Immunogenicity of allogeneic mouse mesenchymal stem cells (MSC)

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

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ABSTRACT

Adult mesenchymal stem cells (MSC) are multipotential cells which can differentiate into various cell types thus giving them utility in tissue engineering regenerative medicine and cell-based therapies. Use of allogeneic MSC offers the prospect of "off-the-shelf" tissue engineered products and tailor-made "designer" therapies with huge benefits to patients and industry. However, their immunological properties are poorly defined and this has hampered their potential clinical utility. Recent studies have suggested that allogeneic MSC are immunoprivileged in addition to possessing immunosuppressive properties. Interestingly, dermal fibroblasts (DF) have also been suggested to be functionally similar to MSC although their immunological properties are controversial.

This study sought to systematically investigate the immunomodulatory properties of allogeneic MSC, namely (i) immunosuppression and (ii) (immunogenicity of allogeneic MSC before and after differentiation using an allogeneic mouse model which employed two genetically distinct strains; Balb/c (H2 d) and C3H (H2 k) which are used as responder (recipient) and stimulator (donor) respectively. Immunosuppressive properties were investigated using adaptations of the one-way mixed lymphocyte reaction (MLR) while the immunogenicity was tested using the lymphocyte transformation assays (LTA). DF were similarly tested for comparison.

MSC and DF were successfully isolated from the bone marrow and abdominal skin respectively of Balb/c and C3H mice, expanded and characterised by flow cytometry. MSC expressed MHC I, Sca-1, CD29, CD44, CD90.2 and CD105 but not MHC II, CD11b, CD34, CD45, CD80 and CD86. In contrast to MSC, DF were negative for CD29, CD44 and CD105. Both MSC and DF successfully differentiated into adipocytes, chondrocytes and osteocytes in the tri-lineage test; the benchmark test for stem cells.

Various parameters including medium changes, cell viability following mitotic inactivation of stimulator cells, type and concentration of serum for medium supplementation, responder to stimulator cell ratio and total cell numbers were tested to determine appropriate conditions for carrying out the MLR and LTA.

With regard to the immunosuppressive properties, both syngeneic and allogeneic MSC significantly suppressed one-way MLR and two-way MLR. DF also exhibited similar suppressive potency. In LTA, allogeneic, but not syngeneic MSC stimulated Balb/c lymphocyte proliferation. Interestingly, both syngeneic and allogeneic DF failed to stimulate lymphocyte proliferation.

Following chondrogenic differentiation, both syngeneic and allogeneic MSC and DF suppressed one-way MLR, albeit with reduced potency. With regard to their immunogenicity, allogeneic MSC and DF, but not syngeneic MSC and DF significantly stimulated lymphocyte proliferation.

Therefore, it was concluded that both allogeneic MSC and DF possess immunosuppressive properties before and after differentiation. Undifferentiated and differentiated allogeneic MSC and differentiated DF however, may not be immunoprivileged. Thus the clinical utility of allogeneic MSC may be limited by their immunogenicity.

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ABBREVIATIONS

^{3}H-thymidine	tritiated thymidine
7-AAD	7 aminoactimomycin D
ACT	Advanced cell therapies
ADM	adipogenic differentiation medium
AMM	adipogenic maintenance medium
ANOVA	analysis of variance
APC	antigen presenting cells
ASC	adult stem cells
ATP	adenosine triphosphate
BMP2	bone morphogenetic protein-2
BMSSC	bone marrow stromal stem cells
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CD40L	CD40 ligand
CDM	chondrogenic differentiation medium
CFSE	5(6)-carboxyfluorescein diacetate succinimidyl ester
CFU-F	colony forming units- fibroblasts
CL	confidence limit
CO_2	carbon dioxide
Con-A	Concanavalin-A
COX	cyclooxygenase
CTL	cytotoxic T-lymphocytes
CTLA-4	cytotoxic T-lymphocyte antigen 4
d.f.	dilution factor
DC	dendritic cells
DMEM-HG	Dulbecco's modified Eagles medium high glucose
DMEM-LG	Dulbecco's modified Eagles medium low glucose
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ESC	embryonic stem cells
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothyocyanate
GAGs	glycosaminoglycans
GDF	growth and differentiation factor
GvHD	graft versus host disease
GvT	graft versus tumour
HBSS	Hanks's balanced salt solution
HDD	high determinant density
HEPES	[N-(2-hydroxyethyl) piperazine N;-(2-ethansulphonic acid)]
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HSC	haematopoietic stem cells
ICM	inner cell mass
IDO	indoleamine-2,3-dioxygenase
IFNγ	interferon gamma
Ig	immunoglobulin
IMDM	Iscove's modified Dulbecco's medium
IPS	induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
ITAM	immunoreceptor tyrosine-based activation motif
ITS	insulin, transferrin and selenium
IUIS	International Union of Immunological Societies
LFA-1	lymphocyte function-associated antigen 1
LL	lower limit
LSC	liquid scintillation counting
LTA	lymphocyte transformation/proliferation assay
MAPC	multipotential adult progenitor cells
MAPK	mitogen-activated protein kinase
MBC	multiple binary complex
mH	minor histocompatibility antigens
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMSC	multipotential mesenchymal stromal cells
MPC	mesenchymal progenitor cells
mRNA	messenger ribonucleic acid
MS	mouse serum
MSC	mesenchymal stem cells
MSD	minimum significant difference
NBF	neutral buffered formalin
NK	natural killer cells
NO	nitric oxide
ODM	osteogenic differentiation medium
ORO	oil red O
PAM	plastic adherence method
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD-1	programmed death-1
PE	phycoerythrin
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PPARγ	peroxisome proliferator-activated receptor gamma
PRR	pattern recognition receptors
PTK	protein tyrosine kinase
RPE	retinal pigment epithelial cells
RPMI 1640	Rosslyn Park Memorial Institute 1640
Runx2	Runt-related transcriptional factor-2
SBP	statistical biological positive
SCID	severe combined immunodeficiency disease
SCNR	somatic cell nuclear reprogramming
SCNT	somatic cell nuclear transfer
SKP	skin precursor cells

TCA	trichloroacetic acid
TCR	T-cell receptors
TGF	transforming growth factor
TGFβ	transforming growth factor beta
TLR	Toll-like receptors
TNFα	tumour necrosis factor alpha
T-regs	Regulatory T cells
tRNA	transfer ribonucleic acid
UL	upper limit
USSC	unrestricted somatic stem cells
VEGF	vascular endothelial growth factor
SE	standard error

SYMBOLS AND UNITS

%	percentage
ºC	degrees Celsius
Ca^{2+}	Calcium
CDT	cell doubling time
CDU	collagenase digestion units
$Ci.mmol^{-1}$	Curie per millimolar
cm^2	square centimetre
cm^3	cubic centimetre
CPM	counts per minute
CPS	counts per second
$g.L^{-1}$	grams per litre
kDa	kilodaltons
L	litre
Log_{10}	logarithm of a number raised to base 10
M	molar
Mg^{2+}	Magnesium
ml	mililitre
mm	milimitre
mM	millimolar
nm	nanometre
nM	nanomolar
psi	pounds per square inch
SI	stimulation index / indices
v/v	volume per volume
w/v	weight per volume
Δ	delta
β	beta
γ	gamma
µCi	micro Curie
$\mu Ci.ml^{-1}$	micro Curie per millilitre
µl	microlitre
µm	micrometre
µM	micromolar
g	gravity
mm^3	cubic millimitre
$g.ml^{-1}$	grams per millilitre
rpm	rotations per minute

1. CHAPTER ONE: INTRODUCTION

1.1. Stem cells

Stem cells are broadly defined as clonogenic cells that can undergo self-renewal and differentiation along multiple lineage pathways capable of forming functional tissues. The existence of stem cells has been known for a long time as cells associated with tissue repair. They are classified into two broad categories namely embryonic and adult according to the source of tissue from which they are derived. Also, stem cells can be classified according to their differentiation hierarchy as totipotent, pluripotent, multipotent and unipotent.

Totipotent stem cells are cells capable of differentiating into any cell type. They are derived from fertilised oocytes within the first three days of zygote formation at the 8-cell stage after which they lose some of their differentiation capacity such as the ability to form extra-embryonic tissue. After loss of totipotency, these embryonic cells develop into cells that form the inner cell mass (ICM) of the growing blastocyst which goes on to form the foetus and the outer extra-embryonic tissue (trophoblast) which form supporting structures such as the placenta and yolk sac (Ringe *et al.*, 2002; Tesar *et al.*, 2007; Choumerianou *et al.*, 2008).

Pluripotent stem cells possess the capacity to differentiate into any cell of the three-germ layers namely the ectoderm, mesoderm and endoderm but are not able to form cells of the trophoblast. They are cells derived from the ICM of the embryo within 5-14 days post fertilisation and are therefore known as embryonic stem cells (ESC). Further differentiation of the ESCs in the maturing foetus gives rise to specialised tissues and organs; the endoderm forming the inner layers such as the gastrointestinal and respiratory tracts, endocrine organs, urinary tract and the auditory system while ectodermal development gives rise to the outer layers such as the skin and the nervous system. The mesoderm layer develops into cells and tissues of the middle layer (mesenchyme) such as the bone, fat, cartilage, skeletal and smooth muscles (Roobrouck *et al.*, 2008).

Adult mesenchymal tissues harbour multipotential stem cells commonly known as mesenchymal stem cells (MSC) whose differentiation potential is limited to the

formation of mesodermal cell lineages typically adipocytes, chondrocytes and osteoblasts. These cells were initially identified in the bone marrow but are now known to be distributed in other mesenchymal tissues. Many adult tissues are now known to be sources of multipotent stem cells that usually bear the name of the tissue from which they are derived from such as adipose-derived stem cells obtained from fat tissue and neural stem cells derived from brain tissue (Dazzi & Horwood, 2007; Choumerianou *et al.*, 2008).

Unipotent stem cells, commonly known as precursor cells, are cells derived from specific tissues with the ability to differentiate into a single cell type of that tissue such as smooth muscle precursor cells that generate smooth muscle tissue. Generally, the description of precursor cells as 'stem cells' is controversial since the latter term has come to describe undifferentiated cells that at least display multipotency. Precursor cells however are differentiated but do display clonogenic properties which make them useful in tissue repair (Körbling & Estrov, 2003).

1.1.1 Pluripotent stem cells

Pluripotent stem cells generally refer to naturally occurring ESC derived from developing embryos. Lately, adult cells genetically manipulated to become pluripotent, known as induced-pluripotent stem (iPS) cells, also belong to this category.

1.1.1.1. Embryonic stem cells (ESC)

ESC are derived from the *in vitro* culture of cells derived from the ICM of vertebrate blastocysts. They were initially isolated from mouse embryos in 1981 (Evans & Kaufman, 1981; Martin, 1981) and almost nearly two-decades later from donated pre-implantation human embryos obtained from *in vitro* fertilisation procedures (Thomson *et al.*, 1998). At this time, a sudden surge in interest into their potential application in cell replacement therapies and modelling of human development and disease ensued. This was in light of earlier evidence that mouse ESC injected into host blastocyst contributed to the development of all the tissues of the three-germ layers of the adult mice (Beddington & Robertson, 1989; Nagy *et al.*, 1990).

The standard test that demonstrates ESC pluripotency is the ability to form well differentiated teratoma-like masses after subcutaneous, intramuscular or

intratesticular injection into immunodeficient adult mice (Lensch *et al.*, 2007). Suspension cultures of human or mouse ESC generate spontaneous cystic cell aggregates called embryoid bodies which contain cells of the three germ layers (Muller & Lengerke, 2009).

Despite the significant potential of ESC for providing cell-based therapies, clinical application has been hampered by ethical and religious concerns from the wider society and only recently, two on-going clinical trials using human ESC-derived retinal pigment epithelial (RPE) cells have been initiated by Advanced Cell Therapies (ACT), an American company. In one of the studies for the treatment of dry-age related muscular degeneration, these cells were first shown to stave-off and even reverse disease progression in animal models. The first results from the phase I-II trials suggested that the cells had not caused any adverse immunological effects and appeared to improve vision four months post transplantation (Schwartz *et al.*, 2012). The other study is the first UK-sanctioned ESC-clinical trial which commenced in November 2011 at Moorfields Eye hospital in London and uses ACT's RPE for the treatment of patients with Stargardt's muscular dystrophy which causes blindness. Although promising, these trials are still at phase I-II and their efficacy remains under investigation. Another clinical trial by an American company Geron, which used ESC-derived oligodendrocyte progenitor cells to restore spinal cord function in patients with spinal cord injuries was discontinued in November 2011 despite having been previously reported to be promising in preliminary studies (http://www.eurostemcell.org/story/clinical-trials-news-january-2012-update).

1.1.1.2. Induced-pluripotent stem cells (iPS)

iPS are defined as adult somatic cells that have been genetically re-programmed to an embryonic stem cell-like state by forced expression of genes and factors that are thought to be responsible for maintaining pluripotency (Jaenisch & Young, 2008; Nishikawa *et al.*, 2008). Whether iPS and ESC differ in any clinically significant way remains unknown but the development of this type of stem cells was born out of the need to generate genetically equivalent patient-specific (isogenic) cells for use in cell therapies without the risk of rejection which would otherwise necessitate use of immunosuppressive therapy and histocompatibility testing (Muller & Lengerke, 2009).

There are several methods for achieving genetic re-programming of mature cells namely somatic cell nuclear transfer (SCNT), direct somatic cell nuclear reprogramming (SCNR), parthenogenesis and cell fusion

1.1.1.2.1. iPS by somatic cell nuclear transfer (SCNT)

The process of SCNT or therapeutic cloning involves the transfer of a somatic cell-derived nucleus into an enucleated oocyte to generate pluripotent cells that are genetically matched to the nucleus donor. The idea that the contents of an enucleated oocyte contain factors that are sufficiently capable of directing development of a complete organism was pioneered in the 1950s and 1960s and demonstrated in frogs (Briggs & King, 1952; Gurdon, 1962). This approach was later used in mammalian oocytes accompanied with nuclear transfer from somatic cells with the resulting artificial zygotes cultured under growth supporting conditions. Cell division and blastocyst formation ensued and whole organisms were produced following uterine transplantation This approach has been successfully used in many species including mouse, bovine and rabbit models (Cibelli, 2007) to produce fully developed offspring that are able to mature to adulthood as was demonstrated by 'Dolly' the famous sheep in 1996 (Wilmut *et al.*, 2007). Notwithstanding the ethical issues surrounding application of this technology to humans, the difficulty of obtaining viable human oocytes coupled to the low efficiency of the procedure has meant that SCNT is unlikely to see the light of day in the clinic.

1.1.1.2.2. iPS by direct somatic cell nuclear reprogramming (SCNR)

To overcome the problems associated with SCNT, another method of generating pluripotent cells using somatic cells was pioneered by Yamanaka and co-workers in 2006 that generated mouse (Takahashi & Yamanaka, 2006) and human (Takahashi *et al.*, 2007) iPS by retrovirus-mediated transfection of four key pluripotent transcription factor genes (*Oct-3/4*, *Sox2*, *Klf4* and *c-Myc*) into fibroblasts to genetically re-programme the cells to a pluripotent state. The cells resembled ESC in morphology, surface antigen and gene expression, epigenetic status of pluripotent-specific genes and telomerase activity. The mechanism by which the trans-acting factors induced pluripotency remains unknown but it is widely thought that *Oct3/4* and *Sox2* synergistically upregulate 'stemness' genes while suppressing

differentiation associated genes while *c-Myc* and *Klf4* are thought to modify chromatin structure to allow binding of *Sox2* and *Oct-3/4* to their targets (Ramalho-Santos *et al.*, 2002; Takahashi & Yamanaka, 2006). Although *Oct4* seems to be important, *Sox2* and *c-Myc* were shown to be replaceable if specific cells such as neural stem cells that constitutively express high levels of *Sox2* and *c-Myc* were used (Kim *et al.*, 2008).

The greatest disadvantage of this technology comes from its use of virus-mediated gene delivery. Risks such as random insertional mutagenesis, incomplete silencing, oncogene activation by *Oct4* and activation of viral transgenes cannot be overlooked and may limit their clinical application despite the use of mechanisms such as *de novo* methylation that silence viral genes. iPS derived from some mice have been shown to have a high propensity to develop tumours. New strategies that are being employed in order to avert these risks include addition of the reprogramming proteins themselves (protein transduction); discovery of small molecules that are able to activate expression of reprogramming factors and so improve the efficiency of the reprogramming process and use of non-integrating vectors that express known reprogramming factors (Muller & Lengerke, 2009).

1.1.1.2.3. Parthenogenesis

Parthenogenesis is a form of asexual reproduction which refers to the growth and development of an embryo without fertilization. In the context of pluripotent stem cells, direct activation of oocytes is potentially more efficient compared to SCNT. This can be achieved by activation of oocytes with chemicals such as alcohol, ionomycin, 6-dimethylaminopurine and cycloheximide in addition to physical stimuli such as electrical shock and low temperature (Muller & Lengerke, 2009). This method was successfully used to generate ESC in various mammalian species including humans (Kim *et al.*, 2007) and mice (Revazova *et al.*, 2007). Parthenogenic ESC can differentiate into cells of all the three germ layers and are capable of forming teratomas *in vitro* and *in vivo* but demonstrate high levels of chimerism which suggests that additional selection pressures are imposed on the cells (Lengerke *et al.*, 2007). Since these cells are genetically similar but not identical to the oocyte donor, they are a less attractive option for clinical application than other

iPS. Another major drawback of these cells is that they are only available for women who are able to donate fresh oocytes (Muller & Lengerke, 2009).

1.1.1.2.4. Cell fusion

Another technique designed to be independent of oocyte or zygote donations uses the reprogramming capacity of embryonic carcinoma cells by fusing them with somatic cells to form hybrid cells capable of forming teratomas while expressing an embryonic identity such as transcriptional factors, surface antigens and enzymes. Such cells however are tetraploid, genetically unstable and immunologically incompatible with the donor (Muller & Lengerke, 2009). Without further modifications to reduce or silence the embryonic chromosomes (Matsumura *et al.*, 2006), it is unlikely that such technology will be clinically applicable.

1.2. Adult stem cells (ASC)

Adult stem cells (ASC) are defined as undifferentiated cells found among differentiated cells in tissues and organs that can self-renew and differentiate to yield some or all of the major specialized cell types of the tissue or organ. Their primary role is to maintain and repair the tissue from which they are derived (Pittenger *et al.*, 1999; Ringe *et al.*, 2002). Usually, they are named according to the tissue source/niches. ASC that are resident in ectodermal tissues include neural, skin and ocular stem cells. Endodermal derived stem cells include pulmonary epithelial, pancreatic and urogenital stem cells. The most popular group of ASC are those derived from mesodermal tissues which include mesenchymal stem cells (MSC) and haematopoietic stem cells (HSC) (Körbling & Estrov, 2003; Mimeault & Batra, 2006). Bone marrow-derived MSC are the focus of the work presented in this study and will be discussed in detail in **Section 1.3**. The bone marrow is known to harbour two distinct stem cell lineages namely HSC and MSC (Koide *et al.*, 2007).

1.2.1. Haematopoietic stem cells (HSC)

HSC are multipotent stem cells which constitute up to 0.5 % of bone marrow cells and are the precursor cells for all the myeloid (monocytes and macrophages, dendritic cells [DC], neutrophils, basophils, erythrocytes, eosinophils and platelets) and lymphoid (B-cells, T-cells and natural killer [NK] cells) blood cells (Gunsilius *et al.*, 2001). They were the first type of stem cell to be discovered and are the best

characterised of all stem cells. Though they are found primarily in the bone marrow of major bones such as femurs, sternum, ribs and hips, they also circulate in peripheral blood where they replenish missing or damaged components of the haematopoietic and immunologic systems (Trigg, 2004).

Morphologically, HSC resemble lymphocytes, with a rounded nucleus and a low nucleus to cytoplasm ratio. They can be isolated by a variety of techniques including density centrifugation and surface antigen expression. To date, there are no specific characteristics that can uniquely identify them. HSC do not express most of the surface antigens expressed by their terminally differentiated progeny thus specific removal of such cells leaves a cell suspension of predominantly HSC (Bonnet, 2003).

Expression of cluster of differentiation (CD) 34 has been widely used as a distinguishing marker for HSC since it is down-regulated during HSC differentiation although it has been later found in non-haematopoietic fibroblasts and vascular endothelial cells. The most commonly used markers aside from CD34 include CD45, CD38, CD90, CD105, CD117 and CD133. Negative selection for CD13 and CD133 distinguishes myeloid cells, $CD4^+$ and $CD8^+$ distinguishes T-lymphocytes and B-lymphocytes respectively and CD71 shows erythroid cells (Gunsilius *et al.*, 2001; Bonnet, 2003). Clinical application of HSC includes treatment of leukaemia, aplastic anaemia, haematological cancers, thalassemias, severe combined immunodeficiency disease (SCID) and graft versus tumour treatment of cancer as well as graft versus host disease (GvHD) which can be achieved by allogeneic HSC transplantation (Gunsilius *et al.*, 2001; Kondo *et al.*, 2003).

1.3. Mesenchymal stem cells (MSC)

1.3.1. Historical background

Before the phrase ‘stem cell’ was used, scientists as early as the early 19^{th} century were familiar with the concept of tissue regeneration and self-repair as demonstrated by phenomena such as bone callus formation after fracture and formation of bone rudiments using autologous marrow at heterotopic sites in mice (Goujon, 1869), repair of blastema generated in amputated amphibians (Hay & Fischman, 1961) and mechanisms leading to wound healing (Marchand, 1901). Though in most cases the former experiments were carried out using bone-free fragments of marrow, the

specific identities of the cells which acted as progenitors for mature bone and therefore non-haematopoietic cells, could not be precisely and accurately described. However, it had been suggested earlier in 1867 by Cohnheim that the bone marrow-derived cells that played a role in facilitating wound repair were non-haematopoietic and had fibroblast-like morphology (Fehrer & Lepperdinger, 2005; Kode *et al.*, 2009). Thus, the hypothesis that 'stem-like' cells took part in normal tissue repair was widely tested in the mid-20th century and provided further insights about the role of bone marrow stromal cells.

However, it was not until after a series of experiments by Friedenstein and co-workers between 1966 and 1976 that the population of bone marrow-derived, clonal, plastic-adherent cells capable of undergoing differentiation into osteoblasts, adipocytes and chondrocytes was defined as 'bone marrow stromal stem cells' (BMSSC) or osteogenic stem cells (Friedenstein *et al.*, 1966; Friedenstein *et al.*, 1970a; Friedenstein & Kuralesova, 1971; Owen & Friedenstein, 1988; Friedenstein, 1990). Because of their fibroblast-like appearance and strong affinity to adhere to tissue culture vessel plastic, these cells were easily distinguishable from the haematopoietic majority. Their clonal nature was determined when cell suspensions seeded at clonal density produced distinct single colonies which were called 'colony-forming units fibroblasts (CFU-F) (Friedenstein, 1970).

When the stem cell niche hypothesis was postulated by Schofield in 1978 it brought to the fore the concept that stem cell self-renewal and differentiation were tightly controlled by their microenvironment and supporting cells (Schofield, 1978). At the time, much focus was on experimental haematology and the bone marrow was therefore widely regarded as a niche for HSC while other cells of the bone marrow determined their behaviour. This opened a new avenue which explored the functional relationships between HSC and the cellular components of the bone marrow and led to the suggestion that a 'second type' of stem cell was present in the haematopoiesis-supporting stroma of bone marrow. The earlier pioneering work by Friedenstein on BMSSC supported this concept albeit with the 'second type' of stem cell being non-haematopoietic. After the successful isolation of ESC from mouse embryos (Evans & Kaufman, 1981; Martin, 1981) and later from human embryos (Thomson *et al.*, 1998), the term 'mesenchymal' replaced 'stromal' or 'osteogenic' to describe the

non-haematopoietic stem cells from bone marrow and hence the current term ‘mesenchymal stem cells (MSC) was born (Caplan, 1991; Pittenger *et al.*, 1999).

1.3.2. Other sources of multipotent stem cells

Bone marrow-derived MSC belong to a wider group of ASC broadly defined as plastic-adherent, non-haematopoietic cells possessing self-renewal properties and the capacity to differentiate *in vitro* into adipocytes, chondrocytes and osteoblasts (Caplan & Bruder, 2001). Despite their demonstrated ability for differentiating into typical mesodermal cell lineages, trans-differentiation and differentiation into non-mesenchymal phenotypes including neuron-like cells (Keilhoff *et al.*, 2006; Choong *et al.*, 2007), astrocytes (Kopen *et al.*, 1999), myocardium (Pittenger & Martin, 2004), endothelial cells (Oswald *et al.*, 2004) and keratinocytes (Sasaki *et al.*, 2008) has been reported as shown in **Figure 1.1**. However, controversy remains as to whether the observed phenotypic plasticity represented functional trans-differentiation.

Though initially isolated from mouse bone marrow (Friedenstein *et al.*, 1976), which for some time was widely thought to be the most abundant source, MSC have since been shown to reside in virtually all adult mouse organs and tissues (da Silva Meirelles *et al.*, 2006). This universal distribution of MSC is widely thought to be necessitated by the need for maintenance of integrity and repair of the tissues from which they are derived from. In humans, several MSC niches have been identified including peripheral and cord blood (Erices *et al.*, 2000; Tondreau *et al.*, 2005; Goldberg *et al.*, 2007), amniotic fluid (Tsai *et al.*, 2004), placenta (Fukuchi *et al.*, 2004), dental pulp (Gronthos *et al.*, 2000), adipose tissue (Zuk *et al.*, 2002), brain (Galli *et al.*, 2003), hair follicles (Yu *et al.*, 2006) and synovial fluid (Fan *et al.*, 2009). However, bone marrow-derived MSC remain to date the better understood and commonly used type of ASC.

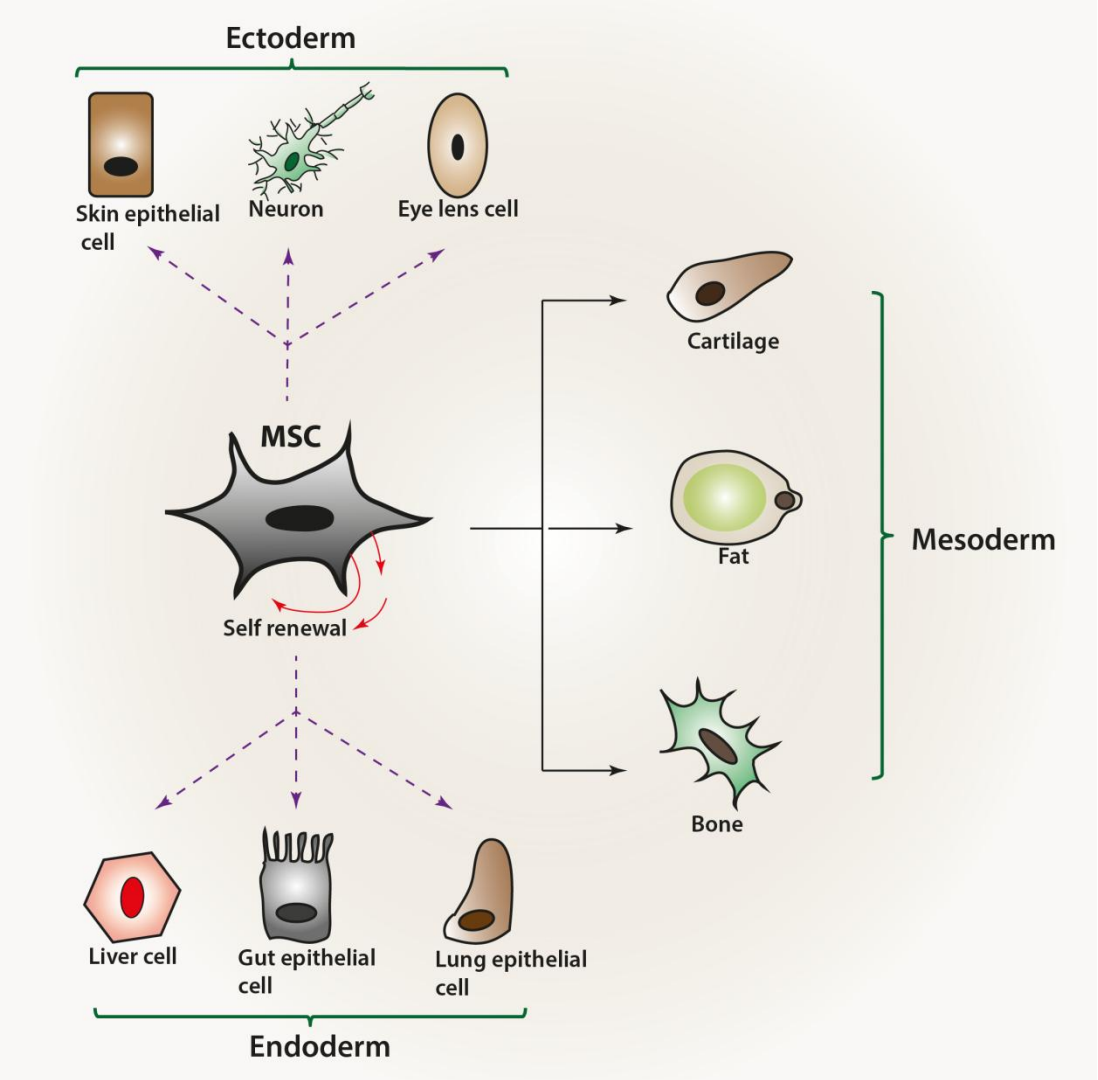

Figure 1.1. Multipotency of MSC. The self-renewal ability of MSC (red arrows) allows them to divide continuously and differentiate into various typical mesodermal cell lineages (solid arrows). *In vitro* trans-differentiation into ectodermal and endodermal cell lineages has been reported (dashed arrows).

1.3.3. MSC characterisation

1.3.3.1. Nomenclature

To date, the defining characteristics of MSC are inconsistent among different laboratories. Variations in isolation methodologies, species, tissue sources, culture media, and culture conditions have resulted in subtle and in some cases obvious differences in the features of the resultant cells. This has made it difficult for direct comparisons on many biological properties to be made for MSC obtained from different research groups. Names such as mesenchymal progenitor cells (MPC) (Sun

et al., 2003), multipotential adult progenitor cells (MAPC) (Jiang *et al.*, 2002b), BMSSC (Bianco *et al.*, 2001) have all been used to refer to MSC and this has led to some confusion regarding their definition. Objections about the inappropriateness of the word 'mesenchymal' have been raised primarily because it was thought that other non-skeletal mesenchymal tissues such as myocardium, smooth muscle and tendon could be generated by MSC but there is no *in vivo* evidence to date to support this notion (Horwitz *et al.*, 2005; Dominici *et al.*, 2006). Furthermore, it is known that many tissue cells previously thought to be derived from MSC have since been shown to originate from different progenitors as evidenced by the presence of different tissue-specific stem cells. However, the name MSC has gained universal acceptance though not precisely accurate in its description of the cells. Other scientific bodies such as the International Society for Cellular Therapy (ISCT) have developed their own criteria and nomenclature for defining human MSC naming them as multipotential mesenchymal stromal cells (MMSC) rather than 'stem cells' with the inclusion of the tissue source from which the cells were derived (Horwitz *et al.*, 2005). The minimum requirements set for human cells to be classified as MMSC by the ISCT (Dominici *et al.*, 2006) are;

- Plastic adherence when maintained under standard culture conditions in tissue culture flasks.
- Expression of specific cell surface antigens (≥ 95%) including CD73, CD90, and CD105 and negative for (≤ 2%) CD34, CD45, CD11b, CD19 and human leukocyte antigen DR (HLA DR) as measured by flow cytometry.
- Demonstration of multipotential differentiation capacity into adipocytes, osteocytes and chondrocytes by *in vitro* staining of cultures.

Though these criteria were set for human derived cells, other mammalian MSC including murine have met these definitions but are still commonly referred to as mesenchymal 'stem cells' rather than 'stromal cells'.

1.3.3.2. Abundance

Current models suggest that postnatal bone marrow, in addition to a variety of other stromal cells, houses MSC and HSC (Koide *et al.*, 2007). MSC have been reported to constitute a small fraction of bone marrow nucleated cells, approximately 1 in 10^6

cells in mice (Sung *et al.*, 2008) and between 0.001 and 0.01 % in humans (Pittenger *et al.*, 1999).

1.3.3.3. Isolation of MSC from bone marrow

The most commonly used approach for obtaining MSC from animal donors such as mouse involves harvesting by flushing the bone marrow with culture medium to remove marrow plugs from compact bone followed by isolation which exploits their physical propensity to adhere to the plastic substrate of cell culture plates. This method is generally known as the plastic adherence method [PAM] (Friedenstein *et al.*, 1970a; Phinney *et al.*, 1999). The non-adherent cells are progressively removed from the cultures during successive medium changes until fibroblast-like cells become prominent in the culture. This is based on the premise that differentiated cells as well as HSC lack longevity in culture under these conditions and are progressively lost with time. This technique has been reported to generate poor yields of mouse MSC as well as being time consuming since cells have to be maintained in culture for a long time and this could potentially affect their biological properties. Studies in both mice and humans have shown that MSC are located in the endosteum or compact bone. This deep location makes it difficult to harvest them even after strong flushing. The heterogeneous cell suspensions obtained from mouse bone marrow have a higher frequency of haematopoietic cells which also are plastic-adherent and therefore could hinder the adherence of MSC. These cells are also known to persist in culture after serial passages due to their ability to bind stromal cells via engagement with adhesion molecules, cytokine receptors and extra-cellular matrix (ECM) proteins even in the absence of exogenous growth factors or cytokines (Short *et al.*, 2001; Baddoo *et al.*, 2003). Most of the murine plastic-adherent stromal cells have been shown to exhibit differentiated phenotypes even in the absence of differentiation inducing culture conditions (Sun *et al.*, 2003). Therefore, cultures resulting from mouse marrow aspirates remain morphologically heterogeneous with cells ranging from narrow spindle-shaped to large polygonal and tightly packed cells (Park *et al.*, 2007c). Despite these disadvantages, the PAM has remained the most commonly used method because it does not involve treating the cells or processing them before plating which could potentially affect the subsequent behaviour of the cells.

1.3.3.4. Phenotypic markers

At present, there is no specific marker or combination of markers that uniquely defines MSC. However, *ex vivo* expanded human and murine MSC have been shown in many studies to commonly and consistently express antigens such as Sca-1, CD29, CD44, CD73, CD90, CD105, CD106 but are devoid of haematopoietic markers CD34 and CD45 or endothelial and macrophage markers CD11b, CD14 and CD31 (Pittenger *et al.*, 1999; Sung *et al.*, 2008). Surface expression of major histocompatibility complex class I (MHC I) in mice or HLA A, B and C in humans has been best described as low, intermediate or partial but MHC II and HLA DP, DQ and DR are completely absent (Le Blanc *et al.*, 2003b; Soleimani & Nadri, 2009). Importantly, both human and murine MSC do not express the immunological co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), CD40 or it ligand CD40L. The commonly used markers for the identification of MSC are listed in **Table 1.1**.

It is noteworthy to mention that there are variations in terms of the actual percentages of cells expressing the same markers by different researchers largely due to differences in MSC tissue source, isolation and expansion methodologies, passage number and donor age and sex. It has been noted that CD45 and CD14 expression by human MSC more than quadrupled between paediatric and older donors though there is currently not enough evidence to support this (Rojewski *et al.*, 2008). It remains unclear how different isolation techniques could affect expression of certain markers but it is well known that manipulation of MSC could result in the up or down-regulation of specific markers. The age and passage number of MSC are important considerations in the characterisation of MSC. It had been shown that senescent MSC expressed normal levels of CD29, CD44, CD90, CD105 and CD166 despite having reduced differentiation capacity. In addition, prolonged culture could lead to down-regulation of certain markers as a result of age, plastic-adherence, cell to cell contact, exposure to xenogeneic growth factors such as foetal calf serum (FCS) used in culture medium and enzymatic manipulation resulting from use of proteolytic cell dissociation solutions (such as trypsin) commonly used during cell passage (Rojewski *et al.*, 2008).

Table 1.1. Common surface markers used in the characterisation of mouse and human MSC

Marker	Mouse	Human	Function	References
Sca-1 (Ly-6A/E)	+		Sca-1 is a member of the Ly-6 antigen family and is the most common MSC marker in mice with both Ly-6 haplotypes. Sca-1 has also been discovered in several non-haematopoietic tissues and can be used to enrich progenitor cell populations other than MSC. It is thought to be involved in regulating both B-and T- cell activation	Baddoo *et al.* (2003); da Silva Meirelles & Nardi (2003); Koide *et al.* (2007); Sung *et al,* (2008); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
MHC I	+/-	+	MHC I molecules are constitutively expressed by all nucleated cells and their function is to bind intracellular peptides for display to cytotoxic $CD8^+$ T-cells. They also function as inhibitory ligands for NK cells.	Jiang *et al.* (2002b); Le Blanc *et al.* (2004a); Jones *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
MHC II	-	-	Expressed only on APC and lymphocytes, displays extracellular antigens to $CD4^+$ T-cells.	Jiang *et al.* (2002b); Le Blanc *et al.* (2004a); Jones *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
CD29	+	+	Membrane receptor associated with cell adhesion and recognition in processes such as tissue repair, immune response and homeostasis.	Baddoo *et al.* (2003); da Silva Meirelles & Nardi (2003); Sun *et al.* (2003); Le Blanc *et al.* (2004a); Chamberlain *et al.* (2007); Sung *et al.* (2008)
CD44	+/-	+	A type of cell adhesion molecule used to identify several types of mesenchymal cells including MSC and HSC. Functions as a bone homing receptor for HSC and MSC migration to the bone marrow. Its expression in mouse MSC has been found to be variable among studies.	Jiang *et al.* (2002b); Baddoo *et al.* (2003); da Silva Meirelles & Nardi (2003); Sun *et al.* (2003); Le Blanc *et al.* (2004a); Chamberlain *et al.* (2007); Sung *et al.* (2008); Soleimani & Nadri (2009)
CD73 (NT5E)	+	+	It is an enzyme that hydrolyses extracellular nucleotides into membrane permeable nucleosides. It is normally used as a marker of lymphocyte differentiation	Chamberlain *et al.* (2007); Jones *et al.* (2007); Schurgers *et al.* (2010)
CD90 (Thy-1)	-/+	+	It is a cell surface protein with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen. Generally used as a marker for a variety of stem cells including MSC and for the axonal processes of mature neurons. Its roles are thought to involve cell-cell and cell-matrix interactions, with implication in neurite outgrowth, nerve regeneration, apoptosis, metastasis, inflammation, and fibrosis.	Baddoo *et al.* (2003); Chamberlain *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* 2010)
CD105 (Endoglin)	+/-	+	It is a type I membrane glycoprotein located on the cell surface of endothelial cells, activated macrophages, fibroblasts and smooth muscle cells as part of the	Sun *et al.* (2003); Le Blanc *et al.* (2004a); Jones *et al.* (2006); Chamberlain *et al.* (2007); Koide

			TGFβ receptor complex. Its main functions include cytoskeletal organisation, affecting cell morphology, migration and vascular remodelling.	*et al.* (2007); Sung *et al.* (2008); Schurgers *et al.* (2010)
CD106 (VCAM-1)	+/-	+	Mainly serves as an adhesion molecule. Mostly found in haematopoietic cells particularly white blood cells where it facilitates binding to the vascular endothelium.	Baddoo *et al.* (2003); Jones *et al.* (2006); Chamberlain *et al.* (2007); Jones *et al.* (2007); Soleimani & Nadri (2009)
CD 13	-	+	It is a transmembrane glycoprotein commonly expressed on granulocytes, myeloid progenitors, endothelial cells, epithelial cells and subset of granular lymphoid cells. It is thought to be involved in the metabolism of many regulatory peptides and functions in antigen processing and the cleavage of chemokines.	Jiang *et al.* (2002b); Jones *et al.* (2006)
CD31 (PECAM-1)	-	-	Commonly found on haematopoietic and endothelial cells and participates in angiogenesis, leukocyte migration, integrin activation and removal of aged neutrophils from the body.	Baddoo *et al.* (2003); Sun *et al.* (2003); Chamberlain *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
CD49e (ITGA5)	+	+	CD49e is an integral protein which binds with CD29 to form the receptor VLA5, which binds the ligands fibronectin and fibrinogen. Its expressed on multiple cell types including thymocytes, mast cells, activated T-cells and splenic B-cells. In addition to its role in adhesion, VLA5 contributes to T-cell co-stimulation.	da Silva Meirelles & Nardi (2003); Koide *et al.* (2007)
CD63	+	+	CD63 is a member of the transmembrane-4 superfamily and mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility and is also known to complex with integrins.	Jones *et al.* (2006)
CD166 (ALCAM)	+	+	A transmembrane glycoprotein of the immunoglobulin family which is commonly expressed on activated T-cells, activated monocytes, epithelial cells, fibroblasts, neurons and melanoma cells. CD166 plays an important role in mediating adhesion interactions between thymic epithelial cells and CD6 cells during intrathymic T-cell development and is now regarded as a marker for cancer stem cells.	Le Blanc *et al.* (2004a); Chamberlain *et al.* (2007); Jones *et al.* (2007)
CD86 (B7-2)	-	-	This protein is a member of the immunoglobulin superfamily commonly expressed by APC. It acts as a co-stimulatory ligand for CD28 (auto-regulation and intracellular association) and CTLA-4 (attenuation of regulation and cellular disassociation) on T-cells. It works in tandem with CD80 to prime T-cells.	Klyushnenkova *et al.* (2005); Jones *et al.* (2006); Chamberlain *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
CD40	-	-	CD40 is constitutively expressed by APC, including DC, B-cells and macrophages and in some cases endothelial cells, smooth muscle cells, fibroblasts and epithelial cells. It functions as a co-stimulatory signal required for their activation. The binding of CD154 (CD40L) on $CD4^+$ T-cells to CD40 activates the APC and induces a variety of downstream effects.	Klyushnenkova *et al.* (2005); Chamberlain *et al.* (2007); Soleimani & Nadri (2009)
CD80	-/+	+/-	It is a protein found on activated B-cells and monocytes that provides a co-	Klyushnenkova *et al.* (2005); Jones *et al.*

(B7-1)			stimulatory signal necessary for T-cell activation and survival. It is the ligand for CD28 (auto-regulation and intercellular association) and CTLA-4 (for attenuation of regulation and cellular disassociation). CD80 works in tandem with CD86 to prime T-cells. The activated protein induces T-cell proliferation and cytokine production.	(2006); Chamberlain *et al.* (2007); Jones *et al.* (2007); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
CD34	-/+	-	It is a cell surface glycoprotein and functions as a cell-cell adhesion factor. It may also mediate the attachment of stem cells to bone marrow ECM or directly to stromal cells. Generally regarded as a marker for distinguishing MSC from HSC.	Baddoo *et al.* (2003); Sun *et al.* (2003); Le Blanc *et al.* (2004a); Chamberlain *et al.* (2007); Koide *et al.* (2007); Sung *et al.* (2008); Soleimani & Nadri (2009)
CD45	-	-	A member of the protein tyrosine phosphatase (PTP) family which acts as a signalling molecule that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. Also regarded as a marker for distinguishing MSC from HSC.	Jiang *et al.* (2002b); Sun *et al.* (2003); Tropel *et al.* (2004); Le Blanc *et al.* (2004a); Chamberlain *et al.* (2007); Sung *et al.* (2008); Soleimani & Nadri (2009)
CD11b (ITGAM)	-	-	It is generally expressed on many leukocytes involved in the innate immune system including monocytes, granulocytes, macrophages, and NK cells. It mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis, cellular and complement activation.	Baddoo *et al.* (2003); Tropel *et al.* (2004); Chamberlain *et al.* (2007); Sung *et al.* (2008); Soleimani & Nadri (2009); Schurgers *et al.* (2010)
CD117 (c-kit)	-	-	It is a cytokine receptor expressed on the surface of haematopoietic stem cells as well as other cell types. CD117 is a receptor tyrosine kinase type III, which binds to stem cell factor (a substance that causes certain types of cells to grow), also known as "steel factor" or "c-kit ligand". Signalling through CD117 plays a role in cell survival, proliferation and differentiation.	Jiang *et al.* (2002b); Baddoo *et al.* (2003); Koide *et al.* (2007); Park *et al.* (2007c); Soleimani & Nadri (2009)
CD271		+	Also known as low-affinity nerve growth factor receptor (LNGFR), it belongs to the low affinity neutrophin receptor and tumour necrosis factor receptor superfamily. Its precise function remains controversial but it is widely thought to be involved in the survival and differentiation of neuronal cells by acting as a sink for neutrophins. It has been found to potentially define a MSC subpopulation and for the enrichment of non-haematopoietic stem cells from human bone marrow and adipose tissue.	Thomson *et al*. (1988); Quirici *et al*. (2002); Jones *et al*. (2002); Kuci *et al*. (2010)
CD146		+	Also known as melanoma cell adhesion molecule (MCAM), it is commonly used as an endothelial cell marker. Its exact function remains unknown but it is thought to form part of the endothelial junction associated with the actin cytoskeleton. Its expression on human MSC is thought to be associated with multipotency with higher expression linked to greater differentiation potential.	Covas *et al*. (2008); Shrugar *et al*. (2009); Russell *et al*. (2010)

STRO-1	+	+	It was originally identified as an antibody for identifying both human and mouse plastic-adherent bone marrow stromal cells but was also later used successfully to identify endothelial cells.	Delorme *et al*. (2006); Lin *et al*. (2008); Yang *et al*. (2008); Ning *et al*. (2011)
CD133 (Prominin)	-	-	It is a marker for HSC, endothelial cells and neural stem cells	Koide *et al.* (2007)

[MHC; major histocompatibility complex. CD; cluster of differentiation. APC; antigen presenting cell. TGFβ; transforming growth factor-beta. VLA5; very late activation antigen-5. CTLA-4; cytotoxic T-lymphocyte antigen-4. ALCAM; activated leukocyte cell adhesion molecule. VCAM-1; vascular cell adhesion molecule-1. PECAM-1; platelet endothelial cell adhesion molecule-1. ITGA5; integrin alpha-5. NT5E; ecto-5'-nucleotidase. NK; natural killer]

1.3.3.5. Expansion

Not enough attention has been put into how different culture media and growth factors used for the growth and expansion of MSC could affect their differentiation potential or expression of cell surface markers. To date, there is no specialised medium for the growth of MSC and different studies have used different types of medium and growth factors. The commonly used media are Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (α-MEM) and Opti-MEM, Iscove's modified Dulbecco's medium (IMDM) supplemented with either FCS, human serum, platelet lysate, low or high glucose, additional amino acids such as L-glutamine, deoxyribonucleotides, fibroblast growth factor (FGF) and many others. Current opinion suggests that expansion medium may only influence the proliferation potency, but it is probable that the effects may go beyond mere cell division. Therefore, the full repertoire of factors that potentially influence the behaviour of MSC in culture remains unresolved. MSC in culture are best described as heterogeneous mixtures of cells with varying proliferation and differentiation capacities. A hierarchical model has been proposed that suggests that MSC cultures lose their clonogenic capacity and multipotency with increasing passaging in culture. It is thought that the majority of the cells *in vitro* are transitional cells with specific lineage potential (Park *et al.*, 2007c)

1.3.3.6. Differentiation

From the previous sections, it has been highlighted that there is no specific marker(s) or isolation method that purely separates MSC from other related cells. To date, the most practical and important presumptive method for defining MSC is the 'functional assay' or 'tri-lineage differentiation assay' that tests the differentiation capacity of the MSC (Pittenger *et al.*, 1999). The capacity of the cells to undergo *in vitro* differentiation to bone, fat, and cartilage under chemical, hormonal or mechanical stimulation is the single critical requirement for identifying putative MSC populations. Even clonal selection does not ensure that a cell population derived from a single colony will be homogeneous after several rounds of division as MSC have been shown to gradually lose their multipotency with time due to several factors which include inherent telomere shortening and experimental conditions (Park *et al.*, 2007c). Efforts to find the major signalling pathways or 'master' regulatory genes that control MSC differentiation are on-going. Though genetic and

or epigenetic programs that govern cell differentiation have remained elusive, much has been learnt from ESC genetics which then led to the development of iPS by Yamanaka and co-workers through genetic reprogramming of mouse (Takahashi & Yamanaka, 2006) and human (Takahashi *et al.*, 2007) fibroblasts discussed earlier in **Section 1.1.12**.

The ability to modulate specific genetic programs in order to maintain a specific differentiation pathway while avoiding spurious differentiation is a pre-requisite for the application of MSC in tissue engineering and regenerative medicine. Some of the known mechanisms that are thought to control the tri-lineage differentiation process in mouse MSC are discussed.

1.3.3.6.1. Adipogenesis

Generally, adipogenic differentiation is induced *in vitro* by culturing confluent MSC in culture medium supplemented with several hormonal and chemicals cues such as: the hormone, insulin; the cyclooxygenase inhibitor, indomethacin, the corticosteroid dexamethasone and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). *In vitro* adipogenic differentiation follows a well characterised two-stage sequence summarised below;

- Determination – involves the commitment of MSC to the adipocyte lineage by changing into preadipocytes which are morphologically indistinguishable from the precursor cells but would have lost their plasticity.
- Terminal differentiation – preadipocytes develop into mature adipocytes by acquiring the ability to synthesize and transport lipids, sensitivity to insulin and secretion of adipocyte specific proteins.

The process is initiated by the expression of the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ), the master adipogenic regulator which promotes adipogenesis whilst suppressing osteogenesis (Nuttall & Gimble, 2004). During the early stages of adipogenic differentiation, PPARγ triggers expression of the CCAAT/enhancer binding proteins (C/EBP) family of transcriptional factors whereby C/EBPβ and C/EBPδ induction is followed by C/EBPα expression which is required for insulin sensitivity in preadipocytes (Rosen *et al.*, 2002; Rosen & MacDougald, 2006). *In vitro*, the binding of PPARγ to specific

cellular ligands induces the transactivation and transrepression of PPARγ thereby promoting adipogenesis whilst blocking osteogenesis. The process is enhanced by increasing intracellular cyclic adenosine monophosphate (cAMP) as a result of IBMX and further binding to specific upregulated nuclear receptors by other differentiation inducing factors in the medium. Insulin exerts its effects by binding with the insulin growth factor-receptor signalling. However the exact link between insulin signalling cascade and the intrinsic adipogenesis cascades remains unknown (Gimble *et al.*, 2008). After 3-9 days the predominantly fibroblast-like MSC are converted to oval, neutral lipid containing cells whose lipid vesicles increase in size with time in culture or through the addition of fatty acids such as oleic, palmitic and linolenic acid to the culture medium (Diascro JR *et al.*, 1998). The differentiated MSC have been reported to exhibit biochemical characteristics similar to those of mature adipocytes (Gimble *et al.*, 1990) including expression of adipogenic genes such as fatty acid-binding protein-4 (FABP4) and lipoprotein lipase as well as secretion of the adipokine, adiponectin and the hormone, leptin (Gimble *et al.*, 2008).

1.3.3.6.2. Chondrogenesis

Chondrogenic differentiation of MSC is induced *in vitro* by the addition of stimulatory compounds including dexamethasone, ascorbate phosphate, pyruvic acid, insulin, transferrin, selenium and transforming growth factor-beta (TGFβ). Unlike adipogenic and osteogenic differentiation which require cell cultures in monolayer, chondrogenic differentiation occurs effectively when MSC are cultured in 3-dimensional conformation such as pellet or micromass cultures (Worster *et al.*, 2001; Goessler *et al.*, 2005; Longobardi *et al.*, 2006; Bernardo *et al.*, 2007). Differentiation can also be achieved through mechanical stimulation and reduced oxygen tension (Malladi *et al.*, 2006; Terraciano *et al.*, 2007) without use of chemical or hormonal factors. The specific signalling pathways that initiate chondrogenic differentiation are still unknown. However, it is thought that the differentiation process is initiated in part by culture medium constituents such as TGFβ, bone morphogenetic protein-2 (BMP2) and growth and differentiation factor (GDF) signalling (Chen *et al.*, 2004; Massagué & Gomis, 2006). Binding of these ligands to their specific receptors initiates signalling through specific intracellular Smad proteins and major mitogen-activated protein kinase (MAPK) cascades which result in the activation of the transcription factors Sox-9 and scleraxis which cause the upregulation of the genes

for ECM proteins and proteoglycans including collagen types II and IX, aggrecan, decorin, versican and cartilage oligomeric matrix protein (Tuan *et al.*, 2003; Baksh *et al.*, 2004). The early changes in the differentiation process are marked by a progressive increase in the levels of the glycosaminoglycans (GAGs) chondroitin-6-sulphate relative to chondroitin-4-suphate in a similar pattern to that observed during the maturation of articular cartilage (Bayliss *et al.*, 1999). Gradually, the MSC lose their fibroblast-like morphology as expression of ECM proteins and biosynthesis of GAGs increase leading to a dramatically altered morphology (Barry & Murphy, 2004).

1.3.3.6.3. Osteogenesis

The *in vitro* osteogenic differentiation of MSC requires confluent monolayer cultures and the presence of stimulatory chemicals such a β-glycerophosphate, dexamethasone, ascorbate-2-phosphate and in some case BMP2 in culture medium. Though the exact mechanisms controlling osteogenic differentiation are not fully elucidated, the differentiation process is considered to be a three-stage process involving proliferation, matrix formation and mineralisation punctuated by expression of osteoblastic phenotype markers (Olsen *et al.*, 2000).

The proliferation stage is highlighted by increased expression of cell cycle genes. One proposed model of osteogenesis suggests that BMP2 promotes osteogenic differentiation by inducing the p300-mediated acetylation of the master osteogenesis regulator Runt-related transcriptional factor-2 (Runx2), which enhances its transactivation capability while increased Wnts protein levels has also been shown to promote the transformation of MSC to osteocytes (Chen *et al.*, 2004; Jeon *et al.*, 2006). Other transcriptional factors thought to regulate the differentiation process include tafazzin, osterix and activating transcriptional factor-4 (Deng *et al.*, 2008). It is hypothesized that crosstalk between BMP2 and Wnts is responsible for the upregulation of Runx2 and together with other signalling pathways leads to the subsequent acquisition of an osteoblastic morphology with upregulation of the enzyme alkaline phosphatase activity which defines the matrix formation stage. Finally, the mineralisation phase is characterised by the deposition of calcium-rich mineralised ECM (Barry & Murphy, 2004; Friedman *et al.*, 2006; Kolf *et al.*, 2007). The differentiated phenotype is also characterised by a large nucleus, enlarged Golgi

apparatus and extensive endoplasmic reticulum to cope with the increased secretion of bone matrix proteins. Expression of markers such as osteopontin and osteocalcin are indicative of differentiated osteoblasts (Deng *et al.*, 2008).

1.4. Clinical application and potential of MSC

The ideal stem cells for clinical application are ESC primarily because of their pluripotency (Pera & Trounson, 2004) but ethical, moral and legal concerns have so far limited their consideration for therapeutic and clinical use (Weissman, 2002) as discussed in **Section 1.1.1.1**. This has led to the growing interest in ASC particularly MSC the use of which does not involve the ethical issues that surround ESC. The last decade has seen an unprecedented interest in the biology of human MSC and exploration of their therapeutic potential. Their self-renewal properties and multilineage differentiation potency have made them candidates for the repair, regeneration and replacement of diseased cells and tissues. MSC offer the options of either being used in their undifferentiated state in cell based therapies or after *ex vivo* differentiation on biomimetic scaffolds in tissue engineering.

Importantly, there has been an accumulation of evidence that proposes that MSC are immunoprivileged in addition to possessing immunosuppressive properties (Le Blanc & Ringden, 2007). Studies carried out using human (Aggarwal & Pittenger, 2005; Klyushnenkova *et al.*, 2005), rodent (Krampera *et al.*, 2003) and primate (Bartholomew *et al.*, 2002) models have shown that MSC failed to elicit a proliferative response from allogeneic lymphocytes in lymphocyte transformation assays (LTA) and suppressed mixed lymphocyte reactions (MLR). Though the mechanisms responsible for their immunomodulatory properties are yet to be resolved, their ability to escape detection by allogeneic lymphocytes (immunoprivilege) has been suggested to be MHC-independent (Le Blanc *et al.*, 2003c). Also, their reported capacity to skew both the innate and adaptive immune responses towards anti-inflammatory/tolerant phenotype (immunosuppression) is thought to be due to a combination of secreted factors and direct cell to cell contact (Kode *et al.*, 2009). In animal models, intravenous administration of MSC has been reported to improve outcomes of renal (Tögel *et al.*, 2005), neural (Zappia *et al.*, 2005) and lung (Ortiz *et al.*, 2003) injury through paracrine effects and a shift from pro-inflammatory to anti-inflammatory cytokine production at the site of injury.

These reported properties would enable the therapeutic application of allogeneic MSC without the risks of rejection normally associated with allogeneic cells and tissues. There is also the potential of the 'universal donor' concept whereby MSC from a single donor might be infinitely expanded and used to commercially manufacture "off-the-shelf" tissue engineered products or individually tailored for multiple patient therapies. Thus both the clinical and commercial potential of allogeneic MSC rest solely on their immunomodulatory properties. Specific applications of MSC can be categorised into three major approaches namely;

- tissue engineering
- local implantation of MSC into diseased tissues
- systemic implantation

1.4.1. Tissue engineering

Tissue engineering is an inter-disciplinary field of regenerative medicine that applies the principles of engineering and biological sciences to understand tissue growth in order to apply this to produce functional tissue for clinical use (Dawson & Oreffo, 2008). It allows for the isolation of a patient's own cells such as MSC, seeding them into biological scaffolds and allowing specific tissues to grow *ex vivo* before implantation back to the patient to repair damage due to disease or trauma. The traditional approach of tissue and organ donation to replace degenerate tissues and organs is increasingly threatened by challenges which include the shortage of donors, physical mismatch of dimensions and importantly, limitations attributable to immunological rejection of donor tissue by the recipient's immune system (Abdallah & Kassem, 2007). However, the reported ability of allogeneic MSC to avoid immune rejection has raised interest in their potential application in tissue engineering procedures. Since MSC can undergo specific guided differentiation, the immunological consequences of differentiated allogeneic MSC would require thorough investigations. Tissue engineering based approaches that utilise MSC would be more suitable for bone, cartilage and adipose tissue engineering since they can easily differentiate into these cell lineages.

1.4.1.1. Bone tissue engineering

Bone defects have been traditionally treated using artificial, autologous and in some cases allogeneic bone grafts. Artificial grafts are mainly limited by their mechanical

properties and biodegradation which should mirror those of natural bone whereas autologous grafts are limited by donor-site morbidity. Immunological rejection poses the major risk for allogeneic grafts (Salgado *et al.*, 2004; Hosseinkhani *et al.*, 2006).

The ability of MSC to undergo osteogenic differentiation under chemical and hormonal stimulation has been exploited with clinical success in the treatment of bone defects by using a tissue engineering protocol that involved seeding *ex vivo* expanded autologous MSC on macroporous hydroxyapatite scaffolds followed by implantation at defect sites. Post-surgery examination revealed callus formation along the implants and good integration at the interface with host bones (Quarto *et al.*, 2001). The authors claimed that since these were 'challenging cases' with multiple previous failed treatments, the success validated the effectiveness of this tissue engineering approach. This clinical trial was conducted following similar successes using autologous and syngeneic MSC in ovine (Kon *et al.*, 2000), canine (Scott *et al.*, 1998) and rodent (Kadiyala *et al.*, 1997) models.

Due to the success with autologous MSC, allogeneic MSC have been used in animal studies mimicking real clinical situations to repair bone defects. In independent studies using a canine model, both allogeneic and autologous MSC seeded on hydroxyapatite tricalcium phosphate scaffolds were used to fill critically-sized bone defects. On examination, the allogeneic MSC grafts enhanced repair of the defects by forming new bone comparable to autologous MSC grafts without need for immunosuppressive therapy or adverse immune response against the allogeneic MSC as measured by the absence of circulating antibodies in sera and infiltrating lymphocytes after histological analysis (Arinzeh *et al.*, 2003; De Kok *et al.*, 2003). This suggested that the *in vivo* osteogenic differentiation process did not alter the immunoprivilege of the allogeneic MSC and thus provided evidence for the potential clinical application of allogeneic MSC. However, in a xenogeneic model in which undifferentiated and osteogenic differentiated human MSC were implanted in immunocompetent mice, the undifferentiated MSC but not the osteogenic differentiated MSC survived the host immune response (Niemeyer *et al.*, 2008). These findings cast a shadow of doubt on the reported immunoprivileged status of differentiated MSC.

1.4.1.2. Cartilage tissue engineering

Due to the poor healing potential of articular cartilage (Tuan *et al.*, 2003), damage due to trauma or degenerative diseases such as osteoarthritis is debilitating to patients and provides a huge challenge to current cartilage tissue engineering procedures. Traditional treatment methods for the restoration of function range from autografting, allografting, periosteal and perichondrial grafting, stimulation of intrinsic regeneration by intentionally drilling full-thickness defects, pharmacological intervention and autologous cell transplantation such as in periosteal flap technique marketed by Genzyme Corporation (Brittberg *et al.*, 1994). However, most of these techniques had higher failure rates in young patients or failed to provide repair to the fully functional state (O'Driscoll, 1998; Tuan *et al.*, 2003). The chondrogenic differentiation capacity of MSC makes them a suitable cell source for cartilage tissue engineering and could be exploited to regenerate cartilage without donor site morbidity.

In separate experiments using rabbit models, autologous MSC seeded on a type I collagen gel and a calcium phosphate and hyaluronan sponge have been used to successfully repair full-thickness cartilage defects in knees and the weight-bearing surface of femoral condyles (Wakitani *et al.*, 1994; Gao *et al.*, 2002). These findings were applied clinically in a study which used autologous MSC embedded in collagen gel to successfully treat patients suffering from osteoarthritis with hyaline cartilage-like tissue forming 8 months post-surgery (Wakitani *et al.*, 2002). Advances in materials science have led to the development of many scaffold materials such as lactic and glycolic acid and their co-polymers, poly(ethylene glycol)-terephthalate and poly(butylene terephthalate) which are highly porous structures to allow for cellular ingrowth, has helped to improve outcomes (Tuan *et al.*, 2003) and more recently attention has been focused on de-cellularisation of natural xenogeneic and allogeneic tissues such as the medial meniscus (Stapleton *et al.*, 2008) for use as biological scaffolds.

1.4.1.3. Adipose tissue engineering

Millions of plastic and reconstructive surgery procedures are carried out every year to repair soft tissue defects that arise from a wide range of trauma and disease conditions such as from tumour resection in mastectomy and carcinoma removal,

traumatic injury such as resulting from deep burns, hereditary and congenital defects such as Romberg's disease and Poland syndrome (Vashi *et al.*, 2008). Current methods for treating adipose tissue defects include autografts, fat-free grafts and alloplastic materials (Girandon *et al.*, 2011), however these techniques have shortcomings such as donor site trauma and morbidity, suboptimal volume retention and poor biocompatibility. In addition, the poor healing of adipose tissue grafts is compounded by the shortage of or premature apoptosis of adipogenic cells (Stosich & Mao, 2007).

The adipogenic potential of MSC can be exploited in tissue engineering approaches using a wide range of both natural and synthetic scaffolds such as injectable or implantable hydrogels, hyaluronic acid, collagen and modified alginate (Gomillion & Burg, 2006). In separate studies, 3D cultures using rat (Neubauer *et al.*, 2005) and human (Hong *et al.*, 2005) MSC loaded onto polylactide-co-glycolide and gelatin sponges respectively successfully generated mature adipose tissues.

Autologous human and murine MSC delivered in a fibrin spray have been reported to accelerate cutaneous wound healing (Falanga *et al.*, 2007). When human MSC were seeded on photopolymerized polyethylene glycol diacrylate scaffolds and implanted in subcutaneous pockets in SCID mice, expression of adipogenic genes and histological staining successfully demonstrated *in vivo* adipogenesis together with retention of shape and dimensions (Alhadlaq *et al.*, 2005; Bauer-Kreisel *et al.*, 2010). Further studies using rabbit MSC implanted into nude mice showed new fat tissue formation (Choi *et al.*, 2005). However, by using immunocompromised mice, these xenogeneic implantation studies only tested the adipogenic potential of MSC but not their immunogenicity. Thus, despite the authors suggesting the potential utility of MSC in adipose tissue engineering, such applications may be restricted to autologous cells only.

1.4.2. Implantation of MSC into diseased tissues

Implantation of MSC at the local site of injury or trauma has been reported in several animal studies which have demonstrated the efficacy of exploiting their multilineage differentiation capacity and immunomodulatory properties. In early studies, autologous MSC were injected in patients with ischaemic limbs due to peripheral artery disease and the findings showed that MSC achieved therapeutic angiogenesis

due their ability to provide endothelial progenitor cells and secretion of angiogenic factors (Tateishi-Yuyama *et al.*, 2002). Badiavas and co-workers used autologous MSC in patients with chronic wounds which did not heal for over one year and in whom other therapies and autologous skin grafting had failed. Complete wound closure and evidence of dermal healing was observed and evidence of engraftment of applied cells and reduced scaring was obtained from biopsies of the healed wounds (Badiavas & Falanga, 2003). In the treatment of patients with fracture non-union, autologous MSC combined with calcium sulphate were applied to the non-healing sites and led to callus formation and bone union (Bajada *et al.*, 2007).

Allogeneic MSC have been tested in small and large animal models particularly in cardiac therapeutics with interesting findings. In two independent studies using porcine models of myocardial infarction, labelled allogeneic MSC were injected into the infarct and peri-infarct areas post-coronary ligation without use of immunosuppressive therapy. The results showed successful intramyocardial engraftment of the allogeneic MSC and their differentiation into cardiomyocytes and endothelial cells without any evidence of allogeneic rejection. The treatment led to preservation of left ventricular function (Caparelli *et al.*, 2001; Makkar *et al.*, 2005). These findings were supported by other independent studies using rodent (Dai *et al.*, 2005) and porcine (Amado *et al.*, 2005; Quevedo *et al.*, 2009) models of myocardial infarction which reported long-term survival, differentiation and engraftment of allogeneic MSC which contributed to the functional improvement of the hosts myocardium without use of immunosuppressants. In light of this evidence and previous findings with autologous MSC (Pouzet *et al.*, 2001), it was thought that the efficacy of allogeneic MSC intramyocardial transplantation could be dose-dependent, thus Poh and co-workers investigated the short and intermediate-term effects of a repeated MSC administration strategy to evaluate the safety of an 'off-the-shelf' allogeneic MSC cell source in a porcine model. They concluded that repeated high dose administration of allogeneic MSC was safe and posed no immunological risk (Poh *et al.*, 2007).

However, Poncelet and co-workers reported that allogeneic MSC were rejected *in vivo* in an allogeneic porcine model despite having been shown to be non-immunogenic *in vitro*. In that study, allogeneic MSC were injected into infarcted areas of the myocardium while allogeneic skin grafts and MSC injected sub-

cutaneously were used as controls without immunosuppression. In all cases, it was found the animals developed donor-specific cellular and humoral allogeneic responses with antibody-complement mediated cytotoxicity (Poncelet *et al.*, 2007). The study highlighted the differences between the *in vitro* and *in vivo* characteristics of MSC suggesting that more research is required to fully understand the mechanisms that control MSC immunomodulatory properties before the prospect of clinical application of allogeneic MSC becomes possible.

1.4.3. Systemic implantation of MSC

The systemic administration of MSC is aimed at exerting the immunoregulatory properties of MSC throughout the body due to their homing and engraftment capabilities at disease or trauma sites where they can differentiate into specific cell lineages to facilitate tissue repair and regeneration (Devine *et al.*, 2001).

Bartholomew and co-workers used a MHC-mismatched primate model and reported that intravenously administered donor MSC prolonged the survival of donor and 3rd party skin grafts (Bartholomew *et al.*, 2002). The same group went further to report that following intravenous infusion of green fluorescent protein (GFP)-tagged autologous or allogeneic MSC resulted in the distribution and engraftment of the cells in 16 different tissues. They concluded that engrafted MSC participated in on-going cell replacement and turnover in the long term (Devine *et al.*, 2003).

However, the results in their first study showed that the rejection of donor and 3rd party grafts was only extended by 4 days after a single or double MSC dose. Thus despite claims by the authors that allogeneic MSC could improve graft survival, such an improvement (4 days) would be of no clinical utility. Even though the improvement in graft survival was attributed to MSC-mediated immunosuppression, the immunogenicity of the donor MSC was not investigated since intravenous administration of antigens is known to be tolerogenic. Interestingly, when animals received donor grafts injected with allogeneic MSC subcutaneously, the authors reported that these grafts failed 4 days post-transplantation as a result of technical reasons. However, it is conceivable that the presence of allogeneic MSC in close proximity to the danger signals produced during grafting could have initiated rapid rejection.

An area that has generated a great deal of interest is the systemic administration of MSC in the treatment of GvHD particularly during allogeneic HSC transplantation for treating different types of leukaemia. In a phase II clinical study, Le Blanc and co-workers reported the treatment of 55 patients with acute GvHD by both haplo-identical and mismatched MSC over a 6-year period. None of the patients suffered any side effects during or after MSC transplantation (Le Blanc *et al.*, 2004c; Le Blanc *et al.*, 2008). Due to myeloablative conditioning which renders the recipient's immune system inactive, infused donor MSC therefore suppressed donor T-cells from mounting immune attack on the recipient's tissues. These studies therefore demonstrated the immunosuppressive capacity of allogeneic MSC but not their lack of immunogenicity.

In a study using a murine model of GvHD however, Sudres and co-workers reported that allogeneic MSC failed to prevent GvHD after intravenous administration in lethally irradiated mice despite showing significant immunosuppressive property *in vitro* (Sudres *et al.*, 2006). These findings therefore contradicted the reports by Le Banc and co-workers mentioned earlier by suggesting that the immunosuppressive effects of the allogeneic MSC were inadequate in stopping donor T-cell alloreactivity despite using MSC doses that had previously demonstrated significant immunosuppression *in vitro*. Though factors such as administered dose or their passage/age could affect the immunosuppressive potency of MSC, it was found that only traces of the infused MSC engrafted in the target organs of GvHD.

Eliopoulos and co-workers using a murine model reported that allogeneic MSC were rejected after subcutaneous implantation in immunocompetent hosts. The rejection was characterised by an increased presence of host-derived CD8 and NK cells infiltrating in the implantation site (Eliopoulos *et al.*, 2005). This study demonstrated that allogeneic MSC were not immunoprivileged. The rejection could have been facilitated by immunological danger signals at the site of implantation which are absent when MSC are infused intravenously.

Similar findings were also reported by Nauta and co-workers who used a murine model for bone marrow transplantation in which recipient mice were either infused with allogeneic or syngeneic MSC under non-myeloablative settings. Though syngeneic MSC significantly enhanced long-term engraftment associated with

tolerance to host and donor antigens, allogeneic MSC were associated with significantly increased rejection. This was further clarified when infusion of allogeneic MSC in naïve mice was met with robust rejection due to a memory T-cell response suggesting that MSC may not be intrinsically immunoprivileged and under appropriate conditions are rejected (Nauta *et al.*, 2006). Furthermore, in a xenogeneic model of myocardial infarction in which human MSC were infused in immunocompetent rats showed significant rejection of the engrafted MSC (Grinnemo *et al.*, 2004).

Collectively, the evidence from both human and animal models discussed thus far has demonstrated the immunosuppressive potency of autologous and allogeneic MSC in immunocompromised settings. However, allogeneic MSC were found to be immunogenic when immunocompetent hosts were used.

1.5. Autologous MSC or allogeneic MSC, does it matter?

From the evidence discussed in **Section 1.4**, it is clear that the genetic origin of MSC, autologous or allogeneic, was important in the design of the experiments and in some cases this was the difference between success and failure of the MSC therapies (Tse *et al.*, 2003; Nauta *et al.*, 2006; Sudres *et al.*, 2006). Ideally, autologous MSC are the most suitable since they pose no immunological risk to the patient. However, in practice there are several challenges associated with this approach.

Firstly, the fraction of MSC obtained from bone marrow aspirate is very small and may require *ex vivo* expansion to increase their numbers to therapeutic levels before they are used in the patient. This time lag between harvesting and application, though more suitable for chronic conditions, is unfavourable in acute conditions. In tissue engineering based applications whereby *ex vivo* expanded MSC have to undergo directed differentiation in culture before use in the patient, this further increases the time lag from cell harvesting to the delivery of treatment. Secondly, patient-derived MSC may be unsuitable due to the risk of them being diseased such as in diseases of the bone marrow or they may be too old such as in elderly patients. These factors make the use of autologous MSC clinically and commercially unattractive.

Allogeneic MSC however overcome all the challenges associated with autologous cells since they can be harvested, expanded and packaged prior to use. There is also the choice of screening potential donors to obtain the best 'quality' of disease-free cells. Tissue engineered products could be manufactured and made available 'off-the-shelf' on demand with huge commercial benefits for industry. The major drawback of allogeneic MSC however, is their inherent risk of being rejected by the recipient immune system which could lead to failure of treatment and potential life-threatening consequences to the patient.

The accumulation of evidence on the immunosuppressive properties and immunoprivileged status of MSC has however led to an increase in studies utilising MSC in allogeneic settings with the hope of potential clinical utility. In order to appreciate the controversy surrounding the immunological status of allogeneic MSC, it is necessary to understand the basis of allorecognition and the mechanisms by which allogeneic cells are rejected by the recipient immune system.

1.6. The immune system – how does it work?

1.6.1. The immune system

All the mechanisms used by an organism to protect itself from foreign environmental agents such as pathogenic microorganisms and their products, chemicals and materials from other organisms in order to prevent corruption of 'self', form the immune system. In vertebrates including humans and mice, the immune system is divided into two parts namely innate and adaptive/acquired immunity.

1.6.1.1. Innate immunity

This is the natural resistance with which an organism is born and includes physical barriers (skin and mucous membranes), chemical agents (cytokines associated with the inflammatory response and complement) and cellular responses (non-specific leukocytes). All these elements combine synergistically to eliminate foreign substances. Though this type of immunity has no memory and generally referred to as non-specific, this is not entirely true as cellular components such as macrophages and DC are able to recognise 'self' from 'non-self' by use of pattern recognition receptors (PRR), for example the well characterised toll-like receptors [TLR] (Akira

et al., 2006). This is particularly important in the elimination of microorganisms that have penetrated or evaded detection by the physical and chemical barriers and also in the initiation of the adaptive immune response.

1.6.1.2. Adaptive/ acquired immunity

This is the specialised branch of the immune system and is characterised by two main features absent in innate immunity; specificity and long-term immunological memory (an increased and rapid response against a previously encountered antigen) against specific foreign antigens. Active adaptive immunity involves the acquisition of immunity through natural encounter with foreign antigens (such as during infection) or through deliberate administration of live or attenuated foreign antigens (such as during vaccination).

The cellular components that participate in this type of immunity are T- and B-lymphocytes as well as antigen presenting cells (APC) such as DC which are also involved in innate immunity. Both types of lymphocytes are formed in the bone marrow from their HSC progenitor cells during lymphoid haematopoiesis. Whereas T-cells migrate to the thymus for maturation, B-cells mature in the bone marrow and spleen. B- and T-lymphocytes mediate the two branches of adaptive immunity namely humoral and cell-mediated immunity. Despite using different mechanisms, these systems collaborate to achieve the goal of protecting the body from foreign antigens (Coico & Sunshine, 2009).

1.6.1.2.1. Humoral immunity

This arm of acquired immunity is mediated by antibodies (immunoglobulin [Ig] molecules) produced and secreted into the plasma by differentiated B-cells (plasma cells). Antibody production in response to specific antigens is initiated when the antigen binds to the specific antibody surface receptor expressed by B-cells which triggers clonal expansion and differentiation leading to the production of the antibodies specific for the stimulating antigen. This process is $CD4^+$ T-cell dependent in most cases. The antibody-antigen complexes are eliminated by phagocytes which may be facilitated by complement activation via the classical pathway (Janeway *et al.*, 2001).

1.6.1.2.2. Cell-mediated immunity

This type of immunity is mediated by T-lymphocytes and does not involve antibodies. The T-cells recognise foreign antigens displayed via molecules of the MHC on APCs by means of surface bound T-cell receptors (TCR) which are specific for MHC-antigen. Broadly, T-cells fall into two sub-populations namely the $CD4^+$ (T-helper) and $CD8^+$ (cytotoxic) cells based on the type of co-receptor molecules associated with the TCR which play an important role in binding of T-cells with APC during antigen presentation, a process that leads to the distinction of 'self' from 'non-self' antigens (Coico & Sunshine, 2009).

In the context of the work carried out in this study, emphasis was focused on the cell-mediated immune response (specifically T-lymphocytes) towards syngeneic (self) and allogeneic (non-self or foreign) cells. Therefore, an explanation of the basis of allorecognition is necessary.

1.7. Basis of immunological response to allogeneic cells and tissues – allorecognition

1.7.1. Antigen presentation

When allogeneic or foreign cells are transplanted into an immunocompetent host, they are rejected by the host immune system. The first step in this process occurs when foreign antigens bound to the MHC molecules are presented at the cell surface by donor or recipient APC for recognition by the recipient's T-cells. This process is known as 'antigen processing and presentation' and includes all the events involved in the generation of peptide epitopes from proteins inside APC and the subsequent binding of these epitopes to MHC molecules and display at the cell surface.

The MHC in humans is a highly polymorphic cluster of genes which encodes protein products (HLA molecules) divided into class I and II. MHC I molecules (HLA A, B and C) are expressed on all nucleated cells while MHC II (HLA DP, DQ and DR) molecules are expressed on specialised APC including DC, macrophages and B-cells. When a protein epitope is bound, they form an immunologically recognisable complex. The APC acquire foreign antigens by phago-, pino- and endocytosis and

process them to yield smaller peptides (epitopes) which become bound to MHC II molecules before they are presented on the cell surface of the APC. Other cells such as dermal fibroblasts (DF) and endothelial cells however, can be induced by interferon gamma (IFNγ) to express MHC II and are referred to as non-professional APC.

$CD4^+$ cells recognise epitopes on APC bound to MHC II and are the predominant mediators of the immune response through secretion of cytokines that can modulate the immune response by exacerbating or suppressing it. $CD8^+$ cells however, recognise epitopes bound to MHC I and are capable of killing the cells bearing the foreign epitopes. $CD8^+$ cells are involved in the destruction of transplanted cells during allograft rejection.

The difference in the nature of antigens presented by MHC I and MHC II molecules is that the former binds intracellular or endogenous antigens synthesized within the cell (generally of viral or parasitic origin present in the cytoplasm) while the latter only binds extracellular or exogenous antigens internalised in acid vesicles taken up by endocytosis (soluble) and phagocytosis (particulate) by professional APC.

When $CD4^+$ or $CD8^+$ T-cells recognise foreign epitopes bound to surface MHC II or I molecules of APC via their T-cell receptors (TCR), the resulting interaction usually leads to T-cell activation which then triggers the immune response. The TCR is comprised of two disulphide bond-linked α and β trans-membrane proteins which are capable of binding epitope but are incapable of transducing the signal downstream. TCR are constitutively linked to the CD3 molecule complex whose intracellular portions contain protein tyrosine kinase (PTK) recognition motif also known as immunoreceptor tyrosine-based activation motif (ITAM). These ITAMs are substrates of *src* family PTKs whose phosphorylation is the initial step in T-cell signalling (Qian *et al.*, 1993; Iwashima *et al.*, 1994).

The binding of MHC-epitope complex by TCR, though being the first signal, is not necessarily sufficient for T-cell activation but requires the presence and participation of co-stimulatory and adhesion molecules which help to strengthen and stabilise the MHC-epitope-TCR complex as explained by the single encounter model (Friedl & Gunzer, 2001). The overall association between the T-cell and APC, known as the

'immunological synapse' (Irvine *et al.*, 2002), brings larger areas of the cells into contact and increases the contact time between the cells. Immunological synapses have been observed to be stable for at least one hour after formation (Friedl & Gunzer, 2001). The CD4 and the CD8 proteins bind to the invariant regions of the MHC II and MHC I respectively to strengthen the binding of the MHC-epitope complex to the TCR. This 'clustering' of the CD4 and CD8 molecules with the TCR complexes is an important process for triggering signal transduction that leads to T-cell activation, cell division and effector functions (Konig & Zhou, 2004).

The most important co-stimulatory interactions are between CD40 and B7 molecules (CD80 and CD86) on the APC with their corresponding ligands CD40L and CD28 or CTLA-4 (cytotoxic T-lymphocyte antigen 4) on the T-cells respectively. The co-stimulatory molecules provide additional signals required for full T-cell activation especially the activation of unprimed T-cells but are thought to be less necessary for activation of primed T-cells or memory T-cells (Coico & Sunshine, 2009). It is thought they help to bring the intracellular molecules involved in T-cell activation into close proximity to the TCR-MHC-epitope complex while dispersing those not involved away from the contact area thereby enhancing and sustaining the signals transduced by the MHC-epitope to the TCR-CD3 complex (**Figure 1.2**).

The adhesion molecules CD2 and LFA-1 (lymphocyte function-associated antigen 1) on the T-cells also bind to their corresponding ligands CD58 and CD54 on APC respectively and these interactions are thought to form an outer adhesive ring whose major function is to provide strong adhesive anchoring of the T-cell to the APC. These adhesive interactions prevent the T-cell and APC moving apart in order to allow the time for the TCR to 'sample' the contents of the MHC-peptide complex. Thus, adhesive molecules stabilize what would otherwise be transient interaction between the cells.

Once, activated, $CD4^+$ cells undergo interleukin-2 (IL-2) dependent clonal expansion and further differentiation. The differentiated effector cells secrete various cytokines which affect the function of multiple cell types of both the innate and adaptive immunity. A population of the dividing $CD4^+$ cells develop to become antigen-specific $CD4^+$ memory cells which are responsible for the swiftness and effectiveness of the secondary T-cell response. The $CD4^+$ memory cells differ from

unprimed $CD4^+$ cells by expressing increased levels of CD44 and reduced levels of CD62l as well as changes of CD45RA to CD45RO. It is currently believed that the B7-CD28 co-stimulation is not a necessity for the stimulation of $CD4^+$ memory cells (Coico & Sunshine, 2009). Once activated by recognition of MHC I-epitope, naïve $CD8^+$ cells also undergo clonal expansion and differentiation into effector cells and memory cells. Clonal expansion is dependent upon IL-2 generated by $CD4^+$ cells. Hence the priming of $CD8^+$ cells requires close proximity of activated $CD4^+$ cells. Effector $CD8^+$ cells also known as cytotoxic T-lymphocytes (CTL) kill the cells bearing the foreign epitopes by two main mechanisms both of which require efferent recognition of MHC I-epitope. One pathway involves the release of granules containing perforins and granzymes into target cells. The second pathway involves activation-induced cell death via the Fas-FasL caspase apoptotic mechanism (Coico & Sunshine, 2009). It is relevant to note at this point that the lack of MHC II and co-stimulatory molecule expression by MSC could have implications for their ability to interact with various cellular components of the immune system.

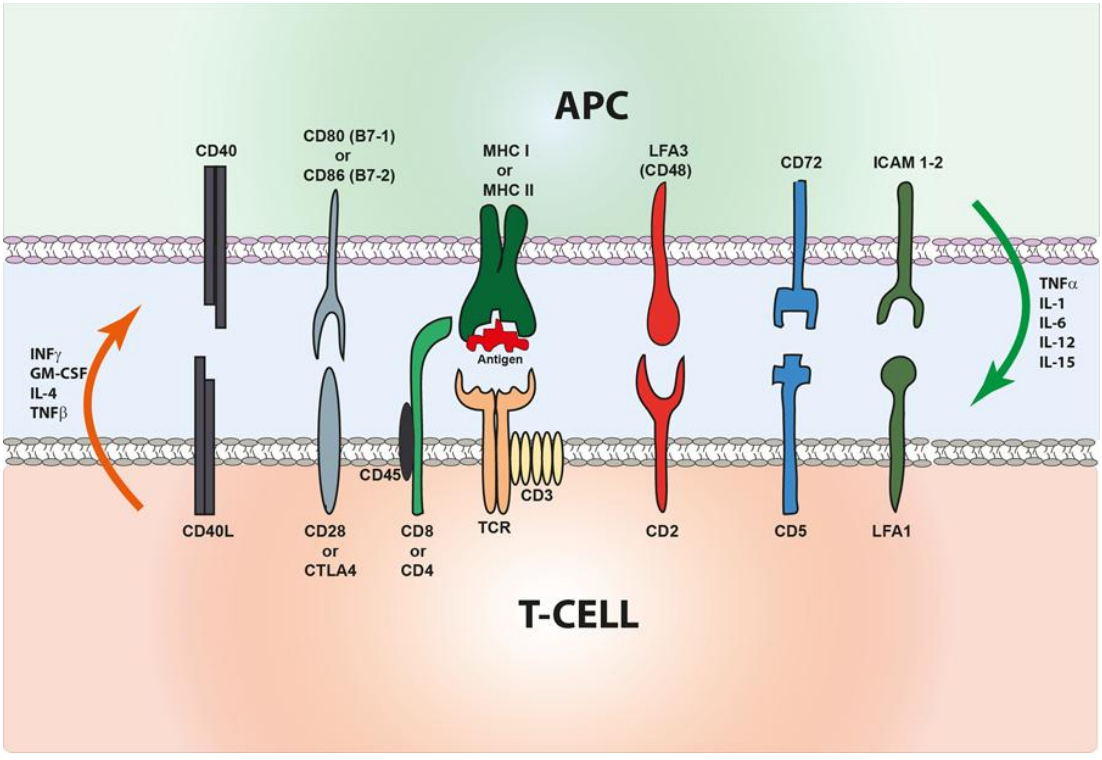

Figure 1.2. Antigen presentation by a professional APC to T-lymphocytes. Processed foreign antigens complexed to MHC I and MHC II are presented by APC to recipient $CD8^+$ and $CD4^+$ T-lymphocytes respectively. Successful antigen presentation is enabled by binding of co-stimulatory molecules to form a strong and long-lasting immunological synapse which leads to activation of the lymphocytes accompanied by secretion of a milieu of immunoactive cytokines and growth factors. Adapted from Coico & Sunshine (2009).

1.7.2. Allorecognition

The transplantation of cells or tissues between genetically distinct individuals of the same species initiates a unique and complex immune response with the purpose of distinguishing 'self' from 'non-self' and the eventual rejection of the allograft. Allorecognition refers to the T-cell mediated recognition of genetically encoded polymorphisms between members of the same species which are present in the MHC molecules. In clinical transplantation, donor-derived polymorphic HLA molecules are recognised as foreign by recipient T-cells which initiate allograft rejection. Unless immunosuppression therapy is employed, the donor allografts are rejected. The rejection can be classified according to its swiftness as acute and chronic depending on the mechanism of the rejection process. Acute and chronic rejection are mediated by T-cells and detected within two weeks to months respectively. A third type of allograft rejection is theoretically possible, known as hyperacute rejection. This is mediated by pre-formed antibodies to donor antigens and occurs within minutes. In clinical transplantation, however, this is mitigated by cross-matching of recipient serum with donor graft (Caballero *et al.*, 2006).

Although MHC incompatibility is responsible for allograft rejection, minor histocompatibility antigens (mH) have also been reported to be responsible for rapid allograft rejection (Game & Lechler, 2002). These antigens are derived from allelically polymorphic proteins which are usually presented in the groove of MHC I and II molecules. Approximately 40 mH antigenic differences are thought to exist between strains of inbred mice and several mH antigens have also been identified in humans. HLA matching can reduce the severity of alloimmune responses in clinical transplantation (25-30 % chance of finding an identical HLA haplotype among family members) but the chances of finding an HLA identical unrelated donor is virtually impossible given the enormous polymorphisms of the MHC system. Immunity against mH antigens is a clinically significant problem evidenced by the need for immunosuppressive treatment of recipients of HLA identical organ grafts and the incidence of GvHD following HLA identical HSC transplantation (Hernandez-Fuentes *et al.*, 1999; Game & Lechler, 2002).

Allorecognition was first demonstrated *in vitro* in MLR experiments in which peripheral blood mononuclear cells (PBMC) from two unrelated individuals were co-

cultured with resulting 'blast-formation' or lymphocyte proliferation resulting from allorecognition (Bain *et al.*, 1964; Bach & Voynow, 1966; Moorhead *et al.*, 1967). MLR have been used as a model in the study of mechanisms involved in allograft rejection and the determination of histocompatibility in tissue matching as they are considered to be the *in vitro* correlate of *in vivo* acute allotransplant rejection.

There are three major pathways of allorecognition namely the direct, indirect and semi-direct pathways. The development of knockout and transgenic mice has allowed the study of the different pathways in isolation. It was historically thought that the direct pathway alone was responsible for allorecognition since the T-cell repertoire was found to contain a high proportion of alloreactive cells (Gökmen *et al.*, 2008).

1.7.2.1. Direct pathway

This pathway involves the recognition of intact donor MHC I and II plus epitope on donor professional APC by the recipient $CD8^+$ and $CD4^+$ T-cells respectively (**Figure 1.3**). Paradoxically, the classic self-MHC restriction property of T-cells would suggest non-reactivity since the donor antigens are not presented in the context of 'self' MHC (Gökmen *et al.*, 2008).

Several models for the direct pathway have been postulated but the widely accepted ones include the high determinant density (HDD) model and the multiple binary complex (MBC) model which differ in the importance given to the presence of peptide antigen in the MHC-epitope complex.

The HDD model proposes that recipient T-cells recognise the exposed unique residues on allogeneic MHC molecules regardless of the nature of epitope in the MHC groove (Portoles *et al.*, 1989). Since donor MHC molecules are present at high density on the cell surface of APC, thus each molecule serves as a ligand for a specific alloreactive T-cell and then there will be a high density of ligands for the T-cells in comparison to the density of MHC-epitope complexes. This high ligand density therefore allows T-cells of lower affinities to respond to the donor MHC leading to a high frequency of alloreactivity. Evidence of this model was demonstrated in independent studies in mice in which blocking the TCR contact regions of allogeneic MHC using synthetic peptides inhibited specific alloresponses

presumably by inhibition of MHC-TCR contact (Schneck *et al.*, 1989; Lombardi *et al.*, 1991). Stronger evidence for this model was demonstrated in mouse $CD8^+$ lymphocytes whereby stimulation occurred in the absence of peptide antigens in the MHC groove (Smith *et al.*, 1997).

The MBC model however proposes that the presence of peptide antigen in the allogeneic MHC groove is a pre-requisite for allorecognition by recipient T-cells in a similar manner to conventional self-restricted responses. The hypothesis is that host T-cells recognise specific antigenic peptides in the donor MHC grooves and differences in allogeneic MHC groove causes different sets of peptides to be presented from homologous proteins. Thus a single MHC mismatch between donor and recipient would be adequate to stimulate an immune response (Game & Lechler, 2002). Another version of the model proposes that any particular cell surface MHC protein is complexed with a naturally arising peptide from the intracellular proteolytic machinery forming a heterogeneous population of binary complexes which can be recognised by many different T-cell clones in the host (Bharat & Mohanakumar, 2007). Therefore, each allogeneic MHC-antigen complex is recognised by a different T-cell and a single MHC incompatibility potentially results in the stimulation of a wide diversity of alloreactive T-cells (Afzali *et al.*, 2007). This hypothesis was supported by evidence that a mutant cell-line incapable of antigen-processing and allostimulation regained the ability to stimulate alloresponse properties after transfection with MHC II-antigen complexes (Weber *et al.*, 1995). In another study, $CD8^+$ T-cells were shown to be specific to a self-peptide presented by foreign MHC I molecules with no evidence of peptide-independent components (Whitelegg *et al.*, 2005).

It is thought that both mechanisms can contribute to the direct pathway with the contribution of each being related to the site and magnitude of differences in MHC molecules between donor and recipient cells (Afzali *et al.*, 2007). The direct recognition of allo-MHC-epitope without the need for antigen processing and presentation with 'self' MHC is thought to be responsible for the rapid and vigorous nature of *in vitro* MLR and *in vivo* acute rejection.

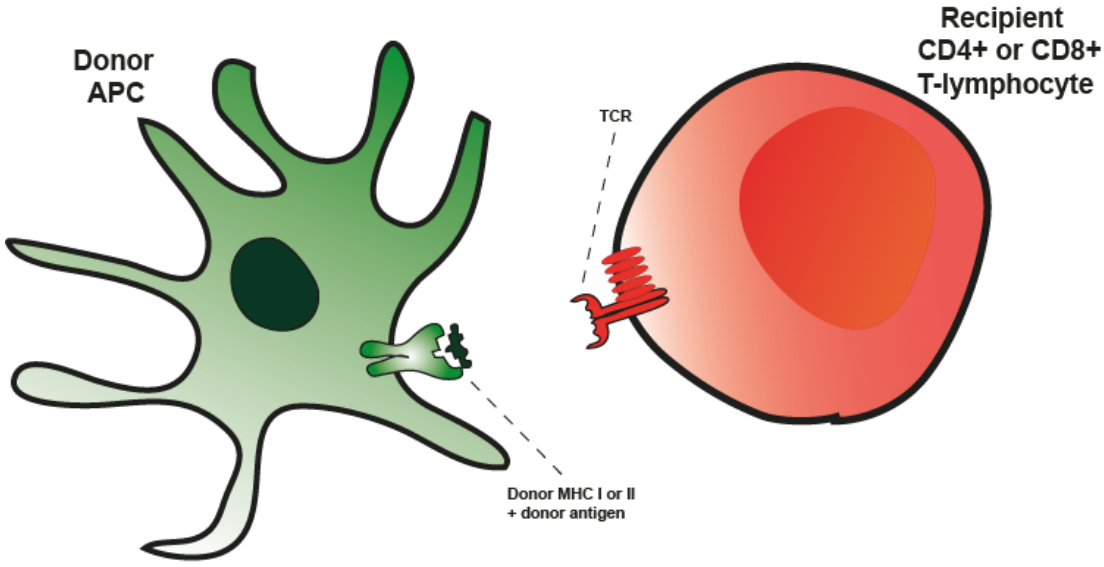

Figure 1.3. The direct pathway of allorecognition. Intact donor MHC-peptide complexes on donor APC are recognised by the recipient's T-cells, with $CD8^+$ cells recognising MHC I molecules and $CD4^+$ cells recognising the MHC II molecules. Adapted from Gökmen *et al*., (2008).

1.7.2.2. Indirect pathway

This pathway involves the presentation of processed donor antigens by recipient APC to recipient T-cells. It differs from the direct pathway by its requirement for antigen processing and use of 'self' MHC during antigen presentation. It is widely thought to be the mechanism involved during chronic allograft rejection (Gökmen *et al.*, 2008). $CD4^+$ T-cells dominate the indirect response as alloantigens shed from the graft are processed by recipient professional APC and presented in the context of 'self' MHC II (**Figure 1.4**). Though this response is chronic, it is amplified through the continuous processing of alloantigens so long the allograft is present.

Prior to discovery of the indirect pathway, allograft rejection was thought to be elicited by the direct pathway alone due to the high frequency of direct pathway alloreactive T-cells in the T-cell repertoire. Analysis of mouse lymph node T-cells showed that over 90% of the allospecific T-cell repertoire was capable of direct allorecognition while only 1-5% accounted for the indirect alloantigen recognition (Benichou *et al.*, 1999). Auchincloss and co-workers provided conclusive evidence of the existence of the indirect pathway by demonstrating that MHC I knock-out recipient mice rejected skin grafts from MHC II knock-out donor mice (Auchincloss *et al.*, 1993). In this case, the recipient mice lacked $CD8^+$ cells capable of

recognising donor MHC I molecules by the direct pathway, thus the recipient $CD4^+$ cells could only be stimulated by donor MHC I indirectly presented in the context of recipient MHC II molecules. This demonstrated that the indirect pathway alone was sufficient to elicit allograft rejection. It has also been shown that recipient APC such as DC, are capable of acquiring and processing intact donor MHC molecules from donor cell debris and subsequently stimulating $CD8^+$ cells by cross priming (Albert *et al.*, 1998; Morón *et al.*, 2004) demonstrating that both $CD8^+$ and $CD4^+$ cells are involved in the indirect pathway. In rodent models, animals immunised with allogeneic MHC II peptides prior to receiving allografts which by definition should stimulate the indirect but not direct pathway, swiftly and strongly rejected the allografts. However, intrathymic injection of the same peptides resulted in the down modulation of the indirect pathway sufficiently to allow prolonged survival of subsequent allografts of the same MHC thus demonstrating the importance of help from $CD4^+$ T-helper cells stimulated by the indirect but not the direct response (Afzali *et al.*, 2007).

The concept of 'passenger cells', in which an allograft brings with it immunostimulatory 'passenger' APC was supported by several studies including the prolonged survival of *in vitro* cultured thyroid tissue in mice presumably due to loss of 'passenger' APC (Lafferty *et al.*, 1976) and permanent survival of rat renal allografts by 'parking' the kidney in an intermediate recipient thereby depleting the allograft of donor passenger cells. However, in later studies, it was shown that inhibition of acute rejection by depleting 'passenger' APC significantly delayed but did not prevent the later development of chronic rejection (Bharat & Mohanakumar, 2007). Despite the rapid decline of direct pathway alloreactive T-cells after transplantation, the indirect pathway continues to present donor antigens to the recipient immune system for as long as the allograft is present due to the continuous influx of donor-processed antigens by recipient APC and this has been shown in several clinical studies involving patients with chronic heart, kidney and lung transplant rejection (Liu *et al.*, 1996; SivaSai *et al.*, 1999).

Since the indirect pathway is a slower mechanism than the direct pathway in eliciting allograft rejection, the current hypothesis is that the direct pathway dominates the early post-transplant period while the indirect pathway becomes prominent with time.

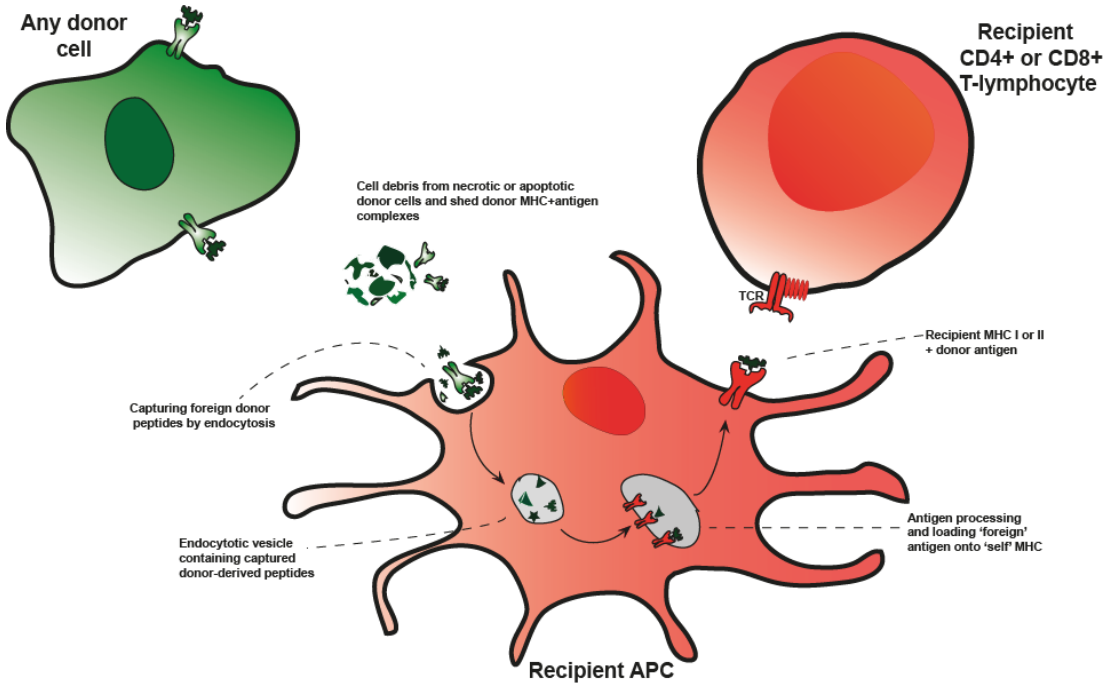

Figure 1.4. The indirect pathway of allorecognition. Antigens from any donor cells, resulting from shed, donor MHC-antigen complexes, apoptotic or necrotic cells are endocytosed, processed and presented to recipient $CD4^+$ and $CD8^+$ T-lymphocytes by recipient APC in the context of recipient's own MHC II and MHC I respectively. The result is presentation of foreign peptide complexed to self MHC to self T-cells. Adapted from Gökmen *et al*., (2008).

1.7.2.3. Semi-direct pathway

This pathway was proposed after the transfer of intact MHC molecules between cells was observed with DC acquiring MHC I and II-epitope complexes by either cell to cell contact or from exosomes secreted by other DC. These DC subsequently primed both naïve $CD8^+$ and $CD4^+$ T-cells (Théry *et al.*, 2002; Jiang *et al.*, 2004). This pathway combines both the direct and indirect pathways.

Traditionally, 'cross-talk' between the direct and indirect pathways (whereby $CD4^+$ with indirect alloreactivity could stimulate or suppress direct pathway $CD8^+$ T-cell responses) was thought to be based on the 4-cell unlinked model in which $CD8^+$ cells are stimulated via the direct pathway by donor cells whereas $CD4^+$ T-helper or T-regulatory cells were recruited by 'self' professional APC via the indirect pathway (Afzali *et al.*, 2007).

However, in the semi-direct pathway, intact donor MHC-epitope complexes are transferred to recipient professional APC thereby allowing the stimulation of $CD8^+$ T-cells by the direct pathway whilst alloantigens picked up by the same recipient professional APC are processed and presented in association with 'self' MHC II to recruit $CD4^+$ T-cells by the indirect pathway (Caballero *et al.*, 2006; Afzali *et al.*, 2007; Gökmen *et al.*, 2008). In this way, both donor-MHC-epitope and self-MHC loaded with donor-MHC derived epitope can be presented on the surface of the same cells thus allowing $CD8^+$ T-cells with direct allospecificity to receive linked help from helper $CD4^+$ T-cells with indirect allospecificity in a 3-cell model (**Figure 1.5**) (Lee *et al.*, 1994; Smyth *et al.*, 2007). This model fills the gaps in which the 4-cell unlinked model is non-compliant (Afzali *et al.*, 2007)

Recent work has confirmed that MHC transfer occurs *in vivo* (Riond *et al.*, 2007) and that allo-MHC can stimulate T-cell responses when delivered in exosomes (Morelli *et al.*, 2004). Importantly, work by Peche and co-workers showed immunological tolerance induction in rats that had received MHC mismatched cardiac allografts through the delivery of donor MHC-epitope complexes in exosomes (Pêche *et al.*, 2006). However the transfer of intact donor MHC-epitope complexes may also provide a means by which $CD8^+$ cells with direct allospecificity continue to be stimulated after donor APC have been depleted, potentially accounting for the longevity of the direct pathway following transplantation in some cases (Gökmen *et al.*, 2008). However, there is no evidence yet to suggest the clinical relevance of this pathway in the context of allograft rejection.

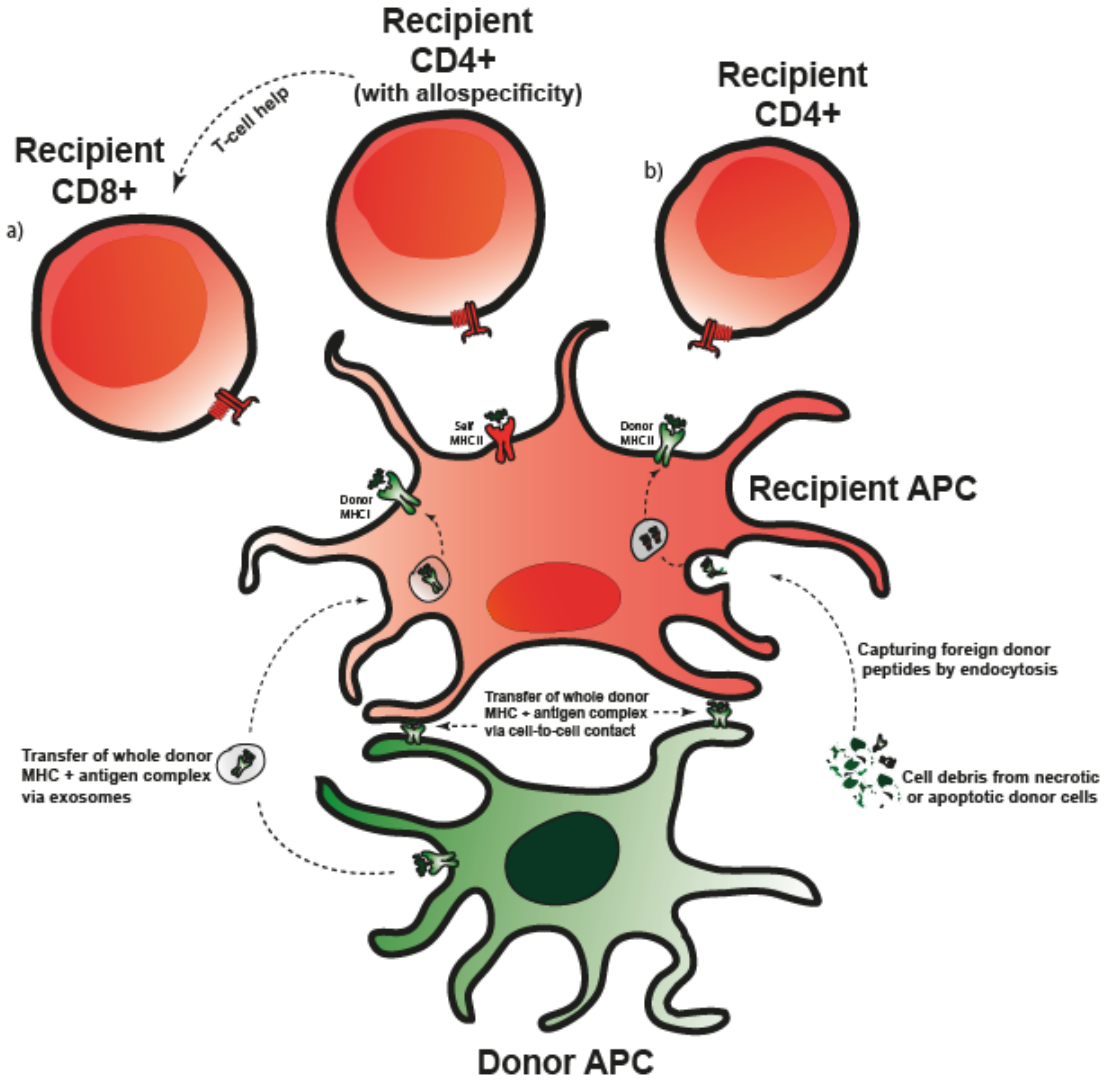

Figure 1.5. The semi-direct pathway of allorecognition. The process results from the transfer of intact donor-MHC-antigen complexes from donor APC to recipient APC by mechanisms involving cell to cell contact or exosomes. Once taken up, recipient APC either presents intact donor-MHC I or II-antigen complex directly to recipient's $CD8^+$ (a) or $CD4^+$ (b) T-cells respectively. Alternatively, recipient APC processes the donor antigen and presents it in the context of self MHC II to recipient's $CD4^+$ T-cells. Once activated, the $CD4^+$ T-cells can help $CD8^+$ T-cells with allospecificity. Adapted from Gökmen *et al*., (2008).

1.8. Immunology of MSC

1.8.1. Immunogenicity of MSC

At this point, it is important to reflect on the perceived immunoprivilege properties of MSC. As was discussed previously, any allogeneic cell that expresses MHC molecules and the appropriate co-stimulatory and adhesion molecules is capable of stimulating allogeneic T-cells via the direct, indirect or semi-direct by mechanisms

described in **Section 1.7.2**. Regardless of the pathway, the cells will be rejected. Considering the MSC phenotype described previously in **Section 1.3.3.4**, their lack of MHC II suggests that they are only potentially capable of stimulating $CD8^+$ T-cells. Their lack of the co-stimulatory molecules CD40, CD80 and CD86 makes them incapable of forming strong and longer lasting immunological synapses with $CD8^+$ cells which makes them hypoimmunogenic or poorly immunogenic and less likely to stimulate $CD8^+$ cells via the direct pathway (**Figure 1.6**). However, in light of the indirect and semi-direct pathways, it is possible to speculate that in the presence of recipient APC which can scavenge any shed MSC debris or MHC I and process them for presentation with "self" MHC II, allogeneic MSC could eventually stimulate $CD4^+$ cells by the indirect pathway. Also, after interaction with recipient APC, direct transfer of intact MHC I or indirectly through exosomes, could lead to stimulation of recipient $CD4^+$ or $CD8^+$ T-lymphocytes via the semi-direct pathway. These possibilities cannot be discounted when investigating the immunogenicity of allogeneic MSC

The evidence that allogeneic MSC are immunoprivileged came from *in vitro* LTA in which allogeneic MSC were co-cultured with PBMC. The assay is based on the rationale that when lymphocytes encounter foreign MHC-epitope complexes, they are activated and proliferate to produce effector cells which participate in the response against the epitope. The LTA will be discussed in detail in **Chapter 4**.

Klyushnenkova and co-workers tested whether human allogeneic MSC elicited a proliferative response against purified T-lymphocytes by measuring the *in vitro* uptake of radioactive tritiated thymidine (^{3}H-thymidine). Over a 10 day period, the mitotically inactivated allogeneic MSC failed to stimulate lymphocyte proliferation. This suggested that the allogeneic MSC were non-immunogenic. Treatment of allogeneic MSC with IFNγ to stimulate MHC I and II expression and retroviral transduction to stimulate expression of co-stimulatory molecules prior to co-culture with T-lymphocytes failed to stimulate lymphocyte proliferation. When mitotically inactivated allogeneic PBMC were used as stimulator cells in the control experiment, significant stimulation of the responder lymphocytes was detected (Klyushnenkova *et al.*, 2005).This evidence suggested that allogeneic MSC were incapable of stimulating T-lymphocytes via the direct pathway of allorecognition. When responder PBMC were co-cultured with allogeneic MSC, lymphocyte proliferation

was detected despite being 60 % less than control (PBMC as stimulator). The authors concluded that allogeneic MSC were immunologically inert despite them having stimulated PBMC albeit lesser than the controls. This partial stimulation could be relevant *in vivo*. In addition, the failure of allogeneic MSC to stimulate purified T-lymphocytes alone may be inadequate to suggest immunoprivilege as the presence of DC and macrophages in unseparated PBMC could facilitate allorecognition via the indirect and semi-direct pathways.

Treatment of human allogeneic MSC with IFNγ to stimulate MHC I and II expression as well as retroviral transduction to stimulate expression of co-stimulatory molecules or use of anti-CD28 antibodies prior to co-culture with T-lymphocytes failed to stimulate lymphocyte proliferation in two independent studies (Tse *et al.*, 2003; Klyushnenkova *et al.*, 2005). This was also confirmed in an allogeneic porcine model (Poncelet *et al.*, 2007). However, following intracardiac injection of allogeneic MSC *in vivo*, they were robustly rejected by the recipient animals which demonstrated that allogeneic MSC were immunogenic. This highlighted the disparities between *in vitro* and *in vivo* studies particularly the time period allowed for experiments. In these studies, the *in vitro* assays (days) were carried out in much less time in comparison to *in vivo* experiments (weeks).

In a murine model, irradiated allogeneic MSC co-cultured with mononuclear cells at a 1:1 ratio failed to stimulate allogeneic T-lymphocyte responses whereas control irradiated allogeneic MNC induced T-lymphocyte proliferation (Sudres *et al.*, 2006). The authors concluded that allogeneic MSC were non-immunogenic. The experiments were carried out in 4 days which is within the standard 3 to 5 day period commonly used in LTA. However, it can be argued that this time was insufficient to detect allorecognition via the indirect and semi-direct pathways as previously discussed in **Sections 1.7.2.2** and **1.7.2.3**.

Le-Blanc and co-workers investigated the immunogenicity of allogeneic human undifferentiated and differentiated (adipocytes, chondrocytes and osteocytes) MSC at different cell densities. In some assays, the allogeneic MSC were treated with IFNγ to stimulate expression of MHC II. The results showed that both undifferentiated and differentiated allogeneic MSC failed to stimulate PBMC after 6 days of co-culture (Le Blanc *et al.*, 2003b). This study was similar to that by Klyushnenkova and co-

workers discussed earlier in which allogeneic MSC stimulated PBMC at day 8 but not at day 6. This illustrates the effect of incubation time for the LTA. Allowing more incubation time could facilitate allorecognition via the indirect or semi-direct pathways whereas shorter incubation may only capture direct allorecognition. This was highlighted in another study which used a rabbit model in which allogeneic MSC failed to stimulate PBMC *in vitro* after 6 days incubation but were rejected *in vivo* after 12 days (Liu *et al.*, 2006). Interestingly, in a separate *in vivo* murine study, transplanted allogeneic MSC were not rejected after 28 days post-transplantation and were found to participate in wound healing (Chen *et al.*, 2009).

Overall, the evidence from *in vitro* studies suggests that allogeneic MSC are immunoprivileged but *in vivo* studies show that they are immunogenic. Thus, to date, the immunogenicity of allogeneic MSC remains controversial.

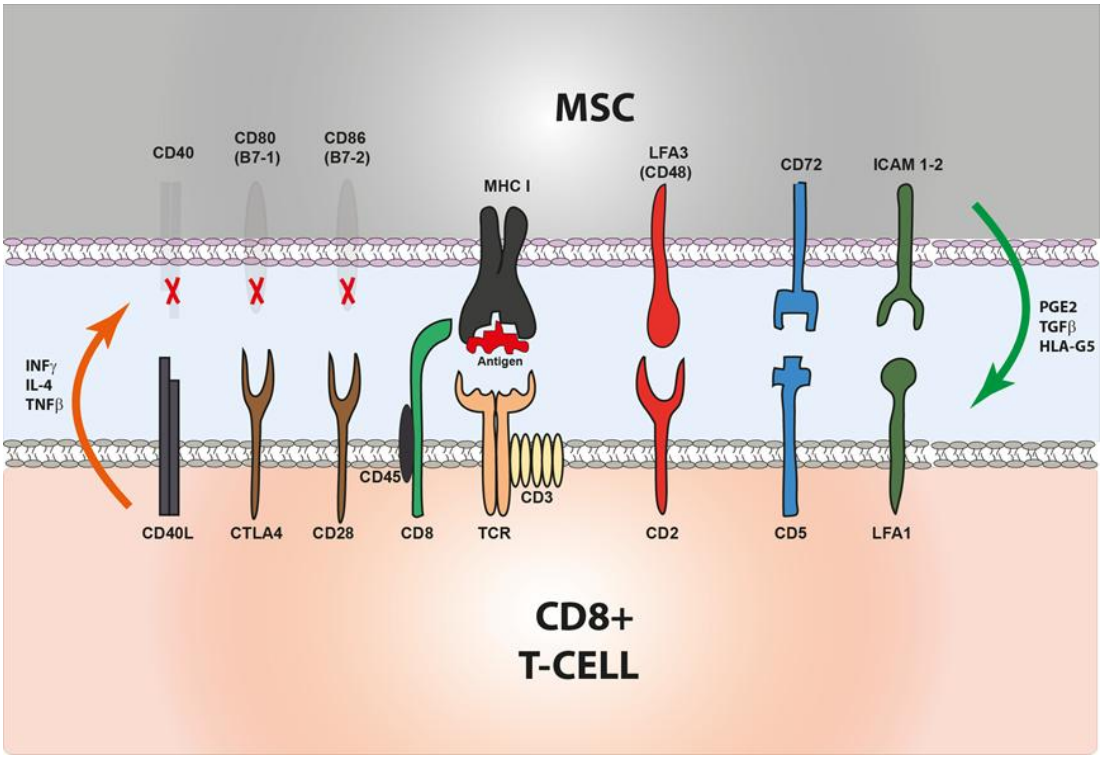

Figure 1.6. MSC antigen presentation. Due to lack of MHC II and co-stimulatory molecules CD40, CD80 and CD86, donor allogeneic MSC are unable to form a stable immunological synapse with recipient $CD8^+$ T-cells. The weak associations are thought to potentially lead to T-cell anergy, tolerance induction and eventual immunoprivilege. Adapted from Coico & Sunshine (2009).

1.8.2. MSC immunosuppression

In addition to being regarded as immunoprivileged, MSC are also generally described as a 'universal' suppressor of the immune system (Chen *et al.*, 2006). Evidence that MHC–mismatched MSC transplanted into a recipient induced tolerance to allogeneic or xenogeneic grafts was first obtained using allogeneic mouse and rat models (Ildstad & Sachs, 1984). Two decades later, these findings were reported for the first time using human bone marrow-derived MSC (Di Nicola *et al.*, 2002). Since then, a great deal of studies using animal and human MSC have been carried out using adaptations of the MLR to test their immunomodulatory properties. The MLR is described in detail in **Chapter 4**.

This assay involves the co-culture of two sets of allogeneic PBMC and results in proliferation of T-lymphocytes due to MHC disparity hence it is regarded as the *in vitro* correlate to allogeneic rejection. The incorporation of MSC in MLR has been widely reported to suppress T-cell proliferation. Although the mechanisms by which they achieve this are not fully understood, MSC are thought to actively interact with individual constituents of both the innate and adaptive immunity inducing a wide range of effects which culminate in immunosuppression, anergy and tolerance induction (Ryan *et al.*, 2005).

1.8.2.1. MSC and T-lymphocytes

Allogeneic MSC have been reported to suppress T-lymphocyte ($CD4^+$ and $CD8^+$) activation and proliferation induced by non-specific mitogens, polyclonal antibodies against CD3 and CD28 and 3rd party alloantigens in many *in vitro* models (Bartholomew *et al.*, 2002; Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Tse *et al.*, 2003; Le Blanc *et al.*, 2003b; Xu *et al.*, 2007). When treated with IFNγ to induce MHC II expression (Le Blanc *et al.*, 2003b), both allogeneic and autologous MSC equally suppressed MLR. This suggested that the immunosuppression was independent of MHC (Le Blanc *et al.*, 2003c). The degree of T-lymphocyte suppression was found to be dose-dependent; with marked suppression observed when a higher (1:10) MSC to T-lymphocyte ratio was used while lower ratios (1:100 - 1:1000) produced a stimulatory effect (Di Nicola *et al.*, 2002; Le Blanc *et al.*, 2003b). This is particularly interesting in clinical application as it suggests that

inadequate doses of allogeneic MSC could be harmful whereas high doses could potentially render patients immunocompromised.

One theory proposes that MSC may have 'veto-like' activity, acting as 'fraudulent' APC which specifically inhibit naïve T-lymphocyte clones that interact with them (Potian *et al.*, 2003). Generally, $CD8^+$ 'veto' cells are known to facilitate graft acceptance by causing the deletion of responding T-lymphocytes in a pathway mediated by TGFβ1 which has been reported to play a role in MSC-induced suppression of $CD8^+$ T-lymphocyte proliferation. Therefore, by acting directly or indirectly through other 'veto' cells, MSC may inhibit T-lymphocytes (Chen *et al.*, 2006).

Cell division arrest in T-lymphocytes exposed to both non-specific and alloantigen stimulation in the presence of either autologous or allogeneic MSC is thought to be at the G_0/G_1 phase of mitosis. Accumulation of T-lymphocytes at the G_0 phase has been detected (Uccelli *et al.*, 2006) which suggested T-lymphocyte quiescence as opposed to T-lymphocyte apoptosis. MSC have also been shown to support the survival of over-stimulated T-lymphocytes which would otherwise undergo Fas/FasL-dependent activation induced cell death (Benvenuto *et al.*, 2007). Thus, the MSC-induced T-lymphocyte anti-proliferative effect is associated with quiescence which can be reversed by IL-2 stimulation (Uccelli *et al.*, 2006).

MSC have been shown to down-regulate CTL-mediated cytotoxicity in MLR when added before the start but not during the cytotoxic phase (Rasmusson *et al.*, 2003; Angoulvant, 2004). MSC pulsed with viral peptides were protected from CTL-mediated lysis, even after IFNγ treatment to increase expression of MHC I (Morandi *et al.*, 2008) suggesting that MSC may affect the afferent phase of alloreactivity and prevent CTL-mediated cytotoxicity. Once CTL are activated, MSC have no effect suggesting they do not affect CTL (Uccelli *et al.*, 2006) but there is little evidence to support this notion.

Another school of thought suggested that MSC induced T-lymphocyte anergy after cell to cell contact. Some T-lymphocyte encounters with MHC-epitope complexes led to clonal anergy. In such cases, whether anergy ensued or clonal expansion was reinstated largely depended on the absence or presence of co-stimulatory signals

respectively. Since MSC lacked the key co-stimulatory signals, it was thought that they achieved immunosuppression by inducing T-lymphocyte anergy. However, experiments using mouse models suggested that allogeneic MSC induced T-lymphocyte tolerance, as opposed to anergy, since the lymphocytes failed to proliferate after the removal of the MSC (Glennie *et al.*, 2005; Zappia *et al.*, 2005). Earlier studies using mouse and human MSC however disagreed with this notion by suggesting that the perceived tolerance was transient and T-lymphocyte responsiveness could be restored (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003).

Another model postulated that MSC exerted immunomodulatory properties by stimulating the development of a subset of $CD4^+$ T-lymphocytes known as T-regulatory cells (T-regs). Naïve $CD4^+$ cells expressing CD25 develop into T-regs whose role is to regulate or suppress other cells in the immune system and are important in cases such as prevention of autoimmune diseases. The two types of T-regs currently known are natural T-regs ($CD4^+$, $CD25^{high}$, [1]$FoxP3^+$) which are produced by the thymus and act in an antigen non-specific manner and induced T-regs ($CD4^+$, $CD25^{+/-}$) which are antigen-specific and formed by differentiation of naïve T-lymphocytes outside the thymus; they include IL-10 secreting T-reg1 and TGFβ secreting TH-3 lymphocytes (Levings *et al.*, 2001b; Sakaguchi, 2004).

In MLR, MSC have been reported to induce IL-10 production which led to an increase in the proportion of cells expressing $CD4^+$ $CD25^{hi}$, $CD4^+$ [2]CTLA4 and $CD4^+$ $CD25^+$ $CTLA4^+$ cells in IL-2 stimulated cultures. CTLA4 is a ligand for the co-stimulatory CD80 and CD86 and is thought to transmit a T-cell inhibitory signal. In contrast, the proportion of $CD25^+$ and $CD38^+$ (a marker for cell activation) lymphocytes decreased in the presence of MSC in mitogen-stimulated lymphocyte cultures (Le Blanc *et al.*, 2004a; Aggarwal & Pittenger, 2005). However, this evidence is controversial as other studies found conflicting results (Aggarwal & Pittenger, 2005; Glennie *et al.*, 2005; Maccario *et al.*, 2005) and this was attributed to the differences in culture conditions (Chen *et al.*, 2006). Also, there is no *in vivo* data to date to confirm this notion. An important consideration is that MSC-mediated induction of T-regs may pose a concern in the clinical application of MSC as excessive T-regs activity may leave the immune system unable to fight infections.

[1] FoxP3 – Forkhead box P3
[2] CTLA4 – cytotoxic T lymphocyte antigen 4

Finally, one of the major ways in which MSC are thought to modulate T-lymphocyte function is by secretion a milieu of soluble immunoactive factors. These factors were reported not to be constitutively expressed by MSC as supernatant from MSC cultures failed to suppress T-lymphocyte proliferation (Le Blanc & Ringden, 2007). This suggested that these factors were only secreted by MSC after 'cross-talk' with other cells. Nitric oxide (NO), indoleamine-2,3-dioxygenase (IDO) and prostaglandin E_2 (PGE_2) are thought to be released by MSC after stimulation by IFNγ produced by T-lymphocytes (Ryan *et al.*, 2007).

IDO is an enzyme that converts tryptophan (essential for lymphocyte proliferation) to kynurenine. Its presence in culture causes tryptophan depletion which would inhibit T-cell proliferation. IFNγ has been reported to stimulate IDO activity in a dose-dependent manner and high IDO activity has been detected in responder T-lymphocytes stimulated by mitomycin C treated PBMC in the presence of MSC. Restoration of tryptophan restored T-lymphocyte proliferation suggesting that IDO activity acted as a T-cell inhibitory effector system (Meisel *et al.*, 2004). On the contrary, when unseparated PBMC were used as responders, tryptophan depletion was not found to be responsible for immunosuppression as apoptotic cell death was not seen which would be expected to result from tryptophan depletion (Aggarwal & Pittenger, 2005).

MSC secretion of PGE_2 when cultured with T-lymphocytes has been reported to stimulate T-regs while suppressing T-lymphocyte response. However, this suppression was reversed in the presence of PGE_2 inhibitors (Tse *et al.*, 2003; Aggarwal & Pittenger, 2005). In mouse models, IFNγ alone or in combination with other pro-inflammatory cytokines such as TNF, IL-1α, IL-1β activated the release of chemokines that attracted T-lymphocytes and produced NO which inhibited T-lymphocyte activation. MSC from mice deficient of the IFNγ receptor IFNγR1 did not exhibit immunosuppressive activity thus emphasizing the role of IFNγ in this model (Ren *et al.*, 2008). Human MSC have been reported to produce HLA-G5 which suppressed T-lymphocyte proliferation, T-lymphocyte cytotoxicity and NK) cell activity but promoted generation of T-regs. Cell to cell contact between MSC and activated T-lymphocytes stimulated IL-10 production which in turn stimulated MSC to express HLA-G5 (Le Blanc & Ringden, 2007).

Other reported soluble factors such as TGFβ, hepatocyte growth factor (HGF), IL-10, haem oxygenase-1, IL-6 and tumour necrosis factor-α (TNFα) though constitutively expressed by MSC, may be overexpressed when MSC are stimulated by cytokines released by T-lymphocytes (Uccelli *et al.*, 2006; Le Blanc & Ringden, 2007; Uccelli *et al.*, 2008). TNFα and IFNγ have been shown to increase MSC production of PGE_2 while IL-6 was shown to inhibit the maturation of DC (Djouad *et al.*, 2007).

However, in a one-way MLR, it was found that neither MSC production of IL-10, TGFβ, PGE_2 nor tryptophan depletion was responsible for T-lymphocyte suppression (Tse *et al.*, 2003).The reasons for conflicting results could be due to different experimental conditions; Tse and co-workers used unseparated mononuclear cells as responder cells (Tse *et al.*, 2003) while other studies used purified T-lymphocytes (Le Blanc & Ringden, 2007). Different culture conditions, reaction kinetics, lymphocyte populations, species-specific differences and MSC doses could significantly contribute to the differences in findings. Most studies tend to use high doses of MSC *in vitro* but such doses are far from physiological conditions where the proportion of MSC in bone marrow to mononuclear cells is less than 1:10 000 (Friedenstein & Kuralesova, 1971).

Importantly, it has been shown that inhibition of any one of the reported molecules does not lead to complete loss of immunosuppression. Thus the relative contribution of the mentioned different factors to the immunosuppressive effects of MSC varies from study to study (Uccelli *et al.*, 2008). Therefore, it is clear that the mechanisms of *in vitro* immunosuppressive effects of MSC are yet to be completely understood as current evidence suggests that none of the soluble molecules discussed has been shown to indispensable. The current view is that MSC employ a combination of cell to cell contact mechanisms and secreted soluble factors to modulate T-cell activity (**Figure1.7**).

1.8.2.2. MSC and B-cells

MSC are thought to modulate B-lymphocyte responses through cell to cell contact and soluble factor mechanisms (Uccelli *et al.*, 2006). In a mouse model, B-lymphocytes stimulated by anti-CD40 antibodies and IL-4 were inhibited by both syngeneic and allogeneic MSC at a high MSC to B-cell ratio of 1:10 to a similar

degree as observed for MSC-mediated T-cell suppression (Glennie *et al.*, 2005). Augello and co-workers employed a similar allogeneic mouse model in series of proliferation and blocking experiments which showed that MSC inhibited both B- and T-lymphocyte proliferation through direct cell contact via the inhibitory molecule programmed death-1 (PD-1) and its ligands PD-L1and PD-L2. This resulted in altered B-lymphocyte secretion of signal transduction molecules and cytokine receptors. In transwell experiments which blocked cell to cell contact, soluble factors secreted by MSC inhibited the proliferation of B-lymphocytes stimulated by pokeweed mitogen (Augello *et al.*, 2005). These findings highlighted the complexity of MSC immunomodulatory mechanisms. When human allogeneic MSC were used at an MSC to B-cell ratio of 1:1, B-lymphocyte proliferation was inhibited at the G_0/G_1 cell cycle phase without induction of apoptotic pathways. Production of the immunoglobulins (IgM, 1gG and IgA) was markedly reduced suggesting that B-lymphocyte differentiation was inhibited by MSC secreted soluble factors. Interestingly, this was only observed at the MSC to B-cell ratio of 1:1 but not at lower MSC ratios of 1:2, 1:5 and 1:10 (Corcione *et al.*, 2006). This was supported by another study in which a ten-fold lower MSC to B-lymphocyte dose apparently increased IgG secretion (Rasmusson *et al.*, 2007).

Allogeneic MSC when cultured at 1:1 ratio with B-cells reduced B-cell expression of the chemokine receptors CXCR4, CXCR5, and CCR7B. Subsequent chemotaxis to their respective ligands CXCL12, CXCL13 and CCL19 respectively was reduced. This demonstrated that MSC inhibited B-lymphocyte chemotactic ability. However, these findings could not be replicated at an MSC to B-cell ratio of 1:10. Despite these effects, B-cell cytokine production and expression of co-stimulatory molecules was unaltered by the presence of MSC (Corcione *et al.*, 2006).

Overall, these studies demonstrated that MSC modulate B-lymphocyte function at multiple levels which include cell proliferation, differentiation to immunoglobulin-producing cells, antibody production and chemotaxis (**Figure 1.7**). In most of the cases, MSC appeared to have a dual effect determined by cell dosage, with high MSC to B-lymphocyte ratio having a suppressive effect while lower ratios had a stimulatory effect. Though the individual studies have supported the potential clinical application of allogeneic MSC, collectively the data are inconclusive and contradictory.

1.8.2.3. MSC and APC and innate immune cells

Although the interactions of MSC and cells of the innate immune system have been less studied, much of the attention has been focused on DC particularly because of their important role as professional APC which link the innate and adaptive immune responses by directly presenting foreign antigens to T-lymphocytes. MSC have been reported to inhibit the differentiation, maturation and activation of DC. Altering DC functions may result in generation of tolerogenic APC (**Figure 1.7**).

MSC have been shown to inhibit the initial differentiation of monocytes to type 1 DC by downregulation of CD1a, CD86, and MHC II and later DC maturation by suppressing CD83 expression (Di Nicola *et al.*, 2002; Zhang *et al.*, 2004; Li *et al.*, 2008). In other studies, $CD34^+$ cord-derived cells failed to develop into $CD14^-$ $CD11a^+$ interstitial DC in the presence of MSC which, in the absence of MSC, would arise from an intermediate $CD14^+$ $CD11a^-$ cell type. In addition to these phenotypical changes, the ability of immature DC to stimulate allogeneic T-lymphocyte proliferation in the presence of MSC has been shown to be significantly hampered (Fibbe *et al.*, 2007). The secretion of TGFβ1 by MSC is thought to inhibit the activation and maturation of human DC (Di Nicola *et al.*, 2002).

Mature DC have been reported to exhibit decreased cell surface expression of MHC II, CD11c, CD83, co-stimulatory molecules and IL-12 production when cultured in the presence of MSC thus severely impairing the antigen presenting function (Aggarwal & Pittenger, 2005; Uccelli *et al.*, 2008). Even after maturation, MSC changed plasmacytoid DC phenotype towards the immature status by downregulating CD83 expression and increasing IL-10 secretion whose knock-on effects led to decreased IFNγ production, increased IL-4 production, downregulation of CD40, CD80 and CD86 co-stimulatory markers and an increase in the number of $CD4^+$ $CD25^+$ T-regs while inhibiting secretion of TNFα. Collectively, these effects led to a state of immunotolerance (Aggarwal & Pittenger, 2005; Maccario *et al.*, 2005). IL-10 producing type II DC have been reported to show minimal or no stimulatory effect in primary MLR and were largely inhibitory towards T-lymphocytes and are by and large functionally and phenotypically classified as inhibitory or tolerogenic cells (Chen *et al.*, 2006).

The mechanism of MSC-mediated DC suppression, though largely unknown, is thought to be mediated by soluble factors such as PGE_2 which is released upon cell to cell contact (Aggarwal & Pittenger, 2005). It has also been noted that DC cell cycle was arrested at the G_0/G_1 phase in the presence of MSC as reported with T-and B-lymphocytes. Thus suppression of lymphocyte proliferation by MSC may not be exclusively due to a direct inhibitory effect on T-lymphocytes but may be associated with the suppressive effect on DC maturation, activation and antigen presentation (Uccelli *et al.*, 2006).

1.8.2.4. MSC and natural killer (NK) cells

NK cells are an integral part of innate immunity owing to their anti-viral and anti-tumour properties. They can lyse target cells without the aid of prior immunisation. They secrete pro-inflammatory cytokines and possess cytolytic activity (Male *et al.*, 2006). Their function is tightly controlled by cell surface receptors that transduce either inhibitory or activating signals. NK-mediated cytolysis is dependent on the expression of specific ligands by the target cells together with aberrant, low-level or absent expression of MHC I which is detected by NK cell-MHC I specific inhibitory receptors (Male *et al.*, 2006; Uccelli *et al.*, 2008).

MSC have been shown to suppress NK cell cytotoxicity by downregulating the expression of NKp30 and NKG2D receptors which are important in NK cell activation and target cell cytolysis (Spaggiari *et al.*, 2006). NK cells proliferate and acquire strong cytolytic activity in the presence of IL-2 and IL-15 however when MSC are introduced, NK cell proliferation and IFNγ production has been shown to be diminished (Aggarwal & Pittenger, 2005; Spaggiari *et al.*, 2006; Selmani *et al.*, 2008). Activated NK cells however have shown significantly high resistance to MSC effects (Spaggiari *et al.*, 2006). IL-15 activated NK cells have been shown to kill both allogeneic and autologous MSC *in vitro* but this was not shown with resting NK cells (Rasmusson *et al.*, 2003). Susceptibility of MSC to NK-mediated killing is largely believed to be due to their low expression of MHC I molecules and expression of several ligands that are recognized by activating NK receptors (Spaggiari *et al.*, 2006). MSC incubation in the presence of IFNγ has been shown to have a protective effect from NK-mediated cytotoxicity through the upregulation of MHC I expression by MSC (Spaggiari *et al.*, 2006; Uccelli *et al.*, 2008). As was

observed with T-lymphocytes, soluble factors such as TGFβ1 and PGE_2 are thought to play a role in the MSC-mediated suppression of NK cell proliferation (Uccelli *et al.*, 2006).

MSC interaction with NK cells has been shown to be dependent on the microenvironment; the presence of excess IFNγ *in vivo* may favour NK suppression by MSC whereas if IFNγ is limiting, the balance shifts towards the destruction of MSC by activated NK cells (**Figure 1.7**), the latter outcome is significant if MSC are to be used in clinical application. Potential application of MSC to help downregulate the abnormal activation of uterine NK cells which are one of the major causes of recurrent pregnancy loss is a promising prospect after the successful *in utero* transplantation of human MSC during the first trimester of pregnancy in a sheep model (Liechty *et al.*, 2000).

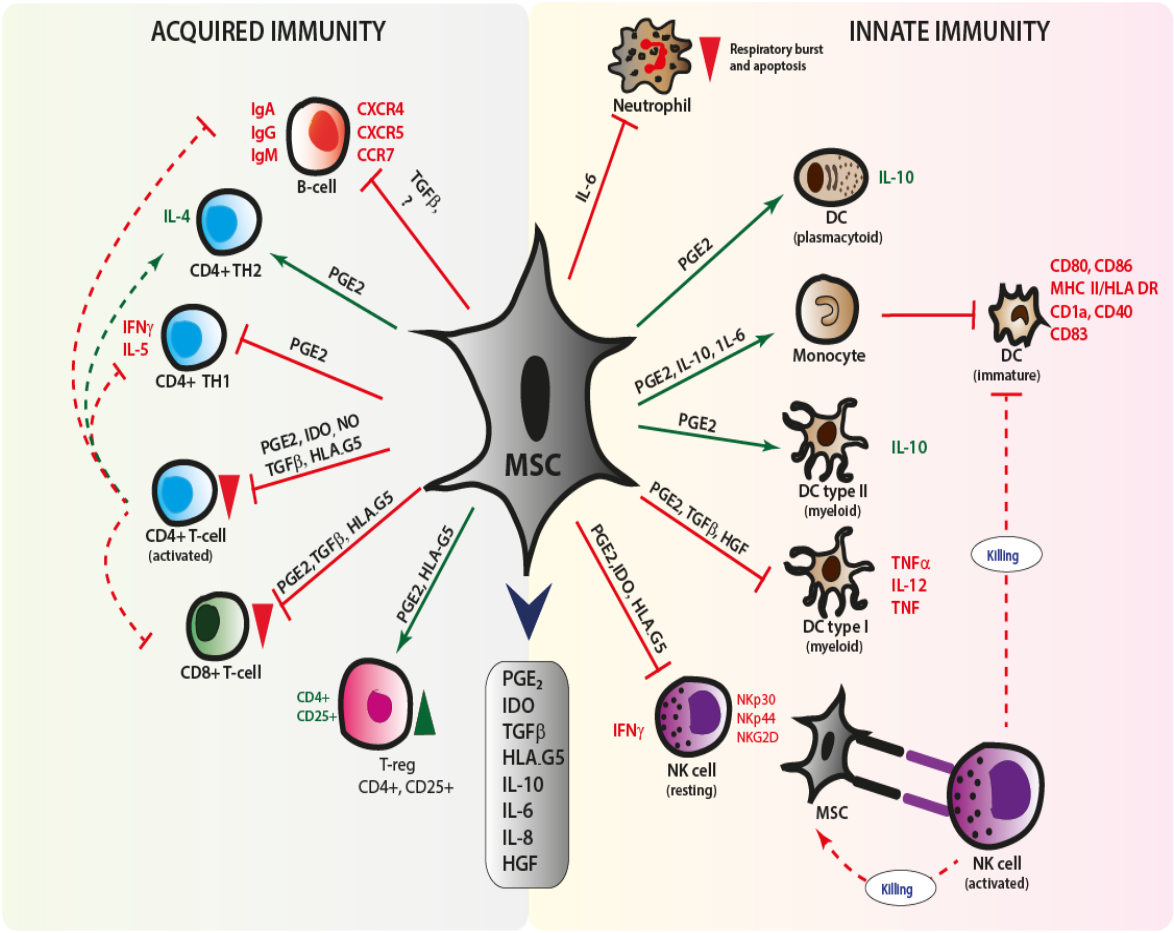

Figure 1.7. Mechanisms of MSC-mediated immunomodulation of the innate and adaptive immune cells *in vitro*. MSC suppress $CD4^+$, $CD8^+$ T and B-lymphocyte stimulation while promoting the expansion of $CD4^+$ $CD\ 25^+$ T-regs which further inhibit immune responses. They also suppress the activation of NK cells and neutrophils as well as inhibiting the growth and maturation of type I myeloid DC while activating IL-10 secretion by type II DC which results in the inhibition of immune responses. MSC achieve these effects by a combination of cell to cell contact and secretion of immunoactive molecules such as PGE_2, IDO, IL-10 and HLA-G5. Adapted from Uccelli, (2006).

1.9. Differentiated MSC and allorecognition

The ability to differentiate into new cell lineages is an important quality of MSC which provides different options for their application. Undifferentiated MSC alone offer so much promise in the field of cell-based therapeutics but differentiated MSC offer a much wider platform for application particularly in tissue engineering and regenerative medicine as discussed earlier. Tissue, repair, regeneration and replacement rely on the ability of MSC to differentiate and form new cells that provide similar biological, physiological and structural functions to the cells/tissues they seek to replace. Therefore, the immunological properties of differentiated MSC

also require thorough investigations. This prospect is more challenging considering that the immunogenicity of undifferentiated allogeneic MSC remains controversial.

MSC can be routinely differentiated into adipocytes, chondrocytes and osteoblasts either by chemical, hormonal or mechanical stimulation as discussed in **Section 1.3.3.6**. This process relies upon the differential expression of lineage specific genes but more importantly their subsequent translation into functional proteins which alter the phenotype and function of the cells. This could have immunological consequences if the change in phenotype brings with it the expression of new surface antigens such as MHC II or secretion of immunostimulatory molecules. Since MSC are considered to be immunoprivileged, the key question would be whether they retain that status even after undergoing terminal differentiation. The challenges posed by the use of differentiated MSC are demonstrated by the work carried out by Chen and co-workers on rat MSC. Following tri-lineage differentiation, chondrogenic, but not adipogenic or osteogenic-differentiated MSC expressed the co-stimulatory molecules CD80 and CD86. The chondrogenic cells subsequently activated human DC and stimulated lymphocyte proliferation in contradiction to their undifferentiated precursors (Chen *et al.*, 2007). In sharp contrast, using a rabbit model, Liu and co-workers showed that osteogenic-differentiated MSC inhibited lymphocyte proliferation significantly more than undifferentiated MSC and secreted more IL-10 and TGFβ. Surprisingly this was reversed following IFNγ treatment. Osteogenic-differentiated MSC's lack of MHC II even after differentiation meant they failed to stimulate allogeneic lymphocytes in co-cultures (Liu *et al.*, 2006). The effects of differentiated MSC on their immunomodulatory properties are discussed widely in **Section 6.4** and **Section 7.3**.

Finally, it is important to note that chemicals used in the differentiation medium can confound the findings in some studies. For instance, indomethacin, a common constituent in most adipogenic differentiation media, is a known inhibitor for cyclooxygenase I and II (COX-I and COX II) enzymes which are involved in the synthesis of PGE_2 hence its increased presence in culture medium supernatants. It could be possible that the presence of indomethacin in culture could affect the production of PGE_2, a key molecule in the immunosuppressive milieu secreted by MSC. This could affect the overall immunomodulatory properties of the differentiated MSC. However, no study to date has investigated the role of chemicals

commonly used in differentiation cocktails. It is important to note at this point that the immunological properties of differentiated MSC are as controversial as their undifferentiated precursors.

1.10. Are MSC immunomodulatory properties unique to stem cells? Enter dermal fibroblasts (DF)

As we have seen so far, the immunomodulatory properties of MSC have been a key area of research over the past decade and studies are focused on bringing them from the 'bench to the bedside'. An important question to ask is whether the inherent hypoimmunogenicity and immunomodulatory properties displayed by MSC in a wide range of human and animal models discussed thus far are a unique property of stem cells or are shared by other related cells. If the latter is true, this would open up opportunities to exploit other cells for clinical use.

Another important finding based on some studies suggested that osteoblasts, chondrocytes and adipocytes obtained after MSC differentiation retained their immunosuppressive and immunoprivileged status (Le Blanc *et al.*, 2003b). This might imply that other differentiated cell types could potentially exhibit similar properties and thus potentially amenable to similar application.

Fibroblasts have long been thought to be related to MSC. In fact, Friedenstein initially described MSC as 'fibroblast-like' cells (Friedenstein *et al.*, 1970a; Friedenstein *et al.*, 1974). Over time, it has been shown that the resemblance goes beyond morphology. Fibroblasts were first thought of as primitive cells (Kindblom & Angervall, 1978) but have recently been shown to express most of the markers used commonly expressed by MSC (Haniffa *et al.*, 2007). They have also been shown to undergo differentiation into the typical mesenchymal cell lineages such as adipocytes, chondrocytes and osteocytes (Wagner *et al.*, 2005; Haniffa *et al.*, 2007; Lorenz *et al.*, 2008). In fact, soon after human MSC were defined at the turn of the century (Pittenger *et al.*, 1999), Toma and co-workers, in a bid to find new MSC niches, isolated cells from the dermis of mouse skin which were capable of neural, adipogenic and myogenic differentiation and long-term self-renewal (Toma *et al.*, 2001) which they later described as skin precursors cells [SKP] (Toma *et al.*, 2005).

In terms of immunomodulatory properties and immunoprivileged status, prior to the MSC-era, fibroblasts from various tissues have been reported to inhibit mitogen or alloantigen-stimulated lymphocyte proliferation and IFNγ secretion (Korn, 1981; Shimabukuro *et al.*, 1992; Donnelly *et al.*, 1993), properties which are associated with MSC as discussed earlier. Importantly, DF have also been shown to secrete immunomodulatory molecules such as PGE_2, IDO, HGF and TGFβ in the presence of stimulated lymphocytes with the suppressive effect enhanced by IFNγ and TNFα (Donnelly *et al.*, 1993; Krampera *et al.*, 2006; Haniffa *et al.*, 2007). MHC II expression by DF was similarly induced by treatment with IFNγ and TNFα (Haniffa *et al.*, 2009). In the presence of IL-6, DF have been shown to inhibit monocyte differentiation into mature DC in a similar manner reported for MSC (Chomarat *et al.*, 2000). However, Saalbach and co-workers demonstrated that DF promoted DC maturation (Saalbach *et al.*, 2007). Direct comparison of MSC and fibroblasts from different tissue sources showed similar *in vitro* immunosuppressive properties (Bocelli-Tyndall *et al.*, 2006; Haniffa *et al.*, 2007). It has also been shown that both cell types induced non-apoptotic T-lymphocyte arrest using a mechanism that arrested cell division at the G_0/G_1 phase (Filer *et al.*, 2006; Jones *et al.*, 2007).

Collectively, the data available in the literature clearly shows that MSC are indistinguishable from fibroblasts and importantly share similar immunomodulatory properties. However, these studies have focused on the immunosuppressive properties of these cells but not their immunogenicity. The most recent attempt at distinguishing MSC from fibroblasts employed the detection of anti-inflamatory and angiogenic factors such as vascular endothelial growth factor (VEGF), HGF and several angiopoietins. The authors concluded that MSC displayed strong angiogenic activity in comparison to DF. Upon priming with TNFα, MSC displayed anti-inflammatory activity whereas DF were pro-inflammatory (Blasi *et al.*, 2011). Despite these variations, the overall immunomodulatory behaviour of MSC and DF remained similar. It is possible to speculate that the immunomodulatory properties described for undifferentiated MSC could be shared by other stromal cells such as differentiated MSC and fibroblasts.

1.11. Aim and objectives

1.11.1. Background

The major challenge hindering the clinical application of allogeneic MSC is the risk of rejection due to immunological incompatibility. As discussed throughout this chapter, there is overwhelming evidence regarding their immunomodulatory properties as tested in both human and animal models under different experimental conditions. These properties are broadly classified as immunosuppression and immunoprivilege.

Immunosuppression is thought to be achieved through mechanisms that involve cell to cell contact and secretion of immunomodulatory molecules. This is thought to result in the inhibition of on-going non-specific and specific immune responses against immunostimulatory mitogens and antigens. This property could be useful in therapeutic interventions to prevent allogeneic transplant rejection.

Immunoprivilege is thought to be due to the inherent hypo- or non-immunogenic nature of MSC as a result of their lack of co-stimulatory molecules (CD40, CD 80 and CD 86) and MHC II as well as partial expression of MHC I. This is thought to make them incapable of stimulating T-cell responses in an allogeneic setting. Many authors believe that immunoprivilege would allow allogeneic MSC to be used as 'universal donor' cells in engineering 'off-the-shelf' tissues and organs that are transplantable across histocompatibility barriers without the risk of rejection.

The studies in support of these claims used *in vitro* human and animal models of allogeneic transplantation by way of MLR to investigate immunosuppression and LTA to investigate immunoprivilege. These tests are the 'gold-standard' immunological methods used for determining alloreactivity of responder lymphocytes to stimulation by allogeneic stimulator cells in histocompatibility tests usually lasting between 3-5 days.

1.11.2. Hypothesis

The lack of co-stimulatory molecules CD40, CD80, CD86 and MHC II expression by MSC implies that they are incapable of stimulating allogeneic lymphocytes by the direct pathway of allorecognition in LTA. However, the hypothesis of this study was that despite their apparent lack of MHC II and co-stimulatory molecules, MSC could

stimulate allogeneic lymphocytes via the indirect and semi-direct pathways (chronic rejection). This is testable *in vitro* by extending the culture period beyond the traditional 3-5 days to 15 days. An allogeneic mouse model system was employed to test this hypothesis.

1.11.3. Why mouse model?

A mouse model system was preferred to human cells for the following reasons;

- There is minimal variation in terms of lymphocyte 'identity' in a mouse model since the same strains were used throughout the study. It may not be possible to obtain lymphocytes from the same human donors over a 3 year period and hence use of different lymphocyte donors can confound the results due to the differences in cell quality and immunological state of the donors at any given time.
- Mouse models allowed better 'quality' MSC to be used in tests unlike human MSC which are generally obtained from elderly patients undergoing hip replacement procedures. MSC can be obtained from healthy and younger sources which are known to demonstrate better stem cell properties than 'mature' MSC.
- Use of genetically mismatched strains allowed the testing of MSC in *bona fide* MLR where the allogeneic MSC are genetically matched to the stimulator lymphocytes. With human cells, it is difficult to obtain both MSC and lymphocytes from the same donor. Thus, most MLR experiments with human MSC use 3rd party MSC which are allogeneic to both the responder and stimulator lymphocytes and though referred to as MLR in most studies, such a system does not represent a true MLR.
- In a mouse model, syngeneic controls (equivalent of autologous) can be set up to allow for more accurate comparison with allogeneic experiments which may not be possible when using human cells.

1.11.4. Aim

This study sought to systematically investigate the immunological properties of allogeneic MSC namely immunosuppression and immunogenicity using a mouse model system which used two genetically distinct strains; Balb/c (H2-*d*) and C3H (H2-*k*) which were used as responder (recipient) and stimulator (donor) respectively.

DF were tested alongside MSC as a control for mesenchymal non-stem cells in order to determine if the immunomodulatory properties were unique to stem cells or were shared by other mesenchymal cells.

1.11.5. Objectives

The objectives of the study were divided into four broad sections namely;

1. To isolate, expand and characterise MSC from bone marrow (femoral) and DF from abdominal skin of Balb/c and C3H mice
2. To define the most suitable conditions for performing MLR and LTA using splenic and lymphoid (inguinal and axillary) mononuclear cells from Balb/c and C3H mice.
3. To investigate the immunosuppressive properties and immunogenicity of allogeneic (C3H) and syngeneic (Balb/c) MSC and DF using adaptations of the MLR (one-way and 2-way) and LTA respectively.
4. To investigate the immunosuppressive properties and immunogenicity of chondrogenic-differentiated allogeneic and syngeneic MSC and DF using adaptations of the MLR (one-way and 2-way) and LTA respectively.

Objective 1 is covered in detail in **Chapter 3**, objective 2 in **Chapter 4**, objectives 3 and 4 in **Chapter 5**.

2. CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.1. Materials

All the equipment, plastic ware, general consumables, chemicals and reagents used in this study are listed in the **Appendix**. Specific reagents and chemicals used in specific experiments including their preparation and storage are listed at the beginning of each chapter.

2.1.1. Animals

Female Balb/c (H2-d) and C3H (H2-k) mice (4-8 weeks) were obtained from Harlan Laboratories, UK and kept under standard animal housing settings in the University of Leeds Central Biomedical Services with food and water administered *ad libitum*.

2.1.2. Glassware

All laboratory glassware used in this study was sourced from Thermo Fisher Scientific, Loughborough, UK unless stated otherwise.

2.1.3. Dissection kits

Dissection kit comprising standard forceps, rat tooth forceps, surgical scissors and scalpel handle (No 4) were supplied by Thackray Instruments, Leeds, UK.

2.2. Methods

2.2.1. Disinfection and sterilisation

2.2.1.1. Washing and disinfection

2.2.1.1.1. Dissection instruments

Dissection kits, cell sieves and glass pestles used for dissection and cell harvesting were washed with running hot water, disinfected with 1 % (v/v) Virkon disinfectant for 20 minutes and washed in running tap water for 5 minutes followed by air drying and dry heat sterilisation.

2.2.1.1.2. Tissue culture cabinets

Class II safety cabinets used for carrying out aseptic techniques for cell harvesting and culture were thoroughly cleaned with 1 % (v/v) Virkon followed by 70 % (v/v) ethanol before and after use. All items entering the safety cabinets including medium bottles, test-tube racks and gloved hands were first sprayed with 70 % (v/v) ethanol as a disinfection step.

2.2.1.2. Dry heat sterilisation

All items and objects were placed in a hot air oven and held at a temperature of 190 ºC for 4 hours.

2.2.1.3. Moist heat sterilisation

Items and solutions unsuitable for dry heat sterilisation were sterilised by autoclaving at 121 ºC for 20 minutes and 15 psi[3] pressure.

2.2.1.4. Filter sterilisation

Solutions unsuitable for autoclaving were filtered through single-use Ministat™ 0.2 μm syringe filters (Sartorius Ltd, UK) using disposable syringes in a class II safety cabinet.

2.2.2. Culture media and reagents

All radioactive ^{3}H-thymidine stocks were purchased, handled, used and disposed of in accordance to the University health and safety regulations for radioactive substances. All assays involving use of radioactive chemicals were carried out in specially designated workspaces.

2.2.2.1. Minimum essential medium (α-MEM)

α-MEM medium was supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine and 100 μg.ml^{-1} penicillin/streptomycin.

2.2.2.2. Dulbecco's modified Eagle's medium High glucose (DMEM-HG) culture medium

DMEM-HG was supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 100 μg.ml^{-1} penicillin/streptomycin.

[3] psi; pounds per square inch

2.2.2.3. Dulbecco's modified Eagle's medium Low glucose (DMEM-LG) culture medium

DMEM-LG was supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 100 $\mu g.ml^{-1}$ penicillin/streptomycin.

2.2.2.4. Cryopreservation medium (cryo-medium)

Cryo-medium was made by supplementing DMEM-LG (for MSC) or DMEM-HG (for DF) with 20% (v/v) FCS, 100 $\mu g.ml^{-1}$ penicillin/streptomycin and 10% (v/v) dimethyl sulphoxide (DMSO).

2.2.2.5. MesenCult™ culture medium

MesenCult™ mesenchymal stem cell basal medium was supplemented with 20 % (v/v) MesenCult™ mesenchymal stem cell stimulatory supplements and 100 $\mu g.ml^{-1}$ penicillin/streptomycin.

2.2.2.6. StemXVivo™ culture medium

StemXVivo™ mouse mesenchymal stem cell medium was supplemented with 100 $\mu g.ml^{-1}$ penicillin/streptomycin.

2.2.2.7. Lymphocyte culture medium

Lymphocyte culture medium was made by supplementing Rosslyn Park Memorial Institute 1640 (RPMI-1640) medium with 10 % (v/v) FCS, 20 mM [4]HEPES buffer, 2 mM L-glutamine and 50 μM β-mercaptoethanol.

2.2.2.8. Lymphocyte transport medium

Transport medium was made by supplementing RPMI-1640 medium with 20 mM HEPES buffer and 100 $\mu g.ml^{-1}$ penicillin/streptomycin.

2.2.2.9. Mitomycin C stock solution

Mitomycin C from *Streptomyces caespitosus* supplied in powder form and classified as a highly toxic substance was opened and weighed in a specially designated 'Poisons room' (Level 4, Garstang South). Stock solutions of 1 $mg.ml^{-1}$ were made by dissolving mitomycin C into sterile PBS without calcium (Ca^{2+}) or magnesium (Mg^{2+}). The 1 $mg.ml^{-1}$ stock solution was aliquoted into clean sterile Bijous which

[4] HEPES; [N-(2-hydroxyethyl) piperazine N;-(2-ethansulphonic acid)] buffer

were individually wrapped in aluminium foil and stored at -20 ºC until use. On day of use, frozen mitomycin C was first thawed in a water bath at 37 ºC before being added into lymphocyte culture medium at the appropriate concentration.

2.2.2.10. Concanavalin-A (Con-A) stock solution

Concanavalin A (Con-A) lyophilised powder was opened and weighed in a specially designated Poisons room. Stock solution was made by dissolving in phosphate buffered saline (PBS) to make a 1 $mg.ml^{-1}$ solution which was divided into 200 µl aliquots and kept at -20 ºC until use.

2.2.2.11. Phytohemagglutinin (PHA) stock solution

PHA lyophilised powder was opened and weighed in a specially designated Poisons room. Stock solution made by dissolving in PBS to make a 1 $mg.ml^{-1}$ solution which was divided into 200 µl aliquots and kept at -20 ºC until use.

2.2.2.12. Camptothecin stock solution

Camptothecin was opened and weighed in a specially designated Poisons room. A 1 $mg.ml^{-1}$ stock solution was made by dissolving in Hank's balanced salt solution (HBSS).

2.2.2.13. 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) stock solution

A 5 mM stock solution was made by dissolving CFSE in DMSO. The solution was divided into 100 µl aliquots which were individually wrapped with aluminium foil and kept at -20 ºC.

2.2.2.14. Low activity ^{3}H thymidine

Low activity ^{3}H-thymidine (specific activity – 6.7 $Ci.mmol^{-1}$; concentration – 1 $mCi.ml^{-1}$) was diluted with RPMI-1640 medium (1:40 dilution ratio) to give a working solution with a final radioactivity concentration of 25 $\mu Ci.ml^{-1}$. The solution was aliquoted into 2 ml vials and stored at 4 ºC until use.

2.2.2.15. Saline solution (0.85 % w/v)

Saline solution (0.85 % w/v) for use during cell harvesting (**Section 2.2.13**) was made by dissolving 0.85 g sodium chloride in 1 litre of distilled water. The solution was kept at room temperature until use.

2.2.2.16. Substrate solution (ATPLite™)

ATPLite™ lyophilized substrate (77 mg) was reconstituted in 5 ml of ATPLite™ substrate buffer at room temperature in a light proof container and used fresh or kept frozen at -20 °C in tubes wrapped with light proof foil.

2.2.2.17. Trichloroacetic acid (10 % w/v) solution (TCA)

TCA (10 % w/v) working solution for use during cell harvesting (**Section 2.2.13**) was made by dissolving 100 g TCA in 1 litre of distilled water. The solution was kept at room temperature in Duran bottle until use.

2.2.3. pH measurement

The pH of solutions was measured using a Jenway 3020 pH meter calibrated using standards of pH 4, 7 and 10. Solutions to be measured were gently stirred and stable readings were taken. To adjust the pH of solutions, 1-6 M hydrochloric acid or 1-6 M sodium hydroxide solutions were added drop wise whilst stirring.

2.2.4. Mouse sacrifice and dissection

Mice were humanely sacrificed under Schedule 1 guidelines by controlled CO_2 asphyxiation and death confirmed by cervical dislocation. The mice were then transferred to the laboratory where dissection was immediately carried out under sterile conditions using sterilised dissection instruments. Mice were first sprayed with 70 % (v/v) ethanol to disinfect the fur and pinned on a dissection board ventral side up.

For MSC isolation, femurs were dissected and all attached epiphyses carefully removed to minimize contamination by other cells before femoral aspiration (described in **Section 3.2.1)**. For isolation of DF, mouse abdominal skin was first shaven clean with a sterile scalpel to remove fur and then small pieces of skin (approximately 2 cm x 2 cm) carefully cut out, soaked in 70 % (v/v) ethanol for 5 minutes to further disinfect them before dispase II digestion as described in S**ection**

3.2.3. For isolation of mononuclear cells, an incision down the mid ventral line of the skin was made and the skin pinned backwards to reveal the areas inside the skin below the four limbs. From each mouse, peripheral lymph nodes (two inguinal and two auxiliary) and the whole spleen were carefully removed and placed in sterile petri dishes containing transport medium before cell harvesting as described in **Section 2.2.11**.

2.2.5. Maintenance and culture of mouse primary cells

All cell culture procedures were carried out under aseptic conditions in a Heraeus Hera Safe Class II bio-safety cabinet (Thermo Electron Corporation, USA. Primary cells cultures (MSC and DF) extracted from Balb/c and C3H mice were cultured in Nunclon™ Δ surface 75 cm^2 (10 ml culture medium) and 125 cm^2 (20 ml culture medium) cell culture flasks with culture medium (DMEM-LG for MSC and DMEM-HG for DF) which was changed every 2-3 days. The cultures were incubated at 37 ºC in an atmosphere of 5% (v/v) CO_2 in air in a SANYO MCO-20AIC (SANYO Biomedical BV, Holland) humidified incubator.

2.2.6. Cell passaging

Upon reaching 80-90 % confluence, culture medium from the flasks was aspirated using sterile glass Pasteur pipettes. The cells were washed twice with PBS without Mg^{2+} or Ca^{2+} (10 ml for 75 cm^2 and 20 ml for 125 cm^2) to ensure removal of culture medium constituents especially FCS which inhibits cell dissociation agents. Hy-Q-Tase™ cell dissociation solution (for MSC) or 0.5 % (v/v) trypsin-EDTA solution (for DF) were added to dislodge and disaggregate the monolayer of cells from the bottom of the flask into suspension (2 ml for 75 cm^2 and 3 ml for 125 cm^2). For MSC cultures, flasks were kept at room temperature in the class II cabinet for 3-5 minutes while for DF, flasks were kept at 37 ºC in 5% (v/v) CO_2 in air in a humidified incubator for 5-8 minutes. Complete cell detachment was checked under an Olympus CK40-SLP light microscope. The resultant cell suspensions were transferred into 50 ml centrifuge tubes to which equal volumes of culture medium were added to inactivate the cell dissociation agents. The cell suspensions were then centrifuged for 10 minutes at 180 *g* and the supernatant discarded while the resulting cell pellet was re-suspended in fresh culture medium and split into a 1:3 ratio. The cells, now assigned the next consecutive passage number, were plated in 75 cm^2 or 125 cm^2

flasks with fresh culture medium added and incubated at 37 ºC in an atmosphere of 5% (v/v) CO_2 in air.

2.2.7. Microscopy

Mouse MSC and DF were analysed for morphology before and during differentiation using an Olympus IX71 inverted microscope set up for phase contrast and images captured using an attached Evolution MP digital camera controlled though Image-Pro® Plus imaging software 4.5 (Media Cybernetics) or an Olympus digital camera controlled through Cell^B image acquisition software (Olympus).

2.2.8. Cell counting by the Trypan blue dye exclusion method

Trypan blue is a dye which when mixed with a cell suspension allows viable cells to be distinguished from non-viable/dead cells by virtue of cell membrane integrity. The dye permeates into non-viable cells due to loss of cell membrane integrity whereas viable cells with intact cell membranes, actively excludes the dye. Thus non-viable cells are coloured blue (and therefore excluded from the count) while viable cells appear colourless when visualised under a light microscope. To perform a cell count, 20 µl of thoroughly resuspended cell suspension was mixed with an equal volume of 0.4 % (v/v) Trypan blue dye in a Bijou vial and 20 µl of the mixture loaded carefully into each chamber of an improved Neubauer counting chamber. Viable cells were counted under an Olympus CK 40-SLP light microscope (x100 magnification) and enumeration aided by a cell counter. The whole chamber had a depth of 0.1 mm and was divided into 9 large squares each with an area of 1 mm^2 and therefore would contain 0.1 mm^3 (10^{-4} ml) volume of cell suspension. The centre square was further divided into 25 small squares each containing 0.004 mm^3 (4×10^{-6} ml).

For counting MSC and DF, viable cells were counted from *n* of the 9 large squares (*n* = number of large squares in which the viable cells count was between 100 and 300) and the mean count contained in 0.1 mm^3 obtained by dividing by *n*. For counting mononuclear cells, viable cells were counted from 5 of the 25 small squares in the large centre square (4 corner squares plus central square). The total count was then divided by 5 to obtain the mean count per square. This value was then multiplied by 25 to obtain the number of cells in 0.1 mm^3. This was then multiplied by 10^4 and the

dilution factor to obtain the number of viable cells per millilitre as shown by the formula below;

$$\textit{Viable count}\left(\textit{cells}.\textit{ml}^{-1}\right) = \textit{Mean cell count per large square} \times 10^4 \times \textit{d.f.}$$

Where d.f. = dilution factor due to dilution of cell suspension with Trypan blue (in this case d.f. is 2).

2.2.9. Cryopreservation of MSC and DF

Mouse MSC and DF cell cultures for future use were harvested from culture flasks (80-90 % confluence) as described in **Section 2.2.6**, resuspended in cold cryo-medium (**Section 2.2.2.4**), quickly counted by the Trypan blue method (**Section 2.2.8**) and adjusted to 10^5 cells.ml^{-1}. The cells were then aliquoted into pre-chilled cryovials (1 ml per vial) which were immediately placed in a Nalgene freezing container filled with isopropanol and placed into a -80 ºC freezer for a minimum of 4 hours to provide a cooling rate of -1 °C.min^{-1}. The vials were then transferred to liquid nitrogen (-196 °C) for long term preservation.

2.2.10. Resurrection of frozen mouse primary cells

Cryovials containing frozen cells (MSC or DF) were removed from liquid nitrogen storage and defrosted in a 37 °C water bath. The cells were transferred into 25 ml centrifuge tubes and pre-warmed culture medium was added to make up 15 ml before centrifugation at 180 *g* for 10 minutes to remove the DMSO which is toxic to cells on prolonged exposure. This washing step was repeated, each time discarding the supernatant and the cells were finally resuspended in fresh culture medium and plated in 75 cm^2 tissue culture flasks in a volume of 10 ml. The flasks were then incubated at 37 °C in 5 % (v/v) CO_2 in air and maintained as described in **Section 2.2.5**.

2.2.11. Isolation of mononuclear cells from mouse peripheral lymph nodes and spleens

Lymph nodes and spleens were isolated from either Balb/c or C3H mice and placed in petri-dishes containing transport medium as described in **Section 2.2.4**. In a class II bio-safety cabinet, the lymph nodes and spleens were placed in a 100 μm fine mesh steel sieve using sterile forceps. They were chopped into smaller pieces using a

scissors and gently teased and pressed through the mesh using a sterile glass pestle and washed with transport medium to release single cells from the tissue matrix which were collected in a clean petri-dish. The resulting cell suspension was transferred into sterile plastic test tubes and left to stand for 15 minutes to allow the debris to settle at the bottom under gravity while the mononuclear cells remained in suspension. The cell suspension was then carefully transferred into fresh test tubes using sterile glass Pasteur pipettes and centrifuged at 450 *g* for 10 minutes with the resulting supernatant carefully decanted leaving behind approximately 0.5 ml fluid and cell pellets at the base of each tube. The cell pellets were then resuspended into the remaining fluid by gently bashing the tubes against each other after which 6.5 ml of lymphocyte culture medium was added to each tube and the contents gently mixed by inversion.

The cell suspensions were then gently layered into test tubes containing 3 ml of Lymphoprep™ gradient solution (10 ml total volume) while ensuring that the cell suspension formed a layer above the Lymphoprep™ and the tubes were centrifuged at 800 *g* for 30 minutes. The Lymphoprep™ gradient separates mononuclear cells which remain in suspension and form a white band at the Lymphoprep™ / transport medium interface from erythrocytes and granulocytes which settle at the bottom of the tube as illustrated in **Figure 2.1**. Mononuclear cells have a lower buoyant density less than 1.077 $g.ml^{-1}$ compared to other blood cells. Using glass Pasteur pipettes, the mononuclear cell bands were carefully removed and transferred to clean test tubes to which fresh lymphocyte culture medium was added to make up the volume in each tube to 10 ml. The cells were then centrifuged at 450 g for 10 minutes to wash off any Lymphoprep™. The supernatant was decanted leaving behind whitish cell pellets of mononuclear cells which were resuspended in 5 ml of lymphocyte culture medium and counted by the Trypan blue method (**Section 2.2.8**) prior to use in assays.

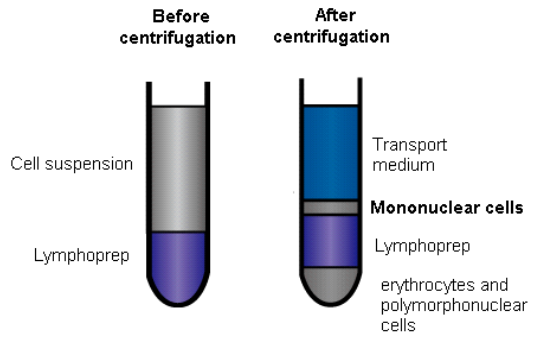

Figure 2.1. Schematic showing separation of mouse mononuclear cells from lymphoid and splenic cell suspensions using Lymphoprep™ density gradient method. After centrifugation, mononuclear cells settle at the Lymphoprep™/medium interface as a whitish band.

2.2.12. Dosing mononuclear cell cultures with ^{3}H-thymidine

Assays involving use of ^{3}H-thymidine were carried out in specially designated workspaces. Cell cultures assayed for ^{3}H-thymidine uptake were spiked with 10 µl per well of low activity ^{3}H-thymidine (25 µCi.ml^{-1}) to give a final concentration of 0.25 µCi per well. Spiking was carried out 16 hours before the end of the specific assay period. After dosing, cell cultures were returned for incubation in a humidified incubator at 37 °C in an atmosphere of 5% (v/v) CO_2 in air. At the end of the culture period, cell cultures were harvested (**Section 2.2.13**) to facilitate the detection of ^{3}H-thymidine incorporated in the DNA.

2.2.13. Cell harvesting using the FilterMate™ cell harvester and scintillation counting using the TopCount™ scintillation and luminescence counter

A FilterMate™ cell harvester was used to process cell cultures to facilitate the detection of ^{3}H-thymidine incorporated into cellular DNA. Cells cultured in U or V bottom 96-wellplates were harvested by vertical vacuum suction onto 96-well UniFilter-96 GF/B plates (Perkin Elmer) pre-wetted with distilled water. These polystyrene plates have wells made of mesh bottoms with glass fibre filters of 1 µm pore size and PEI coating to reduce non-specific binding and Barex plastic coating for compatibility with liquid scintillation counting. The aspirated cells were bound to the glass fibre filters while the medium was sucked through to waste containers. The

cells were then washed with 0.85 % (w/v) saline solution (150 ml per plate) in order to remove medium constituents. A 10% (w/v) TCA solution (100 ml per plate) was aspirated through the bound cells for the purpose of lysing the cells and precipitating the DNA (and other nucleic acids) of the cells. The precipitated DNA remained bound to the glass fibre filters. A final wash with 100 % methanol (150 ml per plate) further precipitated the DNA while washing the TCA and assisted in drying the filters. Plates were removed and left to further dry in the dark at room temperature for at least 45 minutes. Afterwards, 35 µl of MicroScint™-20 liquid scintillation counter (LSC) cocktail (Perkin Elmer) per well was added and the plates sealed with TopSeal™ clear adhesive (Perkin Elmer) followed by radioactivity reading by a TopCount™ NXT scintillation and luminescence counter (Perkin Elmer). Incorporation of ^{3}H-thymidine into cellular DNA was detected as the number of radioactive disintegrations of ^{3}H-thymidine per minute, recorded as counts per minute (CPM) after an initial 10 minute count delay to allow for dark adjustment.

2.2.14. Determination of cell viability using the ATPLite™ assay

The ATPLite™ assay measures the amount of luminescence (light) emitted when cellular adenosine triphosphate (ATP) reacts with D-luciferin in the presence of luciferase to produce luminescence in a reaction summarised in the equation below;

$$ATP + D\text{-}luciferin + O_2 \longrightarrow Oxyluciferin + {}^{5}AMP + {}^{6}PPi + {}^{7}CO_2 + Light$$

The amount of emitted light is proportional to the concentration of ATP released from the cells. In turn, the amount of ATP is related to the metabolic state of cells with viable cells producing more ATP than dead or necrotic cells and since ATP is unstable and is readily degraded outside the cell, it is unlikely dead and necrotic cells will produce ATP. The reaction ensures that ATP released from the cells after cell lysis is not readily hydrolysed by intracellular ATPases or reaction reagents by increasing the pH to 11 thus increasing the stability of ATP. Addition of substrate solution lowers the pH to 7.6 which is favourable for ATP to react with D-luciferase.

On the day of assay, the substrate solution (**Section 2.2.2.16**) was prepared fresh or defrosted at room temperature in a dark cupboard. Meanwhile, cell cultures in 96-

[5] AMP - adenosine monophosphate
[6] PPi - inorganic pyrophosphate
[7] CO_2 - carbon dioxide

well plates were thoroughly mixed and 100 µl of the cell suspension transferred into corresponding wells of a white OptiPlate™ 96-well plate (Perkin Elmer). Mammalian cell lysis solution (50 µl) was added into each well and the plates sealed with TopSeal™ adhesive before they were placed on an orbital shaker at 700 [8]rpm for 5 minutes. Afterwards, 50 µl of substrate solution was added into each well and the plates sealed and covered in aluminium foil to avoid light before being mixed on an orbital shaker at 700 rpm for 5 minutes. The plates were then placed in a TopCount NXT scintillation and luminescence plate reader which was programmed to dark adapt the plates for 10 minutes before measuring the luminescence in each well for 5 seconds and results expressed as counts per second (CPS).

2.2.15. Mitotic inactivation of stimulator cells using mitomycin C

Cells (Balb/c or C3H MSC, DF or mononuclear cells) were suspended in appropriate culture medium supplemented with 10 $\mu g.ml^{-1}$ of mitomycin C in sterile test tubes. The cells were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes. After incubation, the cells were removed from the incubator and washed five times by centrifugation at 180 *g* (MSC and DF) or 450 *g* (mononuclear cells) for 10 minutes with fresh culture medium changes each time to remove the mitomycin C. Cells were resuspended in appropriate culture medium for subsequent assays.

2.2.16. Statistical analysis

All numerical data obtained from replicate samples was analysed using Microsoft® Excel 2010 and GraphPad Prism® version 5.00 software (GraphPad Software Inc, California, USA). Raw data from ^{3}H-thymidine (CPM) and ATP counts (CPS) was first transformed to Log_{10} and analysed by one-way or two-way analysis of variance (ANOVA) for comparing one or two variables respectively. Log_{10} transformation was carried out in order to satisfy the normality assumption required for ANOVA. Statistical significant differences between groups were calculated using the minimum significant difference (MSD) at 95 % confidence level (CL) according to the T-method for equal samples sizes and the T'-method for unequal sample sizes according to Sokal & Rohlf (1981). The MSD is given by the formula;

[8] rpm – rotations per minute

$$MSD = Q_{\alpha=0.05}[k, v] \times SE$$

Where; k – Number of treatment groups

v – Degrees of freedom of MS_{within} groups (from Excel ANOVA table)

SE (standard error) was calculated as follows;

$$\mathbf{SE} = \sqrt{\frac{MS_{within}}{n}}$$

Where; n = number of replicates

MS_{within} = Mean square (within) (from Excel ANOVA table)

The mean values and 95 % CL obtained from Log_{10} transformed data were then back-transformed by taking antilogarithms (10^n; where n is the log_{10} value) and data was plotted as mean values ± 95 % CL, whereby the upper limit (UL) and lower limit (LL) for each mean value were calculated as follows;

$$UL = antilog\ 95\ \%\ CL - antilog\ Mean$$

$$LL = antilog\ Mean - antilog\ 95\%\ CL$$

Graphs were plotted using GraphPad Prism® software.

3. CHAPTER THREE: ISOLATION, EXPANSION AND CHARACTERISATION OF MURINE MSC AND DF

3.1. Introduction

The term 'mesenchymal stem cell' has been and is still used to define groups of non-haematopoietic cells of mesenchymal origin which possess the ability to differentiate into multiple cell lineages of typical mesodermal tissues such as adipose, bone and cartilage. Friedenstein and co-workers were the first group to report on this type of 'fibroblastic-like' cell capable of 'spontaneous bone formation' isolated from Guinea-pig bone marrow (Friedenstein *et al.*, 1970). Later, they discovered that, in addition to clones of these cells forming bone rudiments *in vitro*, they were also able to form cartilage thus making them candidates for osteo-chondrogenic "stem" cells (Friedenstein *et al.*, 1987). Since then, bone marrow stromal cells have been isolated from various mammalian species including postnatal mice (Jiang *et al.*, 2002) and manipulated *in vitro* to yield cells of the adipogenic, chondrogenic and osteogenic lineages with relative ease (Prockop, 1997).

There is little consensus regarding the terminology of MSC partly because of lack of standard procedures for their identification, isolation and characterisation and also because stem cell biology is a relatively new field as discussed broadly in **Section 1.3.3**. Differences amongst research groups in terms of choice of species, methodologies for isolation and conditions for culture led to various names such as BMSSC (Bianco *et al.*, 2001), MPC (Sun *et al.*, 2003), BMSC (Horwitz *et al.*, 2002), MAPC (Jiang *et al.*, 2002) being used to describe them. The most common approach for isolating murine MSC from compact bone is by the PAM described in **Section 1.3.3.3** which involves the flushing of bone marrow with culture medium followed by isolation which exploits the physical tendency of MSC to adhere to the plastic substrate of cell culture flasks (Friedenstein *et al.*, 1970b). This technique was used in this study.

The bone marrow stroma houses two types of stem cells; MSC and HSC in addition to stromal cells which form the majority of the cells (Koide *et al.*, 2007). Due to the presence of multiple cell types in the marrow microenvironment, the isolation and

subsequent characterisation of MSC is rather a complex process due to the following contributing factors;

- MSC constitute a very small proportion of marrow nucleated cells estimated to be 1 in 10^6 cells in mice (Sung *et al.*, 2008).
- Mouse MSC are located in the endosteum whereas HSC tend to exist around the centre of the marrow while other stromal cells are present near the surface of the bone (Short *et al.*, 2001). These deep locations of MSC present difficulties to their harvesting from bone marrow.
- Flushing murine marrow inadvertently yields a heterogeneous cell suspension which possess a higher frequency of haematopoietic cells that also adhere to tissue culture plastic and persist in culture even after serial passages (Baddoo *et al.*, 2003).
- Most murine plastic-adherent stromal cells have been shown to exhibit differentiated phenotypes even in the absence of cell-specific differentiation-inducing factors (Sun *et al.*, 2003).

Therefore, cell cultures resulting from bone marrow aspirates remain morphologically heterogeneous. Efforts to develop new methods that specifically isolate MSC from bone marrow aspirates have been investigated in the last two decades and these include chemical selection (Modderman *et al.*, 1994), negative selection by immunodepletion of contaminating cells (Baddoo *et al.*, 2003) and positive selection utilising cell surface antigens (Nadri & Soleimani, 2007a). The latter two methods are dependent on flow cytometry selection and have made it possible for the phenotype of MSC to be defined based on the expression of and lack of specific markers.

The lack of definitive markers for MSC has led to confusion with regard to the nomenclature for defining them. To date MSC are defined by three main criteria;

- Self-renewal in culture and maintenance of spindle-morphology.
- Expression of and lack of specific surface markers.
- Tri-lineage differentiation into fat, cartilage and bone cells.

The self-renewal capacity is the only phenotypic trait that is considered to be exclusive to all stem cells. The clonogenic potential of MSC can be demonstrated easily by seeding the cells at low density. They divide continuously until confluence thus regular passaging allows the generation of large quantities of cells which is useful for clinical applications. It is important to note that continuous passaging leads to reduced differentiation potential and loss of 'stem' properties. The mechanisms responsible for loss of the intrinsic clonogenic capacity are unknown though telomere shortening is thought to play a part (Park *et al.*, 2007c).

Mouse MSC have been shown to express MHC I, Sca-1, CD29, CD44, CD54, CD73 CD90, CD105 and CD106 while being negative for MHC II, the co-stimulatory molecules CD80 and CD86, the macrophage marker CD11b, the B-cell marker CD19, haematopoietic markers CD34, CD45 and CD133 (Tropel *et al.*, 2004; Sung *et al.*, 2008; Soleimani & Nadri, 2009). However, this criterion does not distinguish MSC from other cells such as DF (Haniffa *et al.*, 2009) and therefore cannot be used in isolation. Even the expression of MHC I has been described as partial (Soleimani & Nadri, 2009) while a few studies have reported the presence of CD80 (Schurgers *et al.*, 2010) on mouse MSC in culture.

The tri-lineage differentiation test is currently the only functional test used to distinguish MSC and has been demonstrated in all studies. When cultured under defined conditions involving chemical and hormonal cues, mouse MSC differentiate into adipocytes, chondrocytes and osteoblasts. The mechanisms that control MSC differentiation are discussed in detail in **Section 1.3.3.6**. Adipogenic differentiation can be achieved by the addition of dexamethasone, indomethacin, IBMX and insulin in culture medium. Intracellular lipid vesicles become visible under light microscopy and accumulate into larger vesicles which can be positively stained with oil red-O after 14-21 days (Tropel *et al.*, 2004). To induce chondrogenic differentiation, high-density cell-pellets or micromass cultures (Denker *et al.*, 1995) are cultured in medium containing ascorbic-2-phosphate, dexamethasone, insulin, transferrin, selenous acid, pyruvic acid and TGFβ. Alcian blue staining shows the presence of glycosaminoglycans which is indicative of cartilage (Tropel *et al.*, 2004). Osteogenesis is achieved by culturing monolayer confluent cells in medium supplemented with ascorbic-2-phosphate, β-glycerophosphate and dexamethasone.

To observe calcium deposition and matrix mineralization, cells stain positive with alizarin red S (Phinney *et al.*, 1999).

It is important to emphasize that despite the tri-lineage test being the cardinal test for MSC multipotency, only a fraction of cells within any MSC population actually undergo differentiation even in cultures obtained from a single clone (Park *et al.*, 2007c). It is not known why some cells fail to undergo differentiation but there is a school of thought that suggests that the differentiation potency diminishes with cell 'ageing'.

DF unlike MSC, can easily be isolated from mouse skin by a combination of dispase and collagenase digestion with the former separating the dermis from the epidermis and the latter loosening the ECM to release individual cells which can be propagated in culture for relatively long periods of time (Kitano & Okada, 1983). DF closely resemble MSC in both morphology and phenotype expressing and lacking the same cell surface markers commonly used for identifying MSC (Haniffa *et al.*, 2009). Interestingly, human DF have been reported to undergo differentiation into adipocytes, chondrocytes and osteoblasts but in most of these studies, tri-lineage differentiation has not been demonstrated in individual experiments (Lysy *et al.*, 2007; Lorenz *et al.*, 2008). The tri-lineage capacity of murine DF however is yet to be demonstrated in a single experiment.

3.1.1. Aims and Objectives

The aim of this part of the study was to isolate and characterise MSC from the bone marrow of Balb/c and C3H mice in order to provide MSC for subsequent immunological studies.

DF were isolated from both Balb/c and C3H mice skin and characterised in the same manner as MSC.

The specific objectives were;

1. To isolate Balb/c and C3H MSC from femoral bone marrow.
2. To expand the bone marrow MSC using different culture media to find the most appropriate followed by morphological characterisation.
3. To determine the phenotype of the MSC using flow cytometry.

4. To determine the potency of the MSC using tri-lineage differentiation into fat, cartilage and bone.
5. To isolate Balb/c and C3H DF from abdominal skin.
6. To characterise the DF as above (3)-(5) for comparative purposes.

3.2. Materials and Methods

3.2.1. Reagents

The following reagents were used and were prepared as described in **Section 2.2.2** unless stated otherwise;

- 3-Isobutyl-1-methylxanthine (IBMX); 100 mg was dissolved in 225 μl of DMSO to make a 2 M stock which was filtered through a 0.2 μm pore size filter. Solution was kept frozen at -20 °C.
- Alcian blue stain solution 1% (w/v) in acetic acid
- Alizarin red S dye; 500 mg of powder was dissolved in 50 ml of distilled water to make a 1 % (w/v) stock solution. The solution was stirred for 2 hours in a flask and filtered through a 0.2 μm pore size filter. The stock solution was covered with aluminium foil and kept in a dark cupboard at room temperature to prevent damage by light.
- Ammonium hydroxide solution; A 10 % (v/v) ammonium hydroxide solution was made by diluting 30 ml of the stock (28 %) with 60 ml distilled water.
- Antibody diluent; PBS supplemented by 0.1% (v/v) sodium azide.
- Ascorbate-2-phosphate; 2.57 g was dissolved in 50 ml of distilled water to make a 200 mM stock solution. Aliquots were stored at -20 ºC.
- Collagenase 1A; 5 mg collagenase 1A dissolved in 10 ml DMEM-HG medium (without antibiotics and FCS) to make a 0.5 $mg.ml^{-1}$ solution. 1 mg of the collagenase 1A contained 62.5 $CDU^{9}.mg^{-1}$.
- Dexamethasone; 25 mg was dissolved in 1274 μl sterile methanol to make a stock of 50 mM which was filtered through a 0.2 μm pore size filter. Stock was stored frozen at -20 °C.
- Dispase II; 5 mg of dispase II dissolved in 10 ml of DMEM-HG medium (without antibiotics and serum) to make a 0.5 $mg.ml^{-1}$ solution containing 5 $U.ml^{-1}$.
- DMEM-HG culture medium
- DMEM-LG culture medium
- Ethanol; 100 %

[9] CDU – collagen digestion units

- FACS blocking buffer (2x); PBS supplemented with 2.5 mM EDTA and 4 % (v/v) FCS.
- Formalin in PBS (10 % v/v): 27 ml of formalin stock (37 %) was diluted with 63 ml of distilled water and 10 ml of 10 PBS.
- Hy-Q-Tase™ cell dissociation solution
- Indomethacin; 100 mg was dissolved in 559 µl DMSO to make a stock of 0.5M which was filtered through a 0.2 µm pore size filter. Stock was stored at -20 °C.
- Insulin; 10 $mg.ml^{-1}$ stock
- Insulin-transferrin-selenium-X (ITS) 100x; stored at 4 ºC.
- Isopropanol; 60% (v/v). 6 ml of 100 % isopropanol was diluted with 4 ml distilled water.
- MesenCult™ culture medium
- Oil red-O solution. 35 mg of Oil red-O powder was dissolved in 10 ml of 100 % isopropanol to make a stock solution of 0.35 % (w/v) oil red-O which was filtered through a 0.2 µm pore size filter and stored in a lightproof container.
- PBS
- Sodium pyruvate 100 mM stock solution; stored at 4 ºC.
- StemXVivo™ culture medium
- Trypsin-EDTA solution; 0.5 % (v/v)
- α-MEM culture medium
- β-glycerophosphate; 10 g was dissolved in 23 ml of distilled water to make a 2 M stock solution which was stored at -20 ºC.
- β-mercaptoethanol

3.2.2. Harvesting and culture of plastic-adherent cells from Balb/c and C3H mice bone marrow

Balb/c and C3H female mice 4-8 weeks old were killed using a schedule (1) method. Femurs were carefully dissected using sterile scissors and forceps and all attached epiphyses were carefully removed to minimize contamination by other cells. Whole marrow plugs were aseptically flushed out using a syringe filled with DMEM-LG

culture medium (18G x 1" hypodermic needle attached to a 2 cm^3 syringe) and collected into sterile petri-dishes. The marrow plugs were carefully dispersed by pipetting up and down and the resulting cell suspensions were plated into T75 tissue culture flasks (10 ml per flask) and incubated for 24 hours in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air to allow adherent cells to attach onto the plastic surface. Non-adherent cells and free-floating marrow debris were removed and discarded from the culture by changing the medium. Few randomly scattered plastic-adherent cells remained in the flasks and were designated passage zero (p0) and maintained in culture with medium changes carried out every 48 hours. Whenever the cells reached 90 % confluence, they were passaged using Hy-Q-Tase™ cell detachment solution to consecutive passage numbers as described in **Section 2.2.6** while some were cryo-preserved as described in **Section 2.2.9**.

3.2.3. Isolation and expansion of Balb/c and C3H mouse DF

Balb/c and C3H 4-8 weeks old female mice were sacrificed using a schedule (1) method. They were thoroughly sprayed with 70 % (v/v) ethanol to disinfect the skin. Using a sterile scalpel blade, abdominal skin was shaved clean of fur with care taken not to cut the skin to prevent contamination. Small pieces of skin approximately 2 cm x 2 cm were aseptically dissected and soaked in 70 % (v/v) ethanol for 5 minutes followed by 3 washes with sterile PBS. The pieces were then carefully placed into petri-dishes with the epidermis side facing upwards. A solution of 0.5 $mg.ml^{-1}$ dispase II enzyme solution in DMEM-HG was carefully added to fully submerge the tissues which were then left overnight for at least 15 hours at 4 ºC. Afterwards, the tissues were carefully placed in fresh petri-dishes and washed 3 times with PBS. Using sterile forceps, the epidermis was gently separated from the dermis. The dermis were placed in sterile 40 ml pots containing 10 ml of 0.5 $mg.ml^{-1}$ collagenase 1A in DMEM-HG medium and incubated on a shaking platform at 37 ºC for 4 hours to allow the dermis to be disaggregated to release single cells. After the dermis had completely digested (no visibly large pieces), the cell suspensions were gently pipetted up and down for a minute followed by the addition of an equal volume of DMEM-HG culture medium to inactivate the collagenase 1A. The cells were washed 3 times by centrifuging at 400 *g* for 10 minutes and then counted by the Trypan blue method descried in **Section 2.2.8**. The cells were then seeded in T75 flasks at 1 x 10^5 cells per flask in DMEM-HG culture medium and incubated at 37 ºC in an

atmosphere of 5 % (v/v) CO_2 in air. The first medium change was carried out after 24 hours to wash off any debris from the p0 cells. Subsequently, medium was changed every 3 days. Upon reaching 90 % confluence, the cells were passaged to the next consecutive number using 0.5 % (v/v) trypsin-EDTA solution for cell detachment as described in **Section 2.2.6** or cryo-preserved as described in **Section 2.2.9** for later use.

3.2.4. Evaluation of different types of culture media for expansion of mouse MSC

In order to determine the most favourable medium which supported MSC expansion, five types of commercially available medium were evaluated for their ability to support cell expansion by means of cell doubling times (CDT). DMEM-LG, DMEM-HG, α-MEM, StemXVivo™, and MesenCult™ culture media were used in the culture of p3 Balb/c and C3H marrow plastic-adherent cells. P2 cells were passaged as described in **Section 2.2.6**, counted by the Trypan blue method (**Section 2.2.8**) and seeded at 1 x 10^5 cells per T75 flask. Into each flask, 10 ml of any of the five media was added and the flasks were cultured at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air.

Medium was changed every 2 to 3 days until the flasks were nearly 100 % confluent at which point the cells were detached as described in **Section 2.2.6** and counted by the Trypan blue method as described in **Section 2.2.8**. CDTs were calculated according to the formula;

$$\boldsymbol{CDT} = (T2 - T1)\, x\, \frac{Log\ 2}{log \frac{N2}{N1}}$$

Where;
- T1 - time at start (hours)
- T2 - time at confluence (hours)
- N2 - final cell count of monolayer
- N1 - initial number of seeded cells

3.2.5. Characterisation of Balb/c and C3H mouse MSC and DF

3.2.5.1. Flow cytometry

Balb/c and C3H bone marrow plastic-adherent cells (p3 and p10) and DF (p6) were analysed for the expression of and lack of a panel of markers commonly used to characterize MSC by flow cytometry.

Upon reaching 95% confluence in culture, the cells were washed twice with PBS and detached using Hy-Q-Tase™ (for plastic-adherent cells) or 0.5 % (v/v) trypsin-EDTA (for DF) as described in **Section 2.2.6**, counted using the Trypan blue method (**Section 2.2.8**) and resuspended in ice-cold PBS at a density of 10^6 cells.ml^{-1}. Aliquots of 100 µl of the cell suspension were placed into sterile 1.5 ml Eppendorf tubes and an equal volume of 2x FACS blocking buffer was added to the cells followed by incubation for 20 minutes on ice. Specific conjugated antibody solutions were prepared with appropriate dilutions in antibody diluent according to **Table 3.1** and kept on ice in the dark. Labelled antibody solutions (100 µl) were added to the cell suspensions, gently mixed and incubated on ice for 1 hour in the dark with occasional flicking to ensure mixing. The cells were then washed 3 times with 300 µl of ice-cold PBS by centrifugation at 3000 *g* for 5 minutes at 4 °C. Two further washes with 500 µl of ice-cold PBS were carried out after which the cell pellets were resuspended in 200 µl of ice-cold PBS. A FACSCalibur (Becton Dickinson) flow cytometer was used to measure the fluorescence intensities of all samples. Data was collected (10 000 events per sample) using CellQuest™ v.3.1 flow cytometry data acquisition software (Becton Dickinson) and analysed using FlowJo™ v.7.6.5 flow cytometry analysis software (Tree Star Inc).

Table 3.1. Conjugated antibodies used for the phenotypic characterisation of Balb/c and C3H bone marrow MSC and DF.

Antibody	Isotype	Clone	Label	Host	Dilution	Supplier
Sca-1	IgG2α, κ	D7	FITC	Rat	1:50	eBioscience
CD29	IgG	eBioHMb1-1	PE	Hamster	1:10	eBioscience
CD90.2	IgG2β, κ	5a-8	FITC	Rat	1:50	Invitrogen
CD80	IgG	16-10A1	FITC	Hamster	1:400	eBioscience
CD86	IgG2β, κ	PO3.1	PE	Rat	1:100	eBioscience
CD86	IgG2β, κ	PO3.1	FITC	Rat	1:100	BioLegend
CD11b	IgG2β, κ	M1/70	FITC	Rat	1:100	eBioscience
MHC I	IgG2β, κ	34-1-2S	FITC	Mouse	1:100	eBioscience
MHC II	IgG2β, κ	M5/114.15.2	FITC	Rat	1:400	eBioscience
CD44	IgG2β, κ	IM7	FITC	Rat	1:200	eBioscience
CD45	IgG2β, κ	30-F11	FITC	Rat	1:20	BioLegend
CD45	IgG2β, κ	30-F11	PE	Rat	1:20	Invitrogen
Isotype control		**Clone**	**Label**	**Host**	**Dilution**	**Supplier**
IgG2α, κ		6H3	FITC	Mouse	1:50	MBL
IgG2α, κ		RMG2a-62	FITC	Rat	1:50	BioLegend
IgG2β, κ		3D12	FITC	Mouse	1:50	MBL
IgG2β, κ		RMG2b-1	FITC	Rat	1:50	BioLegend
IgG2β, κ		3D12	PE	Mouse	1:20	Invitrogen
IgG		RMG1-1	FITC	Rat	1:50	BioLegend
IgG		2E12	FITC	Mouse	1:50	eBioscience
IgG		2E12	PE	Mouse	1:100	eBioscience

[FITC; fluorescein isothyocyanate absorption/ emission max 494-250 nm. PE; phycoerythrin absorption/emission max 496-578 nm. CD; cluster of differentiation; Ig; immunoglobulin; MHC; major histocompatibility complex]

3.2.5.2. Tri-lineage differentiation of Balb/c and C3H MSC and DF

3.2.5.2.1. Adipogenic differentiation

Adipogenic differentiation medium (ADM) was made by supplementing DMEM-LG culture medium (MSC) or DMEM-HG culture medium (DF) with insulin, IBMX, indomethacin and dexamethasone stock solutions prepared as described in **Section 3.2.1** to give final concentrations of 10 $\mu g.ml^{-1}$ insulin, 100 μM indomethacin, 1 μM dexamethasone and 0.5 μM IBMX. Adipogenic maintenance medium (AMM) was made by supplementing DMEM-LG or DMEM-HG culture medium with insulin stock solution (**Section 3.2.1**) to give a final concentration of 10 $\mu g.ml^{-1}$ insulin.

Balb/c and C3H MSC (p10) and DF (p6) cultures were maintained in culture until they reached 90% confluence in T75 flasks. The cells were washed twice with 10 ml per flask PBS without Ca^{2+} and Mg^{2+} and detached from the surface using Hy-Q-Tase™ (MSC) and 0.5 % (v/v) trypsin-EDTA (DF) solution for MSC and DF for 5 minutes at room temperature and 37 °C respectively as described in **Section 2.2.6**. The resulting cell suspensions were washed with DMEM-LG and DMEM-HG culture medium by centrifugation at 180 *g* for 10 minutes. The cell pellets were resuspended in 5 ml of their respective culture medium followed by counting using the Trypan blue dye method (**Section 2.2.8**). Cells were plated in 6-well tissue culture plates at a density of 1 x 10^4 cells.cm^{-2} growth area (1 x 10^5 cells per well) in 3 ml of DMEM-LG and DMEM-HG culture medium. The plates were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in air and maintained in culture until they reached 95 % confluence. Culture medium was replaced with ADM while in control cultures, cells were maintained in DMEM-LG or DMEM-HG culture medium and this was marked as the 1st day of differentiation. In the differentiation cultures, ADM was replaced with AMM after each 48 hour period and this medium interchange was maintained throughout the culture period. Cultures were regularly checked for the presence of lipid vesicles under a light microscope during the differentiation period.

3.2.5.2.1.1. Oil red O (ORO) staining for adipogenesis

At day 14 of adipogenic differentiation, the cells (MSC and DF) were washed twice with PBS and fixed with 10% (v/v) formalin (**Section 3.2.1**) for 10 minutes at room temperature (2 ml per well). Meanwhile, Oil red O (ORO) working solution was prepared by mixing 6 ml of ORO stock solution (**Section 3.2.1**) with 4 ml of distilled water followed by filtering through a 0.2 μm filter. Formalin was removed from the wells and the cells were washed twice with distilled water and twice with 60% (v/v) isopropanol (**Section 3.2.1**) for 5 minutes followed by air-drying at room temperature. Filtered ORO working solution (2 ml per well) was added to the wells and kept for 10 minutes at room temperature in the dark. Afterwards, the ORO solution was removed and the stained cells were immediately washed twice with distilled water. Finally, distilled water enough to cover the cells was added into each well and the cells were viewed under an Olympus BX 71 inverted microscope and images were captured using Cell^B image acquisition software (Olympus).

3.2.5.2.2. Chondrogenic differentiation

Chondrogenic differentiation medium (CDM) was made by supplementing DMEM-LG (MSC) and DMEM-HG (DF) culture medium with 150 μM ascorbate-2-phosphate, 1 mM sodium pyruvate, 0.1 μM dexamethasone, 6.25 $ng.ml^{-1}$ selenous acid, 6.25 $\mu g.ml^{-1}$ transferrin and 6.25 $\mu g.ml^{-1}$ insulin The supplements were prepared as described in **Section 3.2.1**..

Balb/c and C3H MSC (p10) and DF (p6) were cultured in T75 flasks until they reached 90 % confluence. The cells were washed twice with PBS without Ca^{2+} and Mg^{2+} (10 ml per flask) and then detached using Hy-Q-Tase™ (MSC) and 0.5 % (v/v) trypsin-EDTA (DF) for 5 minutes at room temperature and 37 °C respectively as described in **Section 2.2.6**. The resulting cell suspensions were washed with DMEM-LG or DMEM-HG culture medium by centrifugation at 180 *g* for 10 minutes and then counted using the Trypan blue method (**Section 2.2.8**) and finally resuspended in either DMEM-LG or DMEM-HG culture medium at a density of 1 x 10^7 $cells.ml^{-1}$. In order to create micromass chondrogenic nodules in 24-well plates, 10 μl of the cell suspension was carefully placed at the centre of each well using a 20 μl pipette. The plates were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in air for 3 hours to allow the cells to attach. Afterwards, the wells were gently flooded with 1 ml of CDM. Control cultures were cultured in DMEM-LG and DMEM-HG culture medium for MSC and DF respectively. The seeded plates were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in air for 21 days with medium changed each 3 days.

3.2.5.2.2.1. Alcian blue staining for glycosaminoglycans

At day 21 of chondrogenic differentiation, the cells were washed twice with distilled water and fixed with 10% (v/v) formalin for 10 minutes at room temperature (2 ml per well). After fixation, the formalin was removed from the wells and the cells were washed twice with distilled water and then left air dry. The cells were stained with 500 μl of 1 % (w/v) alcian blue staining solution (**Section 3.2.1**) at a pH of 2.5 for 30 minutes. The stain was removed and the cells washed with running tap water for 2 minutes to remove excess stain. To view the cells, 1 ml per well of distilled water was added and the staining viewed with an Olympus BX 71 inverted microscope and images were captured using Cell^B image acquisition software (Olympus).

3.2.5.3. Osteogenic differentiation

Osteogenic differentiation medium (ODM) was made by supplementing DMEM-LG (MSC) and DMEM-HG (DF) culture medium with 0.1 mM of ascorbate-2-phosphate, 10 mM of β-glycerophosphate and 0.0001 mM of dexamethasone. The supplements were prepared as described in **Section 3.2.1**. Balb/c and C3H MSC (p10) and DF (p6) cells were cultured in DMEM-LG and DMEM-HG culture media respectively until they reached 90% confluence. The cells were washed twice with 10ml PBS without Ca^{2+} and Mg^{2+} to remove culture medium and they were detached using Hy-Q-Tase™ and 0.5 % (v/v) trypsin-EDTA for 5 minutes at room temperature and 37 °C respectively for MSC and DF. The resulting cell suspensions were washed with DMEM-LG or DMEM-HG culture medium by centrifugation at 180 *g* for 10 minutes. The cell pellets were resuspended in their respective culture medium, counted by Trypan blue method (**Section 2.2.8**) and plated in 6-well tissue culture plates at a density of 1 x 10^4 cells.cm^{-2} growth area (1 x 10^5 cells per well) in 3 ml of appropriate culture medium. The plates were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in air accompanied by medium changes every 3 days until they reached 95 % confluence at which point culture medium was replaced with ODM in the test wells while control wells were maintained in DMEM-LG or DMEM-HG culture medium for MSC and DF respectively. Medium was changed after each 3 day period for a total culture period of 14 days.

3.2.5.3.1.1. Alizarin red S staining for calcium deposition

Alizarin red S working solution (0.1% w/v) was made by making a 1:10 dilution of the 1% (w/v) stock with distilled water and adjusting the pH to 4.1 with 10 % (v/v) ammonium hydroxide solution. At day 14 of osteogenic differentiation, the cell cultures were washed twice with distilled water and fixed with 10% (v/v) formalin for 10 minutes at room temperature (2 ml per well). After fixation, the formalin was removed from the wells and the cells were washed twice with distilled water and then left to air dry. The cells were stained with 0.1 % (w/v) alizarin red S staining solution (pH 4.1) for 20 minutes (1 ml per well). The cells were washed four times with distilled water to remove excess dye and 1 ml of distilled water was added into each well before viewing the cells with an Olympus BX 71 inverted microscope followed by capturing images using Cell^B image acquisition software (Olympus).

3.3. Results

3.3.1. Harvesting and initial culture of Balb/c and C3H mouse bone marrow stromal cells

Balb/c and C3H 4-8 weeks old female mice were sacrificed and aseptically dissected to separate the femurs. Bone marrow stroma was harvested by femoral aspiration using DMEM-LG culture medium and plated in tissue culture flasks to allow attachment to the plastic surface overnight as described in **Section 3.2.2**.

Three hours post seeding, the cells appeared densely populated (**Figure 3.1A-B**) but after the first medium change 24 hours later, most of the non-adherent cells were lost leaving behind sparse cells (**Figure 3.1C-D**). These cells appeared to exhibit different morphologies including individual spindle-shaped bipolar fibroblastic-like cells, dendritic-like cells and macrophage-like cells (**Figure 3.2A-B**). Most of these heterogeneous cells in the early cultures appeared to become smaller and rounded and were gradually lost during subsequent medium changes (**Figure 3.2C-D**). Some few spindle-shaped fibroblastic-like cells remained attached and were quiescent for some time before gradually dividing and populating the flasks; Balb/c and C3H cells took approximately 3 and 5 weeks respectively to achieve 90 % confluence before the 1st passage. Though these fibroblastic-like cells became more prominent during the initial stages, other morphologies became less apparent.

For the 2nd passage, the cells were seeded at a low density of 0.75 $cm^2.cell^{-1}$ (1 x 10^2 cells per T75 flask) in order to test their clonogenic capacity. The cells initially appeared as sparse colonies of colony forming units-fibroblasts (CFU-F) which gradually grew into small cell clusters (**Figure 3.3A-B**) which assumed a radial growth pattern eventually forming larger populations (**Figure 3.3C-D**).

By late 2nd passage, homogeneous cultures of the fibroblastic-like cells which resembled MSC dominated the cultures and achieved 90 % confluence (**Figure 3.3E-F**) at which point they were passaged and achieved 95 % confluence much faster (within 5 days) compared to the earlier heterogeneous culture.

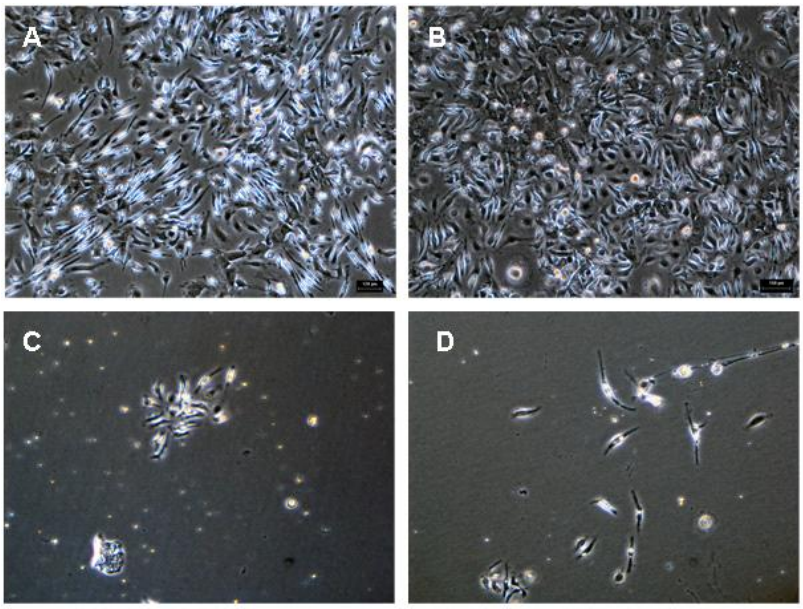

Figure 3.1. Appearance of freshly harvested mouse bone marrow cells before and after the first medium change. Balb/c (A) and C3H (B) bone marrow cells were extracted by femoral aspiration and plated in tissue culture flasks in DMEM-LG culture medium. After 24 hours, the medium was changed with the concomitant loss of non-adherent cells leaving behind few and sparse Balb/c (C) and C3H (D) plastic-adherent cells. Images were captured at x100 magnification using Image-Pro® Plus imaging software (MediaCybernetics).

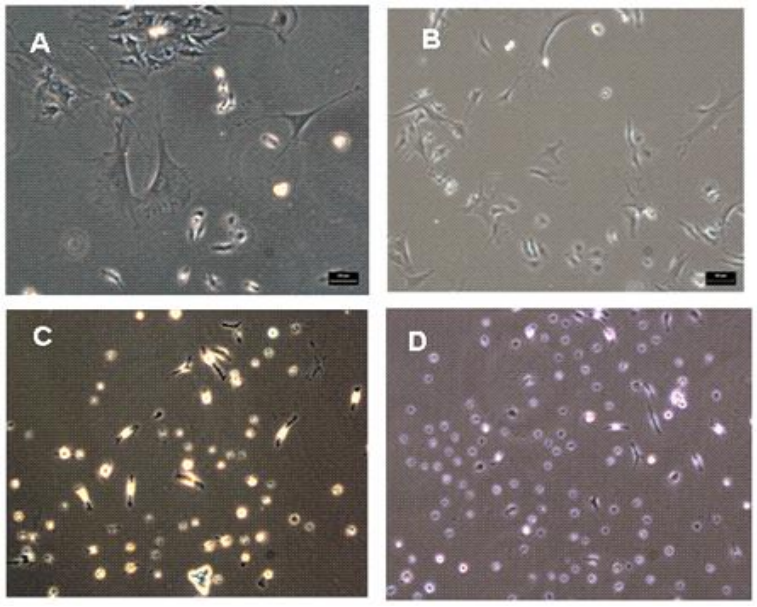

Figure 3.2. Morphological heterogeneity of early cultures of mouse bone marrow-derived plastic-adherent cells. 1st passage Balb/c (A) and C3H (B) cells grown in DMEM-LG culture medium exhibited distinct morphologies. After several days in culture some of the Balb/c (C) and C3H (D) plastic-adherent cells appeared thin and rounded and were eventually lost during subsequent medium changes. Images were captured at x100 magnification using Image-Pro® Plus imaging software (MediaCybernetics).

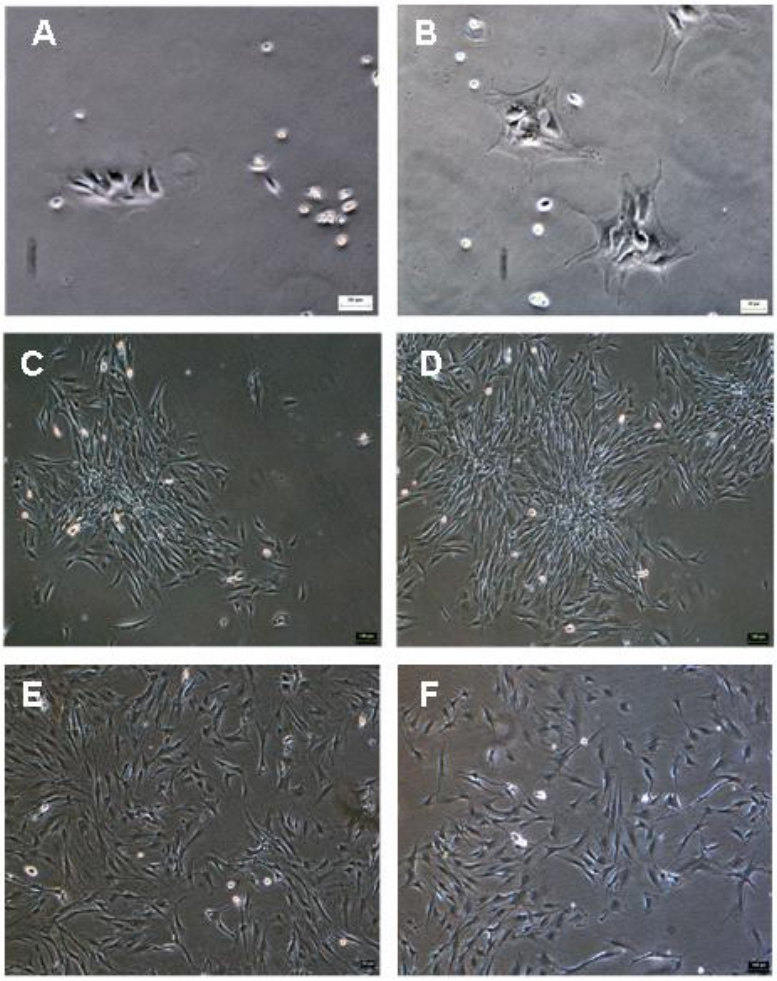

Figure 3.3. Growth and early expansion of mouse bone marrow-derived plastic-adherent fibroblastic-like cells. Persistence of fibroblastic-like cells in Balb/c (left) and C3H (right) cell cultures gave rise to colony forming units (A and B) which gave rise to larger colonies that grew in a radial pattern (C and D). On reaching 90 % confluence, the cells were split to the 2nd passage at low density and after a few days the cells yielded homogeneous cultures of bipolar spindle-shaped fibroblastic-like cells (E and F). Images were captured at x100 (C, D, E and F) and x200 (A and B) magnification using Image-Pro® Plus imaging software (MediaCybernetics).

3.3.2. Appropriate medium for expansion of p3 C3H and Balb/c plastic-adherent bone marrow cells

The type and composition of medium is important for the growth and expansion of MSC. For mouse MSC, different media have been used in different studies hence it was necessary to determine the appropriate culture medium for C3H and Balb/c MSC. Five types of commercially available general purpose (DMEM-HG, DMEM-LG and α-MEM) and MSC-specific (StemXVivo™ and MesenCult®) culture media were tested for their capacity to support growth and expansion of 3rd passage Balb/c

and C3H bone marrow plastic-adherent cells in terms of maintenance of cell morphology and CDT or growth rate.

Cells, except those cultured in α-MEM appeared healthy, maintained their fibroblast-like morphology and were relatively easy to dislodge from the plastic surface during passage using Hy-Q-Tase™ but not 0.5 % (v/v) trypsin-EDTA cell dissociation solution. DMEM-LG and StemXVivo™ cultured cells produced the lowest CDT of 8 hours per doubling which was the fastest rate of cell expansion compared to MesenCult®, DMEM-HG and α-MEM which had CDTs of 24, 30 and 42 hours per doubling respectively (**Figure 3.4**). Cells cultured in α-MEM in addition to having the slowest CDT, appeared thin with an elongated morphology and arranged in a linear pattern in stark contrast to those cultured in DMEM-LG (**Figure 3.5**). These cells were resistant to dissociation by both 0.5 % (v/v) trypsin-EDTA and Hy-Q-Tase™ treatment during passage and could only be dislodged with the aid of a cell scraper. Based on the CDTs and morphological examination, DMEM-LG and StemXVivo™ provided the fastest growth conditions for expansion of both C3H and Balb/c plastic-adherent cells. DMEM-LG was preferred to StemXVivo™ due to cost.

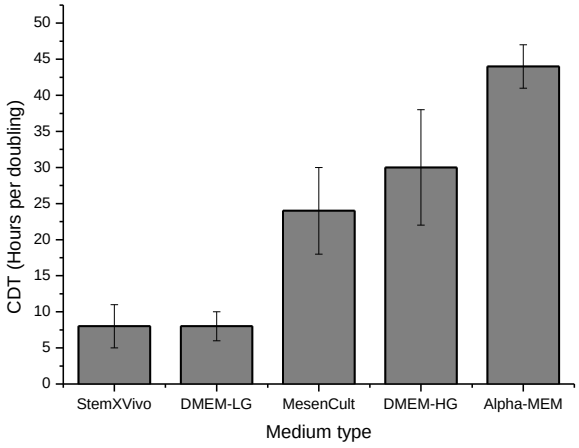

Figure 3.4. Measurement of cell doubling times (CDTs) to determine the appropriate culture medium for the expansion of mouse bone marrow-derived plastic-adherent cells. Balb/c and C3H p3 plastic-adherent cells were cultured in DMEM-LG, DMEM-HG, StemXVivo™, α-MEM and MesenCult® culture media at an initial density of 1 x 10^5.ml^{-1} and medium changed every 3 days in T75 tissue culture flasks. Cells were counted after reaching 95-100 % confluence and CDT computed. The fastest expansion rate was obtained with DMEM-LG and StemXVivo with α-MEM giving the slowest growth rate. Data presented was for C3H cells and is plotted as mean ± 95 % CL of triplicate samples. Results are representative for Balb/c cells.

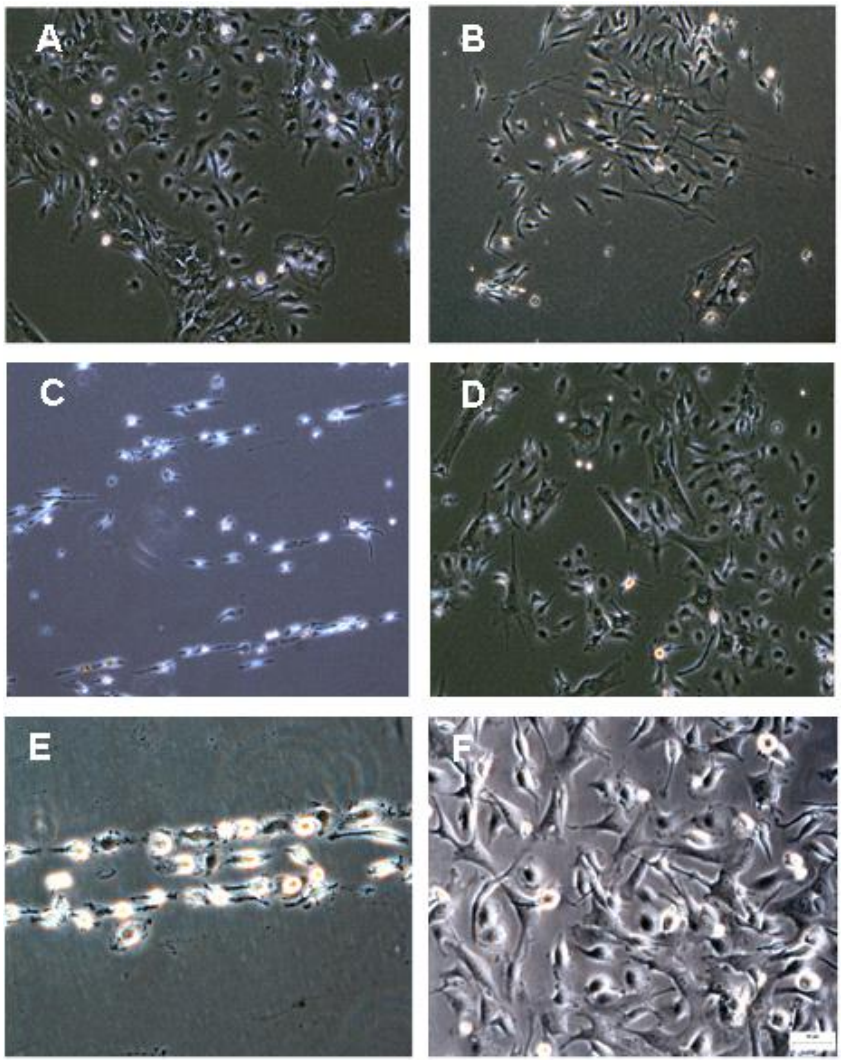

Figure 3.5. Effects of medium type on the growth and expansion of mouse bone marrow-derived plastic-adherent cells. C3H cells (p3) were cultured in α-MEM (left) and DMEM-LG (right) culture media. Initially, the cells exhibited similar morphology (A and B) but after the 1st week, the cells cultured in α-MEM appeared elongated and arranged in a linear pattern (C) whereas those cultured in DMEM-LG (D) retained their bipolar spindle-shaped morphology and were evenly spread across the flask. Prolonged culture in α-MEM resulted in the cells becoming thin and elongated (E) as opposed to those in DMEM-LG cells which appeared healthy (F). Images were captured at x100 (A, B, C and D) and x200 (E and F) magnification using Image-Pro® Plus imaging software (MediaCybernetics).

3.3.3. Expansion of C3H and Balb/c mouse plastic-adherent cells

Having established the most suitable culture medium for cell expansion, both C3H and Balb/c plastic-adherent cells (p3) were expanded in DMEM-LG culture medium. The cells maintained morphological homogeneity consistently over time and were cryo-preserved at every passage until the 14th passage (**Figure 3.6**).

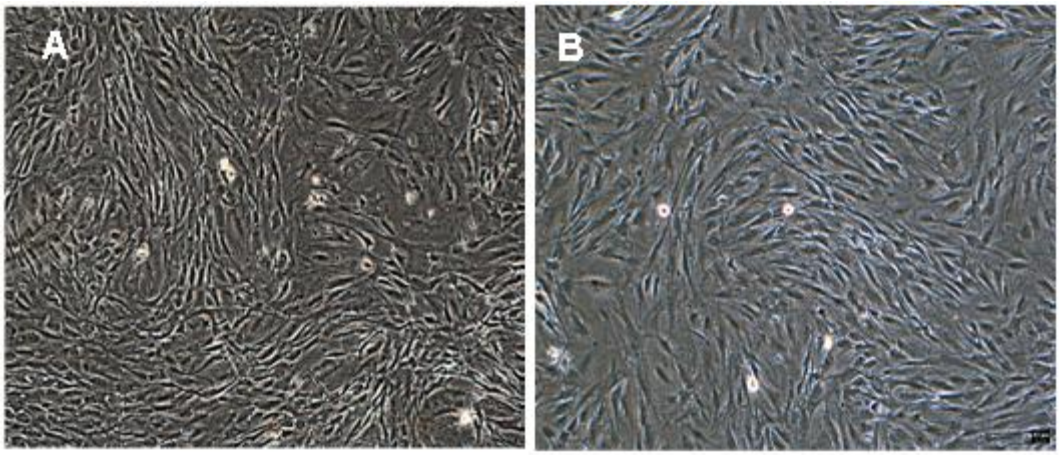

Figure 3.6. Morphological homogeneity of mouse bone marrow-derived plastic-adherent cells after long term culture. Balb/c (A) and C3H (B) p14 cells showing typical bipolar spindle shaped morphology. Images were captured at x100 magnification using Image-Pro® Plus imaging software (MediaCybernetics).

3.3.4. Growth and expansion of Balb/c and C3H mouse DF

DF were isolated from abdominal skin by a double enzymatic digestion process using dispase II and collagenase 1A, washed and cultured in DMEM-HG culture medium. The adherent cells showed typical fibroblastic morphology; bipolar spindle-shaped cells, which reached 95 % confluence after two to three days of seeding and passage. The cells retained their morphology and growth rate in subsequent passages (**Figure 3.7**).

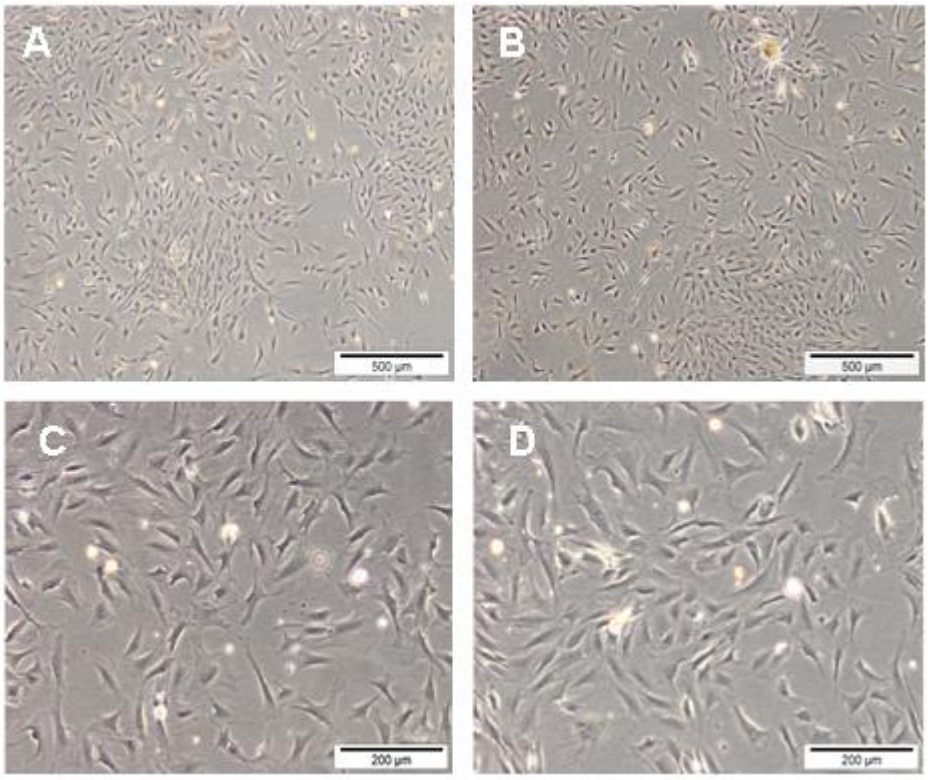

Figure 3.7. Isolation and expansion of mouse DF. Balb/c (left) and C3H (right) mouse abdominal skin were treated with dispase II to separate the epidermis and the dermis followed by collagenase 1A digestion of the dermis to liberate individual cells which thrived in culture giving homogeneous cells (A and B). These cells proliferated in culture for several passages and maintained morphological homogeneity throughout (C and D). Images were captured using Cell^B imaging software (Olympus).

3.3.5. Characterisation of mouse MSC and DF

3.3.5.1. Phenotypic characterisation of Balb/c and C3H bone marrow-derived plastic-adherent cells and DF by flow cytometry

The expression of, or lack of, specific cell surface antigens is commonly used in the characterisation of MSC. Balb/c and C3H bone marrow-derived plastic-adherent cells were analysed by flow cytometry to determine whether they possessed a phenotype consistent with that known for MSC listed in **Table 3.1**. Lower passage (p3) and higher passage (p12) cells were analysed in order to investigate if prolonged culture had an effect on the phenotype of the cells. The same markers were also analysed on Balb/c and C3H (p6) DF in order to assess whether the panel of markers used could be used to distinguish between MSC and DF.

For the bone marrow-derived plastic-adherent cells, the cells were prepared as described in S**ection 3.2.5.1**. Unstained cells were used to define the population of cells of interest from debris and dead cells while isotype control-stained cells were used to set limits for positive and negative cells. All the antibodies used for these analyses were labelled with fluorescein isothyocyanate (FITC) whose fluorescence intensity was detected in the FL-1 channel. Cells exhibiting fluorescence intensity above 10^2 were considered positive for the specific marker whereas those below this threshold were considered negative or not different from the isotype control.

Early passage cells (p3) from Balb/c (**Figure 3.8**) and C3H (**Figure 3.9**) were positive for Sca-1, MHC I, CD29, CD44, CD90.2 and CD105. The cells were negative for MHC II, CD80, CD86, CD11b, CD34 and CD45 (less than 5 %). Late passage (p12) Balb/c (**Figure 3.10**) and C3H (**Figure 3.11**) cells showed a phenotype profile similar to that observed with early passage (p3) cells despite regular passage and extended culture for over 6 months. This showed that the cells maintained their phenotype for prolonged periods.

Expression of CD105 and MHC I was partial in both Balb/c (59 % and 77 %) and C3H (54 % and 75 %) early passage (p3) cells respectively but CD105 was highly expressed (99 %) in p12 cells whilst MHC I expression did not change. CD90.2 expression in p3 Balb/c cells was 67 % and increased to 99 % in p12 cells but it was the same (above 95 %) for both early and late passage C3H cells. CD29 expression in C3H cells however decreased from 100 % in p3 cells to 67 % in p12 cells whilst in Balb/c cells in increased from 88 % to 100 % in p3 and p12 cells respectively.

Despite these variations, the overall phenotype of both early and late passage Balb/c and C3H bone marrow-derived plastic-adherent cells was consistent with the phenotype for MSC reported in many studies. The different specific marker expression profiles between early and late passage cells is also consistent with the heterogeneity of bone marrow cell cultures as was observed throughout the literature. Though the phenotype resembled that of MSC, the cells were further tested to prove their multipotential properties in order to be fully defined as MSC.

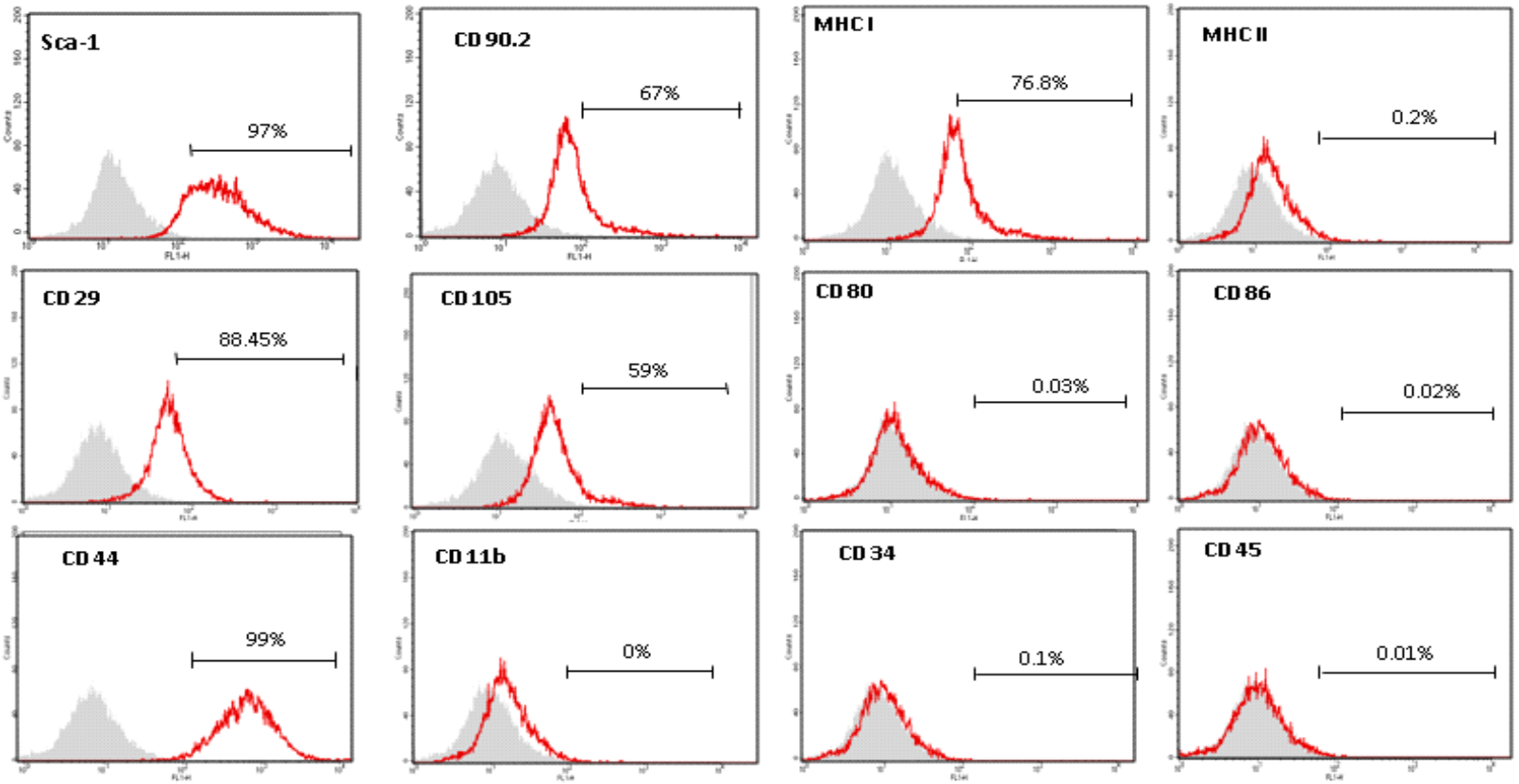

Figure 3.8. Phenotype of Balb/c mouse p3 bone marrow-derived plastic-adherent cells using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated with fluorophore staining above 10^2 considered positive expression of specific marker. Shaded (grey) histograms represented isotype controls, while the red bold lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29, CD44 and CD105 but negative for MHC II, haematopoietic markers CD34 and CD45, co-stimulatory molecules CD80 and CD86 and the macrophage marker CD11b. This profile was consistent with the known MSC phenotype. [CD; cluster of differentiation. MHC; major histocompatibility complex]

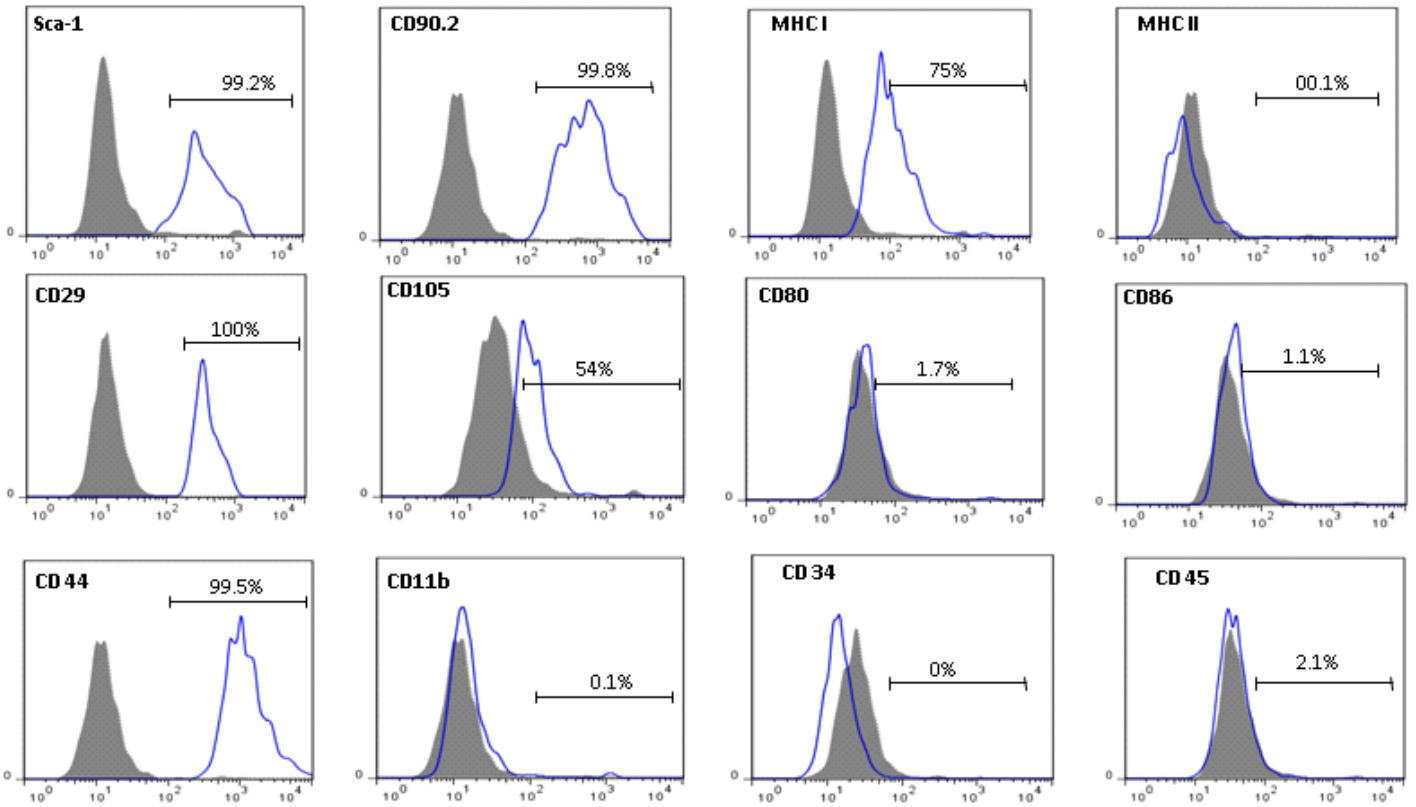

Figure 3.9. Phenotype of C3H mouse p3 bone marrow-derived plastic-adherent cells using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated with fluorophore staining above 10^2 considered positive expression of specific marker. Shaded (grey) histograms represented isotype controls, while the blue bold lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29, CD44 and CD105 but negative for MHC II, haematopoietic markers CD34 and CD45, co-stimulatory molecules CD80 and CD86 and the macrophage marker CD11b. This profile was consistent with the known MSC phenotype. [CD; cluster of differentiation. MHC; major histocompatibility complex]

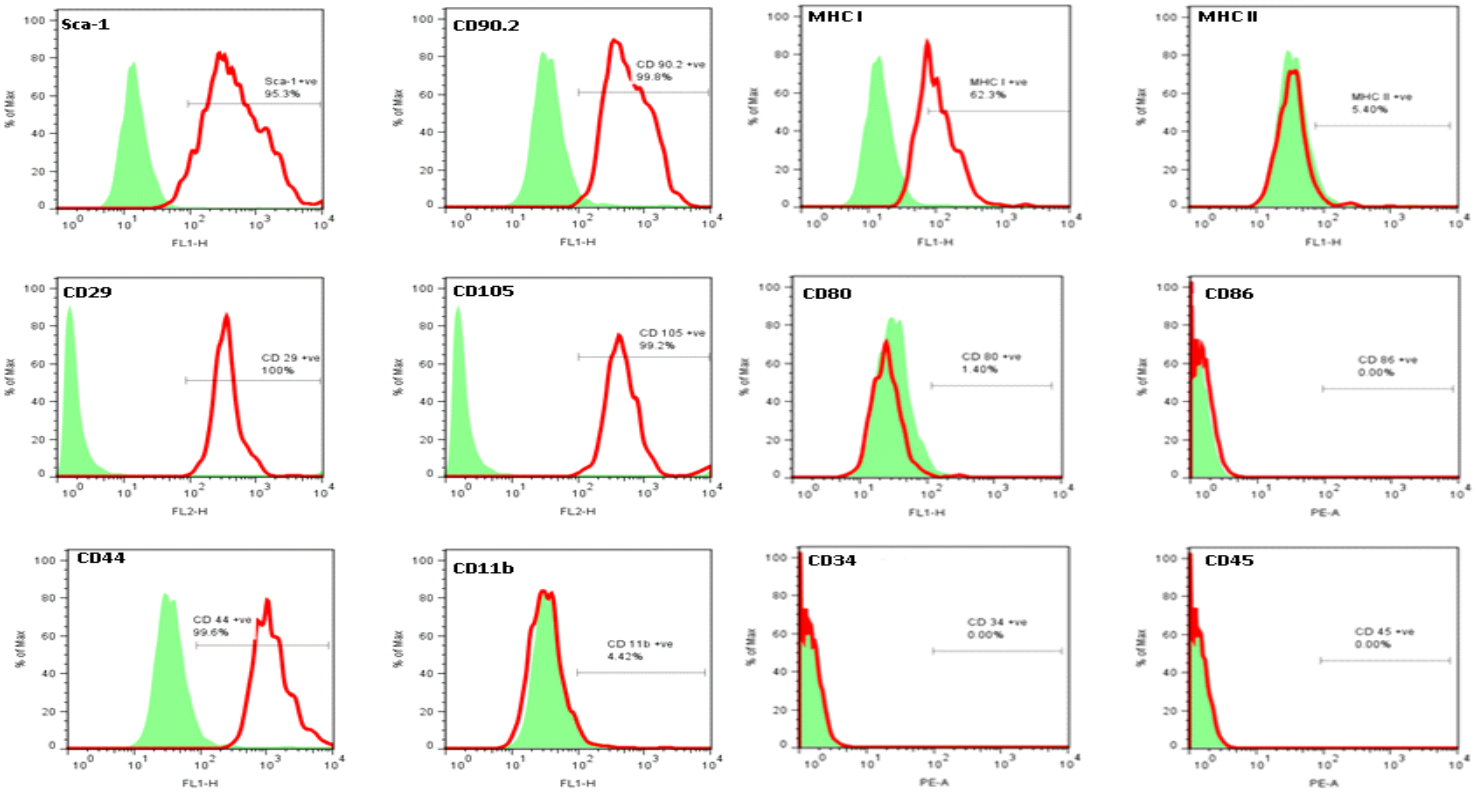

Figure 3.10. Phenotype of Balb/c mouse p12 bone marrow-derived plastic-adherent cells using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated with fluorophore staining above 10^2 considered positive expression of specific marker. Shaded (green) histograms represented isotype controls, while the red bold lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29, CD44 and CD105 but negative for MHC II, haematopoietic markers CD34 and CD45, co-stimulatory molecules CD80 and CD86 and the macrophage marker CD11b. This profile was consistent with the known MSC phenotype. [CD; cluster of differentiation. MHC; major histocompatibility complex]

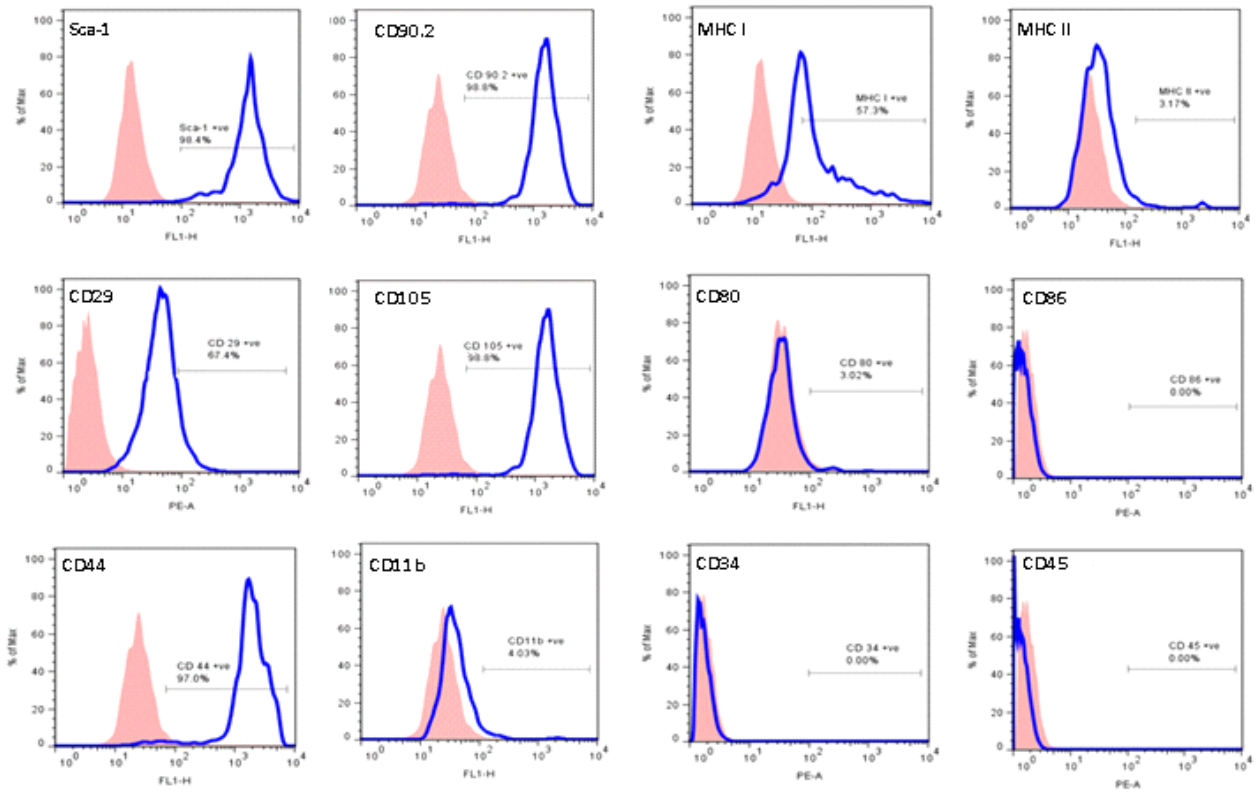

Figure 3.11. Phenotype of C3H mouse p12 bone marrow-derived plastic-adherent cells using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated with fluorophore staining above 10^2 considered positive expression of specific marker. Shaded (pink) histograms represented isotype controls, while the blue bold lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29, CD44 and CD105 but negative for MHC II, haematopoietic markers CD34 and CD45, co-stimulatory molecules CD80 and CD86 and the macrophage marker CD11b.This profile was consistent with the known MSC phenotype. [CD; cluster of differentiation. MHC; major histocompatibility complex]

DF were also analysed for the same markers (**Table 3.1**) as the bone marrow-derived plastic-adherent cells (except CD34) in order to determine whether they shared the same phenotype. Due to their fast growth rate, intermediate passage (p6) Balb/c and C3H DF were analysed by flow cytometry.

Unstained cells were used to distinguish the population of cells of interest from dead cells and debris while isotype control-stained cells were used to set limits for positive and negative cells. All the marker-specific antibodies were FITC-labelled except for CD29 and CD105 which were labelled with phycoerythrin (PE) whose fluorescence was detected in the FL2 channel. Cells which exhibited fluorescence intensity above 10^2 for FITC-labelled or 10^1 for PE-labelled were considered positive for the specific marker whereas those below this threshold were considered negative.

Both Balb/c (**Figure 3.12**) and C3H (**Figure 3.13**) DF were positive for Sca-1, MHC I and CD29 but were negative for MHC II, CD11b, CD44, CD45, CD80, CD86 and CD105 expression. For the positive markers, only Sca-1 had above 95 % expression in both Balb/c and C3H DF. CD90.2, MHC I and CD29 expression was 54 %, 91 % and 90 % respectively in Balb/c DF while for C3H cells it was 78 %, 82 % and 80 % respectively. All the negative markers, except for CD44, had expression less than 5 % in both Balb/c and C3H DF. In the case of CD44, expression was 43 % and 53 % respectively in Balb/c and C3H DF respectively.

The phenotype displayed by DF, though largely resembling that exhibited by bone marrow-derived plastic-adherent cells, showed differences in CD105 and CD44 which were negative in the former but positive in the later.

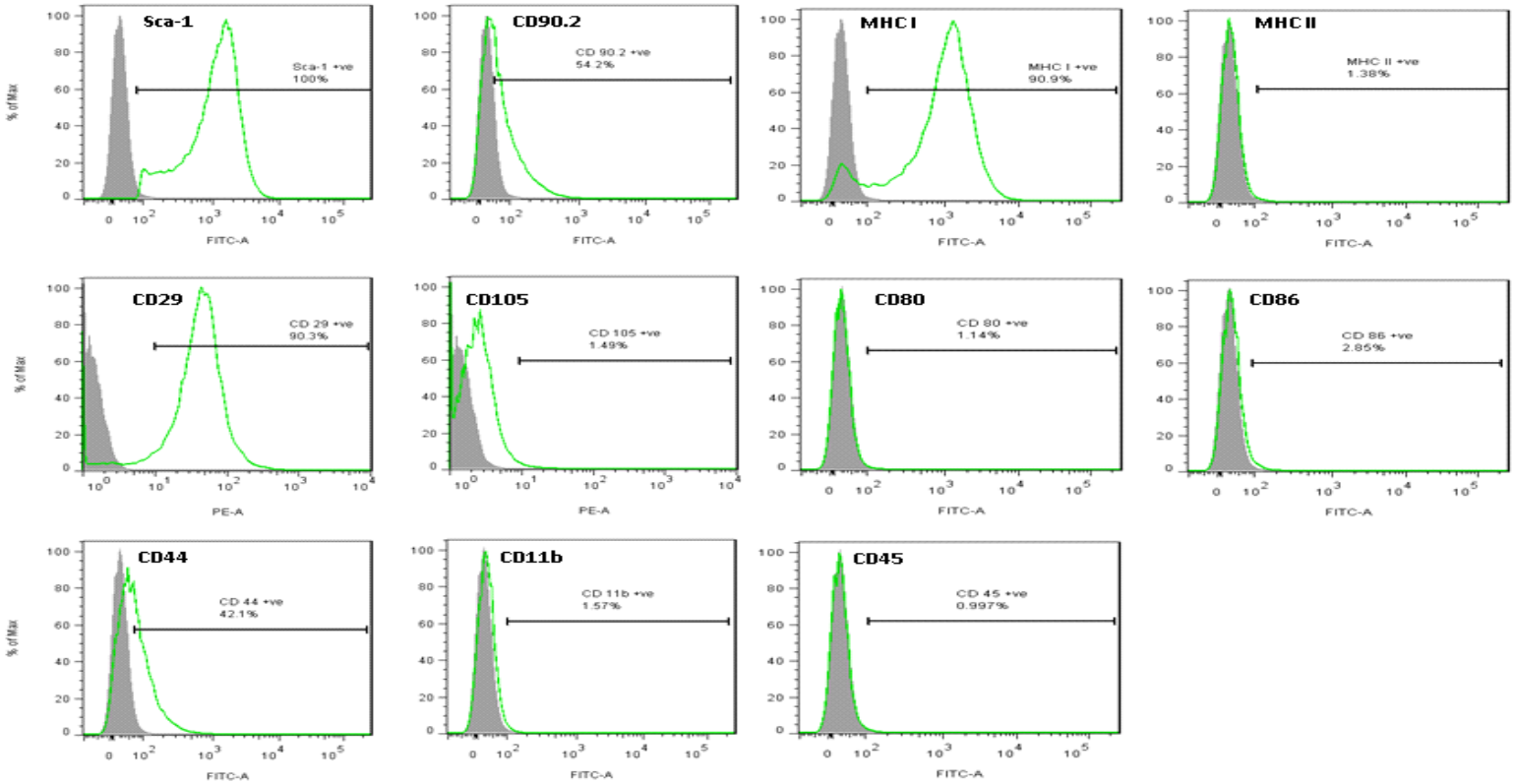

Figure 3.12. Phenotype of Balb/c mouse p6 DF using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated for fluorophore staining above 10^2 and 10^1 considered positive expression of specific markers bound to FITC and PE-labelled antibodies respectively. Shaded (grey) histograms represented isotype controls while the bold green lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29 and MHC I but negative for CD44, CD105, MHC II, CD45, CD80, CD86 and CD11b. [CD; cluster of differentiation; MHC; major histocompatibility complex]

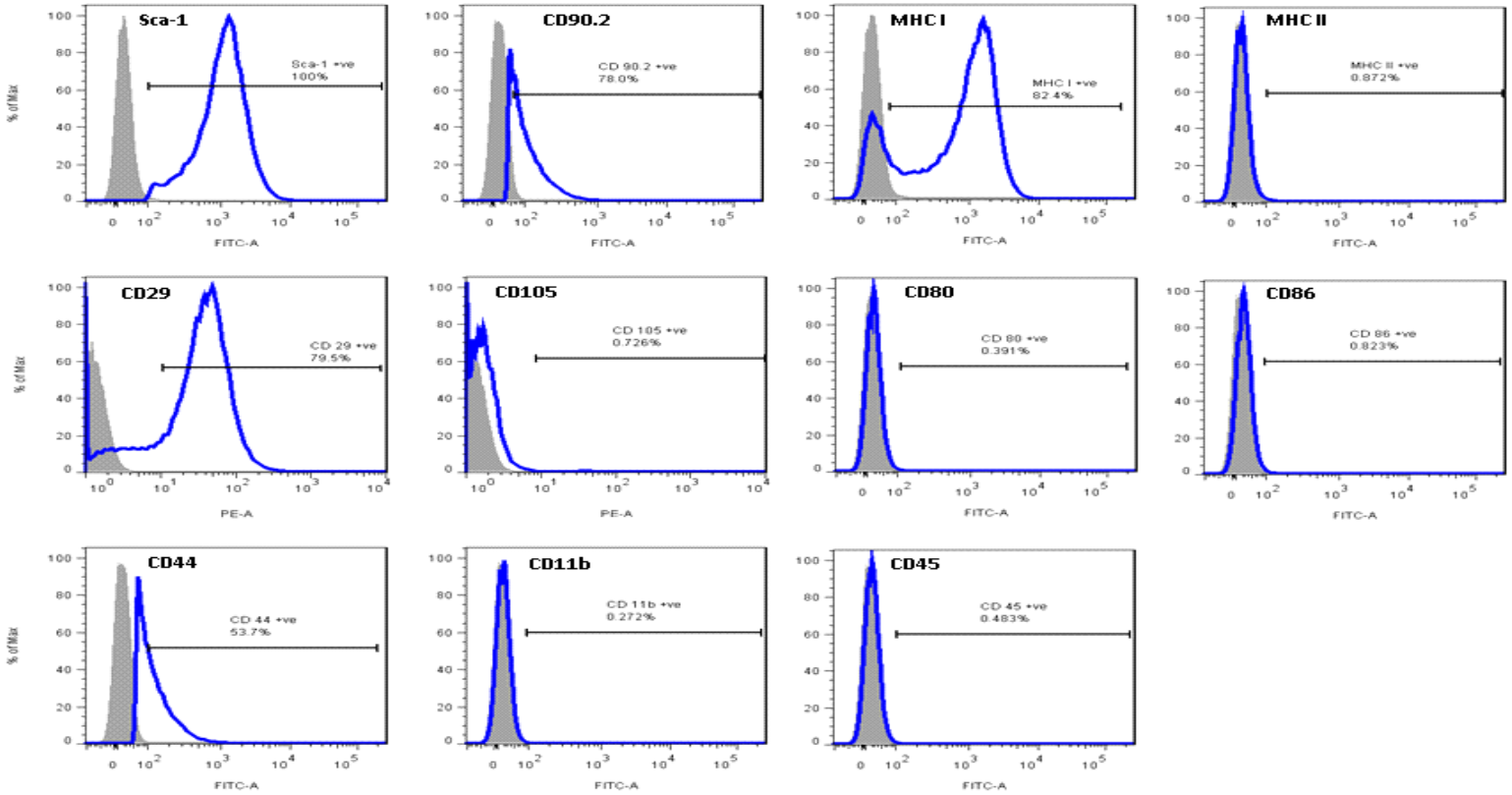

Figure 3.13. Phenotype of C3H mouse p6 DF using flow cytometry. Data shown is representative of 10^4 events acquired and analysed using CellQuest Pro software. Histograms were gated for fluorophore staining above 10^2 and 10^1 considered positive expression of specific markers bound to FITC and PE-labelled antibodies respectively. Shaded (grey) histograms represented isotype controls, while the bold blue lines represented specific marker expression profile. The fraction of positively stained cells is shown above the gate as a percentage of total cells. Cells were positive for the markers Sca-1, CD90.2, CD29 and MHC I but negative for CD44, CD105, MHC II, CD45, CD80, CD86 and CD11b. [CD; cluster of differentiation. MHC; major histocompatibility complex]

3.3.6. Tri-lineage differentiation of Balb/c and C3H bone marrow plastic-adherent cells and DF

In order to define the bone marrow-derived plastic-adherent cells as MSC, the cells should be able to demonstrate multipotential differentiation capacity by changing from their bipolar fibroblastic morphology to fat, cartilage and bone cells. The tri-lineage differentiation test involved treatment of the bone marrow-derived plastic-adherent cells (p10) with specific lineage differentiating media as described in **Section 3.2.5.2** accompanied by morphological examination during the differentiation period followed by histological staining. DF (p6) were also treated in the same way in order to test whether they possessed multipotential differentiation capacity.

3.3.6.1. Adipogenic differentiation

When cultured in ADM, the bone marrow-derived plastic-adherent cells changed from their bipolar spindle-shaped morphology and assumed a rounded morphology while the control cells cultured in non-differentiating conditions (DMEM-LG culture medium only) retained their spindle-shaped morphology. By the 3rd day of culture, small lipid vesicles became visible under light microscopy in both Balb/c (**Figure 3.14**) and C3H (**Figure 3.15**) cells cultured in ADM. With time, these vesicles gradually became larger in size and appeared like 'bunches of grapes' which coalesced to form larger lipid bodies by the 14th day. The adipogenic differentiated cells stained positive with Oil red O (ORO) stain while those cultured in non-differentiating conditions were negative. However, some cells cultured in ADM remained morphologically unchanged throughout the differentiation period and thus were unable to take up the ORO stain.

Similarly, both Balb/c (**Figure 3.16)** and C3H (**Figure 3.17**) DF cultured in ADM accumulated lipid vesicles by the 3rd day and after 14 days, substantial lipid deposits stained positive with oil ORO. Control cells cultured in DMEM-HG culture medium did not develop lipid vesicles and were negative with ORO stain. Thus both the bone marrow-derived plastic-adherent cells and DF demonstrated comparable adipogenic differentiation capacity.

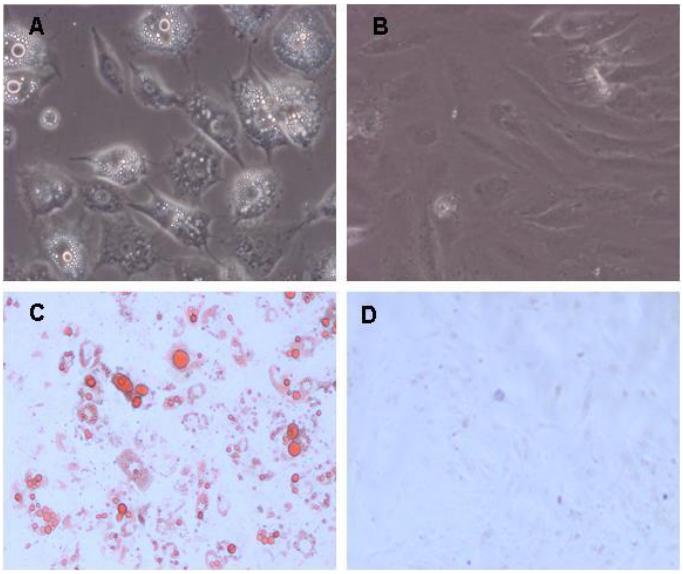

Figure 3.14. Adipogenic differentiation potential of p10 Balb/c mouse bone marrow-derived plastic-adherent cells. The cells were seeded in 6-well plates until confluent and cultured in either ADM and AMM or DMEM-LG culture medium for 14 days. Lipid vesicles accumulated in adipogenic-induced cells (A) while control cells retained their fibroblastic morphology (B). The lipid vesicles stained positive with ORO staining (C) while control cultures were negative (D). Images were captured at x200 magnification (A and B) and x100 (C and D). [ADM; adipogenic differentiation medium. AMM; adipogenic maintenance medium. ORO; oil red O]

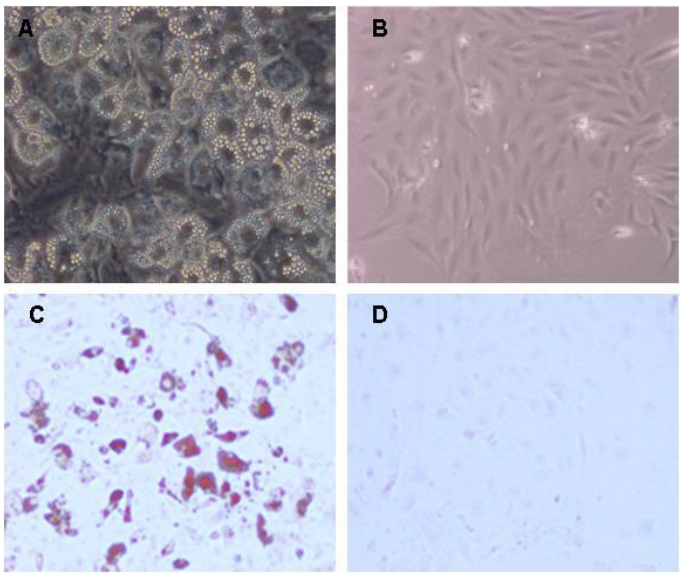

Figure 3.15. Adipogenic differentiation potential of p10 C3H mouse bone marrow-derived plastic-adherent cells. The cells were seeded in 6-well plates until confluent and cultured in either ADM and AMM or DMEM-LG culture medium for 14 days. Lipid vesicles accumulated in adipogenic-induced cells (A) while control cells retained their fibroblastic morphology (B). The lipid vesicles stained positive with oil ORO staining (C) while control cultures were negative (D). Images were captured at x100 magnification. [ADM; adipogenic differentiation medium. AMM; adipogenic maintenance medium. ORO; oil red O]

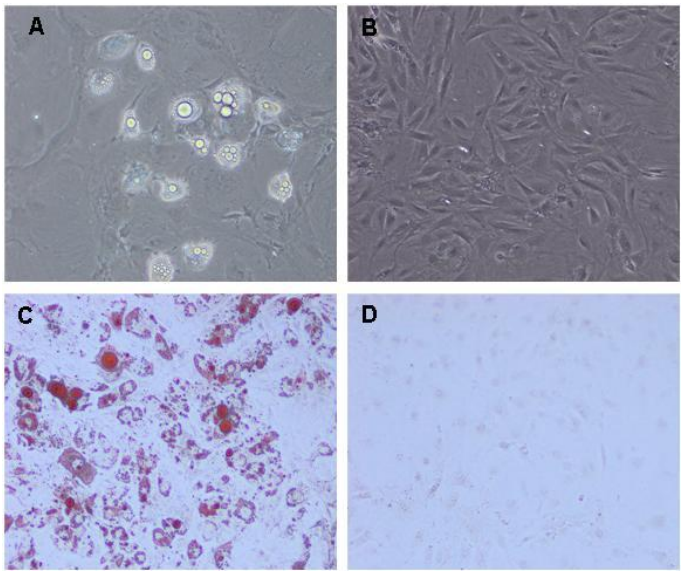

Figure 3.16. Adipogenic differentiation potential of p6 Balb/c mouse DF. The cells were seeded in 6-well plates until confluent and cultured in either ADM and AMM or DMEM-HG culture medium for 14 days. Lipid vesicles accumulated in adipogenic-induced cells (A) while control cells retained their spindle-shaped morphology (B). The lipid vesicles stained positive with ORO staining (C) while control cultures were negative (D). Images were captured at x100 magnification. [ADM; adipogenic differentiation medium. AMM; adipogenic maintenance medium. ORO; oil red O]

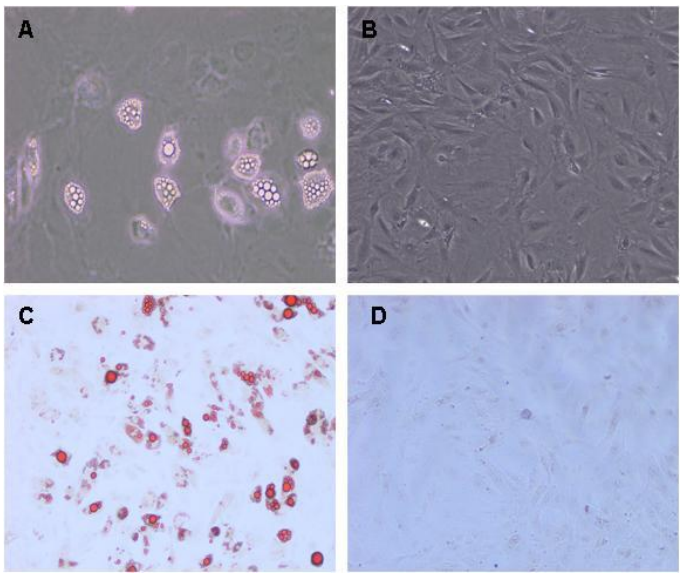

Figure 3.17. Adipogenic differentiation potential of p6 C3H mouse DF. The cells were seeded in 6-well plates until confluent and cultured in either adipogenic induction medium and AMM or DMEM-HG culture medium for 14 days. Lipid vesicles accumulated in adipo-induced cells (A) while control cells retained their spindle-shaped morphology (B). The lipid vesicles stained positive with oil red O staining (C) while control cultures were negative (D). Images were captured at x100 magnification. [ADM; adipogenic differentiation medium. AMM; adipogenic maintenance medium. ORO; oil red O]

3.3.6.2. Chondrogenic differentiation

To achieve chondrogenic differentiation, Balb/c and C3H bone marrow-derived plastic-adherent cells (p10) and DF (p6) were cultured as micro-pellets in CDM for 21 days. Control cells were also cultured as micro-pellets in non-differentiating conditions.

Light microscopy examination of the chondrogenic-induced Balb/c (**Figure 3.18**) and C3H (**Figure 3.19**) bone marrow-derived plastic-adherent cells showed an elongated morphology in comparison to control cells. After 21 days, micro-pellets in chondrogenic medium stained positive with alcian blue dye which stained glycosaminoglycans (GAGs) while control cells cultured in DMEM-LG culture medium were negative for the stain. Interestingly, both Balb/c (**Figure 3.20**) and C3H (**Figure 3.21**) DF cultured in chondrogenic differentiation medium stained positive with alcian blue while cells cultured in DMEM-HG culture medium were negative.

These findings showed that both Balb/c and C3H bone marrow-derived plastic-adherent cells and DF had capacity to different into chondrocytes.

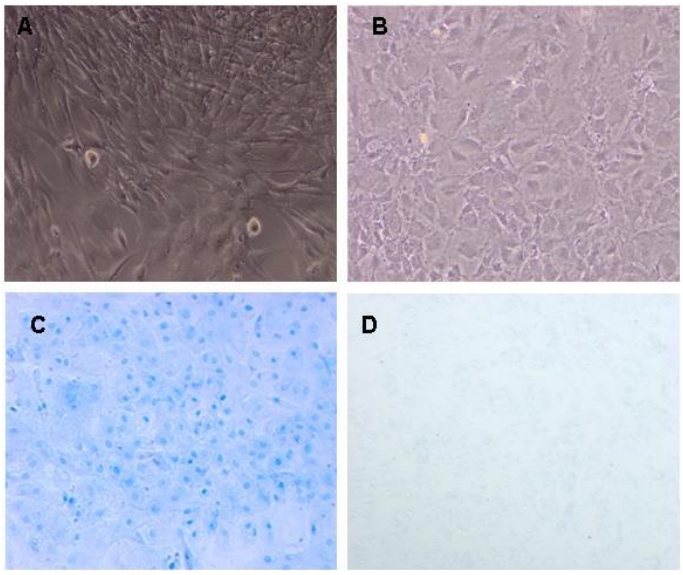

Figure 3.18. Chondrogenic differentiation potential of p10 Balb/c mouse bone marrow-derived plastic-adherent cells. The cells were seeded as micro-pellets in 24-well plates and cultured in CDM or DMEM-LG culture medium for 21 days. Chondrogenic-induced cells were closely packed and assumed an elongated morphology (A) in comparison to control cells (B). Chondrogenesis was detected by alcian blue staining with chondrogenic-induced cells showing substantial GAGs accumulation (C) while control cells were negative (D). Images were captured at x100 magnification. [CDM; chondrogenic differentiation medium. GAGs; glycosaminoglycans]

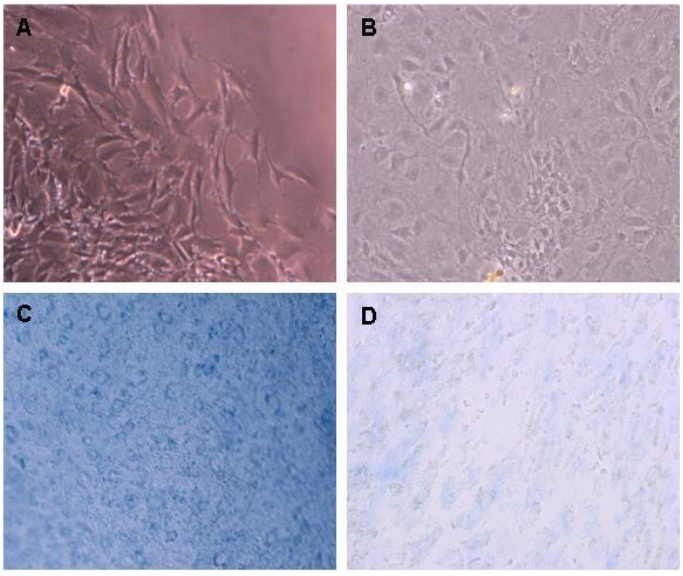

Figure 3.19. Chondrogenic differentiation potential of p10 C3H mouse bone marrow-derived plastic-adherent cells. The cells were seeded as micro-pellets in 24-well plates and cultured in CDM or DMEM-LG culture medium for 21 days. Chondrogenic-induced cells were closely packed and assumed an elongated morphology (A) in comparison to control cells (B). Chondrogenesis was detected by alcian blue staining with chondrogenic-induced cells showing substantial GAG accumulation (C) while control cells were negative (D). Images were captured at x100 magnification. [CDM; chondrogenic differentiation medium. GAG; glycosaminoglycans]

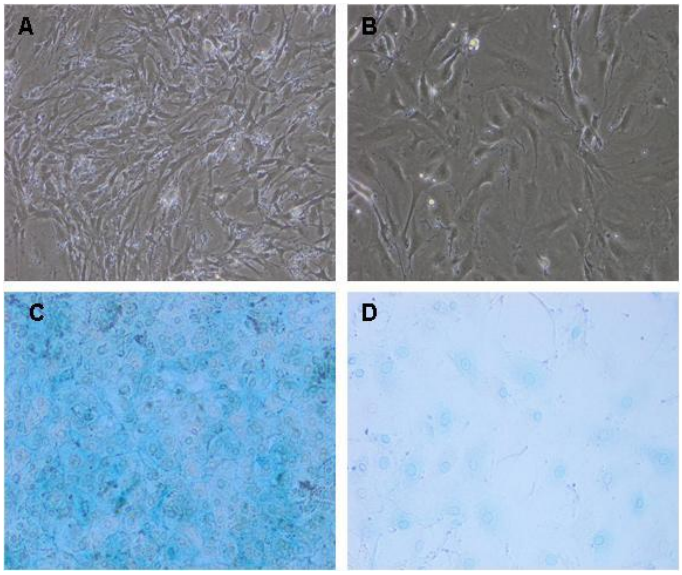

Figure 3.20. Chondrogenic differentiation potential of p6 Balb/c DF. The cells were seeded as micro-pellets in 24-well plates and cultured in CDM or DMEM-HG culture medium for 21 days. Chondrogenic-induced cells were closely packed and assumed an elongated morphology (A) in comparison to control cells (B). Chondrogenesis was detected by alcian blue staining with chondrogenic-induced cells showing substantial GAG accumulation (C) while control cells were negative (D). Images were captured at x100 magnification. [CDM; chondrogenic differentiation medium. GAG; glycosaminoglycans]

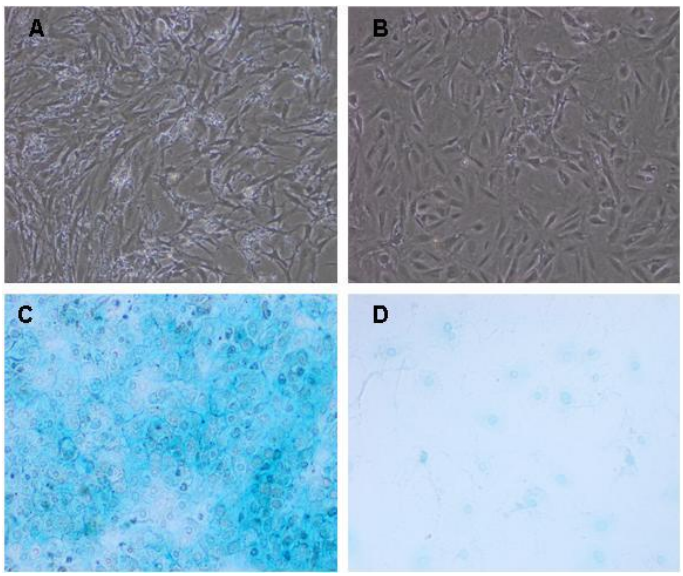

Figure 3.21. Chondrogenic differentiation potential of p6 C3H DF. The cells were seeded as micro-pellets in 24-well plates and cultured in CDM or DMEM-HG culture medium for 21 days. Chondrogenic-induced cells were closely packed and assumed an elongated morphology (A) in comparison to control cells (B). Chondrogenesis was detected by alcian blue staining with chondrogenic-induced cells showing substantial GAG accumulation (C) while control cells were negative (D). Images were captured at x100 magnification. [CDM; chondrogenic differentiation medium. GAG; glycosaminoglycans]

3.3.6.3. Osteogenic differentiation

Osteogenic differentiation was induced by culturing confluent Balb/c and C3H p10 bone marrow-derived plastic-adherent cells and p6 DF in osteogenic ODM for 14 days. Control cells were cultured in DMEM-LG or DMEM-HG culture medium for bone marrow-derived plastic-adherent cells and DF respectively.

Light microscopy examination on the 5th day of both Balb/c (**Figure 3.22**) and C3H (**Figure 3.23**) bone marrow-derived plastic-adherent cells cultured in osteogenic medium showed the cells became smaller and appeared to change morphology by developing dendrite-like projections By the 6th day, mineralisation was visible under the microscope, appearing as whitish plaques between cells while in control cultures, the cells retained their spindle-shaped morphology. After staining with alizarin red S, cells cultured in ODM stained bright orange and this confirmed the presence of calcium deposition while control cells cultured in DMEM-LG culture medium were negative.

Similarly, Balb/c (**Figure 3.24**) and C3H (**Figure 3.25**) DF cultured in ODM also developed dendrite-like projections and stained positive with alizarin red S while control cells cultured in DMEM-HG culture medium were negative after 14 days of culture.

Both the bone marrow-derived plastic-adherent cells and DF from the two mouse strains demonstrated their ability to successfully differentiate into bone cells. Also, the differentiated cells were resistant to dislodging with either 0.5% (v/v) trypsin-EDTA or Hy-Q-Tase™ cell dissociation solutions after 14 days even after incubation for 15 minutes; this is a common phenomenon for bone cells which is a result of extensive mineralisation.

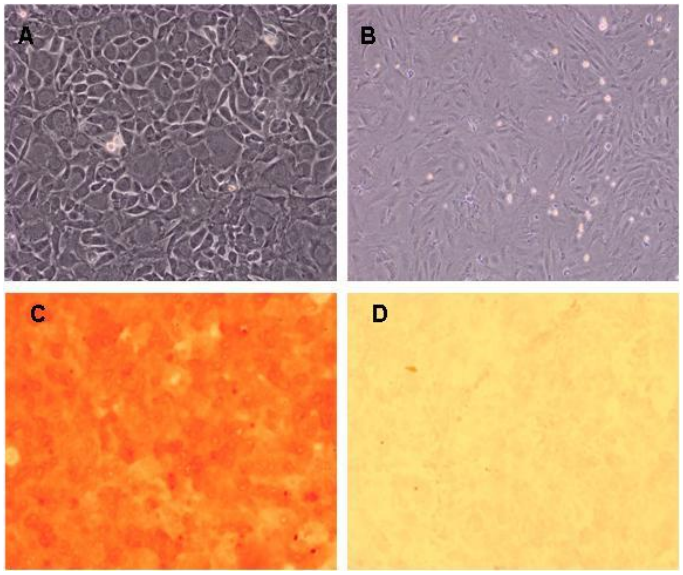

Figure 3.22. Osteogenic differentiation potential of p10 Balb/c mouse bone marrow-derived plastic-adherent cells. The cells were seeded in 6-well plates and cultured to confluence in ODM or DMEM-LG culture medium for 14 days. Osteogenic-induced cells had elaborate whitish boundaries indicative of calcium deposits (A) while control cells showed no morphological changes (B) These calcium deposits stained bright orange with alizarin red S showing extensive mineralisation (C) relative to control cells (D). Images were captured at x100 magnification.[ODM; osteogenic differentiation medium]

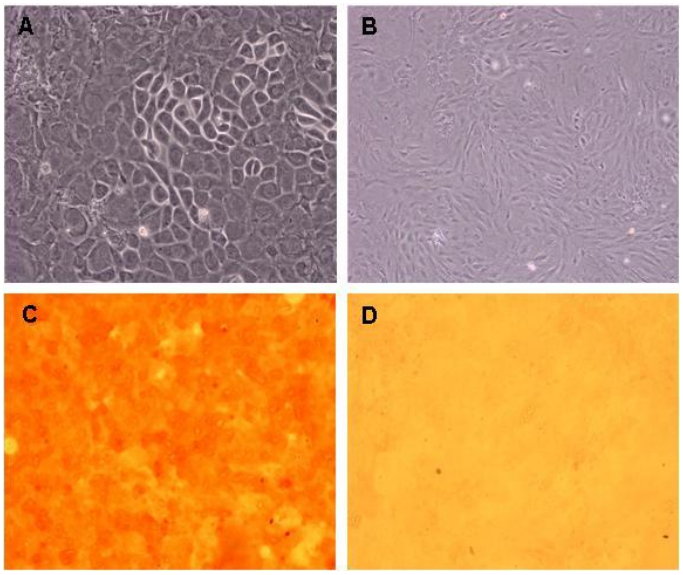

Figure 3.23. Osteogenic differentiation potential of p10 C3H mouse bone marrow-derived plastic-adherent cells. The cells were seeded in 6-well plates and cultured to confluence in ODM or DMEM-LG culture medium for 14 days. Osteogenic-induced cells had elaborate whitish boundaries indicative of calcium deposits (A) while control cells showed no morphological changes (B) These calcium deposits stained bright orange with alizarin red S showing extensive mineralisation (C) relative to control cells (D). Images were captured at x100 magnification. [ODM; osteogenic differentiation medium]

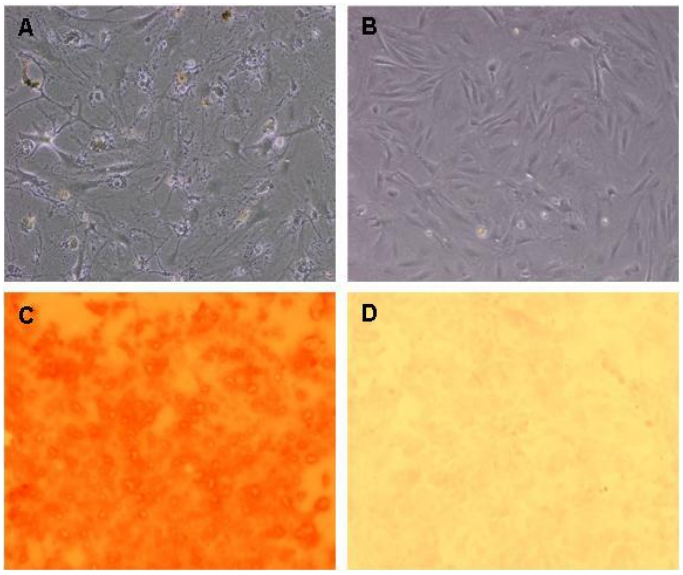

Figure 3.24. Osteogenic differentiation potential of p6 Balb/c mouse DF. The cells were seeded in 6-well plates and cultured to confluence in ODM or DMEM-HG culture medium for 14 days. Osteogenic-induced cells had elaborate whitish boundaries indicative of calcium deposits (A) while control cells showed no morphological changes (B) These calcium deposits stained bright orange with alizarin red S showing extensive mineralisation (C) relative to control cells (D). Images were captured at x100 magnification. [ODM; osteogenic differentiation medium]

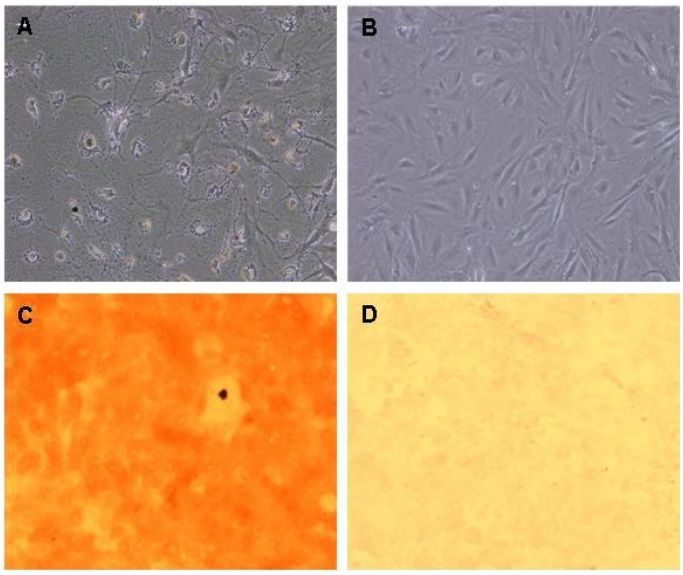

Figure 3.25. Osteogenic differentiation potential of p6 C3H mouse DF. The cells were seeded in 6-well plates and cultured to confluence in ODM or DMEM-HG culture medium for 14 days. Osteogenic-induced cells had elaborate whitish boundaries indicative of calcium deposits (A) while control cells showed no morphological changes (B) These calcium deposits stained bright orange with alizarin red S showing extensive mineralisation (C) relative to control cells (D). Images were captured at x100 magnification. [ODM; osteogenic differentiation medium]

Collectively, the results obtained from the tri-lineage differentiation assay showed that both Balb/c and C3H bone marrow-derived plastic-adherent cells were capable of differentiating into adipocytes, chondrocytes and osteoblasts when stimulated with specific chemicals and hormones added to culture medium. Thus, taken together with the phenotypic analysis, these cells demonstrated the characteristics of cells commonly defined as MSC and were therefore referred to as MSC from this point onwards. Interestingly, DF also demonstrated multipotential properties though their phenotype was slightly different from that of the MSC.

3.4. Discussion

The aim of this chapter was to define the isolation, expansion and characterisation of bone marrow derived MSC from Balb/c and C3H mouse strains which were tested for their immunomodulatory properties in subsequent assays described in **Chapter 5**. Female 4-8 week old Balb/c and C3H mice were used as the source of cells. DF, which were used for comparison, were also isolated from abdominal skin of the two mouse strains and characterised in the same manner as MSC.

Whole bone marrow extracts were obtained by femoral aspiration using DMEM-LG culture medium. The bone marrow-derived cells were cultured overnight to allow the plastic-adherent cells to attach while non-adherent cells were washed off during ensuing medium changes. Few scattered cells with different morphologies including spindle-shaped fibroblastic-like cells, dendritic-like cells and macrophage-like cells remained in the flasks after the initial medium changes. These cultures were marked by a 3-5 week period of minimal growth and expansion particularly during p0 and p1. Several studies have also identified a diverse range of cell types from mouse bone marrow cultures including macrophages, endothelial cells, fibroblasts (Wang *et al.*, 1990), dendritic cells (Inaba *et al.*, 1992), B-cell progenitors, granulocytic and monocytic precursors which were reported to persist in cultures for several weeks (Phinney *et al.*, 1999). Therefore, to overcome the inevitable contamination of MSC with other bone marrow-derived cells, a strategy of prolonged culture of the plastic-adherent cells at low density with regular medium changes was employed based on the postulate that stem cells would continue to proliferate in culture whereas other terminally differentiated cells would be lost over time.

Gradually, some of the morphologically diverse cells appeared smaller, rounded and were eventually lost in ensuing medium changes leaving behind sparsely scattered bipolar spindle-shaped fibroblast-like cells. These cells, known as colony forming unit-fibroblasts (CFU-F), gradually expanded forming colonies which appeared in a distinct radial pattern. Their expansion became rapid by the 2nd passage and cultures appeared homogeneously fibroblast-like. Generally, it was observed that C3H cultures produced more CFU-F than Balb/c cultures though the former took longer to establish than the latter. By the 3rd passage, both Balb/c and C3H cultures appeared homogeneous though this was not interpreted to mean the cells were phenotypically

and functionally identical since previous studies have suggested that other cells, including haematopoietic cells and fibroblasts may persist even after several serial passages (Baddoo *et al.*, 2003) despite cultures appearing morphologically homogeneous. This means that most MSC cultures obtained from bone marrow aspirates are inherently heterogeneous. This notion has been shown to be true in this study particularly following characterisation by flow cytometry as will be discussed later.

At this stage, different types of culture medium were tested to find the most suitable for the expansion of the bone marrow-derived plastic-adherent cells. This was necessitated by the fact that in all mouse MSC studies, different isolation techniques, growth conditions and culture medium have been used. The most common types used include DMEM-HG (Meirelles & Nardi, 2003), DMEM-LG (Jiang *et al.*, 2002), α-MEM (Baddoo *et al.*, 2003) and more recently specialist MSC media such as StemXVivo™ (Sung *et al.*, 2008). These media were tested for their ability to support rapid expansion of the 3rd passage cells by computing the CDT which is the time taken by the cells to divide *in vitro*. The fastest growth rates of 8 hours per doubling were obtained with cells cultured in DMEM-LG and StemXVivo™ followed by those cultured in MesenCult® (24 hours), DMEM-HG (30 hours) and α-MEM (40 hours) respectively. DMEM-LG, instead of StemXVivo™, was chosen as the expansion medium for all cultures on the basis of cost. The unusual linear conformation and subsequent senescence exhibited by cells cultured in α-MEM illustrated the importance of culture medium for successful MSC culture. In addition, the cells cultured in α-MEM were resistant to dislodging using 0.5% (v/v) trypsin-EDTA or Hy-Q-Tase™ cell dissociation solutions and could only be dislodged using a cell scraper. This suggested that either these cells formed stronger adhesions or had mineralised. It is thought that culture medium nutrient composition has a direct effect on cell signalling and consequently impacts cell growth, behaviour (including production of adhesion molecules) and fate (Park *et al.*, 2007c). All the plastic-adherent cells were subsequently expanded in DMEM-LG culture medium over a 6-month period with cells cryo-preserved at different stages until the 15th passage. Though the cells preserved morphological homogeneity from p3 to p15, they were not regarded as MSC.

DF were isolated by digesting small pieces of Balb/c and C3H abdominal skin with dispase II to separate the dermis from the epidermis thus minimising contamination of cells from the dermis with the epidermal keratinocytes. The dermis was subjected to collagenase 1A digestion to release individual fibroblasts which were washed and cultured in DMEM-HG culture medium. Cell expansion was rapid from the onset and cultures were populated by cells showing typical fibroblast morphology and the cells were easy to dislodge with 0.5 % (v/v) trypsin-EDTA solution. Despite the bone marrow-derived plastic-adherent fibroblastic-like cells sharing morphological similarity with DF, the former were however resistant to dislodging using trypsin-EDTA solution during passage and were dislodged using Hy-Q-Tase™ solution. This feature could be potentially used to remove fibroblast contamination from MSC cultures. Despite this characteristic, DF and the bone marrow-derived plastic-adherent cells were morphologically indistinguishable.

The next step was to characterise these bone marrow-derived plastic-adherent cells to determine whether they met the criteria commonly used in the literature to define cells as MSC. Phenotypic examination by flow cytometry using a panel of common cell surface markers showed that both Balb/c and C3H early (p3) and late (p12) passage cells were phenotypically similar, expressing Sca-1, MHC I, CD29, CD44, CD90.2 and CD105 while being negative for MHC II, CD34, CD45, CD80, CD86 and CD11b. This was consistent with other reports (Sun *et al.*, 2003), (Nadri & Soleimani, 2007a) and (Sung *et al.*, 2008). The lack of CD34 and CD45 expression indicated the absence of haematopoietic cells in the cultures and lack of CD11b, a macrophage marker further suggested that the cell cultures were less contaminated with other cells noticed earlier in heterogeneous cultures during the 1st passage. Lack of MHC II and the co-stimulatory molecules CD80 and CD86 is regarded as the an important feature of MSC and a major reason for their widely reported immunomodulatory properties (Le Blanc *et al.*, 2003b). Interestingly, CD105 and CD90.2 expression increased from intermediate levels in p3 cells to over 95 % in p12 cells while CD29 expression was variable between the two mouse species. Such variations have been reported in previous mouse MSC studies but it remains unknown whether these differences have a profound effect on the function of MSC. However, these variations could underline the heterogeneity of MSC cultures as previously alluded.

In comparison, both p6 Balb/c and C3H DF were positive for Sca-1, MHC I and CD29 while negative for CD105, CD44, MHC II, CD34, CD45, CD80, CD86 and CD11b. Despite showing a similar phenotype to the bone marrow-derived MSC, there were differences in CD44 and CD105 expression which was positive in MSC but negative in DF. Blasi and co-workers however, reported that both MSC and DF, were positive for CD44 and CD105 (Blasi *et al.*, 2011).

To date, there is no consensus regarding the definitive phenotype of both MSC and DF since most of the markers used to define MSC are not unique to them but have been shown to be expressed by many different cells of mesenchymal origin. CD105 expression has been reported to be reduced in DF in other studies (Wagner *et al.*, 2005; Lysy *et al.*, 2007) but Haniffa *et al*. (2007) and Lorenz *et al.* (2008) reported similar expression levels to MSC (Haniffa *et al.*, 2007; Lorenz *et al.*, 2008). Interestingly, Jin *et al*. (2007) suggested that down-regulation of CD105 expression in MSC is associated with cell differentiation and recommended that it can be used as a marker for stem cell differentiation status (Jin *et al.*, 2009). In this study however, lack of CD105 expression on DF could not be associated with terminal differentiation as they successfully underwent tri-lineage differentiation; this was also supported by Lysy *et al*. (2007). Though CD105 expression could be a potential marker for distinguishing between MSC and DF, results from this study suggested that CD105 expression in combination with CD44 and CD90.2 expression could be used to distinguish between these two cell types.

For a long time, fibroblasts were thought of as primitive cells in adult tissues (Takahashi & Yamanaka, 2006) but there is increasing uncertainty not only because fibroblasts are now thought to be phenotypically and functionally equivalent to MSC (Haniffa *et al.*, 2007), but also due to the identification of skin-derived precursors (SKP) which were differentiated into cells of mesodermal and neural lineages and retained self-renewal and differentiation potency for over a year (Toma *et al.*, 2001). There have been many reports documenting the existence of sub-populations of stem cell-like cells in mammalian skin (Fernandes *et al.*, 2004; Shih *et al.*, 2005; Toma *et al.*, 2005; Crigler *et al.*, 2007) and hair follicles (Yu *et al.*, 2006). These findings could therefore imply that the stem cell properties displayed by skin-derived DF are

due to the presence of SKP. Thus the lack of specific markers that can distinguish MSC from other mesenchymal cells remains a major challenge in MSC studies.

Tri-lineage differentiation studies of both Balb/c and C3H p10 MSC demonstrated their ability to differentiate into adipocytes, chondrocytes and osteocytes as confirmed by ORO, alcian blue and alizarin red S staining respectively. For adipogenic differentiation, it was observed that lipid vesicle accumulation did not occur in all the cells. This could be interpreted as further demonstration that MSC cultures are heterogeneous with regard to their differentiation capacity. This could be as a result of age-differences within cultures since it is thought that the differentiation capacity of murine MSC declines with mouse age and passage (Kretlow *et al.*, 2008) rather than it being due to heterogeneity resulting from having different cell types. Micromass cultures in chondrogenic medium stained positive with alcian blue while the control cells cultured in DMEM-LG culture medium were negative. Osteogenic differentiation was more prominent as most cells stained orange with alizarin red S due to extensive mineralisation. These cells were resistant to dissociation by Hy-Q-Tase™ or 0.5 % (v/v) trypsin-EDTA solutions even after extended incubation for 15 minutes; a phenomenon attributed to extensive calcification (Yoon *et al.*, 2004).

Cells were grown to confluence before they underwent differentiation as cell to cell contact appeared to be a pre-requisite for the *in vitro* differentiation of the MSC. Close cell to cell contact was thought to play an important role in facilitating adipogenic differentiation as lipid vesicle formation was concentrated in highly confluent areas and osteogenic differentiated cultures were deeply stained in areas of high cell density. This was also illustrated in chondrogenic differentiation where micromass cultures comprising densely populated cells, facilitated GAGs accumulation while in control cultures, cell expansion resulted in dispersal of the cells in the wells.

Curiously, both p6 Balb/c and C3H DF differentiated into adipocytes, chondrocytes and osteoblasts. The tri-lineage differentiation potential was thought to be similar to that shown by MSC. Though there have been few reports of DF differentiation into adipocytes and osteoblasts (Lorenz *et al.*, 2008) and hepatocytes (Lysy *et al.*, 2007), other studies have found them incapable of adipogenic or osteogenic differentiation

(Wagner *et al.*, 2005). Only one study to date (Haniffa *et al.*, 2007) has demonstrated human DF differentiation in the standard tri-lineage test used for defining MSC.

Interestingly, osteogenic and chondrogenic differentiation were achieved in the absence of BMP2 and TGFβ3 respectively in culture media despite them being used in most studies. This raises the question of whether these factors are absolute requirements for achieving differentiation or that their presence may facilitate faster differentiation or indeed triggers a larger fraction of the cells to undergo differentiation. Since differentiation was not quantified in this study, it would have been important to assess the extent of differentiation at various stages in the presence and absence of these factors. The fact that most human MSC studies use cells obtained from older donors and also that in this case mouse MSC obtained from young donors were used may be important in determining the specific chemical and hormonal cues for facilitating differentiation. Although it is known that donor age significantly affects the quality of MSC, there are no studies to date that describe how age related differences could influence the choice and amounts *in vitro* differentiation factors.

To summarize, both early and late passage Balb/c and C3H bone marrow-derived plastic-adherent cells were demonstrated to possess self-renewal properties by undergoing numerous rounds of passaging without observable morphological changes, expressed Sca-1, MHC I, CD29, CD44, CD90.2 and CD105 but did not express MHC II, CD80, CD86, CD34, CD45 and CD11b. Functionally, they showed multipotential capacity by differentiating into adipocytes, chondrocytes and osteocytes. Thus, these cells fulfilled the criteria used in defining MSC and were therefore used in subsequent immunological assays. DF displayed similar properties to MSC such as morphological homogeneity, tri-lineage differentiation capacity but slight phenotypic variations were detected by flow cytometry included absence of CD105, CD44 and CD90.2. The tri-lineage differentiation capacity of mouse DF had not been reported in the available literature to date.

4. CHAPTER FOUR: DEVELOPMENT OF CONDITIONS FOR LYMPHOCYTE TRANSFORMATION ASSAYS (LTA) AND MIXED LYMPHOCYTE REACTIONS (MLR)

4.1. Introduction

Historically, the need to overcome the effects of allograft rejection led to an increased interest in immunology in an effort to improve understanding of the mechanisms underlying immunological competence and histocompatibility (Billingham & Silvers, 1959a). Early studies in the 1960s had reported the appearance and subsequent proliferation of blasts of "immature-appearing" cells in cultures containing human leukocytes and the plant extract phytohemagglutinin (PHA) which was used in the isolation procedure of leukocytes because of its ability to clump red blood cells (Nowell, 1960). Importantly, blastogenesis was detected in co-cultures of allogeneic but not syngeneic murine and rabbit mononuclear cells (Bain *et al.*, 1964; Chapman & Dutton, 1965; Schwarz, 1968). These observations demonstrated that histocompatibility disparities but not matching, triggered leukocyte proliferation.

This provided the basis for the development of the MLR which could be used as a clinical tool for monitoring the immunological competence of the lymphocytes in patients suffering from immunological disorders, or patients undergoing immunosuppressive therapy and organ transplantation (Oppenheim *et al.*, 1968; Janossy & Greaves, 1971). Since then, the MLR has been used as an *in vitro* correlate for immunological tolerance (Schwarz, 1968), allograft survival (Bradley *et al.*, 1972) and in testing diseases such as rheumatoid arthritis (Stastny, 1976), Crohn's disease (Richens *et al.*, 1974) and human immunodeficiency virus [HIV] (Lederman *et al.*, 1998). By definition, MLR refers to a mixture of lymphoid cells from syngeneic, allogeneic or xenogeneic sources.

The commonly used and most informative form of the MLR is the "one-way" MLR (Bach & Voynow, 1966) whereby one set of lymphocytes, known as 'responder' cells are allowed to respond to another set of mitotically inactivated lymphocytes

known as 'stimulator' cells. The lymphocyte proliferative response in the one-way MLR is thus directly attributed to the responder lymphocytes only (Harrison & Paul, 1973). Mitotic inactivation can be achieved by treatment of the stimulator cells with non-lethal doses of mitomycin C or x-irradiation. Both these methods have been shown to be similarly effective (Bach & Bach, 1972). In the 'two-way' MLR, mitotic inactivation is not required as both sets of lymphocytes act as responders and stimulators to each other and contribute to the overall lymphocyte proliferative response. Adaptations of the both the one-way and two-way MLR in which mitotically inactivated non-lymphoid cells are incorporated at the beginning or during on-going cultures have been used extensively in studying the immunomodulatory properties of different cell types. In this study, the immunomodulatory properties of allogeneic MSC and DF were tested and are discussed in detail in **Chapter 5**.

In instances where responder lymphocytes are co-cultured with stimulator cells of non-lymphoid origin or when the stimulating agents are non-cellular such as the non-specific mitogens PHA or Con-A, the capacity of the stimulating cells or agents to induce a proliferative response can be determined. This approach is called the LTA and has been widely used in testing the immunogenicity of different chemicals and cell types (Mills, 1966; Nyfeler & Pichler, 1997). In this study, the LTA was used in investigating the immunogenicity of allogeneic MSC and DF and is discussed in detail in **Chapter 5**.

The proliferative response of the responder lymphocytes in MLR or LTA can be detected and quantified by the incorporation of radioactive tritiated thymidine [^{3}H-thymidine] *in vitro* (Huemer *et al.*, 1968). ^{3}H-thymidine is a radioactive nucleoside and precursor of thymidine found in DNA[10] which becomes incorporated in the DNA strands of daughter cells during mitosis. The uptake or utilisation of ^{3}H-thymidine can be measured to determine the extent of the proliferative response of the responder lymphocytes. This radioactivity is proportional to the number of proliferating cells which in turn is a function of the number of responder lymphocytes that were stimulated to enter the proliferative response (Caron *et al.*, 1965; Börjeson *et al.*, 1966). The amount of ^{3}H-thymidine incorporated into the

[10] DNA – deoxyribonucleic acid

DNA of daughter cells is recorded as the number of radioactive disintegrations or counts per minute (CPM).

Due to the inconsistencies that arise when data is presented as CPM as a result of varying initial cell densities, specific activity of the ^{3}H-thymidine, incubation periods and instrumentation settings, data is often presented as stimulation indices (SI) which are expressed as;

$$SI = \frac{Average\ CPM\ in\ stimulated\ cultures(LTA\ or\ MLR)}{Average\ CPM\ in\ unstimulated\ cultures(control)}$$

Measurement of DNA biosynthesis is the most commonly used method amongst immunologists as recommended by the International Union of Immunological Societies (IUIS) in 1976. Generally, SI between 2 and 3 are regarded as strongly suggesting a positive response and values above 3 are taken as statistical biological positive [SBP] (Frome *et al.*, 2003; Klein *et al.*, 2004). In practice however, a positive SI may not be clinically significant but may merely reflect responsiveness of the responder lymphocytes towards the stimulating agent. It has been argued that CPM data show more consistency and better discriminate between immune and non-immune responses than data expressed as SI (Hicks *et al.*, 1986).

As a result of the variety in specific biological mechanisms other than DNA biosynthesis that occur in transforming cells, a variety of physical and chemical techniques which can detect cell transformations such as protein biosynthesis and other metabolic processes have been developed. Spectrophotometric and fluorimetric based assays such as the reduction of Amalar blue dye (Ansar Ahmed *et al.*, 1994), esterase-mediated hydrolysis of fluorescein diacetate by proliferating lymphocytes (Dotsika & Sanderson, 1987) and the determination of ATP bioluminescence using the luciferin-luciferase reaction (Crouch *et al.*, 1993) have been widely used. More recently, flow cytometry has provided a powerful platform which utilises intracellular fluorescence measurement to determine cell proliferation. The most common approach uses the tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) to quantify overall responder lymphocyte proliferation as well as providing further insight into the history of the dividing lymphocytes (Lyons & Parish, 1994). Using this approach, parameters such as absolute cell numbers (Al-

Rubeai *et al.*, 1997), number of cell divisions undergone by different cell types (Hawkins *et al.*, 2007), distinction between various classes of lymphocytes and cell viability (Jones & Senft, 1985; Quah *et al.*, 2007), which cannot be obtained by the ^{3}H-thymidine method, can be obtained to provide more information with regard to the processes involved during lymphocyte stimulation.

Despite the numerous options available for measuring LTA and MLR, the ^{3}H-thymidine uptake method remains the gold standard and reference for all the other techniques due to its sensitivity and reliability over a long period of time. It is directly related to the DNA of proliferating cells rather than secreted enzymes or fluorescent dyes whose activity is influenced by many *in vitro* factors other than cell division.

Though the LTA and MLR have a long history of clinical application as tests for immunological tolerance and hypersensitivity (Schwarz, 1968; Milovanova, 2007), the reliability and sensitivity of these assays heavily depend on the optimisation of the key methodology variables. The main assumption is that the *in vitro* responses mirror wholly or in part the *in vivo* primary or secondary immunological response (Moorhead *et al.*, 1967). Therefore, it is crucial for both the micro and macro environmental conditions for experiments to parallel those of living organisms. Thus, it is important to define and assess the key variables for MLR and LTA to ensure sensitivity and reproducibility. The work in this chapter was therefore dedicated to defining and assessing the appropriate conditions for carrying out MLR and LTA for determining the immunomodulatory properties and immunogenicity of allogeneic MSC and DF.

4.1.1. Aim and Objectives

The aim of this part of the study was to develop appropriate methodology and conditions for performing LTA and MLR which were used in subsequent assays to investigate the immunological status of allogeneic MSC and DF.

In this model, Balb/c (*H2-d*) mononuclear cells were used as responder cells in LTA assays. They were stimulated with non-specific mitogens (positive control) which tested the effects of various parameters on their capacity to mount proliferative responses. Mitotically inactivated C3H (*H2-k*) and Balb/c mononuclear cells were used as stimulator cells against Balb/c mononuclear cells in one-way MLR as a positive control for the allogeneic and syngeneic response respectively.

The specific objectives were;

1. To determine the type and effective concentration of mitogen (PHA or Con-A) to use as a positive control for Balb/c mononuclear cell stimulation over a 7 day culture period.
2. To determine the effects of replenishing culture medium in on-going cultures up to 21 days.
3. To determine the effective concentration of mitomycin C required for inducing mitotic inactivation in C3H mononuclear cells (stimulator cells).
4. To determine the appropriate type (mouse or FCS) and concentration of serum for culture medium enrichment.
5. To determine the appropriate total cell numbers and ratio of responder (Balb/c) to stimulator (C3H) cells for performing one-way MLR.

Low specific activity ^{3}H-thymidine (6.7 Ci.mmol^{-1}) was used in all the experiments as it was previously found to be more effective than high specific activity ^{3}H-thymidine (20-30 Ci.mmol^{-1}) in LTA involving Balb/c and C3H mononuclear cells in work carried out in the Ingham laboratory (Matthews BJ, 1992).

4.2. Materials and Methods

4.2.1. Reagents

The following reagents were used and were prepared as described in **Section 2.2.2** unless stated otherwise.

- ATPLite™ kit
- Camptothecin stock solution (1 mg.ml^{-1})
- CFSE stock solution (5 mM)
- Con-A stock solution (1 mg.ml^{-1})
- CountBright™ fluorescent counting beads
- FCS
- Low activity ^{3}H-thymidine working solution (25 μCi.ml^{-1})
- Lymphocyte culture medium
- Lymphocyte transport medium
- Methanol; 100 % (Merck)
- Microscint-20 scintillation fluid
- Mitomycin C stock solution (1 mg.ml^{-1})
- Mouse serum (Autogen Bioclear)
- PBS (without Ca^{2+} and Mg^{2+})
- PHA stock solution (1 mg.ml^{-1})
- Saline solution (0.85 % w/v)
- TCA (10 % w/v)
- Viaprobe™ 7-AAD cell viability probe solution

4.2.2. Comparison of PHA and Con-A for the determination of the suitable mitogen and concentration for stimulation of Balb/c mouse mononuclear cells

Mononuclear cells were isolated from Balb/c mouse spleens and lymph nodes, counted and resuspended in lymphocyte culture medium as described in **Section 2.2.11**. The mononuclear cells were seeded in uncoated 96-well U-bottom plates at a density of 1 x 10^5 cells per well in 100 μl volumes. Frozen PHA and Con-A stocks were defrosted and serially diluted in lymphocyte culture medium to create solutions

of 100, 80, 70, 60, 50, 40, 30, 20 and 10 μg.ml^{-1}. PHA or Con-A solutions (100 μl) were added to 100 μl of Balb/c mononuclear cells per well to give cultures containing final concentrations of 50, 40, 35, 30, 25, 20, 15, 10 and 5 μg.ml^{-1} of PHA or Con-A as shown in the experimental setup (**Figure 4.1**). Each mitogen concentration was tested in triplicate wells. Cells without either mitogen were set up as controls for unstimulated and non-dividing cells. The outermost wells of the plates were filled with 200 μl of PBS to minimise evaporation from the test wells during incubation. The plates were cultured in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 3, 5 and 7 days.

For the ^{3}H-thymidine uptake assay, sixteen hours prior to completion of each time point, individual wells were spiked with 10 μl of 25 μCi.ml^{-1} low activity ^{3}H-thymidine to give a concentration of 0.25 μCi per well as described in **Section 2.2.12**. Post spiking, plates were returned for incubation to complete the required culture period. Upon reaching their specific time points, the plates were removed from incubation and the cells were harvested onto glass fibre filter UniFilter-96 GF/B plates and radioactivity counts were read as CPM using a TopCount NXT scintillation plate reader as described in **Section 2.2.13**.

For the ATPLite™ assay, on completion of the incubation period, cultures were briefly mixed on an orbital shaker for one minute to resuspend the cells before they were transferred to white OptiPlate™ 96-well plates. Cell viability was then analysed as described in **Section 2.2.14**. The ATP content was measured in a TopCount NXT luminescence plate reader and data was obtained and recorded as luminescence counts per second (CPS).

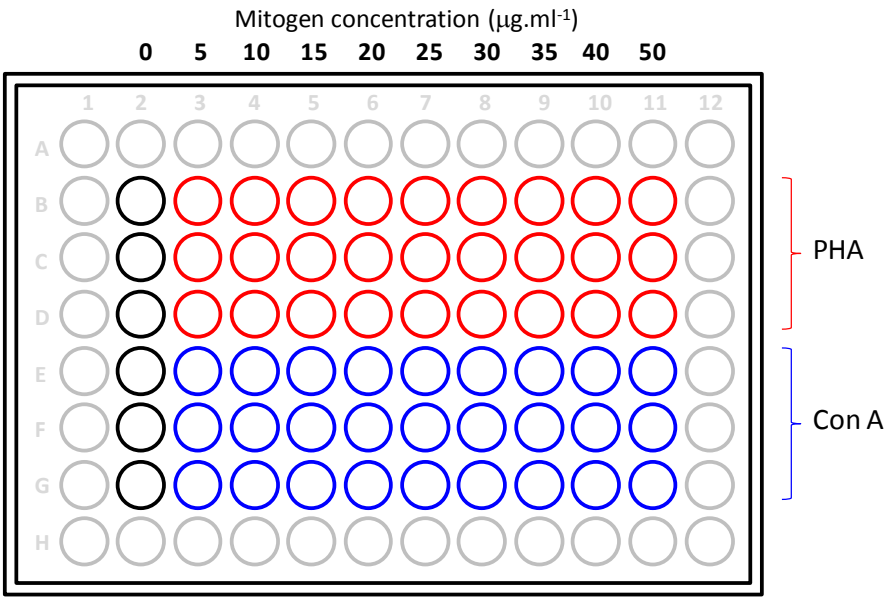

Figure 4.1. Diagram showing the 96-well experimental set up for the comparison of PHA and Con-A in stimulating Balb/c mononuclear cells. Triplicate wells were prepared for each mitogen concentration. The cultures were analysed after 3, 5 and 7 days without medium changes. Unstimulated cells were used as controls for non-dividing cells. Cultures were analysed by the ^{3}H-thymidine uptake assay to determine the most appropriate mitogen and concentration that give highest counts. The ATPLite assay was used to determine cell viability by measuring cellular ATP content. Outer wells (grey) were filled with PBS to minimise evaporation.

4.2.3. Effect of medium changes and extended culture time on the stimulation of Balb/c mononuclear cells with Con-A

Mononuclear cells were isolated from Balb/c mouse spleens and lymph nodes, counted and resuspended in lymphocyte culture medium as described in **Section 2.2.11**. The cells were seeded in uncoated 96-well U-bottom plates at a density of 1 x 10^5 cells per well in 100 μl volumes. Con-A stock was defrosted and serially diluted in lymphocyte culture medium to create solutions of 50, 40, 30, 20 and 10 μg.ml^{-1}. Each well volume was made up to 200 μl by addition of 100 μl of Con-A medium to give final concentrations 25, 20, 15, 10 and 5 μg.ml^{-1} in replicates of six. The outermost wells of the plates were filled with PBS to minimise evaporation from the test wells and the plates were cultured in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 3, 5, 10, 15 and 20 days.

For cultures accompanied with medium changes, 100 μl of medium was aseptically removed from each well (by carefully pipetting medium from the top of the well

while ensuring minimal disturbance to the cell pellet at the bottom of the well) and replaced with 100 µl of fresh culture medium containing the appropriate concentration of Con-A every three days and the plates were returned for incubation. Replicate cultures (without medium changes) were prepared and incubated for similar periods. For each time point, cultures with and without medium changes were tested at the same time. Sixteen hours prior to completion of each time point, individual wells were spiked with 10 µl of 25 µCi.ml^{-1} low activity ^{3}H-thymidine as described in **Section 2.2.12**. Post spiking, plates were returned for incubation to complete the required culture period after which the plates were removed from incubation and the cells harvested and radioactivity counts read as CPM using a TopCount NXT scintillation plate reader as described in **Section 2.2.13**.

4.2.4. Determination of the appropriate mitomycin C concentration for achieving stimulator cell mitotic arrest using C3H mononuclear cells

4.2.4.1. ATPLite™ assay

Lymphoid and splenic mononuclear cells were isolated from C3H mice as described in **Section 2.2.11**. The cells were counted by the Trypan blue method (**Section 2.2.8**). Mitomycin C stock (1 mg.ml^{-1}) was thawed and serially diluted in lymphocyte culture medium to make 10 ml aliquots in sterile test tubes of the following concentrations; 5, 10, 20, 30, 40, 50, 60 and 100 µg.ml^{-1}. Cells (1.8 x 10^{6}) were added to the medium containing mitomycin C corresponding to the eight different concentrations. Untreated (positive control) cells (1.8 x 10^{6}) were suspended in 10 ml culture medium without mitomycin C. The tubes were closed and placed in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes. The cells were removed from the incubator and centrifuged at 450 *g* for 10 minutes. This was followed by five more washes with fresh culture medium, each time discarding the supernatant in order to wash off the mitomycin C. The cells were then plated in uncoated 96-well U-bottom plates at a density of 1 x 10^{5} cells in 100 µl of culture medium per well. The volume in each well was made up to 200 µl by addition of either 100 µl of culture medium (unstimulated group) or 100 µl of culture medium containing 20 µg.ml^{-1} of Con-A to give a final concentration of 10 µg.ml^{-1} per well (Con-A stimulated group). As a positive control for cell death, cells (1.8 x

10^6) were incubated in 10 ml of culture medium containing 10 μg.ml^{-1} camptothecin for 30 minutes followed by five washes by centrifuging in culture medium at 450 *g*. The cells were also plated at a density of 1 x 10^5 cells per well as unstimulated or Con-A stimulated. The experiment was set up as shown in **Figure 4.2**. The plates were kept in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3, 5 and 7 days. Upon reaching the desired time point, the plates were removed from the incubator and 100 μl of cell suspension from each well was transferred to its corresponding well (pipette tips changed for each well) of a white OptiPlate™ 96-well plate and cell viability determined using the ATPLite™ assay as described in **Section 2.2.14**.

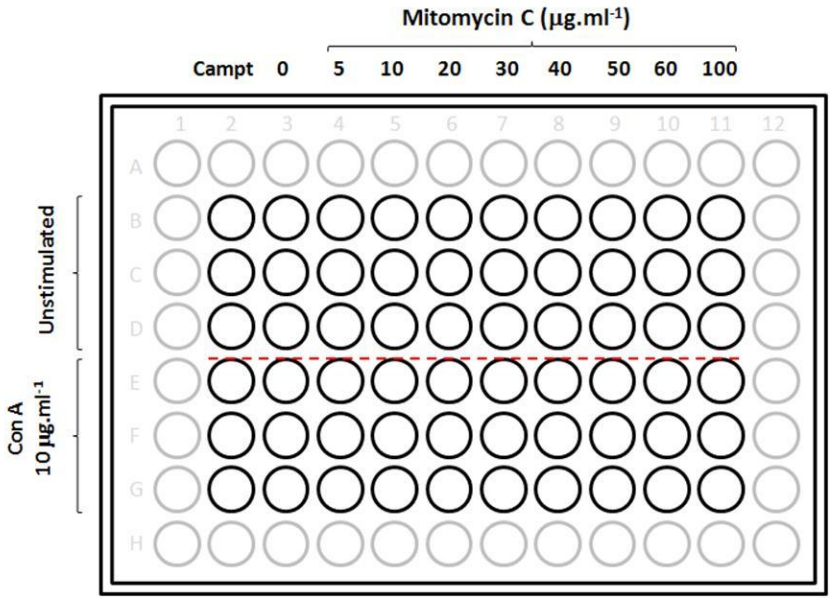

Figure 4.2. Experimental set up showing the 96-well plate plan for investigation of the effects of mitomycin C concentration on ATP content of Con-A stimulated and unstimulated C3H mononuclear cells to determine mitotic inactivation. Each well contained 1 x 10^5 cells in a volume of 200 μl. Camptothecin treated cells (Campt) were used as a positive control for dead cells. Cells in medium only without mitomycin C (0) were used as control for viable non-proliferating cells. Con-A stimulated cells without mitomycin C were positive controls for stimulated proliferating cells. Wells in grey were filled with PBS to minimise evaporation.

4.2.4.2. ^{3}H-thymidine uptake method

Lymphoid and splenic mononuclear cells were isolated from C3H mice as described in **Section 2.2.11** and counted by the Trypan blue method (**Section 2.2.8**). The cells were divided into four groups each comprising 1.8 x 10^6 cells. Each cell group was transferred into test tubes containing 10 ml of lymphocyte culture medium with 0, 5,

10 and 20 μg.ml^{-1} of mitomycin C. The tubes were closed and incubated at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes. Afterwards, the cells were washed five times by centrifuging at 450 *g* for 5 minutes with culture medium changed each time. A positive control for dead cells was prepared by incubating cells 1.8 x 10^6 cells in 10 ml of lymphocyte culture medium containing 10 μg.ml^{-1} camptothecin at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes followed by five washes at 450 *g* for 5 minutes each time discarding the medium. Cells (1 x 10^5 cells in 100 μl of culture medium) were then plated in uncoated 96-well U-bottom plates in triplicate wells as either unstimulated cells or stimulated cells (10 μg.ml^{-1} Con-A) as shown in the experimental set up in **Figure 4.3**.

The plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) $C0_2$ in air for 3, 5 and 7 days. Sixteen hours prior to reaching each specific time point, each well was spiked with 10 μl of low activity ^{3}H-thymidine as described in **Section 2.2.12**. Afterwards, the cells were harvested and ^{3}H-thymidine incorporation was measured using a TopCount NXT plate reader as described in **Section 2.2.13**.

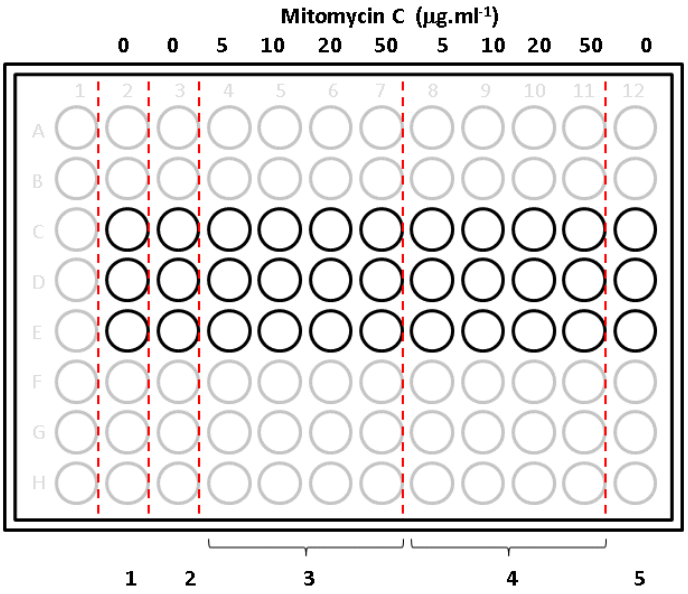

Figure 4.3. Diagram showing the 96-well plate plan for assessing the effects of mitomycin C treatment on C3H mononuclear cells as measured by the ^{3}H-thymidine uptake assay for the determination of mitotic inactivation. The numbers at the bottom of the plate represent the different cell treatments. Treatment 1 (no mitomycin C and no Con-A) was a control for non-proliferating viable cells. Treatment 2 (Con-A stimulated) was a control for stimulated and mitotically active cells. Treatment 3 were the test wells which contained unstimulated cells treated with graded doses of mitomycin C. Treatment 4 were test wells which contained Con-A stimulated cells treated with graded doses of mitomycin C. Treatment 5 were camptothecin treated cells (no mitomycin C, no Con-A) which were used as controls for non-viable/dead cells.

4.2.4.3. CFSE dilution method

4.2.4.3.1. CFSE labelling of C3H mouse mononuclear cells

Lymphoid and splenic mononuclear cells from C3H mice were isolated as described in **Section 2.2.11**. The cells were split into two groups; CFSE labelled (9.9 x 10^6) and unlabelled cells (1.8 x 10^6). The CFSE labelled group was suspended in 9 ml of PBS. Meanwhile under dim light, CFSE stock (5 mM) was thawed and 10 µl was withdrawn and added to 990 µl of PBS to make 1 ml of 50 µM CFSE working solution which was then mixed with the cell suspension in a light proof paper-wrapped test-tube to give a final CFSE concentration of 5 µM in 10 ml. The cells were mixed by vortexing for two seconds followed by incubation for 5 minutes at room temperature to allow CFSE uptake by the cells. After the incubation, the CFSE-labelled cell suspension was diluted with 10 volumes of PBS containing 5 % (v/v) FCS followed by centrifugation at 450 *g* for 5 minutes at 20 ºC to wash off excess

unabsorbed CFSE. The washing steps were repeated thrice to ensure a thorough wash.

4.2.4.3.2. Mitomycin C treatment of CFSE labelled C3H mononuclear cells

In separate light-proof paper wrapped test tubes, CFSE labelled mononuclear cells (1.8×10^6) were transferred into 5 ml volumes of culture medium which were supplemented with mitomycin C to give final concentrations of 5, 10, 20 and 50 $\mu g.ml^{-1}$. The tubes were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes. Afterwards, the cells were washed five times by centrifuging at 450 *g* for 5 minutes. The cells were finally resuspended in 1.8 ml of lymphocyte culture medium.

4.2.4.3.3. Treatment of CFSE labelled and unlabelled C3H mononuclear cells with camptothecin as a positive control for cell death

In separate tubes, unlabelled and CFSE labelled cells (9×10^5) that were used as controls for dead cells were resuspended at 9×10^5 cells in 4.5 ml of culture medium. Into each test tube, 50 µl of 1 $mg.ml^{-1}$ camptothecin was added to give a final concentration of 10 $\mu g.ml^{-1}$. The tubes were incubated at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 30 minutes. After incubation, the cells were washed five times by centrifugation at 450 *g* for 5 minutes with fresh culture medium changed each time. The cells were finally resuspended in 900 µl of lymphocyte culture medium.

4.2.4.3.4. Cell plating

The cells were plated in triplicate wells at a density of 1×10^5 cells in 100 µl of culture medium per well in uncoated 96-well U-bottom plates according to the plan in **Figure 4.4**. For unstimulated cells, a further 100 µl of lymphocyte culture medium was added into each well to give a total culture volume of 200 µl. For stimulated cells, a further 100 µl of lymphocyte culture medium containing 20 $\mu g.ml^{-1}$ of Con-A was added into each well to give a final concentration of 10 $\mu g.ml^{-1}$. The plates were incubated in a humidified incubator at 37 ºC in an atmosphere of 5 % (v/v) CO_2 for 3, 5 and 7 days prior to analysis by flow cytometry. The camptothecin treated cells were used as controls for CFSE labelled or unlabelled dead cells. Cells which had no

mitomycin C, camptothecin or Con-A were controls for viable non-proliferating cells. Controls for proliferating cells (Con-A stimulated) were cultured without mitomycin C.

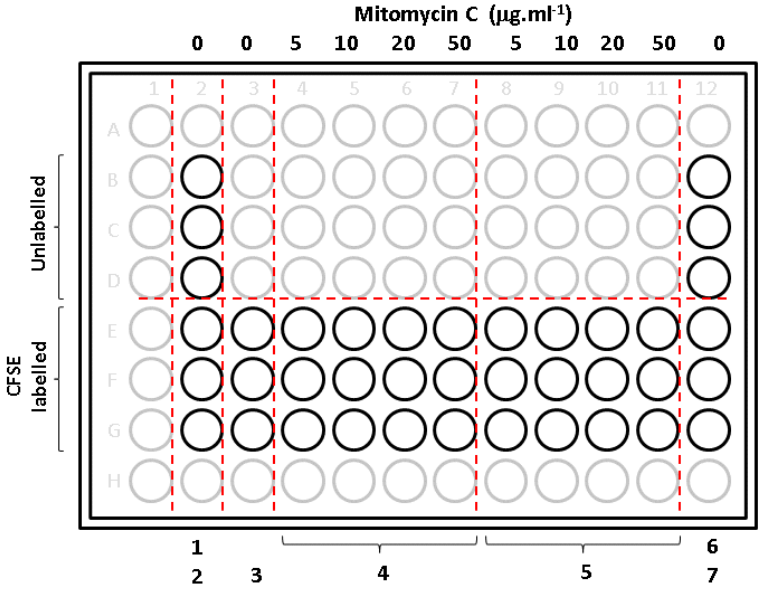

Figure 4.4. Schematic representation of the 96-well plate plan of the CFSE dilution assay for the determination of mitotic inactivation of C3H mononuclear cells by mitomycin C treatment using flow cytometry. The numbers below the plate indicate the different types of cell treatments. Treatment 1 (no mitomycin C, no Con-A) was a control for unlabelled non-proliferating viable cells. Treatment 2 (no mitomycin C, no Con-A) was a control for CFSE labelled non-proliferating cells. Treatment 3 (Con-A stimulated with no mitomycin C) was a control for CFSE labelled mitotically active proliferating cells. Treatments 4 (no Con-A) and 5 (Con-A) were test samples for CFSE labelled unstimulated and stimulated cells respectively treated with similar concentrations of mitomycin C. Treatment 6 and 7 (camptothecin treated; no mitomycin C) were controls for unlabelled and CFSE labelled non-viable cells respectively.

4.2.4.3.5. Flow cytometry analysis of CFSE dilution of C3H mononuclear cells for the determination of mitotic inactivation and cell viability

On completion of the incubation period, CFSE labelled cell cultures were prepared for analysis by flow cytometry using a BD FACSCalibur cytometer. Prior to the analysis, 5 µl of Via-probe™ 7-AAD (7-aminoactinomycin D) probe was added into each well, gently mixed and left for 10 minutes. The 7-AAD solution stained dead cells only thus allowing for the exclusion of non-viable cells from viable cells during analysis. After 7-AAD staining, 50 µl of CountBright™ counting beads were added

into each well and thoroughly mixed. By comparing the ratio of bead to cell events, absolute numbers of cells in the sample could be calculated. The cytometer was set to collect 1000 beads per sample with the side scatter parameter set to logarithmic scale in order to assess bead and cell numbers simultaneously. Data was acquired using CellQuest™ v.3.1 flow cytometry data acquisition software (BD Bioscience) as shown in the schematic (**Figure 4.5**) and analysed using FlowJo™ v.7.6.5 flow cytometry analysis software (Tree Star Inc).

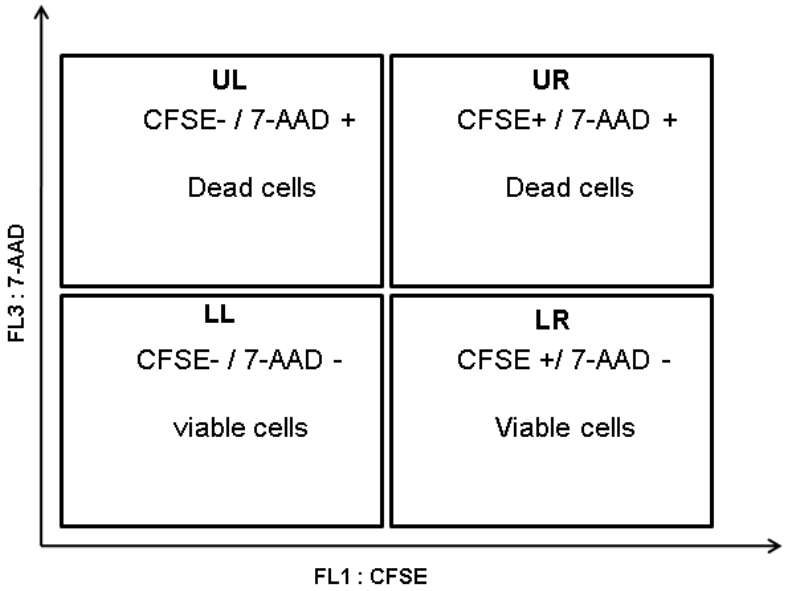

Figure 4.5. Schematic diagram showing the flow cytometer data collection template for the determination of mitotic inactivation of C3H mononuclear cells using mitomycin C. Cells were collected in four quadrants in which viable proliferating or non-proliferating cells collected in LL and LR quadrants according to their CFSE fluorescence intensity. Non-viable/dead unlabelled or CFSE labelled cells stained with 7-AAD collected in the UL or UR quadrants depending on their CFSE fluorescence. [CFSE; 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester. 7-AAD; 7-aminoactinomycin D. UL; upper left. UR; upper right. LL; lower left. LR; lower right]

4.2.5. Comparison of the effects of FCS and mouse serum on Balb/c mononuclear cell stimulation

The rationale for this experiment was to determine the suitable type of serum (FCS or mouse serum) and effective concentration for supplementing lymphocyte culture medium. Mononuclear cells were isolated from Balb/c mouse spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method as described in **Section 2.2.9.** The cells (1.8×10^6 cells per group) were suspended in 1.8 ml of lymphocyte culture medium containing either mouse serum or FCS at 2, 5,

10 and 20 % (v/v) concentrations. Cells cultured in lymphocyte culture medium without serum were used as controls for the need for serum supplementation. Cells cultured in lymphocyte transport medium were also used as controls to show the importance of culture medium for cell growth and proliferation.

The cells were seeded in uncoated 96-well U-bottom plates at a density of 1 x 10^5 cells in 100 µl per well. For unstimulated cells, a further 100 µl of the appropriate culture medium was added to give a final volume of 200 µl per well. For stimulated cells, a further l00 µl of corresponding medium containing 20 µg.ml^{-1} of Con-A was added to give a final concentration of 10 µg.ml^{-1}. Triplicate wells were plated for each type and concentration of serum as illustrated in **Figure 4.6**. The plates were incubated in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 3, 5 and 7 days without medium changes. Sixteen hours prior to completion of each time point, cells were spiked with 10 µl of 25 µCi.ml^{-1} low activity ^{3}H-thymidine as described in **Section 2.2.12**, harvested onto glass fibre filter plates and followed measurement of the radioactivity counts using a TopCount™ NXT scintillation counter as described in **Section 2.2.13**.

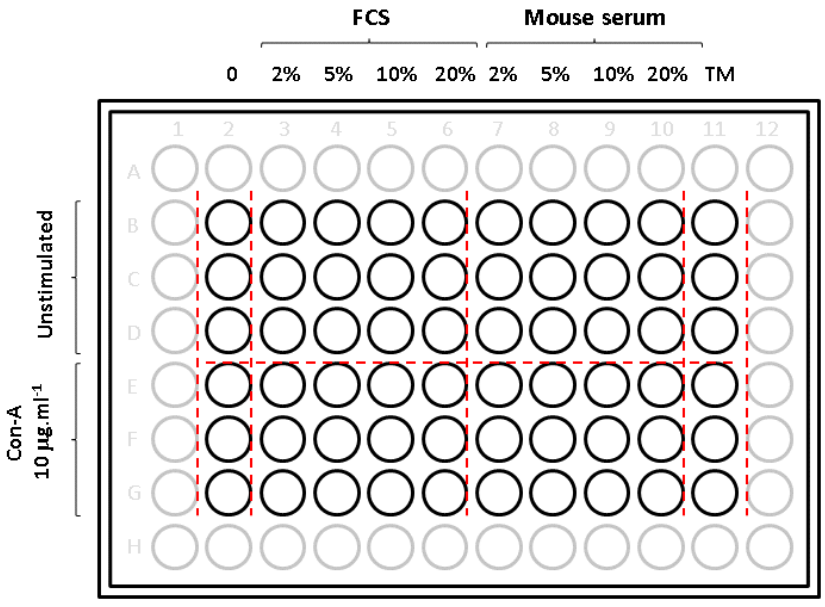

Figure 4.6. Schematic diagram showing the 96-well plate plan for the assessment of the effects of graded amounts of FCS and mouse serum in culture medium on the stimulation of Balb/c mononuclear cells. Unstimulated or Con-A stimulated cells (1 x 10^5 per well) were cultured in lymphocyte culture medium supplemented with graded amounts of FCS or mouse serum. Cells cultured in serum-free medium (0) were used as a control for serum. Cells cultured in transport medium [TM] were a control for culture medium. [FCS; foetal calf serum]

4.2.6. Determination of appropriate cell numbers and ratio of responder and stimulator cells for one-way MLR using 10 % (v/v) FCS and 2 % (v/v) mouse serum as culture medium supplements

Mononuclear cells were isolated from spleens and lymph nodes from Balb/c (responder) and C3H (stimulator) mice as described in **Section 2.2.11** and counted by the Trypan blue method described in **Section 2.2.9**. Balb/c mononuclear cells (1.95×10^7) were suspended in 10.4 ml of lymphocyte culture medium supplemented with 10 % (v/v) FCS or 2 % (v/v) mouse serum.

C3H mononuclear cells (1.23×10^7) were mitotically inactivated by treatment with 10 $\mu g.ml^{-1}$ mitomycin C as described in **Section 2.2.15**. Half of the cells (6.15×10^6) were suspended in 25.7 ml of lymphocyte culture medium supplemented with 10 % (v/v) FCS and the other half was suspended in lymphocyte culture medium supplemented with 2 % (v/v) mouse serum.

Balb/c (responder) and mitotically inactivated C3H mononuclear cells (stimulator) were seeded in uncoated 96-well U-bottom plates at responder to stimulator cell ratios of 1:1, 1:10 and 1:100 in quadruplicates with total cell numbers per well set at 1×10^4, 5×10^4 and 1×10^5 as shown in (**Figure 4.7**). The total volume per well was 200 µl and seeding volumes were computed from a cell density of 5×10^4 cells in 100 µl of either Balb/c or C3H cells which at a 1:1 ratio gave a total cell number of 1×10^5.

Balb/c mononuclear cells, seeded at 1×10^4, 5×10^4 and 1×10^5 cells per well (triplicates) in lymphocyte culture medium containing either 10 % (v/v) FCS or 2 % (v/v) mouse serum with or without 10 $\mu g.ml^{-1}$ Con-A were used as controls for stimulated proliferating cells and unstimulated non-proliferating cells respectively as shown in **Figure 4.8**.

The plates were incubated in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO_2 in air for 3, 6, 9 and 12 days accompanied with medium changes every three days as described in **Section 4.2.3**. Sixteen hours prior to completion of each time point, each well was spiked with 10 µl of 25 $\mu Ci.ml^{-1}$ low activity ^{3}H-thymidine (**Section 2.2.12**), harvested onto glass fibre filter plates and radioactivity counts read on a TopCount™ NXT scintillation counter (**Section 2.2.13**).

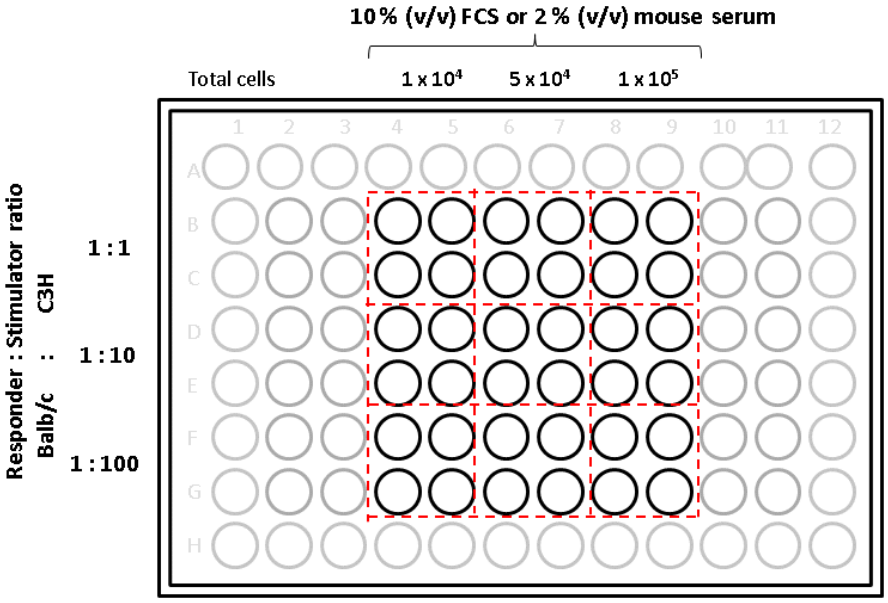

Figure 4.7. Schematic representation of 96-well plate for the one-way MLR for the determination of appropriate responder to stimulator cells ratios and total cell numbers. Balb/c (responder) mononuclear cells and mitomycin C treated C3H (stimulator) mononuclear cells were co-cultured at different cell ratios and different total cell numbers in culture medium supplemented with 10 % (v/v) FCS or 2 % (v/v) mouse serum. Grey wells were filled with transport medium only to minimise evaporation.

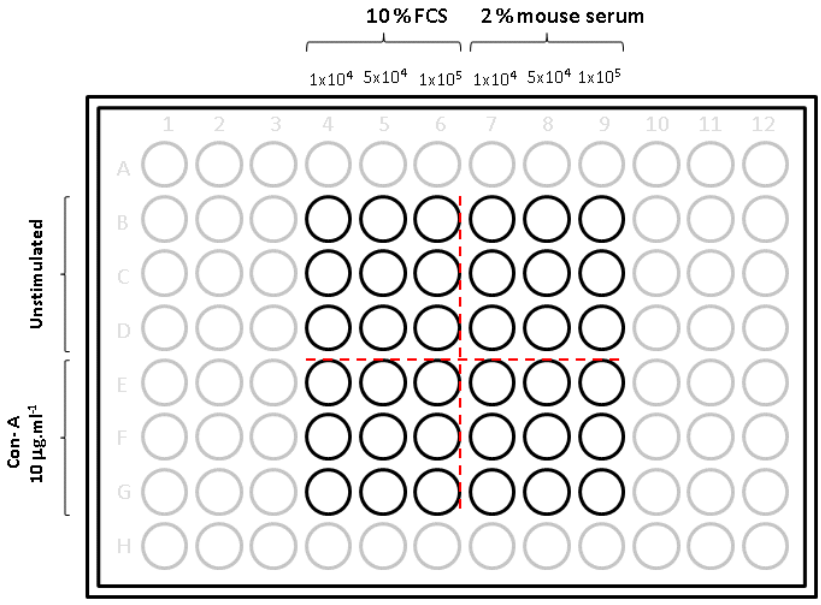

Figure 4.8. Schematic diagram showing the 96-well plate plan for positive (Con-A stimulated) and negative (unstimulated) controls of Balb/c (responder) cells for the one-way MLR. Cells were cultured in medium containing either 10 % (v/v) FCS or 2 % (v/v) mouse serum. Grey wells were filled with transport medium only to minimise evaporation.

4.3. Results

4.3.1. Determination of the suitable type and concentration of mitogen for use as positive control for Balb/c mouse lymphocyte transformation

The magnitude of mitogen induced lymphocyte stimulatory responses depend on the type and concentration of the mitogen. Non-specific mitogens such as PHA or Con-A are the most commonly used mitogens in immunology and are potent lymphocyte stimulatory agents. In this study, PHA and Con-A were compared for their ability to stimulate a fixed number (1×10^5) of Balb/c mouse mononuclear cells over an increasing concentration range for 3, 5 and 7 days. The type and concentration of mitogen that gave the highest ^{3}H-thymidine counts and SI was to be used as the positive control for future LTA assays. The time point at which the highest counts were obtained was regarded as the time point at which lymphocyte proliferation peaked. Cell viability and proliferation (as measured by the intracellular ATP content of mitogen stimulated cell cultures) at all mitogen concentrations and time points were also assessed by the ATPLite assay. The mitogen concentration at which highest counts (CPS) were obtained represented metabolically active viable and proliferating cells.

At day 3, PHA stimulated Balb/c mononuclear cells exhibited a dose-dependent increase in ^{3}H-thymidine uptake with increasing mitogen concentration peaking at 25 $\mu g.ml^{-1}$. For Con-A stimulated cells, ^{3}H-thymidine uptake peaked at 10 $\mu g.ml^{-1}$ and counts were higher than those for PHA at this concentration. Significant differences between PHA and Con-A stimulation were observed at 5, 10, 35 $\mu g.ml^{-1}$ and above. The SI showed that increasing the Con-A concentration above 10 $\mu g.ml^{-1}$ resulted in reduced stimulation. SI less than 3 was recorded at 50 $\mu g.ml^{-1}$ of Con-A (**Figure 4.9**).

At day 5, the ^{3}H-thymidine uptake trend was similar to that observed at day 3 for both PHA and Con-A. Peak responses were observed with 25 and 10 $\mu g.ml^{-1}$ of PHA and Con-A respectively. There were significant differences in counts between PHA and Con-A stimulated cells at all mitogen concentrations except between 25 and 35 $\mu g.ml^{-1}$. The SI for cells stimulated with 35 $\mu g.ml^{-1}$ and above of Con-A were below 3.

At day 7, the counts for Con-A stimulated cells were significantly higher than those for PHA stimulated cells at the 5 and 25 $\mu g.ml^{-1}$ mitogen concentration range. At mitogen concentrations of 30 $\mu g.ml^{-1}$ and higher, the SI for both Con-A and PHA stimulated cells were below 3.

At days 3 and 5, the SI for Con-A stimulated cells were higher than those for PHA stimulated cells at mitogen concentrations between 5 and 20 $\mu g.ml^{-1}$. From 25 $\mu g.ml^{-1}$ onwards, PHA stimulated cells produced higher SI compared to those stimulated with Con-A. At day 7, SI above 3 were recorded for both PHA and Con-A stimulated cells at mitogen concentrations between 5 and 25 $\mu g.ml^{-1}$. Overall, ^{3}H-thymidine counts and SI were highest at day 3 followed by day 5 and day 7 respectively for each given mitogen concentration.

Results from the ATP assay showed that Balb/c mononuclear cells stimulated with PHA at all the mitogen concentrations and Con-A between 5 and 25 $\mu g.ml^{-1}$ produced more ATP compared to unstimulated controls at all the time points (**Figure 4.10**). At day 3, there was a dose-dependent increase in counts at mitogen concentrations between 5 and 25 $\mu g.ml^{-1}$ followed by a dose-dependent decline from 25 $\mu g.ml^{-1}$ to 50 $\mu g.ml^{-1}$. The highest ATP counts at day 3 were obtained at 25 $\mu g.ml^{-1}$ PHA and Con-A. At day 5 and day 7 however, the counts produced at Con-A concentrations of 25 $\mu g.ml^{-1}$ and above fell sharply and from 35 $\mu g.ml^{-1}$ and onwards, the counts dropped below those for the unstimulated controls. This dose dependent decrease in ATP was not observed with PHA at all concentrations. Also, at these time points, ATP counts were higher for cells stimulated with Con-A than PHA at mitogen concentrations between 5 and 25 $\mu g.ml^{-1}$ but above 30 $\mu g.ml^{-1}$ PHA counts were higher than Con-A counts. Therefore, these results showed that higher doses of Con-A drastically reduced cell viability.

Taken together, these data showed that Con-A, at 10 $\mu g.ml^{-1}$, gave the highest ^{3}H-thymidine counts and SI compared to PHA throughout the incubation period. At this concentration, ATP counts were also high showing that cells viability was not affected. Therefore, 10 $\mu g.ml^{-1}$ Con-A was used as a positive control for lymphocyte stimulation in future assays.

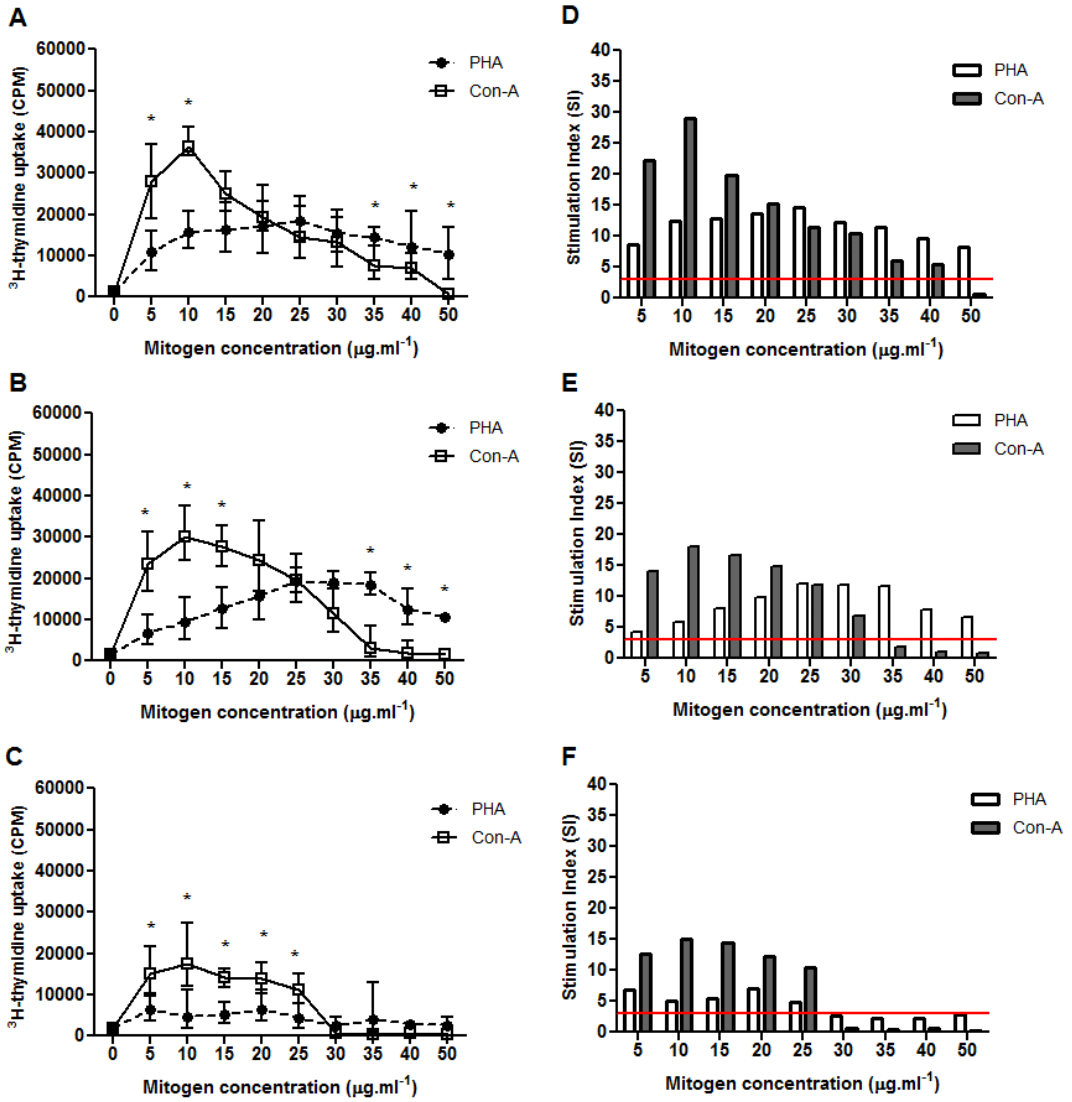

Figure 4.9. Comparison of the effect of PHA and Con-A stimulation of Balb/c mononuclear cells to determine the most suitable mitogen concentration as measured by the incorporation of low activity ^{3}H-thymidine. Balb/c mononuclear cells (1 x 10^5 per well) were stimulated with increasing mitogen concentration (5-50 µg.ml^{-1}) for 3, 5 and 7 days. Unstimulated cells (0) were used as controls for non-proliferating cells. CPM and SI data is shown respectively for day 3 (A and D), day 5 (B and E) and day 7 (C and F). CPM data was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine individual differences between PHA and Con-A at each concentration. Data (n=3) was then back-transformed for presentation and plotted as mean CPM ± 95 % CL (Left). * indicates significant difference between PHA and Con-A for a given concentration. Stimulation is presented as SI for each mitogen (Right). The red line at SI=3 denotes statistical biological positive, SI above 3 are considered significant responses for cells stimulated with PHA or Con-A while those below 3 are not. [CPM; counts per minute.

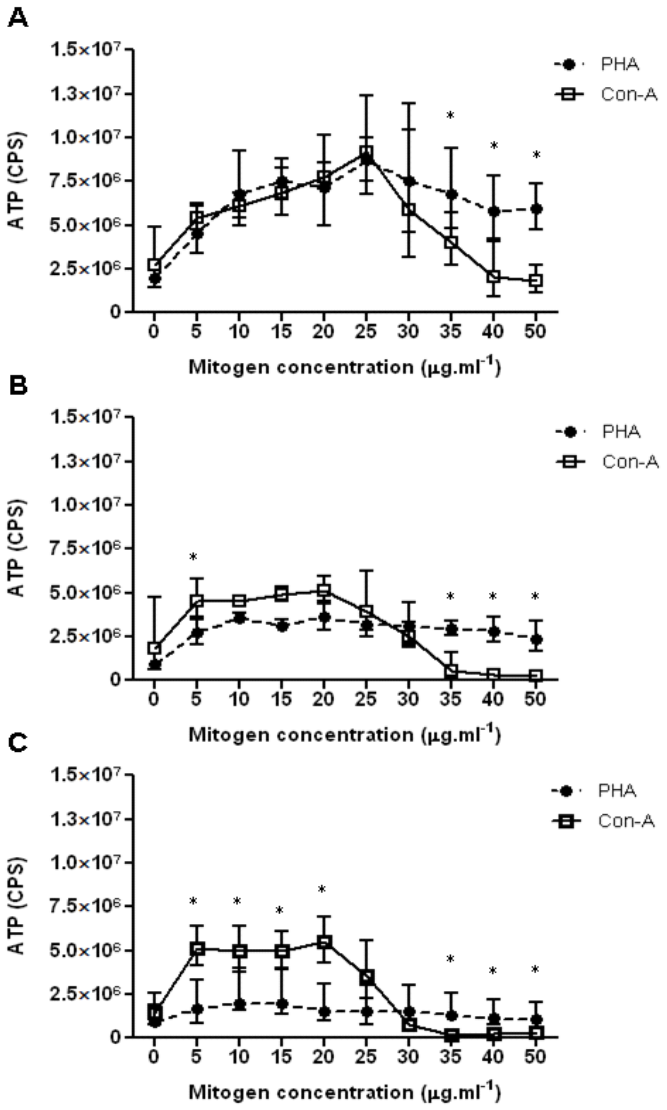

Figure 4.10. Comparison of the effect of PHA and Con-A on Balb/c mononuclear cell viability and proliferation as measured by cellular ATP content. Balb//c mononuclear cells (1 x 10^5 per well) were treated with increasing mitogen concentrations between 5 and 50 µg.ml^{-1} for 3 days (A), 5 days (B) and 7 (C) days. Unstimulated cells (0) were used as negative controls. Data was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine individual differences between PHA and Con-A at each concentration. Data (n=3) was then back-transformed for presentation and plotted as mean CPS ± 95 % CL. * indicates significant difference between PHA and Con-A for a given concentration.

4.3.2. Effects of medium replacement and extended culture period on Con-A stimulated Balb/c mouse mononuclear cells

The rationale behind this experiment was born out of the observation that most LTA and MLR studies in the literature have been carried out between 3 and 7 days. This could limit the detection of responses to the direct pathway but not capture the semi-

direct or indirect pathways of allorecognition which may take longer than 7 days. Over extended culture periods, the availability of nutrients and build-up of toxic metabolic by-products may become limiting. Therefore, this experiment was designed to investigate the effects of culture medium change during an extended assay period.

Balb/c mononuclear cells were stimulated with increasing doses of Con-A (5-25 $\mu g.ml^{-1}$) and cultured with and without medium change. For cultures with medium change, half the culture medium (100 µl per well) was replaced with fresh culture medium every 3 days. ^{3}H-thymidine uptake was measured at days 3, 5, 10, 15 and 20.

The results showed that cultures with medium changes produced higher counts compared to cultures without medium change at all the time points and Con-A concentrations with the exception of day 3 and day 20. Peak counts were obtained at day 5 with 10 $\mu g.ml^{-1}$ of Con-stimulation for cultures with medium changes whereas for cultures without medium changes peak counts were obtained at day 3 with the same concentration of Con-A (**Figure 4.11**). Counts for days 5, 10 and 15 cultures with medium changes were approximately double those without medium changes at all Con-A concentrations. There was a dose–dependent decrease in counts at Con-A concentrations above 10 $\mu g.ml^{-1}$ in both cultures with and without medium changes.

With regard to the stimulation, SI for cultures with medium changes were higher than those for cultures without medium changes at all Con-A concentrations at days 5, 10 and 15. At day 20, all the SI were below 3 except at 10 $\mu g.ml^{-1}$ Con-A in cultures with medium changes. For cultures without medium changes however, SI of 3 and below were obtained at day 10 onwards.

Taken together, these results showed that medium changes after 3 days considerably increased ^{3}H-thymidine uptake in Con-A stimulated cells but not in unstimulated cells. This enabled significant lymphocyte proliferation to be detected up to 20 days at 10 $\mu g.ml^{-1}$ of Con-A. However, without changing the medium, significant lymphocyte proliferation was not detected beyond 10 days. Therefore, future MLR and LTA experiments were carried out with medium changes every 3 days for up to

15 days with the intention to capture the indirect and semi-direct pathways of allorecognition.

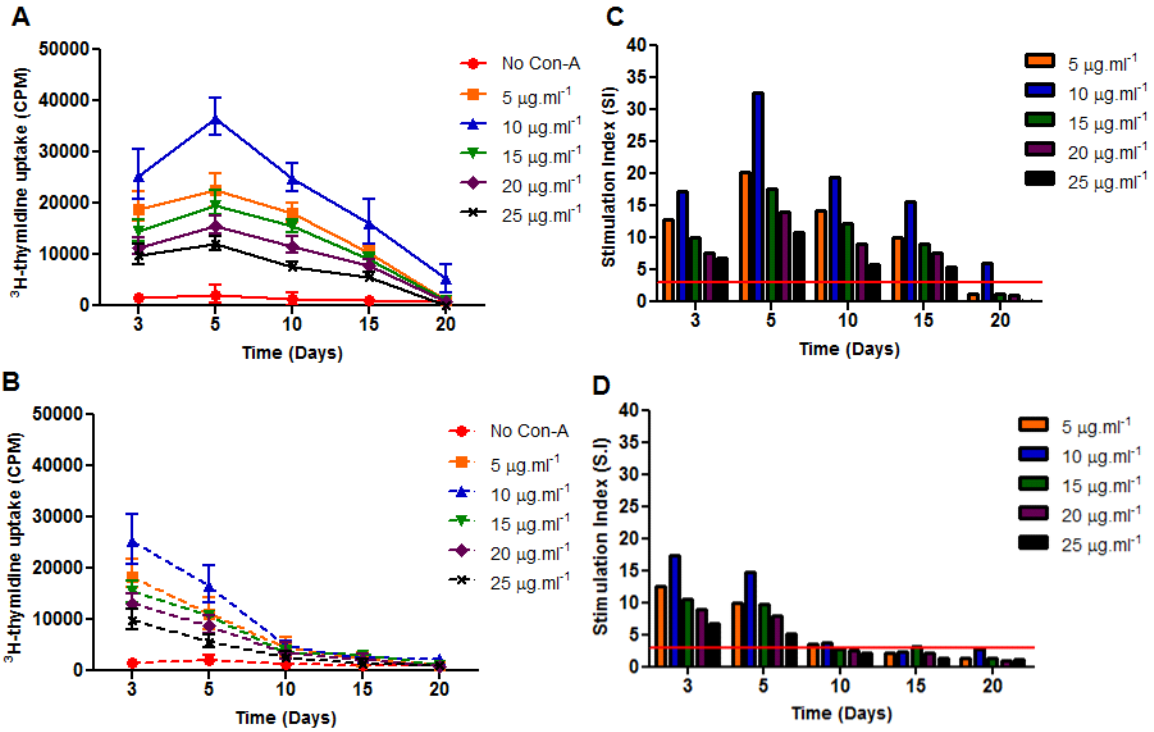

Figure 4.11. Effects of changing culture medium on Con-A stimulated Balb/c mononuclear cells over time as measured by ^{3}H-thymidine uptake. Cells (1 x 10^5 per well) were stimulated with Con-A (5-25 µg.ml^{-1}) and cultured for 3, 5, 10, 15 and 20 days with and without medium changes. The medium changes were carried out every 3 days by replacing 100 µl of medium per well. Unstimulated cells (no Con-A) were used as controls for non-proliferating cells. CPM data (n=6) was Log_{10} transformed to compute upper and lower 95% CL. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for cultures with medium changes (A) and without medium changes (B). Lymphocyte stimulation was presented as SI for cultures with (C) and without (D) medium changes. The red line at SI = 3 denotes statistical biological positive; Only SI above 3 were considered significant responses against Con-A.

4.3.3. Determination of the appropriate mitomycin C concentration for achieving mitotic arrest in stimulator cells

Mitomycin C treatment was the chosen method for arresting cell division in stimulator cells for use in one-way MLR and LTA. In these assays, mitotically inactive but viable stimulator cells provide allogeneic stimulation to responder lymphocytes whose proliferative response can then be measured. Mitomycin C is toxic to cells hence its use must be evaluated to determine the most appropriate working concentration which ensures that stimulator cells are rendered incapable of

mitosis but remain viable. Concentrations between 10 and 50 μg.ml^{-1} mitomycin C have been used in many previous studies but without evidence on the viability of the cells after treatment.

In these experiments, C3H mononuclear cells were treated with increasing concentrations of mitomycin C (5 - 100 μg.ml^{-1}) for 30 minutes and then either stimulated with Con-A (10 μg.ml^{-1}) or left unstimulated. The cells were cultured for 3, 5 and 7 days without medium changes. Lymphocyte proliferation was determined using ^{3}H-thymidine uptake whilst cell viability was tested using the ATPLite™ assay. The CFSE dilution assay was also used to test for cell proliferation and viability. After analysis, the appropriate mitomycin C concentration for inducing mitotic arrest was determined on the premise that when mitotically inactivated, viable C3H mononuclear cells, should be unable to proliferate when stimulated with Con-A.

4.3.3.1. Determination of mitotic inactivation using the ATPLite assay

C3H mononuclear cells were treated with increasing mitomycin C concentrations (5-100 μg.ml^{-1}). Cells treated with 5 μg.ml^{-1} camptothecin were used as positive control for cell death. The results for both unstimulated and Con-A stimulated cells showed that counts decreased with increasing mitomycin C concentration in a dose-dependent fashion at all the time points (**Figure 4.12**). The differences between unstimulated and Con-A stimulated cells at all mitomycin C concentrations were significant, except at 100 μg.ml^{-1} mitomycin C. This showed that Con-A stimulated cells produced more ATP than unstimulated cells.

Since there were significant differences between unstimulated and Con-A stimulated cells between 5 and 60 μg.ml^{-1} of mitomycin C, it was not possible to determine whether or not there was mitotic inactivation. The ideal result would have been no significant differences between counts obtained from stimulated and unstimulated cells at any given concentration of mitomycin C. Although at 100 μg.ml^{-1} of mitomycin C no significant difference was found between stimulated and unstimulated cells, the ATP content of these cells was not significantly different from that of camptothecin treated cells. This indicated that 100 μg.ml^{-1} of mitomycin C was toxic to the cells.

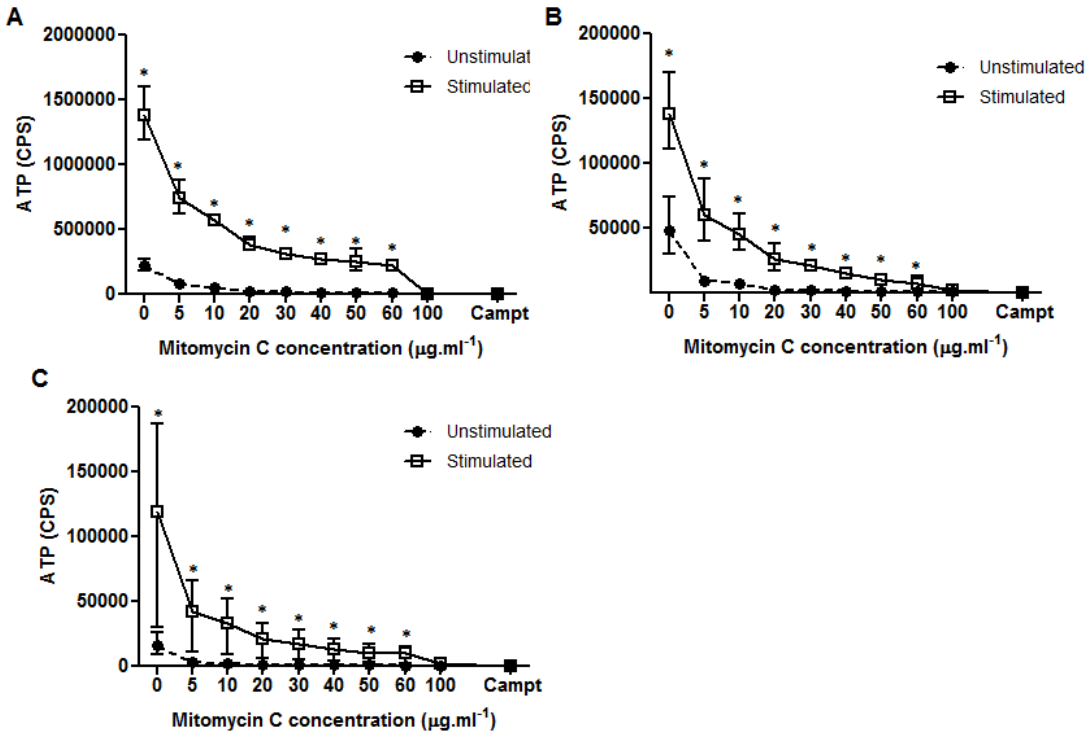

Figure 4.12. Effects of mitomycin C treatment on cellular ATP content of Con-A stimulated and unstimulated C3H mononuclear cells. Cells (1 x 10^5 per well) were treated with increasing doses of mitomycin C (5 – 100 µg.ml^{-1}) for 30 minutes, washed thoroughly and cultured either as unstimulated cells or stimulated (10 µg.ml^{-1} Con-A) for 3, 5 and 7 days. Cellular ATP content was measured as an indicator of cell proliferation and viability. Untreated and unstimulated cells (0) were used as controls for viable non-proliferating cells while untreated and Con-A stimulated cells (0) were used as controls for viable proliferative cells. Camptothecin-treated cells were used as control for dead cells. CPS data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between unstimulated and stimulated cells for a given mitomycin C concentration. Data was then back-transformed for presentation and plotted as mean CPS ± 95 % CL for day 3 (A), day 5 (B) and day 7 (C).* indicates significant difference between unstimulated and Con-A stimulated cells for a given mitomycin C concentration. [Campt; camptothecin]

4.3.3.2. Determination of mitotic inactivation by the ^{3}H-thymidine uptake assay

Results from the ATP assay described in **Section 4.3.3.1** were inconclusive as mitomycin C concentrations between 5 and 60 µg.ml^{-1} gave significant differences between unstimulated and Con-A stimulated cells. Therefore the ^{3}H-thymidine uptake assay was used.

In this experiment, C3H mononuclear cells were treated with mitomycin C (5, 10, 20 and 50 µg.ml^{-1}) and either left unstimulated or stimulated using Con-A (10 µg.ml^{-1}).

The cells were cultured for 3, 5 and 7 days without medium changes. Untreated cells (no mitomycin C), either unstimulated or stimulated were used as controls for viable non-proliferating cells and viable proliferating cell respectively. Camptothecin (10 $\mu g.ml^{-1}$) treated cells were used as controls for dead cells.

The results showed that increasing mitomycin C concentration significantly inhibited ^{3}H-thymidine uptake in Con-A stimulated cells at all the time points (**Figure 4.14**). At days 3, 5 and 7 respectively, significant differences in counts between unstimulated and Con-A stimulated cells were obtained for untreated cells and cells treated with 5 $\mu g.ml^{-1}$, but not for cells treated with 10, 20 and 50 $\mu g.ml^{-1}$ of mitomycin C. Only cells treated with 5 $\mu g.ml^{-1}$ mitomycin C had SI above 3. The SI for cells treated with between 10 and 50 $\mu g.ml^{-1}$ mitomycin C had SI equal to and less than 3 at all the time points. This showed that mitotic inactivation could have been achieved at any of these mitomycin C concentrations.

However, the assay was not informative with regard to cell viability at any given concentration of mitomycin C. Therefore, a further approach to determine the viability of the cells at these mitomycin C concentrations was necessary in order to ensure that future MLR and LTA used viable cells.

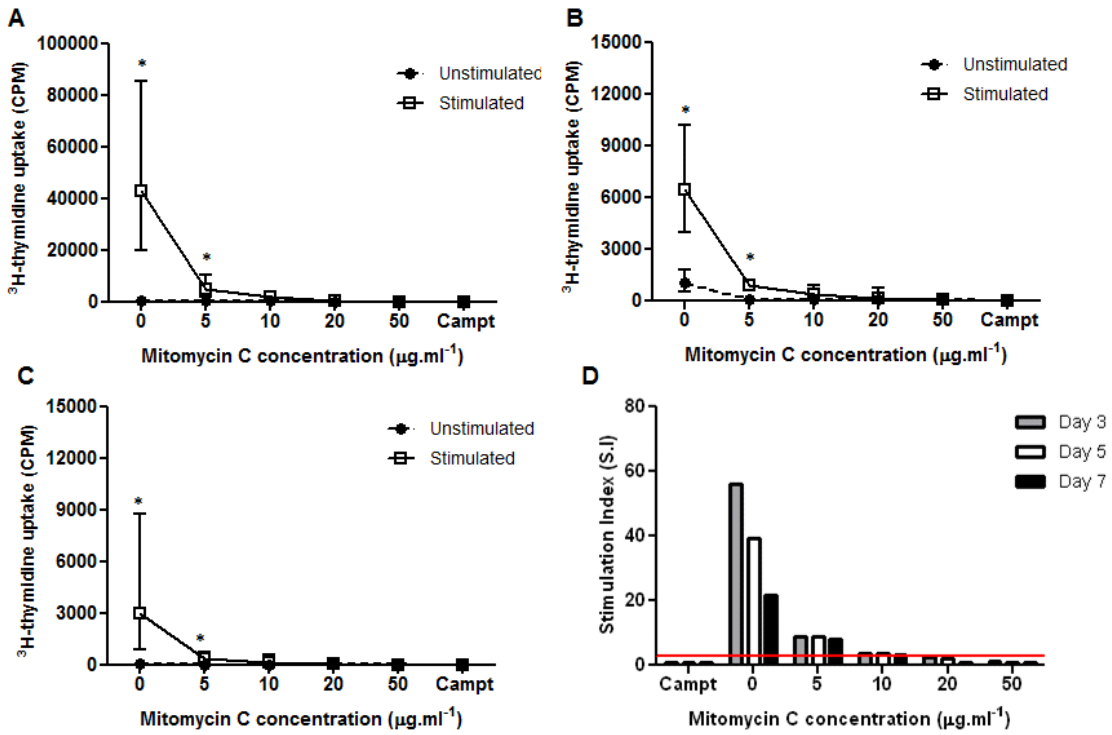

Figure 4.13. Effects of mitomycin C treatment on the proliferation of unstimulated and Con-A stimulated C3H mononuclear cells as measured by ^{3}H-thymidine uptake. C3H mononuclear cells (1 x 10^5 per well) were treated with increasing concentrations of mitomycin C (5 – 50 µg.ml^{-1}) for 30 minutes, washed thoroughly and cultured either as unstimulated cells or Con-A (10 µg.ml^{-1}) stimulated for 3, 5 and 7 days. Untreated and unstimulated cells (0) were used as controls for viable non-proliferating cells while untreated and Con-A stimulated cells (0) were used as controls for viable proliferating cells. Camptothecin-treated cells were used as control for dead cells. CPM data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between unstimulated and stimulated cells for a given mitomycin C concentration. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for day 3 (A), day 5 (B) and day7 (C).* indicates significant difference between unstimulated and stimulated cells for a given mitomycin C concentration. Stimulation is presented as SI (D) for each mitomycin C concentration and time point. The red line at SI=3 denotes statistical biological positive. Only SI above 3 were considered significant responses of C3H mononuclear cells against Con-A stimulation. [Campt; camptothecin]

4.3.3.3. Determination of mitotic inactivation using the CFSE dilution assay

Having determined a broad concentration range (10–50 µg.ml^{-1}) at which mitomycin C had a significant effect on cell division by the ^{3}H-thymidine assay, the CFSE dilution assay was used to determine the most suitable concentration of mitomycin C by determining the cell viability and proliferation capacity of unstimulated and Con-A stimulated cells at these concentrations. Cell division was determined by tracking

intracellular CFSE fluorescence intensity and cell viability was analysed using the viability stain 7-AAD. Absolute cell numbers of unstimulated and Con-A stimulated cells were estimated using fluorescent counting beads. Positive controls for dead cells were set up by treating CFSE labelled and unlabelled cells with camptothecin (10 μg.ml^{-1}). Representative data (n=3) is shown in **Figure 4.14**.

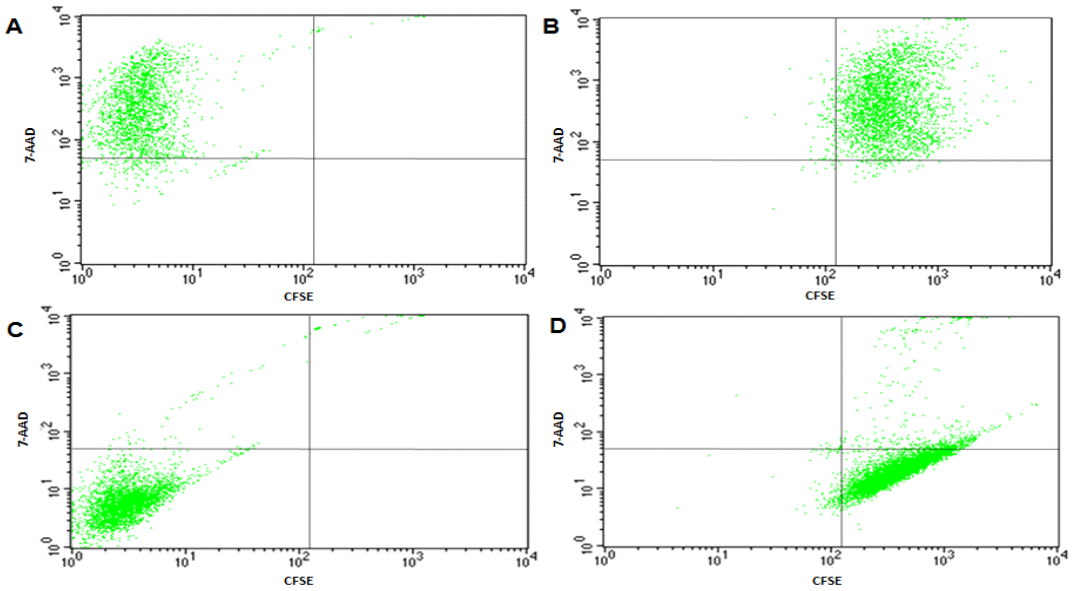

Figure 4.14. Determination of cell viability using CFSE tracking of mitomycin C treated cells by flow cytometry. Unlabelled (A) and CFSE labelled (B) C3H mononuclear cells treated with camptothecin (10 μg.ml^{-1}) were used as controls for CFSE$^-$ 7-AAD$^+$ and CFSE$^+$ 7-AAD$^+$ dead cells respectively. Unlabelled (C) and CFSE labelled (D) viable cells were used as controls for CFSE$^-$ 7-AAD$^-$ and CFSE$^+$ 7-AAD$^-$ viable cells respectively. Absolute cell numbers in each quadrant were estimated by collecting 1000 CountBright™ fluorescent counting beads and then using the bead to cell ratio to obtain the cell numbers. This set up was used to analyse the viability of mitomycin C treated CFSE labelled cells which were either unstimulated or stimulated with Con-A (10 μg.ml^{-1}). Data was acquired using CellQuest™ flow cytometry data acquisition and analysis software v 3.1. The results are representative for three experiments.

For unstimulated cells treated with 5 and 10 μg.ml^{-1} mitomycin C, cell viability was above 90 % but at 20 and 50 μg.ml^{-1} mitomycin C, viability decreased from 80 % to 40 % and 60 % to 30 % at day 3 and day 5 respectively (**Figure 4.15**). At day 7, cell viability dropped from 80 % at 5 μg.ml^{-1} mitomycin C to 20 % at 50 μg.ml^{-1}. These results showed that mitomycin C concentrations above 10 μg.ml^{-1} reduced cell viability.

For Con-A stimulated cells treated with 5 and 10 μg.ml^{-1} mitomycin C, cell viability was above 90 % at day 3 but dropped to 60 % at day 5 and 20 % at day 7. At 20 μg.ml^{-1} mitomycin C, cell viability was 58 % at days 3 and 5 and 20 % at day 7. At 50 μg.ml^{-1} mitomycin C, cell viability dropped to 36 % at days 3 and 5 and 18 % at day 7. For Con-A stimulated control cells (no mitomycin C) however, cell viability was reduced from 100 % at day 3, 76 % at day 5 and 30 % at day 7. The results from the Con-A stimulated controls demonstrated that cell viability dropped considerably over the 7 day period compared to unstimulated control cells (no mitomycin C) in which viability was above 90 % at days 3 and 5 and 80 % at day 7.

These results showed two phenomena; firstly, that stimulation by Con-A reduced the viability of the C3H mononuclear cells and secondly, that treatment of either unstimulated or Con-A stimulated cells with mitomycin C concentrations above 10 μg.ml^{-1} further reduced the cell viability. At 5 and 10 μg.ml^{-1} mitomycin C, cell viability was comparable to that for control cells. In practice, mitotic inactivation is required for unstimulated cells. Therefore, 5 and 10 μg.ml^{-1} mitomycin C appeared to be the most appropriate doses for inducing mitotic arrest.

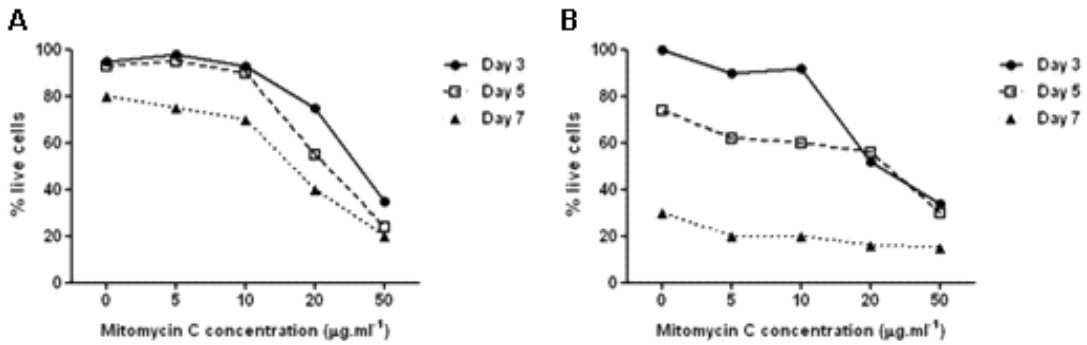

Figure 4.15. Effects of mitomycin C treatment on cell viability of Con-A stimulated and unstimulated C3H mononuclear cells as measured by CFSE and 7-AAD staining assay. Cells were treated with different concentrations (5, 10, 20 and 50 μg.ml^{-1}) of mitomycin C for 30 minutes, washed and labelled with CFSE. The cells were cultured either as unstimulated cells (A) or Con-A stimulated (B) for 3, 5 and 7 days. Cultures were spiked with 5 μl per well of Via-probe™ 7-AAD probe and mixed with 50 μl of counting beads before data was captured by collecting 1000 bead events and computing the fractions of cells in each quadrant. 7-AAD stained cells were regarded as dead while unstained cells were considered as viable. Data was captured and analysed by CellQuest™ software (BD Bioscience) v3.1

Further insights into the status of the cells were obtained by analysing CFSE fluorescence intensities of the cell fractions to determine whether or not the viable cells were mitotically inactivated (dividing cells exhibit CFSE dilution). Data collected using CellQuest™ software was further analysed using FlowJo™ data analysis software.

The results showed that mitotic inactivation was achieved when mitomycin C treated cells (10 $\mu g.ml^{-1}$) failed to respond to stimulation by Con-A as there was no apparent fluorescence shift to the left on histograms. Shifting of fluorescence intensity to the left indicated CFSE dilution which meant that the cells were capable of dividing. At 5 $\mu g.ml^{-1}$ mitomycin C, a shift to the left was noted in Con-A stimulated cells. This showed that at this concentration of mitomycin C, the cells were not completely mitotically inactivated (**Figure 4.16**).

Further analysis of the CFSE dilution profiles for all the samples using the cell proliferation platform of FlowJo™ software showed that between 3 and 14 % of the unstimulated cells treated with different mitomycin C concentrations and control cells underwent mitosis at all the time points (**Figure 4.17A**).

Following stimulation by Con-A however, 80 % of control cells underwent mitosis at day 3, 60 % at day 5 and 42 % at day 7 (**Figure 4.17B**). For stimulated cells treated with 5 $\mu g.ml^{-1}$ mitomycin C, 60 %, 30 % and 20 % of the cells underwent mitosis at days 3, 5 and 7 respectively compared to 18 %, 10 % and 3 % for cells treated with 10 $\mu g.ml^{-1}$ mitomycin C at the same time points. Less than 2 % of the cells treated with 20 and 50 $\mu g.ml^{-1}$ of mitomycin C however, underwent cell division. Based on these results, mitotic inactivation would have been achieved between 20 and 50 $\mu g.ml^{-1}$ mitomycin C but considering the cell viability results, these observations showed that the lack of stimulation was largely due to mitomycin C toxicity.

Further evaluation of the mitotic cell fractions of unstimulated cells revealed that all the cells treated with different mitomycin C concentrations and control cells underwent a single cycle of cell division (**Figure 4.17C**). Analysis of the cells treated with 5 and 10 $\mu g.ml^{-1}$ mitomycin C that had undergone mitosis showed that the dividing cells treated with 10 $\mu g.ml^{-1}$ mitomycin C had gone through one mitotic cycle at all the time points whereas those treated with 5 $\mu g.ml^{-1}$ mitomycin C, cell

division was detected up to the 4^{th}, 7^{th} and 9^{th} division cycles at days 3, 5 and 7 respectively (**Figure 4.17D**).

Taken together, these data showed that treatment of cells with 10 $\mu g.ml^{-1}$ mitomycin C achieved mitotic arrest and maintained cell viability above 90 % for up to 5 days and 70 % for 7 days. A small fraction of these cells (less than 20 %) was found to be capable of only a single round of mitosis following Con-A stimulation for 7 days. Higher concentrations (20 and 50 $\mu g.ml^{-1}$), though inhibiting cell division even after Con-A stimulation, considerably reduced cell viability (below 50 %) whereas lower concentrations (5 $\mu g.ml^{-1}$) left the cells viable but also a sizeable proportion of the cells (up to 60 %) capable of undergoing several (4 to 9) mitotic cycles after 7 days of culture. Hence 10 $\mu g.ml^{-1}$ of mitomycin C was chosen as the appropriate concentration for achieving mitotic arrest and was used in subsequent experiments.

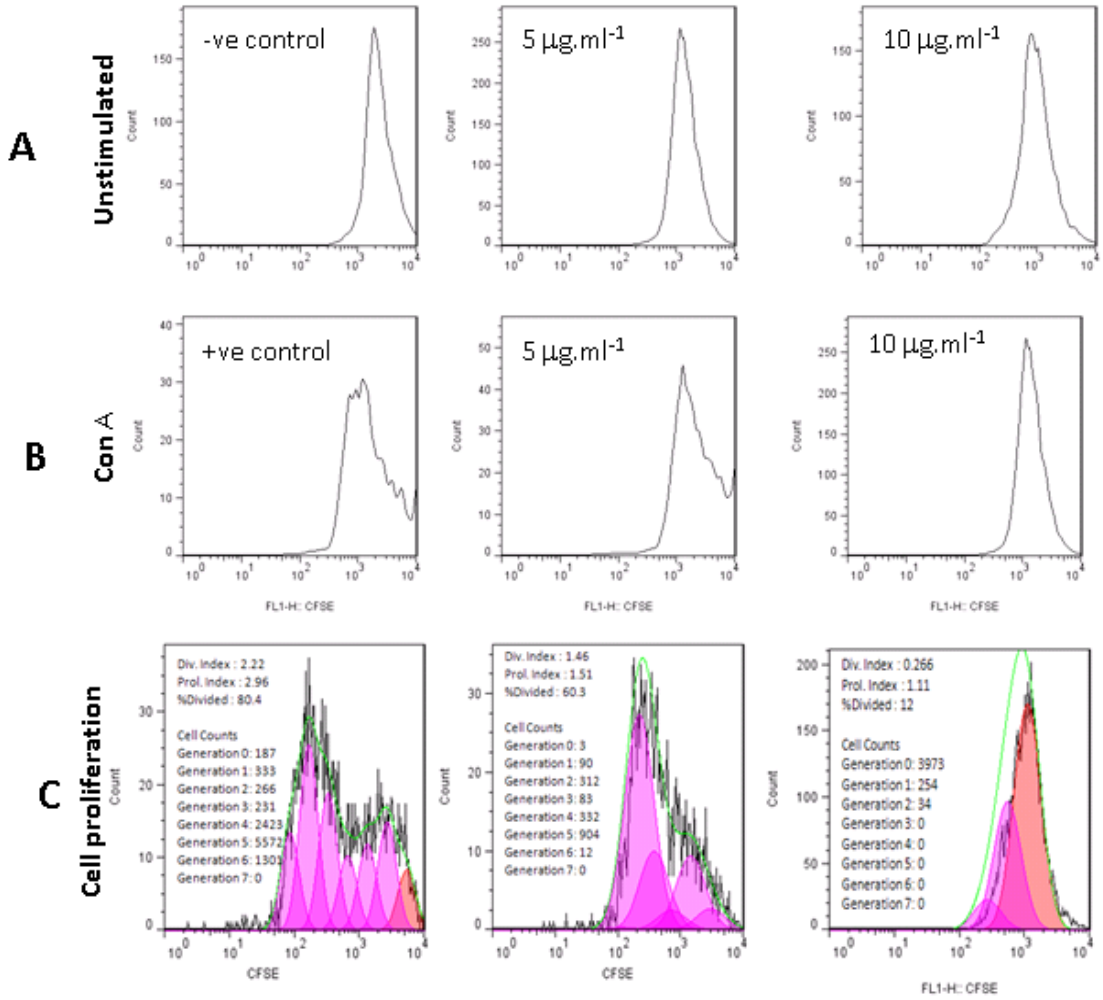

Figure 4.16. CFSE dilution assay for mitomycin C treated C3H mononuclear cells to detect mitosis using FlowJo™ software cell proliferation platform. Representative histograms plotted to show CFSE fluorescence intensities of unstimulated cells (A) and Con-A stimulated cells (B) treated with 5 and 10 µg.ml^{-1} mitomycin C. Positive (Con-A stimulated) and negative (unstimulated) control cells were not treated with mitomycin C. A shift to the left of peak fluorescence intensities denoted CFSE dilution which in turn represented separate cell fractions of daughter cells. The peaks of the Con-A stimulated cells were further resolved using the cell proliferation platform on FlowJo™ to expose groups of cells with the different CFSE fluorescence intensities which corresponded to specific generations of cell division (C). Data is representative of three experiments.

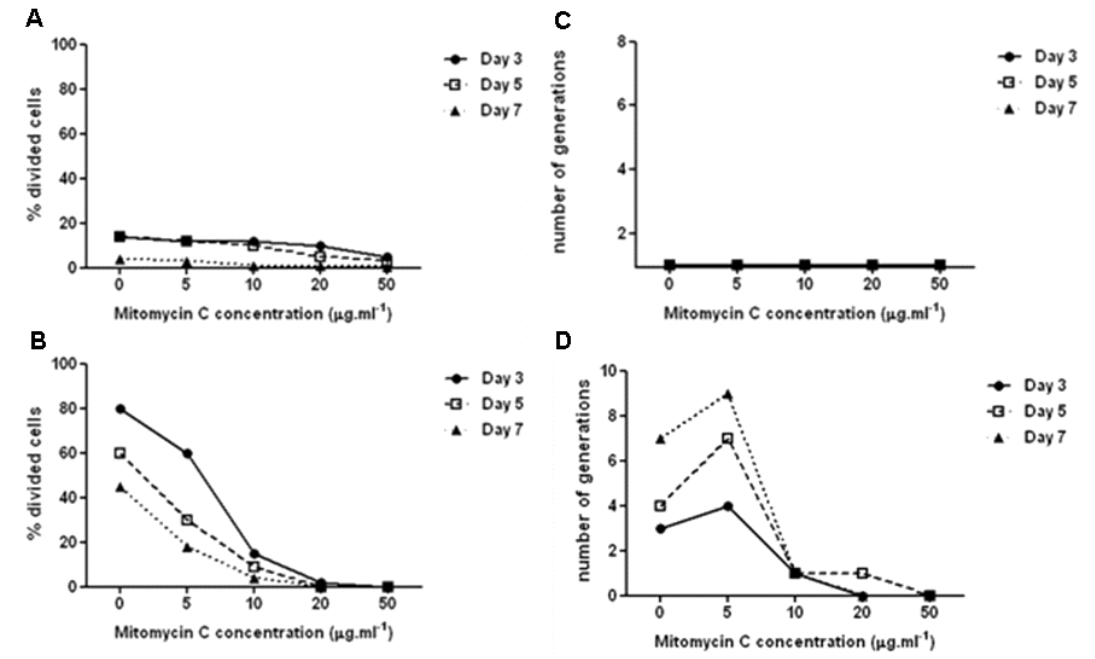

Figure 4.17. Analysis of the effects of mitomycin C treatment on cell division of unstimulated and Con-A stimulated C3H mononuclear cells as determined by CFSE dilution analysis using flow cytometry. Representative data was analysed using the cell proliferation platform in FlowJo™ software v.7.6.5 which resolved the proportion of all cells with CFSE fluorescence intensity lower than control (unstimulated CFSE labelled) cells (mitotic cells). The fractions of divided cells at each mitomycin C concentration are shown for unstimulated (A) and Con-A stimulated cells (B). The number of cell division cycles entered by the dividing cells is shown for unstimulated (C) and Con-A stimulated cells (D) at each mitomycin C concentration. The method used the reduction of cellular CFSE fluorescence intensity relative to the control cells. Halving of the fluorescence intensity was regarded as representative of a single mitotic cycle.

4.3.4. Investigation of mouse serum as an alternative to FCS for supplementing culture medium

Throughout the literature, mouse LTA and MLR have been carried out in culture medium supplemented with 10 % (v/v) FCS. Considering that FCS contains many complex bovine proteins, it is conceivable that in proliferation assays involving mouse lymphocytes that FCS proteins may provide xenogeneic antigens that could initiate responses and give false positive results. For this study, it was important to ensure that any potential stimulatory components of the culture medium were evaluated in order to accurately detect specific allogeneic responses of Balb/c lymphocytes in response to C3H cells. Therefore, commercially sourced heat-inactivated mouse serum at 2, 5, 10 and 20 % (v/v) was used to supplement lymphocyte culture medium in LTA using Balb/c mononuclear cells stimulated with

Con-A (10 $\mu g.ml^{-1}$). For comparison, the same assays were carried out in medium supplemented with FCS at the same concentrations. To check whether serum supplementation was necessary at all, cells were cultured in medium without serum (0 %) as a control for serum supplementation. Similarly, identical cultures were set up in lymphocyte transport medium which is used during the isolation of mononuclear cells. ^{3}H-thymidine uptake was measured at 3, 5 and 7 days without medium changes. Cellular ATP content analysis using the ATPLite™ assay was also carried out to assess cell viability at day 3.

Analysis of day 3 data showed that unstimulated Balb/c mononuclear cells cultured in medium containing graded amounts of FCS produced significantly higher counts compared to those cultured in mouse serum (**Figure 4.18**). For Con-A stimulated cells however, ^{3}H-thymidine uptake increased proportionally with increasing FCS concentration peaking at 20 % (v/v) at day 3 and 10 % (v/v) at days 5 and 7. Conversely, ^{3}H-thymidine uptake decreased inversely with increasing mouse serum concentration with the highest counts obtained at 2 % (v/v). Similar trends were observed at day 5 and day 7. Significant differences in counts were noted at each concentration of FCS and mouse serum. Stimulation indices showed that 10 % (v/v) FCS and 2 % (v/v) mouse serum were optimum for Balb/c mononuclear cells response to stimulation by Con-A. Stimulated cells cultured in 2 % (v/v) mouse serum produced higher counts than cells cultured in 10 % (v/v) FCS at days 3 and 5 but the reverse was true at day 7. Control cells cultured in medium without serum or in lymphocyte transport medium showed no significant differences between unstimulated and Con-A stimulated cells at all the time points. This showed that serum supplementation is an important requirement for lymphocyte transformation.

Analysis of the cellular ATP content of day 3 unstimulated cultures showed that cells cultured in FCS produced significantly more ATP compared to cells cultured in mouse serum at each concentration (**Figure 4.19**). The ATP content was consistently similar at all FCS concentrations. For unstimulated cells cultured in mouse serum however, the ATP levels declined with increasing serum concentration. For Con-A stimulated cells, the ATP levels increased sharply with increasing FCS serum concentration with peak counts at 10 % (v/v) FCS. For Con-A stimulated cells cultured in mouse serum however, ATP levels declined inversely with increasing serum concentration with peak counts obtained at 2 % (v/v). Stimulation with Con-A

had no effect on ATP levels of cells cultured in either lymphocyte transport medium or culture medium without serum.

Taken together, these data showed that increasing FCS concentration increased both ^{3}H-thymidine uptake and ATP content of Con-A stimulated cells with peak counts obtained at 10 % (v/v). The opposite was true for cells cultured in mouse serum in which increasing the serum concentration above 2 % (v/v) was accompanied with a dose dependent decrease in ^{3}H-thymidine uptake and cellular ATP content. Therefore, both 10 % (v/v) FCS and 2 % (v/v) mouse serum were deemed appropriate concentrations and were tested further in subsequent assays.

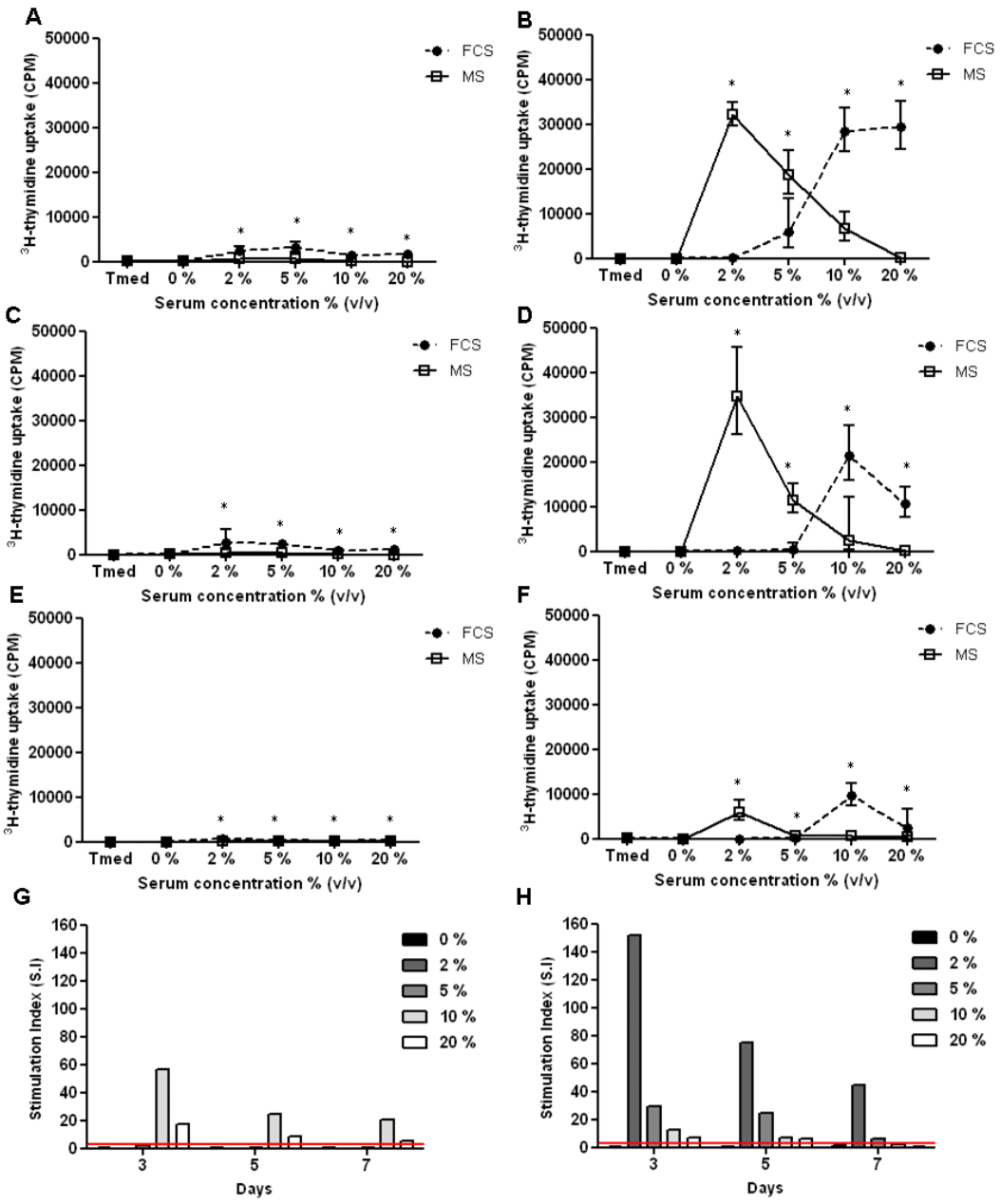

Figure 4.18. Evaluation of mouse serum and FCS concentration on the transformation of Balb/c mononuclear cells. Cells (1 x 10^5 per well) were cultured in medium supplemented with 2, 5, 10 and 20 % (v/v) of either FCS or mouse serum. Cells cultured in serum free medium (0%) and transport medium (Tmed) were used as controls. Cultures were either left unstimulated or stimulated with 10 μg.ml^{-1}Con-A followed by incubation for 3, 5 and 7 days without medium changes. Data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between mouse serum and FCS at the same concentration. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for unstimulated and Con-A stimulated cells at day 3 (A and B), day 5 (C and D) and day 7 (E and F) respectively. * indicates significant differences between FCS and mouse serum at the each concentration. SI were computed and are shown for cells cultured in FCS (G) and mouse serum (H) respectively. The red line at SI=3 denotes statistical biological positive; only SI higher than 3 were considered significant responses of Balb/c mononuclear cells to Con-A. [MS; mouse serum]

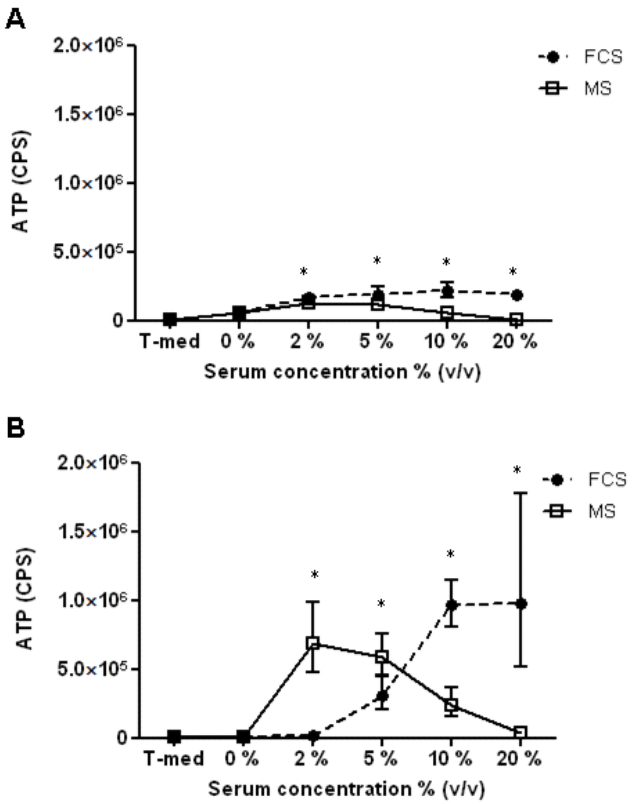

Figure 4.19. Effects of FCS and mouse serum on cellular ATP content of unstimulated and Con-A stimulated Balb/c mononuclear cells. Cells (1 x 10^5 per well) were cultured in medium supplemented with 2, 5, 10 and 20 % (v/v) of either FCS or mouse serum for 3 days. Cellular ATP content was measured as an indicator of cell proliferation and viability. Cells cultured in serum-free medium (0 %) and transport medium (T-med) were used as controls. Data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between cells at in FCS and mouse serum at the same concentration. Data was then back-transformed for presentation and plotted as mean CPS ± 95 % CL for unstimulated cells (A) and Con-A stimulated cells (B).* indicates a significant difference in intracellular ATP content between cells cultured in FCS and mouse serum at the same concentration. [MS; mouse serum]

4.3.5. Determination of appropriate responder to stimulator cell ratios for performing one-way MLR using 2 % (v/v) mouse serum and 10 % (v/v) FCS in culture medium

The purpose of this experiment was to determine the appropriate responder to stimulator cell ratio and suitable total cell numbers for performing one-way MLR. In **Section 4.3.4**, it was found that lymphocyte culture medium containing either 10 % (v/v) FCS or 2 % (v/v) mouse serum was suitable for Balb/c lymphocyte transformation. Therefore, Balb/c mononuclear cells (responder) were co-cultured with mitotically inactivated (10 $\mu g.ml^{-1}$ mitomycin C) C3H mononuclear cells

(stimulator) using culture medium containing either 10 % (v/v) FCS or 2 % (v/v) mouse serum. Responder to stimulator cell ratios were set at 1:1, 1:10 and 1:100 and cultures contained total cell numbers (responder + stimulator) of 1 x 10^4, 5 x 10^4 and 1 x 10^5 cells. The cells were cultured with medium changes every 3 days for 3, 6, 9 and 12 days. The response of Balb/c mononuclear cells to allogeneic C3H cells was tested using the ^{3}H-thymidine uptake assay. SI for the one-way MLR reactions were calculated as follows;

$$SI = \frac{Average\ CPM(Responder + Stimulator)}{Average\ CPM\ (Responder\ only)}$$

The results showed that control cultures of Con-A stimulated Balb/c cells cultured in either 10 % (v/v) FCS or 2 % (v/v) mouse serum were equally stimulated and gave high counts which peaked at day 6 and gradually reduced during the time course. Highest counts were recorded with the highest total cell numbers, in the order 1 x 10^5 > 5 x 10^4 > 1 x 10^4 respectively (**Figure 4.20**).

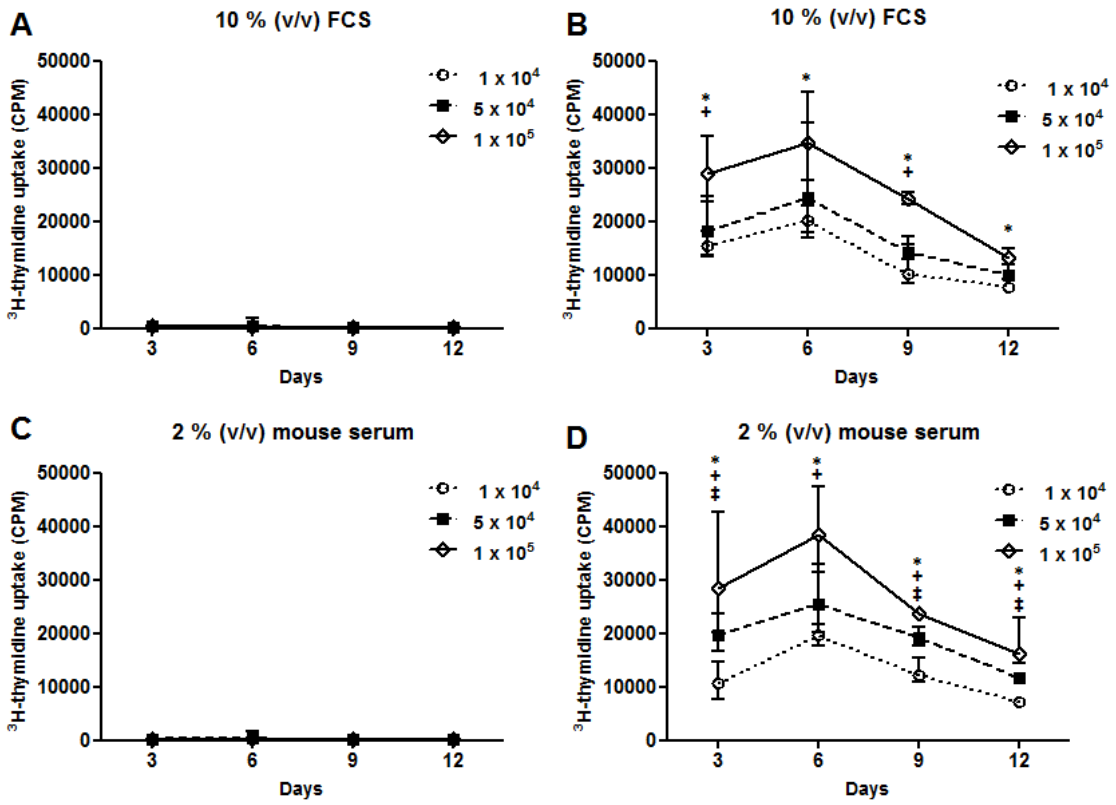

Figure 4.20. Stimulation of different cell numbers of Balb/c mononuclear cells cultured in medium containing 10 % (v/v) FCS and 2 % (v/v) mouse serum as controls for the one-way MLR. Balb/c mononuclear cells were plated at 1 x 10^4, 5 x 10^4 and 1 x 10^5 in medium supplemented with 10 % (v/v) FCS or 2 % (v/v) mouse serum as either unstimulated cells or Con-A (10 μg.ml^{-1}) stimulated cells. The cells were cultured for 3, 6, 9 and 12 days respectively accompanied by medium changes (100 μl replaced with equal volume) at each 3 day period. Lymphocyte proliferation was measured by the incorporation of ^{3}H-thymidine into newly formed DNA of dividing cells. Data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between CPM at each time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for unstimulated cells (A) and Con-A stimulated cells (B) in 10 % (v/v) FCS and unstimulated cells (C) and Con-A stimulated cells (D) in 2 % (v/v) mouse serum.* indicates significant differences between 1 x 10^5 and 1 x 10^4, + indicates significant differences between 1 x 10^5 and 5 x 10^4 and ‡ indicates significant differences between 5 x 10^4 and 1 x 10^4 for a given time point.

For the one-way MLR carried out in culture medium supplemented with 10 % (v/v) FCS, the responder to stimulator cell ratio that gave the highest counts was the 1:1 followed by 1:10 and 1:100 respectively in the order 1:1 > 1:10 > 1:100 at each time point (**Figure 4.21**). In terms of total cell numbers, the highest counts were obtained in cultures containing 1 x 10^5 followed by 5 x 10^4 and 1 x 10^4 respectively in the order 1 x 10^5 > 5 x 10^4 > 1 x 10^4. Peak counts were obtained at day 6 of culture. For one-way MLR carried out in culture medium supplemented with 2% (v/v) mouse

serum however, significantly low counts were obtained for all the responder to stimulator cell ratios in comparison to reactions in 10 % (v/v) FCS at all the points.

In order to determine the extent of the allogeneic response, SI were computed for each different responder to stimulator cell ratio and total cell number at all the time points. The results showed that for MLR in 10 % (v/v) FCS medium, the SI peaked at day 6 and declined thereafter at day 9 and day 12 respectively (**Figure 4.22**). Highest SI were obtained at the 1:1 ratio of responder to stimulator cells followed by 1:10 and 1:100 respectively. At any given time point and cell ratio, the magnitude of the SI was in the order $1 \times 10^5 > 5 \times 10^4 > 1 \times 10^4$.

For cells cultured in 2 % (v/v) mouse serum however, the SI for all the different cell ratios and cell numbers at any given time point were equal to or below 3. This showed that the Balb/c lymphocytes failed to respond to allogeneic C3H mononuclear cell in culture medium containing 2 % (v/v) mouse serum. This was despite prior positive lymphocyte transformation observed when Balb/C mononuclear cells were stimulated with Con-A in 2 % (v/v) mouse serum as described earlier in **Section 4.3.4**.

Taken together, these data showed that the most appropriate conditions for the one-way MLR were achieved at a 1:1 responder to stimulator cell ratio and 1×10^5 total cells in medium containing 10 % (v/v) FCS but not 2 % (v/v) mouse serum. These conditions were used in subsequent assays.

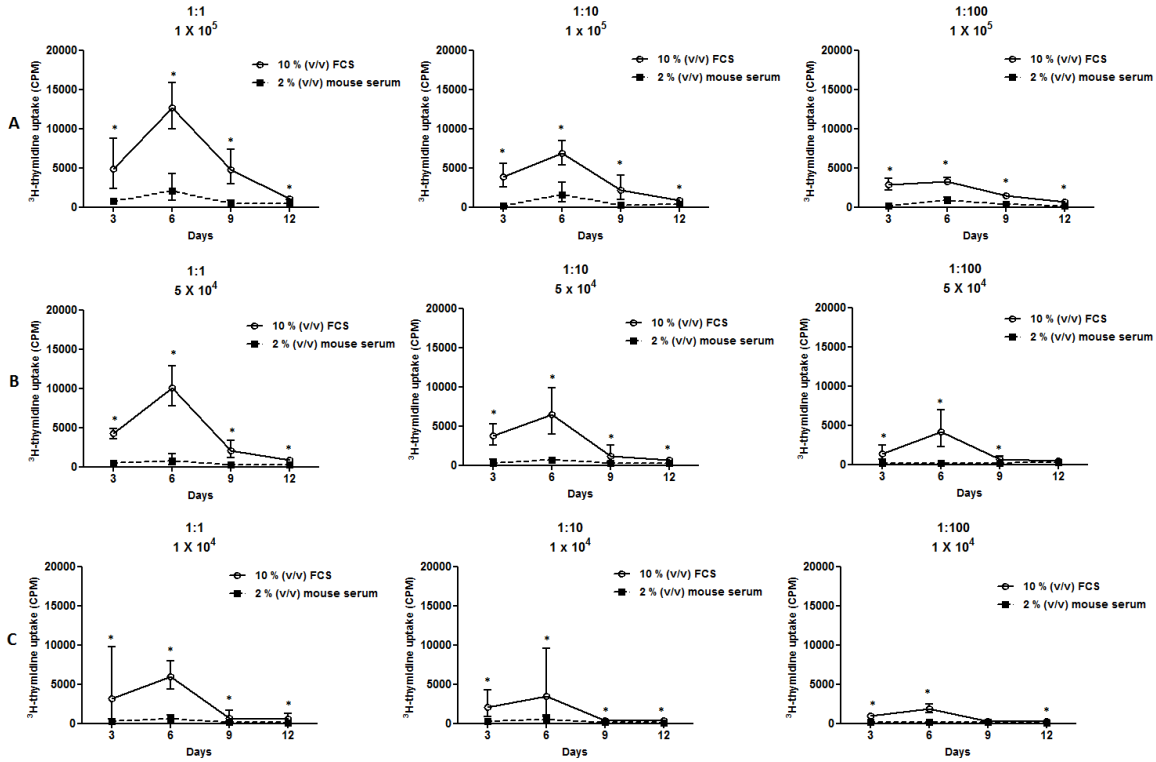

Figure 4.21. One-way MLR in 10 % (v/v) FCS and 2 % (v/v) mouse serum containing medium for the determination of appropriate responder to stimulator cell ratio and total cell numbers. Balb/c (responder) and mitotically inactivated C3H (stimulator) mononuclear cells were co-cultured at ratios of 1:1, 1:10 and 1:100 respectively in cultures containing total cell numbers of 1 x 10^5 (A), 5 x 10^4 (B) and 1 x 10^4 (C) in medium supplemented with either 10 % (v/v) FCS or 2 % (v/v) mouse serum for 3, 6, 9 and 12 days respectively. Medium changes (100 µl replaced with equal volume of fresh medium) were carried out after every 3 days. The allogeneic response of the responder lymphocytes against the stimulators was measured by the incorporation of ^{3}H-thymidine. Data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p<0.05$) by the T method to determine differences between CPM of cells cultured in FCS and mouse serum at each time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL.* indicates significant differences in ^{3}H-thymidine uptake between cells in 10 % (v/v) FCS and 2 % (v/v) mouse serum at a given time point.

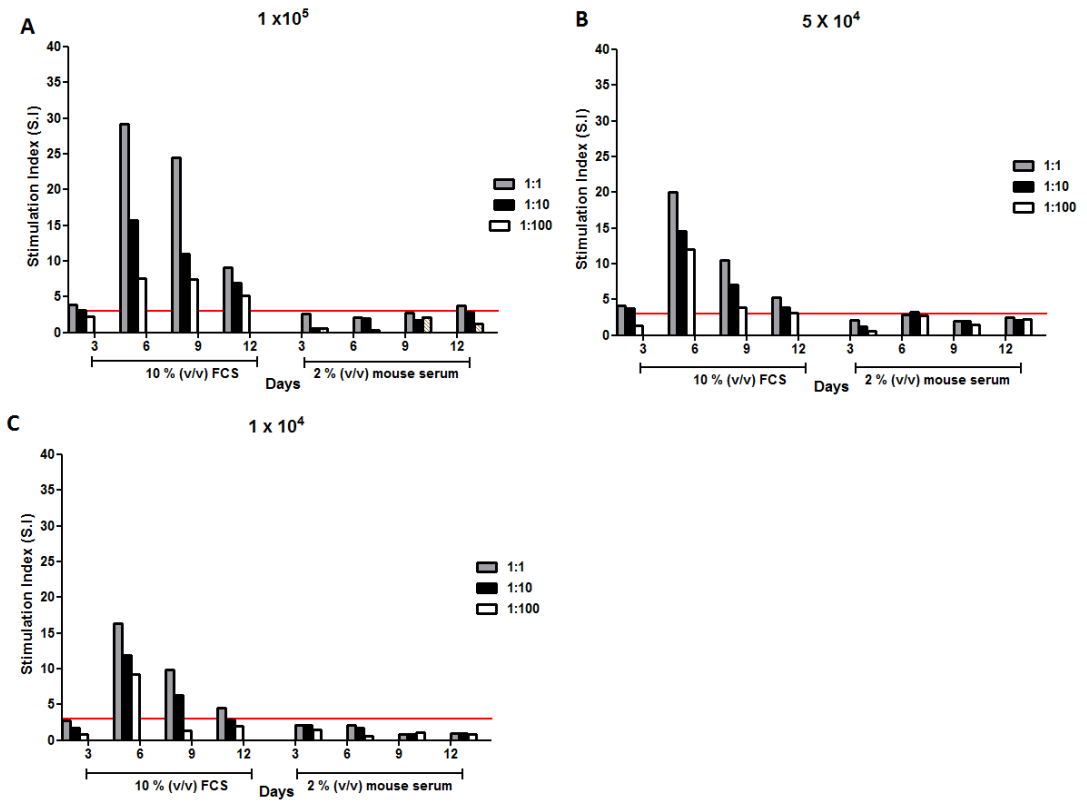

Figure 4.22. Effects of total cell number and different responder to stimulator cell ratios for one-way MLR in 10 % (v/v) FCS and 2 % (v/v) mouse serum culture medium. Balb/c (responder) and C3H (stimulator) mononuclear cells were co-cultured at ratios of 1:1, 1:10 and 1:100 respectively in cultures containing total cell numbers of 1 x 10^5 (A), 5 x 10^4 (B) and 1 x 10^4 (C) in medium supplemented with 10 % (v/v) FCS or 2 % (v/v) mouse serum. The cells were cultured for 3, 6, 9 and 12 days respectively accompanied by medium changes (100 µl replaced with equal volume of fresh medium) every 3 days. The allogeneic response of the Balb/c lymphocytes to C3H stimulators was measured by the incorporation of ^{3}H-thymidine. Results are displayed as SI (ratio between mean CPM of MLR and responder cells only) for each cell ratio at a given time point. The red line at SI=3 denotes statistical biological positive; SI above 3 are considered significant allogeneic responses by Balb/c lymphocytes to C3H cells.

4.4. Discussion

The work carried out in this chapter aimed to test several experimental parameters in order to establish the most suitable conditions for carrying out LTA and one-way MLR which are the key assays employed in the study of the immunogenicity and immunomodulatory properties of allogeneic MSC and DF respectively. As with most immunological assays, the sensitivity and responsiveness of lymphocytes largely depends on *in vitro* micro and macro environmental factors. Therefore, it was important to investigate some of the important variables in order to minimise confounding of the results by these factors. Among the factors investigated were the type and concentration of mitogen to be used as a positive control for lymphocyte proliferation, culture period and medium replacement for longer incubations, effective concentration of mitomycin C for inducing mitotic arrest for stimulator cells, the type and concentration of serum for supplementing culture medium, the ratio of responder to stimulator cells and total cell numbers suitable for sensitivity of the LTA and one-way MLR.

Mouse mononuclear cells were isolated from spleens and lymph nodes of female mice by iso-osmotic density gradient separation at 1.077g.ml^{-1} using Lymphoprep™. This method removed erythrocytes, polymorphonuclear leukocytes and other stromal cells leaving behind a white band of mononuclear cells (Kruisbeek, 2001). The mononuclear cells, which contain lymphocytes, were used in all the experiments instead of separated lymphocytes in order to allow the participation of other accessory cells such as DC in the reactions.

Mitogen stimulated mononuclear cells were used as positive controls in most experiments throughout this study. In order to test the responsiveness of the mononuclear cells, Balb/c mononuclear cells which were used as responder cells, were stimulated with increasing doses of PHA and Con-A to determine the most effective mitogen and appropriate dose. ^{3}H-thymidine uptake and intracellular ATP content were measured after 3, 5 and 7 days to establish the peak time and mitogen concentration at which DNA synthesis and cell proliferation was highest. Peak counts were obtained at day 3 with 10 μg.ml^{-1} of Con-A and 25 μg.ml^{-1} of PHA. It was found that Con-A was effective at low concentrations whereas PHA was effective at higher concentrations. Stimulation using Con-A at 30 μg.ml^{-1} or greater

appeared to have an inhibitory effect on ^{3}H-thymidine uptake. This was further highlighted by the ATPLite™ assay which showed that at these Con-A concentrations, cellular ATP levels dropped significantly below those for control unstimulated cells but this was not the case with PHA stimulated cells. This suggested that these high concentrations of Con-A are toxic to the Balb/c mononuclear cells. This also suggested that despite both mitogens being non-specific, their potency or tolerability by cells was different. It has been suggested that Con-A stimulates a larger proportion of cells that would be otherwise unresponsive to PHA. Early studies on lymphocyte proliferation suggested that unlike PHA, Con-A is capable of stimulating immature T-lymphocytes (Jacobsson & Blomgren, 1974), although this remains unresolved to date.

The results also showed that highest counts and SI were obtained at day 3 followed by day 5 and day 7 respectively. This concurred with other studies which showed that peak DNA synthesis occurred between 3-5 days of lymphocyte stimulation by mitogens (Janossy *et al.*, 1973; De Fries & Mitsuhashi, 1995). Thus, most LTA and MLR from the literature have been carried out between 3 and 5 days. Although this period is suitable when mitogens and other potent stimulators are used, it may be inadequate when less potent stimulators are used. As discussed in **Chapter 1**, MSC have been reported to be poorly immunogenic and failed to stimulate allogeneic lymphocyte *in vitro*. However, the hypothesis of this study suggested that if given adequate time under suitable conditions, MSC could potentially stimulate allogeneic lymphocytes *in vitro* by the indirect or semi-direct pathways.

It has been suggested that capturing the indirect or semi-direct pathways could require up to 10 days or more in culture (Chitilian *et al.*, 1998) or 2-4 weeks *in vivo* (Costa *et al.*, 2004). Therefore, in order to extend the culture period from 3-5 days, the accumulation of toxic metabolic by-products and nutrient depletion may become limiting. It has been shown that lymphocyte stimulation by mitogens and antigens is a highly energy demanding process accompanied by amplified glucose uptake and diminished oxygen intake which results in anaerobic respiration leading to raised lactate levels which inadvertently impact on cell division and viability (Loos & Roos, 1973; Roos & Loos, 1973; Hume *et al.*, 1978). Therefore, a strategy of changing medium during culture was employed in order to extend the culture period to 20

days. This involved the removal of half (100 μl per well) of the culture medium and replacing it with fresh culture medium every 3 day period.

To test this strategy, Balb/c mononuclear cells were stimulated with Con-A at different concentrations (5 to 25 $\mu g.ml^{-1}$) for 20 days with and without medium changes. It was found that medium changing significantly increased ^{3}H-thymidine uptake and shifted peak stimulation from day 3 as observed in cultures without medium changes to day 6. In addition, SI higher than 3 were obtained in cultures with medium changes up to day 15 at all but 10 $\mu g.ml^{-1}$ of Con-A in which SI above 3 were obtained at day 20. In cultures without medium changes, SI higher than 3, were only obtained at day 3 and day 5 but not beyond. This in part could explain why most LTA and MLR in the literature have not been tested beyond a 5 day culture period. The medium change experiments suggested that LTA cultures could be maintained for longer than the standard 5 day period. This strategy was therefore employed in future assays and also in MLR.

It was constantly observed that in Con-A stimulated cultures without medium changes, the phenol red indicator in the culture medium changed from bright red to yellow at day 5 and day 7. When the pH was measured, it was found to be between 5.6 and 6.2. This was not observed in cultures with medium changes. Previous reports have shown that pH drops below 6.6 significantly impacted on lymphocyte proliferation (Frauwirth & Thompson, 2004). The drop in pH could be attributed to the accumulation of lactic acid and other metabolic by-products. For instance, acidic pH could alter protein structure and function of key protein molecules such as enzymes which are involved in vital cellular activities.

The next step in establishing appropriate conditions for LTA and one-way MLR was to determine the concentration of mitomycin C required to induce mitotic arrest while maintaining cell viability in C3H mononuclear cells which were the stimulator cells subsequent one-way MLR. It has been noted in many studies in the literature that a broad range of concentrations of mitomycin C (5 – 120 $\mu g.ml^{-1}$) have been used for achieving mitotic inactivation during MLR. However, most of such studies did not report on the effects of the mitomycin C on cell viability. This is very important since it has been demonstrated that mitomycin C affects cellular mitochondrial DNA which leads to cytotoxicity even at low concentrations (Kulkarni

et al., 2009). It is conceivable that since mitomycin C induces mitotic arrest by cross-linking genomic DNA, mitochondrial DNA, which contains genes that control important metabolic functions including oxidative phosphorylation and synthesis of transfer ribonucleic acid (tRNA) and messenger RNA (mRNA) will also be susceptible to irreversible damage. Arrest of oxidative phosphorylation which provides cellular ATP, starves the cells of vital energy and the inhibition of tRNA and mRNA disables the cells ability to synthesize proteins including key enzymes, hormones, cytokines and other essential molecules. Therefore, it was very important to determine the most suitable concentration of mitomycin C which achieved mitotic inactivation while maintaining cell viability.

C3H mononuclear cells were treated with increasing doses of mitomycin C and cultured for 3, 5 and 7 day without medium changes as unstimulated or Con-A (10 $\mu g.ml^{-1}$) stimulated cells. Using cellular ATP content as a measure of cell viability and mitotic inactivation, it was found that mitomycin C significantly reduced cellular ATP levels in Con-A stimulated cells. However, the data was not informative enough as the ATP levels were not significantly different for cells treated with different doses (5-100 $\mu g.ml^{-1}$) of mitomycin C. Previous studies using Balb/c cells had showed that mitomycin C reduced cellular ATP levels after 48 hours exposure (Pritsos & Briggs, 1996). For this study however, exposure was for 30 minutes yet the ATP content of unstimulated cells was comparable to that of dead (camptothecin-treated) cells. This demonstrated the potency of mitomycin C as an inhibitor of metabolism and respiration. Therefore, this method could not be reliably used to determine the appropriate non-lethal dose of mitomycin C. An explanation for this could be due to the previously mentioned effects of mitomycin on mitochondrial DNA which directly affects the ability of the cells to produce ATP. It is conceivable that once intracellular ATP levels are diminished, cells treated with mitomycin C fail to synthesize ATP thus making ATP measurements a poor indicator of cell viability under such conditions.

It is also possible to hypothesize that once ATP synthesis via aerobic respiration is blocked, a switch to the anaerobic pathway could result in the production of more lactic acid. Accumulation of the lactic acid could lead to a drop in pH and as previously explained. Therefore, it would seem that in static cultures involving

mitomycin C treated cells such as in LTA and one-way MLR, changing the culture medium helps to reduce the accumulation of lactic acid thus allowing cells to survive longer in culture.

Another important factor is the time in which the cells are exposed to mitomycin C. As mentioned previously, Pritsos & Briggs used a 48-hour exposure (Pritsos & Briggs, 1996) while this study used a 30 minute exposure. Although the results have not been presented in this study, analysis of cellular ATP was carried out at 10 minute intervals for 60 minutes using C3H mononuclear cells during preliminary studies. The results showed that cellular ATP levels were similar between untreated cells and cells treated for 10 minutes with between 5 and 120 $\mu g.ml^{-1}$ mitomycin C. However, after 20 minutes, the ATP levels had dropped six-fold and remained constant up to 60 minutes at all the concentrations. This not only demonstrated that mitomycin C inhibits cellular ATP synthesis at all the concentrations tested, but importantly showed that mitomycin C exerts its inhibitory effects after the first 10 minutes of exposure. Thus, a 30 minute exposure was chosen since the ATP content did not change after 20 to 60 minute exposure.

^{3}H-thymidine uptake was then used to determine the effective mitomycin C concentration. Results showed that mitomycin C significantly reduced ^{3}H-thymidine uptake by unstimulated and Con-A stimulated cells at all concentrations used. At mitomycin C concentrations between 10 and 50 $\mu g.ml^{-1}$ there were no significant differences in counts between stimulated and unstimulated cells which suggested mitotic inactivation. This was confirmed by SI values which were less than 3 at these concentrations. However, the range was deemed too broad considering that mitomycin C is cytotoxic. Therefore, despite the results suggesting mitotic inactivation, it had not been established whether the cells were viable or not. Thus, ^{3}H-thymidine uptake alone was not informative in terms of cell viability since similarly low counts were obtained with camptothecin treated cells.

In most previous studies, ^{3}H-thymidine uptake has been widely used for demonstrating mitotic arrest by mitomycin C and x-irradiation as both these methods have been described as similarly effective (Bach & Bach, 1972). However, judging from the results obtained in this study, this method, although suggesting mitotic arrest due to low counts in stimulated cells, did not shed light as to whether this was

due to mitotic inactivation or cytotoxicity. It is possible to hypothesize that both sub-lethal and lethal doses of mitomycin C are likely to inhibit the uptake of ^{3}H-thyidine in different ways. A sub-lethal dose could inhibit both DNA and protein synthesis making the cells incapable of producing daughter cells. However, dead cells also are unable to utilise ^{3}H-thymidine. Thus the results of the ^{3}H-thymidine uptake alone could not be used to determine mitotic arrest without evidence of cell viability.

The problem was solved by using the CFSE dilution assay alongside 7-AAD staining. Flow cytometry analysis of CFSE labelled and 7-AAD stained cells allowed the mitomycin C treated cells (unstimulated and Con-A stimulated) to be analysed for cell viability (only dead cells stained positive with 7-AAD) and cell proliferation (CFSE dilution). Incomplete mitotic inactivation was demonstrated by CFSE dilution in Con-A stimulated cells.

It was shown that mitotic inactivation while maintaining cell viability above 90 % for up to 5 days and 70 % for 7 days was achieved at 10 $\mu g.ml^{-1}$ of mitomycin C. Higher mitomycin C concentrations (20 and 50 $\mu g.ml^{-1}$) drastically reduced cell viability while lower concentrations (5 $\mu g.ml^{-1}$) were insufficient in stopping the cells from undergoing mitosis upon stimulation with Con-A. In addition, less than 16 % of cells treated with 10 $\mu g.ml^{-1}$ mitomycin C, underwent one cycle of mitosis after Con-A stimulation compared to between 20 % and 60 % of cells treated with 5 $\mu g.ml^{-1}$ of mitomycin C, which went up to 9 mitotic cycles over the 7 day period. Approximately 3 to 15 % of negative controls (untreated and unstimulated) also underwent a single mitotic round. This could be attributed to the natural loss of CFSE fluorescence or to a fraction of the cells reacting to some xenogeneic factors in FCS.

CFSE is a fluorescent dye which can label intracellular molecules and is halved with each cell division cycle. This results in a sequential halving of its fluorescence intensity allowing the tracking of up to 10 cell division cycles *in vivo* and *in vitro*. The dye itself is not fluorescent but acquires its fluorescence after fluxing across cell membranes and reacting with intracellular esterases to produce a highly fluorescent but stable dye which forms lasting conjugates with intracellular proteins at neutral pH. 7-AAD is a dye that intercalates permanently with double stranded cellular DNA

of dead cells since it cannot pass across intact membranes of viable cells thus allowing all non-viable cells to be detected and quantified.

These findings showed that previous high concentrations of mitomycin C used in the literature may have been toxic to the cells since none of the studies has reported cell viability analysis following mitomycin C treatment. Using non-viable cells in one-way MLR potentially gives false results as dead cells can indirectly stimulate viable lymphocytes *in vitro* via the indirect and semi-direct pathways. In addition, these findings have also demonstrated the limitations of the ATPLite™ assay which is often used for the determination of cell viability.

Another important parameter considered was the type and concentration of serum for culture medium supplementation. Previous studies have used 10 % (v/v) FCS as the standard supplement but since this study used a mouse model system, it was thought that mouse serum would be more appropriate. Therefore, commercially sourced heat-inactivated mouse serum was compared with FCS at different concentrations in LTA involving Con-A stimulated Balb/c mononuclear cells. The highest counts and SI were obtained at 2% (v/v) mouse serum and 10 % (v/v) FCS. Interestingly, higher mouse serum concentrations had an inhibitory effect on ^{3}H-thymidine uptake whereas conversely, higher FCS concentrations were stimulatory.

Consequentially, both 2 % (v/v) mouse serum and 10 % (v/v) FCS were used in subsequent experiments for the determination of appropriate ratio of responder to stimulator cells for carrying out one-way MLR. Balb/c mononuclear cells were used as responders and mitomycin C (10 $\mu g.ml^{-1}$) treated C3H mononuclear cells were used as stimulators at 1:1, 1:10 and 1:100 ratios. The high stimulator ratios provided different doses of allogeneic antigens thus testing the responsiveness of the responder cells under different allogeneic settings which could arise in applications where high doses of allogeneic cells could be used (such as MSC cell based cell therapies). The 1:1 ratio gave the highest counts and SI followed by 1:10 and 1:100 respectively in 10 % (v/v) FCS medium.

In 2 % (v/v) mouse serum cultures, both the counts and SI were significantly lower with the latter below the significant threshold of 3. This was despite Con-A stimulated positive controls showing significant stimulation in previous experiments. The failure of the one-way MLR in 2% (v/v) mouse serum could be attributed to

inhibitory factor(s) which could be present in the serum. Previous results on serum titration had shown that Con-A stimulation was significantly inhibited at 5 % (v/v) and above of mouse serum. These factors could be inhibitory by blocking antigen presentation to responder cells. Since Con-A controls were positive; this could be because mitogens use different stimulatory pathways to protein antigens. There are currently no studies in the literature over the past two decades that have evaluated the mechanism(s) behind mouse serum based immunosuppression. Only a few early studies in the 1970s and 1980s have reported similar problems with mouse serum in MLR (Veit & Michael, 1973). Several unknown factors thought to be immunoglobulin molecules such as the 7S antibodies (Chalon *et al.*, 1979), gangliosides and all lipoproteins including low and intermediate and high density lipoproteins have been implicated as possible inhibitors (Hsu *et al.*, 1981; Hsu *et al.*, 1982; Marcus, 1984). The mechanism of lipoprotein-mediated immunosuppression has been thought to be as a result of the lipoprotein binding to lipoprotein receptors on T-lymphocytes. It has been shown that more than 70 % of the lipoproteins in mouse serum are high density lipoproteins (Hsu *et al.*, 1982) whereas in other species such as bovine and human the lipoprotein content is different. This could potentially explain why mouse serum suppressed lymphocyte proliferation whereas FCS did not. It remains unknown however, by which mechanism these factors suppress lymphocyte proliferation *in vitro*. Based on the evidence obtained in this study, it is possible to speculate that these factors are more potent against antigenic than non-specific mitogenic stimulation. Therefore, 10 % (v/v) FCS medium supplementation was used in subsequent assays.

In summary, the following conditions were deemed most appropriate for carrying out LTA and one-way MLR using the ^{3}H-thymidine uptake assay; 10 μg.ml^{-1} Con-A as a positive control for lymphocyte stimulation, 10 μg.ml^{-1} of mitomycin C for achieving mitotic inactivation in stimulator cells, medium changes every 3 days for long term MLR and LTA cultures up to 15 days, and responder to stimulator cell ratio of 1:1 with a total cells number of 1 x 10^{5} cells in culture These conditions were used in subsequent LTA and one-way MLR involving MSC and DF which are discussed in **Chapter 5** and chondrogenic differentiated MSC and DF in **Chapter 6**.

5. CHAPTER FIVE: INVESTIGATION OF THE IMMUNOMODULATORY ACTIVITY AND IMMUNOGENICITY OF UNDIFFERENTIATED AND CHONDROGENIC DIFFERENTIATED ALLOGENEIC MOUSE MSC AND DF

5.1. Introduction

The last decade has seen a surge in studies investigating the immunological status of allogeneic MSC in light of their potential application in tissue engineering and regenerative medicine. MSC, in addition to possessing immunosuppressive properties, have also been reported to be immunoprivileged and capable of evading detection by the host immune system when transplanted into an allogeneic host (Bartholomew *et al.,* 2002; Di Nicola *et al.,* 2002; Tse *et al.,* 2003; Le Blanc *et al.,* 2003c).

The ability to differentiate into new distinct cell types makes MSC and their differentiated products attractive for clinical use in the repair, regeneration and replacement of diseased or damaged tissues. Practically, allogeneic MSC would be more useful since cells from a single donor could be infinitely expanded, used to make "off-the-shelf" tissue engineered products or individually tailored as therapies for multiple patients. This "universal donor" concept though attractive is limited by immunological mismatches between donor MSC and the recipient's immune system. Studies using different animal models including mice have found that allogeneic MSC were rejected by hosts and therefore could not be described as intrinsically immunoprivileged (Nauta *et al.,* 2006; Sudres *et al.,* 2006; Poncelet *et al.,* 2007). Thus to date, the immunological status of MSC remains largely unresolved.

As discussed extensively in **Chapter 1**, donor allogeneic cells expressing surface MHC class I and class II molecules and co-stimulatory molecules can present foreign antigens directly to recipient's T-lymphocytes via the direct pathway or indirectly by cross presentation of foreign antigens by the host professional APC via the indirect or semi-direct pathways of allorecognition to elicit a lymphocyte proliferative response. The reported immunosuppressive and immunoprivilege properties of MSC

are widely thought to be in part a result of their phenotype, which is characterised by low expression of MHC I and absence of cell surface MHC II and the co-stimulatory molecules CD40, CD80 and CD86. This phenotype has been thought to be responsible for the inability of MSC to elicit a proliferative response when co-cultured with allogeneic lymphocytes (Di Nicola *et al.*, 2002; Tse *et al.*, 2003; Eliopoulos *et al.*, 2005). Thus on account of this phenotype, it is conceivable that allogeneic MSC failed to stimulate responder T-lymphocytes *in vitro* via the direct pathway but theoretically, it is probable that they will have the capacity to stimulate proliferative responses via the indirect or semi-direct pathways in cultures involving unseparated mononuclear cells containing APC.

A review of the literature showed that various adaptations of the MLR and LTA have been routinely used in assessing the immunological properties of allogeneic MSC using both human cells and animal models. The MLR can be setup as either one-way or two-way, the former involving co-culture of genetically distinct responder lymphocytes and mitotically inactivated stimulator lymphocytes and the latter involving the co-culture of untreated (mitotically active) allogeneic lymphocytes. In the one-way MLR, the ability of the responder lymphocytes to respond to the allogeneic stimulator lymphocytes is measured by the proliferative response of the responder lymphocytes. In the two-way MLR however, the mismatched lymphocytes are both capable of initiating a proliferative response against each other. Mitotically inactivated MSC can be added at the beginning or at different stages of ongoing MLR cultures in order to assess their immunomodulatory properties based on how they affect the course of a MLR.

The LTA involves the co-culture of responder lymphocytes with mitotically inactivated cells (not lymphocytes) which act as stimulator cells, or other stimulatory agents such as antigens or mitogens. In the case of MSC studies, mitotically inactivated allogeneic MSC are used as stimulator cells. Taken together, these adaptations of the MLR and LTA provide a simple approach to determine whether allogeneic MSC are capable of suppressing responder cell proliferative response (immunomodulation) or inducing a proliferative response (or not) in the responding lymphocytes (immunogenicity). These assays and their adaptations have provided much of the current understanding of the immunological status of MSC as previously discussed.

The majority of studies that have suggested that allogeneic MSC are immunoprivileged and immunosuppressive tested mouse MSC in LTA and MLR for 3 to 5 days *in vitro* (Glennie *et al.*, 2005; Sudres *et al.*, 2006; Han *et al.*, 2010). When tested *in vivo* after longer periods however, allogeneic MSC were found to be immunogenic (Nauta *et al.*, 2006; Han *et al.*, 2010). It was hypothesized that the 5 day period generally used for LTA may not have been sufficient to capture the semi-direct and indirect pathways. Thus, extending the incubation period of the MLR and LTA could give further insights into the long term immunomodulatory properties of allogeneic MSC.

The claim by some studies in the literature that allogeneic MSC are immunoprivileged makes them a particularly suitable cell source for tissue engineering applications. In these applications, MSC may be used in their differentiated state. Given that the immunogenicity of undifferentiated MSC is currently controversial, it is also important to investigate whether differentiated MSC are immunogenic. Equally, it is also essential to investigate whether MSC lose or retain their immunosuppressive properties following differentiation into a specific lineage. Therefore, part of the work in this chapter was aimed at investigating the immunosuppressive properties and immunogenicity of allogeneic MSC differentiated into chondrocytes. This is particularly important in cartilage tissue engineering applications using allogeneic MSC.

Tissue engineering aims to provide viable and functional biological products that can replace native tissues lost due to disease or injury. Such products should be able to provide structural and morphological homogeneity with native tissue but importantly should be able to restore the function of the tissue to be replaced. This requirement has contributed significantly to the potential role MSC could play in tissue engineering. The capacity of MSC to differentiate along the chondrogenic lineage offers the potential for living tissues to be engineered *ex vivo* using allogeneic MSC which can then be implanted into patients (Le Blanc, 2006; Nesic *et al.*, 2006).

The increase in life expectancy in developed countries has been accompanied with an increase in musculoskeletal diseases mainly those involving articular cartilage degeneration such as osteoarthritis (Kuo *et al.*, 2006). Various approaches have been developed for cartilage repair as a measure to delay or prevent the need for total joint

replacement. Methods such as autologous chondrocyte implantation/transplantation and matrix-supported autologous chondrocyte implantation use expanded articular chondrocytes (Peterson *et al.*, 2000; Minas, 2001). These methods, though partially successful in small defects, are limited in complicated cases involving larger defects due to the poor proliferation capacity of chondrocytes and existing patho-physiological conditions in patients. Poor vascularisation, nerve and lymph supply and the inherently limited differentiation capacity of articular chondrocytes limits the capacity of damaged cartilage to self-heal (Yuan *et al.*, 2010).

Methods that utilise autologous MSC to provide a source of chondrocytes for cartilage repair may be limited by the patients' own conditions which may not favour use of autologous cells. Moreover, patients would have to go through two procedures; firstly to harvest autologous MSC and secondly to implant the tissue engineered product. These limitations have opened new avenues for use of allogeneic MSC which would allow tissue engineered products to be manufactured to provide ready-made cartilage tissues for repair and replacement and limiting the number of operations patients would have to go through to a one step procedure.

Allogeneic MSC have emerged as potential cells of choice in cartilage tissue engineering because of their multi-differentiation capacity and ease by which they can undergo chondrogenic differentiation *in vitro* as was demonstrated in **Chapter 3.** Chondrogenic differentiation of MSC can be carried out *in vitro* by using cocktails of growth factors and chemicals which are known to induce differentiation along the chondrogenic lineage such as BMP-2 and TGFβ1, insulin and selenium (Worster *et al.*, 2001; Goessler *et al.*, 2005; Longobardi *et al.*, 2006; Bernardo *et al.*, 2007). Mechanical stimulation such as dynamic compression of MSC seeded on biological or synthetic three dimensional scaffolds with or without growth factor cocktails has also been shown to induce chondrogenic differentiation in bioreactors (Campbell *et al.*, 2006; Farrell *et al.*, 2006; Terraciano *et al.*, 2007; McMahon *et al.*, 2008). These approaches have demonstrated that tissue resembling cartilage can be produced using allogeneic MSC which may be of utility for repair and replacement of large defects. However, immune reactivity to allogeneic cells remains the major obstacle for the clinical utility of such products.

The immunological properties of differentiated MSC are largely unknown to date. In a study using a rabbit model, MSC differentiated into osteoblasts were reported to retain their immunoprivileged and immunosuppressive status *in vitro* though the latter was lost after transplantation (Liu *et al.*, 2006). However, in a rat model using MSC differentiated to myocytes for cardiac repair, allogeneic but not syngeneic cells were reported to be immunogenic despite the undifferentiated MSC having been shown to be non-immunogenic *in vitro* prior to differentiation. Increased expression of MHC II was thought to be one of the possible reasons which could have contributed to the loss of their immunoprivilege status (Huang *et al.*, 2010).

It has been reported that differentiated MSC fail to express MHC II after treatment with IFNγ (Le Blanc *et al.*, 2003b). In another study, chondrogenic but not adipogenic or osteogenic differentiated rat allogeneic MSC expressed CD80 and CD86 co-stimulatory molecules and activated DC resulting in an eight-fold increase in lymphocyte proliferation compared to undifferentiated MSC (Chen *et al.*, 2007). This evidence suggested that the differentiation of MSC resulted in loss of their immunoprivilege and immunomodulatory properties. It is also widely accepted that most MSC cultures are heterogeneous due to loss of differentiation potency as a result of ageing (Crisostomo *et al.*, 2006; Izadpanah *et al.*, 2008; Kretlow *et al.*, 2008; Roobrouck *et al.*, 2008; Liu *et al.*, 2012). This often results in some fraction of MSC failing to undergo differentiation *in vitro* as was discussed in **Section 3.4**. Therefore, factors such as the efficiency of the differentiation protocols used by different researchers could lead to differentiated MSC exhibiting different immunological properties. This was demonstrated using a mouse model in a study in which differentiated MSC were found to secrete different types and reduced amounts of immunomodulatory molecules such as TGFβ and IL-10 as well as increased MHC I expression post differentiation (Liu *et al.*, 2012). These changes could potentially alter the immunological properties of differentiated MSC particularly increased MHC I expression.

Taken together, the evidence from the literature indicates that the immunological properties of differentiated MSC are as equally controversial as those of undifferentiated MSC. Given that it is well understood that the process of differentiation brings about physiological, morphological and functional changes, it

was hypothesized that the immunomodulatory properties of undifferentiated MSC would be lost following chondrogenic differentiation. DF, which had previously been shown to undergo chondrogenic differentiation, were also tested alongside MSC.

In **Chapter 4**, the appropriate conditions for performing MLR and LTA were defined as follows;

- 10 $\mu g.ml^{-1}$ Con-A; positive control for lymphocyte proliferation.
- 10 $\mu g.ml^{-1}$ mitomycin C; mitotic arrest of stimulator cells.
- 10 % (v/v) FCS; serum supplement for culture medium.
- Medium replacement every 72 hours of culture (100 µl replaced by fresh 100 µl in a total culture volume of 200 µl per well).
- Responder to stimulator cells ratio of 1:1 in MLR and LTA with a 1 x 10^5 total cells.

Both Balb/c and C3H MSC (p5-6) were incorporated in one-way and two-way MLR and LTA and cultured for 3, 6, 9, 12 and 15 days. Cell proliferation was measured by incorporation of low activity ^{3}H-thymidine. DF from both Balb/c and C3H mice were used as controls for cells of mesenchymal origin. Chondrogenic differentiated MSC and DF were also tested using one-way MLR and LTA in order to determine their immunomodulatory properties and immunogenicity.

5.1.1. Aims and Objectives

The aim of this part of the experimental study was to investigate the immunological properties namely;

i. immunomodulation
ii. immunogenicity

of undifferentiated and chondrogenic differentiated MSC using an allogeneic mouse model with two genetically distinct mouse strains Balb/c (*H2-d*) and C3H (*H2-k*) as responder and stimulator respectively. Immunomodulation was assessed using adaptations of the one-way and two-way MLR in which MSC were added at the beginning of the culture. Immunogenicity was assessed using LTA with mitotically inactivated allogeneic (C3H) and syngeneic (Balb/c) MSC used as stimulators against Balb/c mononuclear cells. Both MLR and LTA were assessed over a 15 day

period. DF were also analysed in the same way as a control. This would determine whether the immunomodulatory properties of MSC were a unique feature of stem cells or a common property shared by other stromal cells of mesenchymal origin.

Specific objectives were;

1. To investigate the effects of mitotically inactivated C3H (allogeneic) and Balb/c (syngeneic) undifferentiated and chondrogenic differentiated MSC and DF in one-way and two-way MLR.
2. To investigate the capacity of mitotically inactivated C3H (allogeneic) and Balb/c (syngeneic) undifferentiated and chondrogenic differentiated MSC and DF to stimulate Balb/c mononuclear cells in LTA.
3. To determine the effects of increasing stimulator cell ratio (MSC or DF) on one-way MLR and LTA.
4. To compare the suitability of using U or V well plates for LTA using MSC as stimulator cells.
5. To investigate the effects of Balb/c (syngeneic) serum on one-way MLR and LTA.

5.2. Material and Methods

5.2.1. Reagents

The following reagents were used and were prepared as described in **Section 2.2.2** unless stated otherwise.

- Alcian blue stain solution 1% (w/v) in acetic acid
- Balb/c serum
- Chondrogenic differentiation medium for DF (described in **Section 3.2.5.2.2**)
- Chondrogenic differentiation medium for MSC (described in **Section 3.2.5.2.2**)
- Collagenase; 0.01 % (v/v)
- Con-A stock solution (1 $mg.ml^{-1}$)
- DMEM-HG culture medium
- DMEM-LG culture medium
- Hy-Q-Tase™ cell dissociation solution
- Low activity ^{3}H-thymidine working solution (25 $\mu Ci.ml^{-1}$)
- Lymphocyte culture medium
- Lymphocyte transport medium
- Methanol; 100 %
- Microscint-20 scintillation fluid
- Mitomycin C stock solution (1 $mg.ml^{-1}$)
- PBS
- Saline solution (0.85 % w/v)
- TCA; 10 % (w/v)
- Trypsin-EDTA solution; 0.5 % (v/v)

5.2.2. Effects of allogeneic and syngeneic MSC on one-way and two-way MLR and LTA

5.2.2.1. Preparation of responder Balb/c mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes from Balb/c mice as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The cells (1.65×10^{7}) were suspended in 16.5 ml of lymphocyte culture medium.

5.2.2.2. Preparation of stimulator C3H mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes of C3H mice as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The cells (1.2×10^7) were suspended in 12 ml of lymphocyte culture medium and divided into two groups. One group of 6×10^6 cells in 6 ml of lymphocyte culture medium was left untreated whilst the remainder of the cells were mitotically inactivated by treatment with 10 $\mu g.ml^{-1}$ mitomycin C as described in **Section 2.2.15**. The cells were then counted by the Trypan blue method described in **Section 2.2.9** and resuspended at 6×10^6 cells in 6 ml of fresh lymphocyte culture medium.

5.2.2.3. Preparation of Balb/c and C3H MSC

Balb/c and C3H MSC (p5) were resurrected as described in **Section 2.2.10** and maintained in DMEM-LG culture medium in 125 cm^2 tissue culture flasks until they reached 80-90 % confluence as described in **Section 2.2.5**. The cells were then detached using Hy-Q-Tase™ cell dissociation solution as described in **Section 2.2.6** and counted by the Trypan blue method described in **Section 2.2.8**. The cells were then suspended at 9×10^6 cells in 9 ml of lymphocyte culture medium. In separate test-tubes, both Balb//c and C3H MSC were divided into two groups of untreated and treated cells. For the untreated cells, 3×10^6 cells were resuspended in 3 ml of lymphocyte culture medium. The remaining cells (treated group) were centrifuged at 180 *g* for 10 minutes and the resulting cell pellets were resuspended in 10 ml of lymphocyte culture medium containing 10 $\mu g.ml^{-1}$ of mitomycin C for mitotic inactivation as described in **Section 2.2.15**. After washing, the cells were then counted by the Trypan blue method described in **Section 2.2.9** and resuspended at 6×10^6 cells in 6 ml of fresh lymphocyte culture medium.

5.2.2.4. Cell plating

The experiments (one-way MLR, two-way MLR and LTA) were set up in uncoated U-bottom 96-well plates at the same time in replicates of six as shown in **Figure 5.1**. The controls were set up in triplicates on separate plates as shown in **Figure 5.2**.

5.2.2.4.1. One-way MLR

For the one-way MLR, responder Balb/c and mitomycin C treated stimulator C3H mononuclear cells were co-cultured by seeding 50 µl containing 5×10^4 cells of each

cell type per well. The wells were made up to a total of 200 μl by adding 100 μl of lymphocyte culture medium. To investigate the effects of allogeneic MSC on the one-way MLR, mitomycin C treated C3H MSC were added to one-way MLR at 1 x 10^5 cells in 50 μl of lymphocyte culture medium per well. Similarly, to investigate the effects of syngeneic MSC on the one-way MLR, mitomycin C treated Balb/c MSC were added to one-way MLR at 5 x 10^4 cells in 50 μl of lymphocyte culture medium per well. The final responder to stimulator to MSC ratio was 1:1:1 and the total volume in each well made up to 200 μl by adding 50 μl of fresh lymphocyte culture medium.

5.2.2.4.2. Two-way MLR

For the two-way MLR, untreated Balb/c and C3H mononuclear cells were co-cultured by mixing 50 μl containing 5 x 10^4 cells of each per well. The wells were made up to a total of 200 μl by adding 100 μl of lymphocyte culture medium. To investigate the effects of MSC on two-way MLR, mitomycin C treated Balb/c or C3H MSC were co-cultured with two-way MLR by adding 50 μl containing 5 x 10^4 cells per well. The final responder to stimulator to MSC ratio was 1:1:1 and the total volume in each well made up to 200 μl by adding 50 μl of fresh lymphocyte culture medium.

5.2.2.4.3. LTA

To investigate the immunogenicity of MSC, responder Balb/c mononuclear cells (5 x 10^4 cells in 50 μl per well) were co-cultured with mitomycin C treated or untreated Balb/c (syngeneic) or C3H (allogeneic) MSC (5 x 10^4 cells in 50 μl per well) at a 1:1 cell ratio. Lymphocyte culture medium (100 μl) was added into each well to give a total volume of 200 μl.

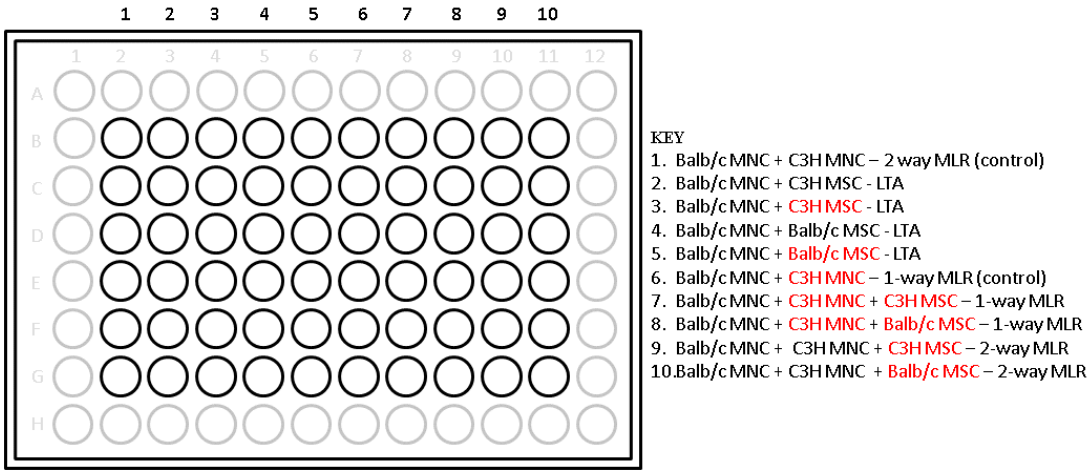

Figure 5.1. Schematic diagram showing the 96-well plate plan for the investigation of the effects of allogeneic and syngeneic MSC on one-way MLR, two-way MLR and LTA as measured by ^{3}H-thymidine uptake. The numbers above the plate correspond to the cell composition shown in the key. The cells in red were treated with 10 μg.ml^{-1} of mitomycin C for mitotic inactivation. All cells were seeded at 5 x 10^{4} per well at 1:1:1 (MLR) and 1:1 (LTA) ratios in replicates of 6. Samples 6, 7 (allogeneic), 8 (syngeneic) represented the one-way MLR; samples 1, 9 and 10 represented the two-way MLR; samples 2, 3 (allogeneic) and 4, 5 (syngeneic) represented the LTA. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.2.4.4. Controls

All the controls were set up by seeding 50 μl containing 5 x 10^{4} cells of each cell type per well in triplicates. A further 150 μl of lymphocyte culture medium was added in each well to give a total culture volume of 200 μl. Cells were either left unstimulated or stimulated by 10 μg.ml^{-1} of Con-A. Unstimulated cells were negative controls for cell proliferation whereas Con-A stimulated cells tested for the proliferative capacity of the cells in the presence of stimulation. Balb/c mononuclear cells tested the proliferative response of the responder cells for both the one-way and two-way MLR and the LTA. Untreated C3H mononuclear cells tested the proliferative capacity of the cells and were controls for the two-way MLR. Mitomycin C treated C3H mononuclear cells tested mitotic inactivation of the stimulator cells in one-way MLR. Mitomycin C treated Balb/c and C3H MSC tested mitotic inactivation of MSC and were controls for the one-way and two-way MLR and the LTA. Untreated and Con-A stimulated Balb/c and C3H MSC tested the proliferative potential of MSC and were controls for the LTA.

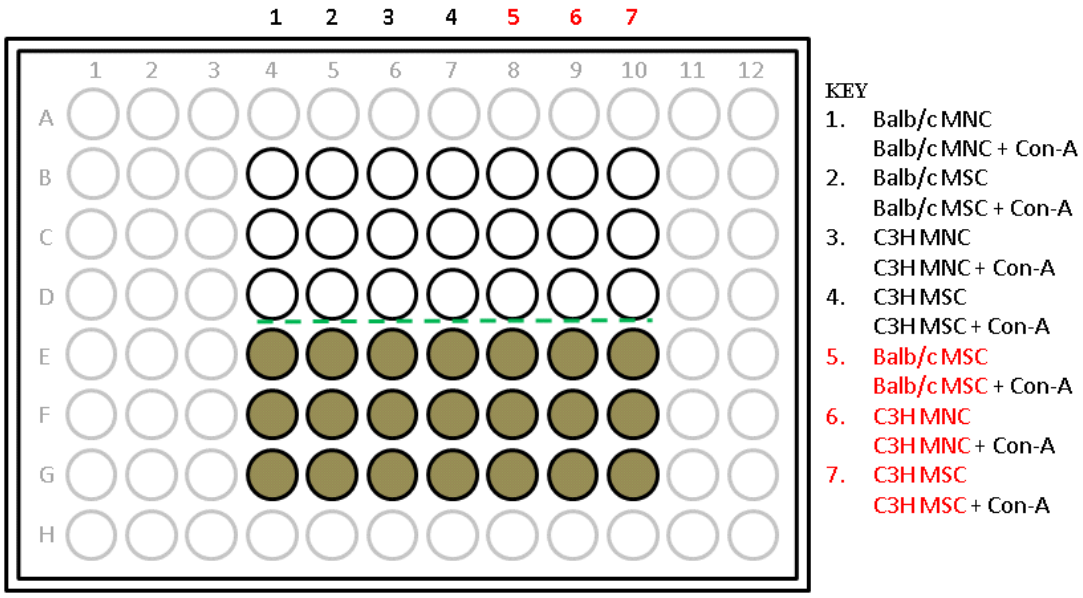

Figure 5.2. Diagram showing the 96-well plate plan for positive (Con-A stimulated) and negative (unstimulated) controls for the one-way MLR, two-way MLR and LTA. The numbers above the plate correspond to the cell composition shown in the key. The cells in red were treated with 10 μg.ml^{-1} of mitomycin C for mitotic inactivation. All cells were seeded at 5 x 10^4 per well in triplicates. Shaded wells represented Con-A stimulated cells while clear wells represented unstimulated cells. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.2.5. Incubation, harvesting and analysis

The seeded plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3, 6, 9, 12 and 15 days with medium replaced every 3 days (100 μl replaced with fresh culture medium) as described in **Section 4.2.3**. Sixteen hours before completion of each time point, each well was spiked with 10 μl of 25 μCi.ml^{-1} of low activity ^{3}H-thymidine as described in **Section 2.2.12**, harvested onto glass fibre filter plates and radioactivity counts read using a TopCount NXT scintillation counter as described in **Section 2.2.13**.

5.2.3. Effects of allogeneic and syngeneic DF on one-way and two-way MLR and LTA

This experiment was carried out as described in **Section 5.2.2** with Balb/c and C3H (p5) DF replacing MSC in one-way MLR, two-way MLR and LTA.

5.2.3.1. Preparation of responder Balb/c mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The mononuclear cells (1.65×10^7) were suspended in 16.5 ml of lymphocyte culture medium.

5.2.3.2. Preparation of stimulator C3H mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The cells (1.2×10^7) were suspended in 12 ml of lymphocyte culture medium and divided into two groups. One group of 6×10^6 cells in 6 ml of lymphocyte culture medium was left untreated whilst the remainder of the cells were treated with mitomycin C as described in **Section 2.2.15** and then counted by the Trypan blue method described in **Section 2.2.9** and resuspended at 6×10^6 cells in 6 ml of fresh lymphocyte culture medium.

5.2.3.3. Preparation of Balb/c and C3H DF

Balb/c and C3H DF (p5) were resurrected as described in **Section 2.2.10** and maintained in DMEM-HG culture medium in 125 cm^2 tissue culture flasks until they reached 80-90 % confluence as described in **Section 2.2.5**. The cells were then detached using 0.5 % (v/v) trypsin-EDTA solution as described in **Section 2.2.6**, counted by the Trypan blue method described in **Section 2.2.8** and then resuspended at 9×10^6 cells in 9 ml of lymphocyte culture medium respectively. In separate test-tubes, the cells were divided into untreated and mitomycin C treated groups. For the untreated groups, 3×10^6 cells were resuspended in 3 ml of lymphocyte culture medium. Cells from the treated group were treated with mitomycin C as described in **Section 2.2.15**. The cells were counted by the Trypan blue method described in **Section 2.2.9** and resuspended at 6×10^6 cells in 6 ml of fresh lymphocyte culture medium.

5.2.3.4. Cell plating

The experiments were set up in uncoated U-bottom 96-well plates as described in **Section 5.2.2.4**. The experiments (one-way MLR, two-way MLR and LTA) were set up at the same time in replicates of six as shown in **Figure 5.3**. The controls were set

up in triplicates on separate plates shown in **Figure 5.4**. Cell numbers and seeding volumes used for plating in **Section 5.2.2.4** were used in this experiment.

5.2.3.4.1. One-way MLR

To investigate the effects of DF on one-way MLR, mitomycin C treated Balb/c and C3H DF were co-cultured with one-way MLR as described in **Section 5.2.2.4.1**.

5.2.3.4.2. Two-way MLR

To investigate the effects of DF on two-way MLR, mitomycin C treated Balb/c and C3H DF were co-cultured with two-way MLR as described in **Section 5.2.2.4.2**.

5.2.3.4.3. LTA

To investigate the immunogenicity of DF, responder Balb/c mononuclear cells (5 x 10^4 cells in 50 µl per well) were co-cultured with mitomycin C treated or untreated Balb/c (syngeneic) and C3H (allogeneic) DF (stimulators) as described in **Section 5.2.2.4.3**.

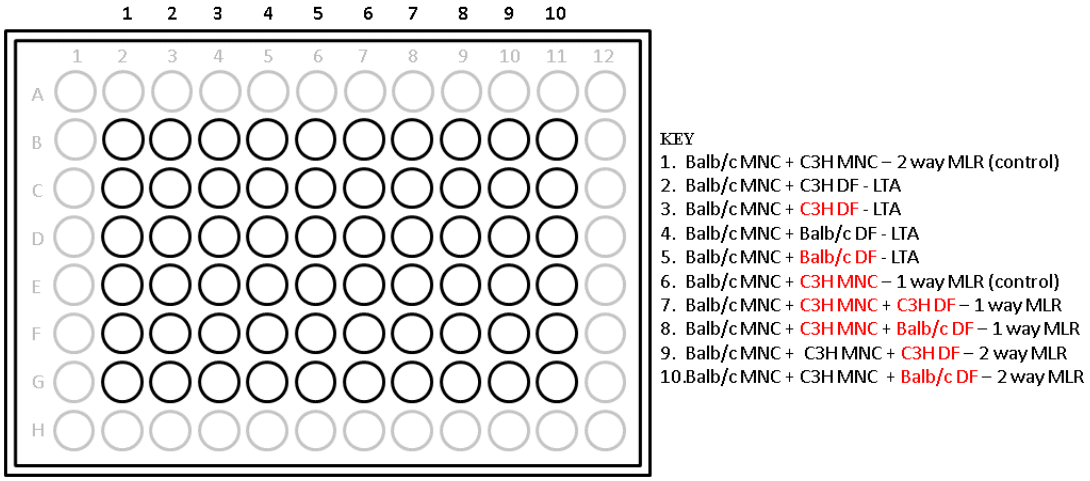

Figure 5.3. Diagram showing the 96-well plate plan for the investigation of the effects of allogeneic and syngeneic DF on one-way MLR, two-way-MLR and LTA as measured by ^{3}H-thymidine uptake. The numbers above the plate correspond to the cell composition shown in the key. The cells in red were treated with 10 µg.ml^{-1} of mitomycin C for mitotic inactivation. All cells were seeded at 5 x 10^4 per well at 1:1:1 (MLR) and 1:1 (LTA) ratios in replicates of 6. Samples 6, 7 (allogeneic), 8 (syngeneic) represented the one-way MLR, samples 1, 9, 10 represented the two-way MLR and samples 2, 3 (allogeneic), 4, 5 (syngeneic) represented the LTA. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.3.4.4. Controls

All the controls were set up by seeding 5 x 10^4 cells in 50 µl per well as described in **Section 5.2.2.4.4**. A further 150 µl of lymphocyte culture medium was added in each well to give a total culture volume of 200 µl. Cells were either left unstimulated or stimulated by 10 µg.ml^{-1} Con-A in triplicates.

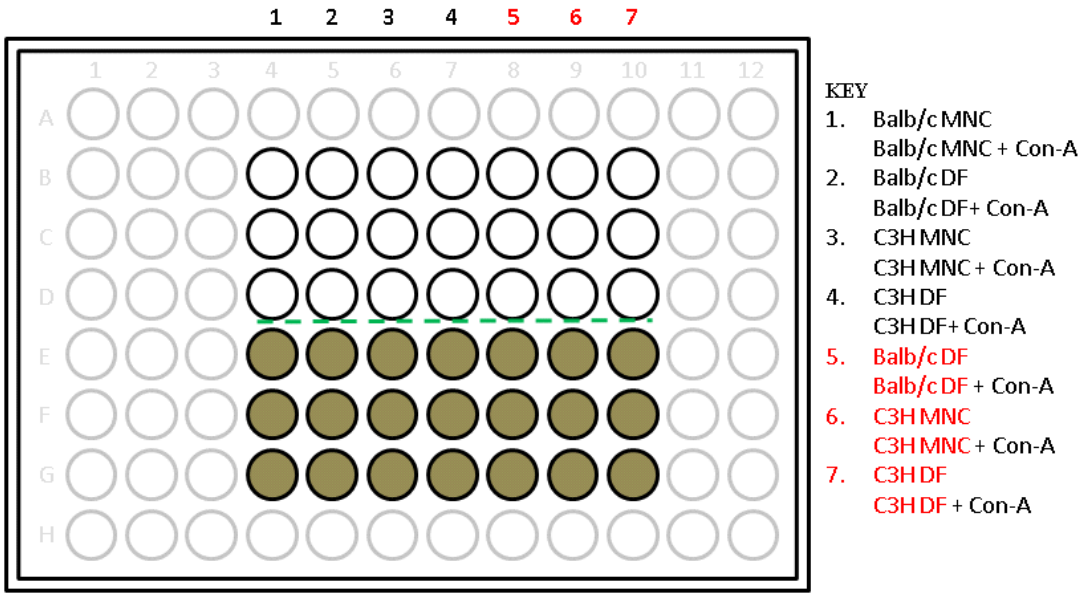

Figure 5.4. Diagram showing the 96-well plate plan for positive (Con-A stimulated) and negative (unstimulated) controls for the one-way MLR, two-way MLR and LTA. The numbers above the plate correspond to the cell composition shown in the key. The cells in red were treated with 10 µg.ml^{-1} of mitomycin C for mitotic inactivation. All cells were seeded at 5 x 10^4 per well in triplicates. Shaded wells represented Con-A stimulated cells while clear wells represented unstimulated cells. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.3.5. Incubation, harvesting and analysis

The cells were incubated, harvested and analysed as described in **Section 5.2.2.5**.

5.2.4. Effect of increasing stimulator cell numbers on the immunogenicity of allogeneic MSC and DF

This experiment was carried out to investigate the effects of increasing stimulator cell numbers (allogeneic and syngeneic MSC and DF) in LTA with Balb/c mononuclear cells as responders.

5.2.4.1. Preparation of Balb/c responder mononuclear cells

Balb/c mononuclear cells were isolated from spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The mononuclear cells (2.55×10^7) were suspended in 25.5 ml of lymphocyte culture medium.

5.2.4.2. Preparation of C3H stimulator mononuclear cells

C3H mononuclear cells were used in the one-way MLR which was a control for alloantigen stimulation. Mononuclear cells were isolated and treated with 10 $\mu g.ml^{-1}$ of mitomycin C to mitotically arrest them as described in **Section 2.2.15**. After washing, the cells were counted and 1.2×10^7 cells were resuspended in 7.2 ml of lymphocyte culture medium.

5.2.4.3. Preparation of stimulator Balb/c and C3H MSC and DF

Balb/c and C3H MSC and DF (p5) were separately detached from tissue culture flasks and treated with 10 $\mu g.ml^{-1}$ of mitomycin C as described in **Section 2.2.15** and **Section 5.2.3.3** respectively The cells were counted by the Trypan blue method described in **Section 2.2.8** and then resuspended at 1.2×10^7 cells in 7.2 ml of lymphocyte culture medium in separate tubes.

5.2.4.4. Cell plating

The experiment was set up in uncoated U-bottom 96-well plates. Two plates were used for each time point. One plate was for the LTA at responder to stimulator ratio of 1:1 in replicates of six and the other plate was for LTA involving responder to stimulator cell ratios of 1:2, 1:3, 1:4 and 1:5 in triplicates.

5.2.4.4.1. Control plate

Responder Balb/c mononuclear cells seeded at 5×10^4 cells in 50 µl per well were co-cultured with 5×10^4 cells in 30 µl of either Balb/c MSC, Balb/c DF, C3H mononuclear cells, C3H MSC or C3H DF giving a responder to stimulator ratio of 1:1 as shown in **Figure 5.5**. Each well was made up to 200 µl by adding 120 µl of lymphocyte culture medium. Con-A stimulated (10 $\mu g.ml^{-1}$) Balb/c mononuclear cells and the one-way MLR were controls for mitogenic and alloantigen stimulation respectively.

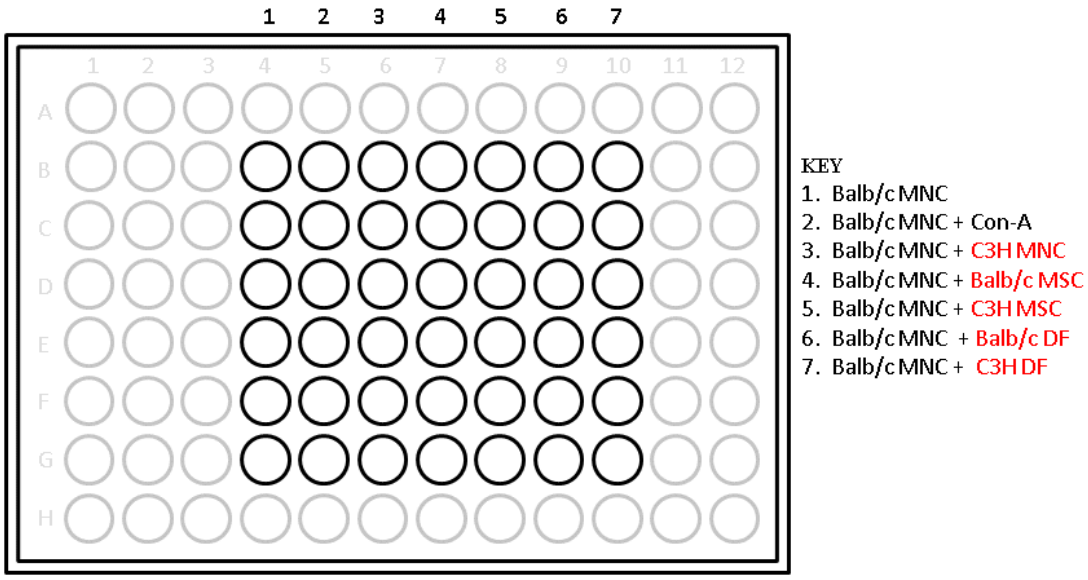

Figure 5.5. Diagram showing the 96-well plate plan for LTA for Balb/c mononuclear cells stimulated by allogeneic and syngeneic MSC and DF. Balb/c mononuclear cells (responders) were seeded at 5 x 10^4 cells per well were co-cultured with an equal number of mitotically inactivated C3H (allogeneic) MSC or DF and Balb/c (syngeneic) MSC or DF in replicates of six. The numbers above the plate correspond to the cell composition shown in the key. Balb/c cells only (1) were a control for unstimulated cells while Con-A stimulated Balb/c cells (2) were a control for proliferating cells. The cells in red were treated with 10 $\mu g.ml^{-1}$ of mitomycin C for mitotic inactivation. The cells were cultured for 3, 6, 9, 12 and 15 days and cell proliferation was determined by the ^{3}H-thymidine uptake assay. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.4.4.2. LTA with increasing stimulator cell ratio

Balb/c mononuclear cells were seeded at 5 x 10^4 cells in 50 µl per well and co-cultured with increasing numbers of stimulator cells as shown in **Figure 5.6**. Stimulator cells were seeded at 1 x 10^5 cells in 60 µl, 1.5 x 10^5 cells in 90 µl, 2 x 10^5 cells in 120 µl and 2.5 x 10^5 cells in 150 µl to give final responder to stimulator cell ratios of 1:2, 1:3, 1:4 and 1:5 respectively. The wells were made up to a final volume of 200 µl by adding lymphocyte culture medium.

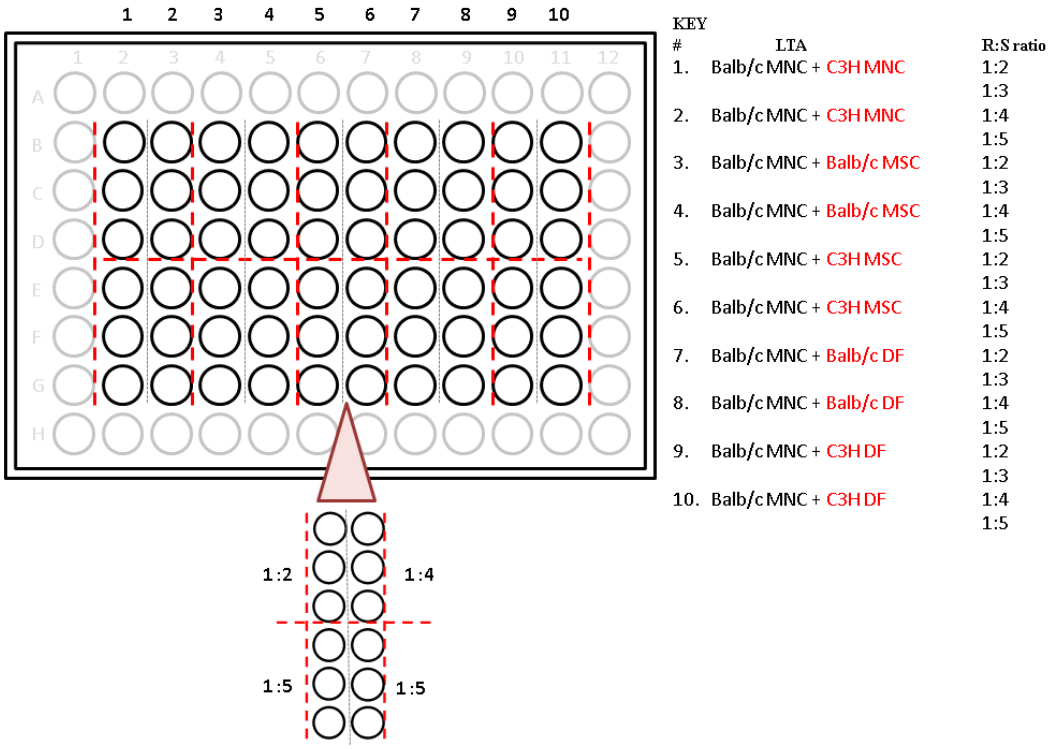

Figure 5.6. Diagram showing the 96-well plate plan for LTA for Balb/c mononuclear cells stimulated by increasing numbers of allogeneic and syngeneic MSC and DF stimulator cells. Balb/c mononuclear cells (responder) were seeded at 5 x 10^4 cells per well were co-cultured with increasing numbers of mitotically inactivated C3H (allogeneic) MSC or DF and Balb/c (syngeneic) MSC or DF to give responder to stimulator cell ratios of 1:2, 1:3, 1:4 and 1:5 in triplicate wells. The numbers above the plate correspond to the cell composition shown in the key. Samples 1 and 2 were one-way MLR which were controls for allogeneic stimulation of responder cells. The cells in red were treated with 10 µg.ml^{-1} of mitomycin C for mitotic inactivation. The cells were cultured for 3, 6, 9, 12 and 15 days and cell proliferation was determined by the ^{3}H-thymidine uptake assay. Outer wells (grey) were filled with PBS to minimise evaporation.

5.2.4.5. Incubation, harvesting and analysis

The seeded plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3, 6, 9, 12 and 15 days with medium replacement, harvested and analysed as described in **Section 5.2.2.5**.

5.2.5. Comparison between U-bottom and V-bottom plates on one-way MLR and LTA using allogeneic and syngeneic MSC and DF

This experiment was carried out to compare between U-bottom and V-bottom plates in order to determine whether well shape influenced the extent of one-way MLR and LTA involving syngeneic and allogeneic MSC and DF.

5.2.5.1. Preparation of Balb/c responder mononuclear cells

Balb/c mononuclear cells were isolated from spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The mononuclear cells (3.3×10^7) were suspended in 33 ml of lymphocyte culture medium.

5.2.5.2. Preparation of stimulator cells

Balb/c and C3H MSC and DF and C3H mononuclear cells were treated with 10 $\mu g.ml^{-1}$ of mitomycin C to mitotically arrest them as described in **Section 2.2.15**. After washing, the cells were counted and resuspended in lymphocyte culture medium at 1.5×10^7 cells in 15 ml respectively.

5.2.5.3. Cell plating

The experiment was set up in uncoated U-bottom and V-bottom 96-well plates. Two plates were used for each time point. Responder and stimulator cells were seeded at 1:1 ratio for LTA and 1:1:1 for one-way MLR in replicates of six. The LTA and one-way MLR were set up on the same plate as shown in **Figure 5.7**.

5.2.5.3.1. One-way MLR

One-way MLR involving mitomycin C treated syngeneic and allogeneic MSC or DF were set up by co-culturing 5×10^4 cells in 50 µl each of Balb/c and mitomycin C treated C3H mononuclear cells with both allogeneic and syngeneic MSC or DF. The total culture volume in each well was made up to 200 µl as described in **Section 5.2.2.4.1**. The one-way MLR without MSC or DF was set up as a control for allogeneic stimulation of responder cells.

5.2.5.3.2. LTA

Balb/c responder mononuclear cells were co-cultured with mitomycin C treated syngeneic and allogeneic stimulator MSC or DF by mixing 50 µl of each containing 5×10^4 cells per well as described in **Section 5.2.2.4.3**.

5.2.5.3.3. Controls

Balb/c mononuclear cells (5×10^4 cells per well) cultured alone or stimulated with 10 $\mu g.ml^{-1}$ Con-A were used as controls for non-proliferating and proliferating

responder cells respectively as described in **Section 5.2.2.4.4**. The one-way MLR was used as a control for antigenic stimulation for responder cells.

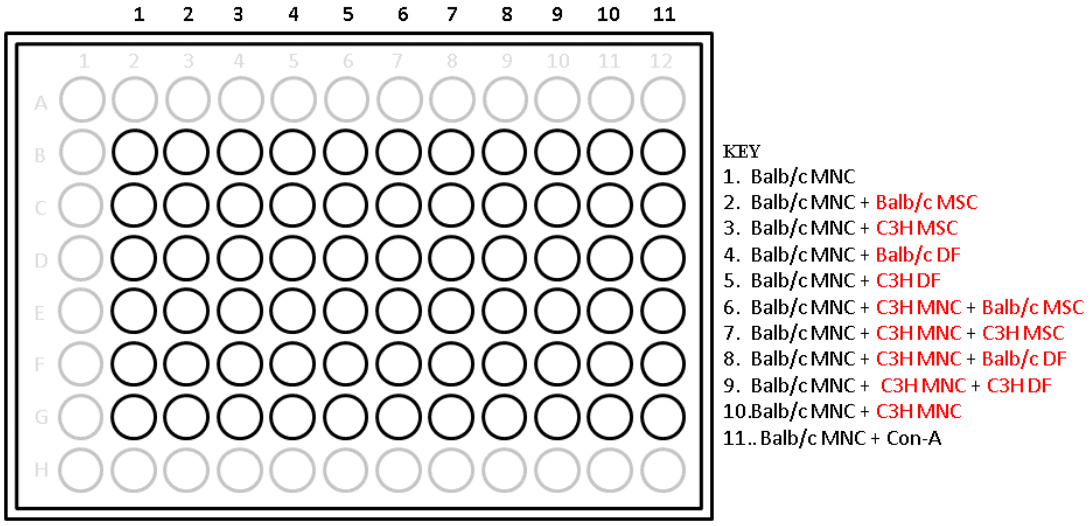

Figure 5.7. Diagram showing the 96-well plate plan for the investigation of the effects of allogeneic and syngeneic MSC and DF on one-way MLR and LTA cultured in U-bottom and V-bottom uncoated plates. Balb/c mononuclear cells (responders) were seeded at 5 x 10^4 cells per well and co-cultured with equal cell numbers of mitotically inactivated allogeneic C3H mononuclear cells, MSC or DF and syngeneic Balb/c MSC or DF to give responder to stimulator cell ratios of 1:1:1 (one-way MLR) and 1:1 (LTA) in replicates of six. The numbers above the plate correspond to the cell composition shown in the key. The cells in red were mitotically inactivated. Balb/c mononuclear cells only (1) were a control for unstimulated responder cells while allogeneic mononuclear cells (10) and mitogen (11) stimulated cells acted as controls for proliferating responder cells. Grey wells were filled with PBS to minimise evaporation.

5.2.5.4. Incubation, harvesting and analysis

The seeded plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3, 6, 9, 12 and 15 days. The cells were then harvested and analysed as described in **Section 5.2.2.5**.

5.2.6. Effect of Balb/c serum on one-way MLR and LTA involving syngeneic and allogeneic MSC and DF

This experiment was carried out to investigate the effects of Balb/c (syngeneic) serum on one-way MLR and LTA using Balb/c mononuclear cells as responders to syngeneic and allogeneic MSC and DF.

5.2.6.1. Preparation of Balb/c serum

Balb/c blood was obtained by cardiac puncture of mice sacrificed according to Schedule 1 procedures described in **Section 2.2.4** (procedure carried out by Professor Eileen Ingham). The blood was collected in sterile tubes (without anticoagulant) and left to stand for one hour to allow clot formation. The clot was carefully removed and the uncoagulated fraction was centrifuged at 1500 *g* at 20 ºC for 10 minutes. The serum was collected as the supernatant and immediately heat treated to inactivate complement by holding the serum in a water-bath at 56 ºC for one hour with gentle swirling every 5 minutes to ensure the serum was evenly heated. The serum was used to supplement lymphocyte culture medium at a concentration of 2 % (v/v).

5.2.6.2. Preparation of Balb/c responder mononuclear cells

Balb/c mononuclear cells were isolated from spleens and lymph nodes as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The mononuclear cells (1.65×10^7) were suspended in 16.5 ml of lymphocyte culture medium supplemented with 2 % (v/v) Balb/c serum.

5.2.6.3. Preparation of stimulator cells

Balb/c and C3H MSC and DF and C3H mononuclear cells were treated with 10 $\mu g.ml^{-1}$ of mitomycin C to mitotically inactivate them as described in **Section 2.2.15**. After washing, the Balb/c and C3H MSC and DF were counted and resuspended in lymphocyte culture medium supplemented with 2 % (v/v) Balb/c serum at 3×10^6 cells in 3 ml respectively. C3H mononuclear cells were resuspended at 7.5×10^6 cells in 7.5 ml of culture medium.

5.2.6.4. Cell plating

The experiment was set up in uncoated U-bottom 96-well plates. Responder and stimulator cells were seeded at 1:1 ratio in LTA and 1:1:1 in one-way MLR in replicates of six as shown in **Figure 5.8**.

5.2.6.4.1. One-way MLR

One-way MLR involving mitomycin C treated syngeneic or allogeneic MSC or DF were set up according to the plan described in **Section 5.2.5.3.1**.

5.2.6.4.2. LTA

Balb/c responder mononuclear cells were co-cultured with mitomycin C treated syngeneic or allogeneic stimulator MSC and DF by mixing 50 µl of each containing 5 x 10^4 cells per well as described in **Section 5.2.2.4.3**.

5.2.6.4.3. Controls

Balb/c mononuclear cells (5 x 10^4 cells per well) cultured alone or stimulated with 10 µg.m^{l-1} Con-A were used as controls for non-proliferating and proliferating responder cells respectively as described in **Section 5.2.2.4.4**.

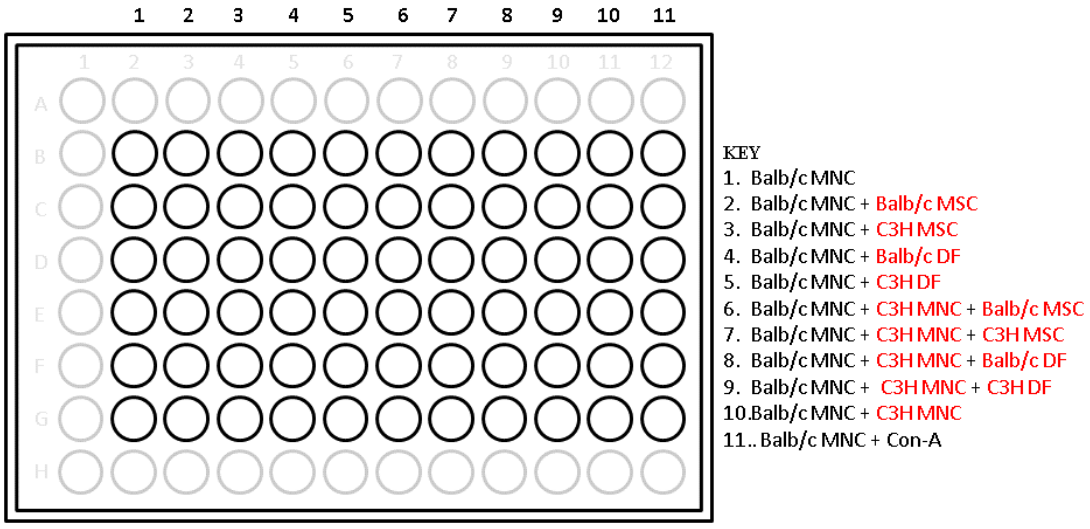

Figure 5.8. Diagram showing the 96-well plate plan for the investigation of the effects of allogeneic and syngeneic MSC and DF on one-way MLR and LTA cultured in medium supplemented with 2 % (v/v) Balb/c serum. Balb/c mononuclear cells (responders) were seeded at 5 x 10^4 cells per well and co-cultured with equal cell numbers of mitotically inactivated C3H mononuclear cells, MSC or DF and Balb/c MSC or DF to give responder to stimulator cell ratios of 1:1:1 (one-way MLR) and 1:1 (LTA) in replicates of six. The numbers above the plate correspond to the cell composition shown in the key. Balb/c mononuclear cells only (1) were a control for unstimulated responder cells while antigen (10) and mitogen (11) stimulated cells acted as controls for mitogen and alloantigen stimulated proliferating responder cells. Grey wells were filled with PBS to minimise evaporation.

5.2.6.5. Incubation, harvesting and analysis

The seeded plates were incubated harvested and analysed as described in **Section 5.2.2.5.**

5.2.7. Chondrogenic differentiation of Balb/c and C3H MSC and DF

Balb/c and C3H MSC (p5) and DF (p5) were resurrected as described in **Section 2.2.10** and maintained in DMEM-LG and DMEM-HG culture medium respectively in 125 cm^2 tissue culture flasks until they reached 80-90 % confluence as described in **Section 2.2.5**. The MSC and DF were then detached using Hy-Q-Tase™ cell dissociation solution and 0.5 % (v/v) trypsin-EDTA solution respectively as described in **Section 2.2.6** after which the cells were washed by centrifuging at 180 *g* for 10 minutes. The resulting pellets for each cell type were resuspended in 10 ml of the appropriate chondrogenic differentiation medium, counted by the Trypan blue method described in **Section 2.2.8** and adjusted to 1 x 10^7 cells in 10 ml of chondrogenic differentiation medium in 50 ml conical tubes. The cells were then centrifuged at 200 *g* for 10 minutes to pellet and the supernatant carefully removed without disturbing the cell pellets. Fresh chondrogenic medium (10 ml per conical tube) was carefully added without disturbing the cell pellets. The tubes were closed loosely to allow aeration and incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air. After each 3 day period, the culture medium was replaced by carefully replacing with fresh chondrogenic differentiation medium for up to 21 days.

5.2.7.1. Preparation of chondrogenic differentiated Balb/c and C3H MSC and DF for the one-way MLR and LTA

After completion of the chondrogenic differentiation time course, culture medium was carefully removed from the tubes and the differentiated MSC and DF cell pellets were washed with PBS to remove remaining medium. In order to dissociate the pellets into individual cells, the pellets were treated with 0.01% (v/v) collagenase (2 ml per pellet) for 15 minutes at 37 °C with regular bashing of tubes against each other until there were no visible clumps of cells. The resulting cell suspensions were mixed with lymphocyte culture medium (10 ml per tube) and centrifuged thrice at 180 *g* for 5 minutes to remove the collagenase. The cells were then mitotically inactivated by suspending them in medium containing 10 $\mu g.ml^{-1}$ of mitomycin C for 30 minutes at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air as described in **Section 2.2.15**. The washed cells were counted by the Trypan blue method described in

Section 2.2.8 and resuspended at 4.5 x 10^6 cells in 4.5 ml of lymphocyte culture medium for each cell type for the one-way MLR and LTA assays. For the Alcian blue staining (**Section 6.2.4)**, 3 x 10^5 cells were resuspended in 30 µl of chondrogenic differentiation culture medium.

5.2.7.1.1. Alcian blue staining

To test whether the MSC and DF had differentiated to chondrocytes, 10 µl containing 1 x 10^5 cells was carefully placed at the centre of each well (3 wells for each cell type) of a 24-well cell culture plate to create micromass nodules as described in **Section 3.2.5.2**.2. The seeded plates were then incubated at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3 hours to allow the cells to attach. Each well was then flooded with 1 ml of chondrogenic differentiation medium and incubated overnight. Afterwards, the medium was removed and the cells were washed twice with PBS and fixed with 10% (v/v) formalin and stained with 1 % (w/v) alcian blue stain as described in **Section 3.2.5.2.2.1**. The cells were viewed using an Olympus BX 71 inverted microscope and images were captured using Cell^B image acquisition software (Olympus).

5.2.7.2. Preparation of responder Balb/c mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes of Balb/c mice as described in **Section 2.2.11** and counted by the Trypan blue method **(Section 2.2.9)**. The cells (1.65 x 10^7) were suspended in 16.5 ml of lymphocyte culture medium.

5.2.7.3. Preparation of stimulator C3H mononuclear cells

Mononuclear cells were isolated from spleens and lymph nodes of C3H mice as described in **Section 2.2.11.** The cells were centrifuged at 450 *g* for 10 minutes and the resulting cell pellet was resuspended in medium containing 10 µg.ml^{-1} of mitomycin C in order to mitotically inactivate them as described in **Section 2.2.15**. The cells were counted by the Trypan blue method described in **Section 2.2.8** and resuspended at 9 x 10^6 cells in 9 ml of fresh lymphocyte culture medium.

5.2.7.4. Cell plating

The one-way MLR and LTA experiments for each time point were set up in the same uncoated V-bottom 96-well plate in replicates of 6 for both chondrogenic differentiated MSC and DF as shown in **Figure 5.9**. Controls were set up in

triplicates as either unstimulated or Con-A stimulated cells except for stimulated Balb/c mononuclear cells which were set up in replicates of 6 as shown in **Figure 5.10**.

5.2.7.4.1. One-way MLR

For this assay, both Balb/c and C3H chondrocyte differentiated MSC and DF were seeded at 5 x 10^4 cells in 50 µl of lymphocyte culture medium per well and incubated overnight for 16 hours at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air to allow the cells to form micro-pellets at the bottom of the wells. Afterwards, 5 x 10^4 cells in 50 µl of each of responder Balb/c mononuclear cells and mitotically inactivated stimulator C3H mononuclear cells were added to the pre-seeded wells. The final responder to stimulator to MSC/DF ratio was 1:1:1 and the total volume in each well made up to 200 µl by adding 50 µl of fresh lymphocyte culture medium.

5.2.7.4.2. LTA

For this assay, the mitotically inactivated chondrogenic differentiated MSC and DF were pre-seeded as described in **Section 5.2.7.1** and incubated overnight. Responder Balb/c mononuclear cells (5 x 10^4 cells in 50 µl per well) were then added to the pre-seeded wells to give a 1:1 responder to stimulator cells ratio. The co-cultures were made up to a final volume of 200 µl by adding a further 100 µl of lymphocyte culture medium.

5.2.7.4.3. Controls

All the controls were set up by seeding 50 µl containing 5 x 10^4 cells of each type per well. Triplicate wells for each cell type (except Balb/c mononuclear cells) were either left unstimulated or stimulated by treatment with 10 µg.ml^{-1} of Con-A. The total culture volume was made up to 200 µl by addition of fresh lymphocyte culture medium. Unstimulated and Con-A stimulated cells served to test whether mitotically inactivated cells were capable of undergoing mitogenic stimulation as a control for the mitotic inactivation procedure. Balb/c mononuclear cells stimulated with Con-A in replicates of 6 were used as a control for lymphocyte proliferation.

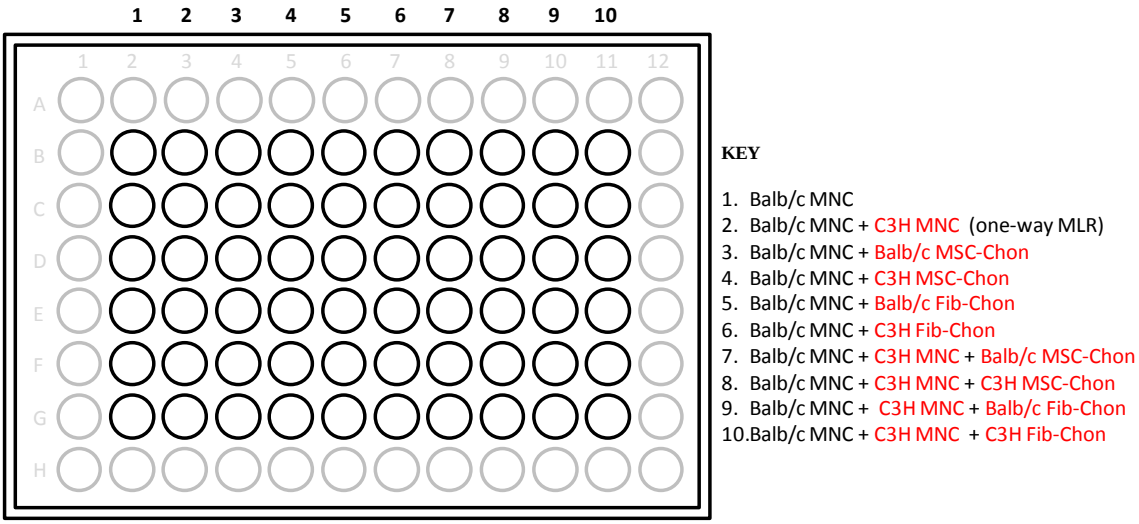

Figure 5.9. Diagram showing the 96-well plate plan for investigating the effects of allogeneic and syngeneic chondrogenic differentiated MSC and DF on one-way MLR and LTA as measured by ^{3}H-thymidine uptake. The numbers above the plate correspond to the cell composition shown in the key. Cells in red were mitotically inactivated using mitomycin C treatment (10 μg.ml^{-1}). All cells were seeded at 5 x 10^{4} per well at 1:1:1 and 1:1 ratio for the one-way MLR and LTA respectively in replicates of 6. Samples 2, 7, 8, 9 and 10 represented the one-way MLR with MSC or DF while samples 3, 4, 5 and 6 represented the LTA. The outer grey wells were filled with PBS to minimise evaporation.

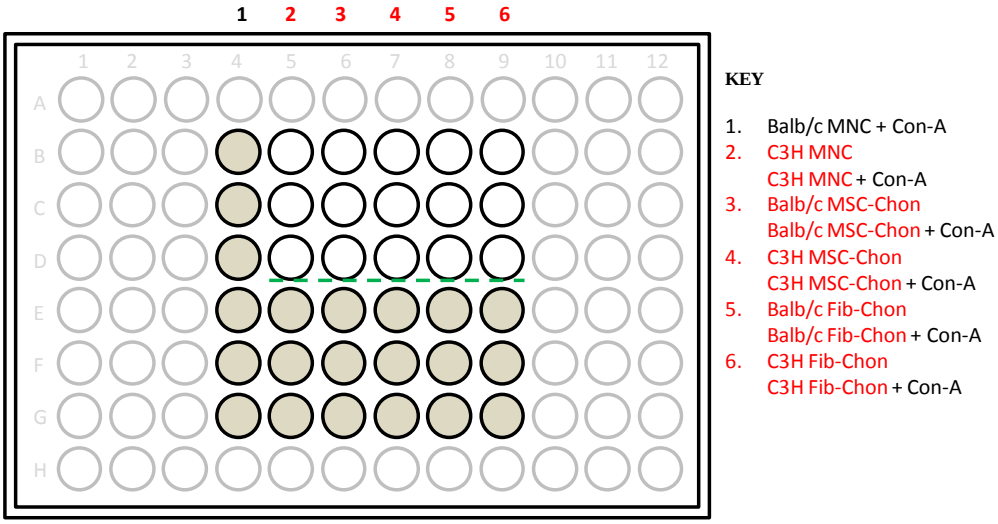

Figure 5.10. Diagram showing the 96-well plate plan for positive (Con-A stimulated) and negative (unstimulated) controls for the one-way MLR and LTA. The numbers above the plate correspond to the cell composition shown in the key. Cells in red were mitotically inactivated using mitomycin C treatment (10 μg.ml^{-1}). Cells were seeded at 5 x 10^{4} per well in triplicates (except for Balb/c mononuclear cells which were in replicates of 6). Shaded wells represented Con-A stimulated cells while clear wells were for unstimulated cells. The outer grey wells were filled with PBS to minimise evaporation.

5.2.7.5. Incubation, harvesting and analysis

The seeded plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5 % (v/v) CO_2 in air for 3, 6, 9, 12 and 15 days with medium replacement and then harvested and analysed as described in **Section 5.2.2.5**.

5.3. Results

5.3.1. Effects of allogeneic and syngeneic undifferentiated MSC on one-way and two-way MLR and LTA

The immunomodulatory properties of both Balb/c (syngeneic) and C3H (allogeneic) undifferentiated MSC were investigated using adaptations of the MLR (one and two-way) and LTA. Mitotically inactivated MSC were added to co-cultures of Balb/c (responder) and mitotically inactivated C3H (stimulator) mononuclear cells in one-way MLR to determine whether syngeneic (Balb/c) and allogeneic (C3H) MSC had any effect on the proliferative response of the responder lymphocytes. In the two-way MLR, mitotically inactivated MSC were added to co-cultures of Balb/c and C3H mononuclear cells to determine whether they had any effect on the proliferative response of mixed lymphocytes. LTA were carried out by co-culturing Balb/c mononuclear cells with mitotically inactivated Balb/c or C3H MSC to test their capacity to stimulate responding lymphocytes (immunogenicity).

With regard to the control cultures for these experiments, monocultures of mitomycin C treated C3H mononuclear cells failed to take up ^{3}H-thymidine with or without stimulation using Con-A during the 15 day culture period. This non-proliferation after stimulation indicated mitotic inactivation of the stimulator cells. Untreated Balb/c and C3H mononuclear cells were significantly stimulated by Con-A with peak counts and SI at day 6 (**Figure 5.11A-B**).

For the one-way MLR, the results for the control experiment showed that responder Balb/c mononuclear cells were notably stimulated by C3H stimulator mononuclear cells. ^{3}H-thymidine uptake peaked at day 6 with a SI of 40. In one-way MLR co-cultured with either Balb/c (syngeneic) or C3H (allogeneic) MSC, ^{3}H-thymidine uptake and SI were significantly ($p<0.05$) suppressed at day 6 onwards (**Figure 5.11C-D**). Suppression of the Balb/c lymphocyte response at day 6 was approximately 90 % for both syngeneic and allogeneic MSC. With regard to the two-way MLR, both Balb/c and C3H MSC significantly suppressed lymphocyte proliferation in a similar trend to that observed for the one-way MLR (**Figure 5.11E-F**). Day 6 SI showed that lymphocyte proliferation in the two-way MLR was reduced by 89 % and 75 % by Balb/c and C3H MSC respectively suggesting that Balb/c MSC exhibited marginally higher immunosuppressive potency compared to C3H MSC.

In LTA, Balb/c and C3H MSC which had and had not been mitotically inactivated were used as stimulators in co-cultures with Balb/c mononuclear cells. The results showed that C3H MSC (allogeneic), but not Balb/c MSC (syngeneic) stimulated Balb/c mononuclear cells (**Figure 5.11G-H**). Stimulation of the Balb/c mononuclear cells with allogeneic MSC (mitotically inactivated and not) was detected at all the time points (SI > 3) except at day 15 (SI < 3). ^{3}H-thymidine uptake by the Balb/c responder cells peaked at day 6 and gradually decreased at subsequent time points. In contrast, co-cultures of Balb/c mononuclear MSC with syngeneic MSC produced counts comparable to those of Balb/c mononuclear cells cultured alone.

When taken together, these results showed that both C3H (allogeneic) and Balb/c (syngeneic) MSC suppressed one-way MLR. Similar suppression was also shown in two-way MLR and demonstrated that mouse MSC, regardless of MHC differences, suppressed lymphocyte proliferative responses. In LTA, C3H MSC, but not Balb/c MSC, stimulated Balb/c mononuclear cells. Thus, allogeneic, and not syngeneic MSC were found to be immunogenic.

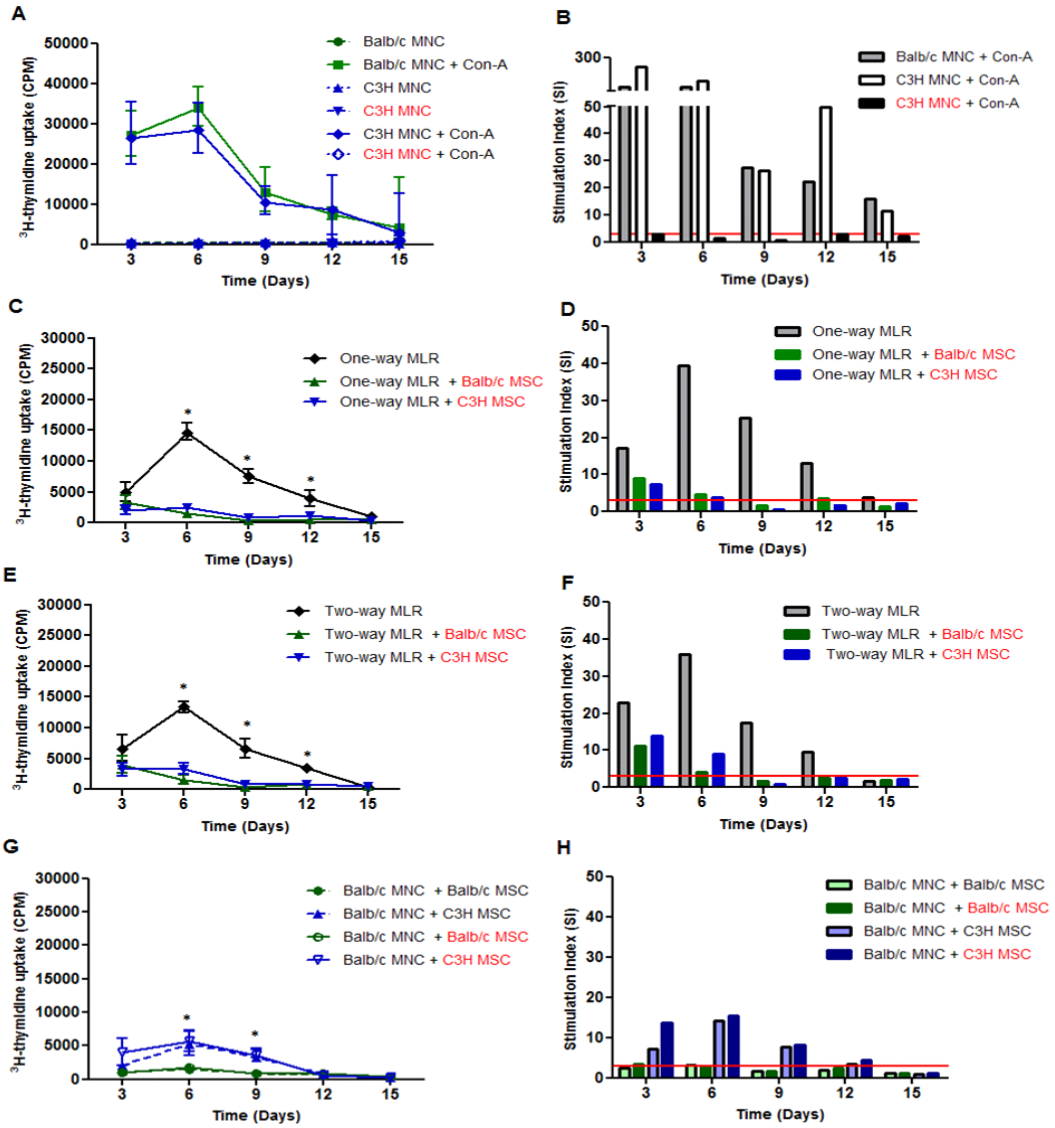

Figure 5.11. Immunomodulation and immunogenicity of C3H (allogeneic) and Balb/c (syngeneic) MSC in one-way and two-way MLR and LTA. Mitotically inactivated Balb/c and C3H MSC (5 x 10^4 cells per well) were co-cultured in one-way MLR at a 1:1:1 ratio. For the two-way MLR, Balb/c and C3H mononuclear cells were cultured with mitotically inactivated Balb/c or C3H MSC at a 1:1:1 ratio. For the LTA, Balb/c mononuclear cells were co-cultured with syngeneic or allogeneic MSC which had and had not been mitotically inactivated at a 1:1 ratio. Balb/c mononuclear cells cultured alone or stimulated with Con-A were controls for non-proliferating and proliferating cells respectively. Culture medium was changed every 3 days by replacing 100 µl with an equal volume of fresh medium and the cells were cultured for 3, 6, 9, 12 and 15 days and lymphocyte proliferation measured by the incorporation of ^{3}H-thymidine. Data (n=6) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p < 0.05$) by the T method to determine individual differences between group means at each time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL and SI for the controls (A and B), one-way MLR (C and D), two-way MLR (E and F) and LTA (G and H) respectively. Mitotically inactivated cells are highlighted in red. The red line at SI=3 denotes SBP; SI > 3 were considered significant. * indicates significant differences in ^{3}H-thymidine uptake; (C) one-way MLR > one-way MLR + C3H / Balb/c MSC; (E) two-way MLR > two-way MLR + C3H / Balb/c MSC; (G) Balb/c MNC + C3H MSC > Balb/c MNC + Balb/c MSC at each time point. [MNC; mononuclear cells]

5.3.2. Effects of allogeneic and syngeneic undifferentiated DF on one and two-way MLR and LTA

Allogeneic (C3H) and syngeneic (Balb/c) DF were also tested for their capacity to modulate one-way and two-way MLR and for their capacity to stimulate lymphocyte proliferation in LTA. The experiments were set up as previously described in **Section 5.3.1** with DF replacing MSC. As previously mentioned, DF were chosen for comparison with MSC because of their mesenchymal origins and provided a test to determine whether the immunomodulatory properties demonstrated by MSC in the previous section were a unique property of MSC or a feature of other cells of the mesenchymal lineage.

With regard to the controls for cell proliferation, monocultures of Balb/c and C3H mononuclear cells stimulated with Con-A showed significant ^{3}H-thymidine uptake in contrast to unstimulated Balb/c and C3H mononuclear cells, which were controls for non-proliferating cells. Highest counts and SI were obtained at day 6 (**Figure 5.12A-B**). Unstimulated and Con-A stimulated mitomycin C treated C3H mononuclear cells produced low counts comparable to those of the unstimulated cells which showed that they were incapable of proliferation.

In the one-way MLR control, Balb/c mononuclear cells were stimulated by mitotically inactivated C3H mononuclear cells with ^{3}H-thymidine uptake and SI peaking at day 6. When Balb/c (syngeneic) or C3H (allogeneic) DF were co-cultured with the one-way MLR, ^{3}H-thymidine uptake was suppressed significantly ($p<0.05$) at days 3, 6 and 9 (**Figure 5.12C-D**). Day 6 control SI was reduced from 28 in the control culture to 7 in the co-cultures with either Balb/c or C3H DF, which represented a 75 % suppression of the Balb/c lymphocyte allogeneic response. A similar trend was observed in the two-way MLR in which approximately 80 % of the control response was suppressed by both Balb/c and C3H (**Figure 5.12E-F**).

In the LTA in which Balb/c responder mononuclear cells were co-cultured with mitomycin C treated or untreated Balb/c and C3H DF, ^{3}H-thymidine uptake was low and not significantly different to that for Balb/c negative control (unstimulated) mononuclear cells. This indicated that both Balb/c and C3H DF failed to stimulate Balb/c mononuclear cells with SI below 3 at all the time points (**Figure 12G-H**).

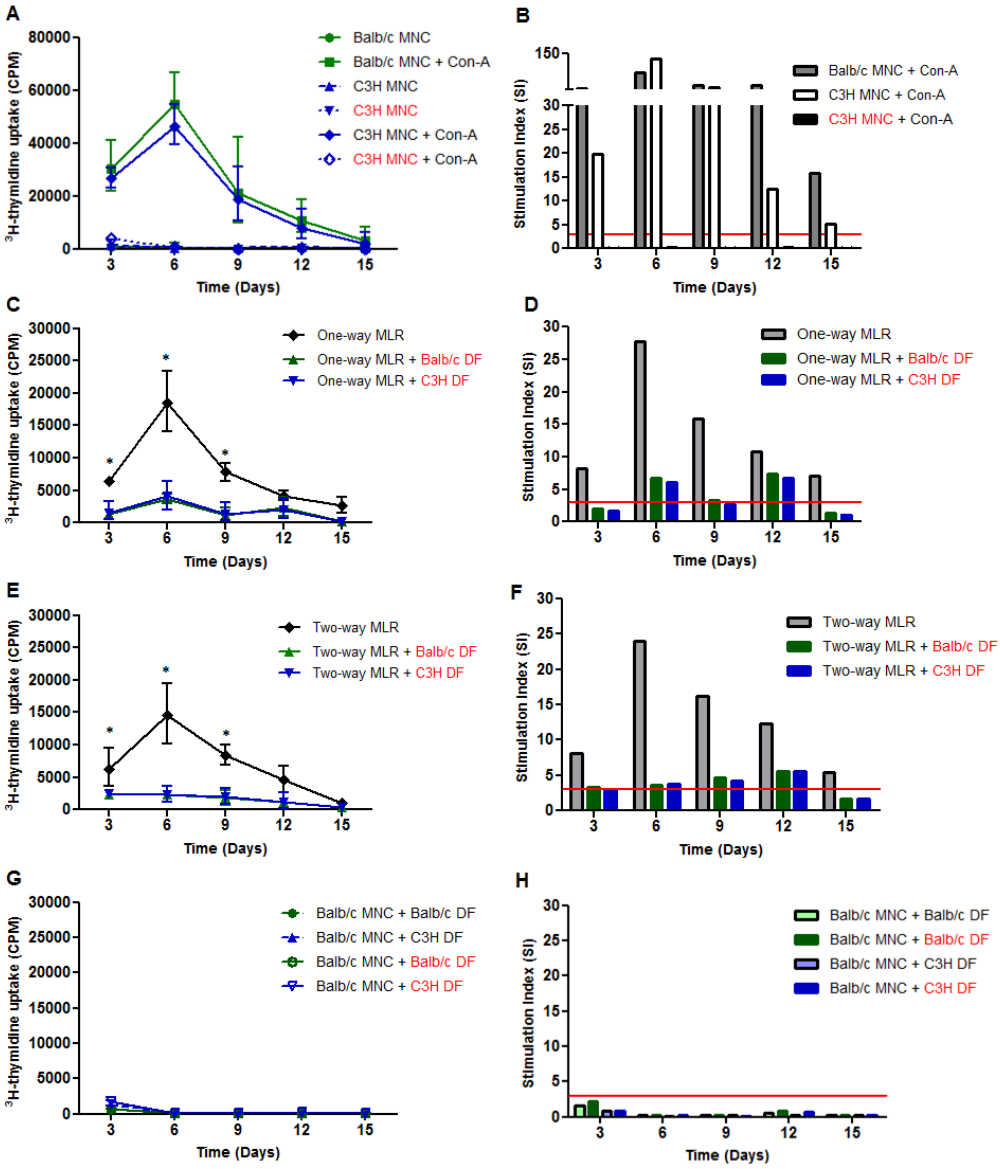

Figure 5.12. Immunomodulation and immunogenicity of C3H (allogeneic) and Balb/c (syngeneic) DF in one-way and two-way MLR and LTA. Mitotically inactivated Balb/c and C3H DF (5 x 10^4 cells per well) were co-cultured in one-way MLR at a 1:1:1 ratio. For the two-way MLR, Balb/c and C3H mononuclear cells were cultured with mitotically inactivated Balb/c or C3H DF at a 1:1:1 ratio. For the LTA, Balb/c mononuclear cells were co-cultured with syngeneic or allogeneic DF which had and had not been mitotically inactivated at a 1:1 ratio. Balb/c mononuclear cells cultured alone or stimulated with Con-A were controls for non-proliferating and proliferating cells respectively. Culture medium was changed every 3 days by replacing 100 µl with an equal volume of fresh medium and the cells were cultured for 3, 6, 9, 12 and 15 days and lymphocyte proliferation measured by the incorporation of ^{3}H-thymidine. Data (n=6) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p < 0.05$) by the T method to determine individual differences between group means at each time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL and SI for the controls (A and B), one-way MLR (C and D), two-way MLR (E and F) and LTA (G and H) respectively. Mitotically inactivated cells are highlighted in red. The red line at SI=3 denotes SBP; SI > 3 were considered significant allogeneic responses. * indicates significant differences in ^{3}H-thymidine uptake; (C) one-way MLR > one-way MLR + C3H / Balb/c DF; (E) two-way MLR > two-way MLR + C3H / Balb/c DF at each time point. [MNC; mononuclear cells]

5.3.3. Effects of increasing stimulator cell ratio on the immunogenicity of allogeneic undifferentiated MSC and DF

The capacity of both allogeneic and syngeneic MSC and DF to suppress one-way and two-way MLR was demonstrated in **Section 5.3.1** and **Section 5.3.2** respectively. However, allogeneic MSC but not DF stimulated Balb/c mononuclear cells. The reactions used equal numbers of responders and stimulators (5 x 10^4 cells at 1:1 ratio). It was thought that increasing the density of stimulator cells in the LTA could potentially increase the stimulatory capacity of the stimulator cells by having more alloantigens available for interaction with responder cells. This was important to investigate further the lymphocyte stimulatory effect observed with allogeneic MSC.

LTA co-cultures comprising Balb/c responder mononuclear cells and mitotically inactivated allogeneic or syngeneic MSC and DF were set up with responder to stimulator cell ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. One-way MLR of Balb/c responder mononuclear cells co-cultured with mitotically inactivated C3H stimulator mononuclear cells were used as controls for stimulated cells. Balb/c mononuclear cells cultured alone were used as controls for unstimulated non-proliferating cells.

With regard to the one-way MLR control, lymphocyte proliferation peaked at day 6 as was seen in previous experiments. Highest counts and SI were obtained with the 1:1 ratio followed by 1:2 in the order of increasing stimulator cell ratio with the 1:5 ratio giving the lowest counts at any given time point (**Figure 5.13A-B).** The differences in counts among the different cell ratios at a given time point were however not significant. SI less than 3 were obtained with the 1:5 ratio at day 12 and at all the ratios (except the 1:1 and 1:2) at day 15. The data showed that the 1:1 ratio was the most appropriate for the one-way MLR and increasing the stimulator cell ratio reduced ^{3}H-thymidine uptake and consequently the SI.

In the LTA, co-cultures of responder Balb/c mononuclear cells with stimulator syngeneic MSC produced counts that were not significantly different from those of the control Balb/c mononuclear cell monocultures at all the time points. Also, the SI at all the time points were less than 3 (**Figure 5.13C-D**). This trend was also observed in LTA involving Balb/c mononuclear cells with syngeneic DF (**Figure 513G-H**) and allogeneic DF (**Figure 5.13I-J**). Co-cultures of Balb/c mononuclear cells with stimulator C3H MSC (allogeneic) however, produced counts that were

significantly different from control Balb/c mononuclear cells cultured alone at day 6 (all ratios), day 9 (all ratios except 1:5) and day 12 (all ratios with the exception of 1:4 and 1:5). The SI at these ratios and time points were above 3 (**Figure 5.13E-F**). The overall trend was that increasing the C3H MSC stimulator cell ratio above the 1:1 ratio was accompanied by a reduction in ^{3}H-thymidine uptake by the responder Balb/c mononuclear cells.

Taken together, the results so far have showed that syngeneic undifferentiated Balb/c MSC and DF and allogeneic C3H DF failed to stimulate Balb/c mononuclear cells at any of the ratios used for the 15 day culture period. Only C3H MSC stimulated Balb/c mononuclear cells. Increasing the C3H MSC cell ratio relative to the responder Balb/c mononuclear cells (above 1:1) however, did not result in increased stimulation.

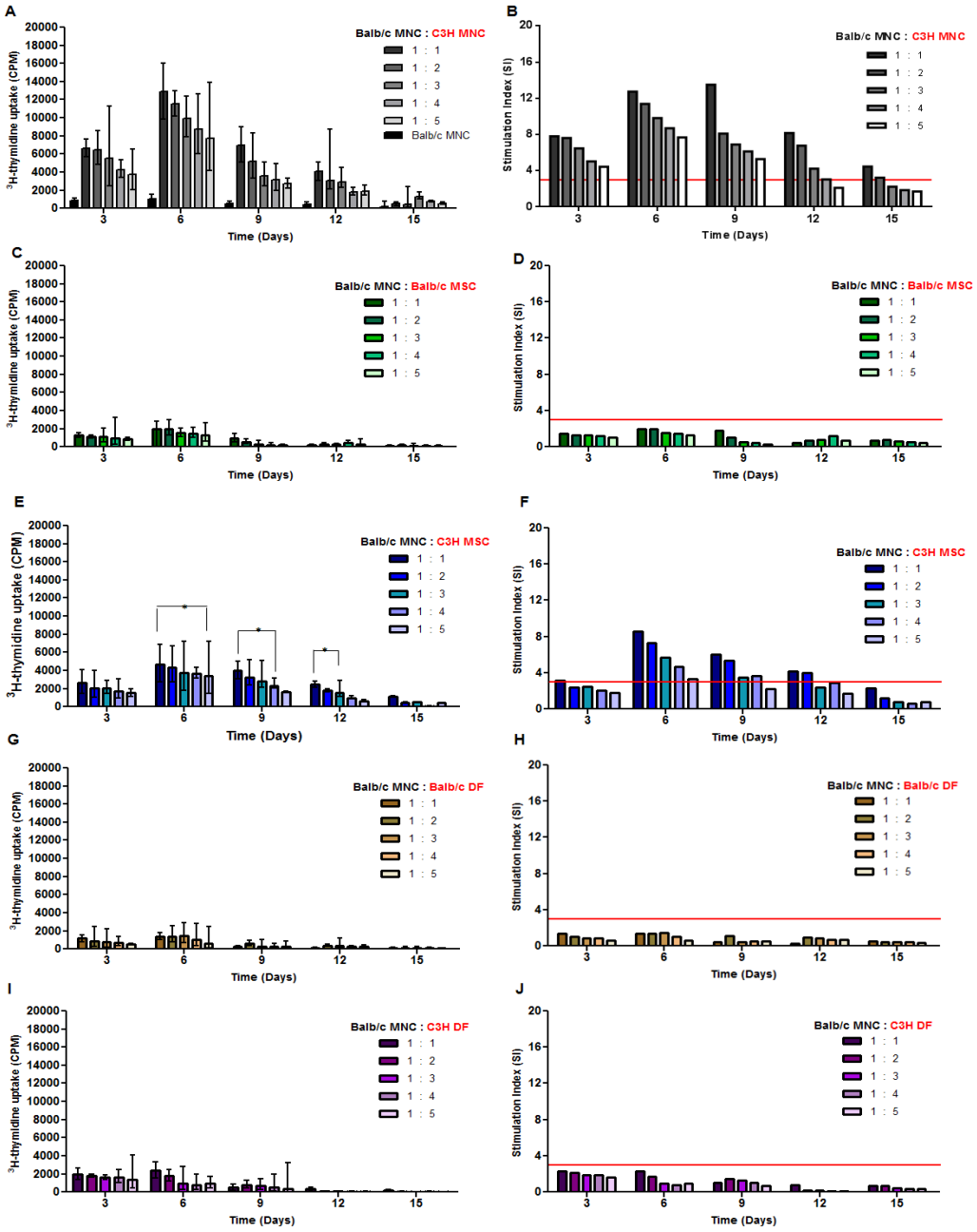

Figure 5.13. Effects of increasing stimulator cell ratios on responder Balb/c lymphocyte transformation by syngeneic and allogeneic MSC and DF. Balb/c mononuclear cells [5 x 10^4 cells per well] were co-cultured with increasing numbers of mitotically inactivated Balb/c and C3H MSC or DF stimulator cells to give responder to stimulator cell ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. Co-cultures of Balb/c and mitotically inactivated C3H MNC (one-way MLR) and Balb/c MNC alone were used as controls for proliferating and non-proliferating cells respectively. Data (n=3) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p < 0.05$) by the T method to determine individual differences between means of different cell ratios. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL and SI for the one-way MLR control (A and B) and co-cultures of Balb/c MNC with Balb/c MSC (C and D), C3H MSC (E and F), Balb/c DF (G and H) and C3H DF (I and J) respectively. * indicates significant differences in ^{3}H-thymidine uptake between LTA involving Balb/c MNC and C3H MSC and control monocultures of Balb/c MNC at a given time point. The red line at SI=3 denotes SBP; SI > 3 were considered significant allogeneic responses. [MNC; mononuclear cells]

5.3.4. Comparison between U-bottom and V-bottom plates on the one-way MLR and LTA using undifferentiated allogeneic and syngeneic MSC and DF

The type and surface coating of cell culture plates used in previous studies in the literature varied from flat bottom, U-bottom and V-bottom coated and uncoated plates. Surface coated (Nunc™ Δ) U-bottom plates were used initially in this study but were discontinued due to cells sticking to the bottom which affected cell harvesting. To address this problem, uncoated U-bottom plates were used for all the MLR and LTA reported thus far. V-bottom plates are thought to provide a narrow and closely packed environment which provides close cell to cell contact which could favour lymphocyte responses. In this experiment, uncoated V-bottom plates were compared to uncoated U-bottom plates in one-way MLR and LTA using C3H (allogeneic) and Balb/c (syngeneic) MSC and DF.

Balb/c and C3H MSC and DF were incorporated in one-way MLR and LTA with Balb/c mononuclear cells as responders at a 1:1 ratio. Con-A stimulated Balb/c mononuclear cells were used as controls for mitogen stimulated cells whereas Balb/c mononuclear cells monocultures were used as controls for unstimulated non-proliferating cells. With regard to the controls for these experiments, ^{3}H-thymidine uptake for Con-A stimulated Balb/c mononuclear cells cultured in U-bottom and V-bottom plates was comparable, with the highest counts obtained at day 6. A similar trend was also observed in the one-way MLR controls. Counts for control unstimulated Balb/c mononuclear cells in both U-bottom and V-bottom plates were not significantly different (**Figure 5.14A**).

In the one-way MLR with Balb/c and C3H MSC or DF, the extent of ^{3}H-thymidine uptake suppression observed with syngeneic or allogeneic MSC (**Figure 5.14B**) and DF (F**igure 5.14D**) was similar in both U-bottom and V-bottom plates. With regard to the LTA co-cultures of responder Balb/c mononuclear cells with Balb/c and C3H MSC (**Figure 2.14C**) and DF (**Figure 5.14E**) as stimulators, the counts obtained using U-bottom and V-bottom plates were not significantly different. Only C3H MSC significantly stimulated Balb/c mononuclear cells at day 6 and day 9 as was previously observed in earlier experiments. Since similar results were obtained with

both U-bottom and V-bottom plates, these results indicated that either may be used in MLR and LTA experiments.

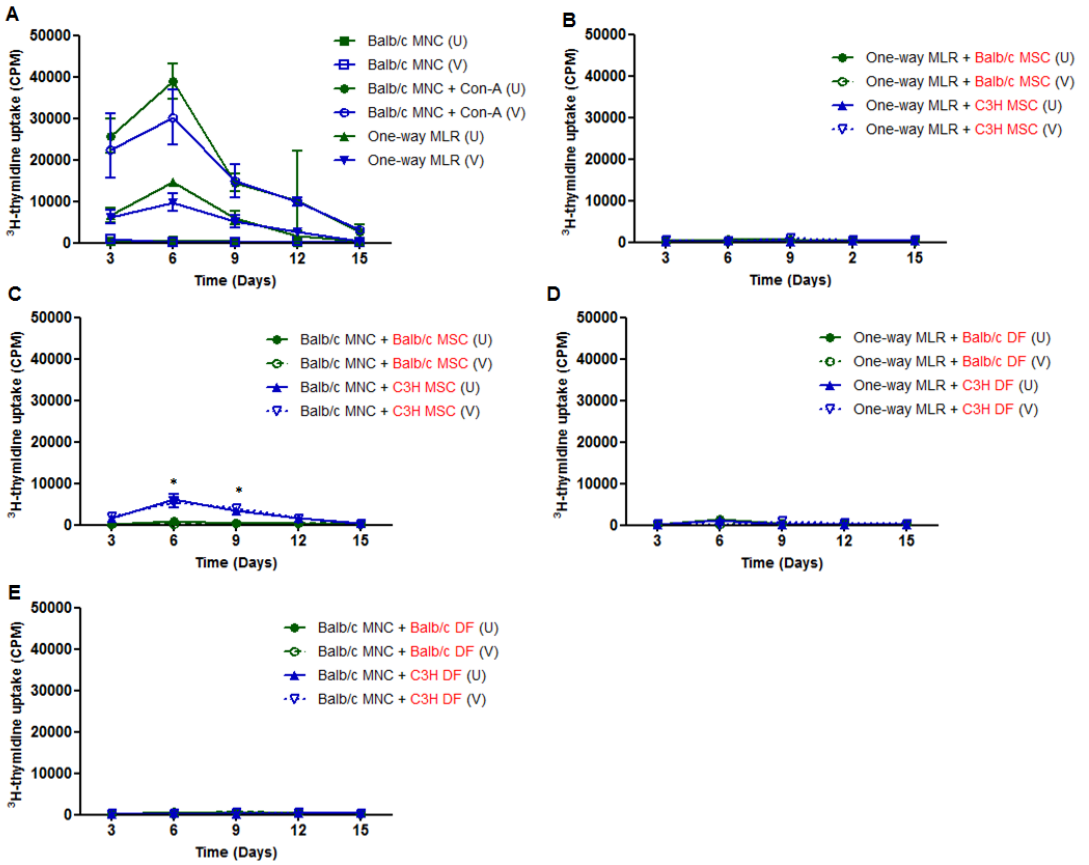

Figure 5.14. Comparison of the effects of syngeneic and allogeneic MSC and DF on one-way MLR and LTA cultured in uncoated U-bottom and V-bottom plates. One-way MLR co-cultures comprising 5 x 10^4 cells per well of each of responder Balb/c mononuclear and mitotically inactivated C3H mononuclear cells were set up in separate uncoated U-bottom and V-bottom plates. Mitotically inactivated Balb/c and C3H MSC or DF were then added to the one-way MLR cultures to give final ratios of 1:1:1. LTA co-cultures of responder Balb/c MNC with syngeneic and allogeneic MSC and DF were also set up at 1:1 ratio. One-way MLR alone and monocultures of Balb/c MNC with or without Con-A were used as controls. The cultures were incubated for 3, 6, 9, 12 and 15 days with medium changed each 3 days (100 µl replaced with fresh medium). Lymphocyte proliferation measured by ^{3}H-thymidine uptake. Data (n=6) was Log_{10} transformed and analysed by 1-way ANOVA followed by calculation of the MSD ($p < 0.05$) by the T method to determine individual differences between means of cells in U and V-bottom plates at a given time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for unstimulated and Con-A stimulated Balb/c MNC and one-way MLR controls (A), one-way MLR with Balb/c and C3H MSC (B), LTA with Balb/c and C3H MSC (C), one-way MLR with Balb/c and C3H DF (D) and LTA with Balb/c and C3H DF (D). * indicates significant differences in ^{3}H-thymidine uptake between Balb/c MNC + C3H MSC and Balb/c MNC only. Cells labelled in red were mitotically inactivated. [MNC; mononuclear cells]

5.3.5. Effect of Balb/c serum on one-way MLR and LTA involving undifferentiated syngeneic and allogeneic MSC and DF

All the MLR and LTA experiments described thus far in this chapter used lymphocyte culture medium supplemented with 10 % (v/v) FCS. Ideally, Balb/c mouse serum would be more suited for these experiments as it is syngeneic to the responder cells. As described in previous experiments in **Section 4.3.5**, commercial heat-inactivated 2 % (v/v) mouse serum inhibited ^{3}H-thymidine uptake in one-way MLR involving responder Balb/c mononuclear cells and mitotically inactivated stimulator C3H mononuclear cells. When the same mouse serum was used in Balb/c mononuclear cells stimulated with Con-A however, significant stimulation comparable to that obtained with 10 % (v/v) FCS was obtained. It was thought that using Balb/c serum would be appropriate and specific for Balb/c lymphocyte responses in comparison to the commercial mouse serum which was obtained from mice from different strains.

In this experiment, 2% (v/v) syngeneic Balb/c serum was used to supplement lymphocyte culture medium which was used in one-way MLR and LTA involving co-cultures of responder Balb/c mononuclear cells and mitotically inactivated Balb/c and C3H MSC or DF at 1:1:1 and 1:1 responder to stimulator cells ratios respectively. Balb/c mononuclear cells cultured alone were used as controls for unstimulated non-proliferating cells while Con-A stimulated Balb/c mononuclear cells were used as control for stimulated proliferating cells.

With regard to the one-way MLR control, C3H mononuclear cells failed to stimulate responder Balb/c mononuclear cells with very low counts recorded at all the time points (**Figure 5.15**). This was similar to the results obtained previously in **Section 4.3.5** in which 2 % (v/v) commercial mouse serum was used to supplement culture medium. Similarly, all LTA and one-way MLR cultures with Balb/c and C3H MSC or DF produced low counts. Only the Con-A stimulated Balb/c mononuclear cell control produced high counts. These results showed that Balb/c serum supplemented culture medium did not support allogeneic stimulation of Balb/c lymphocytes with C3H antigens.

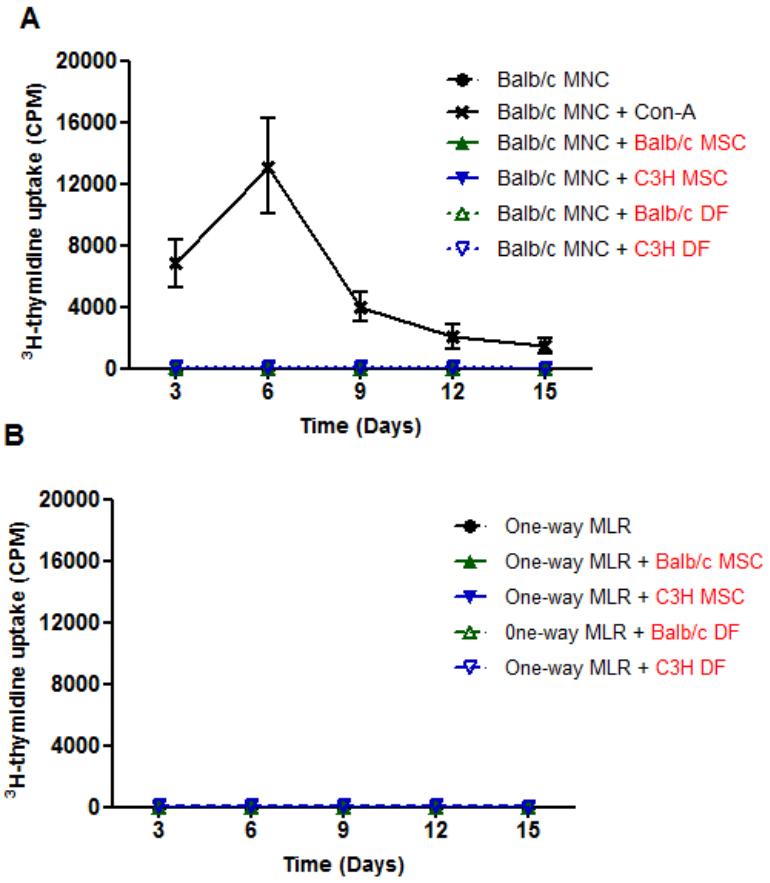

Figure 5.15. Effects of 2 % (v/v) Balb/c mouse serum supplemented culture medium on one-way MLR and LTA involving syngeneic and allogeneic MSC and DF. Balb/c mononuclear cells seeded at 5 x 10^4 cells per well were co-cultured with mitotically inactivated syngeneic Balb/c and allogeneic C3H MSC or DF at a 1:1 ratio in LTA in culture medium supplemented with heat-inactivated 2% (v/v) Balb/c mouse serum. Balb/c mononuclear cells cultured alone or stimulated with Con-A were used as controls for non-dividing and proliferating cells respectively. One-way MLR comprising responder Balb/c mononuclear cells and mitotically inactivated stimulator C3H mononuclear cells were co-cultured with mitotically inactivated Balb/c and C3H MSC and DF in a 1:1:1 ratio. One-way MLR without MSC and DF was used as a control for alloantigen stimulated cells. The cultures were incubated for 3, 6, 9, 12 and 15 days with medium changed each 3 days (100 µl replaced with fresh medium). Lymphocyte proliferation was measured by ^{3}H-thymidine uptake. Data was Log_{10} transformed to calculate 95 % CL. Data was then back-transformed and plotted as mean CPM ± 95 % CL for LTA (A) and one-way MLR (B). [MNC; mononuclear cells]

5.3.6. Effects of allogeneic and syngeneic chondrogenic differentiated MSC on one-way and LTA

MSC and DF were differentiated along the chondrogenic lineage and assessed for their immunomodulatory properties using adaptations of the one-way MLR and LTA. For the one-way MLR, Balb/c (syngeneic) and C3H (allogeneic) chondrogenic differentiated MSC and DF micromass nodules were co-cultured with responder Balb/c and stimulator C3H mononuclear cells. This was carried out in order to determine whether differentiated cells possessed similar immunomodulatory properties to their undifferentiated progenitors. In the LTA, the capacity of the differentiated Balb/c and C3H MSC and DF to stimulate Balb/c lymphocyte proliferation was tested.

After 21 days in chondrogenic medium, Balb/c and C3H MSC and DF stained positive (blue) when stained with alcian blue dye. Positive alcian blue stain is used to identify GAGs which indicated successful differentiation of the MSC and DF to chondrocytes. Control C3H cultures which were cultured in DMEM-LG and DMEM-HG culture medium were negative for the stain (**Figure 5.16**).

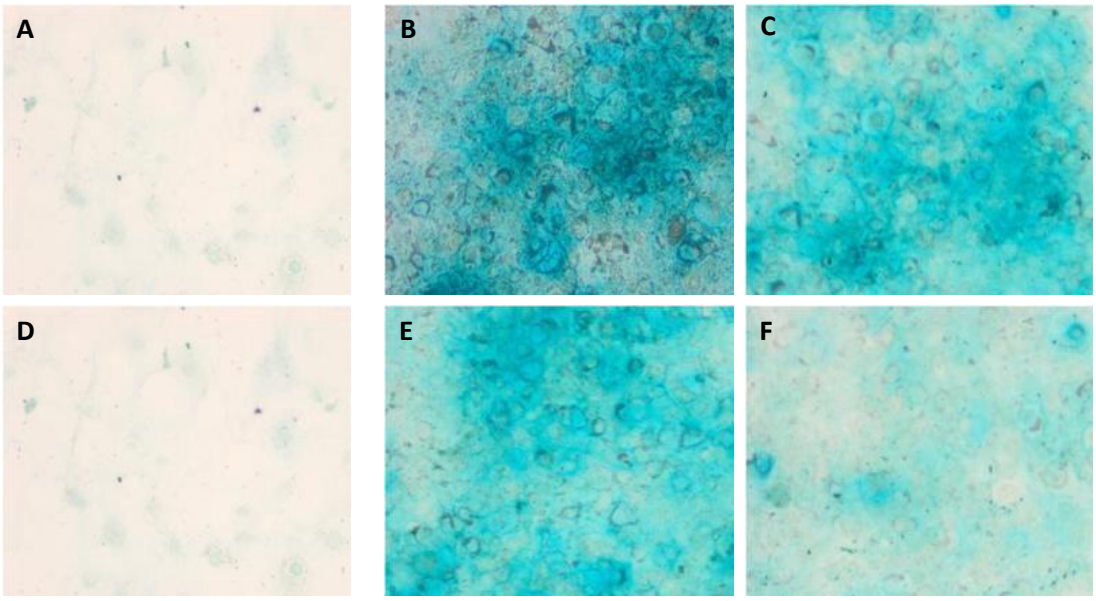

Figure 5.16. Chondrogenic differentiation of mouse MSC and DF. MSC (p5) and DF (p5) were cultured in chondrogenic differentiation medium for 21 days and stained with Alcian blue. C3H MSC (A) and DF (D), which were used as controls were also cultured for 21 days in DMEM-LG and DMEM-HG culture medium respectively and stained negative with Alcian blue. Balb/c and C3H MSC (B and C respectively) and DF (E and F respectively) cultured in chondrogenic differentiation medium stained positive for GAGs with alcian blue. Images were acquired at x100 magnification. [GAGs; glycosaminoglycans]

The differentiated cells and stimulator C3H mononuclear cells were mitotically inactivated by treatment with mitomycin C before they were used in one-way MLR and LTA.

With regard to the control cultures of mitomycin C treated cells, comparisons in ^{3}H-thymidine uptake between Con-A stimulated (**Figure 5.17A**) and unstimulated (**Figure 5.17B**) cells showed no significant differences which demonstrated that the cells were mitotically inactivated. Only responder Balb/c mononuclear cells which were not mitotically inactivated showed significant ^{3}H-thymidine uptake with the highest counts obtained at day 6.

For the one-way MLR, control co-cultures of responder Balb/c mononuclear cells and mitotically inactivated C3H mononuclear cells showed significant ^{3}H-thymidne uptake which demonstrated that Balb/c lymphocyte proliferation in response to C3H mononuclear cells as has been shown in previous experiments. When Balb/c (syngeneic) and C3H (allogeneic) chondrogenic differentiated MSC were cultured in the one-way MLR, significant suppression of ^{3}H-thymidine uptake was observed at days 3, 6 and 9 in comparison to the one-way MLR control with approximately 50 % suppression achieved at day 6 (**Figure 5.17C**). A similar trend was also achieved when Balb/c or C3H chondrogenic differentiated DF were co-cultured in the one-way MLR. Despite the suppression of Balb/c lymphocyte proliferation, SI above 3 were obtained at all the time points except at day 15 (**Figure 5.17E**).

With regard to the LTA in which Balb/c mononuclear cells were co-cultured with allogeneic or syngeneic chondrogenic differentiated MSC and DF, no significant increase in ^{3}H-thymidine uptake relative to monocultures of responder cells, was obtained with either Balb/c (syngeneic) chondrogenic differentiated MSC or DF (**Figure 5.17D**). When C3H (allogeneic) chondrogenic differentiated MSC or DF were co-cultured with responder Balb/c mononuclear cells, significant ^{3}H-thymidine uptake relative to monocultures of Balb/c mononuclear cells, was obtained at days 3, 6 and 9 with the highest counts obtained at day 6. Also, SI equal to above 3 were obtained at these time points with the highest SI of 7 obtained at day 6 with both C3H chondrogenic differentiated MSC and DF (**Figure 5.17F**).

Taken together, the results showed that both syngeneic and allogeneic chondrogenic differentiated MSC and DF equally suppressed one-way MLR. In LTA, allogeneic

but not syngeneic chondrogenic differentiated MSC and DF cells stimulated Balb/c lymphocyte proliferation.

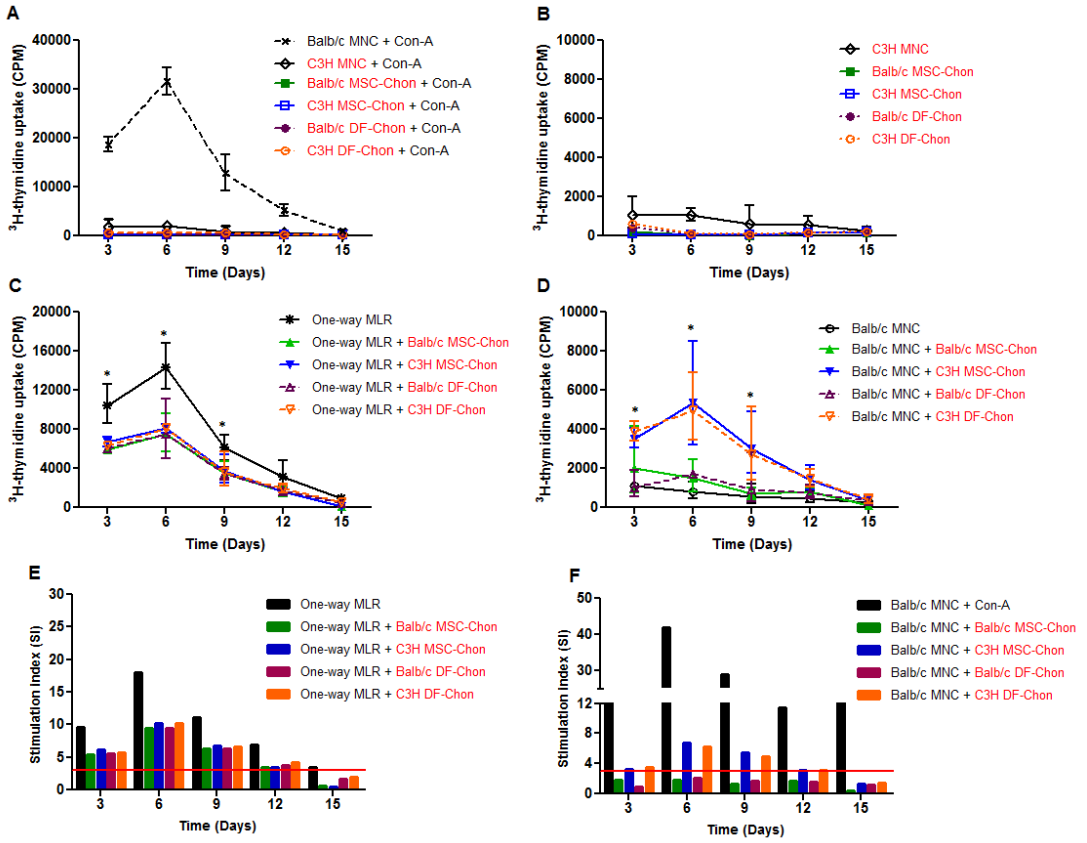

Figure 5.17. Effects of chondrogenic differentiated syngeneic and allogeneic MSC and DF on one-way MLR and LTA as measured by ^{3}H-thymidine uptake. Mitotically inactivated chondrogenic differentiated Balb/c (syngeneic) and C3H (allogeneic) MSC and DF (5 x 10^4 cells per well) were co-cultured in one-way MLR comprising Balb/c and mitotically inactivated C3H mononuclear cells (MNC) at a 1:1:1 ratio. One-way MLR culture only was used as a control for alloantigen stimulation. LTA comprised co-cultures of differentiated Balb/c or C3H MSC and DF with responder Balb/c MNC at 1:1 ratio. Balb/c mononuclear cells (MNC) cultured alone or stimulated with Con-A were used as controls for non-proliferating and proliferating cells respectively. Cultures were incubated for 3, 6, 9, 12 and 15 days with medium changes every 3 days and lymphocyte proliferation was measured by ^{3}H-thymidine uptake. Data (n=6) was Log_{10} transformed and analysed by 2-way ANOVA followed by calculation of the MSD ($p < 0.05$) by the T method to determine individual differences between test and control cells at each time point. Data was then back-transformed for presentation and plotted as mean CPM ± 95 % CL for unstimulated controls (A), stimulated controls (B), one-way MLR (C) and LTA (D). SI were computed for one-way MLR (E) and LTA (F). The red line at SI=3 denotes statistical biological positive; SI above 3 were considered significant. * indicates significant differences between one-way MLR only and one-way MLR + C3H or Balb/c differentiated MSC and DF in (C) and between Balb/c MNC only and Balb/c MNC + C3H differentiated MSC or DF in (D) at each time point.

5.4. Discussion

The purpose of the work carried out in this chapter was aimed at investigating the immunological properties of both undifferentiated and chondrogenic differentiated allogeneic MSC in light of reported evidence that they possess immunosuppressive properties which could potentially be of therapeutic value in clinical procedures such as allogeneic transplantation. In addition, MSC have also been reported to be immunoprivileged which means that donor allogeneic MSC are not rejected in an allogeneic recipient. Therefore, three key questions were investigated in this work, firstly whether undifferentiated MSC possessed immunosuppressive properties and if so, whether the suppression was sufficient to ameliorate the allogeneic rejection and secondly, to test whether MSC were immunoprivileged and incapable of initiating allogeneic rejection. The final question was to investigate whether the properties exhibited by undifferentiated MSC were retained following chondrogenic differentiation.

To answer the first question, syngeneic Balb/c and allogeneic C3H MSC were co-cultured with one-way and two-way MLR comprising mismatched Balb/c and C3H mononuclear cells. For the second question, Balb/c MSC and C3H MSC were co-cultured with responder Balb/c mononuclear cells in LTA to test their capacity to stimulate lymphocyte proliferation. For the third question, MSC were differentiated along the chondrogenic pathway and were similarly tested for their immunomodulatory properties and immunogenicity in one-way MLR and LTA respectively.

For undifferentiated cells, both C3H (allogeneic) and Balb/c (syngeneic) MSC significantly suppressed one-way MLR with 90 % of the proliferative response suppressed at day 6. Similarly, both types of MSC suppressed two-way MLR with Balb/c MSC showing 14 % more suppression compared to C3H MSC when cultured at a 1:1:1 ratio. These findings supported a growing pool of evidence from other *in vitro* studies which used mouse MSC (Djouad *et al.*, 2003; Glennie *et al.*, 2005; Sudres *et al.*, 2006; Xu *et al.*, 2007; Han *et al.*, 2010), human (Di Nicola *et al.*, 2002; Le Blanc *et al.*, 2003c; Klyushnenkova *et al.*, 2005), primate (Bartholomew *et al.*, 2002) and other animal models. Despite using different adaptations of the MLR such as culture period, cell ratios and stimulator cells (3rd party MSC in human studies)

and unseparated or purified responder lymphocytes, the *in vitro* inhibitory potency of allogeneic MSC to controls has been demonstrated to range between 60 – 95 %. Unlike in other previous studies using mouse models, the MLR in this study were carried out for a total of 15 days (compared to an average of 5 days) accompanied by medium replacement (discussed in **Section 4.3.2**). This modification resulted in lymphocyte proliferation as represented by increasing ^{3}H-thymidine uptake, peaking at day 6 in control cultures for the MLR. The SI for the these cultures were above 3 at all the time points except at day 15, which suggested that the Balb/c mononuclear cell allogeneic response to C3H mononuclear cells was detected up to 12 days in culture. Therefore, MSC mediated suppression was sustained for the duration of the cultures. The fact that syngeneic and allogeneic MSC were equally suppressive in one-way MLR suggested that the mechanisms involved were independent of MHC differences. This was previously reported in similar studies which used human MSC (Le Blanc *et al.*, 2003c).

The exact mechanisms behind MSC mediated immunosuppression in MLR remain largely unresolved. It is thought that MSC are able to exert their immunosuppression by a combination of mechanisms which includes secretion of a milieu of suppressive chemicals and cytokines in addition to cell to cell contact mechanisms. Immunosuppressive factors such NO, IDO, PGE_2 and TGFβ have been implicated but some of the evidence is contradictory since some of the factors are not constitutively expressed by all MSC resulting in huge variations across studies (Tse *et al.*, 2003; Meisel *et al.*, 2004; Aggarwal & Pittenger, 2005). This study did not focus on investigating secreted molecules from MLR cultures because the evidence in the literature suggests that of all the secreted immunosuppressive molecules known to be produced by MSC to date, such as IDO, PGE_2 and TGFβ none has been found to be indispensable as discussed in **Section 1.8.2.1**. In fact, it is widely accepted across the stem cell field that experimental conditions and factors such as age and passage number of the MSC and the source of the MSC play a key part in the behaviour exhibited by MSC *in vitro*. Therefore, this study focused on investigating how different MLR parameters such as incubation period and medium changes affected the course of MSC mediated immunosuppression.

With regard to whether allogeneic MSC were immunoprivileged or immunogenic, LTAs were carried out and extended from the usual 5 days to 15 days accompanied by medium changes every 3 day period. Con-A stimulated and unstimulated Balb/c mononuclear cells were used as controls for proliferating and non-proliferating cells respectively. For any response to be deemed stimulatory, the counts would have to be significantly different from those of the control unstimulated Balb/c mononuclear cells as well as the SI having to be above 3. The results showed that C3H MSC (allogeneic) but not Balb/c (syngeneic) MSC significantly stimulated Balb/c responder mononuclear cells using MSC that had and had not been mitotically inactivated. Though the stimulation by C3H MSC peaked at day 6, it was also detected at days 9 and 12. These findings suggested that allogeneic MSC were immunogenic under these experimental conditions and thus contradicted other *in vitro* studies that have reported that allogeneic MSC were immunoprivileged and failed to stimulate allogeneic lymphocytes. Some of these studies did not use the SI as a measure of immunogenicity and therefore based their findings in terms of the counts alone. This approach is not inaccurate but it is based on high counts having to be achieved for a positive stimulatory response. For instance, the counts obtained when Balb/c mononuclear cells were stimulated by C3H mononuclear cells (one-way MLR), were high. The counts obtained when Balb/c mononuclear cells were stimulated with C3H MSC were lower in comparison.

In a study that used human MSC, it was reported that allogeneic MSC were not immunogenic when co-cultured with purified lymphocytes but when they were cultured with unseparated PBMC, the ^{3}H-thymidine counts were significantly higher than for PBMCs cultured alone which suggested that the allogeneic MSC were stimulatory at day 8 but this observation has been largely overlooked in the literature (Klyushnenkova *et al.*, 2005). Due to the inherent hypo-immunogenicity of MSC discussed in **Section 1.8.1** (low expression of MHC I and lack of surface MHC II and co-stimulatory molecules CD80 and CD86), it is probable that they will not be able to stimulate lymphocytes by the direct pathway. However, when PBMC were used, it is also possible that allo-antigens from the allogeneic MSC could be presented to lymphocytes by the APC present in PBMC via the indirect and semi-direct pathways. Therefore, LTA tested within 3-4 days may not capture the indirect and semi-direct pathways.

It is important to note that the stimulatory response shown by C3H MSC was obtained after numerous experiments in which they failed to stimulate Balb/c mononuclear cells. Initially, when the LTA were carried out without medium changes for a 7-day culture period, no stimulatory response was observed. Similar findings were also observed when surface-coated plates were used (Nunc™ Δ). Stimulatory responses were only observed when medium changes and uncoated plates were used. Also, some of the experiments were affected by inconsistent counts particularly for unstimulated control cells which in some cases were too high such that SI, including for Con-A stimulated controls, were drastically reduced. This problem has been reported previously in similar studies using mouse mononuclear cells. Therefore, as discussed in **Section 4.4**, it was necessary to define the most appropriate conditions for performing the LTA and MLR.

Another important consideration for the LTA was the ratio between the responder Balb/c mononuclear cells and the stimulator C3H MSC. Since allogeneic MSC were stimulatory at a 1:1 ratio, it was necessary to investigate whether increasing the stimulator cell ratio in the LTA would lead to an improved allogeneic response. Thus, LTA were prepared in which the ratio of responder Balb/c mononuclear cells to stimulator MSC was increased from 1:1 to 1:5. It was found that ^{3}H-thymidine counts decreased with increasing stimulator cell ratio when C3H MSC were used as stimulators. Similarly, SI values decreased with increasing proportion of allogeneic MSC at all the time points. In previous studies, it was also shown that increasing the numbers of MSC inhibited lymphocyte proliferation in a dose-dependent manner (Le Blanc *et al.*, 2003c; Nauta *et al.*, 2006; Sudres *et al.*, 2006; Jones *et al.*, 2007). Thus, since allogeneic MSC stimulated lymphocyte proliferation at 1:1 ratio, increasing the MSC ratio reduced ^{3}H-thymidine uptake. Therefore, this suggested that at specific cell ratios, allogeneic MSC fail to stimulate lymphocyte proliferation. In a study by Le Blanc and co-workers, it was suggested that at low numbers, allogeneic MSC stimulated lymphocyte proliferation as opposed to suppression observed with higher doses (Le Blanc *et al.*, 2003c). Despite this finding, the authors did not investigate further as to why the MSC were stimulatory at low ratios. This further illustrates how variations in experimental conditions contribute to conflicting conclusions. Most studies in which high ratios of allogeneic MSC were used resulted in MSC failing to stimulate allogeneic lymphocytes (Nauta *et al.*, 2006; Jones *et al.*, 2007).

Studies that have tested allogeneic MSC *in vitro* and *in vivo* using mouse models have also shown allogeneic MSC to be immunogenic *in vivo* despite failing to stimulate allogeneic lymphocytes *in vitro* (Eliopoulos *et al.*, 2005; Nauta *et al.*, 2006; Sudres *et al.*, 2006). This could be due to the fact that high MSC to lymphocyte cell ratios were used *in vitro*, but such MSC doses are less likely to be replicated *in vivo* and therefore allogeneic MSC become stimulatory leading to rejection.

DF were chosen as controls to test whether the properties exhibited by MSC were unique to stem cells or were shared by other cells of mesenchymal origins. Thus, DF were tested in the same way described for MSC. With regard to the one-way MLR, both allogeneic and syngeneic DF suppressed responder Balb/c mononuclear cell response to allogeneic C3H mononuclear cells. Similarly, in the two-way MLR, both C3H and Balb/c DF suppressed lymphocyte proliferation. The suppression exhibited by DF (75 %) though less than exhibited by MSC (90 %) was significant with SI falling below 3.

Slight differences in SI trends between MSC and DF mediated suppression of one and two-way MLR were noticed. When Balb/c and C3H MSC were cultured with one-way and two-way MLR, SI above 3 were obtained at day 3 but dropped below 3 at day 6 onwards. With DF however, SI values were below 3 at day 3 but values above 3 were obtained at day 9. This could suggest that despite both MSC and DF exhibiting immunosuppressive properties, the mechanisms by which they achieve suppression might be different. It has been recently suggested that MSC exhibited anti-inflammatory properties *in vitro* which were absent in DF (Blasi *et al.*, 2011); such differences may potentially affect the mechanisms by which they achieve immunosuppression.

In the last two decades, studies using allogeneic human gingival and corneal fibroblasts have reported their immunosuppressive properties (Shimabukuro *et al.*, 1992; Donnelly *et al.*, 1993). More recently, human primary articular chondrocytes, synovial and dermal fibroblasts have been reported to exhibit dose-dependent lymphocyte inhibitory effects comparable to MSC (Bocelli-Tyndall *et al.*, 2006; Haniffa *et al.*, 2007; Jones *et al.*, 2007). Together with their ability to undergo tri-lineage differentiation and similar phenotype to MSC (discussed in **Chapter 3**), this study has shown that mouse DF possessed comparable immunosuppressive

properties to MSC. Thus, the ability to suppress lymphocyte proliferation may be a property shared by other cells of the mesenchymal lineage.

When DF were tested in LTA, both C3H (allogeneic) and Balb/c (syngeneic) DF failed to stimulate Balb/c mononuclear cells. Unlike allogeneic MSC, allogeneic DF were not immunogenic; with counts obtained not significantly different from those for control cultures and the SI below 3 in all the experiments carried out. Recently, there has been a growing interest in the study of the immunomodulatory properties of DF particularly as more evidence about their similarities to MSC is accumulating. To date, there are a few studies that have investigated the immunoregulatory properties of allogeneic human DF (Haniffa *et al.*, 2007) but none has tested allogeneic DF in LTA to test their immunogenicity. Due to their similarities with MSC, DF and other fibroblasts are now receiving more attention as was reviewed in detail by Haniffa and co-workers (Haniffa *et al.*, 2009) and their potential in tissue engineering and regenerative medicine has not been explored.

The data obtained from MLR and LTA carried out in uncoated U-bottom and V-bottom plates demonstrated that both were equally effective in supporting these experiments unlike the Nunc Δ surface coated plates as previously described in **Section 5.3.4**. Although the counts obtained in the control experiments were marginally higher in U-bottom than in V-bottom plates, there were no significant differences between the two. Importantly, this experiment demonstrated the reproducibility of the assays particularly the stimulation of Balb/c mononuclear cells by allogeneic C3H MSC. Moreover, the counts and overall lymphocyte stimulatory (Con-A control, MLR control and C3H MSC in LTA) and suppressive (Balb/c and C3H MSC and DF in MLR) trends obtained from this experiment were comparable to those obtained in **Section 5.3.1** and **Section 5.3.2**.

When one-way MLR and LTA using Balb/c and C3H MSC and DF were carried out in culture medium supplemented with 2 % (v/v) Balb/c serum (syngeneic to the responder lymphocytes), ^{3}H-thymidine uptake was inhibited in all reactions (including the one-way MLR) except in the Con-A stimulated control. As was observed with commercial mouse serum in **Section 4.3.4,** these findings suggested that mouse serum contains a factor or factors thought to be lipoproteins that suppress lymphocyte proliferation stimulation as previously discussed in **Section 4.4**.

With regard to the commercial mouse serum used previously, it was found out from the suppliers that the serum was a mixture of sera obtained from several mouse strains. Therefore, it was thought that using freshly prepared syngeneic Balb/c serum would favour Balb/c lymphocyte proliferation but this was not the case. Since alloantigen stimulation was achieved in culture medium supplemented with 10 % (v/v) FCS, it is possible to speculate that since FCS has been reported to contain several poorly defined factors such as endotoxins, antigenic peptides, glucocorticoids and other hormones that can influence immune cells such as macrophages, DC and T-lymphocytes (Eske *et al.*, 2009). Therefore, FCS may have the capacity to polarise lymphocytes to proliferate as a result of stimulation from "by-stander" antigens derived from the serum. Importantly, commercial FCS composition varies from batch-to-batch and the variations in some cases have been reported to be significant (Bryan *et al.*, 2011). This could explain in part why variability between different experiments particularly ^{3}H-thymidine counts was in some cases high. This made reproducibility of the experiments very challenging. In some cases, high ^{3}H-thymidine counts were often obtained in negative control experiments which comprised unstimulated cells. In the end, the FCS used in most of the experiments presented in this thesis was obtained from a single batch which had been tested and shown to be non-stimulatory towards negative controls. The problem of lack of reproducibility in mouse MLR and LTA experiments which use FCS for medium supplementation is a widely reported phenomenon and presents major challenges in research.

In a study that used mouse sera obtained from non-pregnant and pregnant mice at different stages of gestation showed that serum obtained from mid-gestation mice significantly suppressed MLR and was accompanied by a notably reduced expression of MHC II molecules on stimulator DC (Shojaeian *et al.*, 2007). This study also reported reduced IFNγ and elevated IL-4 and IL-10 secretion by responder T-cells and implicated progesterone as a potential immunosuppressive factor in the serum since it had been previously shown to inhibit IFNγ secretion (Piccinni *et al.*, 1995). In a different study however, progesterone was shown to promote DC maturation and MHC II expression (Liang *et al.*, 2006) thus making the evidence in inconclusive.

Another study described the existence of immunosuppressive mouse serum factor(s) which were eluted in the gamma-globulin fraction following Sepharose 6B gel filtration chromatography with molecular weights between 100 and 150 kDa which act by blocking IL-2 receptors after internalisation by T-lymphocytes (Lindqvist *et al.*, 1992). It has also been argued that the presence of the hormone leptin in the serum promotes *in vitro* lymphocyte proliferation but its absence led to suppression (Lord *et al.*, 1998).

This is particularly interesting since leptin has been reported to increase the secretion of lipoprotein lipase in human macrophages (Maingrette & Renier, 2003). It is therefore possible to hypothesize that since lipoprotein lipase is required for the metabolism of lipoproteins which have been implicated as potential immunosuppressive factors in mouse serum (**Section 4.4**), the absence of leptin in serum probably due to heat inactivation, could potentially result in the presence of immunosuppressive levels of lipoproteins. It is also interesting to investigate the role of IL-6 in serum-induced immunosuppression since it has been reported to resemble leptin (Fantuzzi & Faggioni, 2000).

Another important component in mouse lymphocyte culture medium is the presence of 50 μM of β-mercaptoethanol. The necessity of this component was tested during the initial stages of this study and it was found that stimulation of Balb/c mononuclear cells with PHA in the absence of or in the presence of amounts less than 50 μM of β-mercaptoethanol was unsuccessful. The role of β-mercaptoethanol in lymphocyte stimulation is unknown but due to its known function as a powerful reducing agent, it is possible to hypothesize that it may be involved in the breaking of disulphide bonds of complex sulphur containing proteins which may aid nutrient uptake or lymphocyte proliferation.

Importantly, it was necessary to investigate the immunosuppressive properties and immunogenicity of chondrogenic differentiated MSC and DF in order to determine whether they retained similar properties to those exhibited by undifferentiated cells. As has been mentioned throughout this study, undifferentiated and in some cases terminally differentiated allogeneic MSC have been reported throughout the literature to be immunosuppressive and immunoprivileged. Crucially, the potential

application of MSC in tissue engineering procedures for tissue repair and replacement requires the use of MSC differentiated into specific lineages.

Theoretically, once terminally differentiated, MSC would lose their immunological and 'stem' properties as they would assume a different morphology and function punctuated by expression of lineage specific proteins and molecules. Thus, the hypothesis for this experiment was that MSC would lose their immunomodulatory properties once they undergo terminal differentiation. Therefore, Balb/c and C3H MSC and DF were differentiated along the chondrogenic lineage and their immunomodulatory properties were tested using the one-way MLR and LTA respectively.

Chondrogenic differentiation of Balb/c and C3H MSC and DF was confirmed by alcian blue staining after 21 days of pellet culture as was previously demonstrated in **Chapter 3**. This assay is the only functional assay to date that clearly demonstrates successful chondrogenic differentiation of stem cells. Complete disaggregation of the pellets required incubation with 0.01 % (v/v) collagenase. The cells were seeded in uncoated V-bottom plates overnight before the one-way MLR and LTA cultures were set up to allow the cells to aggregate into micro-pellets. In **Section 5.3.4**, V-bottom plates were compared to U-bottom plates in one-way MLR and LTA and both types of plates were found to be equally suitable based on the comparable ^{3}H-thymidine uptake counts obtained. Therefore, V-bottom plates were preferred to U-bottom plates as they were thought to be more suitable for the culture of chondrogenic micro-pellets.

With regard to immunosuppressive properties, both chondrogenic differentiated Balb/c and C3H MSC and DF exhibited comparable suppression of the one-way MLR, causing a 50 % reduction in ^{3}H-thymidine uptake in one-way MLR at day 6 whereas in experiments which used undifferentiated MSC and DF, suppression of 90 % and 75 % respectively were obtained. Although the experiments using chondrogenic differentiated MSC and DF and those using undifferentiated MSC and DF were not carried out at the same time, the results appeared to suggest that chondrogenic differentiated MSC and DF suppression of the one-way MLR was less compared to that for undifferentiated MSC and DF. These results concurred with similar findings in a recent study in which mouse MSC differentiated into osteoblasts

were reported to have lost their immunosuppressive capacity. This loss of was attributed to decreased secretion of IL-10 and TGFβ (Liu *et al.*, 2012). Passage number and 'age' of MSC is also thought to lead to loss of MSC immunosuppressive capacity. During differentiation of MSC, the fractions of cells that successfully undergo terminal differentiation vary as a result of the cell 'age'. Therefore, the properties exhibited by MSC cultures at different stages of culture are variable; with long term cultures associated with the gradual loss of differentiation potential and other 'stem' properties (Kretlow *et al.*, 2008). In contrast, other studies have suggested that the immunosuppressive properties displayed by MSC are also inherent in other differentiated cells including chondrocytes (Jones *et al.*, 2007). However, there is little evidence in the literature on the immunosuppressive properties of differentiated MSC. It is probable that the conflicting findings across studies could be accounted for by the variations in the quality or quantity of the cells used. Also, as shown in **Section 5.3.3** and in another study (Le Blanc *et al.*, 2003c), the ratio of responding lymphocytes to stimulator MSC could play a part in determining whether they exhibit an inhibitory or stimulatory response *in vitro*.

With regard to the immunogenicity of the chondrogenic differentiated MSC and DF in LTA, it was shown that allogeneic but not syngeneic chondrogenic differentiated MSC and DF stimulated Balb/c lymphocyte proliferation. Previously, allogeneic C3H MSC, but not DF, stimulated Balb/c lymphocyte proliferation. In this case however, chondrogenic differentiated DF were also found to be immunogenic. There is evidence in the literature that rat MSC differentiation into chondrocytes but not adipocytes and osteocytes resulted in the expression of the co-stimulatory molecules CD80 and CD86 which were partly responsible for the rejection of chondrogenic differentiated MSC following allogeneic transplantation (Chen *et al.*, 2007). In a separate study, MSC differentiation led to surface expression of MHC II and elevated MHC I which led to rejection by allogeneic lymphocytes (Huang *et al.*, 2010). Conflicting results were obtained when osteogenic differentiated MSC were reported to have retained their immunoprivileged status (Liu *et al.*, 2006). It was also reported that human MSC differentiated into chondrocytes, adipocytes and osteocytes failed to stimulate allogeneic lymphocytes *in vitro* (Le Blanc *et al.*, 2003b).

It is likely that during differentiation, specific pathways are switched on depending on the specific properties the cells have to acquire in order to assume new functions.

These pathways could lead to previously immunoprivileged cells becoming immunogenic. This is demonstrated throughout the mammalian developmental process in which immunoprivileged pluripotent stem cells such as ESC, undergo multi-differentiation pathways which lead to immunogenic progeny.

Chondrocytes from various animals, including mice in particular have been reported to constitutively express CD80 and CD86 which potentially could contribute to their immunogenicity (Yuan *et al.*, 2003). This notion was directly contradicted in a report that allogeneic rat cartilage-derived chondrocytes did not constitutively express CD80 and CD86 and were immunoprivileged (Adkisson *et al.*, 2010).

With regard to DF, there are no studies to date that have investigated the immunogenicity of DF using LTA. In general, evidence on the immunomodulatory properties of DF is limited although there is growing interest in this field. These results showed that chondrogenic differentiation of allogeneic MSC and DF resulted in reduced immunosuppressive potency. Both chondrogenic differentiated allogeneic MSC and DF were immunogenic.

In summary, the findings in this chapter have shown that undifferentiated mouse MSC and DF were immunosuppressive in one-way and two-way MLR regardless of histocompatibility differences. Following chondrogenic differentiation both syngeneic and allogeneic MSC and DF were immunosuppressive in one-way MLR although the potency of suppression appeared to be less than that shown by undifferentiated cells. In LTA however, undifferentiated allogeneic MSC (C3H) but not syngeneic MSC stimulated Balb/c responder lymphocytes whilst both allogeneic and syngeneic DF failed to stimulate lymphocyte proliferation. This suggested that despite their immunosuppressive properties, undifferentiated allogeneic MSC may not be immunoprivileged. Following chondrogenic differentiation however, both allogeneic MSC and DF were immunogenic. The failure by undifferentiated DF to stimulate allogeneic lymphocyte proliferation was surprising and would warrant future investigations. Importantly, the properties exhibited by both undifferentiated and chondrogenic differentiated DF suggested that the immunomodulatory properties are shared by other cells of the mesenchymal lineage and this may have implications in research and potential clinical applications in the future.

6. CHAPTER SIX: FINAL DISCUSSION

6.1. Overview

The work carried out in this study was aimed at investigating the immunological properties of allogeneic MSC, namely their immunosuppressive properties and immunoprivileged status, which have been reported in some studies. Other studies however, have reported conflicting results with regard to the immunogenicity of allogeneic MSC particularly in light of their potential in tissue engineering and regenerative medicine. An allogeneic mouse model was used in which Balb/c (responder) and C3H (stimulator) MSC were tested for their immunosuppressive properties using adaptations of the one-way MLR while their immunoprivileged status was tested using the LTA. MSC were isolated from mouse bone marrow and characterised using flow cytometry. The phenotype of the MSC was found to be consistent with that widely regarded as representative of MSC. Both Balb/c and C3H MSC underwent tri-lineage differentiation into adipocytes, chondrocytes and osteocytes which is currently the only functional test for MSC.

It is widely thought that the immunoprivileged status of MSC stems from their phenotype which is characterised by the absence of MHC II and co-stimulatory molecules CD80 and CD86 which play an important role in allorecognition. Thus, in most *in vitro* studies, MSC have been reported to be immunoprivileged on the basis of their failure to stimulate allogeneic lymphocytes *in vitro*. The hypothesis for this study however, speculated that despite their phenotype or hypoimmunogenicity, MSC would stimulate allogeneic lymphocytes via the indirect and semi-direct pathways of allorecognition if cultured for longer than the standard 5 day period used in most studies. Therefore, by allowing for longer incubation under favourable conditions, allogeneic MSC, like all allogeneic cells, would be immunogenic.

In order to perform the one-way MLR and LTA, a significant amount of time was dedicated to defining the most appropriate conditions for performing these assays so that they would capture the indirect and semi-direct responses. Unlike in most studies, the MLR and LTA assays in this study were performed over a 15 day period. A strategy to replace half the culture medium each 3 days as described in **Section 4.2.3** was introduced in order to ensure the availability of nutrients and removal of

metabolic by-products during the longer incubation period. This strategy was shown to significantly increase the late incorporation of ^{3}H-thymidine in cultures stimulated with Con-A in comparison to cultures in which culture medium was unchanged. Peak ^{3}H-thymidine uptake and stimulation was obtained at day 6 and day 3 in cultures with and without medium changes respectively. This strategy was then used in all the MLR and LTA which included MSC. There are currently no reported studies in which medium changes have been carried out in MLR and LTA.

It is widely acknowledged throughout the stem cell field that the major contributing factor to conflicting results is the variability in conditions between studies. Therefore, an effort was made in this study to ensure that the parameters for MLR and LTA were thoroughly tested. One of the key parameters tested was the concentration of mitomycin C (10 $\mu g.ml^{-1}$) used to induce mitotic arrest in stimulator cells. A wide range of concentrations (5 - 100 $\mu g.ml^{-1}$) have been used in various studies. Despite the toxicity of mitomycin C however, none of the studies in the literature have provided evidence for testing the viability of the stimulator cells following treatment. It was found that a balance between maintenance of viability and mitotic inactivation was necessary. Concentrations of mitomycin C above 10 $\mu g.ml^{-1}$ drastically reduced cell viability whereas concentrations below 10 $\mu g.ml^{-1}$ maintained viability but did not effect complete arrest of lymphocyte proliferation upon stimulation. Mitotic inactivation and maintenance of cell viability are crucial factors in MLR and LTA as insufficient inactivation would result in stimulator cells capable of dividing while low cell viability would result in non-viable stimulator cells. Using non-viable stimulator cells may yet provide stimulation of responder lymphocytes via the indirect and semi-direct pathways of allorecognition as discussed in **Section 1.7.2.2** and **Section 1.7.2.3** respectively as a result of antigenic cell debris being presented to responder lymphocytes by responder APC. There is a possibility that some studies in the literature may have used non-viable stimulator cells in their experiments.

Another important factor that warranted investigation was the feasibility of using mouse serum instead of the commonly used FCS for supplementing culture medium. Both commercially sourced mouse serum and Balb/c serum extracted from Balb/c mice blood in the laboratory, were tested at different concentrations in MLR and

LTA. In both cases, both mouse serum and Balb/c serum failed to support Balb/c lymphocyte stimulation by allogeneic C3H mononuclear cells in control one-way MLR despite showing positive results in Con-A stimulated cultures. In Con-A stimulated Balb/c mononuclear cells, mouse serum appeared to inhibit lymphocyte proliferation in a dose-dependent manner. Although similar observations have been noted in the literature and various inhibitory molecules and mechanisms proposed to be the cause as discussed in **Section 4.4** and **Section 5.4**, the exact mechanisms remain unknown to date. With FCS however, lymphocyte proliferation was observed in all one-way MLR and mitogen stimulated controls with increasing FCS concentration resulting in increased ^{3}H-thymidne utilisation which peaked at 10 % (v/v). Although it is possible to speculate that xenogeneic antigenic epitopes in the FCS may have had a stimulatory effect on mouse responder lymphocytes, this was unlikely in the experiments presented in this thesis since control cultures of mononuclear cells alone did not show a proliferative response in FSC supplemented medium. However, as previously discussed in **Section 4.4**, this notion cannot be discounted in general terms since there were experiments in which unstimulated mononuclear cells produced unusually high counts.

Following the establishment of appropriate conditions for performing MLR and LTA, the immunosuppressive properties of MSC were determined using one-way and two-way MLR at a 1:1:1 cell ratio. Both mitotically inactivated allogeneic (C3H) and syngeneic (Balb/c) MSC significantly suppressed one-way MLR with similar potency. The same was observed when either Balb/c or C3H MSC were tested in two-way MLR. Importantly, the suppression was maintained throughout the 15 day culture period. These findings showed that MSC-mediated immunosuppression is non-specific with regard to the differences in MHC and were in agreement with most studies in the literature. When the MSC were differentiated into chondrocytes and then tested for their immunosuppressive properties however, it was found that their suppressive properties decreased following differentiation although it must be stressed that this was observed in separate experiments. For instance, the day 6 SI for the one-way MLR controls were 36 and 18 for the experiments involving undifferentiated MSC and chondrogenic differentiated MSC respectively. However, in cultures with syngeneic and allogeneic undifferentiated MSC and chondrogenic

differentiated MSC respectively, the SI were reduced to 4 in the former and 10 in the latter.

With regard to the immunoprivileged status of MSC, mitotically inactivated or not allogeneic (C3H) and syngeneic (Balb/c) MSC, the findings showed that allogeneic but not syngeneic MSC significantly stimulated Balb/c lymphocytes. It was observed that the stimulation was at its peak at day 6. This evidence suggested that allogeneic MSC were immunogenic and were therefore not immunoprivileged. These findings therefore, contradicted most of the studies in the literature in which allogeneic MSC have been reported to be immunoprivileged. When the stimulator cell (allogeneic MSC) ratio was increased however, the level of stimulation decreased suggesting that high doses of allogeneic MSC relative to the responding lymphocytes would result in allogeneic MSC appearing to be non-immunogenic. Thus, these findings emphasized the importance of cell ratios in these assays. Following chondrogenic differentiation of the MSC, allogeneic but not syngeneic chondrocytes stimulated Balb/c lymphocyte proliferation.

DF were used as controls to test whether the immunomodulatory properties of MSC were unique to stem cells or were shared by other cells of mesenchymal origin. MSC and fibroblasts have been widely reported as sharing morphological and phenotypical similarities and have been suggested to be indistinguishable (Blasi *et al.*, 2011). Thus, DF isolated from Balb/c and C3H mice were tested similarly to MSC. Phenotypic characterisation showed that DF did not express CD90.2, CD105 and CD44 which were expressed by MSC. Following tri-lineage differentiation, DF successfully differentiated into adipocytes, chondrocytes and osteocytes. When tested in one-way MLR, both allogeneic and syngeneic DF suppressed lymphocyte proliferation. Similar suppression was observed when DF were tested in two-way MLR. These findings suggested that, like MSC, DF possessed immunosuppressive properties. However, following chondrogenic differentiation, both allogeneic and syngeneic differentiated DF appeared to have reduced immunosuppressive potency.

Interestingly, when DF were tested in LTA to determine their immunogenicity, both allogeneic (C3H) and syngeneic (Balb/c) DF failed to stimulate Balb/c lymphocyte proliferation. This was also observed when the ratio of stimulator DF to responder cells was increased from 1:1 to 1:5. These results were not expected since allogeneic

MSC had been found to be immunogenic. When the DF were differentiated into chondrocytes however, allogeneic but not syngeneic differentiated DF, stimulated Balb/c lymphocyte proliferation.

In summary, the results obtained in this study showed that both allogeneic and syngeneic MSC were immunosuppressive in one-way MLR. The immunosuppressive properties were also displayed by DF. When tested for their immunoprivileged statuses, allogeneic but not syngeneic MSC, were immunogenic. Surprisingly, allogeneic DF were non-immunogenic under the same conditions used for testing MSC. Following chondrogenic differentiation however, both allogeneic and syngeneic MSC and DF appeared to lose some of their immunosuppressive potency. Both chondrogenic differentiated allogeneic MSC and DF were immunogenic. Therefore, undifferentiated MSC may have utility as immunosuppressive therapies. However, the clinical application of both undifferentiated and differentiated allogeneic MSC may be limited by their immunogenicity. There is need to investigate the potential utility of DF as an alternative for MSC in clinical applications.

It must be emphasized however that these findings were obtained after numerous experiments were carried out in order to create the conditions that were eventually used to obtain the reported findings. Initially, the MLR and LTA produced variable results which were not reproducible. This has been widely reported in studies of this nature using mouse cells. Therefore, during the course of this study, a great deal of effort was invested in testing for the most appropriate conditions for obtaining repeatable experiments. Thus, further experiments discussed in **Section 7.5** which could have been carried out were not completed during the time available.

6.2. Implications of findings

Since the last decade, autologous adult stem cells, particularly bone marrow-derived and adipose-derived MSC, have become widely used as they are affected by neither the ethical obstacles that affect ESC nor the immunological barriers that hinder the use of allogeneic MSC as discussed broadly in **Chapter 1**. The most important feature of autologous MSC is the absence of any immunological risk following application. Since 2008 to date, there have been up to 85 clinical trials (on-going,

completed and currently recruiting) of autologous MSC for the treatment of a wide range of ailments including Parkinson's disease, diabetes mellitus and osteoarthritis (http://clinicaltrials.gov/ct2/home).

Current tissue engineering approaches that utilise autologous MSC have seen some clinical success. In a pioneering case of large organ replacement, autologous MSC obtained from a patient with end-stage bronchomalacia were expanded *ex vivo* and differentiated into chondrocytes which together with epithelial cells, were seeded onto an allogeneic decellularised trachea and developed into a functional organ which was successfully used in the patient (Macchiarini *et al.*, 2008). Importantly, this procedure was characterised by a time lag between harvesting the cells, *ex vivo* expansion and differentiation and bioreactor culture prior to implantation. This delay was not a major problem as in this case, airway replacement is not regarded as an acute life-saving procedure (Grillo, 2002). In acute life threatening conditions however, autologous MSC based approaches, would be unfavourable. Allogeneic MSC would solve this problem as they can be obtained from healthy donors and used to make "off–the-shelf" products either in their differentiated forms in tissue engineered products or in their undifferentiated state as cell therapies with huge commercial benefits for industry as discussed in **Chapter 1**. Allogeneic MSC, as is the case with ESC, face the risk of immunological rejection.

The work carried out in this study therefore sought to investigate the immunological properties of allogeneic MSC in light of recent reports in which allogeneic MSC have been described as possessing immunosuppressive properties in addition to having an immunoprivileged status (Bartholomew *et al.*, 2002; Tse *et al.*, 2003; Ryan *et al.*, 2005; Uccelli *et al.*, 2006). These claims, if true, would enable the application of allogeneic MSC without the risk of immune rejection. The immunosuppressive properties of MSC could be used in the treatment of conditions such as GvHD following HSC transplantation (Toubai *et al.*, 2009) or inflammatory conditions such as Crohn's disease (Newman *et al.*, 2009). The immunoprivileged status would enable the safe application of MSC therapies without immunological risks. Other studies using different *in vitro* and *in vivo* animal models have reported conflicting evidence with regard to the immunoprivilege status of allogeneic MSC (Eliopoulos *et al.*, 2005; Poncelet *et al.*, 2007).

One of the problems that potentially contributed to the conflicting reports with regard to the immunological status of MSC stems from the lack of a universal definition for them. As was discussed previously in **Section 1.3**, the lack of definitive markers for MSC has led to inconsistencies in MSC phenotype. Although the phenotype expressed by the MSC in this study was consistent with that obtained from other studies (Nadri & Soleimani, 2007a; Sung *et al.*, 2008), there were variations with other studies in terms of the expressed markers and the expression levels of specific markers within the MSC cultures. It has been argued that all MSC cultures, including those obtained from single clones, are intrinsically heterogeneous with varying levels of replicative potential (Atala *et al.*, 2009). In both mouse (Kretlow *et al.*, 2008) and human (Stolzing *et al.*, 2008) MSC, it has been demonstrated that MSC "fitness" declined with donor age and passage. These observations, although focused on tri-lineage potential (Kretlow *et al.*, 2008), could have further implications in other aspects of the cells including their immunological properties. Since different studies used MSC obtained from different sources, age and passage, it is conceivable that conflicting findings with regard to the immunological properties of these cells are possible.

In this study, although both early passage and late passage Balb/c and C3H MSC expressed similar phenotypic markers, there were notable differences in the levels of expression for CD105, CD29 and CD90.2 which increased from intermediate to high levels from p3 to p12. These phenotypic variations however, were not further investigated. It was also observed in the literature that attention was not paid to the subtle variations within MSC populations. In light of the potential therapeutic application of MSC, it is important that administered cells are well characterised in terms of their phenotype and potency in order to determine the effective therapeutic doses.

The heterogeneity of MSC cultures also presents a major challenge with regard to the potential application of undifferentiated MSC as cell therapies. As with all therapeutic agents, the potency or dosage of the therapy should be determined using standard tests in order to ensure that effective dosage is administered to bring about the desired effects. With MSC however, it is difficult to determine the relative therapeutic contribution of each cell in culture in light of evidence that MSC potency decreases with age and passage (Kretlow *et al.*, 2008) as well as the existence of

different sub-populations of cells within MSC cultures which exhibit different properties (Crigler *et al.*, 2006; Phinney, 2007). Some of the reported sub-populations derived from MSC cultures include MAPC which were described as pluripotent (Schwartz *et al.*, 2002; Jiang *et al.*, 2002a) and the so-called unrestricted somatic stem cells (USSC) which have also been reported to possess differentiation potential beyond the mesodermal lineages (Kögler *et al.*, 2004). Therefore, with regard to the immunosuppressive properties of MSC, it can be argued that the immunosuppressive properties exhibited by different MSC cultures are always varied due to the types and amounts of immunomodulatory molecules secreted by the different sub-populations thus making potency assays difficult to develop.

In some cases, phenotypic differences have not been found to influence the overall immunomodulatory properties of MSC. In one such study which used a mouse model, $Oct4^+$ and Oct^- MAPC were found to exhibit a similar dose-dependent immunosuppressive effect on allogeneic T and dendritic cells both *in vitro* and *in vivo* (Luyckx *et al.*, 2011). Oct4 is widely used as a marker for ESC and its presence is thought to indicate pluripotency as previosuly mentioned (Jiang *et al.*, 2002a). However, most human MSC isolates are $Oct4^-$ and therefore these findings showed that some differences in marker expression may not alter the immunomodulatory properties of the cells. It is important to note that although similar immunosuppressive potency was observed, the immunogencicity of these cells was not tested.

To date, the exact mechanisms that control MSC immunomodulation are not known which makes it difficult for commercial companies providing "off-the-shelf" therapies based on undifferentiated MSC to define the therapeutic mechanisms which would be important for product description. It is not known also whether the therapeutic properties of MSC cultures are a result of the "typical" MSC or the non-MSC sub-populations or interactions among the different sub-populations. Judging from the evidence, it appears the term MSC is an oversimplification of an otherwise diverse and heterogeneous group of progenitor cells. It is possible therefore, to speculate that commercial companies developing products based on undifferentiated MSC (cell-based) are likely to face more challenges compared to companies making products based on differentiated MSC (tissue engineering-based).

The tri-lineage differentiation assay is often viewed as the gold standard test for distinguishing MSC from other cells which bear phenotypic and morphological resemblance to MSC. This ability had been thought to be a unique feature of stem cells and generally cells that fail this test are not regarded as such (Pittenger *et al.*, 1999). In this study however, DF demonstrated tri-lineage potency similar to bone marrow-derived MSC. During the early stages of this study, this development was not expected as it was thought that only stem cells had the capacity for tri-lineage differentiation. Unlike MSC, the evidence for DF differentiation is controversial. In a study that compared MSC and other cells of mesenchymal origin including primary articular chondrocytes, DF and synovial fibroblasts, only DF, failed to undergo adipogenic and osteogenic differentiation (Jones *et al.*, 2007). Other previous studies had demonstrated DF differentiation into either one or two (French *et al.*, 2004; Wagner *et al.*, 2005; Lorenz *et al.*, 2008) but not all the three cell lineages. More recent studies however, have demonstrated the tri-lineage differentiation capacity of human DF (Junker *et al.*, 2010). This development is particularly interesting as it suggests that DF and other mesenchymal stromal cells may possess similar utility to MSC.

An interesting point to note during differentiation was that the protocols used in this study did not include BMP2 for osteogenic differentiation or TGFβ3 for chondrogenic differentiation. These growth factors have been reported to be essential in stimulating differentiation of MSC and have been used in many MSC differentiation studies. The fact that differentiation was achieved without these factors is particularly interesting. It would have been interesting to determine the extent of differentiation with and without these factors in order to ascertain their necessity as culture medium supplements.

The distinction between MSC and fibroblasts has been a long-standing debate since the discovery of MSC by Friedenstein almost 50 years ago (Friedenstein *et al.*, 1974). In fact, it is still unclear whether fibroblasts can be described as terminally differentiated or precursor cells (Haniffa *et al.*, 2009). Morphologically and phenotypically, MSC and fibroblasts have been reported to be indistinguishable, although in this study MSC but not DF were positive for CD90.2, CD105 and CD44. Although these markers could arguably be used to distinguish mouse MSC from DF in this study, the evidence from other studies suggests that the expression of these

markers in MSC is inconsistent particularly between MSC populations obtained from different sources (Wagner & Ho, 2007). Although DF have been found to express CD90.2, CD105 and CD44 in other studies (Haniffa *et al.*, 2007; Blasi *et al.*, 2011), it is interesting that phenotypic differences have also been found in DF obtained from different body sites (Nolte *et al.*, 2008). Thus, the evidence from the literature clearly demonstrates that our current understanding of the differences between MSC and DF is limited. Thus, despite the subtle differences in phenotype between MSC and DF observed in this study, the morphological and tri-lineage capacity demonstrated by both types of cells suggested that these cells may share similar functional characteristics which potentially could be exploited in the development of clinical therapies.

6.3. Allogeneic and syngeneic MSC and DF are immunosuppressive

Both syngeneic and allogeneic MSC and DF demonstrated significant suppression of the one-way MLR when introduced at the beginning of culture. Similar findings have been reported in a wide range of studies which used human and animal models as discussed in **Section 5.4**. Studies using transwell cultures have demonstrated that the mechanism of suppression involves the secretion of molecules such as IDO, PGE_2, IL-2 and TGFβ and cell to cell contact (Aggarwal & Pittenger, 2005; Uccelli *et al.*, 2006) although the exact mechanisms are yet to be elucidated. Supernatants of MSC cultured alone have been reported to exhibit similar suppression of MLR (Di Nicola *et al.*, 2002; Tse *et al.*, 2003; Liu *et al.*, 2012). This could imply that allogeneic MSC supernatants could be used as potential immunosuppressive therapies. The same cannot be said of DF since it has been demonstrated that supernatants from DF cultures had negligible amounts of immunosuppressive molecules. In fact, cell to cell contact has been shown to be critical for fibroblast-mediated immunosuppression (Sato *et al.*, 1997). Despite differences in the possible mechanisms by which they achieve immunosuppression, this study clearly demonstrated that both allogeneic MSC and DF are immunosuppressive.

The reduced suppressive potency following chondrogenic differentiation however, could suggest that the suppressive properties were dependent on the undifferentiated state. This raises the question whether the chemical supplements used in chondrogenic differentiation medium or that the differentiation process itself

diminished the ability of the cells to suppress lymphocyte proliferation. The latter appears more probable given that the differentiation process changes the cell function and phenotype. This notion was supported by Chen and co-workers who showed that rat MSC partially lost their *in vitro* immunosuppressive properties following chondrogenic differentiation (Chen *et al.*, 2007). Liu and co-workers, using a rabbit skin transplantation model, demonstrated the loss of immunosuppressive potency of autologous osteogenic differentiated MSC *in vivo* (Liu *et al.*, 2006).

Le Blanc and co-workers however, had argued that MSC differentiated into adipocytes, chondrocytes and osteocytes were more potent suppressors of the MLR in comparison to undifferentiated MSC (Le Blanc *et al.*, 2003b). However, their results were highly variable. In some experiments they found that chondrogenic differentiated MSC failed to suppress MLR with lymphocyte proliferation higher than in control MLR (114 %) while in some cases up to 40 % suppression of MLR was observed. Similar variation was also observed in MLR co-cultured with undifferentiated MSC. Although such variation is uncommon, it can be argued that there was need for optimising the experimental conditions in order to minimise inconsistent results as was done in this study. It can be argued therefore, that their conclusion that differentiated MSC are potent immunosuppressors does not fully represent their findings. In this study, suppression of MLR by both syngeneic and allogeneic differentiated MSC was approximately 50 % whereas with undifferentiated MSC it was 90 % which suggested that differentiated MSC lost nearly half of their suppressive potency compared to undifferentiated MSC. This further emphasises the point that differences in experimental conditions may produce conflicting results. The problem of conflicting findings is one of the major challenges affecting stem cell biology and its translation into clinical therapies.

6.4. Allogeneic MSC are not immunoprivileged

Despite the demonstrated capacity of MSC to suppress MLR, the potential clinical utility of allogeneic MSC is dependent on their immunogenicity. This study showed that allogeneic MSC were immunogenic. This is in contrast with many reports in the literature which suggested that they are immunoprivileged (Klyushnenkova *et al.*, 2005; Ryan *et al.*, 2005; Le Blanc & Ringden, 2007; Poncelet *et al.*, 2007; Uccelli *et al.*, 2008; Sensebé *et al.*, 2010).

The basis of MSC immunoprivilege is thought to be due to their lack of MHC II and co-stimulatory molecules CD80 and CD86 as previously discussed in **Section 1.8.1** and **Section 5.4**. This study however, argued that despite their phenotype, allogeneic MSC would be rejected via the indirect and semi-direct pathways of allorecognition which do not require the stimulator cells to express MHC II or co-stimulatory molecules as discussed in **Section 1.7.2**. In order to capture the indirect and semi-direct response *in vitro*, longer incubation periods and culture medium changes would be required. Therefore, medium changes each 3 day period and longer incubations up to 15 days were introduced in this study. To date, this strategy has not been used in any study in the literature. Given that most of the *in vitro* LTA in the literature have been carried out within 5 days without medium changes, it is probable that such conditions did not capture the indirect and semi-direct responses. It is therefore possible to speculate that the discrepancies between *in vitro* and *in vivo* experiments in which allogeneic MSC have been reported to be immunoprivileged in the former but immunogenic in the latter (Nauta *et al.*, 2006; Sudres *et al.*, 2006) could have been partly due to the shorter incubation periods and nutrient depletion encountered *in vitro*.

The importance of experimental conditions was highlighted in this study particularly the effect of changing culture medium. As mentioned previously, experiments in which medium was not changed did not result in notable stimulation of Balb/c lymphocytes by allogeneic C3H MSC. Given that MSC cultured alone have been previously reported to constitutively secrete immunosuppressive molecules (Di Nicola *et al.*, 2002; Liu *et al.*, 2012), it can be argued that when MSC are co-cultured with allogeneic lymphocytes, these immunosuppressive molecules may inhibit allogeneic responses. Without medium changes, an accumulation of inhibitory molecules would therefore quench lymphocyte proliferation in the presence of allogeneic MSC. With medium changes however, the inhibitory molecules would be diluted which would result in the capture of the allogeneic response.

Chondrogenic differentiated C3H allogeneic MSC also stimulated Balb/c mononuclear cells. This was supported by several recent studies in the literature (Liu *et al.*, 2006; Chen *et al.*, 2007; Huang *et al.*, 2010; Technau *et al.*, 2011). Chen and co-workers showed that rat MSC expressed the co-stimulatory molecules CD80 and CD86 in addition to elevated levels of MHC I following chondrogenic

differentiation. They argued that CD80 and CD86 may act as an alternative to MHC II and elicit immune rejection (Chen *et al.*, 2007). In their experiments, chondrogenic differentiated MSC, but not undifferentiated MSC stimulated human PBMC. They also found that CD83 (marker for DC maturation) expression on human DC was elevated in the presence of the chondrogenic differentiated rat MSC. After blocking CD80 and CD86 on the chondrogenic differentiated rat MSC using monoclonal antibodies, CD83 expression on the human DC was significantly reduced. Although this was a xenogeneic model, which is different from the allogeneic model used in this study, they found that removing the DC from the cultures led to a significant reduction in human lymphocyte proliferation. This demonstrated the importance of APC in the stimulation of responding lymphocytes. Despite the authors not mentioning it, this potentially demonstrated the role of the indirect and semi-direct responses in the xenogeneic response. The use of unseparated mononuclear cells in this study allowed the presence of APC to facilitate the rejection of allogeneic MSC. Although an experiment to demonstrate the role of APC was not carried out during this study, Klyushnenkova and co-workers showed that undifferentiated human allogeneic MSC failed to stimulate separated $CD4^+$ and $CD8^+$ lymphocyte proliferation but stimulated allogeneic PBMCs (Klyushnenkova *et al.*, 2005).

Allogeneic C3H DF failed to stimulate responder Balb/c mononuclear cells under the same conditions in which C3H MSC stimulated Balb/c lymphocyte proliferation. This suggested that allogeneic DF are immunoprivileged whilst allogeneic MSC are not. Although DF have been widely used as control cells in MSC studies (Jones *et al.*, 2007), the evidence with regard to their immunogenicity is equally controversial as that for MSC. In previous studies, human DF failed to stimulate a proliferative response in allogeneic lymphocyte whereas allogeneic smooth muscle, endothelial and epidermal cells stimulated significant response (Theobald *et al.*, 1993). Similar findings had been observed using mouse allogeneic DF in earlier studies (Wagner & Wyss, 1973).

Interestingly, this was contradicted in *in vivo* studies in which mouse DF transfected with viral proteins directly induced CD8 lymphocytes in lymphoid organs in the absence of professional APC suggesting that allogeneic DF are capable of initiating allogeneic immune responses (Kundig *et al.*, 1995). Also, in mouse skin transplantation studies in which allogeneic DF were used as feeder cells for

supporting autologous grafts it was shown that allogeneic DF initiated inflammatory responses that led to delayed breakdown of grafts (Hultman *et al.*, 1996). This evidence demonstrated that allogeneic DF are immunogenic. Phenotypic difference between MSC and DF were observed in this study as described in **Section 3.4** and **Section 6.1**. In addition, MSC cultured alone have been reported to constitutively express immunomodulatory molecules (Liu *et al.*, 2012) whereas DF did not (Sato *et al.*, 1997). These differences could imply that MSC and DF use distinct mechanisms to interact with other cells. Chondrogenic differentiated C3H DF however, like chondrogenic differentiated C3H MSC, stimulated Balb/c mononuclear cells.

In a study using human foreskin derived fibroblasts and MSC, it was shown that the immunosuppressive mechanisms employed by these fibroblasts was different from those used by MSC. Unlike the MSC, the fibroblasts were incapable of osteogenic and adipogenic differentiation and did not secrete IDO and NO when co-cultured with PBMC. They however strongly expressed IFNα which suggested that this molecule may be involved in the fibroblast immunosuppressive mechanisms (Wada *et al.*, 2010). This supported earlier findings by Sato and co-workers (Sato *et al.*, 1997) and provided strong evidence that fibroblasts employ different immunomodulatory mechanisms to MSC.

Importantly, the DF used in this study had a shorter *ex vivo* culture period in comparison to MSC due to their fastidious growth and slow growth of MSC. This could potentially have implications to the findings obtained. It is known that prolonged culture periods has affects the properties of *ex vivo* cultured cells. Thus it is possible to speculate that the undifferentiated DF used had less time in culture and hence failed to stimulate allogeneic lymphocytes whereas MSC did. However, following chondrogenic differentiation, which allowed for longer ex vivo culture, DF stimulated allogeneic lymphocytes.

6.5. Future studies

This study investigated the immunogenicity of allogeneic MSC in light of their reported immunoprivilege. Although both undifferentiated and chondrogenic differentiated MSC were found to be immunogenic, further investigations are necessary in order to fully understand the basis of their immunogenicity.

With regard to MSC and DF characterisation, it would be important to quantify the extent of differentiation. For instance, adipogenesis can be quantified by using a simple spectroscopic method to determine the amount of ORO dye taken up by differentiated MSC cultures or computer image analysis which use densitometry to determine the amount of dye taken up by differentiated MSC (Szczepaniak *et al.*, 1999; Chen and Farese, 2002; Foster *et al.*, 2005; Gorjup *et al.*, 2009). Monitoring of calcein intake can be used to quantify the extent of mineralisation during osteogenic differentiation (Uchimura *et al.*, 2003). Quantification of GAGs can be used to determine the extent of chondrogenic differentiation (Lü *et al.*, 2012). Measurement of differentiation would enable systematic analysis of the immunomodulatory properties of MSC during differentiation and can provide important insights on the implications of MSC differentiation in clinical applications.

It would be of interest to investigate the mechanisms that underline the interaction of allogeneic MSC and mononuclear cells. In line with the hypothesis of this study, the interactions between allogeneic MSC and $CD8^+$ or $CD4^+$ lymphocytes in the presence and absence of APC such as DC would provide further insights with regard to the role of the indirect and semi-direct pathways of allorecognition. CD3 specific antibodies could be used to isolate both $CD4^+$ and $CD8^+$ lymphocytes which could then be co-cultured with allogeneic MSC. This would show whether APC are required for the allogeneic response. The absence of APC in MLR and LTA only measures the direct pathways and therefore can be used to determine whether the indirect or semi-direct mechanisms are involved in MSC immunomodulation since APC are required for these pathways. Another approach may involve the co-culture of MSC and MHC I blocked (using specific antibody) $CD4^+$ allogeneic lymphocytes. Since MSC lack MHC II, this experiment would show whether the indirect pathway is involved in allorecognition as the MSC will be incapable of initiating an immune response via the direct pathway.

Use of the antibiotic cerulenin (Falo *et al.*, 1987), or the endoprotease cathepsin S (Riese *et al.*, 1998) which have been reported to inhibit antigen processing and presentation by mouse and human APC, would also enable the determination of the indirect and semi-direct pathways. Since these pathways rely on antigen processing and presentation by responder APC, inhibition of APC activity would exclude the indirect and semi-direct pathways and therefore allow for stimulation to occur only

by the direct pathway. This would allow for the investigation of the immunogenicity of MSC and the role of APC.

The interaction between $CD4^+CD25^+FoxP3^+$ T-regs and MSC remains unknown although it is thought that MSC may promote T-regs proliferation and activation. T-regs can be isolated from mononuclear cell populations using flow cytometry. This could allow experiments such as MLR and LTA to be carried out with and without T-regs in order to determine their role in MSC immunomodulation.

Another approach would involve the detection and measurement of secreted cytokines and other immunomodulatory molecules during LTA particularly before and at the time when lymphocyte proliferation is detected. Although previous studies have implicated several cytokines such as TGFβ, IDO, PGE_2 and NO as having an immunosuppressive role, none of these molecules has been found to be indispensable nor have they been found to play a part in conferring an immunoprivilege status to MSC. In addition, MSC have been reported to constitutively secrete these molecules in monocultures. By measuring their quantities, it would be interesting to investigate whether the allogeneic response is detected with and without medium changes. MSC secretion of TGFβ is particularly interesting since it has been reported to mediate estradiol inhibition of antigen presentation in rat APC. Studies using MSC have shown that TGFβ may also be involved in the inhibition of myeloid DC, $CD4^+$, $CD8^+$ and B cells (Uccelli *et al.*, 2008). IL-10 has been shown to inhibit the antigen presenting capacity of macrophages (Möttönen *et al.*, 1998). Since MSC are known to constitutively secrete IL-10, it would be of interest to investigate whether IL-10 secretion by MSC plays any role in modulating the allogeneic response.

For chondrogenic differentiated allogeneic MSC, it would be necessary to investigate whether the differentiation process results in the expression of co-stimulatory molecules CD80 and CD86 as has been previously reported (Chen *et al.*, 2007). Whether the expression of these molecules alone confers immunogenicity or the presence of APC in mononuclear cells facilitates allorecognition via the indirect and semi-direct pathways warrants further investigation in order to fully understand the mechanisms behind the immunogenicity of differentiated MSC.

With regard to the reduction in immunosuppressive potency of chondrogenic differentiated MSC; measurement of cytokine expression at various stages of MLR may shed more light on the role of the cytokines in mediating allogeneic suppression.

Long term *in vivo* studies to investigate the implications of transplanted allogeneic MSC could be carried out using immunocompetent mice. Both differentiated and undifferentiated allogeneic MSC could be tested.

In light of the failure by C3H DF to stimulate Balb/c mononuclear cells, the immunogenicity of allogeneic DF requires further investigation. Fibroblasts (Boots *et al.*, 1994), but not MSC have been reported to express MHC II and function as APC when exposed to IFNγ. This would provide further insights into the possible differences between MSC and DF mediated immunomodulation.

The distinction between MSC and DF requires further investigation. The phenotypic differences observed in this study require further investigation. This could provide the markers that can be used to distinguish MSC from fibroblasts. Currently, the distinction between MSC and fibroblasts is vague and mostly based on the source which the cells are obtained from. In terms of function, phenotype and morphology, there are more similarities than differences between the two types of cells (Haniffa *et al.*, 2009; Blasi *et al.*, 2011). Given the findings of this study, like MSC, DF could potentially be exploited in tissue engineering and regenerative medicine given the ease by which they can be obtained.

6.6. The future of MSC therapies

The prospect of commercial MSC products reaching the market is growing as the understanding of MSC biology improves. However, there is no evidence to date that conclusively show that allogeneic MSC are immunoprivileged and safe to use either as undifferentiated MSC therapies or differentiated MSC tissue engineered products in immunocompetent patients.

Pluristem Theraupetics Inc, a company based in Israel claimed that its allogeneic MSC product PLX-1 aimed at addressing the global shortfall of matched tissue or bone marrow transplantation, was immunoprivileged (Prather, 2008). However, the testing has not completed the clinical trial phase and to date no evidence of the

efficacy of these cells is known. It was claimed in May 2012 that intramuscular injection of these cells successfully treated a patient with bone marrow aplasia for whom previous allogeneic MSC transplantation procedures had failed. Due to the absence of functional immune cells in the patient, the use of allogeneic MSC was safe but did not prove whether these cells were immunoprivileged.

Prochymal®, an allogeneic MSC based product developed by Osiris Inc for the treatment of severe GvHD (Le Blanc *et al.*, 2008; Wolf & Wolf, 2008) and acute myocardial infarction showed promising results in phase I and II trials but phase III and IV studies failed. Also, phase II trials of the same product for Crohn's disease were discontinued by the company after patients taking placebo fared better than patients treated with the product (Allison, 2009). Despite the failures, Prochymal® was recently registered in May 2012 in Canada as the first "off-the-shelf" allogeneic MSC product for the treatment of severe GvHD in children subject to post clinical studies (http://www.stemcelldigest.net, 2012).

Cephalon Inc, a company based in USA announced its investment of US$1.7 billion in Mesoblast Inc, an Australian company, for the development and commercialisation rights for its allogeneic MSC product Revascor® which is currently under trials for the treatment of congestive heart failure. The product has shown promising phase I and II results (http://www.mesoblast.com, 2012). Recently, TEVA Ltd acquired Cephalon Inc to give the company a value of US$20 billion. This demonstrates the huge commercial investments that are being put into the development of "off-the-shelf" MSC products.

Despite the increased interest in MSC therapies, allogeneic rejection has remained the major challenge with regard to the use of allogeneic MSC. No predictive assays for MSC treatment have been developed to date. This means that the actual performance of MSC therapies cannot be determined. Due to their heterogeneity, the clinical application of undifferentiated MSC faces major challenges. For instance, Mesoblast Inc define their Revascor® MSC as $CD34^{+}CD117^{+}$ (Kocher *et al.*, 2001) whereas Cephalon Inc's Prochymal® are defined as cells expressing more than 90 % of CD73, CD90 and CD105 while being negative for CD34, CD45, CD14 and CD3 (Wolf & Wolf, 2008). The lack of standardisation within the stem cell industry will

continue to hamper progress until MSC are fully defined and the problem of heterogeneity addressed.

6.7. Concluding remarks

Development of autologous MSC therapies as immunosuppressors for treatment of autoimmune diseases is promising. Tissue engineering solutions that utilise autologous MSC, though practically and commercially unfavourable, provide the safest method for tissue repair and regeneration. Despite the practical advantages of allogeneic MSC over autologous MSC and their commercial appeal, their clinical application is hampered by their immunogenicity. Due to the huge investments in MSC research, the immunogenicity of allogeneic MSC will be resolved in the near future. Also, the potential of DF warrants further research into their application in tissue engineering and cell based therapies.

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8. APPENDIX

8.1. General equipment

The equipment listed in **Table 8.1** was used throughout the study

Table 8.1. Equipment used with manufacturer/supplier and model

Equipment	Model/size	Source
Analytical balance	AE50	Mettler Toledo, Leicester, UK
Cell counting chamber	improved Neubauer	Thermo Fisher Scientific, Loughborough, UK.
Cell harvester	FilterMate®	Perkin Elmer, Boston, USA.
Cell sieves	100 μm	Sigma-Aldrich Company Ltd, Dorset, UK
Centrifuge	Harrier 15/80	SANYO Europe BV, Biomedical Division, Loughborough, UK
Centrifuge	Microcentaur	MSE Ltd, London, UK
CO_2 Incubator	MCO-20AIC	SANYO Biomedical Europe BV, Amsterdam, Holland
CO_2 Incubator	MCO-17A	SANYO Biomedical Europe BV, Amsterdam, Holland
Cryo-freezing container	Nalgene "Mr. Frosty" 5100	Thermo Fisher Scientific, Loughborough, UK
Culture medium aspirator	VACUSAFE comfort	Integra Biosciences, Zurich, Switzerland.
Digital camera	Evolution MP	Media Cybernetics, Berkshire, UK.
Digital scale	GR-200-EC	A&D Scales, Devon, UK
Flow cytometer	BD-FACSCalibur™	Becton Dickinson, San Jose, USA.
Flow cytometer	BD-LSRFortessa™	Becton Dickinson, San Jose, USA.
Flow cytometer	Partec PAS III	Cronus Technologies, Hampshire, UK
Fridge	Electrolux ER8817C	Jencons PLC, Bedfordshire, UK.
Freezer (-20 ºC)	Electrolux 3000	Jencons PLC, Bedfordshire, UK.
Freezer (-80 ºC)	MDF U74V VIP™	SANYO Europe BV, Biomedical Division, Loughborough, UK
Hot air oven	OMT225.XX2.C	Weiss-Gallenkemp, Loughborough, UK
Incubator	-	Laboratory and Electrical Engineering Company, Nottingham, UK
Inverted microscope	Olympus, IX71	Olympus optical Co. Ltd., Southall, UK

Light microscope	Olympus CK40-SLP	Olympus optical Co. Ltd., Southall, UK
Liquid nitrogen Dewar	BIO65	Jencons PLC, Bedfordshire, UK.
Liquid scintillation and luminescence counter	TopCount® NXT C9902	Perkin Elmer, Boston, USA.
Orbital microplate shaker	IKA KS130	VWR-Jencons, Leicestershire, UK
pH Meter	3020	Jenway Ltd, Dumor, UK
Gilson pipettes	Gilson P2, P20, P200, P1000	Gilson Inc., Middleton, USA
Pipettes	PIPETBOY *acu*	Integra Biosciences AG, Switzerland
Refrigerated centrifuge	Harrier 18/80	SANYO Europe BV, Biomedical Division, Loughborough, UK
Refrigerated centrifuge	5418	Eppendorf, Cambridge, UK
Water bath	Clifton	Scientific Laboratory Supplies, Nottingham, UK

8.2. General consumables

Disposable and consumable items used in the study are listed in **Table 8.2**.

Table 8.2. Disposable laboratory consumables used with suppliers

Item	Source
BD FACSFlow	Becton Dickinson, Oxford, UK
Benchkote protection paper	Scientific Laboratory Suppliers, Nottingham, UK
CO_2 gas	BOC Ltd, West Sussex, UK
Filter paper Whatman® (grade 1-5)	Scientific Laboratory Suppliers, Nottingham, UK
Glass coverslips MIC3228	Scientific Laboratory Suppliers, Nottingham, UK
Liquid nitrogen gas	BOC Ltd, West Sussex, UK
Ministat 0.2 μm syringe filters	Sartorius Ltd, Surrey, UK.
Nitrile powder free gloves	STARLAB Ltd., Milton Keynes, UK
Parafilm M	Scientific Laboratory Suppliers, Nottingham, UK
Pasteur pipettes	Alpha Laboratories Ltd, Hampshire, UK.
Scalpel blades size 22	Thermo Fisher Scientific, Loughborough, UK
Sheath fluid for flow cytometer	Becton Dickinson, Oxford, UK
Sterile scalpel blades #22	Thermo Fisher Scientific, Loughborough, UK.
18G x 1” hypodermic syringe needles	Terumo Medical Corporation, New Jersey, USA

Trigene	Scientific Laboratory Suppliers, Nottingham, UK
TopSeal™-A self-adhesive for 96-well plates	Perkin Elmer, Berkshire, UK.
Virkon	Bios Europe Ltd, Lancashire, UK

8.3. Plastic ware

Plastic ware used in the study is listed in **Table 8.3**.

Table 8.3. Disposable plastic ware used with sizes and suppliers

Item	Brand & Size	Source
6-well plates (sterile)	Flat-bottom, Nunclon™ Δ surface	Thermo Fisher Scientific, Loughborough, UK.
24-well plates (sterile)	Flat-bottom, Nunclon™ Δ surface	Thermo Fisher Scientific, Loughborough, UK.
96-well glass filter bottomed plates for TopCount™	UniFilter™ GF/B 96	Perkin-Elmer, Berkshire, UK.
96-well plates for TopCount™	OptiPlate™-1536, white, flat-bottomed	Perkin-Elmer, Berkshire, UK
96-well plates	U-bottom, Nunclon™ Δ surface	Thermo Fisher Scientific, Loughborough, UK.
96-well plates (γ-irradiated)	U-bottom, Uncoated	Sterilin Ltd, Staffordshire, UK.
96-well plates	V-bottom, Nunclon™ Δ surface	Thermo Fisher Scientific, Loughborough, UK.
96-well plates (γ-irradiated)	V-bottom, Uncoated	Sterilin Ltd, Staffordshire, UK.
Bijous	5 ml	Scientific Laboratory Suppliers, Nottingham, UK
Cell culture flasks	Nunclon™ Δ surface: 25 cm^2, 75 cm^2 and 125 cm^2	Thermo Fisher Scientific, Loughborough, UK.
Centrifuge tubes	Corning™ 15 ml and 20 ml	Thermo Fisher Scientific, Loughborough, UK.
Cryovials	Cryotub™ 1.5 ml	Thermo Fisher Scientific, Loughborough, UK.
Disposable plastic pippetes (sterile)	Stripette™ 1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Sigma-Aldrich, Surrey, UK.
Disposable plastic pippetes (sterile)	1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Corning Costar Ltd., High Wycombe, UK.
Lids for 96-well Sterilin plates (γ-irradiated)	-	Sterilin Ltd, Staffordshire, UK.

Microfuge tubes	Eppendorf™ 1.5 ml	VWR international, West Chester, UK.
Petri-dishes	50 mm (dia) x 12 mm (H) 100 mm (dia) x 15 mm (H)	Sterilin Limited, Staffordshire, UK.
Pipette tips (non-sterile)	2 µl, 10 µl, 20 µl, 200 µl and 100 µl	STARLAB Ltd., Milton Keynes, UK
Pipette tips (sterile endotoxin free)	2 µl, 10 µl, 20 µl, 200 µl and 100 µl	STARLAB Ltd., Milton Keynes, UK
Plastic syringes	2 ml, 5 ml, 10 ml and 20 ml	Scientific Laboratory Suppliers, Nottingham, UK
Plastic syringes	2 ml, 5 ml, 10 ml and 20 ml	Terumo Medical Corporation, New Jersey, USA
Specimen pots	60 ml, 150 ml and 250 ml	Scientific Laboratory Suppliers, Nottingham, UK
Test tubes	10 ml	Scientific Laboratory Suppliers, Nottingham, UK.
Universal tubes	25 ml	Scientific Laboratory Suppliers, Nottingham, UK

[dia; diameter. H; height. ml; millilitre. µl; microlitre. mm; millimetre. cm^2; square centimetre]

8.4. Reagents and chemicals

Reagents and chemicals used in the study are listed in **Table 8.4**;

Table 8.4. Reagents and chemicals used with suppliers

Item	Source
Alcian blue stain	Sigma-Aldrich, Surrey, UK.
Alcian blue stain (1 % v/v) in acetic acid	Biostain Ready Reagents, Manchester, UK.
Alizarin red S dye	Sigma-Aldrich, Surrey, UK.
Ascorbate -2-phosphate	Sigma-Aldrich, Surrey, UK.
Ammonium hydroxide	Sigma-Aldrich, Surrey, UK.
ATPLite™ assay kits	Perkin-Elmer, Boston, USA.
β-glycerophosphate	Sigma-Aldrich, Surrey, UK.
β-mercaptoethanol	Sigma-Aldrich, Surrey, UK.
Camptothecin	Sigma-Aldrich, Surrey, UK.
CFSE	Sigma-Aldrich, Surrey, UK.
Collagenase 1A	Sigma-Aldrich, Surrey, UK.
CountBright™ fluorescent counting beads	Life Technologies Inc, Paisley, UK.

for flow cytometry; (excitation, UV– 635 nm; emission, 385–800 nm)	
Dexamethasone	Sigma-Aldrich, Surrey, UK.
Dimethyl sulphoxide (DMSO)	Life Technologies Inc, Paisley, UK.
Dispase II	Sigma-Aldrich, Surrey, UK.
DMEM-High glucose (4.5 $g.L^{-1}$) without L-glutamine	Lonza, Slough, UK.
DMEM-Low glucose (1 $g.L^{-1}$) with L-glutamine	Life Technologies Inc, Paisley, UK.
Ethanol (100%)	Thermo Fisher Scientific, Loughborough, UK.
Foetal calf serum (FCS)	Lonza, Slough, UK.
Hank's balanced salt solution (HBSS)	Life Technologies Inc, Paisley, UK.
HEPES buffer	Lonza, Slough, UK.
Hy-Q-Tase™ cell dissociation solution	Thermo Fisher Scientific, Loughborough, UK.
IBMX (3-isobutyl-1-methylxanthine)	Sigma-Aldrich, Surrey, UK.
Indomethacin	Sigma-Aldrich, Surrey, UK.
Insulin (10 $mg.ml^{-1}$) solution	Sigma-Aldrich, Surrey, UK.
Insulin-Transferrin-Selenium-X supplement (ITS)	Life Technologies Inc, Paisley, UK.
Isopropanol	Thermo Fisher Scientific, Loughborough, UK.
L-glutamine	Life Technologies Inc, Paisley, UK.
Lymphoprep™ density gradient medium	Axis-Shield, Kimbolton, UK.
MesenCult™ mesenchymal stem cell basal medium	STEMCELL Technologies, Manchester, UK.
MesenCult™ mesenchymal stem cell medium stimulatory supplements	STEMCELL Technologies, Manchester, UK.
Methanol (100%)	Merck, Middlesex, UK.
Microscint-20™ scintillation fluid	Perkin-Elmer, Berkshire, UK.
Minimum essential medium (α-MEM)	Lonza, Slough, UK.
Mitomycin C	Sigma-Aldrich, Surrey, UK.
Mouse serum	Autogen Bioclear
Neutral buffered formalin (10 % v/v)	Sigma-Aldrich, Surrey, UK
Oil red O dye	Sigma-Aldrich, Surrey, UK.
Penicillin-Streptomycin mixture	Lonza, Slough, UK.
Phosphate buffered saline (PBS) solution	Lonza, Slough, UK.
Phytohaemagglutinin (PHA)	Sigma-Aldrich, Surrey, UK.

Purelab Option distilled water	ELGA LabWater, High Wycombe, UK.
RPMI-1640 medium without L-glutamine	Lonza, Slough, UK.
StemXVivo™ human and mouse MSC medium	R&D Systems, Oxford, UK
Sodium chloride	Merck, Middlesex, UK.
Sodium pyruvate	Helena Biosciences, Gateshead, UK
Trichloroacetic acid (TCA)	Thermo Fisher Scientific, Loughborough, UK.
Trigene	Scientific Laboratory Suppliers, Nottingham, UK
Tritiated (^{3}H) thymidine (Low activity) NET027001MC	Perkin-Elmer, Berkshire, UK.
Trypan blue (0.4%) dye solution	Sigma-Aldrich, Surrey, UK.
Trypsin 0.5% with EDTA (10x)	Life Technologies Inc, Paisley, UK.
Via-Probe™ 7-amino actinomycin-D (7-AAD) cell viability probe	BD Bioscience, Oxford, UK

[EDTA; Ethylenediaminetetraacetic acid. HEPES; N-(2-hydroxyethyl) piperazine N;-(2-ethansulphonic acid. RPMI-1640; Rosslyn Park Memorial Institute. DMEM; Dulbecco's modified Eagles medium. CFSE; Carboxyfluorescein succinimidyl ester]