Adipose-Derived Regenerative Cells and Secretions for the Treatment of Canine Osteoarthritis

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ABSTRACT

The emergence of cellular therapies in the last fifteen years has seen a paradigm shift in healthcare not only in human medicine but in veterinary medicine also. The use of mesenchymal stem cells (MSCs) from bone marrow, cord blood or adipose tissue has been used in the treatment of both equine and canine musculoskeletal disorders since the early 2000's. Little is known however, about the therapeutic mechanisms of these treatments. This thesis documents the cell types and secretion capabilities of adipose-derived therapeutics. It details the evolution of a treatment offering for canine osteoarthritis (OA), from an autologous freshly derived point-of-care treatment to an allogeneic culture-expanded off-the-shelf therapeutic.

Whilst some characterisation of canine MSCs has occurred *in vitro*, there is little known about the cell composition and secretion capabilities of the mixed cell population derived from adipose tissue, known as the stromal vascular fraction (SVF). Preliminary *in vivo* efficacy using the canine SVF was demonstrated in dogs suffering from OA. Whilst historically the differentiation capability of MSCs was thought to be the major driver of therapeutic efficacy, the early treatment response seen in these dogs was far too rapid to be due to tissue regeneration. Furthermore, it is now well established in the literature that the paracrine secretions of MSCs are a key contributing factor to therapeutic effect of these cells.

To determine the cell types and composition of the canine SVF and the canine MSCs themselves, an *in vitro* characterisation utilising cluster differentiation antibodies and flow cytometry was performed. Additionally, the secretion profile of the cell types of the SVF in combination and the MSCs alone was carried out using a multi-plex cytokine analysis approach. This work demonstrated the cells of the SVF secreted therapeutically relevant cytokines and furthermore a correlation between the cells types present and the secretion profile was shown.

To further exploit the apparent therapeutic benefit of the cellular secretions, a concentrated cell culture conditioned medium containing the secretions, was added to culture-expanded MSCs. To determine the efficacy of MSCs cryopreserved with cellular secretions, a mouse model of induced arthritis was utilised. The results of this trial showed that the addition of secretions to MSCs had a superior ability as an

arthritis-relieving agent to that of MSCs alone. Furthermore, the use of MSCs with secretions was shown to have the same clinical efficacy in canine OA as the freshly derived SVF therapeutic.

DECLARATION

I certify that the work in this thesis entitled "Adipose-Derived Regenerative Cells for the Treatment of Canine Osteoarthritis" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by the Regeneus Ltd Animal Ethics Committee in the following applications:

AEC Proposal Number - RE006
AEC Proposal Number - RE011
AEC Proposal Number - RE012

These proposals were acknowledged and approved by the Macquarie University Animal Ethics Committee at their meeting on 17 May 2012.

The University of Queensland's Animal Ethics Committee approved the research presented in Chapter 5. Confirmation of this approval was provided to the Macquarie University Animal Ethics Committee.

Animal Ethics Approval:

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Date

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PUBLICATIONS

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Webster, R.A., Blaber, S.P., Herbert, B.R., Breen, E.J., Vesey, G., *A Comparison of Canine Adipose-Derived Mesenchymal Stem Cells, With and Without the Addition of Cellular Secretions in the Treatment of a Mouse Model of CAIA.* Open Journal of Regenerative Medicine, 2014. Submitted for publication

Webster, R.A., Blaber, S.P., Herbert, B.R., Wilkins, M.R., Vesey, G., *The role of mesenchymal stem cells in veterinary therapeutic – a review.* New Zealand Veterinary Journal, 2012. 60(5): p. 265-272.

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Patent:

Australian patent application AU 2013203072 Entitled *"Therapeutic Methods and Compositions Comprising Cells and Secretions".* Examined and accepted by the Australian Patent Office. An international PCT application has been filed (WO_2013_040649_A1).

ABBREVIATIONS

ABAM	Antibiotic-antimycotic
ACI	Autologous chondrocyte implantation
ADL	Activities of daily living
AD-MSC(s)	Adipose-derived mesenchymal stem cells Delta area under the curve
ANOVA	Analysis of variance
AOFAS	American orthopedic foot and ankle score
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSC(s)	Bone marrow-derived mesenchymal stem cells
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CAIA	Collagen antibody-induced arthritis
CD	Cluster of differentiation
CFU-F	Colony forming units-fibroblasts
CIA	Collagen-induced arthritis
cm	Centimeter(s)
CO_2	Carbon dioxide
CT	Computed tomography
DC	Dendritic cell
DMEM (HG)	Dulbecco's modified eagle medium (High glucose)
DMSO	Dimethyl sulfoxide
EdU	Ethynyl-2′-deoxyuridine
ELISA(s)	Enzyme-linked immunosorbent assay
ESC(s)	Embryonic stem cells
FABP4	Fatty acid-binding protein
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSD	German shepherd dog
GVHD	Graft-versus-host disease
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HSC(s)	Hematopoietic stem cells
HTO	High tibial osteotomy
IBMX	3-isobutyl-1-methylxanthine
IKDC	International knee documentation committee
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
IFATS	International Federation for Adipose Therapeutics and Science
IFN	Interferon
IGF	Insulin-like growth factor
IM	Intramuscular
iPS	Induced pluripotent stem cells
iTRAQ	Isobaric tags for relative and absolute quantitation
IP	Interferon gamma-induced protein
ISCT	International Society Cellular Therapy
IV	Intravenous
KC	Keratinocyte chemoattractant
kg	Kilogram(s)
KOOS	Knee osteoarthritis outcome score
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
M	Molar
M (1 & 2)	Macrophage type 1 or type 2
MCP	Monocyte chemoattractant protein

M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma-interferon
MIP	Macrophage inflammatory protein
mL	Milliliter
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinases
MOCART	Magnetic resonance observation of cartilage repair tissue scoring system
MRI	Magnetic resonance imaging
MSC(s)	Mesenchymal stem cells
n	Number
NaOH	Sodium hydroxide
NK	Natural killer
NO	Nitric Oxide
NRS	Numerical rating scale
NSAIDS	Non steroidal anti-inflammatory drugs
O_2	Oxygen
OA	Osteoarthritis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PGE_2	Prostagalandin E_2
PI	Propidium iodide
PRP	Platelet rich plasma
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T cell expressed and secreted RNA Ribonucleic acid
RCT	Randomized clinical trial
RNA	Ribonucleic acid

ROM	Range of motion
SC	Sub-cutaneous
SD	Standard deviation
SEM	Standard error of the mean
SSC	Side scatter
SVF	Stromal vascular fraction
TGF	Transforming growth factor
Th	T- helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
Treg	T-regulatory cell
UCB-MSC(s)	Umbilical cord blood-derived mesenchymal stem cells
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WAT	White adipose tissue
WBC(s)	White blood cell/s
WORMS	Whole-organ magnetic resonance imaging score
xg	Units of centrifugal force, i.e. earth's gravitational acceleration
α	Alpha
β	Beta
γ	Gamma
µm	Micron
µM	Micromolar

CHAPTER ONE

Introduction

This chapter provides a broad overview of regenerative medicine and an historical summary of the relevant types of stem cells involved in cellular regenerative medicine, in particular, mesenchymal stem cells (MSCs), This introduction discusses the relevant sources of MSCs and the therapeutic properties of these cells. As the aim of this thesis was to investigate the regenerative properties of adipose-derived MSCs for the treatment of canine osteoarthritis (OA), this introduction also outlines the current status of cellular therapy in veterinary medicine and the role of MSCs and their secretions in the treatment OA. Additionally, a review of adipose tissue and its cell types and secretions is provided. Furthermore this chapter discusses the advantages and limitations of two methods to provide an in-clinic MSC cellular therapeutic; a point-of-care freshly derived cellular treatment and a readily available off-the-shelf cryopreserved therapeutic. Lastly this introduction provides an overview of the aims of the research undertaken in this thesis.

Outputs from this Chapter

Webster, R.A., Blaber, S.P., Herbert, B.R., Wilkins, M.R., Vesey, G., *The role of mesenchymal stem cells in veterinary therapeutic – a review.* New Zealand Veterinary Journal, 2012. 60(5): p. 265-272.

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1.1. Regenerative Medicine – Cellular Therapy

The term regenerative medicine was first used just two decades ago when Leland Kaiser used the term in a report written in 1992 entitled "The Future of Multihospital Systems [1]." He described in this report what he thought 21st century health care would entail. He touched on genetic engineering, nanotechnology, and a future of holographic imaging and artificial intelligence diagnosis. In a paragraph entitled "Regenerative Medicine" he stated "A new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems" [1]. Despite Kaiser's prescient terminology, the notion of treating chronic disease by repairing, regenerating or replacing cells, tissue or even organs through biomedicine began much earlier than this. The earliest example of regenerative medicine was the first successful bone marrow transplant (now known as a hematopoietic stem cell transplant) in 1956 to replace cells of the blood after myeloablation [2].

Cellular therapy represents one arm of regenerative medicine and most commonly involves the use of stem cells derived from either adult tissues or embryos. Therapeutic applications are numerous and include cancer vaccines, immune therapy and treatment of pathologies such as heart disease, diabetes, and orthopedic or neurological conditions [3, 4]. Cellular treatments provide a therapeutic option, which in contrast to traditional synthetic drug therapies, do not rely on a single target receptor or pathway for their action. Instead, cellular therapeutics exert regenerative capabilities via a broad range of mechanisms including cell differentiation potential and secretion of soluble molecules such as growth factors and cytokines to stimulate repair and regeneration of damaged cells or tissues. Figure 1.1 provides a chronological summary of key developments in cellular therapy some of which are discussed in the sections below.

The use of stem cells as therapeutics has become one of the largest areas of scientific and medical research worldwide. The use of stem cells for the treatment of multiple diseases has become widespread. There are several different types of stem cells (refer to Section 1.2.1), however, the adult stem cell types, mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), are currently most commonly utilised as therapeutics. The regulatory framework in many jurisdictions as well as the ethical and safety concerns surrounding other stem cell types, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), prevent their use in clinical applications currently. At this time, there are over 5000 clinical trials listed on the American government clinical trials website investigating the use of either MSCs or HSCs in humans for the treatment of a variety of diseases including diabetes, ischemic injuries, liver failure, musculoskeletal disorders, and graft-versus-host disease[5].

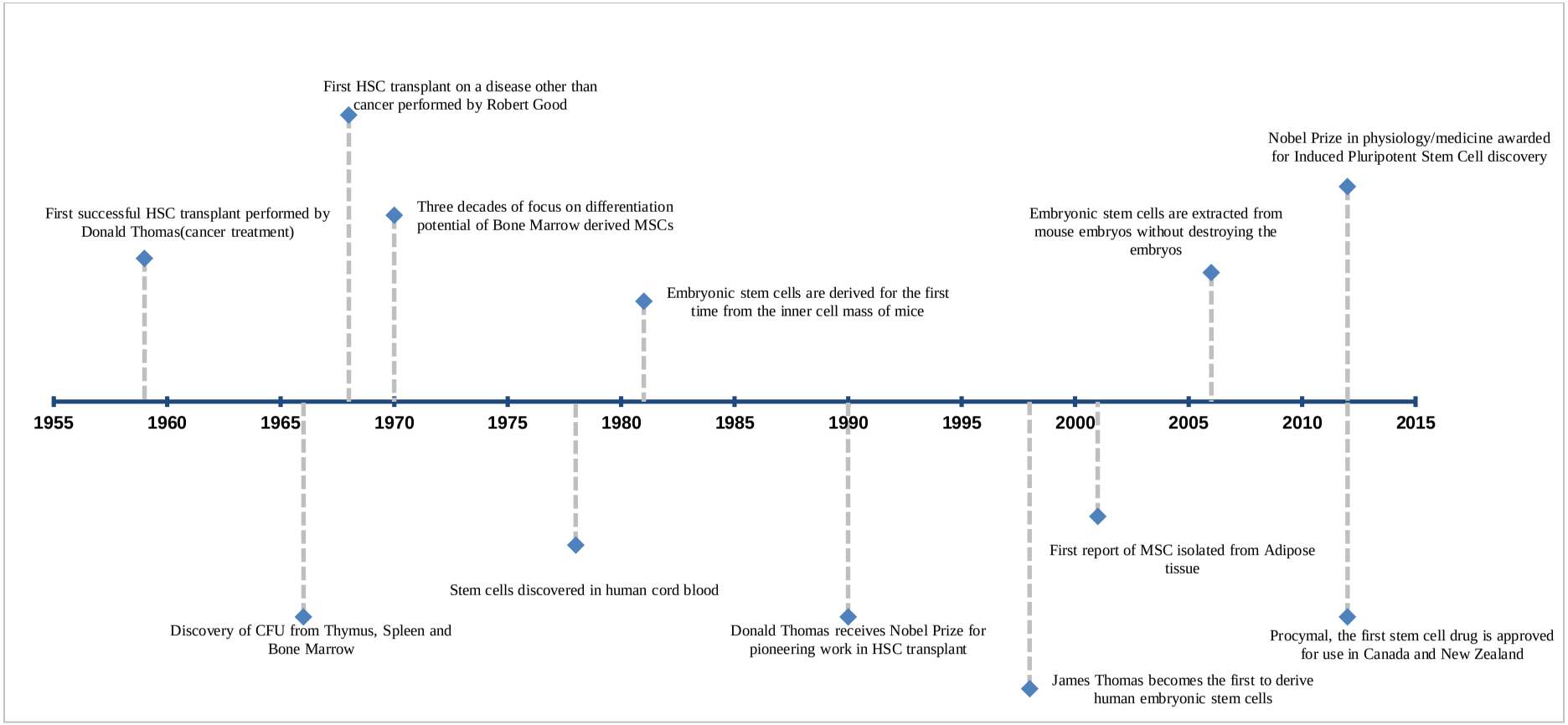

Figure 1.1. Chronological summary of key developments in cellular therapy

1.2. Stem Cells

Stem cells are part of the tissue repair mechanism found in all mammalian tissues [6]. They are unspecialised cells capable of self-renewal through division, sometimes after lengthy periods of inactivity [7]. Under certain physiologic or experimental conditions, stem cells can differentiate into tissue- or organ-specific cells with specialised functions, for example a muscle, blood or neural cell [8]. It is these characteristics that distinguish stem cells from other cell types in the body. There are several types of stem cells, each having key properties that dictate how it will develop.

Germ cells and newly fertilized zygotes are considered to be totipotent, meaning they can form all cell types of the embryo, the adult, and the extra-embryonic tissue such as the placenta [9]. If a stem cell is pluripotent, it can differentiate into any cell type of the three germ layers, the endoderm, ectoderm and mesoderm, but cannot form extra-embryonic tissue. ESCs are exclusively pluripotent, and thus offer a wide range of differentiation capabilities [9]. All other stem cells found within the specialised tissue of the fetus and adult are considered to be multipotent [9]. These cells have the ability to form multiple cell types but are lineage restricted. The remaining stem cell type is unipotent, often referred to as a progenitor cells. These cells possess the property of self-renewal but can only form their own cell type [9].

1.2.1. Types of Stem Cells

1.2.1.1. Embryonic Stem Cells

ESCs were first harvested and culture-expanded from the mouse blastocyst as recently as the 1980's [10, 11]. In 1998 James Thomson and his team were the first to successfully derive human ESCs from a blastocyst and develop the first embryonic stem cell lines [12]. A decade of excitement ensued as Thompson and his team demonstrated the potential of ESCs to regenerate and differentiate into specialised tissues. However, this discovery also initiated the ethical debate surrounding the

destruction of human embryos. As such, whilst ESCs are considered to be of vast therapeutic potential theoretically, their use is fraught with controversy due to the ethical, religious and legislative issues surrounding embryo-derived material. Furthermore, there is risk associated with the therapeutic use of ESCs due to immunological rejection, mutation and the formation of tumours [13, 14]. There are currently no approved treatments using ESCs.

1.2.1.2. Induced Pluripotent Stem Cells

iPSCs, although derived from adult tissue, are not to be confused with adult stem cells. iPSCs are produced by inducing somatic cells to embryonic pluripotency through the forced expression of key transcription factors Oct4, Sox2, Klf4 and c –Myc or Nanog and LIN28. Cells, which only previously had multipotentiality, have much greater differentiation capability after the delivery of transcription factors but have none of the ethical concerns associated with ESCs. The discovery of iPSCs has been a recent one, first reported by Takahashi *et al.,* in 2006 with a landmark publication demonstrating that iPSCs were created from mouse fibroblasts through altering only 4 genes [15]. In 2007 the same research group created iPS cells from human fibroblasts [16] while James Thomson's team later that year, produced iPS cells from human skin [17]. Despite this promising discovery, iPS cells have the propensity to form tumours, and thus are likely to be some time away from being a therapeutic reality [18]. This is in part due to the delivery methods of transcription factors into target cells to induce pluripotency. Initially retrovirus or lentivirus were utilised for transduction of target cells, leading to altered differentiation potential or malignant transformation. More recently adenoviral transduction has been utilised resulting in a reduced risk of tumorogenesis due to their transient expression of transcription factors [19]. Plasmid transfection also exhibits a reduced risk of tumorogenesis but results in a slower rate of iPSCs induction [20].

1.2.1.3. Adult Stem Cells

Adult stem cells are multipotent meaning they can give rise to any cell type of the cell germ layer they are derived from. The use of somatic cells carry none of the

ethical issues associated with that of ESCs as they can be obtained from fully developed adult tissues. Furthermore, adult stem cells pose no tumor formation risk [21]. Broadly speaking adult stem cells can be divided into two groups, HSCs and MSCs.

1.2.1.3.1. Hematopoietic Stem Cells

Alexander Maksimov, a Russian histologist, proposed the term stem cell in 1908. Maksimov was the first to hypothesise a self-renewing HSC capable of transforming into different cell types [22]. More than a century later, bone marrow transplantation is probably the most well understood stem cell therapeutic. HSC's derived from bone marrow have the capability to differentiate *in vivo* into all the cellular components of the blood. Thousands of bone marrow transplants have occurred in hospitals all over the world since the 1960's. These transplants are primarily for cancer patients after radiotherapy or chemotherapy to replace blood cells lost in this process, although HSCs are also utilised for other blood or immune diseases. HSC treatments are performed utilising either autologous or donor matched allogeneic cells. HSCs can be procured directly from bone marrow, umbilical cord blood or from peripheral blood in a mobilised cytokine induced state [13]. *In vivo* differentiation potential of HSCs is essential to their therapeutic ability and this fact has contributed to the perception that differentiation potential is key to all stem cell therapies.

1.2.2. Mesenchymal Stem Cells

1.2.2.1. Nomenclature – A Reflection of Cell Capability

Arnold Caplan coined the term Mesenchymal Stem Cell in 1991 when he proposed the mesengenic process [23]. The mesengenic process details the differentiation potential of MSC's into cell lineages of the mesenchyme (Figure 1.2) [24]. It was largely modeled on what was known to be the crucial therapeutic capability of HSC's for bone marrow transplants, being cell differentiation.

Although MSCs are capable of differentiating into specific cell types, namely adipocytes, osteoblasts and chondrocytes, this is only one arm of their therapeutic capacity. It is now well understood that the paracrine activities of MSCs are a major driver of therapeutic effect [25]. MSCs secrete cytokines, growth factors and chemokines; collectively know as trophic factors. These trophic factors have anti-inflammatory, immune-modulatory, anti-apoptotic, anti-fibrotic, mitotic and migratory properties [26] which are outlined in the following sections.

There have been a number of name changes proposed for the acronym MSC to better reflect the function of these cells. Firstly conjecture as to whether MSC's actually possess what is known to be stemness exists within the research community [27]. *In vitro*, the ability of MSC's to renew themselves and differentiate into cells of the mesenchyme lineage first documented by Friedenstein and colleagues [28] is universally recognised. However, the accepted definition of stemness, an "undifferentiated cell from which a variety of other cell types can develop through the process of cellular differentiation", does not truly reflect the *in vivo* mechanism of action of MSCs [29]. As such, Dominici *et al*., (2006), proposed the name "Mesenchymal Stromal Cell", which has been widely adopted by fellow researchers [30]. Mesenchymal stromal cell aptly describes the cell but does not provide any information about its functionality. In 2010, almost a decade after first naming the MSC, Arnold Caplan provided an alternative name that is more descriptive of functionality, the "Medicinal Signaling Cell" [31]. He proposed that MSCs in fact act as "site regulated drug stores". Another proposed name, in line with Caplan's suggestion is "Multipurpose Secreting Cells". This more fittingly describes the multi-potential nature of these cells in a multitude of both acute and chronic diseases [32]. Regardless of which name best fits the acronym MSC, the therapeutic capabilities described below (Section 1.2.2.4.) demonstrate why MSCs are being pursued as therapeutics in the medical community and furthermore their relevance in the research described in this thesis.

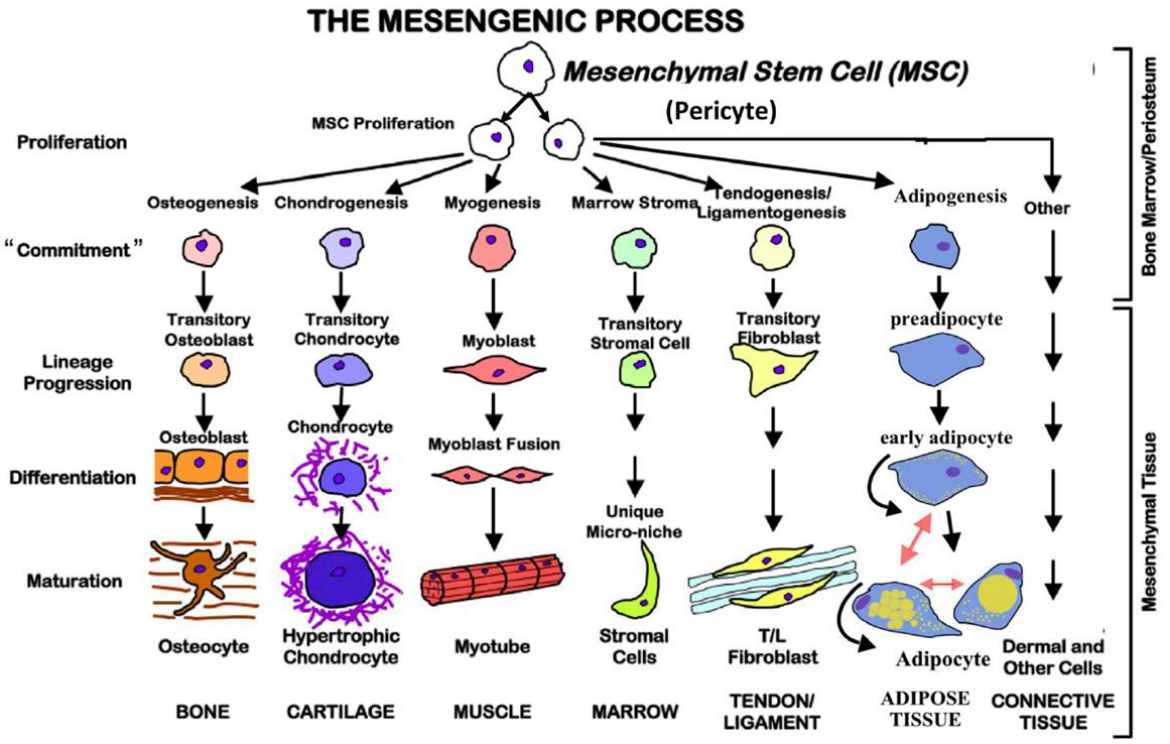

Figure 1.2. The mesengenic process

Image taken from **[24].**

1.2.2.2. Sources of Mesenchymal Stem Cells

MSCs can be obtained from multiple sites including the nasal passage, synovium [33], cartilage, umbilical cord and dental pulp [34]. However for the purpose of this thesis, relating primarily to canines, only the commonly utilised sources of bone marrow and adipose tissue will be explained. MSCs can be readily harvested from these sites in adult mammals and both sites enable a reasonable proportion of MSCs to be harvested. However, the number of MSCs that can be isolated from these tissues can vary, and it should be noted that a known therapeutic dose of MSCs has not been yet defined for any disease in any species.

1.2.2.2.1. Bone Marrow

Bone marrow contains both MSCs and HSCs. HSCs are present at 1 in 10,000 cells throughout life whereas MSC numbers are much lower and decrease with age. MSCs are present in bone marrow at about 1 in every 300,000 cells throughout adulthood [35]. With so few numbers of MSCs being readily isolated from bone

marrow and the difficulty associated with isolating significant amounts of bone marrow, an ex vivo culture step is almost always performed to amplify cell number. The expansion of MSCs in a tissue culture purification process means that all other cell types from the bone marrow, including potentially important immune cells, are lost during this process.

1.2.2.2.2. Adipose Tissue

MSCs are present in adipose tissue at approximately 500-1000 times greater levels than bone marrow [36]. The most likely explanation for this finding is that MSCs, along with other nucleated cell types, are associated with the vast network of dense capillary beds lining adipose tissue [37]. Cells associated with these capillary beds can be easily isolated from adipose tissue by enzymatic or mechanical digestion [8]. A pellet of cells is obtained, known as the stromal vascular fraction (SVF; see Section 1.5.1.), which is comprised of between 1 and 35% MSCs [38, 39]. Adipose tissue is easily harvested from people and companion animals such as dogs either by liposuction or surgical excision.

1.2.2.3. Characterisation of Mesenchymal Stem Cells

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) released a position statement in 2006 detailing a "set of standards to define human MSC for both laboratory-based scientific investigations and for pre-clinical studies" [30]. These set criteria included, plastic adherence, expression of specific surface antigen markers (≥ 95%+ CD105, CD73, CD90 and ≤ 2%+ CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR) and multipotent differentiation potential into osteoblasts, adipocytes, and chondroblasts [30]. Additionally in 2012 the International Federation for Adipose Therapeutics and Science (IFATS) and the ISCT released a supplementary position statement detailing a set criteria specific to adipose derived SVF and MSCs detailed in Table 1.1 [39].

MSCs regardless of their tissue origin possess the ability to proliferate *in vitro* under normal cell culture conditions. This is a feature, which can be exploited to obtain a relatively pure population of MSCs, which can be expanded to achieve a

desired cell number. Furthermore, MSCs can differentiate *in vitro* into a specific cell type, which is achieved by growing the cells in a chemically defined medium [40]. It is important to note that the criterion set was defined primarily for human MSCs and human SVF cells. Although canine SVF cells and MSCs meet many of criteria specified, some canine specific monoclonal antibodies are currently unavailable [41].

Table 1.1. Summary of criteria to identify adipose-derived SVF and culture expanded MSCs

Feature	Assay	Cells of SVF	MSCs[1]
Viability	Vital stain by flow cytometry or microscopy	>70% viable	>90% viable
Immunophenotype	Flow cytometry	Primary stable positive markers for stromal cells: CD13, CD29, CD44, CD73, CD90 (>40%), CD34 (>20%) Primary negative markers for stromal cells: CD31 (<20%), CD45 (<50%)	Primary stable positive markers: CD13, CD29, CD44, CD73, CD90, CD105 (>80%) Primary unstable positive marker: CD34 (present at variable levels) Primary negative marker: CD31, CD45, CD235a (<2%)
Proliferation and frequency	CFU-F	Anticipated frequency: >1%	Anticipated frequency: >5%
Adipogeneic differentiation	Histochemistry	N/A	Histology: oil red O, Nile red or stain specific for lipid inclusions
Chondrogeneic differentiation		N/A	Histology: alcian blue or safranin O
Osteogeneic differentiation		N/A	Histology: alizarin red or von Kossa

Criteria were defined jointly by the International Society for Cellular Therapy and International Federation for Adipose Therapeutics and Science [39].
[1] MSCs were adipose-derived.

1.2.2.4 Therapeutic Properties of Mesenchymal Stem Cells

Although MSCs can differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts, this occurs under specialised chemically induced conditions. Whilst in many cases the differentiated cells morphologically appear as the target cell type, this does not ensure the differentiated cell will be functional [40]. As such, the potential to use *in vitro* differentiated cells in people or animals suffering from disease is still far from being a mainstream therapeutic reality. There are many unknowns regarding the functionality and safety of using ex vivo expanded and differentiated cells [42-44]. However, the research and clinical communities are rapidly embracing the possibilities of MSCs beyond the historical constraints of differentiation. The trophic activities of MSCs through the bioactive molecules they secrete are now well recognised to be a major driver of their therapeutic effect [26]. Cytokines, chemokines and growth factors secreted by MSCs in response to their microenvironment have been shown to suppress inflammation and immune responses, promote angiogenesis and mediate apoptosis and fibrosis [26]. Trophic factors, produced in response to tissue injury or disease, are in turn responsible for the stem cell-driven repair and regeneration process. Figure 1.3 shows the trophic and immune-modulatory functions of MSCs, which will be discussed in turn in Section 1.2.2.4.

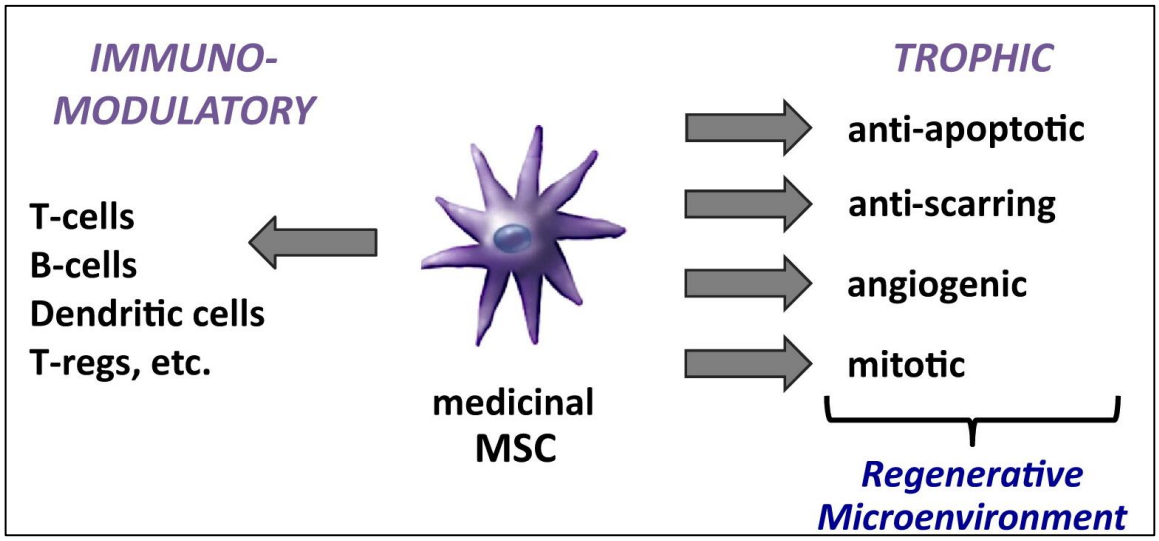

Figure 1.3. The trophic and immune-modulatory functions of MSCs via secreted bioactive molecules.

Image taken from [45].

1.2.2.4.1. Immunomodulatory Properties of Mesenchymal Stem Cells

MSCs are well recognised to be immune privileged as they lack expression of major histocompatibility complex (MHC) class II molecules and have limited expression of MHC class I molecules. This prevents their recognition by T-cells and thus they escape the cytotoxic effects of natural killer (NK)-cells [46]. Their interactions with lymphocyte cells have been demonstrated to attenuate graft-versus-host disease [47] and autoimmune diseases via immunosuppression. As such, MSCs have demonstrated therapeutic relevance in many immune mediated conditions such as Crohn's disease [48], multiple sclerosis [49] and lung disorders, for example asthma [50] and cystic fibrosis [51].

MSCs have been shown *in vitro* and *in vivo* to have an impact on both innate and adaptive immunity via the following:

Suppression of T-cell proliferation – T-cells are responsible for destroying pathogenic or allogeneic stimuli. Once activated, T-cells proliferate and release inflammatory cytokines. Suppression of overactive proliferation of these cells is paramount in immune modulation. Suppression by MSCs is achieved via the secretion of soluble factors including, but not limited to, nitric oxide (NO) [52], prostaglandin E2 (PGE2) and transforming growth factor (TGF)-β which in turn inhibit the production of pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ thus promoting an anti-inflammatory environment. The inhibition of T cell proliferation has been observed by MSCs from both allogeneic and autologous sources [53, 54]. MSCs also lack the co-stimulatory molecules CD80 and CD86, another mechanism proposed for T cell suppression.

Promotion of T-regulatory (Treg) cells – MSCs also promote the generation of functional CD4+ CD25+ or CD8+ Treg cells. Treg cells are anti-inflammatory and prevent hyper-immune responses, which is essential for the treatment of inflammatory or immune driven diseases [55, 56].

Prevention of Dendritic Cells (DC) maturation – MSCs have been shown to interfere with DC proliferation, differentiation and maturation from monocytes or CD34+

progenitor cells. The hampering of DC maturation prevents expression of the co-stimulatory molecules CD40, CD80 and CD86 necessary to activate T-cells [57, 58].

Induction of immature DC phenotype – Another way MSCs manipulate the adaptive immune system is by inducing an immature DC phenotype, which in turn prevents T-cell recognition. Co-culture experiments have demonstrated that MSCs down-regulate expression of co-stimulatory molecules CD40, CD80 and CD86 required for DC maturation. Consequently, this results in a reduction in the secretion of the pro-inflammatory cytokines TNF-α, IFN-γ and interleukin (IL)-12 and up-regulated secretion of the anti-inflammatory cytokine IL-10 [59, 60].

Suppression of Natural Killer (NK) Cell proliferation – MSCs through secretion of PGE2 and TGF-β have been reported to suppress gene expression of IL-2 and IL-15, which are required for NK cell proliferation. This in turn prevents NK cell secretion of IFN-γ thereby reducing NK cell cytotoxicity [55, 61].

Inhibition of activated B cells – B cells function as crucial antigen presenting cells in many autoimmune diseases and can secrete pro-inflammatory cytokines such as IL-6 and IFN-γ. MSCs have been shown to regulate B-cell proliferation, migration and antigen production via arrest at the G0/G1 cell cycle phase [62, 63].

Taken together it is clear that MSCs can suppress the function of immune cells of both the innate and adaptive immune system. This capability is of therapeutic relevance in conditions that evoke an undesired ongoing immune response such as rheumatoid arthritis, leukemia [64], GVHD [47, 65] and transplantation rejection [53]. The modulation of immune response by MSCs is achieved through a combination of soluble factors acting in concert to mediate the inflammatory environment they are introduced to, as well as via direct cell-cell signaling. Figure 1.4 provides a schematic detailing the immune-modulatory properties of MSCs [66].

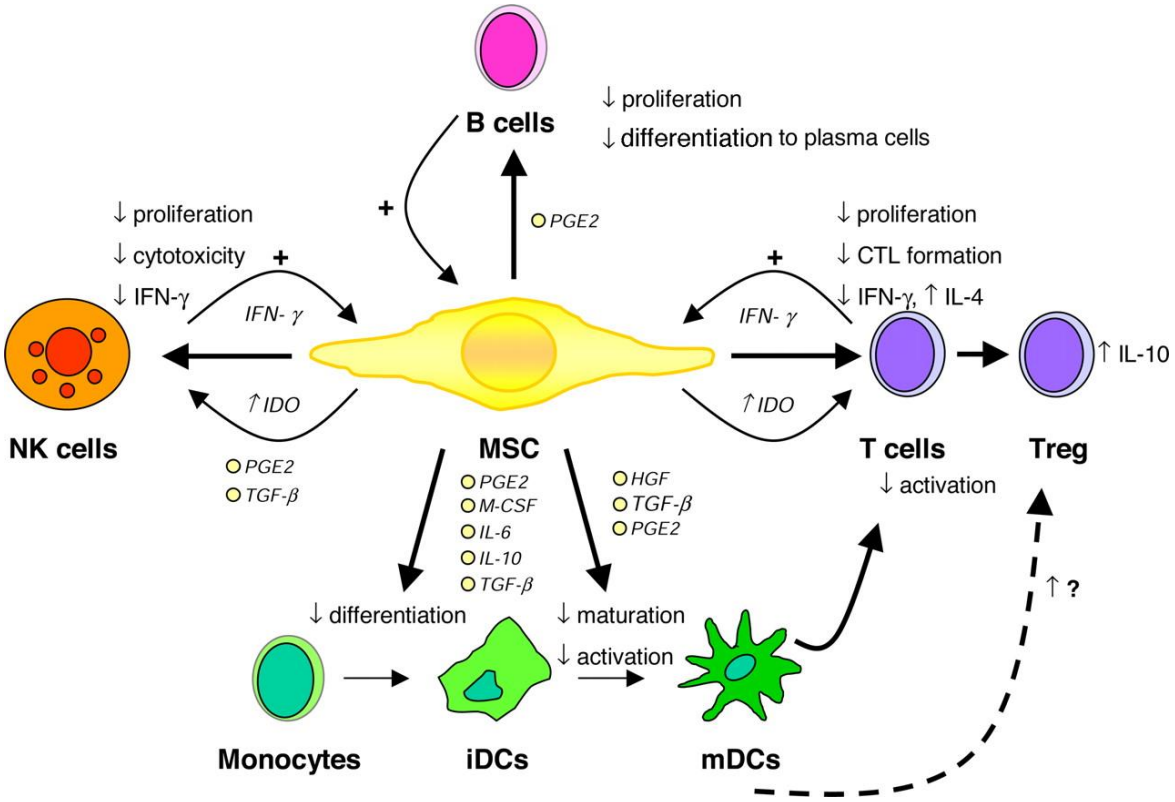

Figure 1.4. Immune-modulatory properties of MSCs

Figure taken from [66].

1.2.2.4.2. Angiogenic Properties of Mesenchymal Stem Cells

MSCs stimulate angiogenesis primarily via secretion of the growth factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Angiogenesis is the process of forming new blood vessels from existing vasculature and plays an important role in ischemic conditions such as stroke, cardiac disease and wound healing. Secreted levels of these bioactive factors from MSCs have demonstrated significant improvements in tissue repair due to increased vascularisation and reduced ischemia-induced cell death in animal models of myocardial infarction [67], corneal ulcers [68] and stroke [69]. Levels of VEGF secreted from MSCs have shown to be regulated by their environment. For example, Rehman *et al.,* (2004) demonstrated that elevated levels of VEGF are secreted by MSCs in hypoxic tissue culture conditions [70]. This highlights that the secretion of trophic factors by MSCs is a dynamic process and is governed by the microenvironment the cells are introduced to.

1.2.2.4.3. Homing Capabilities of Mesenchymal Stem Cells

There are numerous *in vitro* and *in vivo* studies demonstrating the ability of MSCs to home to sites of inflammation and injury. MSCs have the ability to exert their therapeutic effect through not only site directed means, but can home to sites of injury or disease through systemic administration. As administration of cells directly into organs such as the heart carries significant risks, the homing properties of MSCs are attractive and as such have lead researchers to explore other routes of administration.

The migration of MSCs to sites of damage can be attributed to growth factor and chemokine receptors. An *in vitro* study by Rojas *et al.,* (2005) demonstrated that MSC migration is driven by the pro-inflammatory cytokine TNF-α, highlighting that chemo-attraction could direct systemically infused MSCs to inflammatory sites [71]. In accordance, another *in vitro* assay showed MSCs home towards diseased mouse lung tissue but not to healthy lung cells [72]. Another factor suggested to be involved in the trafficking and subsequent adhesion of MSCs to sites of injury is the cell surface antigen CD44. CD44 is a molecule expressed on the surface of MSCs and is recognised to bind to hyaluronan, a major component of extra cellular matrix with increased expression in many diseased tissues [73]. In a mouse model of acute renal failure, MSCs have been shown to migrate to the injured kidney, where hyaluronic acid expression was increased, resulting in recovery of the kidney [74] Conversely MSCs derived from CD44 knockout mice did not localise to the injured kidney and did not induce renal recovery [74].

Many groups have reported the migration of intravenously administered MSCs to diseased sites whereby they have effectively mediated or inhibited the onset of the induced disease. This has been shown in models of heart disease [75, 76], stroke [77, 78], osteogenesis imperfect [79], kidney [80], liver [81] and lung disease [82]. Researchers have shown that although a proportion of introduced MSCs migrate and infiltrate to the site of tissue injury, a proportion becomes entrapped in the lungs suggesting [83] that the trophic mediators expressed by MSCs can successfully mediate disease remotely. Shabbir *et al.,* (2009) tested this hypothesis by injecting MSCs intramuscularly in a hamster heart failure model and found that not only did intramuscular administration of MSCs effectively rescue the failing heart, but that the

administration of MSC secretions alone had a similar effect [67]. These results highlight that the morphological and functional improvement seen in disease after MSC administration is related to the sophisticated cross talk of MSCs and their secretions rather than differentiation of introduced cells into the target tissue type.

1.2.2.4.4. Anti-scarring Properties of Mesenchymal Stem Cells

The body responds to repeated injury through remodeling and fibrosis of damaged tissues to encapsulate the site of an injury. This mechanism is imperative to the repair of wounds on the bodies' external surface. However, fibrosis of injured internal tissues such as the heart, liver, kidney or spinal cord, is a serious problem often resulting in the loss of function of whole or part of a tissue type. MSCs if administered before the development of scar tissue can improve the therapeutic outcome post-injury by promoting repair and regeneration of injured tissue and suppressing fibrosis.

MSCs, via secretion of the anti-fibrotic factors, hepatocyte growth factor (HGF) and adrenomedullin, were shown to attenuate fibrosis in mice in a pre-clinical model of myocardial infarction [84]. MSC implantation also significantly decreased the expression of type I and type III collagen, as well as matrix metalloproteinases (MMP) types 2 and 9, resulting in a decrease in normal collagen degradation and improved heart function [84]. In another mouse model of ischemia and reperfusion injury of adipose tissue, MSCs were shown to secrete HGF in response to expression of bFGF by the injured tissue, which lead to a suppression of fibrosis. Furthermore if up-regulation of HGF was inhibited by suppression of bFGF, or by a neutralising antibody against HGF, increased fibrosis of the tissue was observed [85].

1.2.2.4.5. Anti-apoptotic Properties of Mesenchymal Stem Cells

MSCs also possess the ability, through their paracrine activity, to prevent or reduce apoptosis in the acute phase of tissue injury. This has been demonstrated in animal models of acute renal injury [86], hepatic failure [87] and hind limb ischemia [70]. Togel *et al.,* (2007) in a model of acute renal failure reported that infused MSCs not only attached to the renal microvasculature but significantly reduced apoptosis of

neighboring cells. The renal protective factors recognised for decreased apoptosis and proliferation of surviving cells were determined to be the growth factors VEGF, HGF and insulin-like growth factor (IGF)-1[86]. Rehman *et al.,* (2004) demonstrated that the expression of the anti-apoptotic factors VEGF, TGF-β, HGF, bFGF and granulocyte macrophage colony stimulating factor (GM-CSF) by MSCs were up-regulated in hypoxic conditions *in vitro*. Hypoxia takes place in the early stages of injury. Therefore it is advantageous that under hypoxic conditions MSCs respond to their microenvironment through increased expression of growth factors particularly VEGF which is capable of inducing angiogenesis and has cytoprotective effects [70]. Reduction in cell death was further demonstrated when MSCs cultured under hypoxic conditions were administered into mice subjected to hind-limb ischemia. The MSC treated limbs exhibited reduced necrosis and improved perfusion compared to the limbs of control animals [70]. *In vitro* co-culture experiments utilising two specific models of apoptosis; UV irradiated fibroblasts and incubation of epithelial cells in low pH hypoxic conditions, showed that secretion of stanniocalcin-1 by MSCs was responsible for reduced apoptosis in these models [88].

In summary, MSCs have the ability to regulate immune responses, dampen the inflammatory response to injury, prevent scarring and cell death and have angiogenic properties. MSCs can be isolated from many mammalian tissues and can be administered either in a site specific or systemic fashion. Furthermore the use of MSCs for their trophic activity, which in turn is recognised to lead to the regeneration of tissue, is currently utilised clinically to provide therapeutics for both people and animals alike. Table 1.2 provides a summary of the regenerative capabilities of MSCs.

Table 1.2. Summary of regenerative mechanisms of MSCs

Replacement of damaged or lost cells: MSCs can differentiate into cells types of damaged or lost tissues. Introduced MSCs have been shown to be found in newly regenerated tissues [89] Furthermore, via paracrine signally, MSCs can support resident cells to repair and regenerate damaged or diseased tissues [67, 68]
MSC migration and docking: MSCs home to sites of inflammation and injury even if administered remotely [70]. Furthermore introduced cells may embed with host cells to enhance resident cellular function [68, 90].
Anti-apoptosis: The secretion of the growth factors VEGF, HGF and IGF-1 secreted by MSCs have been shown to prevent or reduce cell death at the time of injury [70] .
Anti-inflammatory effects: MSC secretions, including IL-10 and HGF, from introduced cells have an immediate anti-inflammatory effect suppressing expression of pro-inflammatory cytokines TNF-α, IL-1α and IL-1β [91].
Immune modulation: MSCs are immune privileged, meaning they can avoid a host immune response. MSCs in response to activation by immune cell secretion of pro-inflammatory cytokines can suppress the local immune environment, reducing inflammation and blocking autoimmune responses [47, 53, 92].
Angiogenesis: MSCs are pro-angiogenic which is mediated via secretion of MCP-1, VEGF and bFGF [70].
Anti-scarring: Secretion of HGF by MSCs limits fibrosis if administered in the acute phase of injury prior to scar tissue formation [85].

1.2.2.5 Mesenchymal Stem Cell Secretions

As described in Section 1.2.2.4., the cytokines, chemokines and growth factors secreted by MSCs have been shown to play a crucial role in modulating disease and in turn switching the microenvironment from that of a destructive inflammatory environment to one which facilitates repair and regeneration. Consequently, the secretions from MSCs stimulate endogenous cells to repair and regenerate damaged tissue. As such, understanding the complex secretory activity of MSCs is crucial to understanding their role *in vivo*. MSCs have the capability to alter their secretion activities based on the environment they are introduced to. Instances of this have been demonstrated *in vitro*. An example of this was the up-regulation of growth factor secretions in hypoxic conditions demonstrated by Rehman *et al.,* [70]. This result provides insight into how MSCs may alter their secretion profile when introduced into a host with an ischemic injury.

A number of studies investigating the therapeutic benefits of MSCs have confirmed that secretions are a major driver of the therapeutic effect of MSCs. In particular, administration of conditioned media harvested from culture-expanded MSCs has been demonstrated to have a therapeutic benefit similar to MSCs in induced animal models of corneal eye injury [68] and ischemic reperfusion injury [93]. These examples further emphasise the relevance of MSC secretome in mediating disease. However, given the short half-life of the secretions, the therapeutic effect was shorter than that of cellular therapy [94] and in some instances required repeat application of the secretions [68].

Due to the importance of trophic factor secretions from MSCs, much of the research performed in this thesis focuses on investigating the cytokine, growth factor and chemokine profiles of the cell populations utilised. Table 1.3, adapted from [26], provides an overview of some of the important cytokines, growth factors and chemokines in relation to their trophic and immune modulatory roles. Table 1.4 summarises the cytokines, growth factors and chemokines investigated in the research described in this thesis. These biomolecules are categorized into pro-and anti-inflammatory cytokines, dual role cytokines, growth factors and chemokines.

Table 1.3. MSC biomolecule secretions

Functional effect	Biomolecule	References
Trophic		
Anti-apoptotic	VEGF, HGF, IGF-I, TGF-β, bFGF, GM-CSF	[70, 86, 88]
Angiogenic	bFGF , VEGF , MCP-1, IL-6	[93, 95]
Stimulatory (Mitosis, proliferation and differentiation of resident cells).	LIF , IL-6 , M-CSF	[96]
Anti-fibrotic	bFGF, HGF	[85]
Migration and embedding	CD44 molecule, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, IL-8, IP-10	[97]
Immune-modulatory	PGE_2, TGF-β HGF, LIF	[52, 55, 98, 99]

Table modified from [26].

Table 1.4. MSC biomolecule secretions

Functional Category	Biomolecule Name	Biomolecule Function
Pro-inflammatory	IFN-γ	**IFN-γ** has a wide range of properties which include inducing cytotoxic activity, inhibition of mitosis, inducing apoptosis, increasing expression of MHC class I and II molecules and controlling leukocyte and endothelial cell interactions.
	IL-8	**IL-8** is a potent selective activator of neutrophils and a chemoattractant for neutrophils, natural killer cells, T cells and basophils [100, 101]. IL-8 plays an important role in innate immunity and in providing the first line of defense during the early host response to invading pathogens [102]. IL-8 stimulates the mobilization of progenitor cells from the bone marrow into the circulation [103]. Furthermore, there is some evidence to suggest IL-8 may also be involved in stimulating angiogenesis [104].
	TNF-α	**TNF-α** is considered to be a major pro-inflammatory cytokine. It is recognized as an important early response cytokine with a multitude of effects during both acute and chronic states of inflammation [105-111]. The early expression of TNF-α in response to invading pathogens/tissue injury plays a crucial role in stimulating the production of other cytokines including IL-1, IL-6, IL-8 and the chemokine MCP-1, as well as inducing the production of itself from a variety of cell types [106, 108, 110, 112-115]. This activation of the cytokine network is essential for the recruitment of immune cells, monocytes and macrophages in particular, to the site of infection or inflammation [115]. Furthermore, TNF-α acts on the paracrine level by promoting the expression of adhesion molecules by endothelial cells and the production of MCP-1 by non-immune cells thereby enhancing monocyte recruitment [116]. TNF-α also plays a role in immune-regulation in that it can affect B cell differentiation and stimulate the production of cytotoxic T cells [117-119].

Anti-inflammatory	IL-10	**IL-10** is a potent anti-inflammatory cytokine, which, through its immunosuppressive effects, protects the host from inappropriate immune responses to invading pathogens and autoimmune diseases. It inhibits the production of a number of key pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-12, TNF-α, and IL-8 [120, 121], chemokines including MCP-1, MIP-1α, MIP-1β, RANTES and IP-10, and chemokine receptors. Furthermore, it up-regulates the production and secretion of IL-1Ra [122, 123]. IL-10 indirectly suppresses antigen specific T cell activation and T cell proliferation [120, 121, 124]. In contrast, IL-10 promotes the proliferation, differentiation, survival and isotype switching of B cells.
Chemokines	IP-10	**IP-10**, also known as CXCL10, is induced by IFN-γ and is a potent chemoattractant for a variety of cell types including monocytes/macrophages, eosinophils, natural killer (NK) cells and activated T cells [125-127]. IP-10 is also thought to be particularly important for the recruitment of T cells to sites of infection/inflammation. IP-10 is a potent chemoattractant for monocytes and activated T cells.
	MCP-1	**MCP-1** is a potent monocyte attractant but can also recruit memory T cells and dendritic cells to sites of infection/inflammation [128]. MCP-1 has recently been implicated in the recruitment of monocytes to the bone during the remodeling process [129, 130]. Although the exact mechanism of action is unclear, MCP-1 has been recognized as an angiogenic chemokine. It modulates angiogenesis through its actions on endothelial cells including inducing migration and sprouting of endothelial cells in the absence of inflammation [128]. Furthermore, MCP-1 can up-regulate VEGF [131], a major angiogenic factor.
	IL-7	**IL-7** is not considered a true interleukin as it is primarily produced by non-hematopoietic stromal cells rather than by leukocytes [132, 133]. IL-7 is crucial for T cell development and T cell homeostasis as well as preventing T cell apoptosis [132]. Furthermore, IL-7 may play some role in primary B cell development although it is not absolutely required [134 and references therein]. IL-7 also stimulates the production of inflammatory factors by monocytes [135].

	VEGF	**VEGF** is a potent promoter of angiogenesis and is a crucial rate-limiting component in the complex and coordinated process of new vessel growth and maturation [136]. The primary target of VEGF is endothelial cells and it has been shown to be a survival factor for endothelial cells both in vitro and in vivo [137-140]. VEGF can have mitogenic effects on other cell types [see 141 and references therein], promote monocyte chemotaxis [142], induce colony formation of some granulocyte-macrophage progenitor cells [143], increase B cell production [144], inhibit dendritic cell development [145] and induce vascular permeability [146, 147].
	IL-6	**IL-6** was initially thought to be a pro-inflammatory cytokine, however, numerous studies have shown that IL-6 can be anti-inflammatory and immunosuppressive. It is now considered to be a "multi-functional, pleiotropic cytokine involved in the regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation" [148]. IL-6 can suppress TNF-α and IL-1β production, as well as inducing synthesis of IL-1Ra and therefore mediates an anti-inflammatory response [149, 150]. On the other hand, IL-6 is the main inducer of C-reactive protein and can stimulate hepatic production of other acute phase proteins indicating its role in promoting inflammation [151]. IL-6 can also induce B cell differentiation, survival and antibody production [152], stimulate T cell growth and cytotoxic T cell differentiation [153], promote macrophage and megakaryocyte differentiation [154], direct leukocyte activation and trafficking [148], up-regulate the production of MCP-1 by endothelial cells [155], and is involved in neoangiogenesis [156].

1.2.2.6 Mesenchymal Stem Cells for the Treatment of Osteoarthritis

Due to the ability of MSCs to both mediate an inflammatory cytokine milieu and to initiate endogenous tissue regenerative activities, the treatment of OA is an obvious therapeutic application for MSCs. MSCs not only have the ability to address the structural issues associated with OA but also the underlying inflammatory aspects of the disease (to be discussed in turn in Section 1.3.). Paracrine activities of MSCs can prevent chondrocyte apoptosis, attenuate progression of synovial inflammation, and ameliorate damage to ligaments and menisci, thereby inhibiting further joint destruction. Examples of MSCs for the treatment of OA have been demonstrated in animal models, and in human and canine case studies or clinical trials of OA.

Table 1.4 describes several pivotal studies that utilised MSCs to treat OA in animal models. Table 1.5 provides a current overview of the treatment of musculoskeletal disorders with MSCs in people. The use of MSCs in real clinical disease in animals will be discussed in Section 1.4.1.

Table 1.5. Pivotal studies utilising MSCs in animal models of osteoarthritis.

Condition (n)	Stem cell type	Reintroduction site	Outcome	Ref
Surgically induced partial tear of anterior cruciate ligament injury in rats (98)	Green fluorescent protein (GFP) labeled allogeneic, cultured BM-MSCs	Intra-articular injection	Healing tissue was formed in the area of the transected ligament in which introduced GFP labeled cells were detected.	[157]
Surgically excised medial meniscus and resected cruciate ligament in goats (24)	GFP labeled Autologous, cultured BM-MSCs	Intra-articular injection	MSCs were administered 6 weeks after the surgery to ensure onset of OA. MSCs had a protective effective preventing the onset of OA, additionally GFP labeled MSCs were detected in regenerated meniscal tissue.	[89]
Surgically induced full thickness defects of articular cartilage in New Zealand white rabbits (68)	Autologous, cultured BM or periosteum MSCs	Introduced into the medial femoral condoyle with collagen gel.	Hyaline liked cartilage formed in defects. There was no significant difference between cells from BM or periosteum.	[158]
Arthroscopically induced OA in the middle carpal joint of horses (24)	Autologous uncultured AD-MSCs and autologous, cultured BM-MSCs	Introduced into the OA-affected joint 14 days after arthroscopy.	No significant differences were seen between the two MSC sources	[159]
Surgically induced partial thickness cartilage defect in the femoral condyle of the mini-pig (42)	Autologous, cultured BM-MSCs	Intra-articular injection in combination with hyaluronic acid (HA)	At 6 and 12 weeks post surgery when pigs were sacrificed the MSC treated group showed improved cartilage healing (histologically and morphologically) compared to saline and HA alone control groups.	[160]
Surgically induced anterior cruciate ligament transection injury New Zealand white rabbits (28)	Allogeneic cultured AD-MSCs	Intra-articular injection	MSCs were administered 12 weeks after the rabbit knees were destabilized to ensure onset of OA. Treatment with MSCs resulted in less cartilage degeneration, osteophyte formation, and subchondral sclerosis than the control group at 20 weeks post surgery.	[161]

Table 1.6. MSCs in the Treatment of Human Musculoskeletal Diseases

Condition (n)	Year	Stem cell type	Reintroduction technique	Study type	Pain and/or function	Cartilage Results	Ref
Full thickness articular cartilage defect of the femoral condyle (1)	2007	Autologous bone-marrow derived MSCs	Embedded within a collagen gel	Case study	1 year after surgery, the clinical symptoms improved significantly. Patient attained previous level of activity (31 y.o. judo player) and experienced neither pain or other complications	7 months after surgery, the defect was filled with a hyaline-like type of cartilage tissue which stained positively with Safranin-O	[162]
Degenerative joint disease (1)	2008	Autologous bone-marrow derived MSCs	Intra-articular injection	Case study	At 24 weeks post-injection, the patient experienced increased range of motion (from -2 ° to +3 °) and decreased (by 95%) VAS pain score	At 24 weeks post injection, the patient had statistically significant cartilage and meniscus growth as determined by MRI	[163]
Full thickness articular cartilage defect of femoral condyle, trochlea or patella (36 vs. 36)	2010	Autologous bone-marrow derived MSCs Vs. Autologous chondrocyte implantation (ACI)	Embedded within cell sheets under periosteal patch	Cohort study	Significant improvement 2 years post surgery in both groups for; quality of life assessment, subjective knee assessment and activity level assessment	Histologic evaluation of the biopsy samples taken from patients (n=7) from both groups showed hyaline-like cartilage tissue	[164]
HTO for OA of the knee (12 vs. 12) *(HTO Vs. HTO + MSCs)*	2002	Autologous bone marrow derived MSCs	Embedded within a collagen gel	Case-control	Clinical improvement not significantly different	42 weeks after surgery, the arthroscopic and histological grading score was better in the HTO + stem cells group than in the cell-free control group. MSCs were equally as effective in OA treatment as ACI and required one less surgical procedure	[165]
Articular cartilage defects in the patella-femoral joint (3)	2007	Autologous bone-marrow derived MSCs	Embedded within a collagen sheet (covered with either periosteum or synovium)	Case series	6 months after transplantation, the patients clinical symptoms (pain, crepitus, effusion) were reduced dramatically and were maintained for the course of the follow-up period (17-27 months)	1 year after surgery, MRI revealed complete coverage with; Case 1= fibrocartilage Case 2= unable detect if hyaline or fibrocartilage Case 3= not assessed on MRI	[166]
Meniscal and cartilage injury (1)	2008	Autologous bone-marrow derived MSCs	Intra-articular injection	Case study	At 3 months follow-up, VAS scores decreased from 3.33 to 0.13	At 3 months post injection, MRI analysis demonstrated an increase in meniscus volume	[167]

Talar Osteochondral Lesions (48)	2009	Autologous bone-marrow derived MSCs	Inserted arthroscopically with the addition of a scaffold	Case series	AOFAS score improved from 64.4 to 91.4	Histologic evaluation showed regenerated tissue in various degrees of remodeling	[168]
Full thickness articular cartilage defect of femoral condyle (5)	2010	Autologous bone-marrow derived MSCs	Transplanted with addition Platelet-Rich Fibrin Glue	Case series	Average subjective knee scores for all patients showed statistically significant improvement at 6 & 12 months postoperatively	At 1 post surgery, MRI of 3 patients revealed complete surface congruity with native cartilage, and 2 patients showed incomplete congruity. Arthroscopic scores were 8/12 and 11/12 (nearly normal) for the 2 patients who consented to arthroscopy	[169]
OA of the knee (2)	2011	Autologous adipose derived SVF + PRP, HA and calcium chloride	Intra-articular injection	Case series	Measured physical therapy outcomes, subjective pain and functional status all improved	MRI showed significant positive changes-cartilage regeneration in the patients with OA	[170]
Osteochondral lesions of the knee (20)	2010	Autologous bone-marrow derived MSCs	Embedded with the addition of HA scaffold	Case series	Significant improvement ($p<0.0005$) in both the IKDC and KOOS score from pre to post testing	MRI showed satisfactory growth of bone and cartilage, nearly complete defect filling, and satisfactory integration of the graft in 80% of patients	[171]
Osteochondral lesions of the knee (30)	2013	Autologous bone-marrow derived MSCs	Embedded with the addition of HA or collagen scaffold	Case series	The clinical improvement was statistically significant both for IKDC and KOOS scores at each of the follow-up, with increased clinical improvement over time	MRI at 12 & 24 months showed regeneration of the subchondral bone and cartilaginous tissue in different parameters of the MOCART score.	[172]
Cartilage lesion of the knee (70)	2012	Autologous bone-marrow derived MSCs	Microfracture + Intra-articular injection of MSC and HA	Observational cohort	Significant improvement in mean knee, quality of life and pain assessments	MRI findings were encouraging. Neo-cartilage with good infill and integration. There was also significant reduction in underlying marrow oedema	[173]
Chondral lesions (50)	2013	Autologous peripheral blood cells	Microfracture + Intra-articular injection of cells and HA	RCT	Progressive improvement of the subjective IKDC scores.	Significant statistical improvement in histologic and MRI scores.	[174]

Large traumatic cartilage defects of the knee (2)	2011	Autologous bone-marrow derived MSCs	Re-implanted into defects with collagen scaffold	Case series	KOOS & IKDC showed significant improvement	Arthroscopic assessment showed good defect infill, stiffness and incorporation to the adjacent cartilage	[175]
Knee Osteoarthritis (4)	2011	Autologous bone-marrow derived MSCs	Intra-articular injection	Case series	Walking time for pain to appear improved for three patients. VAS decreased in all patients. Minor improvements for crepitus and ROM	N/A	[176]
Small chondral defects of the knee (5)	2011	Autologous peripheral blood cells	Microfracture + Intra-articular injection of cells and HA	Case series	N/A	Second look arthroscopy and histologic sections confirmed regeneration of articular cartilage	[177]
Chondromalacia Patellae (3)	2013	Autologous adipose-tissue derived stem cells	Intra-articular injection of MSC, HA and calcium chloride activated PRP	Retro case series	VAS pain scores improved 80-90%	MRI revealed improvement of the damaged tissues (softened cartilages)	[178]
Knee osteoarthritis (6)	2012	Autologous bone-marrow derived MSCs	Intra-articular injection	Case series	No local or systemic AEs. Pain, functional status of the knee and walking distance tended to be improved up to six months post-injection, after which pain appeared to be slightly increased and walking ability slightly decreased	MRI (6mths) displayed an increase in cartilage thickness, extension of the repair tissue over the subchondral bone and a considerable decrease in the size of edematous subchondral patches in 3/6 patients	[179]
Knee osteoarthritis (12)	2013	Autologous expanded bone marrow	Intra-articular injection ($40x10^6$)	Pilot phase I/II Study	65-78% improvement in pain within 3 months which was sustained until 12 months. Improvements in functionality and QoL outcomes were smaller although WOMAC outcomes were still significant	Significant reduction in 'poor cartilage index' score as determined by MRI T2 mapping. Quantitative evidence of partial articular cartilage healing.	[180]
Meniscus regeneration and osteoarthritis (55)	2014	Allogeneic expanded bone marrow	Intra-articular injection 3-groups A. MSCs $50x10^6$ B. MSCs $150x10^6$ C. HA	Double blind RCT phase I/II	Significant reduction in VAS at 24 months for group A (p= 0.05) and group B (p=0.04) when compared to the HA control group (C).	Significantly increased meniscal volume (defined as >15%) in 24% of patients in group A and 6% in group B at 12 months post meniscectomy	[181]

Knee osteoarthritis (18)	2013	Autologous infrapatellar fat pad MSCs. (Not cultured)	Intra-articular injection $1.18x10^6$ IFP cells + two subsequent injections of 3mL of PRP	Level IV, therapeutic case series	Significant improvement in WOMAC ($P<0.001$), Lysholm ($P<0.001$) and VAS ($p=0.005$) score at the last follow-up time-point of 26 months	MRI WORMS* analysis resulted in a significantly improved score ($P<0.001$) at the last follow-up time-point. Additionally there was a positive correlation between number of cells injected and clinical and MRI outcomes	[182]
Knee osteoarthritis (30)	2013	Autologous adipose-derived SVF	Intra-articular injection of $4.2 x10^7$ SVF cells (of which 10% were deemed to be MSCs) prepared with 3mL of PRP. Cells were injected same day after arthroscopic lavage	Level IV, therapeutic case series	Significant improvement in Lysholm ($P<0.05$), VAS ($p<0.05$) and KOOS ($p<0.05$) score. K-L grade (OA grade) only worsened in 5 patients during the 24 month study time-frame	16 of the 30 patients had a second look arthroscopy performed. 14 of the 16 patients had an improved or maintained cartilage healing status	[183]

Key: Visual analogue scale (VAS), Magnetic resonance imagining (MRI), High tibial osteotomy (HTO), Autologous chondrocyte implantation (ACI), American orthopedic foot and ankle score (AOFAS), International Knee Documentation Committee (IKDC), Knee osteoarthritis outcome score (KOOS), magnetic resonance observation of cartilage repair tissue scoring system (MOCART), Randomised clinical trial (RCT), Hyaluronic acid (HA).

1.3. The Disease Osteoarthritis

Osteoarthritis, the most common form of musculoskeletal disorder, is a chronic and debilitating disease. Currently it is a disease with no known cure and treatment options are focused mainly on managing the pain associated with the disease. OA is characterised not only by articular cartilage loss but also by inflammation of the synovium, weakening of ligaments and periarticular muscles, subchondral bone sclerosis with subsequent formation of osteophytes (Figure 1.5). The symptoms of OA can vary dramatically between individuals with some patients actually being asymptomatic [184]. However the classic signs of an OA-affected joint include pain, stiffness, joint effusion, and loss of function [184, 185].

OA can be divided into two types, primary and secondary OA. Primary OA is recognised when there is no known trauma or disease process causing the OA and is usually associated with age related wear and tear. Secondary OA is resultant from either injury or illness or has a hereditary or congenital component such as mal-alignment of the joint [186, 187]. Despite these classifications, the etiology of the disease remains undetermined [188]. In general, the development and progression of OA is thought to be due to an imbalance between the degradation and repair mechanisms in both the articular cartilage and subchondral bone.

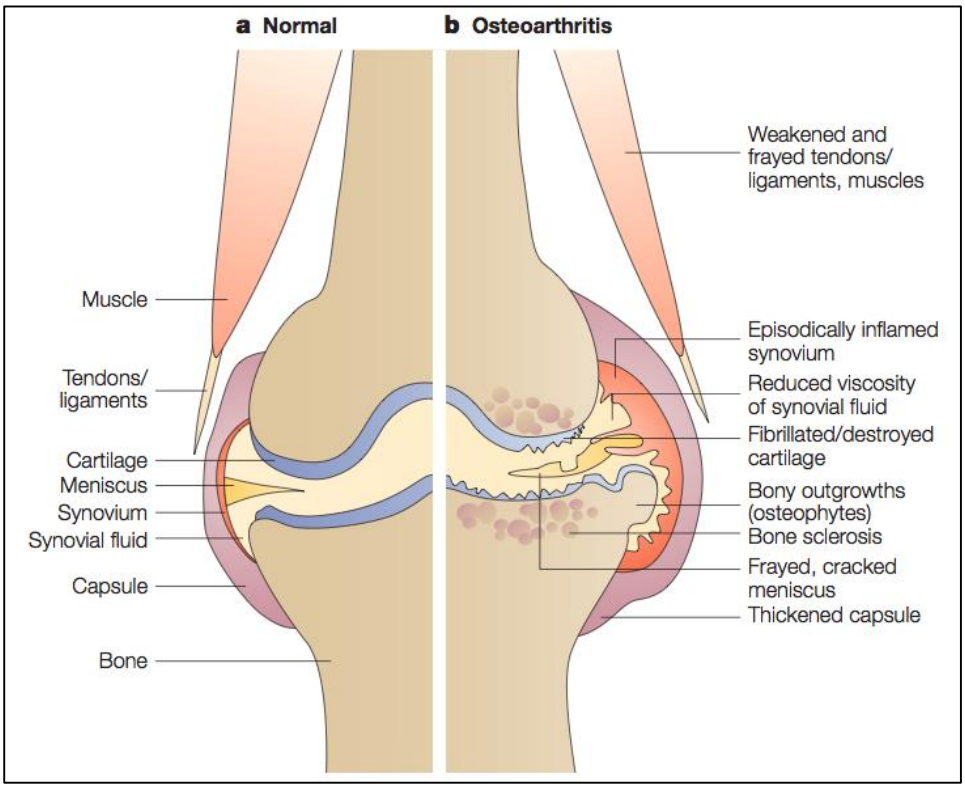

Figure 1.5. The manifestations of osteoarthritis in the joint.

Osteoarthritis of a knee joint (a) normal joint and (b) osteoarthritic joint
Image taken from [189].

1.3.1. Osteoarthritis and Inflammation

OA is classically referred to as a non-inflammatory disease in comparison to other inflammatory arthritic diseases including rheumatoid arthritis and seronegative spondyloarthropathies [190]. However, it is increasingly evident that inflammation plays a major role in OA disease progression [185]. Regardless of whether a patient presents with primary or secondary OA, the synovial membrane becomes inflamed in response to cartilage degradation and changes in the underlying bone [184]. Once the degeneration of the joint has begun, the cells of the synovial membrane attempt to clear the cartilage debris present in the synovial fluid. As a result, the synovial membrane becomes hypertrophic and hyperplasic and the situation turns to that of a self-sustaining cycle of low-grade inflammation [187].

The inflammatory cycle involved in OA development and progression within a joint is incredibly complicated and not well understood. It appears there is a complex positive feedback loop involved whereby many of the cytokines are able to stimulate

the release of each other and themselves, which ultimately feeds the self-sustaining cycle of inflammation. The cytokines and growth factors considered to be involved in this cycle can be categorised as catabolic, anabolic, inhibitory or regulatory based on their roles in cartilage metabolism. The key pro-inflammatory cytokines responsible for the inflammation in osteoarthritic joints are IL-1β and TNF-α. IL-1β is considered the major contributor to cartilage destruction whilst TNF-α drives the inflammatory process. IL-4, IL-10, IL-13 and interleukin 1 receptor antagonist (IL-1Ra) mediate an anti-inflammatory response in OA, whereas IL-6 and IL-8 are considered regulatory cytokines. Growth factors such as IGF-1, TGF, bFGF and bone morphogenic proteins (BMP-2, 4,7,9) constitute the anabolic factors involved in OA. An imbalance of these cytokines and growth factors result in an increase in degradation within the joint and a subsequent decrease in the repair mechanisms.

IL-1β and TNF-α are among the major drivers of inflammation in an OA joint and the neutralisation of these cytokines are a focus of academic and commercial studies to find a cure or at the least, halt the progression of OA. The levels of both IL-1β and TNF-α are present in higher levels in synovial fluid, synovial membrane, chondrocytes, and subchondral bone in patients with OA [191]. Chondrocytes and synoviocytes (cells of the synovial membrane) are known to produce both of these pro-inflammatory cytokines [192]. Both IL-1β and TNF-α have been implicated in the activation of proteases including MMPs responsible for cleaving collagen, MMP1, MMP8 and MMP13, and MMP3, which cleaves proteoglycans [193, 194]. IL-1β has been implicated in inhibiting the synthesis of natural MMP inhibitors known as tissue inhibitor of metalloproteinase (TIMP) [195]. Furthermore, IL-1β and TNF-α not only stimulate the production of other cytokine such as IL-6 [196], IL-8 [197], LIF [198, 199], CCL5 [200] and prostaglandin production from chondrocytes and synoviocytes, but can also stimulate their own production from these cells [185, 187, 195]. IL-1β and TNF-α stimulation of chondrocytes derived from human OA-affected joints caused an increase in chemokine expression including monocyte chemoattractant protein 1 (MCP-1) *in vitro* [201]. Yuan *et al.,* 2001 also illustrated a 62% decrease in proteoglycan synthesis by OA chondrocytes in the presence of MCP-1 when compared to the healthy chondrocyte control. Therefore, these two cytokines directly cause cartilage destruction and indirectly inhibit matrix synthesis. The cumulative effect of

all of the above activities of these two cytokines results in a skew in the normal catabolic and anabolic processes in the joint to favour a more destructive environment. Furthermore, the combination of IL-1β and TNF-α has been shown to induce more advanced cartilage destruction *in vivo* then either cytokine alone [202, 203].

1.3.2. Osteoarthritis in Canines

OA commonly affects the canine pet population. In the US it is estimated that one in every five dogs over the age of one year suffers from osteoarthritis [204]. With an estimated canine population of 72 million in the US this estimate indicates that over 14 million dogs are affected by OA [205]. Management of OA in dogs is challenging as it affects all age groups and can span the lifetime of an animal therefore requiring life long intervention.

Hip and elbow dysplasia are hereditary disorders with symptoms developing primarily in the first 12 months of a dogs life. These disorders result in osteoarthritic pain and lameness commonly requiring surgical intervention and life long management. Canine cruciate ligament disease is the most common orthopedic injury in dogs and in 2003, $1.32 billion was spent in the US alone on managing cruciate ligament rupture in dogs [206]. Despite costly surgical intervention to repair the ligament, cruciate ligament rupture leads inevitably to the onset of OA, which not only has a huge impact on the long-term outcome of the ligament repair, but results in loss of function, pain and lameness. Age related primary OA in dogs is extremely common with over 50% of presenting OA cases being observed in dogs aged between 8-13 years. However, age related onset is dependent on breed. In large breed dogs such as the Rottweiler, the mean onset age is 3.5 years whilst in dogs such as poodles, 9.5 years is the mean [207]. Primary OA in the dog is usually multifocal with all or a combination of elbow, knee, hip and shoulder joints being affected.

Many treatment options currently exist for canine OA. These include weight management through specialised diets, supplements such as chondroitin sulfate, non-steroidal anti-inflammatory drugs (NSAIDs), pentosan polysulphate, other analgesic drugs and surgery [208]. No single treatment provides relief for all patients and many dogs have only a partial reduction in symptoms despite a combination of treatments.

It is well understood that little is to be gained by surgical intervention except in the case of total joint arthroplasty, which is an effective treatment for OA of the coxofemoral (hip) joints [209]. However, this is an invasive and technically demanding surgical procedure with well-recognised complications and a high financial cost. The multi-focal nature of OA in most patients also limits the effectiveness of surgical treatments. Therefore, there is a clear need for an effective, less invasive technique that can treat multiple affected joints in a single procedure but is compatible with all existing treatments. The dual abilities of MSCs to modulate inflammation and incite endogenous tissue regeneration via trophic activity, offer a promising treatment for canine OA.

1.4. Stem Cells in Veterinary Medicine

The last 10 years, has seen the rise of stem cell research and regenerative medicine emerge in the veterinary community. As with human medicine, the veterinary industry is experiencing a paradigm shift in healthcare away from symptom relieving drug therapies towards a cellular repair and regeneration approach. The veterinary communities and regulatory framework that govern veterinary therapeutics are receptive to new technologies. As in human medicine, stem cell treatments are available for animal patients at a limited number of veterinary hospitals around the world. In particular, MSC treatments for animals are commercially available in the US, the UK, UAE, Europe, New Zealand and Australia. However, this type of medicine is not yet mainstream and there is still much to understand regarding the mechanisms of action of stem cells as therapeutics for them to become commonplace. Published literature on the clinical use of stem cells in veterinary medicine is limited to a small number of studies in canine and equine musculoskeletal and immune mediated conditions, which will be discussed in brief.

1.4.1. Results of Mesenchymal Stem Cell Trials in Animals

Published studies in animals utilising MSCs for real clinical disease have focused on the treatment of atopic dermatitis, tendon injury, and OA. These treatments have utilised either bone marrow- or adipose tissue-derived MSCs. These applications are discussed in turn.

1.4.1.1. Atopic Dermatitis

One open, non-controlled, non-blinded pilot clinical trial has been documented for the treatment of canine atopic dermatitis. Canine atopic dermatitis is described as a genetically pre-disposed inflammatory disorder. In this trial an intravenous injection of 1.3 million culture-expanded autologous adipose-derived MSCs per kg was administered. The results showed no significant improvement in clinical signs of atopic dermatitis in the 5 dogs treated, however, 2 of the 5 dogs treated showed a trend toward sustained reduction of visual pruritus score [210].

1.4.1.2. Tendon and Ligament Injury

Three approaches have been used to treat equine tendon and ligament injury. The first was an intra-lesion injection of autologous adipose-derived cells, containing a mixed population of non-cultured cells. Anecdotal clinical results of more than 2,500 horses demonstrated that the therapy was advantageous in horses with tendon and ligament injuries [211]. Smith and Webbon (2005) reported the use of *in vitro* expanded bone marrow MSCs in over 100 horses with superficial digital flexor tendinopathy. This approach showed rapid in-filling of the tendon lesion with no negative side effects [212]. Follow-up of the horses that entered full training (> 1 year) showed a re-injury rate of 18%. The number of horses that entered full training was not reported but a previous study reported a 56% re-injury rate for similar categories of horses that did not receive stem cell treatment [213]. Del Bue *et al.,* (2008) reported the treatment of tendonitis in 16 horses with culture-expanded adipose-derived MSCs in conjunction with platelet-rich plasma. Of the 16 horses treated 14 returned to their full pre-injury function [214].

1.4.1.3. Osteoarthritis

Four reports on the use of adipose-derived MSCs for the treatment of canine OA have shown promising results [215-218]. Two studies involved an intra-articular injection of a mixed population of adipose-derived, uncultured cells. One study was a randomised double-blind trial of 21 dogs with OA of the coxofemoral (hip) joint. The dogs treated with cells had a statistically significant improvement in lameness and functional ability compared with the dogs that received a placebo saline injection [217]. The second study used the same mixed population of adipose-derived cells to treat dogs with arthritic humeroradial (elbow) joints [218]. Improved lameness was observed and this remained evident at the final follow-up time-point of 180 days post-treatment. Two other groups have utilised adipose-derived cultured MSCs to treat elbow [216] and hip OA [215] in dogs. Guercio *et al.,* (2012) treated four dogs with clinically diagnosed lameness associated with OA. MSCs with either HA or PRP were utilised in this study and the dogs of each group demonstrated improvements in lameness, functional disability and pain on manipulation [216]. Vilar *et al.,* (2013) objectively demonstrated, with the use of force plate analysis, that treatment of severe hip OA in eight dogs with culture-expanded adipose-derived MSCs and PRP resulted in reduced lameness [215].

In essence only two sources for obtaining MSCs are currently applicable in small animal veterinary medicine, either bone marrow or adipose tissue. Due to the limited numbers of MSCs derived from fresh bone marrow aspirate in comparison to adipose tissue and the morbidity associated with collection of bone marrow in the dog, the research performed in this thesis has concentrated on the use adipose-derived MSCs and their secretions.

1.5. Adipose Tissue

In the late 1980's adipose tissue was recognised to contain large numbers of precursor cells (now known as MSCs) that could become fibroblastic and were capable of differentiating into adipocytes under *in vitro* culture conditions [219]. Several other key discoveries made in the late 1980's and 1990's, including the identification of the adipokines adipsin, leptin and adiponectin and their role in

glucose and appetite regulation, firmly established adipose tissue as an endocrine organ [220-223]. In 2001 Patricia Zuk and colleagues first suggested that adipose tissue could provide an alternate source of adult stem cells. Their published research demonstrated cells derived from adipose tissue could differentiate *in vitro* into cells of the adipogeneic, chondrogeneic, myogenic, and osteogeneic lineages [8]. Far from hormonally inert, it is now recognised that adipose tissue secretes a variety of cytokines that can act on other organ systems of the body through autocrine, paracrine or endocrine signaling [224]. These findings, coupled with the ease of harvesting large quantities of adipose tissue have lead to this tissue being a desired regenerative cell source.

Adipose tissue is largely comprised of adipocytes with approximately 60-85% of the weight of adipose tissue being the lipid contained within the adipocytes. Each adipocyte is filled with a single large lipid droplet, which is predominately triglyceride. The remaining weight is composed of water (5-30%) and protein (2-3%) [225]. The adipocytes are supported by richly vascularised connective tissue with each adipocyte being in contact with at least one capillary [226]. This dense network of capillaries provides, not only delivery of substrates and oxygen to the tissue, but also a route for the release of hormones and cytokines that act in an endocrine fashion. The non-adipocyte fraction of the adipose tissue is comprised of numerous cell types, which are collectively known as the stromal vascular fraction (SVF). Figure 1.6 is a microscopy image of a section of collagenase digested adipose tissue that is stained with the nucleic acid dye Syto 11. The image shows large lipid filled adipocytes with their nuclei displaced to the periphery of the cell allowing space for triglyceride. Running through the adipose tissue are long strands of connective tissue with a myriad of brightly stained nuclei of the numerous cell types that comprise the SVF. The cell types that comprise the SVF will be discussed in Section 1.5.1.

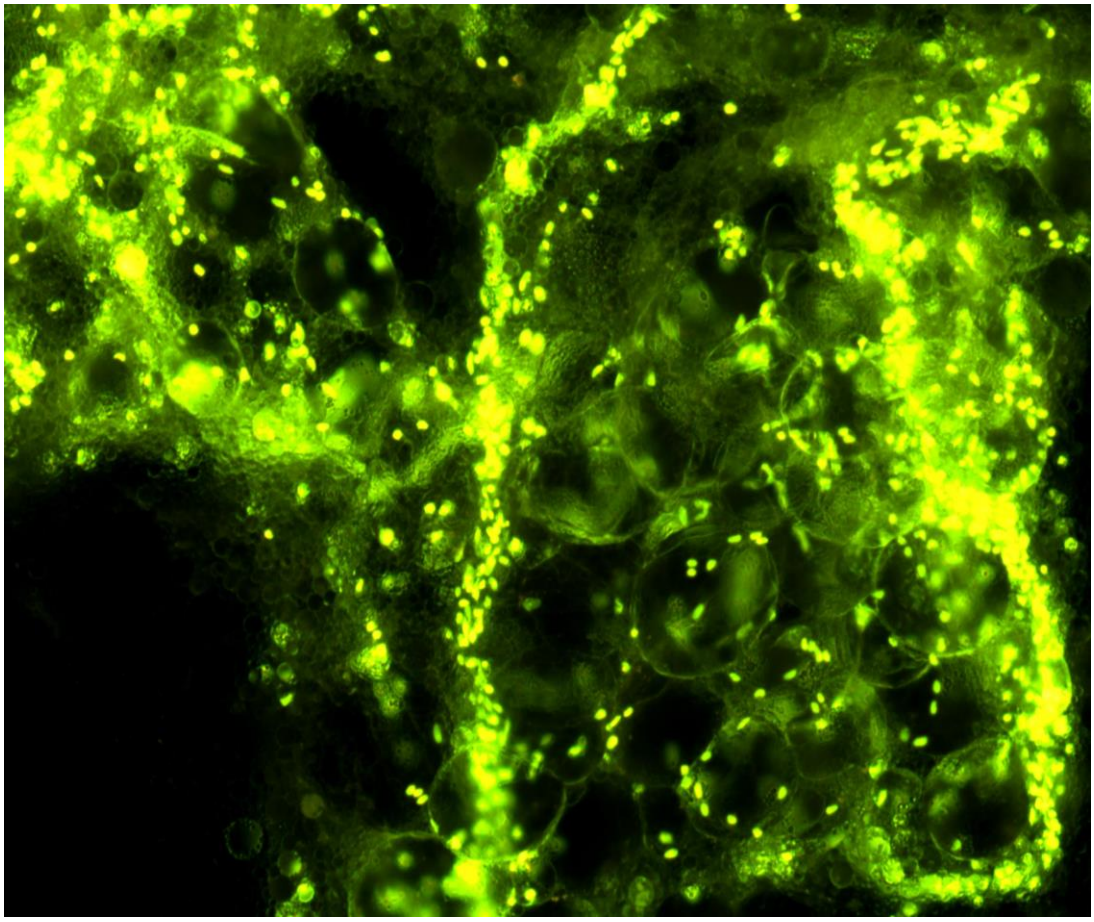

Figure 1.6. Microscopy image of collagenase digested adipose tissue stained with Syto11.

Image taken by Michael Medynskyj and used with his permission.

1.5.1. The Stromal Vascular Fraction

The SVF results from enzymatic digestion or mechanical disruption of adipose tissue. The SVF obtained from human adipose tissue is comprised of multiple cell types including MSCs, HSCs, endothelial cells, pre-adipocytes, smooth muscle cells and white blood cells (WBCs) [36, 49, 227, 228]. Although the SVF has a high proportion of MSCs (refer to Section 1.2.2.2.2.) that have relevance in the treatment OA, the therapeutic use of the entire SVF offers its own benefits. The heterogeneous cell population allows for immediate same day treatment, as no *in vitro* selection or expansion steps are required. Furthermore, it is likely that the non-MSC cell types in the SVF possess therapeutic properties and act in a concerted manner with each other, and the MSCs, as well as with the local cells of the environment they are introduced to. Consequently a therapeutic effect greater than the effect described

with MSC therapy alone has been reported in the treatment of multiple sclerosis [49, 229].

Currently characterisation of the cell types that comprise the canine SVF have not been detailed in the literature. Comprehensive investigations of the human SVF by Varma *et al.,* (2007) and Zimmerlin *et al.,* (2010) using CD markers and flow cytometry do however, offer some insight into the cell types likely to comprise all mammalian adipose tissue. Varma's characterisation determined that, 34.6 ± 17.8 % of SVF cells were positive for MSC surface markers, 12.2 ± 9.5 % were identified as endothelial cells, 10.3 ± 9.9 % mural cells and 37.03 ± 9.5 % expressed cell surface antigen of various types of WBCs [36]. With the use of high speed flow cytometric sorting techniques, Zimmerlin *et al.,* (2010) identified two individual MSC populations and two individual endothelial cell populations [230].

The WBCs identified in the adipose tissue include both granulocytes and lymphocytes. Adipose tissue is a site of intense immune cell activation and interaction so it is not surprising that essentially all of the immune cells are present in the SVF. Of most interest are the immune regulatory macrophages and T regulatory (Treg) cells. Macrophages isolated from non-obese adipose tissue are recognised to exhibit an alternatively activated (M2) phenotype. M2 macrophages have been shown to secrete high levels of anti-inflammatory cytokines IL-10 and IL-1Rα [231], which are recognised to be very relevant as mediators of inflammation in OA as described in Section 1.3.1. In activated immunity, M2 macrophages along with MSCs are thought to prevent co-stimulatory molecule activation inducing Treg expression rather than T helper cell expression. The Treg cells in turn are involved in maintaining macrophages in an M2 phenotype rather than the pro-inflammatory M1 macrophage phenotype. Treg cells are the body's most important defense against inappropriate immune responses and therefore SVF M2 macrophages and MSCs play an important role in not only transplant rejection and immune mediated conditions but also chronic tissue injury such as OA [232].

Due to the highly vascularised nature of adipose tissue it is not surprising that the SVF is comprised of a proportion of the endothelial cells. Endothelial cells are well understood to be involved in angiogenesis and have been shown to reverse induced hind limb ischemia and preserve the affected limb in rodent models [233]. The endothelial cells are likely present in the SVF due to the disruption of blood vessels

during the excision and digestion of adipose tissue. Koh *et al.,* (2011) report that the formation of vasculature created by the SVF is due to the rapid reassembly of endothelial cells. This mechanism to construct a vascular network is relevant in wound healing, tissue graft survival and ischemic conditions [234]. Furthermore Trakteuv *et al.,* (2009) have demonstrated the synergistic relationship between MSCs and the endothelial cells of the SVF. They identified that the combination of MSCs and endothelial cells provided greater neovascularisation than either cell type in isolation [235].

Varma reports approximately 10% of the SVF is made up of mural cells, being pericytes and vascular smooth muscle cells. These cell types are identified by positive cell surface antigen expression of CD146 and CD34 and are negative for CD31, which distinguishes this population from the endothelial cell population. Whilst vascular smooth muscle cells form part of the blood vessel wall, pericytes are found wrapped around endothelial cells. Currently the *in situ* niche of MSCs is not well validated, however based on a seminal paper by Crisan *et al.,* (2008); many researchers now propose that MSCs are in fact a subset of perivascular cells. In support of this theory pericytes, like MSCs, are found in a multitude of organs and tissues throughout the body. Additionally, they express the same cell surface markers and have the ability to differentiate *in vitro* into cells of the osteogeneic, chondrogeneic, and adipogeneic lineages [37]. Furthermore Zimmerlin *et al.,* (2010) reported isolated adipose tissue pericytes had a greater propensity for adipogeneic differentiation than the other characterised SVF cell populations [230].

In summary the SVF contains numerous cell types that work in a collaborative manner to alter the microenvironment of a site of injury or can shift overactive immune responses into a regulatory immune environment.

1.5.2. Expansion and Cryopreservation of Mesenchymal Stem Cells

Adipose tissue is a host tissue of MSCs, which can easily be purified by digestion and in turn MSCs can be isolated through adhesion to plastic in cell culture conditions. The culturing of MSCs prior to therapeutic use has two clear benefits. The cell numbers are amplified and the culturing process proportionally reduces the number of cells that are incapable of reproducing. To culture cells, a cell mixture, in

this example the SVF, is incubated in standard culture media in plastic tissue culture flasks and maintained at 37°C in 5% CO2. The MSCs adhere to the plastic surface of the flask while the non-adherent cells float and are removed. The adherent cells can be expanded over a period of several weeks to achieve a desired cell number. Although MSCs are not the only cell type of the SVF to exclusively adhere to plastic, the MSC portion proliferates faster eventually eliminating all other cell types. It is important to note that post cell culture expansion, the MSC phenotype alters when compared to freshly derived MSCs. Namely MSCs are often CD34+ post isolation and subsequently CD34- post culture [36, 230, 236]. Expansion of the MSCs from the SVF has the benefit of not only amplifying cell number, but can enable tissue engineering approaches for autologous use as seen in examples of MSCs grown on scaffolds of matrices [237]. Additionally, established cryopreservation techniques enable long-term storage of culture-expanded MSCs. Cryopreservation involves the freezing of MSCs at sub-zero temperatures to prevent damage or death of the cells. Biological activity ceases in cells stored in liquid nitrogen at temperatures of -196°C enabling long-term storage of cells. To limit any damage to the cells during the freezing process itself, usually caused by the formation of ice crystals that puncture cell membranes, cryoprotectant agents are utilised.

Once successfully cryopreserved, studies have shown that long-term cryostorage does not affect the stem cell characteristics of MSCs, being differentiation capability, cell surface antigen expression and among other things cell morphology, proliferation and senescence rates [238, 239]. There are however, risks associated with the cryopreservation and the thawing of cells. Most cryoprotectant agents are inherently toxic to cells during the thawing process and at anytime that cells are kept in contact with the cryopreservant pre- or post-thaw. As such, new cryopreservation methods, cryoprotectant agents or a combination of cryoprotectants are constantly being investigated [240, 241].

1.5.3. Providing Adipose-derived MSC Treatment to the Veterinary Clinic: Autologous Freshly Isolated In-clinic Cell Therapies versus Allogeneic Off-the-shelf Therapies

To date, the adipose-derived treatment offerings available in veterinary practice have predominately been autologous in nature. Autologous treatments can be administered in two forms, either as a freshly derived product or as a culture expanded isolated MSC product.

Freshly derived SVF cells can be isolated from adipose tissue within as little as an hour and contain significantly more MSCs per gram than bone marrow. As such the SVF is beginning to be utilised as a point-of-care cellular therapeutic for both animals and people alike. The heterogeneous cell population of the SVF has been demonstrated to be efficacious in canine OA and in human patients suffering from multiple sclerosis [49, 217, 218]. However, providing a freshly derived SVF treatment has its limitations. The process of harvesting the adipose tissue and isolating the SVF is time, space and labor intensive and is subject to variability. Furthermore, significant cost can be incurred for the surgical procedure and the process of preparing the SVF from the fat. Although not necessarily the case in people and horses, to harvest adipose tissue from a dog requires a general anesthetic. The anesthetic and surgical component of an SVF therapeutic for dogs therefore also introduces significant patient morbidity and additional financial cost.

An allogeneic culture-expanded MSC therapeutic is an alternate cellular therapy option. Unlike the mixed cell population of the SVF, the immune-privileged nature of isolated MSCs make allogeneic stem cell therapeutics a possibility. An allogeneic product in-turn limits recipient patient morbidity, as a surgical procedure and an anesthetic episode are not required. Furthermore, adipose tissue can be harvested from healthy donor animals undergoing a routine procedure such as an ovariohysterectomy, which limits donor morbidity. The culture expansion process enables cells from one donor to be expanded exponentially. This in-turn supports consistency of a product, something that is not possible in freshly derived therapeutics that inevitably vary from individual to individual. Whilst the reproducibility and ease of availability of an off-the-shelf allogeneic therapeutic have clear benefits there are some limitations. To provide an immune-privileged

homogeneous cell population, MSCs must be selected for by plastic adherence, in-turn all other cells they normally reside with *in vivo* are lost. An *in vitro* study investigating human liposaspirate cell populations demonstrated that the secretion profile of isolated MSCs was very different to the secretion profile of the SVF. The MSC profile consisted of significantly higher concentrations of some cytokines while the secretion of other cytokines disappeared completely in comparison to the SVF [242]. This is likely due to the loss of cell signaling from the other SVF cells. It is unclear what effect selection of one cell population will have on normal paracrine activity when utilised *in vivo*.

1.6. Project Outline

This thesis explored the therapeutic efficacy of two methods of producing adipose-derived cells and their secretions to provide cellular regenerative medicine for dogs suffering from OA. The first method was an autologous adipose-derived SVF treatment. This treatment utilised not only freshly derived MSCs but also all of the other cells that comprise the SVF. This was a personalised, point of care treatment option. The second method is an 'off-the-shelf' allogeneic MSC treatment option. This offering exploited the proliferation capability and immune-privileged nature of MSCs. The research in this thesis was performed with the overarching aim of providing safe, efficacious and affordable cellular therapeutics for the treatment of canine OA.

1.61. Thesis Aims

The overall aims of the thesis were:

Chapter 3 - To assess if freshly isolated canine SVF cells had a therapeutic benefit in the treatment of clinical canine OA through the follow-up of animals receiving the treatment.

Chapter 4 - To characterise the canine SVF in terms of cell types, proportion of cell types, and secretion profiling.

Chapter 5 - To investigate the therapeutic effect of cryopreserved canine MSCs with the addition of canine adipose-derived cell secretions, versus a standard cryopreservant, in a standardised murine model of arthritis.

Chapter 6 – To perform an *in vitro* analysis investigating the effects of cryopreserving canine MSCs, in a cryopreservant medium containing canine adipose-derived cell secretions versus a standard cryopreservant.

CHAPTER TWO

Materials and Methods

Described in this chapter are the materials, equipment and methods utilised in the process of performing the research described in this thesis.

2.1. Materials

The materials and equipment used in this work are presented in Table 2.1.

Table 2.1. Materials and equipment

Materials/Equipment	Company
0.2µm Nanosep MF centrifugal Devices with Bio-inert® membrane	Pall Scientific, USA
35µm nylon mesh tubes	Becton Dickinson, USA
6-well+lid Greiner CellStar cell culture plates	Greiner Bio-one, Germany
12-well+lid Greiner CellStar cell culture plates	Greiner Bio-one, Germany
Bio-Plex 200 system with version 5.0 software	Bio-Rad, USA
Bio-Plex Pro II magnetic wash station	Bio-Rad, USA
Bio-Plex Pro Mouse 23-plex assay	Bio-Rad, USA
Bio-Plex Pro Mouse 9-plex assay	Bio-Rad, USA
Carl Zeiss Primo Vert inverted microscope	Carl Zeiss Pty Ltd, Germany
Cell culture flasks (T175 cm2)	Greiner Bio-one, Germany
Cell culture flasks four-layer cell factory	Sigma-Aldrich, USA
Cryovials	Thermo Fisher Scientific, USA
FACScan flow cytometer	Becton Dickinson, USA
Filter 0.22µm steripak	Millipore, USA
Filter 400µm steriflip	Millipore, USA
Filter Vivaflow 200	Sartorius, Germany
Flow cytometry tube	Becton Dickinson, USA
HEPA Forma Series II Water jacketed 37°C, 5% CO2 incubator	Thermo Fisher Scientific, USA
Mr Frosty (slow rate freezing device)	Nalgene, USA
TruCount tubes	Becton Dickinson, USA
Water bath	Thermo Fisher Scientific, USA

Table 2.2 contains the source of the reagents and chemicals used in this work.

Table 2.2. Reagents and chemicals

Reagent/Chemical	Supplier
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich Co, USA
1% antibiotic-antimycotic solution (ABAM)	Life Technologies, USA
ACK lysing buffer	Life Technologies, USA
Alizarin Red S	Sigma-Aldrich Co, USA
Anti-canine CD4-FITC antibody	eBiosciences, USA
Anti-canine CD24-FITC antibody	eBiosciences, USA
Anti-canine CD34-PE antibody	eBiosciences, USA
Anti-canine CD44-FITC antibody	eBiosciences, USA
Anti-canine CD45-FITC antibody	eBiosciences, USA
Anti-canine CD90-PE antibody	eBiosciences, USA
Anti-canine CD146-FITC antibody	eBiosciences, USA
Anti- Rat IgG2b K FITC isotype control	eBiosciences, USA
Anti- Rat IgG2a KFITC isotype control	eBiosciences, USA
Anti- Mouse IgG1 K FITC isotype control	eBiosciences, USA
Anti- Mouse IgG1 K PE isotype control	eBiosciences, USA
Anti- Rat IgG2b K PE isotype control	eBiosciences, USA
B-glycerophosphate disodium salt hydrate	Sigma-Aldrich Co, USA
Bio-Plex sheath fluid	Bio-Rad, USA
Bovine serum albumin (BSA)	Sigma-Aldrich Co, USA
Canine serum (CS)	Wongaburra Research Centre, Australia
Collagenase	Sigma-Aldrich, USA
Dexamethasone	Sigma-Aldrich Co, USA
Dimethyl sulfoxide (DMSO)	Life Technologies, USA
Dulbecco's Modified Eagle Medium (DMEM; high glucose (HG))	Life Technologies, USA
Ethanol	Sigma-Aldrich Co, USA
ELISA, quantikine, canine VEGF	R&D Systems, USA
ELISA quantikine, mouse, rat, porcine, canine TGF-β1	R&D Systems, USA
FACS lysing solution	Becton Dickinson, USA

Fetal Bovine Serum (FBS)	Life Technologies, USA
Flow cytometry buffer	eBiosciences, USA
Indomethacin	Sigma-Aldrich Co, USA
Insulin	Life Technologies, USA
Isoflow	Becman Coulter, USA
Isopropanol	Sigma-Aldrich Co, USA
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich Co, USA
Oil Red O	Sigma-Aldrich Co, USA
Paraformaldehyde	Sigma-Aldrich Co, USA
Phosphate buffered saline (PBS)	Life Technologies, USA
Propidium iodide	Sigma-Aldrich Co, USA
Saline	Baxter, Australia
Syto11	Life Technologies, USA
TrypLE express	Life Technologies, USA

The formulations of solutions used in this thesis are presented in Table 2.3.

Table 2.3. Solution formulations

Solution (working concentration)	Formulation
Alizarin Red S solution (2%)	2% solution prepared by dissolving Alizarin Red powder in MilliQ water; pH adjusted to 4.2 with 5M NaOH; 0.2µm syringe filtered
B-glycerophosphate disodium salt hydrate (173mM)	173mM stock prepared by adding 560mg of B-glycerophosphate disodium salt hydrate in 15mL of HG DMEM with 10% CS; aliquots frozen.
Dexamethasone (1µM or 0.1µM)	5mM stock prepared by dissolving dexamethasone powder in 100% ethanol; aliquots frozen; diluted in standard cell culture media to obtain working concentration of 1µM or 0.1µM
FACS Lysing solution (1x)	10x FACS Lysing solution purchased from Becton Dickinson; diluted in isoflow to obtain a 1x solution
Fat digestion solution (0.05%)	25mg collagenase dissolved in 47.5mL of HG DMEM
IBMX (0.5mM)	250mM stock prepared by dissolving IBMX powder in DMSO; aliquots frozen; diluted in standard cell culture media to obtain working concentration of 0.5mM
Indomethacin (200µM)	48mM stock prepared by dissolving indomethacin powder in DMSO; aliquots frozen; diluted in standard cell culture media to obtain working concentration of 200µM
Isopropanol (60%)	99% isopropanol diluted with MilliQ water
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (50 µM)	10mM stock prepared by dissolving L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate powder in HG DMEM with 10% FBS; aliquots frozen; diluted in standard cell culture media to obtain working concentration of 50µM
Oil Red O (0.2%)	0.3% stock prepared by dissolving Oil Red O powder in 99% isopropanol; diluted in MilliQ water to obtain 0.2% solution; incubated at room temperature for 10

	minutes; 0.2µm syringe filtered
Paraformaldehyde (4%)	Paraformaldehyde dissolved in PBS to obtain a 4% solution; heated to 70°C for 10 minutes; pH adjusted to 7 with 5M NaOH
Propidium iodide (10 µg/mL)	Stock solution of 1mg/mL in water purchased from Sigma-Aldrich; diluted in Isoflow to obtain a working concentration of 10µg/mL
Standard cell culture media (FBS)	HG DMEM; 10% FBS; 1% ABAM
Standard cell culture media (CS)	HG DMEM; 10% CS; 1% ABAM

2.2. Methods

2.2.1. Techniques for the Measurement of Secreted Proteins

The ability to identify and measure the quantity of bioactive factors secreted by cells is an important component in understanding cell-cell communication and the role these bioactive factors play in the therapeutic effect of cellular therapy. Whilst there are new techniques such as selected reaction monitoring (SRM, also known as multiple reaction monitoring) being developed and validated for the quantitation of secreted proteins, the most commonly employed approach to measure bioactive factors is immunoassays such as direct enzyme-linked immunosorbent assays (ELISAs).

SRM is a targeted quantitative mass spectrometry technique. In contrast to conventional mass spectrometry techniques designed to detect all peptides in a given sample, SRM aims to detect only the peptide/s of interest. The peptide mixture is analysed using a scanning mass spectrometer and restricting the acquisition mass range to the mass to charge ratio of the ion/s of interest [243]. Whilst SRM is a promising technique, developing these assays for target analytes requires significant optimisation and subsequent validation to identify suitable transitions (pairs or peptide precursor ion and fragment ion mass masses) unique to the peptide/protein of interest [243]. As this approach directly targets the protein/peptide of interest, it is an attractive alternative for measuring proteins for which there are no available antibodies or antibodies are difficult to obtain.

Direct ELISAs rely on antibodies specific for the target analyte. In the case of a sandwich ELISA, the analyte is 'sandwiched' between a capture antibody and an enzyme-linked secondary antibody, which serves as the detection antibody. Addition of a substrate for the enzyme conjugated to the secondary antibody allows for quantitation of the amount of target analyte present through either colorimetric or fluorescent detection means. ELISAs are typically designed to measure and quantitate a single analyte of interest in a complex mixture of proteins. However, in the past 5 years, multi-plex immunoassays have been developed to enable the measurement of multiple analytes of interest in a complex sample within a single assay. This approach utilises Luminex xMAP technology, which involves a set of uniquely coloured beads

that may also be magnetic. Each antibody to a target analyte is conjugated to unique fluorescently coloured beads. The beads are incubated with the sample, followed by biotinylated secondary antibodies and a streptavidin-phycoerythrin (PE) reporter complex. The levels of each analyte can be quantified using a dual-laser flow-based microplate reader system. Using this approach, the lasers first detect the internal fluorescence of the beads followed by the intensity of the fluorescence of the streptavidin-PE reporter, allowing quantitation of a multiple analytes in one sample. This approach enables up to 100 proteins to be quantified in a single sample in a single assay. Multiple companies now offer disease or pathway specific multi-plex panels. This technology therefore has many advantages over the conventional single-plex ELISA approach. In particular, multi-plex assays allow for large numbers of proteins to be detected in a small sample volume (25-50μL) in a more cost and time efficient manner. It is for these reasons that the Milliplex Map 13-plex canine cytokine, chemokine and growth factor panel was used extensively in this thesis to measure and compare secreted levels of these factors from adipose-derived SVF, MSCs and secretions from both cellular populations. This multi-plex approach has also been used in this thesis to determine if it can be used as an objective biological metric to determine the effect of a treatment *in vivo*.

2.2.1.1. Milliplex and Bio-Plex Protocols for Measuring Secreted Proteins

The experiments described in this thesis used the Milliplex Map 13-plex canine cytokine, chemokine and growth factor kit (catalogue number CCYTMG-90K-PX13; Millipore, USA), the Bio-Plex Pro Mouse Cytokine 23-plex (catalogue number M60-009RDPD) kit and the Bio-Plex Pro Mouse Cytokine 9-plex (catalogue number MD0-00000EL) kit (Bio-Rad, USA). Regardless of the kit used, the protocol used to measure the bioactive factors in different sample types was essentially the same. The protocol was followed according to the manufacturers instructions and is described below.

2.2.1.1.1. Canine Milliplex

All reagents were allowed to warm to room temperature prior to use. The standard was prepared by dissolving the provided standard with 250 μL of deionized

water. Six polypropylene microfuge tubes were labeled (12,500pg/mL, 3125pg/mL, 781pg/mL, 195pg/mL, 48.8pg/mL, 12.2pg/mL) and filled with 150µL of assay buffer. The provided standard was vortexed and a 4-fold serial dilution was performed by adding 50µL of the 50,000pg/mL standard to the 12,500pg/mL labeled tube and then 50µL from that tube to the subsequent tube and so forth. The assay buffer alone constituted the 0pg/mL standard. To each well, 200µL of assay buffer was added. The plate was then sealed and mixed on a plate shaker for 10 minutes and the assay buffer subsequently decanted. Samples were filtered through 0.2µm Nanosep MP Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, USA) for 5 minutes at 9000*xg*. A volume of 25µL of each standard, quality control and sample were added to the appropriately labeled well. An additional 25µL of assay buffer and 25µL of cell culture media were added to each well followed by 25µL of vortexed pre-mixed beads. The plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 2 hours at 300rpm. The wells were then washed twice using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. A volume of 25µL of room temperature detection antibody was added to each well. The plate was again sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 60 minutes at 300rpm. To each well containing the detection antibody, 25µL of Streptavidin-PE solution was added. The plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 30 minutes at 300rpm. The wells were then washed twice using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. A volume of 150µL of sheath fluid was added to each well and the plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100 rpm for 5 minutes. The plate was then read and the data acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA).

2.2.1.1.2. Mouse Bio-Plex

All reagents were allowed to warm to room temperature prior to use. The standard was prepared by dissolving the provided lyophilised standard with 500µL of appropriate diluent. Cell culture media was used to dilute the standards when cell culture supernatant samples were being analysed. The provided standard diluent was used when serum samples were being analysed. The standard was vortexed and placed on ice for 30 minutes. Following this incubation, the standards were then prepared by diluting 128µL of the stock standard in 72µL of diluent. A 1:4 dilution series was then performed to produce 8 standards. The concentration of each individual cytokine in the highest standard is provided with each kit. The 10x coupled beads were vortexed and subsequently prepared by diluting the beads 1:10 in assay diluent. To each well, 50µL of the 1x coupled beads was added and washed twice using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. Samples were filtered through 0.2µm Nanosep MP Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, USA) for 5 minutes at 9000*xg*. A volume of 50µL of the standards, blanks and samples were added to each well. The plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 30 minutes at 300rpm. The wells were then washed three times using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. The 10x detection antibody was vortexed and then prepared by diluting 1:10 with detection antibody diluent. To each well, 25µL of the 1x detection antibody was added. The plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 30 minutes at 300rpm. The wells were then washed three times using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. The 100x Streptavidin-PE solution was vortexed and then prepared by diluting 1:100 with assay buffer. To each well, 50µL of the 1x Streptavidin-PE solution was added. The plate was sealed, protected from light and incubated at room temperature on a shaker set at 1100rpm for 1 minute followed by 10 minutes at 300rpm. The wells were then washed three times using the Bio-Plex Pro II magnetic wash station primed with the provided wash buffer. A volume of 125µL of assay buffer was added to each well and the plate was sealed, protected from light and incubated at room

temperature on a shaker set at 1100rpm for 1 minute. The plate was then read and the data acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA).

2.2.2. Flow Cytometry Techniques for Cell-Surface Antigen Profiling

Immunophenotyping is one of the major clinical applications of flow cytometry. It is used in the diagnosis of myelomas, lymphomas and leukemia and is also used to monitor disease progression and the effectiveness of medical interventions [244]. Flow cytometry is a key technology used in this thesis for the cell enumeration, viability assaying and immunophenotyping of adipose-derived SVF cells and MSCs. Immunophenotyping allows an estimation of the relative percentage of certain cell types within a cell population by measuring expression of cell surface antigens. The available canine monoclonal antibodies; CD4, CD25, CD34, CD44, CD45, CD90, CD146 and appropriate corresponding isotype controls (eBiosciences, USA) were used in this thesis for cell surface antigen profiling of both freshly derived SVF cells and cryopreserved MSCs post-thaw. Additionally for each antibody a corresponding isotype was included as a control. Isotype control antibodies are designed to help assess the level of background staining that is inherent in cell-antibody binding assays. In essence the same immunophenotyping protocol was utilised for both the SVF and MSCs however there were some differences in how the SVF and MSC samples were analysed on the flow cytometer due to the SVF being a mixed cell population.

2.2.2.1. SVF and MSC Immunophenotyping Sample Preparation

The supernatant from both freshly derived SVF and post-thaw MSC cell pellets was discarded and cells re-suspended in 1mL of flow cytometry staining buffer (buffered saline solution containing FBS and sodium azide (0.09%) as a preservative; eBiosciences, USA) in a 15mL conical centrifuge tube. Cells were washed twice by centrifugation at 400*xg* for 5 minutes. The supernatant was discarded and cells resuspended in 1mL of flow cytometry buffer. A 50µL aliquot of the resuspended cells was added to 13 flow cytometry tubes (Becton Dickinson, USA). There was 1 flow

cytometry tube for each of the 7 antibodies CD45-FITC (catalogue number: 11-5450-42), CD44-FITC (11-5440-42), CD4-FITC (11-5040-42), CD25-FITC (11-0250-42), CD146-FITC (14-1469-82), CD34-PE (12-0340-42) and CD90-PE (12-5900-42), as well as the following isotype controls, Rat IgG2b K FITC (11-4031) Rat IgG2a kappa FITC (11-4321) Mouse IgG1 K FITC (11-4714), Mouse IgG1 K PE (12-4714) and Rat IgG2b kappa PE (12-4031), and 1 tube for the unstained control. To each of the flow cytometry tubes (other than the unstained control), 5μL of the appropriate conjugated antibody or corresponding isotype control was added. The samples were vortexed for approximately 3 seconds and tubes were placed in the dark at 4°C for 30 minutes. After this incubation, 500μL of flow cytometry staining buffer was added to each flow cytometry tube and centrifuged again at 400*xg* for 5 minutes. The supernatant was discarded and this washing process was repeated a second time. A 1x solution of FACSfix lysing solution (Becton Dickinson, USA) was prepared by diluting FACSfix lysing solution in PBS 1:10. To each tube, 200μL of the 10% FACSfix lysing solution was added. All tubes were run on a FACScan flow cytometer that had a 488nm laser.

2.2.2.2. SVF and MSC Flow Cytometry Analysis

Characterisation of the SVF is difficult due to the presence of cell and tissue debris, cell doublets, and autofluorescence. For this reason the addition of a nucleic acid dye (Syto11 or PI) was required to distinguish the SVF cells from the auto-fluorescence of debris inherent in the sample. Syto11 and PI nucleic acid dyes are compatible with PE and FITC fluorochromes respectively, allowing cell surface antigen characterisation to be performed on a flow cytometer with a 488nm laser. As the nucleic acid dyes were required to identify the SVF cells, the samples were gated on forward scatter channel (FSC) and fluorescence channel (FL)-1 or FL-2 respectively. Fluorescence compensation was not required as there was no spillover fluorescence of Syto11 and PI in the FL-2 and FL-1 channel respectively.

The MSCs that underwent cryopreservation were a culture-expanded cell population that were not associated with any of the cell and tissue debris problems identified with the SVF. Therefore no nucleic acid dyes were required to identify the

MSC population. As such, all MSC samples were gated on FSC versus side scatter channel (SSC).

For both the MSC and SVF samples, the use of an isotype gating control to help distinguish specific from nonspecific binding was utilised. The isotype control was used to determine positivity or negativity for each of the individual CD markers.

2.2.2.3. SVF and MSC Viability Sample Preparation and Flow Cytometry Analysis

An aliquot (50µL or 200µL) of the freshly isolated SVF cells or freshly isolated MSCs was filtered through 35µm nylon mesh topped tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and transferred into Trucount tubes (Becton Dickinson Franklin Lakes, New Jersey, USA) containing Isoflow (650µL), PI (200µL) and Syto11 (100µL). Initially samples were gated on FSC (x-axis) versus FL-1 (y-axis). Total cell events were gated and enumerated based on a total count of 500 Trucount beads. The Trucount beads enabled standardization of gated events regardless of the sample type being analysed. A second plot, showing total gated cell events, was gated on FL-1 (y-axis) versus FL-2 (x-axis). The nucleic acid dye Syto11 permeabilizes all mammalian cell membranes whereas PI is membrane impermeant and is generally excluded from viable cells. As such, the number of cells PI positive were deemed non-viable. The PI positive cells could be distinguished from the total number of Syto11 positive cells to determine viability percentage. Figure 2.1 shows representative dot plots of the described viability protocol.

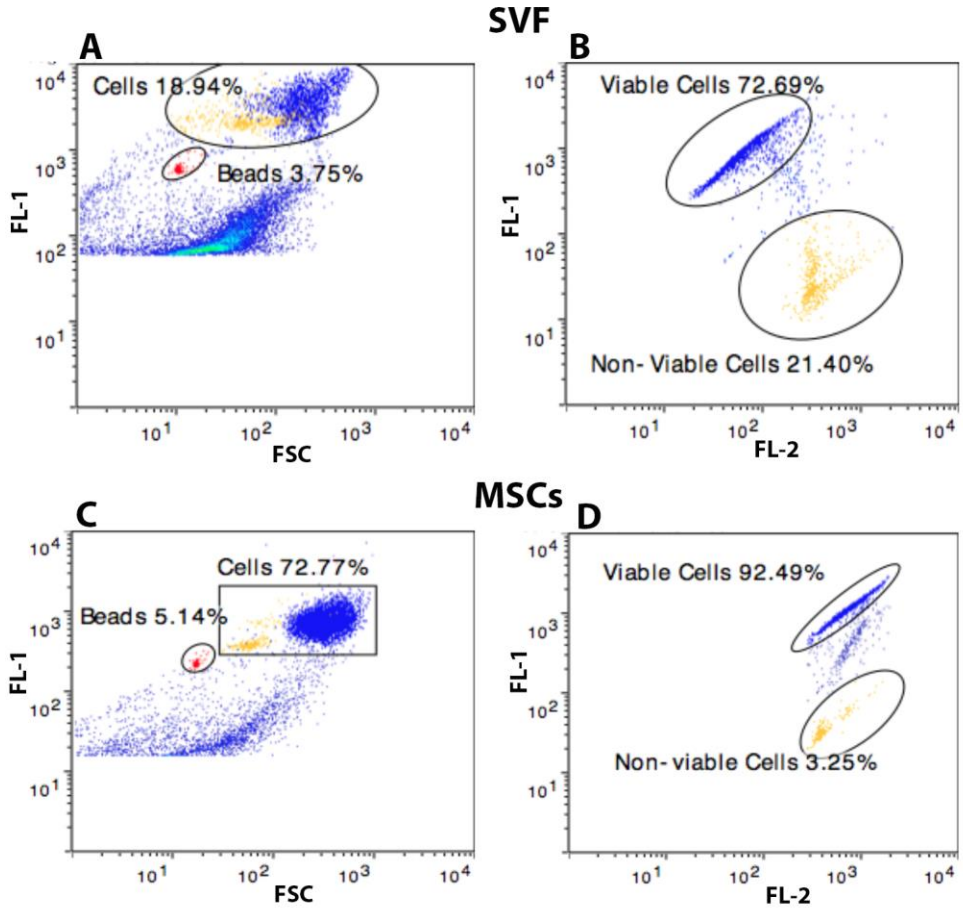

Figure 2.1. Representative flow plots of canine SVF and MSCs viability.

(A-B) show representative flow plots of canine SVF viability. Plot (A) is gated on FL-1 versus FSC. A total of 500 beads were counted (shown in red). Cells were determined by their high FL-1 fluorescence (shown in blue and yellow gate). Plot (B) shows just the 'Cells' population gated on FL-1 versus FL-2. Cells with high FL-1 fluorescence were considered viable (shown in blue gate) and cells with high FL-2 and low FL-1 fluorescence were considered non-viable (shown in yellow gate). (C-D) show representative flow plots of canine MSC viability. Plot (C) is gated on FL-1 versus FSC. A total of 500 beads were counted (shown in red). Cells were determined by their high FL-1 fluorescence (shown in blue and yellow gate). Plot (D) shows just the 'Cells' population gated on FL-1 versus FL-2. Cells with high FL-1 fluorescence were considered viable (shown in blue gate) and cells with high FL-2 and low FL-1 fluorescence were considered non-viable (shown in yellow gate). The results are presented as percentage of total events.

2.2.2.4. Click-iT EdU Proliferation Assay

The Click-iT EdU (Life Technologies, USA) assay was used to determine active cell proliferation over-time. EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into newly formed DNA as cells multiply. Detection of the EdU is based on a copper catalysed covalent reaction between an azide and an alkyne (click reaction). In the Click-iT EdU assay, the alkyne is found in the ethynyl of the EdU while the azide is coupled to an AlexaFluor 488 dye. The addition of the

AlexaFluor 488 dye enabled detection of the percentage of S-phase cells through the use of flow cytometry.

EdU, AlexaFluor 488 azide, DMSO, saponin-based permeabilisation wash reagent and the EdU buffer additive were allowed to warm to room temperature. A 10mM solution of EdU was prepared by adding 8mL of DMSO to lyophilised EdU and mixing well. To prepare a working solution of AlexaFluor 488 azide, 260µL of DMSO was added to the dye and mixed well. A 1X working solution of saponin-based permeabilisation wash reagent was prepared by adding 10mL of the reagent to 90mL of 1% BSA in PBS. To create a 10X stock solution of EdU buffer, 2mL of deionized water was added to the EdU buffer additive.

Pelleted MSCs, were resuspended in 3mL of 1% BSA in PBS and centrifuged at 400*xg* for 5 minutes. The supernatant was discarded and pelleted cells were dislodged by adding 100µL of Click-iT fixative. Cells were incubated for 15 minutes at room temperature protected from light. To each tube, 3mL of 1% BSA in PBS was added and tubes were centrifuged at 400*xg* for 5 minutes. The supernatant was discarded and pelleted cells were resuspended in 100µL of saponin-based permeabilisation wash reagent. Cells were again incubated for 15 minutes at room temperature protected from light. To each tube, 438µL of PBS, 10µL of $CuSO_4$, 2.5µL of AlexaFluor 488 azide and 50µL of EdU buffer additive was added. Cells were incubated for 30 minutes at room temperature protected from light. To each tube, 3mL of the saponin-based permeabilisation wash reagent was added and tubes were centrifuged at 400*xg* for 5 minutes. The supernatant was discarded and cells were resuspended in 500µL of the saponin-based permeabilisation and then analysed on a FACScan flow cytometer with a 488nm laser.

A control sample that was never exposed to EdU for each time-point for each replicate was used to determine gating. Anything above the negative cell population was considered to be actively proliferating.

Each results chapter of this thesis contains a detailed description of the methods used. Table 2.4 contains an index of the methodology used.

Table 2.4. Methods

Method	Refer to Section
Adipogeneic differentiation of MSCs	6.2.2.1.2.
Adipose tissue digestion	3.2.2, 4.2.1, 5.2.1.1, 6.2.1.1.
Bio-Plex analysis of mouse serum	5.2.3.2.
CAIA mouse trial - conducted by TetraQ	5.2.2.
CD marker characterisation of cells	4.2.4, 6.2.2.1.1.
Cryopreservation of MSCs	5.2.1.5, 6.2.1.5
Enumeration and viability testing of cells using flow cytometry	3.2.4, 5.2.1.3, 6.2.1.4
Milliplex MAP analysis of canine conditioned media and serum	4.2.6, 5.2.3.1, 6.2.2.2
MSC isolation and expansion	4.2.2, 5.2.1.2, 6.2.1.2
Osteogeneic differentiation of MSCs	6.2.2.1.2.
Production of secretions from SVF	4.2.5.
Production of secretions from MSCs	5.2.1.4, 6.2.1.3
Click-iT EdU Proliferation assay	6.2.2.1.3.
SVF isolation	3.2.2, 4.2.1, 5.2.1.1, 6.2.1.1

CHAPTER THREE

Evaluation of the Treatment of Canine Osteoarthritis with Autologous Adipose-derived Stromal Vascular Fraction Cells

This chapter documents the outcomes of twenty-six dogs suffering from osteoarthritis that were treated within a newly developed adipose-derived cellular therapy. This is a preliminary chapter investigating the effects of autologous stromal vascular fraction (SVF) therapies. The overall aim of this chapter was determine if there was an efficacious response to an autologous point-of-care adipose-derived therapeutic to assess if further investigation into this cellular therapy for the treatment of canine osteoarthritis was warranted.

3.1. Introduction

OA is a debilitating and painful musculoskeletal disease traditionally characterised by the loss of articular cartilage. It is a multifactorial inflammatory disease that affects multiple joint tissues, including synovium, tendon, and ligament and subchondral bone. Although the onset of OA is not clearly understood, it is evident that pro-inflammatory cytokines, including IL-Iβ and TNF-α, perpetuate the degenerative process of osteoarthritis [191]. These cytokines disrupt the balance of the catabolic and anabolic activities of chondrocytes, eventually leading to the destruction of joint tissues.

OA is the most common articular disease that affects dogs, with an estimated 20% of dogs afflicted at any one time [204]. The current standard of care for OA is drug therapy, usually analgesics or non-steroidal anti-inflammatory drugs (NSAIDs). However, these drug therapies are symptom relieving only and do not address the underlying pathophysiology of the disease. Furthermore, long-term use of these medications has been associated with toxicity issues [245] and often do not provide complete pain relief [246]. As such there is a need for disease modifying treatment options for OA.

Over the last decade cellular therapy has emerged in veterinary medicine in the form of bone marrow- or adipose-derived mesenchymal stem cells (MSCs) or adipose-derived stromal vascular fraction (SVF) cells. The methods currently used to isolate the SVF from adipose tissue are based on techniques first developed by Rodbell in the early 1960's [247]. The methods involve digesting minced adipose tissue with collagenase and then separating the adipocytes and other nucleated cells that comprise the SVF by centrifugation. The SVF contains a range of nucleated cell types including MSCs, hematopoietic stem cells (HSCs), endothelial cells, vascular smooth muscle cells and leukocytes [36, 248]. MSCs are multipotent cells capable of differentiating into all cells of the mesenchyme lineage, namely cartilage, bone, tendon, ligament, muscle and adipose tissue [249]. Adipose tissue is a highly vascularised tissue and contains approximately 500-1000 times more MSCs per gram than bone marrow [250, 251]. Consequently, the use of the adipose-derived SVF provides a source of MSCs that requires no additional ex vivo expansion. There is now a plethora of literature demonstrating the therapeutic mechanisms of MSCs via *in*

vitro assays and in animal models of induced disease. It is now well recognised that the therapeutic effect of MSCs is driven by their paracrine activities [26]. Both MSCs and the SVF have been shown *in vitro* to secrete high quantities of therapeutically important cytokines [242]. These cytokines play a key role in controlling tissue-specific repair processes and have been shown to modulate immune and inflammatory responses, prevent fibrosis and apoptosis and incite resident stem cells to proliferate [26]. Additionally, promising evidence of hyaline cartilage regeneration has been demonstrated utilising MSCs in acute cartilage injury models in rabbits and pigs [158, 160]. These studies showed superior cartilage histology and morphology of MSC treated groups in comparison to control groups [158, 160]. Furthermore, evidence of the slowing of the degenerative changes in OA and protective mechanisms of MSCs by intra-articular administration has been shown in degenerative cartilage injury models both goats and rabbits [89, 161].

Taken together, the dual capabilities of MSCs to regenerate cartilage and modulate the tissue microenvironment, via paracrine activities, has lead to the use of MSCs and the SVF for the treatment of clinical cases of canine and equine musculoskeletal pathologies. A double-blind multicentre trial utilising adipose-derived SVF for the treatment of canine OA of the coxofemoral (hip) joint showed a significant improvement between treated and control dogs in; lameness at walk, lameness at trot, pain on manipulation and range of motion, at all follow-up time-points of 30, 60 and 90 days post-treatment [217]. A further open study detailing the same adipose-derived SVF treatment in canine elbow OA, demonstrated a significant difference in lameness at walk, lameness at trot, functional disability and joint stiffness in the treated dogs post-treatment [218]. Although this study did not have a control group, the follow-up period continued to 6 months. A single intra-articular injection of culture-expanded adipose-derived MSCs in conjunction with platelet rich plasma (PRP) has also been shown to be effective in the treatment of canine hip OA [215]. Vilar *et al*., (2013) treated eight dogs with severe hip OA and utilised force plate to assess therapeutic response. At 6 months post-treatment they reported a significant improvement in peak vertical force and vertical impulse in the treated dogs compared to baseline [215]. Another study investigated the effect of a single intra-articular injection of culture-expanded adipose-derived MSCs in conjunction with either PRP or hyaluronic acid (HA) in four dogs with elbow OA [216]. At 1 month

post injection clinical outcomes in lameness and pain on manipulation improved markedly according to veterinary and owner examination [216]. In horses, Smith and Webbon (2005) reported the use of culture-expanded bone marrow derived MSCs in over 100 horses with superficial digital flexor tendinopathy. This technique showed rapid in-fill of the tendon lesion with no negative side effects [212]. Bue *et al.,* (2008) reported the treatment of tendonitis in sixteen horses with adipose-derived stem cells in conjunction with platelet-rich plasma. Fourteen of the sixteen horses treated returned to their full pre-injury function [252]. The treatment of equine joint, and tendon injuries with either bone marrow or cord blood-derived stem cells has been available in the United Kingdom since 2002 [212]. In the United States, the treatment of musculoskeletal disorders in horses and dogs utilising autologous adipose-derived stromal vascular fraction (SVF) has been available since 2003, with over four thousand horses and more than three thousand dogs treated [217, 253]. The use of adipose- or bone marrow-derived MSCs or SVF have been shown to be both safe and efficacious in the treatment of clinical cases of equine and canine joint or tendon diseases.

3.1.2. Chapter Aims

This chapter describes the use of freshly isolated adipose-derived SVF cells to treat clinical cases of canine OA. Treatment was performed as an autologous same day therapeutic where isolation of the SVF was performed on-site in the veterinary hospital. The immediate cell processing and same day re-introduction of cells differed from other published studies on adipose-derived regenerative cellular therapy for canine OA [217, 218]. It was hypothesised that the rapid re-introduction of the SVF cells would ensure greater cell viability and therefore may lead to a superior therapeutic response than the outcomes described in other treatment regimes.

In collaboration with the industry partner, a rapid in-house procedure to isolate the SVF from adipose tissue for autologous, point-of-care treatment was developed for use in the veterinary hospital. Twenty-six dogs were treated in the first year subsequent to procedure development. These dogs were client-owned pets that were suffering from clinically diagnosed OA. The aim of this chapter was to assess the

efficacy and longevity of this cellular therapy. To achieve this aim, owner questionnaires and veterinary examination scores were collated and analysed.

3.2. Methods

3.2.1. Patient Profile

Twenty-six dogs were treated within the first year of the adipose-derived protocol development. All dogs were client-owned pets presenting to the veterinary hospital due to osteoarthritic signs. The two principal veterinarians of the veterinary hospital treated and performed all examinations for these dogs. Decision to treat was based on clinical presentation of OA, with radiographic or computed tomography (CT) imaging to confirm the diagnosis and grade of OA. OA was graded as previously described utilising a 0-4 scale (0 [normal joint] to 4 [severe degenerative changes]) [217, 218]. Routine haematology, biochemistry and urine analysis was performed for all dogs to ensure overall health. Dogs were excluded from treatment for any pre-existing debilitating disease, or any suspected neurological disease. Dog owners signed a detailed information and consent form prior to treatment.

3.2.2. Adipose Tissue Harvest and Isolation of Cells

All dogs received a general anaesthetic for adipose tissue removal. Adipose tissue was collected by excision under sterile surgical conditions from the inguinal fat pad and ranged from 10 to 40 grams depending on the size and body condition of the dog. The adipose tissue was processed in an onsite laboratory immediately after excision. The tissue was finely minced with scissors and digested with 0.05% Collagenase type I (Sigma Aldrich, St Louis, Missouri, USA) in saline at 37°C for sixty minutes with a gentle mixing by hand every 15 minutes. The digested sample was filtered through a sterile 400µm mesh filter (Millipore, Billerica, Massachusetts, USA) and then centrifuged at 500*xg* for ten minutes. The adipocytes and SVF were washed once in saline, resuspended in saline and then immediately injected into the affected joints.

3.2.3. Intra-articular Injections

Intra-articular injections were performed under sedation with medetomidine hydrochloride. Injection sites were surgically clipped and prepped in an aseptic manner. The joint space was confirmed by the aspiration of synovial fluid. Depending on the size of the joint, between 1mL and 3mL of the cell suspension was injected into each joint.

3.2.4. Cell Analysis

Flow cytometry was used to enumerate the SVF cells and to measure cell viability. An aliquot (100µL) of the cell suspension was mixed with propidium iodide (Sigma Aldrich, St Louis, Missouri, USA) in Isoflow (Beckman Coulter, Brea California, USA) at a concentration of 50µg/mL. A second 100µL aliquot was mixed with Syto11 (Invitrogen) in Isoflow at a concentration of 50µM. Both samples were made up to 1mL with Isoflow and transferred separately into Trucount tubes (Becton Dickinson, San Jose, USA). Samples were analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, USA).

3.2.5. Evaluation of Dogs

3.2.5.1. Veterinary Assessment

The veterinary examination consisted of a 5-point numerical rating scale (NRS) adapted from existing published criteria as previously described [217, 218, 254]. The five categories for each of the nine examination questions are detailed in Table 3.1. As some questions were joint specific, the recorded score for the three questions indicated with an * was determined from the worst presenting joint of each dog. For consistency, the examination was performed by the same veterinarian for all pre- and post-treatment time-points.

Table 3.1. Numerical rating scale scoring profile used for veterinary examination

Question	Score				
	0	**1**	**2**	**3**	**4**
Lameness at walk	Undetectable	Barely detectable lameness	Mild lameness	Moderate lameness	Severe lameness
Lameness at trot	Undetectable	Barely detectable lameness	Mild lameness	Moderate lameness	Severe lameness
Lameness at run	Undetectable	Barely detectable lameness	Mild lameness	Moderate lameness	Severe lameness
Functional disability	Normal activities, no functional disability	Slightly stiff gait or slight functional disability	Stiff gait, clearly does not move freely or moderate functional disability	Very stiff gait, avoids weight bearing on affected limb or severe functional disability	Does not want to walk or is completely functionally disabled
Crepitus*	None	Mild	Moderate	Audible	Extreme
Swelling*	No swelling	Slight inflammation	Moderate swelling	Obviously larger limb	Extremely swollen
Jumping	Jumps easily	Slightly guarded jump	Jumps with a bit of difficulty, or climbs up	Jumps or climbs with great difficulty	Will not try to jump or climb up due to difficulty/pain
Stairs	No difficulty with stairs, climbing or descending	Slightly careful, uses both paws successively	Clearly does not move freely, uses both paws at the same time	Walks stairs with great difficulty bunny-hops	Will not walk stairs due to difficulty or pain
Pain on manipulation*	No pain or limitation when joint manipulated	Mild pain when joint manipulated	Moderate pain when joint manipulated	Severe pain when joint manipulated	Pain at any attempt of joint manipulation

A five point numerical rating scale was utilised for the veterinary examination assessment. This assessment questionnaire was adapted from previously developed scoring systems [217, 218, 255]. A score of 0 was indicative of the best possible result whilst a score of 4 indicated the worst possible result.

3.2.5.2. Owner Assessment

The owner-completed questionnaire was designed as a visual analogue scale (VAS). The owner was asked to place a vertical line along a horizontal 100mm line to reflect their pet's status for each of the 19 questions. The dog owners completed this questionnaire at the same time-points as the veterinarians. This questionnaire was adapted from the following existing questionnaires: the Helsinki Chronic Pain Index [256, 257], the Canine brief pain inventory questionnaire [258], and the VAS designed questionnaire [259] which have since been validated. An example of one of the nineteen owner-completed questions is shown below in Figure 3.1.

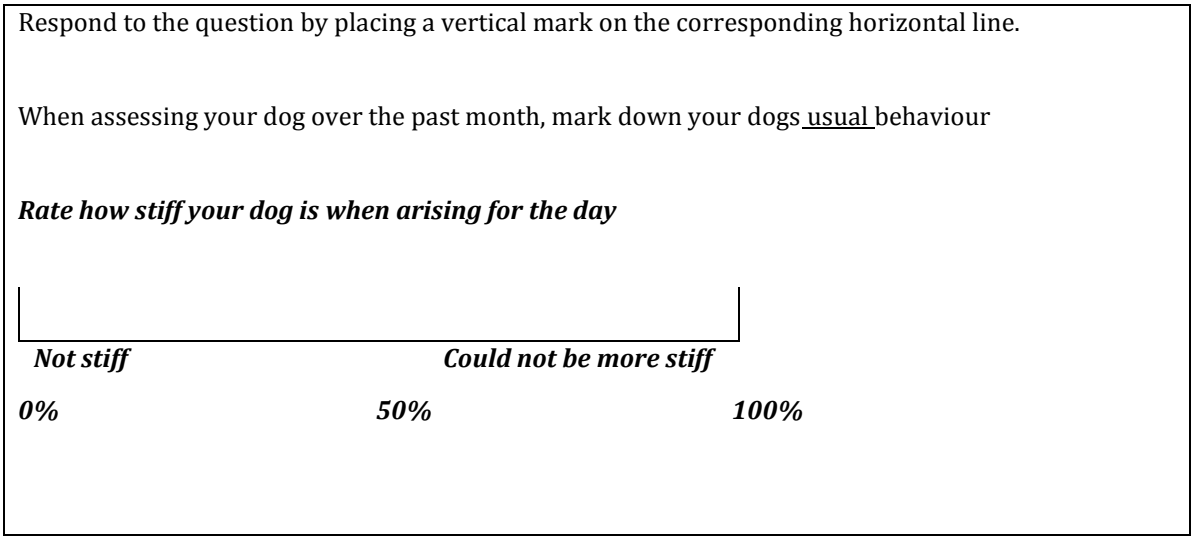

Figure 3.1. Example question from the owner-completed questionnaire.

An example of one of the nineteen questions that comprised the owner completed questionnaire. A VAS was utilised for owner reported outcomes and was adapted from existing validated questionnaires [256-259].

3.2.5.3. Medication Usage

Additionally, veterinarians recorded the medication usage of the treated dogs during the 6 month time frame both pre- and post- adipose-derived cell therapy. The dogs were administered with injectable tramadol and/or an NSAID immediately after the adipose-derived cell therapy procedure. This formed part of the standard level of care after surgical fat removal and intra-articular injections. Therefore, the assessment of medication use after adipose-derived cell therapy started from the 10 day post-treatment visit. Medication use was not restricted during the follow-up

assessment period and veterinarians assessed the dogs' analgesia and anti-inflammatory requirements at each follow-up visit and prescribed medication accordingly.

3.2.6. Data Analysis

The veterinary and owner completed questionnaire scores are presented as the average ± the standard error of the mean (SEM). Longitudinal statistical analysis was performed on the veterinary and owner completed questionnaire results using paired two-tailed t-tests. A p-value < 0.05 was considered statistically significant. The results of these questionnaires are also reported as a percentage change from baseline where the baseline score was normalised to 100%. To assess the outcome of the treatment described in this chapter to previously described results of adipose-derived SVF for the treatment of canine OA related veterinary questionnaire data was obtained and re-produced as a percentage improvement from baseline [217, 218].

3.3. Results

3.3.1. Patient Attributes and Intervention Details

Table 3.2 contains the patient attributes of the twenty-six dogs treated with adipose-derived cell therapy. The average age of the dogs was 9.4 years and ranged from 2 to 14 years. The average number of joints treated was 3.5 joints and ranged from 1 to 7 joints. Twenty-four dogs were medium-large breed dogs; the other two dogs were a miniature poodle and pug. Twenty-three of the dogs treated were geriatric dogs diagnosed with severe degenerative joint disease recognised as grade 4 OA. Two dogs were diagnosed with developmental OA suffering from hip and elbow dysplasia. In one dog diagnosed with grade 3 OA of the stifle, traditional medical therapy was contraindicated as he suffered from gastrointestinal disease as a result of NSAID therapy.

An average of 1.98 million SVF cells was recovered per gram of adipose tissue. The number of cells used for treatment ranged from 14 million to 250 million with an average of 55 million. Cell viability ranged from 50% to 95% with an average of 73%.

Table 3.2. Patient attributes.

Attributes	
Average dog age in years ± standard deviation (range).	9.4 ± 3.5. (2-14)
Sex F/M	20/6
Breed (# of dogs)	Border collie (7), GSD (5), Golden Retriever (3), Standard Poodle (1), Labrador (2) Mix breed (2), Maremma (1), Samoyed (1), Rough collie (1), Spaniel (1), Pug (1), Miniature poodle (1)
Average number of joints treated per individual dog ± standard deviation (range).	3.5 ± 1.4. (1-7)
Types of joints treated (# of joints)	Hip (38), Elbow (22), Stifle (20), Hock (6), Carpus (4)
Grade of OA (# of dogs)	Grade 4, severe OA (23) Grade 3 moderate to severe OA (3)

3.3.2. Safety

The treatment was well tolerated by all dogs and no serious adverse events were reported. One dog presented with swelling at the joint re-injection sites, which resolved after subcutaneous administration of an NSAID.

3.3.3. Clinical Evaluation

Veterinary examinations and owner questionnaires were completed when the dogs presented at the veterinary clinic for consultation. The follow-up assessments were part of the standard level of care of the veterinarians assessing the efficacy of a new treatment offering. The owners were encouraged, but were under no obligation, to return for the follow-up consultations. Therefore, some dogs were lost to follow-up at some time-points. Also a significant number of dogs died between the 6 month and final follow-up time-point assessments. These deaths were considered unrelated to the treatment or its effects and were largely due to the majority of the dogs being

geriatric and suffering from other age related diseases. The number of dogs that presented for data collection at each time point is detailed below (Table 3.3).

Table 3.3. Numbers of dogs with collected follow-up data at each time-point.

Time-point	Number of dogs with follow-up data
10 days	26
1 month	25
2 months	22
3 months	24
4 months	21
5 months	23
6 months	26
9 months	18
Final follow-up time point 12-18 months	9

3.3.3.1. Veterinary Assessment

Figure 3.2 shows the average result of each of the veterinary examination questions at each follow-up time-point. From this figure it is evident that all parameters measured showed a similar trend of a rapid reduction in the score within 10 days of treatment, which was maintained to the final follow-up time-point. These results demonstrate a rapid improvement in lameness, function and pain. Figure 3.2J shows the average of all nine veterinary examination questions.

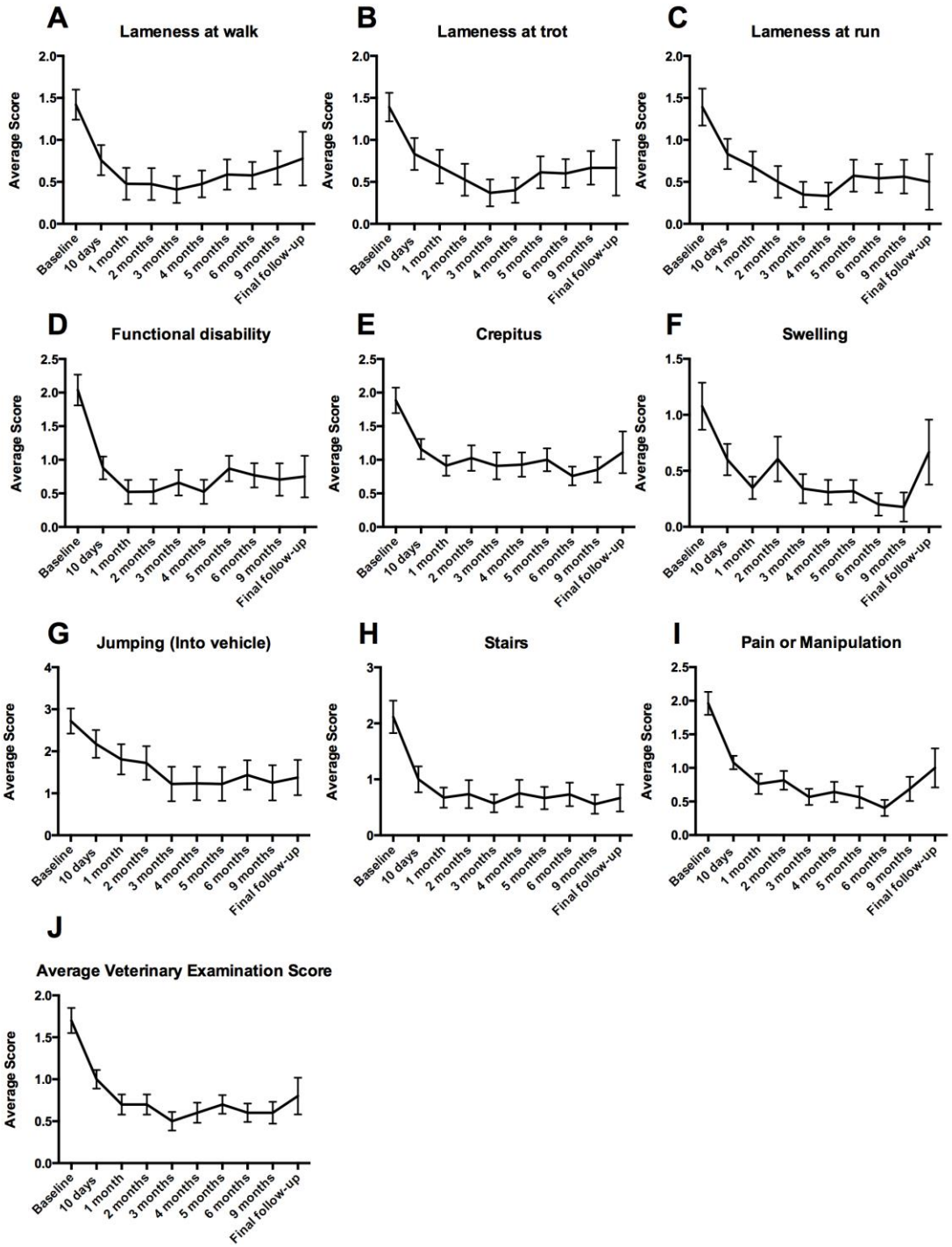

Figure 3.2. Veterinary examination scores.

(A-I) show the average veterinary examination score for each question. See Table 3.1 for the scoring system used. (J) shows the average of all eleven questions. The worst possible score was four and the best possible score was zero. Scores for (E, F and I) reflect the worst performing joint if multiple joints were treated. See Table 3.3 for the number of dogs with follow-up data at each time-point. The results are presented as the average ± SEM.

Table 3.4 displays the average combined veterinary examination question score ± SEM for all follow-up time-points. A two-tailed t-test showed that the score obtained at all post-treatment time-points was significantly lower than the baseline score.

Table 3.4. Average of all veterinary examination questions for dogs treated with autologous adipose-derived cell therapy for OA.

Time-point	Average	SEM	P-value
Baseline	1.7	0.2	
10 days	1.0	0.1	9.52E-08
1 month	0.7	0.1	4.89E-09
2 months	0.7	0.1	1.45E-07
3 months	0.5	0.1	2.04E-08
4 months	0.6	0.1	2.69E-08
5 months	0.7	0.1	3.88E-09
6 months	0.6	0.1	1.97E-08
7 months	0.6	0.1	2.22E-06
Final time-point	0.8	0.2	6.81E-03

Scores were obtained from a 5-point NRS (0 [best] to 4 [worst]) SEM = standard error of the mean. See Table 3.3 for the number of dogs with follow-up data at each time-point. Paired two-tailed t-tests were performed to compare post-treatment scores to baseline.

3.3.3.2. Owner Assessment

In comparison to the veterinary examination scoring system, the owner-completed questionnaire focused more pointedly on the activities of daily living (ADL) and how pain affected these activities. It included questions about exercise, toileting and stiffness after rest. Figure 3.3 shows the average of each of the owner-completed questions at each follow-up time-point. From this figure it is evident that an improvement in all parameters was observed from 10 days post-treatment, which was maintained to the final follow-up time-point. Figure 3.3T shows the average of all 19 owner completed questions.

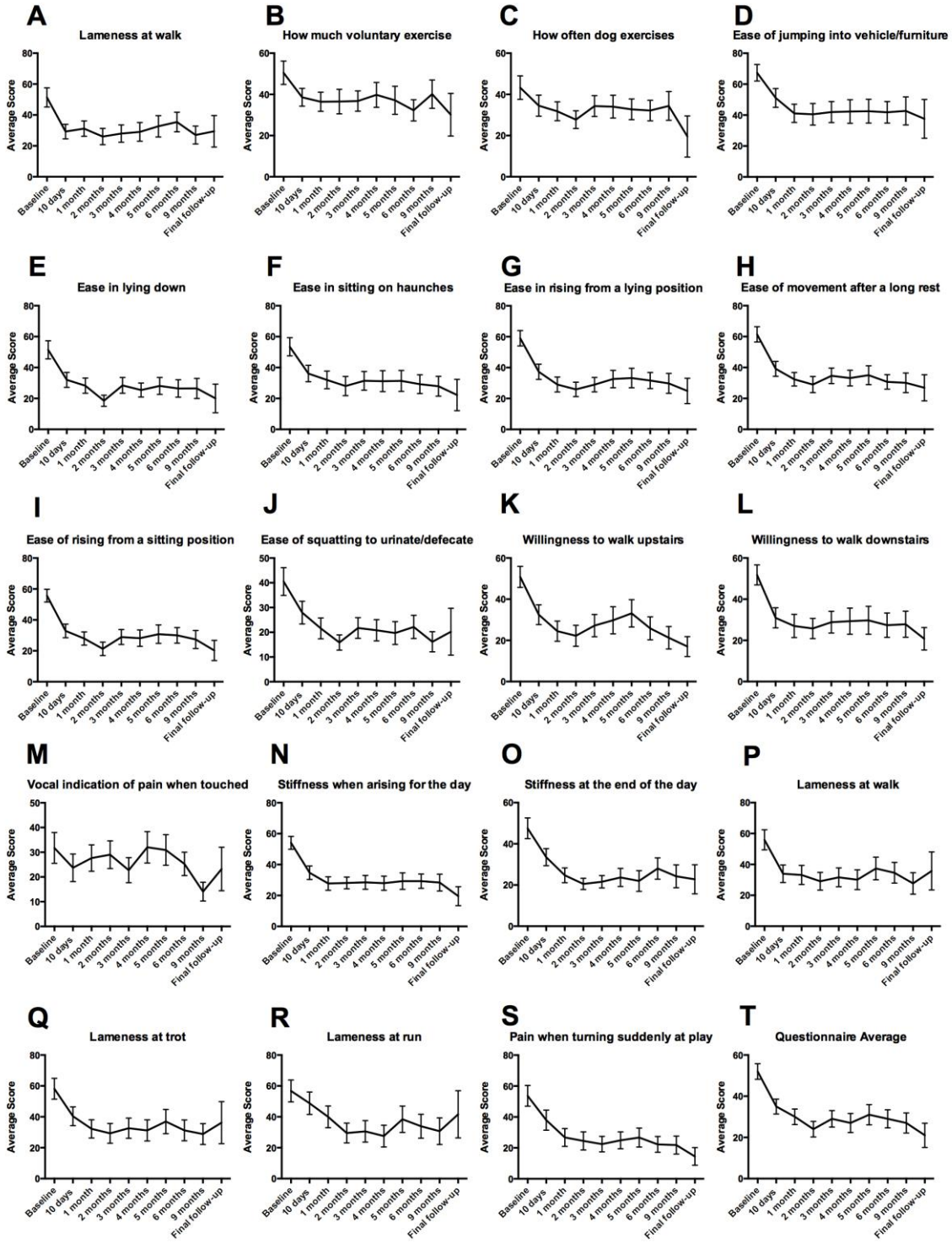

Figure 3.3. Owner completed questionnaire scores.

(A-S) show the average owner reported score for each question. (T) shows the average of all 19 questions. This questionnaire utilised a 100mm VAS to determine the score (100 [worst] to 0 [best]). See Table 3.3 for the number of dogs with follow-up data at each time-point. The results are presented as the average ± SEM.

Table 3.5 shows the average combined owner-completed questionnaire score ± SEM for all follow-up time-points. As with the veterinary examination scores, a two-tailed t-test showed that the scores obtained at all post-treatment time-points were significantly lower than the baseline score.

Table 3.5. Average of all owner-completed questions for all dogs.

Time-point	Average	SEM	P-value
Baseline	52.2	3.8	
10 days	35.2	3.6	1.88E-06
1 month	30.2	3.8	1.21E-06
2 months	24.3	3.8	1.02E-06
3 months	29.3	4.0	1.89E-07
4 months	27.3	4.6	5.71E-07
5 months	31.5	4.9	7.42E-06
6 months	29.1	4.4	2.07E-06
7 months	27.3	4.9	1.75E-04
Final time-point	20.6	5.9	1.25E-02

Scores were obtained from a 100mm VAS with 100 being the worst possible score and 0 being the best possible score. SEM = standard error of the mean. See Table 3.3 for the number of dogs with follow-up data at each time-point. Paired two-tailed t-tests were performed to compare post-treatment scores to baseline.

At the 6 month follow-up time-point, the average improvement in the veterinary reported score was 67% (n=26; Figure 3.4A). At the final follow-up time-point, veterinary examination scoring resulted in an average improvement of 47% from baseline (n= 9; Figure 3.4A). At both the 6 month and final follow-up time-points the veterinarians reported an improvement in all but two dogs. Similarly, the owner completed questionnaire outcome, resulted in an average improvement from baseline of 45% and 44% at the 6 month (n=26) and final follow-up time-points (n=9) respectively (Figure 3.4B). Dog owners reported an improvement in twenty-three of the twenty-six dogs treated at the 6 month follow-up time-point and an improvement in seven of nine dogs at the final follow-up time-point.

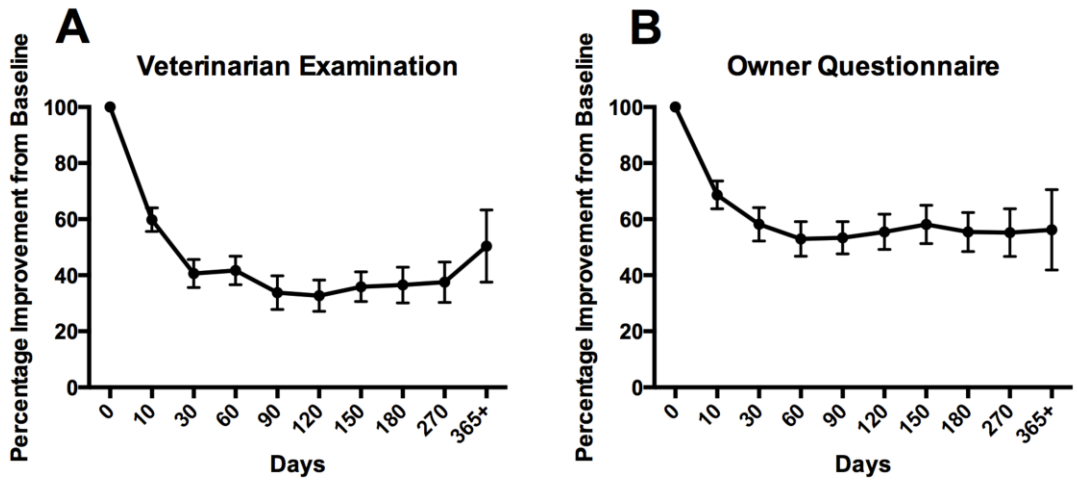

Figure 3.4. Percentage improvement from baseline.

(A) Veterinarian examination and (B) owner questionnaire reported outcomes.

3.3.3.3. Medication Use

The medication usage of each dog was recorded for the 6 month time frame prior to adipose-derived cell therapy and during the 10 day to 6 month post-treatment time frame. The type of medication, and how many dogs were prescribed each different type of medication are complied in Table 3.6. Of the twenty-six dogs treated, two dogs were not prescribed any OA medication in the 6 month time frame prior to treatment. The remaining twenty-four dogs were prescribed between one and three types of the medication during the pre-treatment time frame. At baseline, three dogs were on three types of medication; nine dogs on two types of medication and twelve dogs were taking a single medication, totaling thirty-nine instances of medication use between the twenty-six dogs (Table 3.7). At 6 months post-treatment the total amount of medication use had dropped from thirty-nine instances to ten. A total of eight dogs remained on medication, one dog remained on three types of medication and seven dogs were receiving one type of medication (Table 3.7). Of the eight dogs remaining on medication, two dogs reduced their prednisone usage by half at 6 months post-treatment. Of the three dogs remaining on carprofen, one dog's usage doubled from sub-therapeutic levels of 12.5mg/day to a therapeutic dose of 25mg/day. All other dose rates of medication, for the dogs that continued to be prescribed OA-related medication post-treatment, remained as the pre-treatment dose rate.

Table 3.6. OA-related medication use for all dogs at baseline and 6 months post adipose-derived cell therapy.

Generic medication name (drug type)	**Baseline***	**6 months post-treatment #**
Meloxicam (NSAID)	4	1
Carprofen (NSAID)	11	3
Firocoxib (NSAID)	4	0
Tramadol (Opioid)	2	1
Prednisolone (Corticosteroid)	5	2
Calcium Pentosan Polysulphate injectable (Chondromodulating agent)	11	1
Calcium Pentosan Polysulphate oral (Chondromodulating agent)	2	1
Total	39	10

Data presented are the number of dogs on each prescribed medication.
* Medication administered to the dogs during the 6 month time period prior to treatment.
# Medication administered between 10 days and 6 months post-treatment.

Table 3.7. Number of dogs prescribed multiple, single or no OA-related medication at baseline and 6 months post adipose-derived cell therapy.

	Baseline*	**6 months post-treatment #**
3 types of medication	**3**	**1**
2 types of medication	**9**	**0**
1 type of medication	**12**	**7**
No medication	**2**	**18**
Total	**26**	**26**

Data presented are the number of dogs taking multiple, single or no medication.
* Number of dogs prescribed multiple medication types, a single medication or no medication during the 6 month time frame pre-treatment.
# Number of dogs prescribed multiple medication types, a single medication or no medication between 10 days and 6 months post-treatment.

3.4. Discussion

This chapter reports the collated veterinary and owner reported outcomes of twenty-six dogs treated for OA with a newly developed autologous adipose-derived SVF cells. Adipose tissue was harvested from the inguinal fat pad of the dogs and processed on-site to obtain the SVF, and re-introduced into the dogs' osteoarthritic joints. Only two veterinarians performed this procedure during the 12 month time frame and the assessment of each dog at all time-points remained consistent to the individual treating veterinarian. The dogs were client-owned pets suffering from clinically diagnosed OA that in most circumstances were no longer effectively managed with traditional drug therapy and were not suitable surgical candidates. Most dogs were geriatric and suffering from multi-joint OA.

3.4.1. Autologous adipose-derived cell therapy has a beneficial effect for the treatment of canine osteoarthritis

Currently, existing drug therapies for OA provide symptomatic relief from pain only. Medications such as carprofen, firocoxib and meloxicam have been shown to be very effective in moderating the signs of OA, namely associated pain [260-262]. However, no treatments are recognised to repair cartilage or prevent further destruction of the joint. Whilst pentosan polysulphate, a polysulphated polysaccharide, is described as a chondroprotective agent [263], there is limited evidence of its therapeutic effect in canine OA [264]. With no known cure for OA, disease modifying OA treatments are much coveted.

The owner questionnaire and veterinary examination outcomes in this study showed a rapid and statistically significant improvement in pain and function within 10 days of adipose-derived cell therapy treatment. The improvements observed in these dogs were sustained throughout the follow-up period. Traditionally it was thought that the therapeutic potential of MSCs was due to their multi-lineage differentiation capacity, with the thought that MSCs would differentiate into chondrocytes and mediate OA through this mechanism. However, while newly regenerated cartilage has been demonstrated in small animal models of OA post-cell therapy [158, 160], there is no direct evidence that the MSCs themselves are

differentiating into chondrocytes. In a chronic degenerative large animal model of OA, MSC treatment resulted in a marked reduction in degeneration of the articular cartilage, osteophytic remodelling, and subchondral sclerosis compared to control treated joints [89]. Therefore in this model, not only did the MSCs have a protective effective preventing the onset of OA, but introduced MSCs were detected in regenerated meniscal tissue. However, it is well accepted in the literature that regeneration of cartilage in animal models of OA takes weeks to months to achieve in these models [158, 160]. Therefore, the reduction in pain and increased functionality seen as early as ten days post-treatment in this study was unlikely to be due to tissue regeneration. Additionally, although no medications were restricted or prohibited by the treating veterinarians, Table 3.6 indicates that there was a reduction in the pharmaceutical requirements of all but one dog after adipose-derived cell therapy.

More recently MSCs have been shown to have extensive paracrine activities which mediate their anti-apoptotic, anti-fibrotic, anti-inflammatory and immune-modulatory capabilities [26]. *In vitro*, MSCs and the SVF have been shown to secrete high levels of therapeutically relevant growth factors and cytokines such as VEGF, IL-7, IL-10 and IL-13 [242]. Furthermore, the co-culture of allogeneic or autologous adipose-derived MSCs with chondrocytes and synovicytes derived from human osteoarthritic joints showed that inflammatory cytokines such as IL-6, IL-Iβ and TNF-α were all down-regulated [265]. Therefore, it is possible that the initial rapid improvements observed in this study could be due to the anti-inflammatory and immune-modulatory paracrine actions of the MSCs and other cell types of the SVF, rather than the regeneration of new cartilage [91, 266]. Given the promising evidence in the literature demonstrating that MSCs may halt or reverse destruction of tissues, it is anticipated that in the long-term, the cell therapy administered to the dogs in this study could halt the progression of OA or may even regenerate damaged tissue.

Published studies utilising adipose-derived SVF for the treatment of canine OA have previously demonstrated a therapeutic benefit of SVF administration in canine hip and elbow OA [217, 218]. In these studies the extracted adipose tissue was shipped overnight to a central processing facility where the SVF was isolated and then shipped back to the veterinary hospital. Under a best-case scenario a 72-hour turn-around time from adipose-tissue harvest till SVF administration would have occurred. It was hypothesised, that the point-of-care immediate administration of the

SVF cells described in this chapter would result in an improved therapeutic outcome due to greater cell viability than the methods used in the Black studies. On average over 11 million viable SVF cells were injected per joint in this study, more than double the 5 million viable cells administered per joint reported by Black *et.al* (2007) [217]. To enable a comparison, the results of the consistent veterinary examination questions between this study and the two above mentioned studies were plotted on the same graph. Figure 3.5 shows the average percentage improvement from baseline to the final follow-up time-point for the dogs treated in this study and in the previously described Black studies of canine hip and elbow OA [217, 218]. Additionally the placebo control group from Black *et al*., (2007) was included [217]. Figure 3.5 shows that the twenty-six dogs treated described in this chapter had a greater percentage improvement in; lameness at walk, lameness at trot, functional disability and pain on manipulation than dogs treated in the Black studies. Furthermore, Figure 3.5 also shows that the SVF treatment in all three studies had a clear benefit over a placebo control (Figure 3.5). Whilst some understanding of the efficacy of the SVF treatment described in this chapter can be gleaned from the previously described control group from Black *et al*., (2007) the outcomes described in this study are from multi-joint treated OA cases rather than a controlled cohort of dogs where the treatment of only one consistent joint occurred [217].

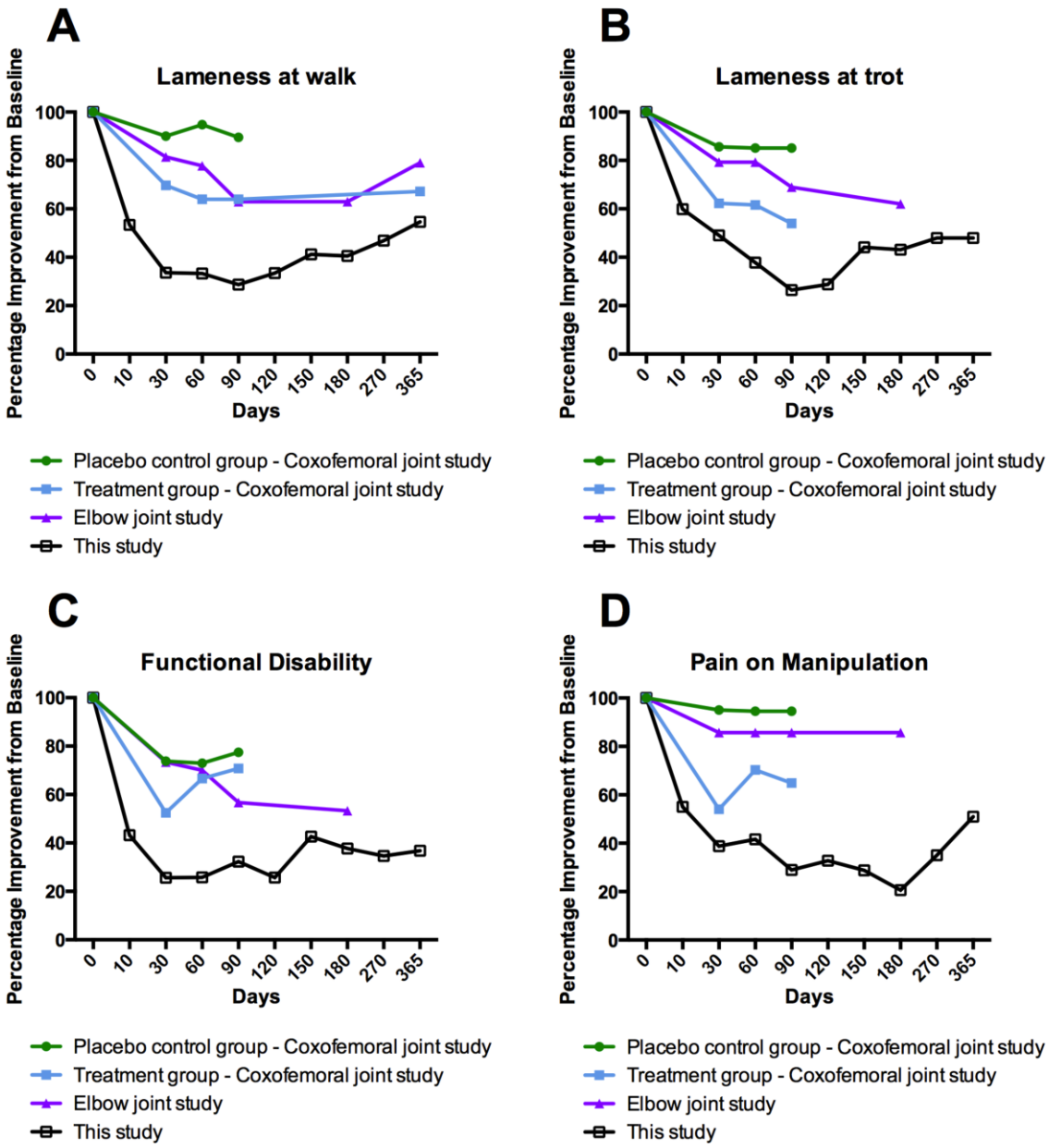

Figure 3.5. Comparison of veterinary examination question results from this study to other published studies.

Percentage improvement from baseline for: A. Lameness at walk, B. Lameness at trot, C. Functional disability and D. Pain on manipulation. The average percentage improvement of all dogs as determined by veterinary examination questionnaires utilised in this study and in previously published studies [217, 218].

3.4.2. Limitations and Future Directions

The apparent clinical improvement and reduction in medication use documented in this chapter and positive results from other similar studies [217, 218] are encouraging, but are far from conclusive. Reported in this chapter are real-world clinical outcomes of data collated from veterinary examination and dog owner questionnaires. The collection of this data was considered to be part of the standard level of care by the veterinarians as part of the introduction of a new treatment regime in their veterinary hospital. As such, there are several major limitations in the design of this study. The gold standard to wholly confirm the efficacy of a treatment involves multiple outcome measures, including objective parameters and a placebo control group [267].

To fully determine the efficacy of the SVF treatment described in this chapter the inclusion of a control group was necessary. However, due to these dogs being true clinical cases of OA, and predominately suffering from multi-joint OA, it would have been unethical to enroll an equivalent group of dogs in a control arm. Furthermore, as the aim of this study was not to determine if adipose-derived SVF treatment was more efficacious than commonly used symptom-relieving agents, it would have been inappropriate to compare this group of dogs to a group receiving an OA symptom-relieving agent such as an NSAID.

This study would have benefited from the addition of force plate or gait analysis to provide an objective outcome measure to assess changes in lameness and gait. These quantitative assessment tools do not suffer from owner placebo and veterinary bias effects. However, objective gait analysis provides only an evaluation of a dog at one particular point in time, of which is outside of a dog's typical environment and where normal behaviours are not exhibited [268, 269]. Therefore, owner-based assessment is recognised to be a powerful tool in assessing changes in an animal's typical behaviour in their typical environment [256-259]. Despite this, a placebo effect has been demonstrated previously when using owner and veterinary questionnaires to measure treatment effect in OA in dogs [270]. Future studies should be designed with both subjective and objective outcome measure to ensure robust data capture.

As this chapter details the results of a real clinical treatment offering, and not a clinical trial, dog owners were encouraged but under no obligation to return for follow-up consultation. Consequently, the number of animals lost to follow-up, particularly at the later time-points of 9 months and 12-18 months post-treatment, further limits the strength of this data. Additionally, repeat radiographic or CT imaging was not performed on these dogs. It would have been ideal to use repeat imaging to determine if any structural changes were occurring in treated joints. It would not have been appropriate or ethical however, to anaesthetise and hospitalise these dogs again for repeat imaging.

The SVF, unlike a pharmaceutical preparation, has no defined active ingredient. As the cell types that comprise the SVF are numerous, it is not known which cell type or types are responsible for the perceived efficacy. Furthermore, as this is a biological treatment, differences between the amounts of adipose tissue extracted, the cell number, cell viability and in all likelihood cell composition, make estimating the therapeutic outcome of a treatment offering such as this difficult. To understand the composition of the canine SVF, and the possible effect the composition may have on therapeutic potential, further characterisation is required.

3.5. Conclusions

Despite the limitations of this study, both safety and preliminary efficacy were established. Furthermore, as hypothesised, point-of-care immediate administration of the SVF appeared to have greater efficacy than that of off-site cellular processing and delayed administration of cells. This chapter reports real-world clinical data captured by veterinarians and dog-owners that was collated for use in this thesis. The sole purpose of collating this data was to determine if further investigation into adipose-derived cell populations and additional product development for the treatment of canine OA were warranted. The preliminary efficacious results demonstrated merit further investigation.

3.6. Acknowledgements

I would like to acknowledge that Veterinarians, Doctors Angus Ross and Jamie Geddes of Ku-Ring-Gai Veterinary Hospital performed the diagnosis of OA, the surgical excision of adipose tissue, intra-articular injections and veterinary consultations and examinations for all twenty-six dogs described in this study. I would also like to acknowledge all of the veterinary nurses, administration staff and all other support staff of Ku-Ring-Gai Veterinary Hospital for their expert nursing care and client communication skills.

CHAPTER FOUR

Characterisation of Canine Stromal Vascular Fraction using Flow Cytometry and Cell Secretion Profiling

Cellular therapy utilising mesenchymal stem cells (MSCs) or adipose stromal vascular fraction (SVF) is emerging in the veterinary space. The previous chapter documented preliminary efficacious outcomes of twenty-six dogs treated with autologous SVF. Whilst canine MSCs have been characterised in vitro, there is little known about the cell composition and secretion capabilities of canine SVF. The aim of this study was to characterise the canine SVF using immunophenotyping and cell secretion profiling. Cell counts, viability and immunophenotyping were performed on fresh canine SVF samples. The conditioned media of the canine SVF was analysed using a panel of fifteen bioactive factors. Furthermore, a correlation was performed to assess the relationship between cell number, cell type and secretion profile.

OUTPUTS FROM THIS CHAPTER

Webster, R.A., Blaber, S.P., Breen, E.J., Herbert, B.H., Vesey, G., *Characterisation of the Canine Stromal Vascular Fraction using Flow Cytometry and Cell Secretion Profiling*. BMC Veterinary Research, submitted March 2014.

4.1. Introduction

Cellular therapy for the treatment of a variety of diseases in the veterinary field is becoming increasingly common. Commercial stem cell derived treatments are available for equines and canines in the US, the UK, UAE, Europe, New Zealand and Australia. These cellular therapies include cultured MSCs [212, 271] and adipose-derived uncultured SVF [217, 218, 272]. There are clinical studies published describing promising therapeutic outcomes from these available therapies in both equines and canines but little is characterised in terms of what comprises the cell population used in these therapies. While some studies have characterised cultured bone marrow or adipose-derived cells in canines, [92, 248, 273, 274] to the author's knowledge there is no literature characterising freshly isolated canine adipose-derived SVF. This is in some part due to the technical difficulties associated with characterising the SVF. In particular, characterisation of the SVF is difficult due to the presence of cell and tissue debris. Detecting and analysing the cells within this background of debris is challenging and complicates performing multiple antibody fluorochrome staining [230, 275].

There are benefits to utilising freshly isolated adipose-derived cells for therapeutics. The SVF from adipose tissue is a rich source of MSCs containing 500 times more MSCs per gram than bone marrow [250, 251, 276] negating any need to expand cells *in vitro* and thereby enabling an in-clinic same day treatment. MSCs are capable of multi-lineage differentiation [30] and possess immunosuppressive capabilities [55]. These therapeutic properties coupled with the relative ease of obtaining large quantities of tissue and the ability to rapidly isolate the SVF for autologous use, has led to a significant increase in focus on this tissue for use in regenerative medicine. Traditionally MSCs were considered to exert their therapeutic effect through their ability to differentiate into cells of the damaged tissue type post-implantation. Recent *in vivo* studies have shown that transplanted MSCs do not always differentiate even though regeneration of the damaged tissue has occurred [68, 277]. Furthermore, it has been demonstrated that the administration of MSC secretions alone can achieve a similar initial therapeutic response to cell therapy [68]. Thus, it is now considered that cellular communications via secretions at the

autocrine, paracrine and endocrine levels, are major drivers of the therapeutic effects of cell therapy.

Despite efficacious reported clinical outcomes of treatments utilising adipose-derived cell therapy in both people and animals [49, 163, 180, 181, 217, 218], there is also usually a portion of non-responding individuals. The reason for the lack of response may be due to various factors including; disease progression, co-morbidities, environmental factors or could be due to composition of the cellular therapy used in the treatment.

To further understand the mechanisms behind the therapeutic effect and therefore gain insight as to why a treatment is not always successful, further definition of the freshly isolated adipose-derived SVF is required. There are limited studies characterising human adipose-derived SVF, but these studies have shown that the SVF is comprised of a number of different cell types. These include white blood cells (WBC) such as T regulatory cells and macrophages, vascular smooth muscle cells, endothelial cells, pericytes and MSCs [36, 227, 228]. These human studies show there is high variability between individuals in the types and proportions of cell types seen. It is of interest to know if canine SVF is comprised of equivalent cell types and proportions and if there is high variability between individuals as is seen in people.

4.1.1. Chapter Aims

The aim of this study was firstly to characterise the cellular composition of freshly isolated canine SVF, using cell surface antigen expression markers, and secondly to assess the secretion capabilities of the SVF using a multi-plex cytokine analysis approach. This type of characterisation approach may be a useful tool in future for predicting the therapeutic outcome of SVF therapy in real clinical cases.

4.2. Methods

4.2.1. Adipose Tissue Harvest and Isolation of Cells

Adipose tissue was collected from the falciform ligament from eight approximately 6 month old female healthy dogs that were undergoing a routine desex procedure. The adipose tissue was washed with saline, minced with sterile scissors and digested with 0.05% collagenase (Sigma-Aldrich, St Louis, Missouri, USA) in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, New York, USA) in a 37°C water bath for 60 minutes. The digested sample was then passed through a sterile 400µm filter (Millipore, Billerica, Massachusetts, USA) and centrifuged at 2000*xg* for 5 minutes. The floating lipid, adipocytes and supernatant were aspirated and discarded. The pelleted SVF cells were resuspended in saline, centrifuged at 2000*xg* for 5 minutes and then resuspended in DMEM. The SVF cell suspension was used for flow cytometry analysis, isolation and expansion of MSCs and for production of conditioned media for analysis of secretions (see below).

4.2.2. Isolation and Culture Expansion of Adherent Cells and Preparation for Flow Cytometry

To obtain adherent cells, a portion of the SVF pellet was seeded into a T75cm^2 flask containing DMEM supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, New York, USA) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, New York, USA) and incubated at 37°C with 5% CO_2. The media was changed every 3 days with the initial media change resulting in the removal of non-adherent cells. When the adherent cells reached 80% confluence, cells were liberated from the flask using TrypLE express (Life Technologies, Grand Island, New York, USA), and diluted in the above cell culture media. Adherent cells at passage 2 were used for the experiments described in this study.

4.2.3. Blood Collection and Preparation for Flow Cytometry

Blood was collected from healthy dogs undergoing routine haemotology and biochemistry screening. Blood was collected by jugular venipuncture using a 22-gauge needle into EDTA vacutainers (Becton Dickinson, Franklin Lakes, New Jersey, USA). Red blood cell lysis was performed using ACK lysing buffer according to manufacturers instructions (Life Technologies Grand Island, New York, USA).

4.2.4. Flow Cytometry

A FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used for all flow cytometry experiments in this study. An aliquot (200µL) of the freshly isolated SVF cells was filtered through a 35µm nylon mesh topped tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and transferred into a Trucount tube (Becton Dickinson Franklin Lakes, New Jersey, USA) containing Isoflow, propidium iodide and Syto11. The total number of viable cells in each of the SVF samples was determined as described in section 2.2.2.3.

Aliquots of blood, adherent cells and SVF cells were each added to 3 tubes containing either; a) isoflow (Beckman Coulter, Indianapolis, Indiana, USA) for analysis with no nucleic dye, b) isoflow with propidium iodide (10µg/mL; Sigma-Aldrich St Louis, Missouri, USA) or c) isoflow with Syto11 (1µg/mL; Life Technologies Grand Island, New York, USA).

Blood, adherent cells and SVF cells were centrifuged at 2000*xg* for 5 minutes. The resultant pelleted cells were resuspended in Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA). The cells were stained with the following antibodies according to the manufactures instructions (eBioscience, San Diego, California, USA): CD45-FITC (11-5450-42), CD44-FITC (11-5440-42), CD4-FITC (11-5040-42), CD25-FITC (11-0250-42), CD146-FITC (14-1469-82), CD34-PE (12-0340-42) and CD90-PE (12-5900-42), as well as the following isotype controls, Rat IgG2b K FITC (11-4031) Rat IgG2a kappa FITC (11-4321) Mouse IgG1 K FITC (11-4714), Mouse IgG1 K FITC (11-4714) and Rat IgG2b kappa PE (12-4031). Cells stained with PE or FITC conjugated antibodies were also stained with Syto11 and propidium iodide respectively to enable detection of the cells within the background of auto fluorescent debris that is present within freshly isolated SVF cells.

4.2.5. Production of Conditioned Media

The volume of each freshly isolated SVF cell suspension required to seed 192,000 viable cells was transferred to a single well of a 6-well plate (Greiner Bio-one, Frickenhausen, Germany). Volumes ranged from 0.3mL to 1.6mL. The volume in each well was normalised to 2.8mL with standard cell culture media consisting of DMEM supplemented with 10% (FBS) and 1% antibiotic-antimycotic solution. The samples were incubated at 37°C with 5% CO_2 for 72 hours without media changes. At 72 hours all samples had reached 80% confluence. The conditioned medium was collected, centrifuged at 4980*xg* for 10 minutes and stored at -80°C.

4.2.6. Cytokine Analysis

Conditioned medium samples were filtered through 0.2µm Nanosep MF Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, Port Washington, New York, USA) for 5 minutes at 9000*xg*. A 50µL portion of each filtered sample was analysed using the Milliplex MAP® Canine Cytokine 13-plex assay which measures IFN-γ, IL-8, IL-15, IL-18, TNF-α, IL-10, IP-10, KC-like, MCP-1, GM-CSF, IL-7, IL-2 and IL-6 (Millipore, Billerica, Massachusetts, USA) according to the manufacturers instructions. The washing steps were performed on the Bio-Plex Pro II magnetic wash station and the data was acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, Hercules, California, USA).

A volume of 50µL or 200µL of each filtered sample were analysed using TGF-β and VEGF ELISAs (R&D Systems, Minneapolis, Minnesota, USA) respectively according to manufacturers instructions.

4.2.7. Statistical Analysis

The Heat map was produced using R's heatmap.2 function. A Kendall's rank correlation was performed to measure the correlation between two variables. The first analysis was performed on the total SVF cell counts of each dog and the number of cells positive for each cell surface marker for each dog. The second analysis was performed on the number of cells positive for each cell surface marker and the fluorescence intensity levels of secreted cytokines. The significance of the Kendall's

rank correlation was determined by using a matrix of p-values for testing the hypothesis of no correlation against the alternative that there is a non-zero correlation. A p-value <0.1 was considered significantly different from zero.

4.3. Results

To enable characterisation of the SVF, distinguishing the cells from the non-cellular background was imperative. Without the addition of a nucleic acid dye, the cells in both canine blood and culture expanded canine MSCs could be clearly distinguished on the flow cytometer using only forward scatter and side scatter (Figure 4.1A and B). However, when using this approach to distinguish cells of the canine SVF from the background, there was no clearly distinguishable population of cells (Figure 4.1C). The nucleic acid dyes propidium iodide (PI) and Syto 11 were trialed individually in an attempt to visualise this nucleated cell population. It is apparent from Figures 4.1F and I that both dyes enable the nucleated cell population to be seen in the SVF samples. Therefore with the use of these nucleic acid dyes the characterisation of the SVF with monoclonal antibodies was possible. An average of approximately 2.3 million SVF cells were isolated per gram of adipose tissue and cell viability ranged from 48.6% to 75.6% (Table 4.1).

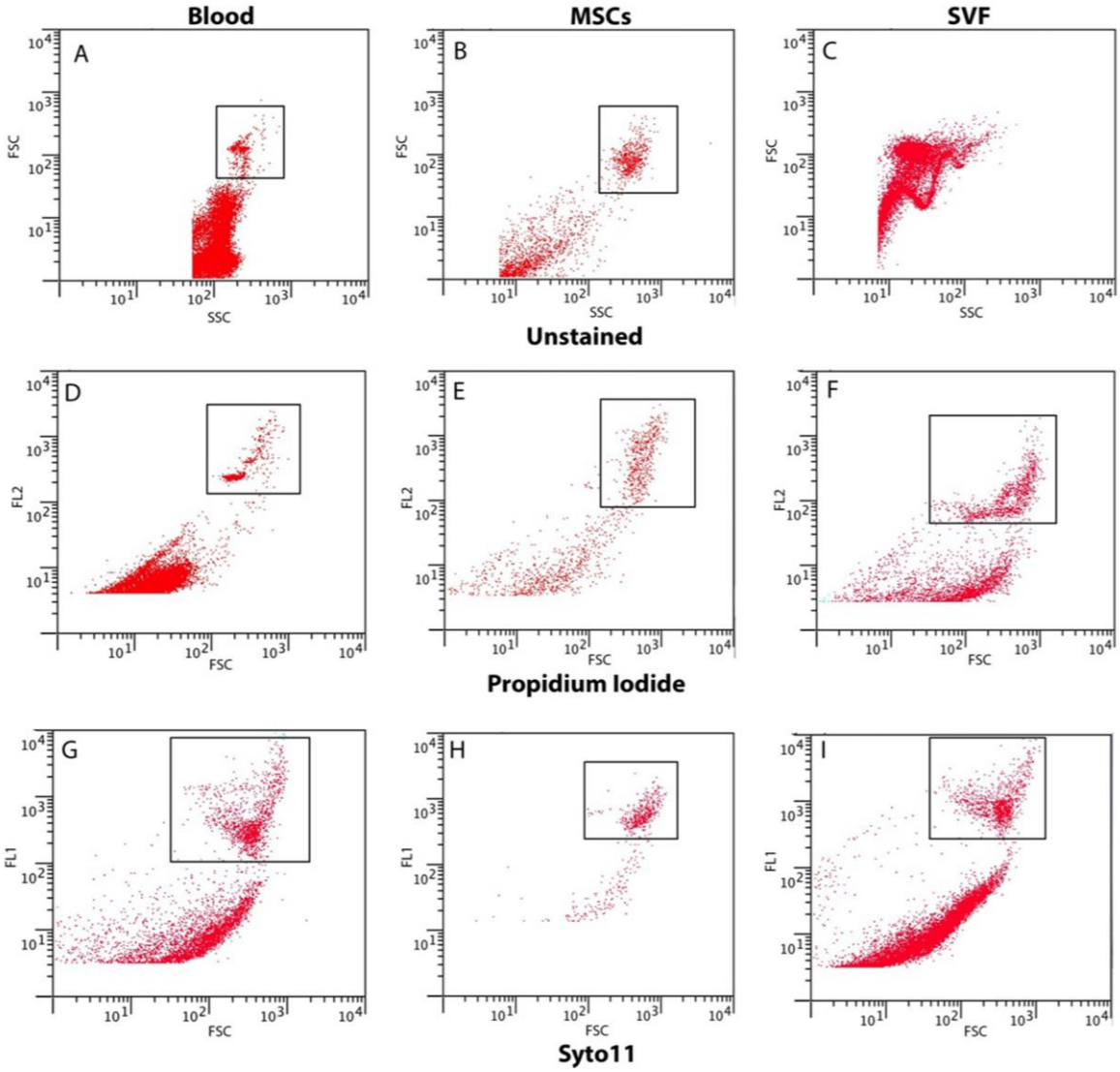

Figure 4.1. Flow cytometry plots of canine bloods, MSCs and SVF.

Canine blood, MSCs and SVF were analysed on a FACScan flow cytometer without nucleic dye (A, B & C respectively), and with the nucleic stain's PI (D, E & F respectively) and Syto 11 (G, H & I respectively). The cellular populations are identified in the square boxes.

Table 4.1. Cell count and viability from canine SVF samples.

Dog	Cells per gram of adipose tissue	Viability (%)
1	1,904,124	75.6
2	2,552,150	72.2
3	3,646,792	63.2
4	1,869,780	48.6
5	2,201,815	62.6
6	3,953,862	60.0
7	809,945	52.9
8	1,151,103	63.0
Average ± SD	2,261,196 ± 1,101,448	62 ± 9

Cells per gram of adipose tissue and viability results from the SVF of eight dogs. The average ± standard deviation values are also shown.

Cell surface antigen staining was used to characterise the cell populations in the canine SVF. Canine blood and MSCs were used as controls for CD marker characterisation prior to characterising the SVF. Consistent with the literature, canine blood was positive for the characteristic white blood cell markers CD4 and CD45 and the cell adhesion marker, CD44 (Figures 4.2 & 4.3). Canine blood was negative for CD25 and the stromal and endothelial cell markers CD146, CD34 and CD90 (Figure 4.2 & 4.3). Similarly as expected, MSCs were negative for the characteristic white blood cell markers and positive for the stromal and endothelial cell markers (Figure 4.2 & 4.4).

CD marker characterisation of the SVF fraction showed varying proportions of cells that were positive for the white blood cell, stromal and endothelial cell markers assessed (Table 4.2). Figure 4.2 shows representative histograms of the positive populations for each marker whereas figure 4.5 shows representative dot plots with full gating strategy the SVF. CD146 was expressed at the highest percentage being on average 33% and CD4 with the lowest average percentage of 8%. From Table 4.2 it is evident that there is high variability between individual dogs as indicated by the large standard deviations. Appendix V figure 9.1 shows the SVF data from all remaining seven dogs of this data set.

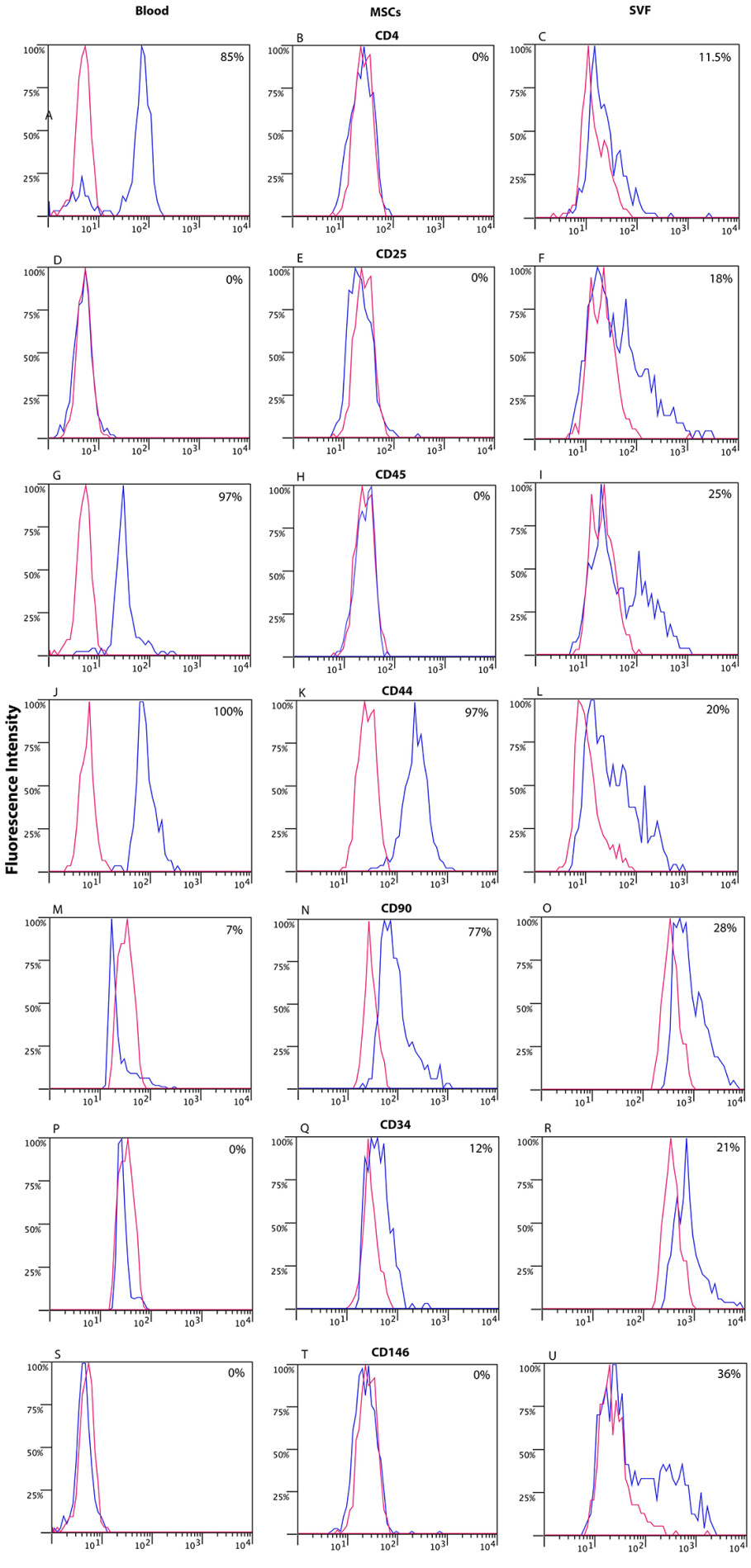
Blood
MSCs
SVF
CD4
CD25
CD45
CD44
CD90
CD34
CD146
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
85%
0%
11.5%
0%
0%
18%
97%
0%
25%
100%
97%
20%
7%
77%
28%
0%
12%
21%
0%
0%
36%
Fluorescence Intensity
100%
75%
50%
25%
10^1
10^2
10^3
10^4

Figure 4.2. Representative phenotypical characterisation of canine blood MSCs and SVF using CD markers and flow cytometry.

Canine blood (A, D, G, J, M, P, S), MSCs (B, E, H, K, N, Q, T) and SVF (C, F, I, L, O, R, U) were analysed on a FACScan flow cytometer for characteristic white blood cell markers, CD4-FITC (A, B & C) CD25-FITC (D, E & F) and CD45-FITC (G, H & I) and for characteristic stromal and endothelial cell markers CD44-FITC (J, K & L), CD90-PE (M, N, O), CD34-PE (P, Q & R) and CD146-FITC (S, T & U). For each cell type and fluorescence conjugate, an unstained control (red line) was included. The blue line represents the stained sample. The percentage of positive cells is shown in each plot.

Table 4.2. Percentage of canine SVF cells positive for CD markers characteristic of white blood, stromal and endothelial cells.

Phenotype	Percentage of positive cells	Characteristic cell type/s (Antibody specificity)
CD45+	26 ± 6	Found on all leukocytes.
CD44+	20 ± 6	Found on immune hematopoietic cells, cell adhesion & migration molecule, MSC and stromal cell marker
CD4+	8 ± 8	T helper cells, monocytes, macrophages and dendritic cells
CD25+	17 ± 6	Immune cells of T & B cell lineages
CD146+	33 ± 11	Cells of the endothelial cell lineage
CD90+	25 ± 7	MSC and stromal cell marker
CD34+	16 ± 11	Marker of hematopoietic progenitor and stem cells

The percentage of positive cells is expressed as the average of eight SVF samples ± the standard deviation.

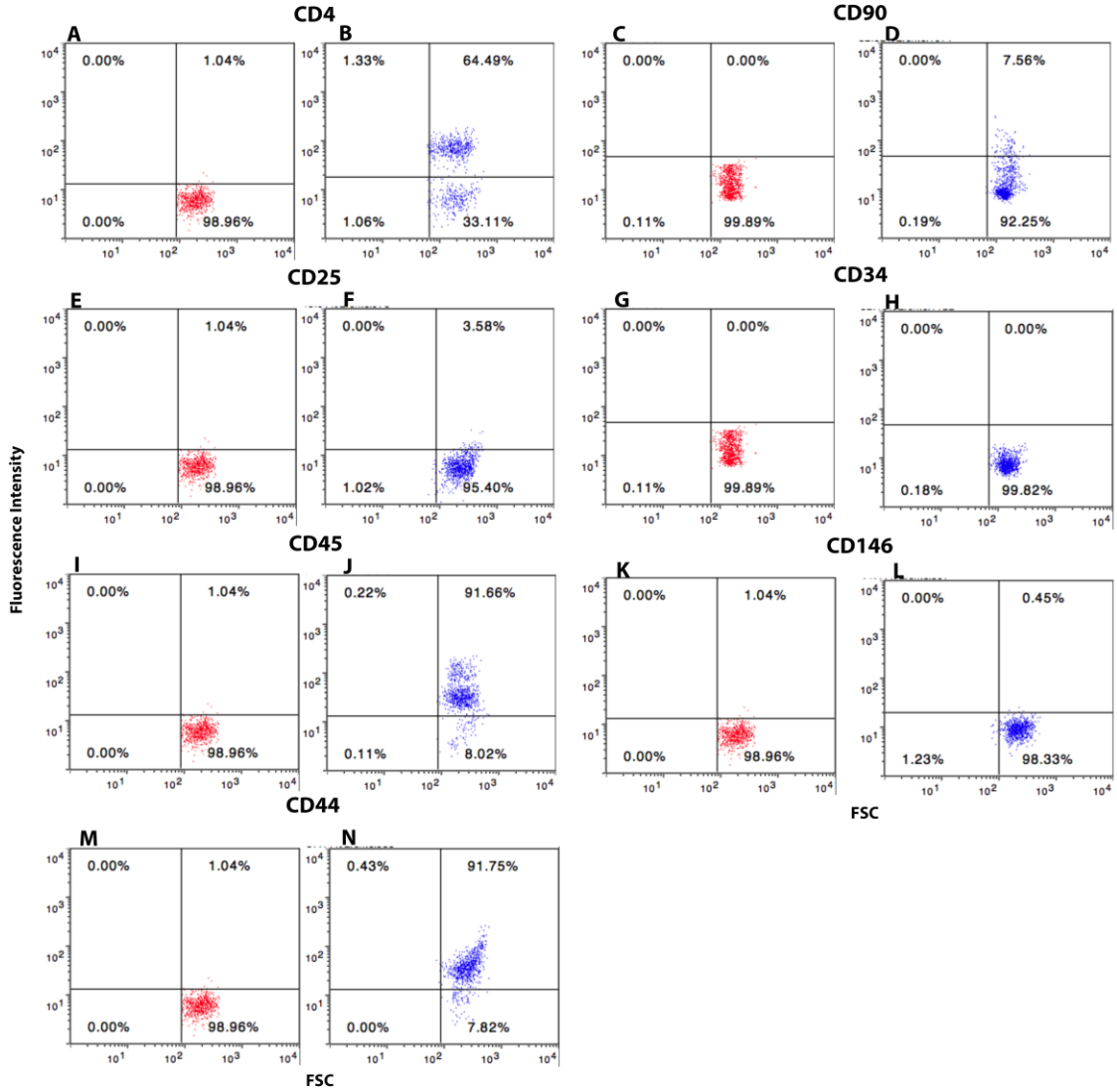

Figure 4.3. Representative phenotypical characterisation of canine blood using CD markers and flow cytometry.

Canine blood was analysed on a FACScan flow cytometer for characteristic white blood cell markers, CD4-FITC (B) CD25-FITC (F) and CD45-FITC (J) and for characteristic stromal and endothelial cell markers CD44-FITC (N), CD90-PE (D), CD34-PE (H) and CD146-FITC (L). For each cell type and fluorescence conjugate, an unstained control (red dot plots A, C, E, G, I, K & M) was included. The blue dot plot represents the stained sample. The percentage of positive cells is shown in the top right quadrant of each plot.

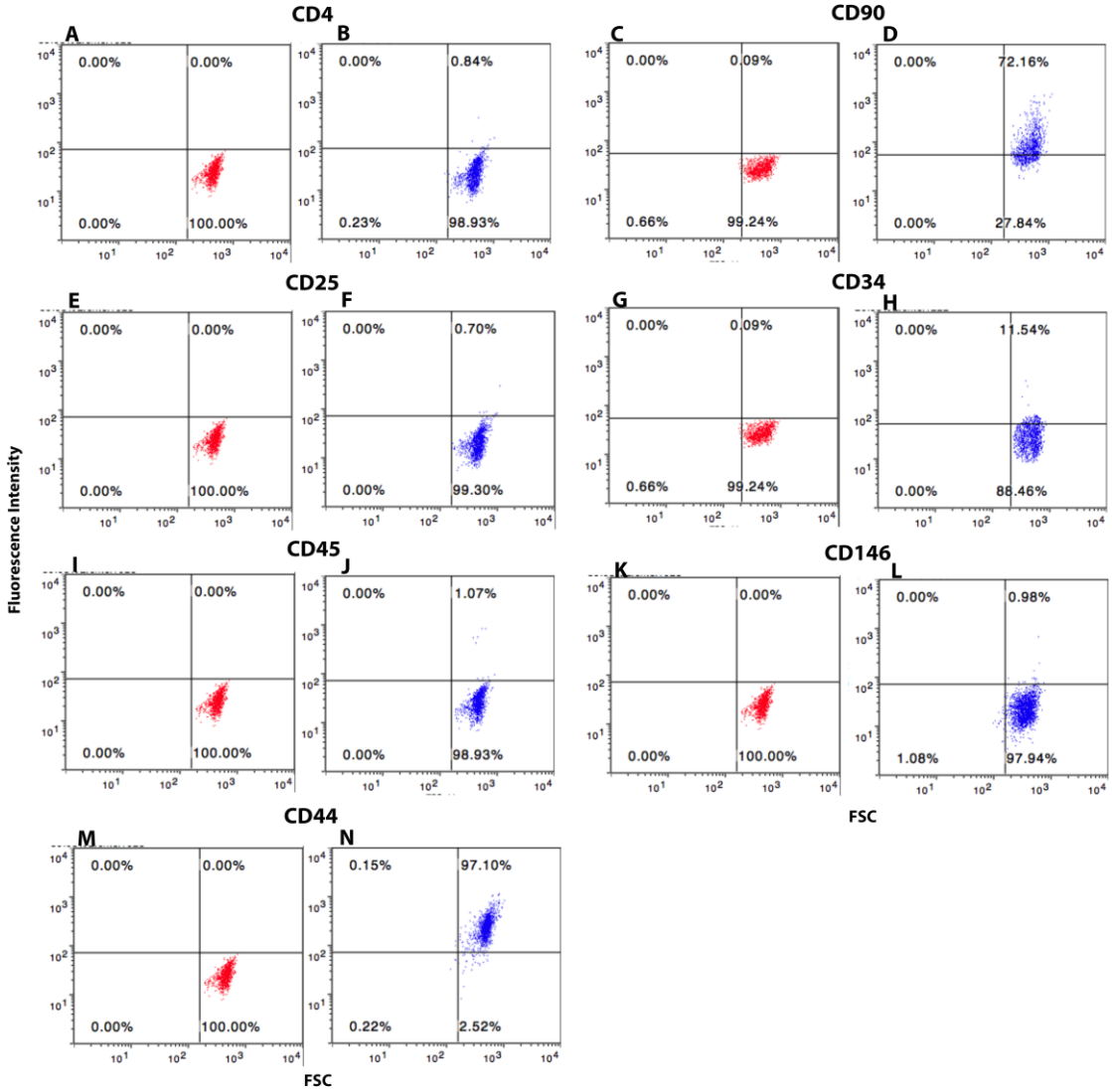

Figure 4.4. Representative phenotypical characterisation of canine MSCs using CD markers and flow cytometry.

Canine MSCs were analysed on a FACScan flow cytometer for characteristic white blood cell markers, CD4-FITC (B) CD25-FITC (F) and CD45-FITC (J) and for characteristic stromal and endothelial cell markers CD44-FITC (N), CD90-PE (D), CD34-PE (H) and CD146-FITC (L). For each cell type and fluorescence conjugate, an unstained control (red dot plots A, C, E, G, I, K & M) was included. The blue dot plot represents the stained sample. The percentage of positive cells is shown in the top right quadrant of each plot.

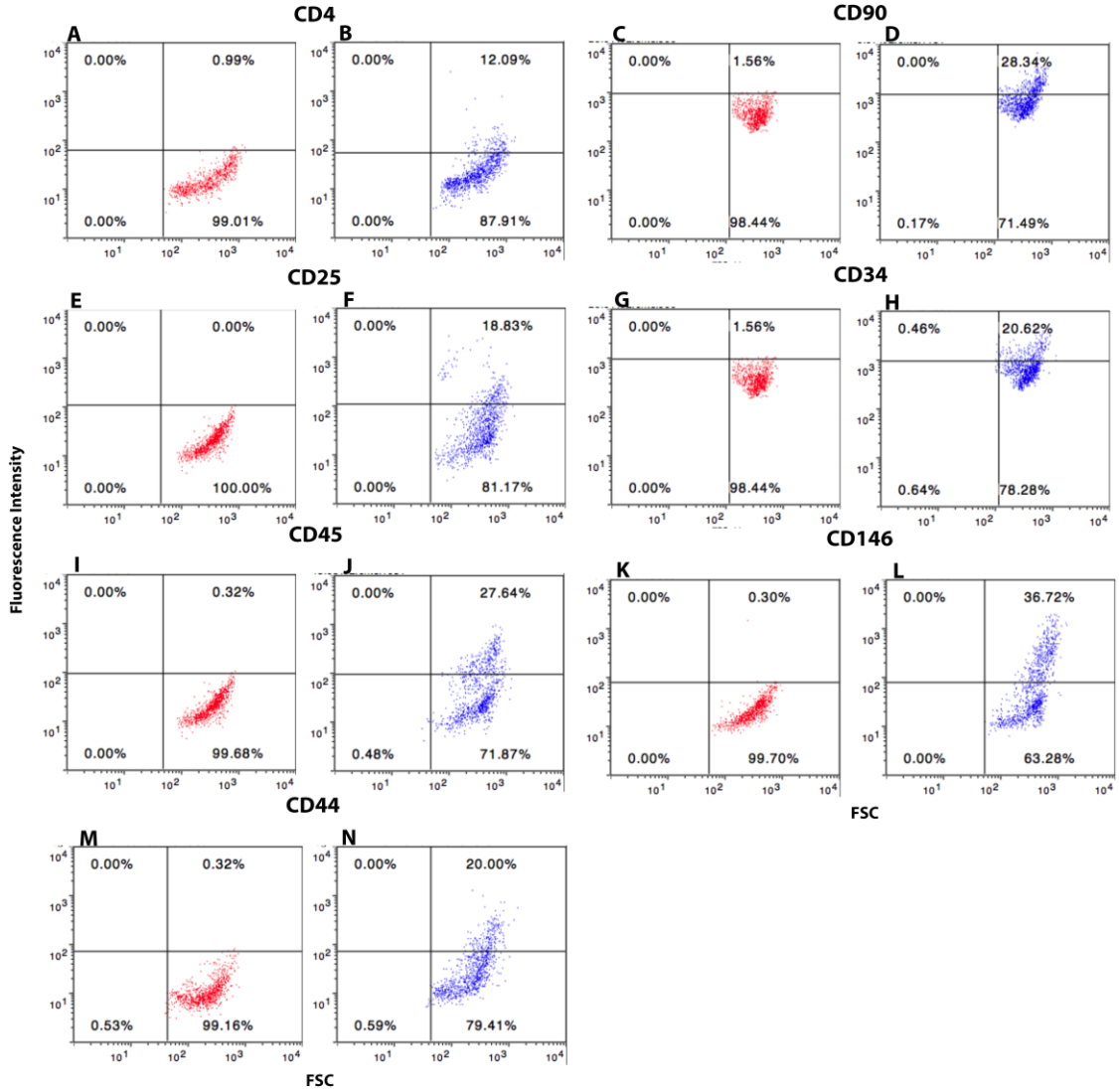

Figure 4.5. Representative phenotypical characterisation of canine SVF using CD markers and flow cytometry.

Canine SVF was analysed on a FACScan flow cytometer for characteristic white blood cell markers, CD4-FITC (B) CD25-FITC (F) and CD45-FITC (J) and for characteristic stromal and endothelial cell markers CD44-FITC (N), CD90-PE (D), CD34-PE (H) and CD146-FITC (L). For each cell type and fluorescence conjugate, an unstained control (red dot plots A, C, E, G, I, K & M) was included. The blue dot plot represents the stained sample. The percentage of positive cells is shown in the top right quadrant of each plot.

Analysis of the conditioned media from the SVF cells for a range of cytokines revealed that 7 cytokines (IL-8, KC-like, MCP-1, VEGF, TGF-β and IL-6) were present at levels higher than 800 pg/mL, and a further 6 cytokines were detected at levels <100 pg/mL. TNF-α and IP-10 were not detected (Table 4.3).

Table 4.3. Cytokines secreted by canine SVF *in vitro*.

Functional Category	**Cytokine Name**	**Cytokine Concentration (pg/mL)**
Pro-inflammatory	IFN-γ	7 ± 6
	IL-8	18152 ± 11156
	IL-15	32 ± 9
	IL-18	16 ± 5
	TNF-α	N.D
Anti-inflammatory	IL-10	116 ± 38
Chemokines	IP-10	N.D
	KC-like	11809 ± 4301
	MCP-1	18753 ± 669
Growth Factors	GM-CSF	47 ± 19
	IL-7	13 ± 8
	TGF-β	831 ± 277
	VEGF	1535 ± 597
Dual Roles	IL-2	22 ± 5
	IL-6	866 ± 1433

Cytokine concentrations are expressed as the average ± the standard deviation (n=8).

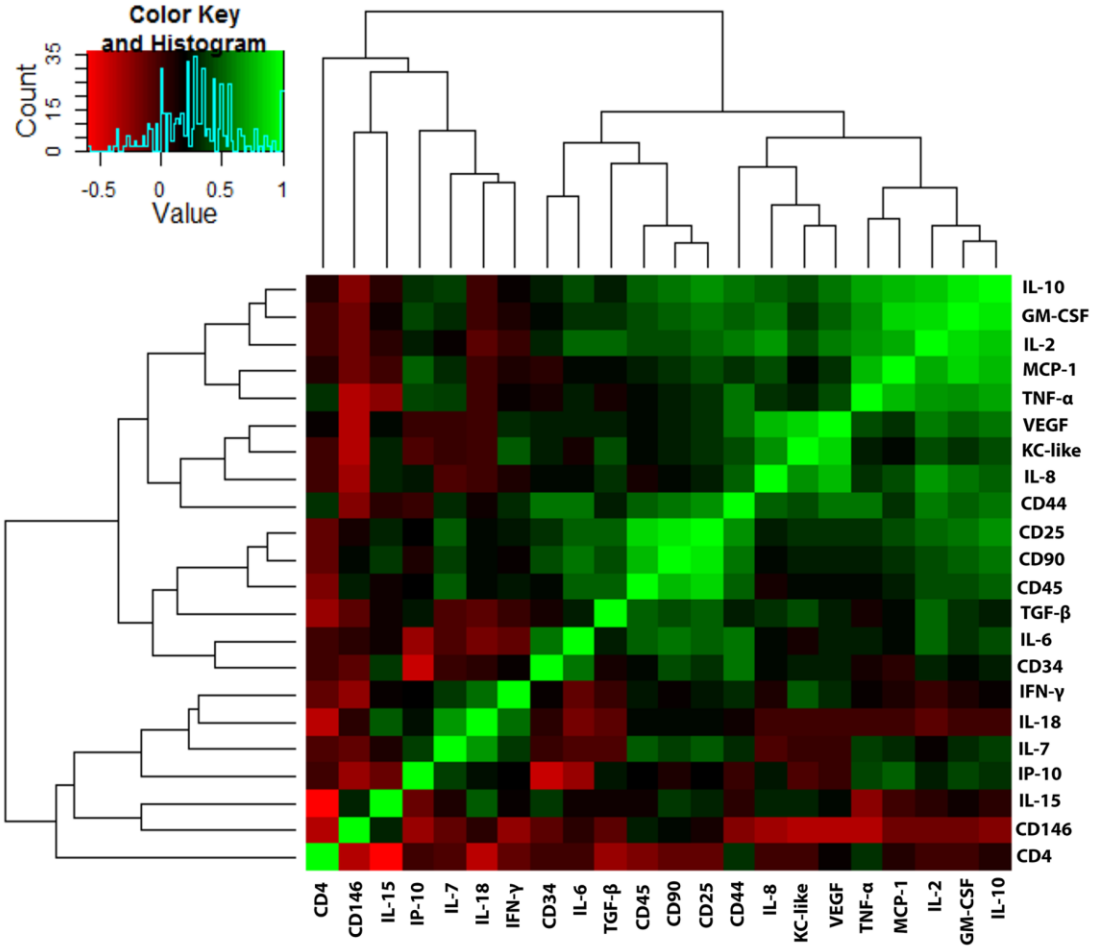

Figure 4.6. Heat map of the relationship between CD marker expression and cytokine secretions from the SVF of 8 dogs.

The relationship between the 15 cytokines, chemokines and growth factors measured (using fluorescence intensity) and CD marker expression profiles of the SVF were investigated using a multi-scale bootstrapping approach. Green indicates a positive correlation whereas red indicates a negative correlation. The dendrogram indicates the clustering patterns of these data.

A hierarchical clustering approach was applied to investigate the relationship between cell surface antigen expression and secreted cytokine levels (Figure 4.6). It is evident from the heat map (Figure 4.6) that there is a strongly positive correlation between CD25, CD45 and CD90 and that a positive correlation between the bioactive factors IL-10, GM-CSF, IL-2, MCP-1, TNF-α and VEGF exists. Interestingly the CD marker CD146 that is expressed at the highest proportion of all of the CD markers measured in this study was negatively correlated to all other CD markers and all cytokines.

As a relationship between certain CD markers was observed, to further investigate if CD marker expression levels correlated to the cell number obtained per

gram of fat, a Kendalls correlation approach was used where the significance of the correlation was also tested. A significant correlation (p-value <0.05) between cell number per gram of adipose tissue and the CD markers CD25, CD45 and CD90 was found (Table 4.4). Additionally a highly significant (p-value <0.01) correlation existed between these three markers to each other (Table 4.4). To further investigate this finding, the correlation between the CD markers CD25, CD45 and CD90 and secreted cytokines was determined (Table 4.5). There was a significant correlation between CD25 expression levels and secreted levels of the growth factors GM-CSF and TGF-β, the anti-inflammatory cytokine IL-10 and the dual role cytokine IL-2. A significant correlation was found between CD90 and secreted levels of IL-10 and the anti- and pro-inflammatory cytokine IL-6. There were a number of positive correlations between the CD markers and the above-mentioned cytokines that were close to significance with a p-value of just above 0.1 (Table 4.5).

Table 4.4. Kendall's correlation values for cell number per gram of adipose tissue and cell surface antigen CD marker expression.

CD Marker	Kendall's Correlation Value	p-value
CD4	- 0.089	0.886
CD25	0.714	**0.019**
CD34	0.500	0.108
CD44	0.500	0.108
CD45	0.714	**0.019**
CD90	0.786	**0.009**
CD146	0.286	0.386

The CD marker expression levels were correlated to cell number per gram of adipose tissue using a Kendall's correlation. The significance of the correlation was tested and correlations with a p-value <0.05 are indicated in bold.

Table 4.5. Kendall's correlation values of CD markers CD25, CD45 and CD90 to secreted cytokine levels.

	CD25	**CD45**	**CD90**	**IL-2**	**IL-6**	**IL-10**	**GM-CSF**	**TGF-β**
CD25	1	**0.857 (0.004)**	**0.929 (0.002)**	**0.519 (0.10)**	0.5 (0.11)	**0.643 (0.04)**	**0.571 (0.063)**	**0.5 (0.004)**
CD45	**0.857 (0.004)**	1	**0.786 (0.009)**	0.445 (0.17)	0.5 (0.11)	0.5 (0.11)	0.429 (0.17)	0.5 (0.11)
CD90	**0.929 (0.002)**	**0.786 (0.009)**	1	0.445 (0.17)	**0.571 (0.06)**	**0.571 (0.06)**	0.5 (0.11)	0.429 (0.17)
IL-2	**0.519 (0.10)**	0.445 (0.17)	0.445 (0.17)	1	**0.519 (0.10)**	**0.815 (0.008)**	**0.889 (0.004)**	**0.519 (0.10)**
IL-6	0.5 (0.11)	0.5 (0.11)	**0.571 (0.06)**	**0.519 (0.10)**	1	0.429 (0.17)	0.357 (0.27)	0.286 (0.39)
IL-10	**0.643 (0.04)**	0.5 (0.11)	**0.571 (0.06)**	**0.815 (0.008)**	0.429 (0.17)	1	**0.929 (0.002)**	0.286 (0.39)
GM-CSF	**0.571 (0.06)**	0.429 (0.17)	0.5 (0.11)	**0.889 (0.004)**	0.357 (0.26)	**0.929 (0.002)**	1	0.357 (0.27)
TGF-β	**0.5 (0.004)**	0.5 (0.11)	0.429 (0.17)	**0.519 (0.10)**	0.286 (0.39)	0.286 (0.39)	0.357 (0.27)	1

The CD marker expression levels were correlated to secreted cytokines. The Kendalls correlation values are shown in the table and p-values are in brackets. The correlation with a p-value <0.1 are indicated in bold. Only the secreted cytokines that showed a significant correlation to CD markers CD25, CD45 and CD90 are shown.

4.4. Discussion

4.4.1. Canine SVF contains a Large Population of Cells that Express MSC Surface Markers

Characterisation of the SVF is difficult due to cell and tissue debris and the inherent autofluorescence of these particles. In this chapter, the addition of a nucleic acid dye (Syto11 or PI) was required to distinguish the SVF cells from the autofluorescence of debris present in the samples. Syto11 and PI nucleic acid dyes are compatible with PE and FITC fluorochromes respectively, allowing cell surface antigen characterisation to be performed on a flow cytometer with a 488nm laser. In this work, Syto11 could be used in conjunction with PE labeled antibodies and PI could be used in conjunction with FITC labeled antibodies. The inclusion of a third fluorochrome was not successful with either of these combinations. Therefore, multi-fluorochrome cell surface antigen staining was not possible.

Others have successfully performed analysis of human SVF using multi-fluorochrome cell surface antigen staining by using a flow cytometer with a UV laser, which enable DAPI to be used in a distinct optical channel [230]. Flow cytometers fitted with UV lasers are not widely available and therefore this approach was not an option. Another successful approach has been the use of destiny gradient centrifugation to purify cells away from cell and tissue debris [36]. This approach requires a relatively large sample size as significant loses of cells occur during the processing. In this study, only small volumes of adipose tissue were harvested and cell numbers were too low after density gradient centrifugation to be analysed by flow cytometry.

The canine SVF cells analysed in this study were positive for the stromal, endothelial and WBC markers, CD90, CD44, CD146, CD34, CD45, CD25 and CD4 in varying proportions. Several research groups have attempted to identify the location of MSCs within adipose tissue [37, 230, 278, 279]. The consensus between researchers is that MSCs *in situ* reside in the perivascular location. Some groups report this being the CD34 positive/CD31 negative phenotype [235], while others describe a CD146 positive population found in the perivascular region exhibiting the biological properties of MSCs once isolated and cultured [280]. Zimmerlin *et al.,*

(2010) utilised high-speed flow cytometry sorting techniques to determine the *in situ* location of MSCs in human adipose tissue [230]. They identified three separate MSC-like populations residing in adipose tissue. All three populations were capable of culture expansion and adipogeneic differentiation that was superior to that of the entire SVF. These three populations were identified as, pericytes, which were CD146+/CD90+/CD34-, outer adventitial adipose stromal cells, which were CD146-/CD90+/CD34+ and a CD34+/CD146+ pericyte subset. Although Zimmerlin and others have suggested that CD34 and/or CD146 positive cells are the MSCs in the adipose tissue niche, it is important to note that expression of both of these surface markers are lost following *in vitro* expansion of adherent cells of the SVF [228]. An average of 16% and 33% of cells of the canine SVF in this study were CD34 and CD146 positive respectively and in-line with the literature the positive percentage of CD34 and CD146 expressed on canine cultured MSCs was negligible. (Figures 4.2 and 4.4). In contrast, studies have demonstrated human and canine cultured MSCs express the stromal markers CD44 and CD90 [92, 228, 248]. Furthermore, these cells have been shown to be capable of multi-lineage differentiation, which is one of the three criteria set by the International Society of Cellular Therapies to define a cell as an MSC [30]. Consequently CD44 and CD90 are recognised as cultured adipose-derived SVF and MSC cell surface antigen markers [39, 281]. In this study CD44 and CD90 were expressed on an average of 20% and 25% of freshly isolated canine SVF cells respectively. Taken together, our results suggest that canine SVF is comprised of a relatively high proportion of cells expressing markers indicative of MSCs. However, it is important to note that whilst MSCs may express CD44, in a mixed cell population such as the SVF, CD44 doesn't necessarily define the MSC population, due to CD44 also being present on hematopoietic immune cells.

The proportion of SVF cells positive for the WBC markers CD45, CD25 and CD4 was also investigated. CD45 surface antigen is expressed on all leukocytes [282], and in this study on average a quarter of the canine SVF was characterised as leukocytes. To further examine the white blood cell fraction of the SVF, the lymphocyte markers CD25 and CD4 were used [283, 284]. CD4 was found on 8% of canine SVF cells, whilst 17% of the canine SVF was positive for CD25 (Table 4.2). Utilised in combination CD25 and CD4 are cluster differentiation markers used to identify Treg cells [284]. Treg cells are a prominent population found in the SVF [49] and the results presented

in this chapter indicate that approximately 8% of the canine SVF are Treg cells Interestingly the percentage of CD4 positive cells from the SVF of the 8 samples investigated in this study ranged from 1-25% (Appendix V). These immune regulatory cells play an important role in cell therapy as once activated these cell types secrete large amounts of TGF-β and IL-10 which have powerful immunosuppressant properties capable of mediating chronic inflammation [285]. Conversely, CD25 in isolation is found on activated T cells and B cells. Whereas, Treg cells limit and suppress immune responses, active T and B cells are responsible for cell-mediated and humoral immune responses respectively.

Several studies have attempted to characterise the cellular composition of the human SVF [36, 227, 228]. Some of the proportions of the described cell types seen in the canine SVF in this study differ from what has been published on the cellular composition of human SVF. While CD146 [228] and CD45 [36, 227] are expressed in similar percentages to that described in human SVF, CD44, CD90 and CD34 [36, 228] are all expressed in lower proportions. A recent study by Feisst *et al.*, (2014) provides the most in-depth characterisation of human adipose SVF [236]. They used an 11-marker flow cytometry panel to compare SVF to dermal cells. Cell proportions, as either adipose stem cells (ASC) or endothelial progenitor cells (EPC), were reported as a percentage of the CD45- population. ASCs accounted for 80% to 93% of the CD45- population with EPCs between 1% and 18%. In Feisst *et al.*, (2014) CD90 was positive in both ASC and EPC, and CD146 positive only in EPCs, but at low expression levels [236]. In contrast, the data presented in this chapter, where CD146 was found at 50% higher frequency than CD90, indicates that EPCs may be present at higher frequency than ASCs in canine SVF. Varying outcomes between cells expressing these CD markers between human SVF and canine SVF are likely due, but not limited to, differences between species, biological variation between individuals, and different fat procurement sites and harvest methods. Additionally complexity arises when trying to perform this type of characterisation in canines due to the lack of available suitable monoclonal antibodies [286].

4.4.2. Canine SVF Secretes Cytokines of Therapeutic Importance

Traditionally, the main mechanism of action of MSCs was considered to be their ability to differentiate into cells of various lineages. However, it is now accepted that cellular secretions are a major driver of the therapeutic effect of cellular therapy. The bioactive factors secreted by MSCs have been widely researched *in vitro* [46, 53, 287]. Furthermore, their role in the therapeutic effect of cellular therapy has been confirmed via *in vivo* studies demonstrating that administration of conditioned medium alone can achieve a similar initial effect to cell therapy [68]. However, there is little known about the complex *in vitro* secretion profile of mixed cell populations such as the SVF or their therapeutic effect *in vivo*. Once cells of the SVF are introduced to culture conditions to select for plastic adherent MSCs, some of the cross talk and feedback mechanisms arising from signals from other cells that comprise the SVF are lost. *In vivo* these feedback mechanisms govern the physiology and functionality of a cell and without this regulation significantly elevated levels of cytokines can be produced from MSCs under *in vitro* tissue culture conditions. Blaber *et al.,* (2012) compared secretions from human SVF and MSCs and reported elevated levels of IFN-γ VEGF, IL-6, IL-7, IL-10 and IL-13 in the conditioned medium of MSCs compared to the SVF [242]. To the author's knowledge there are no reports of the secretion profile from the canine SVF, however Kang *et al.,* (2008) offers some insight into understanding mixed cell population therapeutics in canines. Kang and co-workers demonstrated that co-culture of MSCs and leukocytes, compared to leukocytes alone, resulted in a reduction of the pro-inflammatory cytokine TNF-α, an increased production of IFN-γ, IL-6 and TGF-β but no difference in the production of IL-10 [92]. Therefore, Kang *et al.,* (2008), consistent with Blaber *et al.,* (2012), demonstrate that distinct secretion profiles, presumably arising from cross-talk and feedback mechanisms, are obtained from MSCs held *in vitro* with other cells of the SVF. As the SVF is a commercially available therapeutic utilising a mixed cell population, understanding what the SVF secretes as a whole is important. It was demonstrated that holding the SVF *in vitro* results in the secretion of high levels of the cytokines, IL-8, IL-6, KC-like, MCP-1, VEGF and TGF-β. These results were obtained from holding the SVF in *in vitro* conditions, and may not completely reflect what happens *in vivo* in a joint. The joint is a low oxygen environment, which contains synovial fluid and

numerous other tissue types. In the experiments described in this chapter, cells were held under standard cell culture conditions (~20% oxygen) and were not exposed to signaling from the numerous cell types present in a joint. Regardless, the datasets presented in this chapter may provide some insight into the potential treatment effect of the SVF. In particular, it has been demonstrated that TGF-β is crucial for prevention or repair of cartilage damage [288]. As such, secretion of a wide array of bioactive factors, and direct cell-cell signaling of the SVF with cells of the joint, in particular infiltrating T cells, may mediate the inflammatory cascade evident in osteoarthritis.

4.4.3. Therapeutic Importance

Freshly isolated SVF cellular therapy is now a treatment option for numerous conditions in both people and animals. The most common diseases in dogs' being treated with cellular therapeutics are musculoskeletal and immune-mediated disorders [289]. Although clinical results from these treatments are promising, there are always a proportion of non-responding patients [217, 218 and our unpublished data, 272]. To-date, no study has definitively determined the number of cells, from either isolated MSCs or from mixed cell populations, required to achieve a therapeutic effect. Using cellular therapeutics such as the SVF adds further complication to this approach because it is comprised of multiple cell types. Our study demonstrated a large variability between individual dog SVF profiles, which was consistent with the variability seen in profiling of the human SVF. The variability in the proportion of cell types present in the SVF, and consequently their secretion capabilities, could affect the clinical outcome of a cellular therapy. To gain insight into this possibility, understanding if a relationship exists between the number of cells isolated per gram of adipose tissue, their immunophenotype, and their secretion capabilities was important.

Interestingly CD146 and CD34 despite being expressed on relatively high proportions of cells in this study were not significantly correlated with cell number or with any of the cytokines investigated in this work. It is possible that the panel of cytokines measured was too small to determine the soluble factors that have a relationship with these cell surface antigen types. However, this work demonstrated

that a correlation between cell number and the cell surface antigen markers CD90, CD25 and CD45 exist (Table 4.4). It was also demonstrated that these three cell surface antigen-expressing markers are significantly correlated to one another and a positive correlation between these three markers and the production of the therapeutically important cytokines IL-2, IL-6, IL-10, GM-CSF and TGF-β exists (Table 4.5).

IL-10 is a key anti-inflammatory cytokine, which plays a role in the differentiation and function of T-regulatory cells with its primary function being to regulate immune and inflammatory responses [290, 291]. In this study IL-10 was correlated with the recognised MSC marker CD90 (p-value <0.1) and with the lymphocyte marker CD25 (p-value <0.05). *In vitro,* expanded MSCs have been shown to secrete high levels of IL-10 [242] and T-regulatory cells are well understood to secrete IL-10 [46]. However, Kang *et.al* (2008) report no increased production of IL-10 with the addition of canine MSCs to canine leukocytes. This could have been due to the absence of signals from other stromal vascular cells [92]. IL-2 and IL-6 can have either an anti- or pro-inflammatory function depending on the environment [292] IL-6, like IL-10, is correlated with cells that have CD90 surface antigen expression (p-value <0.1). Kang *et.al* (2008) shows a highly significant increase in the production of IL-6 with the addition of cultured MSCs to canine leukocytes [92]. Human MSCs are also shown to secrete high levels of this cytokine *in vitro* [242]. IL-2 regulates the activities of lymphocytes and as expected correlated (p-value <0.1) with cells expressing CD25. Furthermore, CD25 along with CD122 forms the heterodimer of the IL-2 receptor, with the binding of IL-2 to its receptor resulting in the promotion of lymphocyte mitosis [293]. GM-CSF and TGF-β are growth factors significantly correlated to CD25 in this study. GM-CSF plays a role in cell differentiation of granulocytes and monocytes [284]. Blaber *et al*., (2012) show GM-CSF is measured in higher levels in human SVF than MSCs [242] and no expression of GM-CSF mRNA was detected in canine bone marrow-derived MSCs [92]. TGF-β exerts immunosuppressive effects and is recognised to play a role in the proliferation and differentiation of mesenchymal stem cells [98]. Additionally, the combination of TGF-β and IL-2 *in-vitro* directs the differentiation of T- cells into T-regulatory cells [294]. Collectively, these results indicate that the MSCs, leukocytes and lymphocytes present in the canine SVF, correlate to the number of cells harvested from an individual.

Furthermore, these cells have the ability to secrete levels of the therapeutically important cytokines IL-2, IL-6, IL-10, TGF-β and GM-CSF. Following treatment of a disease or condition with the SVF, it is likely that these cells initially exert a therapeutic response by mediating the immune processes responsible for inflammation through their secretions [66]. In the long-term, it is believed that MSCs constitute the second phase of the therapeutic response by tissue embedding and continuing to secrete the soluble mediators required to enable the process of tissue regeneration to take place [25, 82].

Although this *in vitro* study offers some insight into cell types, proportions and the secretion profile from the canine SVF, it does not completely reflect what may happen *in vivo* in real clinical disease. To determine if cell number is definitively correlated to the cell surface antigens CD90, CD45 and CD25 a study involving significantly more biological replicates would be required. Furthermore, following *in vivo* implantation, the SVF cells are likely to alter their secretion profiles based on the signals they are receiving from the diseased or damaged environment. To determine if IL-2, IL-6, IL-10, TGF-β and GM-CSF are secreted *in vivo*, an analysis of tissue or fluid samples from the host would be required. Furthermore, identification of which cell types were responsible for the production of individual cytokines was not attempted. However, it would be interesting to know for example if CD90 positive cells are always responsible for the production of IL-10. To gain an understanding of this question, a further study where individual cells are sorted and their intracellular cytokine levels immediately analysed, via either cell lysis or intra-cellular cytokine staining, is required.

4.5. Conclusions

This study is the first to characterise freshly isolated canine SVF using cell surface antigen immunophenotyping and cytokine secretion profiling. This study showed that the canine SVF profile is highly variable between individuals, a result that is consistent with literature profiling the human SVF. This study has also demonstrated the number of cells expressing certain cell surface antigens (CD25, CD45, CD90) were directly related to the number of cells harvested. Additionally these cell types were also correlated with the secreted levels of the therapeutically

relevant cytokines IL-2, IL-6, IL-10, TGF-β and GM-CSF. It is possible that the variability in the proportion of cell types present in the SVF, and consequently their secretion capabilities, could affect the clinical outcome of this type of cellular therapy. Future analysis of the canine SVF using multicolour flow cytometry, as has been performed on human SVF, will provide greater knowledge of the canine SVF composition. This will require the use of sophisticated flow cytometers and likely in-house cell labeling with alternative fluorochromes due to the limited selection of canine antibodies currently available. Furthermore, future studies are required to investigate if a correlation exists between the cellular composition and secretion profile of the SVF therapeutic used and clinical outcome.

CHAPTER FIVE

A Comparison of Canine Mesenchymal Stem Cells With and Without Cellular Secretions in the Treatment of a Mouse Model of CAIA

This chapter investigated the potential of culture expanded mesenchymal stem cells (MSCs) with the addition of cellular secretions as an off-the-shelf therapeutic. The previous two chapters explored both the clinical efficacy of an autologous stromal vascular fraction (SVF) point-of-care therapeutic and the characterisation of the cell types that comprise the SVF and the secretion profile produced by these cells. To enable accessibility of a cellular therapeutic to more than just a few expert veterinary practitioners, and to provide a cellular therapeutic that could be consistently produced an off-the-shelf product was required. The previous two chapters highlighted that trophic factor secretions of the SVF are a likely driver of therapeutic benefit. In this chapter an investigation was performed to determine if a combination of cells and concentrated cellular secretions had a greater therapeutic benefit over that of just cells. Experiments were performed using culture expanded MSCs with SVF secretions as a therapeutic treatment in a mouse model of arthritis. The combination of cells and cellular secretions is a novel concept and to the authors knowledge has not been demonstrated previously.

5.1. Introduction

The previous two chapters have focused on the clinical evaluation and *in vitro* characterisation of freshly isolated adipose-derived SVF. It was demonstrated that canine SVF cells might have a rapid and sustained therapeutic effect in the treatment of canine osteoarthritis (OA). It was also hypothesised that the trophic factors secreted by the SVF cells may be responsible for the initial clinical response seen in dogs treated with freshly derived SVF. Furthermore, secretion profiling of conditioned medium from SVF cells demonstrated that they secrete a range of cytokines, growth factors and chemokines that have therapeutic capabilities.

There are many benefits in utilising the SVF as a freshly derived point-of-care treatment. These include the safety associated with using autologous cells, the benefit of a mixed cell population and the fact that the SVF enables provision of an autologous same-day treatment option. However, this approach is also costly and requires the surgical extraction of adipose tissue and potentially up to two anaesthetics in a day. Surgery, which requires a general anaesthetic, is not always safe or appropriate in dogs that suffer from co-morbidities or that are geriatric, of which both are often the case in dogs diagnosed with OA.

An alternative cell therapy approach that is less invasive and potentially less costly is the use of cultured allogeneic cells. Such an approach would involve harvesting adipose tissue from a donor animal, isolating the plastic adherent cells and expanding the number of cells in tissue culture. This approach would eliminate the need for surgical extraction of adipose tissue and a general anaesthetic for the recipient dog.

It has long been established in the scientific literature that culture expanded MSCs derived from bone marrow [28], adipose tissue [8] or a variety of other tissue sources for MSCs [295] have therapeutic potential in the treatment of a range of diseases. MSCs have been shown to be efficacious in the treatment of immune-mediated conditions [47, 49], ischemic injury [296, 297], wound healing [264] and in the context of this thesis, musculoskeletal diseases and injuries, including OA [180, 181, 217, 218]. Until recently, the differentiation ability of MSCs was considered the defining characteristic by which MSCs may alter the pathology of OA, with the hypothesis that introduced cells would differentiate into chondrocytes and replace

damaged cartilage [89]. However, the reality is considerably more complex, and whilst MSCs have been shown to regenerate diseased joint tissues [89, 158] they also address the underlying immune and inflammatory responses related to OA [298] without undergoing differentiation.

MSCs are recognised to be immune privileged as they lack expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules and have only limited expression of MHC-1 [96, 299]. As such, this characteristic prevents their detection by the immune system and in turn prevents immune rejection [53, 300]. This is not only seen in genetically non-identical members of the same species but also between different species. The earliest evidence of this was seen in a xenogeneic model where human MSCs were transplanted, *in utero* into foetal sheep. Despite cells being transplanted after the expected development of immune-competence in the sheep, the transplanted human cells engrafted and survived for at least the length of the study; thirteen months post-transplantation [301]. This phenomenon has been further substantiated in numerous *in vitro* and *in vivo* studies including mouse models of Graft versus Host Disease (GVHD) [65] and prolonged skin graft survival in the baboon [53]. Furthermore, the use of allogeneic culture expanded canine MSCs has been shown to be not only safe but also efficacious in a canine model of segmental bone defects [302]. The use of human MSCs in animal models is now an accepted, and expected, step in the safety and efficacy trial pathway for cellular therapy.

It is generally accepted in the scientific literature that secretions from MSCs have powerful therapeutic effects. In recent years the scientific community has focused not only on the differentiation ability of MSCs but also the regenerative mechanisms of MSCs achieved via secretions. The paracrine actions of MSCs have been shown to have angiogenic [303], anti-fibrotic [82], mitogenic [67, 98], anti-apoptotic [88] and immune modulatory properties [26 and references therein]. It is understood that these paracrine actions may alter a diseased environment and induce regeneration of tissues via activation of resident cells [67, 68, 89]. Furthermore, the administration of MSC secretions alone has been shown to be therapeutic in animal models of corneal eye injury [68], cardiac infarction [67, 304] and ischemic stroke injury [305] albeit with reduced longevity of efficacy compared to cellular therapy. It is clear that MSCs are activated by tissue injury and respond to

damage signals by homing to the site of injury and altering their secretion profile. Increased numbers of MSCs are also found in the blood after injury [306-308].

Harvesting fat, isolating the SVF and subsequent culture steps are analogous to ischemic injury and promote increased secretion of some cytokines and alterations in the overall cytokine profile [309]. In addition, the administration of adipose derived secretions was therapeutic in a mouse model of rheumatoid arthritis [310]. Whilst the production of secretions from harvested tissue or cells is possible in large quantities, the *in vitro* environment is unlikely to exactly match the *in vivo* microenvironment after injury or during a disease process such as OA. This is likely to mean that *in vitro* produced secretions will be different in profile from those secreted by implanted cells responding to the local tissue signalling. The hypothesis in this chapter was that a combination of *in vitro* produced secretions and cells would be therapeutically superior to cells alone.

Animal models of induced disease such as ischemic reperfusion injury [296, 297] or corneal eye injury [68] have helped facilitate an understanding of the therapeutic capabilities of MSCs. Animal models also provide invaluable insight into the efficacy and safety of treatments in early stage development. One such model, described in the literature, is collagen antibody-induced arthritis (CAIA) [254]. This is a model designed to closely mimic human rheumatoid arthritis (RA). The CAIA model is an improved version of the original collagen-induced arthritis (CIA) model [265]. In the CAIA model, arthritis is induced by the administration of a cocktail of monoclonal antibodies, which in turn activates the innate immune system and stimulates the recruitment of macrophages and granulocytes to joints. The addition of antibodies therefore negates the need for the initial T and B-cell response necessary in CIA affected mice for the onset of disease [254]. The immediate activation of immune response leads to a rapid and yet consistent onset of disease [254]. The CAIA model also benefits from the addition of lipopolysaccharide (LPS), which increases disease incidence and severity in comparison to the CIA model [265].

5.1.1. Chapter Aims

The objective of this chapter was to determine if the addition of cellular secretions to culture expanded adipose-derived canine MSCs had a superior clinical

outcome as an arthritis-relieving agent compared to culture expanded adipose-derived canine MSCs alone. This chapter represents the first step in the development of a novel off-the-shelf therapy that combined concentrated cellular secretions and culture expanded adipose-derived MSCs. It was hypothesised that the benefit of cellular secretions would be twofold; an initial contribution to the therapeutic effect whilst also enhancing the paracrine activities of the MSCs. This combination was anticipated to provide a greater therapeutic benefit than that of just MSCs alone.

To determine whether the addition of cellular secretions to culture expanded MSCs had an enhanced therapeutic benefit over MSCs alone, a preliminary study utilising the mouse model of CAIA was performed. The primary outcome of this study was determined by the ability of the treatment actives to ameliorate the clinical signs of arthritis. A secondary outcome consisted of determining whether any differences could be seen in serum cytokine levels between the groups treated with the two treatment articles.

5.2. Methods

5.2.1. Preparation of Test Articles

5.2.1.1. Adipose Tissue Harvest and Isolation of Cells

Adipose tissue was collected from the falciform ligament of a three-year old female Kelpie dog that underwent a routine desex procedure. A total of 13.8 grams of adipose tissue was obtained. The adipose tissue was washed with saline, minced with sterile scissors and digested with 0.05% collagenase (Sigma-Aldrich, St Louis, Missouri, USA) in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, New York, USA) in a 37°C water bath for 60 minutes. The digested sample was then passed through a 400µm filter (Millipore, Billerica, Massachusetts, USA) and then centrifuged at 2000*xg* for 5 minutes. The floating lipid, adipocytes and supernatant were aspirated and discarded. The pelleted SVF cells were re-suspended in saline, centrifuged at 2000*xg* for 5 minutes and then resuspended in 10mL of DMEM.

5.2.1.2. Cell Expansion

The SVF pellet was seeded into a four-layer cell factory (Sigma-Aldrich, St Louis, Missouri, USA) containing 800mLs of standard cell culture media (DMEM supplemented with 10% canine serum (Wongaburra Research Centre, Casino, NSW) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, New York, USA) and incubated at 37°C with 5% CO_2 to obtain adherent cells. After 8 days the adherent MSCs reached 80% confluence, at which time the MSCs were liberated from the flask using TrypLE express (Life Technologies, Grand Island, New York, USA), and diluted in the above cell culture media.

5.2.1.3. Analysis of Cell Numbers and Viability by Flow Cytometry

An aliquot (200µL) of the freshly isolated MSCs was filtered through a 35µm nylon mesh topped tube (Becton Dickinson, Franklin Lakes, New Jersey, USA) and transferred into a Trucount tube (Becton Dickinson Franklin Lakes, New Jersey, USA) containing isoflow (Beckman Coulter, Indianapolis, Indiana, USA), propidium iodide (10 µg/mL; Sigma-Aldrich St Louis, Missouri, USA) and Syto11 (1 µg/mL; Life Technologies Grand Island, New York, USA). The total number and percentage of viability of the MSCs was determined.

5.2.1.4. Production of Cellular Secretions

Cellular secretions were produced from the adipose tissue from the falciform ligament of a 6 month old female Labrador dog that underwent a routine desex procedure. A total of 11.7 grams of adipose tissue was obtained. Isolation of the SVF and subsequent cell expansion procedures were followed as described above. When MSCs reached 80% confluence, the culture supernatant was aspirated and filtered through a steripak 0.22µm filter system (Millipore, Billerica, Massachusetts, USA). Once filter sterilised, the culture supernatant was concentrated 10-fold utilising the viva flow system (Sartorius, Goettingen, German) and again filter sterilised. The 10x concentrated cellular secretions were then stored at -80°C until required.

5.2.1.5. Cryopreservation

MSCs were cryopreserved in 1mL cryovials (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) containing approximately 200,000 (4µL) MSCs in either, 40µL of DMSO (Life Technologies, Grand Island, New York, USA) and 356µl of canine serum, or 40µl of DMSO, 178µL of canine serum and 178µL of 10x concentrated cellular secretions. The cryovials were stored at -80°C in a controlled rate freezing device (Nalgene, Mr Frosty, Sigma-Aldrich, St Louis, Missouri, USA) for 24 hours before being transferred and stored in liquid nitrogen at -196°C. Prior to administration of the test articles to the mice, a further analysis of cell number and viability as described above was performed on thawed MSCs to ensure MSCs were still present and viable after cryogenic-storage.

5.2.2. Collagen-Antibody Induced Arthritis – Mouse Model:

5.2.2.1. Test System and Environment

Nulliparous and non-pregnant female Balb/c (BALB/cJAsmu) mice at six-eight weeks of age were used in this trial. The mice were housed in individually ventilated cages throughout the study with a maximum of ten animals in each cage. The mice were provided with animal bedding (Fibrecycle Pty Ltd., Australia) during acclimatisation. The mice had access to rat and mouse pellets (Specialty feeds, Australia) and water *ad libitum* throughout the study. The mice were also given chewing sticks (Able Scientific, Australia) and Alphatwists (Tecniplast, Australia) as environmental enrichment. The housing temperature throughout the study was 23 ± 3°C, with a humidity of 30-70%, a twelve-hour light/dark cycle and a minimum of fifteen air changes per hour. The mice were allowed to acclimatise to these housing conditions for three days prior to commencement of the trial. Table 5.1 provides a summary of the trial timeline once commenced.

Table 5.1. Time-line design for trial.

Event	Day of Trial
Clinical measurements	0 and 2-13
Administration of antibody cocktail	0
Administration of lipopolysaccharide	3
Onset of clinical arthritis signs	5
Administration of test articles	6
Euthanasia	14
Blood Collection	14

5.2.2.2. Induction of Collagen-Antibody Induced Arthritis

At day 0, each mouse (total of 12 mice) received an intravenous injection of 1.5mg (150µL) of an anti-type II collagen 5-clone antibody cocktail (Table 5.1). This cocktail contains 5 monoclonal antibodies: clone A2-10 (IgG2a), F10-21 (IgG2a), D8-6 (IgG2a), D1-2G (IgG2b), and D2-112 (IgG2b) recognise the conserved epitopes on various species of type II collagen. Clones A2-10, D1-2G, and D2-112 recognise

individual epitopes clustered within the 167 amino acid peptide fragment called LyC1 (124-290) of the CB11 fragment (124-402) of type II collagen. Whereas clones F10-21 and D8-6 recognise epitopes within the 83 amino acid peptide fragments called LyC2 (291-374) of the CB11 fragment of type II collagen.

On day 3, mice received an intra-peritoneal injection of 80µL (40µg/mouse) of lipopolysaccharide (LPS). The subsequent administration of LPS following the antibody cocktail not only increases the severity of the arthritis through the induction of pro-inflammatory cytokines, but also reduces the amount of monoclonal antibody required to induce the arthritis in this model.

5.2.2.3. Treatment Groups

The trial design consisted of two groups, each containing six-mice (Table 5.2). Group 1 received cultured expanded cryopreserved canine MSCs with the addition of 45% concentrated cellular secretions (MSCs+secretions) administered once as an intravenous bolus on day six of the trial. Group 2 received cultured expanded cryopreserved canine MSCs without the addition of cellular secretions (MSCs only) administered once as an intravenous bolus on day six of the trial. Serum was also collected from an additional six mice of the same age and body weight to that of the mice in the treatment groups. These animals were untreated mice and acted as a control group as an indicator of normal physiological serum levels of the bioactive-factors measured.

Table 5.2. Trial design of treatment groups of mice suffering from collagen antibody-induce arthritis.

Group (6mice/group)	Treatment	Route of Administration	Volume Administered	Treatment Regime
Group 1	MSCs+secretions	Intravenous	140µl (70,000 MSCs)	Administered on Day 6
Group 2	MSCs only	Intravenous	140µl (70,000 MSCs)	Administered on Day 6

5.2.2.4. Behavioural Observations

The mice were monitored throughout the trial period (2 weeks) and measurements were taken on days 0 and 2-13. The measurements were taken prior to administration of the collagen antibody cocktail (day 0) and LPS (day 3) and prior to administration of the test articles (MSCs+secretions or MSCs only).

Body weight; paw volume, ankle size and clinical arthritis scores were measured in each mouse throughout the treatment. The paw volume of each mouse was measured using a plethysmometer. The paw size was measured using micro calipers across the hillock (ankle joint) of each hind paw. The mice were assessed for the severity of arthritis using the scale outlined in Table 5.3.

Table 5.3. Assessment criteria for clinical arthritis score of mice suffering from collagen-antibody induced arthritis.

Arthritis Score	Criteria
0	Normal
1	Mild Redness, slight swelling of ankle or wrist, redness and swelling limited to individual joints
2	Moderate swelling of ankle or wrist redness in more than one joint
3	Severe swelling including some digits, ankle and foot
4	Maximal swelling and inflamed involving multiple joints

5.2.2.5. Blood Sampling

Following euthanasia with Lethabarb on day 14, blood was collected from all mice via cardiac puncture. Serum was obtained by centrifugation of the blood. Serum samples from age-matched naïve mice were also obtained as control samples. Serum samples were stored at -80°C prior to analysis using multi-plex cytokine analysis technology.

5.2.3. *In vitro* Analysis

5.2.3.1. Milliplex Analysis of Canine Cytokines and Growth Factors

Serum samples from the mice of both treatment arms and from the naïve mice were filtered through 0.2μm Nanosep MF Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, Port Washington, New York, USA) for 5 minutes at 9000 *x g*. A 25μL portion of each filtered sample was analysed using the Milliplex MAP® Canine Cytokine 13-plex assay which measures IFN-γ, IL-8, IL-15, IL-18, TNF-α, IL-10, IP-10, KC-like, MCP-1, GM-CSF, IL-7, IL-2 and L-6 (Millipore, Billerica, Massachusetts, USA) according to the manufacturers instructions. The washing steps were performed on the Bio-Plex Pro II magnetic wash station and the data were acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, Hercules, California, USA).

5.2.3.2. Bio-Plex Analysis of Mouse Cytokines and Growth Factors

To investigate the effect of both treatment articles in the mice, a total of thirty-two mouse cytokines were measured in the mouse serum samples of both treatment arms, in the naïve mice serum samples and in the canine secretions and serum controls. Canine secretions, canine serum and all mouse serum samples were filtered through 0.2μm Nanosep MF Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, USA) for 5 minutes at 9000*xg*. Samples (50μL) were analysed using the Bio-Plex Pro Mouse Cytokine 23-plex and Bio-Plex Pro Mouse Cytokine 9-plex assays (Bio-Rad, USA) according to the manufacturers instructions. The washing steps were performed on the Bio-Plex Pro II magnetic wash station and the data were acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA).

5.2.4. Data Analysis

5.2.4.1. Data Analysis of CAIA Primary Outcome Measurements

The primary outcome measures; paw volume (cm^3), ankle size (mm), clinical arthritis score and body weight (g) for both treatment groups are presented as the average ± standard error of the mean (SEM). To determine if there was a significant difference between the primary outcome measurements obtained between the two treatment arms, a mixed model ANOVA was used. The data were stratified into two timeframes. The first was days 0-5, which encompassed the induction of CAIA prior to treatment with the test articles. The second was days 6-13, which encompassed the treatment of the mice at day 6 with the test articles and their treatment outcome until the conclusion of data collection at day 13. The mixed model ANOVA included a fixed effect for treatment and a random effect for mouse. Mouse was treated as a random effect because daily readings were recorded for each mouse throughout the trial. The model, in R notation, used was: Score ~ Treatment + (1|Mouse). For this linear model, F-tests were calculated using a type II Wald chi-square. The statistical significance criterion applied was $p < 0.05$.

5.2.4.2. Data Analysis of Mouse and Canine Cytokines, Chemokines and Growth Factors Measured in the Mouse Serum Samples

The mouse serum from the two treatment arms and naïve mice were run on the canine cytokine 13-plex and on the mouse cytokine 23-plex and 9-plex. Concentrations of each cytokine, chemokine or growth factor in pg/mL were obtained and reported as average ± SEM. Two-tailed t-tests were used to determine if the differences in cytokine concentrations measured in the serum between groups were statistically significant. A p-value < 0.05 was considered statistically significant. The canine serum and secretion controls were run on the mouse cytokine 23-plex and 9-plex. Concentrations of each cytokine, chemokine or growth factor are shown in pg/140μL to demonstrate the amount of canine protein injected into the mice and reported as average ± SEM.

5.3. Results

The primary outcome measures of this study were paw volume; ankle size and clinical arthritis score, of the CAIA affected mice. These measurements were used to determine if a combination of MSCs+secretions had a superior ability to ameliorate the clinical signs of arthritis in comparison to culture expanded MSCs only. Secondary outcomes consisted of identifying differences between the two treatment arms in circulating serum cytokine levels.

5.3.1. CAIA Primary Outcome Measures

The ankle size, paw volume and clinical arthritis score of the six mice in each of the two test arms were measured on days 0 and 2-13 (Table 5.1). As anticipated the CAIA model induced the clinical manifestations of arthritis being paw swelling and redness. Figures 5.2 A, C, E and G show the mean (± SEM) for ankle size (mm); paw volume (cm^2), clinical score and body weight (g) respectively. From days 0-5, prior to administration of the test articles, no significant difference was seen in any of the four parameters between the two groups. However, from days 6-13 post administration of the test articles (Table 5.1), a clear reduction in the clinical signs of arthritis was seen in the MSCs+secretions group in comparison to the MSC only test group. A mixed model ANOVA shown in Figure 5.1 showed that there was no significant difference between the two groups in body weight at either the 0-5 or 6-13 day timeframes (Figure 5.1H), however, there was a highly significant difference in ankle size, paw volume and clinical score between the two groups between days 6-13 (Figures 5.1 B, D and F respectively). Treatment with MSCs+secretions compared to MSCs only demonstrated a highly significant reduction in ankle size (p-value = 4.27E-04; Figure 5.1B); paw volume (p-value = 1.86E-04; Figure 5.1D) and overall clinical score (p-value = 1.84E-05; Figure 5.1F). These results demonstrated that culture expanded MSCs+secretions, when administered as a single bolus intravenous dose six days post-CAIA induction, appeared to provide a superior reduction of the signs of arthritis compared to MSCs alone.

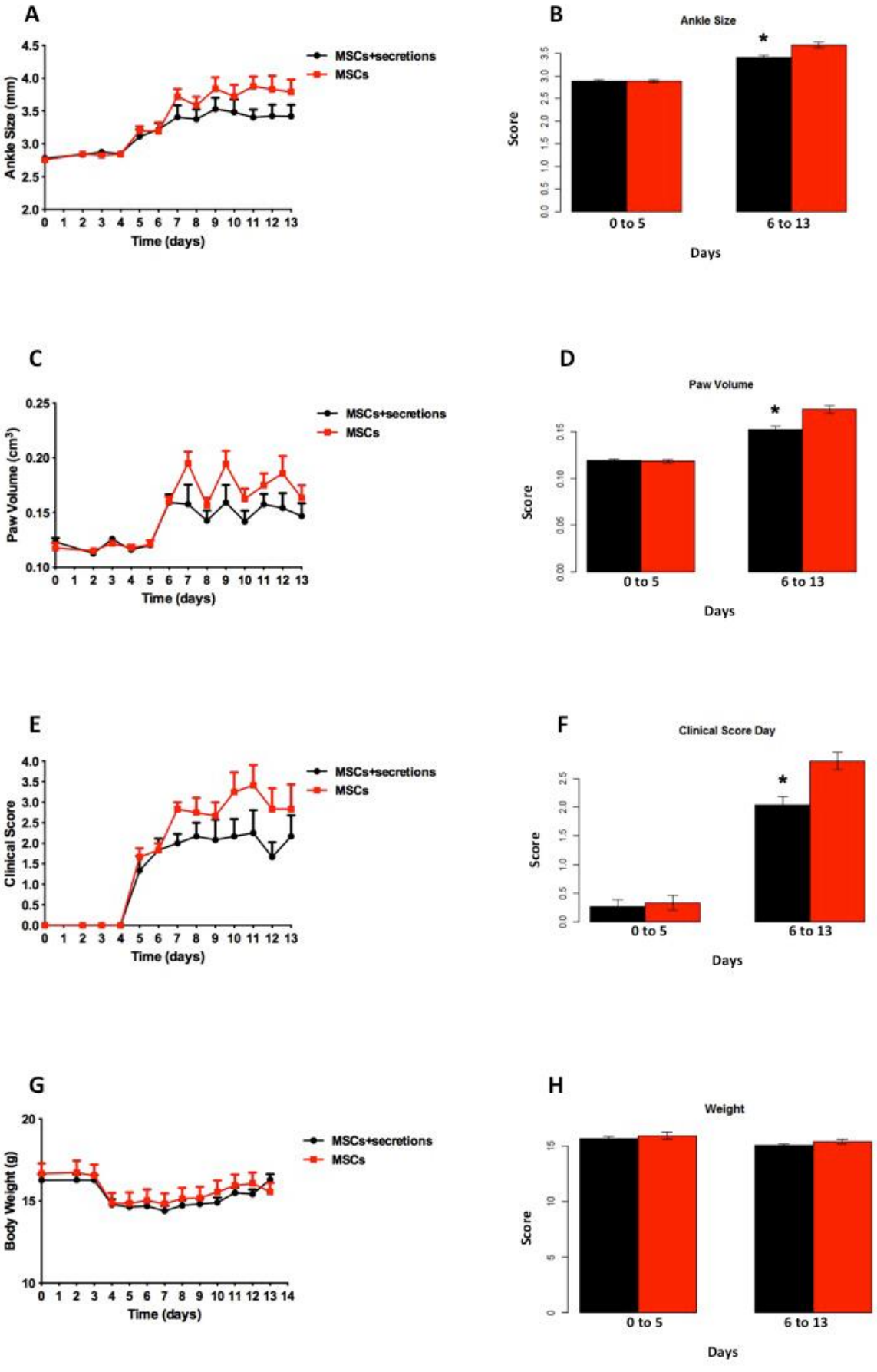
A
Ankle Size (mm)
Time (days)
MSCs+secretions
MSCs
B
Ankle Size
Score
0 to 5
6 to 13
Days
C
Paw Volume (cm³)
Time (days)
MSCs+secretions
MSCs
D
Paw Volume
Score
0 to 5
6 to 13
Days
E
Clinical Score
Time (days)
MSCs+secretions
MSCs
F
Clinical Score Day
Score
0 to 5
6 to 13
Days
G
Body Weight (g)
Time (days)
MSCs+secretions
MSCs
H
Weight
Score
0 to 5
6 to 13
Days

Figure 5.1. Ankle size, paw volume, clinical score and body weight measurements of mice suffering from collagen antibody-induced arthritis treated with canine MSCs+secretions or MSCs only.

Mice suffering from collagen antibody-induced arthritis were subsequently treated with canine culture expanded MSCs+secretions or MSCs only. The mice were assessed for ankle size (A&E) paw volume (B&F) clinical score (C&G) and body weight (D&H) throughout the course of the study to determine the effectiveness of the two treatment arms to ameliorate the signs of arthritis following CAIA induction. The mean ± SEM for all four clinical assessments (A-D) and the results of a mixed model ANOVA (E-H) are graphed. An * denotes a p-value of < 0.05 when comparing the two treatment groups.

5.3.2. Serum Levels of Mouse Cytokines, Chemokines and Growth Factors

Serum was collected from the mice of both treatment arms on day 14 of the trial subsequent to euthanasia. Serum was also collected from an additional six naïve mice to serve as an indicator of normal physiological serum levels of the bioactive-factors measured. A total of thirty-two mouse cytokines, growth factors and chemokines were measured to determine if any differences could be detected at a biological level between the two treatment arms and the naïve mice. Of these thirty-two bioactive factors three were not detected in the mouse serum and a further eight were shown to have some level of cross-reactivity with the proteins present in the canine serum and secretions (Table 5.4). As such, these bioactive factors were not included in any further analysis.

Table 5.4. Mouse bioactive factors not detected or that cross-reacted with canine serum and secretions.

Bioactive factor	Bioactive factor family	Cross-reacted	Not detected
TNF-α	Pro-inflammatory	X	
IFN-γ	Pro-inflammatory		X
IL-1β	Pro-inflammatory	X	
IL-12 (p40)	Pro-inflammatory	X	
IL-12 (p70)	Pro-inflammatory	X	
MIP-1α	Chemokine		X
MIP-1β	Chemokine	X	
Eotaxin	Chemokine	X	
KC	Chemokine		X
GM-CSF	Growth factor	X	
LIF	Dual role cytokine	X	

Mouse bioactive factors either not detected in the mouse serum or which were shown to have some level of cross-reactivity with the proteins present in the canine serum and secretions. (Bioactive factors are measured in pg/mL).

5.3.2.1. Anti-Inflammatory Cytokines

Figure 5.2 shows the levels of the anti-inflammatory cytokines IL-3, IL-4, IL-10 and IL-13 measured in the mouse serum. A higher level of IL-13 (p-value of < 0.1) was seen in the MSCs only group compared to the MSCs+secretions test group. Additionally, the MSCs only group had significantly higher serum concentrations of IL-3, IL-4, IL-10 and IL-13 compared to the naïve mice (p-values < 0.05). There was no significant difference in the serum levels of these anti-inflammatory cytokines between the MSCs+secretions group and the naïve mice.

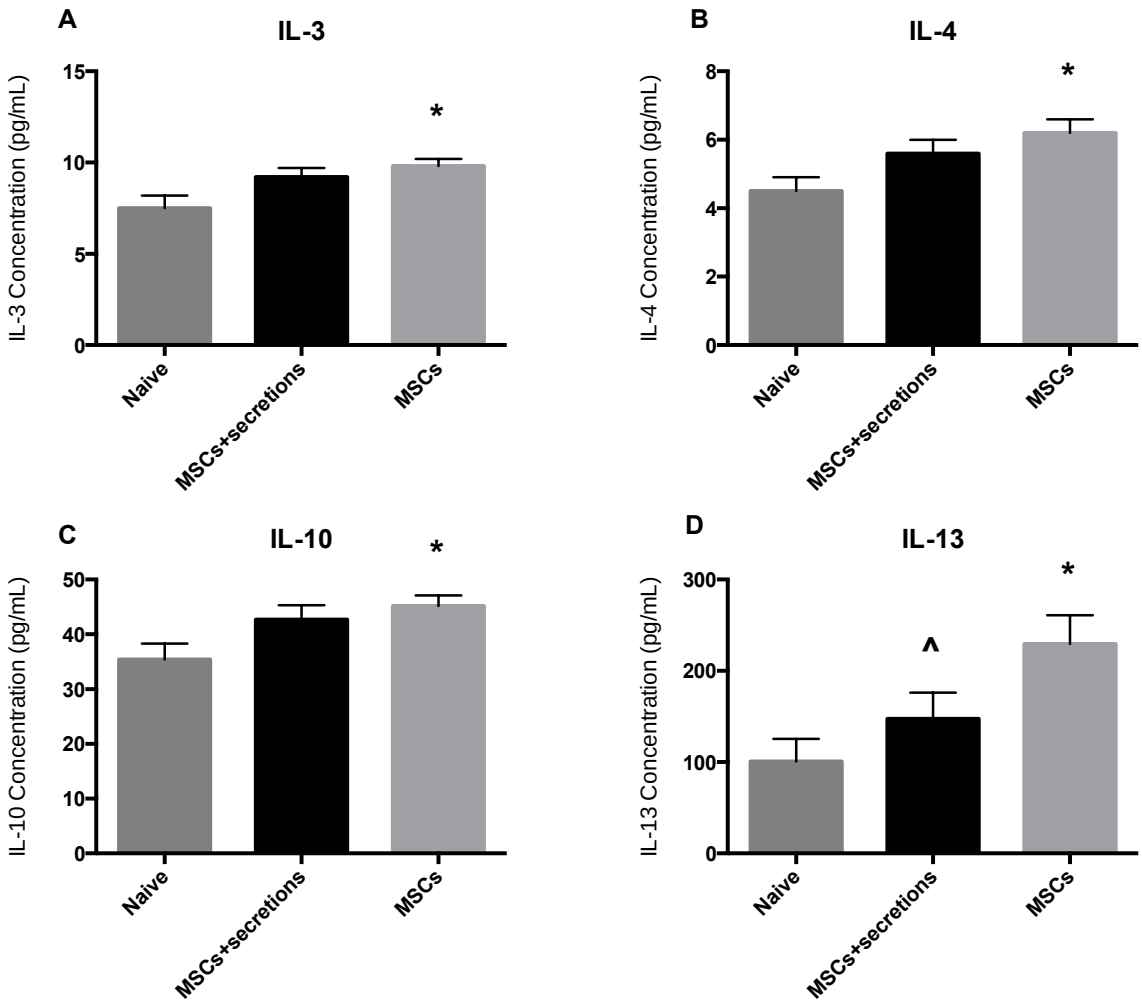

Figure 5.2. Levels of anti-inflammatory cytokines measured in the serum of mice suffering from collagen antibody-induced arthritis following treatment with canine MSCs+secretions or MSCs only.

Serum samples were collected following euthanasia of the mice in both treatment arms and from naïve mice at the completion of the trial. The results of the anti-inflammatory cytokines, IL-13 (A), IL-4 (B), IL-10 (C) and IL-13 (D) in both of the treatment arms and the naïve mice are shown as the mean ± SEM; (n = 6 for all groups). An * denotes a p-value of < 0.05 (students t-test) when comparing test arms to the naïve group. An ^ denotes a p-value of < 0.1 when comparing between the MSCs+secretions and MSCs only test arms.

5.3.2.2. Pro-Inflammatory Cytokines

Figure 5.3 shows the serum concentrations of the six pro-inflammatory cytokines measured in the mouse serum. IL-9, IL-15, IL-17 and IL-18 were detected in both test arms at significantly greater serum concentrations compared to serum levels of the naïve mice (Figures 5.3 C, D, E & F respectively). Additionally IL-1α, and IL-5 (Figures 5.3 A & B respectively) were expressed at a statistically higher serum concentration in the MSCs only group in comparison to the naïve mice. IL-9 (Figure 5.3C) was also expressed at a significantly higher serum concentration in the MSCs only test group (21.1 ± 7.3 pg/mL) compared to the MSCs+secretions group (3.3 ± 3.3 pg/mL). IL-9 was the only cytokine detected at a significantly different serum concentration between the two test arms.

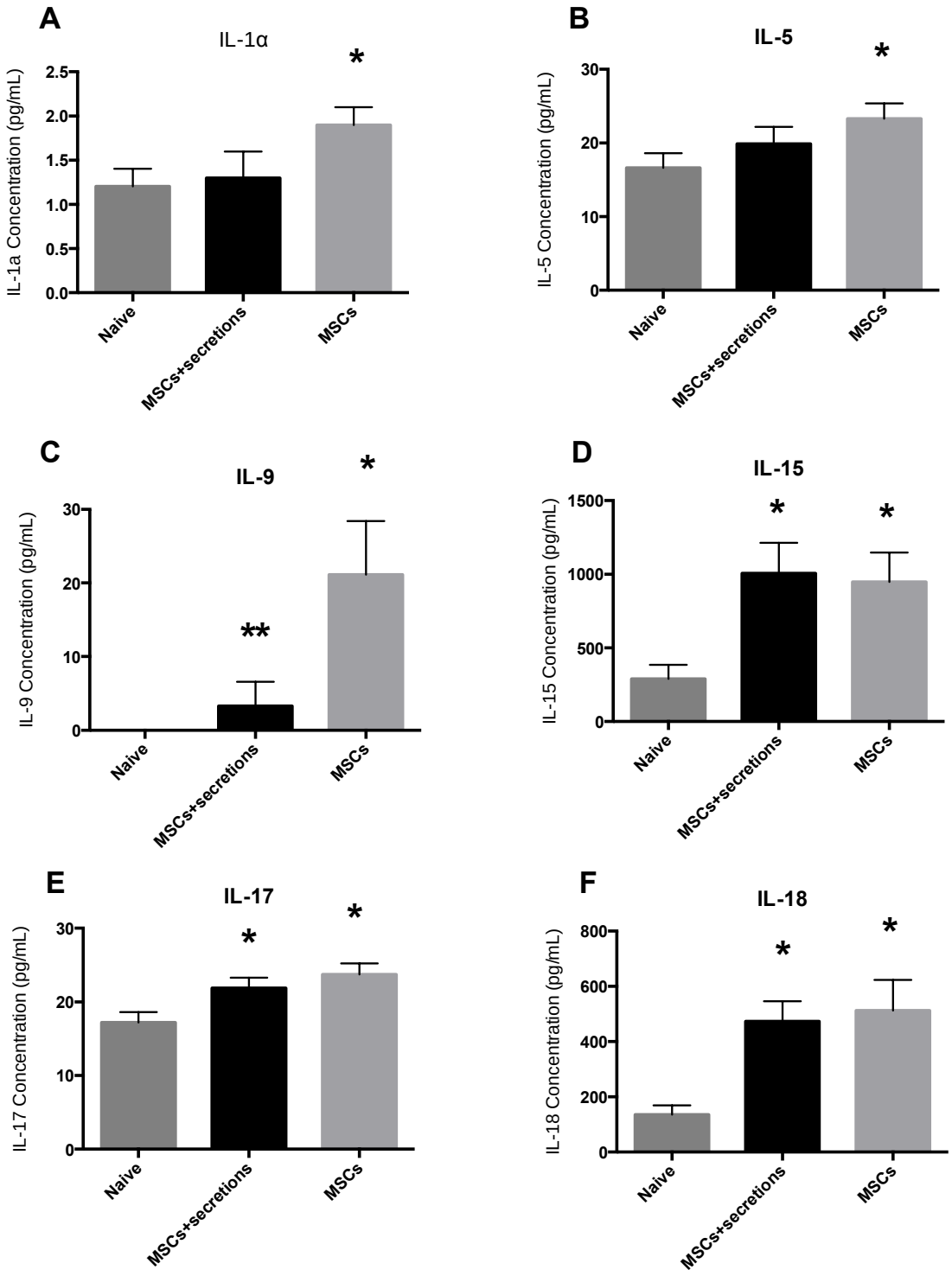

Figure 5.3. Levels of pro-inflammatory cytokines measured in the serum of mice suffering from collagen antibody-induced arthritis following treatment with canine MSCs+secretions or MSCs only.

Serum samples were collected following euthanasia of the mice in both treatment arms and from naïve mice at the completion of the trial. The results of the pro-inflammatory cytokines, IL-1α (A), IL-5 (B), IL-9 (C), IL-15 (D), IL-17 (E), IL-18 (F), in both of the treatment arms and the naïve mice are shown as the mean ± SEM; (n = 6 for all groups). An * denotes a p-value of < 0.05 (students t-test) when the test arms are compared to the naïve group. An ** denotes a p-value of < 0.05 when comparing between the MSCs+secretions and MSCs only test arms.

5.3.2.3. Dual Roles

IL-2 and IL-6 are cytokines that have either an anti-inflammatory or pro-inflammatory role depending on the circumstance. No statistically significant difference was seen in serum levels of IL-2 and IL-6 between the two test arms (Figures 5.4A and B). However, IL-2 was present at significantly increased levels in the serum of the mice in both test groups compared to the serum of the naïve mice (Figures 5.4A). Additionally, the MSCs only group had significantly increased serum levels of IL-6 (12.8 ± 1.0 pg/mL) compared to the serum of the naïve mice (7.8 ± 0.9 pg/mL; Figure 5.4B).

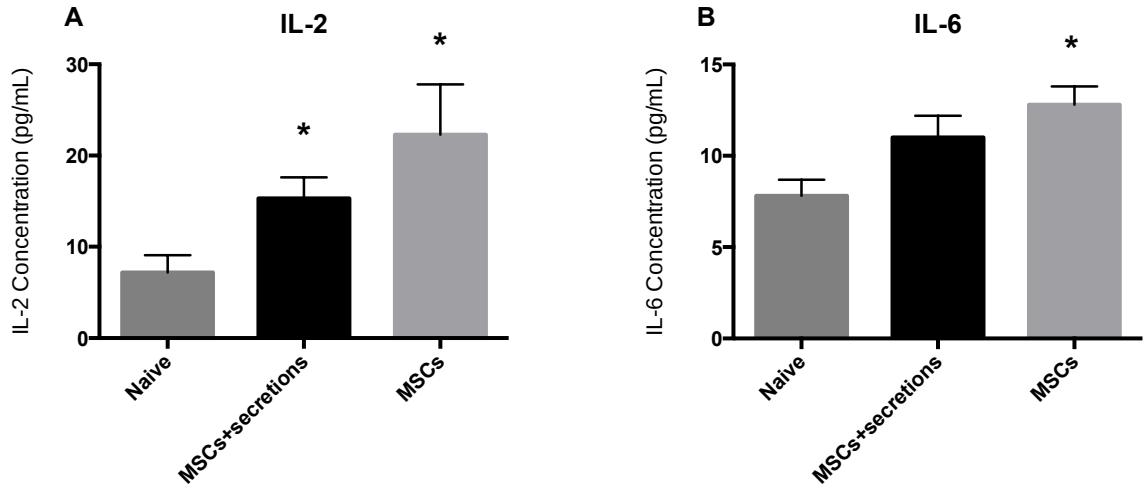

Figure 5.4. Levels of cytokines with dual roles measured in the serum of mice suffering from collagen antibody-induced arthritis following treatment with canine MSCs+secretions or MSCs only.

Serum samples were collected following euthanasia of the mice in both treatment arms and naïve mice at the completion of the trial. The results of the cytokines with dual roles, IL-2 (A) and IL-6 (B) in both treatment arms and the naïve mice are shown as the mean ± SEM; (n = 6 for all groups). An * denotes a p-value of < 0.05 (students t-test) when comparing test groups to the naïve mice group

5.3.2.4. Growth Factors

Figure 5.5 shows significantly increased concentrations in the five growth factors detected in the serum of the mice in one or both test groups compared to the naïve mice. In comparison to naïve mice, G-CSF, bFGF, M-CSF and VEGF (Figures 5.5A, B, C & E respectively) were measured in significantly elevated levels in the serum of mice treated with MSCs+secretions and MSCs only. The MSCs+secretions study arm also had a significantly greater serum concentration of PDGF-bb compared to the naïve mice (Figure 5.5D). There was no significant difference in the serum concentration of any of the five growth factors measured between the two test groups.

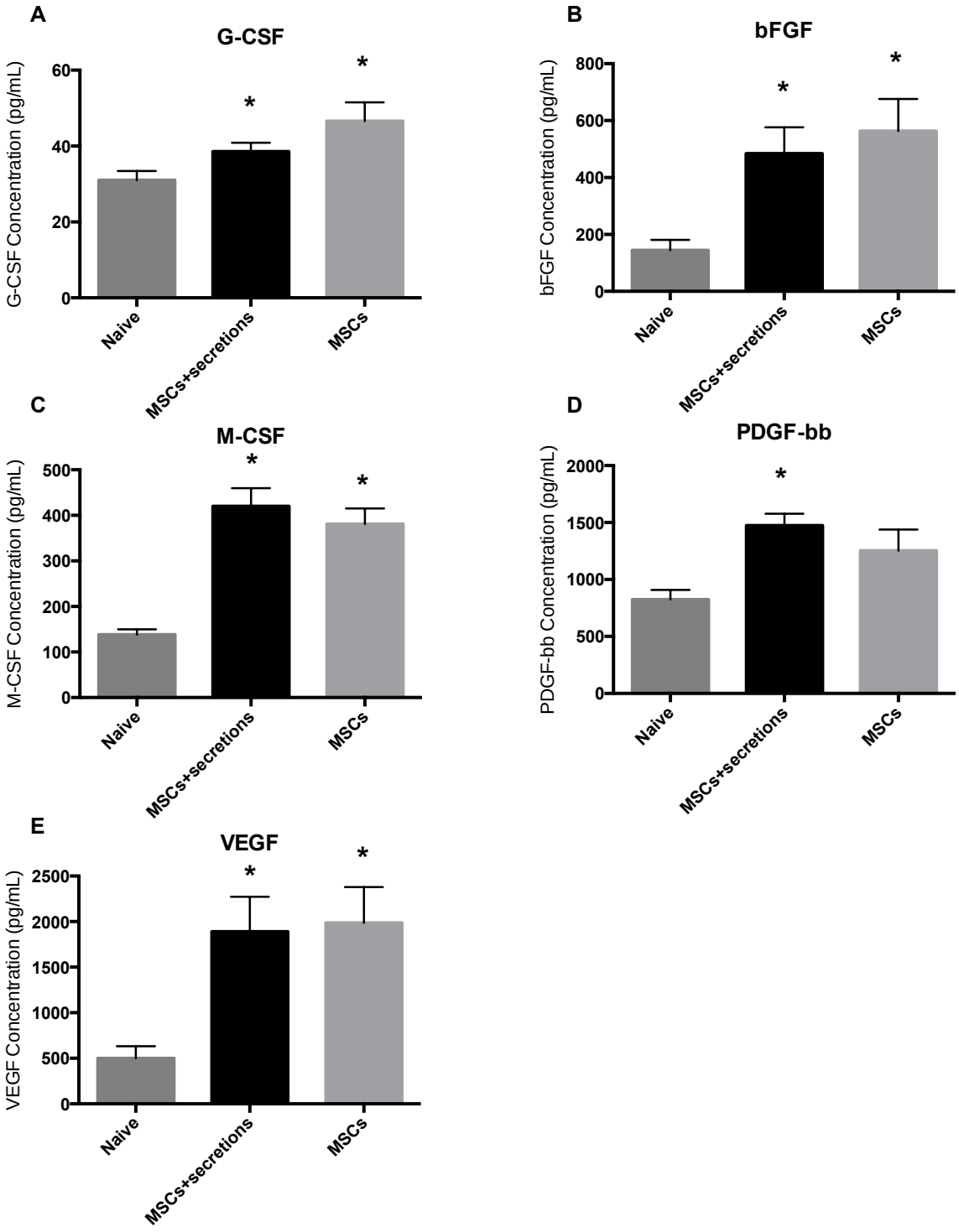

Figure 5.5. Levels of growth factors measured in the serum of mice suffering from collagen antibody-induced arthritis following treatment with canine MSCs+secretions or MSCs only.

Serum samples were collected following euthanasia of the mice in both treatment arms and from naïve mice at the completion of the trial. The results of the growth factors, G-CSF (A), FGF-β (B), M-CSF (C), PDGF-ββ (D) and VEGF (E) in each of the treatment groups and the naïve mice are shown as the mean ± SEM; (n = 6 for all groups). An * denotes a p-value of < 0.05 (students t-test) when comparing test groups to the naïve group.

5.3.2.5. Chemokines

The chemokines MCP-1, MIP-2, MIG and RANTES (Figures 5.6A, B, C & D) were all detected at significantly greater serum concentrations in both test groups compared to the naïve mice. RANTES (Figure 5.6D) was also detected at a higher serum concentration (p-value < 0.1) in the MSCs+secretions group in comparison to the MSCs only test group. MCP-1 (Figure 5.6A) was detected in the MSCs only test group at a significantly higher (p-value < 0.05) serum concentration compared to the naïve mice and also at a greater level (p-value < 0.1) than the MSCs+secretions test group.

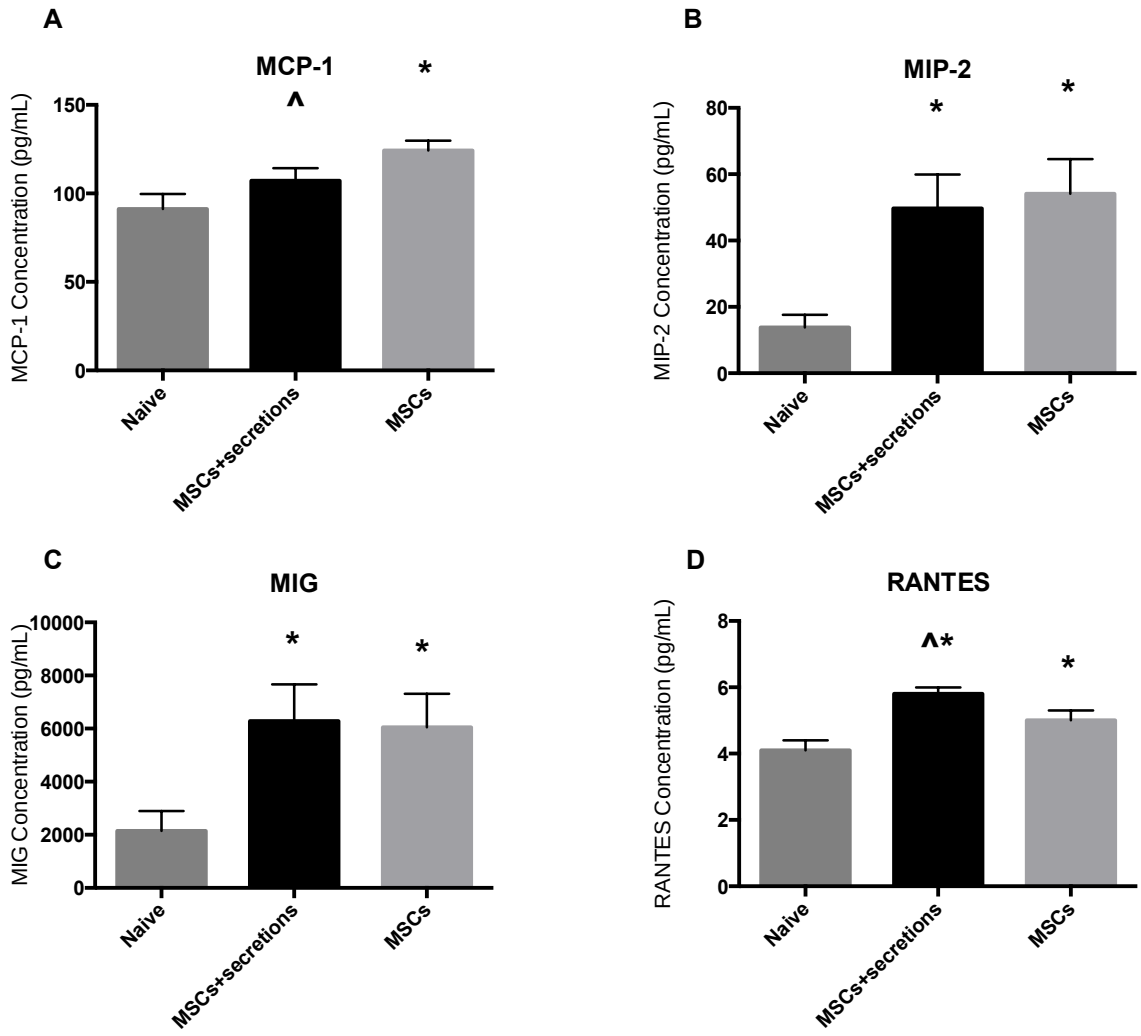

Figure 5.6. Levels of chemokines measured in the serum of mice suffering from collagen antibody-induced arthritis following treatment with canine MSCs+secretions or MSCs only.

Serum samples were collected following euthanasia of the mice in both treatment groups and from naïve mice at the completion of the trial. The results of the chemokines, MCP-1 (A), MIP-2 (B) MIG (C) and RANTES (D) in each of the treatment groups and the naïve mice are shown as the mean ± SEM; (n = 6 for all groups). An * denotes a p-value of < 0.05 (students t-test) when compared to the naïve group. An ^ denotes a p-value of < 0.1 when comparing the two test groups.

5.3.2.6. Summary of the Serum Levels of Mouse Cytokines and Growth Factors

Thirty-two mouse cytokines, chemokines and growth factors, collectively known as bioactive factors, were measured in the serum of the six mice in each treatment group and the six naïve mice. Of these thirty-two bioactive factors, eleven were not included in the analysis due to either, not being detected, or showing cross-reactivity with canine proteins (Table 5.4). A summary of the remaining twenty-one mouse bioactive factors is shown in Table 5.5. The summary shows the bioactive factors that were significantly increased in each of the two treatment groups, in comparison to the measured levels of bioactive factors in the naïve mice serum (Table 5.5). Thirteen of the measured bioactive factors were detected in statistically greater concentrations (p-value < 0.05) in the canine MSCs+secretions test group compared to the naïve mice. Five of these were growth factors, three were chemokines; four were pro-inflammatory cytokines and one was the dual role cytokine IL-2 (Table 5.5). The growth factor PDGF-bb and chemokine RANTES were the only bioactive factors detected in the MSCs+secretions group, at significantly higher concentrations (p-value < 0.05 and 0.1 respectively) than the naïve mice, which were not also measured at a higher concentration in the MSCs only test group.

Twenty of the bioactive factors were detected at a greater serum concentration in the MSCs only test group in comparison to the serum concentrations of the naïve mice. These included all four of the anti-inflammatory cytokines, both of the dual role cytokines IL-2 and IL-6, four growth factors, four chemokines and six pro-inflammatory cytokines (Table 5.5). Seven more bioactive factors were detected at significantly higher serum concentrations in the serum of the MSCs only test group compared to the naïve mice, in comparison to the MSCs+secretions group. These seven were the chemokine MCP-1, IL-6 a dual role cytokine, four were the anti-inflammatory cytokines IL-3, IL-4, IL-10 and IL-13 and two were the pro-inflammatory cytokines IL-1α and IL-5. Additionally, in the serum of the MSCs only test arm, the chemokine MCP-1 (p-value < 0.1) the anti-inflammatory cytokine IL-13 (p-value < 0.1) and pro-inflammatory cytokine IL-9 (p-value < 0.05) were detected at significantly greater serum concentrations in the MSC only group than that of the MSCs+secretions group.

Table 5.5. A comparison of the number of murine serum trophic factors, which were statistically significantly different (p-value < 0.05) between the naïve and the two treatment arms.

Bioactive factor group	MSCs+secretions	MSCs alone
Anti-inflammatory	0	4
Pro-inflammatory	4	6
Dual role cytokines	1	2
Growth factors	5	4
Chemokines	3	4
Total	13	20

Number bioactive factors measured in significantly greater levels in the serum of the CAIA affected mice following treatment with canine MSCs+secretions or MSCs only compared to naïve mice serum.

5.3.3. Detection of Canine Cytokines, Growth Factors and Chemokines in Mouse Serum

An potential advantage of treating the mice suffering from CAIA with canine MSCs and secretions was the ability to measure, not only the murine bioactive factors present in the mouse serum, but also the canine proteins present in the serum of the mice. It was anticipated that this approach could potentially enable a distinction between the functionality of the MSCs+secretions and MSCs only treatment arms, as ascertained by the levels of secreted canine cytokines. To determine this, the serum samples collected from the mice of both treatment arms and the naïve mice were assayed using the Milliplex MAP Canine 13-plex kit. Furthermore, to determine the levels of canine bioactive factors that were administered to the mice via the cryopreservation medium of both test arms, the canine secretions and canine serum were also analysed on the Milliplex MAP Canine 13-plex kit. Of the thirteen cytokines measured, IFN-γ, IP-10 and IL-7 were not detected in the canine serum or secretions. Of the remaining ten cytokines, the amount administered in the 140µL injected into the CAIA affected mice is shown in Figure 5.7A&B.

A significant level of cross-reactivity was present between the mouse serum proteins and the antibodies against seven of the canine cytokines. As the naïve mice never received any substances of canine origin, these bioactive factors were not included in any further analysis. The level of cross reactivity is shown in Table 5.6.

Three non-cross-reacting canine cytokines, GM-CSF, IL8 and MCP-1 were detected in the serum of fewer than half of the mice in the MSCs+secretions group. These cytokines were also present in the highest concentration in the canine secretions used in the MSCs+secretions group (Figure).

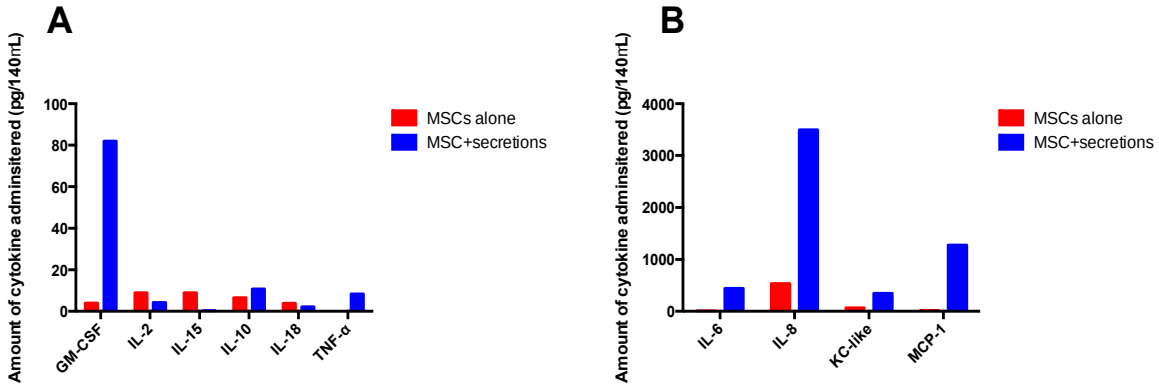

Figure 5.7. Levels of canine bioactive factors measured in the canine secretions and canine serum administered to the mice suffering from collagen antibody-induced arthritis.

The cryopreservation media for the MSCs+secretions and MSCs only test groups were assayed on the Milliplex MAP Canine 13-plex kit to determine the levels of the canine cytokines, growth factors and chemokines in the cryopreservant medium consisting of either 90% canine serum and 10% DMSO (MSCs only) or 45% canine secretions, 45% canine serum and 10% DMSO (MSCs+secretions). (A) bioactive factors measured at <100pg/140µL. (B) bioactive factors measured at >100pg/140µL.

Table 5.6. Level of cross-reactivity of mouse proteins with anti-canine antibodies.

Bioactive factor	Naïve	MSCs+secretions	MSCs alone
GM-CSF	0 ± 0	0 ± 0	35 ± 26
IFN-γ	3 ± 1	4 ± 1	3 ± 1
IL-2	1 ± 1	0 ± 0	25 ± 25
IL-6	1 ± 1	0 ± 0	22 ± 20
IL-7	2 ± 2	0 ± 0	60 ± 60
IL-8	0 ± 0	0 ± 0	149 ± 94
IL-15	7 ± 4	5 ± 5	87 ± 84
IP-10	87 ± 7	62 ± 1	77 ± 6
KC-like	13 ± 2	14 ± 3	18 ± 2
IL-10	7 ± 7	8 ± 5	180 ± 120
IL-18	5 ± 3	0 ± 0	32 ± 26
MCP-1	0 ± 0	0 ± 0	32 ± 32
TNF-α	0 ± 0	61 ± 61	50 ± 39

Cross-reactivity of mouse serum proteins with anti-canine anti-bodies measured in the serum of naïve mice and mice following treatment with canine MSCs+secretions or MSCs only using the canine Milliplex kit. Bioactive factors are measured in pg/mL. Data are presented as the mean ± SEM (n = 6 for all groups).

5.4. Discussion

The purpose of this chapter was to determine if the addition of cellular secretions to MSCs had a therapeutic effect compared to MSCs alone in ameliorating the clinical signs of arthritis. To answer this question, a CAIA mouse model of rheumatoid arthritis was utilised. Two groups of six mice were used in this study. Each mouse received a single intravenous injection of either culture-expanded canine MSCs with secretions (MSCs+secretions group), or culture expanded canine MSCs only (MSCs only group). The primary outcome measures of the trial consisted of the clinical manifestations of arthritis, which were paw volume (cm^3), ankle size (mm) and an overall clinical arthritis score. As a secondary outcome, differences in serum cytokine levels between both groups were investigated and compared to that of age matched naïve mice. Additionally the remaining circulating levels of canine cytokines in both test groups were investigated.

The aim of this trial was not to determine if MSCs in their own right would have a therapeutic effect in the CAIA model, but to determine if the addition of cellular secretions could provide an improved clinical benefit to that of just MSCs only. There is a plethora of literature detailing the therapeutic ability of MSCs in numerous pathologies, such as cardiovascular diseases [311], spinal cord injury [312, 313], bone and cartilage repair [163, 165, 302], and autoimmune diseases [314 and references therein]. Previous research utilising the CAIA model has clearly demonstrated that administration of a cell culture media vehicle control had no effect on the clinical signs of arthritis [315]. Furthermore, administration of MSCs to a model similar to CAIA, collagen induced arthritis (CIA), have been shown to reduce the incidence and severity of arthritis [316] and in some cases, prevent onset of arthritis [54]. Consequently, due to the accepted therapeutic ability of MSCs in these models of arthritis and other conditions, a vehicle control was not included in this trial. There is also considerable evidence that the clinical effect of MSCs is quite distinct from other non-stem cells and therefore a cellular control was not included.

5.4.1. MSCs Combined with Cellular Secretions had a Significantly Greater Effect on the Clinical Signs of OA than MSCs alone

Previously it has been demonstrated in the same CAIA model that cellular secretions alone could significantly reduce the clinical signs of arthritis [315]. Given that cellular secretions are a mixture of therapeutically important anti-inflammatory trophic factors, it was hypothesised that the addition of cellular secretions to MSCs would provide a rapid and sustained improvement in the clinical signs of arthritis in comparison to MSCs only. It was anticipated that the secretions would provide an initial dampening of inflammatory immune responses. As hypothesised, the results of this trial showed that CAIA affected mice in the MSCs+secretions group had a significant improvement in the clinical signs of arthritis, compared to the MSCs only treatment group (p-value of < 0.05). The addition of secretions to the MSCs resulted in a rapid clinical effect that was sustained throughout the length of the trial.

From days 0-5 of the trial, prior to administration of the test articles, daily assessment of mice body weight and the outcome measures, paw volume, ankle size and clinical arthritis score, showed that there was no significant difference between

the mice in the treatment arms. From days 6-13, post administration of MSCs+secretions or MSCs only, there was a significant reduction in the severity of arthritis in all three-outcome measures, in the MSCs+secretions treatment group compared to the MSCs only group (p-value of < 0.05). However, no significant difference in the weight of the mice was seen between the treatment groups. The rapid response in the MSCs+secretions group was evident as early as day 7, one day post intravenous injection of the test articles. This observation may indicate that the secretions portion of the therapeutic was having an effect on the immune and inflammatory response characteristics of CAIA [254]. Not only was the effect observed rapid, but it was also sustained throughout the length of the trial. Had the effect solely been due to an initial therapeutic response provided by the secretions, it would have been likely that the significant difference between the two treatment groups would not have been apparent for the entire length of the trial as the half-life of these proteins are generally less than 12 hours [94]. It could be assumed that after an initial lag, the treatment effect of the MSCs only group would be equivalent to the MSCs+secretions group. The sustained significant difference between both groups suggested that the addition of cellular secretions not only diminished the immune inflammatory responses but that the secretions may also have contributed to MSC survival and function, which in turn led to a sustained therapeutic effect greater than that of just MSCs alone.

5.4.2. CAIA, a Model for T-helper Cell Cytokine Imbalance

The CAIA model is one that closely reflects the disease of RA. RA is an autoimmune disease characterised by an imbalance between T helper cell (Th) type 1 and Th type 2 cytokines, resulting in pain, inflammation and eventual destruction of cartilage and bone [317]. CAIA in mice is induced using a cocktail of antibodies and results in a systemic autoimmune condition that shares many of the pathological features of RA, such as thickened synovial tissue (pannus formation), lymphocyte infiltration, synovitis and cartilage and bone destruction [318].

Although the etiology of RA is unknown, it is well understood that an imbalance between the Th1 and Th2 cytokine secretions exist [319]. Th1 cells, responsible for immune surveillance and initiation of inflammation, are found in

abundance in the synovium and serum of RA patients [320, 321]. Th2 cell types and cytokines are responsible for mediating this type of inflammatory process. Although they are present in the synovium of RA patients, a pronounced chronic inflammation perpetuates [322]. A Th1 cytokine response is characterised by the production of pro-inflammatory cytokines IFN-γ, IL-2 and TNF-α, which are necessary for cellular immune responses [323]. Under normal inflammatory processes, secretion of Th2 type cytokines such as IL-4, IL-6, IL-10 and TGF-β mediate the Th1 response and prevent uncontrolled tissue damage [323].

To alter the cytokine driven imbalance characteristic of autoimmune conditions, many conventional therapies for the treatment of these disorders, such as corticosteroids, attempt to induce a Th2 response to counteract the overexpression of Th1 type cytokines [324]. Corticosteroids are very effective in suppressing Th1 immune responses, however overuse of corticosteroids can have severe undesired side effects such as Cushing's syndrome, bone weakening and diabetes [325, 326]. Prolonged activation or suppression of either the Th1 or Th2 cytokine populations can result in damaging autoimmunity.

5.4.3. Cells with Secretions Induce Wide-Spread Immune Suppression

RA, characterised by a pro-inflammatory cytokine profile, is commonly treated with agents to suppress the Th1 response and induce a Th2 cytokine profile, such as dexamethasone, a synthetic glucocorticoid. A secondary outcome of this study was to determine if the ability of MSCs ± secretions to dampen the clinical response in the CAIA affected mice could be attributed to a clear shift in Th1/Th2 cytokine profiles. To achieve this, serum collected from the CAIA affected mice at the conclusion of the trial was analysed using Bio-Rad Bio-Plex panels for thirty-two cytokines, chemokines and growth factors. The results of this analysis showed no clear Th1 or Th2 profile at the serum level in either of the test groups. Instead, the serum of the mice of both treatment arms contained detectable levels of cytokines associated with both the Th1 and Th2 responses. In particular, the serum collected from the MSCs only treatment group contained significantly increased levels of the pro-inflammatory cytokines IL-1α, IL-5, IL-9, IL-15, IL-17 and IL-18 and a corresponding increase in the anti-inflammatory cytokines IL-3, IL-4, IL-10 and IL-13. In contrast,

only a significant increase in pro-inflammatory cytokines IL-9, IL-15, IL-17 and IL-18 were seen in the MSCs+secretions group. Of the thirty-two bioactive factors measured in the mouse serum, twenty were detected in the serum of the MSCs only group at statistically greater concentrations than that of the naïve mice serum ($p < 0.05$). Conversely, only thirteen of the cytokines, chemokines and growth factors measured were present in detectable levels in the MSCs+secretions group at statistically greater concentrations than that of the naïve mice serum ($p < 0.05$). This result indicated that the serum of the mice in the MSCs+secretions treatment group contained circulating levels of cytokines closer to that of normal physiological levels. This biological outcome correlated with the improved clinical response of the mice in the MSCs+secretions group as ascertained by the primary outcome measurements. Whilst this result was encouraging, it should be noted that blood sampling of the mice was not performed throughout the trial. Therefore there was no way to know what the circulating serum cytokine levels of the mice in both treatment arms were prior to, or during the trial phase. Although the exact mechanism of action is not known, it was evident that neither treatment arm caused an exclusive shift to a Th2 response. Furthermore both treatment arms had differing serum cytokine profiles to each other and to that of the naïve mice controls.

5.4.4. Do Cellular Secretions Assist Introduced Cells to Embed?

A benefit of utilising a xenogeneic system whereby canine MSCs and secretions were introduced into CAIA affected mice was the ability to investigate circulating levels of canine proteins in the serum of the mice. Of the thirteen canine cytokines investigated, only four were measured at detectable levels that showed no level of cross-reactivity to the mouse serum. Of these, bioactive factors GM-CSF, MCP-1, IL-8 and TNF-α were detected in the serum of fewer than half the mice in MSCs+secretions test group, while TNF-α, was detected in the serum of one mouse in the MSCs only group. It is well described in the literature that these trophic factors have only a short serum half-life of less than 24 hours although the exact half-life varies under different reported conditions [94, 327-330]. As such, a straightforward conclusion could be that after a single administration of the test articles, the circulating levels of the administered secretions would be eliminated from the serum of the mice. It then

could be concluded that the levels of the canine bioactive factors measured were from viable MSCs embedded in the CAIA mice. Whilst it is generally reported that cytokines have short half-lives in serum after IV administration, the situation is considerably more complex. For example, in the case of IL-8, the free cytokine has a serum half-life of minutes, however, studies have shown that there is a dynamic equilibrium between monomers, dimers, and cell-surface bound forms of IL-8. In addition, IL8 binds leukocytes and red blood cells, which may significantly increase the actual half-life [331]. Because only three canine cytokines were detected in the serum in less than half of mice in the MSCs+secretions group it is not possible to draw any conclusions about the half-life or mode of action of the canine cytokines.

These data hint, that not only may the addition of secretions have promoted an initial immune suppression in the CAIA affected mice, but also may have assisted in cell survival and embedding in some of the mice. To determine if this theory is indeed correct, tracking and post-euthanasia detection and quantification of the administered cells would be required.

5.4.5. Therapeutic Importance: MSCs and their Secretions are Beneficial in the Treatment of Autoimmune and Degenerative Diseases

The properties of MSCs and their secretions are well understood to have immune-modulatory effects [26]. There are a myriad of both acute and chronic conditions where inflammation is a serious component and requires suppression before the process of repair and regeneration can take place. As such, it could be assumed this type of therapy would be beneficial for not only RA, but for many other autoimmune and degenerative diseases. MSCs and/or their secretions have demonstrated efficacy in disorders such as cardiac infarction [304], bleomycin induced lung injury [82], GVHD [332, 333], Crohn's disease [48 and references therein] and maladies of the musculoskeletal system such as OA [163, 180].

There are many examples of culture expanded MSCs in the treatment of OA. These examples are demonstrated in animal models and in human case studies or clinical trials of OA. In animal models of disease, regeneration of cartilage, tendon or meniscus has been shown utilising either autologous or allogeneic MSCs in mice, goats, horses and rabbits [89, 158, 161, 334]. In people the regeneration of joint

tissues is also demonstrated, as well as increased functionality, reduction in pain and improved quality of life. In this study a superior clinical therapeutic effect has been demonstrated utilising MSCs+secretions than that of commonly used cultured MSCs. It would be of interest to know if MSCs+secretions would have superior efficacy in other animal models such as those described. Although the CAIA model is one of autoimmune disease, many of the disease characteristics are consistent with those seen in chronic degenerative diseases such as OA. Disease characteristics of cartilage and bone destruction are typical within both conditions, as are similarities between cytokine profiles [321, 335]. OA is not traditionally described as a condition characterised by inflammation, however, both joint disorders show predominance of pro-inflammatory cytokines and reduced Th2 cytokine expression [336]. Therefore the combination of MSCs+secretions is likely to assist in altering the Th1/Th2 imbalance in degenerative diseases as well as in immune-mediated disorders. CAIA is a simulated systemic autoimmune disease whereas degenerative disorders such as OA are typically isolated to an affected joint/s. The addition of cellular secretions to MSCs is likely to assist in altering the cytokine profile of the microenvironment of a joint which in turn may enable both resident and introduced cells to undertake the process of tissue regeneration.

In this study the test articles were administered intravenously. For a systemic condition this is arguably the most appropriate mode of administration. However, no tracking of cells or post euthanasia histopathology of the mice in this trial was performed to determine if intravenously administered MSCs migrated toward sites of tissue damage. It has been clearly demonstrated in the literature that MSCs are capable of homing towards sites of inflammation. For an isolated, avascular structure such as a joint however, the intravenous administration of MSCs and/or their secretions may be inferior to that of intra-articular site-specific administration. In a controlled model such as the CAIA model, it is well understood when the clinical manifestations of the simulated disease will occur and for that reason the optimal time to administer test treatments to determine efficacy is also well comprehended. In real clinical cases of disease, whether immune-mediated or degenerative, the progression of the disease and therefore the time in which treatments are administered may be of consequence to the therapeutic outcome.

5.5. Conclusion

MSCs in conjunction with secretions had a superior ability to ameliorate the clinical sign of arthritis in CAIA affected mice than that of just MSCs only. Circulating serum cytokine levels supported this clinical result, in that mice treated with MSCs+secretions showed a cytokine profile closer to that of the serum of the naïve mice. Furthermore, detection of four canine cytokines in the serum of mice in the MSCs+secretions group, suggested that the addition of secretions might also have assisted in cell survival, and potentially the ability of MSCs to embed in host tissues. Further investigation is required to determine if the outcomes of this study, demonstrated in an induced disease model, are achievable in real clinical cases of disease. The outcomes of this study suggest benefit in administering MSCs in combination with secretions over that of MSCs only.

CHAPTER SIX

The Analysis of Culture Expanded Adipose-derived Cells and the Effect of Cellular Secretions as a Cryopreservative

The previous chapter demonstrated an improved clinical outcome in CAIA-affected mice treated with canine MSCs+secretions compared to MSCs only. In this chapter an investigation to determine whether passaged MSCs could be a viable off-the-shelf cellular therapeutic for osteoarthritis in dogs, was performed. The need for an allogeneic off-the-shelf MSC product is apparent when comparing the difference in expense, time and patient morbidity with freshly derived point-of-care SVF treatments. The established technical aspects of cell culture, cryopreservation and storage, enables multiple treatment doses of MSCs to be generated from a single donor. However, given the effort and expense involved in producing an allogeneic off-the-shelf MSC therapeutic on a large-scale, it would be advantageous to determine whether cells derived from a given donor would be beneficial clinically. This involved in vitro characterisation of culture-expanded MSCs focusing on cell recovery and functionality of MSCs+secretions in comparison to MSCs only.

Outputs from this Chapter

Australian patent application AU 2013203072 Entitled "Therapeutic Methods and Compositions Comprising Cells and Secretions". Examined and accepted by the Australian Patent Office. An international PCT application has been filed (WO_2013_040649_A1).

6.1. Introduction

The preliminary outcomes reported in Chapter 3 of this thesis demonstrated an apparent long lasting therapeutic effect in the treatment of canine OA with the use of autologous SVF. Whilst clinically beneficial, the autologous approach to cellular therapy described in this thesis has a number of clinical and commercial limitations. A point-of-care SVF treatment requires a general anaesthesia, a surgical procedure and onsite cell processing capabilities, which are time, space and cost prohibitive. These factors combined, limit mainstream up-take in veterinary practice. An off-the-shelf therapeutic product would overcome these issues and would enable greater accessibility to the market by eliminating the need for expensive equipment, timely surgery and sample processing, in turn meaning a less costly therapeutic. Furthermore, these benefits also mean that, if required, repeat treatments are less prohibitive as tissue removal is not required.

The SVF is comprised of a variety of cell types including immune cells, vascular smooth muscle cells, endothelial cells and MSCs [36, 227, 228]. The majority of these cells express antigens on their surface that are recognised by the immune system of different individuals. The non-immune-privileged nature of these cells prevents the use of the SVF as an allogeneic cell therapy. In contrast, MSCs express low levels of major histocompatibility complex (MHC) class I molecules and lack the expression of MHC class II molecules. MSCs also lack expression of the co-stimulatory molecules CD40, CD80 and CD86, are not subjected to cytotoxic T cell mediated lysis and do not stimulate the proliferation of lymphocytes [53, 300]. Taken together, the immune-privileged nature of MSCs makes them a particularly attractive cellular therapy option for allogeneic or xenogeneic settings. As such, allogeneic MSCs enable provision of an off-the-shelf cellular therapeutic.

It has been clearly demonstrated in the literature that MSCs alone can reduce pain and inflammation and stimulate tissue regeneration in a variety of musculoskeletal conditions in both animal models and human studies [89, 161, 180, 181]. Furthermore, MSCs have been demonstrated to migrate to sites of trauma and injury, when administered remotely [67]. This migratory ability enables administration of MSCs via subcutaneous, intramuscular or intravenous routes and is a clear advantage of MSCs as an off-the-shelf therapeutic. In the dog study described

in this chapter, intra-articular injection was used because the animals were being treated for osteoarthritis.

The ability of MSCs to modulate inflammation and stimulate regeneration is largely due to the secretion of bioactive factors from these cells [26, 92]. Following exposure to damaged or diseased tissue, MSCs respond to the local microenvironment and alter their secretion profile to stimulate repair and regeneration [298]. Numerous studies have demonstrated that administration of MSC secretions alone achieves a therapeutic effect similar to that of cell therapy initially [67, 304, 305]. However, the therapeutic benefit of secretion-based therapy is only short-term in comparison to cellular therapy and consequently requires repeated administration [68]. As the half-life of most cytokines, chemokines and growth factors is minutes to hours [94], the short-term therapeutic benefits of secretion-based therapies are not unexpected. In addition, the effectiveness of secretions in small animal models will be hard to replicate in larger animals because of the increased blood volume and overall size. An exception to this is intra-articular injection, where the joint capsule provides a small volume microenvironment where the short-term effects of secretions and the medium to long-term effects of MSCs can be maximised. Given the potency of secretion-based therapy, there is the potential to further exploit the therapeutic benefit of secretions by optimising the concentration of cytokines and the number of cells in a mixed product.

Providing an off-the-shelf cell-based product requires the ability to store the cells for an adequate length of time. Slow-rate freezing and then storage of the cells in cryovials under liquid nitrogen enables long-term storage of cells in both the research laboratory and the hospital setting. In conventional storage techniques, cells are cryopreserved in a medium containing ~10% dimethyl sulfoxide (DMSO) and ~90% serum, although variations exist [337, 338]. Given that secretions are considered to be a major driver of the therapeutic effect of cellular therapy, and that secretion-based therapy alone has been demonstrated to have beneficial effects in animal models, this study was designed to exploit the potential of secretions in the cryopreservant of an off-the-shelf MSC therapeutic. The previous chapter described the use of canine culture expanded MSCs to attenuate the effects of collagen antibody induced arthritis (CAIA) in mice. In particular it investigated the therapeutic effect of a novel off-the-shelf therapeutic, MSCs+secretions, compared to MSCs cryopreserved

in routinely used cryopreservant media. It was demonstrated in the CAIA model that there was an advanced therapeutic effect of MSCs and secretions in combination, compared to MSCs only in the affected mice. It was not possible however, to decipher if the superior therapeutic effect demonstrated, was due to the secretions alone or the effect of the secretions on the MSCs. Due to the prolonged therapeutic effect observed, it was hypothesised that the cellular secretions enhanced MSC function.

6.1.1. Chapter Aims

The objectives of this chapter were to firstly confirm that the cryopreserved cells remained as functional MSCs, using cell surface antigen profiling and differentiation capacity, and secondly to determine the secretion profile and proliferation capacity of these MSCs. Furthermore, multi-plex cytokine profiling was used to investigate the differences between MSCs+secretions in comparison to the MSCs only. It was hypothesised that including the secretions in the cryopreservation media would enhance the functionality and post-thaw recovery of the MSCs. It was anticipated that the MSCs and secretions in combination would provide a novel off-the-shelf therapeutic for canine OA in terms of both cell quality and immediate post-injection benefit of immune-modulatory secretions.

To address these objectives, adipose-derived cells were expanded and cryopreserved in a mixture of adipose-derived secretions and standard cryopreservant media (allogeneic canine serum + 10% DMSO) and compared to cells in standard cryopreservant media. In order to be commercially viable, a single fat donation must be capable of providing thousands of therapeutic doses of MSCs. This requires repeated passaging of the cells and the prototype therapeutics used in this chapter were expanded to passage three. The following assays were performed on cells from three dogs to characterise the MSC profile and to compare the recovery and functionality of MSCs+secretions and MSCs only.

1. *Confirmation of cell population being MSCs post cryopreservation*
    a. Cell surface immunophenotyping
    b. Adipogeneic and osteogeneic differentiation potential
2. *Recovery and functionality of MSCs populations post cryopreservation*
    a. Cell enumeration and viability
    b. Quantification of actively proliferating cells
    c. Assessment of secretion capabilities

The results in Chapter 5 demonstrated a superior therapeutic effect from the administration of MSCs+secretions compared to MSCs only, in mice suffering from CAIA. This superior efficacy was hypothesised to be due to the cellular secretions having an effect on the MSCs and enhancing their therapeutic capacity. In this chapter it was anticipated that the addition secretions in the MSCs+secretions group may alter the proliferation capability and secretion profile of the MSCs. Therefore, the proliferation and secretion profiling assays were performed on the same samples with the medium present and also with the medium removed by washing. This approach enabled an investigation of whether the presence of the cellular secretions in combination with the MSCs in culture was responsible for any observed changes, or whether the secretions did in fact have an effect on the MSCs during cryopreservation and post-thaw recovery. The four test groups utilised for proliferation and secretion profile assays are described in Table 6.1.

After testing of the MSCs from three dogs, one was selected and used to produce a prototype MSCs+secretions product for a veterinary trial. Data collected from dog owners whose osteoarthritic dogs were treated with the described off-the-shelf MSCs+secretions product was collated and analysed. A preliminary comparison to determine if the off-the-shelf MSCs product had an equivalent or superior therapeutic outcome to the point-of-care SVF treatment offering, described in Chapter 3 of this thesis, was performed.

6.2. Methods

6.2.1. Production

6.2.1.1. Isolation of the Stromal Vascular Fraction

Adipose tissue (34g, 28g, 15g) was collected from the falciform ligament of three approximately 6 month old female guide dog puppies that underwent a routine desex procedure. The adipose tissue was washed with saline, minced with sterile scissors and digested with 0.05% collagenase (Sigma-Aldrich, St Louis, Missouri, USA) in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, New York, USA) in a 37°C water bath for 60 minutes. The digested sample was passed through a 400µm filter (Millipore, Billerica, Massachusetts, USA) and then centrifuged at 2000*xg* for 5 minutes. The floating lipid, adipocytes and supernatant were aspirated and discarded. The pelleted SVF cells were resuspended in saline, centrifuged at 2000*xg* for 5 minutes and then resuspended in 10mL of DMEM.

6.2.1.2. *In vitro* Expansion

The SVF pellet was seeded into a four-layer cell factory (Sigma-Aldrich, St Louis, Missouri, USA) containing 800mLs of standard cell culture media (DMEM supplemented with 10% canine serum (Wongaburra Research Centre, Casino, NSW) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, New York, USA) and incubated at 37°C with 5% CO_2. Media was changed every 3 days with the initial media change resulting in the removal of non-adherent cells. Once adherent MSCs reached 80% confluence, the MSCs were liberated from the flask using TrypLE express (Life Technologies, Grand Island, New York, USA), and passaged in the above cell culture media. MSCs at passage three were used in the experiments described in this chapter.

6.2.1.3. Secretion Production

Culture medium was collected from the passage three MSCs described above after three days in culture. The culture medium was filtered through a steripak 0.22µm filter system (Millipore, Billerica, Massachusetts, USA). Once filter sterilised, the culture medium was then concentrated 10-fold utilising the viva flow system (Sartorius, Goettingen, German) according to the manufacturers instructions and filter sterilised. The 10x concentrated culture medium was then stored at -80°C until required. Throughout this chapter this concentrated culture medium is referred to as cellular secretions.

6.2.1.4. Cell Enumeration and Viability

An aliquot (50 or 200µL) of the freshly isolated MSCs was filtered through a 35µm nylon mesh topped tube (Becton Dickinson, Franklin Lakes, New Jersey, USA) and transferred into a Trucount tube (Becton Dickinson Franklin Lakes, New Jersey, USA) containing Isoflow, propidium iodide (10 µg/mL; Sigma-Aldrich St Louis, Missouri, USA) and Syto11 (1 µg/mL; Life Technologies Grand Island, New York, USA). The total number and percentage of viability of the MSCs pre and post-cryopreservation was determined. Cell counts and viability were also performed post-washing of MSCs to remove the cryopreservation medium. Cell counts and viability data were used to normalise the number of cells seeded into each well or flask.

6.2.1.5. Cryopreservation

MSCs were cryopreserved in 2mL cryovials (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). Each vial was prepared with between one and three million MSCs resuspended in either, 900µL of canine serum and 100µL of DMSO (MSCs only group; Life Technologies, Grand Island, New York, USA) or 450µL of canine serum, 450µL of 10x concentrated cellular secretions and 100µL of DMSO (MSCs+secretions group). The cryovials were stored at -80°C in a controlled rate freezing device (Nalgene, Mr Frosty, Sigma-Aldrich, St Louis, Missouri, USA) for 24 hours before being transferred and stored in liquid nitrogen at -196°C.

6.2.2. Analysis

6.2.2.1. Thawing of MSCs+secretions and MSCs only

For all analyses, an MSCs+secretions and a MSCs only vial were removed from liquid nitrogen and thawed in a 37°C water bath. For proliferation capability and cytokine secretion profiling, half of the volume (500µL) from each of the vials was removed and centrifuged at 2000*xg* for 5 minutes and then resuspended in 500µL of standard cell culture media. These samples became the MSCs+secretions and MSCs only washed groups. The four test groups are described below in Table 6.1.

Table 6.1. Descriptions of cellular test groups used in *in vitro* experiments

Test Group	Description
MSCs+secretions	MSCs cryopreserved in 45% canine serum, 45% concentrated secretions and 10% DMSO
MSCs only	MSCs cryopreserved in 90% canine serum and 10% DMSO
MSCs+secretions washed	MSCs cryopreserved in 45% canine serum, 45% concentrated secretions and 10% DMSO. Centrifuged post-thaw and re-suspended in 500µl of standard cell culture supernatant
MSCs only washed	MSCs cryopreserved in 90% canine serum and 10% DMSO. Centrifuged post-thaw and re-suspended in 500µl of standard cell culture supernatant

6.2.2.1. Confirmation of MSCs

6.2.2.1.1. Immunophenotyping

Cells (600µL) of the MSCs+secretions and MSCs only groups were centrifuged at 2000*xg* for 5 minutes. The resultant pelleted cells were resuspended in Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA). MSCs (50µL) of each group were then stained with the following antibodies according to the manufacturers instructions (eBioscience, San Diego, California, USA): CD45-FITC (11-5450-42), CD44-FITC (11-5440-42), CD4-FITC (11-5040-42), CD25-FITC (11-0250-42), CD146-FITC (14-1469-82), CD34-PE (12- 0340-42) and CD90-PE (12-5900-42), as well as the following isotype controls, Rat IgG2b K FITC (11-4031) Rat IgG2a kappa

FITC (11-4321) Mouse IgG1 K FITC (11-4714), Mouse IgG1 K FITC (11-4714) and Rat IgG2b kappa PE (12-4031). Antibody stained samples were analysed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). All samples were analysed and gated on side scatter (SSC) versus forward scatter (FSC) using the appropriate isotype control to determine the percentage of positive cells to each cell surface antigen.

6.2.2.1.2. Differentiation

Differentiation into osteogeneic and adipogeneic lineages were performed on the canine MSCs+secretions and MSCs only groups. MSCs of both groups were seeded at a density of 5 x 10^3 viable cells per cm^2 in 6-well plates (Greiner Bio-one, Frickenhausen, Germany) for adipogeneic and osteogeneic differentiation. Defined adipogeneic and osteogeneic differentiation media formulations were used as previously described in Table 2.3 and incubated at 37°C with 5% CO_2. The cells received media changes every 3 days. Upon completion of differentiation, cells were washed twice with PBS (Life Technologies, Grand Island, New York, USA) and incubated for 30 minutes with 4% paraformaldehyde (Sigma-Aldrich St Louis, Missouri, USA). For adipogeneic differentiation, the cells were subsequently washed with MilliQ water, incubated with 60% isopropanol (Sigma-Aldrich St Louis, Missouri, USA), stained with 0.2% Oil Red O (Sigma-Aldrich St Louis, Missouri, USA) solution for 5 minutes at room temperature and washed with tap water. For osteogeneic differentiation, the cells were stained with 2% Alizarin red solution (Sigma-Aldrich St Louis, Missouri, USA) for 2 minutes at room temperature and washed 3 times with MilliQ water. Control and differentiated cells were imaged using a Carl Zeiss Primo Vert inverted microscope (Carl Zeiss Pty Ltd, Oberkochen, Germany).

6.2.2.1.3. Proliferation Analysis

Cells of the four test groups; MSCs+secretions, MSCs+secretions washed, MSCs only and MSCs only washed, were seeded into 8 wells of two 12-well plates (Greiner Bio-one, Frickenhausen, Germany) at a density of 6 x 10^3 viable cells per cm^2. All wells were made up to 800µL with standard cell culture media and incubated at 37°C

with 5% CO_2. At 24 hours post seeding 1µL of a 10mM 5-ethynyl-2′-deoxyuridine (EdU; Life Technologies, Grand Island, New York, USA) solution was added to 8 of the 16 wells, the remaining 8 wells acted as the unstained controls for each of the four test groups. At 6 and 24 hours post addition of EdU, MSCs were liberated from the flask using TrypLE express (Life Technologies, Grand Island, New York, USA), and diluted in 1% BSA (Sigma-Aldrich St Louis, Missouri, USA) and PBS. Click-iT® EdU assay processes (Life Technologies, Grand Island, New York, USA) were followed as per manufacturers instructions described in Section 2.2.2.3. All samples were analysed on a FACScan flow cytometer and gated on side scatter (SSC) versus forward scatter (FSC). The control for each sample was used to determine the percentage of actively proliferating cells.

6.2.2.2. Cytokine Secretion Analysis

Cells of the four test groups; MSCs+secretions, MSCs+secretions washed, MSCs only and MSCs only washed, were seeded into 8 wells of two 12 well plates at a density of 4 x 10^3 viable cells per cm^2. All wells were made up to 800µL with standard cell culture media and incubated at 37°C with 5% CO_2. At 3, 6, 9 and 24 hours post-seeding the conditioned medium from the well of each of the four test groups was collected and centrifuged at 2000*xg* for 5 minutes and then stored at -80°C until needed for analysis. Conditioned medium and cryopreservation media samples from each test group were filtered through 0.2µm Nanosep MF Centrifugal Devices with Bio-Inert® Membrane (Pall Scientific, Port Washington, New York, USA) and centrifuged for 5 minutes at 9000*xg*. A 50µL portion of each filtered sample was analysed using the Milliplex MAP® Canine Cytokine 13-plex assay which measures IFN-γ, IL- 8, IL-15, IL-18, TNF-α, IL-10, IP-10, KC-like, MCP-1, GM-CSF, IL-7, IL-2 and IL-6 (Millipore, Billerica, Massachusetts, USA) according to the manufacturers instructions. The washing steps were performed on the Bio-Plex Pro II magnetic wash station and the data was acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, Hercules, California, USA). A volume of 50µL or 200µL of each filtered sample were analysed using TGF-β and VEGF ELISAs (R&D Systems, Minneapolis, Minnesota, USA) respectively according to the manufacturers instructions.

6.2.2.3. Dog Owner Data Collection

As part of an ethics approved study, preliminary data was collected from dog owners whose dogs were treated with the off-the-shelf MSCs+secretions product described in this chapter. This was a multi-centre study performed by veterinarians. Veterinarians were responsible for the diagnosis of OA, the intra-articular injections of the MSCs+secretions product and all follow-up care of the dogs. Treated dogs were administered one vial of the MSCs+secretions product per joint treated. Each vial contained 1mL of product. The number of joints treated per dog ranged from 1-4 joints. Joints treated were hips, elbows and stifles. This thesis reports only on the collation of the owner-reported data. The validated 'Canine Brief Pain Index' questionnaire [258] was provided to owners via an on-line survey. Owners were asked to complete the questionnaire at 10 days, 1, 2, 3 and 6 months post the intra-articular injection of their dog's joint/s. Data reported in this chapter is preliminary only and shows the initial data captured from what is an on-going post marketing surveillance exercise. There is limited data at all time-points as shown in Table 6.2.

Table 6.2. Number of owner completed Canine Brief Pain Index questionnaires at each time-point.

Time-point	Baseline	10 days post-treatment	1 month post-treatment	2 months post-treatment	3 months post-treatment	6 months post-treatment
Number of data points	52	40	28	25	25	15

The number of owner completed questionnaires for dogs treated with MSCs+secretions at each time-point.

6.2.2.4. Statistical Analysis

The percentage of positive cells for the flow cytometry immunophenotyping and proliferation assays is shown as an average ± standard deviation (SD). Cytokine secretion analysis is shown as the average ± standard error of the mean (SEM). A two-tailed t-test was used to test for statistical differences between the four groups. A p-value < 0.05 was considered statistically significant. Canine Brief Pain Index

questionnaire outcomes are shown as an average ± SD. The average was calculated from all dogs included at each time-point for each question (Table 6.2).

6.3. Results

The conditioned media produced from the culture expansion was concentrated and used as a cryopreservation additive. The secretion profile of the conditioned media used for cryopreservation was determined for each dog. Of the fifteen cytokines measured only nine were detected in the conditioned media (Table 6.3).

Table 6.3. Secretion profile of the conditioned media used for cryopreservation of the MSCs+secretions.

Donor	Cytokine								
	IL-2	**IL-6**	**IL-8**	**KC-like**	**IL-10**	**IL-18**	**MCP-1**	**TGF-β**	**VEGF**
Dog 1	26	21	3165	321	18	15	3021	153	904
Dog 2	29	21	2415	363	19	20	9098	104	661
Dog 3	30	19	2511	391	24	16	3590	80	811

Cytokine levels are reported in pg/mL.

For the three biological replicates used in this study, from both the MSCs+secretions test group and MSCs only test group, an initial total cell count and viability was performed utilising flow cytometry and nucleic acid dye staining (Table 6.4). An average total cell count and viability ± standard deviation (SD) of both test groups was also performed to determine if there were any differences between the MSCs+secretions and MSCs only. Whilst it was evident that there was variability between individual dogs, the average cell number and viability between the MSCs+secretions and the MSCs alone groups was comparable (Table 6.4).

Table 6.4. Cell count and viability (%) from three biological replicates of cryopreserved MSCs+secretions and MSCs only.

Donor	MSCs+secretions		MSCs only	
	Cell Count/vial	Viability	Cell Count/vial	Viability
1	1,642,740	79	1,501,665	88
2	2,907,190	69	2,099,405	80
3	2,633,400	74	2,503,820	63
Average	2,394,443	74	2,034,963	77
SD	665,232	5	504,176	13

Total cell count and viability percentage for the cryopreserved MSCs+secretions and MSCs only samples are shown. Average and SD of three biological replicates are reported for both groups.

6.3.1. Confirmation of Mesenchymal Stem Cells

To confirm the presence of MSCs, in accordance with the International Society of Cellular Therapies (ISCT) recommendation for the minimal criteria for defining human MSCs, cell surface antigen profiling and differentiation potential were performed.

6.3.1.1. Cell Surface Antigen Profiling

Consistent with the literature and confirmatory of MSCs, both the MSCs+secretions and MSCs groups were negative for white blood cell, hematopoietic and endothelial cell markers CD4 (Figure 6.1A and B), CD25 (Figure 6.1C and D), CD45 (Figure 6.1E and F), CD34 (Figure 6.1G and H) and CD146 (Figure 6.1I and J). Additionally, MSCs in both groups were positive in varying percentages for the MSC-like markers CD44 (Figure 6.1K and L) and CD90 (Figure 6.1M and N). The percentage of positive cells for each of the three biological replicates for CD44 and CD90 are shown in Table 6.5. On average 93.3 ± 2.9% and 92.3 ± 5.7% of cells in MSCs+secretions vials expressed the CD44 and CD90 cell surface antigen respectively compared to 96.7 ± 4.9% and 89.0 ± 5.6 of cells in the MSCs only group. No significant difference between expression levels of CD44 or CD90 between the two test groups was observed.

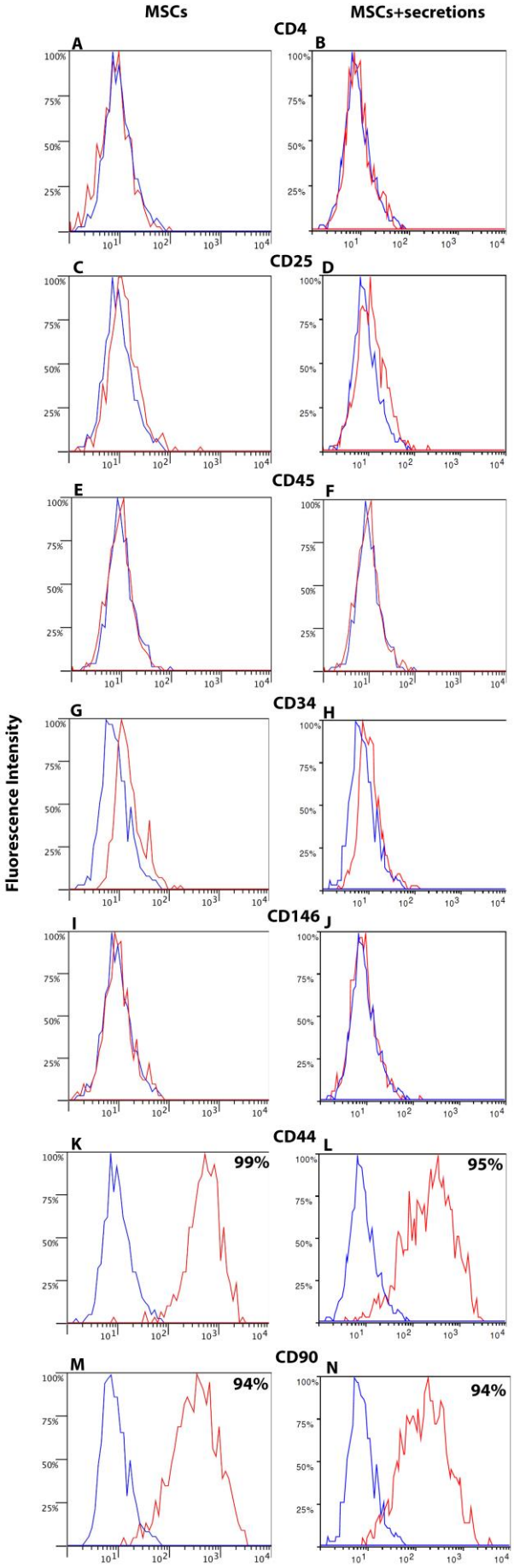
MSCs
MSCs+secretions
CD4
A
B
CD25
C
D
CD45
E
F
CD34
G
H
CD146
I
J
CD44
K
L
99%
95%
CD90
M
N
94%
94%
Fluorescence Intensity

Figure 6.1. Representative phenotypical characterisation of MSCs+secretions and MSCs only utilising CD markers and flow cytometry.

MSCs obtained from the two test groups, MSCs+secretions and MSCs only, were analysed on a FACScan flow cytometer. Characteristic white blood cell markers, CD4-FITC (A & B) CD25-FITC (C & D) and CD45-FITC (E & F) and characteristic hematopoietic and endothelial cell markers CD34-PE (G & H) and CD146-FITC (I & J) and MSC-like markers CD44-FITC (K & L), CD90-PE (M & N) were analysed. For each cell type and fluorescence conjugate, an unstained and isotype control were included. The percentage of positive cells expressing the cell surface antigen for CD44 and CD90 are shown in K-N.

Table 6.5. Percentage of canine MSCs positive for CD90 and CD44 cell surface antigen markers.

Donor	Percentage of positive cells			
	CD44		CD90	
	MSCs+secretions	MSCs only	MSCs+secretions	MSCs only
1	95	99	94	94
2	100	91	97	90
3	100	100	86	83
Average ± SD	93.3 ± 2.9	96.7 ± 4.9	92.3 ± 5.7	89.0 ± 5.6

MSCs obtained from the two test groups, MSCs+secretions and MSCs only were analysed for CD44 and CD90 using a FACScan flow cytometer. The percentage of positive cells is expressed as the average ± SD.

6.3.1.2. Differentiation Potential

To further confirm the presence of MSCs, adipogeneic and osteogeneic differentiation assays were performed. Control MSCs in basal media were negative for calcium deposits following Alizarin red staining (Figure 6.2A). In contrast MSCs from both test groups, MSCs only and MSCs+secretions (Figure 6.2B and C respectively), showed similar staining intensity for calcium accumulation. Similarly, control MSCs were negative for lipid accumulation following Oil Red O staining (Figure 6.2D) whereas differentiated MSCs from both test groups (Figure 6.2E and F), had equivalent lipid accrual.

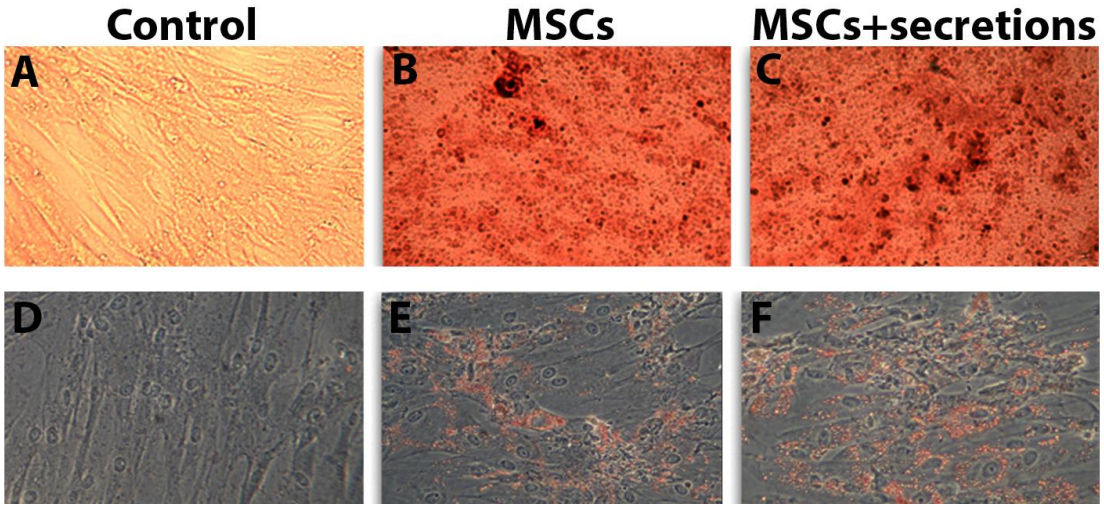

Figure 6.2. Osteogeneic and adipogeneic differentiation of MSCs.

Cells of the test groups, MSCs+secretions and MSCs only, were cultured in standard media for 3 days prior to osteogeneic and adipogeneic differentiation using standard media formulations. Control MSCs were negative for calcium deposits (A) and lipid accumulation (D). MSCs from both test groups exhibited similar osteogeneic (B & C) and adipogeneic (E & F) differentiation potential.

6.3.2. Recovery and Functionality of MSC Populations' post-cryopreservation

Further to confirming the presence of MSCs by plastic adherence, differentiation potential and cell surface antigen expression, an investigation of the secretion profile and proliferation ability of the MSCs was performed. Additionally, a comparison of the proliferation capability and cytokine secretion profile of the two test groups, MSCs+secretions and MSCs was completed. It was hypothesised that the presence of the cellular secretions would have an impact on MSC proliferation and secretion of cytokines. To investigate this the MSCs were seeded in culture immediately after thawing and also after removal of the cryopreservation medium by washing.

6.3.2.1. Proliferation

To ensure that culture-expanded, cryopreserved and stored MSCs were still capable of proliferation and to investigate if the addition of cellular secretions altered the proliferation capabilities of MSCs, a Click-iT® EdU flow cytometry assay was utilised. The percentage of actively proliferating cells was determined at 6 and 24 hours post addition of EdU to MSCs. Figure 6.3 shows representative images of

proliferation activity of MSCs from both test groups before and after removal of the cryopreservation medium at 6 and 24 hours. This example demonstrates that a proportion of MSCs at both time-points were actively proliferating. Furthermore, Figure 6.3E-H show that by 24 hours post addition of EdU, MSCs were undergoing further cell doubling, as shown by the two distinct positive populations and increased fluorescence compared to Figure 6.3A-D. Although this example shows that the MSCs from all test groups were actively proliferating at both 6 and 24 hours post addition of EdU, this was not the case for dog two in this study. The percentage of actively proliferating MSCs from each of the three biological replicates is compiled in Table 6.6. This table shows that there were substantial differences in the proportion of actively proliferating MSCs between each of the three biological replicates. In particular, Table 6.6 shows that the MSCs from donor two in all four test arms were not proliferating at 6 hours post addition of EdU. The variability observed coupled with the small sample size meant that no statistically significant difference could be determined between the two test groups. For biological replicates one and two, no apparent difference in proliferation activity between the four test groups, MSCs+secretions, MSCs only, MSCs+secretions washed and MSCs washed was shown at either time-point (Table 6.6). However, for dog three it may be that the presence of the secretions had an effect on the proliferation of the MSCs even after the removal of cryopreservation medium by washing at both the 6 and 24 hour time-points.

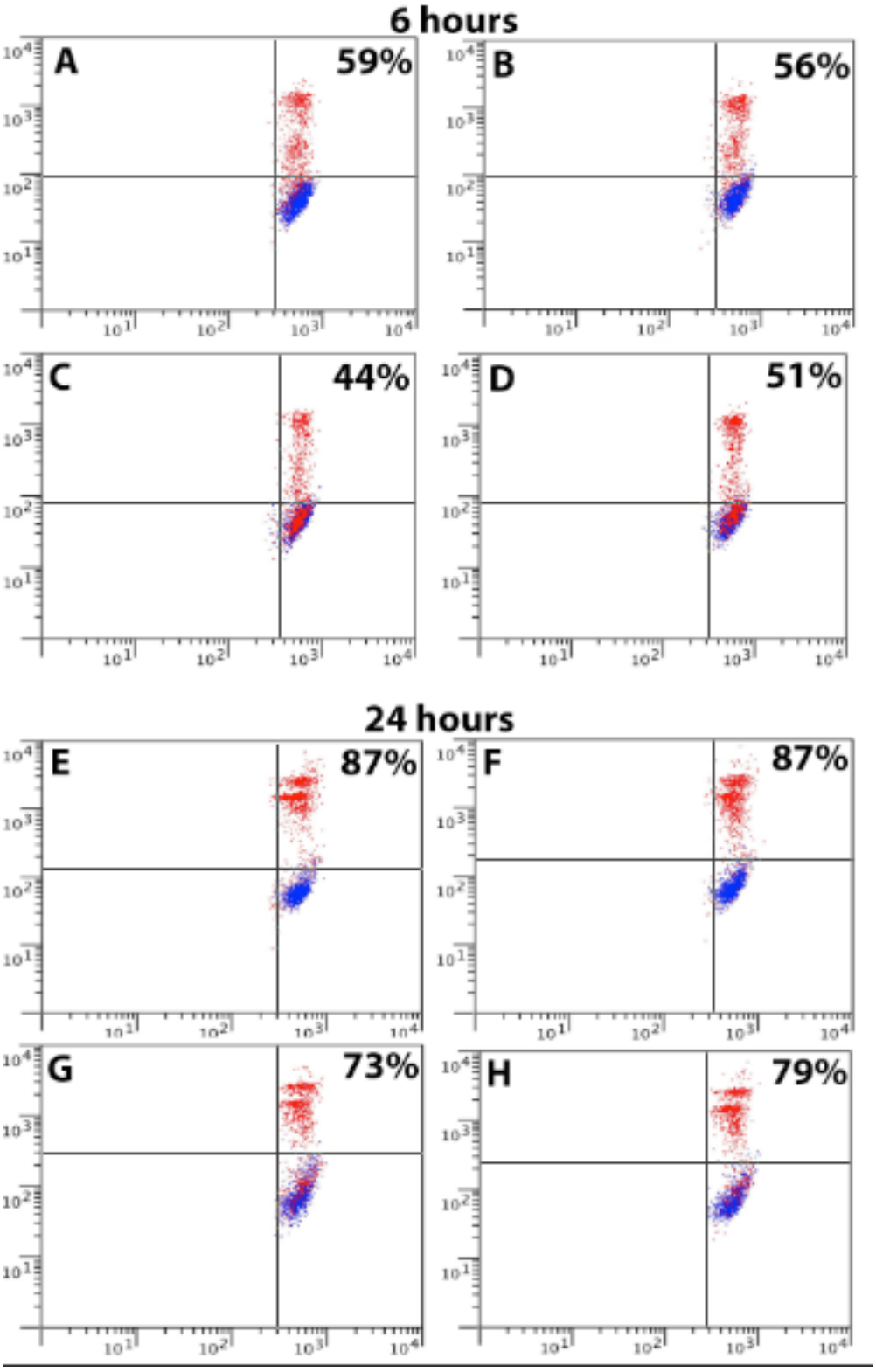

Figure 6.3. Representative scatter plot of actively proliferating MSCs.

MSCs of the four test groups MSCs+secretions, MSCs+secretions washed, MSCs only and MSCs only washed were cultured in standard media for 24 hours prior to administration of EdU. Following addition of EdU, cells remained in culture for a further 6 hours (A-D) and 24 hours (E-H) prior to flow cytometry analyses. The percentage of actively proliferating MSCs in the MSCs+secretions group (A & E), MSCs only group (B & F), MSCs+secretions washed (C & G) and MSCs only washed group (D and H) are shown in red. MSCs of each of the four test groups that were not exposed to EdU are shown in blue.

Table 6.6. Percentage of canine MSCs actively proliferating at 6 hours and 24 hours post addition of EdU.

	6 hours post-treatment with EDU				24 hours post-treatment with EDU			
Dog	**MSCs+secretions**	**MSCs**	**MSCs+secretions post-thaw wash**	**MSCs post-thaw wash**	**MSCs+ secretions**	**MSCs**	**MSCs+secretions post-thaw wash**	**MSCs post-thaw wash**
1	59	56	44	51	87	87	73	79
2	0	0	0	0	17	15	21	18
3	10	4	9	4	30	6	35	10
Average ± SD	23.0 ± 31.6	20.0 ± 31.2	17.7 ± 23.2	18.3 ± 28.4	44.7 ± 37.2	36.0 ± 44.4	43.0 ± 26.9	35.7 ± 37.7

MSCs from the MSCs+secretions, MSCs only, MSCs+secretions washed and MSCs only washed groups were analysed for actively proliferating cells using a FACScan flow cytometer. The percentage of actively proliferating cells are expressed as the average ± the SD.

6.3.2.1. Secretion of Cytokines

To investigate the secretion profile of passaged and cryopreserved MSCs in a medium containing secretions, versus standard cryopreservation medium, MSCs were seeded immediately post-thaw into 6-well plates. Additionally, to eliminate the possibility of the secretions directly contributing to the secretion profile, the cryopreservation medium was also removed from the two test arms of all three donors prior to seeding. Conditioned media was collected from the four groups, MSCs+secretions, MSCs+secretions washed, MSCs only and MSCs only washed, at 3, 6, 9 and 24 hours post seeding. These conditioned media samples were analysed utilising a panel of thirteen canine cytokines that were measured using a Milliplex assay. A further two cytokines (VEGF and TGF-β) were measured using ELISAs. Figure 6.4 contains the results of the five cytokines that were detected in the conditioned media of the four test groups at each time-point. Additionally, the level of each cytokine measured in the two different cryopreservation mediums are included as a baseline reading. For all cytokines, the level measured in the cryopreservation media was negligible in comparison to the amount secreted by the cells once in culture (Figure 6.4A-E; 0 hours). There were no significant differences between detected levels of IL-8 (Figure 6.4A), KC-like (Figure 6.4B) and TGF-β (Figure 6.4D) in each of the four test groups at the time-points analysed. However, the MSCs+secretions test group did secrete significantly more MCP-1 (Figure 6.4C) at 3, 6 and 9 hours in comparison to the MSCs+secretions washed and MSCs only groups (p-values <0.05). By 24 hours in culture however, the increased expression of MCP-1 in the MSCs+secretions was no longer significantly different to the other 3 test groups. Whilst it appears in Figure 6.4E that VEGF levels were significantly higher at all time-points in the MSCs+secretions test group, there was substantial variation in the amount of VEGF secreted between the 3 biological replicates and therefore the results are not statistically significant. Biological variation was observed at all time-points assessed. To demonstrate the level of variation observed in the cytokines measured in the conditioned medium of groups from each donor, the cytokine concentrations at 24 hours are reported in Table 6.7. In this table, the most similar result observed between two donors within a group was MCP-1 in the

MSCs+secretions samples from dog one (2648 pg/mL) and dog two (3044 pg/mL) representing a 1.1-fold difference. The greatest variation was observed in IL-8 in the MSCs group with a 9.7-fold difference between dog one (713 pg/mL) and dog three (6937 pg/mL) at 24 hours.

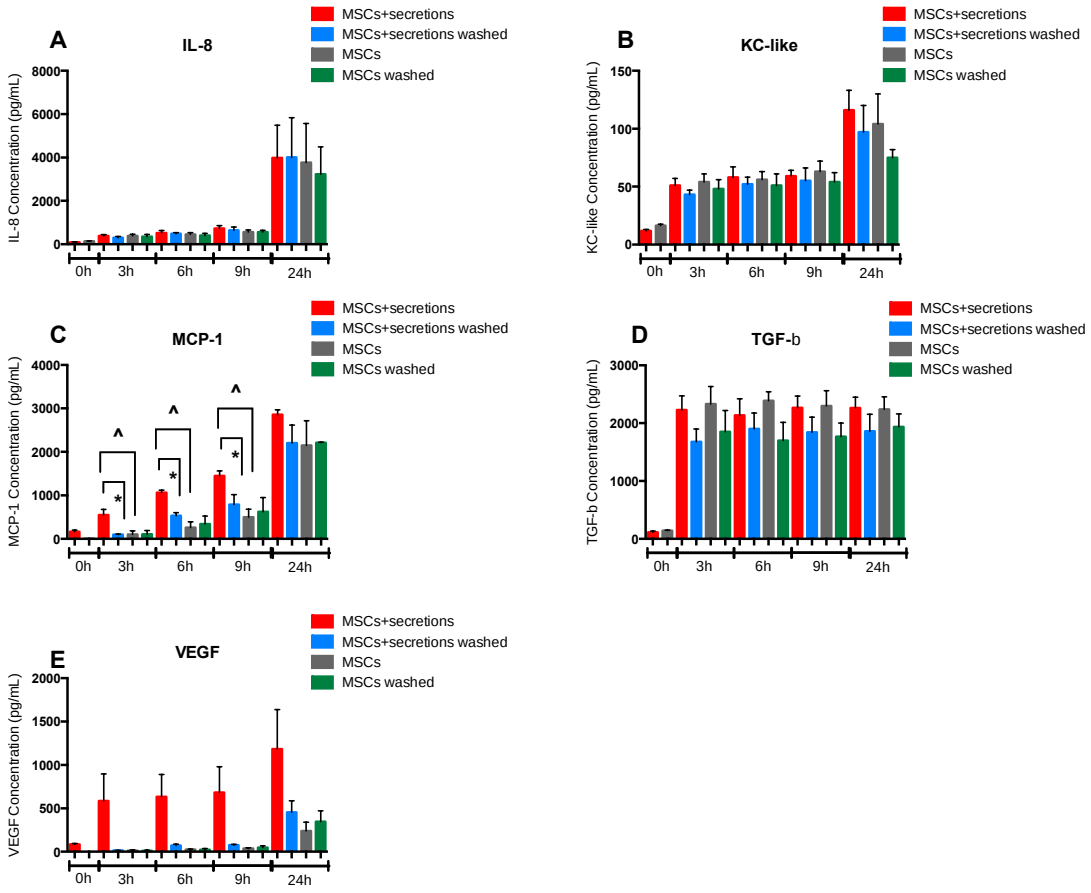

Figure 6.4. Cytokine secretion profiling from MSC conditioned medium.

MSCs of the 4 test groups, MSCs+secretions, MSCs+secretions washed, MSCs only and MSCs only washed were cultured in standard media for 3, 6, 9 and 24 hours. At these time-points the conditioned medium was collected and analysed for a total of 15 cytokines. Additionally samples of the cryopreservation medium were analysed to provide baseline cytokine levels. The cytokines IL-8 (A), KC-like (B), MCP-1 (C), TGF-β (D) and VEGF (E) were detected. The results are presented as an average ± SEM (n=3). Two-tailed t-tests were performed, an * denotes a p-value of <0.05 when MSCs+secretions are compared to MSCs+secretions washed and an ^ denotes a p-value of <0.05 when MSCs+secretions are compared to the MSCs only group.

Table 6.7. Cytokine levels in conditioned medium from MSCs of each donor at 24 hours.

Cytokine	Time-point	Sample	Dog 1	Dog 2	Dog 3	Average ± SD
IL-8	24 hours	MSCs+secretions	1326	6561	4054	3980 ± 2618
		MSCs	713	3661	6937	3770 ± 3114
		MSCs+secretions Washed	942	7257	3830	4010 ± 3161
		MSCs Washed	904	3553	5234	3230 ± 2183
KC-like	24 hours	MSCs+secretions	81	136	131	116 ± 30
		MSCs	65	92	153	104 ± 45
		MSCs+secretions Washed	61	139	92	97 ± 40
		MSCs Washed	61	82	82	75 ± 12
MCP-1	24 hours	MSCs+secretions	2648	3044	2869	2854 ± 198
		MSCs	1083	2341	3021	2148 ± 984
		MSCs+secretions Washed	1794	3028	1780	2200 ± 716
		MSCs Washed	2235	2203	2196	2211 ± 20
TGF-β	24 hours	MSCs+secretions	2606	2220	1961	2262 ± 325
		MSCs	2469	2445	1797	2237 ± 381
		MSCs+secretions Washed	2320	1945	1322	1862 ± 504
		MSCs Washed	2348	1881	1573	1934 ± 390
VEGF	24 hours	MSCs+secretions	938	2067	545	1183 ± 790
		MSCs	118	442	155	238 ± 178
		MSCs+secretions Washed	356	718	288	454 ± 231
		MSCs Washed	591	272	172	345 ± 219

Cytokine data are presented as pg/mL.

6.3.3. Real World Clinical Data Assessment

Data from a cohort of dogs with clinically diagnosed OA was collected, as part of an animal ethics approved study, from all dogs that received an intra-articular injection of MSCs+secretions, the novel off-the-shelf cellular therapeutic described in this chapter. The cells used to produce the MSCs+secretions prototype product were from both dogs one and three.

Figure 6.5 shows the results of the validated 'Canine Brief Pain Index' questionnaire as reported by dog owners at baseline, 10 days, 1, 2, 3 and 6 months

post intra-articular injection. From these results it is clear, that on average, dog owners reported a rapid and sustained decrease in pain associated with normal daily activities (Figure 6.5, questions 1-10) and an increase in improvement in the dogs' overall quality of life (Figure 6.5, question 11). Figure 6.5 also shows that the overall questionnaire-combined average substantially improved from a baseline score of 5.0 $\pm$ 0.15 to a score of 0.57 $\pm$ 0.22 at 6 months post-treatment.

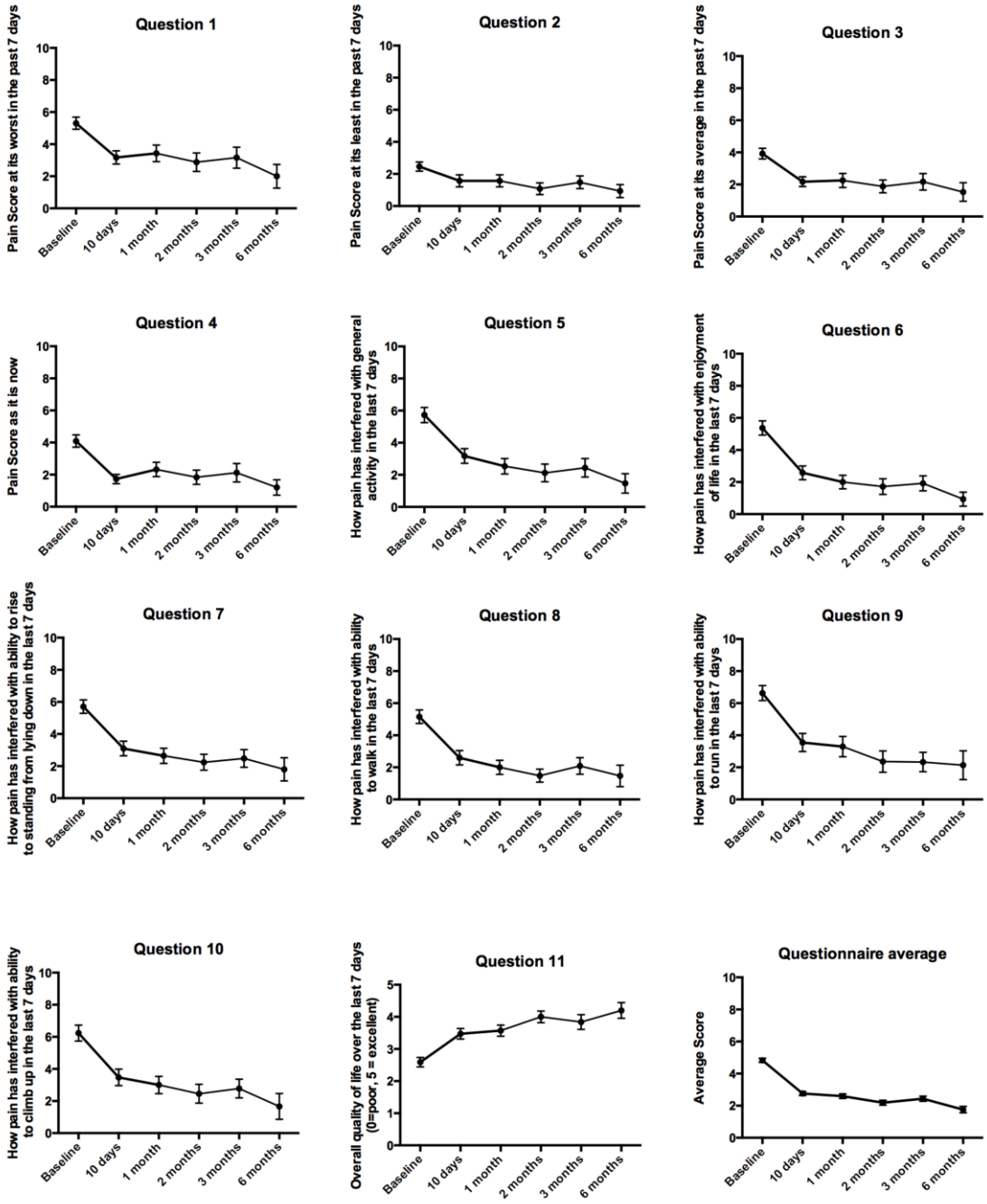

Figure 6.5. Canine Brief Pain Index owner reported outcomes from dogs treated with MSCs+secretions.

Dogs with clinically diagnosed OA received a single intra-articular injection of the novel off-the-shelf MSCs+secretions product described in this chapter. As part of an animal ethics approved study, owner reported outcomes were collected, at baseline prior to injection, 10 days, 1, 2, 3 and 6 months post-treatment.

6.4. Discussion

6.4.1. Confirmation of the Presence of MSCs and Comparison to SVF

To confirm the presence of MSCs, differentiation potential and cell surface antigen profiling was performed on both of the test groups, MSCs+secretions and MSCs only. In accordance with the International Society for Cellular Therapy position statement [39], adipogeneic and osteogeneic differentiation and CD marker expression confirmed the cells to be MSCs. As expected, no differences were seen between the two test arms. Unlike the CD marker profile of the heterogeneous SVF (Chapter 4), the profiles of both test groups in this study were negative for all CD markers except the MSC-like markers CD44 and CD90. Table 6.8 shows the difference between the cell-surface antigen profiles of the SVF characterised in Chapter 4 and the MSCs described in this chapter.

Table 6.8. Percentage of canine SVF cells and MSCs positive for CD markers characteristic of white blood cells, endothelial cells and MSC-like cells.

CD marker	Characteristic cell type/s (Antibody specificity)	MSCs+secretions	MSCs	SVF
CD45+	Found on all leukocytes.	0	0	26 ± 6
CD44+	Cell adhesion molecule, MSC and stromal cell marker	93 ± 3	97 ± 5	20 ± 6
CD4+	T helper cells, monocytes, macrophages and dendritic cells	0	0	8 ± 8
CD25+	Immune cells of T & B cell lineages	0	0	17 ± 6
CD146+	Cells of the endothelial cell lineage	0	0	33 ± 11
CD90+	MSC and stromal cell marker	92 ± 6	89 ± 5	25 ± 7
CD34+	Marker of hematopoietic progenitor and stem cells	0	0	16 ± 11

The percentage of positive cells is expressed as the average ± SD. MSCs+secretions and MSCs only n=3, SVF n=8.

Of the fifteen cytokines measured, only nine were detected in the conditioned media used for cryopreservation in the MSCs+secretions group. Furthermore, only five of the nine cytokines were detected in the conditioned medium of the MSCs of both groups after cryopreservation. The cytokines IL-2, IL-6, IL-10 and IL-18, were no longer detected in the MSCs+secretions conditioned media post-thaw. In all likelihood this was due to these cytokines originally being detected at very low pg/mL ranges (Table 6.3), which were then further diluted with culture media, resulting in the expression of these cytokines decreasing below the detection limit of the Milliplex assay. Furthermore, at three hours post-seeding of MSCs, which was the earliest secretion collection time-point, it could have been that bioactive factors had been consumed by the MSCs. Still, it is important to note that detection of these four cytokines was lost post-thaw as it could be hypothesised that MSCs lose the ability to secrete these cytokines due to the cryopreservation process.

As with the cell surface antigen profile of the SVF and the MSCs, there were differences seen in the secretion profile of the MSCs compared to the SVF. Although the experimental conditions in this chapter differed from those described in Chapter 4, it appeared that with the loss of the heterogeneity of the SVF, the expression of many cytokines decreased below the detection limit of the Milliplex assay. The pro-inflammatory cytokines IFN-γ, IL-15, IL-18 and TNF-α, the dual role cytokines IL-2 and IL-6, growth factors GM-CSF and IL-7, chemokine IP-10 and the anti-inflammatory cytokine IL-10, which were all detected in the conditioned medium of the SVF were not detected in the conditioned medium of the culture expanded cryopreserved MSCs. Interestingly, as mentioned above IL-2, IL-6, IL-10 and IL-18 were detected in the conditioned media used for cryopreservation in the MSCs+secretions group prior to cryopreservation but not post-thaw. Variability between the secretion profiles obtained from the SVF, which contains a mixed cell population, and isolated MSCs has previously been described [242].

6.4.2. The Addition of Cellular Secretions to MSCs did not Generally Significantly Affect Post-thaw Recovery and Functionality

Post-thaw cell counts and viability revealed that the cryopreservation process had limited impact on total cell count and viability. Furthermore, the cell count and

viability assays revealed that the addition of secretions did not generally enhance the post-thaw recovery of the MSCs in this aspect. The total cell count and percentage of viable cells was comparable between the two test groups, even with large variation between individual dogs.

To determine whether the addition of cellular secretions had an enhanced effect on the secretion profile and proliferation capability of MSCs, a washing step for each test group was essential. Therefore, for both test groups, half of each sample was centrifuged to remove the cryopreservation medium. This was done in an attempt to determine if the addition of the cellular secretions had a direct effect on proliferation capability and the secretion profile, or if the addition of cellular secretions actually altered the functionality of the MSCs themselves. The results showed that for dogs one and two the proliferation capacity of the MSCs following thawing was not enhanced by the addition of secretions. In the case of dog three the proliferation was enhanced by the addition of secretions. The percentage of actively proliferating cells between the MSCs+secretions and MSCs+secretions washed test groups from dog three were shown to have 30 and 35 % respectively, actively proliferating cells by 24 hours post addition of EdU (Table 6.6). These levels were considerably greater than the 6 and 10% of actively proliferating cells seen in the MSCs alone and MSCs alone washed test groups respectively (Table 6.6). This result suggests that the addition of secretions, in this example, improved the proliferation proficiency of the cryopreserved MSCs+secretions group despite the removal of the secretions, indicating the secretions improved the post-thaw functionality and recovery of this particular dog's MSCs.

When investigating the secretion profile of the test groups, of the five cytokines detected in the conditioned media, no significant differences were seen between the test groups in KC-like, VEGF and TGF-β, and IL-8 (Figure 6.4). However cryopreservation in medium containing secretions resulted in significantly increased levels of the chemokine MCP-1 at 3, 6 and 9 hours after seeding in comparison to MSCs alone (Figure 6.4). Additionally, significantly greater concentrations of MCP-1 were detected in the conditioned medium of the MSCs+secretions test group in comparison to its washed counterpart. The level of MCP-1 present in the secretions was negligible compared to the amount secreted by the cells in the MSCs+secretions test group. These results indicate that the level of MCP-1 measured was not due to the

MCP-1 in the added secretions, but instead, the addition of secretions had an effect on the MSCs themselves, resulting in a significant increase in its production.

MCP-1 plays a role in the recruitment of monocytes to sites of injury. It is prevalent in serum and synovial fluid in inflammatory pathologies such as rheumatoid arthritis (RA). In a clinical study investigating the levels of MCP-1 in both the serum and synovial fluid of RA and OA patients, a significantly greater amount of MCP-1 was detected in RA patients [339]. However, in an induced OA model in mice, MCP-1 was shown to be central to the pain associated with OA [340].

Despite the *in vitro* finding of increased levels of MCP-1 in the MSCs+secretions group, the administration of canine MSCs+secretions to the CAIA affected mice (Chapter 5), resulted in a significant reduction in host MCP-1 serum levels when compared to the MSCs only test group. It is clear that *in vitro* culture conditions do not always reflect the *in vivo* environment and therefore it is likely that the cells and the presence of the secretions will respond differently when introduced to a host with an inflammatory condition. It is possible that the significant increase in MCP-1 production in the MSCs+secretions test group may occur *in vivo* creating a negative feedback loop which may be partially responsible for the suppressed MCP-1 production in the CAIA host.

6.4.3. Culture Expanded MSCs are Subject to Individual Variation: Implications for Product Development.

Variation between individuals was clearly seen in the proliferation ability and secretion profile of the culture expanded MSCs. The biological variation between the three donors was most clearly observed when assessing the proliferation ability of the MSCs. Biological replicates one and three were shown to be actively proliferating at 6 hours post addition of EdU. However, for donor two, no proliferation was seen until 24 hours post addition of EdU (Table 6.6). Interestingly, donor two was the sample with the greatest level of MCP-1 expression in the conditioned media used for the cryopreservation of the MSCs+secretions group, which was almost three times greater than the MCP-1 detected in the conditioned media of donors one and three (Table 6.3). Previously conditioned media displaying significantly elevated levels of MCP-1, VEGF, MIP-1α, MIP- 1β and MIG compared to control media, was shown to

induce angiogenesis in canine vascular endothelial cells. MCP-1 and MIP-1α increased cell migration. Additionally, MCP-1 alone was shown to have a cell protective effect by reducing caspase-3 activity in H9c2 cells [341]. Donor two also exhibited the greatest level of VEGF secretion post-thaw (Table 6.7). In the MSCs+secretions test group this donor secreted between 2 and 6 times more VEGF than donors one and three respectively at all time-points. VEGF is recognised to influence MSC survival, engraftment and proliferation capability [342]. In a study investigating the role of VEGF in cell replacement after brain injury, VEGF was shown to be responsible for the proliferation of neuronal precursor cells in cultured mouse cerebral cortex cells [343]. Not only was this large variation between individual donors seen in VEGF but also in measured levels of other cytokines such as, KC-like and IL-8 as demonstrated in Table 6.7.

The three biological replicates used in this study were MSCs and secretions derived from the adipose tissue of three individual dogs. All three dogs were approximately 6 month old female guide dog puppies and all adipose tissue was derived from the falciform ligament during a routine desex procedure. All adipose tissue and cells derived from the tissue were processed in exactly the same way in terms of tissue digestion, culture expansion and cryopreservation procedures. Furthermore, for all *in vitro* work performed in this chapter, all experiments were standardised by viable cell number. Despite this, the results of this chapter clearly demonstrate that there was a large degree of biological variation between the three dog samples in proliferation capacity and levels of secreted cytokines.

The level of biological variation shown in the functional assay outcomes from culture expanded MSCs of three donor dogs was not expected. In comparison to the SVF, culture expanded MSCs are a relatively homogeneous population of cells as evident by the CD marker expression profile. Therefore, the proliferation capacity and secretion profile of the MSCs processed in the same manner from dogs of similar characteristics were anticipated to be comparable. An analysis technology capable of genome-wide coverage, such as transcriptomics or quantitative proteomics, may delineate differences between the cells derived from individual donors. Production of a large-scale off-the-shelf allogeneic cell therapy is reliant on a cell line that is clinically useful to warrant the time and expense required in the manufacturing. Therefore, assessment of *in vitro* functionality as a precursor to clinical efficacy is

essential. Furthermore, with the development of new assays, identification of critical functional parameters may aid in determining a clinically ideal cell profile.

6.4.4. Defining a Criteria for Selection of Donors for Production of Culture Expanded Cell Therapies

The data presented in this chapter demonstrated that despite the adipose tissue samples being processed in the same manner and the experiments performed on normalised cell numbers, substantial biological variation between the three donors results was observed. To produce a commercially viable off-the-shelf therapeutic, a single fat donation must be capable of providing thousands of therapeutic doses of MSCs. As such, minimum *in vitro* assay specifications need to be defined for donor cells to meet on a test scale prior to large-scale production. Defining a set of minimum criteria and ensuring donor cells meet these criteria is essential given the effort and expense involved in producing an allogeneic off-the-shelf MSC therapeutics on a large-scale. In this study, dog two had very limited proliferation and the secretion profile was skewed towards MCP-1 in the cryopreservation media and towards IL-8 and VEGF after cryopreservation. In contrast, cells from donors one and three had a proliferation level of > 30% and did not have a skewed secretion profile. The results reported in this chapter from the veterinary trial were MSCs+secretions prototypes produced from the cells of donors one and three. A reduction in pain and improvement in function was observed in the dogs treated with these MSCs+secretions prototypes. Defining minimum proliferation and cytokine profile specifications for MSCs could be an initial set of criteria that could be useful in screening donor cells prior to large scale manufacture. Over time in a production facility it is anticipated that the criteria would be reviewed regularly and altered according to the results from *in vitro* and *in vivo* datasets.

6.4.5. Therapeutic Importance

There are many examples in the literature describing that MSCs exert their therapeutic effect primarily through their paracrine activities. MSCs have been demonstrated to secrete a wide array of bioactive factors that are angiogenic, mitotic,

anti-apoptotic, anti-scarring and immune-modulatory [26]. The importance of MSC paracrine activities has been further confirmed in animal models using MSC conditioned media only, which have demonstrated similar therapeutic effect to that of MSCs [304, 305]. However, the therapeutic effect is not long lasting which is likely due to the transient nature of secretions. In a corneal wound injury model it was demonstrated that three applications of conditioned medium from MSCs achieved an effect similar to that of MSCs themselves but had a superior therapeutic outcome to a single administration of MSC secretions [68].

Given the established importance of secretions in MSC therapeutic activity, it was anticipated that incorporating secretions in the cryopreservant would not only aid post-thaw recovery and functionality of the MSCs, but also enhance the therapeutic capabilities of the cells. Whilst there were no negative effects observed in the *in vitro* experiments, there was no uniform improvement in post-thaw recovery and functionality with the addition of secretions to MSCs. Despite this, there were some scenarios where within a biological replicate, a more favourable result was observed in the MSCs+secretions group over the MSCs only group. There were no situations in which the MSCs alone group appeared to exhibit an improved post-thaw recovery or functionality in comparison to the MSCs+secretions test group. To determine the effect of the presence of the secretions on the MSCs in the cryopreservant, a panel of fifteen cytokines was utilised. Of these fifteen cytokines only MCP-1 secretion was significantly higher in the MSCs+secretions test group, compared to all other groups analysed. It is possible that the presence of the secretions could significantly alter the expression of a number of other cytokines, or other signaling molecules, but currently there are a limited number of assays available for measuring canine cytokines. With the development of further assays for detecting cytokines, such as PDGF, HGF and IL-1Ra, it may be possible to further investigate the effect of cellular secretions on MSCs.

A clinically positive outcome was demonstrated in the CAIA affected mice (Chapter 5) and in the experiments described in this chapter no adverse changes were shown in work up of MSCs+secretions. Consequently, the industry partner in conjunction with partnering veterinarians, collected owner reported outcomes regarding the efficacy of the MSCs+secretions therapeutic in treated dogs. Veterinarians administered a single intra-articular injection of this cellular product to

client-owned dogs with clinically diagnosed OA in an on-going multi-centre study. The MSCs+secretions product was delivered to the veterinary practices in cryopreserved vials that remained in liquid nitrogen storage until just prior to use. In this chapter the initial owner reported data was collated and analysed. The owner reported outcomes shown in Figure 6.5 demonstrate that intra-articular injection of the MSCs+secretions had a clear therapeutic effect with a significant improvement in pain and function. Furthermore, this therapeutic effect was similar to that reported in Chapter 3 following intra-articular injection of the autologous adipose-derived SVF cells (Figure 6.6). It must be noted however that this was not a placebo-controlled study and although validated, the owner-completed questionnaire is a subjective tool only, and a placebo effect has been demonstrated previously when using owner reported questionnaires [270]. Furthermore, clinical outcomes were based only on owner questionnaire results without the addition of veterinary examination outcomes as described in Chapter 3. Additionally, there are limited data points comprising the 6 month follow-up time-point (Figure 6.6B).

Whilst there are distinct differences between the SVF and MSCs+secretions treatment offerings, in terms of cell surface antigen and secretion profiles, both treatments have resulted in encouraging clinical improvements. Preliminary data from both of these cell therapies have demonstrated pain relief and an improved quality of life in dogs suffering from real clinical OA (Figure 6.6A and B).

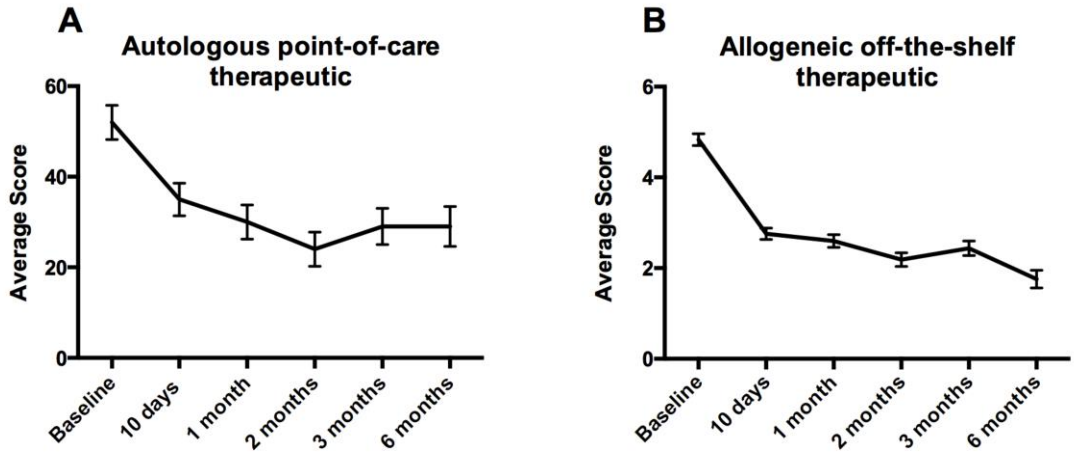

Figure 6.6. Owner reported outcomes from dogs treated with autologous adipose-derived SVF and allogeneic off-the-shelf MSCs+secretions.

Dogs with clinically diagnosed OA received a single intra-articular injection of autologous adipose-derived SVF described in Chapter 3 (A) or the allogeneic off-the-shelf MSCs+secretions product described in this chapter (B). As part of an animal ethics approved studies, owner reported outcomes were collected, at baseline prior to injection, 10 days, 1, 2, 3 and 6 months post-treatment.

6.5. Conclusion

The results presented in this chapter showed that the addition of cellular secretions to MSCs generally had no clear benefit in the post-thaw recovery and functionality of cryopreserved MSCs. However, no negative effects of incorporating the secretions in the cryopreservant were observed. The results demonstrated that significant variability in functional measures of MSCs existed between the three donors. Production of large-scale off-the-shelf therapeutics requires identification of critical functional parameters that may aid in determining a clinically ideal cell profile to warrant the time and cost involved in manufacturing a therapeutic. Furthermore administration of MSCs+secretions resulted in a reduction in pain and an improved quality of life in dogs treated with this novel off-the-shelf allogeneic therapeutic.

6.6. Acknowledgements

I would like to acknowledge that the Veterinarians of Greencross Redbank Plains, Greencross Stud Park, Greencross Jindalee, Greencross Woolloongabba and 4 Paws Vets Neutral Bay performed the diagnosis of OA, intra-articular injections and veterinary consultations and examinations for all dogs treated with the MSCs+secretions product described in this study. I would also like to thank the dog owners for completing the questionnaire described in tis study.

CHAPTER SEVEN

General Discussion

This chapter discusses the findings of this doctoral thesis in the wider context of the regenerative medicine field and proposes further work that warrants pursuing.

7.1. Cellular Therapy: A Paradigm Shift in Medicine

There is a growing need for new ways to repair, replace or regenerate damaged or failing cells, tissues or organs of the body. Significant advances in the medical field over hundreds of years have enabled people to live much longer than would otherwise have been possible. These advances are numerous and examples include the discovery and continued development of antibiotics for the prevention of infection-related deaths, cholesterol and blood pressure lowering pharmaceuticals, the use of stents and xenogeneic valves for heart conditions, and organ transplantation. Furthermore, there have been significant advances in diagnostic technologies, which identify predisposition and early disease onset as well as monitoring disease progression. These advances have improved the management of many conditions. As such, for decades pharmaceutical or surgical interventions have been a mainstay in healthcare offerings. However, with an increase in human life expectancy in almost every country in the world, we are currently faced with an epidemic of age related degenerative diseases where conventional care lacks effective treatments or cures. Failing tissues and organs in conditions such as diabetes, Alzheimer's, OA and macular degeneration are prevalent in an aging population, all of which incur a massive worldwide financial burden and result in decreased quality of life for sufferers.

Cellular medicine is the next step in the evolution of medicine, not only for age related diseases, but also for countless previously untreatable malignant and non-malignant conditions that affect millions of people of all ages worldwide. It is likely that cellular therapeutics will not only provide therapeutic treatments where currently there are none, but will also be utilised to augment current therapeutic regimes. For example cellular treatments will be utilised in conjunction with conventional pharmaceutical and surgical treatment interventions to assist in areas such as wound healing, tissue regeneration and immunosuppression.

The most well understood form of cellular medicine is the use of HSCs for blood cancers, inherited blood disorders and cellular replacement in chemotherapy patients. HSC transplants have been successfully performed since the 1960's. HSCs are, however, limited in regenerative capabilities beyond their role in blood pathologies. HSCs are unable to proliferate and differentiate under *in vitro* conditions

and are not immune-privileged limiting allogeneic use to matched donors only. As such, the scientific and medical communities have looked to other cell types for the use in degenerative chronic diseases as well as acute conditions and injuries.

When the first human ESC lines were established in 1998, the possibility of repairing tissues lost to trauma or disease became a reality. As such, numerous research groups in the worldwide scientific community commenced research into the therapeutic potential of these cells. Although promising, the utilisation of ESCs in regenerative medicine has not become a reality due to ethical, immunological and teratoma formation issues associated with these cells.

Nearly a decade later the discovery of reprogramming a somatic multipotent cell to act as an ESC using a set of four genes only [15], has opened the door to the promise of regenerating or replacing tissues and whole organs once again. The use of iPSCs, although not hampered with any of the ethical concerns of ESCs, is still burdened with some of the roadblocks associated with ESCs. Inconsistencies and inefficiencies in the reprogramming of cells have been reported, as have concerns around the genetic stability and the tendency toward tumor induction of iPS cells. As such, almost monthly, new methods to induce pluripotency while increasing efficiency and minimising tumor propensity are reported. In 2013 the first human clinical trial using iPSCs was approved in Japan [344]. This trial, for the treatment of macular degeneration, is associated with some safety concerns around tumor induction and immunological rejection. However, the low quantity of cells required to treat this condition is believed to minimise this risk sufficiently to warrant the study [344]. Although rapid advances are being made to realise the potential of iPSCs, it is likely some time away before iPSCs become a mainstream therapeutic reality.

MSCs, however, can safely fill the void between the limitations of HSCs, the currently un-exploitable potential of ESCs, and the yet to be fulfilled promise of iPSCs. There is a wealth of literature published on the ability of MSCs to proliferate and differentiate *in vitro*. The immune-privileged status of MSCs through their lack of MHC class II and co-stimulatory (CD40, CD80 and CD86) molecule expression has enabled widespread therapeutic use of MSCs particularly for allogeneic transplantation. Furthermore, a meta-analysis of 32 studies involving a total of 1012 participants treated with MSCs for a variety of conditions including stroke, cardiomyopathy and Crohn's disease, has confirmed the safety of MSC therapy [21].

Taken together, the lack of ethical, immunological and safety concerns associated with MSCs has allowed the regenerative medicine field to proceed to the next stage of cellular therapy.

There are two main therapeutic approaches evolving around the use of MSCs in regenerative healthcare. The first is the tissue engineering approach whereby functional tissues or specialised cells are produced *ex vivo* and subsequently introduced into areas of diseased or damaged tissue. Whilst significant advances have been made in stem cell science and biocompatible scaffolds, it is a scientifically difficult area. As such, whilst this approach holds promise, many challenges are required to be overcome before safe and proven tissue engineered products are available for therapeutic use. The second approach involves stimulating the body's own regenerative capabilities. In contrast to tissue engineering, this area, through the use of autologous or allogeneic adult cell therapy, focuses on controlling the microenvironment in areas of injury. In particular, this approach involves switching from that of a destructive pro-inflammatory environment to an anti-inflammatory one, which supports repair and regeneration of damaged tissues through cell engraftment and paracrine signaling.

Bone marrow transplantation set the scene for the regenerative medicine field and is a recognised and routinely used treatment following myeloablation during cancer treatment regimes. Given the success of this therapy, many subsequent cell therapy studies have focused on stem cell differentiation, which is the main mechanism of action of bone marrow transplants. That is, HSCs within the bone marrow are understood to repopulate all cellular constituents of blood. As such, in the early 1990's researchers were focused on the differentiation of MSCs to unlock their therapeutic potential [23, 345]. This was an understandable emphasis, considering the mode of action of HSCs and main therapeutic potential of ESCs and cellular proliferation is considered a requirement when attempting to replace damaged tissues or organs. The ability of MSCs to differentiate into cells of the adipogenic, osteogenic, chondrogenic, and more recently, myogenic and neurogenic lineages has been demonstrated *in vitro*. In line with the view that the therapeutic potential of MSCs was based on differentiation, the International Society for Cellular Therapy has released multiple position statements on the parameters required to define a cell as an MSC, which includes their ability to differentiate into adipocytes,

chondrocytes and osteoblasts [30, 39]. Despite the efforts of researchers demonstrating the *in vitro* differentiation capabilities of MSCs, *in vivo* investigations of these cells in animal models of induced disease have not confirmed differentiation as their therapeutic mechanism. Introduced MSCs have resulted in regeneration of tissue; amelioration of induced diseases and have been detected in *de novo* tissue identified via cell tracking methods. Rarely, however, have implanted MSCs been shown definitively to differentiate into the target cell type. These results have brought about a rethink of the *in vivo* MSC differentiation hypothesis for tissue regeneration or repair. In the last decade *in vitro* studies, animal model research and clinical use have clearly shown that secretions from MSCs are a key driver in the therapeutic action of these cells, discussed further in Section 7.3.

Although the literature, in recent years, has focused on the immunomodulatory functions of MSCs, the fate of implanted cells remains a poorly addressed question. As allogeneic MSCs progress towards clinical use, regulatory authorities will require data on the fate of implanted cells. There is good *in vitro* and *in vivo* [53, 346] evidence in support of the 'immune-privileged' status of allo-MSCs; however, implanted cells are often not detectable after a few weeks or months. This has led to a number of groups investigating the fate of MSCs that may undergo 'spontaneous' differentiation *in vivo,* which could lead to loss of immune privileged status. Some studies have now documented specific cellular (T-cell) and humoral (B-cell/antibody) immune responses against donor antigens following administration of allo-MSCs. The concept of allo-MSC is likely to be closely studied in human and animal recipients [347].

The majority of animal and human studies using allogeneic MSCs have investigated the effect of a single administration of cells and very few groups have looked at the immune response to multiple doses. On the available evidence, the powerful immunomodulatory effect of MSCs appears to overcome any allo-rejection issues, especially when a single administration of cells is given. The safety of allogeneic MSCs for human use is increasingly accepted; and there are currently 65 active allo-MSC clinical trials registered with the NIH and accessible via clinicaltrials.gov website [348].

7.2. Mesenchymal Stem Cell Therapy: Evolution in Clinical Delivery

Two linked aspects of MSC use are important to consider when intending to use these cells clinically. One is the source of cells, which determines whether cells can be used fresh and uncultured at the point of care, or whether a cell culture step is required to expand cell numbers. The second is the use of either autologous or allogeneic MSCs.

This thesis has explored the product development of both autologous and allogeneic adipose-derived cellular therapeutics. The use of adipose tissue as a source of cells for therapeutic use has two clear benefits over bone marrow, particularly in canine medicine. Firstly, there is a high percentage of MSCs in adipose tissue and as such culture expansion is not necessary to achieve an appropriate number of cells for therapeutic use [49, 217, 218]. Secondly, provision of an off-the-shelf allogeneic cellular therapeutic is best achieved through culture expansion of adipose tissue. Whilst some differences have been observed between bone marrow- and adipose tissue-derived MSCs [349], there is no evidence that either source is therapeutically superior. The superiority of adipose tissue over bone marrow as a source of cells for therapeutic use relates to a reduction in donor morbidity. Adipose tissue can be easily harvested from a dog during a routine desex procedure and, therefore, poses no additional morbidity risk to the donor. In contrast, bone marrow aspiration is not a routine procedure performed in veterinary or human medicine and may result in significant patient morbidity.

The results presented in this thesis are focused on the development of adipose-derived cell therapy for canine OA, and understanding the therapeutic effects of such therapies. The results presented in this thesis progress from an autologous SVF treatment to an off-the-shelf allogeneic MSC therapeutic. Both treatment offerings were novel and demonstrated preliminary apparent clinical efficacy in the treatment of canine OA. There are, however, caveats on these studies; in that they were performed through veterinary clinics and cells were administered to pets suffering from OA. In both cases there was no control group because of the difficulties of obtaining ethics approval for a ‘non-treatment’ group. The alternative, of comparing an anti-inflammatory drug control to a cell therapy in domestic pets was

viewed as equally difficult. There is a risk that the observed efficacy has a placebo component due to the assessments by the owner and/or the veterinarian.

A freshly derived SVF treatment has the clear benefits over an allogeneic off-the-shelf product of being a minimally manipulated, personalised therapeutic. Furthermore, the SVF is comprised of numerous cell types other than MSCs including endothelial cells, vascular smooth muscle cells, pericytes, and white blood cells. The SVF also contains Treg cells [350] that play a key role in modulating the immune system and maintaining tolerance to self-antigens and, therefore, preventing the development of autoimmune diseases. Additionally, the SVF contains pericytes. Pericytes, were traditionally considered to play a key role in integrating and coordinating endothelial cell processes, there is now, however, emerging data demonstrating that pericytes share many of the phenotypical and functional characteristics of MSCs [37]. In particular, pericytes have been demonstrated to have extensive secretion capabilities [351-353]. Characterisation of the SVF is complex due to cell and tissue debris and the inherent autofluorescence of these particles. The addition of a nucleic acid dye (Syto11 or PI) is generally required to distinguish the SVF cells from the autofluorescence of debris present in the samples. Syto11 and PI nucleic acid dyes are compatible with PE and FITC fluorochromes respectively, allowing cell surface antigen characterisation to be performed on a flow cytometer with a 488nm laser. In the work presented in Chapter 3, Syto11 was used in conjunction with PE labeled antibodies and PI in conjunction with FITC labeled antibodies. The inclusion of a third fluorochrome was not successful with either of these combinations. Therefore, multi-fluorochrome cell surface antigen staining was not possible in this thesis.

As the cells of the SVF reside in close-proximity within adipose tissue *in situ*, there are significant cross-talk and feedback mechanisms occurring between these cells, which govern their activities including secretion production. The combination of the multiple cell types that comprise the SVF has been demonstrated to produce a distinct secretion profile to that of MSCs [242]. Although the isolated SVF and culture expanded MSCs described in chapters 4 and 6 respectively, were isolated from different donor dogs, variances were observed between the secretion profiles obtained from these two cell populations. In particular, detection of IL-10 and IL-6, which are characteristic of a Th2 anti-inflammatory profile, were detected in the

secretion profile of the canine SVF (Chapter 4). In contrast, these cytokines were not detected in the conditioned media of the canine culture expanded MSCs (Chapter 6). A Th2 profile and induction of anti-inflammatory M2 macrophages is characteristic of SVF or MSCs responding to inflammatory signaling, found in the microenvironment after injury. The Th2 profile is also observed *in vitro* with mixed cell culture, MSCs and inflammatory cells or freshly isolated SVF [354, 355]. The isolation and 'culture' of SVF mimics tissue injury and is likely to drive the observed Th2 response [355]. In contrast, homogeneous cultures of passaged MSCs are generally not exposed to inflammatory conditions and the secretion of Th2 related cytokines is diminished [354].

Whilst there may be a benefit in using all of the cell types that comprise the SVF, there are a number of factors associated with this therapy that may limit its widespread use as a veterinary therapeutic. The fact that the SVF contains numerous cell types which are not immune-privileged limits this therapeutic to an autologous option only. To harvest the adipose tissue required for SVF isolation, an anesthetic and surgical episode is required. This creates significant patient morbidity and is also costly. Furthermore, more often than not the patient in need of treatment is geriatric, and there are suggestions that MSC and pericyte numbers decrease with increasing age [31, 356].

Allogeneic culture-expanded cellular off-the-shelf therapeutics has the potential to become a widespread therapy option in both the animal and human medical spaces. The major advantage of an allogeneic cellular product is elimination of the requirement to extract adipose tissue from each patient. In the context of this thesis, the adipose tissue was obtained from healthy young guide dogs that were undergoing a routine desex procedure, thus eliminating any additional morbidity risk for the donor dogs in association with the adipose tissue harvest. To be able to provide an inexpensive on-hand product to veterinary practices, the tissue obtained from the donor dogs needs to provide multiple doses from that donor. One of the three characteristics used to define a cell as an MSC, is their ability to adhere to plastic. This characteristic can be exploited to isolate and subsequently purify MSCs from mixed cell populations such as the SVF. Culture expansion and cryostorage of cells are well-established procedures. In the context of this thesis, the production method used to produce the allogeneic of-the-shelf therapeutic investigated in

Chapter 6, has the capability to produce 100,000 doses of MSCs from a single canine adipose tissue donation (Regeneus Ltd, internal data).

Despite the loss of the other potentially therapeutic cells from the SVF, the development of an allogeneic off-the-shelf product utilising a homogeneous cell population has many advantages. In particular, the cost to the end user will be markedly less than the cost of autologous cell therapy. As mentioned, MSCs are immune-privileged enabling the treatment of a large number of patients from a single adipose tissue donation. In line with this, a major advantage of an off-the-shelf therapeutic is the ability to treat a patient who requires immediate intervention (such as renal ischemic injury or stroke) or who is unable to have a general anaesthetic and undergo the surgical procedures required for autologous cell therapy. Furthermore, this type of therapeutic lends itself well to conditions that require repeat treatment. Lastly, the use of a homologous cell population enables the definition and characterisation of a product essential for product consistency and regulatory requirements for product registration.

7.3. Secretions: The Key to Achieving a Therapeutic Effect

As mentioned, the differentiation capacity was considered an indicator of the therapeutic ability of stem cells. In the clinical aspects of this thesis, a rapid (within 10 days) and sustained reduction in pain and improvement in function was reported by both veterinarians and owners. Furthermore, a rapid treatment effect was observed regardless of whether the dogs received autologous or allogeneic adipose-derived therapy for osteoarthritis (Chapters 3 and 6). Animal studies have demonstrated that cartilage regeneration takes weeks to months to achieve in both acute or chronic injury models or real clinical cases of joint disease. The rapid response observed in Chapter 3 was far too early to be due to joint structural changes and is most likely to be a result of mediation of inflammatory cascades in the joint via cellular secretions and paracrine signaling of the introduced cells. A rapid therapeutic response to administration of SVF cells or MSCs has been demonstrated in other published studies [217, 218, 357]. Black *et al.,* (2007) was a blinded study with a saline-injected control group of dogs with OA of the coxofemoral joint [217]. The treatment group was intra-articular injection of autologous adipose derived SVF. The

placebo control group underwent fat harvest and the SVF cells were cryopreserved. The treatment group in this study showed a statistically significant improvement in lameness compared with the blinded, saline-injected, control group and significant improvement over time from baseline. A second study by Black *et al*., (2008) demonstrated very similar results in canines with elbow OA [218]. Importantly, the Black studies observed that the majority of the therapeutic effect on pain and lameness were observed within the first three months and the elbow study followed the animals for 6 months. These published data are very similar to those observed in the studies documented in this thesis in Chapters 3 and 6. It is interesting to note that animals that show a significant, and rapid, response to the treatment maintain that improvement for at least one year. Whilst the animal model literature contains many examples of cartilage regrowth after stem cell administration [160, 161, 358], which may account for the long-term effects observed in dogs, it is not possible to prove this experimentally in domestic pets.

The immunomodulatory functions of MSCs have prompted some groups to change the nomenclature to reflect that, and they are often referred to as 'Small Molecular Factories' or 'Medicinal Signaling Cells'. MSCs have been shown to alter their cytokine secretion profile in a dynamic fashion in response to the microenvironment they are introduced into. In particular, these cells, via their secretions, have the ability to prevent apoptosis of damaged cells, prevent the de-differentiation of cells that use this mechanism as a response to injury, and promote proliferation of endogenous cells in the damaged area. In MSC/chondrocyte xenogeneic co-culture experiments, the MSCs did not produce any type II collagen, but they resulted in an increased production of type II collagen by chondrocytes [359, 360]. In animal models of cartilage damage, MSCs have a positive effect on the production of hyaline-like cartilage. As discussed earlier, MSCs are rarely found to differentiate *in vivo* after intra-articular administration, instead, new cartilage tissue after MSC treatment has improved cell arrangement, subchondral bone remodeling and integration with surrounding cartilage than controls or treatments with other cell types [361]. As such, the potent secretions of MSCs are a major driver of the ability of MSCs to halt or reverse the progression of disease. Furthermore, even if administered remotely, MSCs can change the course of a disease via paracrine signaling activities [67, 362]. As such, MSCs have been shown to be efficacious in a

wide range of induced and clinical pathologies including both autoimmune driven conditions, as well as degenerative diseases.

With the worldwide research focus now firmly on a secretion-driven hypothesis for MSC action *in vivo*, much of this thesis focused on assessing the secretion profile of the cellular populations utilised in both of the real world canine therapeutics. To-date there is limited publications reporting on the cell composition and secretion profile of the SVF and the reports that do exist are limited to human studies. To the author's knowledge, Chapter 4 of this thesis provides the first report characterising both the canine SVF cell composition and its secretion profile. Despite so little being known about this cell population, the SVF is being utilised commercially in canine therapeutics in numerous countries throughout the world and, therefore, understanding its composition is important. In line with publications characterising the human SVF cell composition, in Chapter 4 it was demonstrated that the canine SVF cell composition and secretion profile was subject to significant variability between individual dogs. In particular, in this study, 25 ± 7 % of canine SVF cells were positive for the MSC and stromal marker CD90, and Varma *et al*., (2007) reported 35 ± 18 % of human SVF cells were MSC-like [36]. Furthermore, in this study, a correlation between cell types of the canine SVF (CD25, CD40 and CD90 positive cells) and cytokines secreted by the SVF (IL-2, IL-10, GM-CSF and TGF-β) existed. As such, the individual variability in the percentage of cell types present and in turn the SVF secretion profile may be a source of variability in the therapeutic potential of an SVF treatment on a case-by-case basis.

The ability to further characterise the canine SVF and its secretion profile through the development of new assays, coupled with the long-term follow-up of animals treated, is likely to shed light on whether variation in the SVF cell composition does indeed alter the therapeutic effect. Further investigation using greater numbers of biological replicates and new assays will likely confirm, and uncover, the importance of a correlation between cell types and secretion profiles of the canine SVF.

A plethora of publications report on the paracrine activities of MSCs, and this has lead to researchers investigating the use of MSC secretions alone as therapeutics. The use of MSC secretions has been demonstrated to be efficacious in models of corneal eye ulcers [68], cardiac infarction [304] and stroke [305]. However, the

secretions in isolation only achieve a short-term therapeutic effect in comparison to the MSCs themselves. Whilst MSCs and MSC secretions in isolation have been reported *in vivo*, to the author's knowledge this is the first report of the use of these two elements together. In developing an off-the-shelf MSC product it was hypothesised that the addition of cellular secretions in concert with the MSCs would provide an improved therapeutic product to that of just MSCs or cellular secretions alone. This hypothesis was tested in a CAIA mouse model (Chapter 5). An initial reduction of CAIA clinical symptoms was seen at a significant level in the mice administered with MSCs and secretions in comparison to the mice that only received MSCs. This initial response also continued until the study end-point eight days post administration of the test articles. When investigating canine cytokine proteins in mice eight days after administration, canine MCP-1, TNF-α, IL-8 and GM-CSF were detected in the blood of fewer than half of the MSCs+secretions group test animals. Although high levels of MCP-1, IL-8 and GM-CSF had been administered to the mice in the SVF canine secretions, the half-life of these secretions is often stated to be less than 12 hours [363]. This is likely to be an over-simplification, however, and a dynamic equilibrium between bound and free forms of cytokine may prolong the actual half-life. As such, it is possible that the addition of secretions aided in MSC embedding and had a functional impact on the MSCs; however, additional studies are required to determine the roles of secretions and MSCs in a mixed therapeutic. It would be ideal to treat a cohort of CAIA mice with only canine secretions and assess their detectability in the serum at a series of time-points.

In human OA, cytokine therapy has produced moderate symptom reduction in two published studies [364, 365]. The study by Baltzer *et al.*, (2009) used conditioned human serum to treat OA via intra-articular injection [365]. The process of conditioning the serum involves holding whole blood at 37°C for six hours, which increases the concentration of anti-inflammatory cytokines such as IL-1Ra. The conditioning process does not, however, produce a uniform shift towards a therapeutically favourable profile, as pro-inflammatory cytokines IL-1β, oncostatin M and TNF-α were up-regulated 20.9-fold, 2.9 fold and 10.2-fold, respectively.

The lack of good quality canine monoclonal antibodies is a major limitation of the characterisation of canine conditioned media or serum. There are few ELISA assays and the Milliplex panel used in this thesis is the only one available for dogs.

These limitations make comparisons with human studies difficult and speculative. One finding that is clear from human studies is that the half-life of injected cytokines in synovial fluid is low. Rutgers *et al.*, (2010) reported that the half-life of autologous cytokines was no more than one or two days and the concentration of cytokines such as IL-1Ra was not increased even after repeated injections of conditioned serum [366]. They did not, however, address the issues of free versus bound cytokines.

In comparing the results of Chapters 5 and 6 with the available OA literature it is clear that some caveats must be highlighted. The CAIA model, whilst providing clear data that supports the use of MSCs+secretions, a key issue is the systemic, rather than intra-articular administration of the test articles. Nevertheless, the advantages of synchronised onset of symptoms and the ability to obtain data rapidly make the CAIA model an important tool for early evaluation studies. The lack of broad canine cytokine panels makes comparison with human studies difficult; however, the therapeutic efficacy of cytokine administration appears to be conserved between mouse, dog and human.

If a very limited panel of cytokines is available for analysis it is possible that the analysis may become sidetracked; considering the levels of cytokines that may not be relevant. For example, the chemokine MCP-1, growth factor GM-CSF and pro-inflammatory cytokine IL-8 were present at very high concentrations in the canine SVF secretions used in conjunction with MSCs. These cytokines have been shown to be detrimental in both RA and OA pathologies. In GM-CSF knockout studies in mice the elimination of GM-CSF has shown to improve both RA and OA conditions in induced models [367]. Furthermore, both MCP-1 and IL-8 have been associated with pro-inflammatory conditions and have been negatively associated with both RA and OA [339, 368, 369]. Despite the secretion of these soluble factors, which may not ideal in the treatment of either a RA or OA pathology, the clinical response in the CAIA affected mice was superior in the mice treated with MSCs and secretions rather than just MSCs alone. This may have been due to the production of anti-inflammatory cytokines in response to the elevated levels of these cytokines via cellular feedback mechanisms, or it may be that numerous other cytokines, chemokines or growth factors that are yet to be detected and characterised are playing a role in mediating disease symptoms. In addition, a tight focus on cytokines ignores other components of serum or conditioned media that may play an important role in disease

modification. One such molecule is Alpha-2-Macroglobulin (A2M), which is a naturally-occurring plasma glycoprotein that functions as a pan-protease inhibitor but does not normally reach high levels within the intra-articular joint space [370]. A2M modulates the systemic inflammatory response by its ability to bait, trap, and clear various MMPs and cytokines. An arthritis study has also shown that A2M inhibited enzymatic digestion of cartilage oligomeric matrix protein (COMP) [371].

This thesis was largely focused on characterising the secretion profiles of therapeutically important adipose-derived cell populations. In particular, a panel of cytokines, chemokines and growth factors was measured in the conditioned medium of these cell populations. Whilst these bioactive factors are of therapeutic importance and provide insight into the therapeutic potential of adipose-derived cells, there are a number of paracrine signaling mechanisms that cells use. One such signaling mechanism is the horizontal transfer of microvesicles. Microvesicles contain mRNA, microRNA, transcription factors and proteins from the cell of origin within a circular fragment of membrane. These microvesicles facilitate cell-cell communication through surface-expressed ligands that activate the target cell, and can transfer genetic or material proteins to the target cell [372]. Cells also secrete an array of hormones (prostaglandins), lipid compounds (resolvins and protectins) and chemical mediators (opioid-like peptides) [373, 374]. Therefore, to gain a comprehensive understanding of the signaling mechanisms of adipose-derived cells, and their role in exerting the therapeutic effect of these cells, these additional signaling pathways require investigation.

7.4. Defining Mesenchymal Stem Cells

A contributing factor in the slow path to translation of MSC-based therapies is the heterogeneity of the isolated cells and the lack of standardised methods for the definition of an MSC. The lack of an MSC-specific surface marker is often described as a roadblock in the development of definitively characterised MSCs. As a result, MSCs are typically isolated by plastic adherence, which produces a heterogeneous population of cells that differ in their growth kinetics and differentiation potential. It may be that heterogeneous cell populations increase experimental variability, decrease the efficiency of differentiation, and contribute to conflicting data in the

literature; however, these issues can be significantly reduced by cell selection. The heterogeneous nature of cell populations is evident immediately after tissue harvest and at earlier passages. Using plastic adherent selection and the cell culture techniques described in this thesis, by passage three, there is cell population homogeneity that is sufficient for clinical use. Culture-acquired homogeneity is illustrated in the MSC literature [375, 376] and in this thesis by looking at CD44 and CD90, which are documented MSC markers. In canine SVF, CD44 was present on 20% ± 6% of cells and CD90 on 25% ± 7% of cells. By passage three the level of expression for both CD44 and CD90 was over 90% with small standard deviations.

The issue of defining MSCs has become considerably more difficult with the discovery of their perivascular nature, and residence in essentially all adult tissues, meaning subtle source-specific differences exist between MSCs [377]. The process of defining an MSC product has been further complicated by researchers and companies focusing on differences in surface marker profiles to secure intellectual property protection of functionally similar cell types. New cell characterisation techniques, such as transcriptomics and proteomics, with the capacity to define cells based on hundreds or thousands of molecular signatures or interactions are also being used to seek out subsets of MSCs that have not been described previously or those that have novel functionality [41]. A useful guide to trends in MSC translation was published recently [378]. The US Food and Drug Administration (FDA) analysed Investigational New Drug (IND) clinical trial submissions of mesenchymal stromal (MSC)-based products. The goal of this analysis was to inform cell therapeutic product developers and highlight challenges in translation. Before 2008, the tissue source of MSCs was almost exclusively bone marrow, but had decreased to 55% by 2012. Currently, the most frequent tissue sources include umbilical cord, placenta and adipose tissue.

The selection and culture conditions described in this thesis compare favourably with the trends reported by the FDA. In approximately 80% of FDA submissions, MSCs were cultured in media containing FBS at concentrations between 2-20% [378]. In addition to serum, many protocols included growth factors. The most frequent FBS alternative was human platelet lysate, and replacing animal serum with defined media was in the process development plan of many submissions. The majority of submitted protocols (~90%) included MSC culture under normoxic conditions. Cryopreservation of final product was used in 80% of protocols, with a

common post thaw viability threshold set at 70%, especially for intravenous infusion. There was considerable variability in descriptions of "MSC bioactivity" and proposed potency assays. Fewer than half of submissions described such assays at all.

In 2006 the ISCT proposed a set of markers that must expressed on MSCs: CD105, CD73 and CD90. In addition, they must lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Whilst other markers such as CD271 [379] or STRO1 [380, 381] are included in some FDA submissions, to define MSC subpopulations, the ISCT list was most frequently used to characterise cells by phenotype. The FDA authors noted that there is no clarity across submissions on: definitions, manufacturing, markers, assays and administration of MSC products [378]. The FDA places considerable weight on proof-of-concept animal studies to investigate safety and efficacy.

7.5. Clinical Relevance: the Role of Mesenchymal Stem Cells in the Treatment of Osteoarthritis

Osteoarthritis is the most common joint disease in both people and dogs. Clinical presentation of symptoms and the etiopathophysiology of the disease are similar in both species. Regardless of the cause of OA, be it age-related wear and tear or acute trauma to the joint by disease or injury, immunological instability and joint tissue destruction inevitably result. Currently no pharmacological, non-pharmacological or surgical intervention has been demonstrated to protect or restore joint tissues. As such, there is a critical need for research and medical communities to develop disease-modifying OA treatments. The easy availability and self-renewal capacity of MSCs, coupled with their chondrocyte differentiation potential and immunosuppressive capacities make them an obvious choice for use in the treatment of OA. Unlike pharmaceutical treatments, which are designed to act on one receptor pathway, cells are dynamic and can alter the course of a disease holistically through the secretion of paracrine mediators.

The preliminary real world dog data and animal model results presented in this thesis (Chapters 3, 5 and 6) have shown clinical efficacy in the treatment of OA with either autologous SVF cells or allogeneic MSCs. The dog treatments did not

include control groups and there is a risk of veterinarian or owner driven placebo effect, although the data presented here were similar to canine trials where control groups were used.

It is likely that the SVF and MSC cellular therapies exert disease-modifying potential in a condition such as OA via two modes of action. In the initial phase the pro-inflammatory and tissue destructive microenvironment of the joint is likely mediated by cell-to-cell communication and through the secretion of soluble mediators of the introduced cells. These actions are expected to be responsible for the rapid clinical improvements seen in the dogs and mice reported in results of this thesis. The therapeutic response seen in the dogs however, was not short-lived, as shown by the long-term (6-18 month) clinical results reported in this thesis (Chapters 3 and 6). The long-term apparent clinical improvement indicated that introduced cells might continue to exert reparative properties even after an initial phase response. A recent publication reported that a single intra-articular injection of human MSCs were found locally in joint tissues in mice 6 months after MSC administration [90]. This study highlighted that MSCs can engraft in host tissues and remain locally for extended periods of time, which constitutes the second mode of action. As such, the SVF cells and MSCs utilised in the treatment of canine OA in this thesis may act as site-specific, dynamic mediators of disease, resulting in regeneration of damaged tissues.

Increasingly, domestic dogs are being identified as a good model organism in the research of human diseases [382] Generally today, dogs experience a good level of healthcare and live until old age. Like people, dogs also have highly reactive immune systems that protect them from infectious disease but predispose them to autoimmune disorders. As such dogs experience many of the same healthcare disorders as people. In particular, and relevant to the research performed in this thesis, both dogs and people suffer from OA. The OA disease profile is essentially the same in both species and as such, it can be assumed that treatments successfully developed for canines, can possibly be translated for the treatment of human OA.

7.6. Final Remarks and Future Directions

The novel autologous and allogeneic adipose-derived cellular therapeutics described in this thesis were demonstrated to be both safe and clinically efficacious for the treatment of induced disease in a mouse model and similar to other published studies on canine OA. The real world clinical data presented in response to SVF and MSC treatment in dogs (Chapters 3 and 6) was encouraging, however the data sets were small and lacked sufficient controls to definitively determine the therapeutic efficacy of these treatments.

The aim of this thesis was to investigate safe, efficacious and affordable potential cellular therapeutic options for canine OA. To fulfill this aim several key factors to ensure any cellular therapeutic option could become a mainstream treatment offering required consideration. These key factors consisted of, patient morbidity, efficacy of treatment, expense to end user (pet owner), regulatory environment and the accessibility and consistency of a product. Throughout the product progression described in this thesis, it became apparent that an off-the-shelf allogeneic treatment with the addition of cellular secretions was not only a safe and effective treatment but was likely to best meet regulatory and market requirements.

The off-the-shelf MSCs+secretions product was shown to be efficacious in a mouse model of arthritis and preliminary efficacy was also demonstrated in the treatment of clinical canine OA. Whilst the clinical signs of OA were reduced in both the mice and dogs it was not possible to assess if any structural improvements to the joint had occurred. Future studies should incorporate an imagining or histological component to look for structural or histological changes in joint tissues. Additionally, cell labeling and tracking to determine the fate of introduced cells would provide valuable supporting evidence to the hypothesis that MSCs embed in local tissues.

Variability in cell population and secretion profile between individuals was seen in the characterisation of the canine SVF (Chapter 4) and also in the *in vitro* investigation of MSCs (Chapter 6). The MSCs, which were derived from adipose tissue harvested from three separate dog donors, essentially all had equivalent cell surface antigen profiles and differentiation capabilities. However, significant differences in their proliferation capability and secretion profiles were observed. Traditionally, differentiation capability and cell surface antigen profiling have been utilised in the

identification of an MSC [30, 39]. These assays however, provide no information in relation to how a MSC may function *in vivo*. As such additional analysis tools and functional assays such as the Click-It EdU proliferation assay and Milliplex 13-plex canine cytokine, chemokine and growth factor panel provide an aid to determine if the cells from a given donor will be clinically useful. Furthermore, large-scale analysis tools such as transcriptomics or quantitative proteomics (iTRAQ) may prove useful in uncovering specific characteristics of cells that relate to their suitability as a therapeutic. The development of additional assays to detect other cytokines, chemokines and growth factors such as HGF, IL-4, IL-13 and IGF will provide further information regarding the cellular secretion profile and in-turn potential *in vivo* functionality of an MSC derived cellular product.

Although not possible in the context of the research presented in this thesis, an investigation of the cytokines present at a local level pre- and post-intra-articular injection of cells in the joint would be advantageous. Understanding the inflammatory changes in the joint would provide further insight into the therapeutic mechanisms of cellular therapy. Whilst this is ethically challenging in canine studies, as an anesthetic is required to aspirate synovial fluid from dogs, future human studies could potentially more easily facilitate this type of investigation. Additionally, utilising a xenogeneic model and an assay to quantity bioactive factors, such as the Milliplex 13-plex, could enable detection of introduced cytokines at the site-specific level. The multi-plex assay utilised in this thesis enabled an assessment of not only the secretion profile of the SVF and MSCs, but was also used to investigate the level of circulating canine cytokines remaining in the mice serum of CAIA affected mice providing some information regarding the functionality of the introduced cells (Chapter 5).

There is a place for both an autologous and allogeneic cellular therapy in veterinary medicine. It is likely however, that due to several key factors, cell treatment offerings will move towards an allogeneic off-the-shelf product. An allogeneic product enables accessibility to all patients including those that are unable to undergo an anesthetic and surgical episode for tissue removal. The ability to generate multiple thousands of doses from one tissue donor eliminates patient-to-patient variability and in turn ensures product consistency. Through the use of functional assays and sophisticated molecular analysis tools a cellular profile, which is likely to optimise treatment potential can be identified. To definitively determine

efficacy of the off-the-shelf novel allogeneic MSC product described in this thesis a statistically powered, placebo-controlled trial utilising objective outcome measures such as gait analysis is required. The veterinary community, owners and their animals, will benefit from an allogeneic stem cell treatment, which is proved to be efficacious, readily available, reproducible and affordable. The research presented in this thesis is the first step to providing this treatment.

CHAPTER EIGHT

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CHAPTER NINE

Appendices

Appendix I: Animal Ethics Approval

1. Regeneus AEC Approved Project – RE006

   Evaluation of cultured stem cells for treating lameness in dogs and horses.

   MQ noted collaboration 17 May 2012

2. Regeneus AEC Approved Project – RE 011

   Collection of data to assess efficacy of AdiCell allogeneic cells and adipose cell in dogs

   MQ noted collaboration 17 May 2012

3. Regeneus AEC Approved Project – RE 012

   Collection of adipose tissue from dogs during desex operations.

   MQ noted collaboration 17 May 2012

4. UQ AEC Approved Project – TETRAQ/332/11/Regeneus

   Anti-inflammatory efficacy assessment of REG001 and REG002 in mouse model of monoclonal

   MQ noted collaboration 14 June 2012

From: Animal Ethics <animal.ethics@mq.edu.au>

Subject: Outcome of 17 May 2012 AEC meeting - Collaborative Notification

Date: 31 May 2012 2:56:15 PM AEST

To: Benjamin Herbert <benjamin.herbert@mq.edu.au>

Dear Dr. Herbert,

Ms. Rebecca Webster, PhD Student (Dr. Ben Herbert, Faculty of Science)	Evaluation of cultured stem cells for treating lameness in dogs and horses
	Collection of data to assess efficacy of AdiCell, allogeneic cells and adipose cell secretions in dogs
	Collection of adipose tissue from dogs during desex operations

Ms. Webster notified Macquarie University Animal Ethics Committee of the three collaborative projects under "Regeneus Animal Ethics Committee".

We have been provided copies of the applications, including the Research Proposal and documentation of their approval.

Decision

The Committee noted and approved the above collaborative work.

The above collaborative notification was acknowledged and approved by the Macquarie University Animal Ethics Committee at their meeting on 17 May 2012.

Please note that should the project continue after the initial 12 months, the renewed Animal Research Authority (or other evidence of approval) from the host AEC should be forwarded to the MQ AEC.

Kind regards,

Prof Michael Gillings
Chair, Animal Ethics Committee

Office of the Deputy Vice Chancellor (Research)

Animal Ethics Secretariat

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Appendix II: Animal Ethics Approval

THE UNIVERSITY OF QUEENSLAND

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

ANIMAL ETHICS APPROVAL CERTIFICATE 21-Jun-2012

Activity Details

Chief Investigator:	Professor Maree Smith, Integrated Preclinical Drug Development
Title:	Adipose cells as a treatment for collagen antibody induced arthritis (CAIA)
AEC Approval Number:	CIPDD/225/12/TETRAQ/REGENEUS
Previous AEC Number:	
Approval Duration:	25-Jun-2012 to 25-Jun-2015
Funding Body:	TetraQ, Regeneus
Group:	Molecular Biosciences
Other Staff/Students:	Suzanne O'Hagan, Sunderajhan Sekar, Andy Kuo, Rahul Shankar, Angela Raboczyj, Chiew Yuen Dieu, Kelly Sweeney, Nematullah KHAN, WEI Goh, Arjun Muralidharan
Location(s):	TetraQ - Level 3 Steele Building

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Mice - Outbred	BALB/c	Adults	Female	Institutional Breeding Colony	408	408

Permit(s):

Proviso(s):

Approval Details

Description	Amount	Balance
Mice - Outbred (BALB/c, Female, Adults, Institutional Breeding Colony)		
25 May 2012 Initial approval	408	408

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W www.uq.edu.au/research/rid/

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

Please use this Approval Number:

1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all Project Animals details be made available to Animal House OIC.
(UAEC Ruling 14/12/2001)

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 21-Jun-2012)

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Pages 231-245 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Webster, R., Herbert, B., Blaber, S., Vesey, G. (2011) Mesenchymal stem cells in veterinary medicine, *The Veterinary Nurse*, Vol. 2, No.2, pp. 58-62. https://doi.org/10.12968/vetn.2011.2.2.58

Webster, R., Blaber, S., Herbert, B., Wilkins, M., Vesey, G. (2012) The role of mesenchymal stem cells in veterinary therapeutics – a review, *New Zealand Veterinary Journal*, Vol. 60, No. 5, pp. 265-272, DOI: doi.org/10.1080/00480169.2012.683377

Appendix V: Additional data for figure 4.2 (seven dogs)

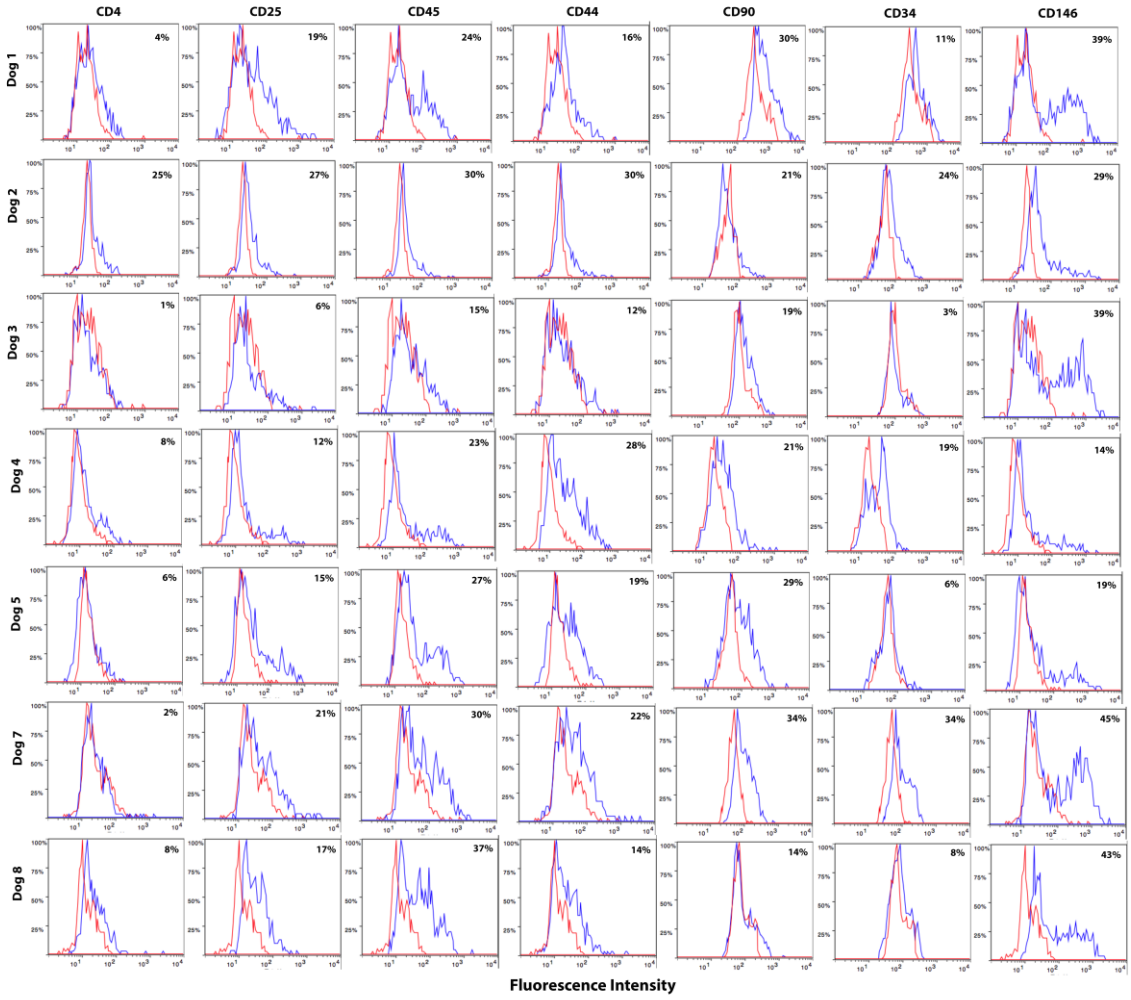

Figure 9.1 Phenotypical characterisation of canine SVF using CD markers and flow cytometry.

Canine SVF was analysed on a FACScan flow cytometer for characteristic white blood cell markers, CD4-FITC, CD25-FITC, CD45-FITC and for characteristic stromal and endothelial cell markers CD44-FITC, CD90-PE, CD34-PE and CD146-FITC. For each cell type and fluorescence conjugate, an unstained control (red line) was included. The blue line represents the stained sample. The percentage of positive cells is shown in each plot.