derived lymphocytes) (Stites, 1991). Antigen presenting cells are cells whose role is to phago/pino-cytose foreign proteins/organisms, and process the antigen into peptide form that is presented on the surface of the cell via the major histocompatability complex (MHC), forming an immunogenic complex. The major histocompatability complex (MHC) is a large genomic region present in most vertebrates. The genes and hence the proteins expressed by the MHC are extremely polymorphic in the population. Cell surface molecules encoded by the MHC present antigens to T-cells allowing the T-cells to recognise 'self' and 'non-self' cells and take action accordingly. In humans the MHC is known as the human leukocyte antigen (HLA) system. T-cells, which are the effector arm of the immune response, can ordinarily only recognise antigens that have been processed and presented with the MHC and associated co-stimulatory molecules present on the surface of these cells. CD4 positive T cells (also known as helper T cells or T_H cells) require antigen to be presented with MHC class II. This subset of T-

cells are the predominant mediators of cytokine secretion, which enhances or suppresses the responses of other lymphocytes (Kimball, 1990).

MHC class I, unlike class II is expressed on all nucleated cells (Stites, 1991). Another subset of T cells, known as CD8 positive T-cells, or cytotoxic T-cells are able to recognise antigen presented on MHC class I and when appropriately activated are able to eliminate the cell.

Monocytes, macrophages dendritic cells and B-cells are able present antigens to T-lymphocytes via ingesting and presenting peptides from foreign proteins via MHC class I and II present on their cell surface and are known as antigen presenting cells.

There are two main differences between MHC class-I and II. MHC class-I molecules present peptides obtained from within the cell, and MHC class-II present peptides derived from proteins in intracellular vesicles and therefore peptides that are derived from exogeneous antigens internalised by antigen presenting cells.

1.3.2 THE ADAPTIVE RESPONSE: T-CELLS AND B-CELLS

When T-cells develop within the thymus, they express a randomly rearranged antigen receptor on their cell surface called the T-cell receptor (TCR) that binds to the MHC molecule expressed by cells of the individual. However the majority of these T-cells, due to the randomly generated nature of the antigen receptor, are unable to bind to the MHC proteins that are expressed by the individual. About 90% of the developing T-cells in the thymus die because they are not able to bind to MHC proteins and do not receive a survival signal (Palmer, 2003). Further maturation of these putative T-cells is dependent on the ability of these cells to bind to antigen presenting cells (APCs) expressing MHC-self peptide complexes, and therefore that the T-cells express a functional TCR. The most strongly reactive T-cells are very tightly controlled, as although a functional TCR is desirable, self-reactive T-cells are not. It is the progenitor T-cells that are weakly reactive that progress further and fully mature, and engagement of a MHC class-I peptide complex results in the maturation of a CD8+ T-cell, whereas the recognition of MHC class-II – peptide results in the maturation of a CD4+ restricted T-cell (Palmer, 2003). If the T-cell is highly reactive and binds strongly to the MHC-self complex, this results in the apoptotic death of the putative T-cell, preserving self-tolerance. Mechanisms exist in the periphery to eliminate any auto-reactive T-cells that may have progressed through maturation and this is mediated through the action of $CD25^{+}CD4^{+}$ regulatory T-cells (Takahashi et al., 1998; Hogquist et al., 2005). Mature naive CD4+ and CD8+ T-cells reside within secondary lymphoid tissues and recirculate in the blood and lymph in a quiescent state until they encounter specific antigen.

B-cells are lymphocytes that are involved in the humoral immune response, and the principle functions of these cells are to make antibodies towards antigens, act as antigen presenting cells, and develop into memory B cells. B-cells are produced in the bone marrow and the maturation process is similar to T-cells with gene rearrangement forming the antigen binding region of the B-cell receptor. In the context of using allogeneic MSCs in patients, the T-cell response plays the central role in immune recognition, therefore will be the main subject focussed on.

1.3.3 THE RECOGNITION OF ANTIGEN BY T-CELLS

The recognition of the MHC-epitope complex by the T-cell receptor is not dependent on the TCR and the MHC-epitope complex alone, accessory proteins are involved. The TCR is also associated with CD3 forming the TCR-CD3 complex and it is the CD3 molecule that initiates the intracellular signalling response after interaction of the T-cell with the MHC-peptide. The actual recognition of the MHC-epitope is mediated by the TCR-CD3 complex, but there are also a number of co-stimulatory molecules that play an important accessory role in the initial recognition of antigen and subsequent activation of naive T-cells by strengthening the interaction between T-cells and the antigen-presenting cell, or playing a role in signal transduction (Kuby, 1997). The T-cell receptor and related co-stimulatory molecules are shown in **Figure 1.3**. Although the recognition of antigen by the CD4+ and CD8+ T-cells is the same, the activation of the T-cells is different, and the two types of T-cell have different requirements before they are activated. The CD4+ T-cell is activated when the MHC class II-epitope is recognised and presented with co-stimulatory molecules. This activates the T-cell which is them able to undergo clonal expansion and mature into memory T-cells and effector cells. The effector cells are able to secrete cytokines that regulate and assist in the active immune response and it is these cells that help in the maturation of B-cells and the clonal expansion of cytotoxic CD8+ T-cells. CD8+ T-cells recognise MHC class I-epitope and also need the presence of co-stimulatory molecules to become activated. In addition to this, these cells also require the help of activated CD4+ T-cells before they themselves can undergo clonal expansion into memory and effector cytotoxic cells. This help is in the form of secreted IL-2, and without this CD4+ T-cell help, CD8+ T-cells cannot respond (Janeway et al., 2005). Effector CD8+ T-cells are able to lyse targeted cells after recognition of the MHC class I-epitope on the target cell via secretion of cytotoxins such as perforin, granzymes, and granulysin.

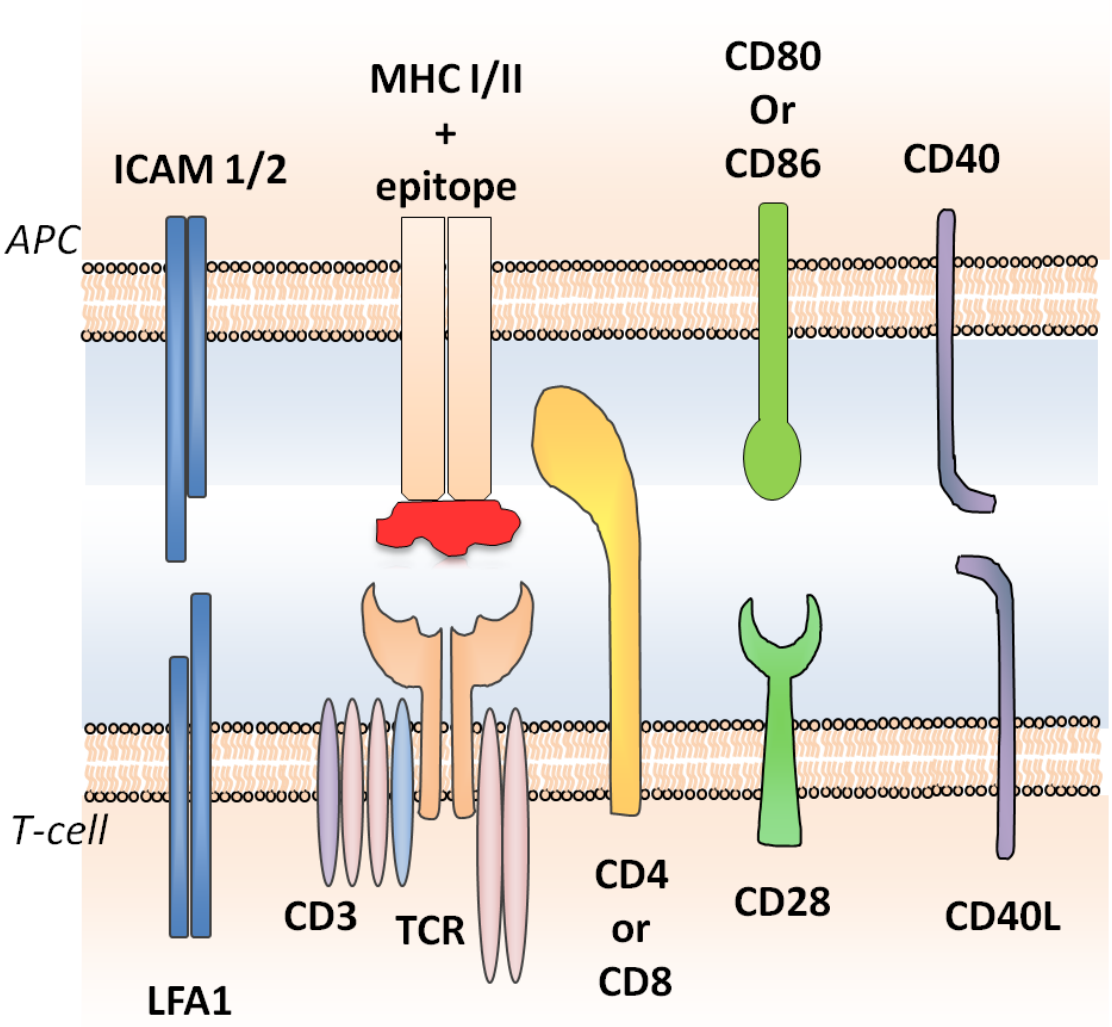


Figure 1.3: T-cell receptor binding. The T-cell receptor and co-stimulatory molecules present on the surface of T-cells involved in the recognition of antigen by T-cells. The T-cell receptor (TCR) recognises antigen presented by MHC molecules, with CD4 determining specificity for MHC class-II, and CD8 for MHC class-I. CD3 associates with the TCR and is involved in downstream signalling following recognition of antigen. Lymphocyte function associated-antigen 1 (LFA1) binds to ICAM-1 on the antigen presenting cell (APC). CD28 binds to the costimulatory molecules CD80 and CD86 expressed by the APC and CD40L binds to the costimulatory molecule CD40.

1.3.4 How T-cells recognise alloantigen

It was shown in the early 1960's that if a mixture of lymphocytes from two different individuals (genetically not identical), were cultured together they were capable of stimulating each other and generating a large number of lymphoid blast cells in-vitro (Bain et al., 1964). It was shown that the strongest reaction occurred (the largest number of cells produced) in the reaction that had discrepancy at the major histocompatability complex. From its initial use, the mixed lymphocyte reaction (MLR) has been used as an important experimental method for investigating the cellular mechanisms involved with the rejection of allografts and more importantly for assessing histocompatability (Stites, 1991).

The process by which donor cells are recognised by host T-cells is called 'allorecognition'. There are 3 forms of allorecognition: direct, indirect, and semi-direct.

The concept of direct recognition is that T-cells are able to recognize intact allogeneic MHC class I and II molecules on the surface of allogeneic cells (Watschinger, 1995; Hornick and Lechler, 1997). There are two hypotheses that are used to explain why the T-cell response to allogeneic MHC is so strong. The first is the multiple binary complex hypothesis; this theory proposes that T-cells with direct allospecificity have dual specificity for both the peptide and the foreign MHC molecule (Hornick and Lechler, 1997). This theory states that due to the extreme diversity of the bound peptides that are displayed by the MHC on the cell surface, one MHC molecule is able to generate several hundred different peptide:MHC complexes, that are each recognised by an individual clone of an alloreactive T-cell. The second theory was proposed later and is known as the high determinant density hypothesis. The principle of this theory is that the ligand of the alloreactive T-cell is the allogeneic MHC molecule itself, with little or no specificity for peptide bound to it (Rogers and Lechler, 2001). The name of this theory is derived from the fact that the determinant density available to the T-cell (number of matching ligands) due to any molecule of allogeneic MHC potentially acting as ligand is very high. The affinity of the alloreactive T-cell's receptor could be much lower than that usually required for a peptide:self MHC complex, and this allows T cells with low, medium and high affinity into the alloreactive T cell repertoire (Hornick and Lechler, 1997; Rogers and Lechler, 2001).

Due to the highly polymorphic nature of the MHC molecules, responder and stimulator MHC molecules could be similar and share conserved sequences within the T-cell receptor contacting surface of the molecule. It is the regions of variation within the peptide binding groove that bind and present different peptides. The response observed therefore is directed at the multiplicity of different peptides bound by the MHC molecule (Hornick and Lechler,

1997). This relates to the multiple binary complex hypothesis. The alternative theory can be explained when the exposed surfaces of the stimulator and the responder MHC molecules are different. Both of these theories seem to invalidate the principle of self-MHC restriction, in that the TCR recognises non-self MHC molecules. In order to explain why there appears to be such a high proportion of alloreactive T-cells there are 2 hypotheses. The first of these is applicable when self and non-self MHC molecules share similar conserved regions in the exposed TCR contacting surface of the molecule and it is difference in the peptide binding region that confer specificity to the binding of different sets of peptides. The alloresponse is therefore generated towards the variability of the different peptides bound by the MHC molecule. The second theory applies when the exposed surfaces of the responder and stimulator MHC are significantly different. The high determinant density hypothesis then provided a better explanation for the strength of the alloresponse and principle of self-MHC restriction is explained by a small number of T-cells whose TCR is specific for self-MHC molecules and by chance cross react with a foreign MHC molecule (Hornick and Lechler, 1997).

Direct antigen presentation does not explain rejection in the absence of passenger leukocytes and therefore in-direct recognition was proposed in the early 1980's (Lechler and Batchelor, 1982). This proposed that the normal pathway for antigen presentation by APCs (alloantigens presented by donor APC to CD4+ T-cells) was an important factor in chronic graft rejection. The theory behind this was that initially, the donor graft could contain enough APC's (dendritic cells) that could migrate to host lymphoid tissue and perform direct alloantigen presentation. Over time this population of donor cells would decrease, and cases where rejection is prolonged (chronic) can therefore be explained by the influx of host APCs. Alloantigens that are shed from a graft are processed as exogenous antigens by the host APCs and are then presented via the host APC MHC class-II and therefore the process of indirect allorecognition is predominantly performed by CD4+ T-cells (Caballero et al., 2006; Afzali et al., 2007). Because of the nature of this response, the pathway of indirect recognition is comparatively slower than direct recognition. Typically in rejection, there is infiltration and proliferation of lymphocytes and monocytes into vascularised grafts before day 6, with full graft rejection occurring by 10-12 days (Kuby, 1997). Interaction and crosstalk between the direct and indirect pathways of recognition (indirect CD4+ T-cells are able to diminish, and amplify the direct pathway CD8+ T-cell responses) have previously relied on the so called 4-cell unlinked model, where the host CD8+ cytotoxic T-cells are stimulated via the direct pathway by the donor cells, while helper or regulatory CD4+ T-cells are recruited through interaction with the host APC presenting allogeneic MHC via the indirect pathway (Afzali et al., 2007). These two methods of

allorecognition do not take into account that CD4+ and CD8+ T-cells are recruited and linked by the same APC.

The final method of allorecognition attempts to explain this. This method of allorecognition is called the semi-direct pathway. In this pathway donor MHC molecules expressing allogeneic antigen are transferred to the host dendritic cells, allowing the stimulation of CD8+ T-cells via the direct pathway and peptides of histocompatibility antigens which are then processed and presented allow the recruitment of CD4+ T-cells via the indirect response (Afzali et al., 2007; Gokmen et al., 2008). This transfer of MHC molecules can occur via cell to cell contact or exosomes, and links direct and indirect allorecognition pathways through a single APC. An overview of the three different methods of allorecognition is shown in **Figure 1.4**.

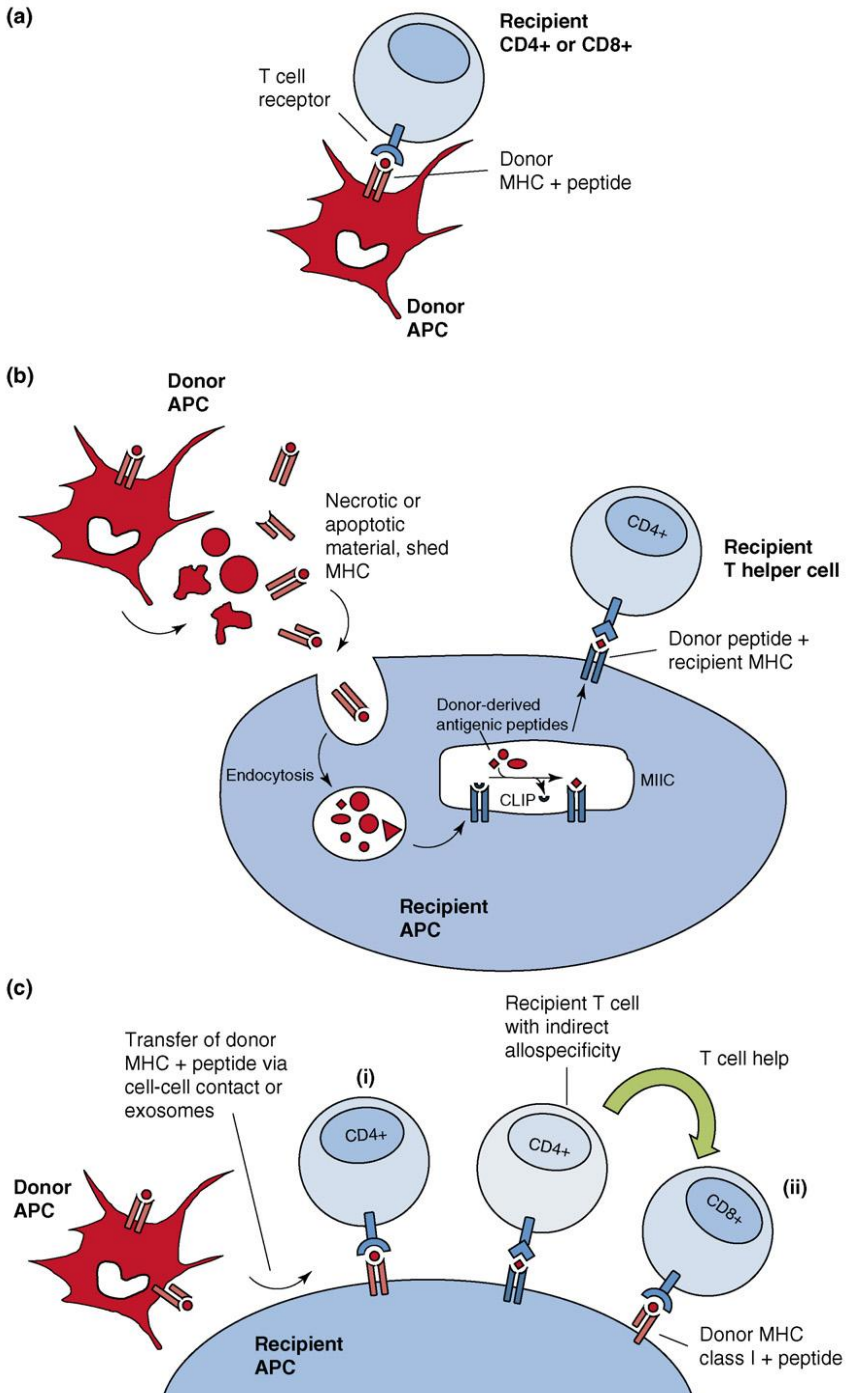


Figure 1.4: An overview of direct, indirect, and semi-direct alloantigen recognition. A) In direct recognition recipient T-cells are able to recognize intact allogeneic MHC molecules present on the surface of the donor antigen presenting cell. **B)** In in-direct alloantigens that are shed from graft cells are processed as exogenous antigens by the recipient APCs, and are then presented via the host APC MHC class-II to CD4+ T-cells. **C)** In semi-direct allorecognition donor MHC molecules expressing allogeneic antigen are transferred to host dendritic cells allowing the stimulation of CD8+ T-cells via the direct pathway, and peptides of histocompatibility antigens are processed and presented allow the recruitment of CD4+ T-cells via the indirect response, which aid in CD8+ T-cell priming. Figure taken from Gokmen *et al.* (2008).

Historically the direct pathway of recognition has been favoured as the dominant pathway in the role of allorecognition, in particular when acute rejection occurs when there is a rapid initiation of the allograft response. The current view however places a lot more emphasis on the role that indirect and semi-direct recognition plays in the role of allorecognition (Rogers and Lechler, 2001). In the role of allorecognition of MSCs, it is believed that these cells are not able to be recognised directly by T-cells due to their lack of co-stimulatory molecule and MHC class II expression. When determining if MSCs are able to elicit an immune response, it is important to be able to determine which part of the response are the MCSs most likely to encounter.

1.4 IMMUNOGENICITY AND IMMUNOMODULATION BY MSCS

It has been well documented that hMSCs express intermediate levels of cell-surface MHC class I molecules, but that MHC class II is not expressed on the cell surface. MHC class II molecules have been shown to be present intracellularly (Klyushnenkova et al., 2005). Le Blanc *et al* showed that when differentiated into bone, fat, and cartilage for 6-12 days, MSCs continued to express HLA class I, but even after the addition of interferon-γ (IFN-γ) which usually causes an increase in both MHC class I and II in MSCs (Klyushnenkova et al., 2005), no surface expression of class II was induced (Le Blanc et al., 2003a).

If allogeneic cells are transplanted into a person with a healthy immune system without the administration of immunosuppressant's, the cells will be recognised by the host's immune system and killed. Human MSCs do not express co-stimulatory molecules CD40, CD80, or CD86 even after IFN-γ administration and as such are protected from T-cell recognition by the direct pathway of recognition (Le Blanc and Ringden, 2007). This is not the only mechanism that recipient T-cells can be activated by; they can also be activated indirectly by recipient antigen presenting cells (APCs). The indirect activation process occurs when foreign antigens released from dead cells are taken up into APCs through endocytosis and are presented on the surface. This presentation of foreign antigen primes T-cells to target and neutralise cells expressing the antigen as described above. When allogeneic lymphocytes are cultured with skin fibroblasts or renal tubular epithelial cells, the lymphocytes have been shown proliferate (Le Blanc and Ringden, 2007). When differentiated MSCs (fat, bone and cartilage) and undifferentiated MSCs were co-cultured with allogeneic lymphocytes for up to 7 days then no proliferation was observed. This occured even in the presence of APCs and after the provision of co-stimulatory signals using both CD80 or CD86 gene transduction in the MSCs, or CD28 specific antibodies (Bartholomew et al., 2002; Le Blanc et al., 2003a; Beyth et al., 2005; Klyushnenkova et al., 2005). As well as the proliferation of lymphocytes, alloreactivity can also be measured by

production of IFN-γ. Studies measuring the IFN-γ production of activated lymphocytes when cultured with MSCs correlated with the proliferation studies; hMSCs did not elicit IFN-γ production in human peripheral blood mononuclear cells (PBMC), but non-immunomodulatory cells such as fibroblasts did (Krampera et al., 2003; Klyushnenkova et al., 2005).

The evidence indicates that MSCs therefore do not induce lymphocyte proliferation, upregulate activation-associated markers, or stimulate IFN-γ production. As well as being protected from the CD4+ T-cell response, MSCs have been shown to escape lysis by CD8+ cytotoxic T-cells (Bartholomew et al., 2002; Rasmusson et al., 2003). Rasmusson *et al* demonstrated that unseparated lymphocytes stimulated *in-vitro* to target PBMCs that were derived from a specific donor could lyse lymphocytes from the same individual, but not the patients MSCs (Rasmusson et al., 2003). The same group also demonstrated the ability of MSCs to both avoid natural killer (NK) cell specific lysis, and induce an abortive activation programme in fully differentiated CD8+ T-cells so that the major effector functions were not activated (Rasmusson et al., 2003; Le Blanc and Ringden, 2007).

1.4.1 MSCs AND T-CELLS

The ability of MSCs to suppress T-lymphocyte activation and proliferation was first demonstrated in 2002 (Di Nicola et al., 2002; Le Blanc et al., 2003a; Tse et al., 2003). T-cells induced by mitogens (Bartholomew et al., 2002; Di Nicola et al., 2002; Krampera et al., 2003; Le Blanc et al., 2003a; Tse et al., 2003), alloantigens (Le Blanc et al., 2003a; Potian et al., 2003; Tse et al., 2003) or antibodies to CD3 and CD28 (Bartholomew et al., 2002; Krampera et al., 2003; Tse et al., 2003) have been shown to have their proliferation suppressed when co-cultured with MSCs. Studies have also shown the ability of MSCs to inhibit the cytotoxic effects of activated cytotoxic T-cells (Potian et al., 2003), but this is thought to be due to an inhibition of proliferation of the T-cells rather than a direct suppression of cytolytic activity (Rasmusson et al., 2003) and this was further demonstrated as cytotoxicity was not affected when MSCs were added after the T-cells became activated.

The suppression observed appeared to be independent of MSC source and the effects remained constant if either allogeneic or autologous MSCs were used (Le Blanc et al., 2003a). It is thought that the suppression observed was due to secreted soluble factors, as when MSCs were separated from PBMCs by a semi-permeable membrane no change in the effect was observed (Di Nicola et al., 2002; Tse et al., 2003). Interestingly, it also seemed that in order for the inhibitory factors to be produced, MSCs needed to be grown in the presence of lymphocytes. This suggested that a dynamic cross-talk between T-cells and MSCs existed before production of the immunosuppressive soluble factors was initiated (Nauta and Fibbe,

2007). Di Nicola *et al* have suggested that hepatocyte growth factor (HGF) and TGF-β mediated suppression in mixed lymphocyte cultures, a finding supported by neutralising antibodies towards HGF and TGF-β allowing T-cells to once again proliferate (Di Nicola et al., 2002). This has however been contested with other groups unable to reproduce the effects obtained (Le Blanc et al., 2004a). Instead different mechanisms are thought to be involved, with specific mechanisms depending on the stimulus.

Another method proposed to explain how MSCs modulate the immune response is though the upregulation of regulatory T-cells (T-regs). These cells are naive T-cells that express CD25 and suppress T-cell proliferation (Blazar and Taylor, 2005). Djouad *et al* have shown that MSCs induced the formation of CD8+ regulatory T-cells that inhibited the proliferation of allogeneic lymphocytes (Djouad et al., 2003). When MSCs were cultured with mitogen stimulated PBMCs, an increase in CD4+CD25+ cells displaying regulatory phenotype (positive for FOX P3) was observed (Djouad et al., 2003). These cells have yet to be functionally analysed and it is possible that they do not have any effect on T-cell proliferation, as other studies have shown that CD4+CD25+ T-regs have no effect on the modulation of T-cell proliferation by MSCs (Krampera et al., 2003).

Indoleamine 2, 3-dioxygenase (IDO), an enzyme catalysing tryptophan breakdown, is also thought to be involved in the inhibition of T-cell proliferation by MSCs (Meisel et al., 2004). When MSCs are exposed to IFN-γ (produced by activated T-cells) they produce IDO which results in the depletion of tryptophan and the production of kynurenine breakdown products. These products have been shown to inhibit lymphocyte proliferation (Nauta and Fibbe, 2007). It is still unclear what role IDO actually plays in T-cell suppression (Aggarwal and Pittenger, 2005); one study found that the degradation of tryptophan into kynurenine products induced apoptosis in T-cells (Plumas et al., 2005), yet other studies have found that MSCs do not induce apoptosis in T-cells (Rasmusson, 2006; Le Blanc and Ringden, 2007).

It has been shown that MSCs constitutively produce prostaglandin E2 (PGE_2) and expression of this mediator is upregulated when MSCs are cultured with PBMCs (Tse et al., 2003). The suppression of PGE_2 synthesis has also been reported to stop the MSC mediated inhibition of T-cell cytokine production and profileration (Aggarwal and Pittenger, 2005), but this effect has been contested by other studies (Rasmusson et al., 2003; Tse et al., 2003).

Although there is widespread use of the term immunosuppressive effects of MSCs most of the evidence to date has only demonstrated the ability of MSCs to inhibit T-cell proliferation *in-*

vitro, therefore primarily affecting the effector arm of the T-cell immune response (Nauta and Fibbe, 2007).

1.4.2 MSCs AND APCs

As discussed previously, APCs are able to present antigens to T-cells via MHC on the cells surface. T-cells can recognise this complex via their T-cell receptor resulting in the activation of the T-cell. MSCs have been shown to reduce the formation of dendritic cells (DCs) from monocyte precursors (Maccario et al., 2005). As DCs are APCs this results in reduced T-cell activation. When DCs were grown in the presence of MSCs their response to maturation signals was impaired and the 'mature cells' did not express CD83 or upregulate their expression of other co-stimulatory molecules (Zhang et al., 2004). Immature DCs grown together with MSCs have been shown to exhibit very poor T-cell activation efficacy (Nauta and Fibbe, 2007). The cytokine secretion profile of DCs is altered when grown with MSCs, with decreased production of pro-inflammatory cytokines IFN-γ, IL-12 and TNF-α and an upregulation of the anti-inflammatory cytokine IL-10 (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Jiang et al., 2005). From the observed effects thus far, it seems that MSC can affect the generation of DCs and their subsequent maturation, in order to generate a subset of DCs that have an immune-inhibitory phenotype. Nauta *et al* also demonstrated that the inhibitory effects of MSCs on DCs are caused in part by soluble factors (Nauta et al., 2006a). Blocking studies carried out on the production of IL-6 and mononuclear-phagocyte colony stimulating factor (M-CSF) by MSCs have shown that although these factors play a part in suppressing DC maturation, they are not solely responsible (Nauta and Fibbe, 2007). PGE_2 has again been proposed to be involved in this process, and the blocking of PGE_2 synthesis restored the secretion of IFN-γ and TNF-α by DCs (Aggarwal and Pittenger, 2005). The increased production of the anti-inflammatory cytokine IL-10 by DCs when cultured with MSCs may also play a role in their ability to suppress T-cell proliferation, as neutralising antibodies to IL-10 increased T-cell proliferation in culture (Beyth et al., 2005).

As well as directly suppressing T-cell proliferation, the disruption of APC maturation and alteration of cytokine expression profile is thought to play a role in indirectly suppressing T-cell proliferation.

1.4.3 MSCs AND B-CELLS

There is little evidence to suggest that MSCs can suppress B-cell proliferation; it has been shown that MSCs can inhibit lymphocyte proliferation induced by *Staphylococcus aureus* Protein A and pokeweed mitogen (Corcione et al., 2006; Le Blanc and Ringden, 2007). As

protein A stimulates the proliferation of T and B-cells and lymphocyte stimulation by pokeweed mitogen is T-cell dependent, inhibition of B-cell proliferation cannot be shown to be separate from T-cells (Le Blanc and Ringden, 2007). These studies have only been carried out in murine models, but human MSCs have been shown to reduce the level of IgM, IgG and IgA produced by activated B-cells when cultured in a 1:1 ratio (Corcione et al., 2006). MSCs were shown to alter the expression of chemokine receptors CXCR4, CXCR5 and CCR7B through downregulation and also reduced the chemotaxis to CXCL12, CXCR4 ligand, CXCL13 and to the CXCR5 ligand, but only when large numbers of MSCs were present (Le Blanc and Ringden, 2007). In fact, at a 10-fold lower dose of MSCs, splenic and blood derived B-cells were actually stimulated towards IgG production as tested by Elispot (Rasmusson et al., 2007). It is possible that in the presence of excess MSCs, the stimulatory path becomes overloaded, suggesting that the nature of the effect is influenced by the magnitude of the stimulus (Le Blanc et al., 2003a). When separated by a semi-permeable membrane it has been shown that the soluble factors released result in the inhibition of proliferation of B-cells, but actual culture supernatant had no effect. This suggests that the inhibitory soluble factors are released only after paracrine stimulation of MSCs by the B-cells (Corcione et al., 2006).

1.4.4 MSC AND NK CELLS

Natural Killer (NK) cells express cell surface receptors that receive signals from the environment and other cells. Depending on this signal NK cells respond by producing effector molecules that can inhibit cell growth, lyse cells, and transmit signals to other cells of the immune system. It has been suggested that MSCs are able to suppress IL-2 and IL-15 driven NK-cell proliferation and IFN-γ production (Rasmusson et al., 2003; Sotiropoulou et al., 2006; Spaggiari et al., 2006), but are not able to inhibit lysis by freshly isolated NK cells. Again the proliferation and cytokine production of IL-15 stimulated NK cells was shown to be inhibited by soluble factors, but the cytotoxicity required cell-cell contact suggesting that different mechanisms exist for NK suppression by MSCs (Sotiropoulou et al., 2006). Although these studies suggest that MSCs are able to decrease the proliferation of NK cells, a 1:1 ratio of NK cells:MSCs was needed in order to get a 50% reduction in growth (Maccario et al., 2005; Spaggiari et al., 2006). At ratios of 10:1 there ceased to be any inhibition of proliferation and at this ratio there is also no inhibition of CTL or NK cell cytotoxic function. Based on the study by Maccario *et al*, only very high concentrations of MSCs downregulated the expansion of CD8+ T-cells, and both Spaggari and Maccario showed that very high concentrations are needed for the downregulation of NK cells (Maccario et al., 2005; Spaggiari et al., 2006). It is not currently possible to use this many cells in *in-vivo* therapies, so at present these studies

have shown that NK-cell modulation by MSCs as a therapeutic treatment *in-vivo* would be minimal.

A summary of the different ways in which MSCs have been reported to affect the immune system is shown in **Figure 1.5**:

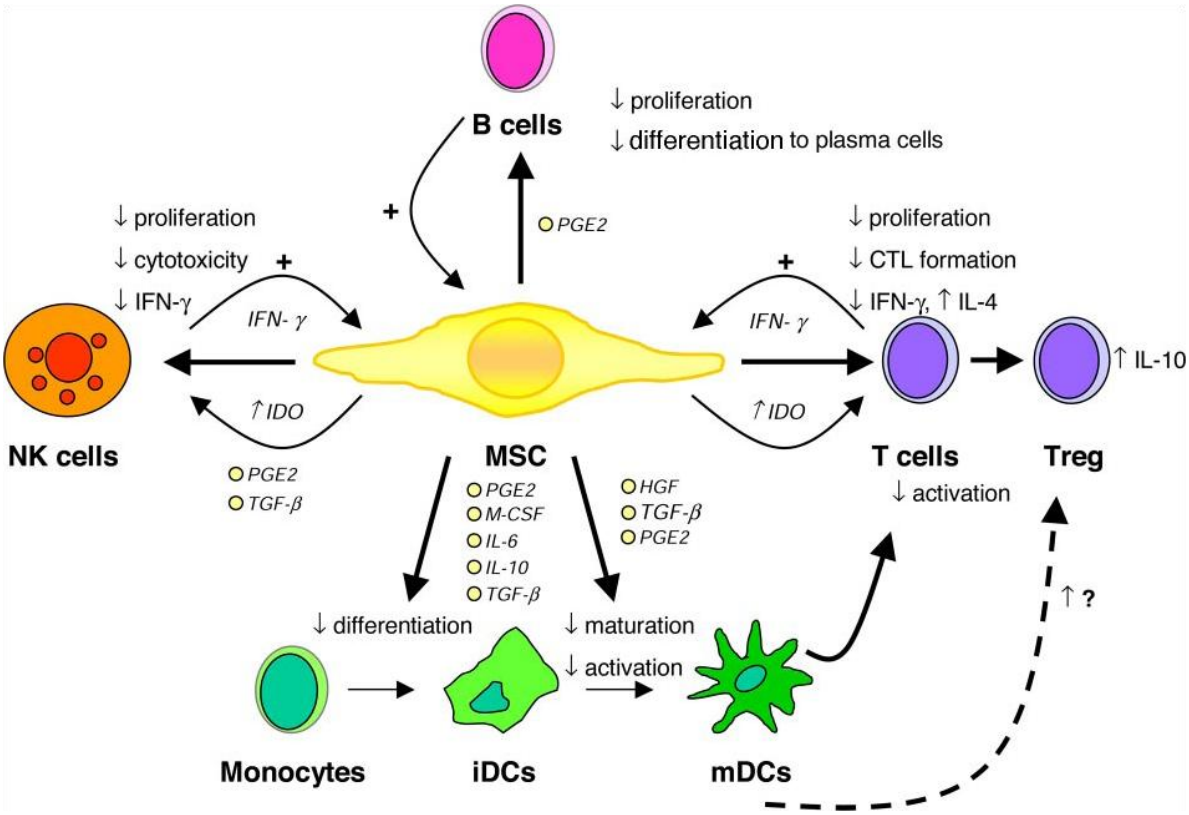


Figure 1.5: An overview of how the factors secreted by MSCs have been reported to affect the immune response. Figure taken from (Nauta and Fibbe, 2007).

1.5 IMMUNOMODULATION BY MSCS IN-VIVO

Although there has been a large amount of work carried out on the immunosuppressive effects of MSCs *in-vitro* there is still limited data on their *in-vivo* effects. Bartholomew *et al* demonstrated in baboons that a systemic infusion of allogeneic MSCs extended skin graft lifespan from 7 to 11 days and failed to elicit an immune response from the host lymphocytes (Bartholomew et al., 2002). The skin grafts were still rejected however. In a human patient with severe aplastic anemia, semi-allogeneic MSCs were given as an infusion after cyclophosphamide treatment (Fouillard et al., 2003). Severe aplastic anemia is an auto-immune disease in which a subset of cases exhibit damage to bone marrow stroma. Analysis of the patient's bone marrow after the infusion demonstrated partial recovery of stroma, and the presence of semi-allogeneic MSC engrafted into the bone marrow itself. LeBlanc *et al* had

more promising results in a study in which fully mismatched allogeneic fetal liver-derived MSCs were infused into a fetus diagnosed with serious osteogenesis imperfecta (Horwitz et al., 2005). No lymphocyte proliferation against the transplanted MSCs was observed, and MSCs were found to have engrafted and differentiated into bone in samples analysed when the patient was 9 months of age. Although this study was conducted in a fetus in whom self-tolerance mechanisms would result in the implanted MSCs being recognised as part of the host, this study still showed that allogeneic MSCs are able to engraft, differentiate and induce tolerance when transplanted into a host with an immature immune system. Koc *et al* conducted two separate studies into the use of MSCs in breast cancer patients and patients suffering from Hurler syndrome and metachromatic leukodystrophy (MLD) (Koc et al., 2002). Hurler syndrome and MLD sufferers develop significant skeletal and neurological defects that hinder their survival. Treatment for these diseases usually involves hematopoietic stem cell transplantation to partially correct some of the clinical manifestations. This is a risky process with the chances of the patient developing graft-versus host disease (GvHD). Following semi-allogeneic MSC infusion, some of the MLD patients exhibited significant improvements in nerve conduction velocity, and bone mineral density was either maintained or improved with no immune rejection of MSCs noted. More recently a clinical trial was carried out in Europe, in which donor MSCs were infused into 14 children undergoing CD34+ HLA-disparate cell transplantation (Ball et al., 2007). Historic controls had shown a graft failure rate of 15% in 47 patients, but in all patients infused with MSCs, hematopoietic engraftment was a success.

LeBlanc *et al* have conducted further studies on using allogeneic MSCs as a treatment/preventative for GvHD. MSCs were given to a patient exhibiting severe GvHD that was unresponsive to all previous therapy (Le Blanc et al., 2004a); After MSC administration the patient made a full recovery due to the rapid immunosuppressive and healing effect on the gut, and one year on from the treatment the patient was still well. A larger scale study lasting over 60 months aimed to assess, using a large number of patients, if MSC infusion could alleviate GvHD occurring after haemotopoietic stem cell transplantation (HSCT). Of 55 patients all with steroid-resistant, acute, severe GvHD, 39 responded to MSC infusion, resulting in alleviated symptoms and higher survival rate (Le Blanc et al., 2008). Over 50% of patients that had a complete response to treatment were alive 2 years after treatment. Although more work needs to be carried out on how the dose of MSC can affect the treatment of GvHD, there are two randomized European phase III trials that have recently started to further investigate the potential of allogeneic MSC as a treatment or preventative measure of acute GvHD following allogeneic stem cell transplantation (Nauta and Fibbe, 2007). A pilot study using 7 patients co-administered with MSCs when undergoing HSCT again had promising results with increased

reconstituted haematopoiesis in patients who had previous graft failures and showing that co-transplantation of MSCs with HSCT has positive effects (Le Blanc and Ringden, 2007). The latest study recruiting patients for a phase III clinical trial for steroid refractory acute GvHD and for newly diagnosed acute GvHD has completed enrolment and the results of this are eagerly anticipated (Trounson et al., 2011). The study is being conducted by a company called Osiris Theraputics and it is their product Prochymal (an intraveneously administered formulation of allogeneic MSCs) that is being evaluated for the phase III clinical trial. This product is also under evaluation in phase II clinical trials for repair of cardiac tissue following heart attack and the repair of lung tissue in patients with chronic obstructive pulmonary disease. A further product, Chondrogen (another injectable formula of allogeneic MSCs) is also being evaluated to treat arthritis in the knee.

It is thought that transplanted MSCs migrate to a wide variety of tissues in the body to mediate immune suppression, and can even be found engrafted within bone tissue (Le Blanc and Ringden, 2007; Nauta and Fibbe, 2007). Allogeneic MSCs have also been demonstrated to engraft into a patient's bone marrow (Fouillard et al., 2003). One study found that in a rat model after intra-venous injection of MSCs the majority of uptake of these cells was in the lungs up to 4 hours after infusion (Barbash et al., 2003).

The use of MSCs in the majority of the above studies does not provide true insight into the immunogenicity of allogeneic MSCs in vivo, since, in these studies most of the patients had been subjected to immunosuppressive therapy prior to receiving the allogeneic MSCs and therefore were not fully immunocompetent. In instances where patients are immunocompetent no long term engraftment or presence of allogeneic cells has currently been demonstrated.

1.6 CLINICAL USES OF MSCS

Much of the knowledge regarding MSCs has been acquired from *in-vitro* experimentation, and more work needs to be done in clinical trials. Animal models do provide a basis on which to make hypotheses, but differences exist between human and animal MSCs in addition to the differences between the responses towards alloantigens. To help monitor progress of ongoing clinical trials and outcomes, a database set up within the European Group for Blood and Marrow Transplantation (EBMT) will hold details of patients treated, cells transplanted and clinical outcomes. Currently, there are over 120 clinical trials involving MSCs for human use **(Figure 1.6)**.

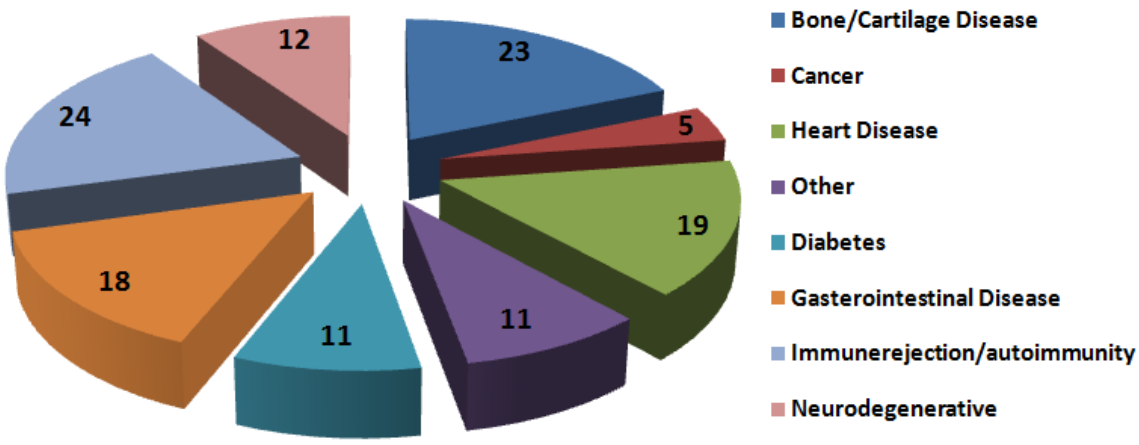


Figure 1.6: Current clinical trials using MSCs in humans, with number of clinical trials on segments, and the clinical area in which the trials are being undertaken. Data from this figure are taken from clinical trials.gov. Adapted from (Trounson et al., 2011)**.**

The majority of these are in Phase I-II, so still in the proof of efficacy and safety studies (Trounson et al., 2011). The majority of these studies use autologous MSCs and the largest area is for the treatment of immune related and auto-immune diseases. Although it is still early days, there are over 400 patients treated using MSCs to reduce inflammation and halt disease progression and further analysis into how MSCs affect the long term stability and progression of diseases/conditions will occur as time progresses.

In addition to using semi-allogeneic and allogeneic MSCs in the clinic, some studies have used autologous MSCs. One study performed demonstrated that autologous MSC infusion when performed with peripheral-blood stem cell transplantation induced rapid hematopoietic recovery (enhancing engraftment) (Koc et al., 2000).

Although there is little evidence for the differentiation of MSCs into functional neuronal cells some clinical trials have been performed injecting autologous MSCs intrathecally into spinal cord cerebrospinal fluid (allowing cells access to the brain and spinal column). This was performed in patients suffering from multiple sclerosis and amyotrophic lateral sclerosis (Karussis et al., 2010). The results of this study suggested that MSCs do exhibit an immunomodulatory effect in-vivo, as there was an increase in regulatory T-cells and a

reduction in the proportion of activated dendritic cells and lymphocytes and a reduction in lymphocyte stimulation by PHA, observed as early as 4 hours post administration.

There are further clinical trials currently underway, evaluating the use of MSCs in bone repair (the healing of non-union fractures), with 36 patients with fracture gaps of larger than 6cm treated with a mixture of stromal, progenitor cells, and autologous MSCs. In tandem with this another trial is underway in which 24 patients with distal tibial fractures have been treated, again using autologous MSCs (Tseng et al., 2008). The follow up results of these studies are awaited.

It has been shown that when expanded *ex-vivo* murine MSCs have dramatically reduced engraftment capacity and homing potential (Rombouts and Ploemacher, 2003). In order to obtain sufficient cells, current clinical trials have used expanded MSCs. With methods to isolate and purify MSCs resulting in ever increasing yields, it is necessary to compare the differences between expanded vs non-expanded MSCs in therapies; it is possible that non-expanded MSCs would migrate and engraft more efficiently to the damaged target tissue resulting in greater level of targeted immunosuppression, improving the outcome of the treatment.

It has been suggested in *in-vivo* studies that MSCs are able to avoid usual alloresponses supporting the possibility of using allogeneic stem cells for future therapies. As mentioned, these studies have usually been performed in immuno-compromised patients. There have also been some studies carried out in animal models that suggest that although allogeneic MSCs can engraft in immune-compromised hosts or at immune-privileged sites, they do in fact initiate an immune response in normal hosts (Nauta and Fibbe, 2007).

The effects of differentiation on the capacity of MSCs to modulate the immune response needs to be determined as there is currently very little data on the immune response towards fully differentiated MSCs. As discussed previously many groups have demonstrated the immunoprivileged nature of undifferentiated MSCs, but in these studies MSCs and lymphocytes have only been cultured together for up to 7 days. The results of the assays performed do indeed indicate that, probably due to the lack of expression by MSCs of MHC class II and co-stimulatory molecules, and also intermediate expression of MHC class I, that MSCs do not stimulate the lymphocytes directly. However, in only performing the assays by culturing MSCs and lymphocytes together for up to 7 days, alternative methods of allorecognition (indirect and semi-direct) may not be given the opportunity to manifest.

In order to robustly test the hypothesis that MSCs are immuno-privileged, it may be necessary to culture MSCs with lymphocytes and APCs over prolonged time periods.

In addition to this, if MSCs are indeed immuno-privileged, there are no 'long term' studies investigating whether once differentiated the MSCs retain their immunoprivileged status, or if they are effectively targeted and eliminated by the host's immune system. The characterisation of MSCs requires the trilineage differentiation into bone, fat and cartilage. Differentiation into cartilage requires 3D culture as spheroids and bone differentiated MSCs are difficult to analyse due to increased expression of cell adhesion factors and calcification. Fat cells however are a very interesting cell type to study following differentiation of MSCs, as *in-vivo* adipose cells have been shown to secrete hormones termed 'adipokines', such as leptin, resistin, and adiponectin (Schaffler et al., 2007). These adipokines are known to exert potent immunomodulatory effects on immune cells (Tilg and Moschen, 2006; Bielby et al., 2007; Schaffler et al., 2007). Adipocytes have been demonstrated to be potent producers of pro-inflammatory cytokines such as TNF-α and interleukin-6, as well as chemokines such as monocyte chemoattractant protein (MCP)-1 (Schaffler et al., 2006; Schaffler et al., 2007).

1.7 AIMS AND OBJECTIVES

The current belief that MSCs are immuno-privileged is based on experiments that do not fully appreciate the response of lymphocytes to alloantigens. The current methodology used to determine the immuno-privileged nature of MSCs involves culturing MSCs with peripheral blood mononuclear cells (PBMCs) for up to 7 days and so limits observations to the effect of the direct pathway of allorecognition. The assay conditions in which MSCs are cultured with PBMCs needs to be optimised to allow the effect of indirect and semi-direct pathways to be assessed. One of the defining characteristics of MSCs is their trilineage differentiation into bone, fat, and cartilage cell types. It was decided to use adipogenic differentiation to assess the effect of PBMCs on differentiated MSCs due to the close relationship of adipose tissue with cells of the immune system *in-vivo*. There are currently many methods used to quantify levels of adipogenesis in MSC cultures, but there is no gold standard method. The most common assays used to determine levels of adipogenesis are to be compared, and the most suitable assay used to determine which MSC donor cells exhibit the highest adipogenic potential before use in the aforementioned culture with PBMCs.

It is our hypothesis that MSCs will not directly stimulate allogeneic lymphocytes in classical lymphocyte proliferation assays due to their lack of expression of immune-regulatory molecules (CD40, CD80, CD86, MHC Class-II), but will be capable of stimulating allogeneic

lymphocytes via the indirect pathway of antigen presentation. In order to test this hypothesis the following aims and objectives were set:

- To isolate MSCs from 4 BM donors before they are expanded, and characterised.
- To differentiate MSCs into adipose cells, before comparing methods of quantifying adipogenesis, with the MSC from the donor exhibiting highest levels of adipogenesis determined.
- To determine the methodology used to perform the one-way lymphocyte transformation assay, and extend the duration of the assay beyond the typical 7 day period previously performed in the current literature.
- To perform the lymphocyte transformation assay using peripheral blood mononuclear cells from 6 healthy volunteers cultured with the MSCs from the 4 bone marrow donors.
- To differentiate the most adipogenic donor MSCs into adipose cells, and perform the lymphocyte transformation assay against the differentiated cells.

Chapter 2: GENERAL MATERIALS AND METHODS

2.1 EQUIPMENT

The equipment used throughout these studies is detailed in **Table 2.1**

Equipment	Company
5% (v/v) CO_2 in air	BOC Ltd., West Sussex, UK.
96 well microplate lid Sterilin	Fisher Scientific, Loughborough, UK.
96 well microplate Sterilin	Fisher Scientific, Loughborough, UK.
96 well U-bottom culture plates	Fisher Scientific, Loughborough, UK.
ABI Prism 7900	Applied Biosystems, Warrington, UK.
ATPLite assay	Perkin Elmer, Berkshire, UK.
Automatic pipettes (Gilson P2-P500)	Anachem, Bedfordshire, UK
Balance-4 figure (AND GR-200)	Jencons PLC, Bedforshire, UK
Cell medium aspirator (Vacusafe comfort)	Integra Biosciences, Zurich, Switzerland
Centrifuge (Harrier 15/80)	SANYO Biomedical Europe BV,
Centrifuge-Micro (MSE Micro Centaur I)	SANYO Biomedical Europe BV, Amsterdam, Holland
Class II safety cabinet (Heraeus 85)	Kendro, Hertfordshire, UK
CO_2 Incubator (MCO-20AIC)	SANYO Biomedical Europe BV, Amsterdam, Holland
Counting Chamber Improved Neubauer	Scientific Laboratory Supplies Ltd (SLS), Nottingham, UK.
FACScan	Becton Dickinson, Oxford, UK.
FilterMate™ cell harvester	Perkin Elmer, Berkshire, UK.
Freezer -25°C (Electrolux 3000)	Jencons PLC, Bedforshire, UK
Fume hood	Whiteley, Hants, UK.

Glass fibre filter plates	Perkin Elmer, Berkshire, UK.
Hot air oven (SANYO OMT225)	SANYO Biomedical Europe BV, Amsterdam, Holland.
Incubator	Laboratory and electrical engineering, Nottingham, UK.
Laminar flow cabinet (Bassaire 06HB)	Jencons PLC, Bedforshire, UK
LB940 Multilabel Reader Mithras	Berthold Technologies, Hertfordshire, UK.
Liquid nitrogen dewer (BIO65)	Jencons PLC, Bedforshire, UK
Liquid nitrogen gas	BOC Ltd., West Sussex, UK
Luminescence counter and liquid scintillation counter (Packard TopCount NX)	Perkin Elmer, Albany street, Boston.
Magnetic stirrer	Bibby Sterlin Ltd, Stone, Staffordshire, UK.
Microplate reader (Multiscan Spectrum)	Thermo Electron Corporation, Waltham, USA
Microscope-Inverted (Olympus, IX71)	Olympus Optical Co (UK) Ltd, Southall, UK.
Microscope-Upright (Olympus, BX71)	Olympus Optical Co (UK) Ltd, Southall, UK.
pH meter (3020)	Jenway Ltd, Dumow, UK.
Pipettor Tips Combitips (1ml, 2.5ml, 5ml, 12.5ml)	Fisher Scientific, Loughborough, UK.
Specimen pot (60 ml, 100 ml, 250 ml)	Scientific Laboratory Supplies Ltd (SLS), Nottingham, UK.
Tissue Culture Flask (25 and 75 cm2)	Fisher Scientific, Loughborough, UK.
Tissue culture plates (6, 12, 48, 96)	Fisher Scientific, Loughborough, UK.
Topcount Glass Fibre 96 Well Plates	Perkin Elmer, Albany street, Boston.
Topseal Applicator	Perkin Elmer, Albany street, Boston.
Universal/centrifuge tube (25 ml)	Scientific Laboratory Supplies Ltd (SLS), Nottingham, UK.
Vacutainer blood collection system	Becton Dickinson, Oxford, UK.
Vortexer (Grant)	Jencons PLC, Bedforshire, UK
Water bath	Fisher Scientific, Loughborough, UK.

Table 2.1: Equipment used with manufacturer.

2.2 MATERIALS

All laboratory consumables were obtained from Scientific Laboratory Supplies Ltd (Nottingham, UK) unless otherwise stated. The materials used are listed in **Table 2.2**.

Materials	Company
10% (v/v) Neutral buffered formalin	Sigma-Aldrich Ltd, Poole, UK.
4',6-diamidino-2-phenylindole (DAPI)	Invitrogen, Paisley, UK.
Acid phenol pH 5	Sigma, Surrey, UK.
Alcian Blue stain	Sigma-Aldrich Ltd., Poole, UK.
ATP-Lite-M® assay	Perkin Elmer, Albany street, Boston.
BD FACSFlow	Becton Dickinson, Oxford, UK.
Camptothecin	Sigma, Surrey, UK.
Crystal violet solution	Sigma, Surrey, UK.
Chloroform isoamylalcohol 24:1 (red)	Sigma, Surrey, UK.
Dimethyl sulphoxide (DMSO)	Invitrogen, Paisley, UK.
DMEM	Lonza, Slough, UK.
DNase I	Ambion, Warrington, UK.
Dulbecco's modified Eagle's medium (DMEM)	Gibco Life Technologies Ltd., Paisley, Scotland
Ethanol (100%)	Fisher Scientific, Loughborough, UK.
Fetal calf serum	Lonza, Slough, UK.
Flat bottom culture plates	Fisher Scientific, Loughborough, UK.
FluoroNunc™ plate	Thermofisher Scientific, Loughborough, UK.
Flurescein diacetate	Fisher Scientific, Loughborough, UK
Formaldehyde (37-40%)	Gentamedical, Tockwith, UK.
Guanidine isothionate solution	Sigma, Surrey, UK.

Hanks balanced salt solution (HBSS)	Invitrogen, Paisley, UK.
High activity tritiated thymidine	Perkin Elmer, Berkshire, UK.
Horse Serum	Stem Cell Technologies, Sheffield, UK
Human AB Serum	Source Biosciences Life Sciences
Hydrocortisone	Sigma, Surrey, UK.
Hy-Q-tase®	Fisher Scientific, Loughborough, UK.
Indomethacin	Sigma, Surrey, UK.
IntraStain, Fixation and Permeabilization Kit for Flow Cytometry	Dako, Cambridge, UK.
Isobutylmethylxanthine	Sigma, Surrey, UK.
Isopropanol	Fisher Scientific, Loughborough, UK
Isopropanol	VWR International West Chester, UK.
L-glutamine	Gibco Life Technologies Ltd., Paisley, Scotland.
Low activity tritiated thymidine	Perkin Elmer, Berkshire, UK.
Lymphoprep	Axis-Shield, Middlesex, UK.
Methanol (100%)	Merck, Middlesex, UK.
Methylated spirits	VWR International West Chester, UK.
Microscint-20	Perkin Elmer, Berkshire, UK.
Mitomycin C	Sigma, Surrey, UK.
N-(2-hydroxyethyl) piperazine N;-(2-ethansulphonic acid) [HEPES] (1M)	Lonza, Slough, UK.
NH Expansion Medium	Miltenyi Biotech, Surrey, UK.
Nile red	Sigma, Surrey, UK.
N-Lauryl sarcosine sodium salt	Sigma, Surrey, UK.
Nuclease free water	Severn Biotech Ltd, Worcestershire, UK.

Oil red-O	Sigma, Surrey, UK.
Omniscript RT Kit	Qiagen, West Sussex, UK.
PBS tablets	Oxoid, Hampshire, UK.
Penicillin-streptomycin mixture	Lonza, Slough, UK.
Percoll	Sigma-Aldrich Ltd, Poole, UK.
Phosphate buffered saline (PBS) tablets	Oxoid, Basingstoke, UK.
Phytohaemagglutinin	Sigma, Surrey, UK.
Purelab Option distilled water still	ELGA LabWater, High Wycombe, UK.
Ribolock RNAse Inhibitor	Fermentas, North Yorkshire, UK.
RNasin	Promega, Southampton, UK.
Rosslyn Park Memorial Institute (RPMI) medium without L-glutamine	Lonza, Slough, UK.
Saponin	Sigma, Surrey, UK.
Sheath fluid (FACs instrument)	Becton & Dickenson, Oxford, UK.
Sodium acetate	Fisher Scientific, Loughborough, UK
Sodium chloride	Merck, Middlesex, UK.
Sodium citrate	Sigma, Surrey, UK.
Sodium hydroxide	Sigma-Aldrich Ltd, Poole, UK.
Sodium pyruvate	Gibco Life Technologies Ltd., Paisley, Scotland
Superscript II	Invitrogen, Paisley, UK.
Trichloroacetic acid	Fisher Scientific, Loughborough, UK
Trigene	Scientific Laboratory Supplies Ltd.
TRIzol® RNA lysis solution	Invitrogen, Paisley, UK.
Trypan Blue (0.4%)	Sigma, Surrey, UK.
Trypsin, 0.5 % with EDTA (10x)	Invitrogen, Paisley, UK.

Virkon	Bios Europe Ltd., Lancashire, UK
β-mercaptoethanol	Sigma, Surrey, UK.

Table 2.2: Materials used with manufacturer.

2.2.1 GLASSWARE

All laboratory glassware was obtained from Fisher Scientific (Loughborough, UK) unless otherwise stated. All glassware was cleaned by immersion in a 1 % (v/v) solution of detergent (Trigene®) overnight. Glassware was subsequently rinsed thoroughly in tap water followed by a final wash in distilled water to remove residual detergent. The glassware was then dried or sterilised by dry heat (Section 2.2.1.1).

2.2.2 STERILE AND DISPOSABLE PLASTICWARE

Tissue culture flasks (25 cm^2 and 75 cm^2) and Nunc branded flat bottomed 96-well plates were purchased from Thermo Fisher Scientific Ltd. Culture flat bottomed 24 well, 48 well and 96 well, and U-bottomed 96-well plates were purchased from Thermo Fisher Scientific Ltd. Universal containers (25 ml) and specimen containers (60 ml, 150 ml and 250 ml) were purchased from Scientific Laboratory Supplies Ltd. 'Stripette' disposable pipettes (1 ml, 2 ml, 5 ml, 10 ml and 25 ml) were supplied by Sigma-Aldrich Ltd. Sterile, endotoxin-free filter and non-filter pipette tips (20 µl, 200 µl and 1000 µl) were purchased from Star Labs. Disposable plastic syringes were purchased from Scientific Laboratories Supplies Ltd.

2.2.3 PHOSPHATE BUFFERED SALINE (PBS)

One PBS tablet (Oxoid) was dissolved in 100 ml of distilled water. The solution was stored at room temperature until required.

2.3 METHODS

2.3.1 DRY HEAT STERILISATION

Items to be sterilised were placed into a hot air oven and held at a temperature of 190 °C for 4 hours.

2.3.2 MOIST HEAT STERILISATION

Objects and solutions unsuitable for dry heat sterilisation were sterilised by autoclaving at 121 °C for 20 minutes at a pressure of 15 psi.

2.3.3 FILTER STERILISATION

Solutions that were unsuitable for moist heat sterilisation were sterilised by filtering through a filter with 0.2 µm pore size (Sartorius Ltd, UK) using a disposable syringe (Scientific Laboratory Supplies Ltd.). Larger filter units (Nunc brand, Fisher Scientific Ltd.) were used for larger volumes. All manipulations were carried out in a class II safety cabinet.

2.3.4 MEASUREMENT OF PH

The pH of solutions was measured using a Jenway 3020 pH meter. The pH meter was calibrated using solutions of pH 4, 7 and 10 made from buffer tablets (Merck Chemicals Ltd.) dissolved in de-ionised water. The pH of solutions was measured using temperature compensation. To adjust the pH of solutions, 1-6 M hydrochloric acid (HCl) or 1-6 M sodium hydroxide (NaOH) was added drop-wise whilst stirring.

2.3.5 MICROSCOPY

Bright-field microscopy was carried out using an Olympus BX51 microscope. Fluorescent microscopy was carried out using the same microscope in combination with a fluorescent vertical illuminator (BX51-RFA) using either the FITC (495/520 nm) or DAPI filters (360/460 nm). Proliferating mammalian cells in tissue culture flasks were viewed using an inverted microscope (Olympus IX71) set up for phase contrast microscopy. Images were photographed using an attached Olympus Digital camera controlled through Cell^B software systems with correct Kohler illumination.

2.3.6 RESURRECTION AND MAINTENANCE OF PRIMARY CELLS AND CELL LINES

Cells were removed from liquid nitrogen storage and rapidly defrosted in a 37 °C water bath. Pre-warmed supplemented medium (10 ml) was added drop wise to the cells prior to centrifugation at 150 g for ten minutes. The supernatant was discarded and the pellet resuspended in 5 ml of supplemented medium. This was transferred to a 75 cm^2 tissue culture flask and the cells incubated at 37 °C in 5 % (v/v) CO_2 in air. The medium was changed every two days until the cells became confluent.

2.3.7 ESTIMATION OF CELL VIABILITY – TRYPAN BLUE DYE EXCLUSION METHOD

The Trypan blue exclusion assay works on the principle that the cell membrane of viable cells is intact and therefore is not permeable to the dye. A cell suspension (90 µl) was mixed thoroughly with 10 µl Trypan blue solution. This solution was then loaded into an Improved Neubauer counting chamber. Cell counts were perfomed by observing both chambers under the inverted microscope. Non-viable cells allow the dye to permeate due to loss of membrane potential on cell death. This results in non-viable cells having a blue colouration and being excluded from the count. To allow an accurate counting method, the cell suspension was diluted so that between 30 and 300 cells were in the counting chamber. The total number of viable cells per ml was calculated as follows:

$$\text{Number of cells.ml}^{-1} = \text{Mean cell count} \times 10^4 \times \text{Original dilution } {}^{10}/_{9}$$

2.3.8 CRYOPRESERVATION OF PRIMARY CELLS AND CELL LINES

Cells were isolated from the tissue culture flasks and a cell count was performed as described above. The cell suspension was then centrifuged for 10 minutes at 150 *g*. The supernatant was carefully aspirated and the cells were resuspended in medium containing 50 % (v/v) fetal calf serum and 10 % (v/v) dimethyl sulphoxide (DMSO) [filter sterilised prior to use]. Cell suspension was then aliquoted (1 ml) into cryovials so that each vial contained 1 million cells. Vials were then transferred into a cryo freezing pot containing isopropanol. This was placed into a -80 °C freezer for 12 hours. Cryovials were then transferred into liquid nitrogen for long term storage.

2.3.9 STATISTICAL ANALYSIS

All numerical data was analysed using Microsoft Excel® and GraphPad Prism®. Data is typically shown as mean values ± 95 % confidence limits, which were calculated using GraphPad Prism software. One-way analysis of variance was used to analyse data with one variable to be examined. Two-way analysis of variance was used to analyse experimental data with two variables. The non-parametric Mann Whitney-U test was used to assess whether two independent variables had equal values and was performed using GraphPad Prism® software. The Spearman rank coefficient was used to determine the statistical significance between two variables, values were calculated using GraphPad Prism® software. Statistical formula were confirmed using Sokal and Rohlf, 1981.

Chapter 3: ISOLATION, CULTURE AND CHARACTERISATION OF MSCs

3.1 INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) have been referred to by various terms since their initial characterization by Freidstein *et al.* over 40 years ago. They have been called mesenchymal stem cells, multipotent stem cells and marrow stromal cells. Due to the diverse nomenclature associated with these cells, the International Society for Cellular Therapy has attempted to clarify the terminology associated with MSCs, and termed the cells multipotent mesenchymal stromal cells. The ISCT also decreed that the source should be included in the terminology, for example, bone marrow or dental pulp derived (Horwitz et al., 2005). As well as nomenclature, the ISCT also defined a set of standard minimum criteria for defining cells as human multipotent mesenchymal stromal cells:

- Plastic adherent under standard culture conditions.
- Be positive for the expression of CD73, CD90, and CD105, and negative for the expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR.
- Be able to differentiate into adipocytes, osteocytes and chondrocytes under specific stimulus in vitro.

As there is presently no single defining marker for multipotent mesenchymal stromal cells; in order for cells *in-vitro* to be classified as multipotent mesenchymal stromal cells, they have to fulfil all of the above criteria. For simplicity, the cells will be termed MSCs throughout this thesis.

Multipotent mesenchymal stromal cells have been shown to be present in many of the tissues in the body, but even with increasing interest in obtaining them from adipose tissue (Uysal and Mizuno; Zuk et al., 2001; Zuk et al., 2002) the most well documented method of isolation of MSCs is from the bone marrow. The bone marrow provides an important niche for many cell types in the body including haematopoietic stem cells, mesenchymal stromal cells, and adipocytes (Salem and Thiemermann, 2010). In fact the population of MSCs within the nucleated cell portion of the bone marrow is between 0.001 % and 0.01 % (Friedenstein et al.,

1974; Jones et al., 2002; Uccelli et al., 2006). Although there are very few MSCs present, they can be isolated, and expanded with relatively high efficiency (Pittenger et al., 1999). The *in-vivo* markers expressed by MSCs within their native niche have proved difficult to determine, due to the low frequency of these cells within bone marrow aspirates. The detection of these cells within the bone marrow previously relied on lengthy *in-vitro* techniques such as the colony-forming unit fibroblast assay (CFU-F) (Castro-Malaspina et al., 1980). This is a retrospective assay that can be variable due to its reliance on factors such as cell seeding density and growth medium. More recently with the prevalence of more sophisticated equipment, techniques such as flow cytometry provide valuable tools that allow a more rapid method to examine the phenotype of MSCs (Jones et al., 2006). A study carried out by Jones *et al*. (2006) showed that a proportion of cells in the bone marrow strongly expressing LNGFR (CD271, **Table 1.1**) and D7-FIB provided exclusively all of the CFU-Fs, providing strong evidence to allow the rapid purification and isolation of MSCs directly from samples of bone marrow (Jones et al., 2006). D7-FIB is expressed on human fibroblasts and epithelial cells. In peripheral blood this cell surface markers is expressed on myeloid cells and a very small number of lymphocytes.

For *in-vitro* culture the standard method for the isolation of MSCs relies on Percoll gradient centrifugation, followed by plastic adhesion of cells isolated from the density interface (Pittenger et al., 1999; Jones et al., 2002). Putative MSCs must then meet the ISCT standard criteria before classification as MSCs.

In order to differentiate MSCs into osteocytes, cells are cultured in medium with the addition of dexamethasone, ascorbic acid, and β-glycerophosphate (Jaiswal et al., 1997). After culture with these supplements in order to determine osteogenic differentiation cells can be stained with alizarin red to detect calcium deposition and matrix mineralisation (Jones et al., 2002). Alkaline phosphatase staining is another popular method for the determination of osteogenesis of MSC cultures (Pittenger et al., 1999).

Adipogenic differentiation can be induced through the addition of indomethacin, 1-methyl-3-isobutylmethylxanthine, and hydrocortisone to the culture medium (Sekiya et al., 2004). The adipogenesis of MSCs in cultures was further enhanced with the addition of horse serum to the differentiation medium. Lipid accumulation in vesicles within cells can be visualised by light microscopy after 1 week, or by staining intracellular lipid content with Oil red-O (Pittenger et al., 1999). Other methods of adipogenic differentiation use culture medium supplemented with insulin or the peroxisome proliferator-activated receptor-ɣ agonist rosiglitazone.

Chondrogenic differentiation can be induced by culturing cells at very high densities (pellet cultures or micromasses) and supplementing culture medium with TGF-β3, ascorbic-2-phosphate, insulin-transferrin-selenium (ITS), sodium pyruvate, and dexamethasone (De Bari et al., 2001). The differentiation into chondrocytes can be visualised through glycosaminoglycan deposition using alcian blue stain (Jones et al., 2002).

3.1.1 AIMS AND OBJECTIVES

The initial aim of this research was to obtain expanded populations of MSCs isolated from fresh human bone marrow aspirates, and to characterise these cells by their morphology and phenotype to establish if the isolated cell population were indeed MSCs. There were 4 objectives:

- To isolate and expand bone marrow derived MSCs in culture
- To observe the morphology of the cells when grown on coated tissue culture plastic flasks.
- To phenotype the cells using flow cytometric analysis against the cell surface markers CD73, CD90, CD105, CD34, CD45, CD14, CD19, and HLA-DR.
- To assess the trilineage potential of the isolated MSCs and stain differentiated populations with alizarin red for calcium content, Oil red-O for intracellular lipid content, and alcian blue for GAG content.

3.2 MATERIALS AND METHODS

3.2.1 EXTRACTION OF HUMAN BONE MARROW AND ISOLATION OF MSCS

Reagents

Penicillin-streptomycin mixture [5000 $\mu g.ml^{-1}$ potassium penicillin, 5000 $\mu g.ml^{-1}$ streptomycin sulphate] (Lonza).

L-glutamine 200 mM (Gibco)

Fetal calf serum (Lonza)

DMEM [4.5 $g.L^{-1}$ glucose without L-glutamine with sodium pyruvate (Lonza)]

Complete DMEM: Complete DMEM medium was made by supplementing DMEM to a final concentration of 10% (v/v) fetal calf serum, 100 $\mu g.ml^{-1}$ penicillin/streptomycin and 2 mM L-glutamine.

NH Expansion Medium (Miltenyi Biotech)

Method

Human bone marrow was extracted from the iliac crest. Samples were collected under full ethical approval (REC 07/Q1205/27) to Dr E. Jones (LIMM; University of Leeds). These samples were taken from theatre at St James Hosiptal, Leeds Teaching Hospital Trust. BM1 and BM2 were obtained via this route, and MSCs isolated according to Jones *et al*. (2002). Briefly, mononuclear cells were isolated from bone marrow using a Lymphoprep™ (Axis-Shield) gradient before purified mononuclear cells were grown in NH medium. Plastic-adherent cells were cultured to passage 0 at 37°C 5 % (v/v) CO_2 in air, before frozen cryovials of isolated MSCs were transferred from LIMM (Leeds Institute of Molecular Medicine) to the IMBE laboratory on dry ice, where they were stored in liquid nitrogen. Cryovials of cells were frozen and resuscitated as described in section 2.3.6-2.3.8.

Bone marrow samples were also donated by patients in the trauma theatre under full ethical approval (REC 06/Q1206/127) to Peter Giannoudis's (St James Hospital, Leeds Teaching Hospital Trust), BM3 and BM4 were obtained through these ethics, and MSCs were isolated using the method described below.

Human bone marrow that was obtained from volunteer patients following admission to clinic with bone fractures of the lower limbs was aspirated from the iliac crest. Marrow was then

mixed in at 1:1 ratio with complete DMEM medium, and cultured for 48 hours at 37°C 5% (v/v) CO_2 in air in T75 tissue culture flasks (Nunc). After this 48 hour period the medium was removed, and the cell monolayer gently washed with PBS before 10 ml NH Expansion Medium was added to the flask and cells were again cultured at 37°C 5% (v/v) CO_2 in air.

3.2.2 EXPANSION OF MSCS

Human MSCs were grown until 90% confluent in T75 tissue culture flasks in 10 ml NH Expansion Medium at 37°C in an atmosphere of 5% (v/v) CO_2. When 90% confluency was reached the medium was removed from the flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. PBS was removed and 2 ml Hy-Q-Tase™ (Fisher Scientific) was added to the flask and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (approx. 10 minutes). When cells had detached the resulting cell suspension was added to 5 ml complete DMEM medium in a 25 ml centrifuge tube, before centrifugation at 150 g for 10 minutes. When centrifugation was complete the supernatant was discarded, and the pellet resuspended in NH Expansion medium (Miltenyi Biotech). The resulting cell suspension was split into double the number of T75 tissue culture flasks before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

3.2.3 EXPANSION OF FIBROBLASTS

Primary human dermal fibroblasts (Cascade Biologics) were grown until 90% confluent in T75 tissue culture flasks in 10 ml complete DMEM at 37°C in an atmosphere of 5% (v/v) CO_2. When 90% confluency was reached the medium was removed from the flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. Following washing the PBS was removed and 2 ml Hy-Q-Tase™ was added to the flask, and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (for approx. 10 minutes). When cells had detached the resulting cell suspension was added to 5 ml complete DMEM in a 25 ml centrifuge tube, before centrifugation at 150 g for 10 minutes. When centrifugation was complete the supernatant was removed, and the pellet resuspended in complete DMEM medium. The resulting cell suspension was split into double the number of T75 tissue culture flasks before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

3.2.4 FLOW CYTOMETRY

Reagents

2x blocking buffer: PBS/2.5mM EDTA/4% (v/v) fetal calf serum

Method

Cells were removed from the surface of the tissue culture plastic using 2 ml Hy-Q-Tase™ per T75 flask. The cell suspension was added to 5 ml complete DMEM and centrifuged at 150 g for 10 minutes. The resulting cell pellet was then resuspended in ice cold PBS, and cells counted using a haemocytometer to give a final cell concentration of $1x10^5$ cells per 100 µl as described in section 2.3.7. Cell suspension (100 µl) was added to clean 1.5 ml eppendorf tubes, and 100 µl ice cold 2x blocking buffer was added before the suspension was incubated on ice for 20 minutes. Primary conjugated antibody (1 µl undiluted as received from the manufacturer) was added to the cell suspension and the eppendorf gently mixed, before the mixture was incubated on ice for 1 hour. Ice cold PBS (300 µl) was added to the suspension and the cells centrifuged at 3000 g at 4°C for 5 minutes to wash.

The supernatant was removed, and the cell pellet resuspended in 500 µl ice cold PBS. The resulting suspension was centrifuged at 3000 g twice more. After 3 washes, the cell pellet was resuspended in 200 µl ice cold PBS, before the fluorescent intensity was measured using the flow cytometer (Becton Dickinson FACSCalibur).

Resulting data was analysed using CellQuest software version 3.1 (BD Biosciences) and 10,000 events were collected per sample. The collection settings used are shown in **Table 3.1**:

The antibodies used are shown in **Table 3.2**.

Param	Detector	Voltage	AmpGain
P1	FSC	E-1	3.37
P2	SSC	335	1.00
P3	FL1	319	1.00
P4	FL2	366	1.00
P5	FL3	381	1.00

Table 3.1: Table of flow cytometry collection settings used for the analysis of MSCs.

Marker	Conjugate	Catalogue Number	Manufacturer	Isotype	Channel
CD105	RPE	MCA1557PET	AbD Serotec	IgG1	FL-2
CD73	RPE	550257	BD Biosciences	IgG1	FL-2
CD90	RPE	MCA90PET	AbD Serotec	IgG1	FL-2
CD45	APC Cy-7	557833	BD Biosciences	IgG1	FL-3
CD34	RPE	MCA1578PET	AbD Serotec	IgG1	FL-2
CD14	RPE	555398	BD Biosciences	IgG2a	FL-2
CD19	RPE	MCA1940PET	AbD Serotec	IgG1	FL-2
HLA-DR	PE Cy-7	335830	BD Biosciences	IgG2a	FL-3
HLA-ABC	FITC	MCA81F	AbD Serotec	IgG2a	FL-1
CD40	FITC	MCA1590F	AbD Serotec	IgG1	FL-1
CD80	FITC	MCA2071F	AbD Serotec	IgG1	FL-1
CD86	FITC	MCA1118F	AbD Serotec	IgG1	FL-1
IgG1	RPE	MG4993	Caltag		FL-2
IgG2	RPE	MG2A04	Caltag		FL-2

Table 3.2: Conjugated antibodies used in flow cytometry to determine MSC phenotype. RPE: R-phycoerythrin absorption/emission max 496/578 nm, APC CY-7: allophycocyanin coupled to cyanin dye CY-7 absorption/emission max 650/785, PE CY-7: phycoerythrin coupled to cyanin dye CY-7 absorption/emission max 596/785, FITC: fluorescein isothiocyanate absorption/emission max 494/520 nm.

3.2.5 ADIPOGENIC DIFFERENTIATION OF HMSCS

Reagents

Isobutylmethylxantine (Sigma): 200 mg isobutylmethylxanthine was added to 1.8 ml dimethyl sulphoxide (DMSO) to make a 0.5 M stock. This solution was filtered through a 0.2 µm pore size filter. This stock solution was aliquoted in 100 µl volumes and frozen at -20°C; 100 µl was added to 100 ml adipogenic medium (i.e. 1:1000) to give a final concentration of 0.5 mM.

Indomethacin (Sigma): A 0.6M stock solution was made by dissolving 100 mg in 467 µl of DMSO. This was filtered using a 0.2 µm pore size filter and frozen in 10 µl aliquots at -20°C; 10 µl was added to 100 ml adipogenic medium (i.e. 1:1000) to give a final concentration of 0.6 mM.

Hydrocortisone (Sigma): 4.854 mg was added to 100 ml PBS to make a 0.1mM stock solution. The solution was filter sterilised using a 0.2 µm pore size filter and frozen at -20°C in 550 µl aliquots; 500 µl was added to 100 ml adipogenic medium immediately before use to give a final concentration of 0.5 µM.

Complete Dulbecco's modified Eagles medium (complete DMEM): Made as described in section 3.2.1.

Method

Adipogenic medium was made by supplementing 100 ml complete DMEM with 10% (v/v) horse serum (Sigma), 100 µl isobutylmethylxanthine, 10 µl indomethacin and 500 µl hydrocortisone. MSCs or fibroblasts were cultured until 90 % confluent in T75 tissue culture flasks. Cells were removed from the surface of the tissue culture plastic using 2 ml Hy-Q-Tase™ per T75 flask. Cell suspensions were added to 5 ml complete DMEM and centrifuged at 150 g for 10 minutes. The supernatant was discarded, and the pellets resuspended in 1 ml complete DMEM. Cells were counted using a haemocytometer and cells to be differentiated were seeded at $4x10^4$ cells per cm^2 growth area in a 24 well plate, and left to attach overnight at 37°C in an atmosphere of 5% (v/v) CO_2 in air. When attached, the culture medium was removed and replaced with adipogenic medium. The medium was replaced twice weekly, and cells were grown up to 21 days. Cultures were fixed and stained as described below on days 0, 3, 7, 14, and 21.

3.2.6 Oil red-O Staining of adipocytes

Reagents

Oil red (Sigma): A 0.5% (w/v) Oil red solution was made by dissolving 50 mg in 10 ml isopropanol.

Formalin (10%): 27 ml formaldehyde/formalin stock (@37%) in 73 ml dH_2O.

Method

Medium was removed from wells and cells were washed twice with PBS. Cells were then fixed in 10% (v/v) formalin in dH_2O for 10 minutes. Stock 0.5 % (v/v) Oil red solution was diluted to a working concentration of 3 parts Oil red to 2 parts water before filtering through a 0.8 µm pore filter and a 0.2 µm pore filter to remove particles. Formalin was removed from wells and the cells washed twice with PBS before addition of filtered Oil red for 10 minutes. Following incubation at room temperature Oil red was removed and wells were washed twice in PBS. The cells were allowed to dry before viewing using an Olympus IX71 inverted microscope using Cell^B image capture software (Olympus).

3.2.7 OSTEOGENIC DIFFERENTIATION OF MSCS

Reagents

Dexamethasone (Sigma): A 50 mM stock solution was made by dissolving 25 mg dexamethasone in 1280 µl 100% ethanol. This solution was dispensed into 10 µl volumes and frozen at -20°C. DMEM was added to the 10 µl volumes (1 ml) to give a 500 µM solution, which was refrozen in 20 µl aliquots, and added to osteogenic medium just before use at a 1:5000 dilution (20 µl in 100 ml medium) to give a final concentration of 10 µM.

Ascorbic-2-phosphate (Sigma): A 200 mM stock solution was made by dissolving 2.57 g ascorbic-2-phosphate in 50 ml dH_2O. This stock solution was aliquoted into 50 µl volumes and stored at -20°C. This was added to osteogenic medium at a 1:2000 dilution (50 µl in 100 ml medium) to give a final concentration of 0.1 mM.

Beta-2-glycerophosphate (Sigma): A 2 M stock solution was made by dissolving 10 g beta-2-glycerophosphate in 23 ml dH_2O. This was frozen in 500 µl aliquots and stored at -20°C. This was added to osteogenic medium at a 1:200 dilution (500 µl in 100 ml medium) to give a final concentration of 10 mM.

Method

Osteogenic medium was made by supplementing 100 ml complete DMEM with 20 µl dexamethasone, 50 µl ascorbic-2-phosphate and 500 µl beta-2-glycerophosphate. MSCs and fibroblasts were cultured until confluent in T75 tissue culture flasks. Cells were removed from the surface of the tissue culture plastic using 2 ml Hy-Q-Tase™ per T75 flask. Cell suspensions were added to 5 ml complete DMEM and centrifuged at 150 g for 10 minutes. The supernatant was discarded, and the pellets resuspended in 1 ml complete DMEM. Cells were counted using a haemocytometer as described in section 2.3.7 and cells to be differentiated were seeded at approx. $3x10^3$ cells per cm^2 growth area in a 24 well plate. The cells were left to attach overnight at 37°C in an atmosphere of 5% (v/v) CO_2 in air. When attached, the culture medium was removed and replaced with osteogenic medium. The medium was replaced twice weekly, and cells were grown up to 21 days. Cultures were fixed and stained as below on days 0, 3, 7, 14, and 21.

3.2.8 ALIZARIN RED STAINING

Reagents

Ammonium hydroxide (10%): A 10% (v/v) ammonium hydroxide solution was made by diluting 30 ml 28% ammonium hydroxide (Sigma) in 60 ml dH_2O.

Alizarin red solution: A 40 mM alizarin red solution was made by dissolving 2 g alizarin red (Sigma) in 100 ml dH_2O .The solution was made to pH 4.1 with 10% (w/v) ammonium hydroxide in dH_2O.

Method

Cells were fixed with 1 ml 10% (v/v) formalin in dH_2O at room temp for 15 min, before the formalin was removed, and the cells washed twice with 1 ml dH_2O. All dH_2O was removed from wells and cells were stained with 500 µl alizarin red solution for 20 minutes with gentle agitation. After 20 minutes the solution was removed, and the wells washed four times with 1 ml dH_2O. The cells were allowed to dry before viewing using an Olympus IX71 inverted microscope using Cell^B image capture software (Olympus).

3.2.9 CHONDROGENIC DIFFERENTIATION OF MSCS

Reagents

Ascorbic-2-phosphate (Sigma): A 200 mM stock solution was made by dissolving 2.57 g ascorbic-2-phosphate in 50 ml dH_2O. This stock solution was aliquoted into 75 µl volumes and stored at -20°C. This was added to chondrogenic medium at a 1:1500 dilution (75 µl in 100 ml medium) to give a final concentration of 1.3 mM.

Insulin-Transferrin-Selenium x100 (Invitrogen) (100 µl in 100 ml medium) to give a final concentration of 1 x.

Sodium pyruvate (Sigma): A 100 mM stock solution was made by dissolving 1.1 g sodium pyruvate in 100 ml dH_2O. This was frozen in 0.5 ml aliquots and stored at -20°C. This was added to chondrogenic medium at a 1:200 dilution 0.5 ml in 100 ml medium) to give a final concentration of 0.5 mM.

Method

MSCs were cultured until 90 % confluent in a T75 tissue culture flask. Cells were removed from the surface of the tissue culture plastic using 2 ml Hy-Q-Tase™ per T75 flask. Cell suspension was added to 5 ml complete DMEM and centrifuged at 150 g for 10 minutes. The supernatant was discarded, and the pellet resuspended in 1 ml complete DMEM. Cells were counted using a haemocytometer, and resuspended at $1x10^7$ cells per ml. Using a 20 µl pipette, a 10 µl volume of suspension was carefully placed in the center of a well of a 24 well plate. The cells were left to attach for 3 hours at 37°C 5% (v/v) CO_2 in air, and after this time had elapsed the wells were flooded with chondrogenic culture medium and the cells were cultured at 37°C 5%

(v/v) CO_2 in air. The medium was replaced twice weekly, and cells were grown up to 21 days. Cultures were fixed and stained as below on days 0, 3, 7, 14, and 21.

3.2.10 Alcian Blue Staining

Reagents

Alcian blue 1 % (w/v) in acetic acid (Biostain Ready Reagents)

Method

Cells were fixed with 1 ml 10% (v/v) formalin at room temp for 15 min, before the formalin was removed, and the cells washed twice with 1 ml dH_2O. All dH_2O was removed from wells and cells were stained with 500 µl 1% (w/v) alcian blue solution for 3 minutes. This was removed, and the wells briefly washed with 1 ml dH_2O to remove excess stain. The cells were allowed to dry before viewing using an Olympus IX71 inverted microscope using Cell^B image capture software (Olympus).

3.3 RESULTS

BM 1 and 2 were isolated using the Lymphoprep method and expanded to passage 0 by Dr. E. Jones at the LIMM. BM 3 and 4 were isolated by the author at the IMBE laboratories at Leeds University. BM 1 was obtained from a donor 69 years old. BM 2 was obtained from a donor 9 years old. BM 3 was obtained from a donor 8 years old, and BM 4 was obtained from a donor 39 years old.

3.3.1 ISOLATION OF CELLS FROM BONE MARROW

A 10 ml bone marrow sample was taken from consenting donors BM 3 and 4 admitted to hospital following bone fracture. The bone marrow was diluted 1:1 in complete DMEM and plated out in a T75 flask. After 48 hours the cell monolayer was washed with 10 ml PBS, before remaining cells were cultured in NH Expansion Medium. Very few numbers of adherent cells were present initially and on average it took one-two weeks for the adherent cells to form colonies.

3.3.2 MORPHOLOGY OF ADHERENT CELLS

The plastic adherent cells exhibited a fibroblastic morphology, and after plating out bone marrow initially formed a sparse single cell population on the tissue culture flask. These single cells produced discreet colonies, and grew in a radial pattern. Colonies could be differentiated visually under light microscopy by the direction of the radial growth, as shown in **Figure 3.1**. This growth pattern only happened at early passages however, with the radial growth pattern becoming less pronounced as the passage number increased.

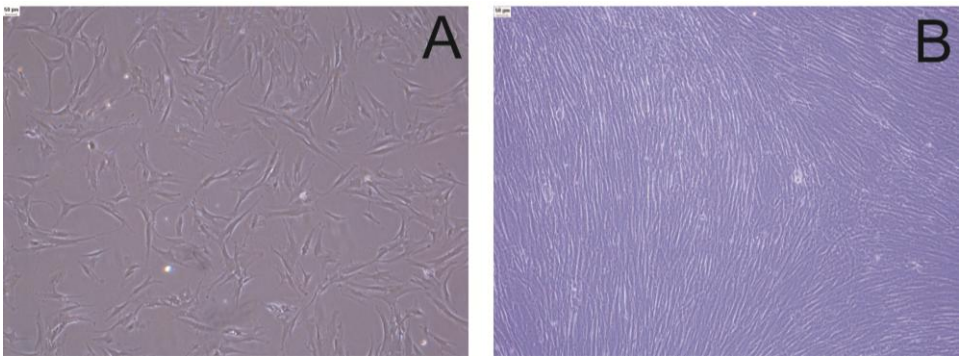


Figure 3.1: Growth of low confluency early passage MSCs, and later passage at 100% confluency. (A) BM3 MSCs freshly isolated from bone marrow following 1 week of culture. **(B)** The radial growth pattern of BM3 MSCs at passage 2, after culture for 12 days from isolation. Cells were grown in NH Expansion Medium. Images are x 40 magnification. Scale bar in the upper left hand corner is 50µm.

3.3.3 PHENOTYPIC CHARACTERISATION OF MSCS USING FLOW CYTOMETRY

BM 1-4 MSCs were expanded up to passage 6 and the phenotype of the cells was investigated using flow cytometry. The events analysed were gated as shown in **Figure 3.2**, in order to discount cell debris and clumps.

The flow cytometric analysis of BM 1-4 MSCs is shown in **Figures 3.3-3.14**. The analysis is displayed as one parameter histograms with the channel the fluorescence is recorded at on the x-axis, and the total count recovered on the y-axis. The gate shown in **Figure 3.2** reduced the likelihood of cell clumps or debris being analysed. The histograms were gated with relative fluorescent intensities of between 10^0-10^1 excluded as being a negative reading, and values above 10^1 considered positive.

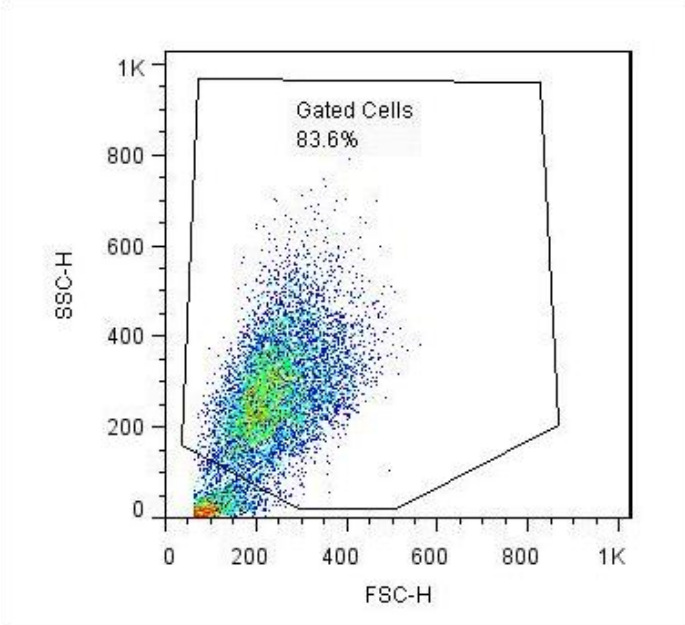


Figure 3.2: Flow cytometric analysis dotplot. The side scatter intensity of cells (representing cell granularity) was plotted against the forward scatter intensity (representing cell size). Each of the dots represents a cell, or 'event' captured. A gate was used to exclude cell debris and clumps.

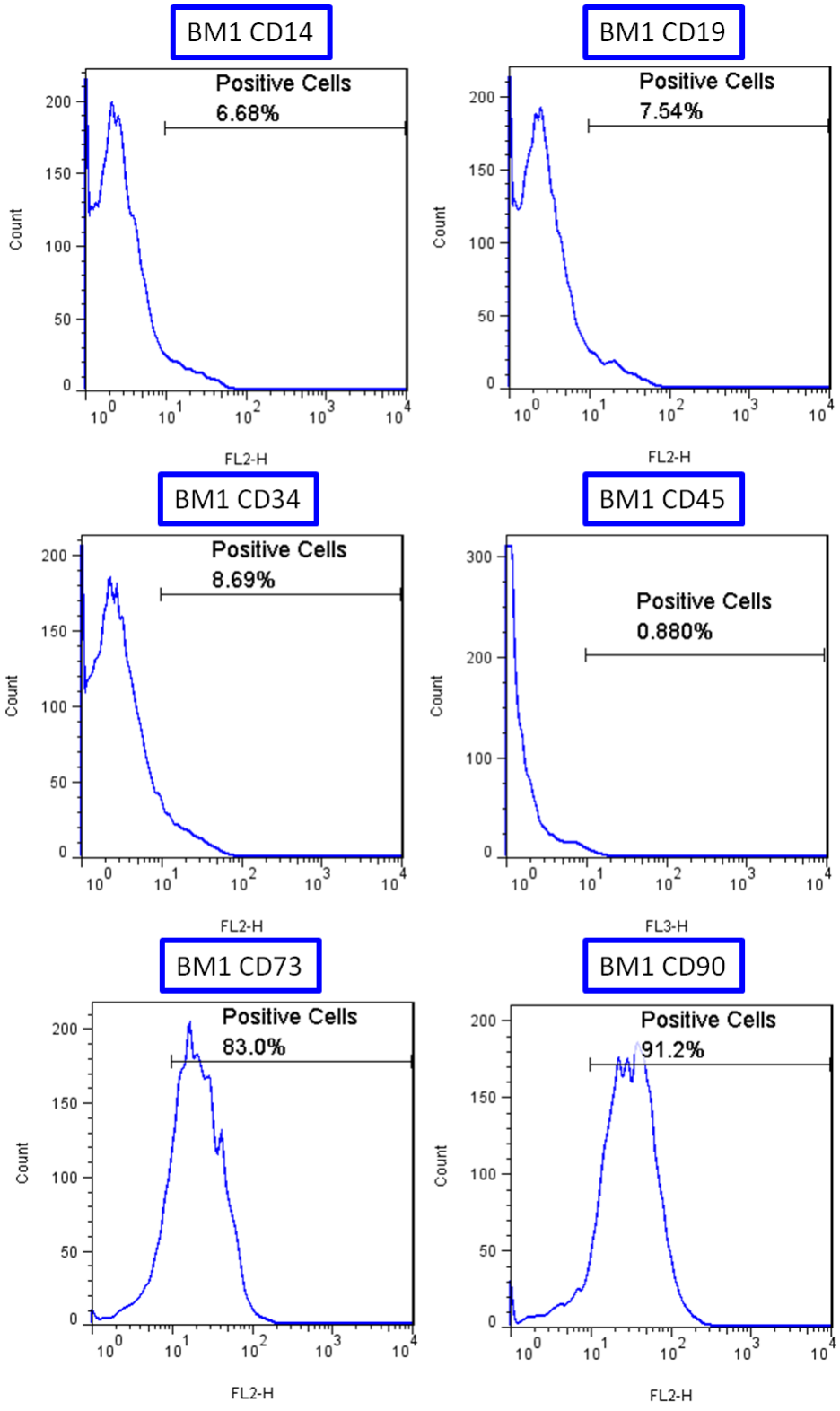


Figure 3.3: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 1 section 1. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD14, CD19, CD34, and CD45, but positively for CD73 and CD90 fulfilling the phenotype criteria defined by the ISCT.

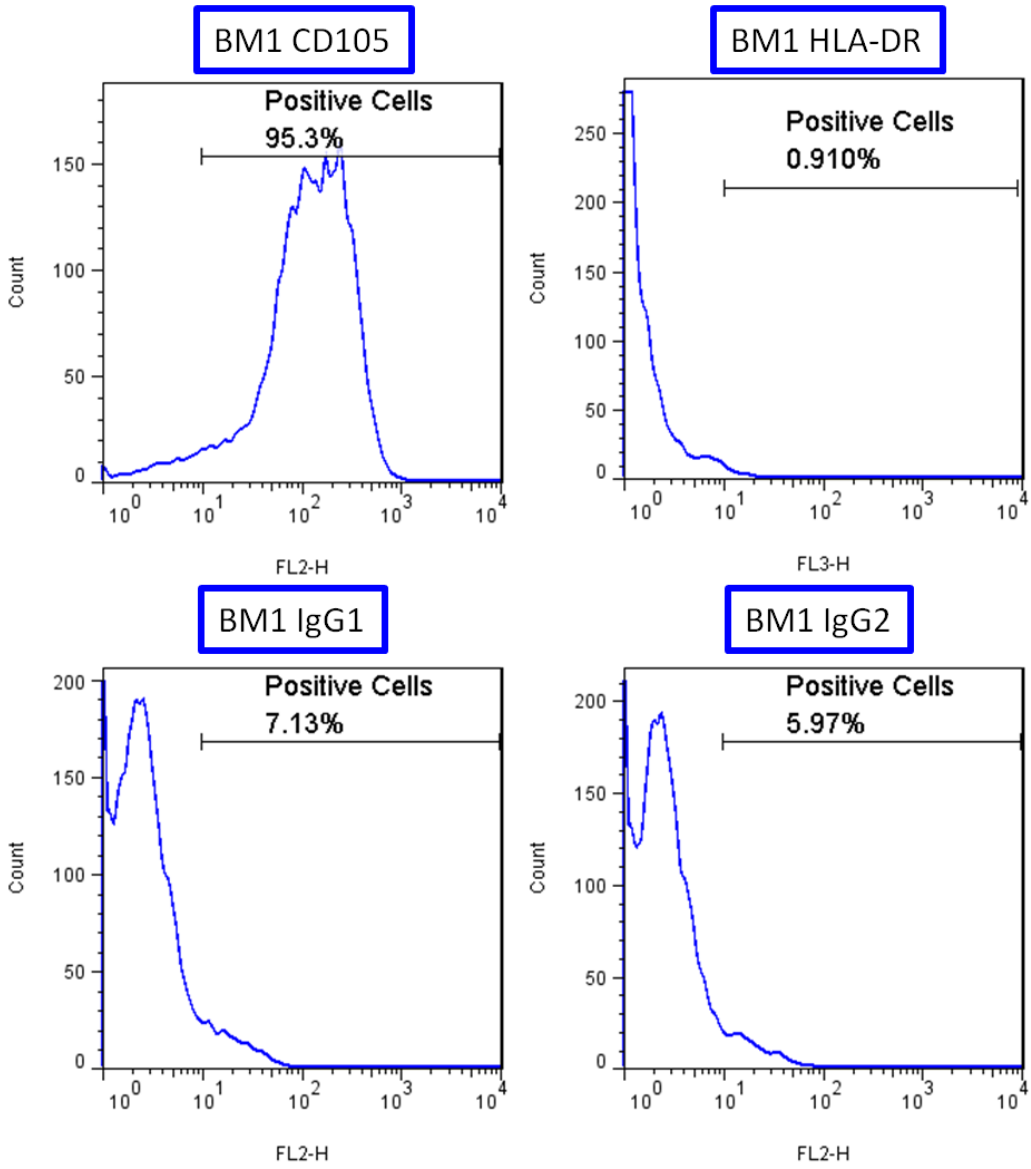


Figure 3.4: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 1 section 2. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for HLA-DR, and the two isotype controls IgG1 and IgG2, but positively for CD105, fulfilling the phenotype criteria defined by the ISCT.

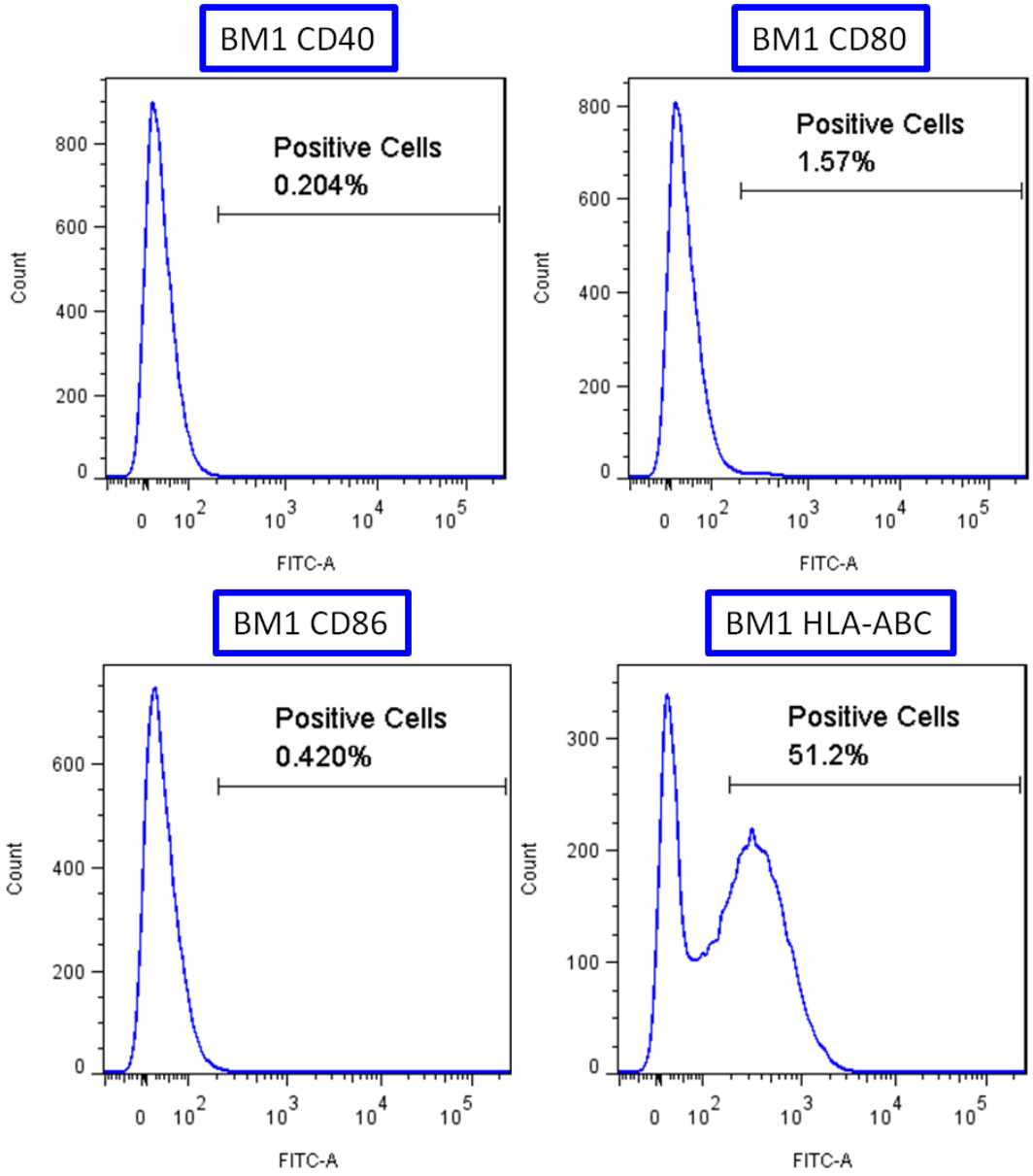


Figure 3.5: Flow cytometry histograms for the immuno-associated cell surface marker expression of the adherent cell population of BM 1 section 3. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^2 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD40, CD80, and CD86, but positively for HLA-ABC, as has been demonstrated in previous studies.

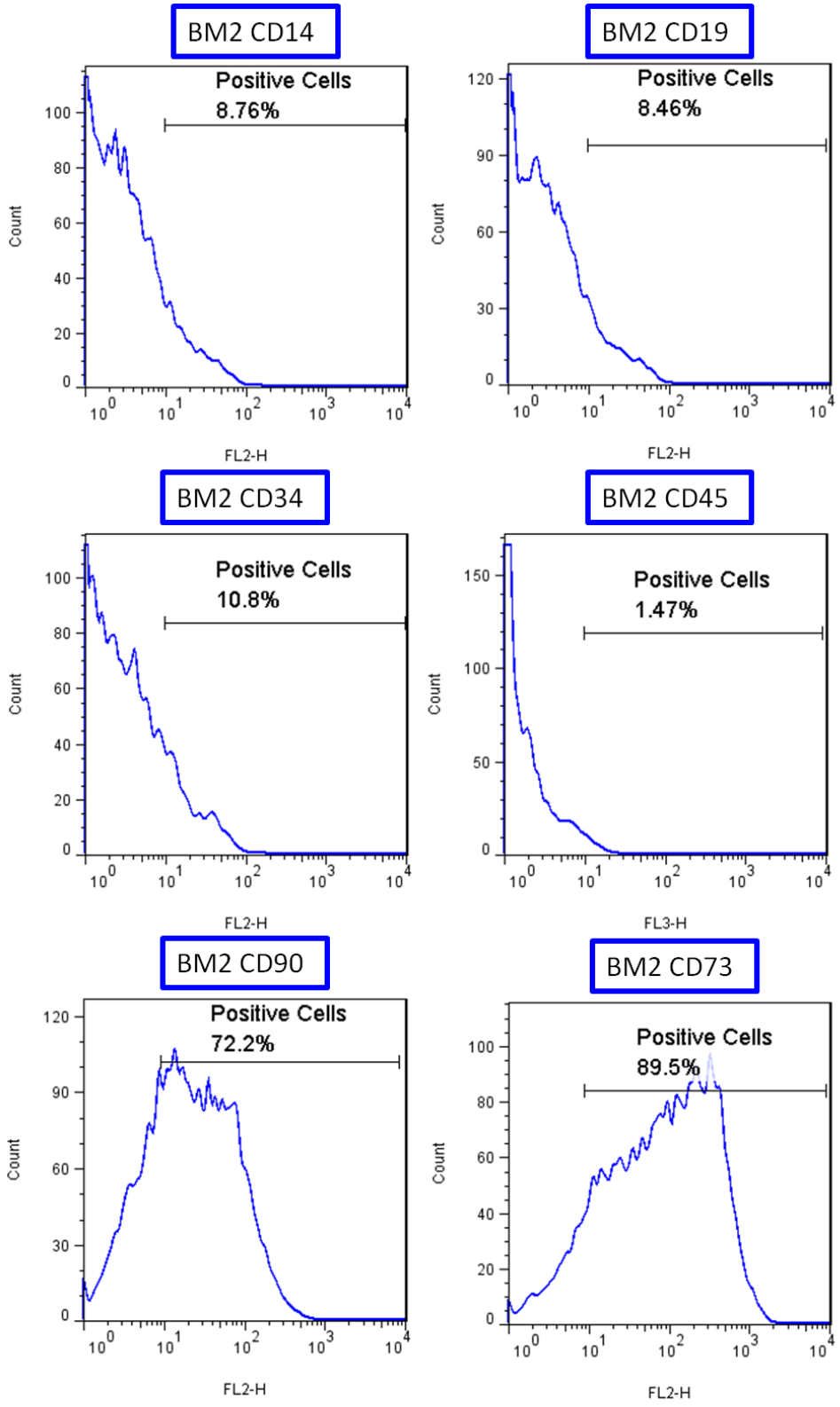


Figure 3.6: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 2 section 1. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD14, CD19, CD34, and CD45, but positively for CD73 and CD90 fulfilling the phenotype criteria defined by the ISCT.

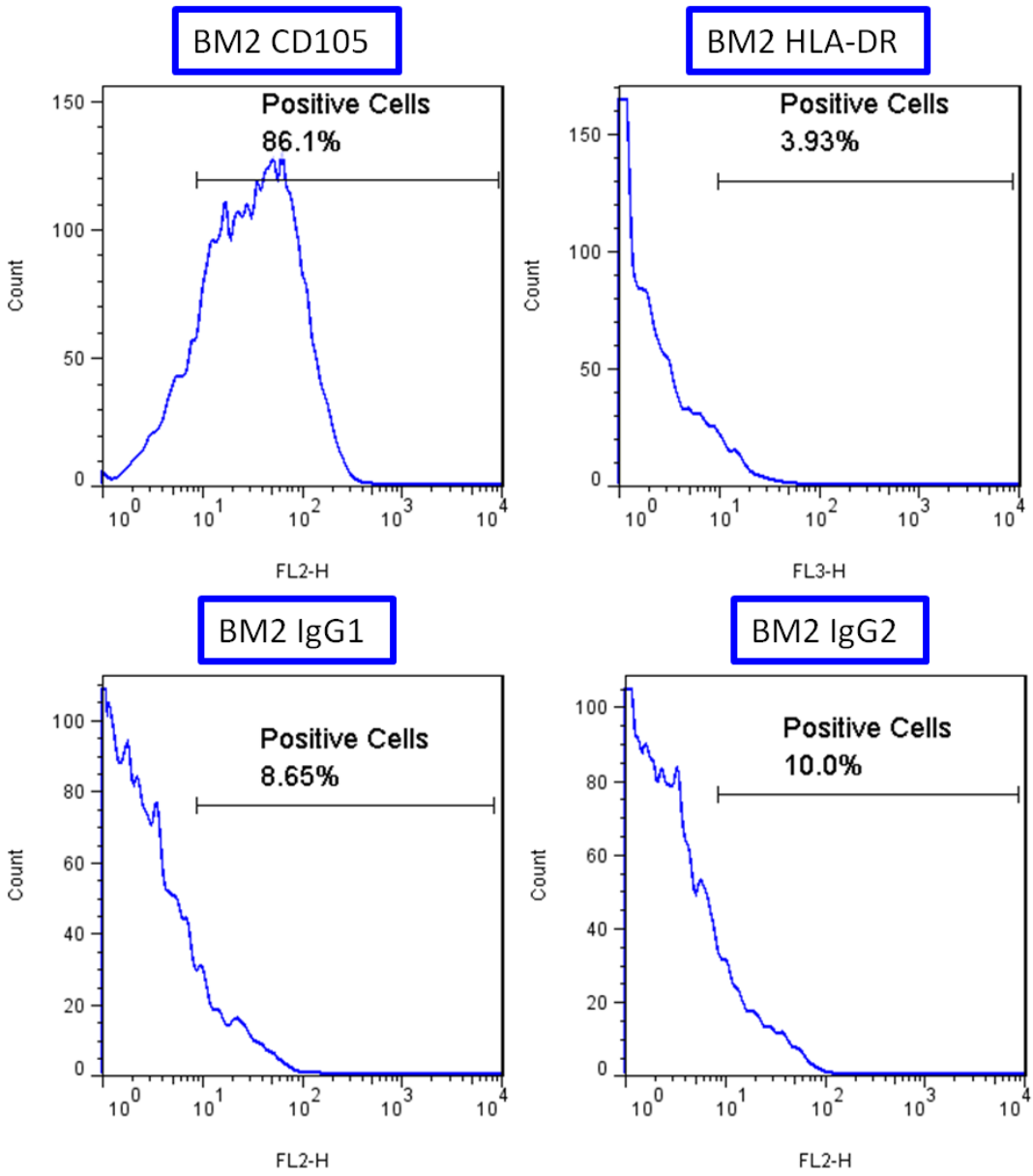


Figure 3.7: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 2 section 2. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for HLA-DR, and the two isotype controls IgG1 and IgG2, but positively for CD105, fulfilling the phenotype criteria defined by the ISCT.

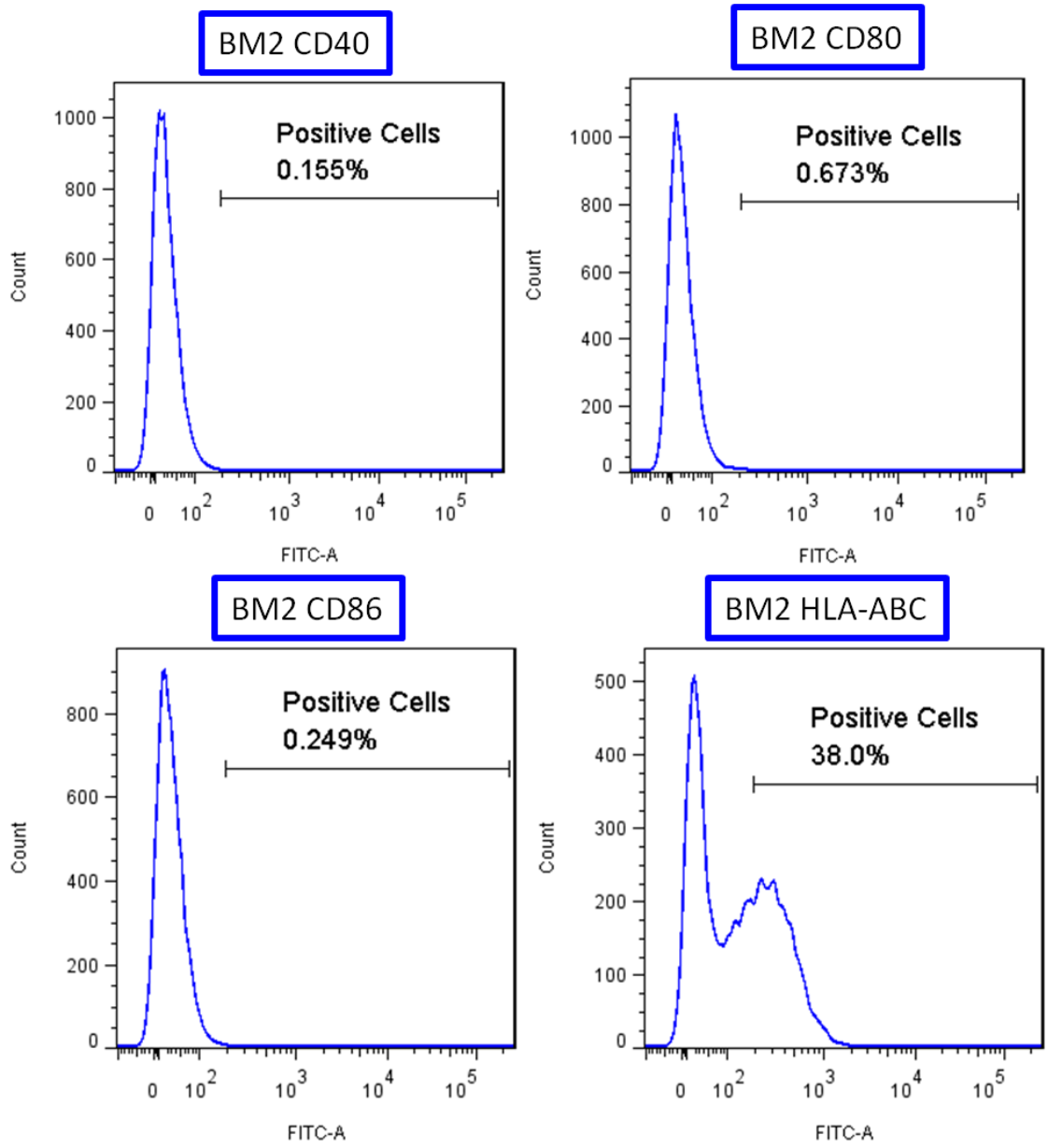


Figure 3.8: Flow cytometry histograms for the immuno-associated cell surface marker expression of the adherent cell population of BM 2 section 3. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^2 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD40, CD80, and CD86, but positively for HLA-ABC, as has been demonstrated in previous studies.

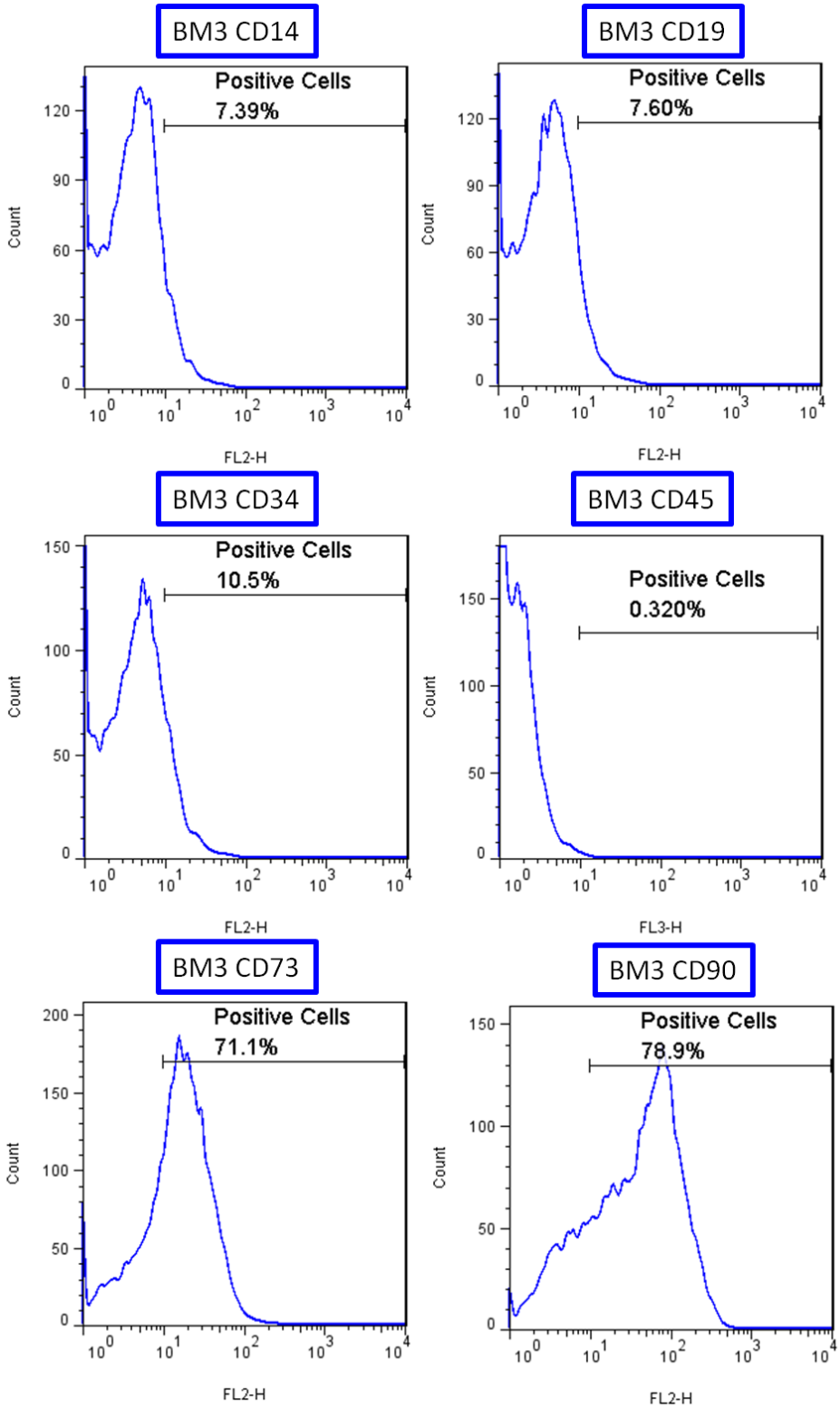


Figure 3.9: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 3 section 1. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD14, CD19, CD34, and CD45, but positively for CD73 and CD90 fulfilling the phenotype criteria defined by the ISCT.

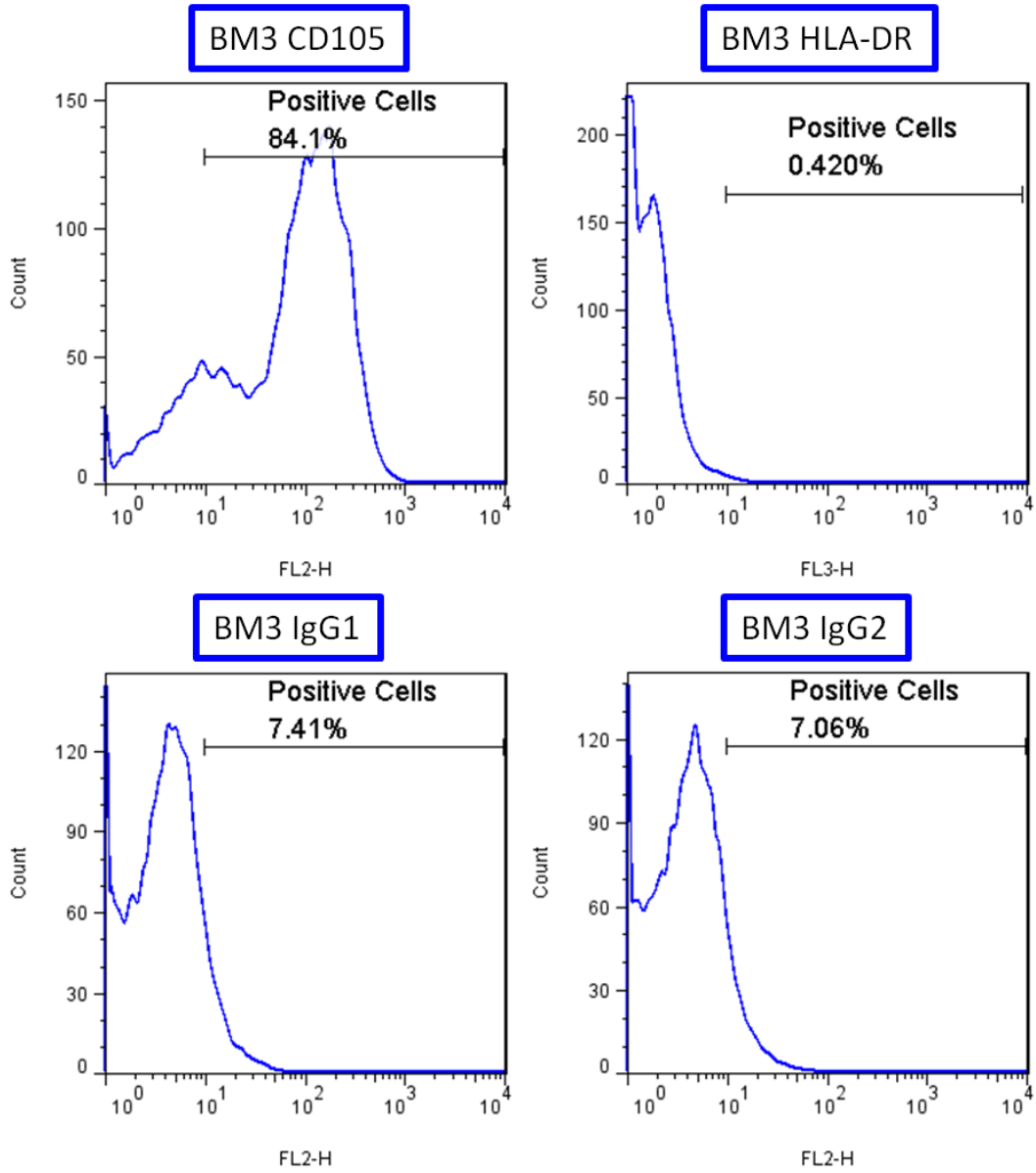


Figure 3.10: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 3 section 2. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for HLA-DR, and the two isotype controls IgG1 and IgG2, but positively for CD105, fulfilling the phenotype criteria defined by the ISCT.

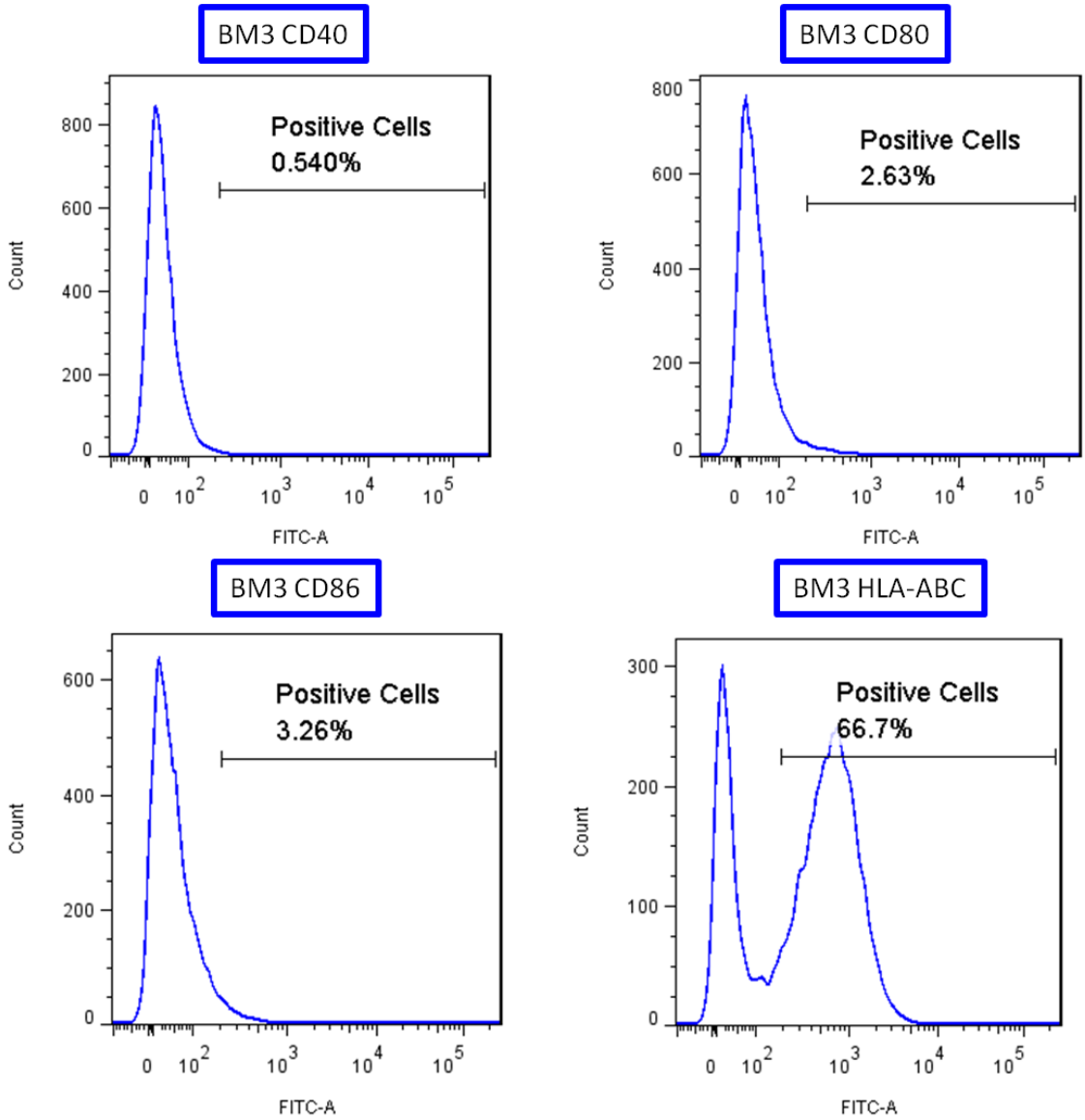


Figure 3.11: Flow cytometry histograms for the immuno-associated cell surface marker expression of the adherent cell population of BM 3 section 3. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^2 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD40, CD80, and CD86, but positively for HLA-ABC, as has been demonstrated in previous studies.

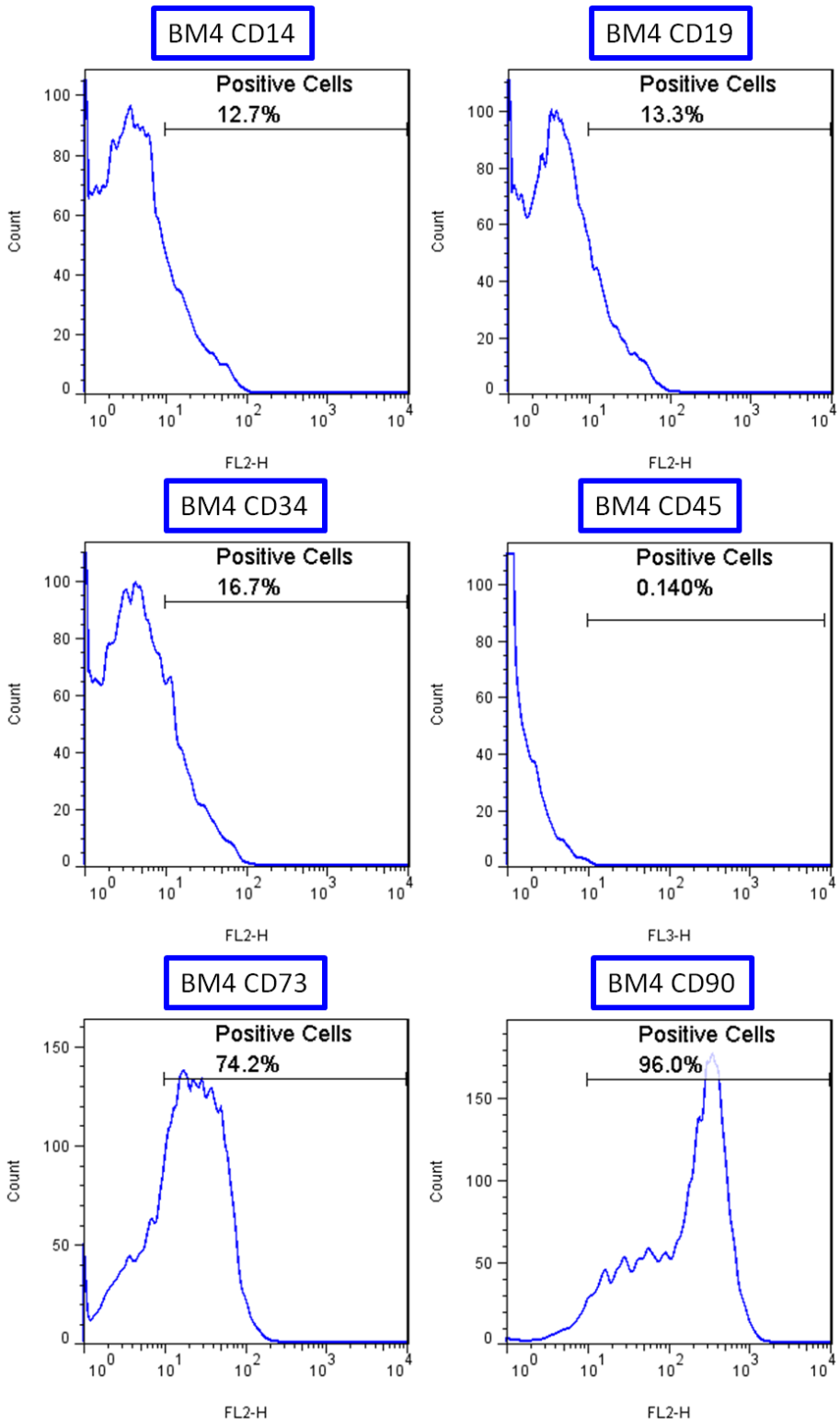


Figure 3.12: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 4 section 1. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD14, CD19, CD34, and CD45, but positively for CD73 and CD90 fulfilling the phenotype criteria defined by the ISCT.

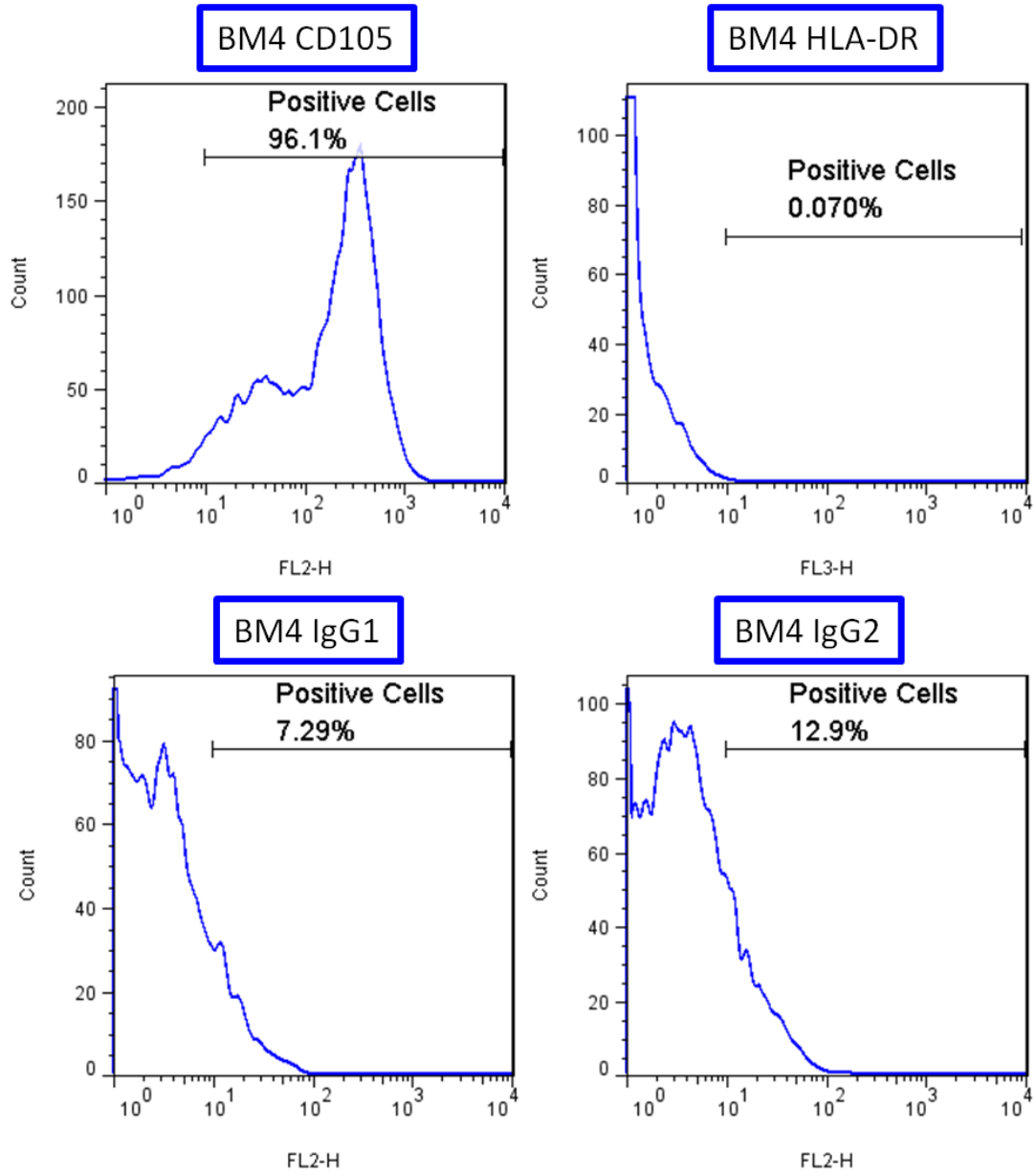


Figure 3.13: Flow cytometry histograms for the cell surface marker expression of the adherent cell population of BM 4 section 2. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^1 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for HLA-DR, and the two isotype controls IgG1 and IgG2, but positively for CD105, fulfilling the phenotype criteria defined by the ISCT.

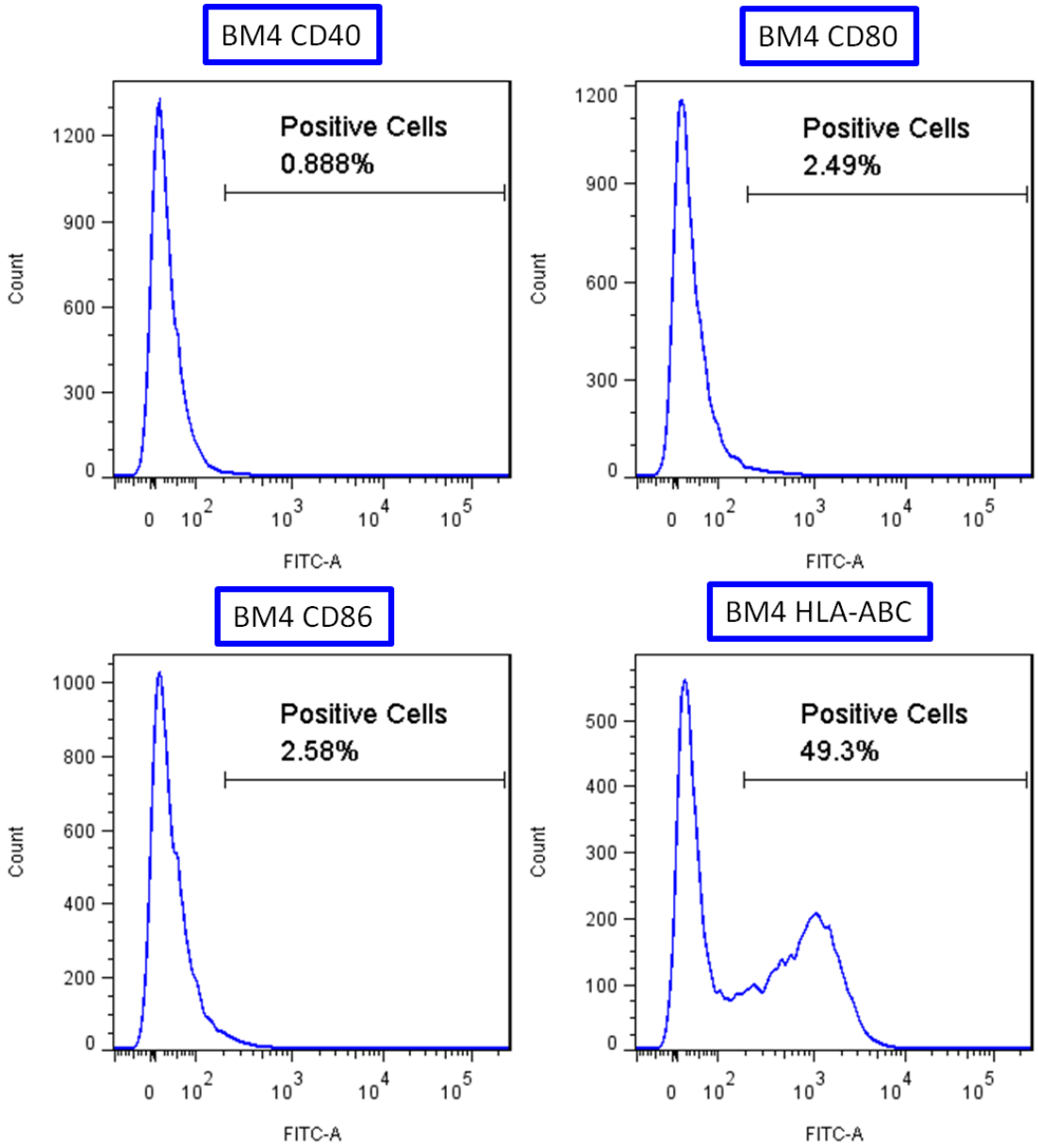


Figure 3.14: Flow cytometry histograms for the immuno-associated cell surface marker expression of the adherent cell population of BM 4 section 3. The text boxes indicate donor bone marrow followed by antigen. Data shown is representative of 10,000 events collected, and the resulting data was analysed in FlowJo ver. 7.6.1.The histograms were gated with staining above 10^2 being deemed positive staining. The percentage of positively staining cells is displayed above the gate. Cells stained negatively for CD40, CD80, and CD86, but positively for HLA-ABC, as has been demonstrated in previous studies.

Flow cytometry was performed in 2 stages. Firstly for the ISCT characteristic markers CD14, CD19, CD34, CD45, HLA-DR, CD73, CD90 and CD105 and secondly for the immuno-associated markers CD40, CD80, CD86 and HLA-ABC. For the second stage when the immuno-associated markers were analysed the laser on the flow cytometer had been replaced. This resulted in the shift of the histogram peaks for the unlabelled cell controls. The histograms were re-gated at 10^2 to account for this. The percentage of positive cells staining for the isotype controls or for the unlabelled MSCs was deducted from the percentage of cells staining positively for the remaining cell surface markers. The mean percentage of positive cells across all 4 BM MSC donors was calculated, and is presented in **Table 3.3** with the separate percentage staining values. If a negative value was obtained, this value was reset to 0% staining.

The markers that have been shown to stain negative had a mean number of positively staining cells less that 5 %. This included CD14, CD19, CD34, CD45, HLA-DR, CD40, CD80, and CD86. The markers that have previously been shown to be positive markers expressed on MSCs stained positive, and these were CD73, CD90, CD105, and HLA-ABC.

	BM1	BM2	BM3	BM4	Mean
CD14	-0.45	0.11	-0.02	5.41	1.26
CD19	0.41	-0.19	0.19	6.01	1.61
CD34	1.56	2.15	3.09	9.41	4.05
CD45	-5.09	-8.53	-6.74	-12.76	0.00
HLA-DR	-5.06	-6.07	-6.64	-12.83	0.00
CD40	-7.7	-9.1	-3.4	-6.8	0.00
CD80	-3.4	-9.8	-3.2	-4.6	0.00
CD86	-1	-6.2	3.6	0.1	0.00
CD73	75.87	63.55	63.69	66.91	67.51
CD90	84.07	80.85	71.49	88.71	81.28
CD105	88.17	77.45	76.69	88.81	82.78
HLA-ABC	46.3	41	49.9	42.4	44.90

Table 3.3: The histogram plots of fluorescent intensity were gated with cells staining greater than 10^1 deemed positive. The percentage of positively stained cells for each antibody is shown. The percentage values obtained have been normalized to the negative isotype controls (CD14, CD19, CD34, CD45, HLA-DR, CD70, CD90 and CD105) or unlabelled cells (CD40, CD80, CD86 and HLA-ABC).

3.3.4 TRI-LINEAGE DIFFERENTIATION ASSAY

The trilineage differentiation potential of bone marrow cells and fibroblasts was investigated by differentiating the cells into bone, fat, and cartilage using specific differentiation medium.

All of the adherent cell populations cultured under differentiation conditions stained positively for their respective differentiation markers (**Figures 3.15-3.26**). In osteogenic cultures, calcium accumulation was only observed later than Day 7, with very little orange/red staining present in cultures differentiated for this length of time or below. Staining appeared to be heterotypic between cells, with areas of localised calcium deposition appearing more intense in different areas of the culture well. Different donor adherent cells also exhibited different osteogenic potential, with cells from donors two and three staining less intensely than donor four for calcium. Adipogenic differentiation could be visualised by day 7 with small lipid vesicles appearing within cells. These vesicles grew increasingly larger in size, appearing at their largest by day 21. Adipogenesis also exhibited the same donor variability, with donor 3 appearing to have the most intracellular lipid staining by day 21. There also appeared to be heterogeneity within the same donor's adherent cells, with not all cells accumulating intracellular lipid droplets. Levels of chondrogenesis also varied between donors; with donor three appearing to have the most GAG deposition by day 21.

BM1 exhibited high levels of osteogenesis (calcium deposition) by day 21 (**Figure 3.15**) with the level of mineralisation so heavy that the cell culture has collapsed in on itself. High levels of adipogenesis were also observed by day 14 and especially by day 21 (**Figure 3.16**).However very low levels of chondrogenesis were observed. The levels of GAG staining appeared to be slightly higher than those of the fibroblast controls (**Figure 3.17**).

BM2 stained for high levels of osteogenesis (calcium deposition) by day 21 but unlike BM1 there were no areas of intense staining (black) and instead an overall increase in calcium level was observed (**Figure 3.18**). Levels of adipogenesis were also high by day 21, but less so than BM1 (**Figure 3.19**). Levels of GAG staining increased from day 0 to day 21, and staining was strongest in chondrogenic medium, with expansion control and fibroblast only staining weakly (**Figure 3.20**).

BM3 had similar levels of calcium staining to BM2, with an overall increase of staining by day 21 (**Figure 3.21**). Staining for intracellular lipids increased until the strongest staining was on day 21 (**Figure 3.22**). Levels of GAG staining increased from day 0 to day 21, and staining was highest on day 21. BM3 was the most chondrogenic and stained the strongest for GAGs by day 21 (**Figure 3.23**).

BM4 high levels of osteogenesis (calcium deposition) by day 21, with both an increase in overall calcium levels, but also small regions of very intense staining appearing black (**Figure 3.24**). Oil red staining showed the presence of intracellular triglycerides in adipogenic media only, with no staining present in expansion medium or in the fibroblast control (**Figure 3.25**). GAG staining was similar to BM2, with levels of staining higher for MSCs in chondrogenic media than both the expansion media and fibroblast control (**Figure 3.26**).

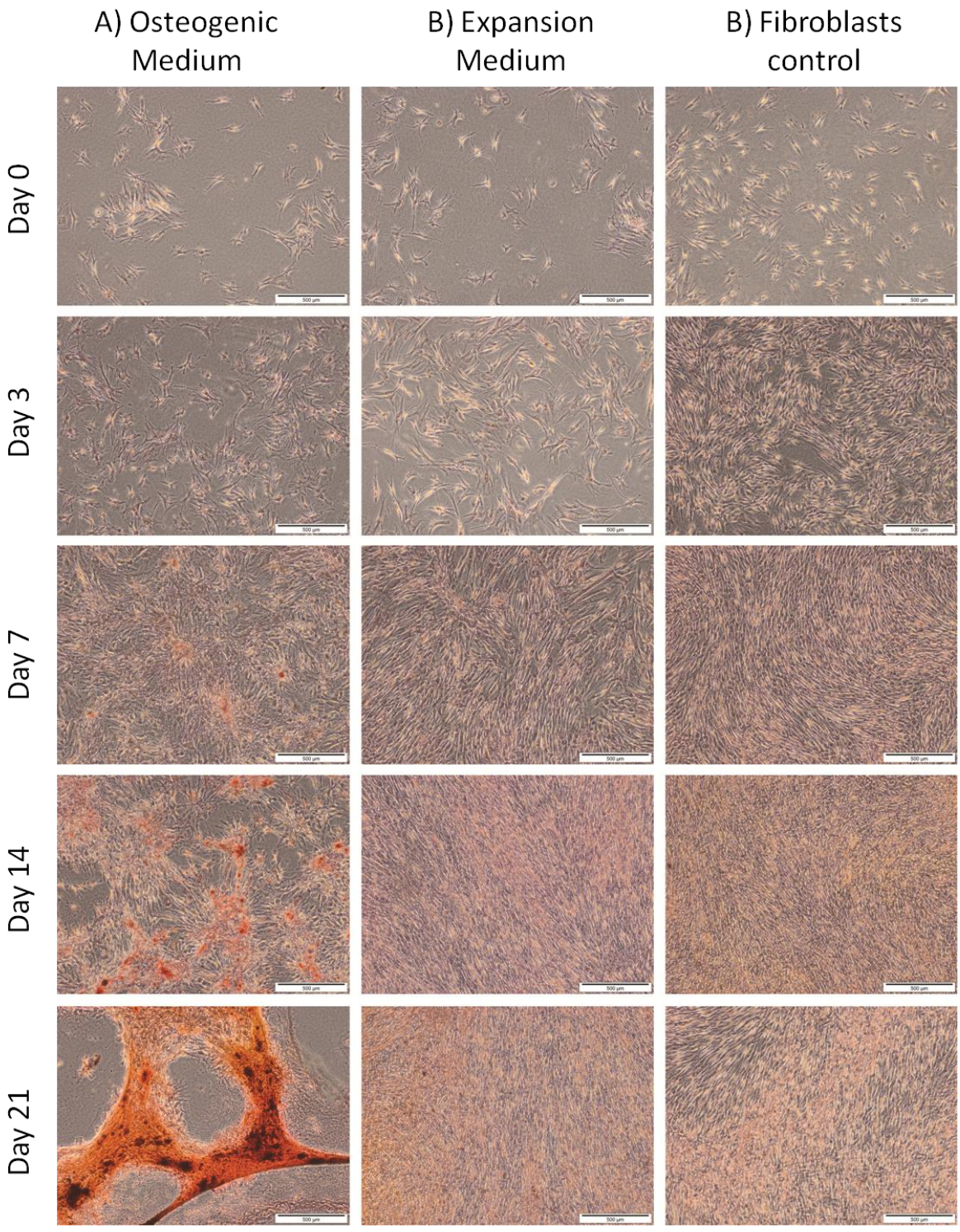


Figure 3.15: The osteogenic potential of donor BM1 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in osteogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in osteogenic induction medium as a control. Calcium deposits stained red/orange, and there is a gradual accumulation of calcium over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

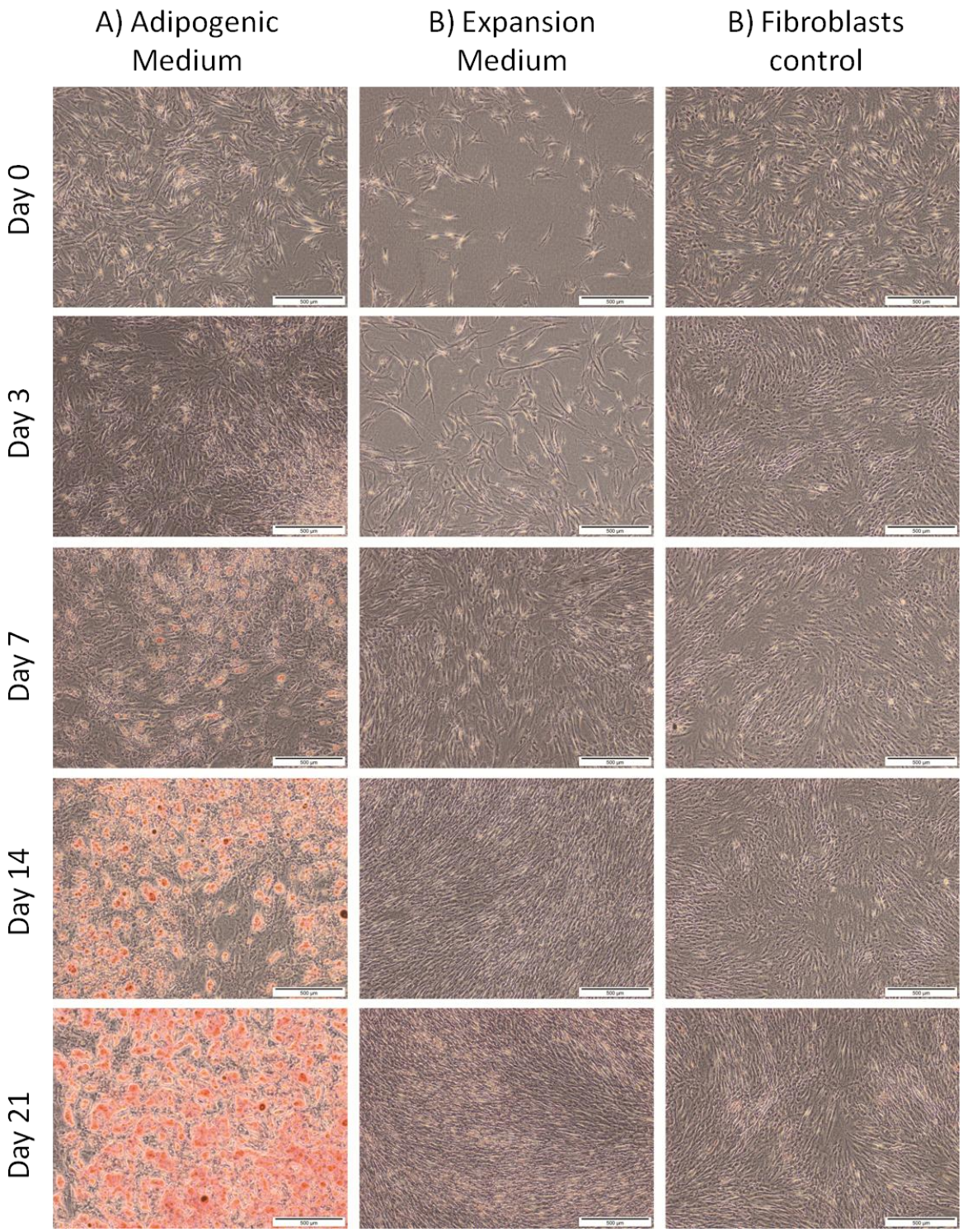


Figure 3.16: The adipogenic potential of donor BM1 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in adipogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Intracellular triglycerides (fat) deposits stained red, and there is a gradual accumulation of fat over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

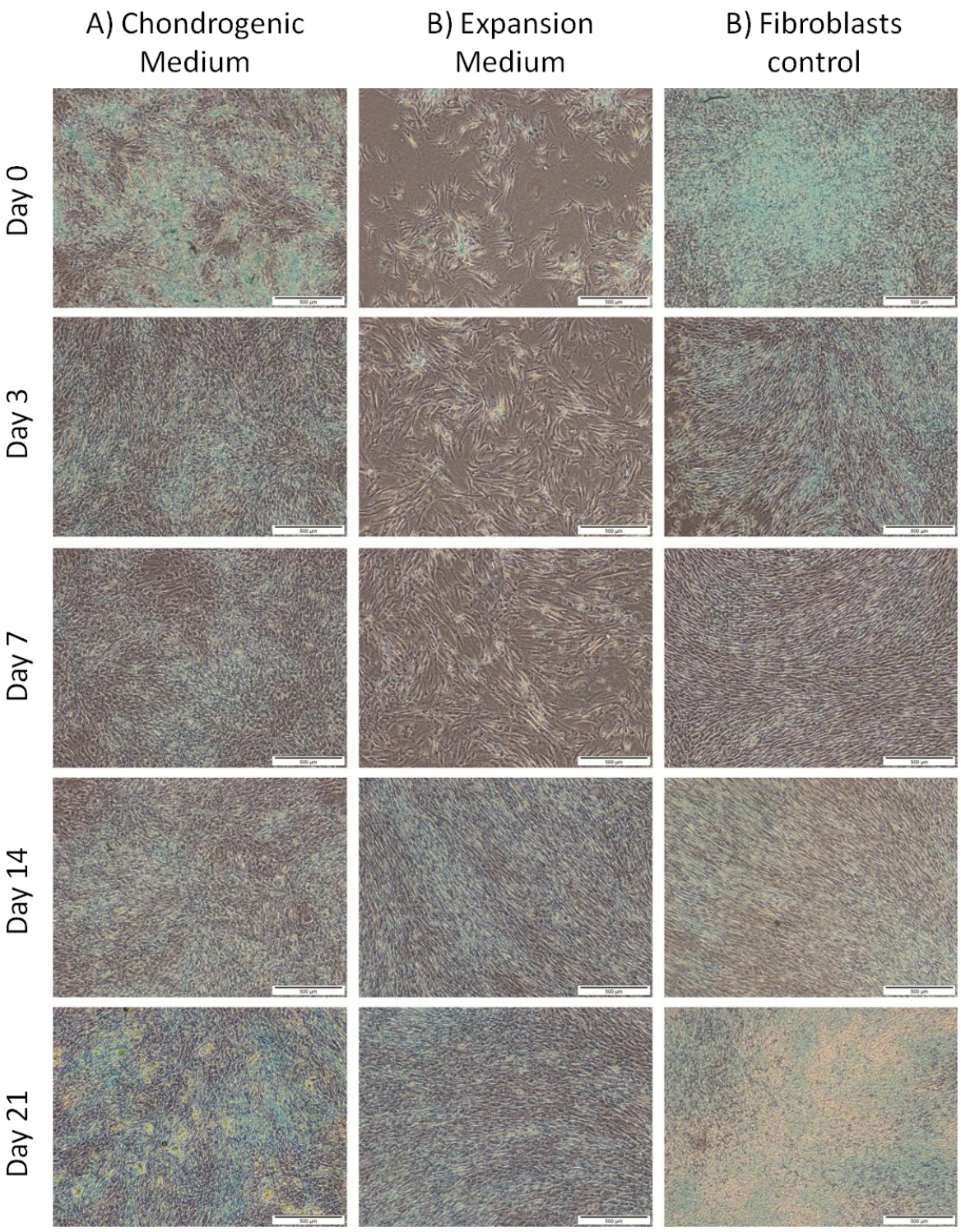


Figure 3.17: The chondrogenic potential of donor BM1 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in chondrogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Glycosaminoglycan (GAG) deposits stained blue, and there is a gradual accumulation of GAGs over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 μm.

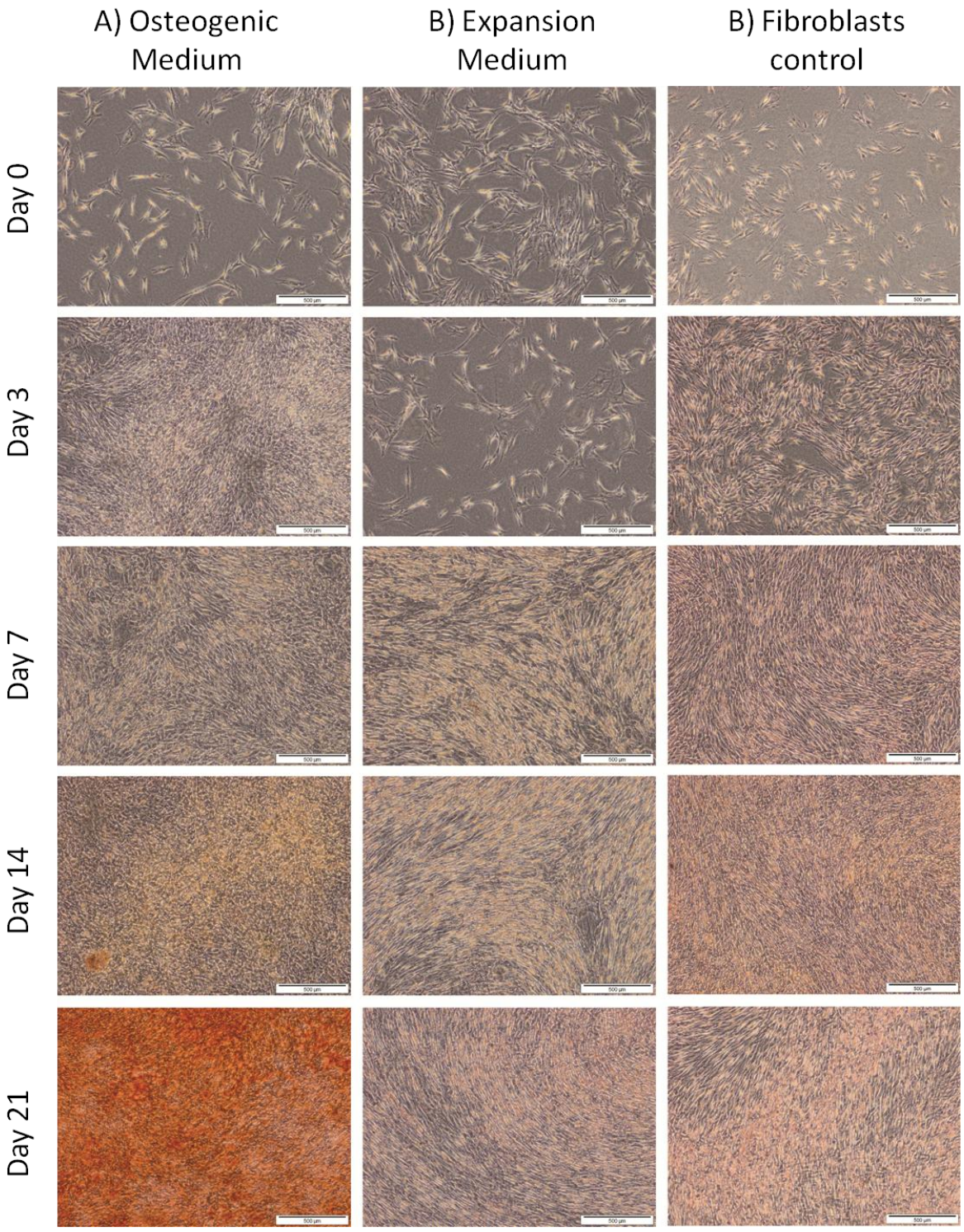


Figure 3.18: The osteogenic potential of donor BM2 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in osteogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in osteogenic induction medium as a control. Calcium deposits stained red/orange, and there is a gradual accumulation of calcium over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

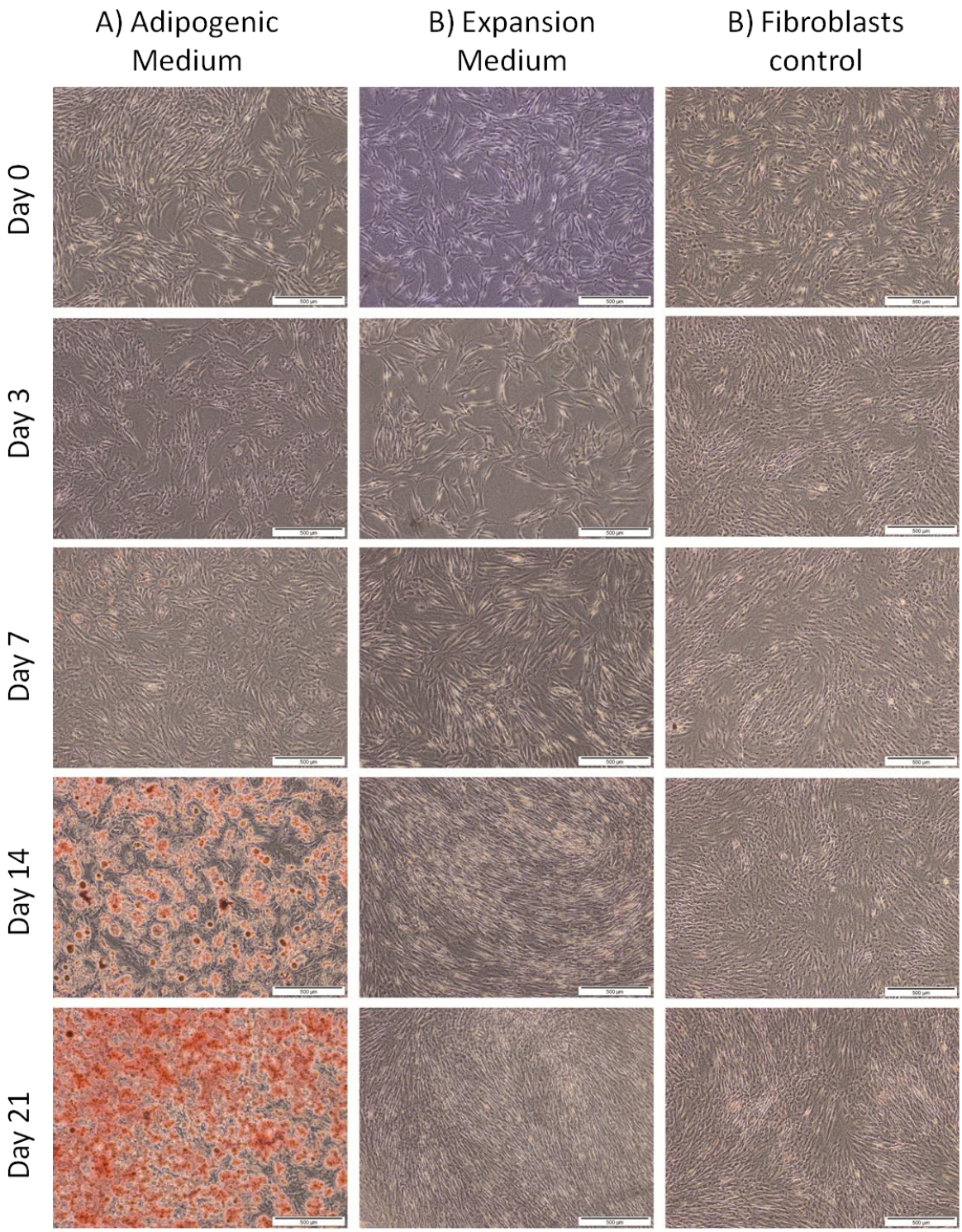


Figure 3.19: The adipogenic potential of donor BM2 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in adipogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Intracellular triglycerides (fat) deposits stained red, and there is a gradual accumulation of fat over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

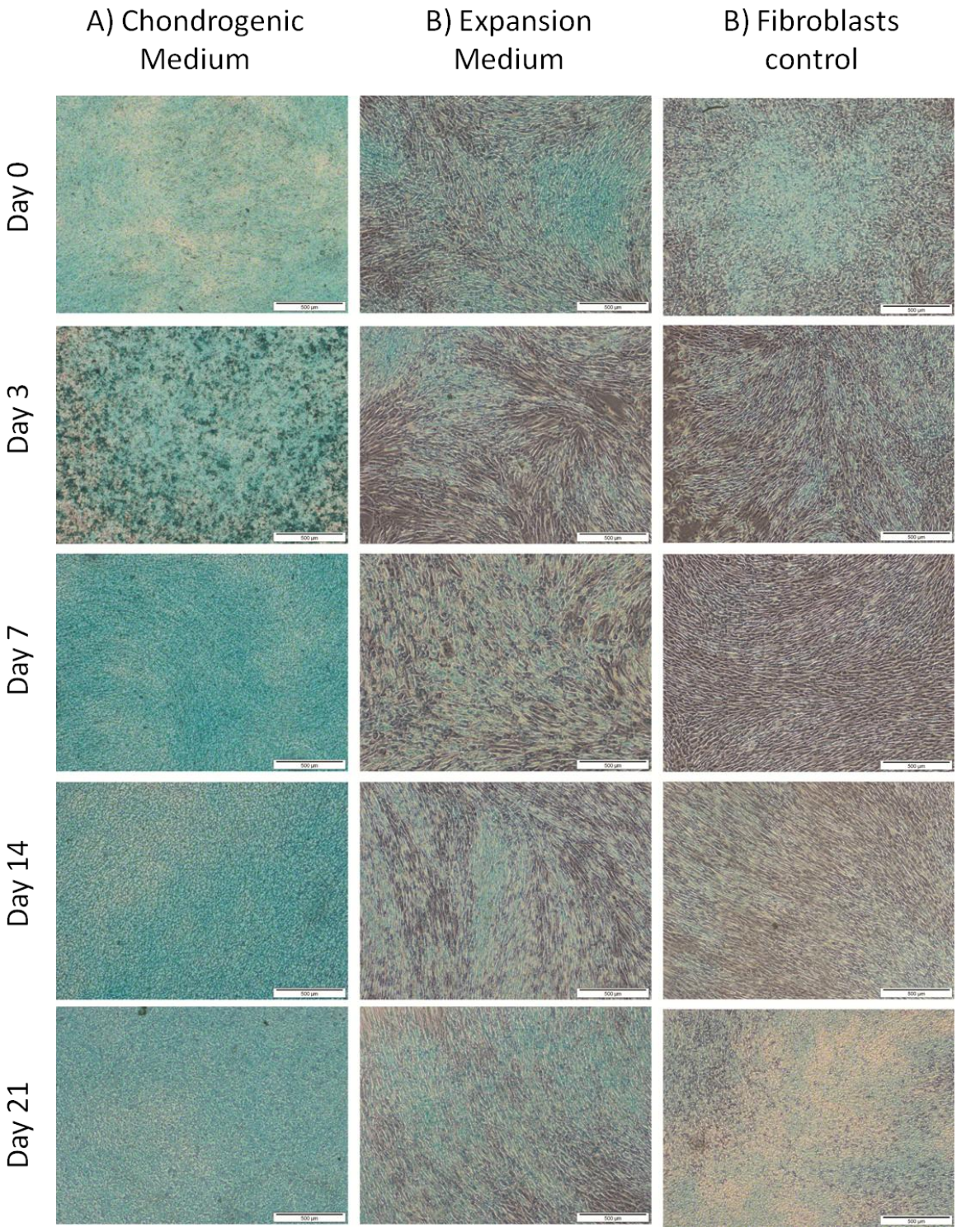


Figure 3.20: The chondrogenic potential of donor BM2 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in chondrogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Glycosaminoglycan (GAG) deposits stained blue, and there is a gradual accumulation of GAGs over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

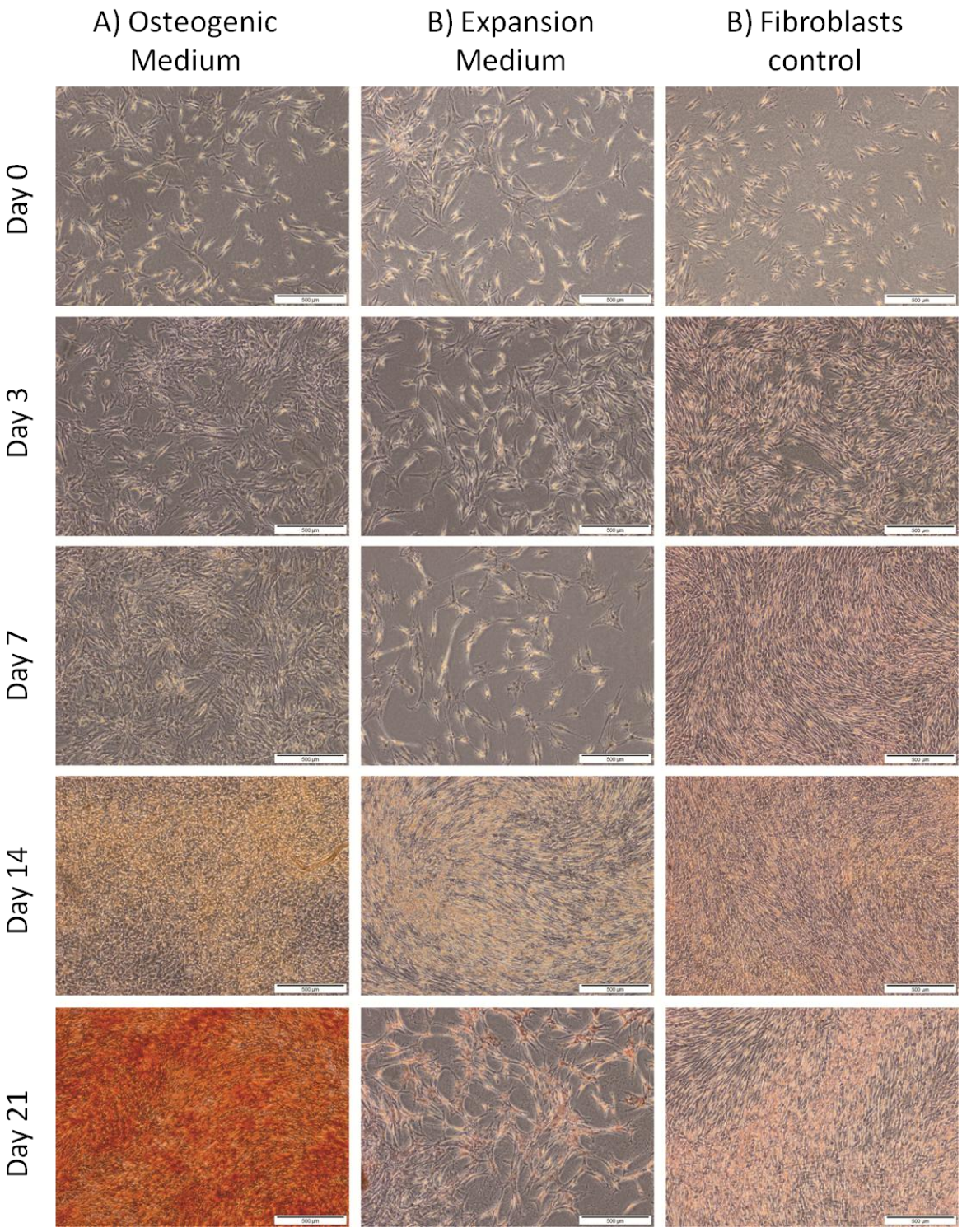


Figure 3.21: The osteogenic potential of donor BM3 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in osteogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in osteogenic induction medium as a control. Calcium deposits stained red/orange, and there is a gradual accumulation of calcium over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

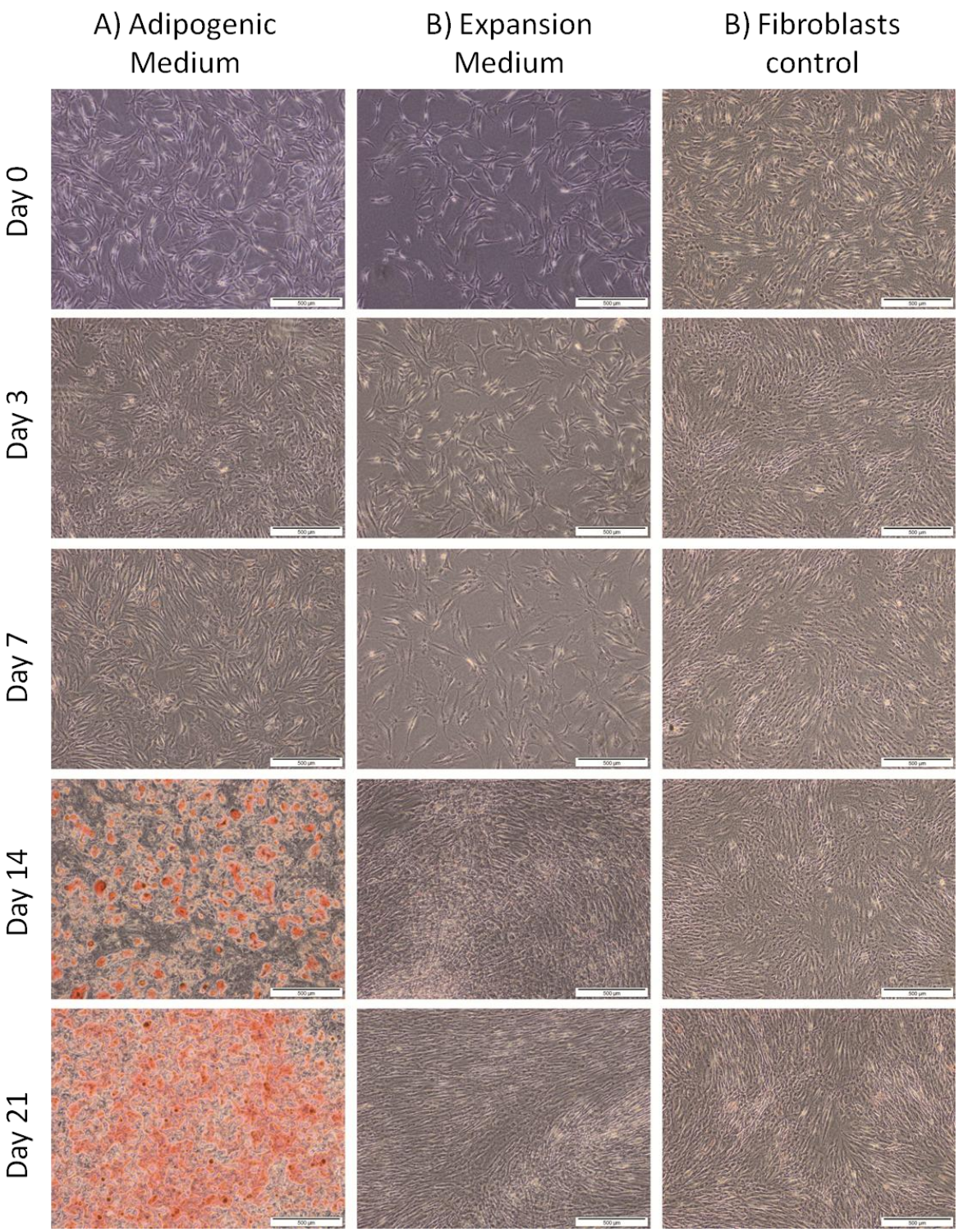


Figure 3.22: The adipogenic potential of donor BM3 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in adipogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Intracellular triglycerides (fat) deposits stained red, and there is a gradual accumulation of fat over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

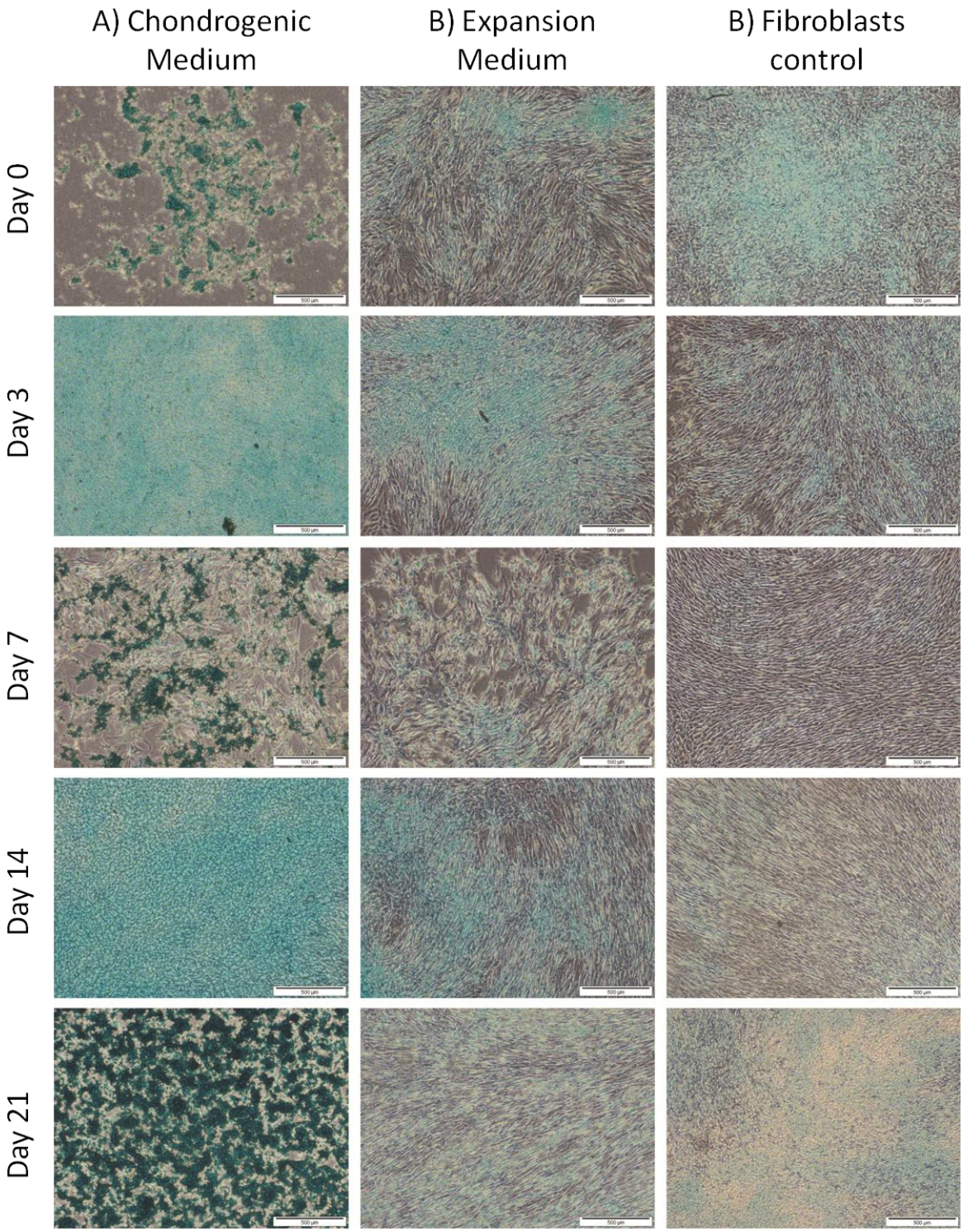


Figure 3.23: The chondrogenic potential of donor BM3 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in chondrogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Glycosaminoglycan (GAG) deposits stained blue, and there is a gradual accumulation of GAGs over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

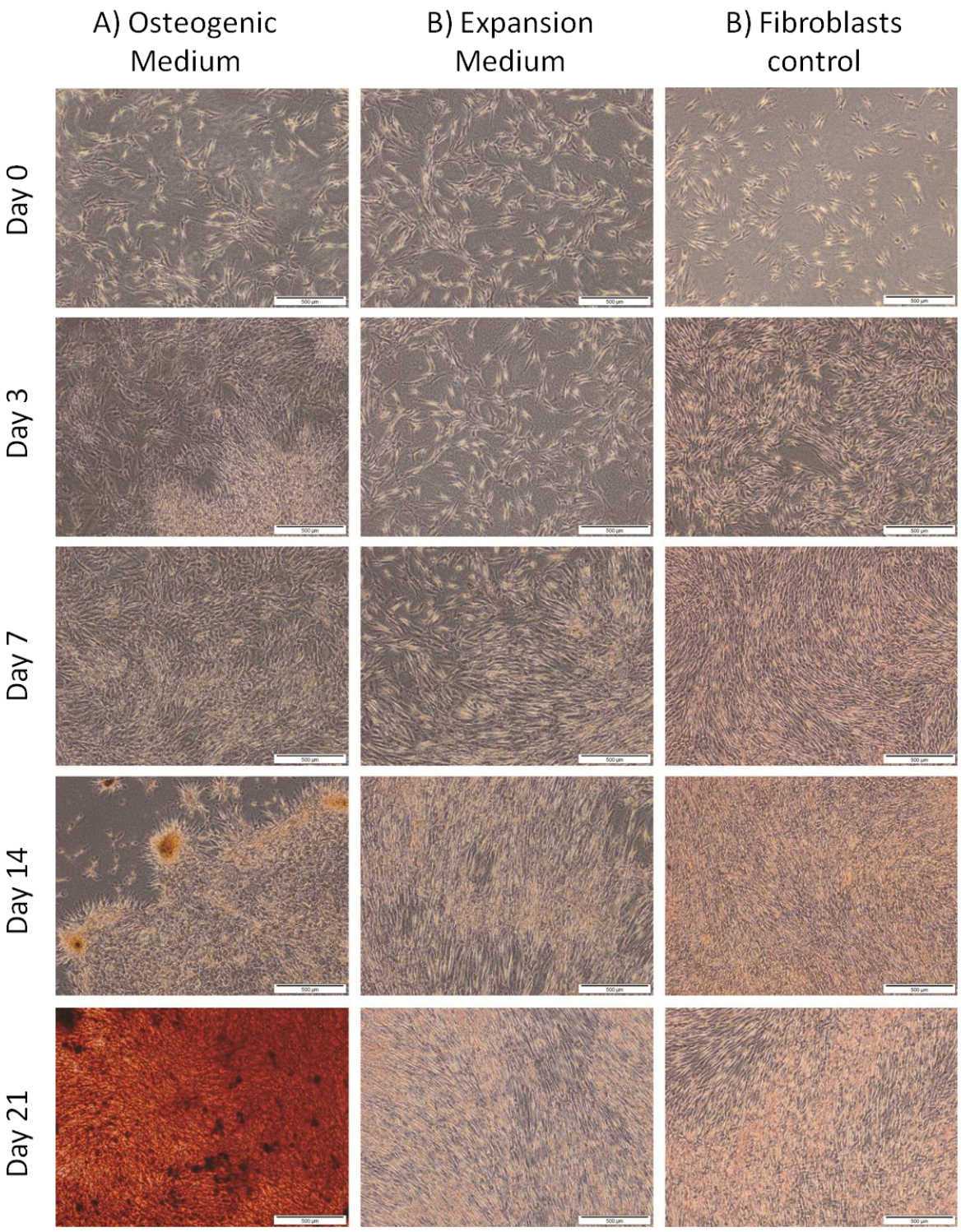


Figure 3.24: The osteogenic potential of donor BM4 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in osteogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in osteogenic induction medium as a control. Calcium deposits stained red/orange, and there is a gradual accumulation of calcium over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

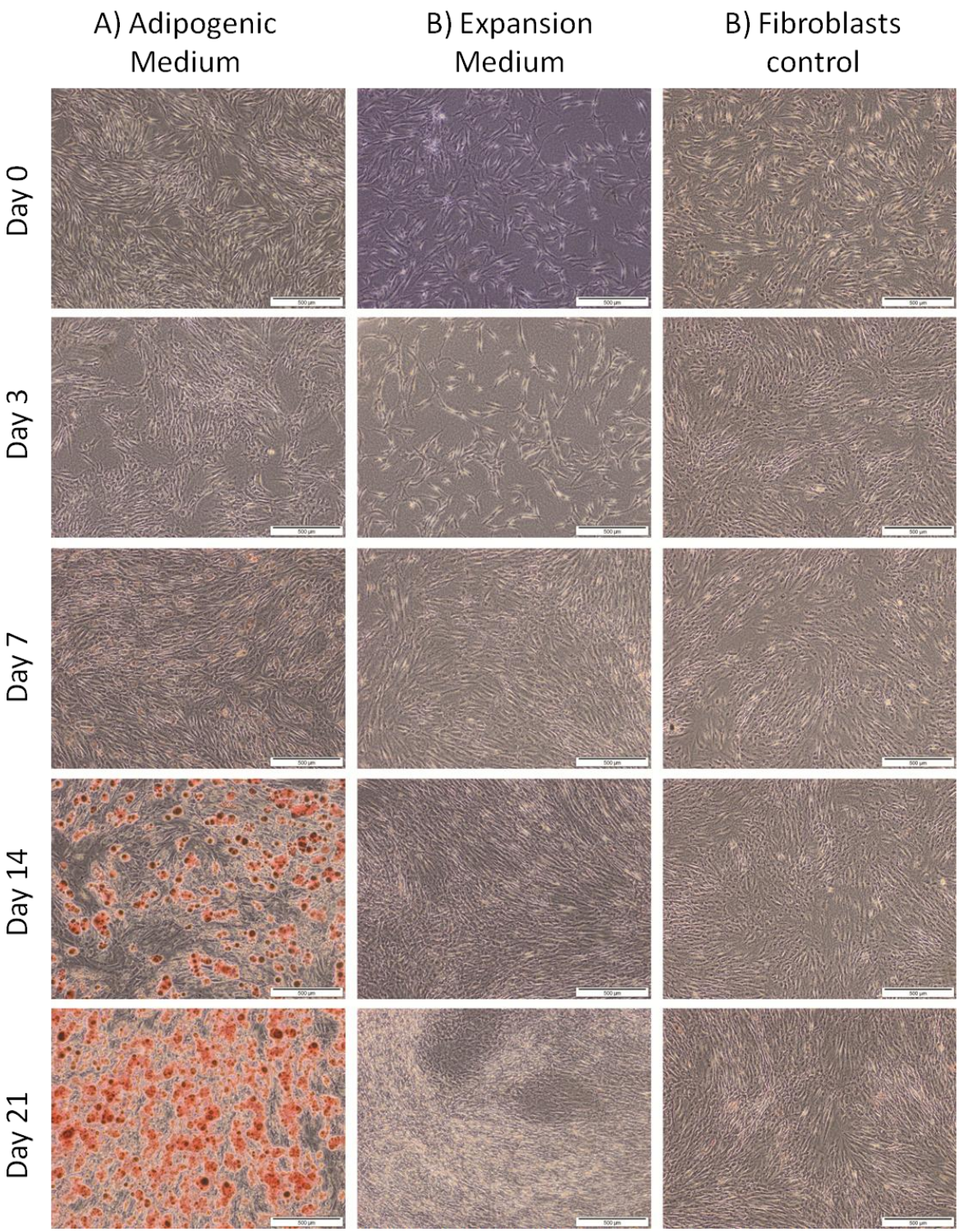


Figure 3.25: The adipogenic potential of donor BM4 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in adipogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Intracellular triglycerides (fat) deposits stained red, and there is a gradual accumulation of fat over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

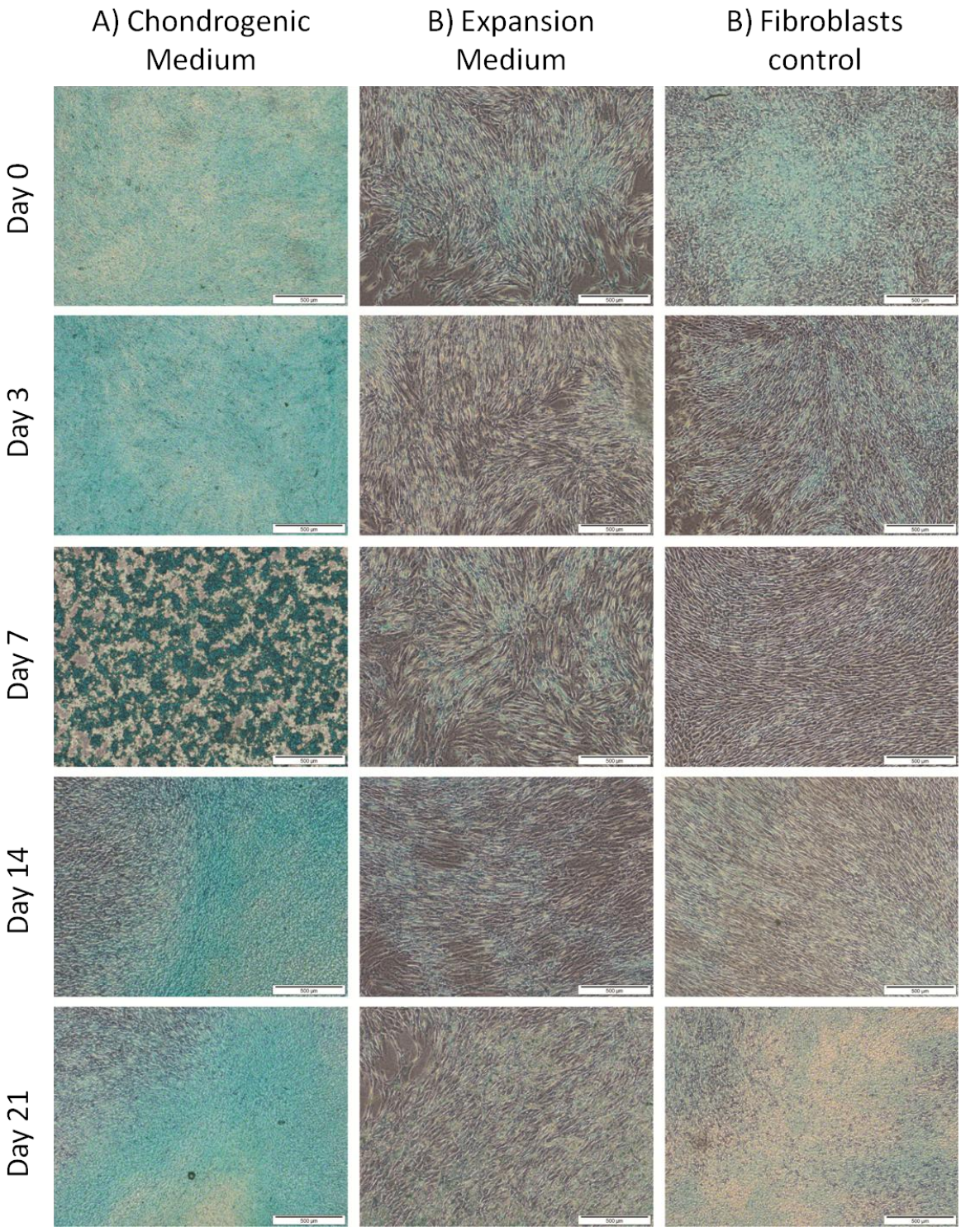


Figure 3.26: The chondrogenic potential of donor BM4 MSCs over 21 days. Adherent cells were seeded into 24 well plates and grown in chondrogenic induction medium (A), or expansion medium (B). Primary human dermal fibroblast cells were grown in adipogenic induction medium as a control. Glycosaminoglycan (GAG) deposits stained blue, and there is a gradual accumulation of GAGs over the timecourse (A), with the greatest staining present on day 21. Images were captured using Cell^B software (Olympus) at x100 magnification. Scale bars represent 500 µm.

3.4 DISCUSSION

Over 40 years ago Freidstein *et al.* documented a population of adherent cells present within the bone marrow that were fibroblast-like, and able to repair damaged bone. Since this initial report, these cells have generated a large amount of interest due to their potential benefits in regenerative medicine, ease of isolation and expansion (Jones et al., 2002), multilineage potential (Pittenger et al., 1999), immune-privileged status and immune-suppressing capabilities (Le Blanc and Ringden, 2007; Nauta and Fibbe, 2007).

It was the aim of this chapter to determine whether the cells obtained from donor bone marrow and used in this thesis were human bone marrow derived MSCs, through the fulfilment of the ISCT criteria.

The radial growth pattern displayed by MSCs when freshly isolated from bone marrow demonstrated their ability to proliferate from single cells into colonies of daughter cells. These colonies of cells can be stained using crystal violet solution and a typically used method of enumerating MSCs in the bone marrow is by the colony-forming units-fibroblastic assay (Castro-Malaspina et al., 1980; Pittenger et al., 1999; Jones et al., 2002). All of the donor bone marrow MSCs exhibited similar morphology: fibroblastic, with long spindle-like cells growing very tightly together. At later passages (p10) the cells started to become senescent, spreading out on the tissue culture plastic and looking more granular.

Two different methods were used to isolate potential MSCs from bone marrow. The first method has been well characterised, and used a Lymphoprep® density centrifugation gradient to separate the mononuclear cell fraction from bone marrow, before these cells were plated out onto tissue culture plastic, and the adherent cells attached and proliferated, while the non-adherent cells were washed away during medium changes (Haynesworth et al., 1992; Pittenger et al., 1999; Jones et al., 2002). The alternate method, also called the direct plating method, used was to mix freshly isolated BM in a 1:1 ratio of complete DMEM growth medium, and plate this mixture out in T75 tissue culture flasks. After 48 hours the cell monolayer was washed and the medium replaced with MSC expansion medium. This method eliminated the need for a Lymphoprep® centrifugation step and as such resulted in a much more streamlined process for obtaining MSCs. When using this method it was not uncommon to find that within the first 48 hours of plating cells out onto tissue culture plastic that the bone marrow and medium mixture coagulated, resulting in a jelly-like layer forming over the top of the cell monolayer. If this layer was forcibly removed during medium changes or during the wash steps then this resulted in a very low MSC yield, with colonies taking > 2 weeks to

appear. However, if this layer was left on the tissue culture plastic, and allowed to gradually dissipate with time, the number of MSCs obtained appeared to be far greater, with colonies appearing in < 1 week, with rapid growth. This could have been due to the presence of beneficial growth factors in the coagulated layer, as this layer would contain a multitude of cells from within the bone marrow, and would more accurately represent the MSC niche from where these cells were originally isolated. It is also possible that the coagulated layer contained platelets that released platelet derived growth factor (PDGF), which has been shown to be a potent mitogen of MSCs (Caplan and Correa, 2011).

The second criteria of the ISCT was the expression (or lack of) of a specific set of cellular markers by MSCs. Flow cytometry was used to allow a quantitative measure of cell marker expression on the adherent cells. A gate was drawn onto the histogram plots, with relative fluorescent intensities of between 10^0-10^1 excluded as being a negative reading, and values above 10^1 considered positive. Percentages of number of cells above 10^1 were given as the number of cells positively staining for a specific antibody. The flow cytometric analysis was divided into 2, with the ISCT characteristic markers CD14, CD19, CD34, CD45, HLA-DR, CD73, CD90 and CD105 analysed primarily and the immuno-associated markers CD40, CD80, CD86 and HLA-ABC analysed later. For the second stage when the immuno-associated markers were analysed the laser on the flow cytometer had been replaced. This resulted in the shift of the histogram peaks for the unlabelled cell controls. The histograms were re-gated at 10^2 to account for this.

The isotype and unstained control positive staining percentages were subtracted from experimental percentages, and mean positive staining percentage calculated.

Fluctuations in donor cell variability (size and granularity) can cause small shifts in histograms, and as the same settings were used for collection throughout phenotyping to allow direct comparisons to be performed, these small shifts can manifest in variations in marker percentage staining.

The histograms obtained for positively stained markers did not have a defined peak, and instead were spread out and had a wide footprint, possibly due to the varied heterogeneous nature of MSCs grown in culture; the size and granularity of MSCs can vary, creating the wide histograms. The differences in size appeared to be a factor of age, with cells of later passages having increasing numbers of larger cells present within their population. The same flow cytometry settings were used to collect data for each of the donor MSCs, to allow differences in cell marker expression to be compared. The fluorescence antibodies for CD45 and HLA-DR

were measured using a different channel (FL-3), and the compensation settings used meant that the peak fluorescence was at 10^0, which resulted in the very low percentage of positively staining cells when compared to the other negative markers. The percentage of positively staining cells were all greater than 65% (except for HLA-ABC), but again due to the large spread out nature of the histograms although the peaks were over 10^1 a portion of the area under the histogram overlapped the negative staining cut off point. All donors' adherent cells were positive for CD73, CD90 and CD105 and negative for the expression of CD14, CD19, CD34, CD45 and HLA-DR. A fluorescently conjugated isotype antibody control was used as a negative control in the flow cytometry to provide a background fluorescent intensity for non-specific primary antibody binding, and the fluorophore for the isotype control was RPE. When performing flow cytometry the isotype control should have the same fluorophore as the test antibody. In this case, the isotype controls used were able to demonstrate that there was no non-specific binding for the positive markers CD73, CD90 and CD105. A limitation of the study was that no FITC labelled isotype control was included. However as the FITC labelled test antibodies used did not demonstrate positive staining, this omission not change the outcome of the results.

Flow cytometry for the co-stimulatory molecules and HLA-ABC demonstrated correlation with previously published literature that MSCs do not express the co-stimulatory molecules CD40, CD80, or CD86, and also do not express HLA-DR. Multipotent mesenchymal stromal cells do express HLA-ABC however, and in agreement with other studies this was shown to be at a low level (Uccelli et al., 2006; Le Blanc and Ringden, 2007; Nauta and Fibbe, 2007; Spencer et al., 2010; Bassi et al., 2011).

The final criteria that the adherent bone marrow population of cells must fulfil in order to be characterised as MSCs is that the cells must be able to differentiate into adipocytes, osteoctytes and chondrocytes using specific culture conditions. All of the trilineage differentiations provided varied levels of differentiation, which was believed to be due to the heterogenic nature of MSCs, and the differences in ages of donor (D'Ippolito et al., 1999; Kretlow et al., 2008).

Adipogenic differentiation was heterogenic across the adherent cell population for each BM donor with a small subset of the cells accumulating no lipid vesicles, even at day 21. The accumulation of lipid vesicles within the cells also appeared to be dependent on the area of the tissue culture well that the cells were grown in; the outer perimeter and the centre of the well displayed highest levels of differentiation. Also, it was noted that adipogenesis occurred in 'patches', with areas of the well appearing very dense for mature fat cells (cells containing

large lipid vesicles) , and other areas having little differentiation. It is possible that where a cluster of adherent cells differentiated into adipocytes, cell signalling molecules or cytokines were released to promote cells in the vicinity to also differentiate. Alternatively, the presence of discreet areas of adipogenesis (or areas where adipogenesis appeared to be noticeably increased when compared to the surroundings) could have been due to cell clumping when the adherent cells were initially plated. Early on during the differentiation time course, as early as day 7 it could be seen that there were a large number cells starting to accumulate lipid vesicles around the circumference of the wells, as well as a dense area of cells in the center of the wells. The differentiation of the adherent cells into adipocytes demonstrated the heterogeneity of the cell population, and the variability of adipogenesis between donors.

Osteogenic differentiation showed a gradual increase in calcium deposition/accumulation as the 21 day timecourse progressed, with the formation of 'bony nodules' or areas of intense staining (almost black rather than red/orange). There was a faint orange tint to the negative control cultures, but this was thought to be due to staining being performed on very dense cell monolayers (MSCs in osteogenic differentiation medium appeared to continue proliferation and differentiation). In some cases the monolayer had started to lift off from the tissue culture plastic or curl around onto itself due to the mineralisation of the culture becoming so heavy. This was most prolific when MSCs were seeded at high densities, such as for osteogenesis. When adipogenic differentiation was performed this was not as large an issue, as MSCs were seeded at lower densities, and began differentiating earlier with little discernable growth phase.

When undergoing osteogenesis the cell population grew until confluent before accumulating calcium, as initially cultures appeared far less dense. When cells were grown in wells already at confluency and differentiation attempted, most of the cell monolayer was lost due to curling or from lifting off the bottom of the well. This was in comparison to adipogenesis, where the population of cells seeded into wells began differentiation instead of initially proliferating.

Chondrogenesis was performed using micromass culture. A large number of cells within a small volume (10µl) were deposited in a tissue culture plate, and this high number of cells attached to the surface of the well, forming a dense micromass of cells. GAG accumulation was used as a marker of chondrogenesis, and levels of staining when the adherent cells were grown in chondrogenic differentiation medium did not appear to be significantly different than cells grown in expansion medium. There was a small amount of residual staining in the cells grown in expansion medium, which could have been due to the regions of very dense cell growth. The chondorogenesis demonstrated by BM MSCs 1-4 was generally low, with weak

increases in staining observed for differentiated MSCs combined with high background staining in the fibroblast control, and also to some extent in the undifferentiated controls. This was perhaps due to the limitations of micromass culture (it is still essentially a two dimensional culture system), and higher levels of chondrogenesis would have possible been observed if pellet culture was used, and MSCs were grown in three dimensional culture. Although levels of chondrogenesis were minimal, it was still possible to observe visible differences between day 0 and day 21 of culture.

In conclusion, the adherent cell population from four bone marrow donors was purified and expanded, before morphological, phenotypic and trilineage differentiation was performed in order to determine if these cells were MSCs. These cells were plastic adherent under standard culture conditions. Flow cytometry demonstrated that these cells were positive for the expression of CD73, CD90 and CD105 and negative for the expression of CD34, CD45, CD14, CD19 and HLA-DR. The adherent cells were also able to differentiate into adipocytes, osteoctyte and chondrocytes.

All adherent cell populations fulfilled the criteria as defined by the ISCT for the definition of mesenchymal stromal cells.

Chapter 4: A COMPARISON OF METHODS USED TO EVALUATE THE ADIPOGENESIS OF MSCs

4.1 INTRODUCTION

Adipose tissue can be divided into two main types, brown adipose tissue and white adipose tissue (Fantuzzi, 2005; Tilg and Moschen, 2006). The major role of brown adipose tissue is nonshivering thermogenesis, whereas the main role of white adipose tissue is energy storage, and this forms the majority of adipose tissue in humans. White adipose tissue is composed of many cell types, including macrophages and fibroblasts, but the major cellular constituents are adipose cells (Fantuzzi, 2005).

MSCs were first shown to differentiate into adipose cells in the early 1980's, when it was demonstrated that the incubation of the fibroblastic cells from bone marrow with hydrocortisone could induce the formation of lipid droplets within the adherent cells (Kaneko et al., 1982). Adipose cells present in white adipose tissue are known to be linked with inflammation and immunity, and have been shown to be able to secrete various pro-inflammatory and anti-inflammatory factors (Tilg and Moschen, 2006) and this is unsurprising given the presence of macrophages within adipose tissue and the often close physical proximity between white adipose tissue and lymphoid tissue (Pond, 2003). The link between immunity/inflammation is not only due to the physical proximity of the cells of the immune system with adipose tissue, but also due to the paracrine factors secreted by adipose cells. In particular, adipose cells have been demonstrated to secrete a specific family of proteins called 'adipokines'. The differentiation of MSCs into adipocytes presents an interesting system for the study of the immunological properties of MSC following differentiation. Mature adipose cells have been shown to secrete adipokines, such as leptin, resistin, and adiponectin (Schaffler et al., 2007). These adipokines are known to exert potent immunomodulatory effects on cells of the immune system (Tilg and Moschen, 2006; Bielby et al., 2007; Schaffler et al., 2007). At the same time adipocytes have been demonstrated to be potent producers of pro-inflammatory cytokines such as TNF-α and interleukin-6, as well as chemokines such as monocyte chemoattractant protein (MCP)-1 (Schaffler et al., 2006; Schaffler et al., 2007).

In order to study the immunological properties of MSC during the process of differentiation into adipocytes, a robust method to quantify and determine the level of adipogenesis *in-vitro*

was required. Various methods have been employed to evaluate adipogenesis, such as oil red-O staining (English et al., 2007; Platt and El-Sohemy, 2009), qrt-PCR of adipogenic markers (Janderova et al., 2003; Fink et al., 2004; Neubauer et al., 2004; Sekiya et al., 2004; Blum and Benvenisty, 2008), flow cytometry of adipogenic markers (Greenspan et al., 1985; Smyth and Wharton, 1992; Dennis et al., 1999; Fink et al., 2004), and Nile red staining (Greenspan et al., 1985).

qrt-PCR is a method that can be used to monitor the progression of a PCR reaction in real time (RT), and quantify (q) the PCR product as it is produced. The process of PCR allows the detection and amplification of specific DNA sequences from a mixture of DNA species. When using rt-PCR however the aim is to look at the levels of mRNA expression in cells, and therefore a reverse transcriptase step to convert mRNA into cDNA is required. This step can be carried out using different methods, from random primers, oligo-dT primers, or even specific sequence primers, and a reverse transcriptase enzyme that creates the cDNA from free oligonucleotides also added to the reaction (Bustin et al., 2005). Theoretically, the more mRNA transcripts for a specific gene there are in a sample, then the higher the number of corresponding cDNA sequences will be present in the final sample to perform PCR. Specific primers used during PCR amplify specific genes/regions of interest, and there are 2 main methods used to quantify the amount of transcript generated from the PCR process; a fluorescent DNA dye, or a specific Taqman® probe. The Taqman® probe is an oligonucleotide specific for the gene/region of interest with a fluorescent molecule attached to the 5' end, and a quencher molecule attached to the 3' end (Heid et al., 1996). The close proximity of the quencher to the probe results in no fluorescence. When the primer anneals to the specific sequence, the Taq polymerase extends the primer along the length of the transcript, and when it reaches the bound Taqman® probe the 5' exonuclease activity of the polymerase cleaves the bound probe, and releases the fluorescent molecule and quencher, allowing the fluorescent molecule to freely fluoresce, and this can then be quantified by the detector. An overview of this method is shown in **Figure 4.1**.

The second widely used method is less specific, and uses a dye (SYBR green) that binds to double stranded DNA and fluoresces, such that as the levels of amplified template increase, the fluorescence increases proportionally. An overview of this method is shown in **Figure 4.2**.

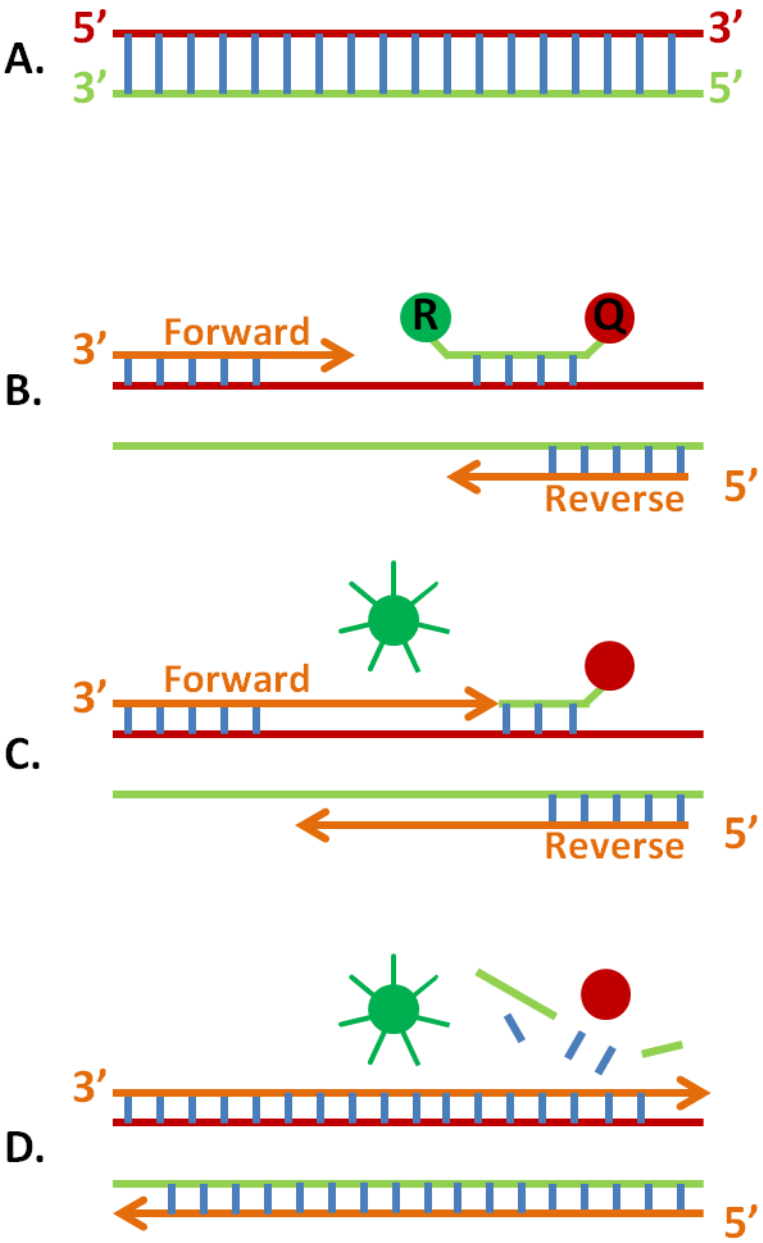


Figure 4.1: An overview of the Taqman® probe. A) A fluorescent reported dye (R) and a quencher (Q) are attached to the 5' and 3' ends of a Taqman® probe, which is sequence specific for the gene of interest. **B)** When the probe binds to the cDNA the close proximity of the quencher molecule prevents the fluorescence of the dye. **C)** During the extension cycle the DNA polymerase cleaves the reporter dye from the Taqman® probe as it travels along the cDNA. **D)** When the dye is free from the close proximity of the quencher, it fluoresces, and this fluorescence is measured. The more cDNA transcripts present the larger the number of Taqman® probes will bind and therefore the higher the fluorescence will be as the reporter dyes are freed.

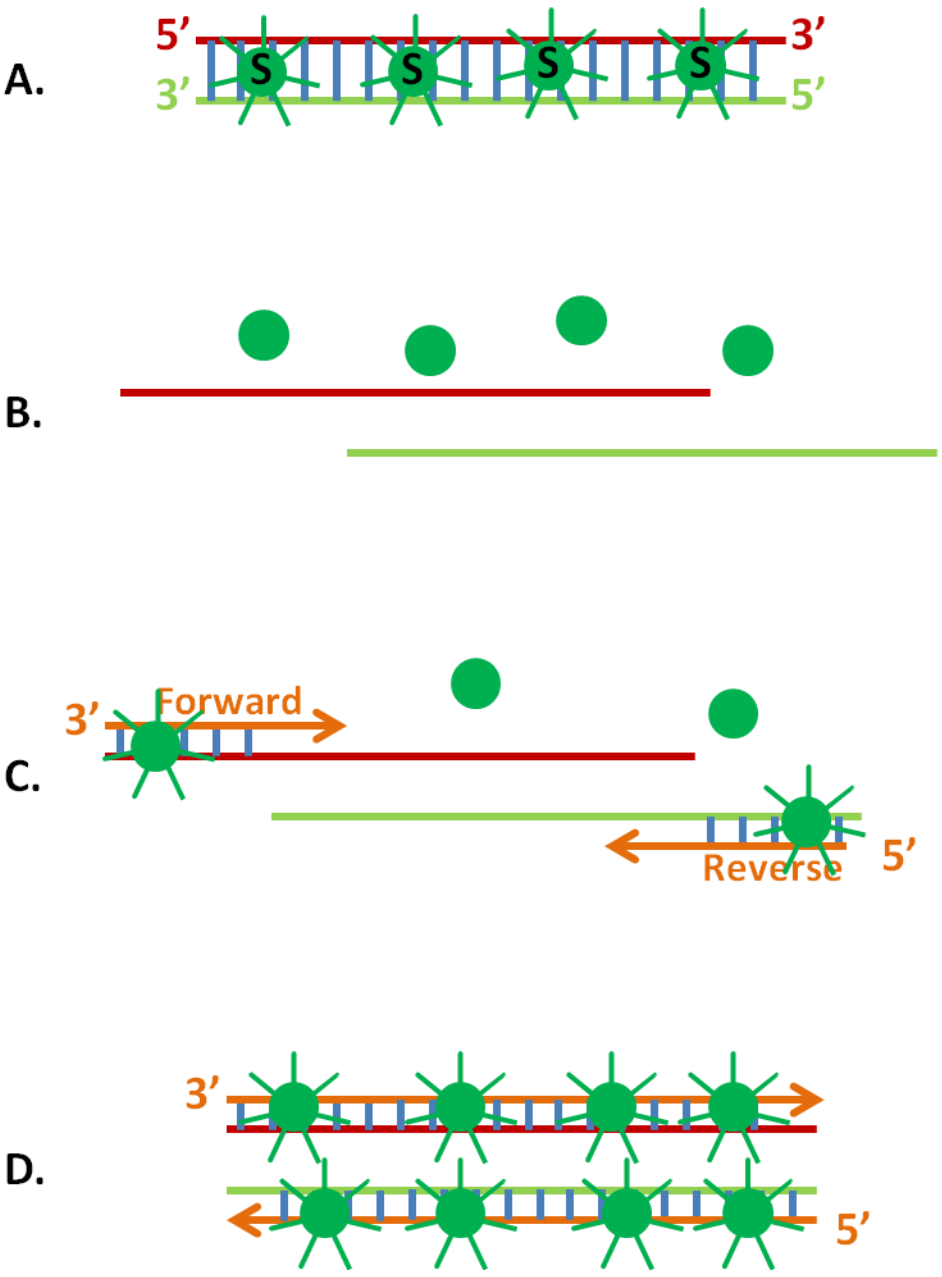


Figure 4.2: An overview of the SYBR green method of measuring qRT-PCR. A) SYBR green (S) binds to double stranded DNA, so in the initial reaction before the denaturisation step the dye is able to bind to the double stranded cDNA and fluoresce. **B)** The denaturisation step causes the cDNA to become single stranded, the the SYBR green is released, and stops fluorescing. **C)** PCR causes the amplification of the specific sequence of interest. **D)** When polymerisation has been completed, there is amplification of cDNA sequence of interest. When the cDNA strands re-anneal and form double stranded DNA, the SYBR green dye binds again to the DNA and fluoresces. Due to the amplification of the sequence of interest there is a larger amount of double stranded cDNA present and this results in the increase of fluorescence that is then measured.

In this study SYBR green was used to measure gene expression, due to the experience of using this technique within the laboratory. Two genes were selected to measure an early transcription factor peroxisome proliferator activated receptor (PPAR-ɣ) and a late marker of adipogenesis, fatty acid binding protein 4 (FABP4) due to their previous reported expression during adipogenesis (Nolte et al., 1998; Janderova et al., 2003; Fink et al., 2004; Neubauer et al., 2004; Sekiya et al., 2004; Blum and Benvenisty, 2008). Both of these transcripts have been shown to increase in adipogenesis. PPAR-ɣ is a nuclear receptor that upon stimulation with ligand binds to retinoid-X receptor and this complex can bind to the DNA and initiate transcription (Nolte et al., 1998). An overview of the response of PPAR-ɣ to ligand is shown in **Figure 4.3**

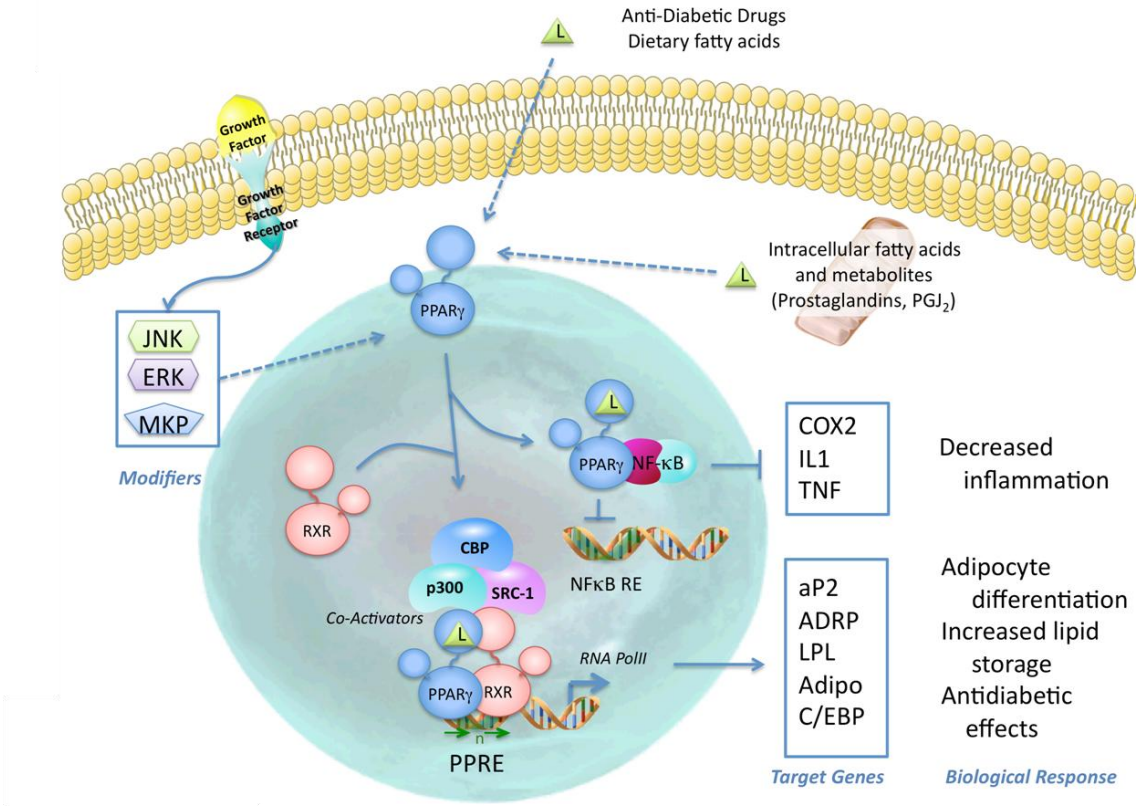


Figure 4.3: An overview of the response of PPAR-ɣ to ligand. Upon stimulation PPAR-ɣ binds to retinoid-X-receptor (RXR), and this complex binds to DNA in the presence of co-activators, to stimulate the expression of adipogenic target genes aP2 (also known as FABP4), adipose differentiation related protein (ADRP), CCAAT-enhancer binding proteins (c/EBP) and low density lipoprotein (LDL) and to block the expression of pro-inflammatory genes COX2, IL-1 and TNF. Image was created by Dr. J. Heuvel for the PPAR resource page in association with the Penn State University; (http://ppar.cas.psu.edu/pathways.html).

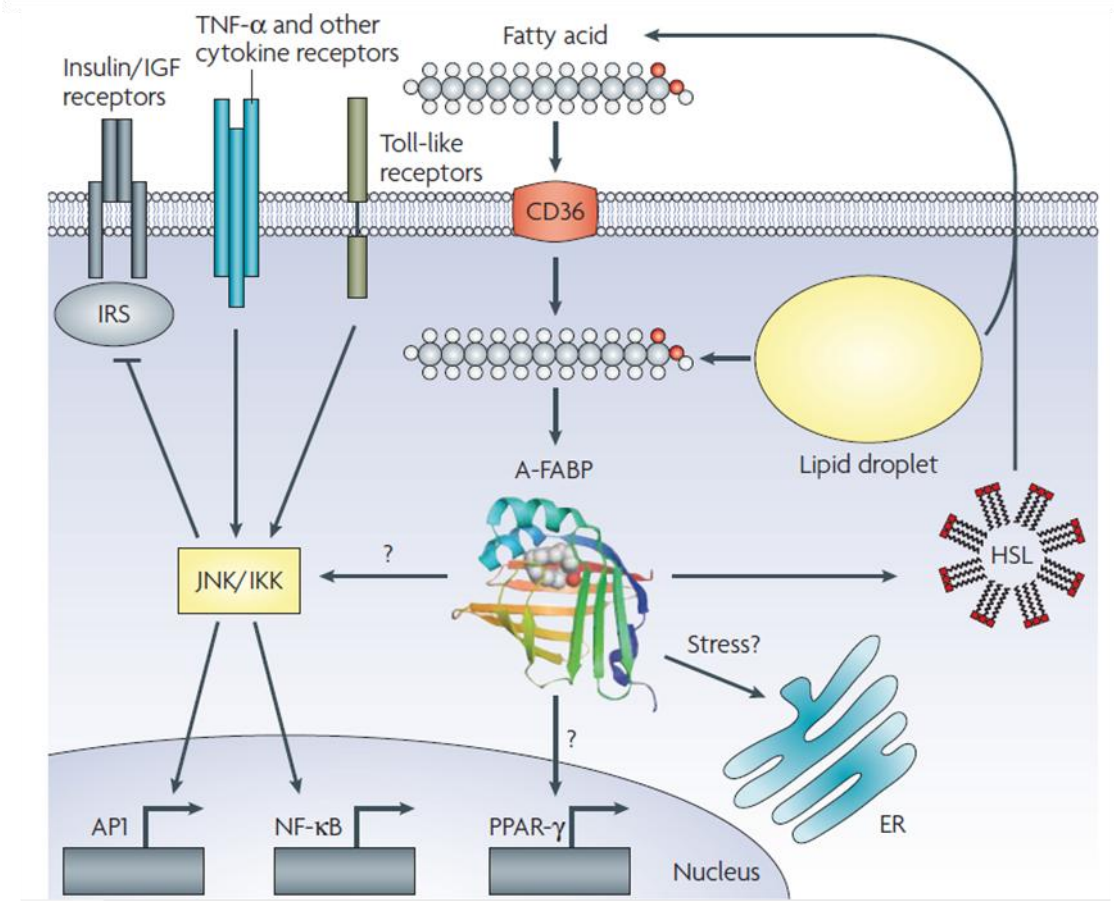


Figure 4.4: Overview of the general functions of FABP4. FABP4 (coloured molecular structure displayed in center of image) interacts with hormone-sensitive lipase (HSL) to affect its catalytic activity, and also integrates into several signalling networks that control inflammatory responses [potentially through the JNK/IKK (inhibitor of kappa kinase)] and insulin action in the adipocyte. FABP4 is able to regulate fatty acid influx, and is thought to be important in controlling lipid hormone production to regulate distant targets. Image taken from Furuhashi et al (2008).

The exact factors involved in the differentiation into mature adipocytes are not currently fully understood, but many different genes have been reported to be involved, with knockout studies demonstrating decreased adipogenesis when these genes are not transcribed or qRT-PCR demonstrating increased levels of gene expression during adipogenesis; CAAT/enhancer binding protein-α (C/EBPα), FABP4, insulin-like growth factor binding protein 2 (IGFBP2) resistin, adiponectin, and lipoprotein lipase, which simulates fatty acid binding and sequestering in mature adipocytes (Hartig et al.; Wu et al., 1999; Rival et al., 2004; Sekiya et al., 2004). FABP4 is also known as fatty acid binding protein (aP2) in other mammals and is a protein involved in the metabolism and intracellular transport of fatty acids in adipose cells; FABP4 is able to bind the fatty acids with a high affinity and re-localise them to other parts of

the cell (Gorbenko et al., 2006). An overview of the general functions of FABP4 is shown in **Figure 4.4**.

In addition to these roles, FABP4 performs the general role of fatty acid binding protein aiding in the transport of lipids to specific compartments in the cell; to lipid droplets for storage, the endoplasmic reticulum for signalling, trafficking and membrane synthesis, to the mitochondria or peroxisome for oxidation, to cytosolic or other enzymes to regulate their activity, to the nucleus for the control of lipid mediated transcriptional programs via nuclear hormone receptors, or other transcription factors that respond to lipids (Furuhashi and Hotamisligil, 2008).

Flow cytometry has been used to measure adipogenesis previously, using Nile red dye (Greenspan et al., 1985; Neubauer et al., 2004). Nile red is a selective fluorescent stain for intracellular lipid droplets at 528-570 nm, and has been coupled with flow cytometry to detect adipocytes through the accumulation of intracellular lipids within the cells (Greenspan et al., 1985; Smyth and Wharton, 1992; Dennis et al., 1999; Fink et al., 2004). Nile red is fluorescent in all organic solvents, but the hydrophobicity of the solvent can change the emission and excitation maxima by up to 60nm (Greenspan et al., 1985). Within intracellular lipid vesicles therefore, the excitation of Nile red occurs at 450-500 nm, and the emission at 528-570 nm.

Oil red-O dye is frequently used for demonstrating adipogenesis, and is widely used as a lysochrome to visualise intracellular lipid droplets (Pittenger et al., 1999; Jones et al., 2002; English et al., 2007; Platt and El-Sohemy, 2009). In addition to its use as a visual representation of adipogenesis, attempts have been made to increase the accuracy and use the amount of Oil red-O staining as a quantifiable indicator of fat. This has been done by visually grading and counting adipogenically differentiated MSCs (English et al., 2007), and also through the spectrophotometric analysis after Oil red-O is removed from the stained cells by isopropanol, and the absorbance measured at 415 nm (Platt and El-Sohemy, 2009).

4.1.1 AIMS AND OBJECTIVES

The aim of this chapter was to differentiate MSCs into adipose cells, and verify that the cell population obtained was adipose cells. In addition to this, a suitable method for quantifying the level of adipogenesis of the MSC population was sought. The objectives were therefore:

- To differentiate MSCs from four donors into adipose cells.
- To verify that the differentiated cell population were adipose cells.
- To assess the suitability of the following methods for measuring adipogenesis:
    1. Oil red-O staining and semi-quantitative scoring.
    2. Real time PCR for PPAR-ɣ and FABP4.
    3. Flow cytometry using intracellular antibody to FABP4 and Nile red.
    4. Fluorescent microplate assay using Nile red and DAPI.

4.2 MATERIALS AND METHODS

4.2.1 ISOLATION AND EXPANSION OF MSCS AND CONTROL SKIN FIBROBLASTS

Human bone marrow MSCs were isolated as described in Chapter 3 section 3.2.1.

Human skin fibroblasts were obtained from the American Type Culture Collection (ATCC) (Teddington, UK) and were expanded as described in Chapter 3 section 3.2.3.

4.2.2 ADIPOGENIC DIFFERENTIATION OF MSCS AND FIBROBLASTS

Adipogenic medium was made as described in Chapter 3 section 3.2.5. Adipogenic differentiation assays were performed in 6-, 24- and 48-well plates, using a constant cell seeding density of $4x10^4$ cells per cm^2. Adipogenic medium was changed twice weekly (½ media changes) during a 21 day time course, and samples were taken for analysis at different time-points on days 0, 3, 7, 14, and 21.

4.2.3 OIL RED-O STAINING FOR SEMI-QUANTITATIVE SCORING OF ADIPOGENESIS

Oil red-O staining was performed as described in Chapter 3 section 3.2.6.

The amount of fat in individual cells was scored semi-quantitatively using a grading scheme developed by English et al. (English et al., 2007). The level of adipogenesis was described by ranking 500 cells in a middle area of the well on their fat content. Ranks were allocated based on the percentage of cytoplasm occupied by fat globules: Grade 1; 0-24%, Grade 2; 25-49%, Grade 3; 50-74%, Grade 4; 75-100%. Semi-quantitative scoring was performed on BM 1, BM 2, and BM 3 donor MSCs, and fibroblasts, with final ranks representing mean values of triplicate wells.

4.2.4 QUANTITATIVE REAL-TIME PCR FOR PPAR-Χ AND FABP4 GENE EXPRESSION

Reagents

Sodium acetate solution (5M): 68.04 g sodium acetate (Fisher Scientific) dissolved in 80 ml dH_2O, adjusted to pH 5.2 using glacial acetic acid (Fisher Scientific). Volume made up to 100 ml using dH_2O.

Sodium citrate (1M): 29.4 g sodium citrate tribasic dihydrate (Sigma) dissolved in 100 ml dH_2O.

Guanidine isothionate solution (Sigma)

N-Lauryl sarcosine solution (5 %): 5 g N-Lauryl sarcosine sodium salt (Sigma) was added to 100 ml dH_2O.

β-mercaptoethanol (Sigma)

RNA lysis buffer was made by combining guanidine isothionate (1 ml) with N-Lauryl sarcosine solution (10 µl), sodium citrate solution (4 µl) and β-mercaptoethanol (5 µl). This solution was stored for up to 2 weeks at 4°C.

Acid phenol pH 5 (Sigma)

Chloroform isoamylalcohol 24:1 (red) (Sigma)

Ethanol 100 % (v/v) (ThermoFisher Scientific)

Ethanol 80 % (v/v) in dH_2O

Nuclease free water (Severn Biotech Ltd)

Method

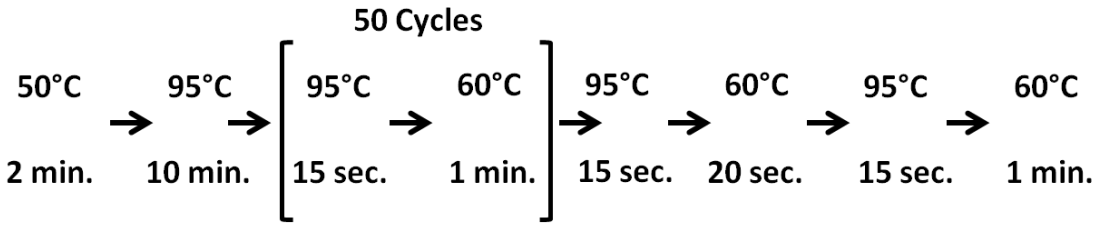
Adipogenic medium was removed from wells of a 6 well plate, and total RNA was isolated from cells using 500µl RNA lysis buffer per well. The lysates were stored in a 1.5 ml eppendorf at -80°C until chloroform-phenol extraction.

Sodium acetate (50 µl), acid phenol (500 µl) and chloroform isoamylalcohol 24:1 (100 µl) was added to the cells in RNA lysis buffer, and the eppendorf tubes were vortexed for 2-3 minutes before an incubation on ice for 10 minutes. This mixing and incubation step was repeated. The solution was centrifuged at 13,000 g in a microfuge for 20 minutes at 4°C. The top aqueous layer (~500 µl) was removed and added to a new 1.5 ml eppendorf tube, before the addition of an equal volume of propan-2-ol. The tube was then inverted several times to mix, before an overnight incubation at -20°C to allow the RNA to precipitate. The sample was then centrifuged at 13,000 g for 30 minutes at 4°C and the supernatant discarded. Ethanol (70 %, 500 µl) was added to each tube to wash the pellet, before centrifugation at 6500 g for 5 minutes at 4°C. The supernatant was removed, and the pellets air dried at room temperature for between 30-60 minutes before the RNA pellet was resuspended in 50 µl nuclease free water. The RNA was treated with 10U DNase I (Pharmacia Biotech AB) and 33U RNasin (Promega UK Ltd) at 37°C for 30 minutes, and at 65°C for 10 minutes to denature the nuclease.

The following procedure was used for creating cDNA; a 1 µg sample of RNA was incubated with 1 µl Oligo(dT) primers (Qiagen) and 12*µl nuclease free water (*volume adjusted so total reaction volume 20µl) before the sample was incubated at 75°C for 10 minutes. After 10

minutes, the reaction was placed on ice. A 1 µl volume of a 100 mM dNTPs set (Qiagen) and 4 µl reaction buffer (Qiagen) were added, and the mixture incubated at 42°C for 5 minutes, before 1 µl Superscript II (Invitrogen) was added and the reaction mixture incubated at 42°C for 2 hours. A final 15 minute incubation at 75°C inactivated the enzyme, and cDNA samples were stored at -20°C.

Real-time PCR was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems) in the presence of SYBR-green. The cycle used was as follows:



Primers were designed by Dr. S. Churchman using Primer Express 2 (Applied Biosystems) and supplied by MWG Biotech. Expression was normalized using a house-keeping gene GAPDH. Primer concentrations were optimized by Dr. S. Churchman and the sequences used are listed later on in this chapter in **Table 4.1**. MSCs from three different donor MSCs were used, and each for each time point the mean cycle threshold (Ct) of each gene of three technical replicates was used to calculate the relative gene expression. The Ct is defined as the number of cycles needed for the fluorescence levels to reach a specific threshold of detection. The Ct is inversely correlated with the amount of template cDNA present in the reaction.

To calculate the relative gene expression the Ct difference between the gene of interest and the housekeeping gene is first calculated. As qRT-PCR is an exponential process this Ct difference is then inverse logged to calculate the relative levels of gene expression when compared to the housekeeping gene ($2^{-\Delta Ct}$).

4.2.5 Flow cytometry for FABP4 and Nile Red staining

On days 0, 3, 7, 14, and 21 adipogenic medium was removed from wells of the 6 well plate and cells were washed twice with PBS.

For Nile red staining cells were harvested by treatment with 1 ml trypsin, 0.5 % (10x) with EDTA (Invitrogen), diluted to 1x in PBS for 5 minutes until cells had detached from the tissue culture plastic. The detached cells were added to 15 ml PBS in a universal, and centrifuged for 10 minutes at 150 g. The supernatant was then discarded and the pellet washed in 10 ml PBS. The cells were centrifuged at 150 g, and the above step repeated, before the pellet was

suspended in 200 µl PBS. The cell solution was transferred to a 96 well U-bottomed plate for staining.

Stock Nile red (Sigma) solution (0.1 mg.ml^{-1}, prepared in methanol) was added to cells at a final concentration of 0.05 µg.ml^{-1} (1:2000 dilution in PBS) (Greenspan et al., 1985) and incubated at 4°C for 20 minutes in the dark. The plate was centrifuged at 13,000 g before the supernatant was removed. Cells were washed in 200 µl BD FACSflow™ again before centrifugation at 13,000 g. The supernatant was discarded, and the pellets resuspended in 200 µl BD FACSflow™ before the fluorescent intensity was measured using the flow cytometer (Becton Dickinson FACScan). Nile red fluorescence emission in adipogenically-differentiated MSCs was detected on the FL2 channel.

FABP4 protein was detected using a purified goat anti-human FABP4 antibody (R & D Systems) at 1:20 dilution in PBS. For intracellular staining with FABP4, trypsinised cells were initially fixed in 100 µl of Intrastain reagent A (Dako). FABP4 antibody was added simultaneously with 100 µl of permeablising reagent/Intrastain reagent B (Dako) for 15 minutes at RT in the dark. After two washes with 200 µl PBS and 13,000 g, cells were incubated with 200 µl biotinilated rabbit anti-goat secondary antibody (1:400 dilution in PBS, Dako) for 15 minutes at RT in the dark. Following 2 further washes in PBS the binding was visualised using streptavidin-FITC (1:50 dilution, BD Sciences). After 2 final washes, the fluorescence were analysed on the FL1 channel with 10,000 events being collected for each sample.

4.2.6 Measure of adipogenic differentiation using Nile red/DAPI staining on a 48 well plate

Reagents

Nile red (1:2000 dilution): 1 µl stock into 100 µl PBS, 4 µl of this solution was diluted into 80 µl PBS.

Paraformaldehyde (10 % v/v): 2 ml 40% (v/v) formaldehyde (Gentamedical) and 8ml PBS.

Saponin (0.2 % w/v): 0.2 g saponin powder (Sigma) in 100 ml PBS.

Method

MSCs and fibroblasts (4x10^4/well) were seeded in a 48 well plate (Nunc) and cultured for 21 days in adipogenic or control NH medium. On days 0, 3, 7, 14, and 21 the level of adipogenesis was determined utilising a luminescent plate reader (LB940 Multilabel Reader Mithras; Berthold Technologies) with each treatment and each day timepoint having six replicate wells.

Culture medium was removed from wells and cells washed twice with PBS. Paraformaldehyde (200 µl) was added to the wells for 30 minutes, before the cell layer was washed with 200 µl PBS. The PBS was removed, and 200 µl PBS was added to each well. A 200 µl volume of crystal violet solution (Sigma) was added to the gaps in between the wells of the plate to minimise light reflection. The background fluorescence was read using 4',6-diamidino-2-phenylindole, DAPI (355/460) and Nile red (485/540) filter sets as per the manufacturer's instructions. These filter sets were used as they provided the closest match to DAPI, excitation at 358 nm, and emission at 461 nm, and Nile red, excitation at 450-500 nm, and emission at >528 nm. Wells were spot read in a 10x10 pattern per well. The PBS was removed from the wells, and 200 µl saponin (0.2% w/v in PBS) was added along with DAPI (Invitrogen) at a final concentration of 1 $\mu g.ml^{-1}$, and Nile red at a final concentration of 1 $\mu g.ml^{-1}$. The plate was then wrapped in foil and incubated at RT for 15 minutes. Following three washes with PBS, 200 µl PBS was added, and plates were again read as above.

Plates were wrapped in foil and stored at 4°C for up to 48 hours if fluorescent microscopy was required. A ratio of adipogenesis was calculated by taking the mean fluorescence reading of the repeats for both Nile red and DAPI stained cells and creating a ratio of Nile red:DAPI fluorescence.

4.2.7 Statistical Correlations

In order to determine whether the methods used for measuring adipogenic progression were comparable, R values were calculated using Spearmans Rank correlation and a two tailed p-value in Graphpad Prism 5™.

4.3 RESULTS

4.3.1 SEMI-QUANTITATIVE SCORING OF ADIPOGENESIS IN MSCS USING OIL RED-O STAINING

Initially the most common method for staining for adipogenically-differentiated MSCs was used (Oil Red staining) and differentiation quantified using a grading system developed by English *et al*, (2007). In this method 500 cells in a central area of the dish were identified using an Oil red-O stain, and the level of adipogenic progression was ranked (1-4) based on the percentage of cytoplasm occupied by fat (**Figure 4.5**).

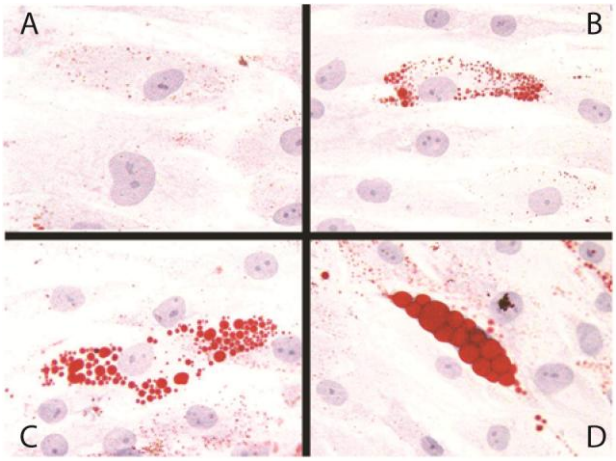


Figure 4.5: Oil red grading scheme: The Oil red grading scheme rates cells based on their fat content; Grade 1 (0-24%), Grade 2 (25-49%), Grade 3 (50-74%), and Grade 4 (75-100%). Images labelled A-D represent each of the Grades; A=Grade 1, B=Grade 2, C=Grade 3, and D=Grade 4. 500 cells in a central area of the dish were identified using an Oil red-O stain, and the level of adipogenic progression was ranked. This process was performed 3 times per well.

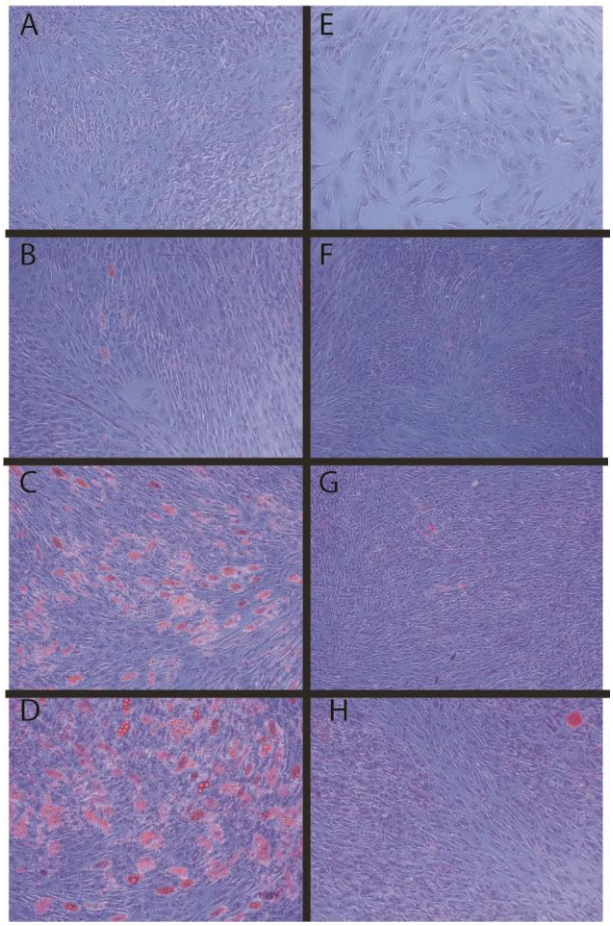


Figure 4.6: Adipogenesis of MSCs and fibroblasts stained with Oil red. Cultures of BM1 MSCs (left panels) differentiated over 21 days into fat and stained with Oil red (A=Day 3 BM1, B=Day 7 BM1, C=Day 14 BM1, and D=Day 21 BM1). Right panels fibroblasts also grown in adipogenic differentiation medium over the same timecourse (E=Day 3 fibroblasts, F=Day 7 fibroblasts, G=Day 14 fibroblasts, and H=Day 21 fibroblasts).

In these experiments MSCs from 3 donors and negative control fibroblasts were grown in adipogenic medium for 21 days and scoring was performed on days 0, 3, 7 14 and 21 post-induction. BM1 MSCs differentiated over 21 days into fat and stained with Oil red (A=Day 3 BM1, B=Day 7 BM1, C=Day 14 BM1, and D=Day 21 BM1) are shown in **Figure 4.6** (left panel), and right panels show fibroblasts also grown in adipogenic differentiation medium over the same timecourse.

Fibroblasts gradually accumulated cells with Grade 1 fat content (**Figure 4.7A**); however they were unable to progress to higher grades and to increase fat accumulation (**Figure 4.7B**). In all MSCs differentiation had similarly begun as a gradual accumulation of Grade 1 cells (by day 7) (**Figure 4.7A**). In contrast to fibroblasts however, MSCs continued to accumulate fat in their cytoplasm and by days 14-21 contained cells with very high fat content (Grades 3-4) (**Figure 4.7B**).

Altogether these experiments confirmed that although fibroblasts had some very low capacity for adipogenesis, they were clearly inferior to MSCs. Secondly, adipogenic progression in MSCs from the same donor was heterogenous, with some cells progressing to Grades 3-4 and others remaining to be Grade 1. Finally, donor-to-donor differences were observed in the rates and the amounts of adipogenesis in MSCs, with Donor BM1 exhibiting less adipogenesis compared to the other two donors (the latter easily progressed to grades 3-4).

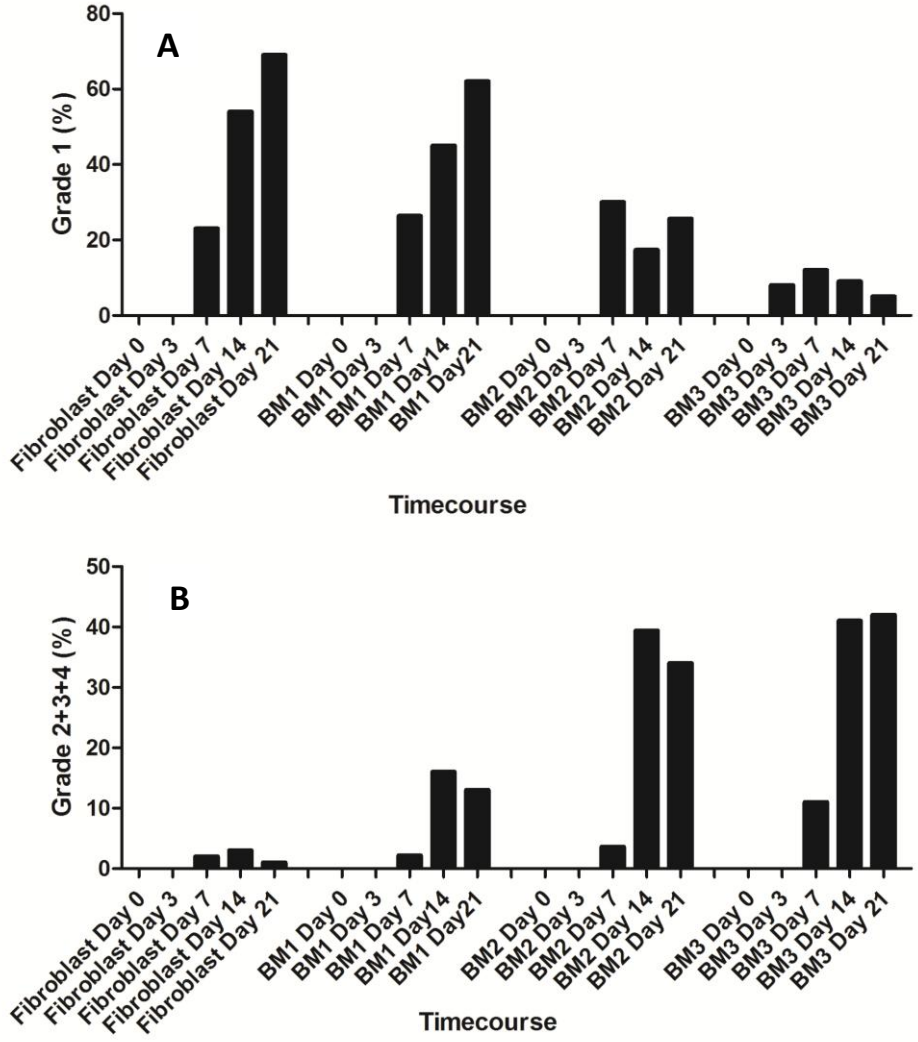


Figure 4.7: Percentage of cells graded based on lipid vacuole accumulation within the cytoplasm. Figure A the percentage of Grade 1 cells within the population. Figure B the number of Grade 2+3+4 (more mature fat cells).

4.3.2 QUANTITATIVE CHANGES IN PPAR-γ AND FABP4 EXPRESSION IN MSCS UNDERGOING ADIPOGENESIS

Adipogenesis-specific peroxisome proliferator activated receptor (PPAR-γ) and the late marker of adipogenesis, fatty acid binding-protein 4 (FABP4) have been previously shown to closely reflect adipogenic progression of MSCs (Janderova et al., 2003; Neubauer et al., 2004; Sekiya et al., 2004; Blum and Benvenisty, 2008). The next aim was to determine whether PPAR-γ and FABP4 mRNA were upregulated in adipogenically-differentiated MSCs and whether these changes paralleled to morphological fat accumulation within the cells. Quantitative real-time PCR was performed on RNA extracted on days 0, 3, 7, 14 and 21 post-induction. An example amplification plot of relative fluorescent intensity against Ct is shown in **Figure 4.8** of day 7 GAPDH, PPAR-γ and FABP4 gene expression. The changes in relative expression levels of the genes were normalised to GAPDH **(Figure 4.9)**. The primer sequences used are displayed in **Table 4.4**.

Primer	Sequence	Concentration
PPARγ F:	cac aag aac aga tcc agt ggt tg	500nM
PPARγ R:	gag gct tat tga gag ctg agt ctt ct	500nM
FABP4 F:	cca taa aga gaa aac gag agg atg at	1000nM
FABP4 R:	tgg aag tga cgc ctt tca tga	1000nM
GAPDH F:	aac agc gac acc cac tcc tc	500nM
GAPDH R:	cat acc agg aaa tga gct tga caa	500nM

Table 4.4: Primer sequences and concentrations used for qRT-PCR.

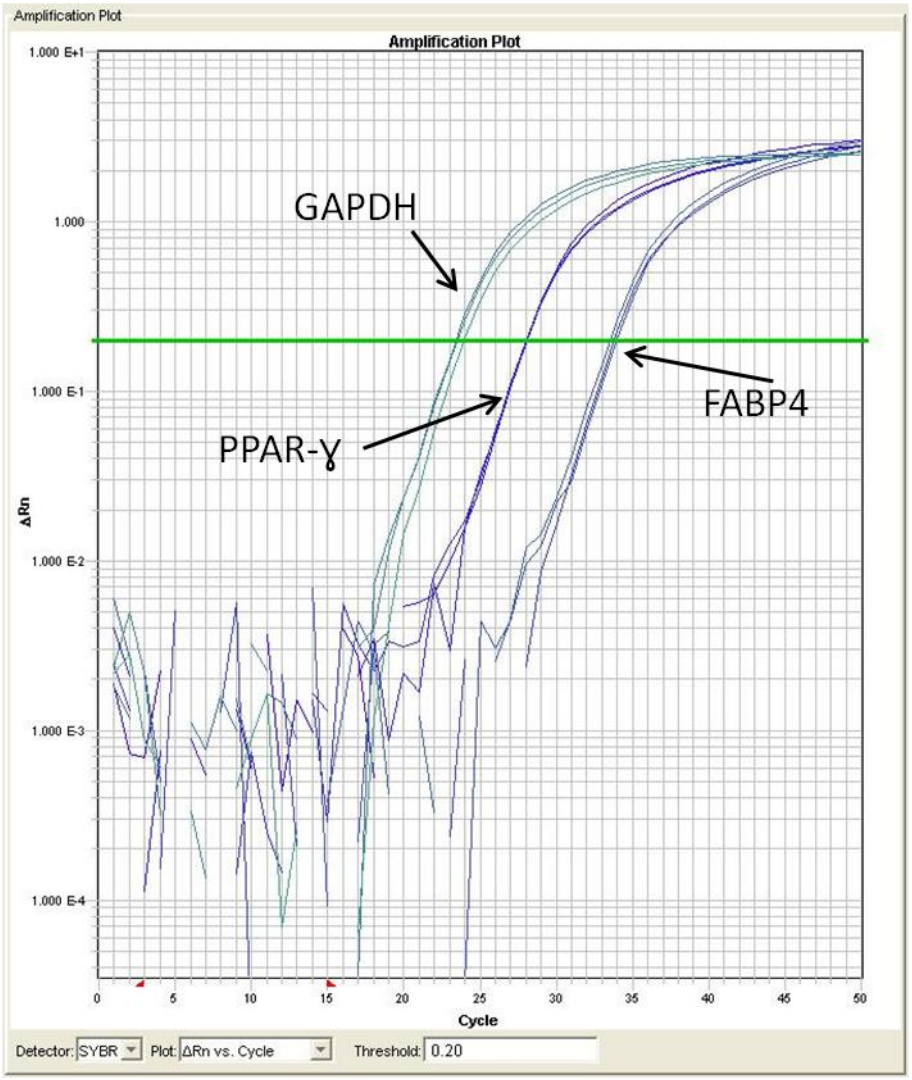


Figure 4.8: The amplification plot of relative fluorescent intensity against Ct for day 7 displaying GAPDH, PPAR-ɣ and FABP4 respectively (n=3).

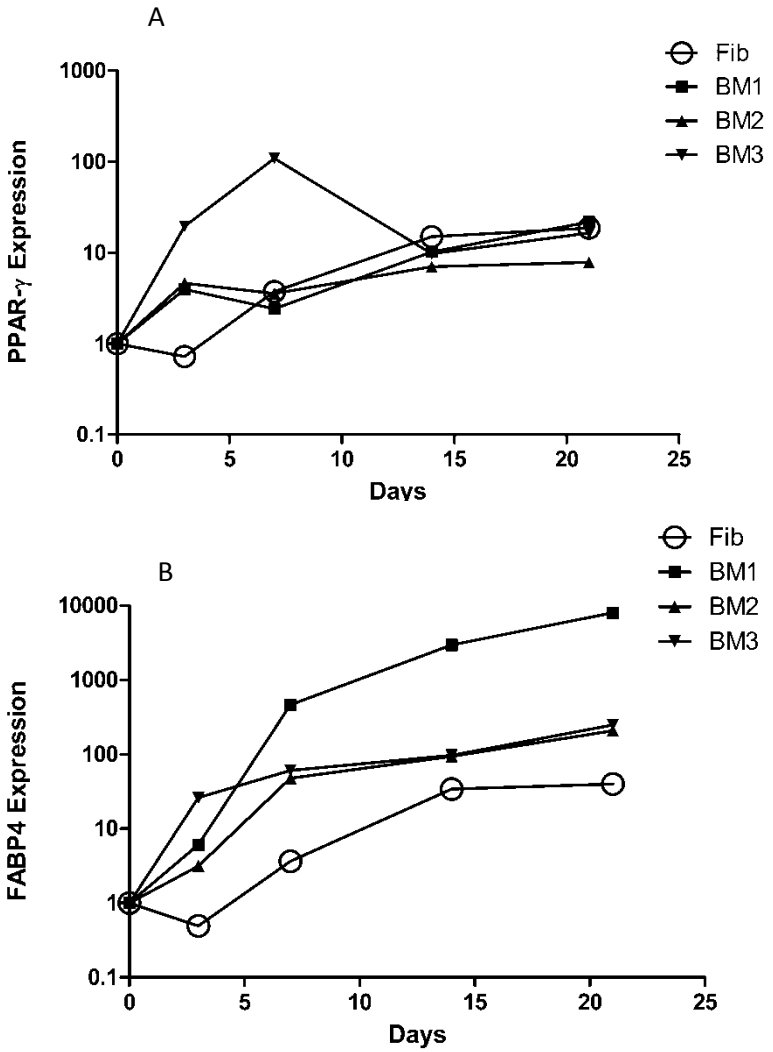


Figure 4.9: Quantitative real-time polymerase chain reaction (qRT-PCR) as a method to measure adipogenesis. Human MSCs and fibroblasts were cultured in adipogenic medium for 21 days. Peroxisome proliferator activated receptor-gamma (PPAR-ɣ) and fatty acid binding protein-4 (FABP4) expression was determined by qRT-PCR performed on RNA taken from cells on days 0, 3, 7, 14 and 21. Levels of gene expression are normalised to housekeeping gene GAPDH and displayed as fold increase over day 0.

The fold increase of PPAR-γ gene expression compared to Day 0 was slightly down-regulated in fibroblasts on day 3, before increasing to approximately 5 fold higher on day 7, 20 fold higher by day 14, and 30 fold on day 21. BM1 PPAR-γ gene expression increased 5 fold by day 3, decreased to 3 fold by day 7 before increasing to 10 fold by day 14, and 20 fold by day 21. BM2 followed a similar trend with expression increasing 5 fold by day 3 before decreasing slightly by day 7, before increasing to 8 fold on days 14 and 21. BM3 PPAR-γ gene expression was up-regulated 30 fold by day 3, increasing to 100 fold by day 7. This decreased to 10 fold by day 14, and 20 fold by day 21.

The fold increase of FABP4 gene expression in fibroblasts compared to Day 0 was slightly down-regulated on day 3, before increasing to approximately 5 fold higher on day 7, and 50 fold higher by day 14 and 21. BM1 FABP4 gene expression increased 8 fold by day 3, 700 fold by day 7 before increasing to 4000 fold by day 14, and 8000 fold by day 21. BM2 followed a similar trend with expression increasing 4 fold by day 3 before increasing to 50 fold by day 7, 90 fold on days 14, and 200 fold by day 21. BM3 FABP4 gene expression was up-regulated 50 fold by day 3, increasing to 70 fold by day 7. This remained the same on day 14, increasing to 200 fold by day 21.

These data showed that PPAR-γ expression generally increased during adipogenesis, however the changes were relatively small and did not reflect adipogenic transformation in MSCs, clearly visible using Oil red staining. In contrast, FABP4 expression rose gradually up to day 21 and generally paralleled microscopic fat accumulation. Nevertheless, the results did not exactly match, as donor BM1 displayed the highest up-regulation in FABP4 mRNA (800-fold) whilst its fat content was the lowest (mainly Grade 1).

4.3.3 Monitoring FABP4 protein accumulation and Nile red staining following MSC adipogenic progression by flow cytometry

Since FABP4 appeared to be a suitable adipogenesis marker by qPCR, the next aim was to monitor FABP4 protein accumulation in MSCs using flow cytometry. This was performed by intracellular labelling with FABP4 antibody on days 0, 3, 7, 14 and 21 post-induction (**Figure 4.10A**, right histograms). The side scatter (SSC) represents the granular intensity of the sample.

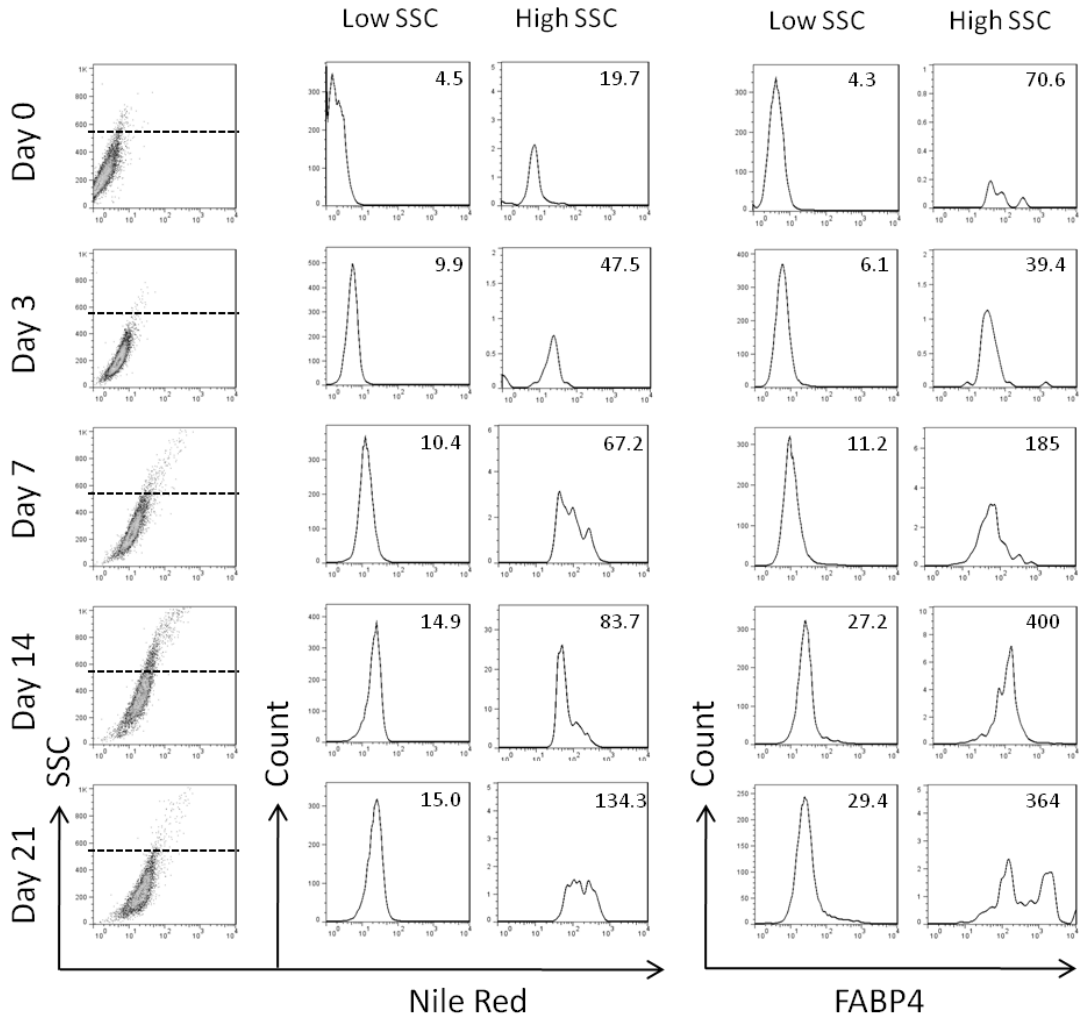


Figure 4.10: Flow cytometry of MSCs cultured in adipogenic medium. (A): BM1, BM2, and BM3 MSCs were cultured in adipogenic medium for 21 days. On days 0, 3, 7, 14 and 21 cells were stained with either FABP4 (right histograms), or with Nile red (centre histograms). The representative dot plots (far left) show the scatter characteristics of the cells. It shows that a population of more granular cells with high side scatter (SSC) increased in number until day 14. The dashed lines shown on the dot plots represent gating used, with events above this line gated into the high side scatter (SSC) category, and below into the low SSC category. The numbers in the top right of the histograms are the geometric means of the relative fluorescent intensities of the cells. This number is an average calculated from the means of BM1-3 in Nile red staining, and is donor BM1 in FABP4 staining. Data shown is representative of 10,000 events per experiment.

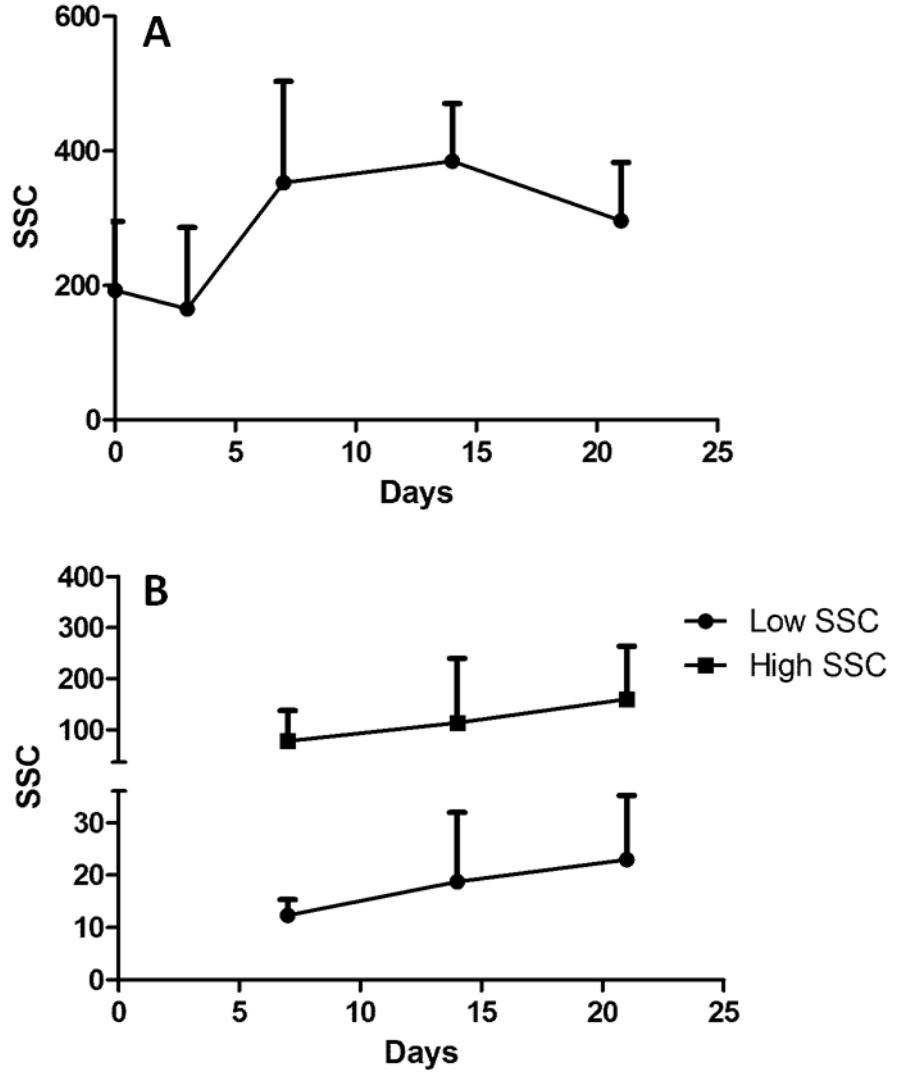


Figure 4.11: Line graphs of side scatter variation following timecourse progression. A) Line graph of SSC shows the increasing granularity of the total MSC population as number of lipid vacuoles in cytoplasm increases as the timecourse progresses. **B)** Split axis line graph compares the granularity of both populations of gated cells, low and high SSC, demonstrating increasing granularity throughout the timecourse. Days 0 and 3 omitted due to the high error encountered when reading fluorescent intensity on the flow cytometer (when gated <10 events were collected for high SSC).

Previous studies have identified marked changes in cell size/granularity profiles upon their adipogenic induction with larger cells being postulated to be more differentiated (Lee et al., 2004). Fatty acid binding protein-4 accumulation was therefore investigated in differentiating MSCs in larger cells (dotted line on dot plots represents gating) and the majority of smaller cells. Indeed, a gradual accumulation of larger cells as the time-course progressed was observed, however they peaked on day 14, not on day 21, (**Figure 4.10**, far right panels).

The shift to the right was observed on the histograms for both the low SSC and high SSC cells, however it was obvious that larger cells contained up to 20-fold more FABP4 at every time-point (**Figure 4.10, far right panels**). Interestingly, smaller cells were not resistant to adipogenesis and also accumulated FABP4 during the whole duration of adipogenesis, albeit at a reduced amount (**Figure 4.10, right panels**). These data clearly confirmed the existence of cellular heterogeneity within the same adipogenesis-driven MSC cultures, first observed by morphological analysis with Oil red. Importantly, flow cytometry for FABP4 and semi-quantitative scoring with Oil red displayed excellent correlation with each other (Spearman R value of $r = 0.978$, $p = <0.0001$, **Figure 4.14**).

Similar trends were observed if Nile Red staining for lipids was used for flow cytometry instead of FABP4 (**Figure 4.10**, centre histograms). This shift in Nile red fluorescence was increased as the time course progressed, with high SSC cells having similarly higher amounts of Nile red fluorescence compared to low SSC cells.

SSC increased throughout the timecourse, indicating an increase in the granularity of the cells, produced by the accumulation of lipid droplets within the cells (**Figure 4.11A**). Relative fluorescent intensity (geometric mean) of high and low SSC populations stained with Nile red were compared, and accumulation of lipid occurs in parallel within both populations, albeit at a much higher level in high SSC cells (**Figure 4.11B**).

The geo-metric mean for days 0 and 3 are omitted, as too few cells were counted to give an accurate representation of fluorescence in the high SSC gate.

Nile red dye and intracellular FABP4 antibody labelling by flow cytometry demonstrated excellent correlation with each other $r = 0.918$, $p=<0.0001$, **Figure 4.14**. Additionally, Nile-red based flow cytometry showed good correlation with morphological semi-quantitative scoring $r = 0.907$, $p = <0.0001$, **Figure 4.14**.

4.3.4 QUANTIFICATION OF MSC ADIPOGENIC PROGRESSION USING MICROPLATE ASSAY WITH NILE RED

Semi-quantitive scoring provided a visual method of quantification of adipogenesis, but it was subjective and very time consuming. qRT-PCR was similarly time-consuming, and measured mRNA levels instead of protein/lipid. Flow cytometry correlated well with morphological lipid accumulation in MSCs, however there was a danger of losing mature floating fat cells during cell trypsinization/centrifugation steps. The utility of a Nile red-based fluorescence microplate assay to measure adipogenesis, using DAPI staining for cell nuclei for normalization was therefore explored (**Figure 4.12**).

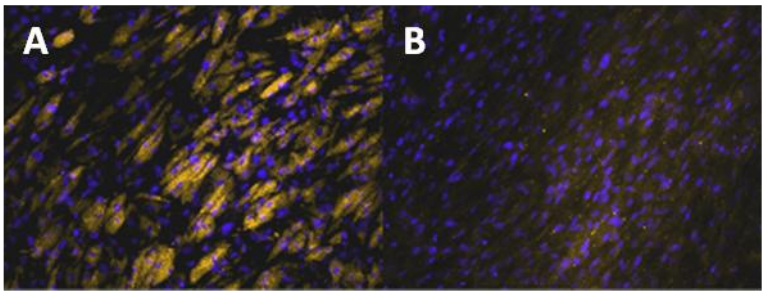


Figure 4.12: Nile red and DAPI staining of MSCs. MSCs and fibroblasts were grown for 0, 3, 7, 14, and 21 days in adipogenic medium, and stained using Nile red dye, and DAPI. Representative images of MSCs (A) and fibroblasts (B) on day 21 are shown. Intracellular lipid droplets in cells stain yellow/gold, and the nucleus of cells is stained blue. Images were taken using an Olympus IX71 inverted microscope using Cell^B image capture software (Olympus) and are x200 magnification.

MSCs or fibroblasts were cultured in adipogenic and normal expansion medium in wells of a 48 well plate. On days 0, 3, 7, 14, and 21 levels of adipogenesis in cells were measured as a ratio of cell number compared to fat content using DAPI or Nile red dye staining, respectively, and normalised to day 0. In order to see if adipogenesis could be measured using DAPI or Nile red only, levels of fluorescence for DAPI only and Nile red only were calculated and normalised to day 0. Non-adipogenic controls were fibroblasts grown in adipogenic medium (**Figure 4.13**).

Levels of DAPI staining remained fairly constant in fibroblasts, and also fluctuated in MSCs after day 3. Nile red staining in fibroblasts remained very low and showed a greater increase in BM 1 - 3 MSCs. Levels of DAPI staining were varied and did not appear to correlate with levels of Nile red staining observed, for example BM demonstrated increasing staining for Nile red but relatively low staining for DAPI, whereas BM 4 stained relatively strongly for DAPI but not Nile red. Levels of adipogenesis were normalised to cell number using the florescent intensity of DAPI staining, and a ratio of Nile red/DAPI was used to measure adipogenesis. Nile red to

DAPI ratios demonstrated minimal adipogenesis in fibroblasts grown in adipo-inductive medium but an increase in adipogenesis to day 21 in BM 1-4 (**Figure 4.13)**.

Both Nile red microplate assays and morphological scoring with Oil Red significantly correlated with each other; r = 0.866, p = <0.0001, **Figure 4.14**.

The adipogenesis of all 4 BM donors and fibroblasts was measured using the fluorescent microplate assay. BM1, 2, and 3 demonstrated adipogenic ratios of increasing to ~10, but BM4 exhibited the highest adipogenesis with ratios to 20 and above (**Figure 4.13**). It was therefore decided that BM4 would be used in later assays where high levels of adipogenesis were required (Chapter 7).

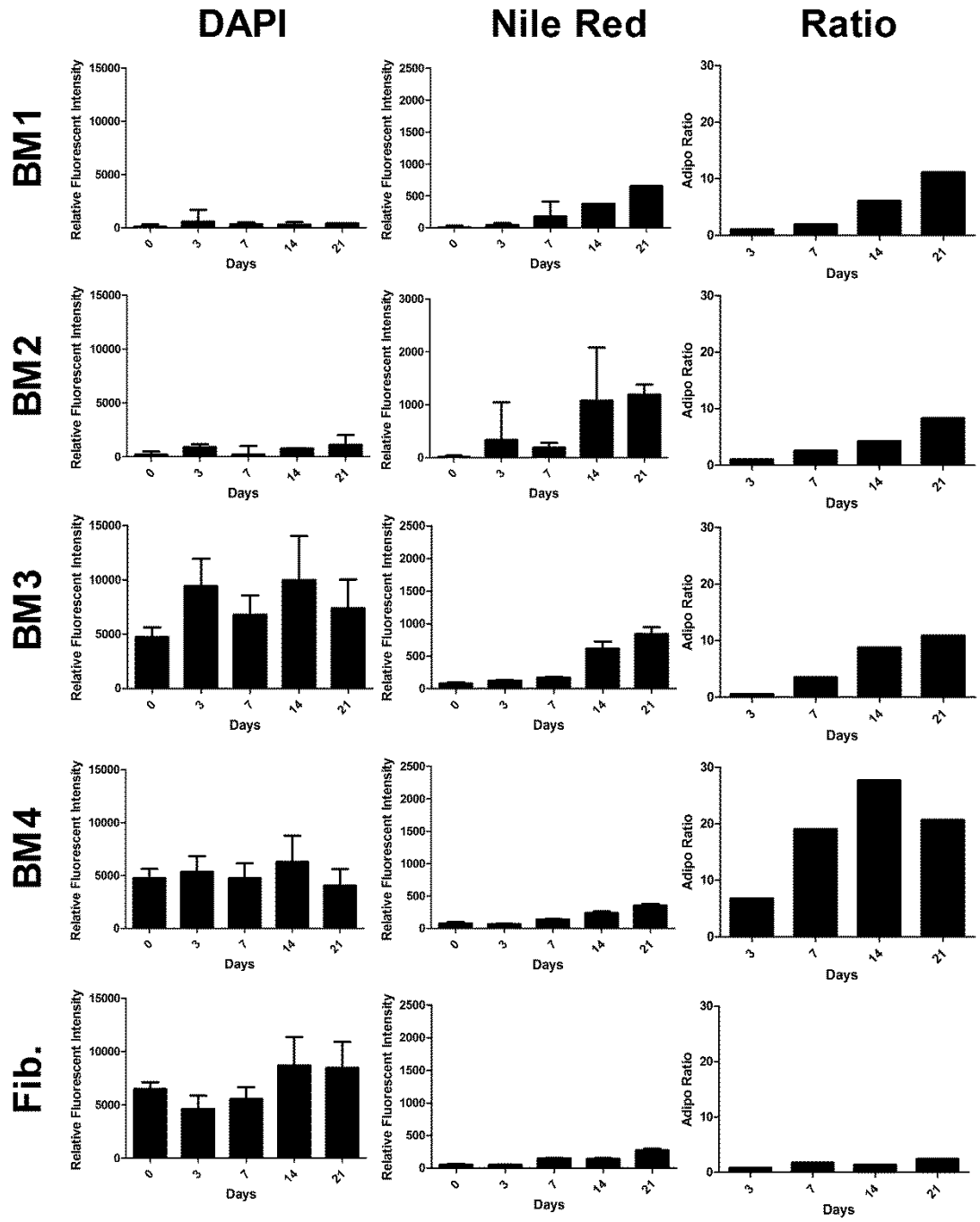


Figure 4.13: Microplate reader as a method of quantifying adipogenesis. Levels of fluorescence for DAPI and Nile red were calculated and normalised to day 0 (n=3) for BM MSCs 1-4 and fibroblasts cultured in adipogenic medium. There was minimal adipogenesis in fibroblasts cultured in adipo-inductive medium, whereas Nile red to DAPI ratios generally demonstrated an increase in adipogenesis to day 21 in MSCs. X-axis values are day timepoints, and the mean values of fluorescence were calculated using Microsoft Excel™ before these were processed into ratios. Error bars shown are ± 95% confidence limits

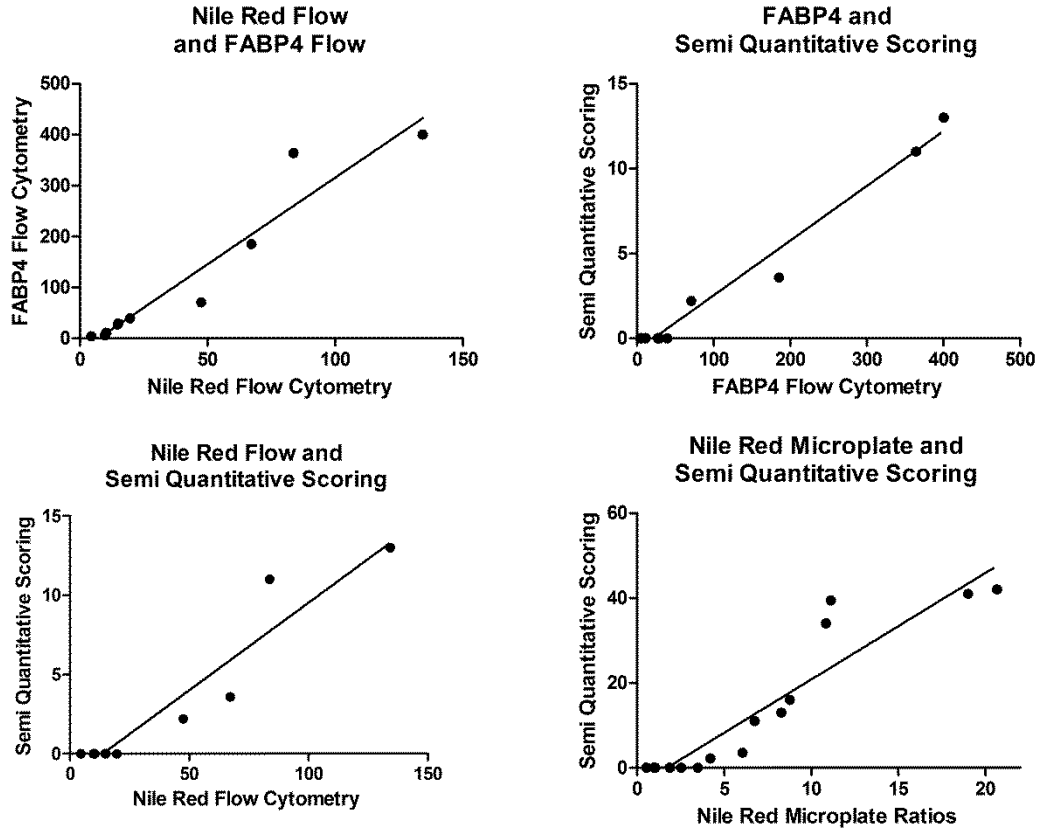


Figure 4.14: Correlation graphs for methods to evaluate adipogenesis in MSCs. Correlation graphs and linear regression analysis were plotted and performed using Graphpad Prism™ FABP4 and Nile red flow cytometry (top left; r^2=0.978), semi-quantitative scoring and FABP4 flow cytometry (top right; r^2=0.918), semi-quantitative scoring and Nile red flow cytometry (bottom left; r^2=0.907) and semi-quantative scoring and Nile red microplate ratios (bottom right; r^2=0.866).

4.4 DISCUSSION

In this chapter several of the previously used methods to measure the adipogenesis of MSCs were compared: semi-quantitative staining with Oil red-O dye, quantitative real-time PCR of fat markers, flow cytometry, and Nile red staining. This was to verify that the population of MSCs was differentiating into adipose cells, and in order to develop a suitable method for quantifying this level of adipogenesis.

The most commonly used staining method for adipogenically-differentiated MSCs (Oil red-O staining was initially used) and differentiation quantified using a grading system developed by English *et al,* (2007). Alternative methods for quantifying the amount of Oil red-O dye present in stained samples are available (such as the absorbance measurement of Oil red-O dye staining the lipid vesicles within cells), but only give an overall level based on total dye content in a sample (Platt and El-Sohemy, 2009). The Oil red-O dye binds to neutral triglycerides and lipids present in cells and the greater the amount of lipids, the greater the volume of staining. Interestingly, semi-quantitative scoring of fibroblasts grown in adipogenic medium suggested that the fibroblasts exhibited a low level of adipogenic differentiation capacity. However, they did not progress into the larger fat cells, with very few Grade 2-4 fat cells present. For MSCs, Oil red-O staining demonstrated an increase in both the percentage of fat-laden cells and the amount of fat per cell during the 21-day time course. Although this method provided a visual method of quantification, it was subjective and very time consuming and as such it was not suitable as a higher throughput method for the quantification of adipogenesis. Morphological analysis using Oil red-O staining showed heterogeneity in the same adipogenesis-driven MSC cultures, with not all cells within the assay differentiating into adipocytes at the same rate, and a subset of the cells failing to differentiate into fat cells.

For quantitative real-time PCR, two genes associated with adipogenesis were selected; peroxisome proliferator activated receptor (PPAR-γ) and the late marker of adipogenesis, fatty acid binding-protein 4 (FABP4). Both of these genes have been shown to increase in expression as MSCs undergo adipogenesis (Janderova et al., 2003; Fink et al., 2004; Neubauer et al., 2004; Sekiya et al., 2004; Blum and Benvenisty, 2008). In contrast to previously published data (Janderova et al., 2003; Sekiya et al., 2004), although PPAR-γ expression generally increased during adipogenesis the changes were relatively small and did not reflect visible adipogenic differentiation in the MSCs. In agreement with previous studies (Sekiya et al., 2004; Boucher et al., 2009) FABP4 expression rose gradually up to day 21. It was noted however that increased expression of FABP4 did not always translate into higher level of visual fat content.

Altogether, FABP4 PCR was highly quantitative and able to track adipogenesis on a transcriptional level, it was time consuming and therefore not considered suitable for a reliable high-throughput measurement of adipogenesis.

To further evaluate the use of FABP4 as a suitable marker for adipogenesis, FABP4 protein accumulation in MSCs was monitored using flow cytometry. Flow cytometry provides information on intensity of staining, size of cells, and cell number. Fatty acid binding protein-4 accumulation in differentiating MSCs was investigated and a gradual accumulation of more granular cells (high SSC) was observed as the time-course progressed, correlating with increasing FABP4 levels in these cells.

Nile red dye has previously been used in flow cytometry to stain adipocytes (Greenspan et al., 1985; Smyth and Wharton, 1992; Dennis et al., 1999; Fink et al., 2004). It is a selective fluorescent stain for intracellular lipid droplets at 528-570nm. When Nile red staining for lipids was assessed by flow cytometry, a shift in intracellular Nile red fluorescence increased as the time course progressed, with higher SSC cells having similarly higher amounts of Nile red fluorescence compared to lower SSC cells.

Staining with both antibodies to FABP4 and Nile red showed similar trends. Both of these methods resulted in the higher mean fluorescence intensity of the higher SSC, more positively staining population of cells peaking on day 14. This population of cells decreased in size from day 14 to day 21. This was in contrast to a previous study which showed an increase in staining as the timecourse progressed, including on the final day 28 (Smyth and Wharton, 1992). This trend was not observed visually when the cells were viewed microscopically, and it is hypothesized that the observed reduction in the larger, more mature fat cells was due to the loss of these cells during the wash steps required during preparation for flow cytometry, due to the increased buoyancy of the cells. Data for high SSC cells for days 0 and 3 was omitted as there were too few cells gated (as there was very little to no lipid accumulation within the cells).

Overall, semi-quantitive scoring provided a visual method of quantifiying adipogenesis, but it was subjective and very time consuming. qRT-PCR was similarly time-consuming, and measured mRNA levels instead of protein or lipid directly. Flow cytometry correlated well with morphological lipid accumulation in MSCs, however there was a danger of losing mature floating fat cells during the preparation steps. Since these methods did not provide robust and time efficient assays for quantifying adipogenic differentiation we explored the utility of a Nile red-based fluorescence microplate assay to measure adipogenesis (Jaiswal et al., 2000). This assay used intracellular Nile red fluorescence for lipid accumulation and DAPI staining for cell

nuclei for normalization. The average fluorescent intensity for DAPI and Nile red for each well was measured, and a ratio of adipogenesis determined based on the number of cells present (DAPI) to amount of intracellular Nile red staining. MSCs stained only with DAPI have increased fluorescence over the timecourse, demonstrating that proliferation continues, even when differentiating. Nile red can be used to measure adipogenesis as a single dye, but due to the proliferation of cells a ratio using Nile red and DAPI together is preferred.

The fluorescent microplate assay provided both highly quantitative measurements and fluorescent images of fat droplet distribution per cell, allowing a numbered measurement to be assigned to differentiated cultures based on their level of adipogenesis, with visual verification of adipocyte content. The results obtained demonstrated the need to normalise the staining of intracellular lipids to cell number to obtain a measured value that takes into account not only levels of adipogenesis, but how this relates to the total cell number present (to distinguish between low cell numbers that are very adipogenic, or a high cell number that are weakly adipogenic).

Previous studies have demonstrated the differentiation heterogeneity of MSCs from different donors undergoing osteogenesis (Phinney et al., 1999; Siddappa et al., 2007). During the timecourse and throughout the various methods employed it was also observed that this variation between donors existed for adipogenesis.

There was strong correlation between all the methods used in this study (Spearmans R-values were between R=0.866-0.978). As Nile red is a chemical dye it is a more cost effective alternative to antibodies and the microplate assay was also found to be the most efficient and simple to perform on a larger volume of samples. Using this method allowed MSCs with greater adipogenesis to be selected for further experiments, where a higher grade of differentiation would be beneficial. It was decided that BM4 would be used in later assays in Chapter 7 where a high degree of adipogenesis was required.

Chapter 5: THE OPTIMISATION OF THE LYMPHOCYTE TRANSFORMATION ASSAY

5.1 INTRODUCTION

An early goal of cellular immunologists was to develop *in-vitro* assays as alternatives to the then only *in-vivo* methods such as skin testing and allograft rejection in small animals. The solution that was developed was the lymphocyte transformation or proliferation assay. For example, if a lymphoid cell population isolated from a donor capable of transferring a delayed type hypersensitivity (DTH) reaction to tuberculin is cultured together with the antigen, the antigen specific lymphocytes will be stimulated to divide (Kimball, 1990). Radioactive thymidine (tritiated 3H) added to the culture medium will be incorporated into the DNA of the cells undergoing mitosis and the levels of radioactivity incorporated into DNA will be indicative of the degree of response. This method, the lymphocyte transformation assay, was the original method developed to study cell mediated immunity *in-vitro*.

It was also shown in the early 1960's that if periphal blood mononuclear cells (PBMCs) from two different individuals (genetically not identical) were cultured together, the cells would stimulate each other and generate a large number of lymphoid blast cells *in-vitro* (Bain et al., 1964). It was discovered that the strongest reaction occurred (the largest number of cells produced) using mixtures of lymphocytes that had high discrepancy at the major histocompatability complex. From its initial use, the mixed lymphocyte reaction (MLR) has been used as an important experimental method for investigating the cellular mechanisms involved with the rejection of allografts, and more importantly for assessing histocompatability (Stites, 1991). The most common form of this reaction is a 'one way' mixed lymphocyte reaction. In this assay the cells from the donors are split into either responder or stimulator cells. The stimulator cells are treated to prevent mitosis either by irradiation, or treatment with mitomycin C (an antibiotic that crosslinks DNA at CpG motifs) (Kimball, 1990; Stites, 1991; Oh et al., 2008; Nasef et al., 2009; Wada et al., 2009; Kuci et al., 2010; Prasanna et al., 2010). This treatment prevents the stimulator cells from dividing and therefore incorporating ^{3}H, but otherwise the cells remain metabolically active. Tritiated thymidine is incorporated into the DNA of the dividing cells, and when these cells are processed and radioactive counts determined, the counts are proportional to the level of response.

The reaction is used to measure the responder lymphocytes reaction to the histocompatibility antigens that are present on the stimulator cells. In order to convert the counts per minute into a practical comparable result, the results from the assay are usually expressed as the stimulation index (SI):

$$SI = \frac{\text{Average counts per minute in test cultures}}{\text{Average counts per minute in control cultures (without stimulator cells)}}$$

The responding cells of the MLR have been shown to be predominantly T-cells, with B-cells also thought to play a small part, as an increase in immunoglobin synthesis has been detected in such assays (Stites, 1991). Although a very useful tool as an *in-vitro* correlate of the transplantation reaction, there are certain flaws to the methodology when used to assess the immunogenicity of non-lymphoid cells.

The one way MLR is an *in-vitro* correlation of the transplantation reaction. This measures the direct pathway of recognition, in which stimulator antigen presenting cells expressing CD80/86, CD40, and foreign MHC class II + epitope can be recognised by responder T-cells, and proliferate. Over recent years, the assay has been adopted to study the immunogenicity of MSCs and studies have shown that MSCs have little capacity to stimulate allogeneic lymphocytes. Strictly speaking, when the one-way mixed 'lymphocyte' reaction is used to test the immunogenicity of MSCs, it is better described as a lymphocyte transformation assay or reaction, since the stimulator cells (MSCs) are not of the lymphoid lineage. Since MSCs do not normally express co-stimulatory molecules or MHC class II it is perhaps unsurprising that they fail to stimulate allogeneic lymphocytes in the classical one-way mixed 'lymphocyte' reaction.

The MLR and LTA are very useful methods as *in-vitro* assays to measure the direct pathway of immune recognition, but there are limitations. A review of the prevailing literature in which the immunogenicity of allogeneic MSCs has been evaluated *in-vitro* revealed that the assay conditions varied considerably in terms of numbers of stimulator MSCs: to responder cells, mitotic inhibition of stimulator cells, method of measurement of lymphocyte proliferation, use of PBMCs or purified T-cells, and types of plate (U, flat, V, coated or uncoated not usually specified) [**Table 5.1**]. In this study uncoated U-bottomed plates were used, due to previous lab experience (Ingham et al., 1993; Ketheesan et al., 1994; Ketheesan et al., 1996b; Ketheesan et al., 1996a).

Moreover, in none of the studies was the incubation period extended for over 7 days.

It was theorised that the assay conditions required optimisation (this chapter) and that the assay should be carried out over an increased time period (Chapter 6) with MSCs as the stimulator cells in order to allow indirect antigen recognition to take place.

5.1.1 AIMS AND OBJECTIVES

The aim of the work in this chapter was to develop the methodology for performing the lymphocyte transformation assay using MSCs as the stimulator cells. There were 5 specific objectives:

- To determine the concentration of PHA (mitogen) for use as the positive control for lymphocyte activation.
- To determine the method for spiking the cells (using tritiated thymidine).
- To determine the concentration of mitomycin C for the mitotic inhibition of stimulator cells.
- To determine the cell number and ratio of responder and stimulator cells in the one way MLR.
- To determine the concentration and source of serum for cell culture medium supplementation.

Type of plate	Method of mitotic inhibition	Responder to stimulator cell ratios	Length of MLR	Measure of cellular proliferation	Were Cells Immunogenic?	Reference
96 well V-bottom plates	Gamma Irradiated	10:0.1 10:1 1:1	5 Days	1µCi/well, 13 hours	MSCs failed to elicit a proliferative response from allogeneic lymphocytes. MSCs added into a mixed lymphocyte reaction, either on day 0 or on day 3, or to mitogen-stimulated lymphocytes, led to a greater than 50% reduction in proliferative activity.	Bartholomew et al., (2002)
96 well plate	Gamma Irradiated	10:1	7 Days	2.5 µCi/well, 24 hrs	In co-culture with allogenic PBMCs, adipo-derived stem cells and MSCs failed to lead to any significant stimulation, including when osteogenically differentiated.	Niemeyer et al., (2007)
96 well plate	10 µg/ml Mitomycin C	1000:1 100:1 10:1	6 Days	BrdU for 18 hrs	MSCs did not provoke allogeneic PBMC proliferation, even when their HLA molecule expression was up-regulated by IFN-ɣ pretreatment. MSCs actively suppressed the allogeneic proliferation of the responder lymphocytes in MLR.	Oh et al., (2008)
24 well plate	Not Fixed	1:1	5 Days	CFSE and flow cytometry	MSC did not deliver a mitogenic signal to purified, unstimulated T cells.	Suva et al., (2008)
96 well plate	Gamma Irradiated	1:0.15	7 Days	1.6 µCi/well, 18hrs	MSC failed to elicit a proliferative response when cocultured with allogeneic PBMC.	Tse et al., (2003)

Table 5.1: A comparison of the methods used to assess the immunogenicity of MSCs

5.2 MATERIALS AND METHODS

5.2.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Reagents:

Transport Medium

Rosslyn Park Memorial Institute (RPMI) medium without L-glutamine (Lonza)

Penicillin-streptomycin mixture [5000 $\mu g.ml^{-1}$ potassium penicillin, 5000 $\mu g.ml^{-1}$ streptomycin sulphate] (Lonza).

L-glutamine 200 mM (Gibco)

HEPES 1 M (Lonza)

RPMI transport medium was made by combining RPMI-1640 with 50 $\mu g.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin, 2 mM L-glutamine and 0.05 M HEPES.

Complete Medium

Human AB Serum (Source Biosciences Life Sciences) – Heat inactivated by heating to 56°C in a water bath for 1 hour, before being frozen at -20°C in 10 ml aliquots.

Fetal calf serum (Lonza)

RPMI complete medium was made by combining transport medium with 10% human or fetal calf serum (v/v).

Method

Human venous blood from healthy volunteers (age range 22-57 years, n=4 female donors, n=2 male) was obtained by venupuncture (30 ml; ethics reference R1508) using the Vacutainer blood collection system (Becton Dickinson) and diluted 1:1 in 30 ml RPMI-1640 transport medium. Diluted blood (7 ml) was layered onto Lymphoprep™ (3 ml) in plastic test tubes. Following centrifugation (800g) for 30 minutes the peripheral blood mononuclear cells (PBMCs) were removed from the interface using endotoxin free sterile glass pipettes, washed three times in RPMI-1640 transport medium (450 g for 30 minutes, 150 g for 30 minutes twice) and resuspended in 10 ml RPMI-1640 complete medium. Cells were counted using the trypan blue dye exclusion assay as described in section 2.3.7.

5.2.2 TITRATION OF PHA AND DETERMINATION OF LYMPHOCYTE TRANSFORMATION USING LOW AND HIGH ACTIVITY ^{3}H THYMIDINE

Phytohaemagglutinin was used as a positive control in all lymphocyte transformation (LTA) assays to show that the PBMCs were capable of mitotic activation

Reagents

High activity tritiated thymidine (specific activity >10Ci.mmol^{-1}) was purchased from Amersham, and diluted 1:10 into RPMI-1640 (0.25 µCi.ml^{-1}) and stored at 4°C until depleted.

Low activity tritiated thymidine (specific activity 5-6 Ci.mmol^{-1}) was purchased from Perkin Elmer, and diluted 1:20 into RPMI-1640 (0.25 µCi.ml^{-1}) and stored at 4°C until depleted.

Complete RPMI-1640 medium

Phytohaemagglutinin (PHA) (Sigma). A stock solution of 1 mg.ml^{-1} phytohaemagglutinin in dH_2O was made. Aliquots (100µl) were frozen at -20°C in 0.5ml eppendorfs. PHA (64 µl) was diluted in 1 ml complete RPMI-1640 medium, and a dilution series created transferring 500 µl PHA in medium, to 500 µl medium to generate concentrations of 64, 32, 16, 8, 4, and 2 µg.ml^{-1} PHA. These dilutions were then added to wells at a 1:1 ratio of PHA to PBMCs in medium to achieve the final concentrations of 32, 16, 8, 4, 2 and 1 µg.ml^{-1}.

Method

Peripheral blood mononuclear cells were isolated from a healthy volunteer and 10,000 cells were cultured in 200 µl of complete RPMI-1640 medium in uncoated U-bottom 96 well plates (VWR) for 0, 3, 5, 7, and 14 days with or without the addition of 1-32 µg.ml^{-1} PHA. Each concentration of PHA was tested in replicates of 5 and cells without PHA, plus PHA without cells were used as controls. The set up of the 96 well plates is shown in **Figure 5.1**. The experiment was carried out twice. Once using low activity and once using high activity ^{3}H thymidine, to determine the extent of lymphocyte transformation.

The 96 well plates were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Sixteen hours before each timepoint, each well of the plate was spiked with 10 µl of low activity tritiated thymidine to give a final concentration of 0.0125 µCi.ml^{-1} in complete culture medium and the cells cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates for determination of the response at each timepoint using a FilterMate™ (Perkin Elmer) cell harvester. In tandem with this on the day of each time point each well of a replicate plate was spiked with 10 µl of high activity tritiated

thymidine to give a final concentration of 0.0125 µCi.ml^{-1} in complete culture medium and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 4 hours. Following this incubation (4 hours post spiking) the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0µgml^{-1}	1 µgml^{-1}	2 µgml^{-1}	4 µgml^{-1}	8 µgml^{-1}	16 µgml^{-1}	32 µgml^{-1}				
C		0µgml^{-1}	1 µgml^{-1}	2 µgml^{-1}	4 µgml^{-1}	8 µgml^{-1}	16 µgml^{-1}	32 µgml^{-1}				
D		0µgml^{-1}	1 µgml^{-1}	2 µgml^{-1}	4 µgml^{-1}	8 µgml^{-1}	16 µgml^{-1}	32 µgml^{-1}				
E		0µgml^{-1}	1 µgml^{-1}	2 µgml^{-1}	4 µgml^{-1}	8 µgml^{-1}	16 µgml^{-1}	32 µgml^{-1}				
F		0µgml^{-1}	1 µgml^{-1}	2 µgml^{-1}	4 µgml^{-1}	8 µgml^{-1}	16 µgml^{-1}	32 µgml^{-1}				
G		Cells in medium only	Cells in medium only	Cells in medium only					32 µgml^{-1} and Medium	32 µgml^{-1} and Medium	32 µgml^{-1} and Medium	
H												

Figure 5.1: Diagram of the 96 well plate experimental wells used to determine the optimal concentration of PHA to stimulate peripheral blood mononuclear cells. Figures indicate concentration of PHA added to 10^4 PBMCs per well, cells in medium only; no PHA, and PHA and medium; no cells.

5.2.3 HARVESTING CELLS USING THE FILTERMATE CELL HARVESTER

Reagents

Saline solution (0.85 % w/v) (NaCl, Merck) in H_2O.

Trichloroacetic acid (10 % w/v) (TCA, Fisher Scientific) in H_2O.

Methanol (100%) (Merck)

Method

A FilterMate™ cell harvester (Perkin Elmer) was used to harvest cells from the U-bottomed 96-well microtitre plates onto 96 well Unifilter GFC plates (Perkin Elmer). The UniFilter 96 plate was pre-wet with 100 ml H_2O. The spiked cells and medium were then aspirated from the plate, and the filter retained the cells. A 0.85 % saline solution (150 ml) was used to wash the cells on the filter membrane. A 10% (w/v) TCA solution (100 ml) was aspirated through the plate, and this lysed the cells, and precipitated the DNA onto the filter membrane. The final wash of methanol (100 ml) aided in the precipitation of any remaining DNA, and also in the drying of the plate. Microscint-20 (Perkin Elmer) was added to each 'well' (35 µl) before radioactive counts were measured on the TopCount™ plate reader. The radioactive counts were measured as counts per minute.

5.2.4 DETERMINATION OF THE OPTIMAL CONCENTRATION OF MITOMYCIN C TO ARREST CELL DIVISION USING THE ATPLITE™ ASSAY TO DETERMINE CELL VIABILITY

Reagents

Mitomycin C stock: Mitomycin C (10 mg; Sigma) was added to 10 ml H_2O to create a 1 $mg.ml^{-1}$ stock solution. This was frozen at -20°C in 100 µl aliquots in 0.5 ml eppendorfs.

Camptothecin stock: Camptothecin (100mg; Sigma) was added to 100 ml HBSS (Gibco) to create a 1 $mg.ml^{-1}$ stock solution.

ATPlite assay (Perkin Elmer)

Methods

<u>L929 Fibroblasts</u>

An immortalised mouse lung fibroblast cell line was obtained from the European Collection of Animal Cell Cultures. L929 cells were cultured in DMEM complete medium (DMEM with 2 mM L-glutamine, 100 $\mu g.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ streptomycin and 10% (v/v) FCS), at 37°C in

a humidified atmosphere containing 5% (v/v) CO2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.3.

The ATPLite™ Assay

The ATPLite assay allows the concentration of adenosine triphosphate (ATP) to be measured by the reaction of ATP with D-luciferin using the enzyme luciferase to produce adenosine monophosphate (AMP), inorganic pyrophosphate (PPi) and light. The reaction is outlined below.

ATP + D-Luciferin + O_2 →Oxyluciferin + AMP + PPi + CO_2 + Light

The light produced is proportional to the ATP concentration. Since the concentration of ATP in cells is reduced when cells undergo apoptosis, higher levels of ATP present within wells correlates with viable cell number. Endogenous ATPases are inactivated by raising the pH to 11 through the addition of cell lysis solution. The subsequent addition of the substrate reduces the pH to 7.6, hence allowing the reaction with D-luciferin and luciferase to occur.

In order to determine the optimum concentration of mitomycin C to arrest the mitotic division of L929 cells, the ATPlite assay was performed after culturing cells following exposure to mitomycin C (MMC).

L929 cells (10,000) were added to wells of a flat bottomed 96 well tissue culture plate (Nunc) in 100 µl DMEM complete medium, and left to attach for 4 hours. A concentration series of MMC was prepared by diluting the stock solution of MMC into complete culture medium to concentrations of 12.5 – 400 $\mu g.ml^{-1}$. One hundred microlitres was added to wells containing 100 µl culture medium and cells to final concentrations of 6.25 – 200 $\mu g.ml^{-1}$ of MMC in replicates of six. Controls were untreated cells in medium only or treated with 4 $\mu g.ml^{-1}$ camptothecin. To treat cells with camptothecin, 4 µl stock solution was added to 996 µl culture medium to create a 4 $\mu g.ml^{-1}$ solution. Culture medium already in wells was removed and replaced with the camptothecin medium. Cells were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air for 1 hour. Following incubation for 1 hour, the MMC medium was removed, and cells were washed twice with PBS, before 200 µl DMEM complete medium was added to wells (cells only and MMC treated). Camptothecin was added to wells at a final concentration of 0.1 $\mu g.ml^{-1}$ as a positive control for cell death, and the plates were incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air. A 96 well plate template for the experimental setup is shown in **Figure 5.2** and six plates were set up.

The ATPlite assay was performed on the cells at days 0, 2, 4, 6, 8, and 10 following exposure to mitomycin C.

ATPLite assay reagents and solutions were allowed to reach room temperature. The luciferase/luciferin substrate (77 mg) was reconstituted with substrate buffer solution (5 ml). The subsequent procedure was carried out under dim light conditions. One hundred microlitres (from 200 µl) of culture medium was removed from a well of the culture plate containing cells, cell lysis solution (50 µl) was added to the well (containing 100 µl of medium) and the plate was placed on an orbital plate shaker (700 rpm) for 5 minutes. Substrate solution (50 µl) was added to the well and the plate was placed on an orbital plate shaker (700 rpm) for 5 minutes. An adherent seal was placed on the plate to prevent contamination of the luminometer detectors and the plate was placed in the TopCount (NXT) luminometer, which was programmed to dark-adapt the plate for 10 minutes and read the luminescence of each well for 5 seconds. The reading output of each well was presented as counts per second (CPS).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
C		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
D		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
E		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
F		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
G		Cells Only	Campto. Treated	200 $\mu g ml^{-1}$	100 $\mu g ml^{-1}$	75 $\mu g ml^{-1}$	50 $\mu g ml^{-1}$	25 $\mu g ml^{-1}$	12.5 $\mu g ml^{-1}$	6.25 $\mu g ml^{-1}$		
H												

Figure 5.2: Diagram of the 96 well plate experimental wells used to determine the optimal concentration of mitomycin C to arrest cell division of L929 cells. Figures indicate concentration of mitomycin C added to 10^4 L929 cells per well, cells and medium, and cells and 0.1 $\mu g.ml^{-1}$ campththecin as a negative control for proliferation.

5.2.5 DETERMINATION OF THE CONCENTRATION OF MITOMYCIN C TO ARREST CELL DIVISION USING FLUORESCEIN DIACETATE TO DETERMINE CELL VIABILITY

Reagents

Fluorescein diacetate (FDA) stock: FDA (20 mg; Fisher Scientific) was dissolved in 20 ml DMSO to create a 1 $mg.ml^{-1}$ stock solution. This was stored at room temperature in the dark.

Fluorescein sodium stock: Fluorescein sodium (20 mg; Fisher Scientific) was dissolved in 20 ml H_2O to create a 1 $mg.ml^{-1}$ stock solution. This was stored at room temperature in the dark.

Method

Primary human dermal fibroblasts (PHDFs) were cultured in complete DMEM (DMEM with 2 mM L-glutamine, 50 $\mu g.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin and 10% (v/v) FCS), at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.3.

Mesenchymal stromal cells from donor BM1 were cultured in NH Expansion medium at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.2.

Fluorescein diacetate is a fluorochrome that has been used to differentiate between live and dead cells in monolayer culture (Krause et al., 1984). This dye is permeable to cell membranes, and in live cells, cell esterases can convert FDA into the fluorescent compound fluorescein. This compound is impermeable and therefore is a useful marker of viable cells in culture (only live cells will fluoresce).

Primary human dermal fibroblasts (10,000) were added to wells of a flat bottomed black 96 well FluoroNunc™ plate (Nunc) in 100 µl culture medium, and left to attach for 4 hours. Following this incubation MMC was added to the wells at 12.5 – 50 $\mu g.ml^{-1}$ in replicates of six. Controls were untreated or treated with 4 $\mu g.ml^{-1}$ camptothecin. Cells were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air for 1 hour.

Following incubation, the MMC medium was removed, and cells were washed twice with PBS, before 200 µl complete DMEM culture medium was added to wells (cells only and MMC treated). Camptothecin was added to wells at a final concentration of 0.1 $\mu g.ml^{-1}$ as a positive control for cell death, and the plates were incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air. FDA staining was carried out on days 0, 2, 4, 8, and 12. FDA (1 µl) was added to wells

and left for 2 minutes at room temperature. Cells were then washed with 200 µl PBS 3 times, before finally adding 200 µl PBS to wells and 200 µl 10 $\mu g.ml^{-1}$ fluorescein sodium to one empty well. Fluorescent intensity was measured on a Fluostar Optima plate reader using the fluorescein sodium as a constant gain adjustment. Settings were set to read at 485 nm excitation, and 520 nm emission for 1 cycle, 1 second cycle time, and 10 flashes per well per cycle.

Mesenchymal stromal cells (10,000) were allowed to attach to wells of a black 96 well FluoroNunc™ plate (Nunc). After 4 hours the attached cells were treated with MMC at the final concentrations shown in **Figure 5.3** for 1 hour. Following incubation for 1 hour, the mitomycin C medium was removed, and cells were washed twice with PBS, before 200 µl NH expansion medium was added to wells (cells only and MMC treated). Camptothecin was added to wells at a final concentration of 0.1 $\mu g.ml^{-1}$ as a positive control for cell death, and the plates were incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air. FDA staining was carried out on days 0, 2, 4, 8, and 12. FDA (1 µl) was added to wells and left for 2 minutes at room temperature. Wells were then washed with 200 µl PBS 3 times, before finally adding 200 µl PBS to wells and 200 µl 10 $\mu g.ml^{-1}$ fluorescein sodium to one empty well. Fluorescent intensity was measured on a Fluostar Optima plate reader using the fluorescein sodium as a constant gain adjustment. Settings were set to read at 485 nm excitation, and 520 nm emission for 1 cycle, 1 second cycle time, and 10 flashes per well per cycle.

A 96 well plate template for the experimental setup is shown in **Figure 5.3**.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}				10µg.ml^{-1} fluorescein sodium		
C		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}						
D		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}						
E		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}						
F		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}						
G		Cells Only	Campto. Treated	50µgml^{-1}	25µgml^{-1}	12.5µgml^{-1}						
H												

Figure 5.3: Diagram of the 96 well plate experimental wells used to determine the optimal concentration of mitomycin C to arrest cell division of 10^4 PHDF and MSCs. Cells grown in medium only were used as a negative control for cell death. Cells grown in 0.1 µg.ml^{-1} camptothecin were used as a positive control for cell death. Figures represent concentrations of mitomycin C used to treat PHDFs and MSCs to inhibit mitosis. FDA was used as a positive stain for cell viability. Fluorescein sodium was used as a constant gain adjustment to allow comparisons between plates.

5.2.6 ONE-WAY MLR TO DETERMINE CELL NUMBERS AND RATIOS OF PBMCS

PBMC were isolated from 2 healthy volunteer's human peripheral blood, and one population was treated with 12.5 $\mu g.ml^{-1}$ MMC for 1 hour at room temperature (the stimulator cells). The cells were centrifuged at 150 g for 30 minutes, and MMC medium was removed and cells were washed in 10 ml PBS twice before they were finally resuspended in 10 ml fresh RPMI-1640 complete medium. Cells were counted using trypan blue dye exclusion (as described in section 2.3.7) and dispensed into a 96 well uncoated U-bottomed plate; untreated PBMCs and MMC treated PBMCs were added to create ratios of 1:1, 1:10, and 1:100 responder to stimulator cells as shown in **Figure 5.4**. The total numbers of cells per well were 10,000, 50,000, and 100,000. The negative control for proliferation was responder PBMCs grown in complete medium only, and positive control was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA. Plates were harvested on days 3, 5, 7, and 12.

Plates were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Sixteen hours before the desired timepoint each well of the plate was spiked with 10 µl of low activity tritiated thymidine (0.0125 $\mu Ci.ml^{-1}$) and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air overnight. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester, before MicroScint-20™ was added and radioactive counts were measured on a TopCount scintillation counter as described in section 5.2.3

A 96 well plate template for the experimental setup is shown in **Figure 5.4**.

	1	2	3	4	5	6	7	8	9	10	11	12
A		Neg. control	1:1	1:1	1:10	1:10	1:100	1:100	Pos. Control			
B	10,000 Cells	**10000** In Medium	**5000** *5000*	**5000** *5000*	**1000** *9000*	**1000** *9000*	**100** *9900*	**100** *9900*	**10,000** + PHA			
C	10,000 Cells	**10,000** In Medium	**5000** *5000*	**5000** *5000*	**1000** *9000*	**1000** *9000*	**100** *9900*	**100** *9900*	**10,000** + PHA			
D	50,000 Cells	**50,000** In Medium	**25,000** *25,000*	**25,000** *25,000*	**5000** *45,000*	**5000** *45,000*	**500** *49,500*	**500** *49,500*	**50,000** + PHA			
E	50,000 Cells	**50,000** In Medium	**25,000** *25,000*	**25,000** *25,000*	**5000** *45,000*	**5000** *45,000*	**500** *49,500*	**500** *49,500*	**50,000** + PHA			
F	100,000 Cells	**100,000** In Medium	**50,000** *50,000*	**50,000** *50,000*	**10,000** *90,000*	**10,000** *90,000*	**1000** *99,000*	**1000** *99,000*	**100,000** + PHA			
G	100,000 Cells	**100,000** In Medium	**50,000** *50,000*	**50,000** *50,000*	**10,000** *90,000*	**10,000** *90,000*	**1000** *99,000*	**1000** *99,000*	**100,000** + PHA			
H												

Figure 5.4: Diagram of the 96 well plate experimental wells used to determine the optimal ratio of responder:stimulator PBMCs, and the optimal total cell number per well. Numbers in bold represent responder cells and italics represent stimulator cells. Ratios of 1:1, 1:10, and 1:100 responder to stimulator cells were used and total numbers of cells per well were 10000, 50000, and 100000. The negative control for proliferation was responder PBMCs grown in complete medium only, and positive control was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA. Plates were harvested on days 3, 5, 7, and 12.

5.2.7 COMPARISON OF THE RESPONSE OF PBMCS IN FETAL CALF SERUM AND HUMAN SERUM

Isolation of autologous human serum: Freshly isolated blood was dispensed into a sterile test tube, and allowed to clot for 2 hours at 37 °C. The test tube was then centrifuged at 2000 g for 10 minutes, and the serum (straw coloured fluid) was removed aseptically and dispensed into a sterile universal.

Method

PBMCs were isolated from healthy volunteer's human peripheral blood, and counted using trypan blue dye exclusion. The PBMCs (25,000, 50,000 and 100,000) were dispensed into uncoated U-bottomed 96 well plates, and either cultured in complete medium, or complete medium with 32 $\mu g.ml^{-1}$ PHA. Two separate timecourses were performed using complete medium supplemented with 10 % (v/v) fetal calf serum, or 10 % (v/v) autologous human serum.

Plates were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 5, 7, and 12 days. Sixteen hours prior the day of the timecourse each well of the plate was spiked with 10 µl of low activity tritiated thymidine (0.0125 $\mu Ci.ml^{-1}$) and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air overnight. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester, before MicroScint-20™ was added and radioactive counts were measured on a TopCount scintillation counter as described in section 5.2.3.

A 96 well plate template for the experimental setup is shown in **Figure 5.5**.

	1	2	3	4	5	6	7	8	9	10	11	12
A		Medium	Medium							Pos. Control	Pos. Control	
B	25,000 Cells	**25,000**	**25,000**							**25,000** + PHA	**25,000** + PHA	
C	25,000 Cells	**25,000**	**25,000**							**25,000** + PHA	**25,000** + PHA	
D	50,000 Cells	**50,000**	**50,000**							**50,000** + PHA	**50,000** + PHA	
E	50,000 Cells	**50,000**	**50,000**							**50,000** + PHA	**50,000** + PHA	
F	100,000 Cells	**100,000**	**100,000**							**100,000** + PHA	**100,000** + PHA	
G	100,000 Cells	**100,000**	**100,000**							**100,000** + PHA	**100,000** + PHA	
H												

Figure 5.5: Diagram of the 96 well plate experimental wells used to determine effects of culturing PBMCs in human or fetal calf serum. PBMCs (25,000, 50,000 and 100,000) were isolated from peripheral human blood and cultured for 5, 7, and 12 days using complete medium supplemented with either 10 % (v/v) fetal calf serum, or 10 % (v/v) human autologous serum. Cells grown in medium only were used as a negative control for lymphocyte stimulation. Cells grown in PHA (32 $\mu g.ml^{-1}$) were used as a positive control for lymphocyte stimulation.

5.2.8 DETERMINATION OF HUMAN SERUM CONCENTRATION FOR USE IN THE LTA

PBMC were isolated from 2 healthy volunteer's human peripheral blood, and counted using trypan blue dye exclusion. The PBMCs (50,000) were dispensed into uncoated U-bottomed 96 well plates. Complete medium was made supplemented with 2, 5, and 10% (v/v) human AB serum. PBMCs were either cultured in complete medium, or complete medium with 32 $\mu g.ml^{-1}$ PHA at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

Plates were cultured for 3, 5, and 7 days. At sixteen hours before the desired timepoint each well of the plate was spiked with 10 µl of low activity tritiated thymidine stock and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air overnight. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester, before MicroScint-20™ was added and radioactive counts were measured on a TopCount scintillation counter as described in section 5.2.3.

A 96 well plate template for the experimental setup is shown in **Figure 5.6**.

5.2.9 DATA ANALYSIS

Data generated in one-way MLR's and LTA's were Log_{10} transformed to satisfy the normality assumption required in order to perform an ANOVA. A post-hoc Tukey test for unequal sample sizes was performed, as recommended by Sokal and Rholf, 1981, which is hithero referred to as the T-method. The Log_{10} transformed data at each time point was analysed by one-way analysis of variance and the minimum significant difference (MSD; $p<0.05$) between each group mean determined using the T-method (Sokal and Rohlf, 1981).

	1	2	3	4	5	6	7	8	9	10	11	12
A		Media + Donor 1 Serum	Media + Donor 1 Serum	Media + Donor 2 Serum	Media + Donor 2 Serum	Pos. Control	Pos. Control	Pos. Control	Pos. Control			
B	50,000 Cells	**50,000 2%**	**50,000 2%**	**50,000 2%**	**50,000 2%**	**50,000-1** + PHA-2%	**50,000-1** + PHA-2%	**50,000-1** + PHA-2%	**50,000-1** + PHA-2%			
C	50,000 Cells	**50,000 2%**	**50,000 2%**	**50,000 2%**	**50,000 2%**	**50,000-1** + PHA-5%	**50,000-1** + PHA-5%	**50,000-1** + PHA-5%	**50,000-1** + PHA-5%			
D	50,000 Cells	**50,000 5%**	**50,000 5%**	**50,000 5%**	**50,000 5%**	**50,000-1** + PHA-10%	**50,000-1** + PHA-10%	**50,000-1** + PHA-10%	**50,000-1** + PHA-10%			
E	50,000 Cells	**50,000 5%**	**50,000 5%**	**50,000 5%**	**50,000 5%**	**50,000-2** + PHA-2%	**50,000-2** + PHA-2%	**50,000-2** + PHA-2%	**50,000-2** + PHA-2%			
F	50,000 Cells	**50,000 10%**	**50,000 10%**	**50,000 10%**	**50,000 10%**	**50,000-2** + PHA-5%	**50,000-2** + PHA-5%	**50,000-2** + PHA-5%	**50,000-2** + PHA-5%			
G	50,000 Cells	**50,000 10%**	**50,000 10%**	**50,000 10%**	**50,000 10%**	**50,000-2** + PHA-10%	**50,000-2** + PHA-10%	**50,000-2** + PHA-10%	**50,000-2** + PHA-10%			
H												

Figure 5.6: Diagram of the 96 well plate experimental wells used to determine the optimal concentration of human AB serum. PBMCs (50,000) from two donors were cultured using 2, 5, or 10% human AB serum, either in complete medium or in complete medium supplemented with 32 $\mu g.ml^{-1}$ PHA. PBMCs gown in medium only were a negative control for stimulation, and PBMCs grown in PHA were a positive control for stimulation. In positive control wells '-1' and '-2' are representative of cells from donor 1 and donor 2 respectively. Plates were cultured for 3, 5, and 7 days.

5.3 RESULTS

5.3.1 TITRATION OF PHA AND DETERMINATION OF LYMPHOCYTE TRANSFORMATION USING LOW AND HIGH ACTIVITY ^{3}H THYMIDINE

Phytohaemagglutinin (PHA) is a lectin found in plants, especially in legumes. It has common uses as a mitogen, a potent stimulator of cell division. In the one-way MLR it is used as a positive control for responder PBMC stimulation. In order to determine the concentration of PHA to be used to stimulate the PBMCs a titration assay was performed using a gradient of increasing concentration of PHA with a fixed number of PBMCs. The stimulation of the PBMCs was measured using tritiated thymidine incorporation into the DNA of the cells, both low activity, and high activity. The stimulation index was calculated as:

$$SI = \frac{\text{Mean cpm in cultures with PHA}}{\text{Mean cpm in control cultures}}$$

The control cultures were PBMCs cultured with no stimulation in medium alone.

Tritiated thymidine incorporation occurs when cells enter mitosis and use the thymidine in the synthesis of new DNA. The radiation is lethal to the cells however and because of this shorter incubation times are required using high activity tritiated thymidine rather than using low activity.

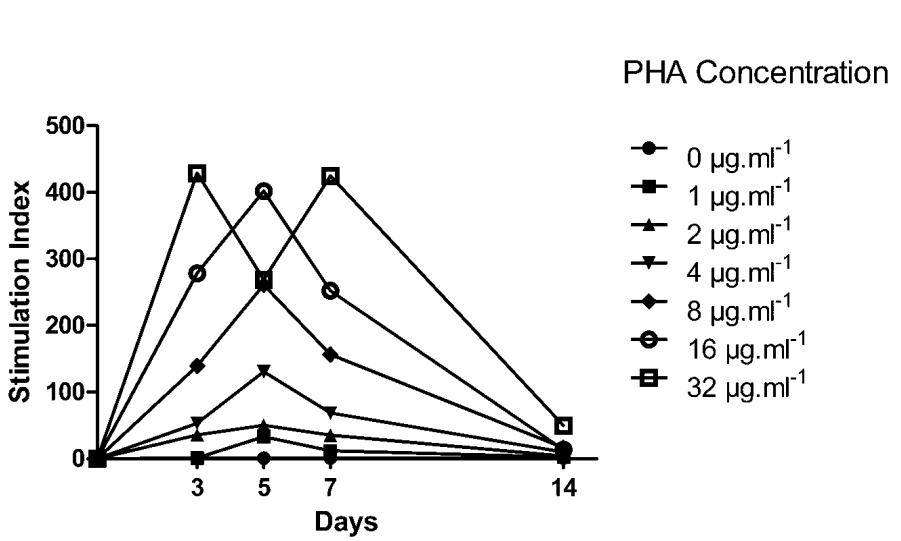


Figure 5.7: The effect of PHA concentration on the stimulation of PBMCs over time as measured by incorporation of high activity tritiated thymidine into DNA (SI). PBMCs were treated with 0, 1, 2, 4, 8, 16, and 32 µg.ml^{-1} PHA. On the day of harvesting the cells were spiked with high activity tritiated thymidine for 4 hours, and counts per minute were determined. Negative control for stimulation was PBMCs cultured in complete medium only in the absence of mitogen. Cells were cultured for 3, 5, 7, and 14 days. Results are displayed as the stimulation index over time.

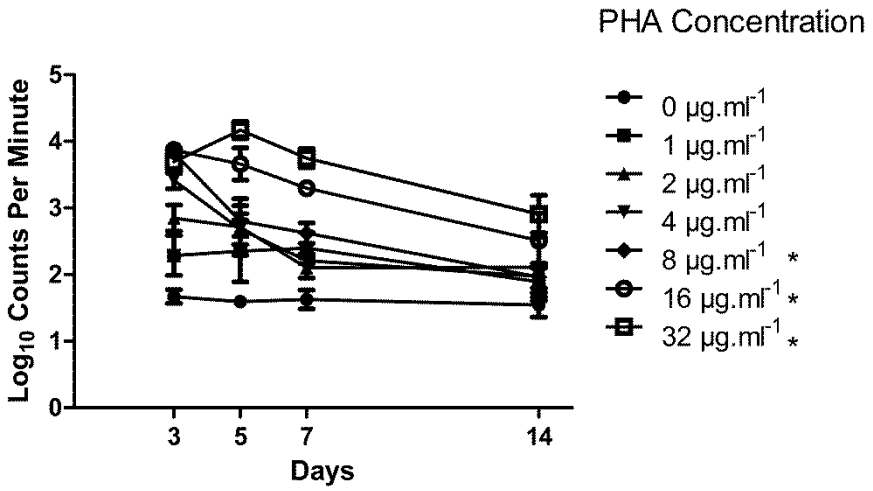


Figure 5.8: The effect of PHA concentration on the stimulation of PBMCs over time as measured by incorporation of high activity tritiated thymidine into DNA (cpm). PBMCs were treated with 0, 1, 2, 4, 8, 16, and 32 µg.ml^{-1} PHA. On the day of harvesting the cells were spiked with high activity tritiated thymidine for 4 hours, and counts per minute were determined. Negative control for stimulation was PBMCs cultured in complete medium only in the absence of mitogen. Cells were cultured for 3, 5, 7, and 14 days. Results are displayed as the mean Log_{10} counts per minute (n=5) over time with 95% confidence intervals. * indicates significant differences ($p<0.05$) compared to 0 µg.ml^{-1} PHA used to stimulate cultured cells as determined by the T-method.

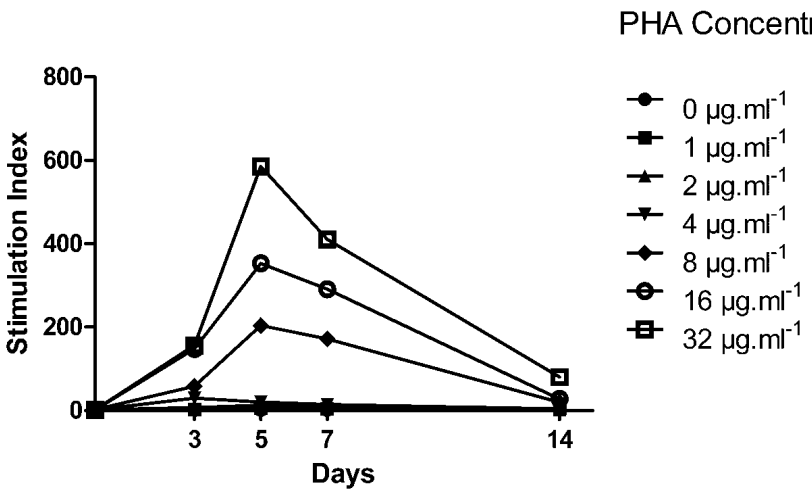


Figure 5.9: The effect of PHA concentration on the stimulation of PBMCs over time as measured by incorporation of low activity tritiated thymidine into DNA (SI). PBMCs were treated with 0, 1, 2, 4, 8, 16, and 32 µg.ml^{-1} PHA. Sixteen hours before the day of harvesting the cells were spiked with low activity tritiated thymidine. After 16 hour incubation, counts per minute were determined using a Topcount ™ scintillation counter. Negative control for stimulation was PBMCs cultured in complete medium only in the absence of mitogen. Cells were cultured for 3, 5, 7, and 14 days. Results are displayed as the stimulation index over time.

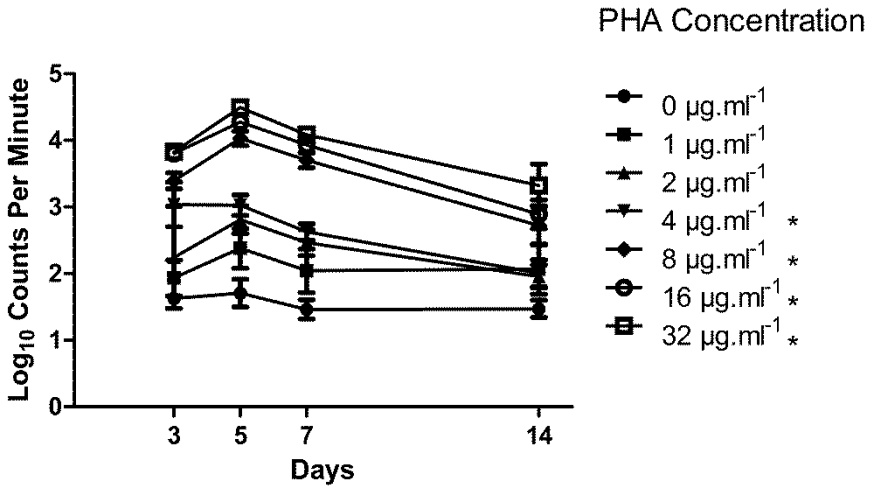


Figure 5.10: The effect of PHA concentration on the stimulation of PBMCs over time as measured by incorporation of low activity tritiated thymidine into DNA (cpm). PBMCs were treated with 0, 1, 2, 4, 8, 16, and 32 µg.ml^{-1} PHA. Sixteen hours before the day of harvesting the cells were spiked with low activity tritiated thymidine. After 16 hour incubation, counts per minute were determined. Negative control for stimulation was PBMCs cultured in complete medium only in the absence of mitogen. Cells were cultured for 3, 5, 7, and 14 days. Results are displayed as the mean Log_{10} counts per minute (n=5) over time with 95% confidence intervals. * indicates significant differences ($p<0.05$) compared to 0 µg.ml^{-1} PHA used to stimulate cultured cells as determined by the T-method.

The results for PBMCs spiked with high activity and low activity thymidine are presented in **Figures 5.7-5.8 and 5.9-5.10** respectively. When PBMCs were stimulated with PHA at 1, 2, or 4 $\mu g.ml^{-1}$ and spiked with low activity thymidine the stimulation index was less than 30. With 8, 16, or 32 $\mu g.ml^{-1}$ PHA stimulation, SI's of 200 or greater were recorded at day 5. The SI's at days 3, 7, and 14 were lower, indicating a peak response at day 5. This pattern of response was also observed when high activity tritiated thymidine was used. Although when the results are displayed as stimulation indices, cells stimulated with 32 $\mu g.ml^{-1}$ PHA did not appear to peak at day 5 (**Figure 5.7**), when the counts per minute were analysed the counts were the highest on this day (**Figure 5.8**).

The stimulation indices were highest when low activity tritiated thymidine was used to spike the cells, and 32 $\mu g.ml^{-1}$ PHA was used to stimulate the cells. These parameters were therefore selected to use in subsequent experiments.

5.3.2 Determination of the concentration of mitomycin C to arrest cell division using the ATPLite™ assay to measure cell number/viability

To arrest mitotic division of cells, mitomycin C (MMC) was used. A range of concentrations of MMC were used (200, 100, 75, 50, 25, 12.5, and 6.25 $\mu g.ml^{-1}$) to assay its effectiveness to arrest the division of an L929 cell line. L929 cells were used in initial experiments to gain an understanding of the concentrations of MMC required to mitotically arrest mesenchymal cells. After treating the cells with MMC, the concentration of ATP present was measured as counts per minute on days 0-10. In the absence of MMC treatment, the L929 cells showed increased ATP levels up to day four of culture, after which the cellular ATP levels remained stationary until day 8, and then declined at day 10 (**Figure 5.11**).

On day 0 directly after treatment with MMC the concentration of ATP (represented by the counts per second) varied between the cultures treated with different concentrations of MMC applied to the cells.

When L929 cells were treated with camptothecin (positive control for cell death) the ATP levels declined from day 0 to day 2, and continued to decline throughout the 10 day culture period. When treated with MMC there was a dose dependent inhibition of ATP levels observed in L929 cells in comparison to the untreated control cells. The cells however, continued to proliferate following treatment with MMC at concentrations of up to 100 $\mu g.ml^{-1}$ MMC. Data is also presented as a percentage relative to L929 cells grown in medium without MMC (**Figure 5.12**). Treatment of L929 cells with mitomycin C at all concentrations caused ATP levels within

cells to fluctuate (demonstrated by deviations from cells in medium only on day 0 after treatment). Due to these fluctuations an alternative method to assess cell viability/growth after mitomycin C treatment was used.

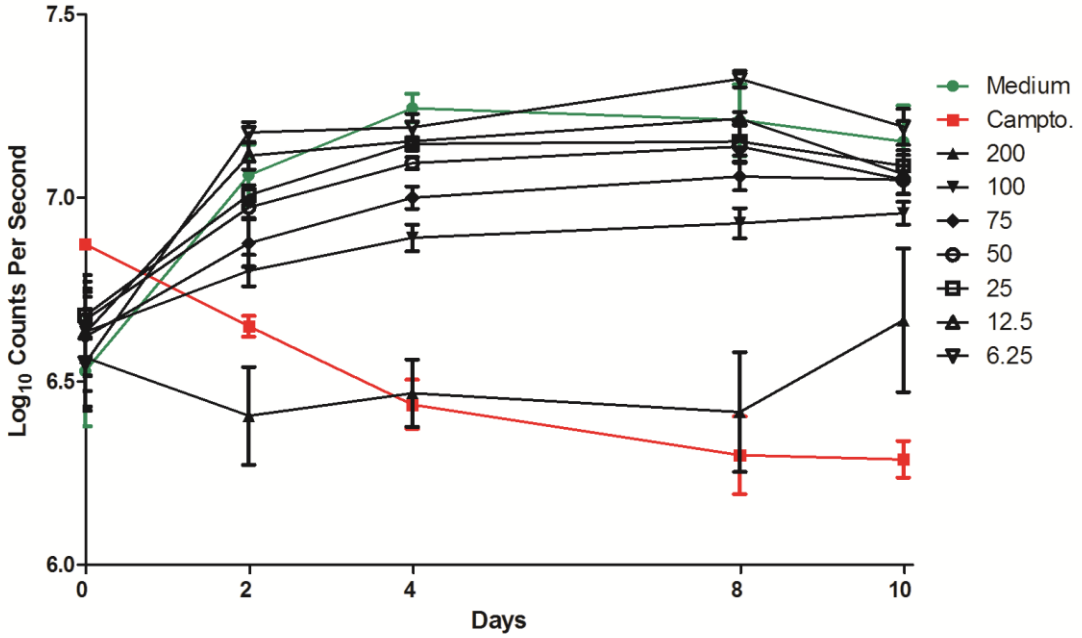


Figure 5.11: The effect of varying concentrations of mitomycin C treatment on the growth of L929 cells over 10 days. L929 cells were treated with MMC (200, 100, 75, 50, 25, 12.5, 6.25 µg.ml^{-1}) for 1 hour, washed and cultured for 10 days. Cell proliferation was determined using an ATPLite assay. Data is expressed as Log_{10} mean (n=6) counts per second ± 95% confidence limits.

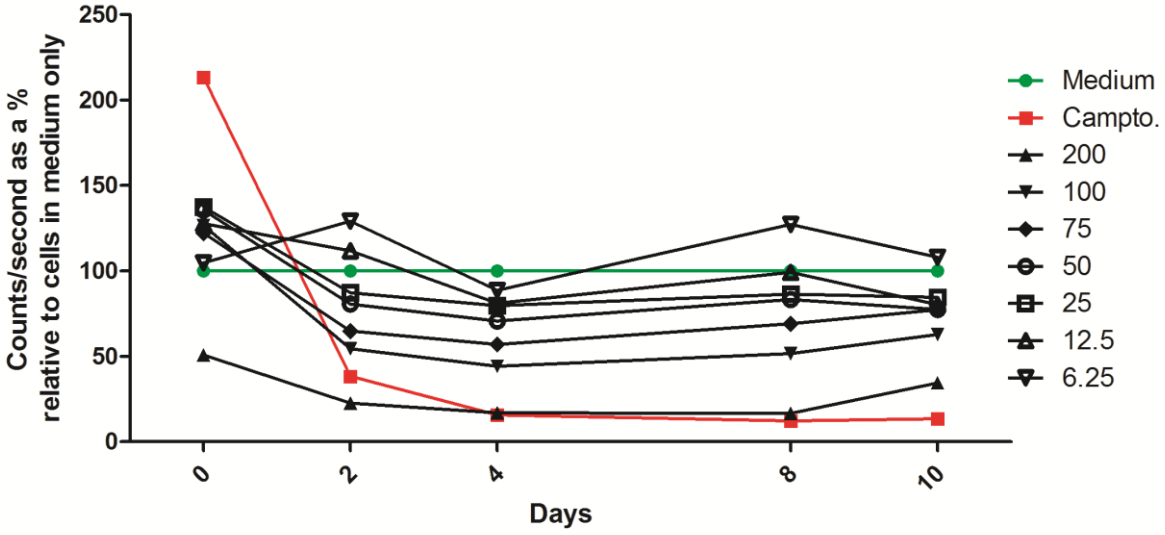


Figure 5.12: The effect of varying concentrations of mitomycin C treatment on the growth of L929 cells over 10 days. L929 cells were treated with MMC (200, 100, 75, 50, 25, 12.5, 6.25 µg.ml^{-1}) for 1 hour, washed and cultured for 10 days. Cell proliferation was determined using an ATPLite assay. Data is expressed as a percentage relative to L929 cells grown in medium alone.

5.3.3 DETERMINATION OF THE OPTIMAL CONCENTRATION OF MITOMYCIN C TO ARREST CELL DIVISION USING FLUORESCEIN DIACETATE

From the experiment with L929 cells described above, it was concluded that this cell line had resistance to mitomycin C treatment and therefore a cell type more closely matching MSCs was chosen for further experiments. Primary dermal human fibroblasts provided a primary cell source to investigate the effect of mitomycin C. In addition, treating cells with mitomycin C was shown to affect the ATP levels of the cells (**Figure 5.12**) so an alternative assay for cell viability was used. In the literature the concentration of MMC used to inhibit mitosis in MSCs is commonly 25 $\mu g.ml^{-1}$. Concentrations of MMC at 50, 25, and 12.5 $\mu g.ml^{-1}$ were therefore selected for further testing. Fluoroscein diacetate was used to stain cells after treatment with MMC as the alternative to the ATPLite assay. Initially PHDFs were used as a primary cell type to assess the effect of MMC treatment on cell proliferation using FDA (**Figure 5.13**). BM1 MSCs were then also treated with MMC (50, 25, and 12.5 $\mu g.ml^{-1}$) and cells stained with FDA on days 0, 2, 4, 8, and 10 (**Figure 5.14**).

PHDFs grown in culture medium without MMC failed to exhibit normal growth, and showed an extended lag period (**Figure 5.13**). Primary dermal human fibroblasts treated with camptothecin showed reduced cell viability from day 0 to day 2, then a sharp decrease in viability to day 4. All three concentrations of mitomycin C used to treat the PHDFs inhibited cell proliferation, but also reduced cell viability at time 0 (**Figure 5.13**).

Mesenchymal stromal cells exhibited a more typical growth curve, with cells grown in medium only proliferating up to day 4, before the number of viable cells dropped on day 8, and remained at this level on day 12. Mesenchymal stromal cells treated with camptothecin remained viable until day 4, but by day 8 there was a marked decrease in cell viability, further decreasing by day 12. As with PHDFs, treatment of MSCs with 50, 25, and 12.5 $\mu g.ml^{-1}$ mitomycin C inhibited cell proliferation but with reduced cell viability at time 0 (**Figure 5.14**).

All 3 concentrations of MMC arrested the mitotic division of both PHDFs and MSCs (no definitive increase in fluorescence) therefore a final concentration of 12.5 $\mu g.ml^{-1}$ was selected to use in further assays to provide the minimum concentration of MMC that would inhibit cell division but maintain cell viability over time in culture.

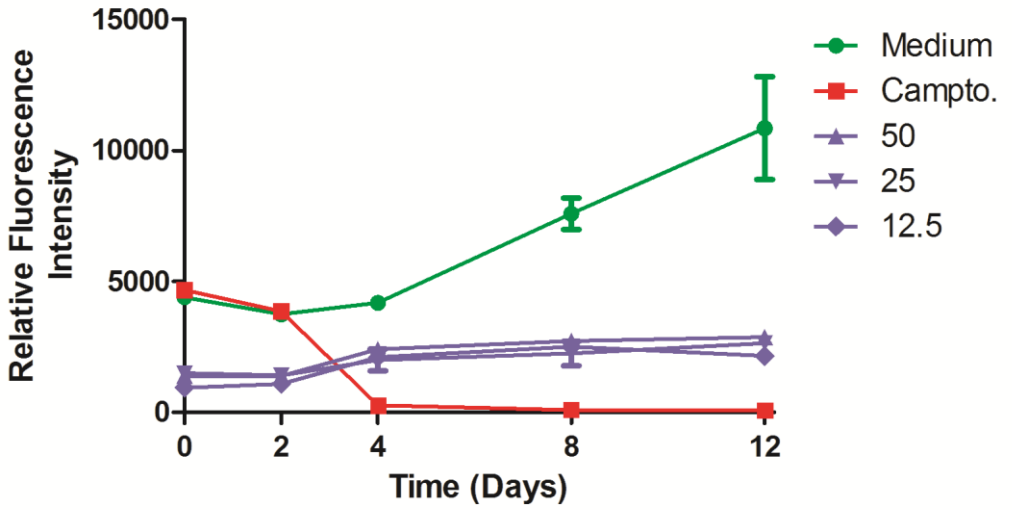


Figure 5.13: Relative fluorescence intensity of PHDFs treated with various doses of mitomycin C. Human dermal fibroblasts were treated with MMC with at 50, 25, and 12.5 µg.ml^{-1} for one hour and then cultured for 0, 2, 4, 8, and 12 days, and the fluorescence intensity after FDA staining was used to assess cell proliferation. The green line is the positive control; cells grown in medium, and the red line is cells grown in 0.1 µg.ml^{-1} camptothecin as an inducer of cell death. Data is displayed as mean relative fluorescent intensity (n=6) ± 95% confidence limits

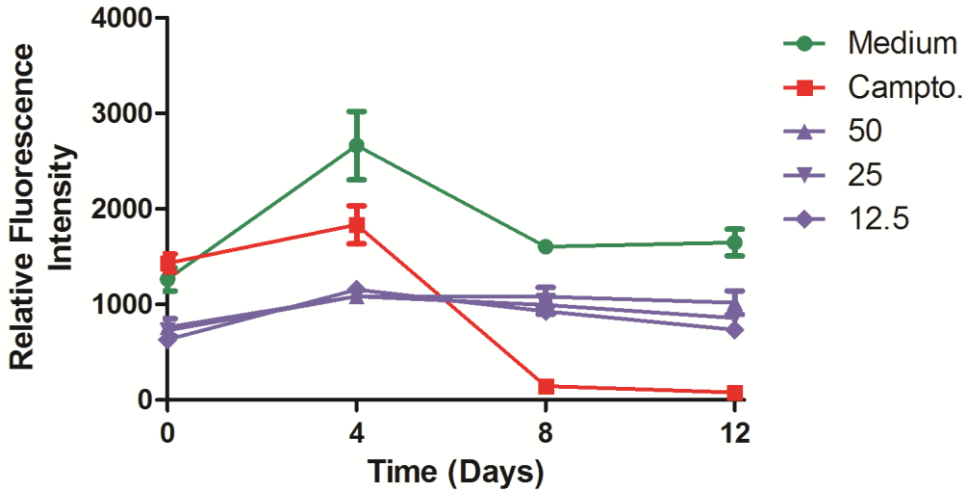


Figure 5.14: Relative fluorescence intensity of MSCs treated with various doses of mitomycin C. Human MSCs were treated with MMC with at 50, 25, and 12.5 µg.ml^{-1} for one hour and then cultured for 0, 4, 8, and 12 days, and the fluorescence intensity after FDA staining was used to assess cell proliferation. The green line is the positive control; cells grown in medium, and the red line is cells grown in 0.1 µg.ml^{-1} camptothecin as an indicer of cell death. Data is displayed as mean relative fluorescent intensity (n=6) ± 95% confidence limits.

5.3.4 ONE-WAY MLR TO DETERMINE CELL NUMBERS AND RATIOS OF PBMCS TO USE TO GENERATE OPTIMUM COUNTS PER MINUTE

In order to determine suitable cell numbers and ratios of responder PBMCs to stimulator cells treated with MMC, experiments were performed at PBMCs to stimulator PBMC ratios of 1:1, 1:10, and 1:100. In addition the number of responder PBMCs was varied between 5000, 25,000 and 50,000 to give total cell numbers (responders plus stimulators) of 10,000, 50,000 and 100,000.

Peripheral blood mononuclear cells treated with PHA as the positive control for stimulation (**Figure 5.15**) gave maximal counts on day 5, before a decline to day 12.

Responder PBMCs (10,000) cultured alone in complete medium without stimulation (**Figure 5.15**) did not appear to proliferate over the 12 day culture period, whereas responder PBMCs (50,000 and 100,000) demonstrated some degree of proliferation from day 3, throughout the culture period to day 12.

When cultured in a 1:1 ratio of responder cells:stimulator cells using 10,000 total cells per well, counts per minute remained stable from day 3 to day 12. However, when cultured at a 1:1 ratio and with 50,000 and 100,000 total cells counts per minute increased from day 3, to peak stimulation at day 7, before counts reduced by day 12 (**Figure 5.15**).

When a 1:10 ratio of responder cells:stimulator cells were cultured together a similar trend to PBMCs cultured at a 1:1 ratio was observed, with 10,000 total cell number demonstrating no increase in counts of the 12 day culture period, but both 50,000 and 100,000 total cell numbers resulted in increased counts from day 3, peaking on day 7, before again reducing by day 12. Counts per minute were slightly less than when a 1:1 ratio was used, but the trend observed was the same (**Figure 5.15**).

When a 1:100 ratio of responder cells:stimulator cells were cultured together no stimulation was observed when 10,000 and 50,000 total cell number were used. When 100,000 total cell number was used, the counts continued increasing to day 12, (**Figure 5.15**).

The data was analysed by one way ANOVA and the MSD (p=0.05) between group means determined at each time point. The results of the analysis are presented in **Figure 5.16**. This revealed that at day 3 for the 1:1 ratio counts per minute for 100,000 total cells were significantly greater than for 50,000 and 10,000 cells. Moreover the counts per minute for the 1:1 ratio with both the 50,000 and 100,000 cells was significantly higher than the counts per

minute for the 1:10 and 1:100 ratios for the same number of total cells. At day 5 and day 7, for cells at 1:1 ratio there was no significant difference between the counts per minute for 50,000 and 100,000 cells, however the counts per minute for both was significantly higher than the counts per minute for 10,000 cells. Again the cells cultured at 1:1 ratio gave significantly higher counts compared to the cells cultured at 1:10 and 1:100 for the same total cell number.

At day 12 the counts were highly variable across all cell cultures. There was no significant difference observed between the cells only negative control and 1:1, 1:10, and 1:100 ratios of stimulator to responder cells, when 10,000, 50,000 and 100,000 total cells were used; except for 50,000 cells at 1:100 ratio, this ratio and cell number had a significantly lower counts per minute.

The highest counts per minute were observed when a 1:1 ratio of responder:stimulator PBMCs was used, and both 50,000 and 100,000 total cell number demonstrated high stimulation. Using 50,000 cells in downstream applications it was possible to conserve the number of cells required to perform the assays, allowing a greater 'n'number to be utilised within the requirements of using cells before passage 6 (using 100,000 cells would double the number of cells needed per assay). It was therefore decided to use a 1:1 ratio, and a 50,000 total cell number in further experiments.

The increase in counts of the PBMCs in medium only (**Figure 5.12**) indicated potential stimulation from the fetal calf serum, therefore it was decided to perform the LTA using human serum, and compare counts to an LTA using fetal calf serum.

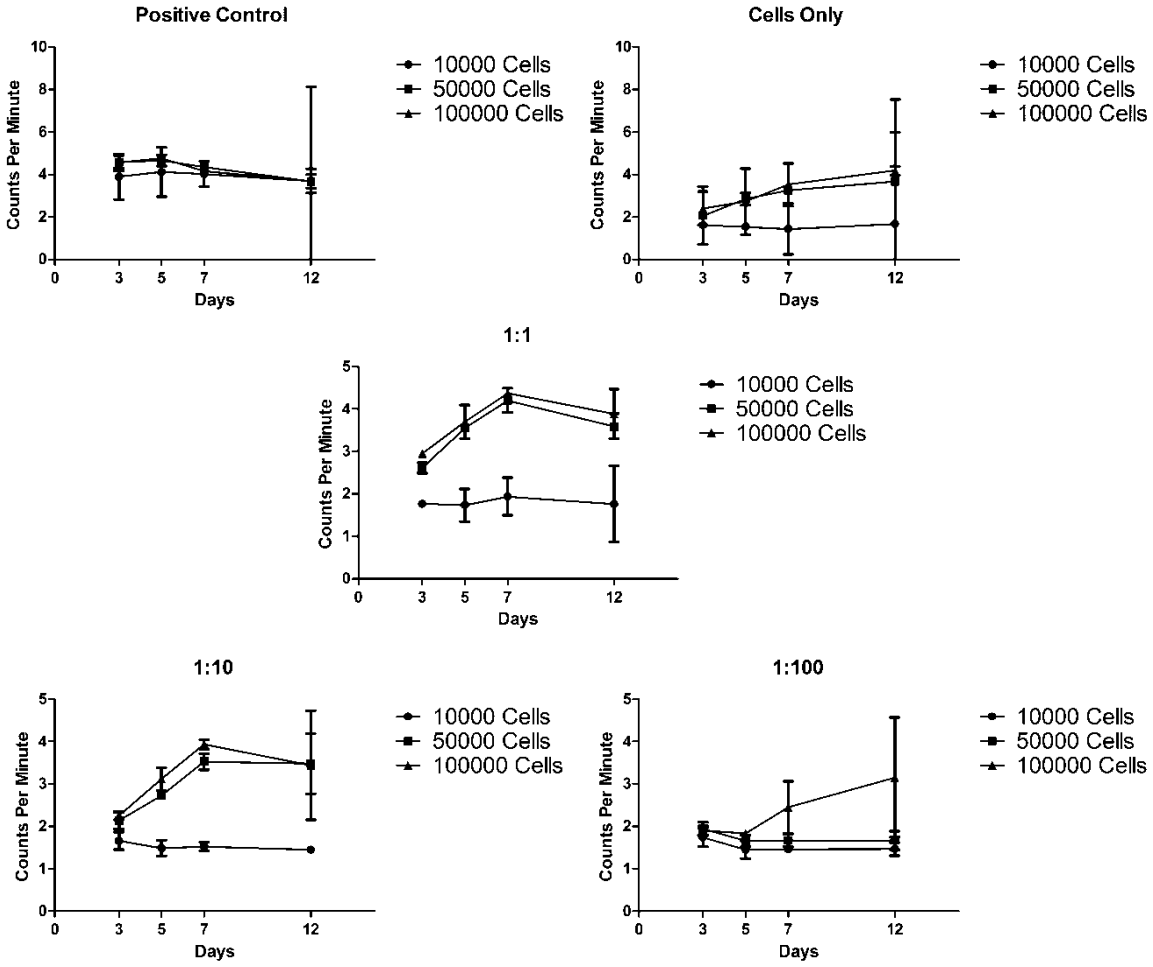


Figure 5.15: A one-way MLR to determine the ratio of responder:stimulator PBMCs, and the total cell number per well that generated the optimum counts per minute (stimulation). Untreated PBMCs (10,000, 5000, 1000, and 100) and mitomycin C treated PBMCs (5000, 9000, 9900, and 10,000) were cultured in ratios of 1:1, 1:10, and 1:100 responder to stimulator cells. The total numbers of cells per well were 10,000, 50,000, and 100,000. The positive control for stimulation was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA. The negative control for stimulation was responder PBMCs cultured in complete culture medium (Cells only). Data is expressed as mean Log_{10} counts per minute (n=4) ± 95% confidence limits.

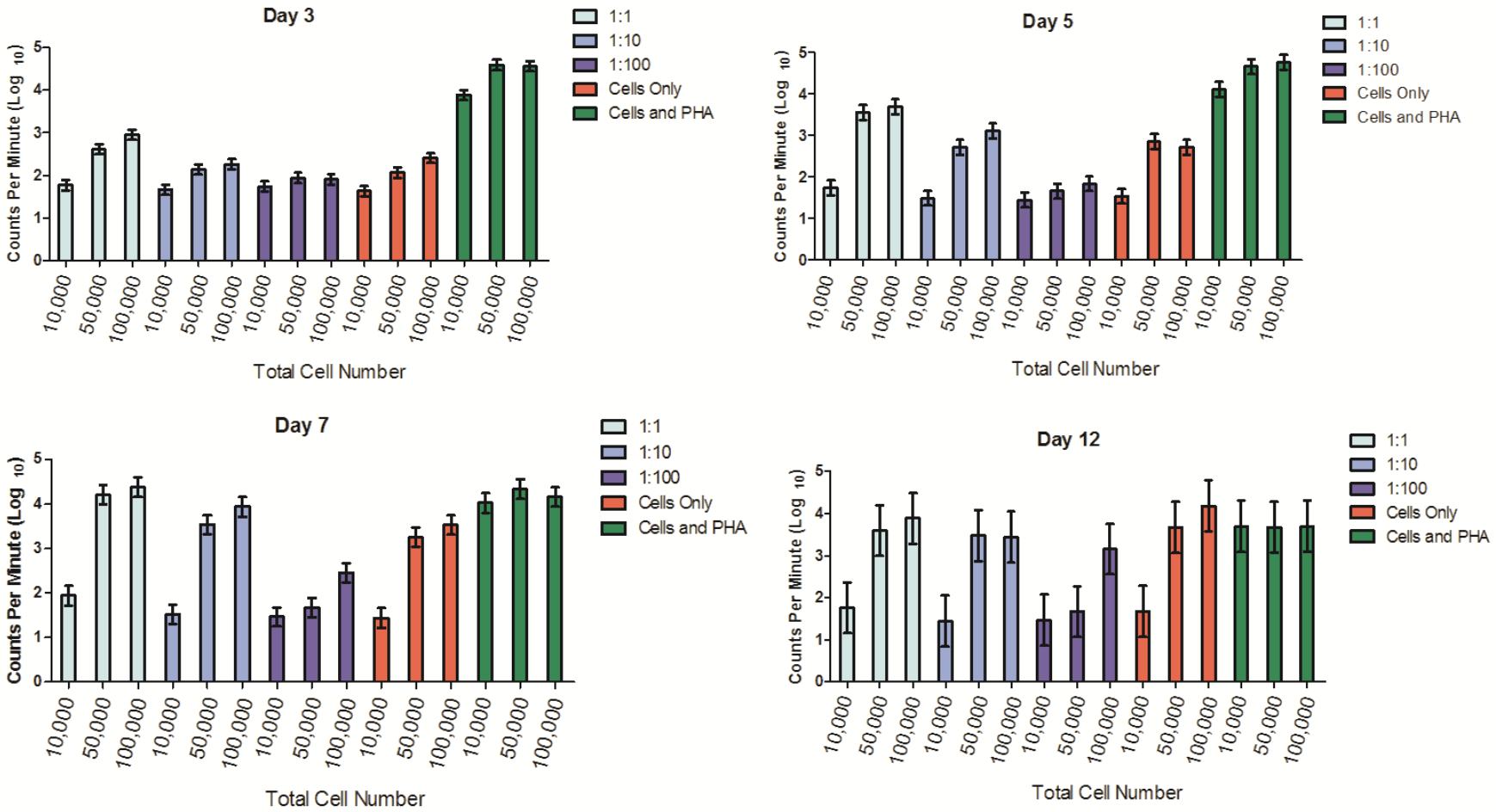


Figure 5.16: Grouped bar chart of Log_{10} counts per minute for a one-way MLR to determine the ratio of responder:stimulator PBMCs and the total cell number per well that generated the optimum counts per minute (stimulation). Untreated PBMCs and mitomycin C treated PBMCs were cultured in ratios of 1:1, 1:10, and 1:100 responder to stimulator cells. The total numbers of cells per well were 10000, 50000, and 100000. The positive control for stimulation was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA (Cells and PHA). The negative control for stimulation was responder PBMCs cultured in complete culture medium (Cells only). Data is expressed as mean Log_{10} counts per minute (n=4). Data (log_{10} cpm) at each time point was analysed by one-way ANOVA and the MSD ($P<0.05$) calculated using the t-method. Error bars are plotted as ± ½ MSD ($p<0.05$). If error bars from different group means do not overlap, then there is significant difference at $p<0.05$.

5.3.5 A LYMPHOCYTE TRANSFORMATION ASSAY TO DETERMINE IF USING HUMAN AB SERUM AS AN ALTERNATIVE TO FETAL CALF SERUM IN COMPLETE MEDIUM CHANGES THE RESPONSE OF PBMCS WHEN STIMULATED WITH PHA

Day 5 was selected as the starting day for analysis, as typically when PHA was used to stimulate PBMCs, peak stimulation occurred on this day.

Peripheral blood mononuclear cells were cultured with and without PHA stimulation at 25,000, 50,000, and 100,000 cells per well for 5, 7, and 12 days in medium supplemented with FBS or human AB serum. In PBMC cultures without PHA no significant stimulation was observed and the counts per minute obtained remained low (**Figure 5.17**). In the presence of PHA, when 25,000 cells were used, the highest stimulation observed was on day 5, with counts per minute decreasing on day 7, and further by day 12. Peripheral blood mononuclear cells cultured at 25,000, 50,000 and 100,000 cells per well showed resulted in the same growth characteristics, with highest stimulation on day 5, and lowest by day 12.

As there was no overall difference in Log_{10} counts per minute between 25,000, 50,000 or 100,000 PBMCs when stimulated with PHA, this further supported the use of 25,000 responder PBMCs in future assays as this agrees with previous data of maximal counts observed when 25,000:25,000 PBMCs were used in the MLR.

In addition to this, counts in the presence of AB serum were significantly higher than when fetal calf serum was used in complete medium, for 25,000, 50,000 and 100,000 PBMCs. It was decided to use human AB serum as a supplement in complete medium for PBMCs due to the generation of higher positive control counts when this serum is used.

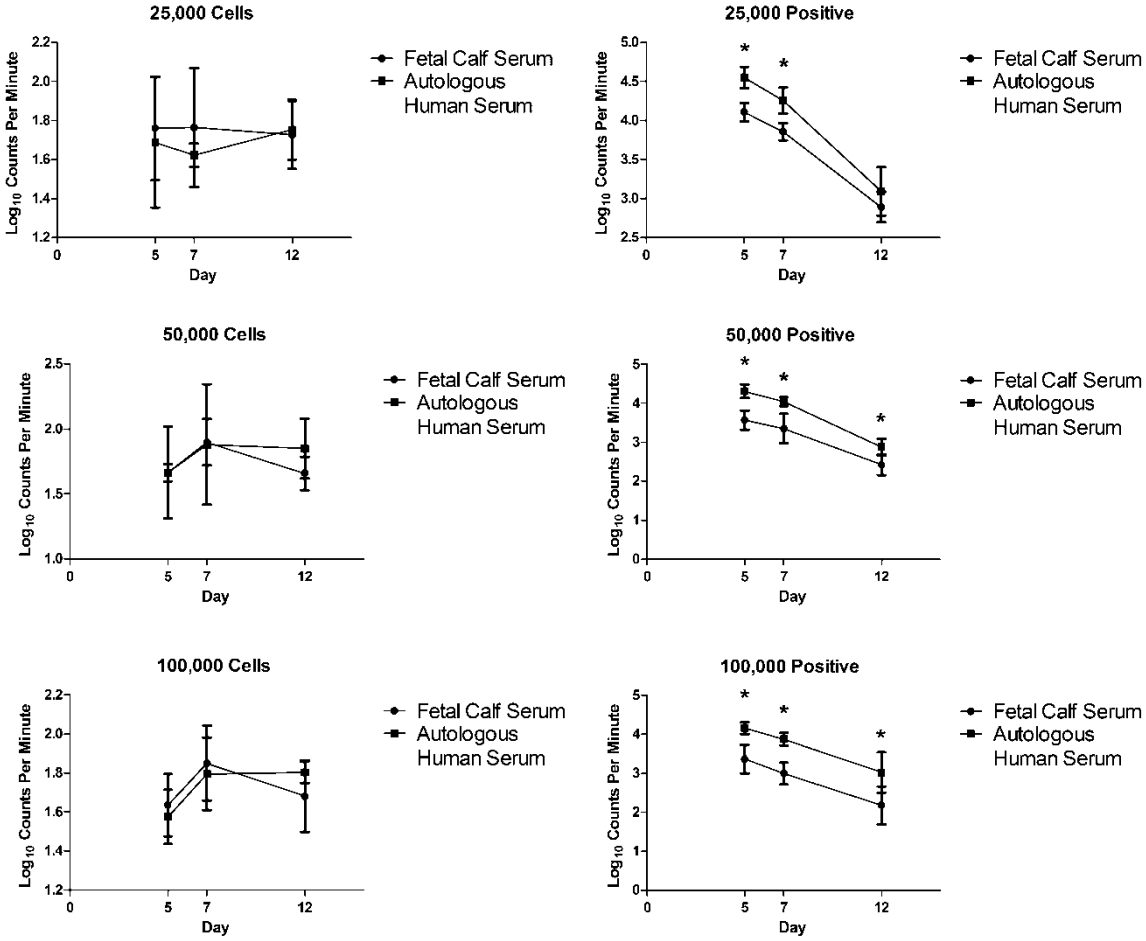


Figure 5.17: Counts per minute of PBMCs cultured without stimulation, and in the presence of PHA using both fetal calf serum, and human AB serum supplemented complete medium. PBMCs (25,000, 50,000, and 100,000) were cultured for 5, 7, and 12 days and the counts per minute of both stimulated and unstimulated PBMCs was compared. Data is expressed as mean Log_{10} counts per minute (n=4) ± 95% confidence limits. Data (log_{10} cpm) for cells cultured in FCS and human AB serum supplemented medium were compared at each time point for stimulated and unstimulated cells by one-way ANOVA. Significant differences between fetal calf serum and autologous human serum are indicated by * = $p<0.05$.

5.3.6 THE EFFECT OF HUMAN AB SERUM CONCENTRATION IN COMPLETE MEDIUM ON STIMULATED (PHA TREATED) AND UNSTIMULATED PERIPHERAL BLOOD MONONUCLEAR CELLS

Both stimulated and unstimulated PBMCs from both donor 1 and donor 2 failed to display any significant proliferation when cultured in complete medium containing 2, 5, or 10% human AB serum, as measured by Log_{10} counts per minute and one way analysis of variance as determined by the T-method (**Figure 5.18**).

When PBMCs were stimulated with PHA and cultured in complete medium supplemented with human AB serum, all three concentrations of serum for both donors displayed similar trends. The counts per minute increased from day 3 to peak on day 5, before counts dropped by day 7.

The concentration of serum used to supplement medium in lymphocyte transformation assays in the literature is typically 10%. It was therefore decided to keep the concentration of serum used as 10% due to the lack of any significant difference between 2, 5, or 10% serum supplementation.

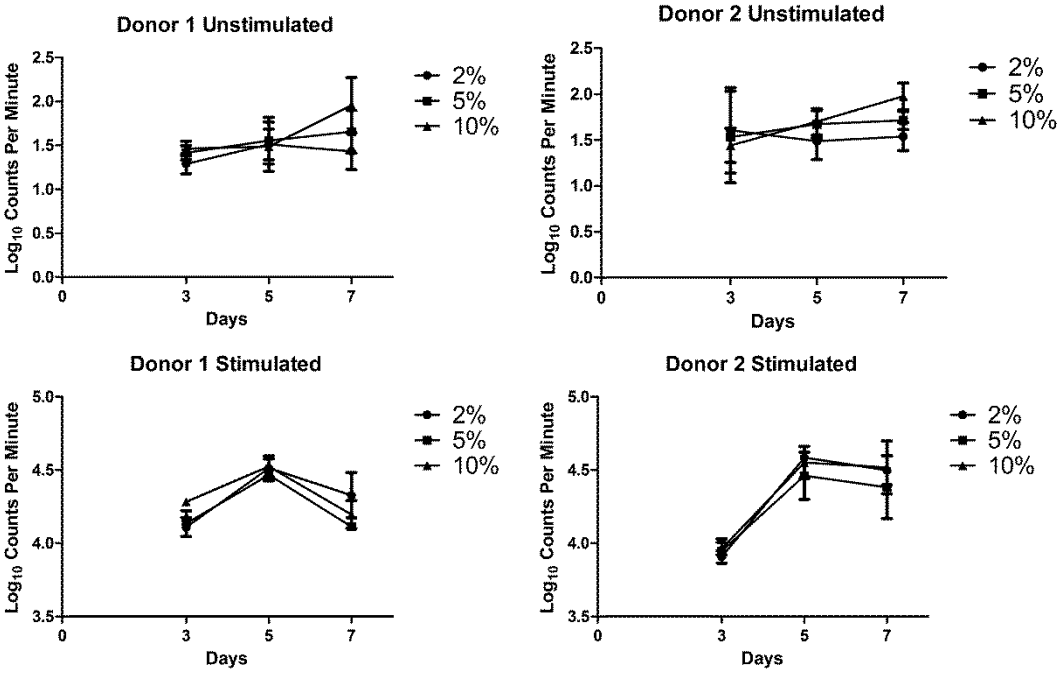


Figure 5.18: Counts per minute of PBMCs cultured in complete medium supplemented with 2, 5, and 10% human AB serum, and either stimulated with PHA, or unstimulated (medium alone). PBMCs (25,000) were cultured for 5, 7, and 12 days. Data (counts per minute) was Log_{10} transformed. Log_{10} data for each donor at each time point, stimulated and unstimulated was compared by one-way ANOVA ($p<0.05$) using the T-method.

5.4 DISCUSSION

There were 5 main objectives for this part of the study. These were the determination of the concentration of PHA (mitogen) for use as the positive control for lymphocyte activation and of the method for spiking the cells (using tritiated thymidine), the determination of the concentration of mitomycin C for the mitotic inhibition of stimulator cells while maintaining their viability and cell number and ratio of responder and stimulator cells in the one way MLR, and finally the determination of the concentration and source of serum for cell culture medium supplementation.

Primarily PBMC were isolated using a Lymphoprep® density centrifugation gradient. This commercially available preparation has been widely used for the isolation of mononuclear cells from peripheral blood, based on the lower buoyant density of mononuclear cells compared to the poly-morphonuclear granulocytes or the erythrocytes. The mononuclear cell population has a buoyant density below 1.077 $g.ml^{-1}$, therefore centrifugation using a medium of this density causes the granulocytes and erythrocytes to pass through the solution, but mononuclear cells remain at the interface. These cells were then aspirated using glass Pasteur pipettes, and the mononuclear cells were then washed to remove traces of Lymphoprep®.

A positive control for stimulation of cultured PBMCs was required to ensure the functional capacity of the isolated cells. A range of concentrations of PHA, a plant lectin that induces mitosis, was used to stimulate cultured PBMCs to proliferate. Proliferation was measured using tritiated thymidine (^3H) incorporation, both high activity and low activity. Tritiated thymidine is a low energy beta (β) emitter; low activity ^3H has a lower specific activity, and high activity ^3H has a higher specific activity. The higher the specific activity is, the higher the number of decays per second, per amount of ^3H.

The high activity ^3H required a shorter incubation time with the cells (4 hours) in comparison with the low activity ^3H (16 hours). The low activity ^3H provided significantly higher counts per minute over the high activity, and therefore was selected for future experiments. A PHA concentration of 32 $\mu g.ml^{-1}$ gave the highest counts, and this was selected to use as a positive control for lymphocyte stimulation in further assays. The use of proper controls is vital when calculating the stimulation index in lymphocyte proliferation assays, as in negative control counts a variation between 100-200 (which in actual counts per minute terms is a very small difference) can cause the stimulation index to as much as halve in value.

In assays to determine the immunogenicity of MSCs, the MSCs act as the stimulator cells. The mitotic ability of these cells needed to be inhibited, so that the incorporation of ^{3}H measured was only due to the proliferation of the responder lymphocytes. In order to do this, the chemical compound mitomycin-C (MMC) was selected; this compound has been used to inhibit proliferation of stimulator cells in MLRs (Oh et al., 2008; Nasef et al., 2009; Prasanna et al., 2010). Initially concentrations of MMC between 6.25 and 200 $\mu g.ml^{-1}$ were used to treat the L929 cell line, and the effect of MMC on the proliferation of these cells over 10 days was measured using the ATPLite® assay. The basis of this assay is that ATP levels are measured, and increasing concentrations of ATP are used signify increasing cell numbers. Initial treatment with MMC caused the concentration of ATP (represented by the counts per second) to vary between the different concentrations of MMC applied to the cells. ATP levels were higher in MMC treated cells on day 0. This is in agreement with previous studies that found MMC also was able to affect mitochondrial DNA and ATP levels within cells treated with MMC can vary extensively due to this (Maehara et al., 1987; Pritsos and Briggs, 1996). Hence, an alternative method of assessing cell proliferation was used. Fluorescein diacetate is a membrane permeable compound, that upon entering viable cells is converted using cell esterase's into the fluorescent fluorescein (Krause et al., 1984). This compound is unable to pass across the cell membrane, and the end result is that viable cells fluoresce. The rationale in using this compound to assess cell proliferation was that if cells are able to divide, the fluorescence will increase, but if cells are unable to proliferate, no increase in fluorescence will occur. In order to determine the concentration of MMC that would arrest cell division in PHDFs 3 concentrations of MMC (50, 25, and 12.5 $\mu g.ml^{-1}$) were selected based on the literature. All three concentrations appeared to inhibit the proliferation of the PHDFs, while maintaining viability. The same assay was repeated on MSCs, and displayed similar results (all three concentrations inhibited cell proliferation and maintained cell viability). The concentration of MMC selected to use in downstream assays was 12.5 $\mu g.ml^{-1}$ as this was the lowest concentration (so least cytotoxic) that inhibited proliferation of both PHDFs and MSCs. Whereas MSCs exhibited a typical growth curve, the PHDFs exhibited a delay in growth due to the low seeding density used, and did not start proliferating until after day 4, causing the atypical growth curve in **Figure 5.13**.

The optimum cell number and ratio of PBMCs that generated the highest counts was determined using a one way MLR. Ratios of 1:1, 1:10, and 1:100 of responder: stimulator cells were selected for testing since in a traditional tissue engineering application, in which MSCs are expanded from an allogeneic donor and seeded onto a scaffold prior to implantation, it is

likely that the APCs of the host will encounter the MSCs at a high cell density. This scenario was recreated *in-vitro* using ratios of MSCs, in excess of donor PBMCs.

It was shown that the highest counts were obtained when a ratio of 1:1 responder cells to stimulator cells was used. Optimum counts per minute were also obtained when 100,000 total cell number per well was used, however, 50,000 total number of cells also showed high counts per minute comparable to 100,000 cells.

The final factor used in the LTA that required determination was the type, and concentration of serum used in the complete culture medium. When developing the assay, PBMC cultured in medium plus fetal calf serum showed some proliferation. It was hypothesised that the foreign proteins (immunogens) in the fetal calf serum might stimulate the PBMCs to proliferate. Human AB serum was used as an alternative. The serum from donors of blood group AB contains no antibodies to either A or B antigens, and provided a commercial alternative to using autologous serum. The serum was heat treated to inactivate the serum complement proteins before it was used in the LTA or MLR assays. A comparison between fetal calf serum, and human AB serum supplemented medium revealed no significant difference in lymphocyte proliferation in unstimulated cultures. However, the positive control counts were significantly higher when human serum was used; therefore human AB serum was selected to be used in further assays. The effect of serum concentration also was assessed on the proliferation of PBMCs. Previously 10% FCS has been used in LTA (Nasef et al., 2009; Wada et al., 2009; Prasanna et al., 2010), or 5-10% human serum (Bocelli-Tyndall et al., 2007; Niemeyer et al., 2007) so LTAs were performed using 2, 5, and 10% (v/v) human serum in complete RPMI culture medium. There was no significant difference between the concentrations of serum used, so 10% was chosen for future assays.

In conclusion the developed LTA used 32 $mg.ml^{-1}$ PHA as positive control for stimulation, a ratio of 1:1 25,000 PBMCs to MSCs, 12.5 $mg.ml^{-1}$ mitomycin C to mitotically inactivate the responder cells, and 10% (v/v) human AB serum in RPMI-1640 complete culture medium. Low activity trititiated thymidine was used to determine the incorporation of thymidine into the DNA of responder cells.

Chapter 6: ASSESSMENT OF THE IMMUNOGENICITY OF MULTIPOTENT MESENCHYMAL STROMAL CELLS USING LYMPHOCYTE TRANSFORMATION ASSAYS OVER 21 DAYS

6.1 INTRODUCTION

The lymphocyte transformation assay is routinely used to assess the proliferative response of peripheral blood mononuclear cells (PBMCs) to antigenic stimulation. In this study the ultimate aim was to assess the response of PBMCs to mesenchymal stromal cells (MSCs).

The immunogenicity of MSCs is controversial. These cells have been reported to be immuno-privileged and have immunosuppressive properties (Bartholomew et al., 2002; Le Blanc et al., 2003b; Tse et al., 2003). Given the ability of these cells to differentiate into bone, fat, and cartilage cell types (Prockop, 1997; Pittenger et al., 1999; Barry and Murphy, 2004; McGonagle and Jones, 2008) and potential applications in tissue engineering, the question regarding the immunogenicity of MSCs is important to fully explore in both *in-vitro* and *in-vivo* systems.

The potential application of MSCs for use in tissue engineering has generated a large amount of interest, as cells with trilineage potential that would remain immunologically inert after allogeneic transplantation would be prime candidates for regenerative medicine applications. This would enable donor banks of MSCs to be established and used to populate scaffolds before implantation, or used to repopulate scaffolds which are then cultured under differentiation conditions to direct cells down the desired lineage, before implantation. The question arises regarding whether tissue engineered constructs based on allogeneic MSCs would be accepted by the recipient's immune system.

As discussed previously, MSCs have been shown not to stimulate a proliferative response when co-cultured with allogeneic lymphocytes. Upon review of the literature, the methodology used to investigate the immunogenicity of MSCs has limitations. The assay used to demonstrate the immune-privileged nature of MSCs has primarily been the classical lymphocyte transformation assay, in which MSCs have been co-cultured with allogeneic lymphocytes for up to 7 days (Bartholomew et al., 2002; Tse et al., 2003; Niemeyer et al., 2007; Oh et al., 2008; Suva et al., 2008). MSCs do not express co-stimulatory molecules CD40, CD80, or CD86, and lack expression of cell surface MHC class-II (Le Blanc and Ringden, 2007). Hence the lack of stimulation when MSCs are co-cultured with lymphocytes for up to 7 days is

unsurprising, as in all likelihood MSCs will not be recognised via the direct pathway of allorecognition. In theory however, MSCs should have the capacity to be recognised by the indirect and semi-direct pathways of allorecognition in which alloantigens are taken up or transferred to recipient antigen presenting cells and presented to recipient CD4+ T-cells. The CD4+ T-cells would then be able to recognise the presented antigen as 'foreign', and proliferate in response to this. This proliferation would be measurable via the incorporation of tritiated thymidine into the T-cell DNA.

It was hypothesized that, by extending the culture time of the LTA it would be possible to measure indirect and/or semi-direct antigen presentation, and that this would give a clearer indication of the immunogenicity of MSCs. Primary dermal human fibroblasts were used as a control cell type due to their mesenchymal lineage and availability.

In the previous chapter methods for carrying out the LTA were developed and the following parameters established.

- 32 $mg.ml^{-1}$ PHA as a positive control for cell stimulation
- PBMCs (25,000) and MSCs (25,000) cultured in a 1:1 ratio of responder: stimulator cells.
- Mitomycin C treatment (12.5 $mg.ml^{-1}$) for one hour to arrest mitosis of MSCs.
- Human AB serum (10% (v/v)) in complete culture medium.
- U-shaped, uncoated 96 well tissue culture plates.

In addition to this methodology developed in the previous chapter, it was decided to culture the MSCs/PHDFs with PBMCs for 3, 5, 7, 12, 16, and 21 days before spiking with low activity tritiated thymidine for 16 hours prior to harvesting.

6.1.1 AIMS AND OBJECTIVES

The aims of this chapter were to assess the capacity of human bone marrow derived MSCs for stimulation of allogeneic lymphocytes in the LTA over an extended culture period of 21 days. There are two main objectives:

- To determine the lymphocyte transformation response of PBMCs from six donors stimulated by MSCs from 4 unrelated donors in LTA assays for up to 21 days.
- To compare the lymphocyte transformation response of PBMCs from six donors when stimulated with MSCs and PHDFs.

6.2 MATERIALS AND METHODS

6.2.1 THE LYMPHOCYTE TRANSFORMATION ASSAY

Reagents

Phytohaemagglutinin (1 mg.ml^{-1} in dH_2O)

Phosphate buffered saline

Hy-Q-Tase™ cell detachment solution

NH expansion medium (Section 5.2.1)

Transport medium (Section 5.2.1)

Complete medium (Section 5.2.1)

Mitomycin C (1 mg.ml^{-1} in dH_2O)

Method

Bone marrow 1-4 MSCs were cultured in NH Expansion medium up to passage 6, at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.2.

Multipotent mesenchymal stromal cells were cultured in NH expansion medium until confluent in 4 T75 tissue culture flasks. When confluency was reached the medium was removed from the flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. PBS was removed and 2 ml Hy-Q-Tase™ was added to the flask, before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (approx. 10 minutes). When cells had detached the resulting cell suspension was resuspended in 5 ml complete medium, and 62.5µl MMC stock was added to give a final concentration of 12.5 µg.ml^{-1}. Following 1 hour incubation the cells were centrifuged at 150 g for 10 minutes, the mitomycin C medium removed, and cells were washed twice with 10ml complete medium. Finally MSCs were resuspended in 5ml complete medium before counting using trypan dye exclusion as described in section 2.3.7. The MSCs from BM 1-4 (25,000) were dispensed into wells of 6 uncoated U-bottom shaped 96 well tissue culture plates as shown in **Figure 6.1** (24 plates in total).

Primary human dermal fibroblasts were cultured in M-DMEM at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.3, and 25,000 were

dispensed into the well of 6 uncoated U-bottom shaped 96 well tissue culture plates as shown in **Figure 6.2**, using the same method as the MSCs.

Peripheral blood mononuclear cells were isolated from 6 healthy volunteer's human peripheral blood as described in section 5.2.1. Following isolation PBMCs were counted using trypan dye exclusion as described in section 2.3.7. These cells (25,000) were dispensed into the wells of 24 uncoated U-bottom shaped 96 well tissue culture plates as shown in **Figure 6.1**.

The negative control for proliferation was responder PBMCs grown in complete medium only, and positive control was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA. Phytohaemagglutinin stock (64 µl) was diluted in 1 ml complete RPMI-1640 medium, and added to wells at a 1:1 ratio of PHA to PBMCs in medium to achieve a final concentration of 32 $\mu g.ml^{-1}$ in the wells shown in **Figure 6.1**.

Plates were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Sixteen hours before the desired timepoint each well of the plate was spiked with 10 µl of low activity tritiated thymidine stock and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air overnight. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester, before 35 µl MicroScint™ was added and radioactive counts were measured on a TopCount scintillation counter as described in section 5.2.3. Plates were harvested on days 3, 5, 7, 12, 16 and 21. On day 12, 100 µl of the complete medium in the wells was removed from the remaining plates, and replaced with 100 µl of fresh complete medium.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**MSCs in medium**			
C	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**MSCs in medium**			
D	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**				
E	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**				
F	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**				
G	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**				
H												

Figure 6.1: Diagram of the 96 well plate experimental wells used to determine the stimulation of peripheral blood mononuclear cells when cultured in complete medium only (negative control for stimulation), in the presence of PHA (positive control for stimulation), and co-cultured with mitomycin C treated MSCs. MSCs in complete medium only are also used as a negative control for proliferation. Donor 1, 2, and 3 represent the donor PBMCs. Plates were also prepared using the same format with PBMCs from donors 4, 5, and 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
C	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
D	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
E	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
F	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
G	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + PHDFs**	**25,000 + PHDFs**	**25,000 + PHDFs**	
H												

Figure 6.2: Diagram of the 96 well plate experimental wells used to determine the stimulation of peripheral blood mononuclear cells when cultured in complete medium only (negative control for stimulation), in the presence of PHA (positive control for stimulation), and co-cultured with mitomycin C treated PHDFs, and MSCs. Donor 1, 2, and 3 represent the donor PBMCs. Plates were also prepared using the same format with PBMCs from donors 4, 5, and 6.

6.2.2 THE LYMPHOCYTE TRANSFORMATION ASSAY CO-CULTURING PERIPHERAL BLOOD MONONUCLEAR CELLS AND MSCS IN V-BOTTOM UNCOATED 96 WELL TISSUE CULTURE PLATES

In addition to performing the LTA in uncoated U-bottom 96 well tissue culture plates, the same methodology as described in section 6.2.1 was used, but uncoated V-bottom plates were used as an alternative.

6.2.3 ANALYSIS OF DATA

Data (counts per minute) from the lymphocyte transformation assays was $\log_{10}$ transformed and is presented as $\log_{10}$ counts per minute ± 95% confidence limits. The $\log_{10}$ counts per minute at each timepoint (day 3, 5, 7, 12, 16, and 21) were compared by one-way analysis of variance and the MSD calculated using the T-method ($p<0.05$). Data was also used to calculate the stimulation indices from the following equation:

$$SI = \frac{\text{Average counts per minute PBMCs + MSCs (or PHDFs)}}{\text{Average counts per minute of PBMCs cultured alone}}$$

6.3 RESULTS

6.3.1 THE CO-CULTURE OF MSCS AND PHDFS WITH PBMCS IN THE LYMPHOCYTE TRANSFORMATION ASSAY

Peripheral blood mononuclear cells were isolated from the blood of 6 healthy volunteers. These cells were co-cultured with MSCs isolated from 4 donor bone marrows. The counts per minute were $\log_{10}$ transformed to conform to the requirements of analysis of variance (variances shown to be homogeneous after $\log_{10}$ transformation). The counts obtained for the positive control cultures with PHA were omitted from analyses since these were included to ensure that the assays were valid (PBMCs were functioning by responding to the mitogen) and it was not part of the experimental design to compare the level of response of the MSCs or PHDFs to the level of response to the mitogen.

The results are presented in **Figures 6.3-6.50**. The results have been presented as both the $\log_{10}$ counts per minute for PBMCs at each time point and the stimulation indices at each time point for each donor PBMC vs each bone marrow derived MSC and PHDF. This allowed comparison of the data presented in each form.

6.3.1.1 BM1

When PBMCs from donor 1 were cultured with PHA, maximal counts were observed on days 3-5 after which the response declined over the remaining 21 day culture period. This response observed when PBMCs were stimulated with PHA was observed for all PBMC donors in all BM 1-4 cultures. When PBMCs from donor 1 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3, 5, 16, and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When PBMCs from donor 1 were cultured with PHDFs significantly higher counts were recorded only on day 3 compared to unstimulated PBMCs (**Figure 6.3**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.4**. This showed stimulation indices above 3 for PHA stimulated PBMCs from day 3, demonstrating the ability of the PBMCs to proliferate. The proliferation of PBMCs was repeated for all 3 PBMC donors upon stimulation with PHA. Stimulation indices above 3 were observed for PBMCs from donor 1 in response to BM1 MSCs at days 3 (SI of 3.18), 5 (SI of 3.83), 7 (SI of 4.38), 12 (SI of 12.31), 16 (SI of 12.95), and 21 (SI of 3.88). For PBMC from donor 1 in response to PHDFs, a stimulation index of above 3 was recorded at days 3 (SI of 3.27) and 12 (SI of 10.66).

When PBMCs from donor 2 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3, 5, 12, 16, and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA).

When PBMCs from donor 2 were cultured with PHDFs significantly higher counts were recorded on days 3, 5, and 7 compared to unstimulated PBMCs (**Figure 6.5**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.6**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM1 MSCs at days 5 (SI of 5.12), 7 (SI of 3.30), 12 (SI of 30.80), 16 (SI of 19.05), and 21 (SI of 7.42). For PBMC from donor 2 in response to PHDFs, a stimulation index of above 3 was recorded at days 5 (SI of 4.07), 7 (SI of 10.45), 12 (SI of 14.87), 16 (SI of 5.93), and 21 (SI of 10.03).

When PBMCs from donor 3 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3, 5, 12, 16, and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When PBMCs from donor 3 were cultured with PHDFs significantly higher counts were also recorded on days 3, 5, 12, 16, and 21 compared to unstimulated PBMCs (**Figure 6.7**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.8**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM1 MSCs at days 5 (SI of 6.43), 7 (SI of 20.60), 12 (SI of 23.07), 16 (SI of 5.88), and 21 (SI of 11.15). For PBMC from donor 3 in response to PHDFs, a stimulation index of above 3 was recorded at days 3 (SI of 3.66), 5 (SI of 3.63), 7 (SI of 15.61), 12 (SI of 12.99), 16 (SI of 6.28), and 21 (SI of 7.57).

When PBMCs from donor 4 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3, 5, and 13 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When PBMCs from donor 4 were cultured with PHDFs significantly higher counts were also recorded on days 3, 5, and 13 compared to unstimulated PBMCs (**Figure 6.9**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.10**. This showed stimulation indices above 3 for PBMCs from donor 4 in response to BM1 MSCs at days 13 (SI of 33.40), and 18 (SI of 7.00). For PBMC from donor 4 in response to PHDFs, a stimulation index of above 3 was recorded at day 13 (SI of 16.28).

When PBMCs from donor 5 were cultured with with BM1 MSCs, significantly higher counts were recorded on days 3, 5, and 18 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When PBMCs from donor 5 were cultured with PHDFs significantly higher counts were also recorded on days 3, 5, and 18 compared to unstimulated PBMCs (**Figure 6.11**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.12**. This showed no stimulation indices above 3 for PBMCs from donor 5 in response to BM1. For PBMC from

donor 5 in response to PHDFs, a stimulation index of above 3 was recorded at days 3 (SI of 3.27), 13 (SI of 4.30), and 18 (SI of 3.12).

When PBMCs from donor 6 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When PBMCs from donor 6 were cultured with PHDFs significantly higher counts were also recorded on days 3 and 5 compared to unstimulated PBMCs (**Figure 6.13**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.14**. This showed stimulation indices above 3 for PBMCs from donor 6 in response to BM1 MSCs at days 3 (SI of 3.31), 5 (SI of 3.64), and 13 (SI of 6.28). For PBMC from donor 6 in response to PHDFs, a stimulation index of above 3 was recorded at days 3 (SI of 4.70) and 5 (SI of 6.40).

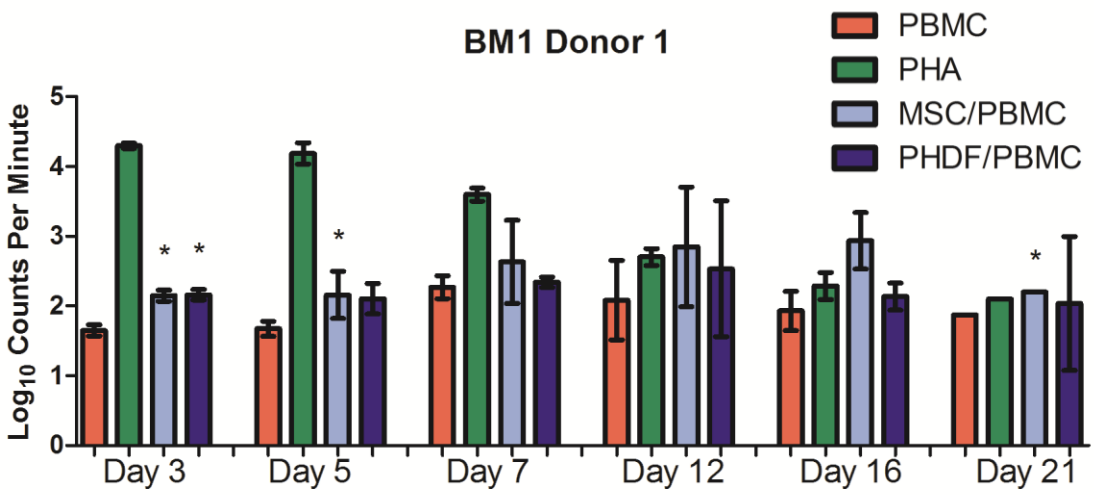


Figure 6.3: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

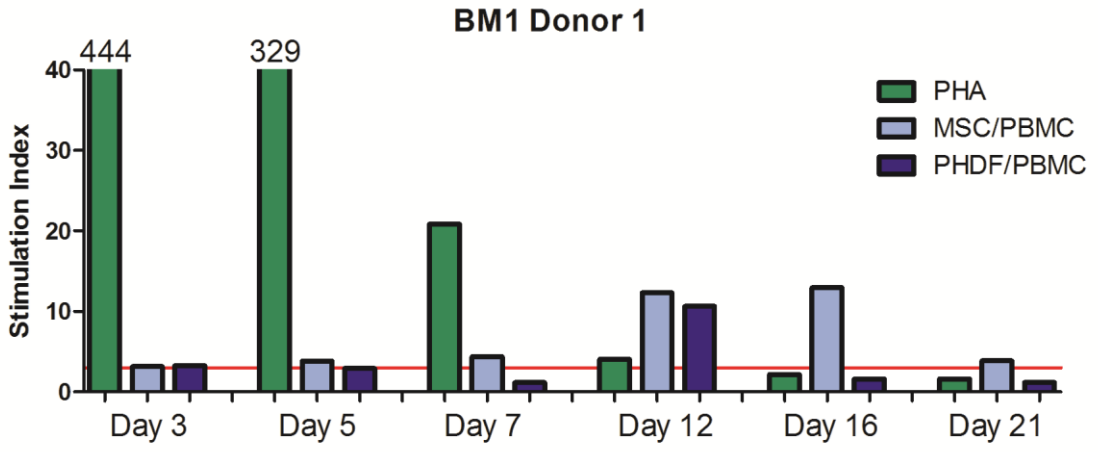


Figure 6.4: The stimulation index (SI) of donor 1 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

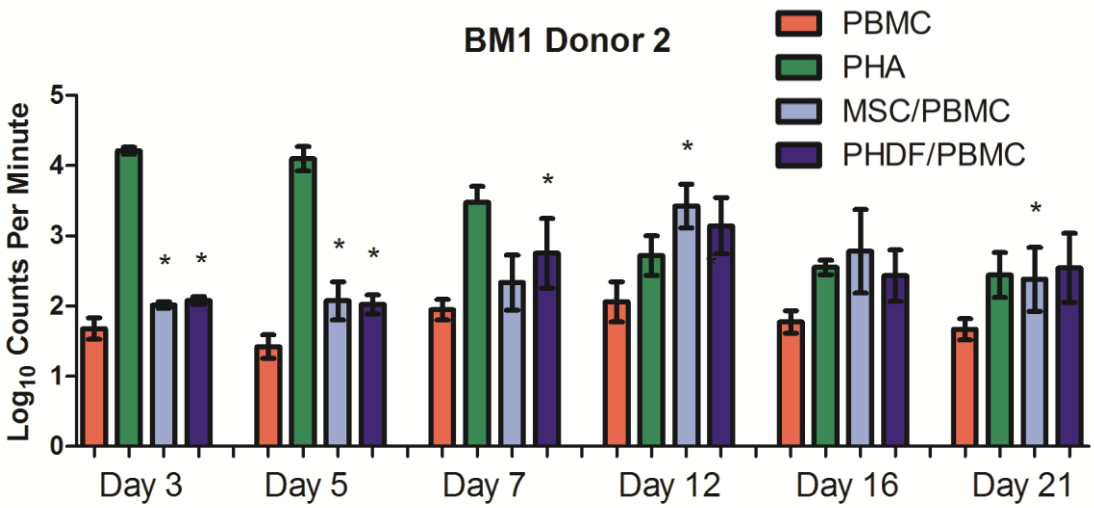


Figure 6.5: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

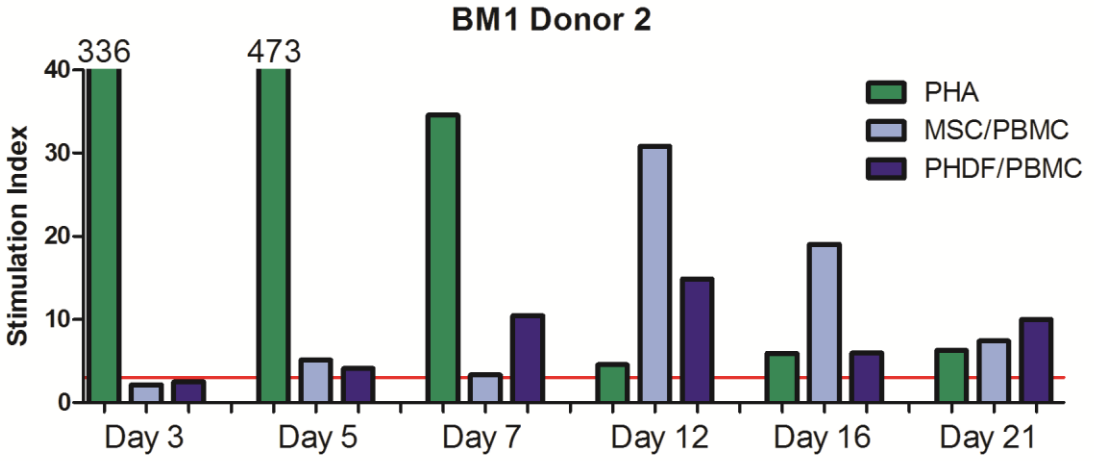


Figure 6.6: The stimulation index (SI) of donor 2 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

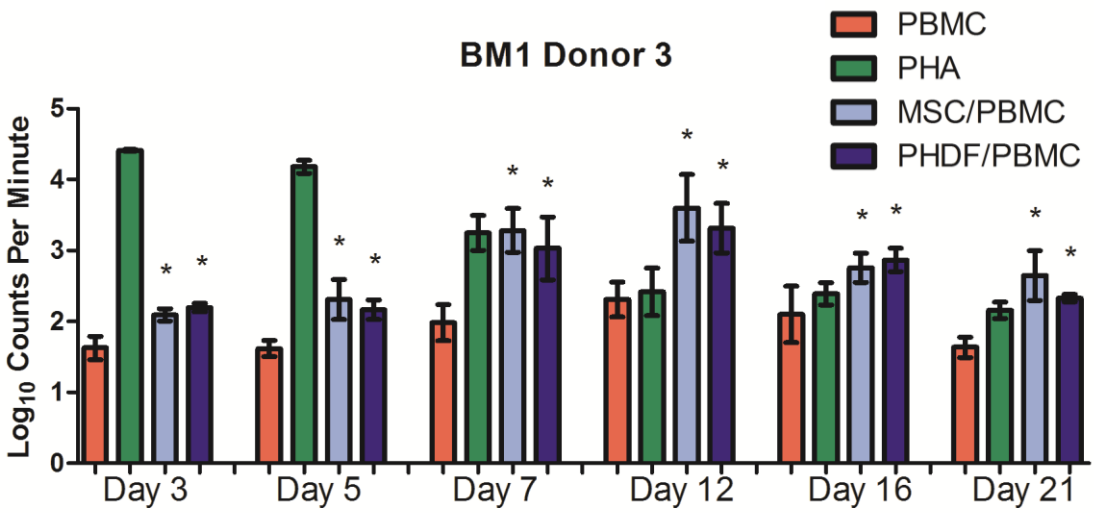


Figure 6.7: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

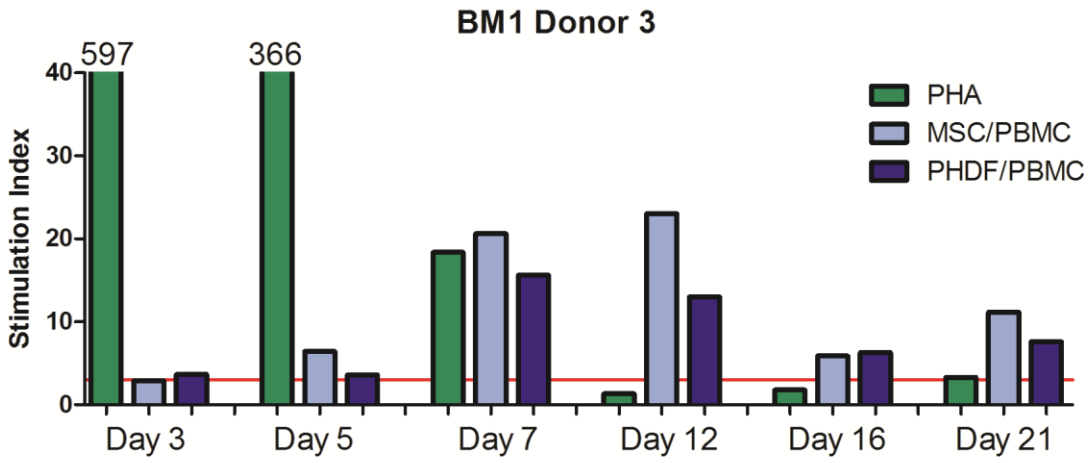


Figure 6.8: The stimulation index (SI) of donor 3 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

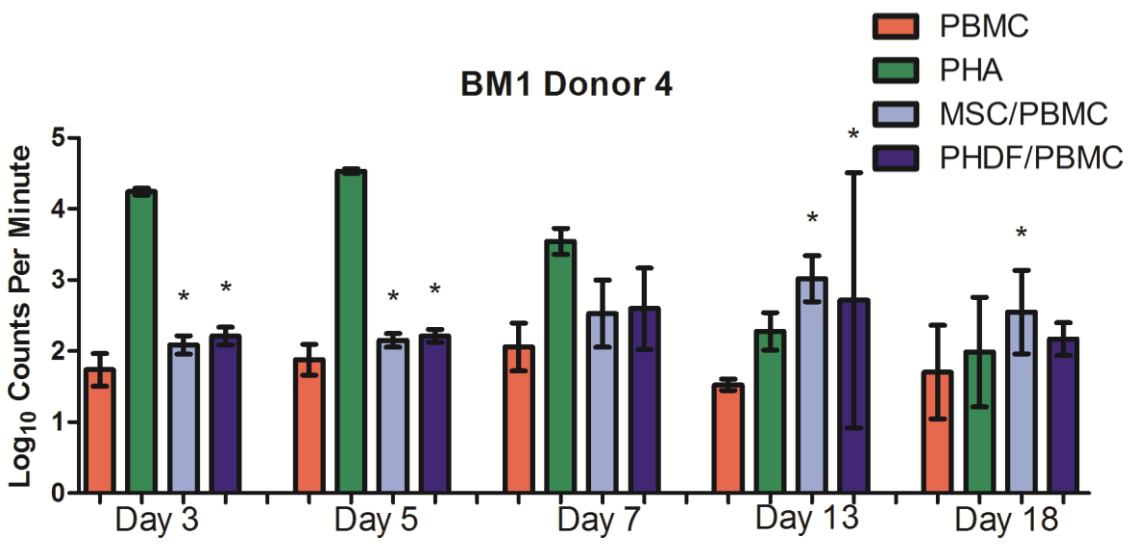


Figure 6.9: Counts per minute of donor 4 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

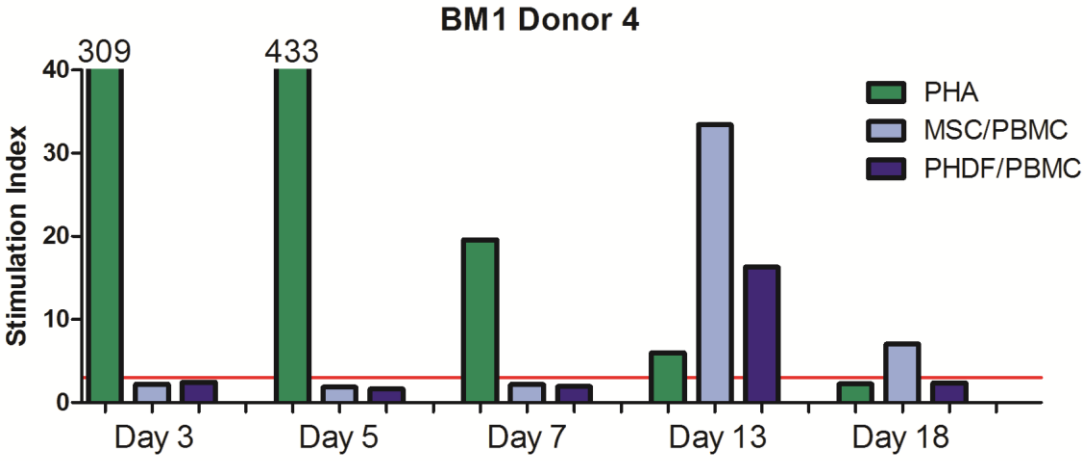


Figure 6.10: The stimulation index (SI) of donor 4 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

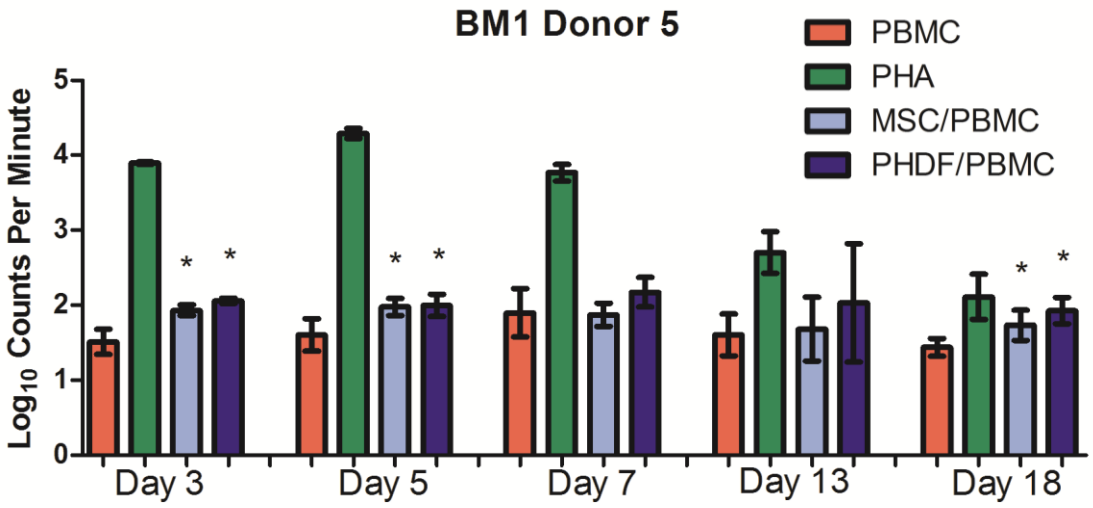


Figure 6.11: Counts per minute of donor 5 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

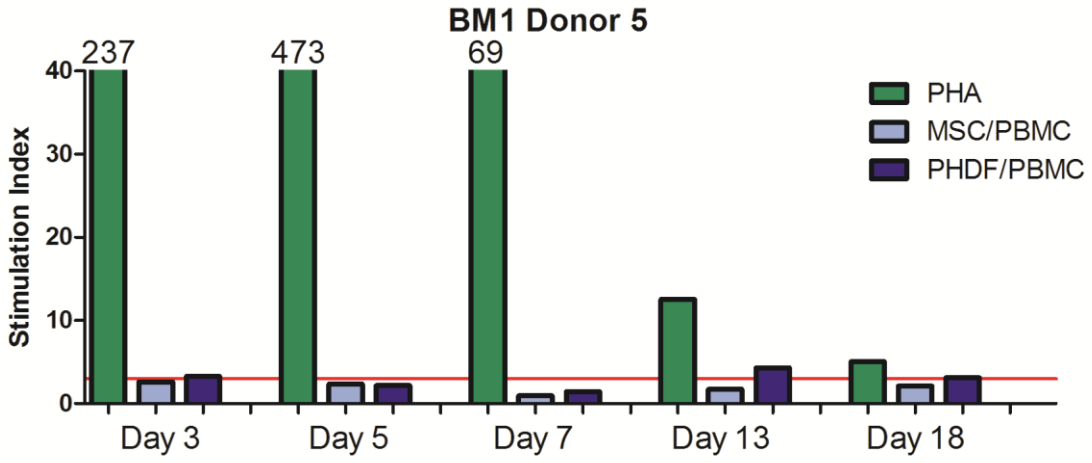


Figure 6.12: The stimulation index (SI) of donor 5 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

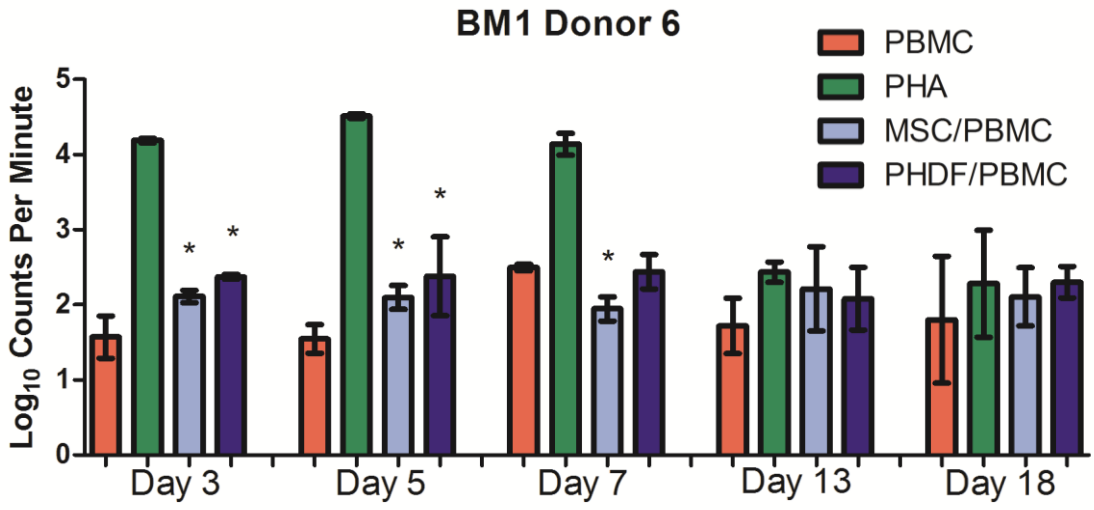


Figure 6.13: Counts per minute of donor 6 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

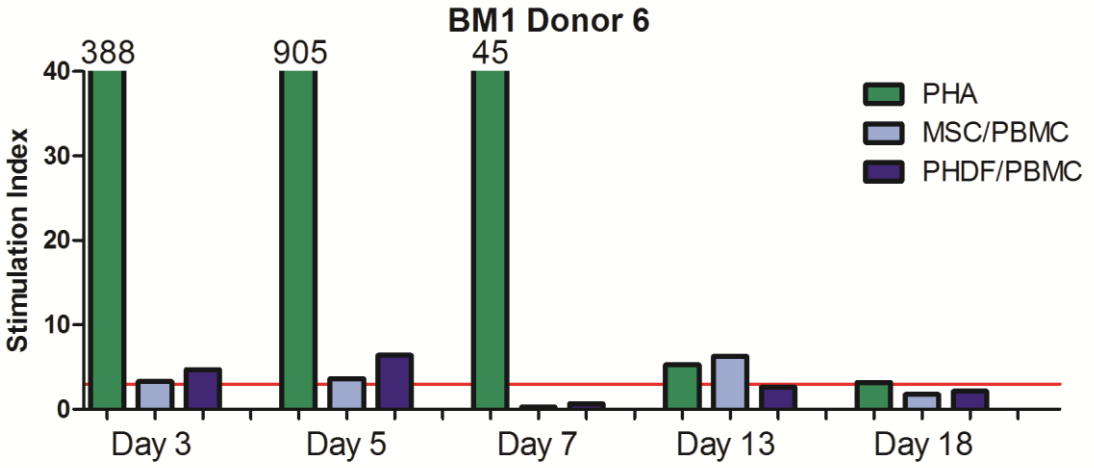


Figure 6.14: The stimulation index (SI) of donor 6 PBMCs cultured with PHA (positive control for stimulation), or with donor BM1 MSCs or PHDFs, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.3.1.2 BM2

When PBMCs from donor 1 were cultured with BM2 MSCs, significantly higher counts were recorded on days 3, 5, 12, and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to unstimulated PBMCs (**Figure 6.15**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.16**. This showed stimulation indices above 3 for PBMCs from donor 1 in response to BM2 MSCs at days 5 (SI of 3.45), 7 (SI of 11.92), 12 (SI of 4.69), 16 (SI of 14.96), and 21 (SI of 4.00). For BM2 MSCs cultured in medium alone no stimulation indices of above 3 were recorded.

When PBMCs from donor 2 were cultured with BM2 MSCs, significantly higher counts were recorded on days 5, 12, and 16 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to unstimulated PBMCs (**Figure 6.17**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.18**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM2 MSCs at days 12 (SI of 5.87) and 16 (SI of 6.02). For BM2 MSCs cultured in medium alone no stimulation indices of above 3 were recorded.

When PBMCs from donor 3 were cultured with BM2 MSCs, significantly higher counts were recorded on days 3 and 7 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to unstimulated PBMCs (**Figure 6.19**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.20**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM2 MSCs at days 5 (SI of 3.64), 12 (SI of 4.04), 16 (SI of 3.30) and 21 (SI of 7.91). For BM2 MSCs cultured in medium alone no stimulation indices of above 3 were recorded.

When PBMCs from donor 4 were cultured with BM2 MSCs, significantly higher counts were recorded on days 3, 5 and 7 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.21**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.22**. This showed stimulation indices above 3 for PBMCs from donor 4 in response to BM2 MSCs at days 7 (SI of

13.04), 12 (SI of 5.48), 16 (SI of 36.00) and 21 (SI of 7.42). For BM2 MSCs cultured in medium alone a stimulation index above 3 was recorded on day 5 (SI of 3.29).

When PBMCs from donor 5 were cultured with BM2 MSCs, significantly higher counts were recorded on days 3, 5, 7 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.23**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.24**. This showed stimulation indices above 3 for PBMCs from donor 5 in response to BM2 MSCs at days 3 (SI of 3.87), 5 (SI of 5.34), 7 (SI of 9.33), 12 (SI of 8.57), 16 (SI of 6.62) and 21 (SI of 6.95). For BM2 MSCs cultured in medium alone stimulation indices above 3 were recorded on day 3 (SI of 3.23) and 5 (SI of 5.87).

When PBMCs from donor 6 were cultured with BM2 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.25**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.26**. This showed stimulation indices above 3 for PBMCs from donor 6 in response to BM2 MSCs at days 3 (SI of 3.10) and 5 (SI of 4.72). For BM2 MSCs cultured in medium alone a stimulation index above 3 was recorded on day 5 (SI of 8.43).

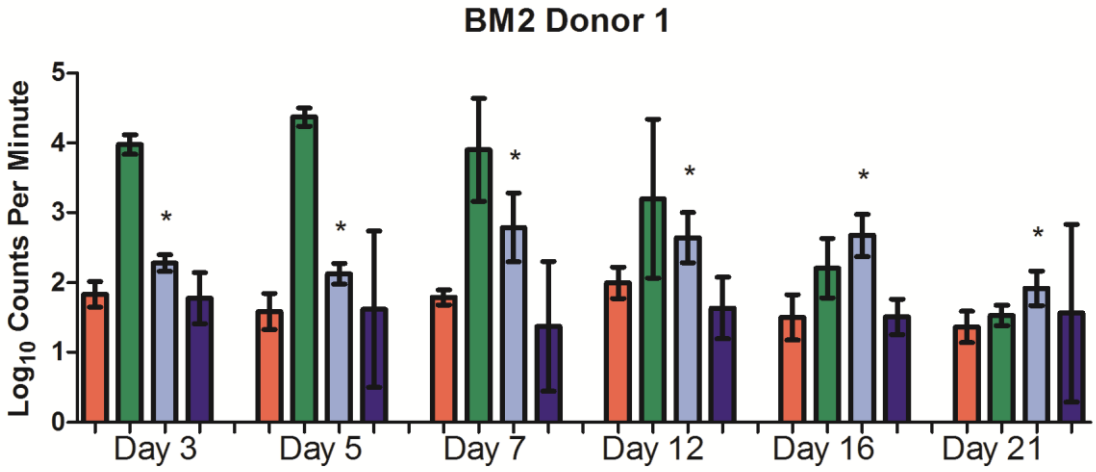


Figure 6.15: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

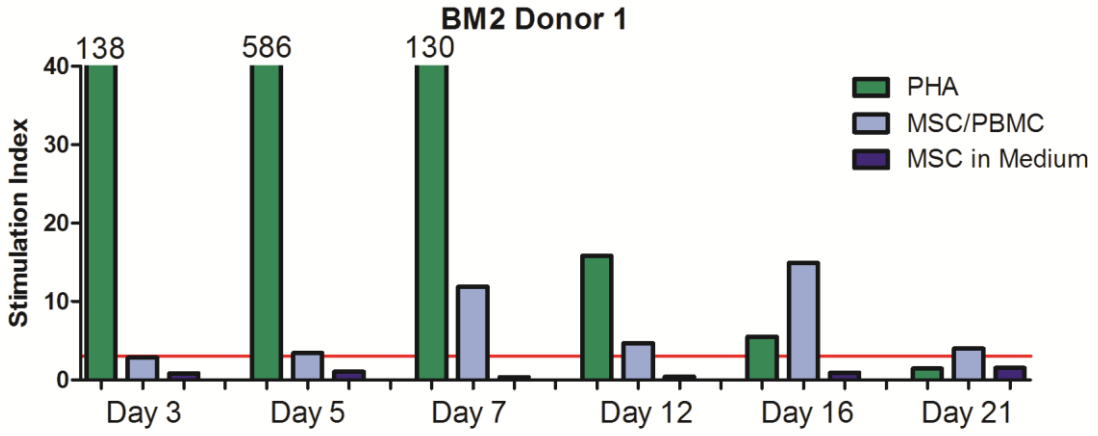


Figure 6.16: The stimulation index (SI) of donor 1 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

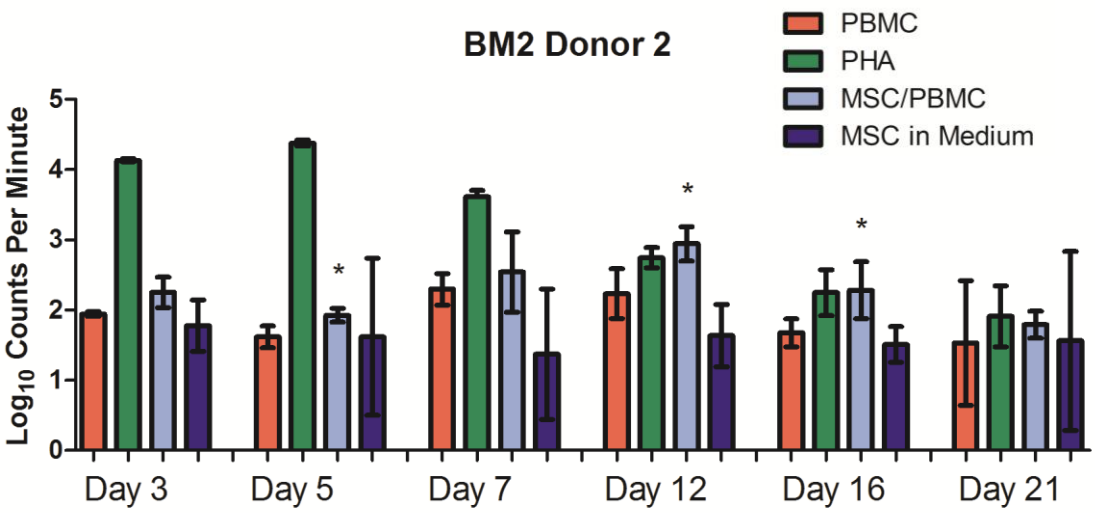


Figure 6.17: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

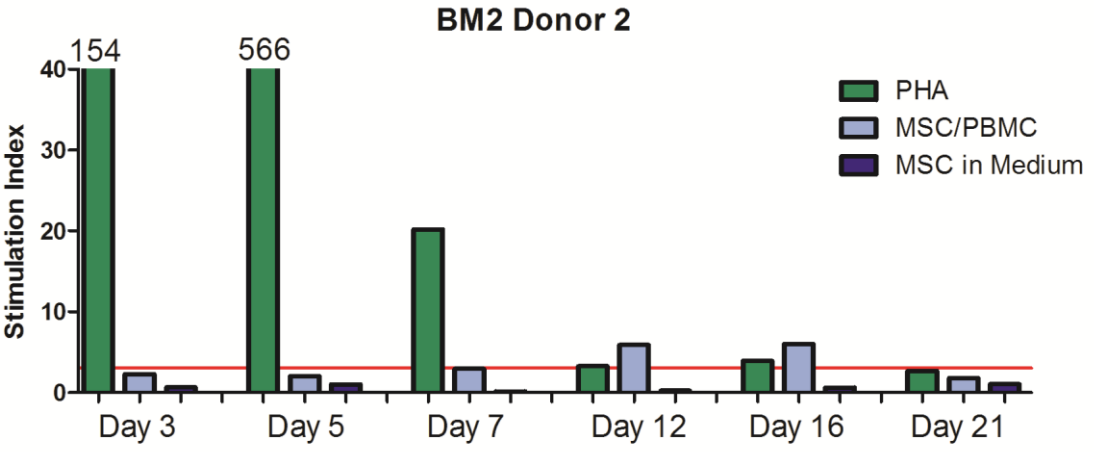


Figure 6.18: The stimulation index (SI) of donor 2 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

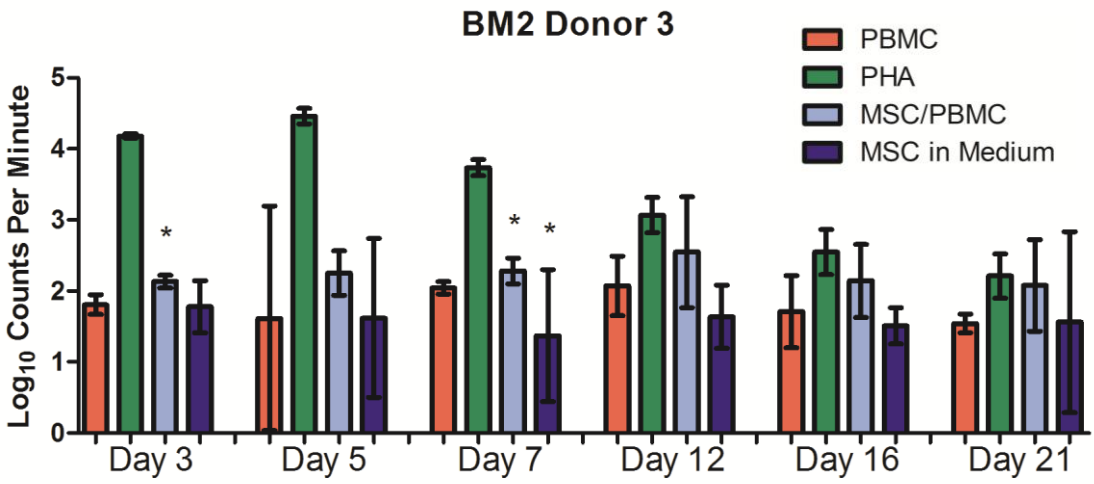


Figure 6.19: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

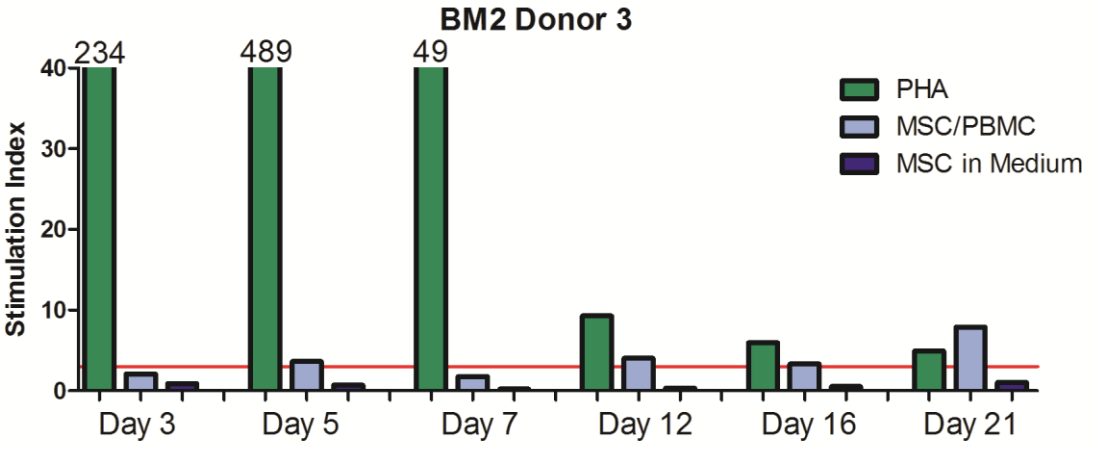


Figure 6.20: The stimulation index (SI) of donor 3 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

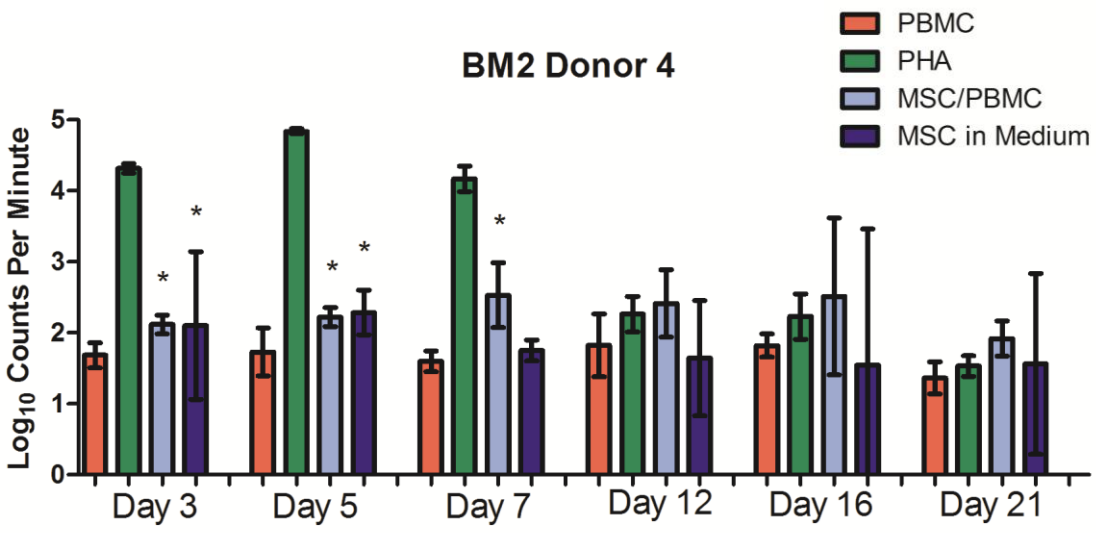


Figure 6.21: Counts per minute of donor 4 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

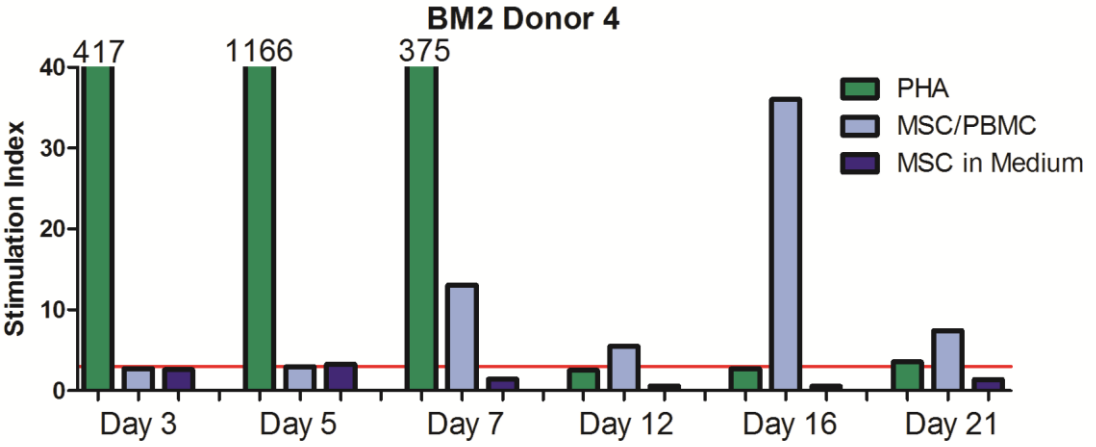


Figure 6.22: The stimulation index (SI) of donor 4 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

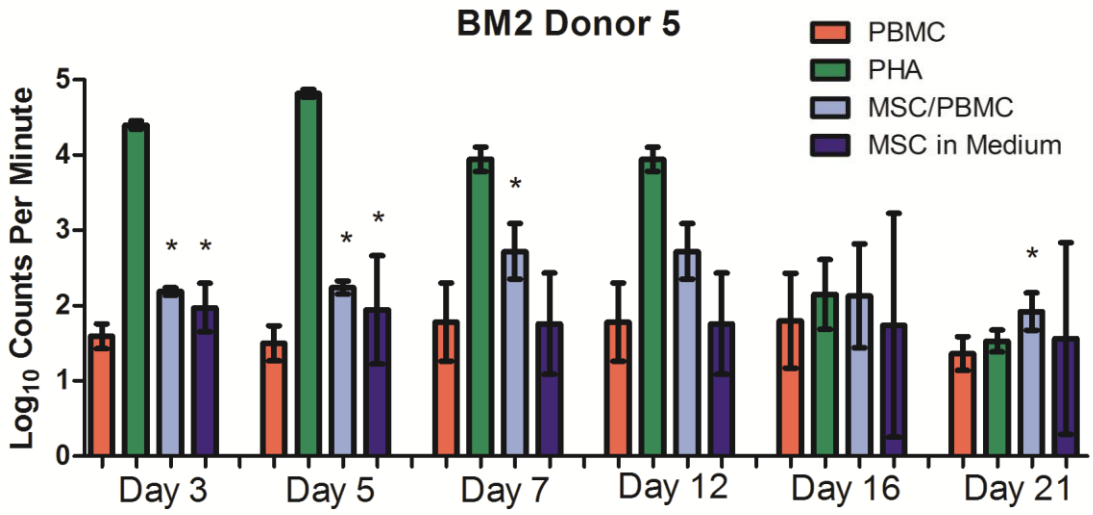


Figure 6.23: Counts per minute of donor 5 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

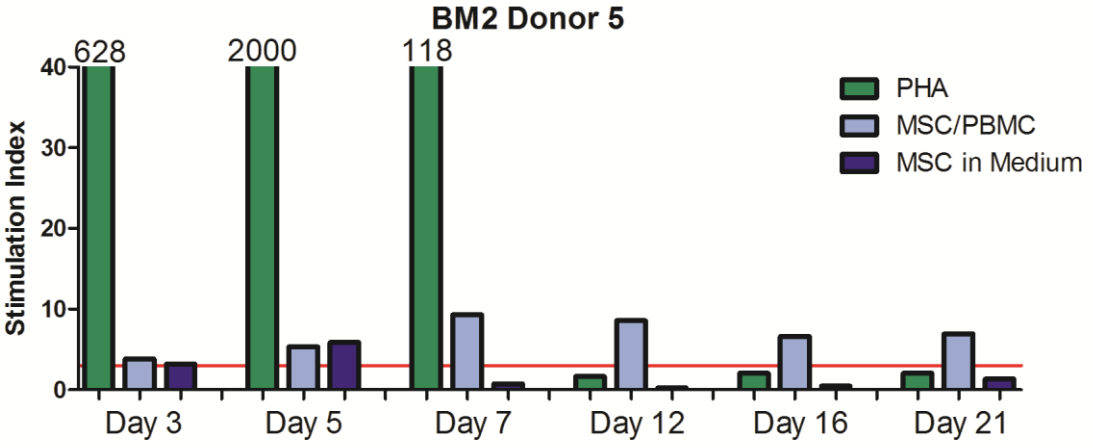


Figure 6.24: The stimulation index (SI) of donor 5 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

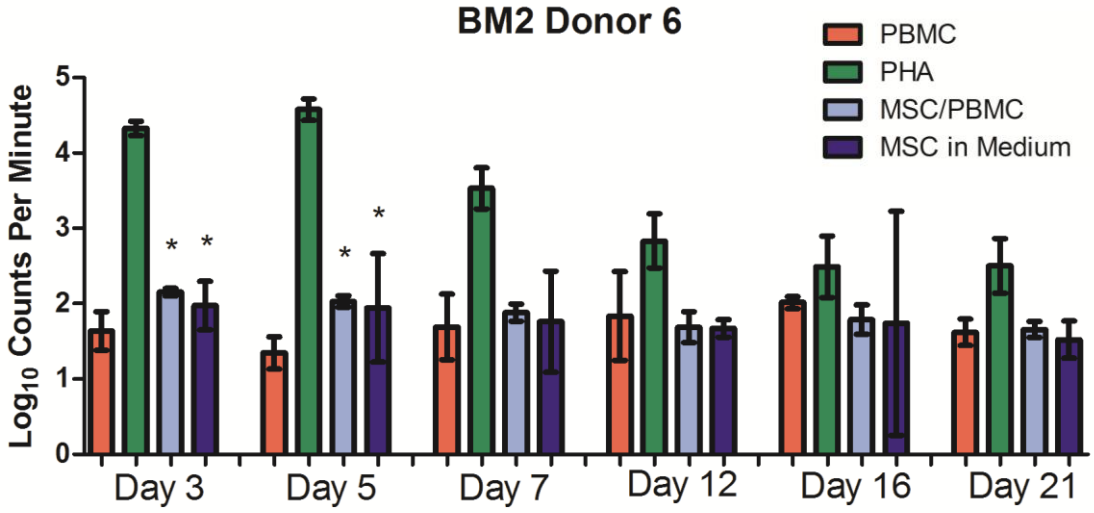


Figure 6.25: Counts per minute of donor 6 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

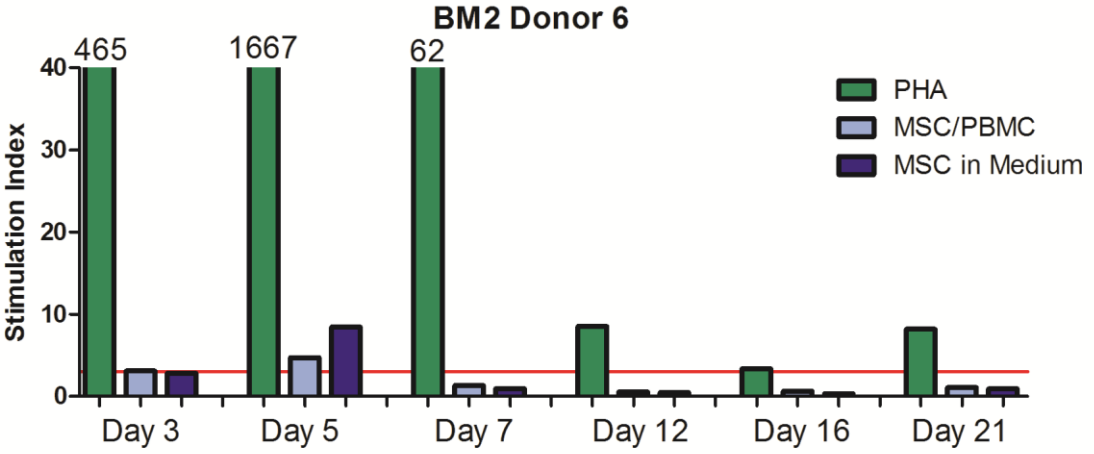


Figure 6.26: The stimulation index (SI) of donor 6 PBMCs cultured with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.3.1.3 BM3

When PBMCs from donor 1 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3, 5 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone significantly higher counts were recorded on day 21 compared to the unstimulated PBMCs (**Figure 6.27**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.28**. This showed stimulation indices above 3 for PBMCs from donor 1 in response to BM3 MSCs at days 5 (SI of 3.26), 16 (SI of 11.94) and 21 (SI of 3.15). For BM3 MSCs cultured in medium alone stimulation indices above 3 were recorded on days 16 (SI of 4.71) and 21 (SI of 3.27).

When PBMCs from donor 2 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3, 5 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.29**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.30**. This showed no stimulation indices above 3 for PBMCs from donor 2 in response to BM3 MSCs. For BM3 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 3 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3, 5, 7 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone significantly higher counts were recorded on day 12 compared to the unstimulated PBMCs (**Figure 6.31**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.32**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM3 MSCs at days 3 (SI of 3.02), 5 (SI of 3.43) and 21 (SI of 3.77). For BM3 MSCs cultured in medium alone stimulation indices above 3 were recorded on day 16 (SI of 4.56).

When PBMCs from donor 4 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs (**Figure 6.33**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.34**. This showed stimulation indices above 3 for PBMCs from donor 4 in response to BM3 MSCs at days 3 (SI of

3.79), 7 (SI of 6.24), 12 (SI of 4.71) and 16 (SI of 4.78). For BM3 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 5 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.35**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.36**. This showed stimulation indices above 3 for PBMCs from donor 5 in response to BM3 MSCs at days 5 (SI of 3.41), 16 (SI of 19.15) and 21 (SI of 9.87). For BM3 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 6 were cultured with BM3 MSCs, significantly higher counts were recorded on days 3, 5 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM3 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.37**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.38**. This showed stimulation indices above 3 for PBMCs from donor 6 in response to BM3 MSCs at days 5 (SI of 3.18) and 16 (SI of 4.77). For BM3 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

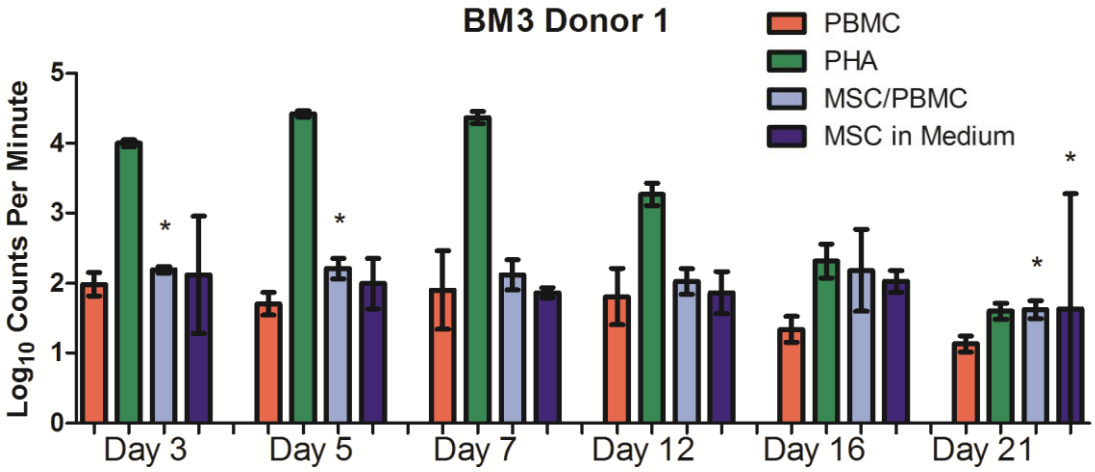


Figure 6.27: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

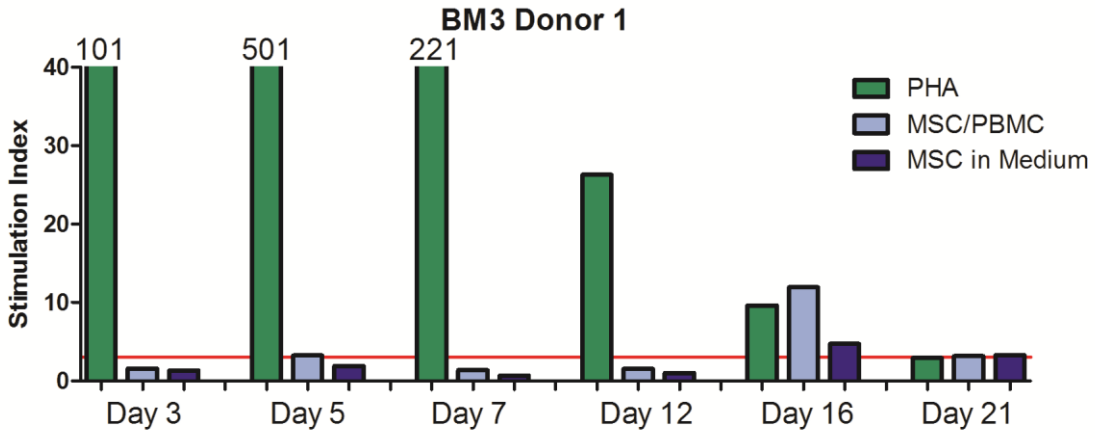


Figure 6.28: The stimulation index (SI) of donor 1 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

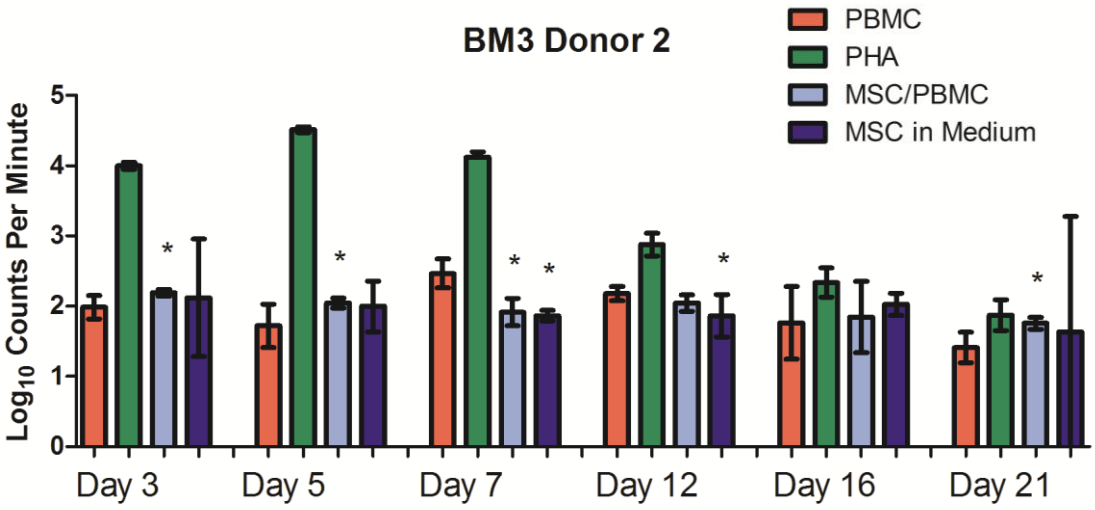


Figure 6.29: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

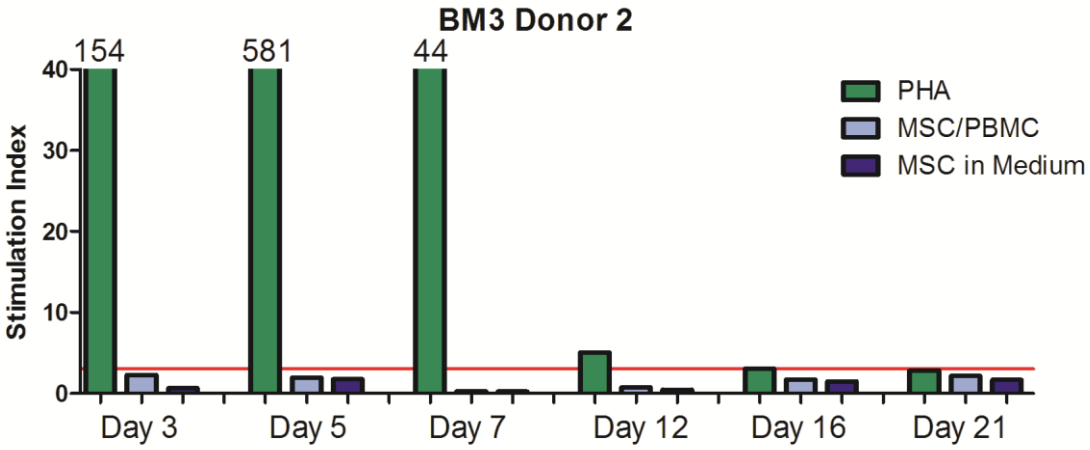


Figure 6.30: The stimulation index (SI) of donor 2 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

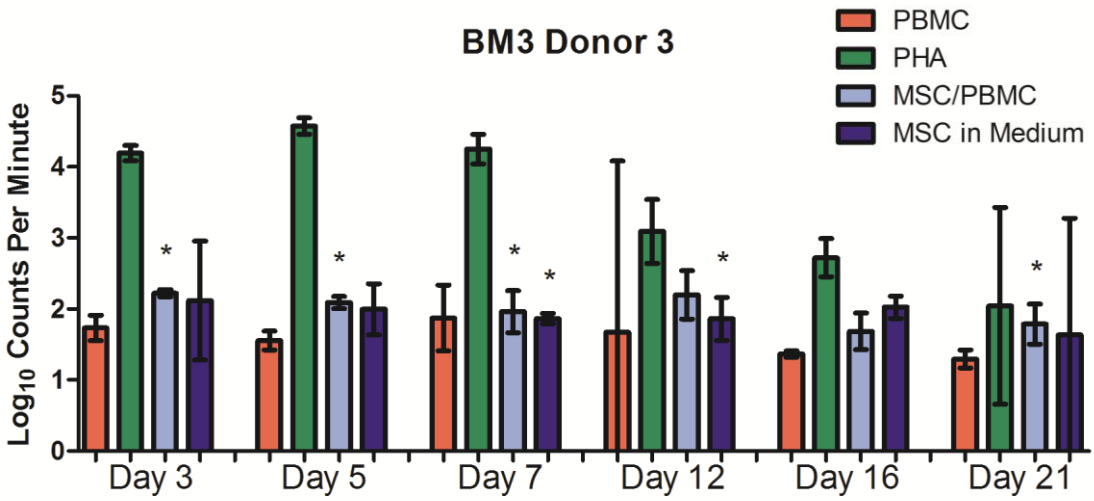


Figure 6.31: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

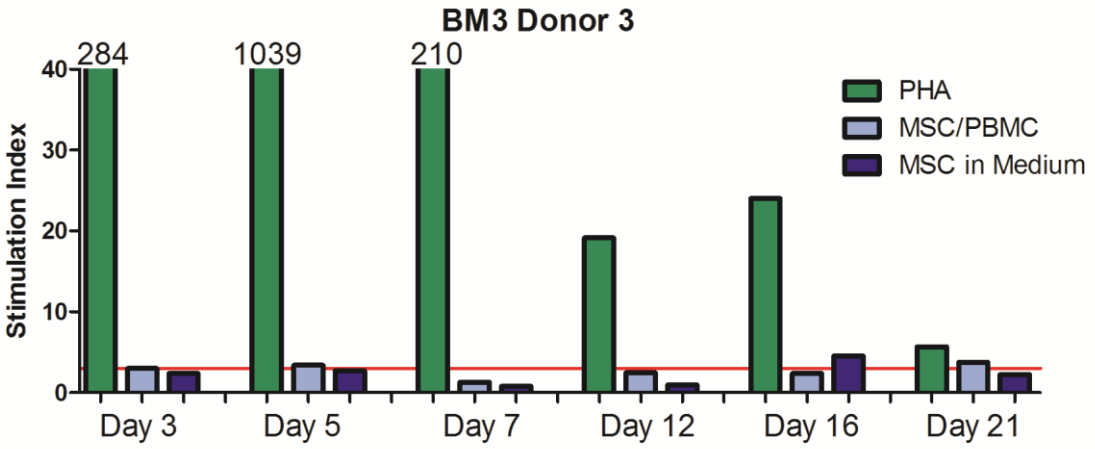


Figure 6.32: The stimulation index (SI) of donor 3 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

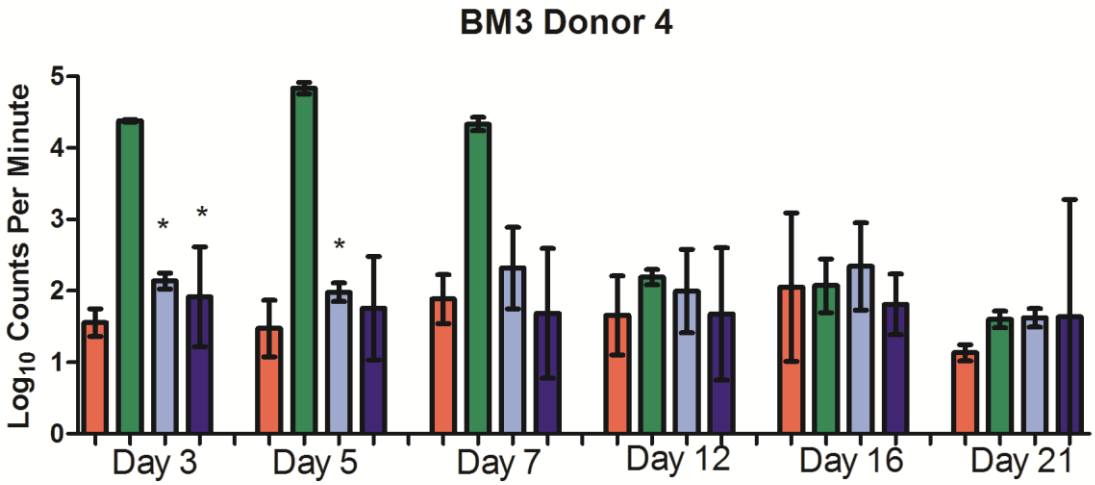


Figure 6.33: Counts per minute of donor 4 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

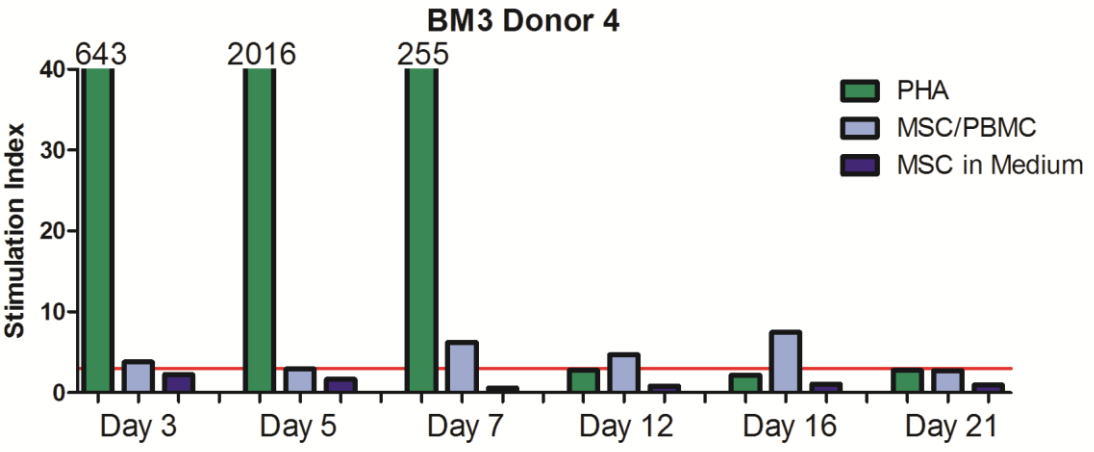


Figure 6.34: The stimulation index (SI) of donor 4 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

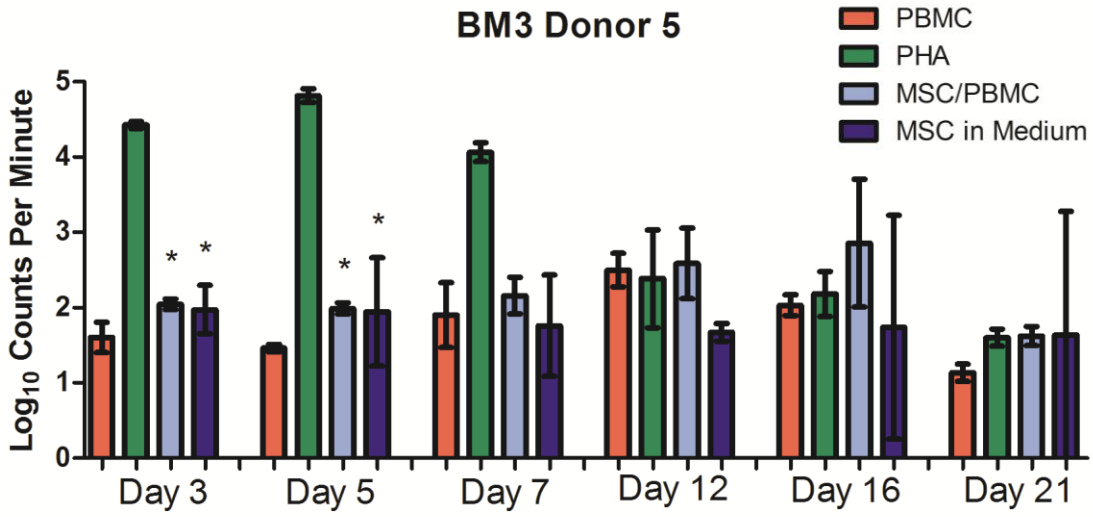


Figure 6.35: Counts per minute of donor 5 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

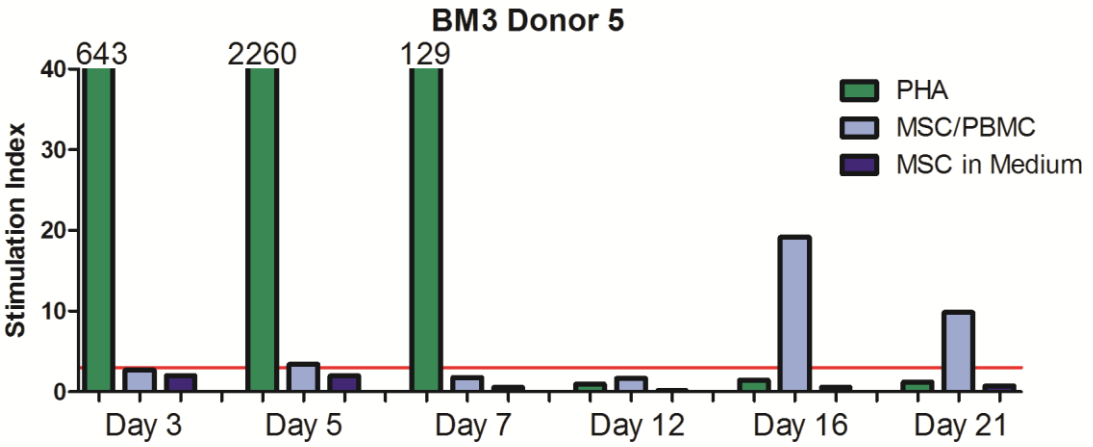


Figure 6.36: The stimulation index (SI) of donor 5 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

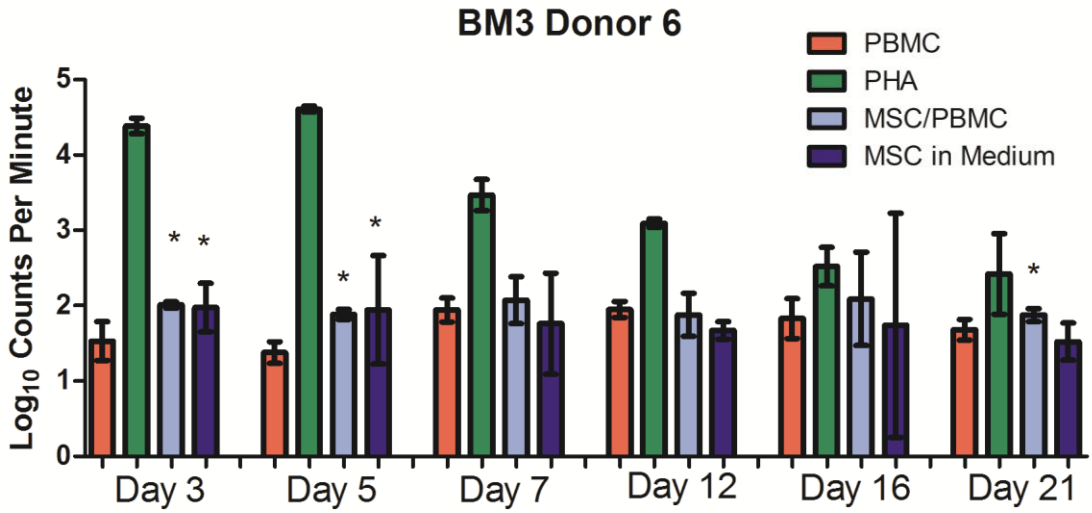


Figure 6.37: Counts per minute of donor 6 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM3 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

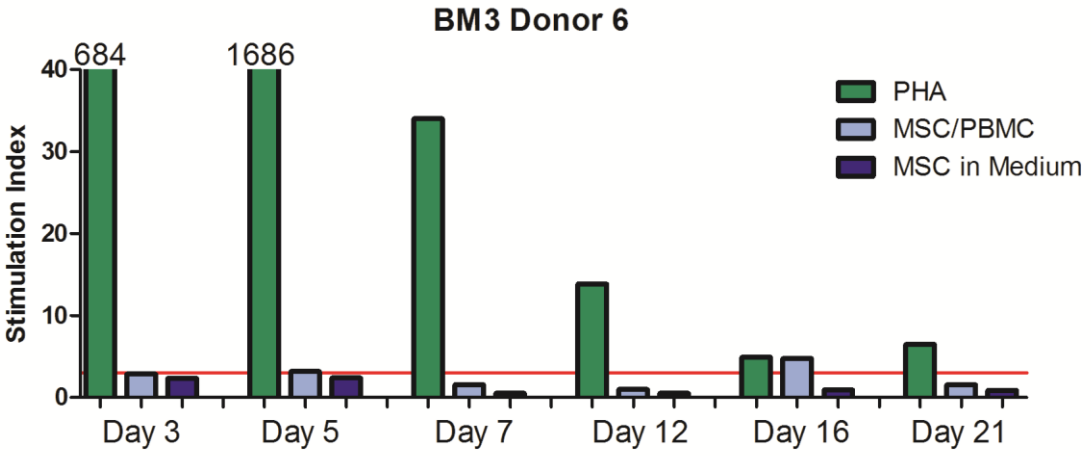


Figure 6.38: The stimulation index (SI) of donor 6 PBMCs cultured with PHA (positive control for stimulation), with donor BM3 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.3.1.4 BM4

When PBMCs from donor 1 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3, 5, 7, 12, 16 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.39**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.40**. This showed stimulation indices above 3 for PBMCs from donor 1 in response to BM4 MSCs at days 5 (SI of 6.89), 7 (SI of 7.53), 12 (SI of 11.69), 16 (SI of 4.20) and 21 (SI of 6.36). For BM4 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 2 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3, 5 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.41**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.42**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM4 MSCs at day 16 (SI of 3.11). For BM4 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 3 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3, 5 and 12 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs (**Figure 6.43**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.44**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM4 MSCs at days 3 (SI of 3.25), 5 (SI of 3.14), 7 (SI of 8.66), 12 (SI of 13.50) and 16 (SI of 14.40). For BM4 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 4 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3, 5 and 7 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.45**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.46**. This showed stimulation indices above 3 for PBMCs from donor 4 in response to BM4 MSCs at days 3 (SI of

3.44), 5 (SI of 5.83), 7 (SI of 6.88), 12 (SI of 6.17), 16 (SI of 15.08) and 21 (SI of 5.77). For BM4 MSCs cultured in medium alone a stimulation index above 3 was recorded on day 3 (SI of 3.32).

When PBMCs from donor 5 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3, 5, 7 and 21 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.47**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.48**. This showed stimulation indices above 3 for PBMCs from donor 5 in response to BM4 MSCs at days 3 (SI of 3.40), 5 (SI of 4.31), 7 (SI of 9.39), 16 (SI of 14.85) and 21 (SI of 9.11). For BM4 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

When PBMCs from donor 6 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone significantly higher counts were also recorded on days 3 and 5 compared to the unstimulated PBMCs (**Figure 6.49**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.50**. This showed stimulation indices above 3 for PBMCs from donor 6 in response to BM4 MSCs at days 3 (SI of 3.06), 5 (SI of 3.18) and 7 (SI of 4.16). For BM4 MSCs cultured in medium alone no stimulation indices above 3 were recorded.

A summary table of the maximal stimulation indices observed when MSCs from BM1-4 were cultured with PBMCs from donor 1-6 is shown in **Table 6.1**. When SI's are grouped together and displayed in tabular format, it appears that donor 6 responses to MSCs and to PHDFs are relatively low when compared to responses from the other PBMC donors (SI <6.5). Other PBMC donors demonstrate SI's of greater than 14. BM 1-4 responses appear to be variable throughout the PBMC donors, with strong stimulation observed with certain donors, while relatively low SI's are observed with others.

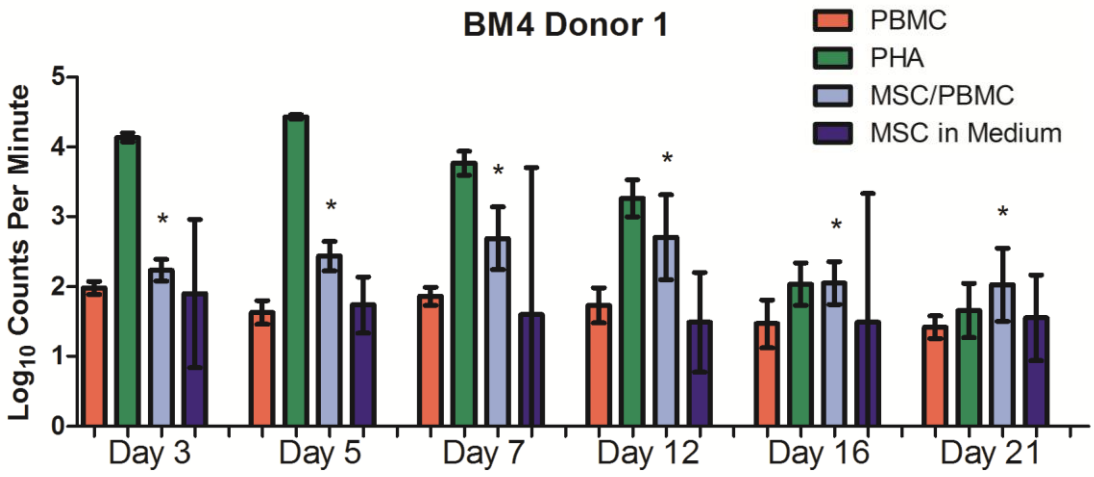


Figure 6.39: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

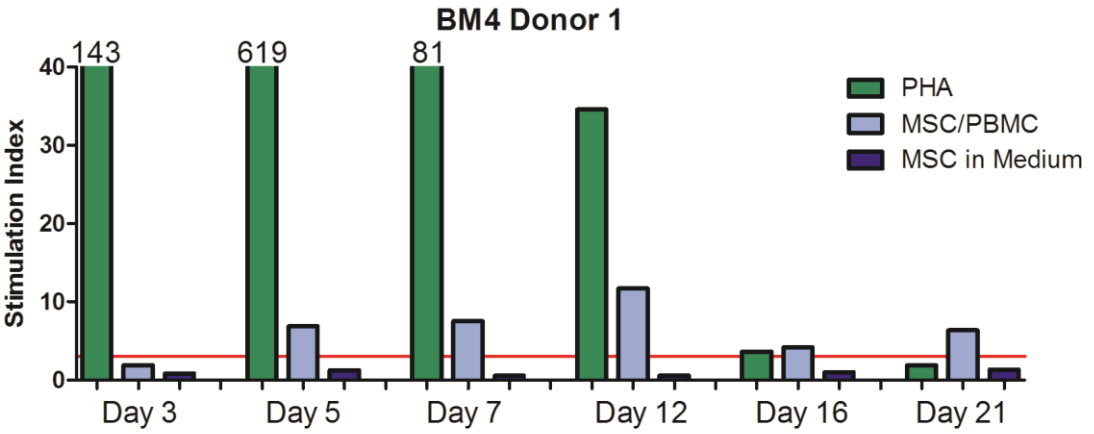


Figure 6.40: The stimulation index (SI) of donor 1 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

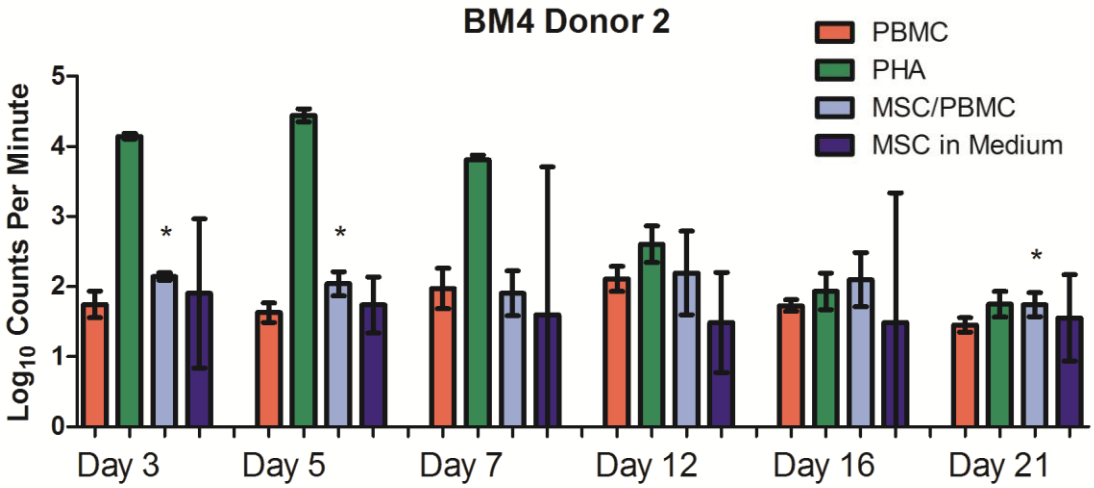


Figure 6.41: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

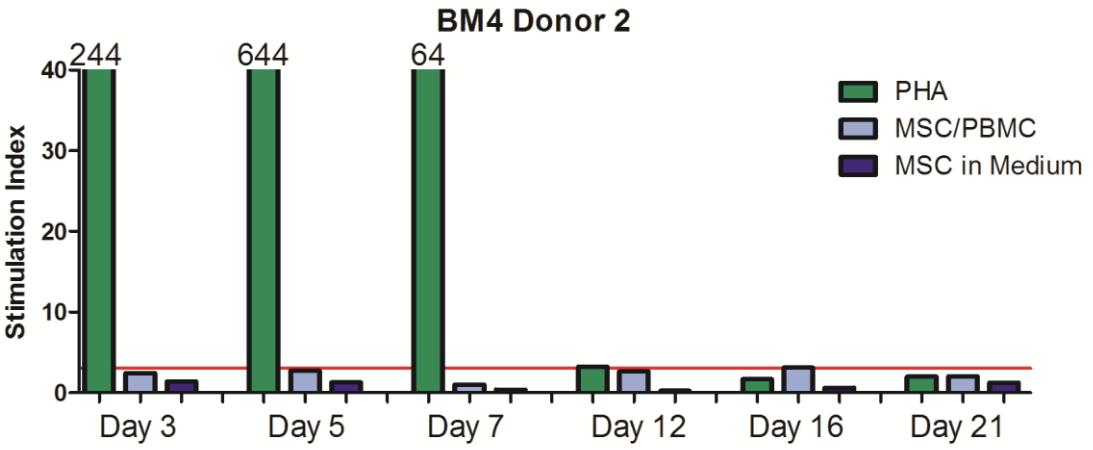


Figure 6.42: The stimulation index (SI) of donor 2 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

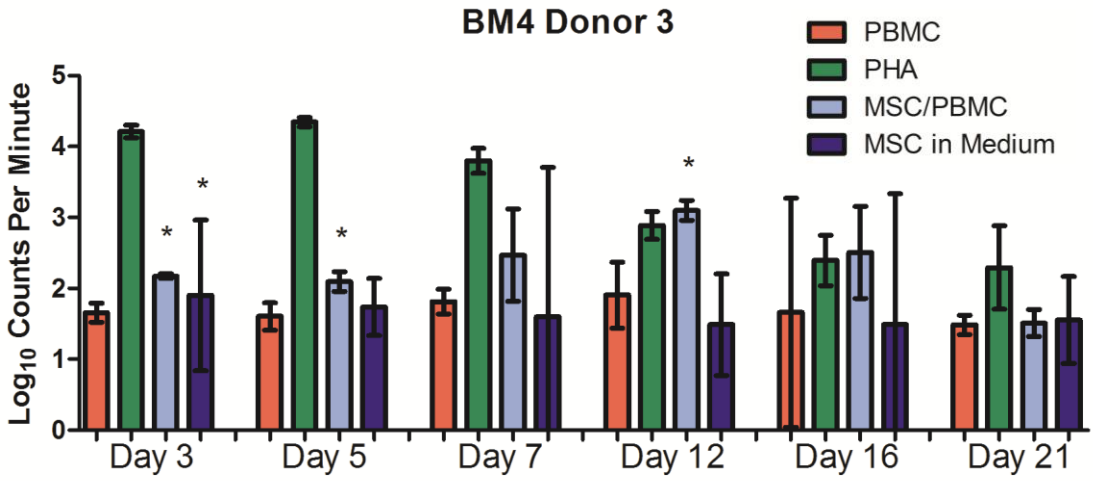


Figure 6.43: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

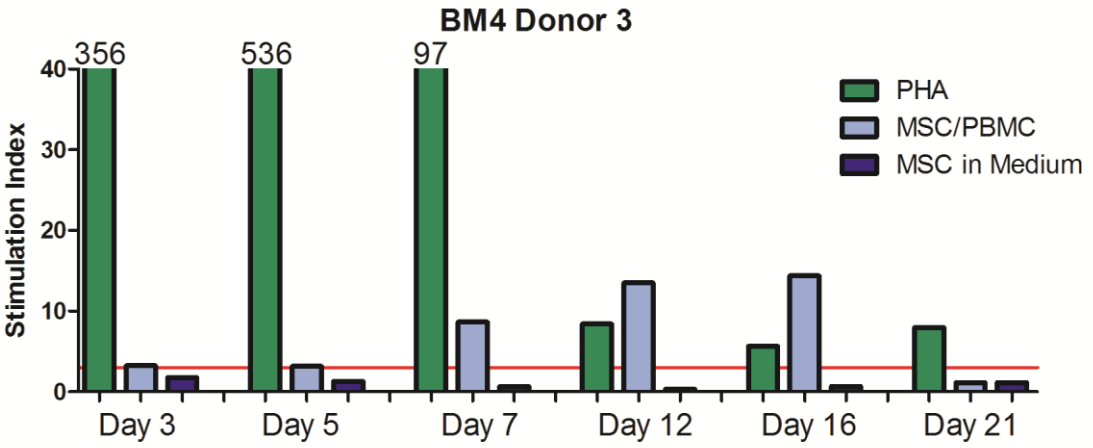


Figure 6.44: The stimulation index (SI) of donor 3 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

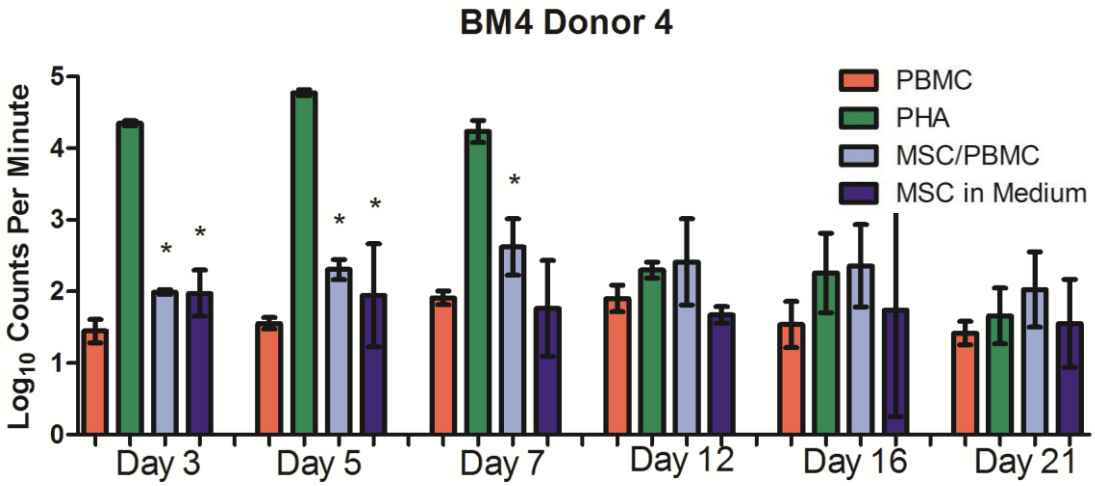


Figure 6.45: Counts per minute of donor 4 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

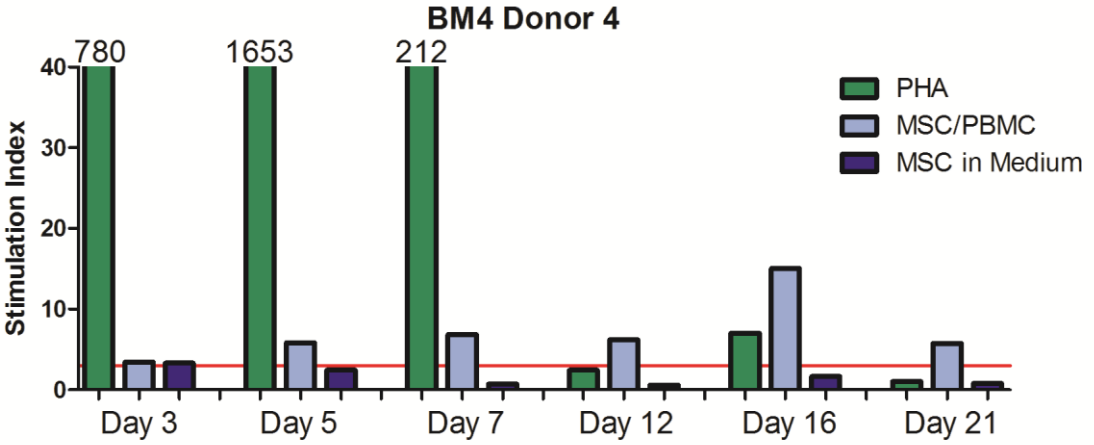


Figure 6.46: The stimulation index (SI) of donor 4 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

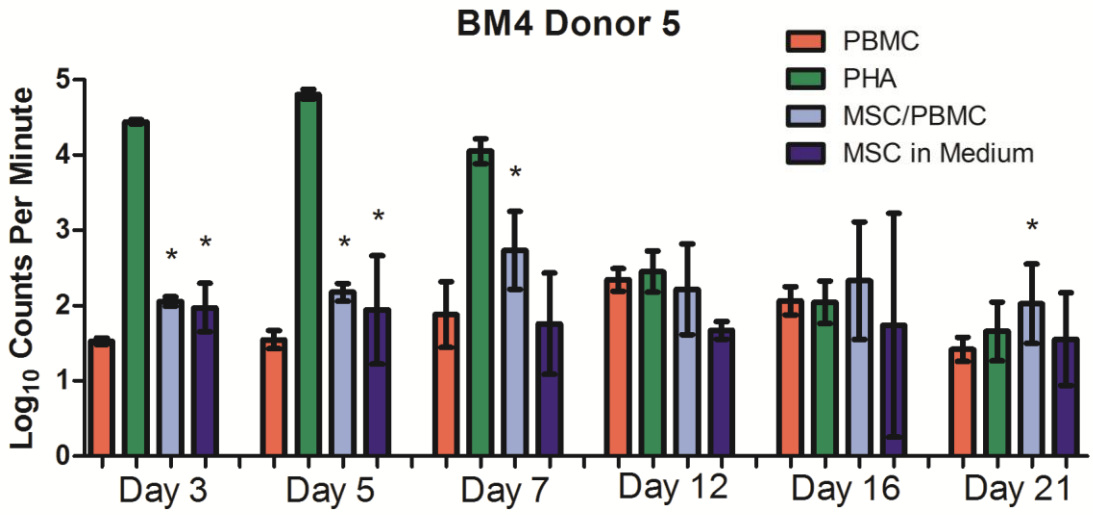


Figure 6.47: Counts per minute of donor 5 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

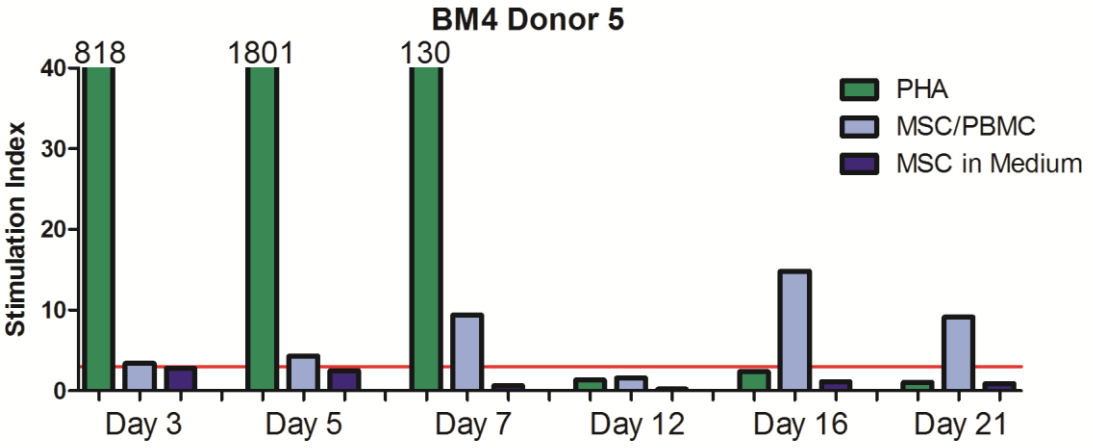


Figure 6.48: The stimulation index (SI) of donor 5 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

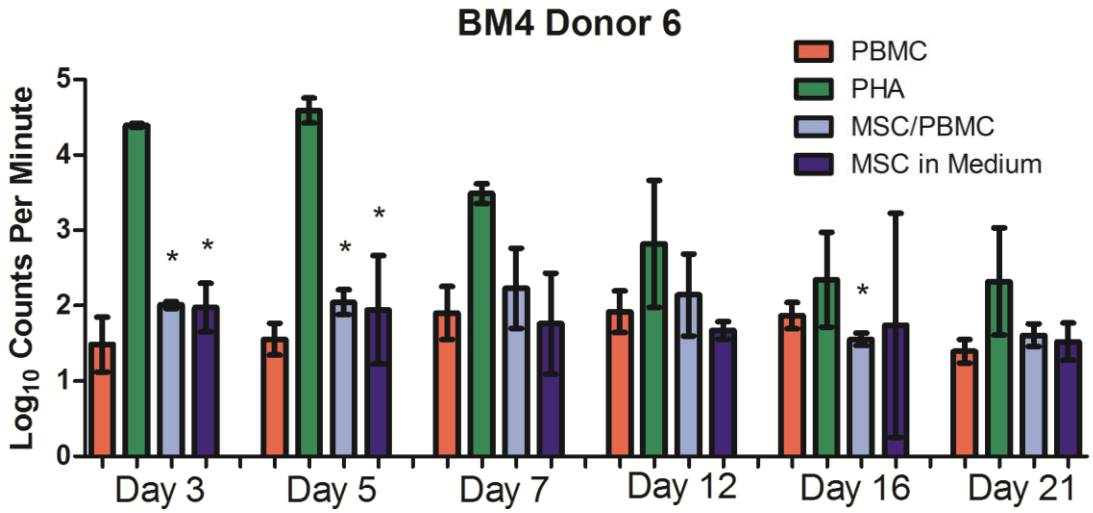


Figure 6.49: Counts per minute of donor 6 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 21 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

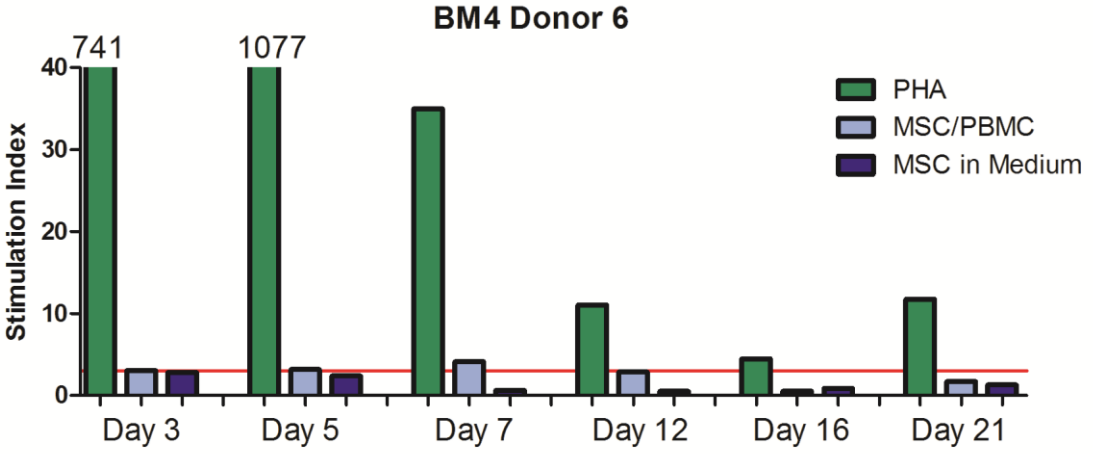


Figure 6.50: The stimulation index (SI) of donor 6 PBMCs cultured with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
PHDFs	**10.66** *Day 12*	**14.87** *Day 12*	**15.61** *Day 7*	**16.28** *Day 13*	**4.30** *Day 13*	**6.40** *Day 5*
BM1	**12.95** *Day 16*	**30.80** *Day 12*	**23.07** *Day 12*	**33.40** *Day 13*	-	**6.28** *Day 13*
BM2	**14.96** *Day 16*	**6.02** *Day 16*	**7.91** *Day 21*	**36.00** *Day 16*	**9.33** *Day 7*	**4.72** *Day 5*
BM3	**11.69** *Day 16*	-	**3.77** *Day 21*	**7.48** *Day 16*	**19.15** *Day 16*	**4.77** *Day 16*
BM4	**11.69** *Day 12*	**3.10** *Day 16*	**14.40** *Day 16*	**15.08** *Day 16*	**14.85** *Day 16*	**4.16** *Day 7*

Table 6.2: Summary table of the PHDFs and MSCs (BM1-4) stimulating PBMCs (donors 1-6) with a positive stimulation index (>3), showing the maximum SI, and day of maximal stimulation. Green cell colour represents positive SI value, and red represents no significant stimulation recorded on days 3-21 of culture.

6.3.2 THE CO-CULTURE OF MSCS WITH PBMCS IN THE LYMPHOCYTE TRANSFORMATION ASSAY PERFORMED IN 96 WELL UNCOATED V-BOTTOM TISSUE CULTURE PLATES

The data presented in the previous sections indicated that overall, the MSCs were capable of stimulating PBMCs in the extended duration lymphocyte transformation assay. However, at time periods greater than 7 days the counts showed a high degree of variability as indicated by the large error bars (in **Figures 6.45, 6.47 and 6.49** for example). It was though that this might be due to the culture conditions not being ideal and that culture in V-bottomed plates might provide more favourable conditions. Hence the experiments with PBMCs from the previous healthy donors (1-3) and MSCs from bone marrow donors BM1, 2, and 4 were repeated using V-bottomed 96 well tissue culture plates.

6.3.2.1 BM1

When PBMCs from donor 1 were cultured with PHA, maximal counts were observed on days 3-5 after which the response declined over the remaining 16 day culture period. This trend of PHA stimulation was observed throughout all the PBMC donors and in all BM MSC cultures. When PBMCs from donor 1 were cultured with BM1 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM1 were cultured in complete medium alone significantly higher counts were not recorded compared to the unstimulated PBMCs (**Figure 6.51**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.52**. This showed stimulation indices above 3 for PHA stimulated PBMCs from day 3, demonstrating the ability of the PBMCs to proliferate. The proliferation of PBMCs was repeated for all 3 PBMC donors upon stimulation with PHA. Stimulation indices above 3 were observed for PBMCs from donor 1 in response to BM1 MSCs at day 3 (SI of 3.92). For BM1 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 2 were cultured with BM1 MSCs, significantly higher counts were recorded on days 3 and 12 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM1 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.53**).

The stimulation indices were calculated and are presented in **Figure 6.54**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM1 MSCs at day 3 (SI of 3.02). For BM1 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 3 were cultured with BM1 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM1 were cultured in complete medium alone significantly higher counts were not recorded compared to the unstimulated PBMCs (**Figure 6.55**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.56**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM1 were not recorded. For BM1 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

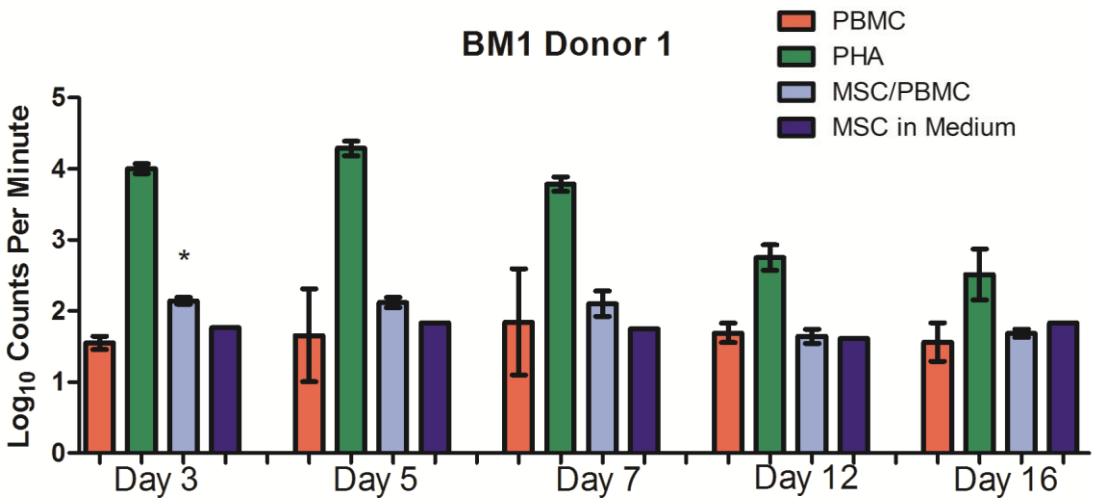


Figure 6.51: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM1 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean $\log_{10}$ counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

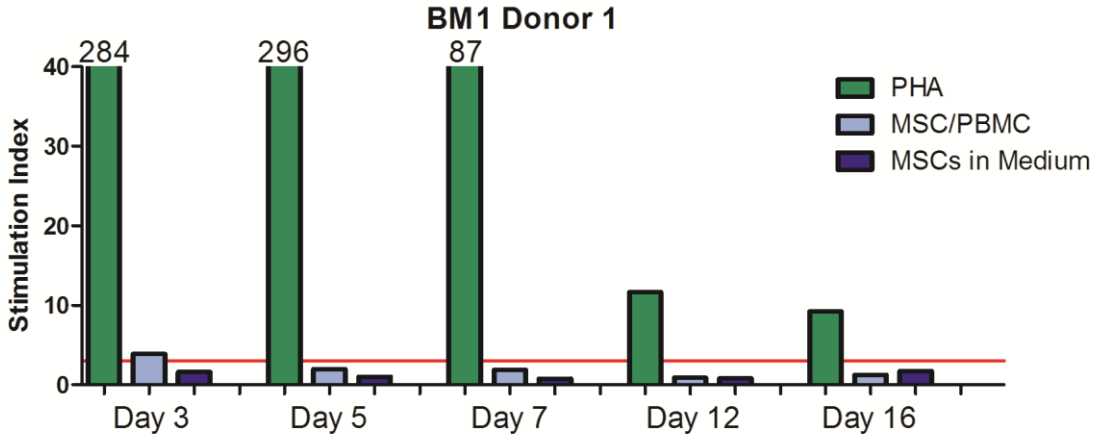


Figure 6.52: The stimulation index (SI) of donor 1 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM1 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

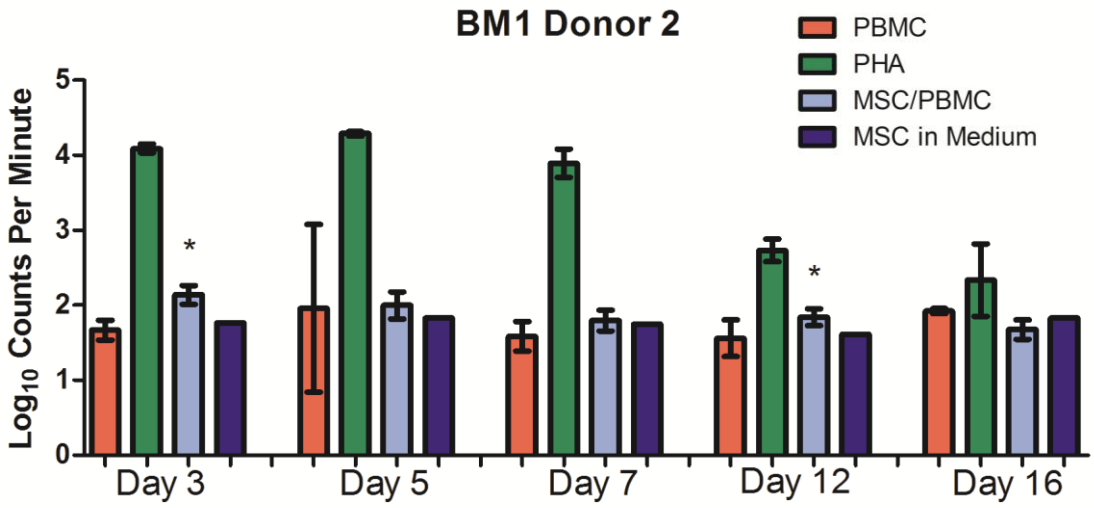


Figure 6.53: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM1 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

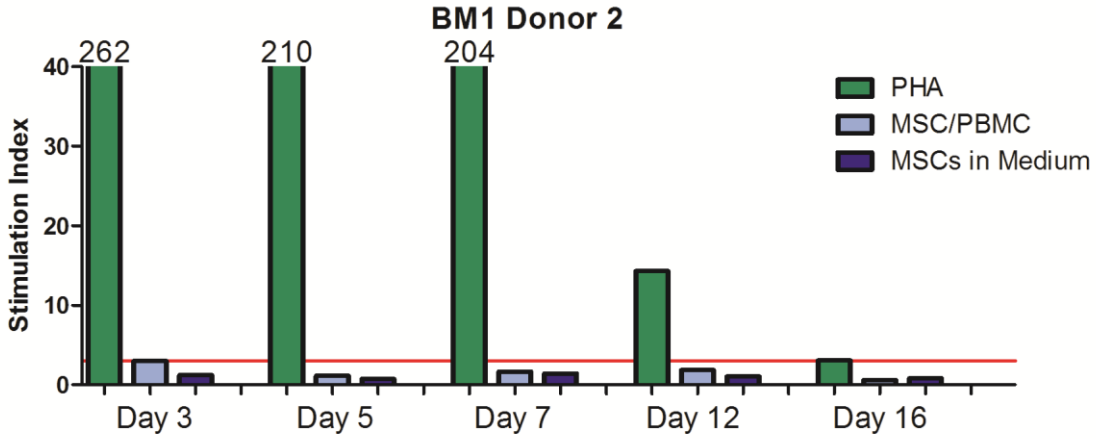


Figure 6.54: The stimulation index (SI) of donor 2 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM1 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

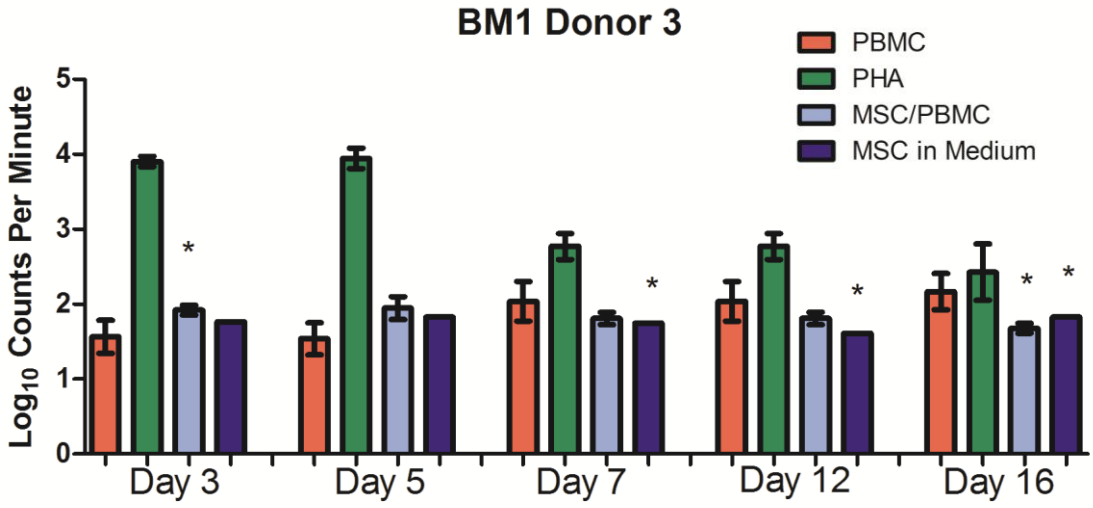


Figure 6.55: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM1 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

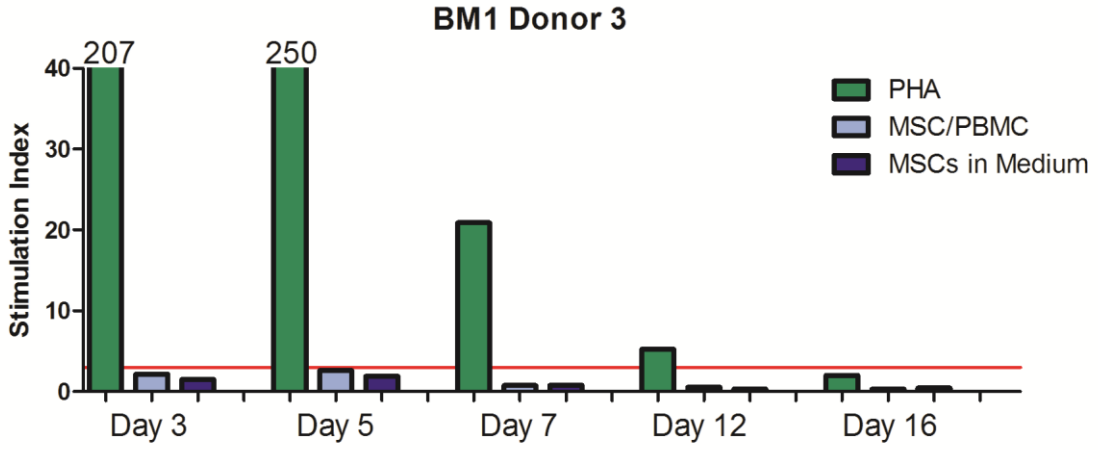


Figure 6.56: The stimulation index (SI) of donor 3 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM1 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.3.2.2 BM2

When PBMCs from donor 1 were cultured with BM2 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.57**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.58**. This showed stimulation indices above 3 for PBMCs from donor 1 in response to BM2 MSCs at day 3 (SI of 3.12). For BM2 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 2 were cultured with BM2 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.59**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.60**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM2 MSCs were not recorded. For BM2 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 3 were cultured with BM2 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM2 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.61**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.62**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM2 MSCs at day 3 (SI of 4.25). For BM2 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

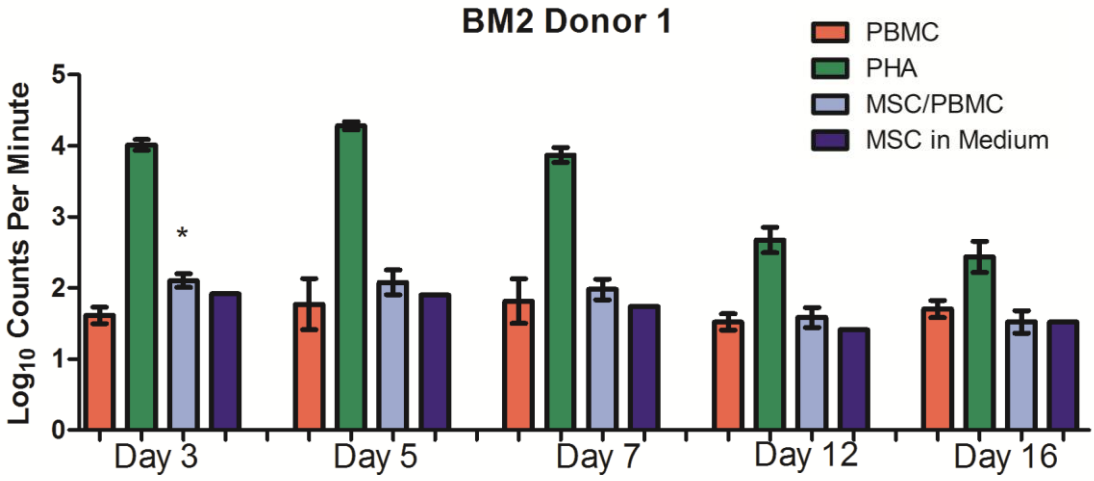


Figure 6.57: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

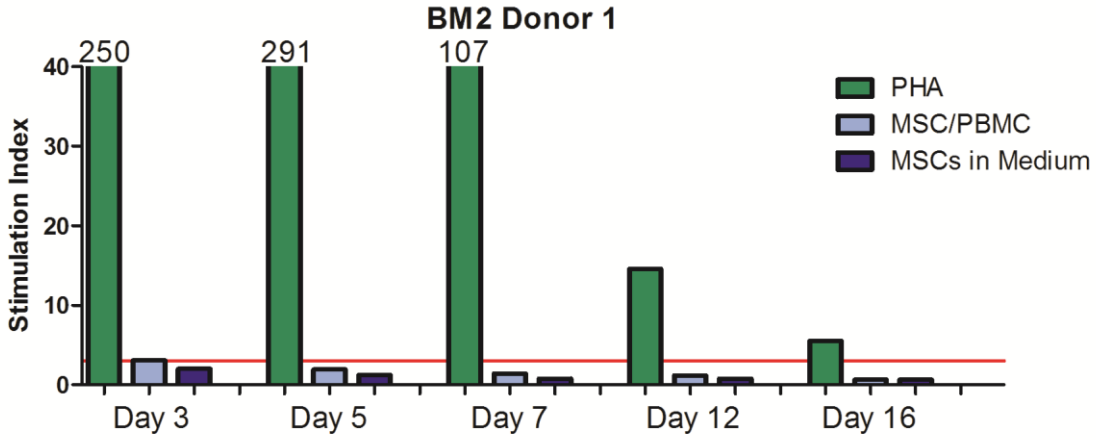


Figure 6.58: The stimulation index (SI) of donor 1 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

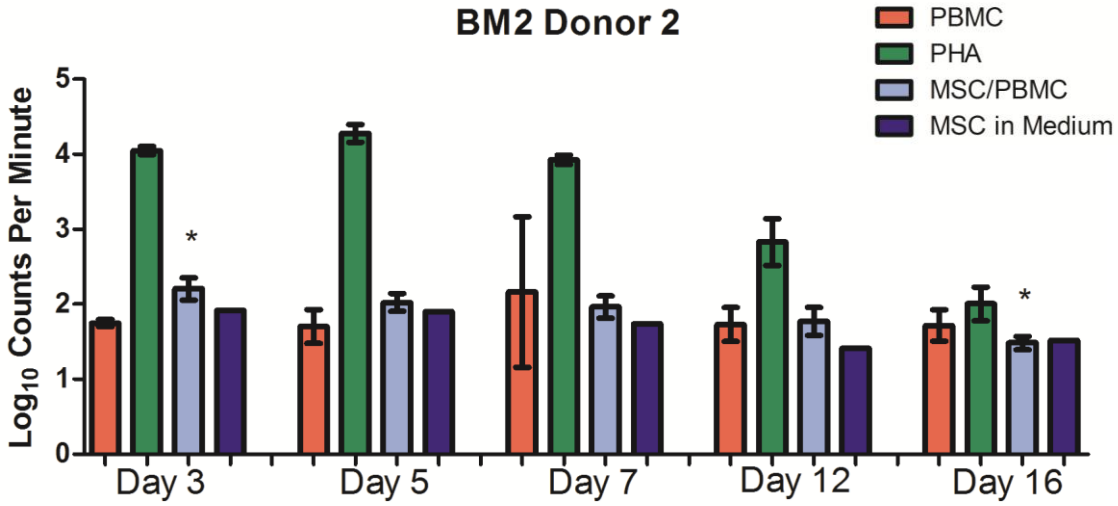


Figure 6.59: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

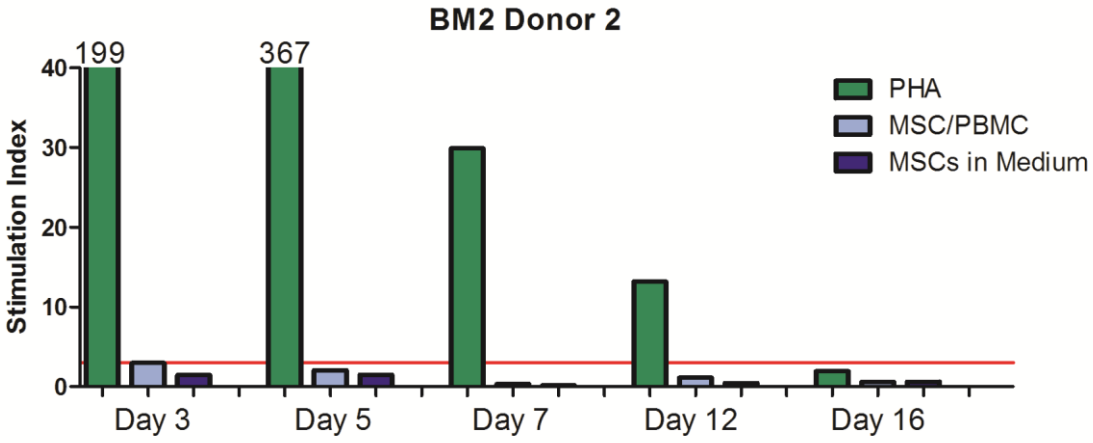


Figure 6.60: The stimulation index (SI) of donor 2 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

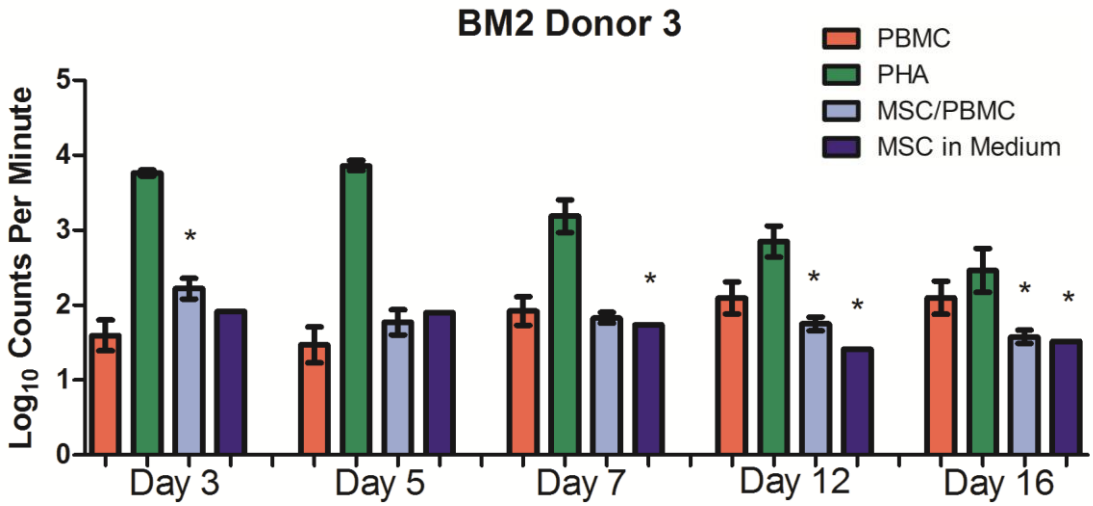


Figure 6.61: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM2 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

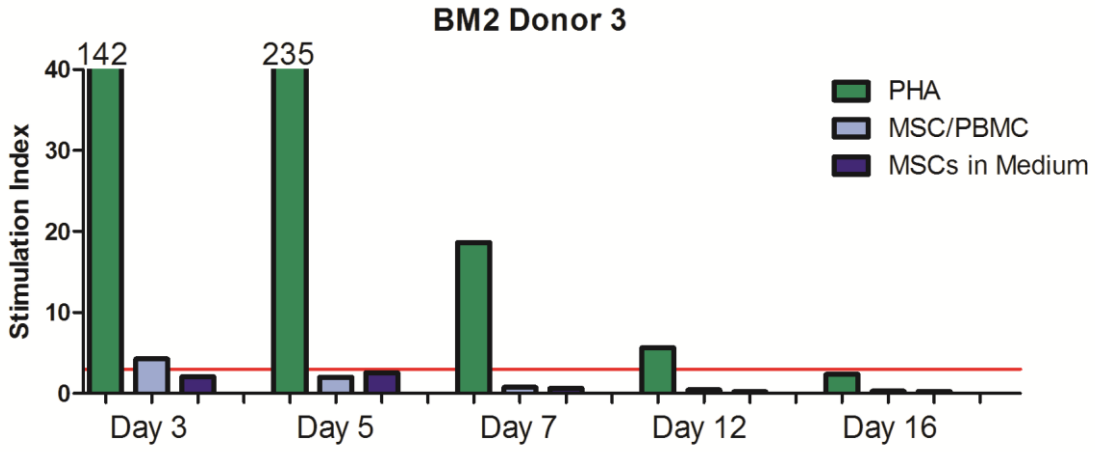


Figure 6.62: The stimulation index (SI) of donor 3 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.3.2.3 BM4

When PBMCs from donor 1 were cultured with BM4 MSCs, significantly higher counts were not recorded compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.63**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.64**. This showed stimulation indices above 3 for PBMCs from donor 1 in response to BM4 MSCs. For BM4 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 2 were cultured with BM4 MSCs, significantly higher counts were recorded on days 3 and 5 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.65**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.66**. This showed stimulation indices above 3 for PBMCs from donor 2 on days 3 (SI of 3.38), 12 (SI of 10.96) and 16 (SI of 3.12) in response to BM4 MSCs. For BM4 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

When PBMCs from donor 3 were cultured with BM4 MSCs, significantly higher counts were recorded on day 3 compared to the unstimulated PBMCs ($p<0.05$ ANOVA). When MSCs from BM4 were cultured in complete medium alone there was no difference in counts compared to the unstimulated PBMCs (**Figure 6.67**; $p<0.05$ ANOVA).

The stimulation indices were calculated and are presented in **Figure 6.68**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to BM4 MSCs. For BM4 MSCs cultured in medium alone stimulation indices above 3 were not recorded.

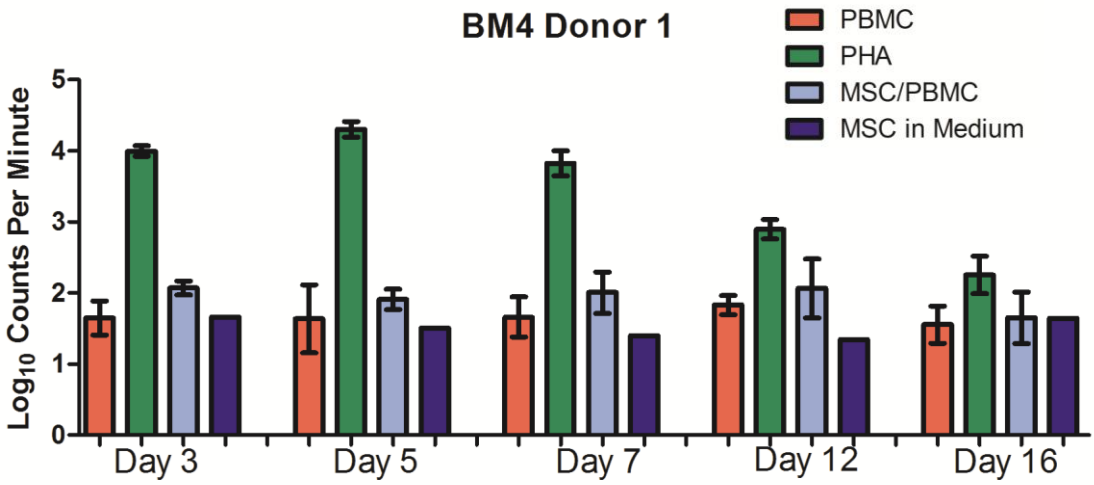


Figure 6.63: Counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

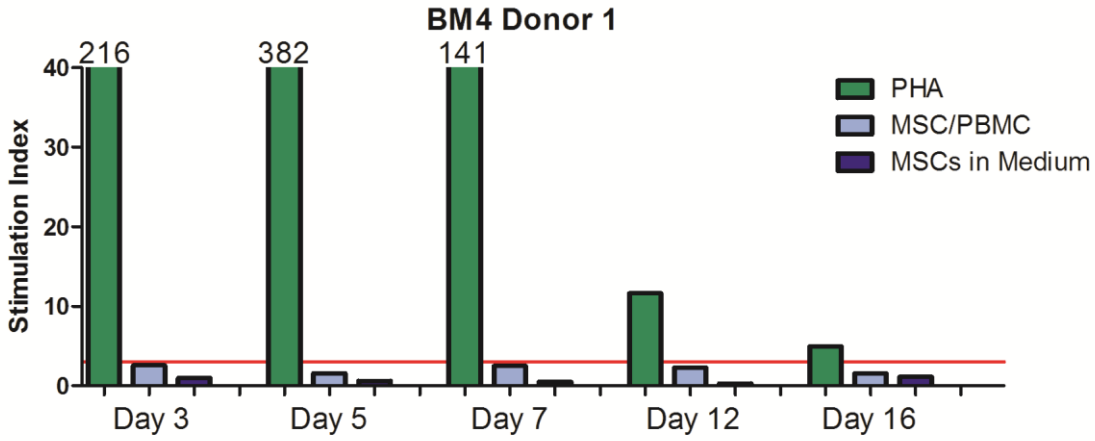


Figure 6.64: The stimulation index (SI) of donor 3 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

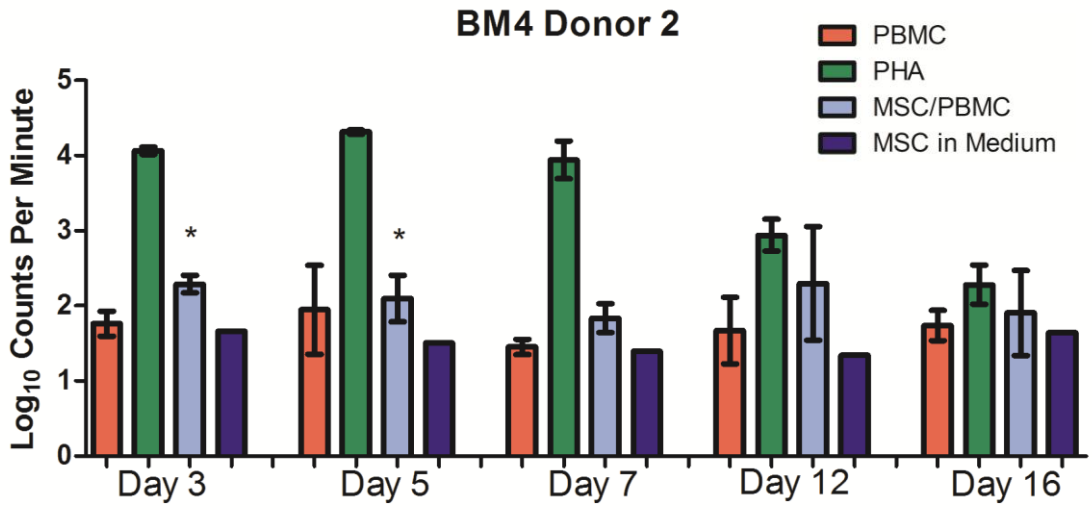


Figure 6.65: Counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

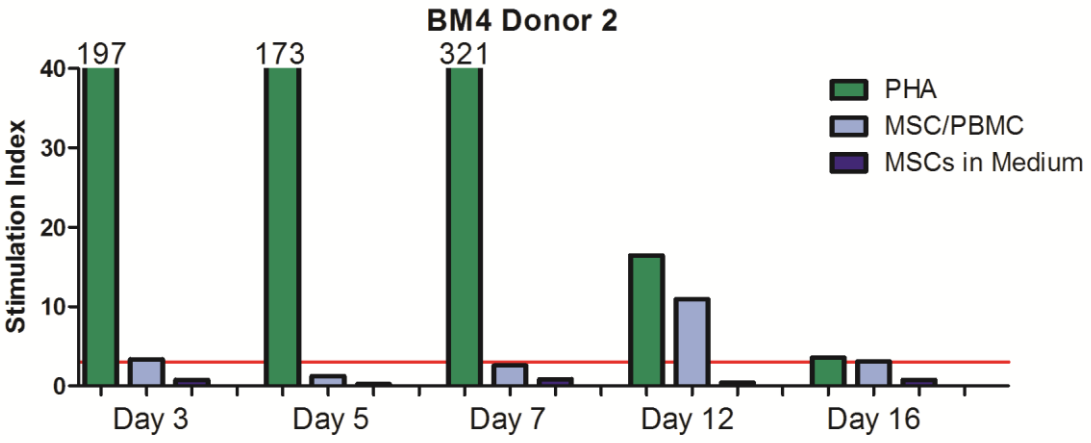


Figure 6.66: The stimulation index (SI) of donor 3 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM2 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

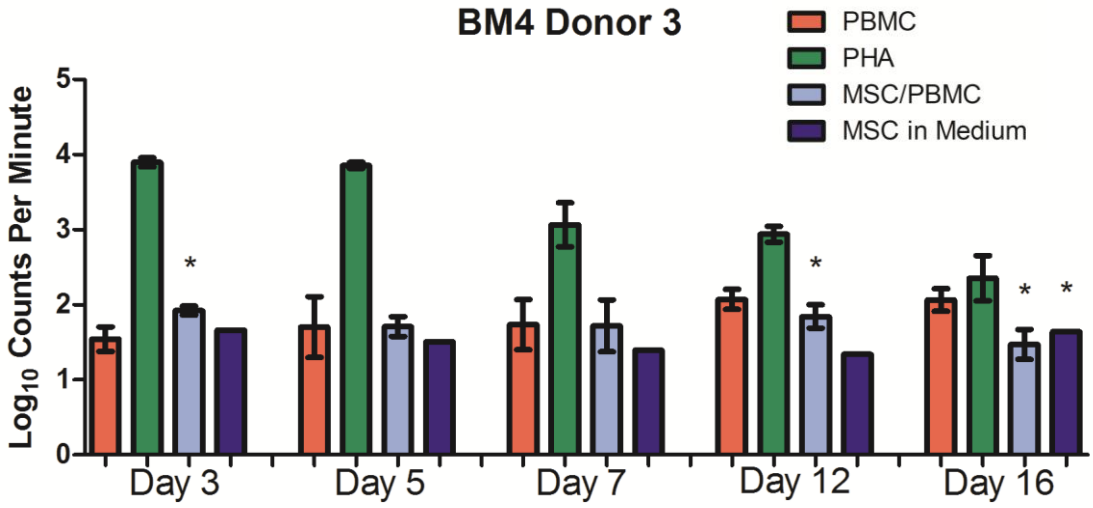


Figure 6.67: Counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with donor BM4 MSCs or MSCs cultured in complete medium alone for 16 days. Data is expressed as the mean log_{10} counts per minute (n=3-4) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant differences ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

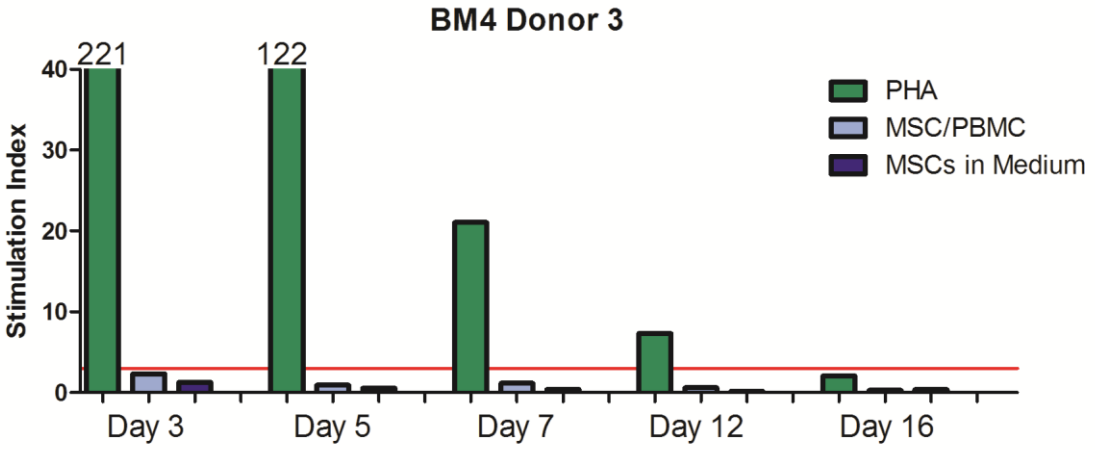


Figure 6.68: The stimulation index (SI) of donor 3 PBMCs cultured in V-bottom plates with PHA (positive control for stimulation), with donor BM4 MSCs, or MSCs cultured in complete medium alone, for 21 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

6.4 DISCUSSION

The aim of the work in this chapter was to perform LTAs by culturing MSCs from 4 donors with peripheral blood mononuclear cells isolated from 6 donors over a culture period up to 21 days. In addition to this, LTAs using human dermal fibroblasts as stimulator cells were also performed.

There have been conflicting reports in the literature about the immunological effects of MSCs. In addition to the reported ability of these cells to down regulate an active immune response *in-vitro* (Bartholomew et al., 2002; Le Blanc et al., 2003b; Bocelli-Tyndall et al., 2007; Nasef et al., 2009; Wada et al., 2009; Kuci et al., 2010; Prasanna et al., 2010), MSCs have also been reported to be immune-privileged. When allogeneic MSCs have been cultured with PBMCs *in-vitro* no proliferation has been observed (Bartholomew et al., 2002; Tse et al., 2003; Jones et al., 2007; Niemeyer et al., 2007; Oh et al., 2008; Suva et al., 2008). As discussed in Chapter 5, there is very little homogeneity between the methods used by different research groups to measure the immunogenicity of MSCs, and the methods that have been used so far to assess the immunogenicity of MSCs have limitations. The majority of these studies have involved culturing MSCs with the PBMCs for less than 7 days and therefore may have only measured the direct pathway of alloantigen recognition. Here, the duration of the assay in which MSCs were co-cultured with PBMCs was extended up to 21 days, in an attempt to incorporate measurement of indirect allorecognition.

To maximise HLA incompatability, bone marrow MSCs were used from 4 donors and cultured with allogeneic PBMCs from 6 healthy volunteers. Stimulation of PBMCs was assessed through the incorporation of radioactive thymidine into the DNA of proliferating cells. The data from these assays was expressed both as $\log_{10}$ counts per minute and as the stimulation index. The counts per minute were $\log_{10}$ transformed to normalise the data, so that statistical analysis (one-way ANOVA) could be performed and significant differences in counts of radioactive thymidine when cells were cultured with and without stimulation by MSCs could be determined. The stimulation index is a method of measuring stimulation of cells that utilises PBMCs cultured in medium only as the negative control for stimulation, and the counts per minute obtained for the PBMCs following stimulation are divided by these counts. A stimulation index above 3 is considered to be a positive response (Ivanyi and Lehner, 1970; Hirose et al., 1989; Kimball, 1990; Gupta et al., 1996; Satoskar et al., 1998).

In order to assess the stimulation of PBMCs in culture, both negative and positive controls for stimulation were needed, these being PBMCs cultured in complete medium alone and PBMCs cultured with PHA to stimulate proliferation.

In the assays reported here, all six donor PBMCs responded to PHA. For the majority of PBMCs stimulated with PHA, maximal stimulation occurred on days 3-5 as observed in Chapter 5.

Statistical analysis using one-way ANOVA of $\log_{10}$ counts per minute provided a method of determining which counts were significantly greater when PBMCs were cultured with MSCs or PHDFs compared to those of PBMCs cultured in medium alone. Due to the variability of counts obtained within replicate wells data was often not significant at longer culture durations. There was a general trend that the data from the counts per minute which were significantly different by ANOVA also gave SI>3. The converse however was not always the case. In some cases the stimulation indices were greater than 10, resulting in positive SI, but when the $\log_{10}$ counts per minute were analysed, no statistical significance was observed (BM2, donor 4, day 16, **Figures 6.21-6.22**). Although variability between replicates was a factor that affected the analysis, the calculation of stimulation indices was dependent on good negative controls. As negative control counts values were generally low, small variations in negative control values had a very large effect on the SI. For example, negative control counts per minute of between 50 and 100 were normal and both very low. However, the effect a fluctuation of between 50 and 100 had on the stimulation index was to halve the SI.

It was important therefore to display the data as both counts per minute, and stimulation indices in order to assess the capacity of the MSCs to stimulate the PBMCs.

In some instances (**Figure 6.3**; day 12) mean $\log_{10}$ counts per minute were higher when PBMCs were cultured with MSCs compared to unstimulated controls, but due to the variability in counts the difference between the means were not statistically significant.

However, relying on purely statistical analysis did not give a good representation of stimulation; in many of the assays in which undifferentiated MSCs were cultured with PBMCs statistical difference between means was recorded early on days 3 and 5, and yet the SI's were less than 3. Hence this was not considered to be positive stimulation.

In a few cases the MSCs cultured alone incorporated low levels of tritiated thymidine. When this was observed it was usually early in the culture duration at days 3-5. This indicated that the MSCs may have been capable of incorporation of some thymidine into DNA despite the mitomycin C treatment. The counts however were extremely low compared to the counts obtained in test wells with PBMCs and did not affect the overall interpretation of the data.

When both significant increases in $\log_{10}$ counts per minute and the SI were used to assess proliferation, stimulation of PBMCs by MSCs was observed with the strongest stimulation usually between days 12-16. Levels of stimulation when measured by the SI were greater than 30 in some instances, and there were only two instances out of the twenty four MSC/PBMC combinations when stimulation was not observed. Extending the duration of the LTA has not been attempted before, and in doing this it was clearly shown that MSCs are capable of stimulating allogeneic lymphocytes to proliferate *in-vitro.* This provided evidence to dispute the prevailing hypothesis that MSCs are immuno-privileged. Lower stimulation also occurred for some MSC/PBMC combinations on days 7 and 21. The data was also in agreement with previous studies in that little stimulation was observed up to day 7 (Bartholomew et al., 2002; Le Blanc et al., 2003b; Tse et al., 2003). These findings highlight the need to extend the duration of LTA's in order to assess the immunogenicity of allogeneic cells which have no capacity for direct antigen presentation.

The age of the MSC donor did not appear to affect the stimulatory capacity of the cells observed, with the oldest donor's cells (BM1, age 69 years old) stimulating 3 PBMC donors with SI's between 20-30, but then stimulating another PBMC donor's cells with an SI of 6.28, and failing completely to stimulate the cells of one of the other PBMC donors. Equally, one of the young donor's cells (BM2, age 9 years old) had the highest SI (36) observed when cultured with PBMC. PBMCs from donor 6 did demonstrate consistently lower stimulation when cultured with all 4 BM MSCs, with the SI failing to rise above 6.4. The stimulation of these cells by PHA however produced SI's as high and higher (in some cases) than the other PBMCs donors. The remaining PBMC donors did not exhibit any difference in stimulation however.

Primary human dermal fibroblasts were used to represent a mature differentiated mesenchymal cell type to use in the LTAs for comparative purposes. When PHDFs were cultured with PBMCs, there was a general trend of the stimulation of PBMCs in co-cultures, but not to the same extent that MSCs stimulated the PBMCs. Primary human dermal fibroblasts were originally cultured as a cell type predicted to stimulate a response in allogeneic PBMCs. In the recent literature skin fibroblasts have been shown to be able to differentiate into bone, fat and cartilage *in-vitro* and express similar phenotypic markers to those expressed by MSCs (Haniffa et al., 2007; Cappellesso-Fleury et al., 2010; Blasi et al., 2011), and also have similar effects of inhibiting the proliferation of stimulated PBMCs *in-vitro*. In the LTAs using extended culture durations surprisingly it was discovered that allogeneic MSCs stimulated PBMCs to a greater extent than the PHDFs.

There was a high degree of variability between wells of the same experimental plate, and it was hypothesized that this may have been due to the culture conditions, and that if MSCs and PBMCs were cultured such a way to encourage the cells to be in even more intimate contact, they would have increased cell-cell interactions, and more consistent reactions. In an attempt to encourage closer proximity between the cells in co-culture, uncoated V-bottom 96 well tissue culture plates were used, and the same LTA's using MSCs and PBMCs were performed.

This however resulted in almost no significant stimulation of the PBMCs when cultured with MSCs, and the stimulation indices obtained for PBMCs treated with PHA were much lower than those observed when uncoated U-bottom 96 well tissue culture plates were used. It was hypothesized that the reason for lower counts when V-bottom wells were used was due to the lack of optimisation in these plates. As shown in the previous Chapter, to gain optimum counts the assay conditions had to be modified from those commonly reported in the literature. With more time, further optimization of the V-bottom plates may have yielded a solution to the variability between wells, but unfortunately time was a limiting factor and it was decided to continue using the U-bottom tissue culture plates.

In conclusion, it was demonstrated in this chapter, in agreement with published literature that PBMCs cultured with allogeneic MSCs for up to 7 days exhibit little to no proliferation. However, when the duration of the assay is extended beyond the 7 days it was shown that allogeneic MSCs stimulated proliferation of PBMCs in 22 out of 24 cases. The stimulation observed was generally higher when MSCs were cultured with PBMCs compared to PHDF's. It was also important to confirm positive SI values with statistical analysis of the counts per minute (tritiated thymidine), as the SI is very dependent on negative control values. Finally, changing the plates from U-bottomed to V-bottomed encouraging the cells to be in intimate contact did not solve the issue of variability observed between replicates.

Chapter 7: ASSESSMENT OF THE IMMUNOGENICITY OF ADIPOGENICALLY DIFFERENTIATED MULTIPOTENT MESENCHYMAL STROMAL CELLS USING LYMPHOCYTE TRANSFORMATION ASSAYS OVER 16 DAYS

7.1 INTRODUCTION

Before the discovery of leptin in 1995, adipose tissue was regarded as a passive and silent organ, whose role in the body was to store excess energy as triglycerides, and to release this energy as fatty acids (Schaffler et al., 2006; Schaffler et al., 2007). Since then adipose tissue has become increasingly recognised as an endocrine organ that secretes a wide variety of cytokines, hormones, chemokines and growth factors, that have an effect on many aspects of the bodies physiology, including appetite, fertility, metabolism, vascular function and, specifically of interest to this study, immunity and inflammation (Schaffler et al., 2006).

There is a need for engineered adipose tissue in the clinic. Subcutaneous adipose tissue defects can arise from congenital abnormalities, oncologic resection and traumatic injury. Soft tissue reconstruction is currently carried using methods such as autografting, fat free grafting, and implantation of alloplastic materials (Girandon et al., 2011). These methods all suffer from disadvantages such as donor site morbidity and graft resorption. A preferred strategy is one that promotes the regeneration of adipose tissue, rather than the replacement of the damaged tissue. In tissue engineering research, this strategy is usually performed through the use of a biocompatible scaffold, seeded with cells of an appropriate phenotype. There are a variety of scaffolds both natural and synthetically created, with an increasing number being developed, but the cells seeded onto the scaffold are usually adipogenic precursor cells (Girandon et al., 2011). These cells can either be purified from adipose tissue, or more commonly adipose derived stem cells or multipotent mesenchymal stromal cells have been used (Zuk et al., 2001; Zuk et al., 2002; De Ugarte et al., 2003; Girandon et al., 2011). The adipogenic potential of MSCs is well documented, and is in fact one of the differentiation characteristics that defines a mesenchymal stromal cell in trilineage differentiation assays (Horwitz et al., 2005).

There are some issues that need to be addressed regarding the future clinical use of tissue engineered adipose tissue utilising MSCs. Firstly, will it be necessary to utilise autologous MSCs to seed the scaffolds? This would involve patients undergoing two procedures to a) harvest the MSCs, expand the cells, and differentiate the cells or seed the scaffold, and b) implantation. Secondly, can allogeneic cells be used that allow the process to be reduced to a one stage process with the potential for commercialisation? Due to the conflicting evidence regarding the immuno-privileged nature of MSCs, one might suspect that allogeneic cells could be used. As shown in the previous chapter however, MSCs even when undifferentiated are able to stimulate the proliferation of allogeneic peripheral blood mononuclear cells.

Adipose tissue has been shown to function as an immunological organ, and in-vivo up to 10% of the stromovascular fraction of adipose tissue has been reported to be CD14+CD31+ macrophages (Fantuzzi, 2005). Adipose tissue has also been shown to secrete various pro-inflammatory cytokines (IL-6 and TNF-α), chemokines (monocyte chemoattractant protein-1) and adipose tissue specific 'adipokines' that may have direct immunological effects. Three of the main adipokines secreted by adipocytes are leptin, adiponectin, and resistin (Fantuzzi, 2005; Tilg and Moschen, 2006). Leptin is pro-inflammatory, and has been shown to increase both T-cell proliferation and the T_H1 response (increased secretion of IL-2 and interferon-ɣ) (Fantuzzi, 2005; Tilg and Moschen, 2006). Adiponectin is anti-inflammatory, and has been shown to reduce secretion of cytokines IL-6 and interferon-ɣ and expression of the transcription factor NF-kB, while promoting secretion of the anti-inflammatory cytokine IL-10 in human macrophages, monocytes and dendritic cells (Tilg and Moschen, 2006). Resistin is another pro-inflammatory adipokine, increasing expression of IL-6, IL-12 and NF-kB by macrophages, but its effect on the adaptive immune response are not yet fully determined. In addition, in humans the expression of resistin is predominantly by macrophages as opposed to adipose cells (Fantuzzi, 2005; Tilg and Moschen, 2006). Two cytokines released by adipose tissue that have received most interest are IL-6 and TNF-α, both are pro-inflammatory;

IL-6 is involved in the promotion of B-cells ability to secrete immunoglobins, and TNF-α is involved in the activation of NF-kB and MAPK pathways.

Interleukin-6 has been shown to be a multi-functional cytokine able to influence various inflammatory reactions. It has been shown to be involved in promoting growth and survival of various cell types, inducing differentiation of monocytes to macrophages, and the recruitment of peripheral blood monocytes to areas of inflammation (Hallbeck et al., 2001). Interleukin-6 exhibits a large number of pleotrophic effects, and one of the main effects *in-vivo* is the promotion of acute-phase proteins by the liver. Several of these proteins mimic the action of

antibodies, but have a broad specificity for pathogen-associated molecular patterns (PAMPs); specific proteins associated with various pathogens (Janeway et al., 2005). In the context of the *in-vitro* LTA the effects of IL-6 observed are the effects this cytokine has on T and B-cells. Interleukin-6 has been shown to increase T-cell activation, and increase the ability of B-cells to secrete immunoglobulins (promote the differentiation of B-cells to plasma cells), as well as act as a growth factor for the fully differentiated plasma cells (Kimball, 1990). With regards to T-cells, IL-6 has been shown to be an activating factor that is able to provide the second (antigen-nonspecific) signal that is required in addition to the immunogen for T-cell activation (Stites, 1991).

Tumor necrosis factor-α binds to two receptors present on many cell surfaces, TNFRI and TNFRII. TNFRI is ubiquitously expressed on mammalian cells and is involved with regulation of apoptosis, inflammation, differentiation and cell proliferation (Szlosarek and Balkwill, 2003). TNFRII is mainly involved with endothelial and haematopoietic cell signalling. The binding of TNF-α to TNFRI ultimately leads to the activation of two main transcription factors, NF_kB and c-Jun (Chen and Goeddel, 2002).The transcription factor NF_kB is able to protect cells from apoptosis (Livolsi et al., 2001), and promote the survival and maturation of B and T-cells, and myeloid dendritic cells (Hayden and Ghosh, 2011). The transcription factor c-Jun products form one half of the translational promoter complex, and when bound to products of the opposing half c-fos forms the complex activator protein-1 (AP-1). Both of these transcription factors are stimulated by the presence of TNF-α (Angel and Karin, 1991). Activator protein-1 has been shown to increase expression of transforming growth factor α and β, and IL-2, and translation stimulated by AP-1 trends towards proliferation and differentiation of cells (Angel and Karin, 1991).

There are many factors that may affect the immunology of adipogenically differentiated in comparison to undifferentiated MSCs (stem cell vs mature cell, secretion of pro-inflammatory adipokines and cytokines, expression of different cell surface markers) and it was hypothesized that the capacity of MSCs to stimulate a proliferative response in allogeneic PBMCs would increase when MSCs were differentiated into adipose cells. During the process of differentiating MSCs into adipose cells, it was noted that the mature culture of differentiated cells was a heterogeneous population of mature adipocytes, and undifferentiated MSCs. These undifferentiated cells could have an effect on the stimulation of PBMCs when differentiated MSCs are used in co-culture with PBMCs. A method to separate the mixed cell population was initially sought, in order that assays using populations of fully mature fat cells could be performed, as opposed to assays with a mixed population of cells.

7.1.1 AIMS AND OBJECTIVES

The aim of the work described in this Chapter was to test the hypothesis that the immunogenicity of MSCs would increase following their differentiation into adipose cells.

Objectives:

- To develop a method for the isolation of mature differentiated adipose cells from 21 day differentiated MSC cultures.
- To determine the capacity of BM4 donor MSCs to stimulate the proliferation of allogeneic PBMCs from 3 donors before and after differentiation into adipose cells in culture using the LTA.

7.2 MATERIALS AND METHODS

7.2.1 PURIFICATION OF MATURE ADIPOCYTES FROM CULTURED MSCS USING DENSITY CENTRIFUGATION

Previous experiments performed in Chapter 2 demonstrated the buoyancy of adipose cells, and the more mature fat cells were lost during the wash steps involved in the staining process for flow cytometry. To separate the mature adipose cells, it was hypothesized that centrifugation for a reduced time would enable the separation of the cell population, with the MSCs containing very little to no intracellular lipids drawn to the bottom of the centrifuge tube by the centripetal forces, whereas the mature fat cells containing a large volume of intracellular lipids would resist the centripetal forces due to their reduced density, and instead float to the surface.

Reagents

Phosphate buffered saline

Hy-Q-Tase™ cell detachment solution

NH expansion medium (section 3.2.2.)

Adipogenic medium(section 3.2.5.)

Complete DMEM medium(section 3.2.1.)

Method

BM2 donor MSCs were isolated and cultured in NH Expansion medium at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.2. MSCs were expanded until 80% confluent in 4 T75 tissue culture flasks. When MSCs were 80% confluent the NH expansion medium was replaced with 10 ml adipogenic differentiation medium. Adipogenic medium was changed twice weekly (½ media changes) over a 21 day period.

After differentiating the MSCs for 21 days, the adipogenic medium was removed from the cultured cells and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. The PBS was removed and discarded, and 2 ml Hy-Q-tase™ cell detachment solution was added before incubation at 37°C for 5 minutes (until all cells were in suspension). The cells were added to 10 ml complete DMEM culture medium in a polythene test tube and centrifuged at 150 g for 5 minutes. The medium was decanted and the cell pellet resuspended in 5 ml PBS. The resulting

cell suspension was centrifuged at 150 g for 1 minute, and samples (100 µl) of supernatant removed using a 1 ml syringe. Samples were taken from the top of the meniscus, 1 cm below the meniscus, and 2 cm below the meniscus. Each sample was placed onto a SuperFrost Plus® glass slide and covered with a glass coverslip, before examination using microscopy to locate mature adipogenically differentiated MSCs.

7.2.2 CEILING CULTURE SEPARATION OF ADIPOGENICALLY DIFFERENTIATED MSCS

Reagents

As in section 6.1.1.

Method

As above (section 6.1.1), BM4 donor MSCs were isolated and cultured in NH Expansion medium at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.2. MSCs were expanded until 80% confluent in 4 T75 tissue culture flasks. When MSCs were 80% confluent the NH expansion medium was replaced with 10 ml adipogenic differentiation medium. Adipogenic medium was changed twice weekly (½ media changes) over a period of 21 days.

After differentiating the MSCs for 21 days, the adipogenic medium was removed from the cultured cells and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. The PBS was removed and discarded, and 2 ml Hy-Q-tase™ cell detachment solution was added before incubation at 37°C for 5 minutes (until all cells were in suspension). The flask was then filled completely with complete DMEM culture medium, and a solid screw top was used instead of the usual filter cap to seal the flask. The flask was then incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air for 4 hours to allow the more buoyant fat cells to attach to the roof of the flask, and the less buoyant stromal cells to remain attached to the bottom of the flask. After 4 hours, the flask was drained of medium, and turned upside down. Hy-Q-tase™ cell detachment solution (1 ml) was added to the flask before incubation at 37°C for 5 minutes (until all cells were in suspension). The 1 ml cell suspension was then added to 1 ml NH expansion medium, and 100 µl of this was dispensed into wells of a U-shaped coated 96 well tissue culture plate. This plate was inverted, and fat cells were left to attach overnight at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Following overnight incubation, the medium was replaced with fresh NH expansion medium, and plates were viewed using microscopy to examine the purity of separated differentiated MSC population.

7.2.3 THE ADIPOGENESIS OF BM2 MSCS CULTURED IN ADIPOGENIC MEDIUM IN UNCOATED AND COATED U-BOTTOM 96 WELL TISSUE CULTURE PLATES

Reagents

Complete DMEM medium

Adipogenic medium

Phytohaemagglutinin (1 mg.ml^{-1} in dH_2O)

Phosphate buffered saline

Hy-Q-Tase™ cell detachment solution

NH expansion medium

Transport medium

Complete medium

Nile red (1:2000 dilution): 1 µl stock into 100 µl PBS, and diluting 4 µl of this solution into 80 µl PBS.

Paraformaldehyde (10% v/v in PBS)

Saponin (0.2% w/v in PBS)

Method

Multipotent mesenchymal stromal cells from donor BM4 were cultured in NH expansion medium until 100% confluent in 2 T75 tissue culture flasks. When 100% confluency was reached the medium was removed from the flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. PBS was removed and 2 ml Hy-Q-Tase™ was added to the flask, before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (approx. 10 minutes). When cells had detached the resulting cell suspension was resuspended in 5 ml adipogenic medium (supplementing complete DMEM with 10% v/v horse serum, 0.5 mM isobutylmethylxanthine, 0.6mM indomethacin and 50 pM hydrocortisone), before counting using trypan dye exclusion as described in section 2.3.7. The MSCs (25,000) were dispensed into wells and left to attach for 4 hours in U-bottom coated 96 well tissue culture plates (Nunc), and uncoated U-bottom 96 well tissue culture plates (Fisher Scientific) and differentiated for 21 days at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in

air. Six repeats of n=6 were prepared and adipogenic medium was changed twice weekly (½ media changes) over a 21 day period.

On day 21 the culture medium was removed from wells and cells washed twice with PBS. Paraformaldehyde (200 µl) was added to the wells for 30 minutes, before the cell layer was washed with 200 µl PBS. The PBS was removed and 200 µl fresh PBS was added to each well. A 100µl volume of crystal violet solution was added to the gaps in between the wells of the plate to minimise light reflection. The background fluorescence was read using 4',6-diamidino-2-phenylindole, DAPI (355/460) and Nile red (485/540) filter sets as per the manufacturer's instructions. These filter sets were used as they provided the closest match to DAPI, excitation at 358 nm, and emission at 461nm, and Nile red, excitation at 450-500 nm, and emission at >528 nm. The overall average fluorescent intensity for each well was read. The PBS was removed from the wells, and 200µl saponin (0.2% w/v in PBS) was added along with DAPI at a final concentration of 1 $\mu g.ml^{-1}$ and Nile red at a final concentration of 1 $\mu g.ml^{-1}$. The plate was then wrapped in foil and incubated at room temperature for 15 minutes. Following three washes with PBS, 200µl PBS was added, and the fluorescence of the wells were again read as above. A ratio of adipogenesis was calculated by taking the mean fluorescence reading of the repeats for both Nile red and DAPI stained cells and creating a ratio of Nile red:DAPI fluorescence (using the methodology developed in Chapter 4 section 4.3.4).

7.2.4 THE LYMPHOCYTE TRANSFORMATION ASSAY CO-CULTURING PERIPHERAL BLOOD MONONUCLEAR CELLS AND MSCS AND THE SEPARATION OF ADIPOCYTES FROM PBMCS WHEN CULTURED TOGETHER IN THE LTA USING FLUORESCENCE ACTIVATED CELL SORTING (FACS).

For these experiments eight 96 well plates were prepared as shown in **Figure 7.1** (4 plates for day 3, 7, 12, and 16 (x2)), with 4 plates set up to use with tritiated thymidine incorporation to assess PBMC proliferation, and the replicate 4 plates set up to sort the mixed MSC and PBMC population via FACS, before RNA isolation for qRT-PCR analysis.

Reagents

Complete DMEM medium

Adipogenic medium

Phytohaemagglutinin (1 $mg.ml^{-1}$ in dH_2O)

Phosphate buffered saline

Hy-Q-Tase™ cell detachment solution

NH expansion medium

Transport medium

Complete medium

Mitomycin C

2x blocking buffer: PBS/2.5mM EDTA/4% (v/v) human AB serum

TRIzol® RNA lysis solution

Anti-human CD45 APC Cy-7 conjugated antibody (BD Biosciences)

Method

MSCs from BM4 were cultured in NH Expansion medium at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. Every 3 days, medium was removed and replaced with fresh. Cells were passaged as previously described in section 3.2.2.

MSCs from donor BM4 were cultured in NH expansion medium until 100% confluent in 4 T75 tissue culture flasks. When 100% confluency at passage 6 was reached the medium was removed from each flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. PBS was removed and 2 ml Hy-Q-Tase™ was added to each flask, before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (approx. 10 minutes). When cells had detached the resulting cell suspension from all flasks was combined and resuspended in 5 ml complete medium before counting using trypan dye exclusion as described in section 2.3.7. The MSCs (25,000) were dispensed into wells of 8 U-bottom coated 96 well tissue culture plates as shown in **Figure 7.1**, seeding only in columns 6-8, and the remaining cells were cryopreserved for 18 days until they were resuscitated and grown in 2 T75 tissue culture flasks as described in section 2.3.6-2.3.8. The MSCs in the eight 96 well tissue culture plates were differentiated for 21 days in adipogenic medium at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. The medium was changed twice weekly (½ media changes).

Mitomycin C stock (250 µl) was added to 20 ml complete medium to form a solution with a concentration of 12.5 $\mu g.ml^{-1}$. This MMC medium was added to wells (200 µl) and the cells incubated for 1 hour at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air.

Following the 1 hour incubation, medium was removed, and the cell monolayer was gently washed twice with 200 µl complete medium.

Resuscitated MSCs grown in the two T75 tissue culture flasks were cultured until 100% confluency was reached, then medium was removed from the flask and the cells were washed with 10 ml PBS without Ca^{2+} or Mg^{2+}. PBS was removed and 2 ml Hy-Q-Tase™ was added to the flask, before incubation at 37°C in an atmosphere of 5% (v/v) CO_2 until cells had detached from the surface of the flask (approx. 10 minutes). When cells had detached the resulting cell suspension was pooled and resuspended in 5 ml complete medium, and 62.5µl MMC stock was added to form the final concentration of 12.5 $\mu g.ml^{-1}$. Following 1 hour incubation the cells were centrifuged at 150 g for 10 minutes, the mitomycin C medium removed, and cells were washed twice with 10ml complete medium. Finally cells were resuspended in 10 ml complete medium before counting using trypan dye exclusion as described in section 2.3.7. Mitomycin C treated MSCs (25,000) were then dispensed into wells of the eight U-bottom coated 96 well tissue culture plates as shown in **Figure 7.1**, seeding in columns 9-11.

Peripheral blood mononuclear cells were isolated from 3 healthy volunteer's human peripheral blood as described in section 5.2.1. Following isolation PBMCs were counted using trypan dye exclusion as described in section 2.3.7. These cells (25,000) were dispensed into the wells of the U-bottom coated 96 well tissue culture plates. Phytohaemagglutinin stock (64 µl) was diluted in 1 ml complete RPMI-1640 medium, then added to wells at a 1:1 ratio of PHA to PBMCs in medium to achieve the final concentration of 32 $\mu g.ml^{-1}$ in the wells shown in **Figure 7.1**.

The negative control for proliferation was responder PBMCs grown in complete medium only, and positive control was responder PBMCs stimulated with 32 $\mu g.ml^{-1}$ PHA. Plates were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

7.2.4.1 THE LTA

For the assessment of PBMC proliferation in four of the eight plates, sixteen hours before the desired timepoint each well of the plate was spiked with 10 µl of low activity tritiated thymidine stock at a final concentration of 0.0125 $\mu Ci.ml^{-1}$ and cultured at 37°C in an atmosphere of 5% (v/v) CO_2 in air overnight. Sixteen hours post spiking the cells were harvested onto glass fibre filter plates using a FilterMate™ (Perkin Elmer) cell harvester, before 35µl MicroScint™ was added and radioactive counts were measured on a TopCount scintillation counter as described in section 5.2.3. Plates were harvested on days 3, 7, 12, and 16. On day 12, 100 µl medium was removed from all wells of the 96 well tissue culture plates,

and frozen in polypropylene 96 well plates at -80°C for later analysis, before 100 µl fresh complete medium was added.

7.2.4.2 CELL SEPARATION

The remaining 4 tissue culture plates were utilised for the separation of adipocytes from PBMCs for RNA expression analysis. The addition of PBMCs and undifferentiated MSCs to the 96 well tissue culture plates was day 0. On days 3, 7, 12, and 16, 150 µl medium was removed from the wells, and was stored in a 96 well polypropylene plate at -80°C. On day 12, 100 µl of the medium in all wells was removed, and 100 µl fresh complete medium added. Medium removed was stored in a 96 well polypropylene plate at -80°C.

To wells containing single cell populations (PBMCs alone and with PHA) 100 µl TRIzol® RNA lysis solution was added. This was incubated for 5 minutes at room temperature, before removing and freezing at -80°C.

Blocking buffer solution (2x) was made (4ml). To this 15 µl primary antibody against CD45 was added. This solution (100 µl) was added to wells containing mixed populations of cells (the adipogenically differentiated MSCs, and undifferentiated MSCs) and incubated on ice for 1 hour. Following incubation, 100 µl ice cold PBS was added to the wells, and the plate was centrifuged at 3000 g for 1 minute. The supernatant was removed from the wells, and 200 µl ice cold PBS added, before the plate was centrifuged again at 3000 g for 1 minute. The PBS was removed from the wells, and 100 µl Hy-Q-tase™ cell detachment solution was added. The plate was then incubated in the dark on ice for at least 30 minutes before cell separation was performed.

Lymphocytes and MSCs were separated by fluorescence activated cell sorting on a BD FACSAria II flow cytometer. Scatter parameters were established using either pure lymphocyte or MSC preparations prior to sorting the mixed samples. Lymphocytes were identified by a low forward (size) and side scatter (granularity) signal and positive staining for APC-Cy7 conjugated CD45. MSCs were identified by their high scatter properties. Detector voltages for the scatter parameters were set so that both lymphocyte and MSC populations were visible on the same scatter plot (forward scatter-area (FSC-A) voltage = 75, log scale, side scatter-are (SSC-A) = 346, log scale). APC-Cy7 fluorescence was excited with the 633nm laser and collected through a 780/60nm band pass filter. Detector voltage for the APC-Cy7 signal was established using unstained cells (voltage = 808, log scale). Cell sorting was performed with a 100 µm nozzle and sheath pressure of 20 psi. Cells were identified from a bivariant dot plot of side scatter vs APC-Cy7 fluorescence. Lymphocytes were identified as having a low side scatter and high

fluorescence signal in the APC-Cy7 plots, while MSCs were identified as having a high side scatter and low fluorescence signal in these plots and gated appropriately on the plots. Cells were sorted at a rate of 3000-5000 events per second and a flow rate below 5 (the flow rate is an arbitrary value. On this specific FACSAria II the actual flow rate was approximately 1 µl.second^{-1}).

A sort mask was applied to ensure maximal cell purity ('purity' mask). Lymphocytes and MSCs were collected into separate wells of a 96 well plate containing 50 µl TRIzol® lysis solution and plate positioning was controlled by an automated cell deposition unit (ACDU) robotic arm within the flow cytometer. Plates containing TRIzol® and cell populations were stored at -80°C for future gene expression analysis using qRT-PCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25, 000 + MSCs**	
C	Donor 1	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	
D	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	
E	Donor 2	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	
F	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	
G	Donor 3	**25,000 in medium**	**25,000 in medium**	**25,000 + PHA**	**25,000 + PHA**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + Adipo**	**25,000 + MSCs**	**25,000 + MSCs**	**25,000 + MSCs**	
H												

Figure 7.1: Diagram of the 96 well plate experimental wells used to determine the stimulation of peripheral blood mononuclear cells when cultured in complete medium only (negative control for stimulation), in the presence of PHA (positive control for stimulation), and co-cultured with adipogenically differentiated BM4 MSCs (Adipo), and undifferentiated BM4 MSCs (MSCs). Donor (1), (2), and (3) represent the donor PBMC.

7.2.5 ANALYSIS OF DATA

Data (counts per minute) from the lymphocyte transformation assays was $\log_{10}$ transformed and is presented as $\log_{10}$ counts per minute ± 95 % confidence limits. The $\log_{10}$ counts per minute at each time point (3, 7, 12, and 16) were compared by one-way analysis of variance and the MSD calculated using the T-method ($p<0.05$). Data was also used to calculate the stimulation indices from the following equation:

$$\frac{\text{cpm PBMCs + MSCs (or adipo)}}{\text{cpm PBMCs alone}}$$

7.3 RESULTS

7.3.1 PURIFICATION OF MATURE ADIPOCYTES FROM CULTURED MSCS USING DENSITY CENTRIFUGATION

When planning to perform the LTA using adipogenically differentiated MSCs, a pure population of mature fat cells to co-culture with PBMCs was desired. However, when MSCs were differentiated into fat, the final population of differentiated cells was heterogeneous, with some MSCs remaining fibroblastic rather than developing intracellular lipid globules within the cytoplasm and differentiating into mature fat cells. A method to separate the mature fat cells from the fibroblastic MSCs was sought, and due to the observations in Chapter 4 about the increased buoyancy of mature adipogenically differentiated MSCs the separation of this cell population using centrifugation was initially used.

Centrifugation of adipogenically differentiated MSCs was performed at 150 g for 1 minute, and samples of supernatant taken from various areas surrounding the meniscus. However, no mature fat cells were observed in any of the samples of supernatant observed at 20x magnification using an Olympus microscope.

7.3.2 CEILING CULTURE SEPARATION OF ADIPOGENICALLY DIFFERENTIATED MSCS

The next method used to attempt separate mature adipose cells from undifferentiated MSCs again utilised the observed buoyancy of the adipose cells. Adipogenically differentiated MSCs were cultured in a T75 tissue culture flask before separation into coated U-bottom 96 well tissue culture plates. When the mixed population of cells was detached from the tissue culture plastic and the flask filled with medium, undifferentiated MSCs adhered to the bottom of the flask (**Figure 7.2**), and the more buoyant adipose cells floated and attached to the roof of the flask (**Figure 7.3**). Further treatment of the adipose cells with cell detachment solution allowed the cells to be dispensed into coated U-bottom 96 well tissue culture plates (**Figure 7.4**).

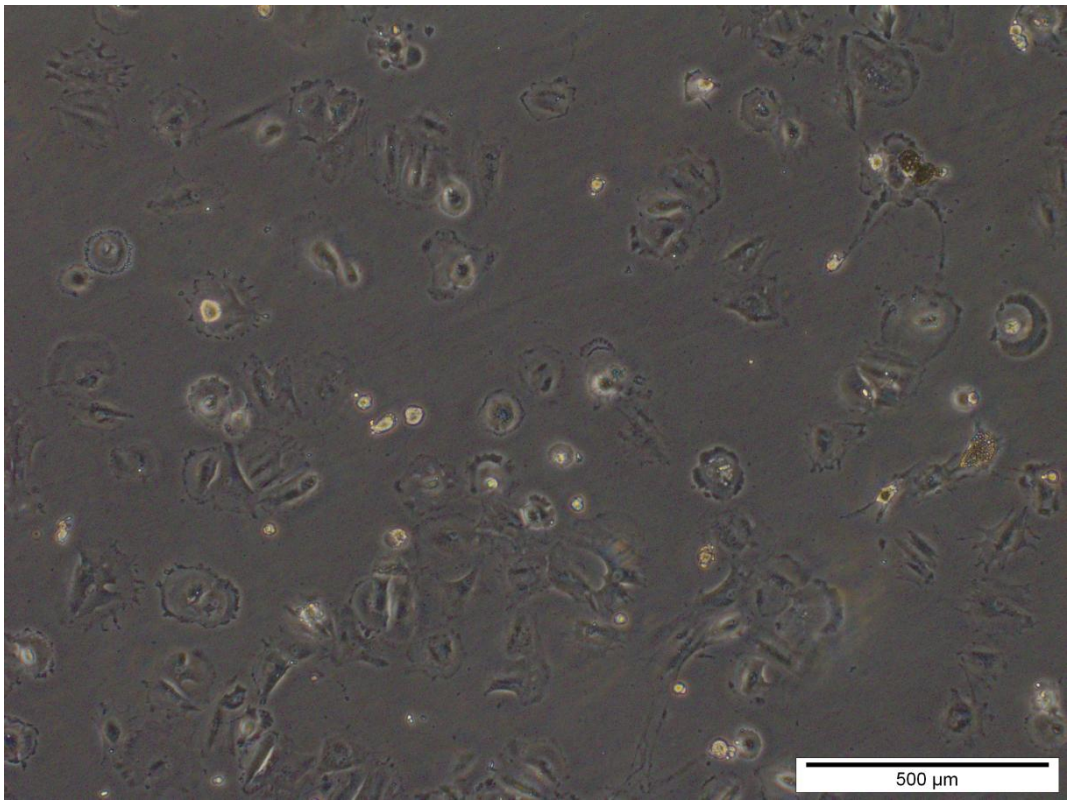


Figure 7.2: Undifferentiated MSCs grown in adipogenic medium for 21 days attached to the bottom of the tissue culture flask following detachment using Hy-Q-tase™ cell detachment solution, and filling the flask with culture medium.

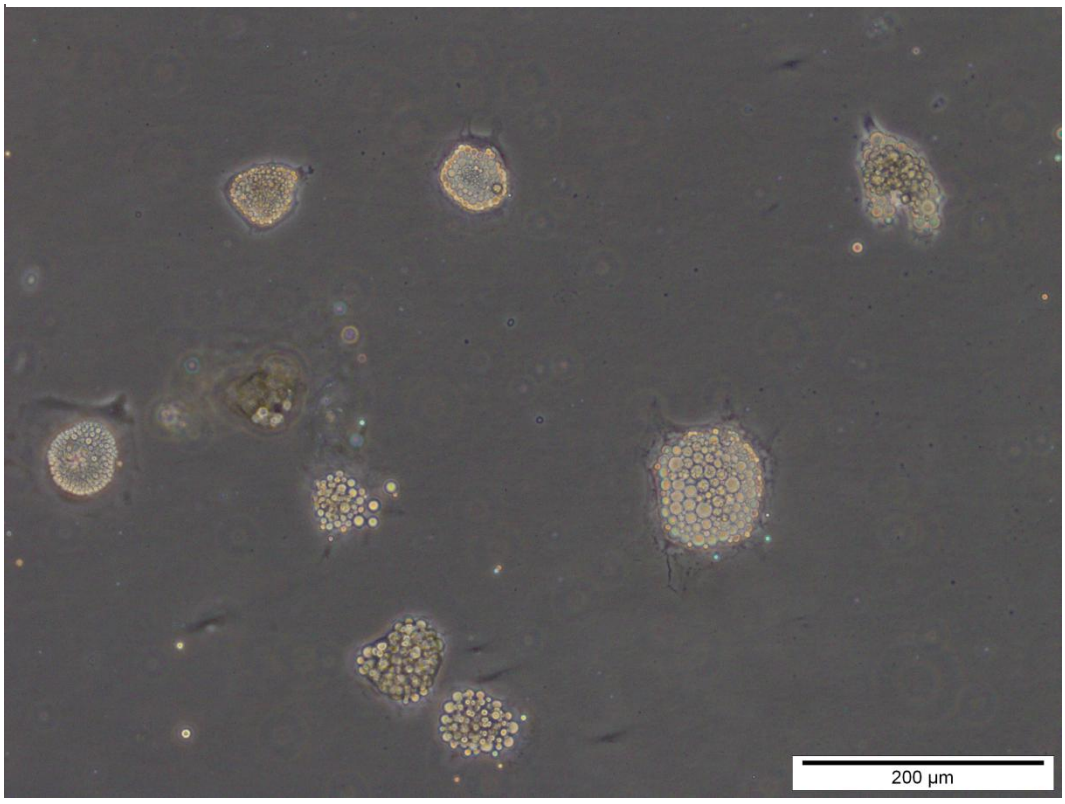


Figure 7.3: Adipogenically differentiated MSCs grown in adipogenic medium for 21 days attached to the roof of the tissue culture flask following detachment using Hy-Q-tase™ cell detachment solution, and filling the flask with culture medium.

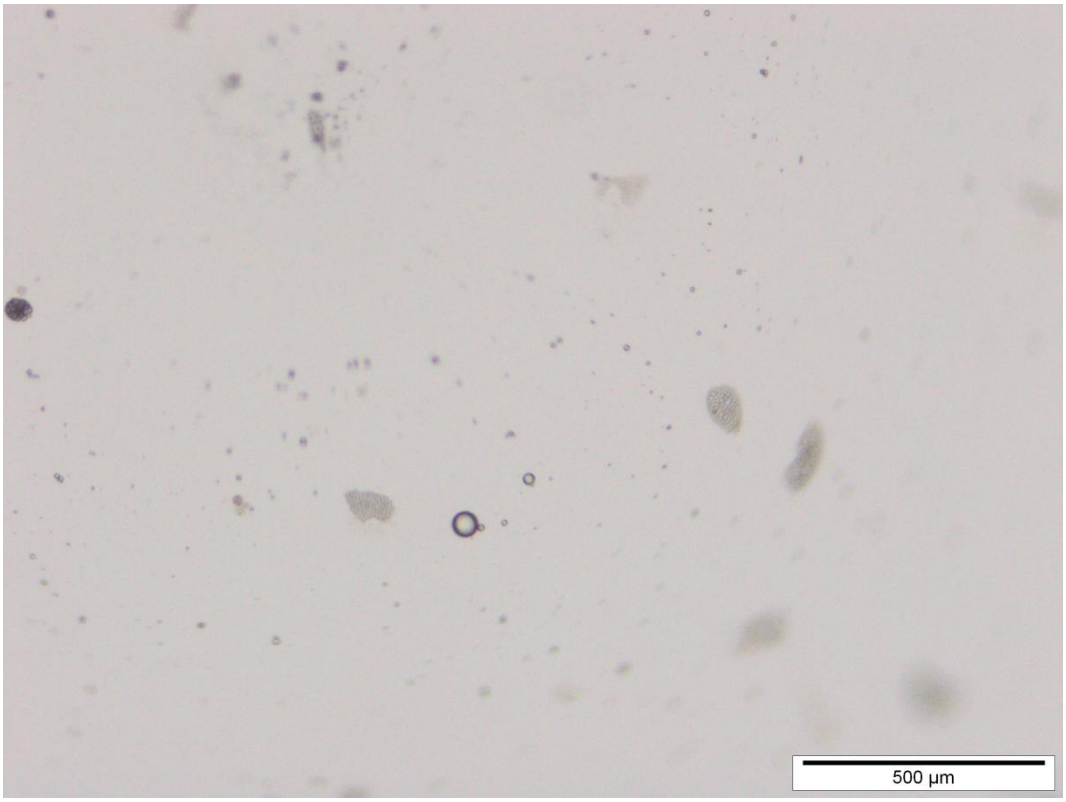


Figure 7.4: Adipogenically differentiated MSCs following detachment using Hy-Q-tase™ cell detachment solution and ceiling culture attachment to U-bottom 96 well tissue culture plates.

Although it was possible to separate adipogenically differentiated MSCs from undifferentiated MSCs using ceiling culture (**Figure 7.3**), and then dispense them into U-bottom plates (**Figure 7.4**), the total number of mature adipose differentiated MSCs was very low. Therefore, although it was possible to obtain a pure population of mature adipose cells using this method, due to the small number of cells and the difficulty in assessing cell number it was not practicable to use this approach to isolate the number of cells required for the LTAs. The decision was therefore made to perform the LTAs using MSCs from donor BM4 differentiated into adipocytes over 21 days and not separated. Donor BM4 was the most adipogenic MSC donor observed in Chapter 4. It was theorised that any effect the undifferentiated MSCs would have in the LTA would be minimal when this donor was used, especially when combined with the evidence in Chapter 5 with regards to the effect of undifferentiated MSCs on PBMCs.

7.3.3 THE ADIPOGENESIS OF BM2 MSCS CULTURED IN ADIPOGENIC MEDIUM IN UNCOATED AND COATED U-BOTTOM 96 WELL TISSUE CULTURE PLATES

To assess the difference in adipogenesis between BM2 MSCs cultured in coated and uncoated U-bottom 96 well tissue culture plates a direct comparison was made using 25,000 BM4 MSCs differentiated for 21 days in adipogenic medium. The levels of adipogenesis were measured on day 21 using the fluorescent microplate reader assay developed in Chapter 4. The ratio obtained when uncoated wells were used was 1.17, compared to 6.11 when coated wells were used (**Figure 7.5**).

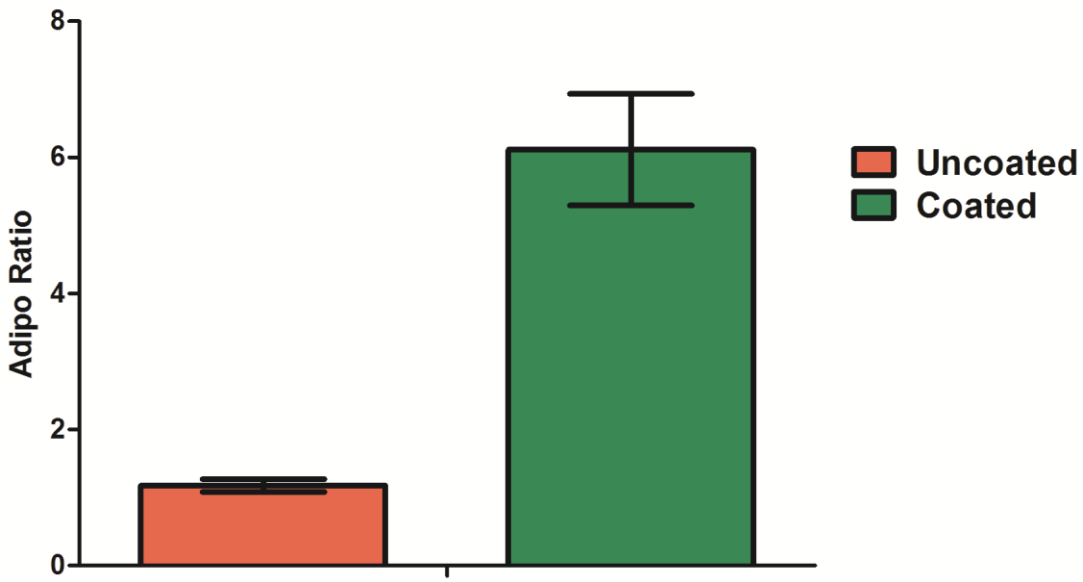


Figure 7.5: Differences of adipogenesis when BM4 MSCs are cultured in adipogenic medium on day 21 in uncoated, and coated U-bottomed 96 well tissue culture plates. Data is expressed as mean (n=6) ± 95% confidence limits. A significant difference was observed (p=0.0022) between uncoated compared to coated wells as determined using an unpaired Mann-Whitney U test.

When adipogenic differentiation was performed in uncoated U-bottomed 96 well tissue culture plates, the initial population of MSCs dispensed into the wells formed a pellet of cells at the bottom of the well. This pellet of cells did exhibit good levels of differentiation (when viewed under bright field microscopy and when stained with Nile red), but medium changes were difficult to perform, as the pellets did not attach to the surface of the wells, and the act of withdrawing and replacing medium from these wells disturbed the pellet, with the pellet becoming more fragile and easier to disrupt as differentiation progressed.

Due to the higher levels of adipogenesis, and easier culture method (for medium changes and higher retention of mature adipocytes) the coated plates were chosen to perform the LTA following the differentiation of MSCs into adipocytes.

7.3.4 THE LYMPHOCYTE TRANSFORMATION ASSAY CO-CULTURING PERIPHERAL BLOOD MONONUCLEAR CELLS AND MSCS

Peripheral blood mononuclear cells were isolated from the blood of 3 healthy volunteers. These cells were co-cultured with BM2 donor MSCs, both undifferentiated and differentiated into adipose cells for 3, 7, 12, and 16 days. As previously, the counts per minute obtained were $\log_{10}$ transformed to conform to the requirements of analysis of variance (variances shown to be homogeneous after $\log_{10}$ transformation). The counts obtained for the positive control cultures with PHA were omitted from analyses since these were included to ensure that the assays were valid (PBMCs were functioning by responding to the mitogen) and it was not part of the experimental design to compare the level of response to the MSCs to the level of response to the mitogen.

When PBMCs from donor 1 were cultured with PHA, maximal counts were observed on day 3, after which the response declined over the remaining 16 day culture period. This response to PHA stimulation was repeated for all 3 PBMC donors. When PBMCs from donor 1 were cultured with BM2 MSCs differentiated into adipose cells, significantly higher counts were recorded on days 3 and 16 compared to the unstimulated PBMCs ($P<0.05$ ANOVA). When PBMCs from donor 1 were cultured with undifferentiated BM2 MSCs significantly higher counts were recorded on days 3 and 7 compared to unstimulated PBMCs (**Figure 7.6**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 7.7**. This showed stimulation indices above 3 for PHA stimulated PBMCs from day 3, for all 3 PBMC donors. Stimulation indices above 3 were recorded for PBMCs from donor 1 in response to BM2 MSCs differentiated into adipose cells at days 3 (SI of 6.4), 12 (SI of 4.4) and 16 (SI of 12.3). For PBMC from donor 1 in response to undifferentiated BM2 MSCs, a stimulation index above 3 was recorded at days 3 (SI of 4.4) and 16 (SI of 10.7).

When PBMCs from donor 2 were cultured with BM2 MSCs differentiated into adipose cells, significantly higher counts were recorded on days 3, 12, and 16 compared to the unstimulated PBMCs ($P<0.05$ ANOVA) . When PBMCs from donor 2 were cultured with undifferentiated BM2 MSCs significantly higher counts were recorded on days 3 and 12 compared to unstimulated PBMCs (**Figure 7.8**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 7.9**. This showed stimulation indices above 3 for PBMCs from donor 2 in response to BM2 MSCs differentiated into adipose cells at days 3 (SI of 5.0), 12 (SI of 3.3) and 16 (SI of 30.8). For PBMC from donor 2 in response to undifferentiated BM2 MSCs, a stimulation index above 3 was also recorded at days 3 (SI of 3.6), 12 (SI of 10.4) and 16 (SI of 14.9).

When PBMCs from donor 3 were cultured with BM2 MSCs differentiated into adipose cells, significantly higher counts were recorded on all days 3, 7, 12, and 16 compared to the unstimulated PBMCs ($P<0.05$ ANOVA). When PBMCs from donor 3 were cultured with undifferentiated BM2 MSCs significantly higher counts were also recorded on all days 3, 7, 12, and 16, compared to unstimulated PBMCs (**Figure 7.10**; $p<0.05$, ANOVA).

The stimulation indices were calculated and are presented in **Figure 7.11**. This showed stimulation indices above 3 for PBMCs from donor 3 in response to differentiated BM2 MSCs at days 3 (SI of 9.0), 7 (SI of 3.2), 12 (SI of 20.6) and 16 (SI of 23.1). For PBMC from donor 3 in response to undifferentiated BM2 MSCs, a stimulation index above 3 was recorded at days 3 (SI of 3.8), 12 (SI of 15.6) and 16 (SI of 13.0).

A summary table for the stimulation indices of adipogenically and undifferentiated MSCs from donor BM2 when cultured with donor 1-3 PBMCs is shown in **Figure 7.12**. There was initial low trend stimulation on day 3 in adipogenically differentiated cultures, before stimulation dropped to 3.2 or below on day 7 for all cultures. The stimulation of PBMCs increased to greater than 3 by day 12, and again by day 16. The stimulation observed for adipogenically differentiated MSCs in the majority of cultures was higher than in the undifferentiated cultures.

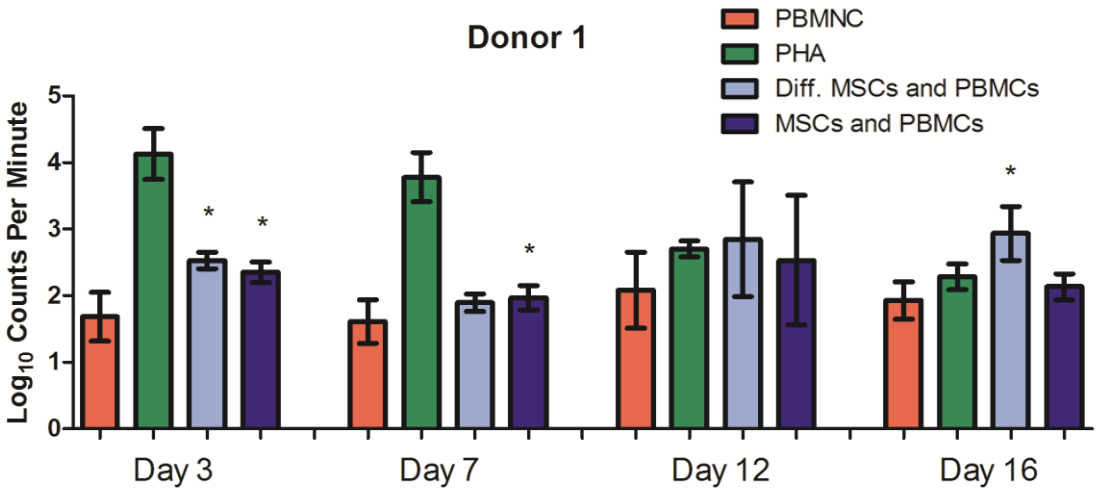


Figure 7.6: Log_{10} counts per minute of donor 1 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs. Data is expressed as mean (n=6) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant difference ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

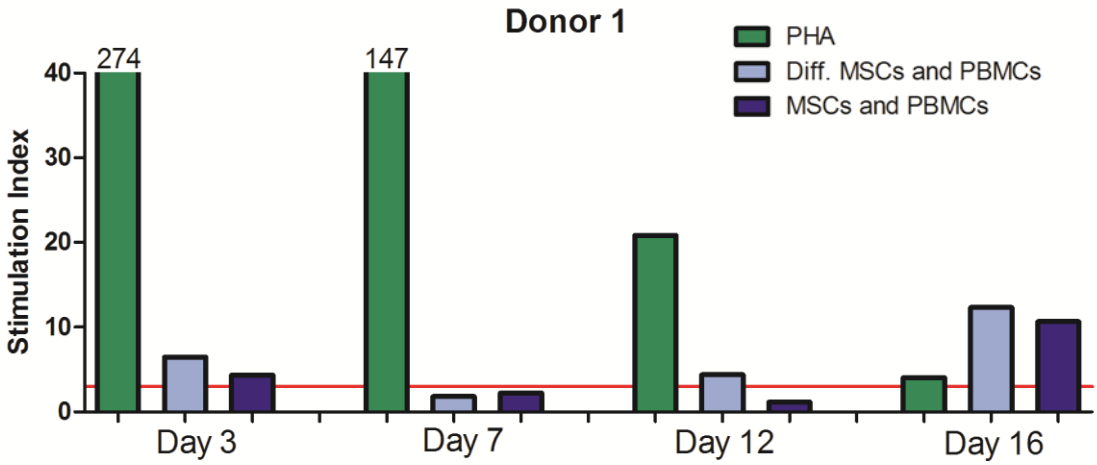


Figure 7.7: The stimulation index (SI) of donor 1 PBMCs cultured with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs for 16 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

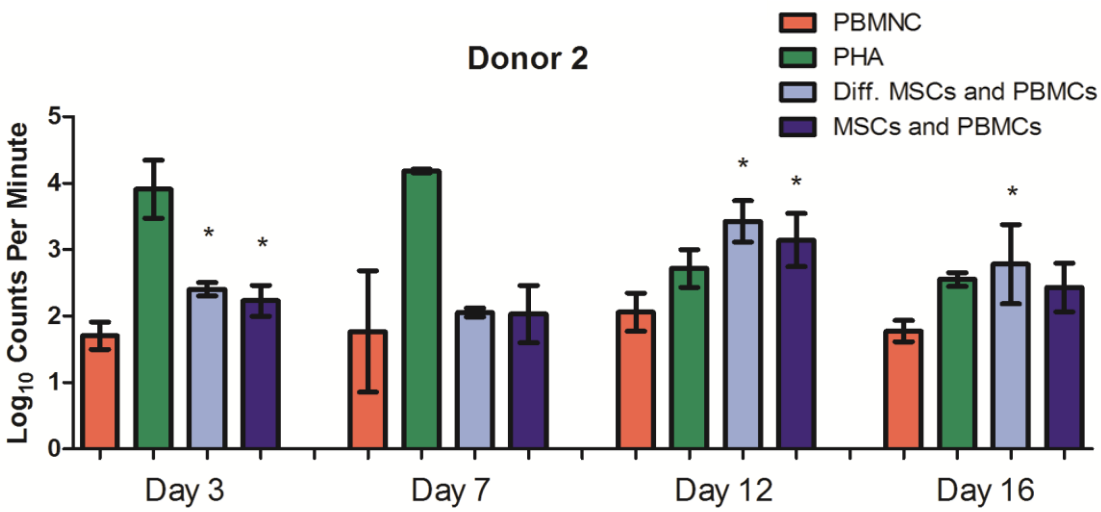


Figure 7.8: Log_{10} counts per minute of donor 2 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs. Data is expressed as mean (n=6) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant difference ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method.

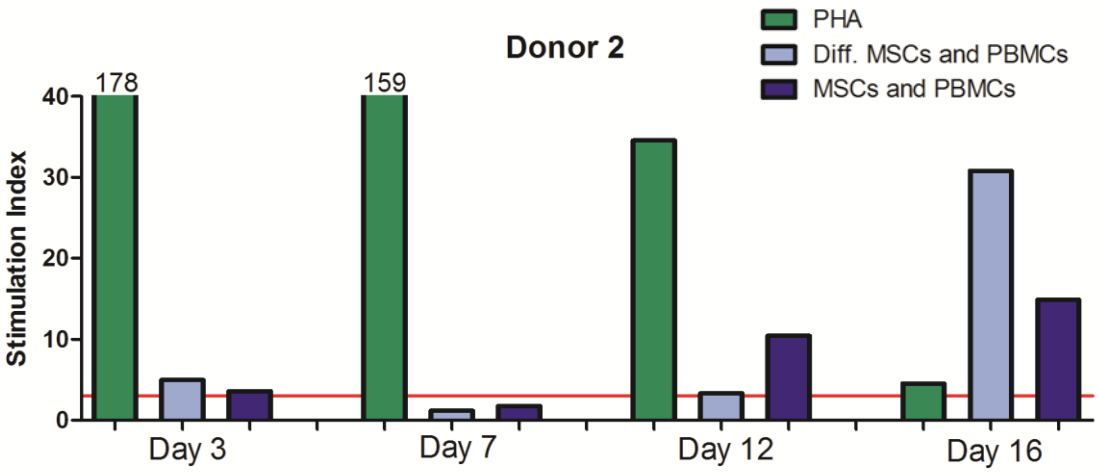


Figure 7.9: The stimulation index (SI) of donor 2 PBMCs cultured with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs for 16 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

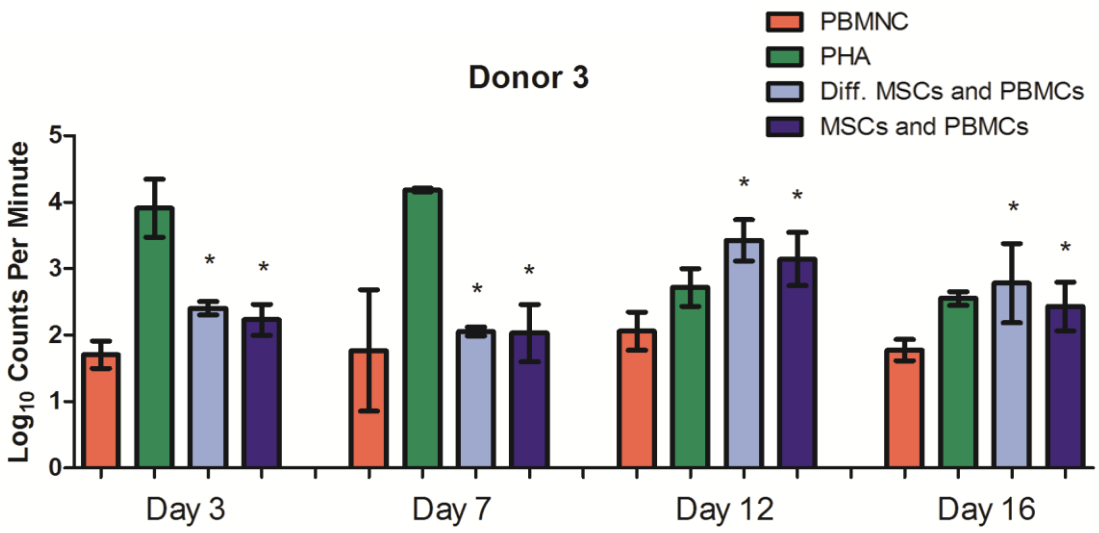


Figure 7.10: Log_{10} counts per minute of donor 3 PBMCs cultured in complete medium only (negative control for proliferation), with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs. Data is expressed as mean (n=6) ± 95% confidence limits. Data was analysed by one-way analysis of variance at each time point. * indicates significant difference ($p<0.05$) compared to the PBMCs cultured in medium alone as determined by the T-method

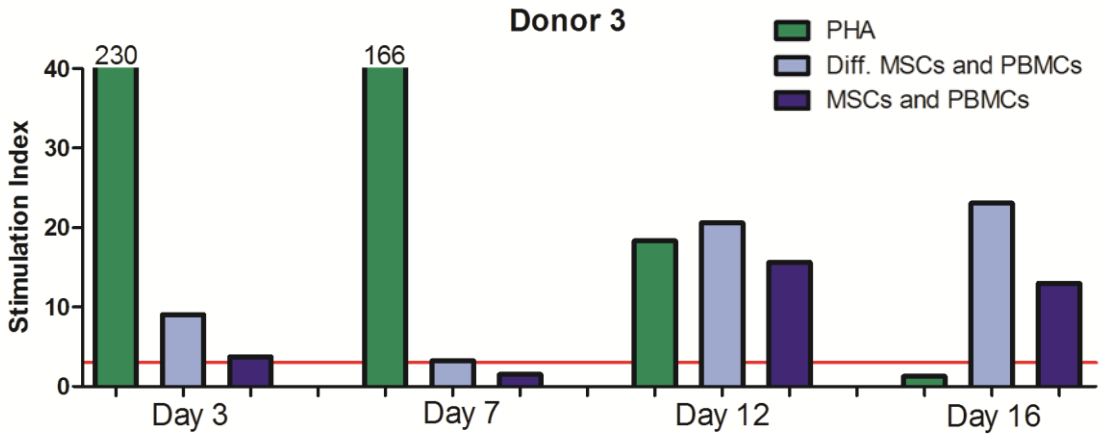


Figure 7.11: The stimulation index (SI) of donor 3 PBMCs cultured with PHA (positive control for stimulation), with BM2 MSCs differentiated into adipose cells, or with undifferentiated BM2 MSCs for 16 days. The red line intersects the y-axis at 3, and values above this height (SI>3) are considered positive stimulation indices.

	Day 3		Day 7		Day 12		Day 16	
	Adipo.	Undiff.	Adipo.	Undiff.	Adipo.	Undiff.	Adipo.	Undiff.
Donor 1	6.4	4.4	<3	<3	4.4	<3	16.0	12.7
Donor 2	5.0	3.6	<3	<3	3.3	10.4	30.8	14.9
Donor 3	9.0	3.8	3.2	<3	20.6	15.6	16.0	13.0

Figure 7.12: Summary table of the adipogenically differentiated (Adipo.) and undifferentiated MSCs (Undiff.) from donor BM2 stimulating PBMCs (donors 1-3) with a positive stimulation index (>3), detailing the SI, and day of stimulation. Green cell colour represents positive SI value, and red is no significant stimulation.

7.3.5 THE SEPARATION OF ADIPOCYTES FROM PBMCS WHEN CULTURED TOGETHER IN THE LTA USING FLUORESCENCE ACTIVATED CELL SORTING (FACS).

In addition to measuring the stimulation of PBMCs using tritiated thymidine incorporation, co-cultures of MSCs and PBMCs were also fluorescently labelled using an anti-CD45 antibody and separated using a FACSAria II FACS cell sorter. Separated populations were resuspended in TRIzol® RNA lysis solution, and stored at -80°C. All culture medium removed from the wells on the tissue culture plate was stored in a polypropylene 96 well plate at -80°C. Cells stored in TRIzol® would be able to be subjected to qRT-PCR for expression of genes associated with the immunology of adipose tissue, and the immune response of T-cells. The culture medium was stored to enable the future assessment of the content of the medium for secreted soluble factors that could be present and affecting the response of the PBMCs towards both the differentiated and undifferentiated MSCs.

7.4 DISCUSSION

There were two aims of this chapter, to develop a method for the isolation of mature differentiated adipose cells from 21 day differentiated MSC cultures and to determine the capacity of BM4 donor MSCs to stimulate the proliferation of allogeneic PBMCs from 3 donors before and after differentiation into adipose cells in culture using the LTA.

When this study commenced it had been reported in the literature that MSCs were immuno-privileged (Bartholomew et al., 2002; Tse et al., 2003; Jones et al., 2007; Niemeyer et al., 2007; Oh et al., 2008; Suva et al., 2008). If undifferentiated MSCs were immune-privileged then it was hypothosized that when these cells were differentiated they would lose this immune-privileged phenotype, as the cells would be effectively changing from a multipotent stem cell into a mature terminally differentiated adipose cell (any characteristics of the undifferentiated MSC that allowed it to escape recognition by the immune system would change on its differentiation into the mature cell type in theory, since otherwise if the source of this tissue *in-vivo* derives from the MSCs, then the tissues themselves would retain their immune-privileged capabilities).

When planning to perform the LTA using adipogenically differentiated MSCs it was desirable to utilise a pure population of mature fat cells. However, when MSCs were differentiated into fat, the final population of differentiated cells was heterogeneous, with some MSCs remaining fibroblastic rather than developing intracellular lipid globules within the cytoplasm and differentiating into mature fat cells.

Previous experiments performed in Chapter 2 had demonstrated the buoyancy of adipose cells, with the more adipogenically differentiated MSCs exhibiting more buoyancy than undifferentiated MSCs. Mature fat cells were lost during the wash steps involved in the staining process for flow cytometry. In order to separate the mature adipose cells, it was hypothesized that centrifugation for a reduced time would allow the separation of the cell populations, with the MSCs containing very little to no intracellular lipids drawn to the bottom of the centrifuge tube by the centripetal forces, whereas the mature fat cells containing a large volume of intracellular lipids would resist the centripetal forces due to their reduced density, and instead float to the surface of the PBS they were suspended in. When this method was performed no adipose cells were found at the surface of the meniscus of the PBS, or up to 2 cm below it. This was thought to be due to the speed of centrifugation, and the density of the PBS used to suspend the MSCs. Instead of remaining buoyant in the solution, the centripetal force used was still great enough over the shorter time used to cause the mature adipose cells

to collect at the bottom of the centrifuge tube, with the more fibroblastic less differentiated MSCs.

The next method that was used to separate the differentiated from undifferentiated MSCs was a modified version of the ceiling culture protocol originally developed in 1986 (Sugihara et al., 1986). The mixed population of differentiated MSCs was detached from the surface of the tissue culture plastic and the flask filled with medium. When the detached cells separated out, the mature adipogenically differentiated MSCs floated to the top of the flask and attached, while the less differentiated MSCs sank and attached to the floor of the flask. This was a successful method of isolating the mature fat cells, as when the roof and floor of the flask were viewed using bright field microscopy the roof had only differentiated cells containing a large volume of multilocular fat, whereas the floor of the flask did not have any mature adipose cells attached. Although a population of mature differentiated adipose cells could be isolated in a tissue culture flask, the problem remained of how to transfer these cells into a U-bottomed 96 well tissue culture plate, in which the LTA was to be performed. The buoyancy of the fat cells was a hindrance, since if these cells were detached from the flask and transferred directly to the plate, the cells would not attach as they would float on the surface of the medium. It was theorised that if the mature fat cells cultured on the ceiling of the flask were detached, and a small volume of medium containing the fat cells dispensed into the culture plate then the surface tension of the liquid would keep the medium from running out of the wells. This would allow the fat cells to float upwards and attach to the plate. This method was successful in that when the wells of the tissue culture plates were viewed using brightfield microscopy mature fat cells could be seen attached to the wells. However, this method was not a viable way to separate the differentiated MSCs for use in the LTA, as for this assay a precise number of cells was required. Centrifugation steps followed by cell counting would introduce the problems seen in the flow cytometry wash steps, with increased loss of cells and difficulty in counting due to the fragile and buoyant nature of the fat cells.

Due to the difficulty in obtaining a large enough population of pure adipose cells, and also following the stimulation of PBMCs observed when co-cultured with undifferentiated MSCs (if undifferentiated MSCs stimulate PBMCs then the presence of them in the LTA using mature differentiated MSCs was less of an issue) it was decided to perform the LTA using one of the more adipogenic BM MSCs. BM4 was selected for this as MSCs from this donor demonstrated consistently high levels of adipogenesis as measured using semi-quantitative scoring using oil red-O, and the fluorescent plate reader assay.

As discussed in Chapter 5, when performing the LTA, the optimum 96 well tissue culture plates were uncoated U-bottomed plates. Culturing cells in these plates is difficult when medium changes are needed, as the cells are not well adhered to the tissue culture plastic, therefore any removal of medium from the wells poses a very high risk of removing cells. Differentiating MSCs into adipose cells in these wells requires regular medium changes twice weekly, and the increasing buoyancy of the cells coupled with the poor adhesion to the plastic made medium changes without losing cells extremely difficult. This was further demonstrated by the reduction in the ratio of adipogenesis when BM4 MSCs were cultured for 21 days in both coated and uncoated U-bottomed 96 well tissue culture plates. Adipogenesis in coated wells was higher (6.11) than uncoated (1.17). Although the optimum plate to perform the LTA was the uncoated (Ingham et al., 1993; Ketheesan et al., 1994; Ketheesan et al., 1996b; Ketheesan et al., 1996a) for this assay higher levels of adipogenesis were desired, and therefore the coated plates were chosen to perform the differentiation of MSCs into adipose cells before the LTA was performed.

As in the previous chapter both the counts per minute and the stimulation indices were considered in order to determine the extent of the response of the PBMCs in the LTA's. The PBMCs from all three donors tested gave significantly higher counts on days 3 and 16 when stimulated with MSCs differentiated into adipocytes compared to cells cultured without stimulation (ANOVA). The PBMCs from donor 2 also gave significantly higher counts on day 12, and PBMCs from donor 3 on all days. When PBMCs from donor 1 were cultured with undifferentiated MSCs significantly higher counts compared to cells cultured without stimulation on days 3 and 7 were recorded. The PBMCs from donor 2 also gave significantly higher counts on days 3 and 12, and PBMCs from donor 3 on day 3, 12 and 16.

Stimulation observed with undifferentiated MSCs on day 3 may have been due to low level non-specific stimulation (stimulation index was <4), whereas significant stimulation on day 3 observed in adipogenically differentiated MSCs (between 4-9), could have been due to the production of adipokines and pro-inflammatory cytokines by the fat cells following MMC treatment.

When the SI were calculated for the LTA's with differentiated MSCs, PBMCs from all donors were stimulated on days 3, 12, and 16, with donors 2 and 3 exhibiting significant stimulation on all days. For undifferentiated MSCs stimulation was observed on days 3 and 12 for all donors, with donors 2 and 3 also exhibiting stimulation on day 16.

Although significant, the counts per minute recorded in the LTA's were lower than those recorded in Chapter 6. This was most probably due to the use of coated plates, as opposed to

uncoated. The positive control counts were noticeably higher when uncoated plates were used. In order to allow comparison between undifferentiated and adipose differentiated MSCs, and due to the use of different tissue culture plates than those used in Chapter 6, both adipose differentiated and undifferentiated MSCs were used in the LTA using the coated plates. The highest SI observed was with the differentiated MSCs and donor 2 PBMCs on day 16 (SI=30.8). The highest SI obtained for co-cultures of donor 1-3 PBMCs with undifferentiated BM2 MSCs was 15.6 (donor 3 day 12), and again this provided strong evidence for the stimulation of PBMCs by allogeneic undifferentiated MSCs.

When the SI was compared between the differentiated and undifferentiated MSCs, adipogenically differentiated MSCs exhibited a trend towards having higher SI than their undifferentiated counterparts. This supported the hypothesis that following differentiation into adipocytes, the immunogenicity of the MSCs increased and could be explained by the relationship that adipose tissue has been reported to have with the immune system and the secretion of various pro-inflammatory factors (Fantuzzi, 2005; Tilg and Moschen, 2006). This increase in stimulation also supported the theory of the loss of any immuno-privileged nature of the MSCs as they change from immature stem cells, to mature terminally differentiated adipose cells.

Unfortunately, time was a limiting factor in these experiments and the additional experimental work that was planned to aid understanding potential mechanisms could not be completed, specifically involving two types of analysis, gene expression and the production of cytokines. During these LTA's on each day that cells were harvested the medium from replicate cultures was removed, and stored at -80°C in polypropylene 96 well plates. This was stored so that ELISA's to detect cytokines secreted by MSCs that may have an effect of the stimulation of PBMCs could be performed. As discussed chapter 1, the soluble factors secreted by MSCs are thought to be heavily involved in how they down regulate the immune response. Indoleamine 2,3-dioxygenase (Meisel et al., 2004) and prostaglandin E_2 (Tse et al., 2003; Aggarwal and Pittenger, 2005; Yanez et al., 2010) have both been strongly suggested to play a large role in how MSCs inhibit activated T cell proliferation and pro-inflammatory cytokine production. The presence of both of these secreted factors in the co-culture medium could be determined to assess how quantities changed as the length of time the MSCs were in co-culture increased.

In addition to storing culture medium for performing ELISA's for cytokines, co-cultures of differentiated and undifferentiated MSCs with PBMCs were separated and stored in RNA lysis buffer. The aim of this was to enable the expression of adipose-specific adipokines leptin and adiponectin, and also the expression of two pro-inflammatory cytokines IL-6 and TNF-α, the

co-stimulatory molecules CD40, CD80, and CD86, and MHC class-I and II to be assessed by qRT-PCR (qRT-PCR was chosen to determine levels of expression as opposed to ELISA as FACS enabled a pure population of the differentiated and undifferentiated MSCs to be separated from the PBMCs, so expression in the two cell populations could be determined, rather than the combined expression as an ELISA using the culture medium would determine).

In conclusion, a suitable method to allow the separation of adipogenically differentiated MSCs that allowed the accurate enumeration and dispensing into wells of a 96 well plate was not determined. It was possible to separate the mature differentiated MSCs using modified ceiling culture separation, but the handling of the separated cells for further assays in which a specific number was required was difficult. The LTA was therefore performed on a heterogeneous differentiated MSC population, in coated U-bottom tissue culture plates, again to aid in the generation of a suitable number of mature adipose cells to be co-cultured with PBMCs. It was found that when the adipogenically differentiated MSCs were co-cultured with PBMCs, the PBMCs did exhibit significant stimulation. Levels of stimulation were also more frequent, and to a higher level in adipogenically differentiated cultures than the undifferentiated counterparts. As has been shown in the previous chapter (Chapter 6) maximal stimulation was observed at 12-16 days. Extending the LTA to assay for the immunogenicity of MSCs has not been performed before, and it is through doing this that it has been possible to demonstrate that MSCs may not be immuno-privileged as indicated in the prevailing literature, and MSCs when differentiated exhibit greater immunogenicity than when in their naive undifferentiated state.

In order to maximize the available data generated from the LTA, the culture medium was retained for future analysis of cytokine content (such as IDO and PGE_2) by ELISA. In addition, the sorting of the PBMCs from the MSCs allows the future analysis of gene expression by qRT-PCR for such genes as the co-stimulatory molecules, MHC class-I and II, pro-inflammatory cytokines IL-6 and TNF-α, and the proinflammatory adipokines leptin and adiponectin.

Chapter 8: FINAL DISCUSSION

All stem cells exhibit three important characteristics that define them as a stem cell. They are unspecialised cells, that are capable of self renewal, and they can differentiate into other specialised mature cell types. There are three main categories of stem cells, these being totipotent stem cells such as those from the fertilised zygote (can differentiate into all tissues, including extra-embryonic), pluripotent stem cells such as those found in the blastocyst inner cell mass (embryonic stem cells, have the potential to differentiate into any cell within the body), and finally multipotent stem cells such as the bone marrow derived stem cells used in this study (these cells can only differentiate into a limited subset of defined tissue types). There is also a further subset of 'stem cells' which are known as induced pluripotent stem cells (iPS). These cells have the characteristics displayed by embryonic stem cells, but their pluripotency is induced by the forced expression of genes, such as the transcription factors Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007).

With regard to their use in the clinic as regenerative cell therapies or in tissue engineering applications, ESCs will always be allogeneic due to the means by which they are derived. Undifferentiated and differentiated ESCs express MHC class-I (Drukker and Benvenisty, 2004) and their immunogenicity has yet to be fully explored *in-vitro* and *in-vivo*. One of the drivers for the development of methods to generate patient derived iPS cells has been that these cells have the potential to provide an unlimited supply of autologous pluripotent cells for regenerative medicine applications. However, there are issues regarding the safety of iPS cells such as their potential for tumorgenicity which remain to be resolved.

Adult multipotential mesenchymal stem cells (MSC) can be derived from the patient requiring therapy, expanded *in-vitro* or used with minimal manipulation to treat the patient from whom they are derived (autologous). On the other hand, they may be isolated from donors, expanded *in-vitro* and used to treat allogeneic recipients. There are minimal issues regarding the safety of using autologous adult MSC in clinical applications. Moreover, the accumulating evidence that allogeneic MSC have immunosuppressive and immune-evasive properties has led to their use in some clinical applications.

It is not surprising then that the majority of clinical trials that have been undertaken or are currently recruiting utilise MSCs. On clinicaltrials.gov, there are 13 trials registered for iPS cells, the majority of which are still active or recruiting and 17 trials for ESCs, again with the majority active or recruiting. However there are 188 studies involving MSC with a mixture of

completed, active and recruiting trials (source clinicaltrials.gov, accessed September 2011). The majority of trials involving ESCs and iPSCs focus on the potential of these cells for non-mesodermal applications (such as nerve cell or retina regrowth) which MSC would not be suitable for. Currently MSCs represent an easier to obtain and safer alternative for mesenchymal applications.

In the early 1960's, a population of adherent cells present within the bone marrow was documented (Friedenstein et al., 1968). These cells were fibroblastic, were able to repair damaged bone and were called osteogenic progenitors. In the early 1990's Arnold Caplan then coined the term mesenchymal stem cell and applied this to these cells cultured *ex-vivo* (Caplan, 1991). The *in-vitro* trilineage assays demonstrating MSCs multipotency and the expression of CD73 and CD105 were published by Mark Pittenger in 1999 (Pittenger et al., 1999), before the lack of standardised nomenclature and characterisation prompted the declaration by the ISCT of the criteria that currently defines the description for cells to be recognised as MSCs (Dominici et al., 2006). These cells have been widely studied and have been acclaimed for their potential uses within the field of regenerative medicine for their advantages such as ease of isolation and expansion (Jones et al., 2002) and immune-privileged status and immune-suppressing capabilities (Le Blanc and Ringden, 2007; Nauta and Fibbe, 2007). The majority of studies on the immuno-regulatory properties of MSCs have investigated the ability of MSCs to reduce an active ongoing immune response. This capability has been demonstrated frequently *in-vitro* (Bartholomew et al., 2002; Di Nicola et al., 2002; Djouad et al., 2003; Krampera et al., 2003; Le Blanc et al., 2004a; Bocelli-Tyndall et al., 2007; Nasef et al., 2009; Kuci et al., 2010), and also *in-vivo* (Le Blanc et al., 2004b; Le Blanc et al., 2008).

The main area of successful use of MSCs in the clinic is for the treatment of autoimmune conditions, in particular for GvHD and Crohns disease. With regard to GvHD, MSCs were first used to treat a patient with treatment-resistant grade IV GvHD resulting in a significant clinical improvement (Le Blanc et al., 2004b). Following on from this study a phase II trial in 55 patients used MSCs again to treat severe GvHD, and reported significant improvements in the majority of patients treated (Le Blanc et al., 2008). The therapeutic effects of MSC in the treatment of GvHD appeared to be independent of donor-HLA match. There are currently 23 clinical trials using MSCs to treat GvHD, the majority of which utilise allogeneic MSCs (clinicaltrials.gov accessed September 2011). On the other hand, a recent study demonstrated that although there was an increase in initial survival, there was no change in the long term (>2 years) survival in severe GvHD patients after allogeneic MSC administration (Remberger et al., 2011). This is in contention with another recent study that used a commercial product based on expanded allogeneic MSCs (Prochymal™; Osiris Theraputics) in the treatment of severe

GvHD in paediatric patients which demonstrated favourable results with 5/12 patients still alive 611 days post-treatment (Prasad et al., 2011).

Currently, there are 8 clinical trials underway that are utilising MSCs to treat patients with Crohns disease (clinicaltrials.gov accessed September 2011), with 50% of these using Prochymal™. This product is also undergoing phase III clinical trials for treatment of GvHD and Crohns disease, and is also being developed for the repair of cardiac tissue following heart attack.

Although allogeneic MSCs appear to have significant promise based on the current clinical results, it is necessary to determine the repeatability and safety of using allogeneic MSCs in additional clinical applications, such as their use in tissue engineering of bone, cartilage and fat, in order to progress potential solutions from research level to clinical application. Very little work has currently been performed investigating the inherent immunogenicity of allogeneic MSC at different stages of their differentiation. The work undertaken in this study was to increase knowledge of the immunogenicty of allogeneic MSC and thus gain a better understanding of their potential clinical applications through refining and developing methodologies for the *in-vitro* assessment of the adipogenesis of human bone marrow MSCs, and to refine the methodology currently used to assess the immunogenicity of human bone marrow MSCs *in-vitro*. Tissue engineered products using allogeneic MSCs do exist on the market (Osteocel™ from Nuvasive for example, an allograft cellular matrix containing MSCs) however it is quite difficult to find clinical data on long term post-operative results. As allogeneic MSCs are starting to make their way into the clinic, it becomes important to assess what happens to these cells when they proliferate and differentiate after implantation.

The first stage in the investigation was to obtain expanded populations of MSCs isolated from fresh human bone marrow isolates, and to characterise these cells by their morphology and phenotype to establish if the isolated cell populations were indeed MSCs. In order to do this, fresh bone marrow isolates were processed to isolate the adherent cell population and these cells were expanded in culture. The morphology of the cells when grown on coated tissue culture plastic flasks was observed and the cells were phenotyped using flow cytometric analysis against cell surface markers. The trilineage potential of the isolated MSCs was observed, and populations were stained with alizarin red for calcium content, Oil red-O for intracellular lipid content, and alcian blue for GAG content.

Putative MSCs isolated from bone marrow were plastic adherent, exhibited fibroblastic morphology and formed distinct colonies when expanded. Using flow cytometry for analysis of cell surface marker expression these cells were positive for CD73, CD90, CD105, and HLA-ABC

and negative for the expression of CD14, CD19, CD34, CD40, CD45, CD80, CD86, and HLA-DR. The pattern of expression of these cell surface markers is replicated throughout the literature (Barry and Murphy, 2004; Dominici et al., 2006; Uccelli et al., 2006; Chamberlain et al., 2007; Nauta and Fibbe, 2007).

The MSCs exhibited trilineage potential, and were able to differentiate into adipocytes, osteoctytes and chondrocytes using specific culture conditions. In the trilineage differentiation assays the MSCs showed varied levels of differentiation, which was believed to be due to the heterogenic nature of MSCs, and the differences in the ages of the donors (D'Ippolito et al., 1999; Kretlow et al., 2008). The chondrogenic differentiation observed using the micromass technique was minimal. Increased staining was observed for all bone marrows from day 0, but at variable levels between the bone marrow donors. BM1 demonstrated the least chondrogenesis and this could have been due to the age of the donor (69). Levels of chondrogenesis may have been imporoved the the cells had been grown in true three dimensional pellet culture.

All of the donor adherent cells isolated fulfilled the criteria as defined by the ISCT for the definition of a mesenchymal stromal cell; MSCs were plastic adherent under standard culture conditions, expressed CD73, CD90, and CD105, and did not express CD14, CD19, CD34, CD45, and HLA-DR, and were able to differentiate into adipocytes, osteoblasts and chondroblasts (Dominici et al., 2006).

This fulfilled the aims of the first part of the study, and after determining that the cells used were MSCs, the next step was to perform further analysis of the adipogenesis of the MSCs.

Adipogenically differentiated MSCs were chosen as a mature differentiated cell type to use to study the immunology of differentiated allogeneic MSCs as native adipose tissue has been shown to function as an immunological organ and as a tissue is able to secrete various pro-inflammatory cytokines (IL-6 and TNF-α), chemokines (monocyte chemoattractant protein-1) and adipose tissue specific 'adipokines' that have direct immunological effects (such as leptin, adiponectin, and resistin). Although the production of TNF-α is mainly due to the macrophages present in adipose tissue, adipocytes have been reported to contribute up to one third of the circulating IL-6 in obese patients (Tilg and Moschen, 2006).

Donor MSCs were differentiated into adipose cells using adipogenic differentiation medium and several published methods for measuring adipogenesis were assayed for suitability; Oil red-O staining of cells and semi-quantitative scoring, real-time PCR using the markers of adipogenesis PPAR-ɣ and FABP4, flow cytometry using an intracellular antibody towards FABP4

and the chemical dye Nile red, and finally a fluorescent microplate reader assay using Nile red and DAPI. A quantitative method of assessing adipogenesis was required, as in the final stages of this study the aim was to assess the proliferative response of PBMCs when cultured with the differentiated MSCs. Since, *in-vivo*, adipose cells are believed to contribute to the secretion of pro-inflammatory cytokines, the MSCs with the greatest adipogenic potential were sought.

Initially Oil red-O staining was used to stain the intracellular lipid droplets and the differentiation quantified using a grading system (English et al., 2007). Alternative methods for quantifying the amount of Oil red-O dye present in stained samples were available (the most common being the absorbance measurement of Oil red-O dye staining the lipid vesicles within cells using spectrometry), but this method only gave an overall level based on total dye content in a sample (Platt and El-Sohemy, 2009). The Oil red-O dye binds to neutral triglycerides and lipids present in cells and the greater the amount of lipids, the greater the intensity of staining. It was found that fibroblasts appeared to exhibit low levels of differentiation potential, with fibroblasts cultured in adipogenic medium developing small quantities of intracellular lipids. This remained at a low level though and using the grading scheme very few Grade 2-4 fat cells were present. In MSCs, Oil red-O staining demonstrated an increase in both the percentage of fat-laden cells and the amount of fat per cell. Although this method provided a visual method of quantification, it was subjective and very time consuming and as such it was not suitable as a higher throughput method for the quantification of adipogenesis.

For quantitative real-time PCR, two genes associated with adipogenesis were selected; peroxisome proliferator activated receptor (PPAR-γ) and the late marker of adipogenesis, fatty acid binding-protein 4 (FABP4). Both of these genes have been shown to increase in expression as MSCs undergo adipogenesis (Janderova et al., 2003; Fink et al., 2004; Neubauer et al., 2004; Sekiya et al., 2004; Blum and Benvenisty, 2008). In contrast to previously published data (Janderova et al., 2003; Sekiya et al., 2004) although PPAR-γ expression generally increased during adipogenesis the changes were relatively small and did not reflect visible adipogenic differentiation in the MSCs. However in agreement with previous studies FABP4 expression rose gradually up to day 21 (Sekiya et al., 2004; Boucher et al., 2009). It was noted however that increased expression of FABP4 did not always translate into a higher level of visual fat content.

It was concluded that qRT-PCR was highly quantitative and able to track adipogenesis on a transcriptional level, but it was time consuming and labour intensive and therefore not considered suitable for a reliable high-throughput measurement of adipogenesis.

To further evaluate the use of FABP4 as a suitable marker for adipogenesis, FABP4 protein accumulation in MSCs was monitored using flow cytometry in differentiating MSCs using an intracellular antibody. A gradual accumulation of more granular cells was observed as the time-course progressed, correlating with increasing FABP4 levels in these cells. In addition to this Nile red dye was also used to stain for lipids using flow cytometry and Nile red fluorescence increased as the time course progressed, with the more granular cells having higher amounts of Nile red fluorescence compared to the less granular cells. Both of these methods resulted in the higher mean fluorescence intensity of the more granular positively staining population of cells peaking on day 14 and decreasing on day 21. This was in contrast to a previous study which showed an increase in staining as the timecourse progressed, including on the final day 28 (Smyth and Wharton, 1992). The reduction in the positively staining population was not observed when the cells were viewed microscopically and it was hypothesized that the reduction in the more granular, more mature fat cells was perhaps because of the loss of these cells during the wash steps required in preparation for flow cytometry, due to the increased buoyancy of the cells.

After comparing the various methods that were routinely used to assess adipogenesis in differentiated MSCs it was concluded that semi-quantitive scoring provided a visual method of quantifiying adipogenesis, but it was subjective and very time consuming. qRT-PCR was similarly time-consuming, and measured mRNA levels instead of protein or lipid directly. Flow cytometry correlated well with morphological lipid accumulation in MSCs, however there was a danger of losing mature floating fat cells during the preparation steps.

Since these methods did not provide a robust and efficient assay for quantifying adipogenic differentiation, the utility of a Nile red-based fluorescence microplate assay to measure adipogenesis was explored. This assay used intracellular Nile red fluorescence to quantify lipid accumulation, and DAPI staining for cell nuclei for normalization. The average fluorescent intensity for DAPI and Nile red for each well was measured and a ratio of adipogenesis determined based on the number of cells present (represented by DAPI fluorescence) to amount of intracellular Nile red staining. MSCs stained only with DAPI were found to have increased fluorescence over the timecourse, demonstrating that proliferation of MSCs continued even when differentiating. It was also found that Nile red could be used to measure adipogenesis as a single dye, but due to the aforementioned proliferation of cells, the ratio using Nile red and DAPI together was preferred.

The fluorescent microplate assay provided both highly quantitative measurements and fluorescent images of fat droplet distribution per cell and allowed a numbered measurement

to be assigned to differentiated cultures based on their level of adipogenesis, with visual verification of adipocyte content. This method had the additional advantage of being high throughput and cost effective.

Previous studies have demonstrated the differentiation heterogeneity of MSCs from different donors undergoing osteogenesis (Phinney et al., 1999; Siddappa et al., 2007). During the timecourse and throughout the various methods employed it was also observed that this variation between donors existed for adipogenesis. MSCs from different donors varied in their adipogeneic differentiation potential and morphological analysis of the mature adipose cells using Oil red-O staining showed heterogeneity in the same adipogenesis-driven MSC cultures, with not all cells within the assay differentiating into adipocytes at the same rate and a subset of the cells failing to differentiate into fat cells. From this it appears that there a certain subset of the MSCs that possess the ability to differentiate into adipocytes, while the remaining cells are unable to progress into mature fat cells. This may be due to preconditioning of the bone marrow MSCs that even at this stage of development in the stem cell population there exists a number of cells whose fate has already been predetermined to such a degree that differentiation into adipocytes cannot be achieved in 21 days. It is possible that given longer these remaining cells would differentiate into mature adipose cells, or it is also possible that the method of culture would need to be adapted to promote the adipogenesis of these cells. Adipogenesis *in-vivo* does not occur in a two dimensional monolayer and our method of culture may preclude MSCs that are not predisposed for adipogenesis from turning into fully mature adipose cells.

The microplate assay was also found to be the most efficient and simple to perform on a larger volume of samples, and as Nile red is a chemical dye it was a more cost effective alternative to using antibodies. Using this method allowed MSCs with a greater adipogenesis to be selected for further experiments, in which a higher grade of differentiation would be beneficial.It was found that BM 4 exhibited the highest levels of adipogenesis as measured using the microplate assay.

As mentioned previously, the immunogenicity of MSCs is controversial. These cells have been reported to be immuno-privileged and have immunosuppressive properties (Bartholomew et al., 2002; Le Blanc et al., 2003b; Tse et al., 2003). Given the ability of these cells to differentiate into bone, fat, and cartilage cell types (Prockop, 1997; Pittenger et al., 1999; Barry and Murphy, 2004; McGonagle and Jones, 2008) and potential applications in tissue engineering, the question regarding the immunogenicity of MSCs is important to fully explore in both *in-vitro* and *in-vivo* systems. There are various methods used in the literature to study the

immunogenicity of MSCs; by tracking T-lymphocyte responder cell divisions using CFSE and flow cytometry or BrdU incorporation, or looking at the effect of MSCs on other immune cells such as NK-cells or B-cells (Sotiropoulou et al., 2006; Oh et al., 2008; Suva et al., 2008; Asari et al., 2009). The most commonly used method and the one most viable for the determination of rejection by the adaptive response is the lymphocyte transformation assay. The methods used in the literature, however, have been highly variable, with different numbers of stimulator MSCs to responder cells, different methods for mitotic inhibition of responder cells, the method of measurement of lymphocyte proliferation, the use of PBMCs or purified T-cells, and the types of plate (Bartholomew et al., 2002; Tse et al., 2003; Niemeyer et al., 2007; Oh et al., 2008; Suva et al., 2008). Most importantly though, the incubation time used has not been extended beyond 7 days in any of the studies, and so may not give enough time for the indirect immune response to occur.

The next stage therefore was to develop the methodology to assess the proliferation of PBMCs when cultured in lymphocyte transformation assays with MSCs. The LTA can be used for the *in-vitro* assessment of the immune response of peripheral blood mononuclear cells (PBMCs) towards an antigen, and was originally developed as an *in-vitro* alternative to the then previously only *in-vivo* assays to measure cell mediated immunity. In addition to using MSCs in the LTA, human dermal fibroblasts were also used as a differentiated control cell type, to compare levels of stimulation.

There were 5 main objectives that were required for the development of the LTA, the determination of the concentration of PHA (mitogen) for use as the positive control for lymphocyte activation, which method to use to for spiking the cells (using tritiated thymidine), the determination of the concentration of mitomycin C for the mitotic inhibition of stimulator cells while retaining cell viability, the determination of the cell number and ratio of responder and stimulator cells in the LTA and finally the determination of the concentration and source of serum for cell culture medium supplementation.

Peripheral blood mononuclear cells were isolated using a Lymphoprep® density centrifugation gradient and PHA was selected to treat PBMCs as a positive control for stimulation. The proliferation of cells when stimulated was measured using tritiated thymidine (^{3}H) incorporation (high and low activity). The low activity ^{3}H provided significantly higher counts per minute over the high activity, and therefore was selected to use in experiments. A PHA concentration of 32 $\mu g.ml^{-1}$ also gave the highest counts from the concentrations used and was selected to use as a positive control for lymphocyte stimulation.

In the LTA, the cell population acting as the stimulator cells is the MSCs. The mitotic ability of these cells required inhibition, in order that the incorporation of ^{3}H measured was only due to the proliferation of the responder (lymphocyte) cells. Previously cell division has been mitotically inhibited using gamma irradiation (Bartholomew et al., 2002; Tse et al., 2003; Niemeyer et al., 2007). Other studies have used the chemical compound MMC, in concentrations between 10 and 25 $\mu g.ml^{-1}$ (Oh et al., 2008; Nasef et al., 2009; Prasanna et al., 2010). These studies did not report upon whether the treated MSCs were unable to proliferate, or were indeed viable, after treatment with MMC. It was decided to evaluate concentrations of MMC in the 10-25 $\mu g.ml^{-1}$ range and determine if the MSCs treated were still viable but unable to divide in culture. In order to do this MSCs and fibroblasts were treated with 3 concentrations of MMC (50, 25, and 12.5 $\mu g.ml^{-1}$) and the proliferation measured using fluorescein diacetate. It was found that all 3 concentrations of MMC inhibited the proliferation of cells, so 12.5 $\mu g.ml^{-1}$ was selected for subsequent use since this was the lowest (and therefore least cytotoxic) that inhibited the proliferation of both cell types whilst the cells remained viable.

The optimum cell number and ratio of PBMCs that generated the highest counts was determined using a one way MLR, and it was shown that the highest counts were obtained when a ratio of 1:1 responder cells to stimulator cells was used. In contrast with previous studies optimum counts per minute were also obtained when 100,000 total cell number per well was used (as opposed to Tse *et al.* who used 100,000 PBMCs and 15,000 MSCs (Tse et al., 2003), LeBlanc *et al*. who used 100,000 PBMCs and between 10-40,000 MSCs (Le Blanc et al., 2003b) and Bartholomew *et al*. who used 100,000 PBMCs and 100,000 MSCs (Bartholomew et al., 2002). The majority of the previous literature therefore used 100,000 PBMCs before the addition of MSCs to the culture, however in this study it was found that 50,000 total number of cells also demonstrated high counts per minute comparable to 100,000 cells with no significant difference between the two cell densities.

Finally the type and concentration of serum used in the complete culture medium was determined. A comparison assay of fetal calf serum and human AB serum showed no significant difference in counts per minute in the negative controls, however, the positive control counts were significantly higher when human serum was used; therefore human AB serum was selected to be used in subsequent LTAs. Previously 10% FCS has been used in LTAs (Nasef et al., 2009; Wada et al., 2009; Prasanna et al., 2010), or 5-10% human serum (Bocelli-Tyndall et al., 2007; Niemeyer et al., 2007) so LTAs were performed using 2, 5, and 10% (v/v) human serum in complete RPMI culture medium. No significant difference was observed between the concentrations of serum used, so 10% was chosen. It was also important to avoid

the use of FCS, since this is of xenogeneic origin and might be capable of stimulating PBMCs in negative controls without PHA or stimulator cells.

The developed LTA used 32 $mg.ml^{-1}$ PHA as positive control for stimulation. The ratio of PBMCs to MSCs was 1:1 and 25,000 cells were used for each (giving a total cell number of 50,000 per well). In order to mitotically inhibit the responder MSCs the cells were incubated with 12.5 $mg.ml^{-1}$ mitomycin C for one hour. The culture medium used was RPMI-1640 supplemented with 10% (v/v) human AB serum. In order to determine levels of stimulation, test cultures were spiked with low activity tritiated thymidine before the stimulation index was calculated by dividing the negative control counts per minute into test counts per minute. For further analysis of the data, Log_{10} counts per minute in control and test wells were compared using one-way ANOVA, and the minimum significant difference ($p<0.05$) between group means determined (Sokal and Rohlf, 1981).

Once the conditions for the assay had been determined, lymphocyte transformation assays were performed using 4 donor MSCs with PBMCs isolated from 6 donors. In addition to this, LTAs using human dermal fibroblasts were also performed. The MSCs/PHDFs were cultured with PBMCs for 3, 5, 7, 12, 16 and 21 days before spiking with low activity tritiated thymidine and the stimulation determined.

Previous studies of the immunogenicity of MSCs using similar LTAs have only reported the data from tritiated thymidine incorporation as the stimulation index (Le Blanc et al., 2003b). A stimulation index greater than 3 is considered significant (Ivanyi and Lehner, 1970; Hirose et al., 1989; Kimball, 1990; Gupta et al., 1996; Satoskar et al., 1998). It has been reported that the SI is a problematic indicator of lymphocyte proliferation because, being a ratio, it weighs unstimulated values so heavily (Herbert et al., 1994). The main problem found with the unstimulated values in LTA's is that they vary a great deal. In order to combat this, unstimulated controls were performed on a minimum of four replicates and mean counts were used. When the LTA was being optimised discrepancies did occur between the repeat values, but the substitution of fetal calf serum for human AB serum minimised the variances observed. In addition, the log_{10} counts per minute obtained in the LTA's were statistically analysed (ANOVA) to determine significanct differences between unstimulated and stimulated cultures. Significant stimulation observed using the SI generally correlated with the significant differences in the counts. Using the SI as a method to determine stimulation allowed comparisons to be made to previous studies of MSC and PBMC LTA's.

As discussed previously, the calculation of the SI is dependent on good negative controls. The negative control for stimulation was PBMCs cultured in medium alone, and these cultures

were performed in replicates of four, and the mean used to calculate the SI. It was found that PHDFs and MSC from all BM donors (1-4) stimulated all of the PBMC donors, with 2 exceptions; BM1 with donor 5 PBMCs, and BM3 with donor 2 PBMCs. This is in strong contention, with the majority of the literature in which MSCs failed to stimulate allogeneic PBMCs in LTAs (Bartholomew et al., 2002; Le Blanc et al., 2003b; Tse et al., 2003). Even in the more recent literature, it is still the commonly held belief that allogeneic MSCs are immuno-privileged (Bassi et al., 2011; English and Mahon, 2011). That this is the widely held opinion is not surprising, given that in the majority of the studies in which LTAs have been performed using allogeneic MSCs, the incubation period of co-culture is usually less than seven days. The highest levels of stimulation observed in this study were usually observed after day 7, with the greatest stimulation observed at days 12-16. Given the low/lack of MHC class-II and co-stimulatory molecule expression this, as mentioned previously, is not especially surprising. Previous studies have focused on the ability of MSCs to stimulate the direct response through limiting the culture of PBMCs with MSCs for less than 7 days. As discussed in the Introduction, the indirect and semi-direct pathways of allorecognition rely on the alloantigens that are shed from the graft cells, which are then processed as exogenous antigens by recipient APCs before presentation to naive T-cells. This process was theorised to take longer than direct recognition and by extending the duration of the LTA up to 21 days it has clearly been demonstrated that MSC have the capacity to stimulate the proliferation of allogeneic PBMC *in-vitro*. It is hypothesised that the stimulation observed was due to indirect and semi-direct pathways of recognition of the allogeneic MSCs.

In addition to production of various anti-inflammatory cytokines, MSCs have also been shown to produce growth and proinflammatory factors, such as bFGF, IL-6, and TNFα, which may also explain some of the stimulatory effect of MSCs on PBMCs *in-vitro* (Crop et al., 2010). Il-6 and TNF-α mainly augment the specific immune response indirectly through the stimulation of the maturation of dendritic cells or B-cells (Janeway et al., 2005), appropriate recognition of antigen by lymphocytes would still be necessary for a proliferative response.

Very few studies have investigated the effect of how the differentiation of MSCs affects the stimulation of PBMCs. In one study bone marrow MSCs were differentiated into osteocytes for 14 days, before performing the LTA for 7 days. They found no significant stimulation with either allogeneic undifferentiated or differentiated MSCs (Niemeyer et al., 2007). An earlier study used undifferentiated MSCs and MSCs differentiated into bone, fat, and cartilage following differentiation for 6 to 12 days, before performing the LTA for 6 days. This study found that there was no proliferative response observed when undifferentiated MSCs were used, and also no response when adipogenic, osteogenic, or chondrogenic differentiated MSCs

were co-cultured with PBMCs (Le Blanc et al., 2003a). When the LTA was performed in this study using differentiated MSCs it was found that adipogenically differentiated MSCs significantly stimulated PBMCs, and they did this at a higher level than undifferentiated MSCs. The stimulation observed however was not as strong as might have been hypothesized, but this could have been due to the use of tissue culture plates that were not optimal for LTAs (U-bottomed coated 96 well tissue culture plates had to be used due to the nature of the differentiated mature fat cells).

In light of these findings, it is relevant to discuss why the therapeutic use of allogeneic MSCs in the clinic has proved successful. As a cellular therapy MSC have been shown to be able to aid in the alleviation of symptoms in conditions such as GvHD and Crohns disease. In the treatment of patients with these conditions, allogeneic MSCs are generally administered intravenously, a route that would not instigate danger signals and is known to be tolerogenic. Moreover, the patients treated are often immunocompromised or immunosuppressed. There is very little evidence to suggest that allogeneic MSCs survive in the longer term in immunocompetent patients. The potential use of MSCs in tissue engineering applications poses very different issues. In tissue engineering applications such as the replacement of bone, cartilage or fat, a high density of allogeneic MSCs would be delivered with a scaffold into a surgical wound, instigating danger signals. It is therefore contended that in this context, the allogeneic MSC would be recognised and eliminated by the recipient immune system.

More recently there have been a small number of studies that have shown that MSCs do in fact stimulate PBMCs in murine (Nauta et al., 2006b) and rat models (Huang et al., 2010) and using human MSCs (albeit adipose tissue derived) significant stimulation of PBMCs in co-culture was also observed (Crop et al., 2010). Nauta *at al.* demonstrated the existence of memory T-cells towards allogeneic MSCs 4 weeks after MSC administration. Huang *et al*. demonstrated increased MHC class I and II expression after differentiation of allogeneic MSCs into myogenic, endothelial or smooth muscle type lineages resulting in increased cytotoxicity when cultured with allogeneic leukocytes. In the *in-vivo* experiments allogeneic MSCs were implanted into infarcted rat myocardium and these cells were eliminated within 5 weeks after implantation. Any functional benefits obtained from the treatment were lost within 5 months. They concluded that the long term ability of allogeneic MSCs to preserve function on the infarcted heart is limited due to the differentiation of the MSCs, from an immuno-privileged phenotype into an immunogenic state (Huang et al., 2010). Crop *et al.* demonstrated that allogeneic MSCs did stimulate proliferation of PBMCs after 7 days of co-culture (4 fold increase) but also showed that autologous MSCs stimulated a 3 fold increase in PBMC proliferation. Interestingly, in these experiments, MSC were removed after 7 days of culture

and the PBMC cultures that were maintained demonstrated an increase in proliferation (up to 25 fold) that was not observed if MSCs remained in the cultures with PBMCs for 14 days. PBMCs were cultured with MSCs at a 5:1 ratio (50,000:10,000) (Crop et al., 2010). Although this study is one of the first in extending the duration of the LTA past 7 days (up to 14) some stimulation of PBMCs was observed from day 0. However the lack of negative control data and the fact that the data was presented as the median results of four experiments make this study difficult to evaluate. As shown in Chapter 6, the specific responses of PBMCs to MSCs can vary between donor to donor, so a greater number of experiments should be performed to clarify the results obtained (Crop et al., 2010).

Interestingly, when PHDFs were co-cultured with PBMCs they produced significant stimulation, and the stimulation was comparable to that observed with MSCs. For some of the BM donor MSC the stimulation indices were higher than for fibroblasts when tested against the same PBMC donor. This may actually not be all that surprising as there have been reports of fibroblasts exhibiting similar characteristics to MSCs. Skin fibroblasts have been shown to be able to differentiate into bone, fat, and cartilage *in-vitro* and express similar phenotypic markers and also have similar effects on inhibiting the proliferation of stimulated PBMC (Haniffa et al., 2007; Cappellesso-Fleury et al., 2010; Blasi et al., 2011). It is starting to emerge that fibroblasts are more similar to MSCs than previously believed, and share many of the characteristics that were thought to be distinguishing features of MSCs (such as the trilineage potential, and immuno-regulatory properties) . The similarity between fibroblasts and MSCs may be dependent on the specific fibroblasts obtained; in this study fibroblasts did not demonstrate trilineage differentiation, but were overall less immunogenic than MSCs (albeit still stimulating PBMCs). It is difficult to distinguish between two cell types (fibroblasts and MSCs) as both cell types appear to demonstrate the same morphology and express the same cell surface markers. It is possible that there is a resident population of multipotent cells residing in the mesenchymal tissues with mature fibroblast cells, and that the methods used for the isolation and culture of “fibroblasts” favours selection of cells with the characteristics of MSC.

8.1 Future Work

Following on from the conclusions that undifferentiated MSCs stimulate PBMC and adipogenically differentiated MSCs also stimulate the proliferation of PBMCs, a number of unanswered questions regarding the immunogenicity of MSCs remain to be addressed. Future studies should address the mechanisms of interaction between MSC and PBMCs in short and long term cultures of undifferentiated and differentiated MSC. This could be carried out by

investigating cytokine secretion and gene expression by both the stimulatory cells and responder cells in the LTAs over a culture period of up to 21 days. Any changes in expression by undifferentiated MSCs of the co-stimulatory molecules, MHC class-I and II, or the secreted factors such as PGE_2 or IDO when undifferentiated MSCs are co-cultured in LTAs would aid in the determination if this plays any role in the stimulation of PBMCs observed.

It would also be of interest to determine the phenotype and function of the lymphocytes that respond to allogeneic MSC in LTAs. It will be important to determine whether the responding cells are CD4+, IFN-ɣ producing, CD 8+ cytotoxic cells or indeed $CD4^+CD25^+Foxp3^+$ regulatory T-cells.

Whether allogeneic MSCs are able to survive long term *in-vivo* needs to be addressed. Since it may not be possible to perform such studies in man, such studies should be carried out in a range of animal models. Currently there is little evidence to suggest that allogeneic MSC survive in the longer term. There are already clinical products that use allogeneic MSCs such as Prochymal™ and Osteocel™ but clinical data on these treatments is hard to find. Long term survival studies tracking MSCs *in-vivo* would offer more conclusive evidence of whether MSCs are immuno-privileged or eliminated by the immune system.

It will also be important to determine whether the repeated treatment of patients with allogeneic MSC leads to a diminuation of therapeutic effect. If allogeneic MSC are immunogenic *in-vivo*, then repeated exposure of a given patient to a range of alloantigens expressed by therapeutic MSC would theoretically lead the induction of alloantigen specific memory T-cells. Hence, the therapeutic effects of allogeneic MSC could be ablated.

8.2 FINAL WORDS

MSCs do stimulate allogeneic PBMCs and are not therefore immune to the attentions of cells of the immune system. They are not strongly immunogenic however and therefore when placed into non-inflammatory environments they may be able to 'remain hidden' and avoid immediate detection and elimination by the immune system. Low (not negative) expression of co-stimulatory molecules and HLA-DR with positive expression of HLA-ABC could account for the lack of initial immune response and explain why it is currently believed that allogeneic MSCs are immuno-privileged. MSCs are able to produce anti-inflammatory cytokines and factors that decrease the inflammatory response (IDO, PGE2). In addition to this, the MSCs are able to affect the maturation of immune cells such as DCs and T-cells and stimulate the production of a regulatory phenotype of T-cells. The use therefore of allogeneic MSCs in the traditional engineering setting may be limited and a very cautious approach should be taken

before allogeneic MSCs are used to aid in the regeneration or creation of tissue replacements. Where allogeneic MSCs do appear to currently excel is as a transient anti-inflammatory treatment (such as in the treatment of GvHD or Crohns disease), or to aid in the reduction of inflammation (such as to aid engraftment of HSCs after transplantation).

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