DISSERTATION

# IMMUNE MODULATORY AND ANTIMICROBIAL PROPERTIES OF MESENCHYMAL STROMAL CELLS DELIVERED SYSTEMICALLY

Submitted by

Valerie Johnson

Department of Microbiology, Immunology, and Pathology

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Doctoral Committee:

Advisors: Steven Dow,
Co-Advisor: Mark Zabel

Anne Avery
Ron Tjalkens

ABSTRACT

IMMUNE MODULATORY AND ANTIMICROBIAL PROPERTIES OF MESENCHYMAL STROMAL CELLS DELIVERED SYSTEMICALLY

Mesenchymal stem cells (MSCs) were first cultured approximately 50 years ago and have been the subject of intense research over the last 20 years as their potential therapeutic properties have become apparent. These cells have the ability to adhere to plastic and are easily expanded. The are considered multipotent in that they can differentiate into bone, cartilage and adipose tissue under certain growth conditions. It was initially thought that these cells would have the ability to regenerate into new tissues facilitating the growth of new cartilage and bone. Initial studies quickly determined that although this was not the case, these cells had beneficial effects in several disease processes. These cells are well-known for their ability to suppress T cell proliferation and so were considered immunosuppressive. As such, in addition to being utilized in regenerative therapies, they were tested in diseases where the immune system was contributing to pathology of disease such as graft versus host disease and auto-immune disorders. The scientific community rapidly learned that these cells had the ability to respond to their environment and rather than being consistently immunosuppressive they were, in fact, immunomodulatory and could increase immune function or decrease it depending on environmental cues. We now know that MSC express toll-like-receptors (TLRs) as do cells of the immune system and therefore are capable of interacting with their environments to either upregulate or downregulate immune function. This creates a host of possibilities for therapeutic utility of these cells.

In 2010 a seminal paper was published describing the antimicrobial properties of these cells in a cecal ligation and puncture animal model of sepsis. This promising study prompted our laboratory to further investigate the antimicrobial properties of these cells in problematic infectious issues such as biofilm infections and multi-drug resistant infections. In Chapter 2 we describe the pre-activation of MSC taking advantage of the TLR expression on these cells to prime them for antibacterial activity. These pre-activated MSC (aMSC) were demonstrated to act synergistically with commonly utilized antibiotics to achieve resolution of a biofilm infection in a mouse model that was unresponsive to antimicrobial therapy alone and indeed to antimicrobial therapy combined with resting MSC. This increased efficacy could be attributed to the increased migration of activated MSC to sites of inflammation in addition to their secretion of antimicrobial peptides and enhancement of innate immunity in the form of increased phagocytosis of bacteria by neutrophils. In addition to this increased antibacterial activity of the innate immune system, the primed cells elicited an increase in wound healing phenotype of macrophages (from an M1 or inflammatory phenotype to an M2 or healing phenotype) in the tissues. All of these mechanistic actions combined resulted in resolution of the biofilm infection and healing of the tissues.

To further explore this potential of these pre-activated cells, *in vitro* experiments were performed utilizing canine MSC and multi-drug resistant (MDR) bacteria to investigate the bactericidal activity of these cells when combined with commonly utilized antibacterial pharmacologic agents. In chapter 3 we describe the results of these experiments. It was discovered that canine MSC when combined with antimicrobial agents acted synergistically *in vitro* to enhance bacterial killing. With these findings, a clinical trial was designed and completed utilizing preactivated MSC (aMSC) in naturally occurring multidrug resistant

infections in dogs who had failed conventional therapy including antibiotics and surgery. All dogs that completed this trial experienced improvement of their clinical signs. Approximately 75% of dogs experienced resolution of infection in addition to resolution of clinical signs. To further investigate the mechanism involved in this process canine MSC were tested for secretion of common antimicrobial peptides (AMPs) to determine if they might be responsible for the observed *in vitro* and *in vivo* effects. The cells demonstrated secretion of multiple AMPs previously implicated in antimicrobial effect of MSC. In addition, activated cells were shown to migrate more readily to an inflammatory stimulus thus increasing likelihood of migration to areas of infection. They also demonstrated increased secretion of chemotactic factor IL-8 known to increase neutrophil migration to the site of infection. Further enhancement of the innate immune system by secreted factors from both activated and resting MSC was demonstrated *in vitro* utilizing primary canine macrophages. We demonstrated both increased macrophage phagocytosis and bacterial killing when primary canine macrophages were exposed to secreted factors from these cells. Interestingly, when exposed to secreted factors from aMSC, primary canine macrophages displayed properties of both inflammatory M1 macrophages and anti-inflammatory M2 macrophages. Canine MSC also demonstrated increased wound healing ability *in vitro* as assessed by increased by increased proliferation, metabolism and migration of canine fibroblasts. Together, this demonstrates that aMSC delivered systemically increases microbial clearance in the face of multidrug resistant or biofilm infections, increased wound healing and resolution of infection in clinically applicable spontaneous wound models.

With the success of systemic treatment of wound infections and obvious effects on the immune system, we wondered if systemic therapy might be superior over local intra articular injection in spontaneously occurring osteoarthritis which often involves multiple areas of

inflammation. The majority of clinical trials involving MSC therapy for osteoarthritis have involved intra-articular injection. Although local inflammation is certainly occurring there is evidence that this process involves systemic inflammation. Additionally, in age related osteoarthritis there are often multiple joints affected. As such, it seems that systemic therapy with an agent that reduces inflammation globally may be more appropriate and effective than local therapy. To test this hypothesis, we utilized an accepted animal model of age-related spontaneous osteoarthritis (OA), the Hartley-Dunkin guinea pig model of OA. Results of this study, detailed in chapter 4, clearly demonstrate an improvement in clinical signs of osteoarthritis as assessed by gait analysis. In addition, we demonstrated a decrease in both intra-articular and systemic inflammation by immunohistochemistry analysis of inflammatory markers and serum and synovial fluid inflammatory markers.

In conclusion systemic MSC have many immunomodulatory actions that can be of benefit in multiple conditions both infectious and non-infectious. The ability of these cells to respond to their environment can be utilized to enhance their activity when utilized for biofilm and multi-drug resistant infections. Systemic administration decreases overall inflammation and has the advantage of homing to sites of inflammation increasing the therapeutic benefit of this treatment in cases of osteoarthritis involving multiple joints and systemic inflammation. Further research into systemic administration and pre-activation of these cells for treatment of osteoarthritis is warranted based on our preliminary results. The translational benefit of this therapy for both infection and arthritis is considerable as naturally occurring models of disease were utilized and displayed significant symptom alleviation and resolution of previously untreatable infections. This represents a new arena of novel therapy for multiple disease processes that currently are responsible for substantial morbidity and mortality.

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# CHAPTER 1

## Review of the Literature

### Defining Mesenchymal Stem Cells

The presence of a type of cell in the bone marrow that gives rise to a lineage of cells other than hematopoietic cells was first identified by Friedenstein in 1968.[1] He then identified that when bone marrow was placed into culture nonhematopoietic cells would grow. These cells had a fibroblast like morphology and would appear within a week and exponentially proliferate.[1] He called these cells colony-forming-unit-fibroblasts or CFU-F and noted their ability to differentiate into bone when cells were grown from bone marrow and then transplanted under the renal capsule of rabbits.[2] In 1991, Caplan coined the term mesenchymal stem cell, comparing these cells to mesoderm cells of the embryo that migrate and differentiate into bone, cartilage and connective tissue.[3] Indeed he hypothesized that these cells could be expanded *in vitro* and delivered back to the donor as a therapeutic modality. Today there are thousands of clinical trials and more than 50,000 publications utilizing these cells for amelioration of a plethora of disease processes.[4]

The term mesenchymal stem cell (MSC) is still widely used today although debates about the accuracy of the term stem cell abound. Mesenchymal stromal cell is also commonly, and perhaps more accurately, utilized to describe this type of cell. The Society for Cellular Therapy addressed the issue in 2005 proposing multipotent mesenchymal stromal cell as a more accurate term for these cells.[5] It is generally thought that the term stem cell is misleading as these cells cannot differentiate into every cell that the embryonic mesoderm gives rise to which leads to misconceptions in the general public as the term is utilized for a variety of products that are not

the same.[6] Indeed, the original assumption that these cells would differentiate into other tissues *in vivo* has long since been proven not to be the case and currently it is well known that these cells function therapeutically by modulation of other cells in the body.[7] It is because of this fact that the author of the term mesenchymal stem cell proposed in recent years that the name be changed to medicinal signaling cell.[8] In spite of the multiple proposed terms mesenchymal stem cell remains the most commonly used and some believe that the simplest solution is to keep the terminology unchanged.[6] Regardless of the nomenclature, it has become standard to define MSC according to characteristics proposed in 2006 by the International Society for Cellular Therapy. These characteristics include the ability of the cells to adhere to plastic, the ability to differentiate into bone, cartilage and fat and the possession of certain surface markers when analyzed by flow cytometry. The definition states that MSC be >95% positive for surface markers CD105, CD73 and CD90 and <2% positive (or negative) for surface markers CD45, CD34, CD14 or 11b, CD79α or 19, and HLA-DR.[9] Since this time multiple other markers have been identified and antigen of the bone marrow stromal-1 antigen (STRO-1), vascular cell adhesion molecule 1/CD106 and melanoma cell adhesion molecule/CD146 are also commonly reported to identify MSC.[10-12]

**Origin and Properties of MSC**

Mesenchymal stem cells can be derived from a variety of adult tissues. The most common tissues utilized for generating these cells are bone marrow, adipose tissue and umbilical cord.[13,14,15] However, generation of these cells has also been described from dental pulp, blood, spleen, heart, liver, hair follicles and skin and it is likely that multiple adult tissues are capable of producing these cells under certain conditions.[16-18, 19,20,21] Although all MSC cell lines appear

phenotypically similar and share properties that define them as MSC according to the ISSC guidelines, there are documented differences in differentiation potential, proliferation, immunologic function and expression of surface markers. While both bone marrow and adipose derived MSC are easily propagated, there is increased morbidity associated with the process of obtaining bone marrow. In addition, it has been demonstrated that adipose derived cells proliferate faster, can be passaged longer without approaching senescence while maintaining chromosomal stability and secrete more bioactive molecules such as VEGF and IL-8.[15,22-24] They additionally exhibit differences in immune function. In a comparison of bone marrow and adipose tissue MSC in dogs, it was demonstrated that adipose derived MSC inhibit T cell proliferation by a different mechanism than do bone marrow derived MSC.[25] Umbilical cord is a noninvasive mechanism for obtaining a stable MSC population, however these cells have been demonstrated to lack the ability to differentiate into the adipocyte lineage and so technically do not fit the ISSC criteria.[15] It has often been assumed that such differences can be attributed to differences in tissue processing and cell culture conditions. While this likely plays a role in the observed differences there is clearly variability between MSC from different sources and donors.[26,27,6] While these differences evoke questions regarding the feasibility of production of a consistent biologic product for pharmacologic purposes it also raises the question of the origin of the actual precursor in the donor tissue that gives rise to this cell type.

While MSC have been studied by tens of thousands of researchers over the past 50 years the actual source of this cell type remains unclear.[4] The most closely related cell type that is commonly believed to be the origin of the MSC is the pericyte. Pericytes are supporting cells that surround the vasculature. They are attractive as the common precursor to the MSC as they are found in all tissue types and share many common markers with MSC such as CD146 and Alk

Phos.[4] There are also differences, however. Researchers have demonstrated that pericytes can form branched hollow structures in vitro that resemble vascular structures while MSCs cannot.[28] One study investigated the fate of pericytes in formation of incisors and found a population of MSC that was not of pericyte origin as well as a population that was.[29] Others have postulated that all MSC are pericytes but the converse statement is not true.[30] A recent study conducting molecular sequencing on pericytes of various tissues postulates that pericytes of distinct tissues have distinct genotypes and that the differences in MSC from various tissues reflects their tissue of origin.[31] Certainly pericytes and MSC share common markers and some common functions that suggest that if they are not the origin of MSC they may be related.

In spite of the differences between cells derived from various sources, species and individual donors, there are many properties of these cells that appear to be universal. Irrespective of cell origin or species, MSC have a limited capacity for proliferation and after multiple passages their proliferation rates decrease and the morphologic appearance of the cell changes. These changes are thought to be the process of aging or senescence. It has been demonstrated in multiple species (mice, humans and cats) that the age of the donor plays a role in this process. Cells from older individuals display lower proliferation rates and enter senescence earlier.[32,33 34-36] It has been demonstrated that senescent cells do not have the same disease modification capacity as do young proliferating cells.[37,38] In addition, as the cells age they begin to display genomic instability which has caused increased concern regarding the risk for malignant transformation but there have been no documented instances of malignant transformation of MSC in recipients.[26]

Another property that appears to be consistent and indeed one that makes it attractive for therapeutic purposes is the lack of immune rejection of these cells when administered to a non-

related recipient animal of the same species (allogeneic) or even to another species (xenogeneic). Growth of cells from the same animal (autologous) is time consuming taking weeks to achieve numbers suitable for therapeutic applications. Therefore, this attribute of MSC is pivotal to the development of this immunotherapeutic product. The escape from immunosurveillance is attributed to the low expression of MHC molecules on MSC and their general tendency to dampen down the immune response.[39] Despite the lack of overt evidence of rejection, several studies have reported a humoral response of the host against MSC, particularly after multiple injections or when donor and recipient have mismatched major histocompatibility complexes.[40] It is known that MSC that are transplanted by any route do not remain viable in the recipient or engraft into the site, but rather exert their effects through immunomodulation of host cells. It is possible that an immune response could decrease the effect of future MSC injections if indeed a cytotoxic immune response was triggered by repeat injections as suggested by some studies.[41] The presence of a multitude of current and historical human clinical trials has underscored that administration of these cells is safe. A meta-analysis in 2012 that included 8 trials and 349 patients identified transient low grade fever as the only adverse reaction reported more in the treatment group than the placebo group.[42] An updated review evaluating only systemically administered MSC evaluated 55 studies and 2,696 human patients. As previously reported, they identified fever as an adverse reaction but also found the relative risk of death to be lower in the patients treated with MSC.[43] These studies would suggest that this therapy is safe but a more difficult question to answer is whether it is efficacious. Even among human clinical trials utilizing intravenous MSC therapy dose administered and number of doses varied widely.[43] In animal models of acute lung injury higher doses have demonstrated increased benefit with no adverse events.[44] The extensively researched immunomodulatory effects and homing ability of

these cells would suggest that intravenously delivered cells at the higher published dose ranges (5-10 x $10^6$/kg) are most likely to be efficacious.

**Immunomodulatory Properties of MSC**

MSC were initially considered as an attractive source of therapy for regenerative purposes. It was thought that their ability to differentiate into multiple cell lineages *in vitro* would translate to engraftment and differentiation in host tissue thus making it an attractive therapy to replace cartilage and bone. It is now well accepted that when delivered systemically these cells are short lived. It is in fact their immunomodulatory properties that are responsible for their effect *in vivo*.[45,46] One of the major ways that MSC interacts with the environment is through toll-like receptors(TLRs).[47] These receptors are present on immune cells and are the main way that the immune system responds to danger signals such as viruses, bacteria and other signals from the body. MSC derived from different tissue sources express different levels of TLRs but it is consistently reported that all MSC express TLRs 1-6.[48] This mechanism is one way that MSC interact with their environment and ligation of these receptors affects the factors that these cells secrete (commonly referred to as the MSC secretome).[49]

MSC were initially considered immunosuppressive due to their ability to suppress T cell proliferation which is still considered a hallmark of these cells and is commonly the method by which activity of these cells is measured. However, it is now known that these cells are immunomodulatory and can have pro-inflammatory as well as anti-inflammatory properties depending on their environment.[48] Of particular importance is the effect of ligation of TLR3 and TLR4 receptors of MSC. It has been demonstrated that ligation of TLR4 on MSC by lipopolysaccharide (LPS) induces a pro-inflammatory phenotype of MSC. This TLR4 primed MSC does not inhibit T cell proliferation and secretes pro-inflammatory factors such as CCL5

(RANTES), IL-6 and TNFα.[50] When TLR3 is ligated on MSC by agonist polyinosinic-polycytidylic acid (Poly I:C) the cell assumes a more anti-inflammatory phenotype and as such suppression of T cell proliferation is unchanged. In addition, this primed cell secretes anti-inflammatory cytokines such as IL-10 and IL-12 and dramatically increases secretion of chemokine CCL2 (MCP-1).[48] This is of particular importance as CCL2 has been demonstrated to be critical for the function of MSC in an allergic airway model.[51] This concept of stem cell phenotype and interaction with its environment is fundamental to their therapeutic potential in multiple ways. The ability to interact with the host and respond to cues appropriately means that the cells can respond to signals and processes happening in the body and change their phenotype as needed. In addition, it creates an opportunity to direct the cell towards a desired phenotype. Multiple approaches have been utilized to change the phenotype of MSC to increase activity including ligation of TLRs, coculture with inflammatory cells or bacteria and genetic modifications.[52,53,54-56] Further understanding of the how these cells interact with the immune system is critical to improve our ability to use them therapeutically.

The interaction of MSC with T cells has historically been utilized to help define this cell type and assess immune activity of this cell. MSC inhibit both CD4+ and CD8+ T cell proliferation and induce the formation of regulatory T cells.[57,58,59] Multiple studies have reported increased numbers of T regulatory cells following infusion of MSC *in vivo*.[60-62] It has been demonstrated that cell contact is necessary for induction of T regulatory cells and $PGE_2$ and TGFβ have also been associated with this process.[63] Notch receptors are also known to be involved in induction of T regulatory cells and additional studies have shown that they also play a part in the MSC interaction with T cells.[64,65] The ability of MSC to suppress T cell proliferation has been demonstrated for a wide variety of species.[66,67,68] However, it has been demonstrated in

multiple species that MSC from different species do not all utilize the same mechanism for suppression of T cells.[69] In human MSCs, multiple pathways have been demonstrated to be involved in T cell suppression including PD-1, cyclo-oxygenase and soluble MHC but indoleamine 2,3-deoxygenase (IDO) is considered the main pathway.[70,71] In mouse MSC nitric oxide appears to be the dominant pathway utilized for T cell suppression.[72-74] In a study comparing mechanisms of T cell suppression by canine MSC it was discovered that TGFβ and adenosine were the main signaling pathways involved but interestingly, bone marrow MSC utilized the cyclooxygenase pathway in addition to TGFβ and adenosine while adipose derived MSC did not. This suggests that MSC from different sources even from the same species may utilize different pathways to effect T cell suppression.[25]

In addition to suppression of T cell proliferation multiple studies have demonstrated an increase in regulatory T cells following MSC administration.[75-77] Studies have reported the importance of both cell-cell contact for this effect and soluble mediators such as TGFβ and PGE2.[59,63] Notch receptors are known to be involved in the development of T regulatory cells and MSC express these receptors and these have also been implicated in the induction of T regulatory cells in the presence of MSC.[78] Recently the phenomenon of mitochondrial transfer as a mechanism of MSC immunomodulatory activity has been explored. Court et al. demonstrated that MSC transfer mitochondria to both T cells and B cells and this transfer induces a T regulatory phenotype in T cells.[79] While the effect of MSC on B cells has not been as well studied, it is known that MSC also inhibit B cell proliferation causing a halt in plasma cell production and subsequent production of IgG, IgM and IgA. Co-stimulatory molecules and cytokine production by B cells does not appear to be affected.[80,81]

T and B cells are not the only immune cells that adopt a regulatory or alternate phenotype when exposed to MSC. It has been demonstrated that antigen presenting cells such as dendritic cells and monocytes also adopt an alternate phenotype.[82] Dendritic cells in this regulatory state promote immune tolerance and downregulate inflammation.[83] Multiple studies have examined the role of MSC on monocytes and it is generally accepted that exposure to MSC induces macrophages to polarize to an M2 or wound healing, anti-inflammatory type of monocyte.[84-87] Although there is a preponderance of evidence that macrophages adopt a phenotype that is considered less inflammatory there are also multiple reports that macrophages exposed to MSC or secreted factors from MSC exhibit enhanced phagocytosis.[88] This may be due to a combination of secreted factors and cell contact dependent factors as this property has been demonstrated to occur in the presence of MSC conditioned medium alone but one group demonstrated that mitochondrial transfer from MSC to macrophages was responsible for increased phagocytic action.[89] Thus there may be multiple pathways by which MSC effect this enhancement.

The other main effector of the innate immune system is neutrophils and there have been several studies documenting various effects of MSC on neutrophils. In general, it is agreed that MSC enhance the longevity of neutrophils and decreased the production of reactive oxygen species.[90] There have been variable and conflicting reports regarding the effect of MSC on neutrophils and it is likely that these differences are due to species differences and also depend on the environment of the neutrophils. *In vitro* studies using human MSC demonstrated increased neutrophil phagocytosis of E Coli and increased anti-bacterial activity of neutrophils.[91,92] A study using equine MSC described a decrease in reactive oxygen species (ROS) production by neutrophils exposed to MSC which others have described. However, in

this study they demonstrated decreased neutrophil extracellular trap (NET) formation by MSC primed neutrophils, where other studies using human MSC demonstrated an increase in NET formation.[93,94] A study that examined the properties of neutrophils after ligation of TLR3 and TLR4 on MSC demonstrated that when neutrophils were exposed to primed MSC they expressed different properties. TLR3 ligation caused in increase in ROS production, increased longevity of the neutrophils and both TLR3 and TLR4 ligation increased MSC secretion of IL8, IL6 and granulocyte-monocyte colony stimulating factor (GM-CSF). TLR3 ligation dramatically upregulated adhesion molecule CD11b, an important adhesion molecule for neutrophil adhesion to endothelium and extravasation.[95,96]

**Pre-conditioning of MSC**

As described above MSC function can change in response to multiple environmental signals including ligation of surface TLRs.[50] Ligation of TLR3 on MSCs has been demonstrated to affect cytokine secretion by activation of nuclear factor kappa B (NFkB). Waterman described this polarization into an MSC1 or pro-inflammatory type MSC or MSC2 or anti-inflammatory MSC induced by ligation of TLR4 and TLR3 respectively.[48] TLR3 activated MSC were demonstrated to produce increased anti-inflammatory cytokine IL-10, increased monocyte chemoattractant protein 1 (MCP-1 or CCL2), increased IL1 receptor antagonist (IL-1RA). Ligation of both receptors increased MSC migration towards an inflammatory stimulus but TLR3 ligation caused markedly more migration than cells primed by TLR4. TLR 4 primed cells demonstrated increased CCL5 (RANTES) production, a chemokine involved in monocyte and leukocyte migration. Additionally, TLR4 ligation decreased the ability of MSC to suppress lymphocyte proliferation. Although these changes in phenotype can be observed *in vitro* it

remains largely unknown what the effect is *in vivo*. Our studies have demonstrated enhanced antimicrobial properties in both an animal model and naturally occurring infections utilizing TLR3 primed MSC.[55] A recent study reports increased wound healing in a rodent wound model utilizing TLR4 primed MSC demonstrating that the activity of these cells *in vivo* may be different than what is predicted based on *in vitro* studies. [97] Other activation strategies have been assessed to enhance MSC function prior to administration.[56] Hypoxia is a commonly utilized pre-conditioning strategy that is used to simulate the conditions present in a wound bed or area of tissue injury.[53] This is thought to be protective and enhance the longevity and improve migratory ability of the MSC. Genetic modification of MSC to increase VEGF expression has been shown to increase wound healing properties of MSC.[98] Pre-activation with other mediators such as interferon γ (IFNγ) has been demonstrated to enhance the immunosuppressive properties of MSC and bears further investigation as well.[54]

**Antibacterial properties of MSC**

Multiple animal models have demonstrated benefit of MSC therapy in sepsis and a variety of other infectious diseases such as tuberculosis, mastitis and pneumonia.[44,62,87,88,92,94,99-115] The beneficial effects of MSC in sepsis appear to be multifactorial and involve not only a direct antibacterial effect but also a dampening down of the overwhelming inflammatory response. Models of a sepsis have demonstrated increased bacterial killing with improved survival and mechanisms thought to be involved include enhanced phagocytosis by monocytes, decreased pro-inflammatory cytokines, an increase in anti-inflammatory cytokine production by monocytes, decrease in organ injury, and enhanced activity of neutrophils.[87,99,102,116,117] Several common themes have emerged from studies of MSC and infection or sepsis. First there appears

to be an improved response when antibiotics are combined with MSC therapy. Animal models utilizing a cecal ligation and puncture model of polymicrobial sepsis found greatly increased survival when antibiotics and stem cells were combined.[112,117] In chapter 2 we describe this synergistic effect in a mouse model of biofilm wound infection and demonstrated *in vitro* that the direct antibacterial effect of MSC are partially abolished by blocking activity of cathelicidin antimicrobial peptides. These peptides are known to work synergistically with antibiotics and so may be responsible for the observed synergistic effect of MSC and antimicrobial agents.[118] Antimicrobial peptides that have been demonstrated to be expressed by MSC include cathelicidins (LL-37), β-defensins, hepcidins, lipocalin and surfactant protein D (SPD).[94,119] Antimicrobial peptides have a wide range of activity. LL-37 and the cathelicidin peptides act directly on bacteria to insert holes in the membrane and thus effect direct killing.[120] However, they also have substantial immunomodulatory effects such as chemotaxis (recruitment of neutrophils and macrophages), and wound healing effects by stimulating angiogenesis and migration of keratinocytes.[121-123] Hepcidin interferes with iron absorption of bacteria.[124] Defensins also create holes in bacterial membranes but have additional immune modulatory function involved in monocyte secretion of cytokines and angiogenesis.[120] *In vitro* experiments in equine cells support the role of MSC secreted AMPs in direct bacterial killing. A recent study utilizing *S. aureus* and *E Coli* found that the factor responsible for killing was less than 30kd for *E Coli* and less than 10kd for *S aureus* and that antibodies that block the action of common antimicrobial peptides partially blocked the antimicrobial activity of MSC secreted factors.[125] In further support, an *in vitro* study found increased killing of imipenem resistant *Pseudomonas aeruginosa* that was partially attributed to antimicrobial peptide LL-37.[115] These findings support the role of AMPs in bactericidal action of MSC but other studies have

documented that, at least *in vivo*, the immune system is equally important in the protective effect of MSC. [91,112]. The efficacy of MSC against bacterial infections is likely multifactorial involving augmentation of the immune system as well as secreted antimicrobial factors.

**MSC in Osteoarthritis**

Osteoarthritis is a complex disease process that involves inflammation and degeneration of the joint with loss of cartilage and exposure of subchondral bone worsening pain and increasing inflammation. It is a leading cause of disability world-wide.[126] Clinical trials utilizing MSC for treatment of arthritis are common with more than 22 clinical trials completed to date that report use of MSC for treatment of osteoarthritis. There are over 70 trials registered worldwide. All of these studies have delivered the cells via intra-articular injection, however, much variability exists regarding source, dose and follow-up which make results of the trials difficult to compare and interpret.[127,128,129] In addition, age related OA is now known to be a systemic problem involving oxidative stress, systemic inflammation, reduced ability of chondrocytes to initiate repair as they age.[130,131] Therefore systemic treatment may be beneficial to treat the disease process occurring throughout the body. In a study of dogs with age related osteoarthritis, systemic treatment with MSC improved behavior and activity scores.[132] A study in human patients demonstrated systemic downregulation of inflammation that persisted for 3 months following intra-articular injection of MSC. They demonstrated increased T regulatory cells in the blood of patients following local injection of MSC that persisted for 3 months.[76] It then follows that systemic therapy may be of increased benefit to further decrease systemic inflammation.

Multiple studies demonstrate increased comfort in human patients with OA following intra-articular injection of MSC.[133,134] The purported benefit of MSC in OA is multifactorial and involves downregulation of inflammation and change of the phenotype of chondrocytes from hypertrophic and fibrotic to a proliferative state. It has also been demonstrated that MSC protect chondrocytes from apoptosis.[135] *In vitro* experiments of MSC in coculture with synoviocytes and chondrocytes have shown that MSC downregulate production of inflammatory cytokines IL1β, TNFα and IL-6 from these cells only when they are overexpressing them as they would in pathologic disease states.[135] Additional studies have documented similar downregulation of inflammation and rodent models have shown increased cartilage regeneration.[136,137] The possibility of cartilage regeneration from MSC therapy is the subject of much debate but a recent meta-analysis reported consistently improved pain and mobility scores among all groups of treated patients with improved outcomes if cells were utilized earlier in the disease process.[134] Further investigations into the mechanism of action, dose and best route of administration of MSC are clearly needed but there appears to be a clinical benefit of treatment that can be optimized with better understanding of the effect.

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# CHAPTER 2

## Activated Mesenchymal Stem Cells Interact with Antibiotics and Host Innate Immune Responses to Control Chronic Bacterial Infections


### Summary

Chronic bacterial infections associated with biofilm formation are often difficult to resolve without extended courses of antibiotic therapy. Mesenchymal stem cells (MSC) exert antibacterial activity *in vitro* and in acute bacterial infection models, but their activity in chronic infection with biofilm models has not been previously investigated. Therefore, we studied the effects of MSC administration in mouse and dog models of chronic infections associated with biofilms. Mice with chronic *Staphylococcus aureus* implant infections were treated by i.v. administration of activated or non-activated MSC, with or without antibiotic therapy. The most effective treatment protocol was identified as activated MSC co-administered with antibiotic therapy. Activated MSC were found to accumulate in the wound margins several days after i.v. administration. Macrophages in infected tissues assumed an M2 phenotype, compared to untreated infections which contained predominately M1 macrophages. Bacterial killing by MSC was found to be mediated in part by secretion of cathelicidin and was significantly increased by antibiotics. Studies in pet dogs with spontaneous chronic multi drug-resistant wound infections demonstrated clearance of bacteria and wound healing following repeated i.v. administration of activated allogeneic canine MSC. Thus, systemic therapy with activated MSC may be an effective new, non-antimicrobial approach to treatment of chronic, drug-resistant infections.

**Background**

Chronic implant and wound infections continue to be a major source of morbidity and mortality in patients, driven in part by the increasing prevalence of drug-resistant bacteria and by the greater incidence of diseases such as diabetes mellitus that predispose to chronic infections [1-4]. Infections characterized by the development of bacterial biofilms, which often develop on the surface of implants such as catheters, orthopedic devices, or partially devitalized tissues, are particularly difficult to manage with antibiotic therapy alone, often requiring weeks to months of continuous therapy. Despite aggressive antibiotic therapy, in many cases biofilm-infected devices or implants must be removed to fully resolve these chronic infections. Thus, there is a strong unmet need for alternatives to conventional antibiotic therapy for management of chronic infections [5]. Current alternative therapies for management of chronically-infected wounds include the use of antibiotic impregnated implant materials or biological scaffolds, administration of biofilm disrupting agents, and administration of immunotherapy together with antibiotics [6,7].

It has been previously established that mesenchymal stem cells (MSC) exhibit antimicrobial properties. These effects have been demonstrated to be both direct and indirect. [8] For example, MSC have been shown to secrete antimicrobial peptides, including cathelicidins, lipocalin-2 and beta-defensins [9-14]. In some studies, secretion of the cathelicidin LL-37 by MSC was shown to be enhanced by bacterial products, indicating that MSC can upregulate antimicrobial activity in the presence of infection. [9] Mesenchymal stem cells have also been shown to interact with the host innate immune system to increase antibacterial activity. For example, several studies have demonstrated increased phagocytosis and killing properties of monocytes and neutrophils following exposure to MSC-secreted factors, and it has been

observed that MSC also suppress inflammation in sepsis models. [15] [16-26] The potential of MSC therapy for systemic infection management is also supported by a recent study which found that MSC acted synergistically with antibiotics to enhance survival in a mouse model of sepsis.[24] Other studies have demonstrated wound healing properties of MSC, including stimulation of angiogenesis, activation of resident stem cell populations, reduction in inflammation, recruitment of immune cells, and suppression of scarring. [27-31]

Other studies have examined whether pre-activation of MSC with Toll-like receptor (TLR) agonists or cytokines can alter their antimicrobial properties. [32] For example, it was found that activation of MSC with TLR3 ligands triggered release of factors that enhanced neutrophil survival. [33] [17] In addition, TLR3 agonists were shown to stimulate greater release of neutrophil chemokines than other TLR agonists and have also been shown to stimulate the migratory properties of MSC. [33-36] These previous findings therefore suggest that it may be possible to manipulate the antimicrobial and immunological properties of MSC *ex vivo* prior to therapy to stimulate greater clinical benefit in the setting of chronic infections.

The utility of treatment with MSC has been demonstrated in animal models of acute infection and wound healing (ref). [10,25,26,28-31,37-39] [12]However, to date there is no published experimental or clinical evidence that administration of activated MSC can be effective for infection management in two common clinical scenarios: treatment of chronically-infected implants, and treatment of infections involving multidrug resistant bacterial infections. [40] Therefore, we used a murine *S aureus* implant infection model to assess the effect of systemic treatment with TLR-activated MSC and concurrent antibiotic therapy for treatment of chronic implant infections. These studies were conducted to optimize treatment conditions and to elucidate microbiological and immunological mechanisms of activity. In addition, proof-of-

concept studies of combined activated MSC and antibiotic therapy were conducted to assess the efficacy of activated MSC and antibiotic therapy in pet dogs with spontaneous multi-drug resistant infections.

The findings reported here indicated that systemic administration of activated MSC was effective in controlling chronic implant infection when co-administered with antibiotic therapy, and that the effect involved both direct and indirect antibacterial and immunological mechanisms. Importantly, the pilot studies in dogs with spontaneous chronic infections demonstrated that repeated i.v. administration of activated allogeneic MSC could be employed effectively with antibiotics for treatment of chronic, drug-resistant infections. These findings suggest that systemic treatment with activated MSC may represent an important new approach to managing chronic infections with multi-drug resistant bacteria.

**Materials and Methods**.

*Bacteria and biofilm preparation.*

The Xen36 strain of *Staphylococcus aureus*, expressing the luciferase gene for bioluminescent imaging, was purchased from Caliper Life Sciences (Perkin Elmer, Santa Clara, CA). Bacteria were propagated in LB medium (BD Falcon) and utilized to coat mesh implanted material during the log phase of growth.

*Mice.*

Mice used in these studies included 8-12 week old CD-1 mice, male and females, and were purchased from Charles River Laboratories. Adipose tissues from transgenic mice expressing GFP under the ubiquitin promoter were kindly provided by Dr. William Janssen, National Jewish Hospital, Denver, CO. [41]. CCR2-gfp reporter mice (on the C57Bl/6 background)

were provided by Dr. Eric Pamer (Memorial Sloan Kettering, NY)[42]. Animals were maintained in sterile microisolater cages under controlled temperature and humidity and were fed sterilized food and water. All procedures involving mice in this study were approved by the Institutional Animal Care and Use Committee at Colorado State University.

*Clinical trial design for wound infection study in pet dogs.*

Dogs enrolled in a pilot clinical study of MSC therapy for wound infection management were client-owned animals that presented to the Colorado State University Veterinary Teaching Hospital for treatment of multidrug resistant chronic wound infections. These studies were approved by the Colorado State University Institutional Animal Care and Use Committee and by the Clinical Review Board at the Veterinary Teaching Hospital. Inclusion criteria involved presence of a documented infection with a multi-drug resistant organism in a site where cultures could be obtained on a regular basis for quantitative analysis. In addition, the infection must have failed conventional treatment for a period of greater than 2 weeks. To fulfill this criteria, animals must have failed treatment with antibiotics and surgical debridement as evidenced by continued symptoms of infection and positive culture of organisms at the site. Animals were excluded from the trial if there was any evidence of systemic disease in which treatment with MSC may be contraindicated, such as neoplasia or that may affect immune function, such as Cushing's disease or organ failure. Animals were also excluded if the site of the infections was such that obtaining quantitative cultures would not be possible.

Dogs enrolled in the trial were treated with allogeneic MSC pre-activated with 10ug/ml of poly I:C (InVivoGen, San Diego CA) x 1hr at a dose of $2x10^6$ cells per kg body weight. Cells were then washed 3x in DPBS (Sigma-Aldrich, St. Louis, MO) and re-suspended in DPBS at a volume of 10ml (30 x $10^6$ cells or less), 15 ml (30-45x$10^6$ cells) or 20ml (>45x$10^6$ cells).

Sodium heparin (Fresenius Kabl USA, Lake Zurich IL) was added immediately prior to injection at 200IU per 10ml. Cells were delivered by intravenous (i.v.) infusion over 15 minutes. Heart rate and respiratory rate were monitored during and for 10 minutes after infusion. Antibiotic therapy was kept consistent for the duration of the study and continued for 1 week after the last injection was administered. Dogs were treated every 2 weeks for a total of 3 treatments, and quantitative cultures were obtained prior to each treatment. Cultures were obtained by fine needle aspirate of 4 quadrants of the infected area. The aspirates were placed in 1ml of TSB (trypticase soy broth, Sigma Aldrich) and quantitative cultures obtained by 10 fold dilution in PBS and plating on LB agar (Luria Broth, Sigma Aldrich). Dogs were considered for additional MSC injections if there was a significant decrease in bacterial infection as evidenced by a significant decrease in number of bacteria in the wound or eradication of 1 or more but not all species of bacteria present at the infection site. Two weeks after the final treatment, quantitative cultures were again performed, and patients were then monitored by follow up phone calls every 2-3 months. Dogs were considered to have a positive response if clinical signs improved and bacterial counts decreased significantly over the treatment interval. The infection was considered eradicated if the animal had more than 2 negative cultures at least 2 weeks apart and the infection site was fully healed with resolution of clinical signs.

*Generation of adipose-tissue derived MSC*.

Adipose tissue-derived MSC were generated from abdominal and inguinal adipose tissues collected from mice. To prepare MSC cultures, adipose tissues were minced under sterile conditions, incubated in a 1 mg/ml solution of collagenase (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% $CO_2$ for 30 minutes, and then triturated. The cell suspension was then centrifuged at 1,050 X G to pellet the stromal vascular fraction (SVF), which was resuspended in MSC

culture medium. MSC culture medium consisted of low glucose DMEM (InVitrogen/Gibco, Carlsbad, CA) supplemented with essential and non-essential amino acids (InVitrogen/Gibco), glutamine (InVitrogen, Gibco), 15% heat-inactivated FBS (Cell Generation, Ft. Collins, CO), and penicillin and streptomycin solution (InVitrogen/Gibco). Cells in medium were allowed to adhere in tissue culture flasks (BD Falcon, Bedford, MA) for 72hr, after which the non-adherent cells were removed and fresh medium was added. When cells reached 80-90% confluence, they were passaged using trypsin-EDTA solution (InVitrogen/Gibco). Cells for i*n vivo* experiments were used at passage 2-3 for all experiments. The cell surface phenotype of MSC was determined by flow cytometry, and the ability of the MSC to undergo tri-lineage differentiation was assessed as described previously [43].

Canine MSCs were generated from adipose tissue obtained from the inguinal region of healthy, young purpose bred research hounds. The adipose tissue was frozen in 1 gm aliquots in freezing media consisting of 75% FBS, 15% MSC medium, and 10% DMSO at -80 degrees within 2 hours of sample collection. Freshly-cultured MSC were generated from frozen adippose tissue aliquots for each treatment, and the cells used between passages 2 and 5 for all dogs. The cryopreserved adipose tissue was thawed and rinsed in DPBS twice. Subsequently the tissue was minced, digested in collagenase, the SVF collected and culture-expanded as described above for mice. At the time of adipose tissue collection, blood was obtained from each donor dog for a complete blood count and chemistry profile. Additional testing for infectious disease included serology to test for *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Dirofilaria immitis*, *Ehrlichia canis* (4DX, Idexx laboratories), and PCR was performed to detect the presence of *Hemoplasma spp*, *Ehrlichia spp*, *Bartonella spp*, and *Rickettsial spp* at the state veterinary diagnostic laboratory. All donors had normal CBC and chemistry profile and were negative on

all infectious disease testing. In addition, each stem cell culture was tested for bacterial, fungal, and mycoplasma contamination at the time of stem cell administration using in house cultures (blood agar, McConkey agar, Sabourad Dextrose agar – BD Falcon, and mycoplasma agar – Udder Health systems, Bellingham WA). Bacterial cultures were incubated at 37°C for 48hr, mycoplasma cultures were incubated at 37°C, 5% $CO_2$, for 7 days, and fungal cultures were incubated at room temperature in the dark for 30 days. No contamination was detected in any culture. Cell surface phenotype was assessed via flow cytometry and tri-lineage differentiation was performed as previously described[43].

*Staphylococcus infected mesh model and IVIS imaging.*

To assess the effects of MSC on chronic wound infections, we utilized a chronic *S. aureus* implant infection model previously developed by our laboratory. [44] Briefly, surgical mesh material (Covidien, Mansfield MA) was cut into 0.5 mm squares, then incubated with Xen36 bacteria in sterile 10 mm petri dishes for 24h with gentle rotary shaking at 37°C. The mesh was then washed in PBS prior to implantation into subcutaneous tissues. Mice were anesthetized with isoflorane, mesh was inserted surgically into a skin flap created in the subcutaneous space over the shoulder, and then the wound was closed with surgical staples. Mice were imaged using an IVIS Spectrum live animal imager (Caliper Life Sciences) to detect and quantify bacteria growing on implanted mesh. We previously demonstrated that the luminescent signals obtained from mice with implants of Xen36 *S. aureus* were linearly correlated with the bacterial burden, as determined by conventional bacterial CFU determination.[44]

*IVIS imaging of injected MSC.*

To track the distribution of MSC *in vivo*, cells were labeled with the far red dye DiR (DiIC18(7) (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) (Molecular Probes and ThermoFisher Scientific, Waltham, MA) by incubation at a concentration of 2.5 uM in PBS at 37°C for 30 min, followed by washing and resuspension in DPBS prior to injection. Animals were injected with 1 X $10^6$ labeled MSC in 200µl DPBS with heparin (100 IU/ml) by the tail vein route, and mice were then briefly anesthetized and subjected to IVIS imaging at the indicated time points. Images were acquired by the IVIS imager, and images were reconstructed using IVIS image software, which was also used to quantitate the density of cells in vivo, using reference images produced with known quantities of DiR-labeled MSC.

*Bacterial quantitative culture from in vitro assays and wound samples.*

Bacteria numbers were quantitated by plating serial, log10 dilutions of culture supernatants obtained by gentle pipetting, and then plating on LB agar quadrant plates (Sigma-Aldrich) and counting bacterial colonies 24hr after culture at 37°C. The $\log_{10}$ CFU was determined numerically from plate counts. For quantitation of bacteria in wound tissues of mice, tissues at the infection site were collected and weighed and then homogenized in PBS using a tissue homogenizer. The supernatants of these homogenates were then serially diluted and plated, and colonies with the typical appearance of *S. aureus* were quantitated.

For determination of the bacterial burden in wound tissues of dogs in the clinical trial, 4-quadrant fine needle aspirates were collected from around the wound perimeter, inoculated into LB medium, then serially diluted and plated on LB plates for quantitation and Trypticase Soy Blood Agar and MacConkey plates (BD Falcon, San Diego CA) for bacterial growth and enumeration. Plates were incubated for a minimum of 48hr at 37°C to confirm absence of bacterial growth. Animals with septic arthritis underwent aspiration of the affected joint under

sedation every 2 weeks during the study period. Joint fluid was also evaluated cytologically. Bacteria were speciated by morphology and biochemical testing, and bacterial CFU were determined by manual counting. Bacterial sensitivities were performed by the Colorado State veterinary diagnostic laboratory (200 West Drake Road, Fort Collins CO 80523).

*Bacterial killing assay.*

To assess the ability of MSC to kill bacteria, cells or conditioned medium (CM) from MSC were utilized. CM was generated by plating $5x10^5$ cells per well in a 24 well plate with 500ul per well of antibiotic free media and incubating at 37°C. Conditioned media was collected 24 h after the cells were plated and were immediately frozen at -80°C and thawed immediately before use. MSC or CM were inoculated with log phase *S. aureus* cultures, typically at a concentration of 1 X $10^5$ CFU bacteria per ml of CM or per ml of cells in 24-well plates, in complete MSC culture medium, but without antibiotics. Cells in culture were inoculated with bacteria at a multiplicity of infection (MOI) of 10:1 (bacteria per cell). Co-cultures were incubated at 37°C for 3hr, then numbers of viable bacteria was determined.

To assess the potential role of cathelicidins in MSC-mediated bacterial killing activity, MSC CM was incubated with an anti-cathelicidin antibody (Abcam, San Francisco CA) at 50 µg/ml for 10 minutes prior to addition of bacteria. An irrelevant rabbit IgG (Abcam, San Francisco CA) was used at the same concentration in parallel cultures as a control for specific cathelicidin activity neutralization.

The ability of MSC or MSC CM to augment antibiotic activity was performed by assessing bacterial killing activity or MSC or CM, with or without the addition of low concentrations of cefazolin. In addition, studies of enhancement of antibiotic activity were also done using recombinant LL-37 (InVivoGen), with or without low concentrations of cefazolin.

*Neutrophil bacterial phagocytosis assay.*

Neutrophils were collected from the peritoneal cavity of mice 24hr following intra-peritoneal injection of 1 ml aged thioglycollate, as described previously [45]. For assessment of the effects of MSC CM on neutrophil phagocytosis, $1x10^6$ neutrophils were incubated with MSC-CM for 1hr at 37°C. The neutrophils were washed twice in PBS, seeded in 4-well chamber slides (BD Falcon), inoculated with log phase *S. aureus* at an MOI of 1:1 for 30 min at 37°C, and then treated with gentamicin (300ug/ml) for 30 minutes to eliminate extracellular bacteria. The neutrophils were then washed twice with PBS, fixed with 1% paraformaldehyde, and immunostained for detection of intracellular *S. aureus* using an anti-staphylococcus antibody (Genway Biotech, San Diego CA) and with a fluorescently conjugated antibody to Ly6G (eBioscience, San Diego CA). The relative numbers of phagocytosed *S. aureus* per neutrophil were determined by microscopy with an Olympus IX-83 confocal microscope, using appropriate lasers and filters and quantitated by image analysis software (Image J).

*Monocyte migration assay*.

Monocytes were collected from the peritoneal cavity of mice 72 h after intra-peritoneal administration of 1 ml aged thioglycollate, as described previously [45]. Monocytes collected from the peritoneal cavity by lavage with PBS were washed and then resuspended in complete medium. Migration was measured using Boyden chambers (BD Falcon) with 8 um pore diameter. Monocytes ($2.5 X 10^5$ cells per well) were added to the top chamber in complete medium, MSC CM was added to the bottom chamber, and the assay was run for 4hr at 37°C. Recombinant mouse CCL2 (100 ng/ml; R&D Systems Inc., Minneapolis, MN) was used as a

positive chemokine control. The numbers of migrated cells were determined by first removing cells from the top of the membrane with cotton swabs, then removing the filter membrane, staining with crystal violet, and manually counting the number of monocytes adherent to the bottom of the membrane, averaging at least 5 random high power fields per sample. Data were displayed as average number of migrated cells per membrane per treatment condition.

*Tissue histology.*

Wound tissues were collected, immersion fixed in 10% neutral buffered formalin, and paraffin-embedded for routine histological processing. Tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for histopathological evaluation. Images were captured using an Olympus IX-83 confocal microscope and Olympus SC30 camera.

*Immunohistochemistry and fluorescent imaging.*

For immunofluorescence evaluation of MSC trafficking to sites of infection, wound tissues were immersion fixed in 1% paraformaldehyde-lysine-periodate fixative (1% paraformaldehyde in 0.2M lysine-HCL, 0.1M anhydrous dibasic sodium phosphate, with 0.21% sodium periodate) for 24hr at 4°C. Following fixation, tissues were placed in a 30% w\v sucrose solution for 24hr at 4°C, prior to embedding and freezing in O.C.T. compound (Tissue Tek, Sakura Finetek USA, Inc., Torrance, CA). Embedded tissues were sectioned to a thickness of 5μm for immunostaining. Tissue sections were immunostained with a rabbit antibody to GFP (Life Technologies) to enhance GFP signal intensity, followed by incubation with a donkey anti-rabbit IgG antibody, conjugated to AF647 (Jackson ImmunoResearch, West Grove, PA). To identify macrophages with an M1 functional phenotype, tissues were permeabilized with 0.01% Triton X and immunostained using an anti-iNOS antibody (Thermo Fisher Scientific, Waltham, MA), followed by incubation with a secondary donkey anti-rabbit cy3 conjugated antibody

(Jackson ImmunoResearch). Macrophages with an M2 phenotype were identified using anti-arginase antibody (Santa Cruz Biotechnology Inc., Dallas, TX) followed by incubation with a secondary donkey anti-goat antibody conjugated to AF647 (Jackson ImmunoResearch).

Immunofluorescent staining for cathelicidin production by MSC was performed by seeding $2x10^5$ MSC onto each well of a 4-chamber slide and incubating overnight at 37°C followed by permeabilization with 0.01% TritonX with subsequent incubation with a rabbit anti-cathelicidin antibody (Abcam, San Francisco CA) followed by incubation with a donkey anti-rabbit IgG antibody conjugated to AF555 (Jackson ImmunoResearch).

For imaging of MSC labeled with DiR or DiD dyes, the tissues were processed similarly and examined with an Olympus IX-83 confocal microscope, using appropriate lasers and filters. Image analysis was performed using CellSens software (Olympus).

*Cytokine assays.*

Cytokine concentrations in supernatants were assayed using commercial ELISA assays performed according to manufacturer's directions. The ELISA kit for murine CCL2 detection was purchased from R&D Systems Inc.

*Statistical analyses.*

Statistical comparisons between data sets with two treatment groups were done using nonparametric t-tests (Mann-Whitney test). Comparisons between 3 or more groups were done using ANOVA, followed by Tukey multiple means post-test. Tests for synergy in bacterial killing were performed using a 2-way ANOVA, according to a previous approach. [46] Statistical analyses were performed using Prism5 software (GraphPad, La Jolla, CA). For all analyses, statistical significance was determined for $p<0.05$.

*Study approval*.

All studies involving animal use were approved by the Colorado State University Institutional Animal Care and Use Committee and use of pet animals enrolled in clinical trials was additionally reviewed by the Clinical Review Board at the Veterinary Teaching Hospital at Colorado State University. Prior to enrollment of an animal in the clinical trial, owner informed consent was obtained.

**Results**.

*Effects of i.v. treatment with activated MSC on wound bacterial burdens*.

Previous studies have demonstrated that topical administration of MSC can improve wound healing and infection control [10,29,31]. Other studies have evaluated the effectiveness of MSC administration for control of acute infections, including pneumonia and sepsis, in mouse infection models [9,19 13,24,26,47-50]. However, the use of MSC to treat chronic bacterial infections associated with biofilms has not been evaluated previously. We used a biofilm model that utilizes the s.c. implantation of surgical mesh coated with luciferase-expressing *S. aureus* to assess the effects of MSC treatment on chronic bacterial infections[44]. In this particular model, treatment with antibiotics is ineffective in reducing bacterial infection at the wound site. Six different treatment groups of mice (n = 5 per group) were used in the study: 1) untreated control animals, 2) animals treated with antibiotics only (orally-administered amoxicillin-clavulanic acid), 3) animals treated with MSC only, 4) animals treated with activated MSC only, 5) animals treated with MSC plus antibiotics, and 6) animals treated with activated MSC plus antibiotics. Mesenchymal stem cells were administered i.v. by tail vein injection 3 times, at 3-day intervals, at a dose of 1 X $10^6$ cells per injection per mouse. The systemic route of administration of MSC

was selected because initial studies determined that local s.c. injection of activated MSC around the site of the infected mesh, in combination with antibiotic therapy, was ineffective in clearing infections (data not shown).

The results of *in vivo* bioluminescence imaging and direct bacterial counts revealed that administration of antibiotics or MSC alone (activated or resting) did not significantly reduce the bacterial burden at the wound site (**Figure 2.1**). Likewise, the combination of antibiotic therapy and administration of non-activated MSC was also ineffective (**Figure 2.1**). The combination of TLR3 ligand-activated MSC with antibiotic therapy was found to be the only treatment that significantly reduced the bacterial burden at the wound site, based on both bioluminescence imaging and direct bacterial enumeration by convention culture (**Figure 2.1A-C**). Previous studies have also reported that activation of MSC with the TLR3 ligand polyinosinic polycytidylic acid (polyI:C) increased MSC antibacterial activity in mouse sepsis models [51]. Thus we conclude that systemic administration of MSC activated *in vitro* prior to administration interacted synergistically with antibiotic therapy to efficiently reduce bacterial numbers in chronic wounds associated with biofilms.

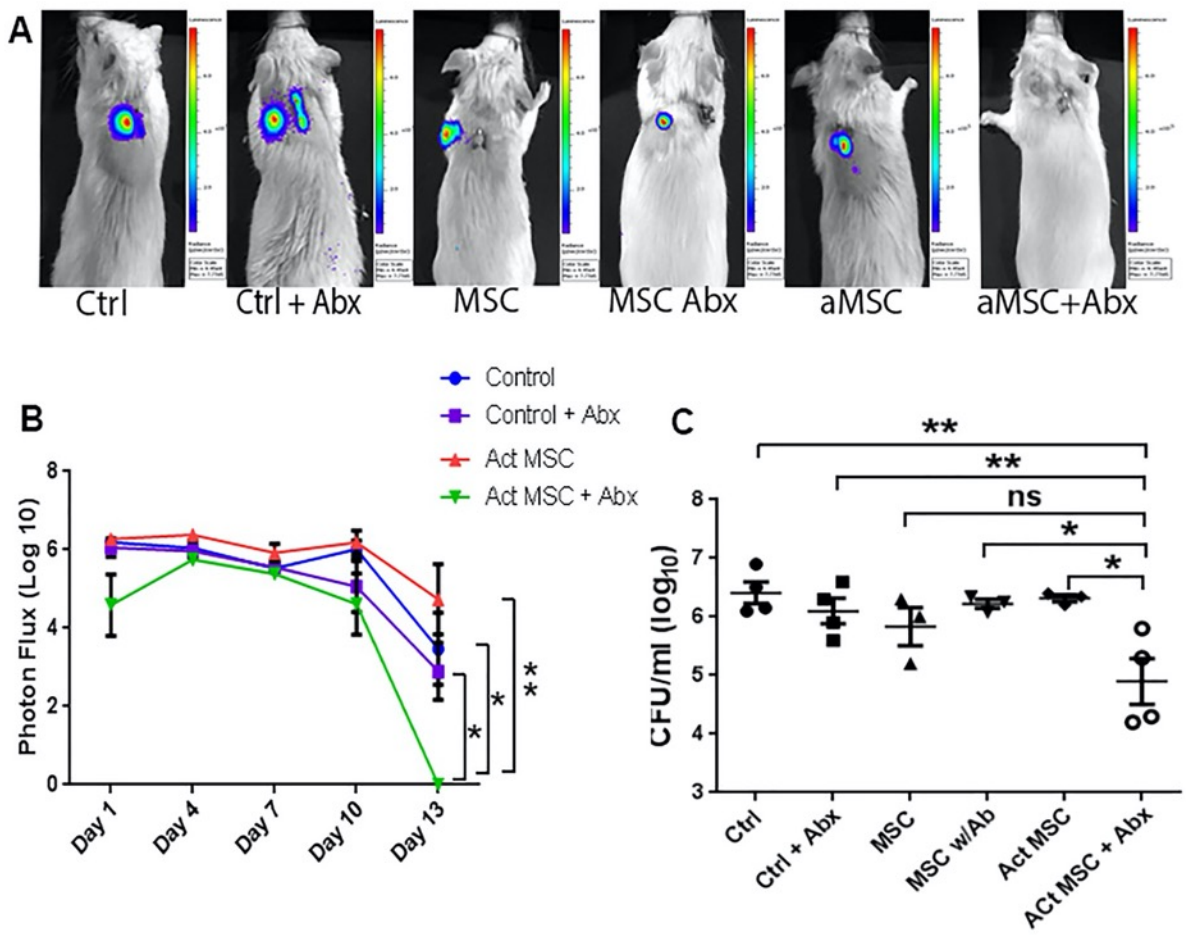



**Figure 2.1**. *Effects of MSC and antibiotic administration on bacterial infection in mouse chronic wound implant infection model*. CD-1 mice (n = 5 per group) were implanted with surgical mesh on which biofilms has been established with a luciferase-expressing strain (Xen36) of *S. aureus*, as described in Methods. On day 2 after implant placement, mice were randomly assigned to the following treatment groups: untreated controls; treatment with antibiotic (amoxicillin-clavulanic acid, continuous treatment in drinking water) only; treatment by i.v. administration of untreated MSC; treatment by i.v. administration of activated MSC; treatment by administration of untreated MSC plus antibiotics; and treatment with activated MSC plus antibiotics. Mice were imaged by IVIS bioluminescence imaging every 2-3 days following mesh implantation to assess the effects of treatment on bacterial burden. In (A), representative IVIS images of wounds in one mouse of each treatment group (n = 5 animals per group) are depicted. Quantitative mean photon intensity from each group of treated animals over time is depicted in (B). Similar results were obtained in 2 additional experiments. In (C), the mean bacterial burden in wounds were determined on day 14 of infection by quantitative counting, as described in Methods, and compared statistically by ANOVA and Tukey multiple means comparison. * denotes $p < 0.05$

*Impact of MSC treatment on wound histology*.

Histologically, infected tissues from the implant infections in antibiotic-only treated animals were characterized as chronic, purulent inflammation with abscess formation with intense neutrophilic infiltrates **(Figure 2.2A-C**). In contrast, infected tissues from animals treated with activated MSC and antibiotics contained primarily a mild monocytic cellular infiltrate (**Fig 2.2 D-F**). Grossly, infected tissues from animals treated only with antibiotics contained large abscesses centered around the implanted mesh material (**Figure 2.2A**). Conversely, infected tissues from animals treated with activated MSC plus antibiotics were relatively free of purulent material and reactive tissues, such that implanted mesh could be readily visualized in the implanted tissues (**Figure 2.2D**). These findings suggested that systemic administration of MSC was also associated with substantial improvement in infected tissue healing.

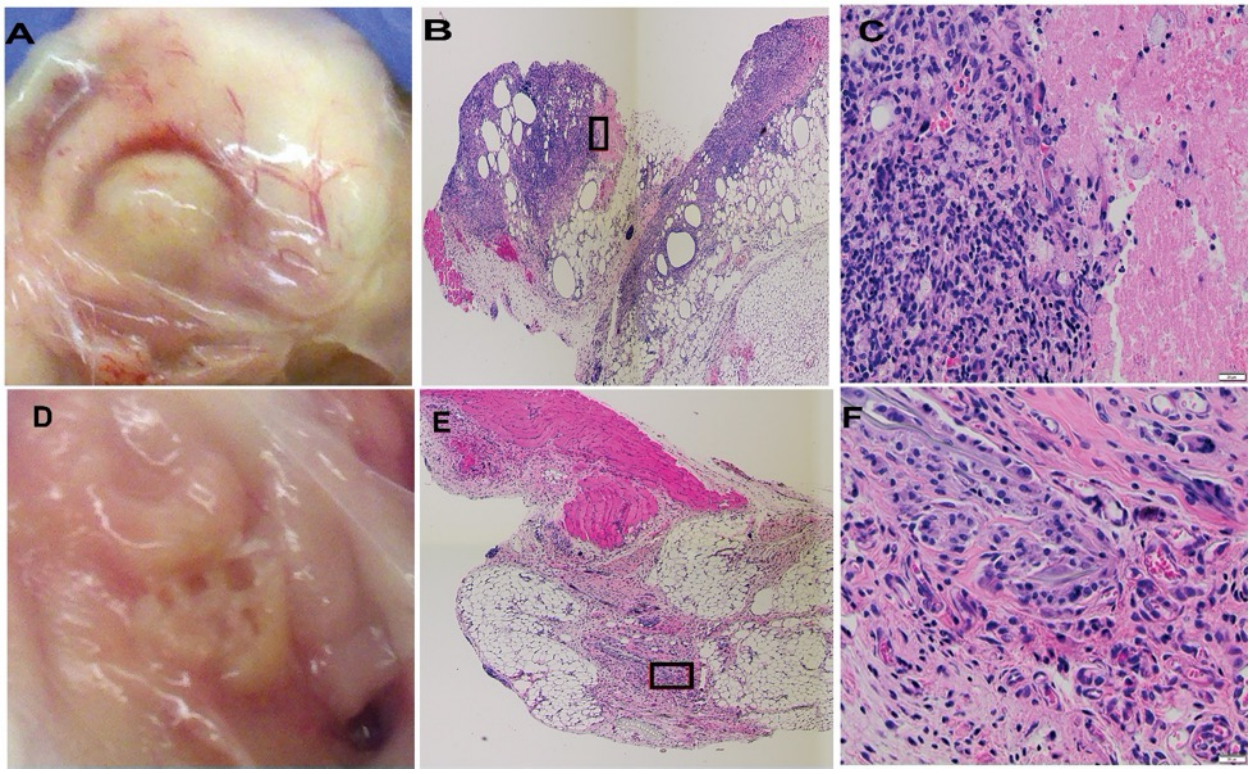



**Figure 2.2**. *Effects of activated MSC administration on wound healing in mouse chronic infection model*. Mice with *S. aureus* infected mesh implants were treated as described in Figure 1. At the completion of the study (day 14), mice were euthanized and skin was dissected to reveal the implant site. In (A), representative photograph of a wound treated with antibiotics only, and corresponding representative photomicrographs of H&E sections (B,C) revealing suppurative inflammation at the wound site. In (D) representative photograph of a wound in a mouse treated with activated MSC plus antibiotics, with implanted mesh visible in wound bed. In (E,F) representative photomicrographs of H & E sections from a mouse treated with activated MSC plus antibiotics, revealing mild monocytic inflammation.

*Migration of MSC to sites of infection following i.v. administration*.

Previous studies have found that the majority of MSC injected i.v. initially lodge in the lungs, therefore, we used several studies to investigate whether MSC would reach in fact be capable of reaching the site of chronic infection in our model [52]. In one study, mice were injected with MSC generated from the adipose tissues of GFP transgenic donor mice to allow for in vivo tracking. [41] For this study, animals with established implant infections were injected twice (3 days apart) i.v. with activated gfp-MSC (1 X $10^6$ cells per injection), starting on day 2 after the infected mesh was implanted. After 2 i.v. injections of gfp-MSC, infection tissues were excised 48hr after the last injection and immunostained for detection of $gfp^+$ cells. Substantial numbers of injected $gfp^+$ MSC were observed in the infected tissue margins of treated mice (**Figure 2.3E,F**), thereby providing evidence that i.v. injected MSC were in fact able to migrate efficiently to the infection site. Mice were also injected with DiD-labeled MSC (both activated and non-activated) for live animal imaging assessment of the effect of activation on MSC migration. We found that activated MSC accumulated to a significantly greater degree in infected tissues than non-activated MSC, as assessed by confocal microscopy **(Figure 2.3A-D**).

MSC were labeled *in vitro* with the far-red fluorescent membrane dye DiR (Molecular Probes) for live animal imaging (Thermo Fisher Scientific). Mice with s.c. implanted *S. aureus* infected implants were injected i.v. at 3-day intervals with DiR-labeled MSC (1 X $10^6$ cells per injection) for a total of 4 injections and imaged daily (**Figure 2.3G-I**). We found that DiR-labeled MSC could be readily detected in the lungs within hours of injection followed by the appearance of labeled MSC in the spleens of mice within 24hr of injection. Beginning at 48hr after injection, DiR-labeled MSC could also be detected accumulating around the margins of infection sites in treated mice (**Figure 2.3G-I**). The number of MSC that accumulated around

infected implants increased over time during the 20 days of monitoring. We concluded therefore that i.v. injected MSC did in fact traffic efficiently to infected implants, likely recruited in response to chemokines produced by inflamed tissues, and that activated MSC migrated more efficiently than non-activated cells.

The chemokine stromal cell derived factor -1 (SDF-1, or CXCL12) is a major chemokine regulating MSC recruitment, and high levels of SDF-1 are produced by inflamed tissues. [53-56] To assess the effects of MSC activation on their migratory behavior, we used Boyden chambers to assess MSC migration to an SDF-1 gradient. These studies revealed that poly I:C activation significantly increased MSC migration to the SDF-1 stimulus and that activation was associated with significant upregulation of CXCR4 expression (data not shown). Thus, enhanced migration may explain in part why activated MSC are more effective than resting MSC for treatment of chronic infections.

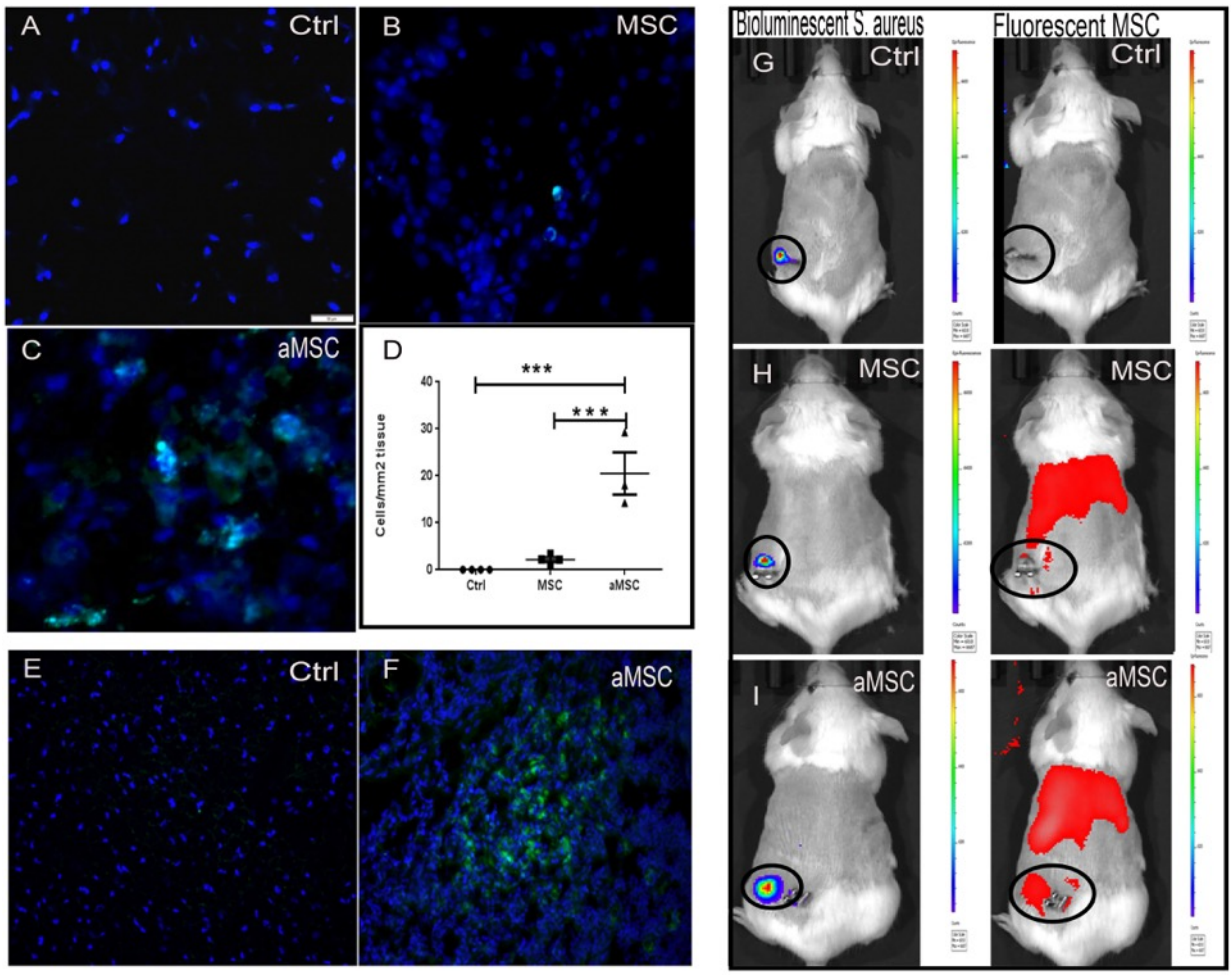



**Figure 2.3.** *Migration of labeled MSC to infected wound sites following i.v. administration*. In (A-C) representative wound tissues from animals receiving no MSC (A), resting MSC (B) and activated MSC (C). MSC were labeled with the fluorescent membrane dye DiD and detected via fluorescent microscopy ($DiD^+$ cells light blue in these images). Animals were injected with labeled MSC 24 hours after implantation of infected mesh and received a second injection 3 days later. Animals were sacrificed 2 days after the second injection and tissues were evaluated histologically. Quantitative counts of $DiD^+$ cells revealed significantly more cells present in tissues from animals receiving activated MSC (D). All animals in this study received antibiotics. Counts were compared using ANOVA with * depicting $p<.05$. In (E,F), wound tissues from a control animal and an animal injected i.v. with activated GFP-transgenic MSC to allow detection in wound tissues. Cells were administered 2 separate injections 3 days apart, and 48h later, the wound tissues were collected and evaluated by fluorescence microscopy for detection of GFP+ cells (F). In other studies, MSC (activated or non-activated) were labeled with the fluorescent dye DiR, and live mice were imaged using an IVIS imager (G-I). In the first 24h after injection, DiR+ cells were detected in the lung and spleen. By 48h post-injection, $DiR^+$ cells were apparent in the region of the infected wound. The cells accumulated at the wound sites and images were taken 3 days after the third MSC injection (H, I). There were also more activated DiR+ cells localized in the region of the wound than in mice injected with non-activated cells. Similar results were obtained in one additional animal study.

*Antimicrobial activity of MSC and interaction with antibiotics.*

Previous studies have reported that MSC can kill multiple different species of bacteria directly, including *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* [12,13,47,57] [9,19,58]. To assess whether MSC or MSC-secreted factors could kill *S. aureus* directly in our system, MSC were incubated with *S. aureus* and the effects on bacterial viability determined 3h later (**Figure 2.4**). Incubation of bacteria with MSC resulted in significant direct killing of *S. aureus* (**Figure 2.4A**). Moreover, conditioned medium (CM) from MSC cultures also elicited significant bacterial killing (**Figure 2.4B**), suggesting that MSC secreted factors were primary mediators of the rapid, MSC-mediated antibacterial activity. However, it is important to note that MSC activation with poly I:C did not increase the antibacterial activity (data not shown). Thus, MSC constitutively produced factors with antibacterial activity, and TLR activation was not required to induce production of these factors.

Results from the mouse chronic implant infection model revealed a strong interaction between activated MSC and antibiotics in terms of eradicating bacteria in chronically infected tissues (see Figure 2.1). Therefore, we next investigated whether MSC secreted factors could potentiate the bactericidal activity of antibiotics. To assess this interaction, MSC cultures were inoculated with *S. aureus*, alone or together with sub-therapeutic concentrations of the beta-lactam antibiotic cefazolin, and the effects on bacterial viability were analyzed (**Figure 2.4C**). These studies revealed that when bacteria were exposed to MSC or to MSC CM, they became more susceptible to antibiotic killing. This significant enhancement of antibiotic activity by MSC was also observed with other classes of antibiotics, including penicillins, aminoglycosides, carbapenems, and fluoroquinolones (data not shown). This phenomenon suggested a general, non-specific mechanism of enhancement of antibiotic activity by MSC produced factors.

Antimicrobial peptides, including cathelicidins, are secreted by a number of cell types including MSC and are capable of direct bacterial killing, typically by induction of membrane pores [59]. One cathelicidin (CAP-18 or LL-37 in humans) or cathelicidin-related antimicrobial peptide (also referred to as CRAMP in mice), has previously been identified as a mediator of MSC-elicited bacterial killing in human MSC. [9] Therefore, we investigated the role of CRAMP in MSC-induced killing of *S. aureus* in our system. First, it was determined using immunocytochemistry with an anti-CRAMP antibody that murine adipose-derived MSC expressed intracellular CRAMP **(Figure 2.4D**). In addition, CRAMP neutralization studies revealed that a significant portion of MSC *S. aureus* killing activity was CRAMP-dependent **(Figure 2.4F**). Next, it was determined that incubation of *S. aureus* with sub-lethal concentrations of LL-37 sensitized the bacteria to killing by cefazolin (**Figure 2.4E**). CM from MSC also induced synergistic killing of *S. aureus* when combined with cefazolin and other classes of antibiotics (data not shown). These results suggest that the demonstrated synergistic control of bacterial infection observed following *in vivo* administration of activated MSC is mediated in part by the local interaction of MSC-secreted antimicrobial peptides such as CRAMP with antibiotics at the wound site.

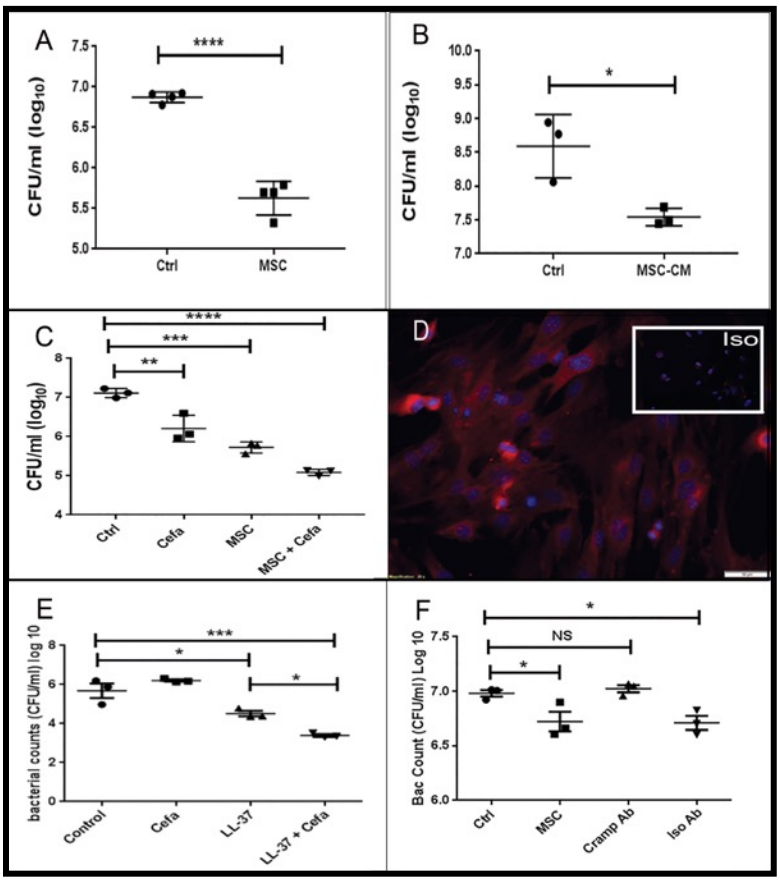



**Figure 2.4**. *Bacterial killing by MSC mediated in part by the antimicrobial peptide Cramp*. The ability of MSC to kill *S. aureus* was assessed by co-culture of bacteria directly with MSC (A) or with MSC CM (B). MSC (5 X $10^5$ cells/well) were co-cultured with bacteria (MOI=2) in triplicate wells of 24-well plates for 3h, then bacteria were collected from supernatants resuspended by gentle pipetting, and CFU were determined by serial dilution and manual counting. Bacteria were incubated directly in MSC CM for 3h in (B). In (C) synergistic killing of *S. aureus* by MSC CM and cefazolin. Cefazolin was added at a dose of 50ng/ml and bacteria added at 1x$10^6$ per well to 1ml of MSC-CM or media alone in a 24 well plate and incubated for 3 hours at 37 °C, * denotes $p < 0.05$ as assessed by ANOVA and Tukey multiple means post-test. Synergy assessed via two way ANOVA for detection of significant interaction, as described previously [46]. Immunocytology was used to assess intracellular expression of the antimicrobial peptide Cramp by MSC in (D), as described in Methods. Cells were immunostained with an irrelevant antibody (isotype panel insert) or with an anti-Cramp antibody (D) and cells were evaluated by fluorescence microscopy. Synergistic killing between antimicrobial peptide LL-37 and beta lactam antibiotic demonstrated in (E). Human LL-37 at 30ug/ml was incubated with 50ng/ml Cefazolin for 3 hours with 1x $10^6$ CFU/ml of S. *aureus*. In (F), MSC were co-cultured with bacteria and the effects of Cramp neutralization on bacterial killing were assessed Synergistic killing of bacteria was demonstrated between MSC and cefazolin (C) and between LL-37 and cefazolin (E). * denotes $p < 0.05$ as assessed by ANOVA and Tukey multiple means post-test. Similar results were obtained in two additional experiments.

*Stimulation of neutrophil phagocytosis by activated MSC*.

The preceding studies established that MSC were capable of killing *S. aureus* directly by producing antimicrobial peptides, but these studies did not however rule out a contribution of additional indirect effects of MSC leading to overall control of bacterial infection *in vivo*. In particular, activation of host innate immune defenses by systemically administered MSC would be a likely candidate for additional mechanisms of MSC antimicrobial action *in vivo*. To investigate the interaction of MSC with host innate immune responses, we first assessed the effects of MSC on neutrophil activity. Neutrophils recovered from the peritoneal cavity of mice were incubated with CM from poly I:C-activated and non-activated MSC to assess effects on bacterial phagocytosis. It was observed that CM from MSC significantly increased neutrophil phagocytosis of *S. aureus* and that this phagocytosis-enhancing effect was increased when neutrophils were incubated with CM prepared from poly I:C-activated MSC (**Figure 2.5 A-D**). These data indicate that MSC can release factors that increase the antibacterial activity of neutrophils. The effects of MSC on neutrophil antibacterial activity may be enhanced by systemic administration, as compared to local or topical administration, due to increased potential for interaction with immune cells present in circulation or in tissues.

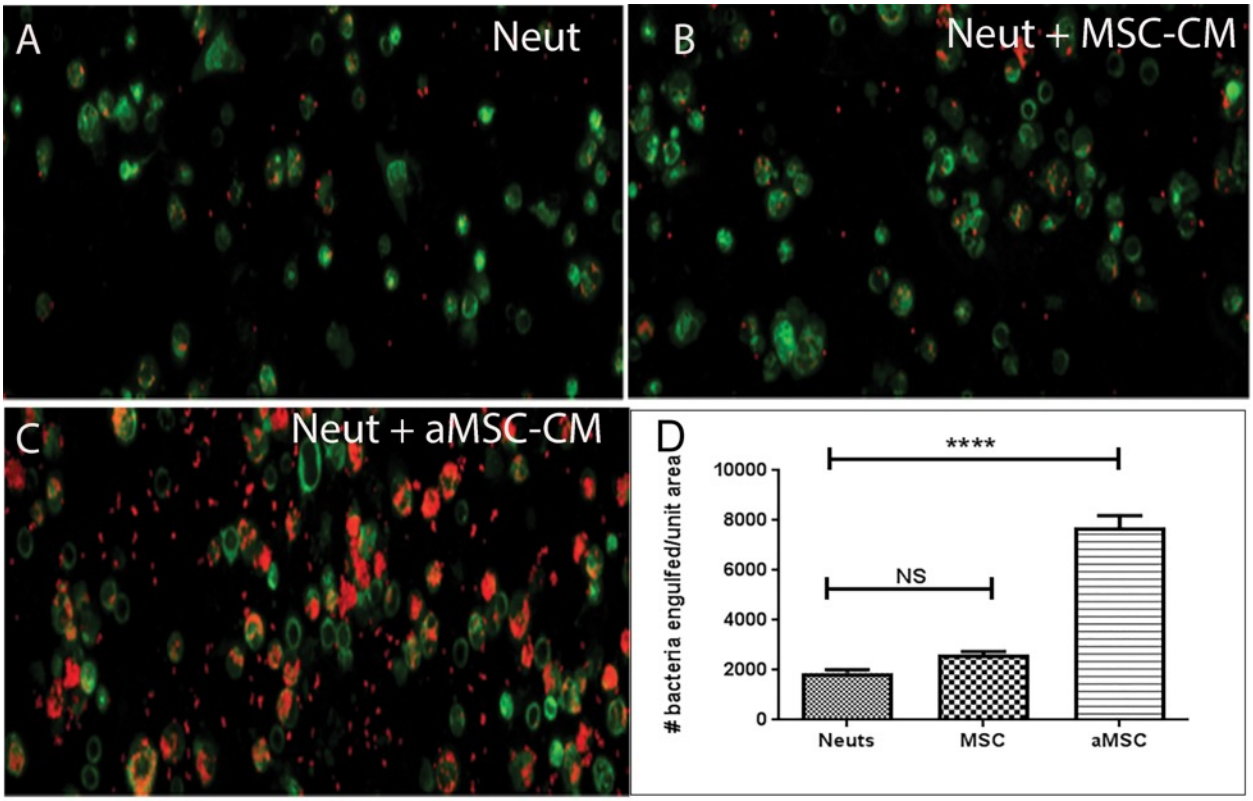



**Figure 2.5**. *Incubation of neutrophils with MSC-CM increases phagocytosis of S. aureus.* The effect of MSC secreted factors on neutrophil phagocytosis was assessed by first incubating mouse neutrophils (derived from peritoneal lavage as noted in Methods), then assessing neutrophil phagocytosis of *S. aureus*. Following incubation neutrophils were infected with *S. aureus* at an MOI of 1 for 30 minutes and extracellular bacteria was killed by the addition of gentamycin. Neutrophils were stained with CD11b (green) and S. *aureus* stained with anti-staph antibody (red). Neutrophils incubated with medium alone (A), resting MSC-CM (B) or activated MSC-CM (C). Image analysis was done using Image J software (NIH). Statistical analysis was performed using way ANOVA and Newman-Keul post test. * indicates $p<.05$. Similar results were obtained in two additional experiments

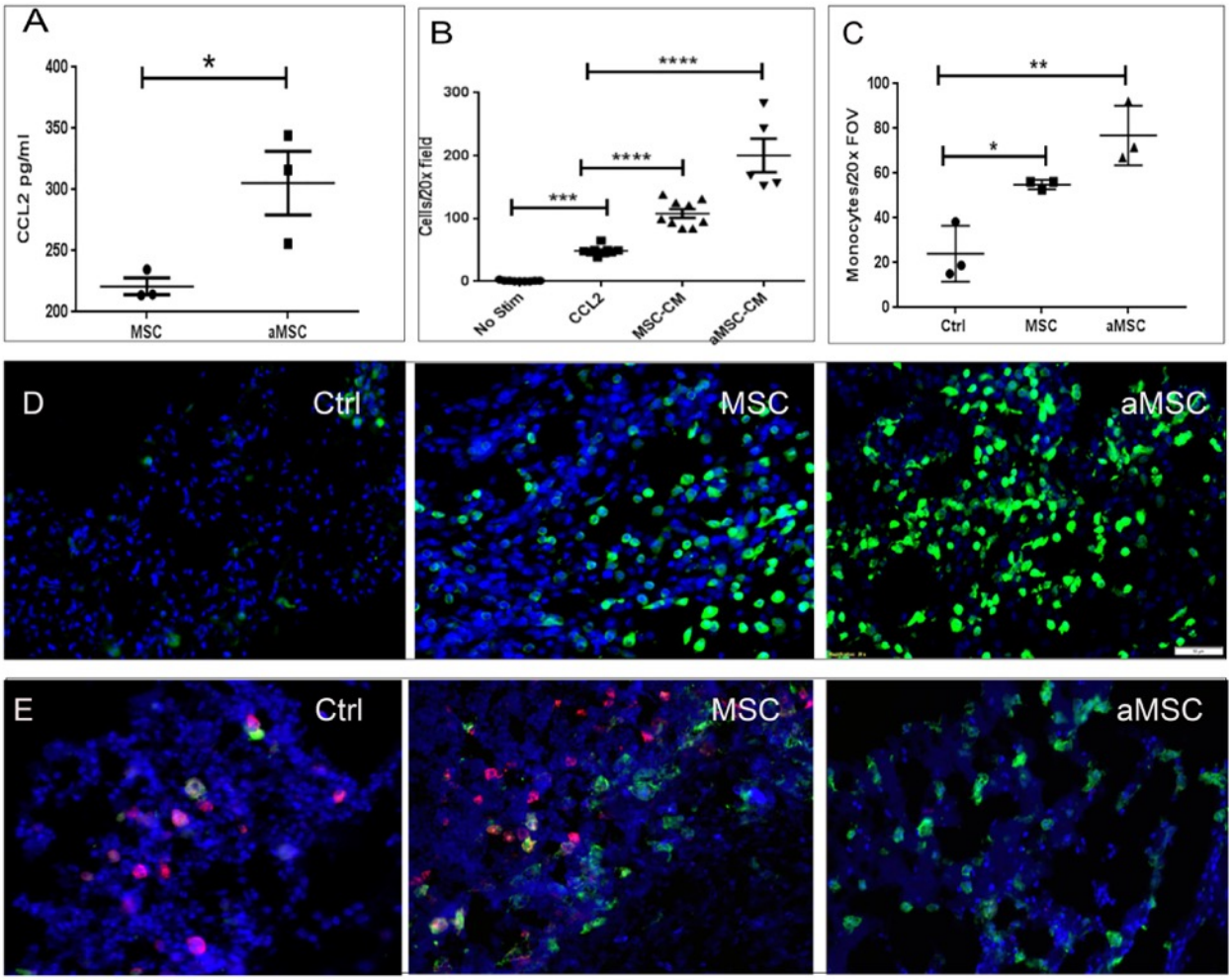
*Monocyte recruitment and macrophage differentiation in wounds in response to MSC administration*.

In addition to neutrophils, monocytes and macrophages play a key role in innate immune control of wound infections. Therefore, the impact of MSC administration on monocyte and macrophage responses in wound tissues was evaluated (**Figure 2.6**). *In vitro*, MSC were found to produce significant amounts of the chemokine CCL2, which is the major chemokine responsible for recruitment of inflammatory monocytes [60]. Significantly more CCL2 was produced by activated MSC (**Figure 2.6A**). Migration of monocytes isolated from the peritoneal cavity of mice to CM from poly I:C activated and resting MSC (aMSC-CM and MSC-CM, respectively) was assessed using Boyden chamber migration assays. Conditioned medium from activated MSC induced significantly greater monocyte migration than CM from non-activated MSC (**Figure 2.6B**).

To determine whether MSC administration triggered monocyte recruitment to infected tissues *in vivo*, CCR2-GFP reporter mice were used to track the migration of inflammatory monocytes expressing CCR2, a marker of inflammatory monocytes[60 42]. Mice with *S. aureus* infected implant infections were injected i.v. twice with MSC (3 days apart), and 24hr later the recruitment of gfp+ inflammatory monocytes to infected tissues was assessed using immunohistochemistry and confocal microscopy (**Figure 2.6D**). A marked infiltrate of CCR2-$GFP^+$ monocytes was observed in infected tissues of mice injected with both non-activated and activated MSC. Consistent with the *in vitro* migration data, the numbers of $CCR2^+$ monocytes was significantly greater in infected tissues of mice treated with activated MSC than in mice treated with non-activated MSC or in control animals (**Figure 2.6C**).

Inflammatory monocytes rapidly differentiate into macrophages once they enter inflamed tissues. [61] In tissues, these macrophages can be classified as either M1 or M2 macrophages based on their cytokine and biochemical profiles, and it is known that macrophages with an M2 phenotype play an important role in wound healing [62]. For example, M2 macrophages, characterized in part by expression of the enzyme arginase, promote wound healing by secretion of growth factors including VEGF and EGF that stimulate wound angiogenesis and epithelialization, respectively [63]. In contrast, M1 macrophages produce factors such as nitric oxide synthase and function primarily to generate antimicrobial activity. [64,65] Mesenchymal stem cells have been shown previously *in vitro* to secrete factors that regulate macrophage phenotype and stimulate macrophage M1 to M2 differentiation [33]. However, the effects of MSC administration on macrophage phenotypes in chronically-infected tissues have not been previously investigated. Therefore, macrophage phenotypes in infected tissues was assessed, using iNOS and arginase expression to identify M1 and M2 macrophages, respectively. We found that the majority of macrophages in untreated infected tissues expressed an M1 phenotype ($iNOS^+$), while in infected tissues from mice treated with activated MSC, most macrophages expressed an M2 phenotype ($arginase^+$). Macrophages from infected tissues of animals treated with non-activated MSC expressed a mixed phenotype of $iNOS^+$ and $arginase^+$ macrophages (**Figure 2.6E**). Quantitatively, infected tissues from mice treated with non-activated MSC contained a roughly equal proportion of M1 and M2 macrophages, while the ratio of M2 to M1 macrophages was significantly increased in infected tissues of mice treated with activated MSC (**Figure 2.6E**). Infected tissues with greater numbers of M2 macrophages would be expected to heal more effectively, consistent with the physical and histological appearance of infected tissues from mice treated with activated MSC (see Figure 2). These findings indicate that MSC can

interact extensively with the host innate immune system during tissue infection and healing, and this interaction likely contributes substantially to the overall antimicrobial effects observed in




MSC-treated animals.

**Figure 2.6**. *Mesenchymal stem cells increase monocyte migration and change the phenotype of macrophages in tissues.* Production of CCL2 by MSC was measured in CM from resting MSC and pIC activated MSC (A). The effects of MSC CM on monocyte migration was assessed using Boyden chambers and monocytes isolated from the peritoneal cavity of thioglycollate-treated mice, as described in Methods. Cell migration data was quantitated using Cell Sens software, Olympus). MSC-CM significantly stimulated monocyte migration compared to medium alone, and CM from activated MSC triggered significantly more migration than CM from non-activated MSC (B). In mice treated with MSC in vivo, monocyte recruitment to infected wounds was assessed using CCR2-GFP reporter mice. Monocytes were present in relatively small numbers in tissues of untreated mice (D), whereas the numbers of infiltrating monocytes were significantly higher in mice treated with MSC (E) and further increased in mice treated with activated MSC (F). These results were verified using tissue samples previously collected from non-GFP reporter mice at the same time point. Monocytes were stained with F4/80 and

quantified using Cell Sens software, Olympus) Statistical analysis performed using ANOVA with Tukey multiple means comparison, * denotes $p<.05$.

*Clinical evaluation of MSC therapy dogs with chronic infections with MDR bacterial*.

A pilot study of MSC antimicrobial therapy was conducted in pet dogs with spontaneously-occurring, multi-drug resistant (MDR) infections to determine whether the observations from the mouse implant infection model could be translated to a realistic large animal model (**Figure 2.7**). The pilot study enrolled 7 pet dogs that were initially evaluated at the Colorado State University Veterinary Teaching Hospital for management of chronic, non-responsive infections containing MDR strains of bacteria. Study animals included the following: 3 dogs with post-operative stifle infections, one dog with an infected traumatic foot wound, one dog with infected pacemaker leads, one dog with an infected bone plate and osteomyelitis, and one dog with deep pyoderma (**Table 2.1**). All of the animals had been extensively pre-treated with antibiotics (for at least 2 weeks) prior to study entry, without evidence of treatment responses.

The 6-week MSC antimicrobial study protocol is described in Figure 2.7. Briefly, study animals were required to remain on the original ineffective antibiotic during the entire 6-week study. Quantitative bacterial cultures were obtained from multiple aspirates of infection sites (except joints, where a single sample was obtained) prior to initiating treatment with MSC, and at 2-week intervals thereafter. Each animal received a series of 3 infusions of allogeneic, poly I:C-activated canine MSC derived from adipose tissue of healthy young, purpose-bred donor dogs. Each treatment consisted of a slow i.v. infusion of cells over 15 minutes via a peripheral vein catheter, at a dose of $2 \times 10^6$ cells per kg body weight. Infection site healing was assessed by serial photographs of the lesions, or in the case of joint infections, by serial cytologic examination of synovial fluid samples.

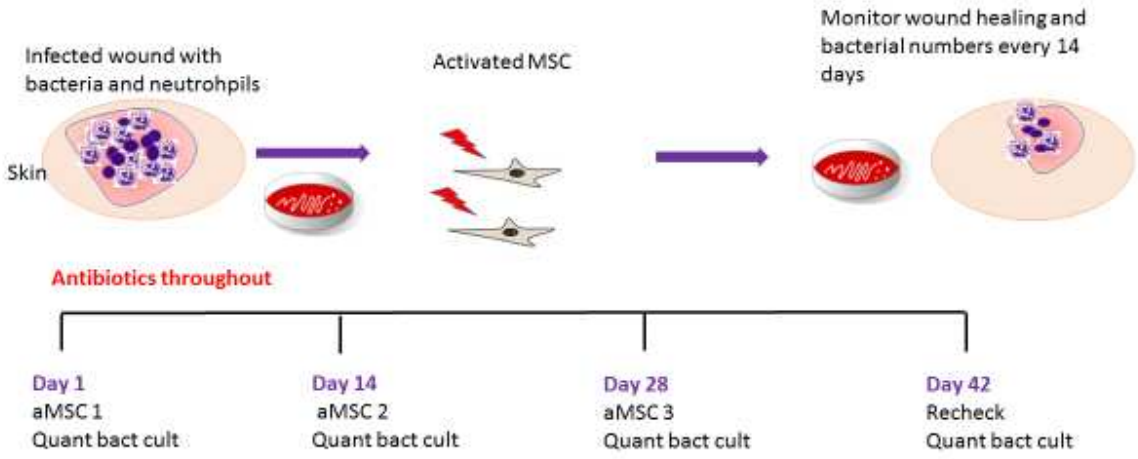



**Figure 2.7**. *Study design for pilot trial in pet dogs with chronic spontaneous infections*. Pet dogs with naturally-occurring chronic infections were enrolled (following owner consent) into a clinical trial evaluating the safety and efficacy of activated allogeneic canine MSC in combination with conventional antibiotic therapy. Criteria for study entry and exclusion are described in Methods. Infection sites were subject to quantitative culture prior to the start of treatment and at 2 weeks intervals during a series of 3 i.v. MSC infusions. Dogs remained on the original ineffective antibiotic treatment during the 8-week trial period.

Infusions of activated allogeneic MSC were well-tolerated clinically by study animals, with no notable adverse effects noted during or after infusion. In one dog (dog 6), the owner felt that the dog was more lethargic for 24 hours following infusion of MSC. As the dog had a history of cardiac dysrhythmias the dog was hospitalized for 24 hours following the next i.v. infusion with EKG monitoring, and arrhythmias or behavioral abnormalities were not noted while the dog was under observation. It was concluded that the stress associated with evaluation and treatment may have been responsible for the lethargy following the first MSC infusion. In 2 of the 3 dogs with stifle infections (Dogs 1 and 2; **Table 2.1**), signs of infection (lameness, pain on joint palpation) were completely resolved by the completion of the 6-week study period.

Clinical improvement was first apparent within 2 weeks of MSC infusion in the treated dogs, and further improvement was noted after the second and third infusions in dogs 1 and 2. Dog 3 had persistence of lameness although pain on palpation of the stifle resolved. In dog 3, the final cause of lameness was determined to be persistent flexion of the joint and tendon contracture. After institution of physical therapy in dog 3, clinical improvement was noted. In dogs 1 and 2, there was no evidence of infection recurrence or need for additional treatment for more than 12 months of follow-up. Dog 3, which originally had a draining tract presumably from the stifle joint, had recurrence of infection 4 months after completing the MSC treatment. In this instance, a different organism was isolated (*Acinetobacter)*. This dog subsequently underwent surgery and had the stifle implant removed, and this dog has done well in the last 4 months of follow-up.

**Table 2.1**. Patient data from 7 pet dogs with spontaneous, chronic infections with MDR bacteria treated with activated MSC.

| Dog | Infection site | Infection duration | Organism(s) | Bacteriologic response (8 weeks) | Clinical Response (8 weeks) |
|---|---|---|---|---|---|
| 1 | Post-operative stifle infection | 12 months | MRSP | Eliminated | Resolved |
| 2 | Post-operative stifle infection | 6 months | MRSP | Eliminated | Resolved |
| 3 | Draining tract stifle | 4 months | MRSP | Eliminated | Resolved |
| 4 | Soft tissue injury- paw | 4 weeks | PA, EC | Eliminated | Resolved |
| 5 | Infected bone plate | 3 months | MRSP , EC, Crny, Kleb | Eliminated (except MRSP) | Improved |
| 6 | Cervical abscess from pacemaker lead | 24 months | MRSP- 2 strains | Unchanged | Improved |
| 7 | Deep pyoderma - paws | 9 months | MRSP | Eliminated | Resolved |

PA = *Pseudomonas aeruginosa*, EC = *Eschericia coli*, MRSP= methcillin resistant *Staphylococcus pseudointermedius*, Crny= *Corynebacterium sp,* Kleb = *Klebsiella sp*

Initial evaluation of synovial fluid samples from the affected stifle of dogs 1 and 2 revealed marked inflammatory cell infiltrates prior to treatment (**Figure 2.8A**). Following treatment with activated MSC, serial evaluation of synovial fluid and revealed progressive reduction in overall cellularity and a shift from neutrophilic inflammation to moderate monocytic inflammation, and the synovial fluid was judged to be normal by 8 weeks after treatment (**Figure 2.8A**). Dog 3 had cultures performed at the draining tract (by fine needle aspirates) on day 0 and on days14, 28, 42 days post treatment. All cultures became negative after the initial day 0 culture.

In Dog 4 (Table 2.1) with traumatic soft tissue infection of the paw, there was a progressive clearance of both strains of MDR bacteria (*E coli* and *Pseudomonas aeruginosa*) from the wound bed over time following MSC treatment (**Figure 2.8B**). Both strains of bacteria were resistant to the treatment antibiotic (amoxicillin/clavulinic acid) throughout the 6-week study. The infected paw healed rapidly during the MSC treatment period (**Figure 2.8C**).

Following MSC treatment in Dog 5 (Table 2.1; infected bone plate and osteomyelitis), 2 of the 3 MDR bacterial strains were eliminated, except for *S. pseudointermedius*. The infected bone plate site was clinically improved, though complete skin coverage of the exposed plate was not achieved. However, the dog was able to retain the bone plate until bone healing progressed enough to allow eventual removal of the implant at 22 months.

In the dog 6 (infected pacemaker leads for 2 years of continuous antibiotic treatment, Table 2.1), the infected lead site became bacteriologically sterile following the initial 3 MSC treatments (data not shown), though the site did not fully heal. An additional 3 MSC treatments were administered, along with surgery to further debride the wound and remove a portion of the pacemaker lead, at which point the infection was resolved.

Study dog 7 (recurrent, deep pyoderma; Table 2.1) was treated with a series of 3 i.v. infusions of MSC, along with cephalexin. Prior to treatment, skin cultures revealed high growth of methicillin resistant *Staphylococcus pseudointermedius*. Following 3 i.v. administrations of MSC, skin cultures became negative within 6 weeks, and clinical signs of pyoderma resolved by the completion of the 8-week study. However, signs of pyoderma recurred 6 months later.

The spontaneous infections in dogs in this study, which are representative of similar chronic, MDR infections in humans, provided an important opportunity to assess the overall safety and potential effectiveness of activated systemic MSC therapy in a large animal model. This pilot study established the safety of activated MSC in dogs, and provided some evidence for efficacy, though it is acknowledged that a larger randomized clinical trial would be required to absolutely establish efficacy. A number of prior studies have established the safety of i.v. delivered allogeneic MSC for treatment of diverse conditions ranging from cardiac infarction to autoimmunity and chronic inflammatory disorders. [66-68] Moreover, the TLR3 agonist pIC has been administered safely to humans as a vaccine adjuvant and non-specific immunotherapeutic for. [69] [70] Therefore, we believe these results provide a strong rationale for the evaluation of TLR3 activated allogeneic MSC as a novel treatment for chronic, drug-resistant infections in humans.

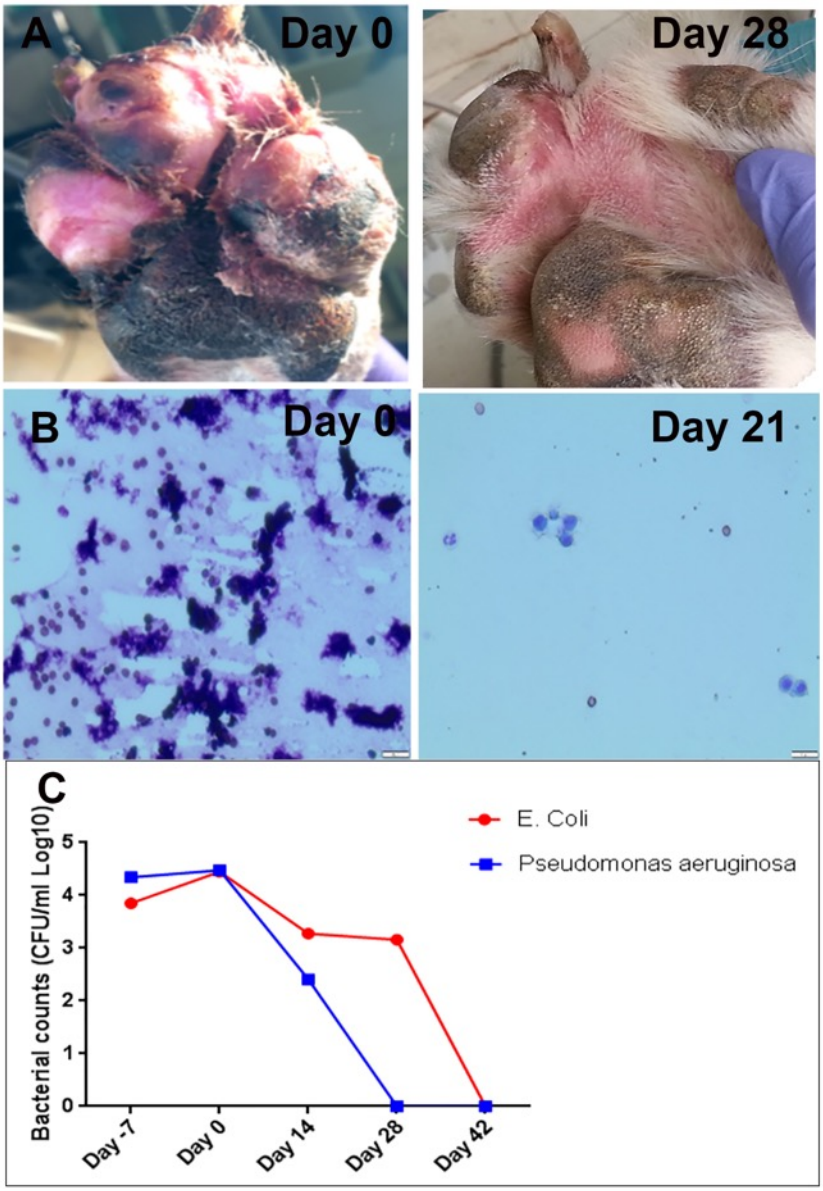



**Figure 8**. *Treatment with activated allogeneic MSC in dogs with spontaneous chronic MDR infections*. Pet dogs with chronic wound infections unresponsive to prolonged antibiotic therapy were enrolled in a clinical trial designed to evaluate the effects of systemic administration of activated canine MSC, as described in Fig 7. In (A) serial cytological evaluation of joint fluid samples obtained from a dog with septic arthritis prior to treatment and at week 4 of treatment with activated MSC. The dog developed post-operative septic arthritis with MDR *S. intermedius* and had been unresponsive to several months long courses of antibiotic treatment over a period of 12 months. The animal become clinically normal at the completion of the 6-week study and the joint fluid also remained normal cytologically. In (B) serial quantitative cultures of a soft tissue infection from traumatic paw injury shows progressive clearance of two multidrug resistant organisms during MSC treatment. In (C), photographs of the paw wound from that same dog prior to treatment and 4 weeks after MSC treatment.

**Discussion**

Chronic infections, especially those associated with implanted foreign material such as catheters or metallic devices, remain a major problem clinically as these infections are very difficult to resolve with antibiotic therapy alone [71] [72] [73] [74]. Our findings suggest that systemic administration of activated MSC, when combined with conventional antibiotic therapy, offer one solution to the problem of chronic, drug-resistant infections. Our studies revealed a strong positive interaction between activated MSC and conventional antibiotics, as well as between activated MSC and the host innate immune response to infection. The net result of the interactions between MSC and antibiotic therapy was significant clearance of bacteria even from heavily infected synthetic implant materials such as mesh coated with *S. aureus* biofilms. Moreover, MSC administration also produced other benefits, including apparent stimulation of wound healing and reduction of infection-associated inflammation and fibrosis.

Multiple, complementary mechanisms of action (both direct and indirect) likely account for the ability of activated MSC to help control wound infections. As has been reported by others, our studies showed that MSC secrete antimicrobial peptides [11] [10] [9]. We also identified indirect mechanisms of enhanced bacterial elimination by MSC involved interactions of activated MSC with the host innate immune response, including both neutrophils and monocytes. For example, MSC were noted to secrete factors which enhanced neutrophil bacterial phagocytosis, resulting in more effective bacterial killing. In addition, activated MSC stimulated monocyte recruitment to wounds and the differentiation of macrophages into an M2 phenotype, which has been associated previously with accelerated wound healing[63] [64]. Previous studies by our lab and others have also demonstrated that MSC can elicit recruitment of monocytes and macrophages into tissues[75] [76] (Takeda K, Webb T and Dow S. manuscript submitted).

Importantly, these neutrophil and monocyte effects were enhanced by pre-activation of the MSC in the current studies. The effects of activated MSC on monocyte recruitment and differentiation may play an important role in both antimicrobial activity and in stimulation of wound healing.

Despite the noted propensity for systemically administered MSC to lodge in the lung, in our study we observed that i.v. administration of MSC resulted in effective control of deep-seated bacterial infections in sites distant from the site of MSC administration [52]. Moreover, we found that local injection of MSC directly into the wound site was not as effective as i.v. delivery for infection control (data not shown). The potential increased interaction of MSC with neutrophils and monocytes when administered i.v. as compared to local administration into wound sites may in part explain the greater efficacy of systemic MSC administration in these cases. For example, MSC were observed to accumulate in the spleen as well as in wound tissues following i.v. administration, and localization of MSC in secondary lymphoid organs such as the spleen may further enhance systemic activation of innate immune defenses. The presence of MSC in the spleen may also modulate the host adaptive immune response to bacterial infection (eg, stimulation of humoral immune responses to bacteria), though this issue was not specifically addressed in this study. Indeed, systemic administration of MSC may be critical to the activation of other immunomodulatory cells that play crucial roles in the wound healing process [75].

While MSC injected i.v. lodged initially in the lungs as has been seen previously, *in vivo* imaging studies showed that substantial numbers of MSC migrated from the lungs to the wound site over the next 24-72 hours. The stimulus for MSC recruitment into wounds likely includes chemokines such as SDF-1, produced locally by inflamed wound tissues. [53,77] In addition, activation of MSC with poly I:C facilitated MSC recruitment to sites of chronic infection, an effect that may be attributed in part to up-regulation of the receptor CXCR4, for which SDF-1 is

the principal ligand. These findings demonstrate that i.v. injected MSC remain viable in the lungs and can migrate efficiently from this site to localize at infected wounds over a period of several days. The migratory potential of systemically administered MSC is particularly relevant for treatment of wounds in sites that cannot easily be reached for direct MSC injection (eg, bone infections, implant-associated infections).

In the era of increasing antibiotic resistance, there is a clear need for new, non-antibiotic approaches to controlling drug-resistant infections. Previous studies have determined that MSC secrete antimicrobial peptides such LL-37 [10 12 78 79 58]. Other investigations have demonstrated that antimicrobial peptides, including cathelicidins, can augment the antimicrobial activity of certain antibiotics[80]. However, these earlier studies did not specifically examine the interaction between MSC-secreted factors and antibiotics with respect to augmented bacterial killing. Our assays revealed that the mouse cathelicidin CRAMP was one of the factors produced by MSC that significantly increased staphylococcus killing. Moreover, in other studies we have observed that MSC-secreted factors can reduce high level antibiotic resistance in bacterial strains with multi-drug resistance (Johnson V, manuscript in preparation). These findings suggest that activated MSC therapy would be particularly useful in the management of deep-seated infections with highly drug-resistant strains of bacteria, where treatment options other than antibiotic therapy may not exist.

The translational relevance of studies conducted in rodent models is not always assured. Accordingly, we completed a pilot study in dogs with spontaneous chronic wound infections where we observed wound infection clearance and healing in dogs treated with repeated i.v. infusions of activated allogeneic MSC, without evidence of toxicity or adverse effects. The apparent beneficial effect from MSC administration was observed in wounds in diverse locations

(eg, joints, cutaneous wounds, infected surgical implants) and with diverse, highly drug-resistant strains of bacteria. These results suggest that systemic administration of activated, allogeneic MSC may be a viable option for the treatment of chronically infected wounds in other species such as humans. Allogeneic MSC have been safely administered to humans for a number of conditions previously, and their use as a treatment for chronic infections wound not represent a unique risk. [81,82]Patients with infected implants or other wounds associated with biofilm formation and highly drug resistant strains of bacteria (eg, infected ulcers in diabetic patients) that have failed conventional antibiotic therapy represent patient populations that may benefit from a short-course of systemic activated MSC therapy. As the need for non-antimicrobial solutions to management of chronic infections grows, use of activated MSC may represent a new solution for some patients with few other viable options.

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# CHAPTER 3

## Evaluation of Activated Mesenchymal Stem Cell Treatment In A Pilot Study Of Canine Multi-Drug Resistant Bacterial Infections


### Summary

Over the past several decades, antimicrobial resistance has increased markedly in part due to overuse of antibiotics. These infections now currently have very limited therapeutic options often resulting in extensive surgery, limb amputation or death. New pharmaceutical agents are of limited value as the bacteria develop resistance to new therapeutics faster than new agents can be developed. Novel treatments are necessary to enhance immune clearance of infection thus circumventing the ability of bacteria to develop resistance to antimicrobial drugs. Mesenchymal stem cells (MSCs) have been demonstrated to have antimicrobial activity and studies in our lab have demonstrated clearance of biofilm infections not responsive to antibiotic treatment when pre-activated stem cells were combined with antibiotics. We hypothesized that pre-activated mesenchymal stem cells (aMSC) would be effective at clearing multidrug resistant (MDR) infections *in vitro* and *in vivo*. Results of these studies suggest that aMSC have anti-bacterial activity against MDR infections and can be effectively utilized to treat infections in which conventional therapies have failed. Our previous work in a mouse model of biofilm infection revealed great potential for treatment of drug resistant or biofilm infections. In this study we report the continuing successful treatment of canine multi-drug resistant infections. Canine mesenchymal stem cells demonstrate synergy with conventional antibiotics and pre-activation of these cells increases migration to the site of inflammation thus concentrating them at the site of infection. These cells work with the innate immune system to increase macrophage

phagocytosis and killing. In addition, these cells demonstrate robust production of anti-microbial peptide LL-37 which has been previously demonstrated to work synergistically with antibiotics. Activated MSC have a variety of influence on the innate immune system which provide increased anti-microbial action. This treatment has great potential for improving therapy for patients infected with drug resistant bacteria for which current therapies are ineffective.

**Background**

An increase in bacterial resistance over the past few decades has been the cause of a worldwide health crisis with more people dying from methicillin resistant *Staphylococcus aureus* (MRSA) than AIDS worldwide. [1]An estimated 94,000 infections and 18,600 deaths occur from MRSA in the US per year. Other drug resistant pathogens are on the rise also and production of new therapeutics has resulted in little benefit to combat this issue as bacteria develop resistance far more rapidly than new drugs can be developed and tested. [2 3] Biofilm infections constitute an additional challenge as bacteria that adhere to an organ or an implant enter a different metabolic state and even if susceptible to antibiotics they can be difficult or impossible to eradicate. [4,5] Multiple strategies have been utilized to combat these difficult to treat infections but still infections and resulting morbidity and mortality continue to rise.[6] It is clear that novel treatments are necessary to overcome these pathogens.

One promising strategy to combat this issue is harnessing the immune system to better fight off infection, which circumvents the problem of rapid development of resistance by bacteria to new pharmaceuticals. In the past decade, the antimicrobial properties of mesenchymal stem cells (MSC) have been recognized. [7-9 10 8,11-13] MSC are a cell type derived from adult stromal tissue with fat and bone marrow being the most common sources. [14] Adipose tissue is easy to obtain and multiple studies have demonstrated similar or improved immunomodulatory function with adipose derived MSC.[15-18] Mesenchymal stem cells have been demonstrated to decrease bacterial counts and improve survival in multiple models of infectious disease such as sepsis and pneumonia and have been demonstrated to have wound healing properties.[10,19 20-22] These cells have been demonstrated to migrate to sites of inflammation and have both local and systemic effects.[23 24] MSC function to suppress inflammation through downregulation of T cell

proliferation, increase in T regulatory cells, and by changing the phenotype of macrophages from an inflammatory M1 to a wound healing M2.[25,26] The antibacterial effect of MSC has been attributed to both a direct effect through secretion of anti-microbial peptides such as cathelicidins (LL-37), defensins, hepcidins and others, and indirect effects on the immune system.[27-29] MSC have been demonstrated to decrease bacterial load by increasing magrophage phagocytosis, increasing neutrophil phagocytosis and increasing the respiratory burst in neutrophils.[7,30-32]

A benefit of cellular therapy with MSC is that these cells are alive and able to interact with their environment to respond to subtle clues and act appropriately in a way that pharmacologic agents cannot. MSC interact with their environment utilizing toll-like-receptors (TLRs) which are present on most immune cells in the body. MSC have been consistently demonstrated to express TLR 1-6.[33,34] Multiple reports have demonstrated changes in MSC phenotype and function with ligation by TLR agonists.[33,35,36] The ability to modify the action of these cells could be utilized to make them more efficacious to treat the disease they are being utilized for.[37,38] Indeed in a previous mouse model of biofilm infection we demonstrated that pre-activation of MSC by TLR3 agonist Poly I:C caused resolution of the infection when administered with antibiotics. In this model antibiotics alone and antibiotics with resting MSC did not eradicate the infection.[26] To determine if pre-activation of MSC would be advantageous against multi-drug resistant infections also we administered pre-activated MSC to canine patients with naturally occurring multi-drug resistant infections. The preliminary results of this study were favorable, and the study was continued with further success. *In vivo,* this therapy was successful with multiple different types of infections and with animals on multiple classes of antibiotics. To predict *in vivo* efficacy adipose derived MSC were tested with various classes of antimicrobials with gram positive and gram negative bacteria *in vitro*. In addition, these cells

were tested with various immune effector cells to determine the mechanism by which they may assist in eradication of multi-drug resistant and biofilm infections *in vivo*. In addition to the observed antibacterial effect, MSC are known to assist in wound healing.[39,40] This property of stem cells may also be beneficial in open wounds. *In vitro* models of wound healing demonstrated a beneficial effect when treated with secreted factors from aMSC. This therapy represents a promising novel therapeutic option to treat infections that currently have no therapeutic options.

## Materials and Methods

*Clinical trial design for wound infection study in pet dogs*

This clinical trial was designed as previously described (Johnson et al.) Dogs enrolled in a pilot clinical study of MSC therapy for wound infection management were client-owned animals that presented to the Colorado State University Veterinary Teaching Hospital for treatment of multidrug resistant chronic infections. These studies were approved by the Colorado State University Institutional Animal Care and Use Committee and by the Clinical Review Board at the Veterinary Teaching Hospital. Inclusion criteria involved presence of a documented infection with a multi-drug resistant organism in a site where cultures could be obtained on a regular basis. In addition, the infection must have failed conventional treatment for a period of greater than 4 weeks. To fulfill these criteria, animals must have failed treatment with antibiotics and surgical debridement as evidenced by continued symptoms of infection and positive culture of organisms at the site. Animals were excluded from the trial if there was any evidence of systemic disease in which treatment with MSC may be contraindicated, such as neoplasia, or that may affect immune function, such as Cushing's disease or organ failure.

Dogs enrolled in the trial were treated with allogeneic adipose derived MSC pre-activated with 10 ug/ml of poly I:C (InVivoGen, San Diego CA) × 1 hr at a dose of $2 \times 10^6$ cells per kg body weight. Cells were then washed 3× in DPBS (Sigma-Aldrich, St. Louis, MO) and re-suspended in DPBS at a volume of 10 ml ($30 \times 10^6$ cells or less), 15 ml ($30–45 \times 10^6$ cells) or 20 ml ($>45 \times 10^6$ cells). Sodium heparin (Fresenius Kabl USA, Lake Zurich IL) was added immediately prior to injection at 200 IU per 10 ml of cell solution. Cells were delivered by intravenous (i.v.) infusion over 15 minutes. Heart rate and respiratory rate were monitored during and for 10 minutes after infusion. Antibiotic therapy was kept consistent for the duration of the study (and consisted of the last antibiotic the animals had been treated with before concluding failure of antibiotic therapy) and continued for 1 week after the last injection was administered. Dogs were treated with MSC every 2 weeks for a total of 3 treatments, and quantitative cultures were obtained prior to each treatment where possible. Cultures were obtained by fine needle aspirate of 4 quadrants of the infected area or by other means where this was not feasible. The aspirates were placed in 1 ml of TSB (trypticase soy broth, Sigma Aldrich) and quantitative cultures obtained by 10 fold dilution in PBS and plating on LB agar (Luria Broth, Sigma Aldrich).

Dogs were considered for additional MSC injections if there was a significant decrease in bacterial infection (but not full bacterial clearance) as evidenced by a significant decrease in number of bacteria in the wound or eradication of 1 or more but not all species of bacteria present at the infection site. Two weeks after the final treatment, quantitative cultures were again performed, and patients were then monitored by follow up phone calls every 2–3 months. Dogs were considered to have a positive response if clinical signs improved and bacterial counts decreased significantly over the treatment interval. The infection was considered eradicated if the

animal had more than 2 negative cultures at least 2 weeks apart and the infection site was fully healed with resolution of clinical signs.

*Generation of canine adipose derived MSC*

Canine adipose MSC used in clinical trial was generated as previously described (Johnson et al.). Adipose tissue was obtained from the inguinal region of healthy, young purpose bred research hounds. The adipose tissue was frozen in 1 gm aliquots in freezing media consisting of 75% FBS, 15% MSC medium, and 10% DMSO at −80 degrees within 2 hours of sample collection. Freshly cultured MSC were generated from frozen adipose tissue aliquots for each treatment, and the cells used between passages 2 and 5 for all dogs. The cryopreserved adipose tissue was thawed and rinsed in DPBS twice. Subsequently the tissue was minced, digested in collagenase, the stromal vascular fraction (SVF) collected and culture expanded as previously described. [26] At the time of adipose tissue collection, blood was obtained from each donor dog for a complete blood count and chemistry profile.

Additional testing for infectious disease included serology to test for Anaplasma phagocytophilum, Borrelia burgdorferi, Dirofilaria immitis, Ehrlichia canis (4DX, Idexx laboratories), and PCR was performed to detect the presence of Hemoplasma spp, Ehrlichia spp, Bartonella spp, and Rickettsial spp at the state veterinary diagnostic laboratory. All donors had normal CBC and biochemistry profiles and were negative on all infectious disease testing. In addition, each stem cell culture was tested for bacterial, fungal, and mycoplasma contamination at the time of stem cell administration using in house cultures (blood agar, McConkey agar, Sabourad Dextrose agar – BD Falcon, and mycoplasma agar – Udder Health systems, Bellingham WA). Bacterial cultures were incubated at 37 °C for 48 hr, mycoplasma cultures were incubated at 37 °C, 5% CO2, for 7 days, and fungal cultures were incubated at room

temperature in the dark for 30 days. No contamination was detected in any culture during the study. Cell surface phenotype was assessed via flow cytometry and tri-lineage differentiation was performed as previously described. [41]

*Activation of canine MSC*

Immediately prior to administration, canine MSC was activated with poly-cytidylic acid (pIC) 10 ug/mL (InvivoGen, San Diego, CA); for 2 hours at 37°C in a 5% $CO_2$ incubator. MSC were detached from flasks by trypsinization. Sodium heparin (Fresenius Kabl USA, Lake Zurich IL) was added at 100IU/mL to MSC to prevent clumping.

*Bacterial quantitative culture in vitro*

Bacteria numbers were quantitated by plating serial, log10 dilutions of culture supernatants obtained by gentle pipetting, and then plating on LB agar quadrant plates (Sigma-Aldrich) and counting bacterial colonies 24 hr after culture at 37 °C. The log10 CFU was determined numerically from plate counts.

*Bacterial Killing assay in vitro*

BKA was performed as previously described. [26] To assess the ability of MSC to kill bacteria, cells or conditioned medium (CM) from MSC were utilized. CM was generated by plating $5 \times 10^5$ cells per well in a 24 well plate with 500 ul per well of antibiotic free media and incubating at 37 °C. Conditioned media was collected 24 h after the cells were plated and were immediately frozen at −80 °C and thawed immediately before use. MSC or CM were inoculated with log phase *Stapyhylococcus aureus* (*S. aureus*) cultures, typically at a concentration of $1 \times 10^5$ CFU bacteria per ml of CM or per ml of cells (multiplicity of infection of 10:1 bacteria per cell) in 24-well plates, in complete MSC culture medium, but without antibiotics. Co-cultures were incubated at 37 °C for 3 hr and numbers of viable bacteria were determined. Addition of

various antibiotics was determined by performing MIC cultures of bacteria with the antibiotic and antibiotic was utilized at a sub-inhibitory concentration.

*Bacterial growth and storage*

Bacteria were obtained from multi-drug resistant (MDR) bacteria cultured from canine patients at the CSU Veterinary Diagnostic Laboratory. Bacteria were isolated and one colony used inoculate 75ml TSB. The culture was then incubated at 37°C shaking at 200 RPM until turbid. The saturated culture was then mixed with 15% sterile glycerol and frozen at -80°C in 1ml aliquots. For use in bacterial killing assays an overnight culture was started using an ice scraping of the frozen aliquot of bacteria in 5ml TSB. The saturated culture was then inoculated (100ul) into fresh TSB (5ml) and grown according to a previously established OD curve. OD curves were determined by serial quantitative culture of bacteria over the log phase of growth with corresponding OD readings.

*Minimum Inhibitory Concentration (MIC)*

Minimum inhibitory concentration for each antibiotic was determined by diluting a .5 McFarland standard for each bacterial isolate 50 times and adding 100ul per well to a 96 well round bottom plate. The antibiotic was diluted and various concentrations added in equal volume to the bacteria and incubated overnight at 37°C. The minimum inhibitory concentration was identified as the concentration at which no bacterial pellet could be visually identified in the well.

*Bacterial quantitative culture from wound samples*

For determination of the bacterial burden in wound tissues of dogs in the clinical trial, 4-quadrant fine needle aspirates were collected from around the wound perimeter, inoculated 1ml LB medium, then serially diluted and plated on LB plates for quantitation and Trypticase Soy

Blood Agar and MacConkey plates (BD Falcon, San Diego CA) for bacterial growth and enumeration. Plates were incubated for a minimum of 48 hr at 37 °C to confirm absence of bacterial growth. Animals with septic arthritis underwent aspiration of the affected joint under sedation every 2 weeks during the study period. Joint fluid was also evaluated cytologically. Animals with nasal infections underwent periodic surveillance of bacterial populations in the affected nares by placing a culture swab intranasally and submission of culture to the veterinary diagnostic laboratory. Bacteria were speciated by morphology and biochemical testing, and bacterial CFU were determined by manual counting. Bacterial sensitivities were performed by the Colorado State veterinary diagnostic laboratory (200 West Drake Road, Fort Collins CO 80523).

*Assessment of bacteriologic and clinical response*

The dogs in the clinical trial were assessed at the end of the study period (8 weeks)to determine if a bacteriologic response and/or a clinical response was present. The criteria for a bacteriologic response was decrease in 50% or more of quantitative bacterial counts in animals where quantitative bacterial counts could be performed. In animals where quantitative cultures were not possible they were considered a responder if they had a negative culture and a partial responder if there was a decrease in the number of bacterial species present in a polymicrobial infection. A clinical response was defined depending on the type of infection and expected resolution. For dogs with open wounds, the wounds were measured at each two week timepoint and a clinical response defined as greater than 50% contraction of the wound by the 8 week endpoint. For dogs with joint infection a clinical response was defined as reduction of inflammation in the joint as assessed by numbers of inflammatory cells in joint fluid (>50% decrease in cell count or resolution of inflammation in the joint). The two dogs with nasal

infections were assessed by owners who scored the infections weekly with a numeric score. The score was based on four categories that the owner would rate 0-4. The categories included appearance of discharge (no discharge, serous discharge, colored discharge or colored discharge with blood), frequency of discharge (never, rare, intermittent or constant), frequency of sneezing (never, less than once per day, more than one per day, more than 4 times per day) and odor (none, mild, moderate, strong). The four categories were added together and a clinical response defined as a decrease in the total score by more than 50% at 8 weeks.

*MSC Migration Assay*

MSC migration towards an inflammatory stimulus was assessed utilizing transwell migration chambers with an 8um pore diameter (BD Falcon) and MSC migration assessed by placing 500ul of MSC media with 100ng/ml SDF-1 (R&D Systems Inc., Minneapolis, MN) in the bottom chamber in the control well and 100ng/ml SDF-1 with 500ul conditioned media from resting MSC and pre-activated MSC (aMSC in the other chambers). Cells and conditioned media were performed as described above. The top well was seeded with $1x10^5$ MSC in 200ul of MSC media and the cells allowed to migrate for 4 hours while incubating at 37°C 5% $CO_2$. After 4 hours the cells were removed from the top of the chamber by gentle agitation with a damp swab and then allowed to dry. The membranes were then fixed in 2% methanol and stained with crystal violet. The migrated cells were counted manually and an average of ten fields at 10x utilized for each replicate. Three replicates were performed per group. Microscopy was performed using the light microscopy function of the Olympus IX83 spinning disk confocal microscope.

*Measurement of MSC secreted cytokines*

Conditioned media of activated and resting MSC was prepared as described above and frozen at -80°C until use. Samples were thawed on ice and utilized immediately. MSC media was utilized as a negative control. Samples were run and analyzed as directed using a commercially available canine VEGF and IL-8 ELISA kits (R&D Minneapolis MN).

*Detection of AMP expression by MSC using immunocytochemistry*

ICC staining of canine MSC for LL37 was performed as previously described. [20]Briefly, 10,000 cells were seeded on coverslips (Chemglass Life Sciences LLC, Vineland, NJ) in 24-well cell culture plates overnight, then fixed with 4% PFA(Paraformaldehyde) (Fisher Scientific, Hampton, New Hampshire) for10 minutes, washed with PBS and permeabilized with 0.1% Triton X-100(Sigma-Aldrich,). Slides were then blocked using 5% v/v normal donkey serum and then incubated with primary antibodies, diluted appropriately. Primary antimicrobial peptide antibodies used include Anti-Surfactant protein D antibody (ab203309 1:200 dilution), Anti-Lipocalin-2 / NGAL antibody (ab63929), Anti-beta 2 Defensin antibody (ab9871), Anti-Hepcidin antibody (ab134790), Anti-Cathelicidin antibody (ab180760) all at 1:100 dilution. All antibodies were purchased from Abcam (Cambridge, MA). Specificity controls for immunostaining included rabbit IgG from nonimmune rabbits diluted to the same concentration as primary antibodies. Following primary antibody incubation, chambers were washed and incubated with secondary antibody donkey antirabbit or anti goat Cy3 (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania) and counter stained with DAPI. Visualization of fluorescence staining was done using an Olympus IX83 spinning disk confocal microscope.

*ELISA for LL37 production*

Measurement of LL-37 production in MSC-CM and aMSC-CM were performed on supernatants generated by plating 100,000 canine MSC in 24 well plates (0.5mL) with a 48 hour incubation. Samples were run in triplicate with CM from 3 separate dogs. A commercially available ELISA kit was utilized for the measurements according to manufacturer's instructions (Lifespan Biosciences, Seattle WA).

*Primary macrophage culture*

Canine macrophages were grown in 10ng/mL of human M-CSF as previously described.[42] Briefly, monocytes were collected from the blood of 3 unrelated dogs of various breeds using the Lympholyte-Poly (Cedarlane, Peterborough, United Kingdom) separation gradient according to manufacturer's instructions. Monocytes were separated by adherence to plastic cell culture dishes overnight and differentiated to macrophages for 7 days in DMEM containing NEAA, essential amino acids, 10% FBS, Pen/Strep, Glutamax and 10ng/mL of recombinant human M-CSF (PeproTech, Rocky Hill NJ). Media was changed every 3 days, removing non adherent cells each time.

*Macrophage bacterial phagocytosis assay*

Quantitative phagocytosis over time was performed using the IncuCyte ZOOMsystem (Essen BioScience Inc, Ann Arbor, Michigan). Log phase *S. aureus* cultures were first fixed and stained using the pH rodo Red Phagocytosis Particle Labeling Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Macrophages were incubated in 24-well plate wells, with MSC growth media or MSC CM or pIC activated MSC CM for 48 hours, and then *S. aureus* was added at an MOI of 25:1 (bacteria to cells).Images (9 per well) were collected every 15 minutes using a 10x objective, and analyzed using IncuCyte S3 Software (Essen BioSci-ence Inc).

*Macrophage polarization*

After 5~7 days of differentiation, canine macrophages were polarized to an M1 or M2 phenotype with a 24 hour incubation with 20ng/mL of canine IFNg (R&D Systems Inc., Minneapolis, MN) for M1 polarization and 20ng/mL each of recombinant canine IL-13 and IL4 (R&D Systems Inc., Minneapolis, MN) for M2 polarization.

*Macrophage bacterial killing*

Bacterial cultures of canine MRSP were grown in MSC growth media overnight to obtain a saturated culture. A sample of this culture was then diluted 1:1000 then grown to log phase monitoring the optical density (OD). During log phase the bacterial count (CFU/ml) was calculated according to a previously established OD curve then diluted to the appropriate concentration. Macrophages were infected with MRSP at an MOI of 3 CFU/cell in 100uL of pre-warmed HBSS containing $Ca^{++}$ and $Mg^{++}$ containing 10% dog serum then incubated for up to 3 hours. Numbers of viable bacteria were determined by plating log10 serial dilutions on LB agar 4 quadrant plates (Thermo Fisher Scientific) and manual counting of colony forming units after 24 hours of incubation at 37°C.

*MSC migration assay*

Canine MSC were generated as described above and harvested at passage 3 for migration assay. Migration was measured using Boyden chambers (BD Falcon) with 8 um pore diameters. Canine MSC (2.5 × 105 cells per well) were added to the top chamber in complete medium, MSC-CM or aMSC-CM was added to the bottom chamber, and the assay was run for 4 hr at 37 °C. Recombinant human SDF-1 (100 ng/ml; R&D Systems Inc., Minneapolis, MN) was used as a positive chemokine control. The numbers of migrated cells were determined by first removing cells from the top of the membrane with cotton swabs, then removing the filter

membrane, staining with crystal violet, and manually counting the number of monocytes adherent to the bottom of the membrane, averaging at least 5 random high power fields per sample. Data were displayed as average number of migrated cells per membrane per treatment condition.

*Immunohistochemistry for macrophage polarization*

Canine macrophages were generated as previously described on 8 well glass bottom chamber slides with 2x $10^6$ PBMC allowed to attach overnight.[42] On day 5, macrophages were incubated with media only, MSC-CM, and aMSC-CM prepared as described above for 48 hours with the addition of 10ng/mL M-CSF. The chamber slides were incubated at 37°C with 5% $CO_2$ for 3 hours then washed three times with phosphate buffered saline (PBS). Slides were then fixed with 4% PFA. Slides were blocked with donkey serum (Jackson ImmunoResearch Laboratories Inc. West Grove PA) and incubated overnight with primary antibody anti-CD 206-PE, clone 3.29B1.10 (Beckman Coulter, Pasadena CA) or rabbit polyclonal anti-iNOS (Invitrogen, Carlsbad CA). Following washing with PBS slides were stained with secondary antibody donkey anti rabbit Cy3 (Jackson ImmunoResearch Laboratories Inc. West Grove PA) for one hour followed by DAPI for 10 minutes. Examination of slides for fluorescence was performed with an Olympus IX-83 confocal microscope and image analysis performed using CellSens software (Olympus).

*Measurement of cytokine production in macrophages incubated with MSC-CM*

Triplicate wells of primary bone marrow macrophages from 3 canine donors were plated in 24 well plates as previously described. MSC-CM and aMSC-CM were prepared as described above. Positive control wells were stimulated with Lipopolysaccharides (LPS) at 200ng/mL (MilliporeSigma, Burlington MA) for 24 hours. Following 48 hour incubation with MSC-CM,

macrophage media was collected and frozen at -80 until time of analysis. Cytokine levels were assessed utilizing commercially available kits for canine cytokine measurement of IL-10 and TNFα (R&D, Minneapolis MN).

*Fibroblast Assays for proliferation, metabolism and migration*

Fibroblast migration was assessed via the IncuCyte® Scratch Wound Assay according to manufacture protocol. Canine fibroblasts were grown from skin samples from three different healthy dog donors as previously described.[41] Briefly 10,000 canine fibroblasts were plated in a 96 well culture plate and allowed to attach overnight. Uniform scratches were then made in the center of each well of fibroblasts using the WoundMaker tool. Control wells had fresh media added and resting and activated MSC-CM added to the test chambers. The area of cell coverage of the defect was measured at 12, 24 and 48 hours.

Fibroblast metabolism was assessed by MTT assay. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), 5uL of MTT reagent at 5mg/mL (Sigma) was added directly to fibroblast plated as described above, and plate was incubated at 37C 5% $CO_2$ for 2 hours. MTT Stop reagent containing 0.01M HCL was then added to stop the reaction. Crystals were solubilized and colorimetric reading was obtained on a 96 well plate reader.

Fibroblast proliferation was measured using Edu incorporation following manufacturer's instructions for Click-iT™ EdU Flow Cytometry Assay Kit (Thermo Fisher Scientific, Waltham MA) with a 24 hour incubation with 10uM Edu prior running the assay. Edu incorporation was measured using a Beckman Coulter Gallios Flow Cytometer and analyzed with FlowJo™ v9.

*Statistical Analysis*

Statistical analysis was performed using Graph Pad Prism 8.0 software (Graph Pad, La Jolla CA). For comparison of two treatment groups a t-test was performed (Mann-Whitney test).

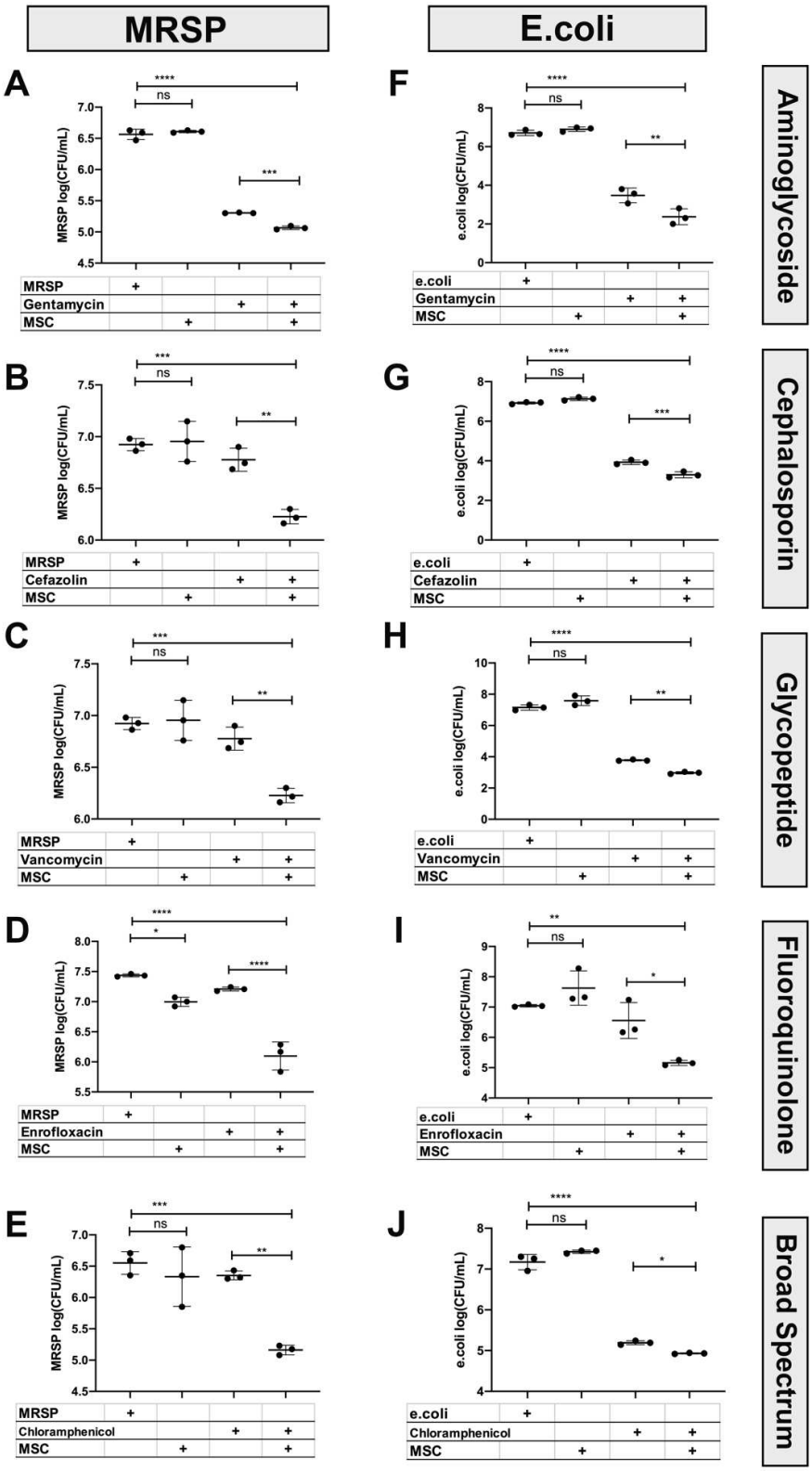
For comparison of more than two groups a two-way ANOVA was run with a Tukey post-test. Tests for synergy were performed using a two-way ANOVA as previously described.[43]

## Results

### Canine MSC exhibit synergistic bactericidal activity when combined with certain classes of antibiotics *in vitro*

The first studies were conducted to identify classes of antibiotics that might exhibit synergistic or additive activity with bactericidal factors secreted by activated MSC, and to also identify classes of antibiotics where there might be antagonistic activity or no activity. These studies were done to guide antibiotic selection for the MSC clinical trial to be undertaken in dogs with drug-resistant bacterial infections. These studies used conventional bacterial killing assays to assess the interaction of antibiotics and MSC secreted factors. To determine if MSC would be effective against multi-drug resistant bacteria in dogs, we utilized bacterial isolates from CSU veterinary diagnostic laboratories that were multi-drug resistant and had been isolated from canine infections. We utilized a gram positive bacteria (*Staphylococcus pseudointermedius*) and a gram negative bacteria (*Escherichia coli*) as these bacteria are commonly isolated from canine patients and may interact differently both with MSC and antibiotics. We tested representatives from commonly prescribed classes of antibiotics and determined a subinhibitory dose for each antibiotic with the bacteria we were using by determining the minimum inhibitory concentration of that bacteria with each antibiotic. We found that MSC interacted synergistically with the antibiotics from each class tested. Our results show that canine MSC combined with commonly used antibiotics exhibit strong bactericidal activity against both *S. aureus* and *E. coli* **(Figure 3.1*)***. MSC alone did not exhibit significant killing, but when combined with commonly used

antibiotics *in vitro*, there was a significant decrease in total bacterial count, showing synergistic increase of bacterial killing between MSC and gentamycin (**Figure 3.1a,f*(*,** cefazolin ***(*Figure 3.1 b,g),** vancomycin (**Figure 3.1 c,h**), enrofloxacin (**Figure 3.1 d,i**) and chloramphenicol (**Figure 3.1 e,j**)


MRSP
E.coli
A
MRSP log(CFU/mL)
MRSP
Gentamycin
MSC
F
e.coli log(CFU/mL)
e.coli
Gentamycin
MSC
Aminoglycoside
B
MRSP log(CFU/mL)
MRSP
Cefazolin
MSC
G
e.coli log(CFU/mL)
e.coli
Cefazolin
MSC
Cephalosporin
C
MRSP log(CFU/mL)
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e.coli log(CFU/mL)
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e.coli log(CFU/mL)
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Enrofloxacin
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Fluoroquinolone
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MRSP log(CFU/mL)
MRSP
Chloramphenicol
MSC
J
e.coli log(CFU/mL)
e.coli
Chloramphenicol
MSC
Broad Spectrum

**Figure 3.1. Interaction of antibiotics and MSC secreted factors for induction of bactericidal activity.**
*In vitro* bacterial killing assays showing synergistic effects between MSC and antibiotics. BKA performed as described in material and methods. Left column shows BKAs performed with canine MRSP isolate, right column shows BKA results using canine *E.coli* isolate. Bars represent mean and SD, with $\log_{10}$ bacterial CFU/mL on y axis. Synergy was computed using prism interaction factor. Each antibiotic used is listed in lower grid for **a,f)** Gentamycin **b,g)** Cefazolin **c,h)**Vancomycin d,i) Enrofloxacin **e,j)** Chloramphenicol Comparisons between three or more groups were done using one-way ANOVA, followed by Tukey multiple means post-test. statistical significance was determined for $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$.

**Activated canine MSC ameliorated clinical signs in dogs with multi drug resistant (MDR) bacterial infections at various sites**

Previous studies in a mouse model of biofilm infection reported efficacy of pre-activated MSC (aMSC) when combined with antibiotics. Our studies *in vitro* suggested that MSC may act synergistically with various antibiotic classes to combat MDR infections. To test efficacy *in vivo*, we conducted a clinical in dogs with naturally occurring multidrug resistant infections that had not responded to conventional therapy for at least two weeks. Results of this study show improvement in clinical response in 88% of patients at the 8 week conclusion of the trial. Fifty percent of dogs had complete resolution of bacterial infection with negative cultures at 8 weeks. Twenty five percent of dogs had a partial response with a decrease in bacterial numbers or eradication of some species of bacteria in a polymicrobial infection. Twenty five percent of dogs had no change in bacterial response at 8 weeks **(Table 3.1)**. This response rate is similar to what we reported previously in a clinical trial with seven dogs enrolled in which 5/7 cleared their infections.[26] Dogs were enrolled after review of record and culture results. Enrollment required a positive culture with an MDR bacteria at the time of enrollment and at the time of the first MSC injection if enrolled before treatment. All dogs had to have failed conventional therapy with conventional antimicrobials for greater than two weeks prior to initiation of treatment and

were required to remain on the antibiotic that they had been receiving over that two week period with no change in antibiotics throughout the course of the trial. Bacterial cultures were collected and quantified by performing quantitative cultures of FNAs of 4 quadrants of the wound as described in methods and the bacteria was identified and counted using colony morphology to identify different types of bacteria. Bacteriologic and clinical responses were evaluated at 8 weeks as described in methods as this was the predetermined endpoint of the study. However, several dogs were evaluated and treated for longer than the study period and results documented and described below.

Dog number one was a 9 year old neutered male Newfoundland treated for osteosarcoma of his forelimb with a limb spare surgery. At the time of enrollment, he had been treated with multiple antibiotics and had gastrointestinal side effects with many of them and was currently being treated with Cephalexin. He had an open wound along the forelimb with exposure of the bone plate. Previous cultures had revealed multiple MDR bacteria including *Staphylococcus pseudointermedius, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Corynebacterium species*. At time of enrollment he had heavey growth of *Proteus mirabilis* and MRSP. He was continued on cephalexin and treated with systemic aMSC every two weeks. At the time of the second injection he had surgery to revise the wound as the skin had started to grow under the exposed bone plate. By week 8 he had improved clinically with less discharge and the surgery site from week 2 had partially dehisced but was contracting. At this time he was terminated from the study but area of incision dehiscence was not closed and subsequently became re-infected with the previous staphylococcus as determined by discharge and skin necrosis. At this point he was re-enrolled in the study and continued to receive MSC injections every 2 weeks until full closure of the wound He received a total of 9 injections and the wound

fully resolved with resolution of bacterial infection and did not recur until the time of his death from unrelated causes 2 years after initiation of therapy **(Fig 3.2a,b Fig 3.3)**.

Dog number two was a 1.5 year old female spayed Great Pyranees who had sustained trauma with pelvic fractures and an open wound on the left thigh. The wound was treated as an open wound and showed signs of infection for which she was treated with marbofloxacin. Methicillin resistant *Stapylococcus pseudointermedius* (MRSP) was subsequently diagnosed on bacterial culture and she was changed to chloramphenicol. Based on the sensitivity of the MRSP, the infection should have resolved, however she continued to have ongoing clinical signs of infection with redness, swelling, discharge and necrosis of surrounding skin over the following two weeks after starting this antibiotic. At the time of the first injection MRSP was again cultured from the open wound and aMSC therapy initiated. At the time of the second injection Pseudomonas aeruginosa was additionally cultured (with MRSP). Over the course of the 8 weeks of the study both bacteria were eliminated and the wound healed uneventfully **(Fig 3.2c,d Fig 3.3)**.

Dog number three was a 5 year old neutered male Labrador retriever who had previously sustained an inguinal wound from lawn edging. The wound was initially closed and subsequently dehisced and a multidrug resistant *Escherichia coli* (*E. coli*) and MRSP were identified on bacterial culture. He was placed on amoxicillin/clavulanic acid and marbofloxacin and a closed suction drain placed. Following removal of the drain the area dehisced and he was enrolled in the MSC study. At the time of enrollment MRSP and *Pseudomonas aeruginosa* were cultured. He was continued on the two antibiotics for the duration of the trial. Only MRSP was cultured throughout the remainder of the trial and the wound was treated as an open wound until

a healthy granulation bed was present. Following closure of the wound it healed uneventfully and further bacterial cultures were negative **(Fig 3.2e,f, Fig 3.3).**

Dog number four was a 17 year old female spayed Border Collie with a history of a decubital ulcer infected with MRSP. Surgery had been performed previously to close the area which subsequently dehisced and culture revealed MRSP. Throughout the duration of the trial the open wound contracted but continued to culture positive for MRSP. At the 8 week visit the wound was almost completely healed and the MRSP colony count decreased but colonies were still detected via FNA of the wound **(Fig 3.2g, Fig 3.3)**. The owner reported that the wound closed but reopened at a later date. This dog was lost to follow up after the report which was obtained at 12 weeks after starting therapy.

Dog number five was a 2 year old neutered male Anatoli Shepard who was adopted with a deep pyoderma and osteomyelitis of the left anterior carpal region. He had been previously treated with chloramphenicol, and then enrofloxacin with continued positive cultures for MRSP and limited contraction of the wound. His initial culture showed heavy growth in all four quadrants of the wound. The cultures were negative at the time of the second injection and the wound was healed fully by 8 weeks and the dog was subsequently lost to follow up **(Fig 3.2 h, Fig 3.3)**.

Dog number six was a 4 year old male neutered wire haired Griffon who was obtained by new owners with a chronic history of lameness and swollen tibiotarsal joint. Joint cultures performed at that time revealed growth of MRSP. He was treated with clindamycin for 30 days and lameness improved but recurred immediately after discontinuing antibiotics. Over the next 6 months he received several courses of antibiotics with variable response of the lameness and culture remained positive for MRSP. He was enrolled in the clinical trial and remained on

clindamycin. Quantitative cultures were not performed as the joint was not amenable to 4 quadrant cultures. Joint fluid samples were obtained for culture at each visit and qualitative cultures performed by the veterinary diagnostic laboratory. The culture was positive at the time of his first treatment with aMSC but all subsequent cultures were negative and owner reported that his lameness had resolved by the second aMSC treatment. He completed the study and continued to be clinically asymptomatic until approximately 1 year following treatment when lameness recurred and culture again revealed MRSP. He was treated with antibiotic therapy at that time based on culture results and subsequently lost to follow up.

Dog number seven was a ten year old male neutered Labrador retriever who had received radiation therapy for nasal chondrosarcoma 1 year prior to enrollment in the clinical trial. At the time of enrollment, he was experiencing severe malodorous discharge from both nostrils with stertorous breathing and intermittent epistaxis. Bacterial culture revealed heavy growth of 3 strains of MRSP, 3 *Enterococcus*, *Arcanobacter, Streptococcus* and *E. Coli.* Quality of life had declined such that the owner was considering euthanasia. He was continued on the most recent systemic antibiotic he had received (marbofloxacin) for the duration of the trial. Following the first three injections at two week intervals, he showed marked improvement in clinical signs as assessed by significantly decreased odor and amount of discharge. Due to location quantitative cultures were not performed and owner and veterinarian assessment of clinical signs as well as types of bacteria cultured from nasal swabs was utilized as an indicator of improvement. Clinical signs began to worsen one month after discontinuation of the aMSC therapy at which time it was reinstituted monthly for 3 months and then every other month with similar improvement of clinical signs. Intermittent cultures continued to reveal presence of MRSP and streptococcus. One year after enrollment in the clinical trial he developed a draining tract over

the maxillary region with tissue necrosis. Biopsy revealed presence of a histiocytic sarcoma and euthanasia was elected.

Dog number eight was an 11 year old spayed female American Eskimo who had received radiation therapy for a nasal squamous cell carcinoma 1 year prior to enrollment in the clinical trial. She had been on trimethoprim sulfamethoxazole for 1 month prior to initiation of treatment with aMSC. Nasal swabs consistently cultured positive for *Staphylococcus pseudointemedius* which was initially methicillin sensitive and converted to a methicillin resistant strain 6 months after starting aMSC therapy. Previous cultures had revealed infection with *E. Coli*, *Klebsiella pneumoniae*, *Corynebacterium*, and *Enterococcus.* At the time of enrollment in the trial she cultured positive for *S. pseudointermedius* and *E. Coli*. She was initially treated with the standard doses of systemic aMSC every 2 weeks for three treatments. Amelioration of her clinical signs was noted by the owner in the form of decreased discharge and decreased stertor. This increased after discontinuing aMSC therapy. Therapy with aMSC was reinstituted at monthly intervals for 3 months and then bimonthly intervals for 6 months. She was continued on antibiotics for the initial 6 months of treatment. The dog continued to culture positive for *E. Coli* intermittently and *S. pseudointermedius* of two different strains one of which developed methicillin resistance. Quantitative cultures were not performed due to location of infection and intermittent cultures were performed to assess flora at the site. Clinical signs continued to remain at bay and at the time of this writing the dog is not currently receiving antibiotics or stem cells and is clinically doing well as assessed by minimal nasal discharge with no odor.

**Table 3.1 Summary of bacterial responses in clinical trial**
Clinical trial in dogs with multidrug resistant infections reveals amelioration of bacterial infection in dogs with wounds. Two dogs treated with chronic nasal infections showed improvement of clinical signs but bacteria were not eliminated. MRSP = methicillin resistant Staphylococcus pseudointermedius EC = Escherichia coli PA = Pseudomonas aeruginosa Crn = Corynebacterium species PR = Proteus species Ent = Enterobacter, Entc= Enterococcus species, Strep = Streptococcus species, Kleb pn = Klebsiella pneumoniae

| Dog | Infection Site | Duration | Bacteria | Clinical Response at 8 weeks | Bacteriologic Response at 8 weeks |
|---|---|---|---|---|---|
| **1** | Post op limb spare surgical site dehiscence | 1 year | MRSP<br>PR | **Responder** – 50% wound contraction | **Nonresponder** – Proteus and MRSP decreased by 10% |
| **2** | Post op wound repair dehiscence | 6 weeks | MRSP<br>PA | **Responder** – 75% wound contraction | **Responder** – eliminated infection |
| **3** | Post op wound repair dehiscence | 8 weeks | MRSP<br>PA<br>EC | **Responder** – fully healed | **Responder** – eliminated infection |
| **4** | Decubital ulcer | 4 weeks | MRSP | **Responder** – 80% contraction of wound | **Nonresponder** – no change in quantitative count |
| **5** | Wound antebrachium | 8 weeks | MRSP | **Responder** – fully healed | **Responder** – eliminated infection |
| **6** | Infection in tibiotarsal joint | 6 months | MRSP | **Responder** – joint cytology normal at 8 weeks | **Responder** – eliminated infection |
| **7** | Nasal infection post radiation | 1 year | MRSP- 3 strains EC, Entc, Strep | **Nonresponder** – 10% improvement in nasal score | **Partial Responder** - eliminated all bacteria but MRSP |
| **8** | Nasal infection post radiation | 1 year | MRSP- 2 strains EC, Entc, Kleb pn,, Crn | **Responder** – 50% improvement in nasal score | **Partial Responder** - - **e**liminated all bacteria except MRSP and *E Coli* |

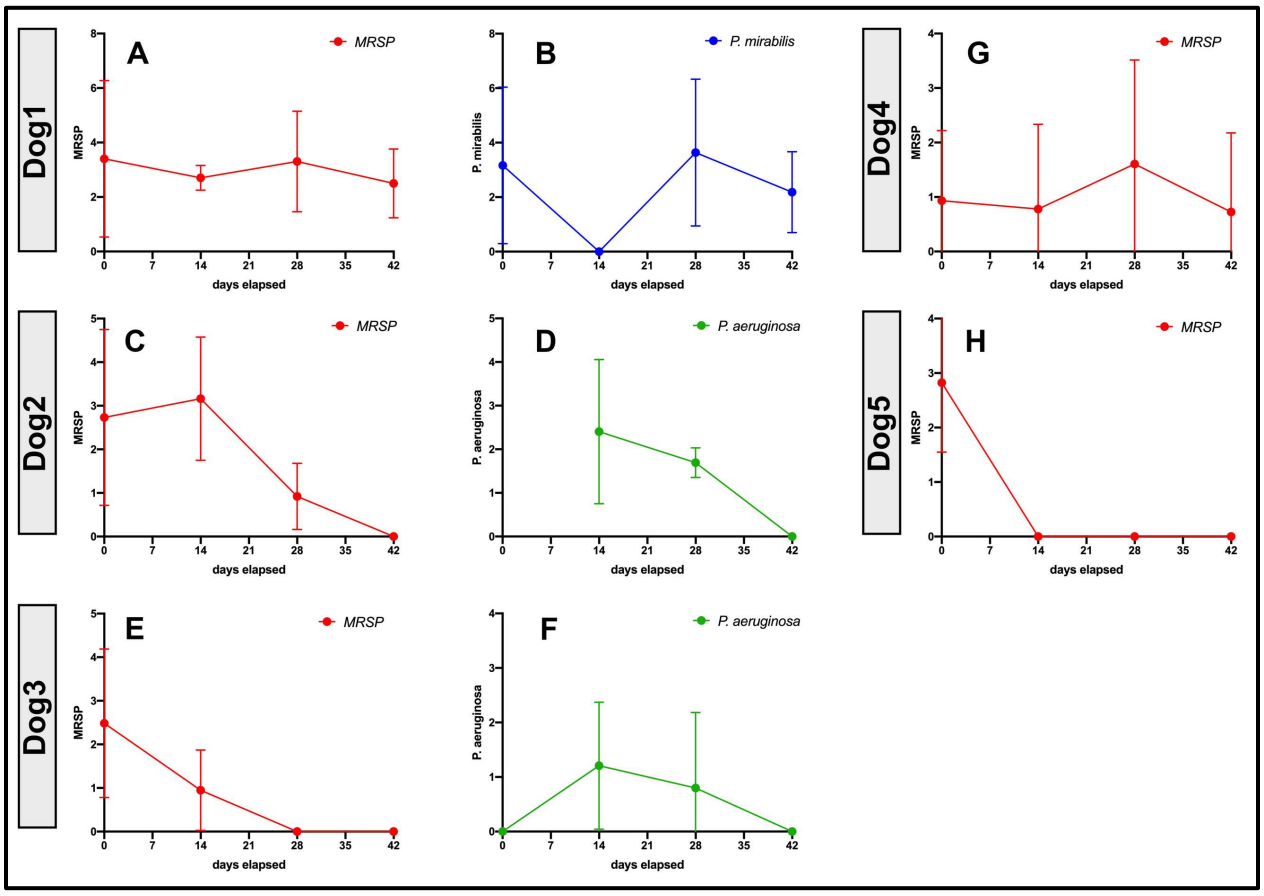


**Figure 3.2 Bacteriologic response at 8 weeks in patients with infections amenable to quantitative bacterial cultures show bacteriologic response in 3/5 dogs with wounds**
Aspirates were taken from 4 quadrants of each wound from dogs 1 through 5 as described in methods, x axis shows days elapsed since treatment from first aMSC treatment at day 0 and 2 weeks following 3rd aMSC injection at day 42. Y axis shows bacterial counts average and standard deviation for 4 quadrants surrounding the wounds. **a**) bacterial counts of MRSP (in red) for dog1 **b**) *Proteus mirabilis* (blue) from dog 1 **c**) MRSP counts from dog 2 (in blue) **d**) *Pseudomonas aeruginosa* counts for dog 2 (in green) **e**) MRSP counts for dog 3 (in red) **f)** *Pseudomonas aeruginosa* counts for dog 3 (in green) **g**) MRSP counts (in red) for dog 4 **h**) MRSP counts (in red) for dog 5


**Figure 3.3 Images of patients with open wounds demonstrating clinical response at 8 weeks.** Clinical response defined as greater than 50% reduction in diameter of open wound at 8 weeks.

**Canine MSCs express and secrete antimicrobial peptides**

Previous studies in human, rodent, and also equine models have shown that LL37 (cathelicidin) antimicrobial peptide (AMP) as well as other antimicrobial peptides including hepcidin, lipocalin, Human βdefensin, and Surfactant Protein D are essential for MSC mediated killing in both gram negative and positive bacteria, typically by the penetration of bacterial membrane.[28,44,45] In addition, AMPs are known to act synergistically with antibiotics and effect direct killing which may explain the synergistic effect of MSC and antibiotics demonstrated *in vitro.* To investigate the mechanisms of bacterial killing, we looked at the expression of these antimicrobial peptides in canine MSCs. We found that canine adipose derived MSC expressed high levels of LL37 by immunohistochemistry (**Figure 3.4a**), and also secreted LL37 peptide into media. (**Figure 3.4b**). Canine MSC also expressed high levels of lipocalin, which is commonly found on mucosal and macrophages **(Figure 3.4f)**. [46] Also found in low levels were beta defensin **(Figure 3.4c)**, hepcidin **(Figure 3.4d)** and surfactant protein D **(Figure 3.4e).** Activation of MSC with the TLR3 ligand pIC did not increase expression of antimicrobial peptides above that of resting MSC (data not shown).

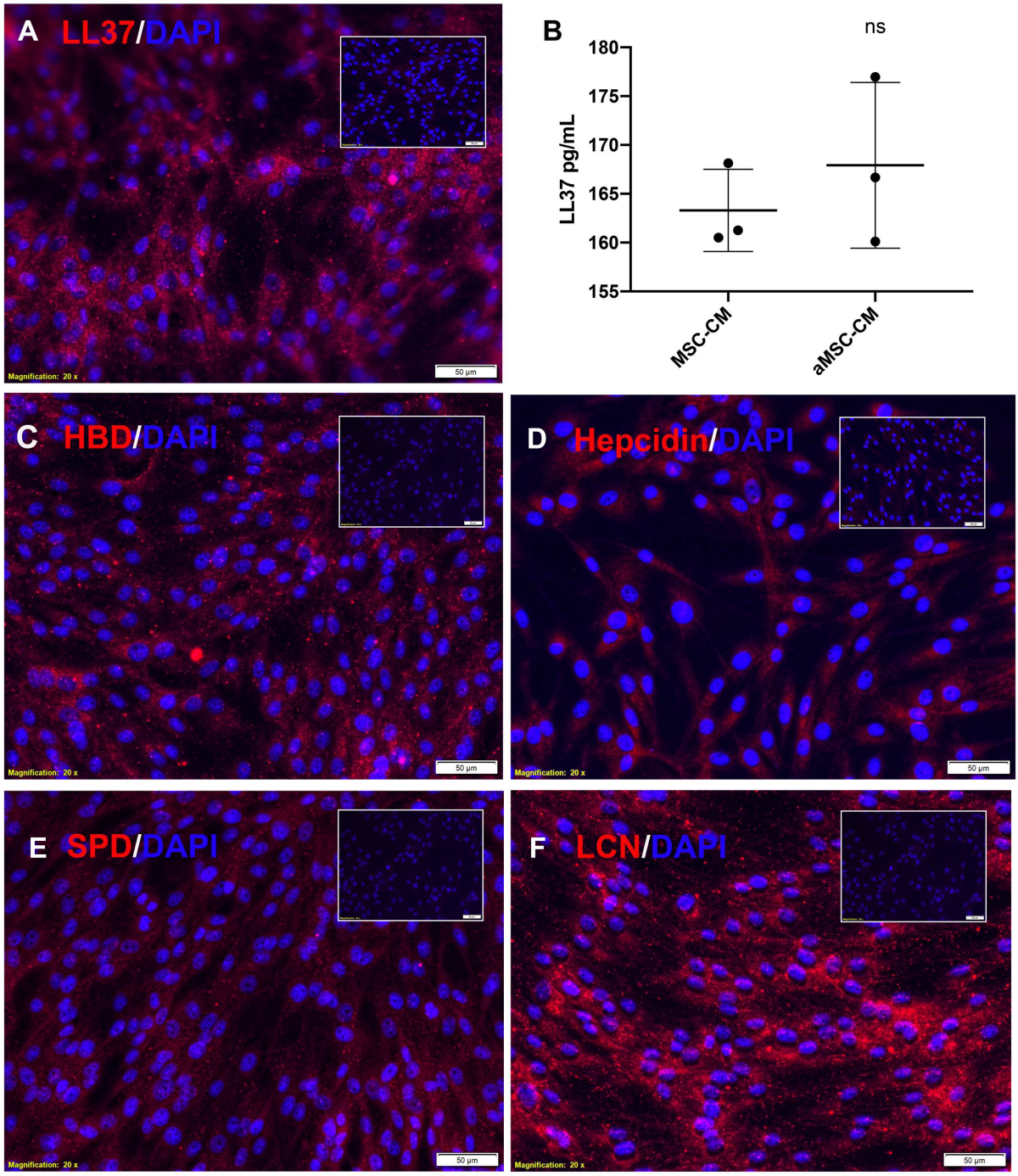


**Figure 3.4 expression of antimicrobial peptides in canine MSC**
Canine MSCs were immune stained with polyclonal antibodies against AMP as described in methods. All panels show AMP staining in red, nuclei stained blue with DAPI and isotype control insert. Scale bar and magnification on bottom for **a)** LL37 **b)** secretion of LL-37 measured by ELISA **c)** human beta-defensin **d)** Surfactant protein D **e)** hepcidin and **f)** Lipocalin. LL-37 ELISA performed using MSC conditioned media (activated and resting) from 3 separate dogs and each dot represents average value for 3 technical replicates from each dog. Significance computed by nonparametric t-tests (Mann-Whitney test)

**MSC activation enhances migratory ability**

Previous studies in our lab using a mouse biofilm infection model have shown that activating mouse adipose derived MSC increases the number of MSC that migrate to the site of infection following IV delivery. [26] Multiple studies have demonstrated that MSC express the chemokine CXCR4 which is critical in MSC migration towards an inflammatory stimulus as this chemokine directs migration to the molecule SDF-1 produced at the site of inflammation. [47] [48,49] To determine if pre-activating canine MSC would increase migration towards a site of inflammation we utilized an *in vitro* transwell migration assay. Migration of canine MSC towards inflammatory molecule SDF-1 was significantly increased when cells were pre-activated with poly I:C (Fig 3.3). Infection causes ongoing inflammation and release of SDF-1.[50] Therefore it would be expected that increased stem cell migration towards the site of inflammation and infection would increase numbers of stem cells at that site (**Figure 3.5**).

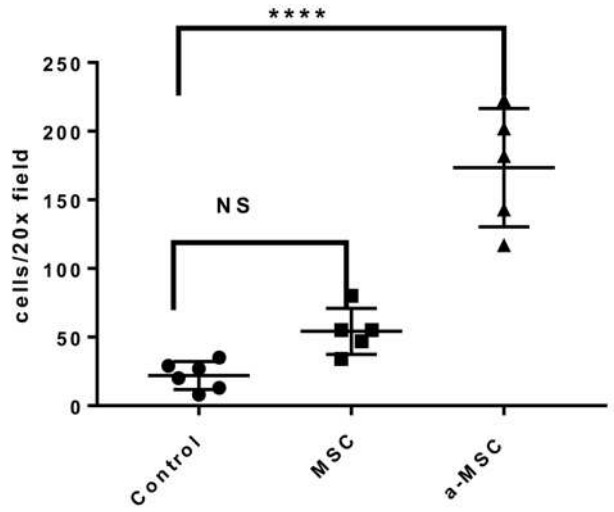


**Figure 3.5 Activation with pIC increased migratory ability of MSC**
Migration assay was performed *in vitro* as described in methods. Graph shows cells per field of crystal violet stained MSC that migrated across membrane towards a recombinant SDF1 gradient. X axis shows pIC activated MSC (aMSC), resting MSC and control with base media only.

**Activation of MSC with TLR 3 agonist influences cells of the innate immune response involved in bacterial clearance and wound healing.**

In addition to having enhanced migratory abilities, activated MSCs have been demonstrated to have effects on inflammatory cells that affect wound healing.[21] It has been previously demonstrated that pre-activation of MSC by ligation of TLR3 increases longevity and bacterial killing of neutrophils.[36] MSC are well known to secrete Vascular Endothelial Growth Factor (VEGF), a cytokine that is important in increasing blood flow and thus delivery of immune cells such as neutrophils to fight infection and macrophages to clean up debris and promote wound healing.[21,51,52] To determine if MSC increased secretion of factors that would affect humoral immunity and wound healing we investigated presence of chemokine IL-8 which promotes migration of neutrophils and VEGF which would promote formation of vasculature that could increase delivery of humoral immune cells. We found that pre-activating canine MSC with poly I:C increased the secretion of IL-8 from MSC 20 fold **(Fig 3.6a).** Both resting and activated MSC secreted VEGF and there was no difference in amount secreted when cells were pre-activated **(Fig 3.6b)**. There were no detectable levels of inflammatory cytokines IL-1β, TNFα or anti-inflammatory cytokine IL-10 (data not shown).

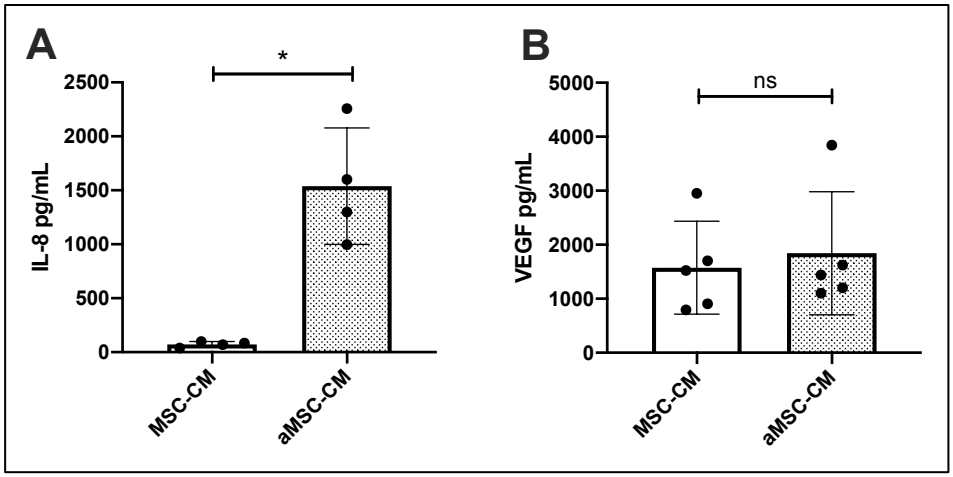


**Figure 3.6 canine MSCs upregulate IL8 with TLR-3 activation and both resting and activated MSC secrete VEGF**
**a)** IL-8 ELISA results showing untreated (white bar) and activated MSC (checkered bar) **b)** VEGF ELISA results. * denotes $p < 0.05$ as determined by nonparametric t-tests (Mann-Whitney test).

**Canine MSCs secrete factors that enhance macrophage phagocytosis and bactericidal activity**

Effects of MSC on the innate immune response are not limited to neutrophils. Multiple studies in rodent models have demonstrated the effects of MSC on monocytes and the subsequent promotion of bactericidal activity through increased macrophage phagocytosis.[7,53,54] To determine if canine MSC increased phagocytosis to a greater degree when pre-activated canine macrophages were incubated with conditioned media from resting or aMSC. A 2 day Incubation with resting MSC conditioned media as well as aMSC conditioned media significantly increased phagocytosis over a period of 9 hours (**Figure 3.7a,b**). Both resting and activated MSC showed increased phagocytosis compared with control with resting MSC ingesting more bacteria than resting MSC. However, in addition to increasing phagocytosis, aMSC-CM also increased the bactericidal effect of canine monocyte derived macrophages when incubated with live MRSA, while resting MSC-CM did not. (**Figure 3.7c**).

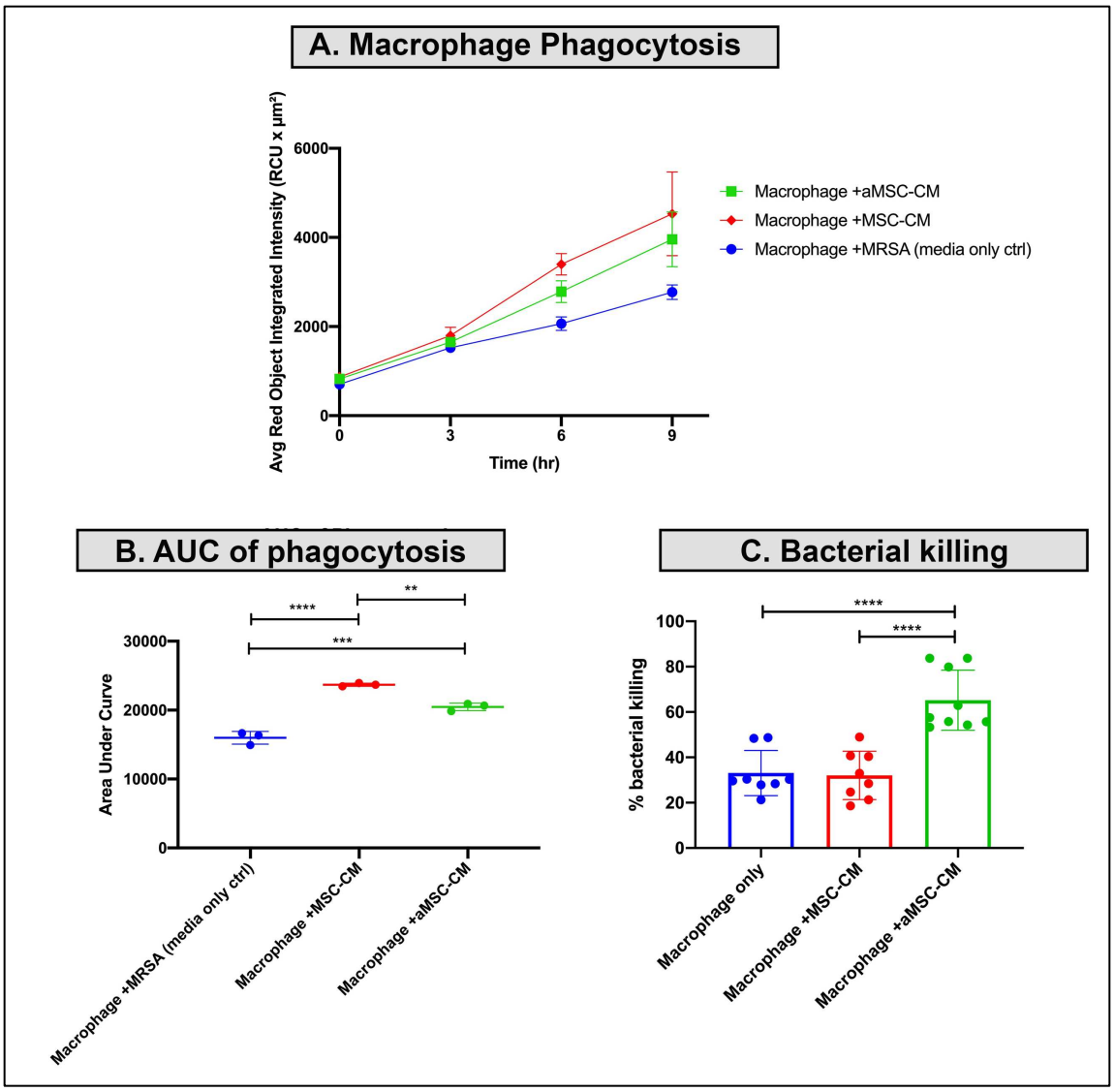


**Figure 3.7 Increased phagocytosis of MRSA by canine macrophages incubated with supernatant from MSC (MSC-CM) and increased killing of bacteria in macrophages when incubated with aMSC-CM.**

**a)** Intensity of phagocytosed bacteria indicated by a change in color following pH changes in the lysosome. X axis shows incubation time and y axis shows "number" of phagocytosed bacteria represented in pixel counts as described in methods. **b)** area under curve (AUC) calculations of incucyte phagocytosis data over a 9-hour period. Color legend indicated in top right with no MRSA control (blue), MSC conditioned macrophage (red), and activated MSC conditioned macrophage (green). **c)** Percent bacterial killing measured by CFU/mL when macrophages were conditioned with MSC-CM then incubated with live MRSA. Y axis shows percent bacterial killing by macrophage only (blue), MSC conditioned macrophage (red), activated MSC conditioned macrophage (green). Comparisons between three or more groups were done using one-way ANOVA, followed by Tukey multiple means post-test.

**MSC secreted factors induce alteration of macrophage polarization towards a cell with both M1 and M2 properties.**

In addition to enhancing phagocytosis and bacterial killing, MSC have been reported to alter the activation state of macrophages. Multiple groups report an increased M2 or wound-healing, anti-inflammatory type macrophage after interaction with MSC.[55,56] *In vivo* studies in a mouse model of *S. aureus* biofilm infection demonstrated increased M2 macrophages in the tissue of mice treated with activated MSC when compared to resting MSC therapy and mice treated with antibiotics alone.[26] To determine the effect of resting and activated MSC on canine macrophages we co-incubated canine primary macrophages with conditioned media of both resting and pre-activated canine MSC. Unpublished studies on our lab have validated CD206 and iNOS as canine cross-reactive markers for M2 and M1 polarized macrophages respectively. Using established protocols, we compared primary macrophages polarized to an inflammatory M1 or alternatively activated, anti-inflammatory M2 phenotype to macrophages incubated with MSC-CM or aMSC-CM (**Figure 3.8**).[57] Our results show that MSC incubation of macrophages did not significantly increase expression of CD206, a M2 marker compared to resting macrophages (**Figure 3.8a**). Compared to resting MSC, factors from pIC activated MSC did not significantly increase CD206 or iNOS (**Figure 3.8b**). When comparing M2/M1 ratio, there was no significant difference in MSC conditioned macrophages compared to M0 resting macrophages. However, pIC activated MSCs caused a shift towards the M1 phenotype indicated by the lowered ratio of CD 206 to iNOS (**Figure 3.8c**). Macrophages exposed to MSC media also displayed increased production of anti-inflammatory cytokine IL-10 (**Figure 3.9a**), and while there was no significant difference between resting and aMSC, neither treatment increased major inflammatory cytokine TNFa (**Figure 3.9b**). These somewhat paradoxical results

demonstrate that *in vitro* aMSC have effects on macrophages that may have elements of both inflammatory (M1) and anti-inflammatory (M2) macrophages. It is known that macrophages display plasticity and *in vivo* their activation states are fluid. [58] Therefore, aMSC may activate macrophages along the continuum of M1 to M2 activation and possess both inflammatory and anti-inflammatory properties.

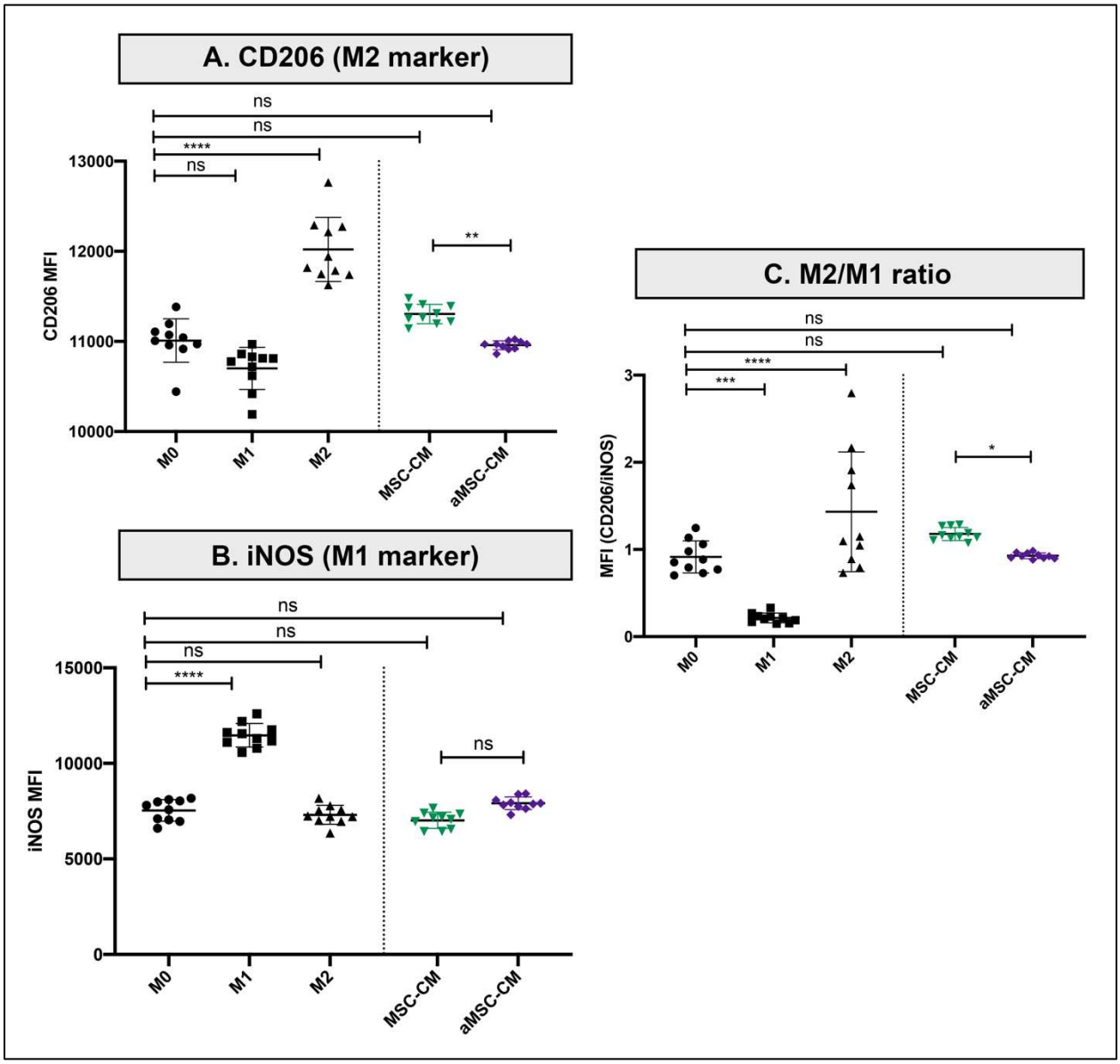


**Figure 3.8 Polarization of canine primary macrophages with MSC-CM**
**a)** Expression of M2 marker CD206 with mean florescent intensity on y axis (MFI). Columns in order show M0 resting macrophages, M1 inflammatory macrophages, M2 alternatively activated macrophages. Then separated by dotted line, MSC-CM conditioned macrophages in green and aMSC-CM conditioned macrophages in purple. **b**) Expression of M1 marker iNOS **c)** M2/M1 ration computed by dividing CD206 MFI by iNOS MFI.. Comparisons between three or more groups were done using one-way ANOVA, followed by Tukey multiple means post-test. statistical significance was determined for $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$

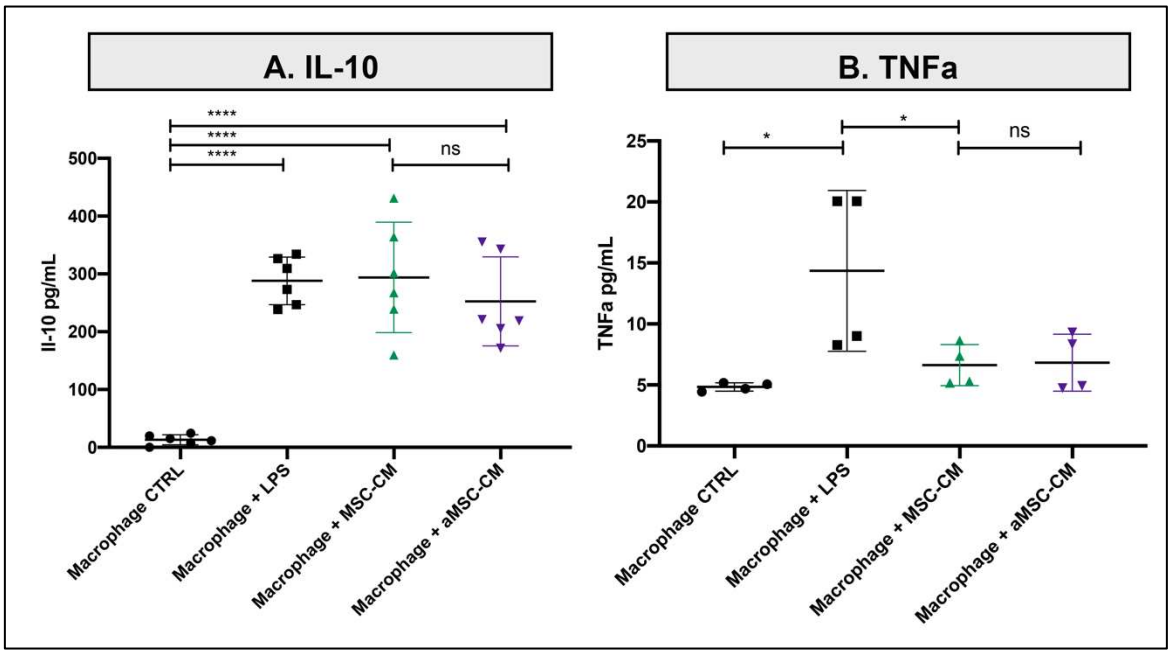


**Figure 3.9 Cytokine secretion profile of macrophages conditioned with MSC-CM**
**a)** IL-10 secretion measured using canine specific ELISA. MSC-CM conditioned macrophages in green and aMSC-CM conditioned macrophages in purple **b)** TNFa secretion measured using canine specific ELISA. Comparisons between three or more groups were done using one-way ANOVA, followed by Tukey multiple means post-test. statistical significance was determined for $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$.

## Canine MSC secrete soluble factors to increase fibroblast metabolism and fibroblast migration

Healing of an infected wound requires bactericidal activity to clear the infection, macrophage influx to clear area of debris and also requires epithelialization to restore the wound barrier and prevent re-infection with environmental bacteria or normal skin flora.[59] MSC have been demonstrated to have wound healing properties mostly attributed to increased angiogenesis and accelerated epithelialization.[60,61] Fibroblasts are also an important component of wound healing and act through secretion of extracellular matrix proteins.[62] Therefore, we examined the effect of MSC secreted factors on canine skin derived fibroblast proliferation, metabolic activity and migration (**Figure 3.10**). Canine skin fibroblasts incubated with aMSC-CM but not resting

MSC-CM significantly increased proliferation as assessed by Edu incorporation (**Figure 3.10a**). Both resting and activated MSC-CM significantly increased fibroblast metabolic activity up to two fold compared to untreated fibroblasts over a 24 hour period (**Figure 3.10b**). A fibroblast scratch assay was utilized to assess migration in response to MSC secreted factors (**Figure 3.10c,d**). Fibroblasts incubated with resting but not activated MSC-CM demonstrated increased ability to migrate across a defect at12 and 24 hours following creation of the defect, indicating increased mobility important for healing large wound areas. Increased fibroblast migration, metabolism and proliferation *in vitro* indicate that canine MSC have a beneficial effect on wound healing.

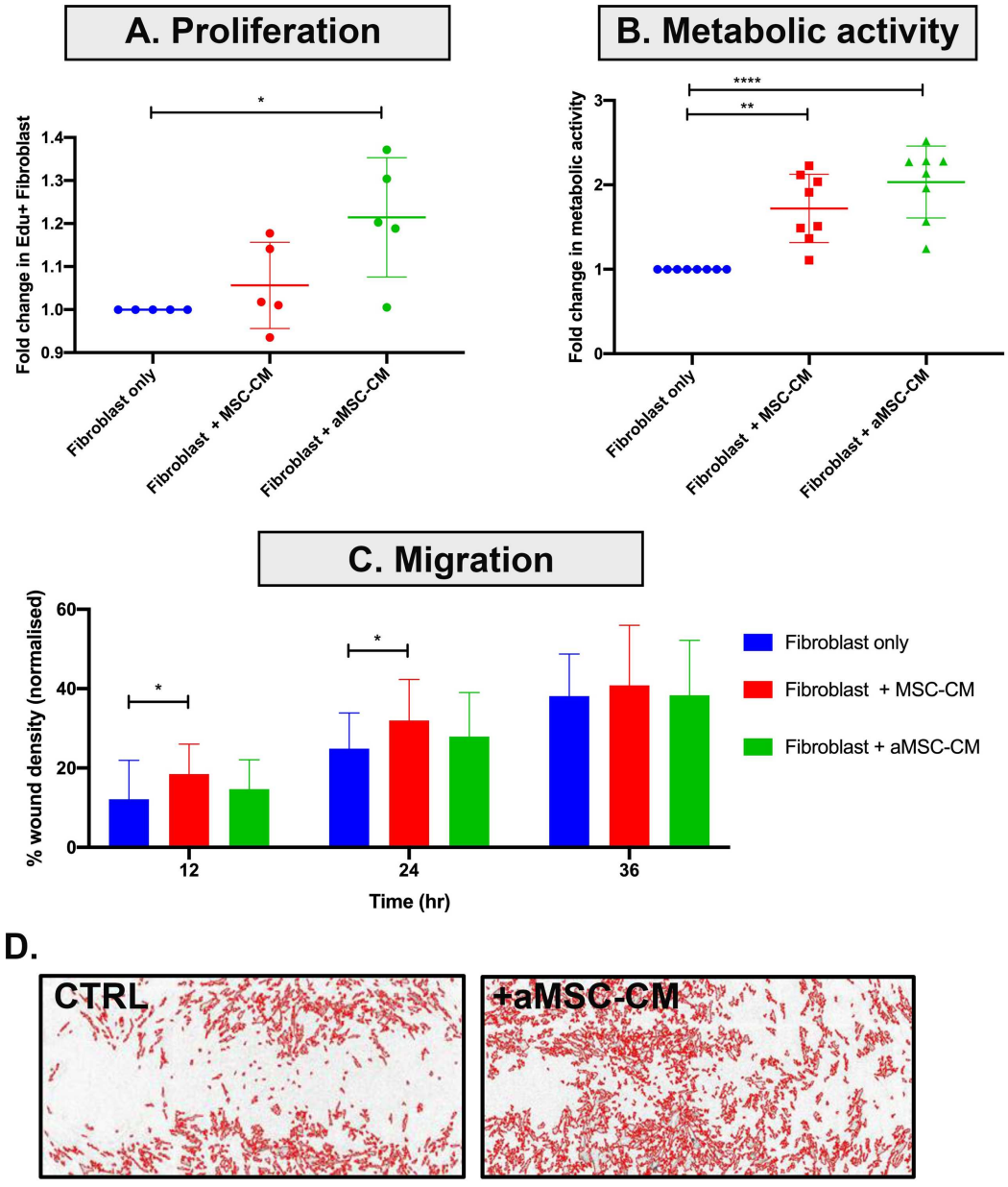


**Figure 3.10 Secreted factors from MSC increase factors associated with wound healing**
**a)** Canine skin fibroblasts were incubated with MSC-CM as described in methods. Green dots show the fold change in Edu incorporation by flow cytometry after a 24 hour incubation period. Purple dots show Edu incorporation in aMSC-CM. Each experiment was completed with 3 donor fibroblast lines using MSC derived from 2 separate dogs. **b)** Fold change in metabolic activity measured by MTT assay. Statistical comparisons between three or more groups were done using one-way ANOVA, followed by Tukey multiple means post-test. statistical significance was determined for $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$. **c)** Coverage of created defect by fibroblast scratch assay with visual representation in **d)**. Coverage of the created defect measured by incucyte as described in methods at specific timepoints. Green bars show fibroblast incubated with MSC-CM while purple bars show MSC incubated with aMSC-CM. Significance was

computed using 2-way ANOVA, with Tukey's multiple comparisons test to compare means within each time point. statistical significance was determined for $*P \leq .05$.
for $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$. Each experiment was completed with 3 donor fibroblast lines using MSC derived from 2 separate dogs. Significance was computed using 2-way ANOVA, with Tukey's multiple comparisons test to compare means within each time point. statistical significance was determined for $*P \leq .05$.

**Discussion**

Mesenchymal stem cells have anti-infective, immunomodulatory and wound healing properties.[9,23,63,64,65] In this pilot clinical trial in dogs with multidrug resistant infections we have established that pre-activated MSC (aMSC) have a beneficial effect on resolution or management of naturally occurring infections that have not responded to conventional therapy. As might be expected, there are some infections that could not be resolved. Patients experiencing multi-drug resistant infections with normal flora such as post radiation nasal infections, could not fully eliminate the infection as the region harbored the bacteria as a normal flora. However, other contaminating bacteria at the site of the wound could be eliminated and their clinical signs dramatically improved such that the comfort of the animal improved to an acceptable level. In cases where the infection was contained in a small wound or a joint, complete resolution was achieved during the eight week clinical trial. In cases where closure of the wound was delayed it was necessary to continue stem cell treatment at a biweekly interval until such time as closure was achievable. As with previously described cases these dogs carried a multidrug resistant staphylococcus as normal skin flora and the bacteria would opportunistically invade and cause infection after discontinuation of aMSC therapy. A solution to this (which was performed in Dog #1) was to continue biweekly infusions of aMSC until such time as the wound could be completely closed. In this dog complete resolution of the infection was achieved by this method.

A previous study conducted in a rodent model demonstrated resolution of biofilm infection utilizing activated mesenchymal stem cells together with antibiotics.[26] This study did not demonstrate consistent bacterial killing *in vitro* with MSC alone but did demonstrate synergistic killing with antimicrobial agents even when the bacteria had acquired resistance to that antimicrobial agent. Antimicrobial peptides are part of the host innate immune response and

are produced by several cell types. They have been demonstrated to work synergistically with common antimicrobial agents and are also produced by mesenchymal stem cells.[66,67,27] In fact, LL-37, a cationic antimicrobial peptide, has been demonstrated to be partially responsible for the antimicrobial effect in a rodent model of pneumonia and other studies have demonstrated the beneficial effect of antimicrobial peptides during MSC therapy.[68,69,29,46] In this study we demonstrated that canine MSC express antimicrobial peptides (AMPs) LL-37, hepcidin, human β-defensin, lipocalin and surfactant protein D. It is likely that production of these AMPs is responsible for the observed synergistic effect *in vitro* with MSC and antibiotics but further testing would be necessary to demonstrate this association. When measured by ELISA, there was no difference in the amount of antimicrobial peptide LL-37 secreted by activated versus resting MSC. Previous studies in a mouse model demonstrated increased migration of cells to sites of infection when MSC are preactivated.[26] In this study we demonstrate increased migration of canine MSC towards and inflammatory stimulus when activated. It is likely that these cells perform *in vivo* in a similar manner as in the mouse model. Therefore, we could expect that increased numbers of cells at the site of infection would then result in an increased secretion of antimicrobial peptides at that site.

It is likely that the observed clinical response is due to a combination of effects involving antimicrobial peptides, immunomodulation, anti-biofilm effects and wound healing effects. We demonstrated that secreted factors from MSC resulted in increased phagocytosis and killing by canine macrophages *in vitro.* Both resting and activated MSC demonstrated increased phagocytosis, however, only activated MSC demonstrated increased killing of bacteria by macrophages. This is consistent with what has been demonstrated in rodent models of sepsis and other animal models.[8,7] In a mouse model of biofilm wound infection we demonstrated an

increase in M2 or wound healing macrophages in the tissues of mice that had received activated MSC and antibiotics.[26] When canine macrophages were exposed to MSC conditioned media we observed a trend towards increased M1 or inflammatory macrophages when macrophages were exposed to activated conditioned media only. However, these same macrophages when exposed to aMSC-CM secreted more IL-10, an anti-inflammatory cytokine generally associated with an M2 or wound healing macrophage. They also did not secrete TNFα which is generally associated with an M1 or inflammatory macrophage. Although macrophages are classified *in vitro* utilizing surface markers generally associated with inflammatory and anti-inflammatory macrophages, we know this process to be a continuum and as such macrophages *in vivo* are likely in a state of flux and not entirely inflammatory or anti-inflammatory.[58,70] Indeed, some studies have demonstrated that exposure of macrophages to MSC induce an alternative state that has properties of both types of macrophages although it is skewed towards M2.[71] Further supporting a wound healing role of MSC were the response of canine fibroblasts to aMSC secreted factors. Fibroblasts demonstrated increased proliferation, migration and metabolism which would translate to wound healing *in vivo*. It is likely that *in vivo* exposure of MSC to multiple ongoing biological processes leads to a continuum of an effect on macrophages that may involve elements of promoting M1 or M2 macrophages depending on the environment. Indeed, the ability of the stem cell to respond to its environment is likely responsible for the success of this therapy.

Neutrophils also play a large role in infection and previous studies in mice have demonstrated increased neutrophil phagocytosis, increased longevity and increased production of oxygen free radicals in neutrophils when the neutrophils were exposed to conditioned media from activated stem cells.[26,32,30,36] We examined the conditioned media of resting and activated MSC and discovered an increased secretion of IL-8, a chemokine that promotes recruitment of

neutrophils. Increased neutrophil migration together with enhanced bactericidal function likely plays a role in the demonstrated bactericidal activity. Vascular endothelial growth factor (VEGF) is a well-known product of MSC and is thought to play a critical role in the wound healing properties of MSC.[72] Canine MSC were demonstrated to secrete VEGF as previously described but there was no difference in secretion between resting and activated. However, as previously mentioned, increased migration of the activated cells to the wound could cause an increase in local VEGF as more cells are available to secrete the factor.

Limitations of this study include the lack of a control group or a group that received resting MSC. The population we were treating had no other therapeutic options which ethically precluded us from including a no treatment group. Resources were not available to treat unlimited patients so the decision to utilize activated MSC alone was based on previous studies in a mouse model as well as *in vitro* effects of activated cells on immune function.

It is likely that the observed clinical response of resolution or decrease of infection with healing of infected wounds represents a result of a combination of factors likely involving increased antibacterial activity from antimicrobial peptides, increased macrophage killing and phagocytosis, and increased neutrophil migration and killing. It likely also involves an increased wound healing phenotype of immune cells with downregulation of inflammation and increased epithelialization. Previous studies in a mouse biofilm model demonstrated that activated mesenchymal stem cells did not have increased direct anti-bacterial activity *in vitro* when compared to resting MSC. However, more cells migrated to the site and therefore the improved activity of activated over resting MSC was attributed to an increase number of cells at the site. However, merely injecting cells at the site would negate the systemic effect on immune cells which likely also plays a large role in the observed effects. More studies are needed to determine

the role of pre-activation of MSC as well as the effect of systemic response to MSC infusion to determine best route of administration and dose to achieve maximal antibacterial and wound healing effect.

This study demonstrates that allogeneic activated mesenchymal stems are efficacious in treating naturally occurring multi-drug resistant infections in dogs. The mechanism is multifactorial and likely consists of local and systemic effects of the cells on the immune system and wound environment. This novel immunotherapy shows great promise to treat the growing number of resistant infections for which there is currently no therapeutic option.

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# CHAPTER 4

## Intravenous Injection of Adipose-Derived Stem Cells Improves Gait Outcomes in a Rodent Model of Primary Osteoarthritis


### Summary

Osteoarthritis remains a leading cause of morbidity among the aging population yet treatment remains mostly symptomatic aimed at reducing pain associated with osteoarthritis.[1]. Mesenchymal stem cells (MSC) have been demonstrated to have immunomodulatory and anti-inflammatory properties that may be beneficial in both alleviation of symptoms and interruption of pathologic changes that occur during osteoarthritis[2]. Multiple studies have evaluated MSC as a therapeutic modality to treat osteoarthritis. [3] The aim of this study was to evaluate allogeneic adipose derived MSCs in a well-known model of spontaneous osteoarthritis utilizing the Hartley guinea pig model. Systemic administration of MSC to affected guinea pigs resulted in no adverse effects. Intravenously delivered MSC were effective in reduction of symptoms of osteoarthritis as assessed by gait analysis. In addition, there was significant decrease in inflammatory cytokines both systemically and in the joint. Systemic administration of MSC has beneficial anti-inflammatory effects and provides symptomatic relief when utilized in a naturally occurring model of osteoarthritis with multiple joints affected.

**Background**

Osteoarthritis (OA), particularly of the hip and knee, currently burdens greater than 242 million people globally.[4] In the U.S., OA is one of the most common diagnoses in general practice and is the leading cause of physical disability in adults.[1] Despite intensive research, there are currently no pharmacologic therapies that ameliorate joint pathology or prevent progression of this condition. Further, symptomatic treatment for OA has limited efficacy, as the underlying pathology often continues to progress, far surpassing the ability of current pharmaceuticals to manage pain and inflammation. There continues to be a large population of people with severe symptoms from osteoarthritis that are not alleviated by current available pharmaceuticals and where surgical intervention and other therapies such as physical therapy and weight loss are not effective.[5,6] Thus, there is a critical need to evaluate alternative therapies which provide analgesic effect and also halt or reverse the pathologic disease process ongoing in affected joints.

Pathologic changes associated with OA include loss of articular cartilage and changes to the synovium and subchondral bone. These changes are facilitated by loss of homeostasis in the joint with increased pro-inflammatory mediators and chemokines predominating as they are released from damaged or diseased cartilage. Increased inflammation both advances the pathology of this disease and leads to the clinical signs of pain and further damage to the joint thus perpetuating the pathologic process.[7] Many mediators have been implicated in this disease process include inflammatory cytokines TNFa, IL-1β, IL-6, chemokines IL-8 and MCP-1 and prostaglandins such as PGE2.[8] *In vitro* studies suggest that MSC have an anti-inflammatory effect on chondrocytes and decrease secretion of inflammatory mediators.[9] Studies from animal models suggest that intra-articular injection of MSC both ameliorate clinical signs of OA and

have chondroprotective effects.[10,11] Commercially available biologic products derived from non-standardized autologous donor sources are being utilized as a new cellular therapy to reduce joint inflammation and alleviate clinical signs of osteoarthritis. Few conclusions can be drawn from these applications as the technique for collection and processing of these treatments varies widely.[3] Adipose tissue-derived MSCs share similar properties with bone marrow-derived MSCs but are easier to collect for clinical application, with higher isolation yields.[12] To date, most MSC trials have delivered intra-articular injections of MSCs to individual joints.[13] However, when administered intravenously MSC have additional immunomodulatory effects which may exert increasing benefit as this pathologic process rarely involves one joint and MSC are known to home to sites of inflammation.[14] Systemic administration of MSC in a clinical trial in dogs resulted in improved activity and behavior scores.[15]

The focus of the current study was to demonstrate symptom modification following intravenous injection of MSCs in the Hartley guinea pig model of spontaneous OA and assess biomarkers commonly associated with osteoarthritis.[16] Most laboratory models OA are injury induced models utilizing surgery, chemicals, and/or genetic manipulation, and may not adequately recapitulate the predominant type of OA (non-traumatic) diagnosed in people.[17] In contrast, the Hartley guinea pig, is a well-established, naturally occurring model found in an outbred animal, with OA pathology that is histologically similar to nontraumatic human disease.[18] They are also similar to humans in their nutritional requirement for Vitamin C, which is key when considering pathology related to inflammation and oxidant activity.[19,20] Our hypothesis was that, compared to a vehicle control group, MSC would have symptom-modifying effects on OA-prone guinea pigs and would reduce inflammatory biomarkers associated with

OA. An additional aim of this study was to determine if MSC have any effect on the pathologic process of OA.

**Materials and Methods**.

*Animals.*

All procedures were approved by the university's Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Twelve10-month-old male Dunkin-Hartley guinea pigs were purchased from a commercial vendor (Charles River Laboratories, Wilmington, MA). Animals were maintained at Colorado State University's Laboratory Animal Resources housing facilities and were monitored daily. All guinea pigs were housed singly in solid bottom cages and kept in 12-hour light/dark cycles. They were provided with food and water *ad libitum*. Animals were assessed by a veterinarian weekly; no clinical signs associated with systemic disease were noted throughout the course of the study

*Study design.*

After three weeks of acclimation to the treadmill-based gait analysis system, animals were randomly allocated to one of two treatment groups: 1) injection vehicle control (control), and 2) MSCs. All animals were harvest at 12 months of age, a time when this strain of guinea pigs has recognized signs and histological changes consistent with moderate knee OA.[21]

*Tissue Sources and Harvesting Techniques.*

Four-month-old guinea pigs served as donors for MSC cultures. Guinea pig adipose derived MSC were grown from abdominal adipose tissue aseptically collected at necropsy. The

adipose tissue was isolated, pooled, and cultured as previously described.[22,23] Cells adhered to plastic and displayed typical MSC morphology and expansion properties.

*Adipose derived stem cell (MSC) culture.*

MSCs were expanded using conventional methods.[23] Cell Surface Marker analysis was performed by single color-flow cytometry. Adherent cells were analyzed for expression of MSC markers on a Beckman Coulter CyAn ADP flow cytometer operating with Summit v4.3 software for data collection. Cells were individually incubated for 30 mins at 37℃ with the following mouse monoclonal antibodies: human CD73 (clone AD2, APC conjugated, Biolegend, San Diego, CA ); human CD44 (clone 1M7, APC conjugated, Biolegend); human CD90/Thy1 (clone OX-7, FITC-conjugated, Abcam); human CD29 (clone TS2/16, PE-CF594-conjugated, Biolegend); and human CD45 (clone H130, PE-CF594-conjugated, Biolegend). Unstained cells, as well as cells stained with identical isotype antibodies, served as controls. Analysis was done with FlowJo (version 10.0.8; Software, Ashland, OR). Samples were run in biological triplicates with two technical duplicates.

*Single-color flow cytometry.*

For cell surface marker analysis, cells were harvested, resuspended in 100 µL HBSS containing specific antibodies, and incubated at 4°C for 30 min. Samples were analyzed using FACSCanto II low cytometer (BD Biosciences) and FCS Express software (De Novo). Antibodies were used at concentrations as recommended by the manufacturer.

*Intravenous administration of MSC to Hartley guinea pigs with advanced OA.*

Animals were induced for general anesthesia using 3% isofluorane mixed with oxygen, followed by maintenance of anesthetic plane with 1-1.5%. Guinea pigs received vehicle or MSC intravenously in alternating peripheral veins (left and right cephalic and saphenous veins) at a

dose of $2x10^6$ cells per animal in 1ml of sterile injectable heparinized saline. Guinea pigs received a total of 4 injections separated by 1-week intervals: 3 injections involved unlabeled cells with the final injection containing fluorescently labeled MSCs such that the location of distributed cells could be tracked/determined.

*Fluorescently Labeled Mesenchymal Stem Cell Tracking.*

For detection of MSC migration to various tissues (spleen, lung and liver), MSCs were labeled using a cell membrane dye for the final intravenous injections. MSCs were incubated with DiD (Vybrant DiD cell labeling solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 15 minutes, washed three times in PBS to remove unincorporated dye and then resuspended in PBS and heparin prior to administration. Tissue processing was performed as reported previously.[22] Sections of tissue were and frozen at -80 °C, cryosections of tissue were then examined via confocal microscopy for the presence of labeled cells as previously reported.[22]

*Gait analysis in Hartley guinea pig OA model.*

Animals were acclimated to the gait analysis system (Mouse Specifics, Inc., Framingham, MA, USA) over 3 weeks. Training and data collection were performed during the same period (10AM to 1PM) and involved the same handlers (SCP & RBM). All procedures were executed in the dark (except for light emitted from the digigait treadmill and computer screen), as this is the environment in which the animals were most willing to run on the treadmill system. The order of which animals were utilized for gait analysis at each time point was randomly selected.

Baseline gait analysis was performed the day immediately prior to the first injection. Subsequent data was collected 3 days after each injection, with final time point assessed 2 weeks following the fourth injection. This allowed for a total of 5 time points.

*Tissue Harvest*.

At the time of harvest, animals were anesthetized with a mixture of isoflurane and oxygen, as above. Body weights were reported at this time. Thoracic cavities were opened, and circulating whole blood was collected with 20-gauge butterfly catheter via direct cardiac puncture. After blood collections, anesthetized animals were placed in a carbon dioxide chamber for euthanasia. Hind limbs were removed at the coxofemoral joint. The left limb was placed in 10% neutral buffered formalin for 48 hours and then transferred to PBS for storage. Prior to decalcification, femur length was measured using calipers. Limbs were then transferred to a 12.5 % solution of ethylenediaminetetraacetic acid (EDTA) at pH 7 for decalcification. EDTA was replaced twice weekly for 6 weeks.

*ELISA (Enzyme-linked Immunosorbent Assay).*

Enzyme-linked Immunosorbent Assay for monocyte chemoattractant protein-1(MCP-1), tumor necrosis factor alpha (TNFα) and prostaglandin ($PGE_2$) on serum and synovial fluid. Serum from whole blood and joint fluid were collected at the time of euthanasia. Serum and joint fluid were stored at -80 °C until analysis. MCP-1, TNFα and $PGE_2$ analysis and quantification were conducted via ELISA using MCP-1 (ABclonal Science, Woburn, MA), TNFα (R&D systems, Minneapolis, MN), and $PGE_2$ analysis kit (ABclonal Science, Woburn, MA). ELISAs were performed in accordance with the manufacturer's instructions. The assay was conducted in technical triplicate for each sample.

*Histologic Grading of OA.*

After decalcification, coronal sections of the knee were paraffin embedded and sections (5 μm) were taken from the center of the medial tibial plateau and stained with toluidine blue. Two blinded, independent evaluators (KSS and MFA) performed histological grading of four

serial coronal sections using adapted Mankin criteria based upon species-specific features of OA.[24,25] This semiquantitative histologic grading scheme is based on articular cartilage structure, proteoglycan content, cellularity and tidemark integrity. Scores from each of the four anatomic locations were summed to obtain a total knee joint OA score for each guinea pig.

*Immunohistochemistry for TNF on liver, kidney, and synovium tissue.*

Immunohistochemistry (IHC) was performed using an automated immunostainer (Bond RXm, Leica Biosystems, Buffalo Grove, IL) on section of liver, kidney, and whole knee joints using a monoclonal mouse to TNF (Abcam ab1793) at a concentration of 1.75µg/ml. Briefly, tissues were embedded in paraffin and cut 5-µm sections, slides were deparaffinized in xylene, cleared, and rehydrated in graded ethanol. Antigen retrieval was performed by heating slides in a citrate-based solution for 7 hours in desert chamber incubator at 55˚C. Normal goat serum (10%) was used as a blocking agent. Slides were incubated in primary antibody overnight at 4°C, followed by a 30-minute incubation with a biotinylated goat anti-mouse secondary antibody. Bone marrow hematopoietic cells and macrophages frequently exhibited TNF staining, serving as internal positive controls for each section. Exposure to secondary antibody, alone, did not result in any positive immunostaining. Mouse IgG control (Thermo Scientific, Waltham, MA) at a concentration of 1.75 µg/ml served as a negative control for each animal tissue. Sections were counterstained with hematoxylin, cover slipped, and viewed by light microscopy. Integrated optical density (OD) was calculated from analysis of four 20x images per tissue sections immunoassayed for TNF or IgG control, providing a mean intensity value with any background immunostaining from each area removed.

*Sample size calculation.*

Group size and power were determined using the statistical software at www.stat.uiowa.edu/~rlenth/Power. Based upon previous work, histological assessment of OA was selected as the principle outcome. Using a within group error of 0.5 and a detectable contrast of 1.0 in a linear regression model, power associated with Tukey/HSD post-test (alpha=0.5) was calculated as 0.9 with a sample size of 6 per experimental group.

*Statistical analyses.*

Data for total body weights, femur length, histologic OA scores, stride length, maximal speed, serum protein (MCP-1, TNF & PGE2) values, and synovial fluid protein (TNF & PGE2) were subjected to, and passed, normality testing via the Kolmogorov-Smirnov test. For comparison between two groups analysis was performed utilizing a nonparametric t test. Statistical significance was set at $P < 0.05$. All statistical analyses were performed with Prism (version 7.0; Graph Pad Software, La Jolla, CA).

## Results

### General description of guinea pigs.

All animals in both groups survived the duration of the study. There were no differences in body weight between treatment groups. To ensure that differences in gait analysis were not attributable to variations in skeletal properties, femur lengths from all animals were measured. Total body weight ranged between 1014-1250 g (control), and 888-1248 g (MSC) guinea pigs. Femur lengths were between 40.23-43.99 mm (control), and 40.31-42.61 mm (MSC). There were no statistical differences in body weights or femur lengths between groups.

### Guinea pig adipose derived MSC display surface markers consistent with MSC

Adipose derived stem cells surface markers were analyzed by flow cytometry analysis after passage 2 to confirm that phenotype was consistent with MSC. MSC were positive for cell surface markers CD44, CD 90 and CD29 and negative for CD73 and CD34 as previously described for guinea pig adipose derived MSC **(Figure 4.1).** [23,26]

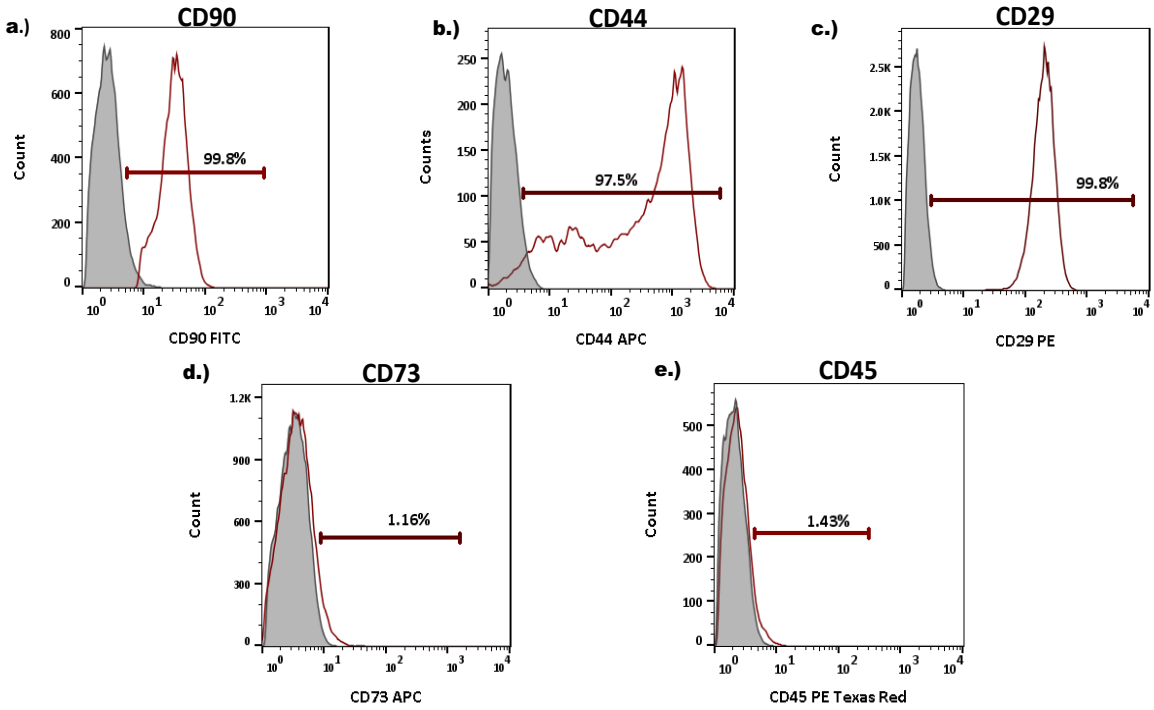


**Figure 4.1: Immunophenotypic characterization of guinea pig MSC by flow cytometry.** Positive expression results of surface marker expression a) CD90 b) CD44 c) CD29 and negative surface marker expression of d) CD73 and e) CD45. Match isotype staining shown in grey while red histograms depict staining with relevant, directly conjugated primary mAbs

**MSC Administered i.v. distribute to lung, liver and spleen**

MSC utilized in osteoarthritis research are generally administered directly in the joint and thought to have an effect locally. [27] A previous studies in dogs with naturally occurring osteoarthritis demonstrated symptomatic improvement with i.v. MSC therapy. [15] Therefore, we hypothesized that systemic administration would result in a decrease in systemic inflammation and increased symptom relief as multiple joints are often affected in age related OA. To

determine the location of intravenously injected MSCs two weeks post injection, cryogenically prepared tissue sections were examined at 14 days after administration of MSC labeled with the lipophilic dye DID. Tissue sections were examined with an Olympus IX-83 confocal microscope, using appropriate lasers and filters. Image analysis was performed using CellSens software (Olympus).

Labeled MSC were detected in the lungs, spleen, and liver of all animals injected with labeled cells **(Fig. 4.2)**. Fluorescently labeled cells were not detected in the lung, spleen and liver of control animals as expected.

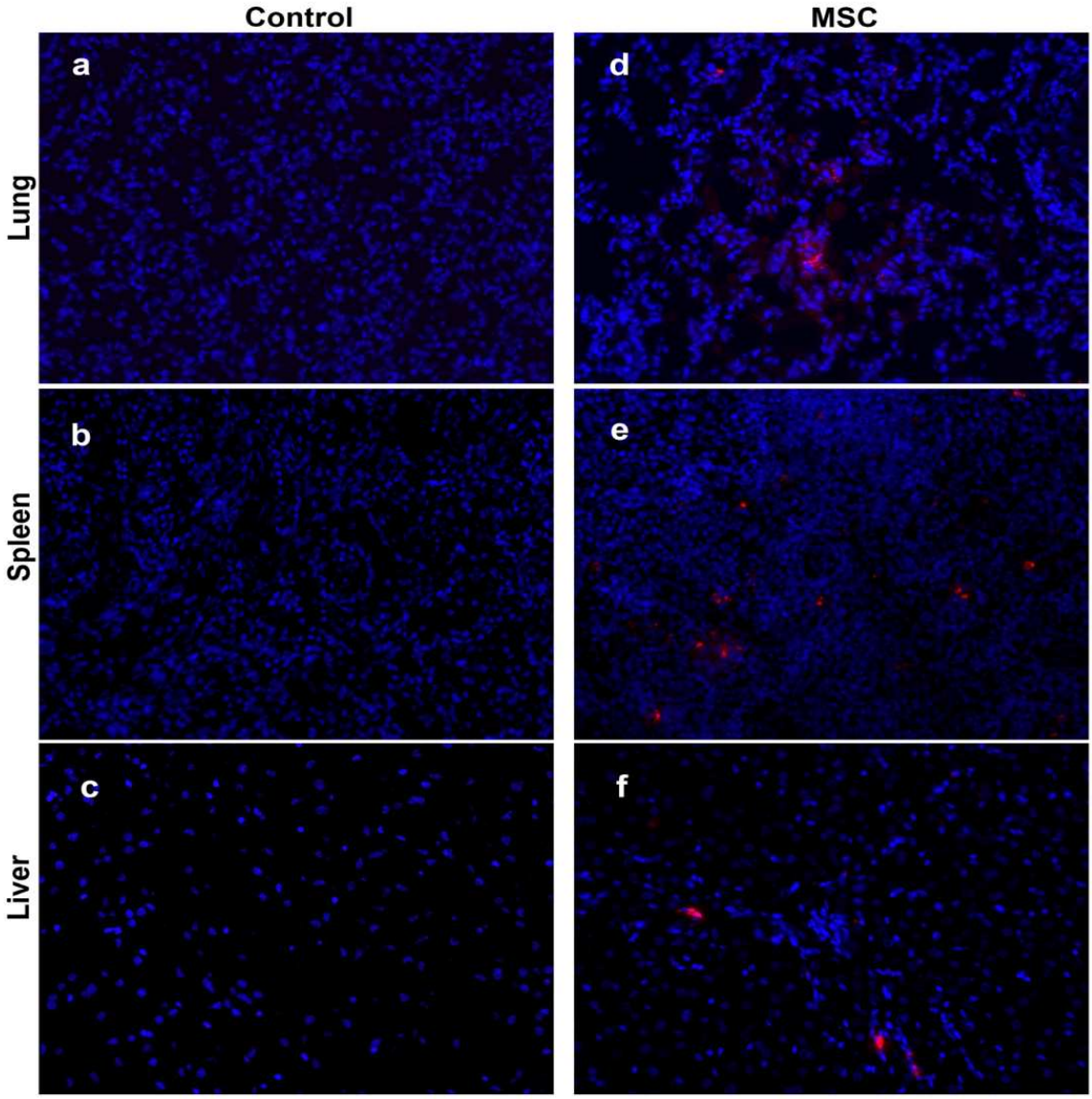


**Figure 4.2 MSC distribute to lungs, liver and spleen**
MSC were labeled a lipophilic fluorescent dye (DiD) and administered intravenously 14 days prior to analysis of tissues. Labeled cells (red) with Dapi nuclear stain (blue) were identified in the lungs, liver and spleen in all animals that received MSC therapy. a) control tissue for lungs with no MSC. b) control spleen tissue with no MSC c) control liver tissue with no MSC. d) Lung with labeled MSC shown in red e) spleen with labeled MSC shown in red f) liver with labeled MSC shown in red.

***No change in histologic score of OA in animals treated with MSC***.

In addition to symptomatic relief, some studies demonstrate improved cartilage regeneration with MSC therapy. [28] To determine if there was any change in joint pathology

sections of joints were examined at the end of the study. OA was assessed by histological assessment of joints using the OARSI grading scheme to evaluate articular cartilage abnormalities. Representative lesions from control and MSC treated group are presented (**Figure 4.3**). Notable evidence of OA included proteoglycan loss with hypocellularity and regions of chondrocyte clustering in animals in all treatment groups. Tidemark duplication was common among all animals. No statistical differences in whole joint OA scoring were present in the treatment group when compared to the control group (**Figure 4.3c**).

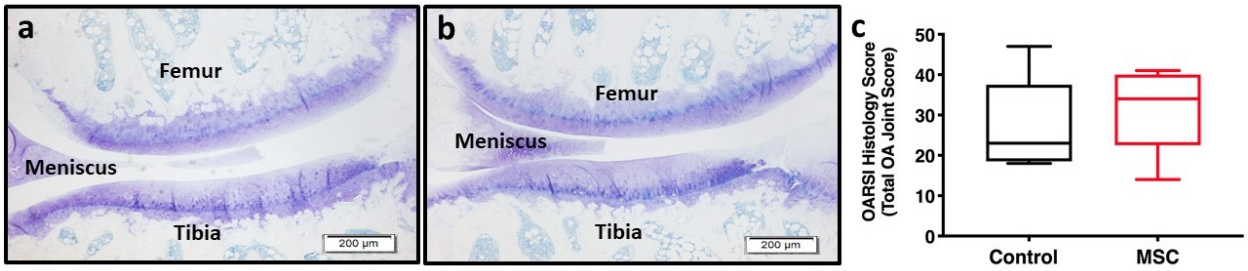


**Figure 4.3 Histologic assessment of OA**
Representative lesions from each group a) control with no treatment and b) MSC. Graphical representation of OARSI histological scoring in c) identified no statistical difference between groups.

**MSC treatment decreases systemic inflammatory cytokines**

Intravenous delivery of MSC is known to decrease systemic inflammation in multiple disease processes. [29] To determine if this therapy decreased systemic inflammation in this model of age-related OA we assessed serum levels of cytokines commonly associated with this disease process. ELISAs were utilized to measure systemic levels of MCP-1, TNFα, and PGE2. We identified significantly lower serum concentrations of MCP-1 ($p<.05$), TNFα ($p<.01$) and $PGE_2$ ($p<.001$) in guinea pigs treated with MSC (**Figure 4.4a-c**). To determine if systemic stem cell treatment decreased local inflammation within the joint, synovial fluid was assessed for levels of TNFα and $PGE_2$. Both TNFα ($p<.01$) and $PGE_2$ ($p<.05$) concentrations were significantly lower in synovial fluid of MSC treated guinea pigs ($p<.01$) when compared to control (**Figure 4.4d,e**).

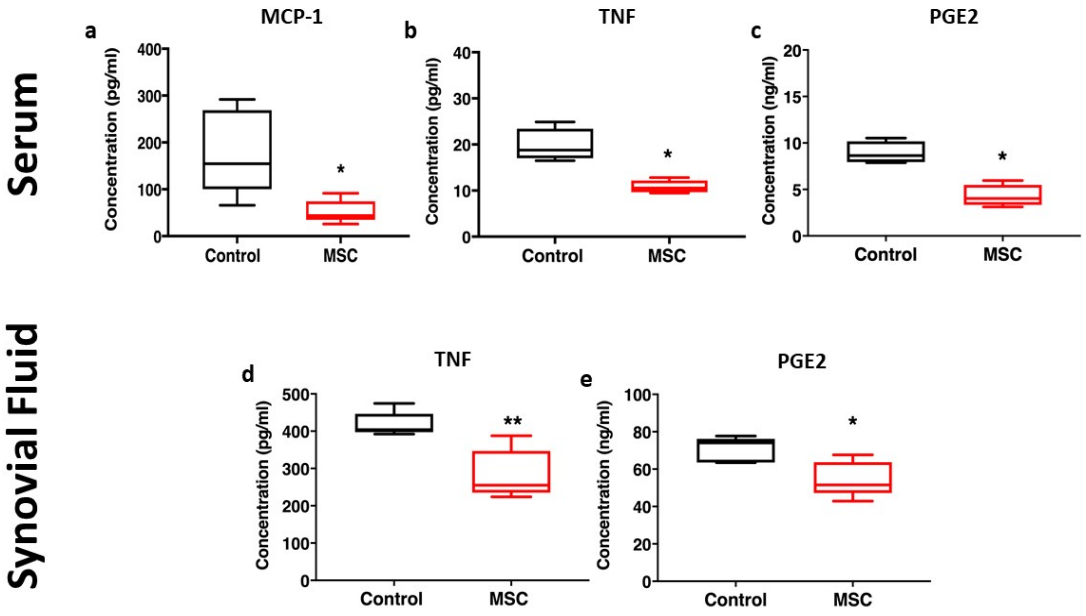


**Figure 4.4 Assessment of secreted protein by ELISA**
Serum concentration of a) MCP-1 b) TNF and c) PGE2. Synovial fluid concentration of d)TNF and e) PGE2 control untreated group shown in black bars, MSC group in red an PIC treated group in blue. Statistical analysis using t test. Significance was noted for * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, and **** $p<0.0001$.

**Improvement in symptomatic control of OA demonstrated by gait analyses**

Downregulation of inflammation by MSC is only of utility if it is accompanied by symptom modification as that is currently the mainstay of OA therapy in humans at this time. [4,1] Guinea pigs treated with systemically delivered MSC demonstrated a statistically (P=0.04) longer stride length immediately prior to termination than control guinea pigs (**Figure 3.4a**). In addition, a significantly higher maximal speed was present in MSC treated animals (P=0.05) when compared to control guinea pigs (**Figure 3.4b**).

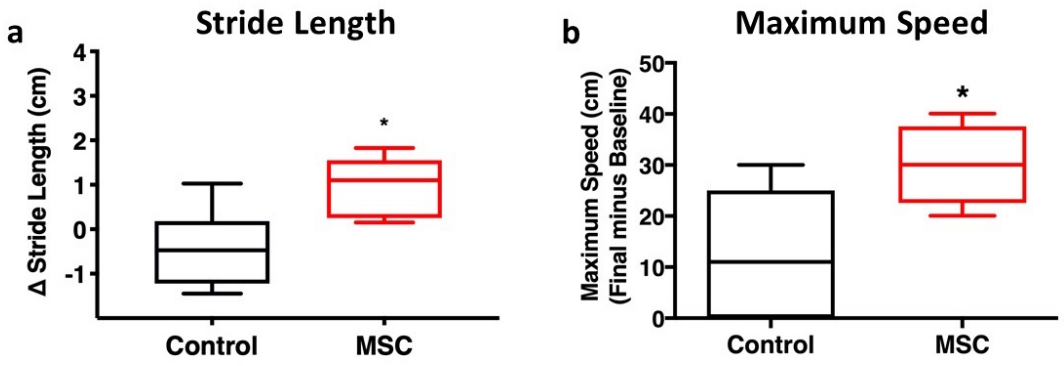


**Figure 4.4 Gait analysis**
Gait analysis including a) stride length control untreated group shown in black bars, MSC group in red and b) normalized maximum speed. Comparisons between 2 groups was done using t-test. Significance was noted for * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, and **** $p<0.0001$.

***MSC administration decreases systemic inflammation in tissues***.

It is known that in aging individuals with OA systemic inflammation is present and contributes to the pathology of the inflammation at the level of the joint. [30] To determine if systemic treatment with MSC affected inflammation intra-articularly and systemically, we examined sections of joint tissue and kidney tissue to evaluate both synovial and systemic inflammation, respectively. TNFα was present in the tissue surrounding the joint capsule indicating inflammation in the joint. There was significantly lower TNFα expression in the synovial capsule in animals treated with MSC suggesting a decrease of local inflammation in affected joints. (**Figure 4.5**).

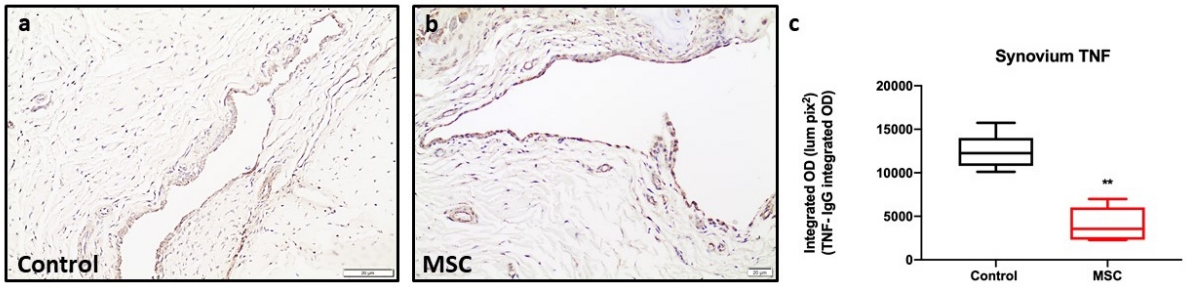


**Figure 4.5 Decrease in TNFα expression in the synovium following MSC treatment**
Red color in tissue positive for TNFα by IHC a) representative photo from control untreated group b) representative from MSC group, c) graphical representation of TNFα positive areas as defined by um/pixel. Comparisons performed using t-test. Significance was noted for * p< 0.05, ** p< 0.01,

In addition, there was a significant decrease in TNFα in the kidney between the treated and control group indicating that systemic inflammation was decreased in treated animals. TNFα levels in tissue was identified utilizing immunostaining with strong and diffuse positive stain within control guinea pig kidney (**Figure 4.6a**). Treatment with systemic MSC showed significantly less immunostaining (p<.001) (**Figure 4.6b**). IgG control stained tissue was used as a negative control quantitatively account for background staining within each animal.

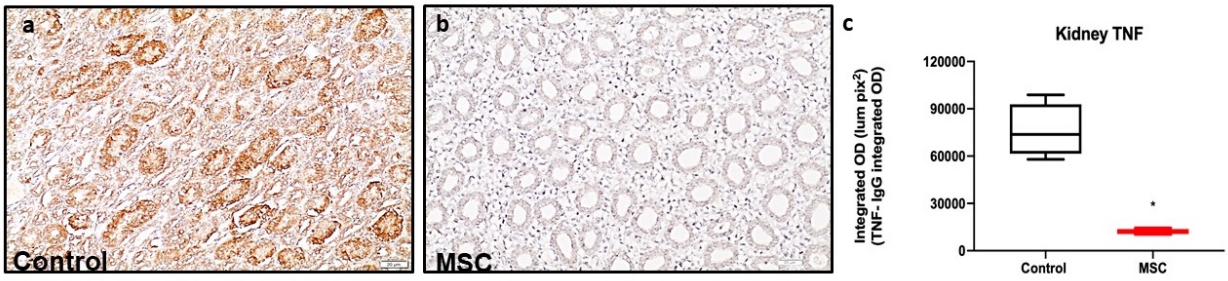


**Figure 4.6 TNFα by IHC within the kidney demonstrates decreased TNFα in MSC treated group**
IHC for TNFα in guinea pig kidney identified by red brown stain a) representative photo from control untreated group b) representative from MSC group and c) representative from PIC activated MSC group. d) Bar graph of TNF positive areas as defined by um/pixel. Statistical analysis utilizing t-test. Significance was noted for * p< 0.05, ** p< 0.01.

**Discussion**

This study demonstrates that intravenous injection of MSCs induces both amelioration of symptoms and decrease in systemic and local inflammation in a laboratory model of spontaneous OA. OA is a complex process which involves loss of homeostasis in the joint, increased inflammatory mediators and loss of cartilage. The inflammation results in both discomfort and a change in the joint environment such that cartilage is resorbed and not replaced. This causes further inflammation and leads to a progressive deterioration in the joint perpetuating the disease process and resulting in the observed symptoms of pain.[30] Reduction of inflammation and increased comfort and pain relief are the goals of treatment to provide symptomatic relief of the patient; however, mere reduction in pain does not ameliorate the pathologic process occurring in the joint. Thus, novel therapeutic interventions should be aimed at both symptom reduction and alteration of the joint microenvironment to promote alteration of the pro-inflammatory milieu that encourages further cartilage loss.[1,4] MSC therapy is currently a promising therapy to accomplish both symptomatic relief and reprogramming of the joint microenvironment. [28,31,32] In this study, we demonstrated symptomatic relief of naturally occurring osteoarthritis in the Hartley guinea pig model after intravenous therapy with allogeneic mesenchymal stem cells. Treated animals showed increase in stride length and challenge speed when compared with controls. This study did not demonstrate a statistical difference in histologic OA score. This lack of improvement may be explained by the brief duration of the study as histologic changes would likely take longer than 6 weeks to manifest a histological change.

Mesenchymal stem cells have been utilized for multiple orthopedic indications in humans and other animals including osteoarthritis, ligament and tendon injuries. [32-35]The majority of MSC therapies are delivered locally at the site of the injury or intra-articularly in the most

affected joint. [27] Systemic administration of MSCs in other diseases has demonstrated that these cells track to sites of inflammation and thus this route may be advantageous in diseases involving multiple sites of inflammation. [14,15,22,36]

Intravenous injection of MSCs in this model resulted in decreased levels of inflammatory cytokines MCP-1, TNFα and PGE2 in serum and TNFα and PGE2 levels were decreased in the joint fluid of treated animals. Increases in systemic and joint TNFα have been correlated with pain in human patients with osteoarthritis.[37] Modulation of the PGE2 pathway has been demonstrated to ameliorate inflammation in osteoarthritis.[9,11] Systemic inflammation in general was decreased as demonstrated by decreased TNFα in the kidneys. OA is now known to be a systemic disease and therefore decreasing overall inflammation is a mechanism by which mesenchymal stem cells can assist in restoring homeostasis and thus promoting and environment that downregulates inflammation and supports retention of cartilage and formation of new cartilage.[9,8]

This model utilized repeated doses of systemic MSC for treatment of osteoarthritis in a naturally occurring model of OA. Several studies have demonstrated that repeated injections of MSC are superior to one injection. [38,15] Indeed the animals in this study demonstrated a marked decrease in both lameness and systemic and local inflammation. A more thorough comparison of number and timing of injections would be necessary to definitely determine the ideal dose, frequency and route of administration. However, these results suggest that further research into this area is indicated.

In summary, inflammatory modulation in idiopathic spontaneous OA utilizing intravenously administered stem cells may provide a therapeutic approach that can manage clinical signs associated with the disease and ameliorate systemic and local inflammation.

Further research is necessary to provide additional insight into ideal dosing intervals and effect of MSC on joint pathology. The aged Hartley guinea pig model with spontaneous OA is an innovative approach to investigate osteoarthritis that more closely approximates the pathology of age related osteoarthritis in humans and is a valuable in vivo screening technique for studying this process.

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# CHAPTER 5

## Final Conclusions and Future Directions

The advent of biological therapy which has its roots in the discovery that cells could be recovered from the bone marrow that could be expanded in culture and utilized therapeutically is an exciting novel arena of therapy.[1] This therapy has its' roots in transplantation of hematopoietic stem cells which began in the early 1940s and became refined and commonly utilized in clinical cases by the late 1980s. The history of hematopoietic stem cell transplant is interesting in that they were initially utilized as a therapy in patients with few other options long before there was understanding of the biology behind the therapy. Indeed, the rodent models of hematopoietic stem cell therapy failed to highlight the issues that would plague this therapy later such as graft versus host disease and much was learned along the way.[2] In the 1960s, Friedenstein et al. discovered a population of cells in the bone marrow that could be expanded *in vitro*, and this was the beginning of what we know today as the adult derived mesenchymal stem cell.[3] In 1995, a phase I clinical trial was conducted using autologous bone marrow culture expanded MSC collected from an individual and reinfused intravenously.[4] This study reported the use of these cells to be safe and a recent meta-analysis that evaluated intravenous administration of MSC in 55 randomized clinical trials with over 2000 patients confirms that premise and in fact showed decreased risk of death in the patients who received MSC therapy.[5] The initial discovery that these cells could grow into bone marrow, adipose tissue and cartilage under the correct conditions led to a concept of regenerative medicine utilizing these tissues as a source of new cartilage or bone for orthopedic disease.[6] This concept has undergone a radical change as we begin to understand the immunomodulatory properties of these cells.[7] Indeed as we

began to understand the potential of this therapy the range of disease processes where they may be of benefit has expanded exponentially.[8] These cells have been demonstrated to have a wide range of applications due to their ability to sense the environment, home to sites of inflammation and effect changes on immune cell function.[9] With the known properties of decreasing inflammation and immunomodulation it is perhaps not surprising that this therapy would be beneficial in systemic inflammation and sepsis as part of the pathologic process involves an overwhelming systemic inflammatory response.[10-12] However, the direct antibacterial properties of MSC was perhaps not as intuitive and appears to have multiple mechanisms many of which are not fully understood.[13]

The possibility of a novel method to treat infection that relies on the immune system comes at a critical juncture in the evolution of bacteria and infection. The development of antibiotics led to a dramatic enhancement of medical practice that saved innumerable lives. However, this era was followed by the rapid evolution of multidrug resistant bacteria and biofilm infections with development of resistance occurring faster than new pharmacologic agents can be manufactured.[14-16] Thus a new strategy is imperative that does not rely solely on the development of novel pharmacologic agents alone. Multiple studies have documented a benefit of mesenchymal stem cells in sepsis and pneumonia and multiple mechanisms of action have been proposed.[13] In chapter 2 we describe the concept that MSC have increased activity against biofilm infections in a mouse model when they are pre-activated and combined with antibiotics. Other studies have confirmed our findings that MSC interact synergistically when combined with antibiotics.[17,18] In our study we detected multiple effects of MSC on the immune system of treated mice such as increased presence of monocytes at the site of infection and a change in phenotype of tissue macrophages from an inflammatory M1 macrophage in untreated mice to a

wound healing or M2 macrophages in treated mice. We discovered that pre-activation of MSC with a TLR3 agonist caused increased migration of the MSC to the site of infection and that resolution of infection occurred only in those mice treated with both activated MSC and antibiotics. We also demonstrated increased neutrophil phagocytosis *in vitro* when mouse neutrophils were preincubated with secreted factors from activated MSC. We identified production of antimicrobial peptides from mouse MSC and were able to partially reverse the direct killing of bacteria by MSC by blocking cathelicidin, an antimicrobial peptide. Other studies have examined antimicrobial peptides and their role in the antibacterial effect of MSC and blocking cathelicidins partially reversed the therapeutic effect of MSC in a mouse model of pneumonia.[19,20] Despite the multiple effects we and others have documented the actual contribution of these effects to resolution of the infection is unknown. To further dissect out the role of MSC and activated MSC on infection multiple modalities could be utilized. The role of antimicrobial peptides could be examined using genetically modified mouse models with antimicrobial peptides knockouts. Antimicrobial peptides that have been identified as potential effectors of the antimicrobial activity of stem cells include the cathelicidins, β-defensins, lipocalin and hepcidin.[17,21,19,22]. The easiest method to compare the effects of these antimicrobial peptides would be to grow cells from knockout mice utilizing cramp knockout mice (the mouse homolog of LL-37), Lcn2 knockout mice (lipocalin), Hpc knockout mice (hepcidin) and Defb1(beta-defension) knockout mice.[23-26] These knockout cells would be utilized in immunocompetent wild type mice as described in Chapter 2 to detect if there is a difference in outcome when MSC do not express antimicrobial peptides and which antimicrobial peptides are important. Another method of determining the effects of MSC activation on infection would be to examine transcriptome analysis of activated and nonactivated MSC when exposed to various

types of bacteria with and without antibiotics present and examine which genes are upregulated. Following this approach further in vivo studies could examine the effect of various immune effector cells on the observed resolution of biofilm infection.

Monocytes have been demonstrated in our model to undergo a change in phenotype and multiple others have demonstrated that monocytes are critical for MSC actions both in infection and other disease processes.[27,28] One method to determine the importance of macrophages in this process would be to deplete the macrophages using clodronate prior to treatment.[29] In this way we could compare side by side wild type mice utilized in the original study with and without macrophages. Our study described in chapter 2 also detected increased neutrophil phagocytosis by activated MSC and this was proposed to be a part of the antibacterial effect of these cells. Employing a similar method, I would propose a model of depleting neutrophils in the wild type mice utilizing a well characterized Ly6G antibody depletion method.[30] The final area that should be included in this investigation is the production of cytokines and chemokines. It is well known that MSC have effects on production of cytokines and chemokines from immune cells and indeed it is thought to be both an orchestrator of the immune modulatory effects and have direct beneficial effects in the setting of sepsis and infection.[9,12] Examination of cytokine profiles in mice treated with activated versus resting stem cells, with and without antibiotics both at early time points and later at resolution of the infection would engender a better understanding of immune regulation and inflammatory status as a result of MSC administration.

In chapter 3 we examine the effects of aMSC therapy in a spontaneous model of canine multidrug resistant infection and examine canine resting and activated MSC *in vitro*. An advantage of utilizing spontaneous canine models is that the pathogenesis and treatment effect is far more analogous to what might be seen in human patients enabling greater translational

potential.[31] Drawbacks include a limited availability of interventions and manipulations but there is still a great potential to further explore our initial findings. Multiplex cytokine analysis is available and well validated in canines and could be a useful tool to explore the immunologic changes associated with administration of activated versus resting cells systemically. A major drawback of our study was the lack of a control group. Designing and implementing a multi-center clinical trial utilizing a more homologous patient population would increase the number of subjects available and also reproducibility. Orthopedic implant infections are the cause of a lot of morbidity and mortality in human patients.[15] Therefore a multicenter trial evaluating normal standard of care for naturally occurring implant infections compared with patients treated with activated MSC could be extremely valuable. In addition to measurement of bacterial infection, the addition of cytokine analysis pre and post treatment could lead to a greater understanding of the immunologic effect of this therapy. In addition, cells could be labeled with a membrane dye and the site of the infection examined for cells with a punch biopsy to determine if a large portion of cells migrate to the site. Alternatively we could track the cells after i.v. administration utilizing MRI. With use of new technology it is now possible to utilize MRI to visualize exactly where MSC migrate following administration. Using small iron microparticles that MSC take up by endocytosis we can utilize simple imaging to visualize where cells are going. This would be an exciting new way to further delve into the process of the function of these cells in the setting of infection.[32]

An additional consequence of the advances in health care is the increased longevity and associated increase in age-related osteoarthritis (OA). It is estimated that more than 200 million people are affected with this condition world-wide and it is associated with increased costs of health care.[33] [34] Despite the burden of this condition, treatment remains mostly symptomatic and

indeed in many patients the currently available therapeutics are ineffective.[35] With the advent of biological therapies multiple clinical trials have been initiated to test this promising new avenue of therapy.[36] The results of these trials have been variable in part due to lack of consistency in isolation, culture expansion, dose and route of administration.[37] The vast majority of clinical trials for OA utilize intra-articular injection of MSC.[38,39] In age related OA there are generally multiple joints affected and it is now known that in systemic inflammation plays a significant role inpeople affected with age-related OA.[40] In chapter 4 we described systemic administration of MSC in a well-known guinea pig model of naturally occurring osteoarthritis. Systemic administration in this model was demonstrated to alleviate clinical signs of multifocal osteoarthritis and decrease both local and systemic inflammation. Following these initial promising results there is much more that could be accomplished utilizing this and other models. A simpler approach to determine the effect of systemic versus local injection on osteoarthritis would involve utilizing a mouse model of induces osteoarthritis. The intra-articular tibial plateau fracture provides a reproducible mouse model that is often utilized to simulate injury induced OA. Although it does not recapitulate all the factors of age-related OA it would be interesting to investigate local vs systemic therapy in this model first as larger numbers of animals could be utilized without the complicating factor of multiple affected joints. If systemic MSC were determined to be more effective or even equally effective in this model it might be worthwhile evaluating local vs systemic injection in the Dunkin-Hartley guinea pig model as we did in Chapter 4 to see if additional benefit is derived from systemic therapy over direct injection of the most affected joints. Examination of the immune response to local vs systemic injection in this model would be of particular interest. This could be accomplished utilizing transcriptome analysis to determine how MSC therapy affects inflammatory and immune pathways when

utilized systemically and locally. In addition, this would be a perfect model to examine the longevity and migration of MSC delivered locally and systemically utilizing the previously mentioned novel iron nanoparticle technology to label the MSC and performing whole body MRI of the guinea pigs to determine where the cells go in the body and how long they stay there.

Macrophages have been demonstrated to play an important role in osteoarthritis with inflammation in the joint stimulating production of M1 or inflammatory macrophages. It has been demonstrated that M2 macrophages are necessary for chondrogenesis and this alteration of the state of the macrophage likely contributes to the loss of cartilage in this condition.[41] MSC therapy is known to affect the polarization of macrophages with a tendency to polarize them towards and M2 phenotype.[42] It is possible that this is an important mechanism in the demonstrated clinical benefit and chondroprotective effect of MSC therapy. To further investigate this effect several methods could be utilized. We could examine macrophages in synovial fluid utilizing the Dunkin-Hartley guinea pig model after optimizing M1/M2 staining in guinea pig macrophages using conventional markers such as iNOS and arginase. Additionally, the previously mentioned mouse model of traumatic OA would be a good model to study the effect in as we could study treatment of OA after depletion of macrophages to see if the protective effect is lost. Subsequently we could examine the joint of the mouse and stain tissues for classic M1/M2 markers after local and systemic treatment with MSC. Simultaneously *in vitro* experiments with mouse macrophages preconditioned by MSC media could be conducted. Coculture experiments utilizing conditioned macrophages and chondrocytes both resting and stimulated by an inflammatory stimulus (LPS) could be utilized to evaluate changes in secretion of inflammatory cytokines by chondrocytes. Chondrocyte proliferation and apoptosis could also be evaluated in this model to determine if preconditioned macrophages have a protective or

stimulatory effect on chondrocytes. Macrophages play an important role in the pathology of OA. The effect of MSC on that process has not been examined in great detail and bears further investigation.

In summary, MSC therapy is a versatile and effective therapy and much remains to be discovered regarding potential therapeutic targets, modulation to improve activity and how they interact during disease and in healthy individuals. These cells have the unique ability to respond to their environment and the potential of these therapies is in the infancy of exploration. There is much to be learned and exciting possibilities for treatments of disease that currently have limited therapeutic options.

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# APPENDICES

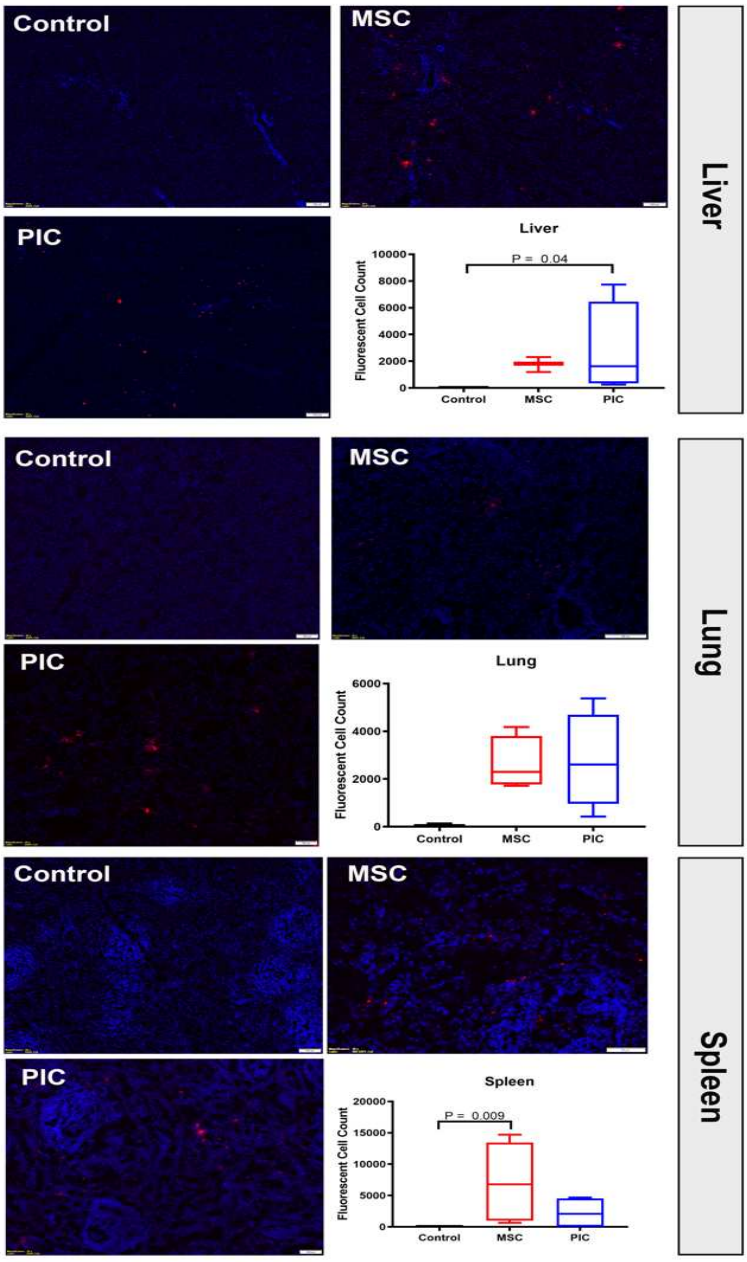
## Supplemental Material for Chapter 4

Previous studies have demonstrated that MSC can be polarized by pre-activation with toll like receptor (TLR) agonists prior to transplantation to enable improved immunomodulation when required.[39-41] A model using MSC pre-activated with TLR3 agonist poly I:C demonstrated anti-biofilm activity and improved migration to sites of inflammation in animals treated with pre-activated cells when compared to animals treated with resting MSC. [22] Therefore, we hypothesized that pre-activating the MSC could be beneficial for treatment of osteoarthritis as increased migration to sites of inflammation would be expected to increase the effect. However, in this model, a similar systemic and local anti-inflammatory effect was demonstrated with both resting and activated cells and both groups demonstrated similar symptomatic relief. There was no difference in the number of engrafted cells identified in the organs. Thus, it appears that, for osteoarthritis, preactivation with a TLR3 agonist does not provide increased benefit for symptom relief or anti-inflammatory effect.

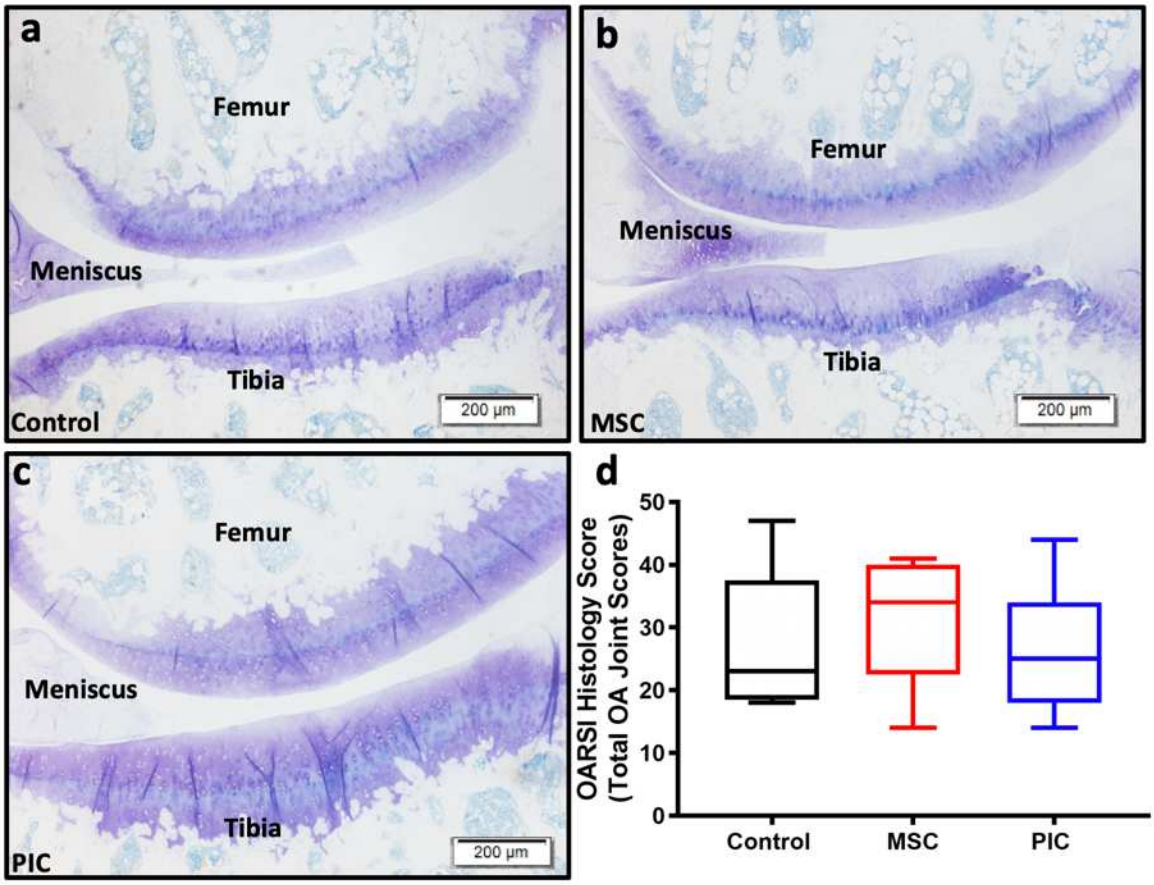
*Supplemental Materials and Methods for Chapter 4:*

An additional group of 6 Hartley strain guinea pigs was purchased and housed as described above and treated with adMSC preactivated with TLR 3 ligand poly IC. Activation of adMSC was accomplished by exposure of expanded adMSC to 10ug/ml of poly (I:C) (InvivoGen, San Diego, CA), for two hours prior to harvest as previously reported[4]. Experimental outcomes were performed as described above utilizing preactivated MSC.

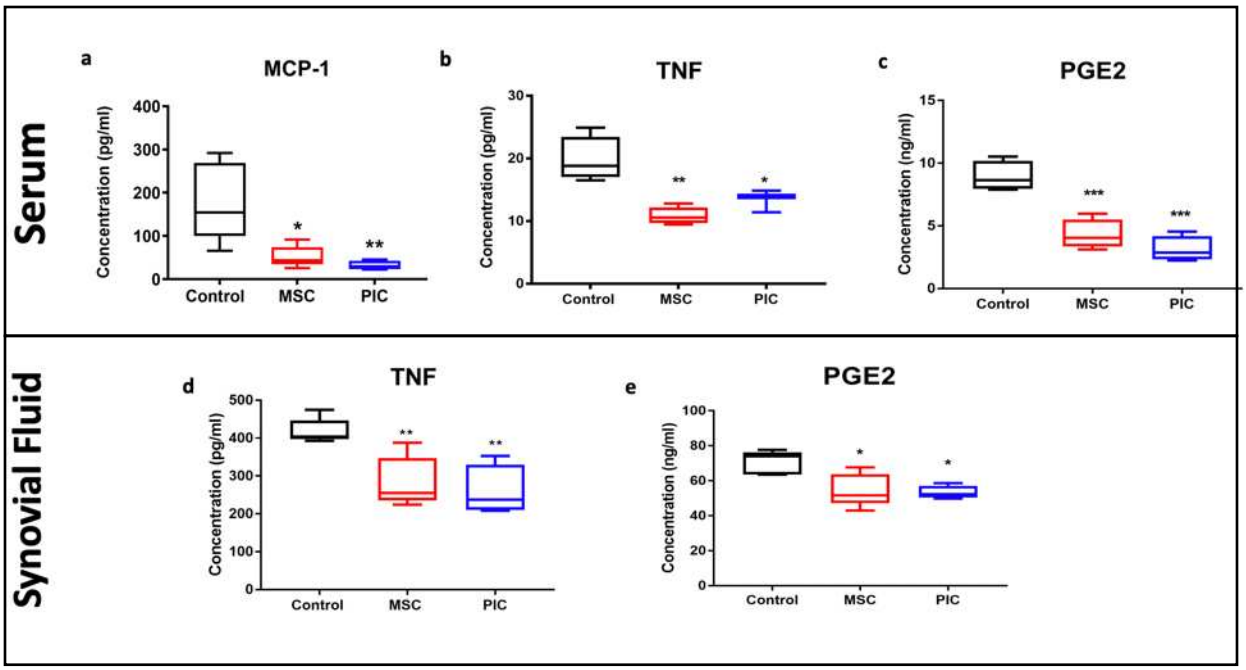
*Supplemental Results for Chapter 4:*



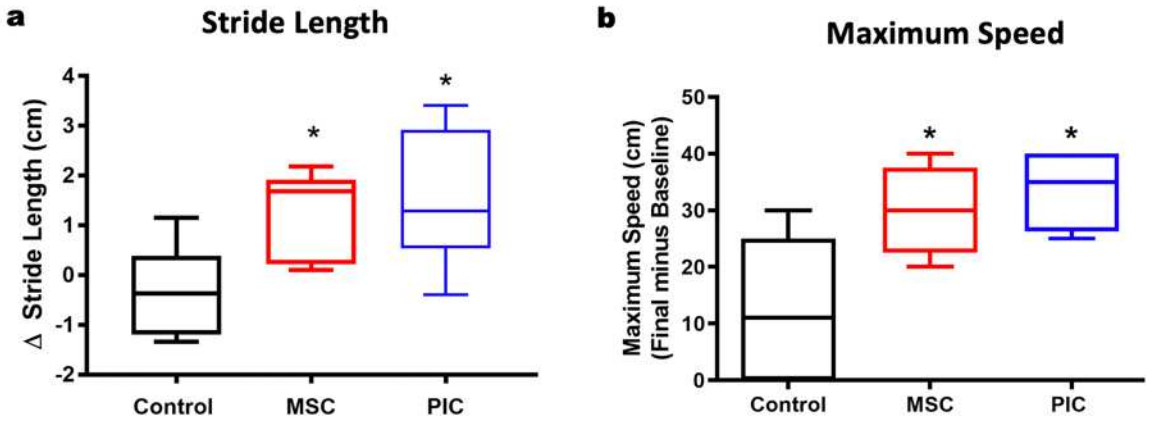

**Supplemental Figure S4.1 Distribution of injected MSC in tissue**
MSC and preactivated MSC (PIC) were labeled a lipophilic fluorescent dye (DiD) and administered intravenously 14 days prior to analysis of tissues. Labeled cells (red) with Dapi nuclear stain (blue) were identified in the lungs, liver and spleen in all animals that received MSC therapy. There was no difference in number of cells in tissues between MSC and PIC.




**Supplemental Figure S4.2 Histologic assessment of OA**
Representative lesions from each group a) control with no treatment b) MSC and c) preactivated MSC (PIC) D) Graphical representation of OARSI histological scoring identified no statistical difference between groups.

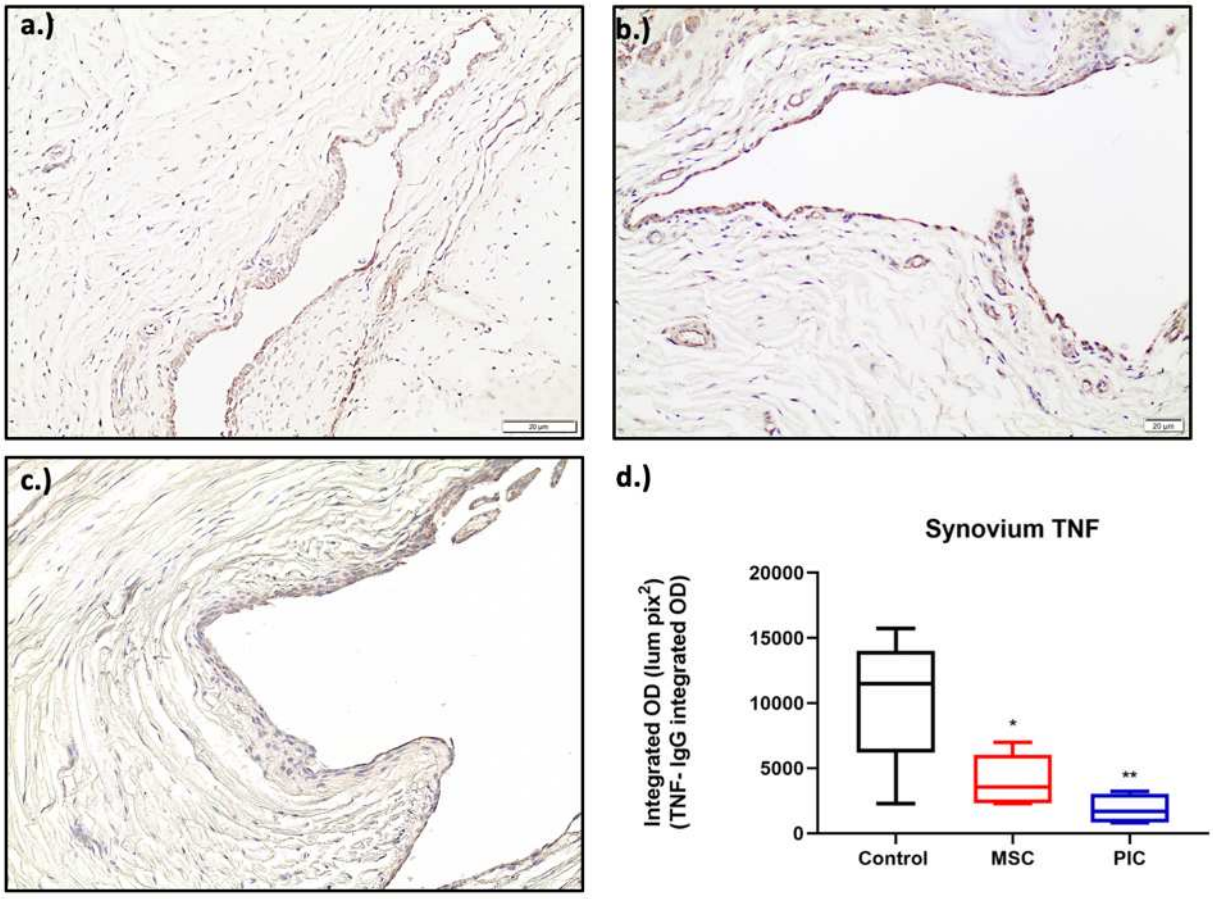



**Supplemental Figure S4.3 Significant decrease in serum and synovial inflammatory markers in both preactivated (PIC) and resting MSC.**
Serum concentration of a) MCP-1 b) TNF and c) PGE2. Synovial fluid concentration of d) TNF and e) PGE2. Control untreated group shown in black bars, MSC group in red, and PIC treated group in blue. Comparisons between 3 or more groups were done using ANOVA, followed by Tukey multiple means comparison post-test. Significance was noted for * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, and **** $p<0.0001$.



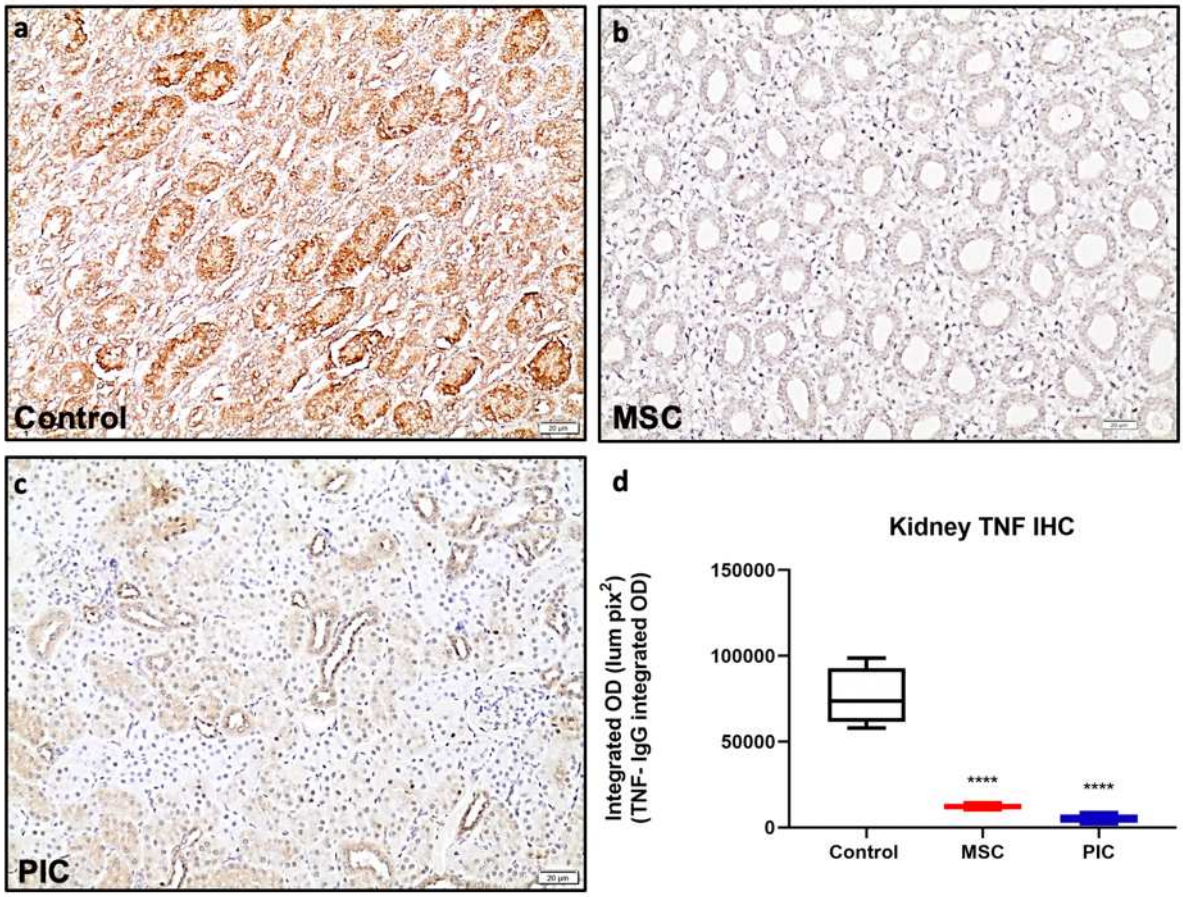

**Supplemental Figure S4.4 Gait analysis reveals increased stride length and maximum speed in guinea pigs treated with both activated (PIC) and resting MSC.**
Gait analysis including a) stride length and b) normalized maximum speed. Control untreated group shown in black bars, MSC group in red and PIC treated group in blue. There was no difference between MSC and PIC but both were significantly increased compared with control

animals. Comparisons between 3 or more groups were done using ANOVA, followed by Tukey multiple means comparison post-test. Significance was noted for * p< 0.05, ** p< 0.01, *** p< 0.001, and **** p<0.0001.




**Supplemental Figure S4.5 TNFα expression decreased in the synovium following MSC treatment**

Red color in tissue positive for TNFα by IHC. a) Representative photo from control untreated group, b) representative from MSC group, and c) representative from PIC activated MSC group. d) Shows graphical representation of TNFα positive areas as defined by um/pixel. TNFα was significantly lower in tissues of guinea pigs treated with activated (PIC) cells compared with those treated with MSC although both were significantly lower than control animals. Comparisons performed using ANOVA, followed by Tukey multiple means comparison post-test. Significance was noted for * p< 0.05, ** p< 0.01.




**Supplemental Figure S4.6 Expression of TNFα by IHC within the kidney demonstrates decreased TNFα in treatment groups**

IHC for TNFα in kidney of guinea pigs identified by red brown stain a) representative photo from control untreated group b) representative from MSC group and c) representative from PIC activated MSC group. d) Bar graph of TNF positive areas as defined by um/pixel. Statistical analysis utilizing ANOVA, followed by Tukey multiple means comparison post-test. Significance was noted for * $p < 0.05$, ** $p < 0.01$,