

**The Flip Side of Osteoimmunity: Crosstalk Among Stem Cells, BMP-2 and Innate Immune Cells, and the Control of Osteoblastogenesis**

By

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## ABSTRACT

Management of bone reconstruction in the presence of inflammation is a challenging and costly clinical problem. It can result in extended repair times, poor bone healing, delayed unions, non-unions, revision surgery, and in the extreme, amputation or death. Acute inflammation can impair clinical reconstruction outcomes by compromising the early stages of bone repair and regeneration, while chronic inflammation exacerbates these effects leading to further significant loss of bone and associated function. Inflammation usually involves cells of the innate immune response, including neutrophils, macrophages and dendritic cells. Using J774a.1 (a mouse macrophage cell line) and fetal skin dendritic cells, I determined that lipopolysaccharide (LPS) induced a pro-inflammatory phenotype, which creates a microenvironment that inhibits bone morphogenetic protein 2 (BMP-2) induced stem cell osteoblastogenesis. However, the addition of an anti-inflammatory/immunoregulatory cytokine Interleukin 10 (IL-10), in the presence of LPS, directs this classical inflammatory response toward an alternative activated microenvironment that is permissive for BMP-2 osteoblastic differentiation. As BMP-2 can direct both osteoclastogenesis and osteoblastogenesis, the preference toward bone resorption or formation/repair thus appears to be influenced by the inflammatory microenvironment. When BMP-2 is in a predominately classic inflammatory microenvironment, it tends to promote osteoclastogenesis and when in an alternatively activated microenvironment, BMP-2 induces osteoblastogenesis. Thus, controlling the microenvironment with IL-10 and/or similar immunoregulatory cytokines may determine the speed and success of BMP-2 mediated bone regeneration, especially under classic inflammatory conditions associated with situations such as infection and multi-tissue trauma.

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## LIST OF ABBREVIATIONS

|               |   |
|---------------|---|
| 1400W         | N-(3-(Aminomethyl)benzyl)acetamidine                            |
| ALP           | Alkaline phosphatase  |
| APC           | Adenomatous polyposis coli                                      |
| APCs          | Antigen presenting cells  |
| BMP           | Bone morphogenetic protein                                      |
| CGRP          | Calcitonin gene related peptide                                 |
| CM            | Conditioned media   |
| cPTIO         | Carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide |
| DAMPS         | Damage associated molecular patterns                            |
| DCs           | Dendritic cell  |
| DMD           | Duchenne muscular dystrophy                                     |
| DMEM          | Dulbecco's Modified Eagle's Media                               |
| EU            | Endotoxin unit  |
| FACS          | Fluorescence activated cell sorting                             |
| FBS           | Fetal bovine serum  |
| FSDCs         | Fetal skin dendritic cells                                      |
| GCSF          | Granulocyte colony stimulating factor                           |
| GMCSF         | Granulocyte macrophage colony stimulating factor                |
| GSK-3         | Glycogen synthase kinase 3                                      |
| HIF           | Hypoxia inducible factors                                       |
| HMSCs         | Human mesenchymal stem cells                                    |
| HO            | Heterotopic ossification  |
| HO-1          | Heme-oxygenase-1  |
| ICAM-1        | Intercellular adhesion molecule 1                               |
| IFN- $\gamma$ | Interferon gamma  |
| IL            | Interleukin   |
| IL-10R1       | Interleukin 10 receptor 1                                       |
| IL-10R2       | Interleukin 10 receptor 2                                       |
| iNOS          | Inducible nitric oxide synthase                                 |
| IP-10         | Interferon inducible protein-10                                 |
| IRAK          | Interleukin 1 receptor associated kinases                       |
| IRF           | Interferon regulatory factor                                    |
| JAK1          | Janus kinase-1  |
| KC            | Keratinocyte chemoattractant                                    |
| LIX           | LPS induced CXC chemokine                                       |
| LPS           | Lipopolysaccharide  |
| MAPK          | Mitogen activated protein kinase                                |
| MCP-1         | Monocyte chemotactic protein-1                                  |

|                  |  |
|------------------|--|
| mDC              | Myeloid dendritic cell   |
| MDSC             | Muscle derived stem cells  |
| MHC              | Major histocompatibility complex                                 |
| MIP-1 $\alpha$   | Macrophage inflammatory peptide 1 alpha                          |
| MIP-2            | Macrophage inflammatory protein 2                                |
| MMP9             | Matrix metalloproteinase-9                                       |
| NF- $\kappa$ B   | Nuclear factor kappa-light-chain-enhancer of activated B cells   |
| NMF              | Non-negative matrix factorization                                |
| NO               | Nitric oxide   |
| PAMPS            | Pattern associated molecular patterns                            |
| PBS              | Phosphate buffered saline  |
| pDC              | Plasmacytoid dendritic cell                                      |
| PGLPS            | Porphyromonas gingivalis lipopolysaccharide                      |
| PRRs             | Pattern recognition receptors                                    |
| PS               | Penicillin-streptomycin  |
| PTH              | Parathyroid hormone  |
| RANKL            | Receptor activator for NF- $\kappa$ B ligand                     |
| RANTES           | Regulated upon activation, normal T-cell expressed, and secreted |
| SARM             | Sterile a and HEAT-Armadillo motifs containing protein           |
| SNAP             | S-Nitroso-N-Acetyl-D,L-Penicillamine                             |
| SP               | Substance P  |
| STAT             | Signal transducer and activator of transcription                 |
| TGF- $\beta$     | Transforming growth factor beta                                  |
| T <sub>H</sub> 1 | T helper cell 1  |
| T <sub>H</sub> 2 | T helper cell 2  |
| TIR              | Toll-interleukin-1   |
| TIRAP            | TIR domain containing adaptor protein                            |
| TLRs             | Toll-like receptors  |
| TNF- $\alpha$    | Tumor necrosis factor alpha                                      |
| TP-STACS         | Topology-preserving stochastic active contour scheme             |
| TRAM             | TRIF related adaptor molecule                                    |
| TRIF             | TIR domain containing adaptor inducing interferon b              |
| TYK2             | Tyrosine kinase-2  |
| VEGF             | Vascular endothelial growth factor                               |

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# CHAPTER 1: Introduction

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## **1.1 Introduction**

Inflammation, driven by the cells of innate immune system, represents a dynamic balance between conflicting physiological goals of immune host defense which is often associated with tissue loss and the maintenance of tissue and tissue repair. Immune dysregulation involving the skeletal system is often plays a key role in pathologies such as osteomyelitis, rheumatoid arthritis, osteoarthritis, osteoporosis and periodontal disease, which can result in brittle bones leading to an increased risk of fracture, as well as delayed repair, non-unions, tooth loss, implant loosening and failure, heterotopic ossification (HO), amputation, and even death. Motivated by the need for new therapeutic strategies to treat diseases mediated by both the skeletal and immune systems, the new field of osteoimmunology has emerged to better understand crosstalk between these systems [2-6]. Thus far, the main focus of osteoimmunology center on the investigation of osteoclasts and metabolic bone disease as related to pathologic bone resorption [9, 10]. However, there is increasing interest in elucidating the immune system's interaction with osteogenesis, as it is an important consideration for bone repair and reconstruction therapies, especially when the injury site is unduly compromised by trauma and/or infection. Thus, this thesis focuses on how the immune system affects BMP-2 mediated bone formation during inflammatory situations and how the use of anti-inflammatory cytokine, IL-10 could potentially remedy this situation. This body of work will detail the contributions I have made to the field of osteoimmunology.

Currently, bone morphogenetic proteins are used clinically to treat fractures and non unions [14, 15]. Emerging evidence suggests that bone morphogenetic protein 2 (BMP-2), delivered with or without antibiotics, can recover some aspects of bone regeneration during infection, however, its response remains severely blunted when compared to non-infection

controls [16, 17]. Counteracting this effect cannot be addressed by simply increasing the BMP-2 dosage because the pharmacological doses (milligram levels) in current clinical use are increasingly associated with undesirable side effects [18-21], including HO. HO, associated with extensive trauma, with or without infection, can be exacerbated by BMP-2 augmented fracture repair, presumably due to the soluble BMP-2 diffusing away from the targeted delivery site to the surrounding damaged soft tissues [22, 23].

The management of bone reconstruction in the presence of bacterial infections represents one of the most challenging and costly clinical problems in Orthopaedic, Craniofacial, Neurosurgical, and Dental medicine as it involves considerable morbidity, extended repair times, poor bone healing, delayed union, non-unions, revision surgery, extended hospital stays and in the extreme, amputation and death [24-42]. Infection-associated pathologies include periodontitis [24-28], osteomyelitis [29-33], and bacterial arthritis [34-36], as well as those associated with orthopedic and oral implant hardware. For example, most long bone osteomyelitis, an acute or chronic bone infection, is associated with open fractures, where bacteria are introduced at the time of injury (during extensive trauma due to war [37, 38]) or inadvertently introduced during treatment. Osteomyelitis can spread from the bone to neighboring tissues such as skin, muscle, tendon and ligament, thus management of the infection is crucial. Recent U.S. combat casualty statistics for the occurrence of osteomyelitis in injuries involving the extremities range from 5 to 15% [39-42]. As this bacterial infection will inhibit the initial phases of bone regeneration and therefore, poses a significant complicating factor in the development and deployment of therapies using osteogenic growth factors for enhancing bone regeneration in repair and reconstruction procedures. Furthermore, such traumatic injuries typically involve extensive damage to surrounding soft tissues, especially associated muscle, nerves and vasculature,

compromising both innate and acquired immune response of the bone and resolution of the infection. Thus, bone repair and reconstruction in the presence of infection remains a significant clinical challenge. Thus, a better understanding of the relationship between the immune cells, the infection-associated immunoregulatory cytokines, the effects potential therapeutic osteogenic growth factors and stem cells is required to advance bone regeneration technologies.

## **1.2 Immune system**

The Latin term *immunis*, meaning “exempt” gave rise to the word commonly associated with the human body’s defense mechanisms against foreign pathogens, and other environmental agents: immunity. There are 2 different types of immunity, the innate and the acquired. The innate immune system is comprised of multiple components, such as physical barriers such as the coughing and body surfaces, for example, skin, and mucus membranes of the digestive tract. The innate immune system is also comprised of cells such as, granulocytes, macrophages and dendritic cells that participate in phagocytosis and secretion of chemokines. The acquired immune system involves the activation of T and B lymphocytes by foreign antigens [43]. These T and B cells have different functions but are similar in that they are antigen specific. In this thesis, I choose to focus on the interactions of the cells of the innate immune system with stem cell differentiation under inflammatory and non-inflammatory conditions.

## **1.3 Cells of the innate immune system**

Cells of the innate immune system include granulocytes such as neutrophils, macrophages, dendritic cells and mast cells. Neutrophils, the most abundant leukocyte found in the blood stream, are the first responders to site of inflammation via chemotaxis. Macrophages reside within tissue and as monocytes in the blood stream. In response to inflammatory cues,

they secrete a variety of cytokines and chemokines, serve as antigen presenting cells (APCs) as well as clear the site of tissue debris. Dendritic cells were named for their morphological similarity to neuronal dendrites, but have no connection to the nervous system. Dendritic cells primarily serve as a key link between the innate and acquired immune systems as APCs. Dendritic cells present antigens to activate T cells, triggering the acquired immunity. In addition to their antigen presenting behaviour, dendritic cells also secrete a wide variety of cues in response to foreign pathogens. The interactions of macrophages and dendritic cells with stem cells were the foci of this study.

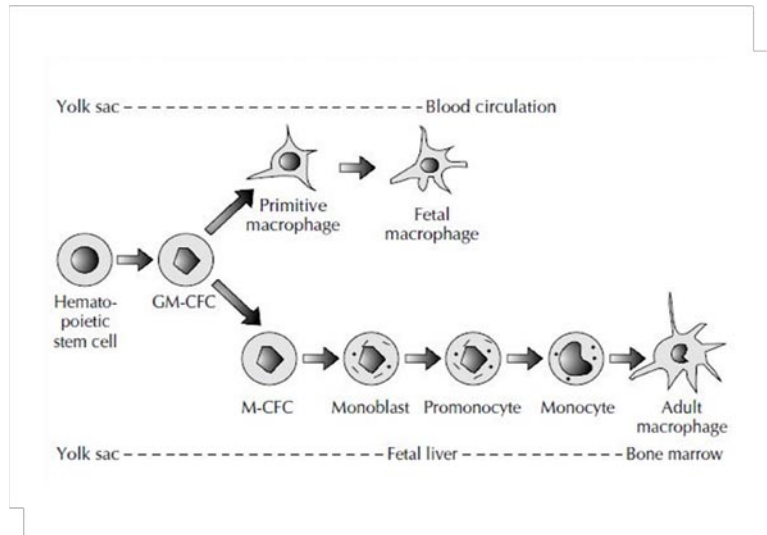
### **1.3.1 Macrophages**

Macrophages are a heterogeneous subset of mononuclear cells residing throughout essentially all tissues. Macrophages are activated in response to such environmental cues as trauma, foreign body reaction and infection. Originally, macrophages were thought of as the garbage men of the body, arriving on scene to clear the site of cell debris and foreign species, such as bacteria. However, macrophages play a more complex role than merely phagocytosis; their role in inflammation and remodeling will be examined in greater detail in the following sections.

#### **1.3.1.a Macrophage development**

Macrophages are a critical part of the body's immune response, carrying out multiple biological functions, such as angiogenesis, clearing of cellular debris, and inflammation [44]. Macrophages arise from a granulocyte-macrophage progenitor. The most immature adult macrophage arises from a monoblast that leaves the bone marrow compartment, and enters the blood stream. It divides once to give rise to 2 promonocytes. Promonocytes divide once to give rise to 2 monocytes. These cells circulate in the blood stream until extravasation, forming a

heterogeneous subset of mononuclear cells residing throughout essentially all tissues, e.g. Kupffer cells in the liver (Figure 1). Fetal macrophages are thought to go through a separate route of development, differentiating from yolk sac derived primitive macrophages prior to adult



**Figure 1:** Fetal and adult macrophage development undergo different routes. Reprinted from [7].

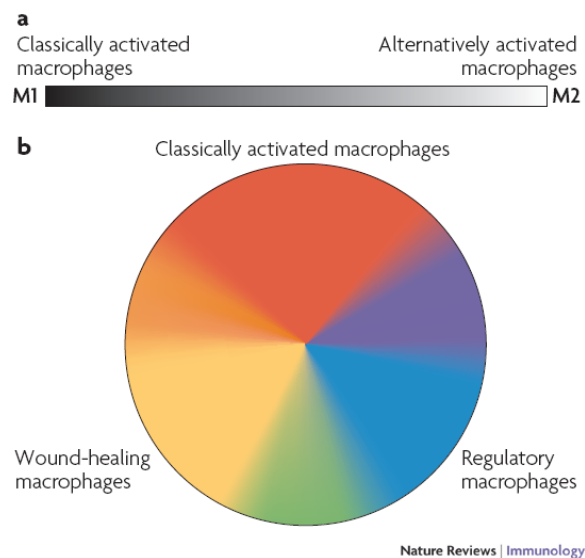
monocytic development. This separate line of development includes chickens, zebrafish, *Xenopus* and *Drosophila*[7].

### 1.3.1.b Macrophage classification

Macrophages exhibit extensive plasticity, enabling them to respond to environmental signals, changing phenotype and function, as altered by the adaptive nature of the innate immune system. Macrophages are activated in response to such environmental cues as trauma, foreign body reaction and infection. A nomenclature is evolving to characterize macrophage heterogeneity based on their functional properties, cell surface markers, and cytokine production. This classification was originally borrowed from T helper cell type 1 or 2 ( $T_H1/T_H2$ ) nomenclature [43]. Exposure to tumor necrosis factor alpha ( $TNF-\alpha$ ), interferon gamma ( $IFN-\gamma$ ) or lipopolysaccharide (LPS) (alone or in concert) results in M1 or the classically activated macrophage. M1 is characterized by the high capacity to present antigen, produce high levels of interleukins (IL) 6, 12 and 23, low levels of IL-10 and toxic intermediates such as nitric oxide

(NO). These macrophages are potent effector cells integrated in  $T_H1$  responses, killing microorganisms and tumor cells, and producing large amounts of inflammatory cytokines.

M2 refers to alternatively activated macrophages. M2 cells are a diverse group representing an ‘alternatively activated’ pathway that exhibits high levels of mannose or other scavenger receptors, produces arginase in the place of arginine, express inflammatory suppressive effects via IL-4, IL-10 and/or IL-13, and generally facilitates tissue repair. The most common designations of M2 cells are M2a, M2b, and M2c subtypes [44-47], where M2a is induced by IL-4 or IL-13, M2b is induced by exposure to agonists of toll like receptors and immune complexes and IL-1, and M2c is induced by IL-10 and glucocorticoid hormones. Recently, these opposing macrophage types have been described as polar extremes along a continuum of macrophage activation phenotypes including M2a, M2b and M2c, as macrophages in various disease states have been found to share characteristics between M1 and M2 subtypes



**Figure 2:** Macrophage activation as a polar opposites or a continuous range. **A.** A linear polarized presentation of M1 and M2 macrophages. **B.** Mosser and Edwards’ understanding of macrophage activation where macrophage types are thought of as a continuum, where colors such as green may represent tumor- associated macrophages, which share characteristics of both regulatory macrophages and wound healing macrophages. Reprinted from [13].

(Figure 2) [13]. The increased presence and persistence of M1s over M2s has implicated in chronic inflammation as well as graft rejection [48].

### **1.3.1.c Macrophage function**

Wound healing involves a series of steps: activation of the clotting mechanism, secretion of chemokines that attract immune cells to the site of injury, the influx of neutrophils and subsequently, macrophages, debridement of the injured tissue, initiation of neovascularization and the stimulation of remodeling and regeneration. Initially, macrophages were thought of simply as the cell population that replaced neutrophils as the main wound phagocyte to remove and degrade injured tissue prior to the reparation. However, Leibovich and Ross [49] showed that depletion of local tissue macrophages and circulating blood monocytes showed a lack of tissue debridement, and more interestingly, a delay in fibroblast proliferation and wound fibrosis, indicating that macrophages play a more complex role than anticipated. Since then, macrophages have been shown to be implicated in a variety of situations illustrated below.

Macrophages have been implicated in the success or failure of implant integration. Valentin *et. al.* [50] determined macrophage participation was crucial to the degradation and remodeling of extracellular matrix scaffolds in rats. Furthermore, the Badylak group has demonstrated that different extracellular matrices elicit different responses from macrophages, and that the prolonged presence of alternatively activated M2s over classically activated M1 macrophages determined if the scaffold would be remodeled or encapsulated respectively [51]. However, inflammation associated M1 macrophages must not be removed entirely from the healing process. Inflammation and macrophages are critical in musculoskeletal repair and reconstruction [52]. Non-specific inhibition of macrophages with systemically delivered anti-inflammatory drugs can have deleterious effects on repair and reconstruction depending on the

nature of the anti-inflammatory drug [53-56]. Nonetheless, a prolonged inflammatory response will not provide an implant with the appropriate network of blood vessels required for nutrient delivery and waste removal. As angiogenesis is critical to the success of graft integration as well as other processes such as bone and fracture repair, the role that macrophages types play in the angiogenic process must not be overlooked [57, 58].

Macrophages have also been implicated in pro-tumor and anti-tumor behaviors. Tumor cells multiply quickly, often creating areas that are beyond reach of simple diffusion. These hypoxic areas release chemokines such as monocyte chemotactic protein 1 and granulocyte macrophage colony stimulating factors (GM-CSF) that attract macrophages. These macrophages display tumoricidal activity towards tumor cells but in areas of hypoxia, they upregulate hypoxia inducible factors (HIFs) 1 and 2. HIFs are transcription factors that activate genes that lead to angiogenesis and tumor metastasis [59]. Furthermore, macrophages play a role in atherosclerosis. Atherosclerosis, the thickening and hardening of the arteries, involves an ongoing inflammatory response. A decreased presence of macrophages has been correlated to a reduction in the progression of the disease [60].

#### **1.3.1.d. Macrophage secretions**

Macrophages secrete multiple products in response to various stimuli. For example, M1 cells when exposed to inflammatory stimuli can result in the secretion of cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6. M2a and M2c cells are characterized by low production of pro-inflammatory cytokines, and increased secretion of IL-10 and decreased secretion of IL-12 [61]. M2bs (monocytes exposed to immune complexes and LPS) are slightly different in their secreted cytokine profile as they continue to produce inflammatory cytokines at high level while retaining the high IL-10 and low IL-12 expression profile, and promotion of T<sub>H</sub>2 differentiation. This

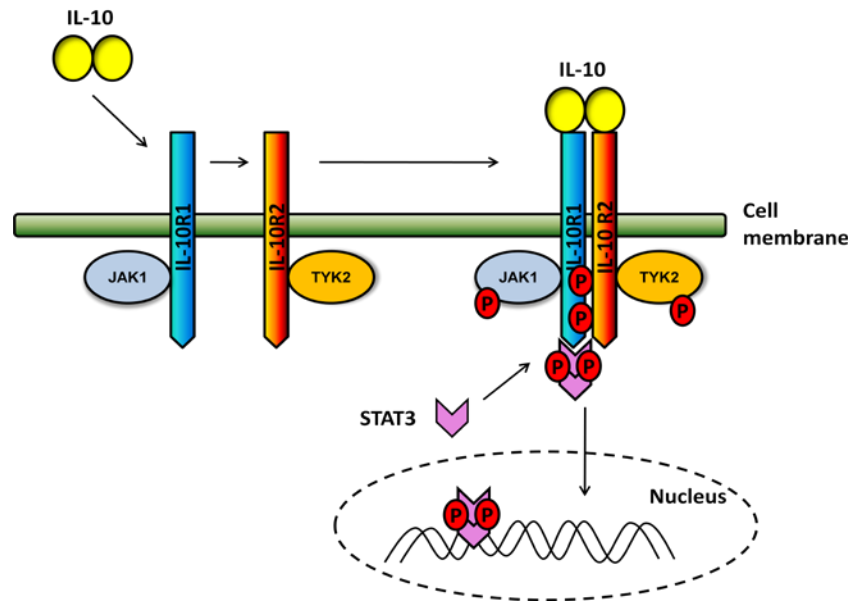
unique cytokine profile allows M2b cells to serve a protective function against LPS toxicity [61, 62]. Some of these secreted cytokines that play important roles will be discussed in further detail below.

#### **1.3.1.d.i Tumor Necrosis Factor- $\alpha$**

Tumor necrosis factor alpha, a cytokine secreted in the inflammatory milieu plays a central role in diverse immune and inflammatory processes. Aberrations in TNF- $\alpha$  production *in vivo* may be pathological. The potential of TNF- $\alpha$  to induce chronic inflammatory disease has been exemplified in animal models, where deregulation of its production leads to the development of various pathologies [63]. Unregulated TNF- $\alpha$  expression has been implicated in a variety of diseases from Alzheimer's disease [64], depression [65] and Crohn's disease (inflammatory bowel disease) [66]. TNF- $\alpha$  has been shown to induce osteoclast differentiation and activation leading to increased bone resorption, tipping the bone remodeling homeostasis which can result in osteoporosis [67-69]. Additionally, it decreases ALP expression in BMP2 induced osteogenesis in C2C12 cells [70], promotes osteoblast apoptosis, and suppresses matrix protein expression such as osteocalcin [71, 72]. TNF- $\alpha$  is able to mediate this inhibition via Msx2, via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation. Msx2 is a member of the homeobox transcription factor family [70], where haploinsufficiency can result in parietal foramina and a gain of function mutation causes craniosynostosis in humans [73, 74]. In contrast, TNF- $\alpha$  induces alkaline phosphatase (ALP) expression in vascular smooth muscle cells via MSX2 induction [75], and BMP-2 expression in articular chondrocytes [76, 77].

### 1.3.1.d.ii Interleukin 10

IL-10, a 5 exon gene, is located on chromosome 1 in humans [78, 79]. The protein, 178 amino acids long, is a homodimer. IL-10 interacts with the IL-10 receptor complex and acts via the JAK-STAT pathway [80] (Figure 3). IL-10R is a member of the class II interferon receptor-like cytokine receptor family, and is composed of two ligand-binding subunits (IL-10R1) and



**Figure 3:** IL-10 activation pathway via STAT3. IL-10 binds to its receptors which then phosphorylate associated Jak1 and Tyk2. Subsequently, Jak1 and Tyk2 phosphorylate IL-10R1 to serve as a docking site for STAT3. STAT3 phosphorylation results in dimerization and translocation into the nucleus. IL-10R is a tetramer that is depicted as a dimer in the figure for ease of representation.

two accessory signaling subunits (IL-10R2) [81-83]. The binding of the IL-10 ligand to the IL-10R1 phosphorylates receptor-associated, Janus Kinase-1 (JAK1) and Tyrosine Kinase-2 (TYK2) (constitutively associated with IL-10R1 and IL-10R2 respectively). Subsequently, JAK1 and TYK2 phosphorylate tyrosine residues (Y446 and Y496) on the intracellular domain of the IL-10R1 chain [83]. These phosphorylated sites serve as docking sites for the latent transcription factors, signal transducer and activator of transcription 1, 3, 5 (STAT1, 3, and 5) [84]. STATs are

transcription factors that reside in the cytoplasm in monomeric inactive forms [85]. STAT3 has been shown to be involved in IL-10's mediation of TNF $\alpha$  suppression [86]. STAT3 binds via its Src Homology-2 domain and homodimerization is triggered as a result of tyrosine-phosphorylated by the receptor-associated JAKs. Dimerized STAT3 translocates from the cytoplasm to the nucleus where it binds with high affinity to STAT-Binding Elements in the promoters of various IL-10-responsive genes. Stimulation of IL-10R-expressing cells, such as macrophages, with IL-10 results in Jak1 and Tyk2, Stats 1 and 3 activation [87].

Continual secretion of inflammatory cytokines can lead to inhospitable environments that could delay and impair the regenerative process. Interleukin 10 (IL-10) is a pleiotropic cytokine that regulates lymphoid and myeloid cell function [88]. It was discovered during a screen of factors produced by T<sub>H</sub>2 cells that inhibited T<sub>H</sub>1 cells cytokine production. In particular, IL-10 greatly inhibits tumor necrosis factor- $\alpha$  and interferon gamma, thus favoring the induction of immunoregulatory T cells. Both TNF- $\alpha$  and IFN- $\gamma$  are involved in pro-inflammatory responses and activating the M1 classical inflammatory macrophage phenotype, driving the further secretion of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, GM-CSF, macrophage inflammatory peptide 1 $\alpha$  (MIP-1 $\alpha$ ), generation of nitric oxide, and upregulation of antigen expression such as CD80 and CD86; all this leads to further perpetuation of the inflammatory environment [89]. IL-10 is able to inhibit TNF $\alpha$  secretion by interfering with TNF $\alpha$  translation and mRNA stability via the p38/mitogen activated protein kinase (MAPK) pathway [90]. Although it is clear that the p38/MAPK pathway is involved, the mechanism by which IL-10 targets this pathway is unclear, presumably, it is via STAT3 activated factors [84, 86]. Nonetheless, inhibition of p38/MAPK pathway affects TNF $\alpha$  mRNA translation by inhibiting LPS-induced ribosomal clustering and coupling of TNF- $\alpha$  mRNA in macrophages, without affecting TNF mRNA accumulation [91]. Additionally, Heme

Oxygenase 1 (HO-1) also plays apart in the IL-10 mediated inhibition of TNF $\alpha$  production. IL-10 is a potent inducer of the gene, HO-1 in murine primary macrophages and J774a.1s via a p38 MAPK pathway. HO-1 is involved in the biosynthesis of heme, catalyzing a reaction producing carbon monoxide, free iron, and the heme precursor, biliverdin [92]. Using an antisense oligonucleotide, HO-1 synthesis was inhibited, reversing the inhibitory effect of IL-10 on LPS induced TNF- $\alpha$  [92]. In addition to TNF- $\alpha$ , HO-1 was found to be critical to IL-10's inhibitory effect on LPS-induced expression of inducible nitric oxide synthase and matrix metalloproteinase-9 (MMP9). MMP9 expression in macrophages has been implicated in inflammation-related issues ranging from collagen matrix breakdown leading to cardiac rupture [93], to allergic lung inflammation [94], and chronic eczematous skin diseases [95]. Carbon monoxide, a product of HO-1's degradation of heme also mediates anti-inflammatory effects via protection from free radical stress. Mice exposed to low levels of carbon monoxide (250ppm) an hour pre and 24 hours post surgery had reduced IL-1 $\beta$  and inducible nitric oxide synthase (iNOS) expression, while rats exposed to carbon monoxide experienced decreased levels of IL-6, IL-1 $\beta$ , iNOS and Cox2 [96, 97].

Villalta *et. al.* [98] examined the use of IL-10 as a candidate for Duchenne muscular dystrophy (DMD) treatment. DMD is caused by a genetic deletion of dystrophin, resulting in the weakening of the muscle cell membrane. Dystrophin loss results in increased muscle contraction induced injury, muscle degeneration and immune cell infiltration. A heterogeneous mix of neutrophils, mast cells, T cells and eosinophils have been reported but number of macrophages present exceeds the rest. Glucocorticoids are commonly prescribed but often result in deleterious side effects such as increased susceptibility to infection and cushingoid appearance. The authors reported that the ablation of IL-10 expression in mdx mice resulted in increased muscle damage

in vivo and reduced mouse strength. Furthermore, IL-10 itself had no effect on muscle cell numbers; rather IL-10 affected the numbers of M1 versus M2 macrophages. Their results show that IL-10 plays a significant role in muscular dystrophy by increasing M2 macrophage activation to affect muscle differentiation.

IL-10 has been implicated in scarless fetal wound healing. Fetal wound healing has decreased inflammation and a lack of fibroplasia. In contrast, adult wound healing is often characterized by a strong inflammatory response that leads to scar formation. Peranteau et. al. [99] determined that fetal wounds produce less inflammatory cytokines, and thus introduced IL-10 via a lentiviral vector to adult wounds. At 3 days, they found that wounds expressing Lenti-IL-10 demonstrated decreased inflammation, notably decreased levels of IL-6, monocyte chemoattractant protein -1 and heat shock protein 47. Additionally, at 3 weeks, these wounds showed a lack of abnormal collagen deposition, and restoration of normal dermal architecture. Addition of IL-10 to the wound site was able to create an environment conducive for wound healing.

IL-10 has also been demonstrated to directly increase survival of both cortical and cerebellar granule neurons [100, 101], astrocytes [102], microglia [103], and progenitor and differentiated oligodendrocytes [104]. Unfortunately, IL-10 has also been implicated in the growth and survival of cancer cells, including non-Hodgkin's lymphoma [105], Burkitt lymphoma [26, 106] and non-small cell lung cancer [107]. This is compounded by the immunomodulatory effects of IL-10 on macrophages, as IL-10 encourages monocytes down the M2c pathway [108], an immunoregulatory pathway, foregoing the classic inflammatory pathway desired for cancer cell death. Thus IL-10 is a double edged sword whose application must be guarded carefully.

### **1.3.2 Dendritic cells**

Dendritic cells (DCs) were first described by Ralph Steinman, as a striking population of cells with a dendritic shape in the spleen [109]. In the 1970s, DCs were not considered to be key antigen presenting cells as macrophages were more abundant [110]. DCs proved to be a tricky population to study due to the scarcity even in lymphoid organs, however cytokine driven in vitro expansion methods have made it easier to investigate the role of DCs [111]. It is now clear that DCs serve as the bridge between the innate and acquired immune systems.

#### **1.3.2.a Dendritic cells development**

Dendritic cells are well equipped to be antigen presenting cells. They are equipped with cytoplasmic “dendrite like” extensions for enhanced T cell interaction, adhesion molecules for T cell contact, an efficient antigen-processing system, including endosomes and lysosomes, and high surface levels of MHC molecules and costimulator molecules for peptide presentation and T cell activation. DCs are derived from hematopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. These cells are characterized by high endocytic activity and low T-cell activation potential [109, 111-113]. Immature dendritic cells constantly sample the surrounding environment for pathogens such as viruses and bacteria. Once they have come into contact with a presentable antigen, immature dendritic cells phagocytose pathogens and degrade their proteins into small pieces and upon maturation present those fragments at their cell surface to T and B cells. However, DCs are hard to study as they lack distinctive cell surface markers. Thus, a combination of the absence and presence of certain markers have been used to identify dendritic cells. These include the presence of co-stimulatory molecules such as CD80 (upregulated in mature DCs) and CD86 (upregulated during early DC maturation), adhesion molecules, CD50, CD54, CD11c and the absence of lineage markers such

as CD19 (B cell), CD68 (macrophage), CD66b (granulocyte) and CD56 (natural killer cells) [110].

### **1.3.2.b Dendritic cell function**

Like macrophages, there are dendritic cell subsets. Most briefly, they are divided into 2 camps, myeloid dendritic cell (mDCs) and plasmacytoid dendritic cells (pDCs), which can be further subdivided based on marker expression. pDCs are thought to release large amounts of interferons in response to microbial nucleic acids [114], thus playing a role in antiviral immunity, while mDCs respond to a variety of pathogenic structures, and play a role in T cell stimulation [115]. In this study of dendritic cells, I focus on the mDCs.

Dendritic cells respond to foreign pathogens via pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs). TLRs recognize specific chemical signatures found on subsets of pathogens. Recognition of these foreign pathogens stimulates DCs to secrete IL-12, a proinflammatory cytokine that results in a  $T_H1$  response, thus these DCs are often referred to as D1. However, IL-10 is able to block this DC maturation process by interfering with production of IL-12 and other costimulatory molecules via inhibiting Inhibitor of kappa B kinase that activates transcription factor, NF- $\kappa$ B activation, thus limiting the  $T_H1$  response [116]. Infact, IL-10 not only prevents DC maturation in vitro, it also increases IL-10 production by dendritic cells, inducing the formation of a tolerogenic dendritic cell that induces immunoregulatory T cells [117]. Furthermore, dendritic cells pretreated with IL-10, then stimulated with LPS displayed decreased levels of NF- $\kappa$ B activation [116]. Additionally, Steinbrink *et. al.* [118] found that these IL-10 pretreated DCs displayed a decreased capacity to stimulate CD4<sup>+</sup> T cells, and induced a state of alloantigen specific anergy in these T cells, resulting in decreased T cell

secretion of IL-2 and IFN- $\gamma$ . Interestingly, mature dendritic cells do not respond to IL-10 [118] which validates the need for early introduction of IL-10 into the site of inflammation.

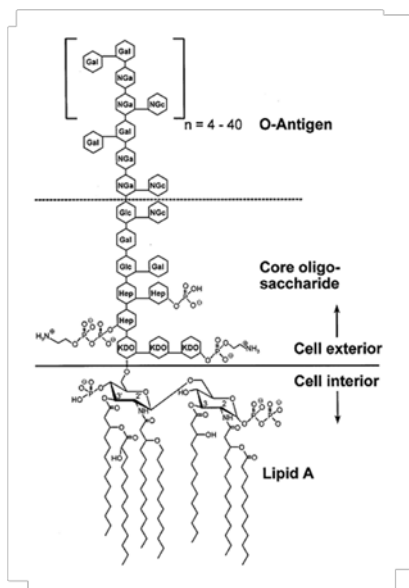
## **1.4 Inflammation**

Inflammation is a process that has evolved to respond to tissue damage. This damage could be due to endogenous or exogenous factors, for example, tissue necrosis, or physical, mechanical, chemical or biological injuries. Biological injuries often include infection by microorganisms that could occur due to breaks in the skin or the lack of sterility during surgery. Inflammation has always been described by its trademarks, swelling (tumor), heat (calor), redness (rubor), pain (dolor) and subsequent loss of function in the inflamed area can be attributed to several cellular mechanisms that are initiated by and in response to the tissue damage [43].

Produced by hepatocytes, acute phase proteins activate, blocking nerve impulses to axons while acting on vascular endothelial cells, causing increased vascular permeability and expression of endothelial cell adhesion molecules, resulting in leukocyte adhesion and extravasation. Degranulating neutrophils and activated macrophages respond quickly to the site of inflammation, secreting cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-1, 6 and 8 in response to the environment. These cytokines induce fever, further leukocyte chemotaxis from the bone marrow, blood vessels and spleen, adhesion and extravasation [43]. In addition, macrophages and dendritic cells are also carrying out antigen presenting duties while clearing the area of cellular debris and foreign pathogens. Should this acute inflammatory response continue, chronic inflammations can occur, in cases such as tuberculosis [119, 120], rheumatoid arthritis [121-124], glomerulonephritis [125, 126] and atherosclerosis [127, 128].

### 1.4.1 Inflammatory stimuli

There are multiple stimuli that can provoke an inflammatory response, for example, a cut, a burn, a corrosive chemical, or onslaught of an infection by microorganisms. These various stimuli will trigger the inflammatory cascade. Currently, some of the more commonly used agents to study inflammation caused by infection is LPS. Negatively charged LPS can be found on the outer membrane of Gram negative bacteria such as *Escherichia coli* and *Porphyromonas gingivalis*, helping to stabilize the membrane structure and can determine the bacteria's responsiveness to antibiotics [129]. LPS, also known as lipoglycans, are endotoxins made up of O antigen, Lipid A and an oligosaccharide. In *E. coli* alone, there are over 160 different O antigens [11]. The Lipid A domain is responsible for most of the toxicity caused by lysed Gram negative bacteria; cell membrane fragments containing Lipid A fragments result in fever, diarrhea, and septic shock (Figure 4).

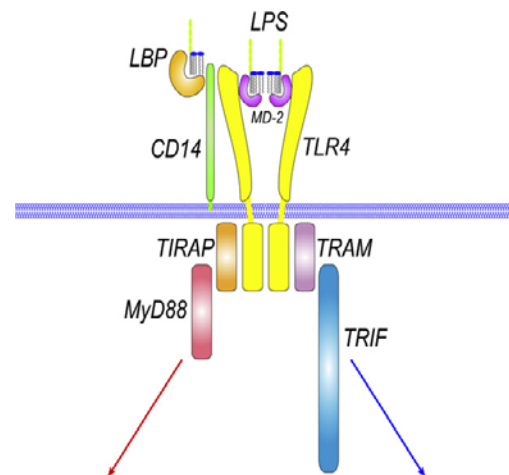


**Figure 4:** Gross structure of intracellular and extracellular domains of lipopolysaccharide. Reprinted from [11].

Toll is a type I transmembrane receptor with a leucine rich repeat and an extracellular domain similar to that IL-1R [130]. TLRs are said to function as PRRs recognizing pathogen associated molecular patterns (PAMPs) such as LPS [131]. For example, lipoteichoic acid (major component of the Gram positive bacterial cell wall) and peptidoglycan (forms the bacterial cell wall) are recognized by TLR2/TLR6 complex, viral DNA rich in unmethylated CpG islands is recognized by TLR9, and viral double stranded RNA interacts with TLR3 [8]. TLR4 can recognize several PAMPs, including LPS from Gram

negative bacteria, fusion protein from respiratory syncytial virus [132], and the protein envelope from the mouse mammary tumor virus [133], as well as endogenous molecules such as heat shock proteins [134] and hyaluronic acid [135].

For TLR recognition of LPS, LPS is said to be captured by the LPS binding protein, a soluble shuttle protein, which then facilitates the association of LPS to CD14 on the cell wall [136, 137]. As CD14 lacks a



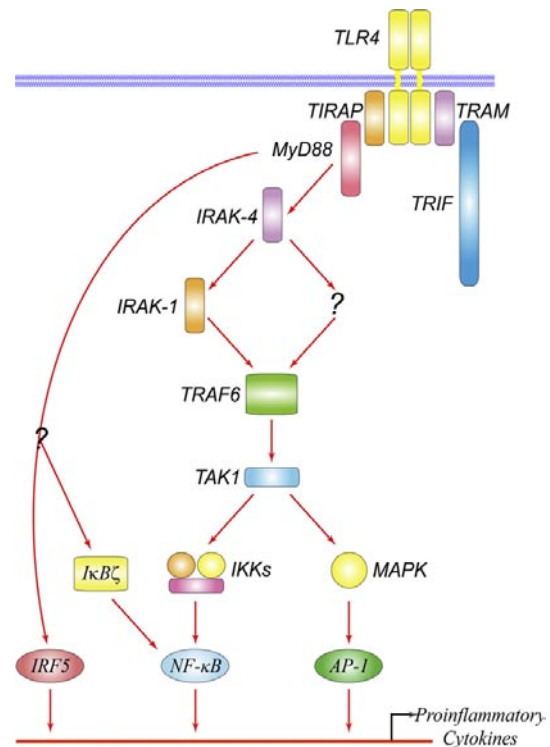
**Figure 5:** Mechanism of LPS activation of TLR4. LPS binding protein binds LPS and delivers it to CD14. CD14 facilitates LPS binding to TLR4/MD2 complex. Subsequent TLR signaling occurs either via a MyD88 dependent or independent pathway. Reprinted from [8].

transmembrane domain, the signal is said to be relayed into the cell via toll like receptor (TLR) 4 and possibly TLR2 [138], though studies have also indicated that TLR2 better recognizes Gram positive bacteria cell wall preparations [139] and a TLR2 knockout has no effect on LPS signaling (Figure 5) [130]. CD14 shuttles LPS to the TLR4/MD2 complex, and upon LPS recognition, TLR4 oligomerizes, and recruits downstream protein adaptors, myeloid differentiation primary response gene 88 (MyD88), Toll-interleukin-1(TIR) domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing interferon b (TRIF), TRIF related adaptor molecule (TRAM) and sterile a and HEAT-Armadillo motifs containing protein (SARM) [140]. TLR4 signaling then proceeds via a MyD88 dependent or independent pathway [141]. In the MyD88 dependent pathway, activation of MyD88 results in the activation of IL-1R-associated kinases (IRAKs), transcription factors NF- $\kappa$ B, interferon regulatory factor (IRF) 5,

and AP1 (via the MAPK pathway), causing the upregulation of pro-inflammatory cytokines and associated genes (Figure 6) [142].

In the MyD88 independent pathway, TRIF mediates the signaling to activate transcription factor IRF3, and later NF- $\kappa$ B and MAPK as well. However, the MyD88 independent pathway results in the increased expression of Type 1 interferon genes [143-145] responsible for antiviral and antibacterial activities [146, 147]. In addition to the production of pro-inflammatory cytokines and interferon species, LPS induced activation of macrophages results in the rapid production of IL-10 alongside TNF $\alpha$  with synchronous kinetics [148]. This endogenous and quick release of IL-10 during endotoxemia is a natural anti-inflammatory response by the body in attempt to control pro-inflammatory cytokine production and LPS toxicity and prevent LPS induced lethality [149, 150]. However, this endogenous release of IL-10 may not be sufficient to overcome to LPS induced inflammation.

Microorganismal stimuli are often treated with a course of antibiotics for four to six weeks, such as penicillin, ampicillin and gramicillin. Most often, this will kill the microorganisms via one or more of the following mechanisms, inhibition of cell wall synthesis; inhibition of protein synthesis, alteration of cell membranes and/or inhibition of nucleic acid synthesis. For example, penicillin and related  $\beta$ -lactam antibiotics result in bacterial death through enzyme-mediated lysis that occurs after the drug



**Figure 6:** MyD88 dependent pathway leads to induction of proinflammatory cytokines. Reprinted from [8].

causes the bacterium to form a defective cell wall [151]. Release of the bacterial cell wall components including LPS will result in the classical activation of immune cells down the inflammatory pathway, and while some inflammation is conducive to the regenerative process, minimal amounts of LPS can induce adverse reactions. Intravenous injections into mammals of this endotoxin at 1ng/ml induced pyrogenic reactions and septic shock [152]. Septic shock can affect any part of the body, including the heart, brain, kidneys, and liver, which can lead to organ failure and ultimately, death. Mortality due to septic shock is approximately 25%-50% [153], depending on the patient's age, overall health, number of failed organs and rate at which medical therapy was delivered [154].

## **1.5 Stem cells**

Stem cells are unspecialized cells that can renew themselves through cell division, or under the right guidance cues, they can be induced to differentiate into tissue specific cells. Some organs that undergo regular wear and tear due to harsh conditions, for example, the gastrointestinal tract, stem cells regularly divide to repair and regenerate damaged tissue. Stem cells exist in embryonic and adult forms. Embryonic stem cells are thought of to be totipotent when isolated at the blastocyst stage; these cells have the capability to become any type of cell in the body [155]. Adult stem cells are pockets of cells that reside in discrete locations, such as the bone marrow, the brain and muscle. These stem cells are not totipotent and are more limited in the range of differentiated tissue, for example, mesenchymal stem cells can differentiate into bone, cartilage and adipose cells.

Stem cells are critical to the reparative and regenerative process after inflammation. The longer the inflammatory environment lingers, the slower the regenerative process. This is due to

the fact that stem cells are highly sensitive to their environment. The presence of reactive oxygen species such as nitric oxide and inflammatory cues such as TNF- $\alpha$  will delay the healing process as this creates an inhospitable environment for the stem cells, inhibiting proliferation and differentiation, activating the apoptotic process, prolonging inflammation. The literature has shown that TNF- $\alpha$  has been shown to induce DNA nicks in mouse embryonic stem cells [156] and affect proliferation and apoptosis in human mesenchymal stem cells [157].

Various stem lines (C2C12s, MC3T3s, human mesenchymal stem cells and muscle derived stem cells) were tested to see the effects of the environment on their differentiative potential in inflammatory environments.

#### **1.5.1 C2C12 cells**

C2C12 cells, a mouse myoblast culture subcloned in Helen Blau's lab around 1980 are commonly used to study osteoblastic differentiation [158]. Under myogenic conditions, such as low concentrations of horse serum, high confluency, or myogenic growth factors such as fibroblast growth factors and endogenous insulin-like growth factor 1, C2C12s differentiate to form myotubes [159-161]. However, the addition of BMP-2 to the C2C12 culture converts the cells towards an osteoblastic lineage by suppressing the transcriptional activities of MyoD and myogenin [162, 163].

#### **1.5.2 MC3T3 cells**

MC3T3 cells were selected as another suitable mouse progenitor cell line to study osteoblastic differentiation as they are often used and could provide confirmation to the experiments conducted on the C2C12 clones [164-166]. MC3T3 cells when cultured in growth media containing ascorbic acid and  $\beta$ -glycerophosphate for 21 days undergo cell differentiation

and mineralization. However, the addition of melatonin to this growth media speeds up cellular differentiation and mineralization from 21 days to 12 days. Additionally, it has been shown that melatonin increased gene expression of bone sialoprotein, alkaline phosphatase, osteontin and osteocalcin in a concentration dependent manner [167]. Melatonin is a major hormone released from the pineal gland (found in the vertebrate brain) [168]. Its secretion levels are regulated by light, with plasma concentrations approximately 50 times higher at night than levels reached during the day [169, 170]. Melatonin regulates a wide range of physiological and pathophysiological processes, for example, hypothalamic control of circadian rhythms [171], sexual development [172], and influence cellular differentiation [173-176]. Interestingly, melatonin also possesses anti-inflammatory properties. Melatonin helps to reduce tissue damage during inflammation by directly scavenging toxic free radicals, reactive oxygen and nitrogen species such as the hydroxyl radical ( $\cdot\text{OH}$ ), peroxynitrite anion ( $\text{ONOO}^-$ ), and hypochlorous acid ( $\text{HOCl}$ ) [177-183].

### **1.5.3 Muscle derived stem cells**

Muscle derived stem cells (MDSCs), considered to be predecessors of satellite cells, are a population of cells isolated via the pour plate method from skeletal muscles. MDSCs exhibit both multipotentiality and self-renewal capabilities. Satellite cells (muscle stem cells) are myogenic precursors that can regenerate muscle, but are thought of as committed to the myogenic lineage [184]. MDSCs are able to regenerate both skeletal and cardiac muscle, and when genetically modified *ex vivo* to express certain target growth factors, MDSCs are able to differentiate into osteogenic and chondrogenic lineages, and are able to promote the repair of bone and cartilage [185].

#### **1.5.4 Human mesenchymal stem cells**

Human mesenchymal stem cells (HMSCs) are thought of as multipotent cells that reside in the adult marrow. Cell morphology is characterized by a small cell body with a few long and thin cell processes. They can proliferate in an undifferentiated state or when exposed to appropriate cues, such as transforming growth factor  $\beta$ , differentiate into chondrocytes [186]. Previously, HMSCs were thought to mainly differentiate into various lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma [186]. However recent studies have shown that HMSCs also have the capability to differentiate into the neuron-like, hepatocyte-like, and epithelial-like cells [187, 188]. However, there is some doubt to the functionality of the neuron like cells derived from HMSCs [189].

As these 4 different cell types represent various states of differentiative potential, it will be interesting to see if cell types react similarly to inflammatory conditions.

#### **1.6 Bone**

Bones are extremely versatile tissues. They come in different shapes; there are long bones, such as the tibia and femur, short bones, such as the ankle and wrists bones, flat bones, such as the sternum, and irregularly shaped ones, like the hip bone. Bones must be strong and not brittle to protect the body's organs, support the body's weight and the forces exerted by contracting and relaxing muscles. Additionally, they store fat and house the marrow where hematopoiesis occurs. There are two types of bones: cortical and cancellous bone. Cortical bone, as its name suggests, is compact and dense, while cancellous bone looks spongy, with lots of open pockets. Bones are able to react dynamically to its environment and the forces that they experiences. Wolff's Law, developed by German surgeon, Julius Wolff states that bones in a

healthy person will adapt to the forces, such as gravity and the push and pull from attached muscles. Thus, hospitalized patients who require bed rest, or astronauts in space will experience a loss in bone mass in addition to muscle atrophy due to the lack of force placed upon the bone. Additionally, bone remodeling takes place in response to the level of calcium in the blood. The lack of calcium in the blood stimulates the brain to release parathyroid hormone into the blood. This in turn activates osteoclasts to break down the bony matrix, releasing calcium into the blood.

### **1.6.1 Bone biology**

During childhood, long bones grow quickly as the epiphyseal plates have yet to be sealed. These plates are replaced by bone after puberty and bones are no longer able to grow. These bones are comprised of a shaft (diaphysis) that is mostly compact bone. The diaphysis is covered by a fibrous connective tissue membrane known as the periosteum, while the ends of the bones are known as epiphyses. In adults, the shaft of the diaphysis contains mostly adipose tissue (yellow marrow), while red marrow is found in the cavities of spongy trabecular bone. Cortical bone is actually made up of Haversian systems, a network of Haversian canals with blood vessels and nerves, surrounded by lamellae and osteocytes residing within the lacunae in the matrix. Smaller canals radiate outward from the central canals, allowing for sufficient nutrient supply to all osteocytes [190]. Additionally, hydroxyapatite, also known as calcium phosphate, provides the mineral component to bone, ensuring that it can withstand compressive forces while collagen fibers within the osteoid provide bone with the ability to withstand tensile forces.

### **1.6.2 Osteogenesis and remodeling**

Bones are formed via the process of osteogenesis. Osteogenesis is a process that is tightly regulated by osteoblasts (bone forming cells), osteoclasts (bone resorbing cells) and osteocytes

(bone cells that reside within the lacunae of the matrix). Osteoblasts arise from mesenchymal progenitor cells that are also able to differentiate into myocytes, adipocytes and chondrocytes [191]. Thus, biological cues such as bone morphogenetic proteins, transforming growth factor beta, platelet derived growth factor BB, the biophysical environment such as matrix deformation, and transcription factor control (Runx2/ Osterix) are critical in ensuring an osteoblastic fate for the progenitor cell. These progenitor cells that commit to an osteoblastic fate go through the following developmental stages, proliferation, differentiation, matrix maturation and mineralization. The proliferation phase is marked by increased type I collagen and histone H4 expression, alkaline phosphatase and bone sialoprotein (component of mineralized tissues) peak during extracellular matrix maturation and osteocalcin during mineralization. Osteocalcin, a hormone secreted by osteoblasts, causes the pancreas to secrete more insulin while increasing the body's sensitivity to insulin is often used as a marker of bone formation [192]. In addition to laying down bone matrix proteins, osteoblasts also help to regulate osteoclast maturation, resulting in bone resorption [193].

Osteocytes are osteoblasts that are mineralized in the matrix that they have created. Due to the influence of the environment and factors such as transforming growth factor beta (TGF- $\beta$ ), osteoblasts develop long spiny processes known as canaliculi as they transition into osteocytes. Together, these osteocytes communicate via gap junctions, thus creating an environment where they are able to respond to external stimuli. Osteocytes are able to transport cell signaling molecules, nutrients and waste products in response to strain derived flow of interstitial fluid.

Osteoclasts are multinucleated cells derived from the fusion of monocytic precursors in the presence of macrophage colony stimulating factor, TGF- $\beta$  superfamily members, the receptor for NF- $\kappa$ B (RANKL), intercellular adhesion molecule 1 (ICAM-1) and inflammatory cytokines

[194]. Osteoclasts, identified by their ruffled cell membrane on bone [195] and tartrate resistant acid phosphatase [196-198], must proliferate and commit to the osteoclast phenotype, before degrading the organic and inorganic components of bone. Bone remodeling is critical to regeneration and repair of the body's skeleton. It is also a delicate balance between osteoblasts laying down new bone matrix and osteoclasts resorbing bone. This dynamic interaction is tightly regulated by local and systemic endocrine factors. The lack of osteoblastic action can result in osteopetrosis, or stone like bone [199]. Conventional dendritic cells are able to differentiate into functional osteoclasts when injected into osteopetrotic mice lacking osteoclastic function[200].

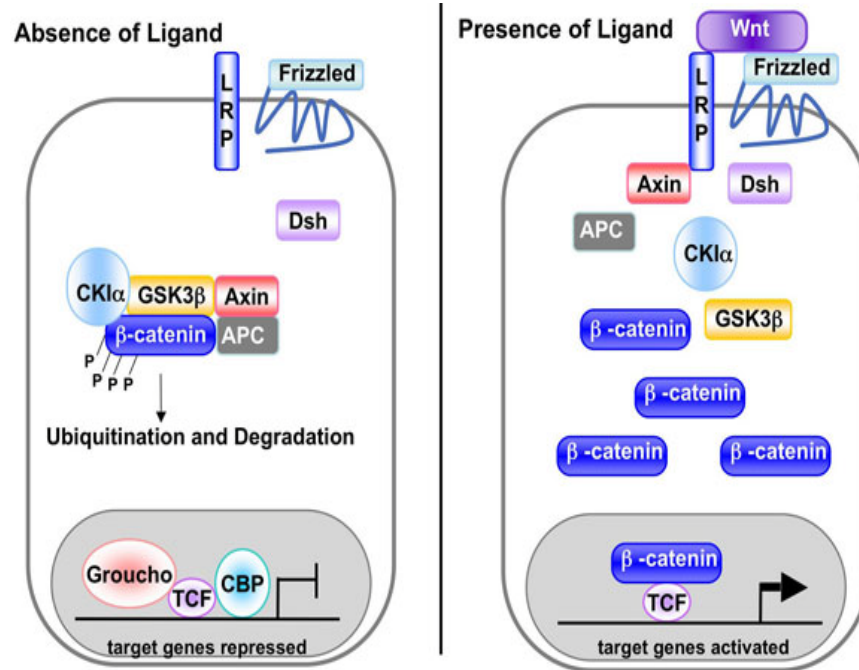
### **1.6.3 Osteogenic Signalling**

As discussed in the previous section, the formation of osteoblasts and osteoclasts are highly regulated by multiple factors. Two of these pathways involving bone morphogenetic proteins and Wnt proteins are discussed in further detail below.

#### **1.6.3.a Wnt signaling pathway**

The Wnt pathway is a network of proteins that are heavily involved in embryogenesis and cancer but are also involved in other processes such as bone regeneration[201]. Via the canonical pathway, the Wnt ligand binds to its cell surface receptor, Frizzled, which in turn activates Dishevelled. Dishevelled inhibits a complex of proteins including Axin, glycogen synthase kinase 3 (GSK-3) and adenomatous polyposis coli (APC), which subsequently inhibits the degradation of beta-catenin. This allows for a pool of cytoplasmic beta-catenin to stabilize, allowing for some beta-catenin to translocate to the nucleus, interacting with the transcription factor and lymphoid enhancer binding factor families of transcription factors to modulate expression of target genes [202-204].

A reduction in Wnt signaling, due to overexpression of Wnt antagonists, deficiency of Wnt ligands, or mutations in genes encoding Wnt receptors results in the loss of bone [205, 206]



**Figure 7:** Canonical Wnt signaling pathway. The lack of the Wnt ligand allows for beta-catenin to be sequestered, ubiquitinated and targeted for degradation, resulting in the suppression of target gene expression. Binding of the Wnt ligand prevents the formation of the degradation complex, allowing for a stable pool of cytoplasmic beta-catenin. This allows beta catenin to translocate into the nucleus, and interacting with transcription factors to upregulate target gene expression. Reprinted from [12] .

while increased Wnt signaling results in increased bone volume, abnormal bone density, sclerosing bone dysplasia [207, 208]. Canonical Wnt signaling can achieve osteogenesis through upregulation of Runx2 expression, via the stabilization of beta-catenin. Additionally, beta-catenin, together with TCF proteins (downstream DNA binding proteins of the canonical Wnt pathway) regulate osteoblast expression of Osteoprotegerin, a major inhibitor of osteoclast differentiation (Figure 7) [209, 210]. Thus, the Wnt pathway is heavily involved in osteogenesis. As expected, there is much crosstalk between the Wnt and BMP2 pathways; BMP2 induced expression of proteins, such as inhibitor of DNA binding/differentiation 1 is downregulated in

Wnt 3a overexpressing C2C12s (mouse myoblast progenitor cells) due to the interaction between beta-catenin and Smad proteins [211] .

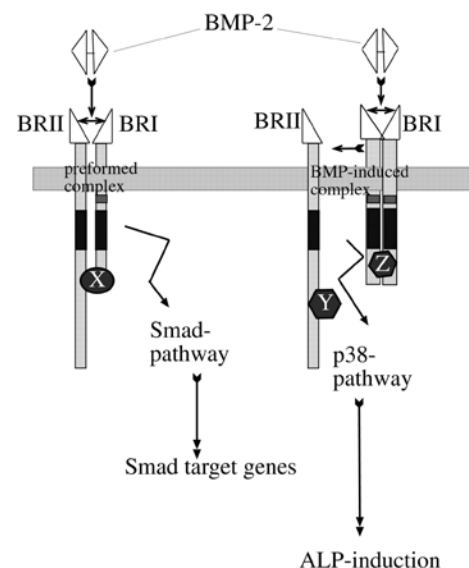
#### **1.6.3.b Bone morphogenetic proteins**

Bone morphogenetic proteins are a group of secreted signaling proteins that were identified by their ability to induce ectopic bone [212]. Evolutionarily conserved, the amino acid sequence of *Drosophila* protein *decapentaplegic* is approximately 75% identical to human BMP-2, and functionally interchangeable with regards to its ability to induce bone formation ectopically [213]. Subsequently, via molecular cloning, BMPs were determined to be part of the TGF- $\beta$  superfamily [214] and to date around 20 BMP family members have been identified and characterized [215]. BMPs are critical to embryonic development and postnatal growth. For example, BMP-2 is crucial during murine embryogenesis, and mice lacking functional BMP-2 die due to malformation of the proamniotic canal and defective cardiac development [216]. BMP-7 null embryos exhibit problems with eye development, while initial outgrowth of the optic vesicle, formation of optic cup and lens induction appear normal, they degenerate over time, indicative of the role of BMP-7 in the maintenance of these optical structures [217]. Additionally , BMPs have been implicated in multiple aspects of development, such as somite patterning, establishment of dorsal-ventral, left-right axis, primary neural induction and regionalization of the brain, cartilage and bone formation [218]. BMPs are synthesized as precursors, which then undergo processing, and are proteolytically cleaved to yield carboxy-terminal mature protein dimers [218] that signal through serine/threonine kinase receptors, composed of type I and II subtypes.

Currently, bone morphogenetic proteins (BMP) are used as therapeutics for healing bone deficits. In particular, BMP-2 and BMP-7 are approved by the Food and Drug Administration; BMP-2 in 2002 [14], marketed as INFUSE® by Medtronic INFUSE® has been approved for use with lumbar tapered fusion device [219] and BMP-7 in 2001 [15], marketed by Stryker Biotech as OP-1 for long bone non union applications. While these and other clinical studies using recombinant human BMP-2 have been successful, their effective healing ranged from 1.7-3.4 mg per scaffold [220-224]. Such quantities of BMP-2 are  $10^6$  to  $10^9$  times higher than regular nanogram physiological concentrations. Such large quantities of the growth factor can be deleterious to the body. While there are a number of studies stating the efficacy of INFUSE® [225, 226], there are just as many studies indicating precautionary placement of INFUSE® in unauthorized sites such as, posterior cervical and thoracic placement, due to the multiple unwanted side effects, including exuberant or ectopic bone formation, paralysis, bowel-bladder and sexual dysfunction, respiratory failure, excessive bleeding, scars, fetal developmental complications, inflammation of adjacent tissues and even death [18, 21, 219].

### 1.6.3.b.i Bone morphogenetic protein 2

Of the 20 members of the BMP family, BMP-2 has received a lot of attention due to its osteogenic ability. Bone morphogenetic protein 2 is a disulfide linked homodimer that binds to its cell surface receptor and can activate its signaling cascade via 2 independent



**Figure 8:** BMP-2 pathways. The pathway on the left shows the canonical BMP-2 pathway via Smad phosphorylation. The pathway on the right shows the alternate BMP-2 pathway that uses the p38 pathway for alkaline phosphatase (ALP) induction. Reprinted from [1].

pathways [1]. BMP-2 mediates its action via two types of transmembrane serine/threonine kinase receptors (BMP-2RI and BMP-2RII) which can form heteromeric complexes before and after ligand binding [227]. The first pathway is where BMP-2 dimerizes and binds to a preformed receptor complex of type I and II, this in turn phosphorylates the receptor Smads 1, 5 and 8 (Figure 8). These form heteromeric complexes with Co-Smad, Smad4, and this complex translocates to the nucleus and alters the expression of target genes, such as Runx2, Osterix, alkaline phosphatase, Type I collagen, and bone sialoprotein. The second pathway is where BMP-2 binds to BMP-2RII which then recruits BMP-2RI, initiating a phosphorylation cascade via the mitogen activated kinase (MAPK) p38 pathway [1, 228, 229]. MAPK components are distinct, activating only specific targets, however MAPK pathways are responsible for transducing multiple stimuli and thus these pathways are susceptible to crosstalk and inhibition. BMP-2 induced osteoblastic differentiation is associated with increased production of a large amount of extracellular matrix proteins including type I collagen, bone sialoprotein and osteocalcin, high levels of ALP activity [162], transcription factors, Osterix and Runx2, and responsiveness to calcitropic hormones such as parathyroid hormone (PTH) and  $1\alpha,25$ -dihydroxyvitamin D3 [230].

As BMP-2 is a direct promoter of both osteoblastogenesis [231-239] and osteoclastogenesis [240-244], the preference toward bone resorption or formation/repair is influenced by the inflammatory microenvironment. Thus, the associated macrophage contribution (M1 or M2c) to the repair site microenvironment will preferentially determine relative effectiveness of therapeutic BMP-2 as an osteogenic agent. Thus, controlling the microenvironment with IL-10 and/or similar immunoregulatory cytokines may be important toward the speed and success of BMP-2 mediated bone regeneration.

In addition to control of osteogenesis, BMP-2 has been shown to induce the release of neuroinflammatory molecules, substance P (SP), and calcitonin gene related peptide (CGRP) from peripheral nerves after traumatic injury [245]. CGRP and SP subsequently recruit mast cells to the site of inflammation to undergo degranulation. Mast cells are resident tissue immune cells that are similar to basophils. Upon degranulation, mast cells tend to release proteoglycans such as heparin(anticoagulant), serine proteases, serotonin (serves as a vasoconstrictor), and histamine [246]. Mast cells also secrete eicosanoids such as platelet activating factor and prostaglandin D2, in addition to cytokine, eosinophil chemotactic factor [246].

#### **1.6.3.b.ii Crosstalk between bone morphogenetic protein 2 and the components of inflammation**

The inflammatory milieu released by cells of the innate immune system is able to influence osteoblastogenesis via BMP-2 because of the regulatory crosstalk between the receptor pathways. Regulatory crosstalk occurs between the TLR and BMP receptor signaling pathways due to overlap between NF- $\kappa$ B and MAPK-directed TLR pathways and/or the BMP/Smad and BMP/MAPK p38 pathways [247-250]. For example, LPS stimulation of TLR4 results in microRNA miR-155, which results in inhibition of Smad1/5 in the canonical BMP/Smad pathway driven by BMP-2,-4,-6 [251]. Additionally, crosstalk between secreted inflammatory cytokines such as TNF- $\alpha$  can interfere with the BMP-2/Smad pathway. Yamashita et. al. antagonized the TNF- $\alpha$ / MAPK pathway using Simvastatin, thus rescuing the BMP-2/Smad pathway, allowing for BMP-2 osteoblastogenesis in C2C12 cells [240]. IL-1 $\beta$ , a cytokine commonly associated with inflammation was found to have opposing effects to TNF- $\alpha$  in MC3T3 cells; IL-1 $\beta$  significantly enhanced BMP-2 and BMP-4 induced ALP expression while TNF- $\alpha$  inhibited ALP induction [252]. Thus, it is apparent that the BMP-2 signaling pathway is

dependent on the parallel pathways that have been activated due to the cytokines profile in the surrounding environment.

#### **1.6.4 Inflammation and bone pathology**

Inflammation affects the balance between bone formation and resorption through the release of pro-inflammatory cytokines such as IL-1 and TNF $\alpha$ . These inflammatory cytokines induce expression of RANKL and ICAM-1 on osteoblasts; RANKL stimulates osteoclast maturation, while ICAM-1 results in firm adhesion allowing for cognate interaction. Thus, pro-inflammatory neutrophils, classically activated macrophages and activated dendritic cells secrete signals that act via osteoblasts to induce osteoclast maturation, upregulating bone resorption, resulting in secondary osteoporosis [193]. Secondary osteoporosis is caused by other underlying medical conditions such as kidney failure [253], rheumatoid arthritis [254], multiple sclerosis [255], and chronic obstructive pulmonary disease [256]. This additional loss in bone density caused by an imbalance of bone homeostasis is detrimental to the body.

While secondary osteoporosis may have a different etiology, it shares similar symptoms with traditional osteoporosis. Traditional osteoporosis can be due to a variety of causes including genetics, nutritional abnormalities, or a drop in estrogen in women during menopause, or a drop in testosterone in men. It can result in bone pain and tenderness, kyphosis (stooped posture), fracture of spinal bones resulting in back or neck pain, and a loss of height. X ray, bone mineral density tests and spine computed tomography can be used to identify the onset of osteoporosis. It is estimated that 1 out of 5 women in America over the age of 50 has osteoporosis [257].

In contrast, inflammation is also associated with HO. Heterotopic ossification is the formation of bone in soft tissue often due to traumatic injuries. It is a frequent complication

following central nervous system disorders, including brain injuries, encephalitis, spinal cord lesions, multiple and traumatic injuries, hip surgery and burns. Additionally, there are hereditary causes such as fibrodysplasia ossificans progressiva, progressive osseous heteroplasia and Albright's hereditary osteodystrophy. [258]. According to recent statistics from the military, as many as 60% of traumatic injuries, resulting from bomb blasts, have associated HO [245]. HO can result in a limitation of the range of joint motion, impacting the day to day activities of patients who already suffer pain and disability due to their original lesion. In addition the patient can further experience increased contractures and spasticity, pressure ulcers and increasing pain [258].

The causes of HO are still unknown apart the common thread of BMP-2 expression in the area of HO [259]. As HO is often complication of traumatic injury, and traumatic injury and inflammation often go hand in hand, inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  are released at the site of injury. In cartilage explants cultures, it was found that IL-1 $\beta$  and TNF- $\alpha$  increased BMP-2 levels both intracellularly and extracellularly, and immunohistochemistry revealed the co-localization of BMP-2 and newly synthesized procollagen within the same cells [76]. Additionally, BMP-2 has been shown to be elevated upon muscle injury and changes in blood flow, as well as released when bone is injured [260-262]. In addition to BMP-2's osteogenic influences, it can act directly on sensory neurons and induce the release of neuroinflammatory molecules, SP, and CGRP from peripheral nerves after traumatic injury. This causes nerve remodeling and the migration of osteogenic and other stem cells from the nerve, resulting in heterotopic ossification [245]. HO is an interesting phenomena as it is linked with inflammation despite the fact that BMP-2 can serve to activate osteoclasts in an inflammatory environment [241], BMP-2's osteoblast inducing actions are curtailed in an inflammatory

environment [70], and that inflammatory environments induce apoptosis [263]. Currently available prophylactic therapies for inhibiting HO, including radiation and NSAIDs, remain controversial, and any potential therapy must be balanced with its potential inhibitory effects on bone repair [264, 265].

## **1.7 Overview of the present study**

The goal of this dissertation is to examine the crosstalk between cells of the immune system and stem cells undergoing osteoblastogenesis due to bone morphogenetic protein 2 (BMP-2) under inflammatory or anti inflammatory conditions. The central hypothesis of this dissertation is that the cells of the innate immune system, such as macrophages and dendritic cells will inhibit BMP-2's ability to induce osteoblastogenesis in stem cells under inflammatory conditions while permitting BMP-2's osteogenic capabilities when IL-10 is co-applied during inflammatory conditions. While this work in this thesis mostly applies to bone regeneration, it is hoped that these data will apply to other cells of the innate immune system, such as neutrophils, and stem cells differentiating down other lineages, such as tendon.

### **Specific Aims:**

**Aim 1: To determine the effect of macrophages on C2C12 progenitor cells under inflammatory and non-inflammatory conditions.**

Monocytes are said to differentiate down the classically activated (M1) inflammatory pathway or towards the alternatively activated, immunoregulatory pathway depending on the stimuli. To understand the crosstalk between macrophages, C2C12s and BMP2, we had to determine if we could control macrophage activation with stimuli such as LPS and IL-10, then analyze the changes in macrophages secretions due to these stimuli, and subsequently, determine

if the macrophage secretions under these different conditions will affect BMP2 induced osteoblastogenesis in C2C12s.

**Aim 2: To determine the effect of dendritic cells on C2C12 progenitor cells under inflammatory and non-inflammatory conditions.**

Dendritic cells are the primary antigen cell type, activating T cells down the  $T_H1$  or  $T_H2$  pathway. Th1 cells produce IFN- $\gamma$ , TNF- $\alpha$ , and IL-12, and are thought to cause macrophage activation, differentiation of T-cells to a cytotoxic (CD8+) phenotype, and graft rejection. In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, cytokines that inhibit activation of dendritic cells and the classic inflammatory pathway [265-267]. Thus dendritic cell activation can lead to chronic inflammation or a remodeling phenotype. This study aims to i) determine if dendritic cell activation can be modulated by stimuli such as LPS and IL-10, ii) analyze dendritic cell secretions under these different conditions and ii) determine if dendritic cell secretions under these different conditions will affect BMP2 induced osteoblastogenesis in C2C12s.

**Aim 3: To determine the effects of macrophages and dendritic cells on different types of stem cells.**

The different stem cell types I have selected to study in this portion of the thesis are known to react similarly to BMP2's osteogenic cues, however they may react differently to secreted macrophage and dendritic cells products as they are at various stages of pluripotency. This study aims to determine if C2C12s, MC3T3s, muscle derived stem cells and human mesenchymal stem cells react similarly to inflammatory secretory products under osteogenic conditions.

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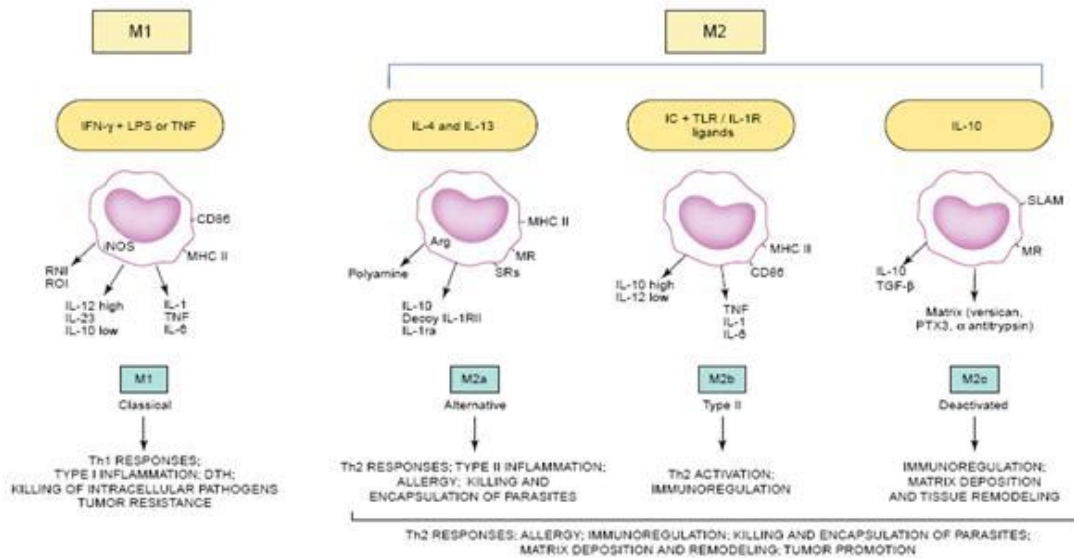
## Chapter 2: Crosstalk between macrophages, BMP-2 and stem cells in inflammatory and non inflammatory environments.

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## 2.1 Introduction

Wound repair and tissue regeneration are highly dependent upon the differentiation of stem cells. Stem cell differentiation is regulated by multiple factors: target cytokines direct stem cell migration from stem cell niches to wound sites and appropriate growth factors influence stem cell differentiation. A hospitable environment is required for these processes to take place. The initial and prolonged tissue environment is often regulated by the cells of the innate immune system, including neutrophils, macrophages and dendritic cells. Together these cells are the first responders to stimulants such as tissue damage, infection, and bioengineered implants, and can contribute to an inflammatory or regenerative environment.

Macrophages are a critical part of the body's immune response, carrying out multiple biological functions, such as angiogenesis, clearing of cellular debris, and inflammation. They arise from granulocyte-macrophage progenitors that leave the bone marrow and then circulate in the bloodstream prior to maturation. When exposed to stimulants such as lipopolysaccharides (commonly found in the bacterial cell wall) and proinflammatory cytokines such as interleukin  $1\beta$ , these monocytes are activated to form the classic inflammatory macrophage, commonly referred to as M1. M1s are characterized by the high capacity to present antigens, produce high levels of interleukins 6, 12 and 23, low levels of interleukin 10 and toxic intermediates such as nitric oxide. These macrophages are potent effector cells integrated in  $T_H1$  responses, killing micro-organisms and tumour cells, and producing large amounts of inflammatory cytokines. Monocytes exposed to IL-4, 13 and 10 develop into alternatively activated macrophages, referred to as M2 [1]. M2s are generally characterized by the low production of pro-inflammatory cytokines, regulate inflammatory responses and adaptive immunity, scavenge debris and promote angiogenesis, tissue remodeling and repair. Recently, these opposing macrophage types have



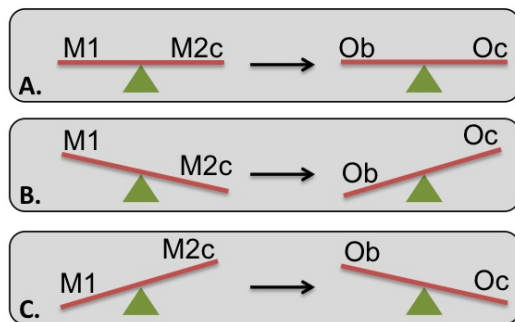
**Figure 1:** Differences in stimulants, secreted products and responses of M1, M2a, M2b and M2c phenotypes. Image reprinted from [1].

been described as polar extremes along a continuum of macrophage activation phenotypes including M2a, M2b and M2c (Figure 1).

Studies have shown that the increased presence and persistence of M1s over M2s have implications in chronic inflammation. Chronic inflammation can not only delay the regenerative process, it can lead to transplant necrosis and implant rejection. Persistent inflammation is a hallmark of multiple diseases such as tuberculosis, atherosclerosis, and rheumatoid arthritis. As persistent inflammatory macrophages are deleterious to the wound site, inflammation must be controlled there. For example, monocytes costimulated by both LPS and IL-10 display decreased mRNA expression of IL-1 $\alpha$ , IL-6 and TNF- $\alpha$ . IL-10 is widely regarded as an anti-inflammatory cytokine. It is said to block the expression of pro-inflammatory genes and enhance the production of anti-inflammatory molecules. IL-10 acts via the JAK-STAT pathway, where a dimeric IL-10 binds to its receptor, activating tyrosine kinases and recruiting STAT3 [2]. STAT3 is a crucial player in mediating the anti-inflammatory downstream effects of IL-10. Thus, the use

of IL-10 in conjunction with inflammatory stimulants could alter the wound environment dramatically.

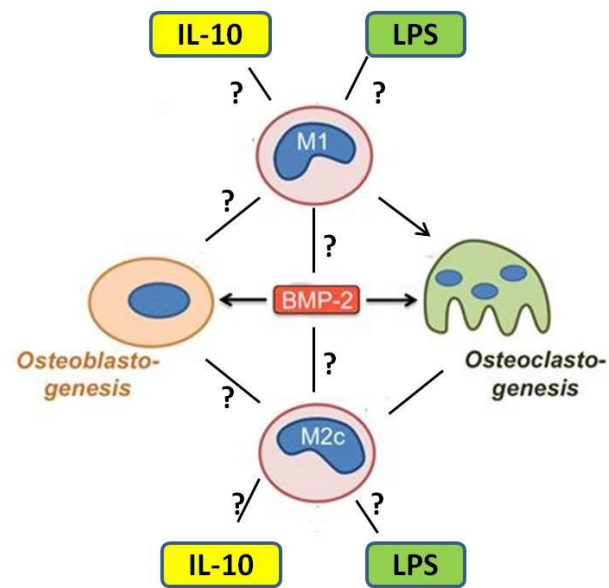
To study the effects of the wound environment on stem cell differentiation, we chose to focus on the effects of bone morphogenetic protein 2's on progenitor cell type C2C12 in osteoblastogenesis. Stem cells are critical to the reparative and regenerative process after inflammation. The longer the inflammatory environment lingers, the slower the regenerative process. This is due to the fact that stem cells are highly sensitive to their environment. The presence of reactive oxygen species such as nitric oxide and inflammatory cues such as TNF- $\alpha$  can delay the healing process as this creates an inhospitable environment for the stem cells, inhibiting proliferation and differentiation, activating the apoptotic process, and prolonging inflammation. Currently, there is much interest in the use of the growth factor, bone morphogenetic protein 2 for bone regeneration therapies. BMP-2 has been successful in



**Figure 2:** Effects of M1 and M2c ratio upon BMP-2's actions. **A.** The balance between osteoblastogenesis and osteoclastogenesis is dependant upon the ratio of M1 to M2c. **B.** Predominance of classic inflammatory M1s results in osteoclastic actions of BMP-2. **C.** Increase in M2c over M1 during the remodeling/repair phase results in tissue regeneration as BMP-2's actions now favor osteoblastogenesis.

regenerating bone when added exogenously to cell cultures *in vitro* and within biomaterials *in vivo*. BMP-2's osteogenesis effects with regards to C2C12s in culture are well documented. BMP-2 acts via the Smad pathway to induce the expression of markers of osteoblastogenesis such as alkaline phosphatase, collagen type I, osteopontin and osteocalcin. However, BMP-2's osteoblast inducing capabilities are dependent upon cellular environment. Under inflammatory

conditions where M1 macrophages predominate, BMP-2 has osteoclast inductive actions [3], resulting in an increase in loss of bone mass, and causing further delays in regeneration [4]. However, when M2c macrophages predominate, BMP-2's role switches to that of osteoblastogenesis (Figure 2) [5]. Thus, inflammation not only is able to affect progenitor cell recruitment and proliferation and differentiation, it can also have unwanted side effects on pleiotropic growth factors intended for regenerative purposes. Hence it is critical to gain a better understanding of the crosstalk between these different players in an inflammatory wound site: the stimuli, the innate immune cells, the progenitor cells and their directive growth factors (Figure 3).



**Figure 3:** Investigating the crosstalk between BMP-2, macrophages and bone cells. Lines ending in ↑ indicate stimulation; ⊥ indicates inhibition; and ? indicates interactions to be determined in this chapter.

To study the crosstalk between BMP-2, macrophages, and stem cells in inflammatory and anti-inflammatory

situations, we chose representative cell lines J774a.1, a mouse macrophage like cell line and C2C12s, a mouse myoblastic progenitor cell line. J774a.1 cells are a monocyte/macrophage cell line isolated from a female BALB/c mouse commonly used to study macrophage activation [6-8], while C2C12s are a mouse progenitor cell line that is predisposed towards muscle but is able to differentiate to bone under the influence of BMP-2 [3]. I determined if: i) IL-10 could potentially be used as an anti-inflammatory agent to improve a harsh extracellular environment caused by LPS mediated macrophage activation ii) what products are secreted by macrophages

in inflammatory and anti-inflammatory conditions and iii) how the classical inflammatory activation of macrophages affects BMP-2's induction of stem cell differentiation.

## **2.2 Methods and Materials**

Escherichia coli lipopolysaccharide was purchased from Sigma (St Louis, MO). Bone morphogenetic protein 2 was purchased from Medtronic (Minneapolis, MN). Interleukin 10 was a gift from Dr. Xin Xiao Zheng. FITC-anti-CD86 and APC-anti-CD86 were purchased from BD Pharmingen (San Diego, CA), anti-CD163 and anti F4/80 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and secondary antibody, DyLight 594 Donkey Anti Rabbit and DyLight 649 Donkey Anti Goat were purchased from Jackson Laboratories (West Grove, PA). Phosphate buffered saline (PBS) was purchased from Fisher Scientific (Pittsburgh, PA). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. All experiments were performed three times in triplicate unless noted otherwise to ensure statistical significance.

### **2.2.1 Cell culture**

J774a.1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin and streptomycin. Mouse C2C12 cells (ATTC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Media (DMEM), 10% fetal bovine serum and 1% penicillin-streptomycin (PS). All cells were kept at 37°C, 5% CO<sub>2</sub> in a humidified incubator.

### **2.2.2 Surgery and nitrocellulose implantation**

Nitrocellulose discs (3mm) (Millipore, Billerica, MA) were sterilized in 100% ethanol for 1 minute, washed twice in sterile filtered deionized water, then washed a final time in sterile

filtered PBS before drying overnight under sterile conditions. PBS, 1 $\mu$ g of IL-1 $\beta$  (ProspecBio, Ness-Ziona, Israel) or 1 $\mu$ g of IL-10 were hand printed onto both sides of the nitrocellulose disc, allowed to dry for 10 minutes, then washed with sterile filtered PBS to rinse off unbound cytokine. C57BL/6 mice from Harlan Sprague Dawley (Indianapolis, Indiana) were used between weeks 8 -10 after birth. The mice were anesthetized for surgery using intraperitoneal sodium pentobarbital (80mg/kg). For subcutaneous implants, small bilateral skin incisions (<1cm) were made over the hip and shoulder distal from the dorsal midline and subcutaneous pouches were created with blunt dissection. Nitrocellulose discs were implanted subcutaneously and skin incisions were sutured closed. Surgeries were performed on heating pads and each mouse was allowed to recover from anesthesia on a heating pad before returning to its housing unit. Analgesic (Ibuprofen, ~0.15 mg/ml in drinking water) was administered post-surgery. The dietary habits, general health status, and the surgical sites of mice were monitored daily. Any animal showing signs of infection or uncontrollable pain was immediately euthanized and removed from the study. All operations were in accordance with institutional animal use and care regulations.

### **2.2.3 Euthanasia and specimen harvest**

At the predetermined time of sacrifice, mice were anesthetized by exposure to isofluorane (Med-Vet International, Mettawa, IL) in a closed chamber (approximately 30s of exposure), then euthanized by cervical dislocation. The nitrocellulose discs were explanted and washed with PBS to get rid of debris. The specimens were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes, then blocked with 1% bovine serum albumin (BSA) (Sigma, St Louis, MO) for 1 hour prior to primary antibody staining using F4/80, CD86 and CD163 (1:250 dilution) overnight at 4°C. Specimens were then treated

with secondary antibodies DyLight 594 and DyLight 649, for 1 hour at room temperature prior to imaging using a Zeiss Axioplan2 (Thornwood, NY) imaging microscope.

#### **2.2.4 Flow cytometry**

J774a.1 cells were treated with LPS, IL-10, or LPS and IL-10 for 24 or 48 hours prior to cell surface marker analysis using a Becton Dickinson FACS DIVA. To prepare cells for FACS analysis, J774a.1s were washed with phosphate buffered saline (PBS; Fisher Scientific, Pittsburgh, PA), then blocked with 1% BSA in PBS for 1 hour at 4 °C. Cells were washed in PBS and then incubated with the CD86 antibody (1:250 dilution) for 1 hour at 4 °C. Subsequently, cells were prepared for flow cytometry by washing and straining through a cell strainer to remove clumps.

#### **2.2.5 Confocal microscopy**

To image the cells using confocal microscopy, J774a.1 cells were washed with PBS, fixed in 4% paraformaldehyde for 15 minutes, blocked with 1% BSA in PBS for 40 minutes, then incubated with anti CD86 and anti CD163 antibodies (1:250 dilution) overnight at 4 °C. Subsequently the cells were washed and treated with the DyLight 594 secondary antibody (1:200 dilution) for 1 hour at room temperature. Cells were washed prior to imaging. Confocal images were captured on a Zeiss LSM 510 (Thornwood, NY) using a 20x objective.

#### **2.2.6 Conditioned media experiments**

##### **2.2.6.a Liquid phase experiments**

$1 \times 10^6$  J774a.1 cells in tissue culture were treated using the following concentrations of biological response modifiers for 24 or 48 hours: 100 ng/ml LPS, 100 ng/ml IL-10 or 100 ng/ml each of IL-10 and LPS. Conditioned media was harvested 24 or 48 hours post treatment and kept

frozen prior to use.  $12 \times 10^4$  C2C12 cells were treated with regular or conditioned media, with or without 100 ng/ml of BMP-2, or 10ng/ml tumor necrosis factor alpha (Isokine, Kopavogur, Iceland). The conditioned media was refreshed at day 2 of culture and cells were stained for alkaline phosphatase activity at day 4 of culture.

#### **2.2.6.b Solid phase experiments**

Fibrin coated coverslips were printed with 20 overprints of BMP-2 at 100 $\mu$ g/mL in 4 1x1mm patterns. The coverslips were then left to dry and the area around the pattern was etched by a diamond tipped pen for later identification. The coverslips were washed in PBS for 5 minutes and left overnight in RPMI 1640 containing 1% penicillin streptomycin. The following day, C2C12s (Passage 15 and under) were seeded at  $30 \times 10^4$  cells per coverslip and cultured in J774A.1 48 hour conditioned media with 1 $\mu$ g/mL of aprotinin. A conditioned media change was done at ~48 hours post-seeding and cells were stained for alkaline phosphatase at ~4 days post seeding.

#### **2.2.7 Alkaline phosphatase staining**

Alkaline phosphatase activity (SIGMAFAST) was detected according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Brightfield images of the cells were captured using a 10X and 20X objective on a Leica DMIL LED microscope (Buffalo Grove, IL).

#### **2.2.8 Griess assay for nitrate reductase**

Cell culture media was harvested from J774a.1 cells treated with 100 ng/ml of LPS, 100ng/ml of IL-10 or both for 24 and 48 hours (conditioned media). The conditioned media were assayed for levels of nitric oxide following the manufacturer's instructions using a Griess assay (Biotium, CA), and read using a Tecan Safire<sup>2</sup> plate reader (Durham, NC).

### **2.2.9 Wright's staining of primary chicken monocytes**

Peripheral blood was harvested from White Leghorn chickens at Eichner's farm (Wexford, PA) via intravenous puncture. Mononuclear cells were isolated from the blood using Histopaque 1077 (Sigma, St Louis, MO) following manufacturer's protocols. Adherent monocytes were obtained after an overnight plate down at 37°C in RPMI 1640, 10% chicken serum, 1% penicillin-streptomycin. Monocytes were scraped off and plated in 6 well plates at  $\sim 1 \times 10^6$  cells/ well. Wells were treated using the following LPS concentrations: 0ng/ml, 100ng/ml, and 200ng/ml for 24 hours, then stained with Wright's stain. Wright's stain was purchased from Medex Supply (Monsey, NY) and staining was carried out as per manufacturer's instructions; cells were imaged on a Leica DMIL LED microscope (Buffalo Grove, IL).

### **2.2.10 Computational image analysis**

#### **2.2.10.a Segmentation**

Images obtained were segmented into single-cell regions using Topology-Preserving Stochastic Active Contour Scheme (TP-STACS) [9], a modified version of the STACS algorithm [10]. Briefly, the algorithm begins by creating a contour around the nuclear stain of the image. Using the protein image, the contour grows until it reaches an equilibrium state. More simply, the algorithm can be imagined as hills and valleys. The algorithm starts at the top of the hill until it reaches sea-level.

#### **2.2.10.b Calculation of Average Fluorescence**

The average fluorescence in each cell region was calculated. The fluorescence ratio between CD86 and CD163 expression was also calculated for each cell region. This can also be

expressed as the M1:M2c ratio, as CD86 is a marker for M1 differentiation and CD163 is a marker for M2c differentiation.

#### **2.2.10.c Quantification of Alkaline Phosphatase Staining.**

Quantification of alkaline phosphatase staining was done by blind spectral unmixing using non-negative matrix factorization. Simple linear unmixing is defined by:  $V = W \times H$ , where  $V$  is the source image, an  $(m \times n)$ -by- $c$  matrix (the number of colors,  $c$ , is 3 for RGB images),  $W$  is the color-bases matrix, a  $c$ -by- $r$  matrix (where  $r$  is the number of sources to be separated), and  $H$  is the unmixed image of the same size as the source image. Because of experimental variation, the spectra of immunocytochemical dyes are often not consistent across every image, making simple linear unmixing inappropriate. Thus, I used a method demonstrated by Newberg *et. al.* [11] that uses non-negative matrix factorization (NMF) to blindly unmix the images. Briefly, NMF assumes that each stain contributes non-negatively to the overall image intensity. This method has been shown to be effective in unmixing brightfield images [11]. Blind spectral unmixing by NMF uses a different color matrix,  $W$ , for each image.  $H$  is randomly initialized and NMF is used to solve for the non-negative matrix factors  $W$  and  $H$  by iteratively minimizing the distance between  $V$  and  $W \times H$ . The post-processing of  $H$  into a new unmixed image was similar to that of the linear unmixing, in which each channel was scaled and then remapped into the image data channels. The unmixed image was summed to determine the amount of ALP staining.

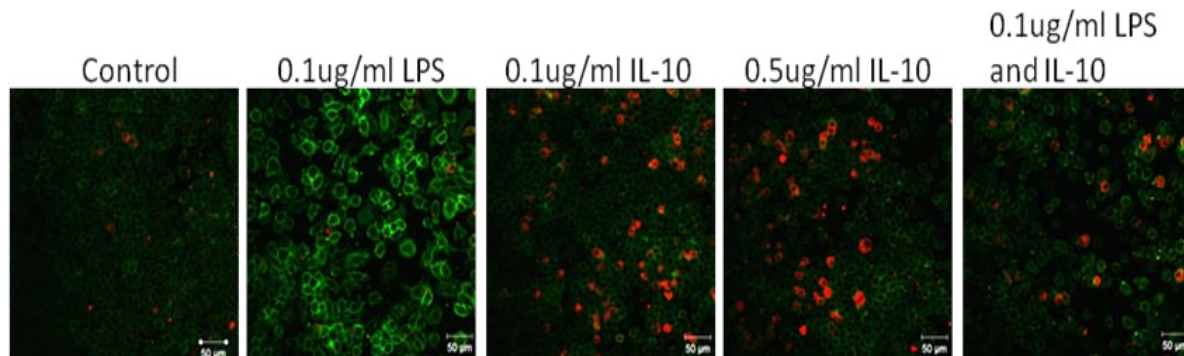
#### **2.2.11 Milliplex/MAP cytokine/chemokine assay**

Control, 100ng/ml LPS, 100ng/ml IL-10 and 100ng/ml of LPS and IL-10 conditioned media were harvested from J774a.1 cells after 24 or 48 hours. Secreted components were

analyzed using the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, Billerica, MA), which was run in accordance with the manufacturer's instructions.

## 2.3 Results

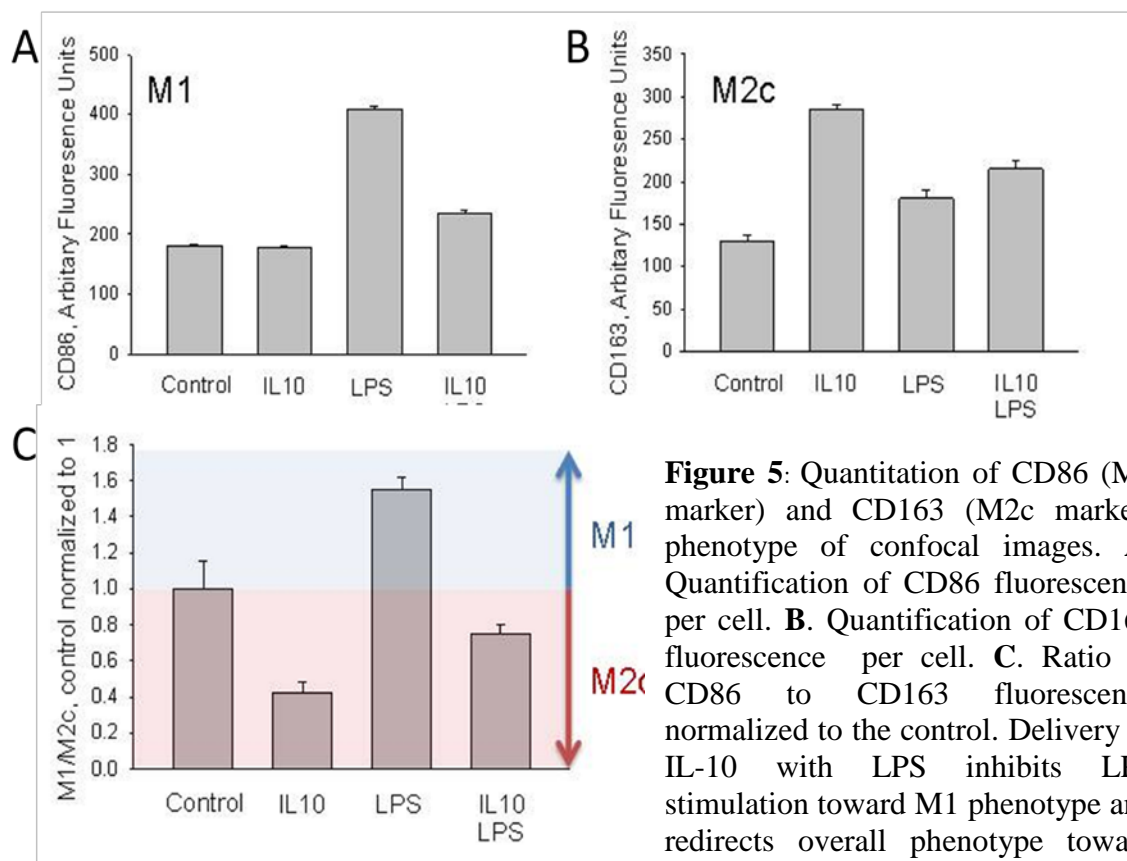
### 2.3.1 M1 and M2c macrophage activation



**Figure 4:** IL-10 influences macrophage phenotype after stimulation with LPS. Confocal images of J774A.1 cells treated with LPS and/or IL-10 for 24 hours, then assayed for M1 and M2C marker expression. Pseudocolored red, CD163, an M2C marker. Pseudocolored green, CD86, an M1 marker. LPS activates J774A.1 toward an M1 phenotype, resulting in an upregulation of CD86 and not CD163. Treatment with IL-10 alone does not upregulate CD86, and results in an increase of CD163+ cells. Co-treatment of LPS with IL-10 resulted in a decrease in CD86. Notably, there was also an increase in CD163+ cells. Images were captured at 20X and the scalebar indicates 50 µm.

To determine whether macrophage activation could affect BMP-2's induction of osteoblastogenesis in stem cells, I had to ascertain that I could indeed activate macrophages. J774a.1 cells were exposed to 100ng/ml of LPS (inflammatory M1 stimulator) and/or 100ng/ml of IL-10 (anti-inflammatory M2c stimulator) for 24 or 48 hours, then stained for M1 (CD86) and M2c (CD163) markers and assayed via confocal microscopy, flow cytometry, cell morphology using Wright's stain and for nitric oxide secretion using the Griess assay.

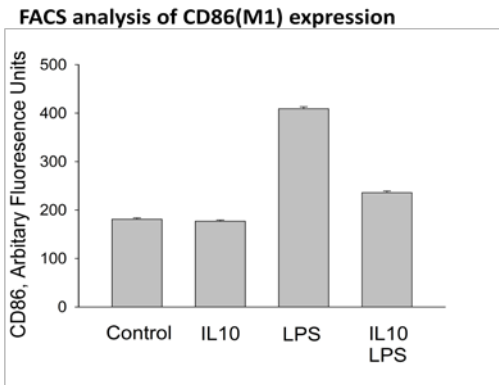
Figure 4 shows confocal images of J774a.1 cells stimulated with 100ng/ml LPS, 100ng/ml IL-10, 500 ng/ml IL-10 and 100ng/ml of LPS and IL-10 after 24 hours. Qualitatively, the images indicate that macrophages treated with LPS express increased CD86 (M1 marker) over the control group but not CD163, while macrophages treated with IL-10 did not exhibit the same increase in CD86, but rather expressed an increase in CD163 (M2c marker). Interestingly, macrophages treated with LPS and IL-10 displayed an increase in CD163 and a slight increase in



**Figure 5:** Quantitation of CD86 (M1 marker) and CD163 (M2c marker) phenotype of confocal images. **A.** Quantification of CD86 fluorescence per cell. **B.** Quantification of CD163 fluorescence per cell. **C.** Ratio of CD86 to CD163 fluorescence normalized to the control. Delivery of IL-10 with LPS inhibits LPS stimulation toward M1 phenotype and redirects overall phenotype toward M2c in J774a.1 cells. That is, in mixtures, IL-10 dominates.

CD86 as compared to the LPS only treatment, indicative of IL-10 anti-inflammatory properties. To verify if qualitative estimates were true, the images captured via confocal microscopy were segmented using a topology-preserving STACS algorithm and the fluorescence for each individual cell region was averaged, maintaining spatial registration of immunostained adhered

cells. CD86 and CD163 fluorescence for each cell was quantified and tabulated (Figure 5A and 5B). IL-10 increases CD163 expression and decreases CD86 expression when co-delivered with LPS. When a ratio of CD86 to CD163 expression per cell is compared, a ratio value  $<1$  is indicative of an increased presence of M2c macrophages, while a ratio value  $>1$  indicates

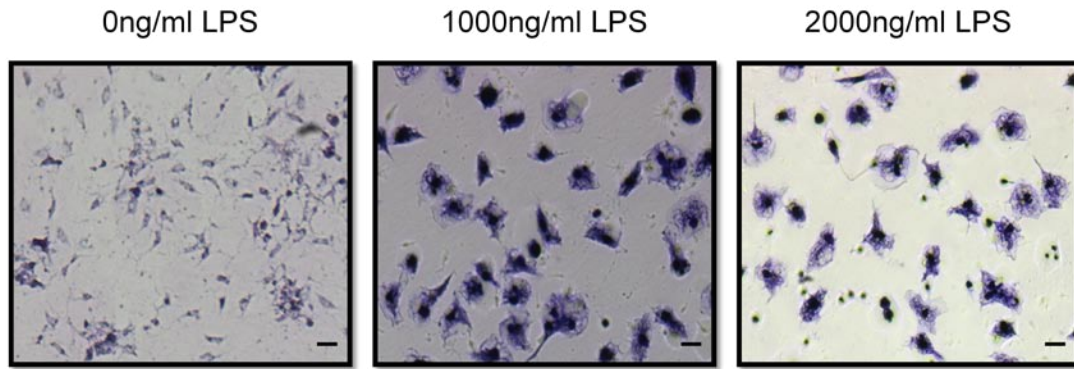


**Figure 6:** FACS analysis of J774A.1 cells assayed for M1 (CD86) expression 24 hours post treatment. IL-10 is able to reduce LPS induced CD86 expression, verifying my quantitative computational analysis of cells on coverslips.

increased M1 macrophage presence (Figure 5C). When applied alone, inflammatory stimuli LPS shifted the ratio towards that of an M1 phenotype (ratio of 1.5), while IL-10 shifted the ratio towards that of an M2c phenotype (ratio of 0.4), when co-applied, IL-10 decreased the ratio from 1.5 to 0.8, shifting it from an M1 to M2c phenotype, overriding the effects of LPS.

Subsequently, to verify the computed quantification of fluorescence, flow cytometry of similarly treated LPS and IL-10 treated J774a.1 cells

was run to measure CD86 fluorescence (Figure 6). Fluorescence activated cell sorting (FACS) analysis revealed that LPS upregulated CD86 expression while IL-10 does not. Additionally, IL-10 abrogated LPS induced CD86 expression. These quantitative measurements confirm that IL-10 reduces expression of inflammatory markers, thus I was able to i) use IL-10 as an anti-inflammatory agent against inflammatory stimuli such as LPS, ii) IL-10 not only decreases CD86 expression, but alters the ratio of M2c to M1 macrophages when co-applied with LPS and iii) create inflammatory and anti-inflammatory macrophages with the J774a.1 cell line *in vitro*. Additionally, these results also help to address several issues that may have arisen due to the nature of the cell line or the inflammatory stimuli. J774a.1 cells are known to secrete low levels

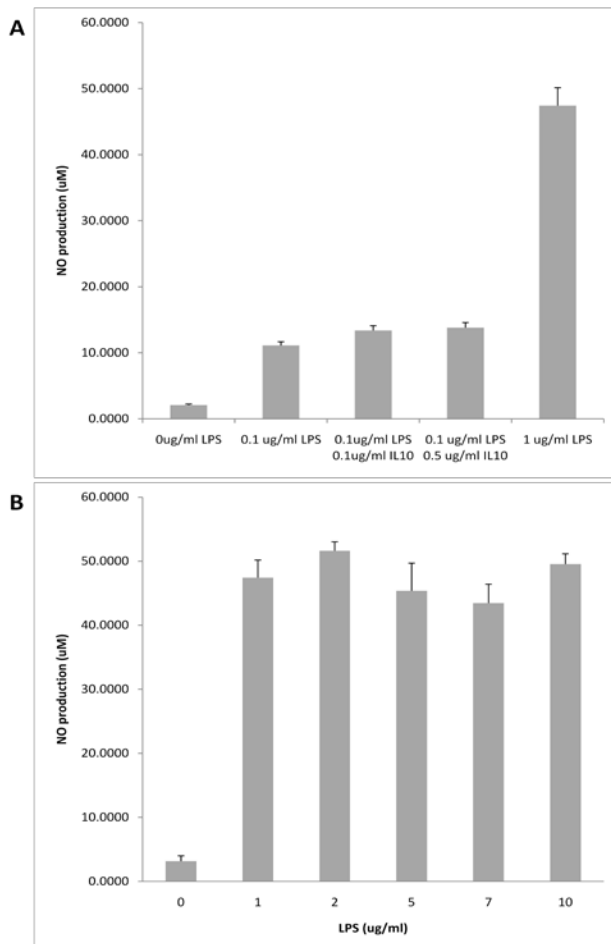


**Figure 7:** Wright's stain of LPS activated primary chicken monocytes. Activated macrophages increase in size and vesicularity. Images captured with 20X objective, scale bar represents 20  $\mu\text{m}$ .

of IL-1 $\beta$  [12]; IL-1 $\beta$  is traditionally thought of as a proinflammatory cytokine, but is now known to be a M2b macrophage stimulator. However, the J774a.1 cell line responded to the pro- and anti-inflammatory stimuli, thus macrophage activation due to autocrine signaling caused by low level secretions of IL-1 $\beta$  does not seem to influence the results. Additionally, LPS stimulates the secretion of inflammatory cytokines as well as IL-10. However, IL-10 secretions are not high enough to function similarly to that of the exogenous IL-10, which suppresses the LPS mediated inflammatory phenotype.

In addition to changes in cell surface marker expression (CD86, CD163), classically activated monocytes developing into macrophages also experience a change in morphology, specifically cell shape and size. Unactivated J774a.1s are round in shape with minimal and small projections, and are small in size ( $\sim 10\ \mu\text{m}$  in diameter), while cells activated with LPS increase in size and exhibit increased vesicularity (Figure 3). These changes are similar to the changes exhibited by primary monocytes. In Figure 7, isolated primary chicken monocytes were isolated from venous blood and treated with different doses of LPS: 0 ng/ml, 1000 ng/ml and 2000ng/ml. Activated monocytes were then stained with Wright's stain to visualize changes in cell

morphology. The primary cells displayed a similar increase in size (~10  $\mu\text{m}$  to ~100  $\mu\text{m}$ , roughly a 10 fold increase in size) and vesicularity upon exposure to *E. coli* LPS, further validating the authenticity of cell line, J774a.1's reaction.



**Figure 8:** Concentration of nitric oxide in the media of J774a.1 cells treated with different concentrations of LPS and IL-10 for 24 hours. **A.** Dose dependent increase in NO secretion observed. IL-10 does not decrease LPS induced NO production. **B.** LPS induced NO secretion plateaus after 1  $\mu\text{g/ml}$  of LPS.

Upon LPS stimulation, activated macrophages produce reactive nitrogen intermediates such as nitric oxide (NO) via inducible nitric oxide synthase (iNOS) [13, 14]. iNOS induction results in the secretion of NO, which at high concentrations can cause severe damage to foreign pathogens as well as host tissue [15]. To determine if J774a.1 cells secrete NO, J774a.1s were stimulated with increasing doses of LPS for 24 hours, and the media was assayed using Griess reagents. The Griess assay allows for the photometric detection of nitrite. As NO is unstable at physiological conditions and often oxidizes to a mixture of nitrite and nitrate, nitrate is reduced to nitrite enzymatically via

nitrate reductase so that the total amount of nitrite can be measured via the Griess assay [16-18].

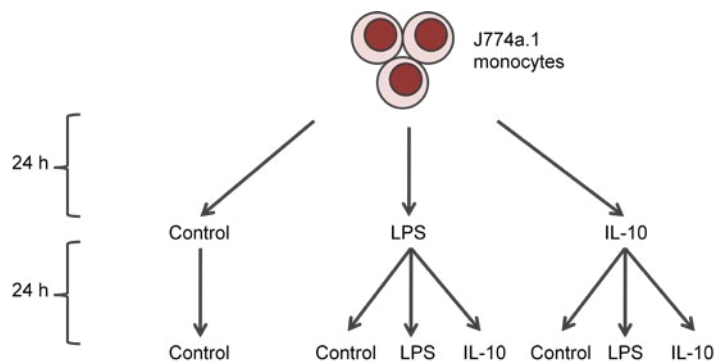
I found a dose dependent increase in NO secretion from 0 to 1  $\mu\text{g/ml}$  of LPS (Figure 8A), and

insignificant differences in NO secretion at dosages greater than 1 µg/ml of LPS (Figure 8B), hence I chose to activate the monocytes at a commonly used dosage of 0.1 µg/ml of LPS. Interestingly, equal (0.1 µg/ml) or greater (0.5 µg/ml) dosing of IL-10 did not diminish NO secretions when co treated with 0.1 µg/ml of LPS (Figure 8A). This is in contrast to the decrease in CD86 expression as seen with the flow cytometry results seen in Figure 6. Though IL-10 does not decrease NO secretions from LPS activated monocytes, this does not inhibit IL-10's ability to abrogate other markers of LPS induced inflammation in macrophages.

### 2.3.2 Reversal of activated macrophage phenotypes.

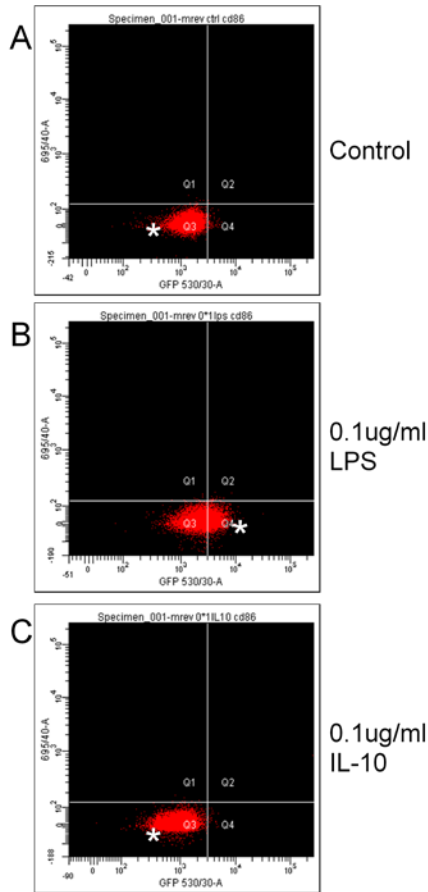
Monocytes respond to chemical cues, resulting in extravasation from the blood vessel to site of injury, developing into M1, M2a, M2b or M2c macrophages depending on the cues they encounter. As the persistence of M1 macrophages can delay the regenerative process, it would be beneficial if M2c macrophage development could be encouraged early on, while decreasing but not removing the presence of M1 macrophages.

As demonstrated in *in vitro* experiments in the section before, IL-10 could serve as a



**Figure 9:** Experimental setup to determine extent of macrophage plasticity. Cells were treated with 100ng/ml of LPS, or 100ng/ml of IL-10, and analysis was conducted via flow cytometry.

potential therapy for skewing the M2c:M1 macrophage ratio at the wound site if delivered locally. However, while this is a promising idea, it would be moot if IL-10 had to be co-delivered together with the insult, thus limiting the scope of this therapy to situations such as

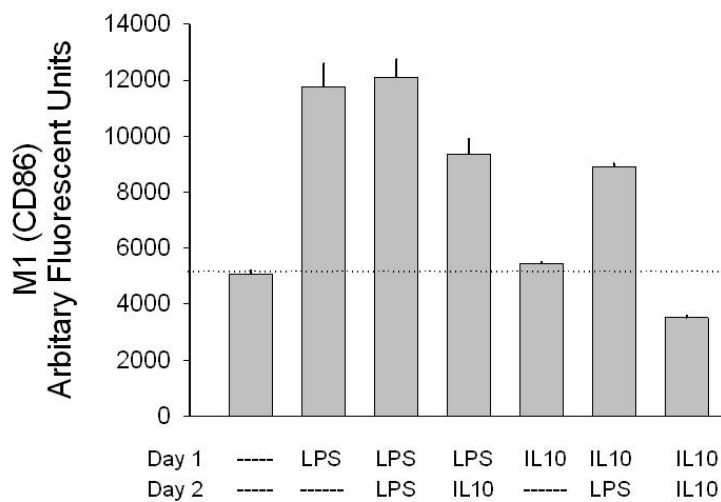


**Figure 10:** CD86-FITC profile of J774a.1 cells. **A.** Control group. **B.** LPS treated cells. **C.** IL-10 treated cells. Cells were isolated from the quadrants marked with an asterisk for each treatment group.

implants and surgery, and eliminating situations such as infections and trauma. It would be beneficial if IL-10 could be introduced as an anti-inflammatory therapy post infection, or post trauma, that would affect the differentiation of new monocytes and influence the phenotype of differentiated macrophages. For this idea to be successful, differentiated macrophages must be dynamic and responsive to IL-10 treatment post LPS stimulation.

To determine the plasticity of macrophage phenotype, I carried out the following experiment (Figure 9): J774a.1 monocytes were divided into 3 treatment groups: control, 100ng/ml of LPS or 100ng/ml of IL-10 for 24 hours to activate them towards the M1 or M2c pathway. After 24 hours of treatment, FACS analysis using a CD86-FITC antibody (M1

marker) is used to verify that the monocytes have been activated. Within the IL-10 group, cells that are CD86<sup>low</sup> are sorted into 3 equal and separate groups for another 24 hours of treatment (Figure 10A, 10C), while CD86<sup>high</sup> cells from the LPS group are likewise, sorted into 3 equal and separate groups (Figure 10B). Each group of 3 receives the same treatment for another 24 hours: control, 100ng/ml LPS or 100ng/ml IL-10. Subsequently, the cells are prepared for FACS analysis using a CD86-APC antibody to identify new CD86 expression. Thus, I was able to investigate if activated macrophages would still be able to respond to a changing environment.



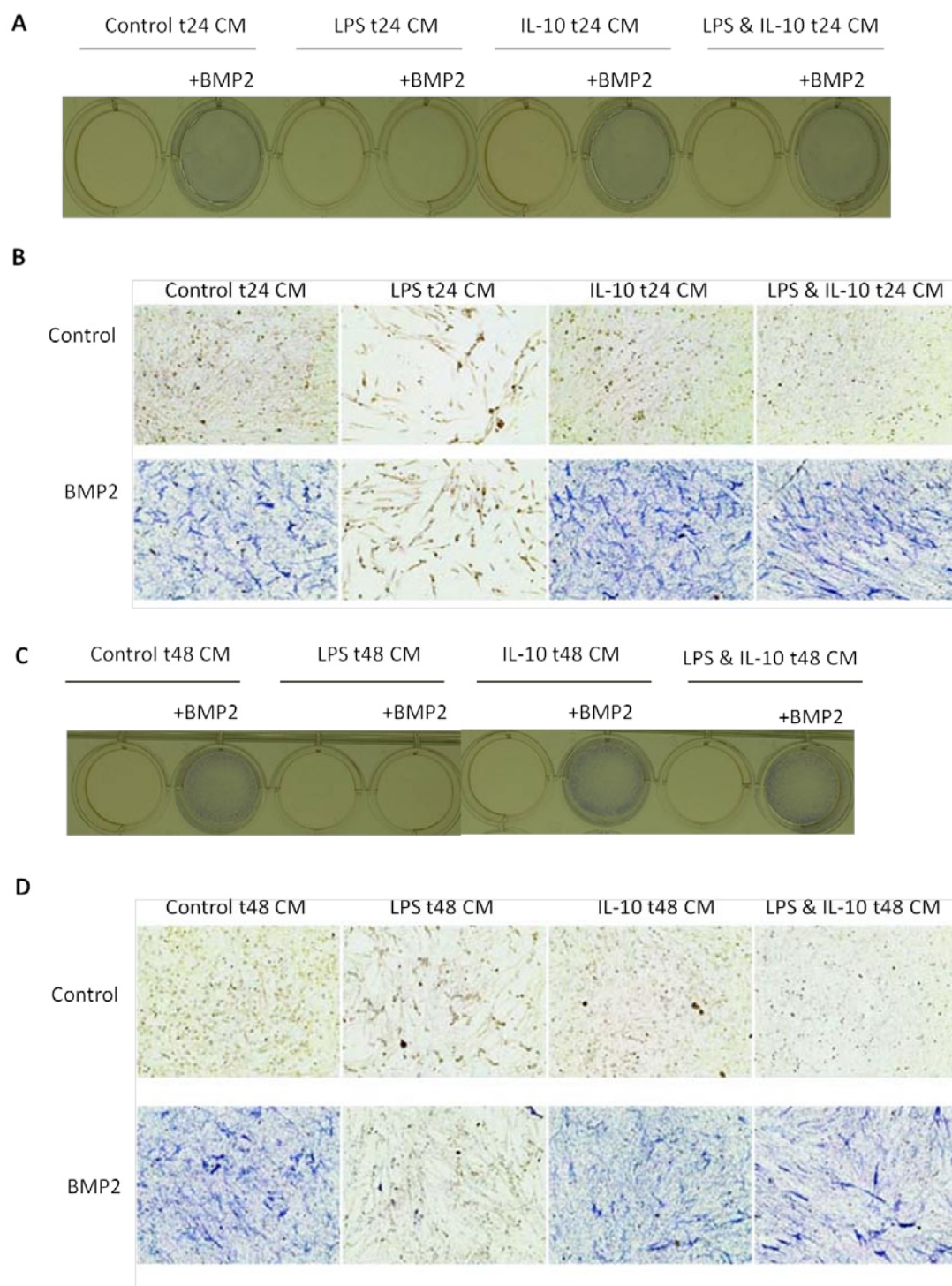
**Figure 11:** Plasticity of M1 phenotype. J774a.1 cells were cultured for 24 hours in either control media, 100 ng/ml LPS or 100 ng/ml IL-10. Using FACs, cells positive for CD86-FITC were isolated from each treatment group and then recultured for another 24 hr as indicated for control media, LPS or IL-10. Using CD86-APC flow, under control conditions the phenotype did not change, LPS did not further enhance M1, while IL-10 reversed M1 expression. LPS reversed the inhibition by IL10 whereas continued IL-10 further reversed M1.

LPS stimulated CD86<sup>high</sup> cells maintain their CD86<sup>high</sup> phenotype with or without continued LPS stimulation, however a 24 hour treatment with IL-10 decreases CD86 expression. IL-10 stimulated CD86<sup>low</sup> cells continued to be CD86<sup>low</sup> without continued IL-10 stimulation, and expressed decreased amounts of CD86 with continued IL-10

stimulation. CD86<sup>low</sup> cells upregulated their CD86 expression upon exposure to LPS, similar to levels of IL-10 treated CD86<sup>high</sup> cells (Figure 11). This suggests that IL-10's effects linger despite its absence from the treatment, and continued IL-10 treatment over time is capable of greater suppression of inflammation. Hence, macrophages are able to respond to IL-10's anti-inflammatory effects despite prior LPS stimulation, indicative of the level of plasticity macrophage phenotype can exhibit.

### 2.3.3 Effects of conditioned media on stem cell differentiation

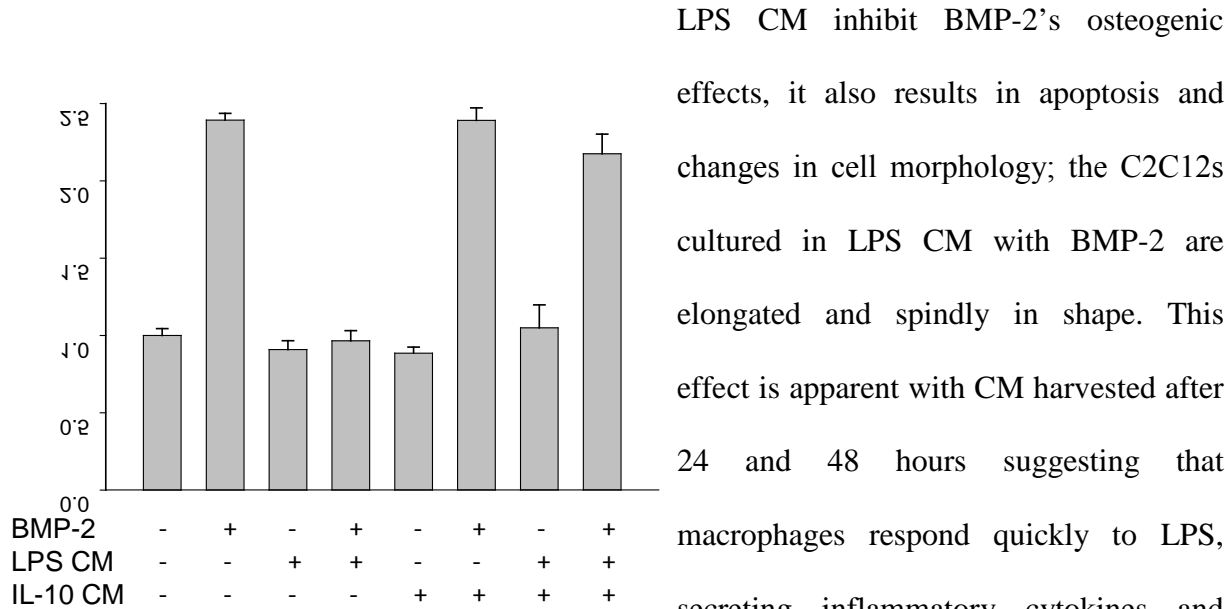
The ability to control macrophage phenotype bodes well for the potential use of IL-10 in a clinical setting. However, how would the ability to control macrophage phenotype affect BMP-



**Figure 12:** *In vitro* ALP activity of C2C12 cells exposed to J774a.1 cells 24 (t24)(A&B) and 48 (t48)(C&D) hour CM derived in the presence of control media, 100 ng/ml LPS, 100 ng/ml IL-10, or 100ng/ml of LPS and IL-10. Non-CM control and CMs were added in the presence of 100 ng/ml BMP-2 for 3 days then stained for ALP. Magnification-10x.

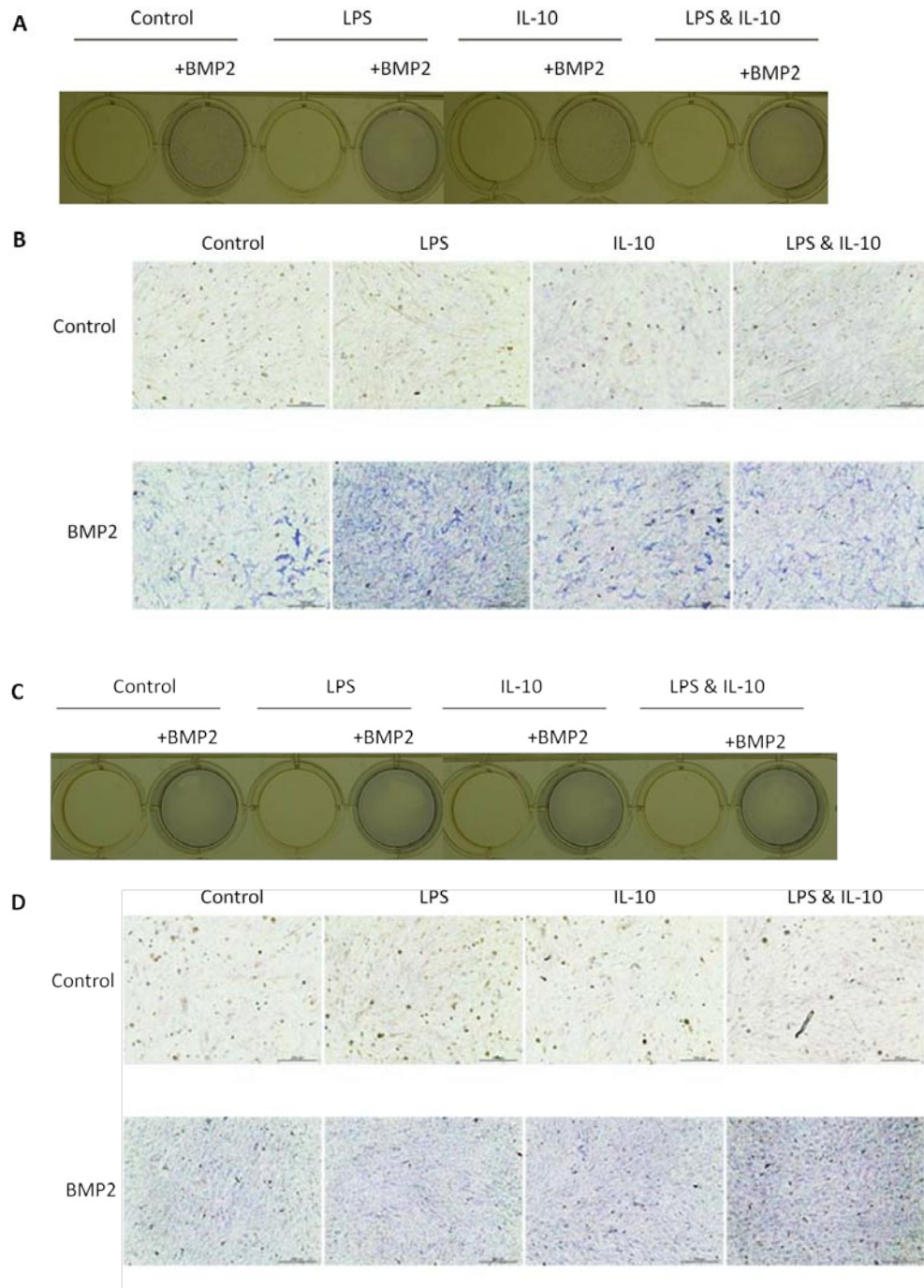
2 directed osteoblastogenesis? I cultured J774a.1 cells in the presence of control media, LPS, IL-10 or LPS and IL-10, and collected conditioned media (CM) at 24 or 48 hr. This CM was then incubated with C2C12 stem cells and with BMP-2 (100ng/ml), and stained for alkaline phosphatase after 3 days as a standard marker for osteoblast differentiation.

Figure 12 illustrates the effects of LPS CM on BMP-2 directed osteoblastogenesis; not only does



**Figure 13:** *In vitro* ALP activity of C2C12 cells exposed to macrophage 24 hr CM with and without BMP-2. CM derived from J774a.1 cells stimulated by control media, LPS or IL-10 was

LPS CM inhibit BMP-2’s osteogenic effects, it also results in apoptosis and changes in cell morphology; the C2C12s cultured in LPS CM with BMP-2 are elongated and spindly in shape. This effect is apparent with CM harvested after 24 and 48 hours suggesting that macrophages respond quickly to LPS, secreting inflammatory cytokines and other cues that will not only prevent the recruitment of progenitor cells, but will



**Figure 14:** Brightfield images of *in vitro* ALP activity of C2C12 cells exposed to 100 ng/ml LPS, 100 ng/ml IL-10, or 100ng/ml of LPS and IL-10 in the presence/absence of 100 ng/ml BMP-2 for 3 days then stained for ALP. A & B. Experiment carried out in RPMI1640. C & D. Experiment carried out DMEM. Direct treatment of LPS and IL-10 does not affect BMP-2 directed osteoblastogenesis. Magnification - 10x.

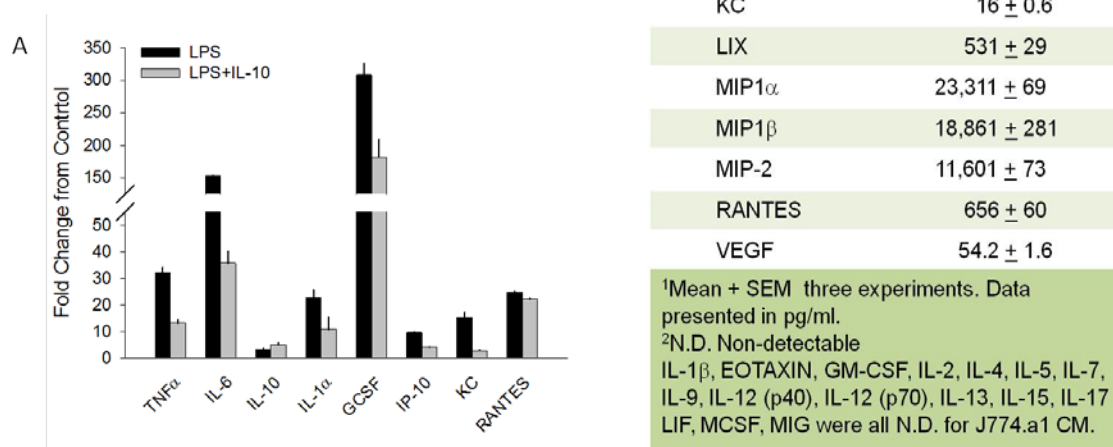
qualitative estimates and revealing that LPS CM inhibits ALP expression ~2.5 times as compared to control 24 hour CM (Figure 13).

As IL-10 and LPS were not removed from the harvested CM, I needed to determine if the apoptotic and anti-differentiative effects seen on the C2C12 cells were due to the secretions of macrophages after encountering LPS and/or IL-10 or the interactions of residual LPS and IL-10 in the conditioned media with the C2C12 progenitor cells themselves. To determine the effect of LPS and IL-10 on BMP-2's osteogenic effects on C2C12s, I treated the progenitor cells with LPS and/or IL-10 directly (i.e. no conditioned media) in RPMI (same media as the conditioned media) and found LPS had no direct effect on BMP-2 directed osteoblastogenesis as evidenced by the strong ALP staining in all cell wells treated with BMP-2 (Figure 14A & B). C2C12 cells are cultured in DMEM, and to ensure that the change in media did not affect its ability to respond to BMP-2, the same experiment was replicated in DMEM. LPS also had no direct effect on C2C12s cultured in DMEM (Figure 14C & D). Additionally, I also determined that direct treatments using IL-10 with LPS CM and BMP-2 was unable to rescue ALP expression in C2C12s, suggesting that IL-10's anti-inflammatory effects primarily target the macrophage secretory pathways (data not shown). Thus these experiments validate the effects observed with LPS CM and LPS and IL-10 CM. The lack of ALP staining is due to the secretions of the activated macrophages and not LPS and IL-10 directly, so it is important not to discount the immune system and its ability to influence the wound environment from the start, and its potential to delay the regenerative process.

#### **2.3.4 Conditioned media analysis**

To determine the secretory products of activated macrophages, conditioned media at 24

**Figure 15:** Partial analysis of 48 hour J774a.1 conditioned media components. Using the Milliplex MAP cytokine/chemokine assay, control, LPS, LPS and IL-10 conditioned media harvested from J774a.1 were analyzed for concentrations of cytokines involved in inflammation. **A.** Graph illustrating the cytokines with significant fold changes in expression in LPS CM over control CM. **B.** Baseline cytokine concentration in 48 hour J774a.1 conditioned media.



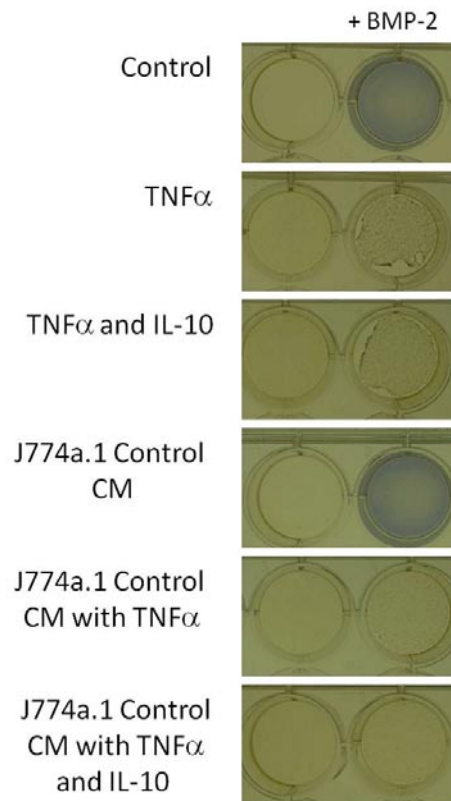
and 48 hours were harvested and 32 different cytokines involved in inflammation were analyzed using the Milliplex MAP mouse cytokine/chemokine panel. Results from the 24 hour conditioned media were similar to that of the 48 hour conditioned media; hence I have presented the data for the 48 hour conditioned media in Figure 15.

Control and IL-10 CM did not result in increased secretion of these cytokines. LPS stimulation resulted in a significant increase in secreted tumor necrosis factor alpha (TNF- $\alpha$ ), IL-6, IL-10, IL-1 $\alpha$ , granulocyte colony stimulating factor (G-CSF), interferon gamma induced protein 10 (IP-10), keratinocyte chemoattractant (KC) and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), while IL-10 and LPS CM resulted in significant

decreases as compared to LPS CM in the above mentioned cytokines and chemokines except for IL-10. This can be attributed to the residual IL-10 present in the CM because of the treatment.

TNF- $\alpha$  expression is one of the components of CM that is significantly affected by IL-10's addition to LPS. LPS CM is ~30 fold higher in TNF- $\alpha$  expression than control CM, while IL-10 and LPS CM resulted in a ~10 fold increase over control CM, i.e., a 2 fold decrease as compared to LPS CM. This significant decrease in TNF- $\alpha$  expression could contribute to the increase in ALP expression when C2C12s are cultured in LPS and IL-10 CM. TNF- $\alpha$  is a pleiotropic cytokine that stimulates the acute phase reaction involved in an inflammatory response. TNF- $\alpha$  is involved in apoptosis [19], increases or inhibits tumorigenesis depending on the situation [20], inhibits viral replication *in vitro*, and regulates immune cells [21], yet increased TNF- $\alpha$  protein levels *in vivo* have been linked to increased viral levels and myocardial lesions [22].

To determine if TNF- $\alpha$  inhibited BMP-2's osteogenic effects, I added TNF- $\alpha$  and BMP-2 directly to C2C12s and stained for ALP after 3 days of treatment (Figure 16). I saw that TNF- $\alpha$



**Figure 16:** TNF- $\alpha$  inhibits BMP-2's osteogenic effects on C2C12. C2C12s were treated with or without TNF- $\alpha$  (10 ng/ml), BMP-2 (100 ng/ml), IL-10 (100 ng/ml) and J774a.1 t24 CM for 3 days prior to ALP staining. IL-10 is unable to rescue TNF- $\alpha$ 's inhibition of BMP-2 induced osteogenesis.

inhibited BMP-2

induced ALP

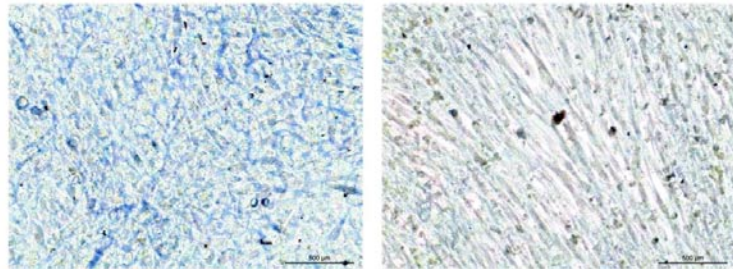
expression in regular  
media and in 24 hour

J774a.1 control CM.

IL-10 was unable to  
rescue the phenotype,

as in the results obtained with IL-10 and LPS CM.

J774a.1 CM  
with BMP-2



**Figure 17:** ALP stained 10X Images of C2C12s treated with TNF- $\alpha$ . TNF- $\alpha$  inhibits differentiation but does not induce apoptosis. Scale bar represents 500  $\mu$ m.

A closer look revealed that TNF- $\alpha$  inhibited differentiation of C2C12s but did not induce apoptosis (Figure 17). While this is contradictory to the results (TNF- $\alpha$  induced apoptosis) obtained by Thammasitboon *et. al.* [23], my target cell type (C2C12) differed from theirs (MC3T3), hence my results are not directly comparable. Thus, I maintain that TNF- $\alpha$  accounts for the inhibition of osteoblastogenesis but not the apoptosis seen in LPS CM treated C2C12s, thus other components of CM are responsible for the cell death observed in LPS CM treated C2C12s.

### 2.3.5 Issues with tissue engineered constructs and macrophage activation

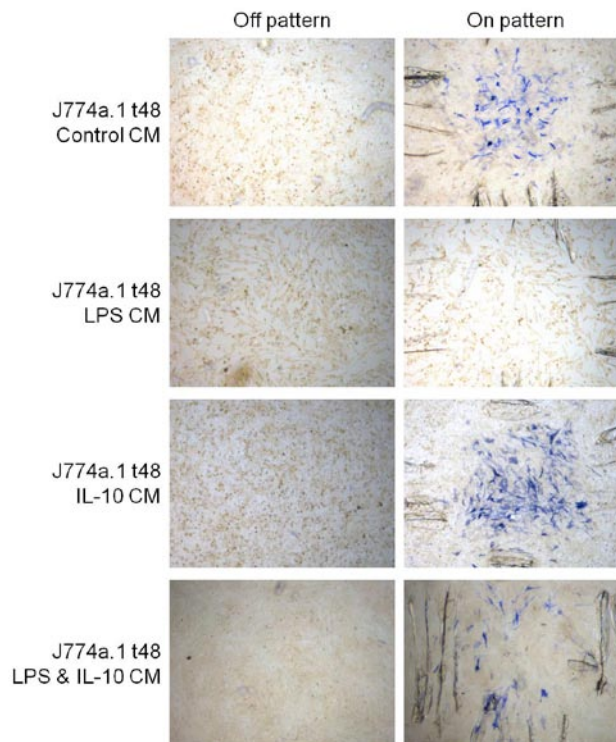
#### 2.3.5.a Immobilized BMP-2 and J774a.1 conditioned media

Previous sections described experiments where BMP-2 was added to the culture media (liquid phase experiments), however, many implants strive to immobilize BMP-2 to provide continuous osteogenic signaling, avoiding issues of soluble BMP-2 diffusing away from the construct, and high and non-physiological concentrations of BMP-2 that can cause unwanted side effects such as exuberant or ectopic bone formation [24-26]. Such implants may employ

different immobilization techniques, including covalently linking the growth factor to the construct [27], or taking advantage of the heparin present in the engineered tissue construct and the heparin binding sites of the growth factor [28].

Immobilized BMP-2 on DermaMatrix implanted into a 5mm cavarial defect induced bone formation within 2 weeks [29],

however, if that same tissue implant were placed into an inflammatory environment, the implant might not be as successful, and the initiation of the regenerative process might take much longer. To determine how successfully such implants will fare in different environments, I used a bio-ink jet printer designed by Phil Campbell and Lee Weiss to create a biopattern of BMP-2 atop fibrin coated glass coverslips. C2C12s were cultured on these biopatterned coverslips, then cultured in J774a.1 CM for 3 days prior to evaluation by ALP staining (solid phase

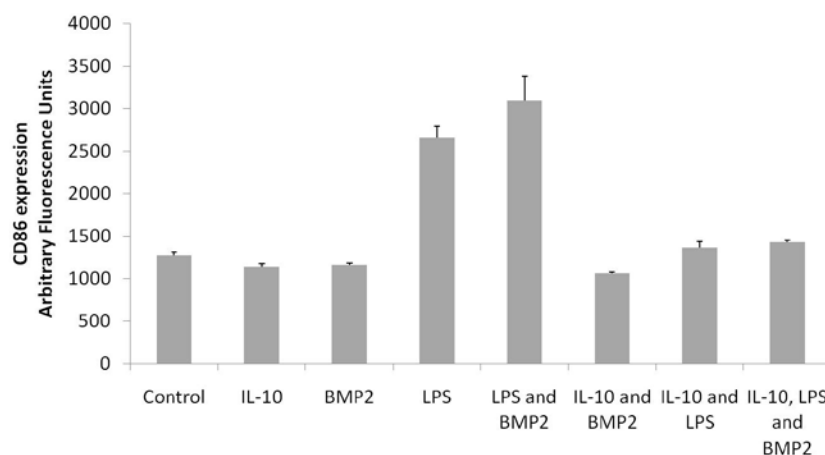


**Figure 18:** Brightfield 4X images of C2C12s on/off immobilized BMP-2, cultured in J774a.1 48 hour CM. LPS CM prevents BMP-2 osteogenesis while IL-10 rescues ALP expression when cotreated with LPS.

experiments). I found that the results from the solid phase experiments mimicked that of the liquid phase experiments. C2C12 cells lacked ALP staining on the BMP-2 patterns in LPS CM, but not on IL-10 and LPS CM (Figure 18). Thus, implants placed in an inflammatory environment could result in delayed or even complete inhibition of the regenerative process.

### 2.3.5.b Macrophage interaction with BMP-2

Use of IL-10 to dampen the effects of LPS, thus creating an anti-inflammatory environment is critical to the regenerative process, however, if the monocytes were classically activated down the inflammatory pathway by a growth factor present in the tissue engineered implant (e.g. BMP-2), that would further delay the regenerative process. I treated J774a.1 cells with BMP-2 for 24 hours, then assayed CD86 (M1 marker) expression via flow cytometry to



**Figure 19:** Flow cytometry analysis of CD86 expression of J774a.1 cells exposed to either 100ng/ml IL-10, and/or 100ng/ml LPS and/or 100ng/ml of BMP-2. BMP-2 does not activate increase CD86 expression of J774a.1. Increase seen with LPS and BMP-2 treatment is not significantly different from the increase activation due to LPS treatment.

determine if BMP-2 could classically activate macrophages.

Expression of CD86 in J774a.1 cells did not increase when they were treated with

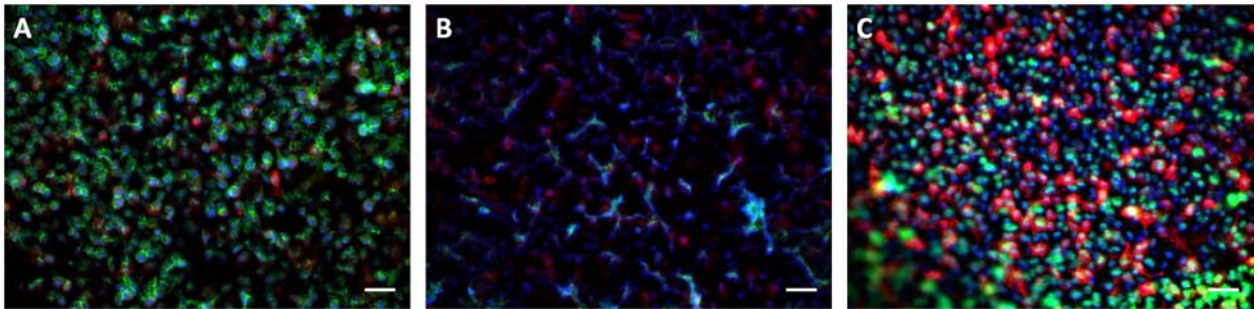
BMP-2; in fact the expression level was close to that of the control group and IL-

10 stimulated group. BMP-2 did enhance CD86 expression significantly when coupled with LPS or IL-10 treatment. BMP-2 did not inhibit IL-10 from abrogating LPS induced CD86 expression (Figure 19). Thus, BMP-2 does not activate monocytes down the inflammatory pathway towards the M1 phenotype.

### 2.3.6 Macrophage activation *in vivo*

*In vitro* experiments are isolated, dealing with one cell type at a time and 1 or 2 stimulants in each experiment, while *in vivo* processes are more complex due to the interplay of multiple cell types, from resident dendritic cells in the skin (Langerhans cells) to early responding neutrophils and together with stimulation from degranulating neutrophils, or surrounding stroma as well as implanted cytokines [30].

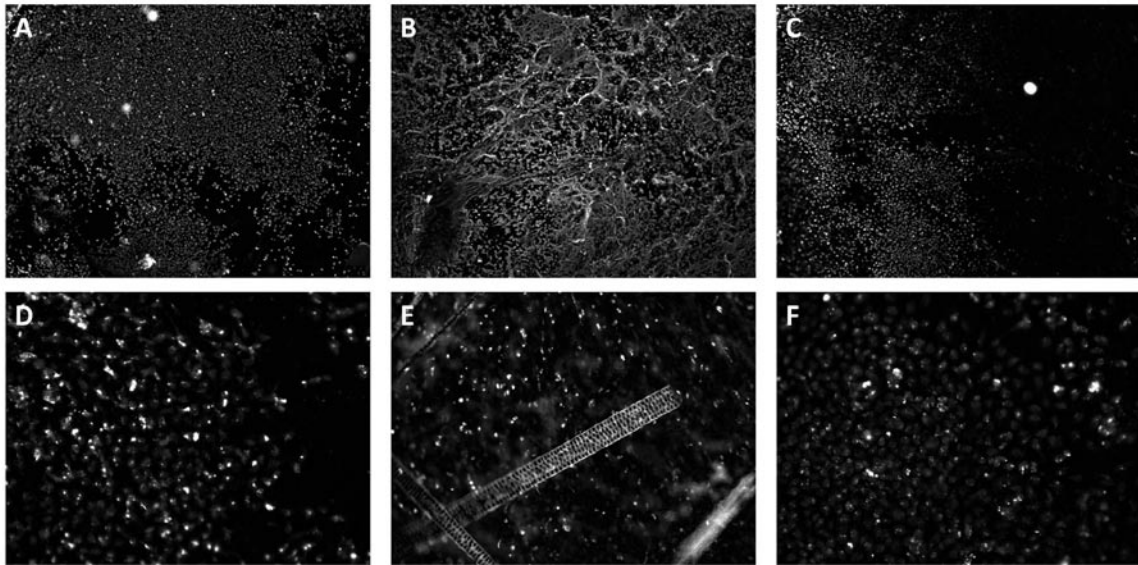
Having controlled LPS-induced macrophage activation *in vitro* with IL-10, I shifted my focus to the control of macrophage phenotype *in vivo*, to determine if my *in vitro* data could be replicated *in vivo* using C57BL/6 mice. 3 different types of 5mm nitrocellulose discs, control, IL-10 and IL-1 $\beta$  were implanted subcutaneously in mice and harvested 48 hours later and stained for nuclei, CD86 (M1 marker), CD163 (M2C marker), and F4/80 (macrophage marker). I



**Figure 20:** Immunofluorescent staining of 5mm bioprinted nitrocellulose discs implanted subcutaneously in mice. Discs were harvested 48 hours post implantation. **A.** Control nitrocellulose. **B.** IL-1 $\beta$  on nitrocellulose. **C.** IL-10 on nitrocellulose. Pseudocolored green, F4/80 indicative of macrophages, pseudocolored blue, nuclei, and pseudocolored red, CD163 (M2C). Scale bar represents 100  $\mu$ m.

switched to the IL-1 $\beta$  as it was not permissive to use LPS *in vivo* under the approved IACUC protocol. IL-1 $\beta$  is thought of traditionally as a pro-inflammatory cytokine, and was expected to behave similarly to LPS.

Nitrocellulose discs by themselves attract a large number of macrophages as evidenced by the F4/80 staining, and a large number of these macrophage express weak CD163 expression (Figure 20A). In the IL-1 $\beta$  printed discs, there is a small number of macrophages (lack of F4/80 staining within cellular boundaries), and CD163 staining is random and not confined to cell boundaries. Additionally, DAPI nuclear staining demarcating nuclei was irregular and the DAPI



**Figure 21:** Immunofluorescent staining of 5mm bioprinted nitrocellulose discs implanted subcutaneously in mice. Discs were harvested 48 hours post implantation. **A.** DAPI stain of control nitrocellulose disc. **B.** DAPI stain of IL-1 $\beta$  on nitrocellulose disc. Staining pattern is similar to neutrophil extracellular traps **C.** DAPI stain on nitrocellulose disc. Imaged at 5X **D.** CD86 staining of control nitrocellulose disc. **E.** CD86 staining of IL-1 $\beta$  nitrocellulose disc. **F.** CD86 staining of IL-10 nitrocellulose disc. Images captured at 20X.

label showed long processes that appear to connect nuclei (Figure 20B). IL-10 treated nitrocellulose discs displayed numerous CD68+ cells with strong CD163 staining as compared to the control, indicating an increase M2C macrophages (Figure 20C).

An overview of the nitrocellulose discs at a 5X magnification confirmed the difference in surface topology (Figure 21 A, B & C). DAPI or 4', 6-diamidino-2-phenylindole is a fluorescent stain that strongly binds to the A-T rich regions of DNA. In live cells, this staining appears as an

intact round shape, indicative of the nuclei, however IL-1 $\beta$  printed nitrocellulose discs is a mix of round shapes as well as long fibrous structures that are absent from IL-10 and control nitrocellulose discs. As a result, it was difficult to obtain CD86 staining for IL-1 $\beta$  as compared to the control and IL-10 printed nitrocellulose discs. However, CD86 staining for control and IL-10 printed nitrocellulose discs showed similar levels of fluorescence, indicating that IL-10 does not increase CD86 expression over the CD86 expression stimulated by nitrocellulose material (Figure 21 D, E & F).

## **2.4 Discussion**

Resident and recruited macrophages are said to be the major producer of chemokines at wound sites, and are thought to be the second immune cell type to show up after neutrophils, phagocytosing cell debris as well as foreign pathogens, while serving critical functions in tissue repair, including collagen degradation and the promotion of angiogenesis [31] while also presenting antigen to T helper cells [32]. I have shown that these macrophages can be activated towards an M1 phenotype is the presence of a stimulant such as LPS (representative of a bacterial infection) and that this M1 phenotype can be dampened in the presence of IL-10. Furthermore, the presence of IL-10 upregulates the M2c phenotype to speed up the process of remodeling and regeneration, shortening the healing process. Fortunately, IL-10 is also able to abrogate CD86 (M1 marker) expression of macrophages post LPS treatment, indicating that IL-10 therapies may be administered after the immunological insult and still achieve positive results.

Inflammatory macrophages also alter stem cell differentiation. Conditioned media collected from stimulated macrophages were inhibitory or permissive to BMP-2's osteogenic

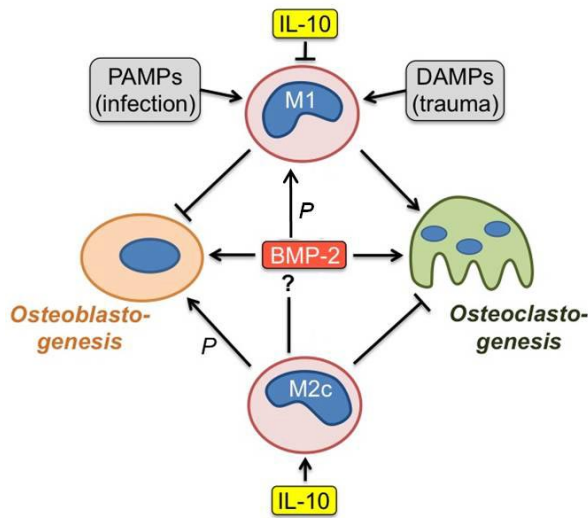
effects. LPS CM not only prevented ALP expression in C2C12s, it also resulted in morphological changes, a lack of cell proliferation and apoptosis. LPS CM is a simpler version of the inflammatory milieu that progenitor cells would experience when entering a wound site, hence if LPS CM alone is able to inhibit BMP-2 osteoblastogenesis, the extracellular environment at a wound site will be much more inhospitable to stem cell differentiation due to other factors such as neutrophilic degranulation, as well as chemokines secreted by neighboring stroma and other immune cells, such as dendritic cells. However, IL-10 & LPS CM rescues ALP expression, suggesting that locally immobilized IL-10 on implanted tissue engineered constructs might help to alter the local wound environment by minimizing secretion of proinflammatory cytokines, and reversing differentiated macrophages. It must be noted that IL-10's effects are focused on immune cells and not progenitor cells. It is most unlikely that progenitor cells will encounter proinflammatory cytokines without any immune cells *in vivo*.

Analysis of the conditioned media revealed some of the components of conditioned media that inhibit BMP-2 osteoblastogenesis. However, this list is not comprehensive and the existence of other cytokines should be investigated. Nonetheless, the list of 32 cytokines provided some interesting leads. In addition to the secretion of TNF- $\alpha$  and IL-10 upon LPS stimulation, significant changes were also detected in the levels of secretion of IL-6, IL-1 $\alpha$ , GCSF, IP-10, KC and RANTES. IL-6 is able to act as an inflammatory and anti-inflammatory cytokine [33], and is secreted by T cells and macrophages in response to situations such as trauma, infections or burns. As a pro-inflammatory cytokine, osteoblasts secrete IL-6 to stimulate osteoclast development [34], yet as an anti-inflammatory cytokine, IL-6 is able to inhibit effects of TNF- $\alpha$ . It is important to investigate effects of IL-6 as its increased expression has been seen in patients with metastatic cancer [35], diabetes [36], atherosclerosis [37], and

rheumatoid arthritis [38]. IL-1 $\alpha$ , one of the 9 members of the IL-1 family, is proteolytically processed and released in response to injury, and is said to induce apoptosis in addition to its other functions (e.g. hematopoiesis) [39]. Additionally, IL-1 $\alpha$  is said to have a synergistic effect with TNF- $\alpha$  as well as inducing further secretion of TNF- $\alpha$  [40]. Release of IL-1 $\alpha$  in response to LPS stimulation may be responsible for the apoptotic effect observed with LPS CM cultured C2C12s. GCSF, thought of as a proinflammatory cytokine, stimulates the maturation of granulocytes from the bone marrow [41], and results in the rapid mobilization of neutrophils in response to tissue insult [42, 43]. IP-10 plays multiple roles; as a chemoattractant for macrophages, dendritic cells, natural killer cells and T cells, promoter of T cell adhesion, as well as of angiogenesis and antitumoral activity [44, 45]. KC, also known as IL-8, is a chemoattractant for neutrophils, macrophages and other leukocytes. Expression of IL-8 can cause rolling monocytes to adhere to vascular endothelium, resulting in extravasation and migration to the site of injury [46]. Last but not least, RANTES, is a chemoattractant for monocytes and memory T cells [47]. All of these secreted cytokines and chemoattractants serve to prolong the inflammatory reaction by either increasing secretions of pro-inflammatory mediators or attracting more neutrophils, macrophages and other immune cells. The influx of new immune cells would then be subjected to the barrage of pro-inflammatory cytokines that would then continue this inflammatory state, which could ultimately lead to chronic inflammation.

*In vivo* experiments revealed that the M2c phenotype could be upregulated locally at the implant site by IL-10, however, what was most interesting was the presence of the fibrous DAPI stained structures seen on the surface of IL-1 $\beta$  nitrocellulose discs. These fibrous structures are reminiscent of neutrophil extracellular traps (NETS). Neutrophils employ various methods to kill

foreign pathogens(e.g. LPS): degranulation, resulting in the secretion of antimicrobials, engulfment of pathogens and the formation of NETS [48]. To form NETS, neutrophils must degranulate and release chromatin to form an extracellular fibril matrix. NETS are networks of extracellular fibers, primarily composed of neutrophil DNA, that ensnare and bind pathogens while minimizing toxicity to the host [49]. The formation of neutrophil NETS makes it more



**Figure 22:** Relationships between M1, M2c, BMP-2 and its effects on osteoblastogenesis. *P* indicates a permissive but not necessary direct promotion. Lines ending in ↑ indicate stimulation; ⊥ indicates inhibition; and ? indicates regulation is currently unknown. PAMPs – Pathogen associated molecular patterns, DAMPS- Damage associated molecular patterns.

complicated to evaluate the type of cell present on the surface of the implant, thus other methods of evaluation must be explored to determine cell phenotype. Additionally, further studies should be done to determine if local inflammation can be overcome with immobilized IL-10 on tissue engineered constructs, and if this would permit osteoblastogenesis via BMP-2 despite an inflammatory environment.

My experiments have demonstrated that under inflammatory conditions, activated macrophages are able to influence BMP-2

induced osteoblastogenesis due to the pro-inflammatory chemokines secreted. However, this influence can be curtailed with the use of interleukin 10. Thus, IL-10 provides new avenues for treatments of situations involving chronic and acute inflammation. As bone remodeling is a balance between bone formation (osteoblastogenesis) and bone destruction (osteoclastogenesis),

it is critical to assess the contribution and determine the role that the immune system plays when one side is favored over the other.

These set of experiments determined the crosstalk between macrophages, BMP-2 and stem cells (Figure 22). PAMPS, such as LPS, encourage M1 formation, while IL-10 is able to inhibit M1 formation encouraging M2c formation. BMP-2 is does not encourage or inhibit M1 formation, but we have yet to determine formally BMP-2's effect on M2c formation. Lastly, the secretions from classic inflammatory M1s inhibit BMP-2 mediated osteoblastogenesis and M2c is permissive to BMP-2's osteogenic actions. Thus, my thesis has helped to connect and define the crosstalk that occurs between macrophages, BMP-2, and stem cells on the flip side of osteoimmunology.

## **2.5 Acknowledgements**

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## Chapter 3: Crosstalk between dendritic cells, BMP-2 and stem cells in inflammatory and non-inflammatory environments.

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### **3.1 Introduction**

The extracellular environment during inflammation is established by the cells of the innate immune system, including neutrophils, macrophages, dendritic cells and natural killer cells. Implants placed in such inflammatory situations, especially implants infused with BMP-2 may experience a delay in the efficacy of BMP-2 due to the acute or chronic inflammatory milieu. In this chapter, I focus on the role that dendritic cells play in inflammation, and how it affects BMP-2's osteogenic inducing action on C2C12s.

Inflammation is a critical part of the healing process. It arises in many situations, such as chemical burns, pathogenic insult, and surgically implanted tissue engineered constructs [8]. It involves cells from both the innate and acquired immune system from initially responding neutrophils to the later cytotoxic T cells. For example, surgically implanting tissue engineered constructs might result in the introduction of foreign pathogens. The recognition of foreign pathogens leads to the activation of the innate immune system, triggering a cascade of events that includes the secretion of pro-inflammatory cytokines and chemokines for pathogen toxicity and further immune activation. However, these inflammatory cytokines do not target the pathogen exclusively and often causes host tissue damage, thus exacerbating the problem. As undue and prolonged inflammation can severely damage surrounding host tissue, implant acceptance and remodeling will be further delayed. There are many ways to abrogate inflammation; one of them is to control the activation of the cells that play pivotal roles in the inflammatory process.

The dendritic cell is especially interesting because it connects the innate with acquired immune system and is capable of initiating a primary immune response. Dendritic cells are often discussed in their antigen presenting capacity. This is rightly so as the primary immune response relies on the efficient activation and priming of naïve T cells by dendritic cells [13-15]. However,

in addition to their primary role as antigen presenting cells, dendritic cells play a huge role in initiating the innate immune response, resulting in inflammation. Immature DCs are located strategically in external environment contact areas such as the skin (Langerhan cells), or the airway or the gastrointestinal mucosa where they are able to recognize foreign pathogens via pattern recognition receptors and phagocytize these foreign pathogens [16]. DCs continually sample their environment via macropinocytosis and receptor mediated endocytosis. Like macrophages and neutrophils, dendritic cells phagocytize pathogens but do not degrade antigens so completely which results in a conservation of antigenic peptides, hence an increased presentation on major histocompatibility complexes (MHC) to T cells. Common stimulants known to activate dendritic cell maturation include lipopolysaccharide and interferon gamma. Exposure to these stimulants results in maturation of the dendritic cell, marked by an increased expression of MHC molecules and the co-stimulatory molecules, CD80 and CD86 [17]. This multistep maturation process results in secretion of cytokines and chemokines that influence the microenvironment as well as attract the innate immune system's first responders, neutrophils and macrophages to the site of infection. Subsequently, dendritic cells migrate to the draining lymph nodes, where they activate T cells, the effectors of acquired immunity and immunological memory [18]. Thus, dendritic cells play a key role in the inflammatory process as they are able to recruit, activate and act as a bridge between the innate and acquired systems.

Pleiotropic cytokine IL-10 is increasingly used to modulate inflammation. Traditionally thought of as an anti-inflammatory mediator via the downregulation of proinflammatory cytokine production, MHC class II antigens and co-stimulatory molecules expression, IL-10 is also said to mediate inflammation, enhancing natural killer function, leading to pathogen destruction and increased antigen availability [9]. Nonetheless, studies show that IL-10 resulted

in reduced NF- $\kappa$ B in monocytes, T cells [10, 11] and dendritic cells. Bhattacharyya *et. al.* determined that pretreatment of IL-10 prior to LPS stimulation decreased NF- $\kappa$ B translocation in myeloid dendritic cells, suppressing dendritic cell activation, decreasing dendritic cell capability to activate T cells [12].

BMP-2 is most often regarded as an osteogenic growth factor. Infact, BMP-2's osteogenic effects on C2C12 cell cultures [1], and in *in vivo* settings such as, cavarial defects [2, 3] are well documented in the literature. BMP-2 is so successful that it is used as an osteogenic growth factor in implants within the human body [5]. However, BMP-2 is capable of inducing osteoblastogenesis as well as osteoclastogenesis; together with RANKL, BMP-2 supports osteoclast precursor differentiation and fusion [6]. RANKL (a member of the tumor necrosis factor family) expression, in turn, increases in the presence of inflammation [7]. Thus, during inflammatory situations, BMP-2's function switches from osteoblastogenesis to osteoclastogenesis, altering the original intentions of osteogenic implants, such as Medtronic's INFUSE® bone graft.

To study the crosstalk between dendritic cells, stem cells and BMP-2, I used fetal skin dendritic cells (FSDCs) as a representative cell line for dendritic cells and C2C12s as a representative stem cell line. FSDCs, isolated from fetal mouse skin, are cells of a myeloid lineage that display a dendritic morphology. They possess a macrophage/immature DC-like surface phenotype and priming capacity *in vivo*, but require further differentiation and activation signals to express their antigen presenting potential *in vitro* [19]. C2C12 is a mouse progenitor cell line that is predisposed to develop into muscle but is able to differentiate into bone under the influence of BMP-2 [20]. I determined whether IL-10 could potentially be used as an anti-inflammatory agent to improve a harsh extracellular environment caused by LPS mediated

dendritic cell activation, then I determined what products are secreted by dendritic cell in inflammatory and anti-inflammatory conditions and finally, I determined how the classical inflammatory activation of dendritic cells affected BMP-2's induction of C2C12 differentiation.

## **3.2 Methods and materials**

Escherichia coli lipopolysaccharide was purchased from Sigma (St Louis, MO). Bone morphogenetic protein 2 was purchased from Medtronic (Minneapolis, MN). Interleukin 10 (IL-10) was a gift from Dr. Xin Xiao Zheng. FITC-anti-CD86 was purchased from BD Pharmingen (San Diego, CA). Phosphate buffered saline was purchased from Fisher Scientific (Pittsburgh, PA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. All experiments were performed three times in triplicate unless noted otherwise to ensure statistical significance.

### **3.2.1 Cell culture**

FSDC cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 8mM Glutamax and 1% penicillin and streptomycin (complete culture media). Mouse C2C12 cells (ATTC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Media, 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were kept at 37°C, 5% CO<sub>2</sub> in a humidified incubator.

### **3.2.2 Flow cytometry**

FSDC cells were treated with LPS, IL-10, or LPS and IL-10 for 24 or 48 hours prior to cell surface marker analysis using a Becton Dickinson FACS DIVA. To prepare cells for FACS analysis, FSDCs were washed with phosphate buffered saline (Fisher Scientific, Pittsburgh, PA), then blocked with 1% BSA in PBS for 1 hour at 4 °C. Cells were washed in PBS and then

incubated with the CD86 antibody (1:250 dilution) for 1 hour at 4 °C. Subsequently, cells were prepared for flow cytometry analysis by washing and straining through a cell strainer to remove clumps.

### **3.2.3 Conditioned media experiments**

1 x 10<sup>6</sup> FSDC cells in tissue culture were treated using the following concentrations of biological response modifiers for 24 or 48 hours: 100 ng/ml LPS, 100 ng/ml IL-10, 100 ng/ml each of IL-10 and LPS, or pretreated with 10 µM of N-(3-(Aminomethyl)benzyl)acetamidine (1400W) (Cayman Chemical, Ann Arbor, Michigan). FSDCs were pretreated with 1400W 30 minutes prior to other treatments. Conditioned media was harvested 24 or 48 hours post treatment and kept frozen prior to use. 12x10<sup>4</sup> C2C12 cells were treated with regular or conditioned media, with or without 100 ng/ml of BMP-2, 25 µM of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) (Invitrogen, Carlsbad, CA) , and 200 µM of S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) (Invitrogen, Carlsbad, CA). SNAP, an NO donor, was added daily as it has a half life of 6 hours. The conditioned media was refreshed at day 2 of culture and cells were stained for alkaline phosphatase activity at day 4 of culture.

### **3.2.4 Immobilized LPS and IL-10 experiment**

5mm DermaMatrix (Synthes, West Chester, PA) discs were soaked in ethanol for minutes, followed by 3 five minute washes of PBS, then allowed to dry. 50 ng of LPS, 50 ng of IL-10 and 50 ng/ml of LPS and IL-10 were printed on each DermaMatrix disc and allowed to dry. Subsequently, discs were washed in PBS to remove non-adherent LPS and IL-10, then dried. FSDCs are then seeded at 1 x 10<sup>6</sup> cells per DermaMatrix disc and cultured in complete RPMI 1640 culture media. At 24 or 48 hours, the media is harvested to determine nitric oxide concentration using the Griess Assay.

### **3.2.5 Alkaline phosphatase staining**

Alkaline phosphatase activity (SIGMAFAST) was detected according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Brightfield images of the cells were captured using a 10X and 20X objective on a Leica DMIL LED microscope (Buffalo Grove, IL).

### **3.2.6 Quantification of alkaline phosphatase staining**

Quantification of alkaline phosphatase staining was done by blind spectral unmixing by non-negative matrix factorization. Simple linear unmixing is defined by:  $V = W \times H$ , where  $V$  is the source image, an  $(m \times n)$ -by- $c$  matrix (the number of colors,  $c$ , is 3 for RGB images),  $W$  is the color-bases matrix, a  $c$ -by- $r$  matrix (where  $r$  is the number of sources to be separated), and  $H$  is the unmixed image of the same size as the source image. Because of experimental variation, the spectra of immunocytochemical dyes are often not consistent across every image, making simple linear unmixing inappropriate. Thus, I used a method demonstrated by Newberg *et. al.* [21] that uses non-negative matrix factorization to blindly unmix the images. Briefly, NMF assumes that each stain contributes non-negatively to the overall image intensity. This method has been shown to be effective in unmixing brightfield images [21]. Blind spectral unmixing by NMF uses a different color matrix,  $W$ , for each image.  $H$  is randomly initialized and NMF is used to solve for the non-negative matrix factors  $W$  and  $H$  by iteratively minimizing the distance between  $V$  and  $W \times H$ . The post-processing of  $H$  into a new unmixed image was similar to that of linear unmixing, in which each channel was scaled and then remapped into the image data channels. The unmixed image was summed to determine the amount of ALP staining.

### **3.2.7 Griess assay for nitrate reductase**

Cell culture media was harvested from FSDCs cells treated with 100ng/ml of BMP-2, 100 ng/ml of LPS, 100ng/ml IL-10 and combinations of these stimulants for 24 and 48 hours

(conditioned media). The conditioned media were assayed for levels of nitric oxide following the manufacturer's instructions using a Griess assay (Biotium, CA), and read using a Tecan Safire<sup>2</sup> plate reader (Durham, NC).

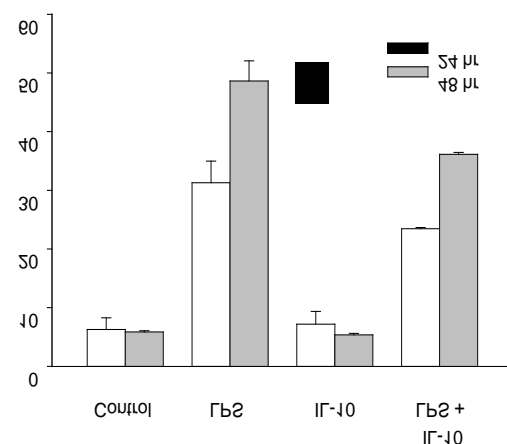
### 3.2.7 Milliplex/MAP cytokine/chemokine assay

Control, 100ng/ml LPS, 100ng/ml IL-10 and 100ng/ml of LPS and IL-10 conditioned media were harvested from FSDC cells after 24 or 48 hours. Secreted components were analyzed using the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, Billerica, MA) and run in accordance with the manufacturer's instructions.

## 3.3 Results

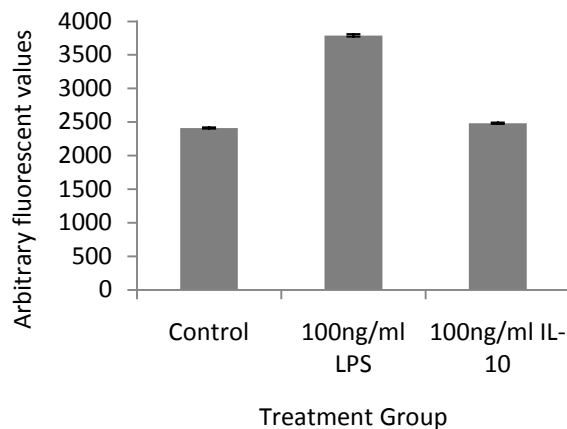
### 3.3.1 FSDC activation

To determine if I could recreate an inflammatory environment, I treated FSDCs with 100ng/ml of LPS and assayed the secretion of nitric oxide after 24 and 48 hours using the Griess assay. Nitric oxide is generated by phagocytes as part of the immune response. Additionally, I also co-treated the FSDCs with 100ng/ml of IL-10 and LPS to determine if IL-10 could abrogate LPS-induced inflammation. The results (Figure 1) indicate LPS induces a ~6.5 fold increase in NO secretion after 24 hours and a ~10 fold increase after 48 hours as compared to baseline. IL-10 does not increase



**Figure 1:** Measurement of nitric oxide production after exposure of FSDCs to LPS (100ng/ml) and IL-10 (100ng/ml) for 24 or 48 hours.

NO production at 24 or 48 hours but IL-10 does decrease LPS induced NO secretion significantly. Expression of nitric oxide at 24 hours after IL-10 and LPS treatment increased only ~5 fold, while expression at 48 hours increased only ~8 fold. Higher dosages of IL-10 (500ng/ml, 1000ng/ml) did not further decrease LPS (100ng/ml) induced NO secretion (data not shown). Nitric oxide secreted as part of an immune response is secreted as free radicals, which can cause DNA damage, and bacterial toxicity [22]. This IL-10 mediated decrease in NO secretion dampens but does not eliminate inflammation. Additionally, the data suggest that IL-10



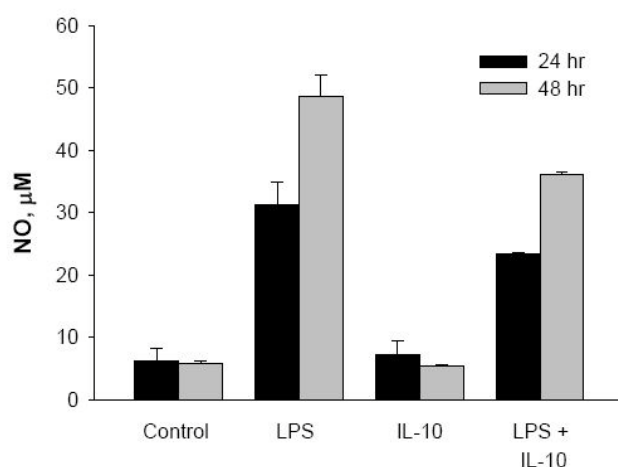
**Figure 2:** CD86 expression of FSDCs after 24 hours of treatment with LPS or IL-10. IL-10 does not increase CD86 expression while LPS does increase CD86 expression.

does not play an inflammatory role but an anti-inflammatory one with respect to dendritic cells, as for macrophages. As inflammation is the first step in the regenerative process, it is optimal that I can control dendritic cell activation with IL-10 to prevent chronic and undue inflammation.

In addition to the secretion of nitric oxide, I sought another method for

separating activated and immature dendritic cells from each other. I investigated the expression of co-stimulatory molecule CD86 expression under inflammatory and anti-inflammatory conditions. I stimulated FSDCs with LPS and IL-10 for 24 hours, and assayed CD86 expression via flow cytometry (Figure 2). I find that FSDCs treated with LPS express a ~1.5 fold increase in CD86 expression while FSDCs treated with IL-10 display no increase in CD86 expression. Thus, in addition to NO secretion, CD86 may be used as a marker for FSDC activation.

Implants often encounter inflammatory responses due to residual LPS, as traces of LPS are retained by almost all surfaces, despite careful washing. This is dangerous as minute amounts of LPS can result in endotoxin shock and tissue damage [23]. It is difficult to rid implants of endotoxins as they are extremely stable molecules and are resistant to extreme temperatures and pH. Much research has been conducted to study various methods to rid implants of LPS, some of these cleaning methods are washing with Triton X-100, LPS affinity resins, and use of ultra filtered reagents [23, 24]. While these methods are fairly successful, they are not always compatible and applicable to tissue engineered implants, and there may still be residual amounts of endotoxin. Thus in addition to the washing and sterilization, alternative methods to control inflammatory response must be employed. The addition of IL-10 to LPS in culture media proved to be successful at abrogating the inflammatory response, as well as altering dendritic cell activation and macrophage phenotype, thus I wanted to determine if immobilized IL-10 would perform similarly, helping to dampen the inflammatory response locally, as compared to a systemic treatment with IL-10 which would affect the whole body's immune system. I



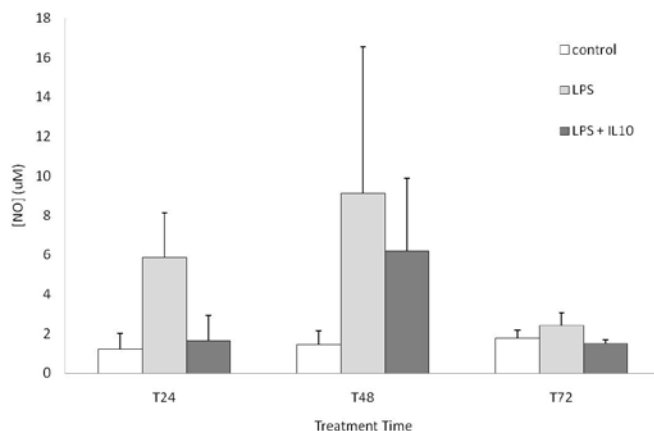
**Figure 3:** NO secretion by FSDC seeded on DermaMatrix containing immobilized LPS and IL-10. Bars represent  $\pm$  SEM.

immobilized LPS (~50ng) and IL-10 (~50ng) onto 5mm DermaMatrix discs via handprinting. Discs were rinsed, seeded with FSDCs and incubated for 24 or 48 hours. The media were then assayed for nitric oxide secretion. Griess assay results indicated that immobilized LPS behaved similarly to the LPS added to culture media, resulting in an increase

in NO secretion. Likewise, immobilized IL-10 does not result in secretion of NO, and immobilized IL-10 is able to abrogate secretion of immobilized LPS stimulated NO secretion at 24 and 48 hours (Figure 3).

### 3.3.2 FSDC reversal

Exposure of immature dendritic cells to stimuli such as LPS results in activation and maturation. This activation and maturation process results in the secretion of proinflammatory cytokines and chemokines attracting other innate cell types, and the migration of mature dendritic cells to the draining lymph nodes to activate  $T_H0$  cells towards the  $T_H1$  phenotype. To be able to control inflammation, I have to control dendritic cell activation and modify the responses of activated dendritic cells.

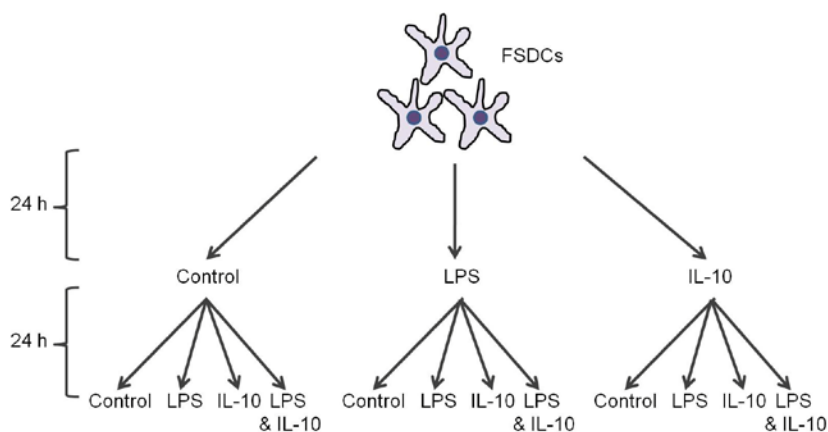


**Figure 4:** FSDC subjected to 24 hours of LPS stimulation continue to secrete NO for an additional 24 hours. IL-10 decreases LPS stimulated NO secretion. [LPS] = 100ng/ml, [IL-10] = 100ng/ml.

To gain a better understanding of dendritic cell behavior post activation, I stimulated FSDCs for 24 hours with LPS and IL-10, then removed the stimuli to determine if FSDCs would continue to secrete NO after the removal of the stimuli. FSDCs were cultured in either 100ng/ml of LPS or 100ng/ml of LPS and IL-10 for 24 hours, then the cells were washed

with PBS and then placed in regular culture media, and after incubation for another 24 or 48 hours, the media were assayed for nitric oxide production using the Griess assay. I find that FSDCs secrete NO 24 hours after LPS stimulation and NO production is abrogated with the

addition of LPS plus IL-10. Removal of LPS and LPS plus IL-10 stimuli did not inhibit NO production, LPS treated FSDCs continue to secrete NO for an additional 24 hours at increased levels. Cells treated with LPS plus IL-10 also continued to secrete NO for an additional 24 hours at increased levels as compared to the T24 time point (Figure 4). At T72, 48 hours after the removal of stimulation, NO secretion decreases for both the LPS treated group and the LPS and IL-10 treated group to levels similar to that of the control group. Thus, this increase in secreted NO concentration at T48 suggests that dendritic cells continue to respond to LPS and IL-10 stimuli for at least 24 hours after stimulation but eventually downregulated inducible nitric oxide synthase expression, thus decreasing secreted nitric oxide. Thus, FSDCs may be dynamic in their response to the environment, regardless of activation.



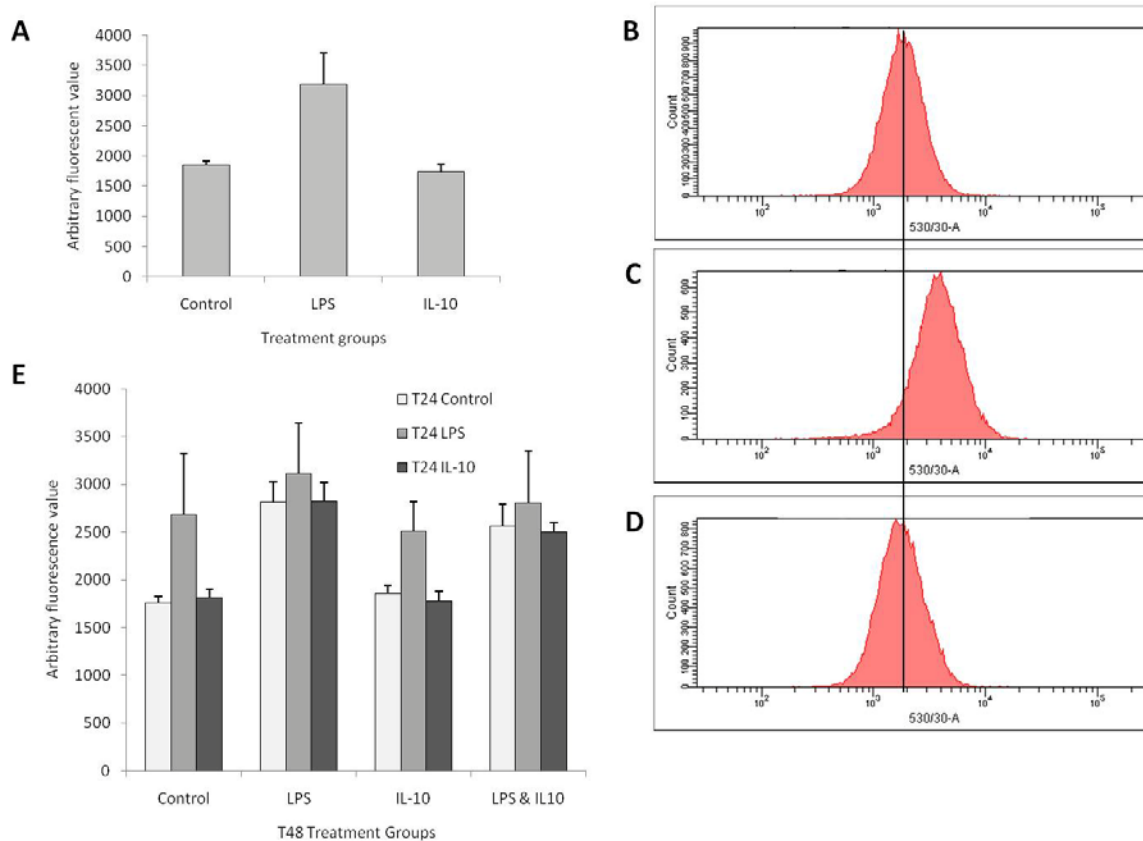
**Figure 5:** Experimental setup to determine extent of FSDC plasticity. Cells were treated with 100ng/ml of LPS, or 100ng/ml of IL-10, and analysis was conducted via flow cytometry.

Mature human dendritic cells are reported to be non-responsive to IL-10 [25], to determine if this is truly the case, the following experiment was carried out (Figure 5): FSDCs were divided

into 3 treatment groups: control, 100ng/ml of LPS or 100ng/ml of IL-10 for 24 hours for activation. After 24 hours of treatment, FACS analysis using a CD86-FITC antibody was used to determine if the dendritic cells have been activated. As FSDCs are extremely sensitive to the preparative process as well as fluorescence activated cell sorting (FACS), I chose to analyze a

subset of the population at each time point instead of sorting for CD86<sup>high</sup> and CD86<sup>low</sup> cells. Each group of 3 received the same treatment for another 24 hours: control, 100ng/ml LPS, 100ng/ml IL-10, or 100ng/ml of LPS and IL-10. Subsequently, the cells were prepared for FACS analysis again using a CD86-FITC antibody. Thus, I was able to investigate if activated FSDCs would be able to respond to a changing environment.

Analysis of CD86 expression after 24 hours reveals that LPS treatment resulted in an increase of CD86 expression, while IL-10 treatment did not (Figure 6A). Expression profiles in Figure 6B,C &D confirm the shift in peak of fluorescence measured using a 530/30 filter. Figure 6E demonstrates the plasticity of FSDC cells measured using CD86 expression. As the costimulatory molecule, CD86 is a marker for dendritic cell activation and maturation, increases or decreases in expression will indicate if FSDCs are able to adapt to a changing environment. IL-10 treatment 24 hours later of LPS-activated FSDCs displayed a decrease in CD86 expression as compared to LPS treatment of LPS treated FSDCs. Likewise, IL-10 plus LPS treatment also decreased CD86 expression of LPS-activated FSDCs. However, as expected, the IL-10 plus LPS treatment was not as effective as IL-10 alone in reducing the expression of CD86. LPS-treated cells that received no treatment exhibited a decrease in CD86 expression, similar to the decrease in NO expression exhibited in Figure 4. However, the lack of continual LPS treatment did not decrease CD86 expression as effectively as IL-10 treatment.



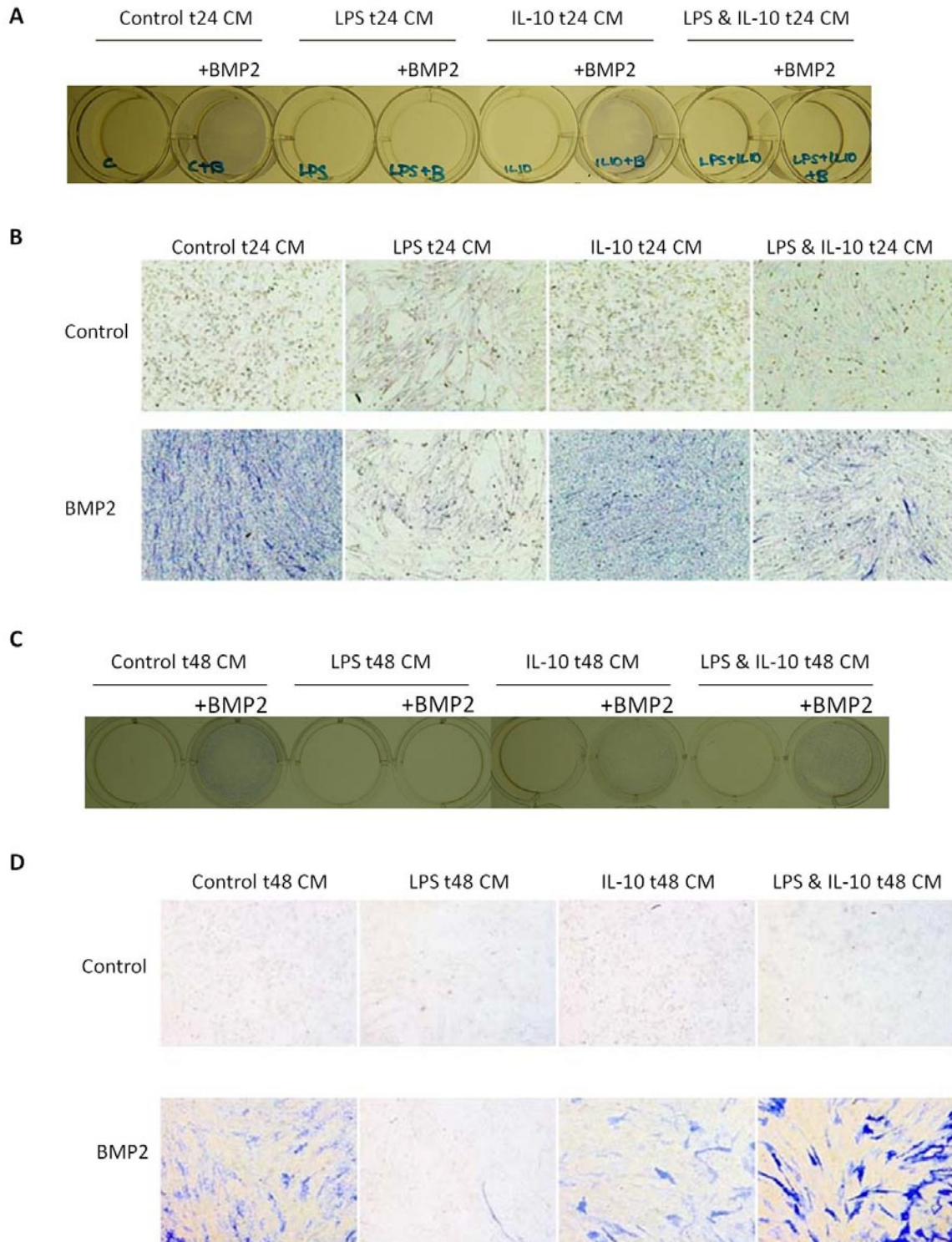
**Figure 6:** Analysis of FSDC plasticity. [LPS] = 100ng/ml, [IL-10] = 100ng/ml. **A.** CD86 profile after 24 hours of treatment. **B,C,D.** Histogram of CD86 expression of Control, LPS, and IL-10 treated FSDCs at 24 hours. **E.** Analysis of CD86 expression by FSDCs at 48 hours of treatment.

Treatment of FSDCs with IL-10 did not prevent FSDCs from upregulating CD86 expression with LPS stimulation. LPS plus IL-10 stimulation of IL-10 treated cells also upregulated CD86 expression but not to the extent of LPS only treatment. As expected, LPS treatment of IL-10 treated cells did not achieve the same level of CD86 expression as LPS treatment of LPS treated cells. Thus, according to CD86 expression, FSDCs are able to respond to IL-10 treatment post activation and maturation, hinting at a level of dendritic plasticity. Further evaluation of other markers of dendritic maturation such as co-stimulatory molecule, CD40 and cluster of differentiation marker, CD83 should be carried out to confirm the extent of plasticity in dendritic cells.

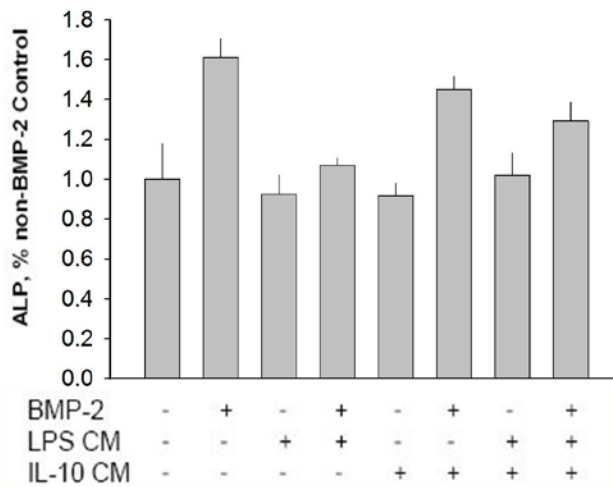
### 3.3.3 FSDC conditioned media experiments

I have demonstrated the ability to use IL-10 to suppress dendritic cell activation; however is this suppression sufficient to rescue BMP-2 directed osteoblastogenesis under inflammatory conditions? I cultured FSDCs in the presence of 100 ng/ml LPS, 100 ng/ml IL-10 or 100ng/ml LPS and IL-10, and collected conditioned media at 24 or 48 hr. This CM was then incubated with C2C12 stem cells and with BMP-2 (100ng/ml), and stained for alkaline phosphatase after 3 days as a marker for osteoblast differentiation.

Figure 7 demonstrates the effects of LPS CM on BMP-2 directed osteoblastogenesis. LPS CM inhibits BMP-2 directed osteoblastogenesis, encourages C2C12 apoptosis and changes in cell morphology; the C2C12s cultured in LPS CM with BMP-2 are elongated and spindly in shape. This effect is apparent with CM harvested after 24 and 48 hours suggesting that the FSDCs responded quickly to the LPS stimulant, secreting inflammatory cytokines and other cues that prevent the recruitment of progenitor cells, kill stem cells present within the vicinity and reduce the efficacy of BMP-2. Hence, using BMP-2 with implants that elicit an inflammatory response will render the effects of BMP-2 ineffective till inflammatory conditions subside. IL-10 CM, and IL-10 and LPS CM do not inhibit BMP-2's actions as evidenced by increased ALP expression. Thus, IL-10 interferes with LPS stimulation of dendritic cells, and the LPS plus IL-10 conditioned media is permissive to C2C12 survival, proliferation, and differentiation. The images in Figure 7B were unmixed and ALP staining was quantified, verifying the qualitative estimates and revealing that FSDC LPS 24 hour CM inhibits ALP expression ~1.45 times as compared to control 24 hour CM (Figure 8).



**Figure 7:** *In vitro* ALP activity of C2C12 cells exposed to FSDC cells 24 (t24)(A&B) and 48 (t48)(C&D) hour CM derived in the presence of control media, 100 ng/ml LPS, 100 ng/ml IL-10, or 100ng/ml of LPS and IL-10. Non-CM control and CMs were added in the presence of 100 ng/ml BMP-2 for 3 days then stained for ALP. Magnification-10x.



**Figure 8:** Quantification of *in vitro* ALP activity of C2C12 cells exposed to FSDC 24 hour CM with and without BMP-2. C2C12s were cultured in CM derived from J774a.1 cells stimulated by 100ng/ml LPS or 100ng/ml IL-10, then stained for ALP in 4 days. Data are presented as ALP staining normalized to BMP-2 control.

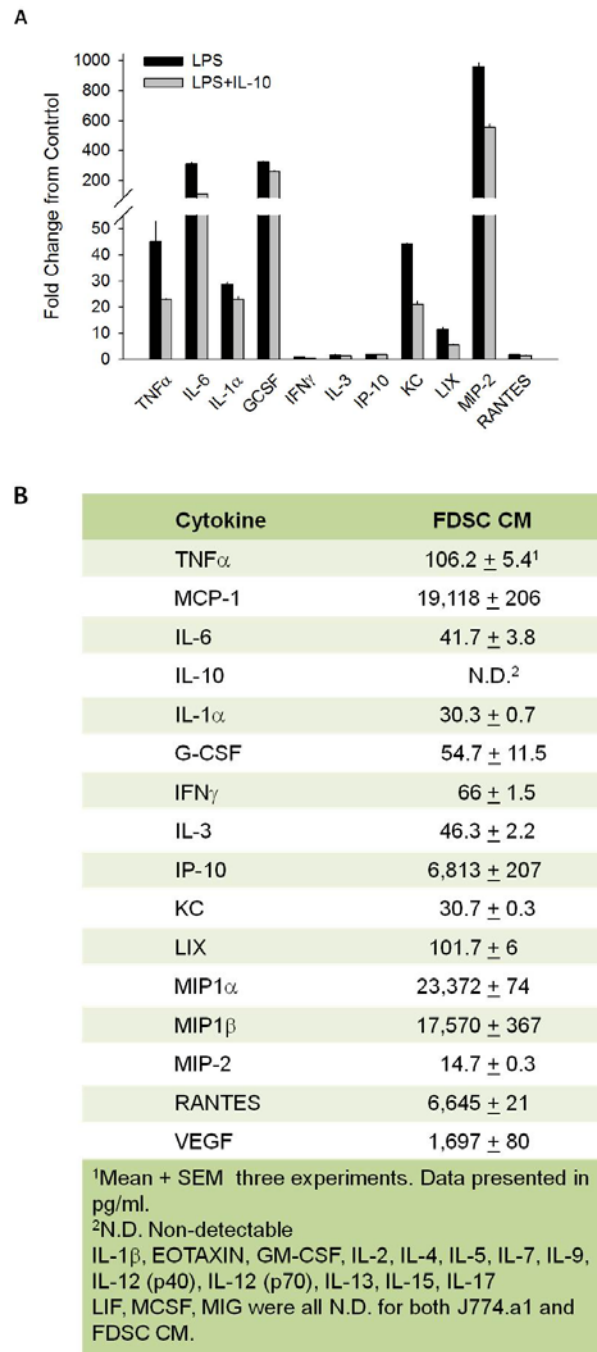
As discussed previously in Chapter 2, C2C12s respond not to LPS stimulation, but to the products secreted by dendritic cells upon LPS stimulation. Likewise, IL-10 is unable to rescue ALP expression when C2C12s are cultured in FSDC LPS CM and treated with 100 ng/ml IL-10 (data not shown), similarly suggesting that IL-10 anti-inflammatory actions target FSDCs and their secretions and not the C2C12s.

### 3.3.4 FSDC conditioned media analysis

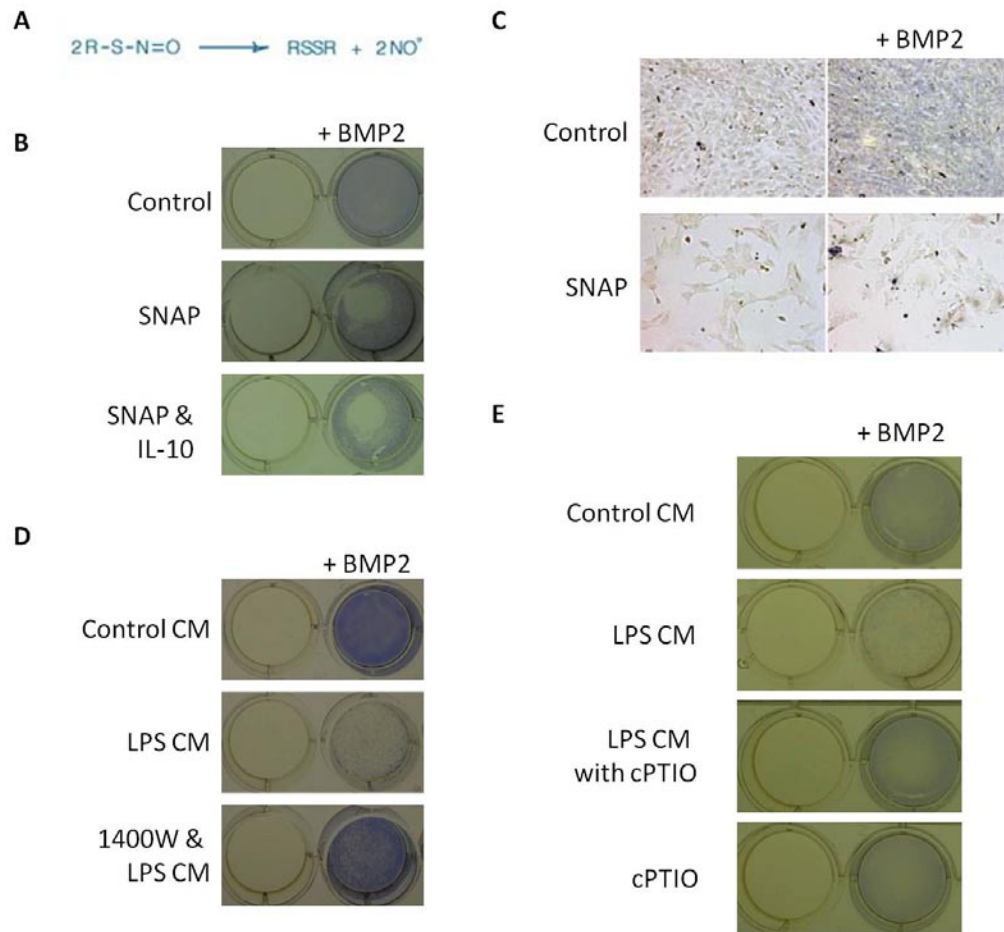
Determining the components of conditioned media can provide a window as to how inhibition of BMP-2 mediated osteogenesis may occur. To determine the secretory products of activated FSDCs, conditioned media at 24 and 48 hours were harvested and 32 different cytokines involved in inflammation were analyzed using the Milliplex MAP mouse cytokine/chemokine panel. As results from the 24 hour conditioned media are similar to that of the 48 hour conditioned media, I have presented the data from for the 48 hour conditioned media in Figure 9.

Control and IL-10 CM did not result in increased secretion of these cytokines. LPS stimulation resulted in a significant increase in secreted tumor necrosis factor alpha, IL-6, IL-1 $\alpha$ , granulocyte colony stimulating factor (G-CSF), keratinocyte chemoattractant (KC), LPS-induced CXC chemokine (LIX), macrophage inflammatory protein 2 (MIP-2) and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), while IL-10 and LPS CM resulted in significant decreases as compared to LPS CM in the above mentioned cytokines and chemokines.

In addition to the cytokines and chemokines included in the Milliplex/MAP array, nitric oxide secretion is upregulated by FSDCs upon LPS stimulation. To understand the effects of NO upon BMP-2 directed osteoblastogenesis, I utilized NO donor, SNAP, NO radical scavenger, cPTIO, and inducible nitric oxide synthase



**Figure 9:** Partial analysis of 48 hour J774a.1 conditioned media components. Using the Milliplex MAP cytokine/chemokine assay, control, LPS, LPS and IL-10 conditioned media harvested from FSDC were analyzed for concentrations of cytokines involved in inflammation. **A.** Graph illustrating the cytokines with significant fold changes in expression in LPS CM over control CM. **B.**



**Figure 10:** NO effects on BMP-2 directed osteoblastogenesis. **A.** Mechanism of spontaneous NO release by SNAP. Image reprinted from [4]. **B.** SNAP inhibits BMP-2 induction of ALP expression. [SNAP] = 200 $\mu$ M, [IL-10] = 100ng/ml. **C.** SNAP treated cells are less dense than control cells, suggesting apoptotic effect of NO. Images captured at 20X. **D.** C2C12s treated with control, LPS and 1400W and LPS FSDC 24 hour CM. 1400W treated FSDCs generates LPS CM hospitable to BMP-2 induced osteoblastogenesis. [1400W] = 10  $\mu$ M. **E.** C2C12s treated with LPS CM and NO radical scavenger, cPTIO. Addition of cPTIO rescues BMP-2 induced ALP expression. [cPTIO] = 25 $\mu$ M.

inhibitor, 1400W. SNAP spontaneously releases NO under physiological condition (Figure 10A), while 1400W is a slow, tight binding inhibitor of iNOS [26].

To determine if NO can affect BMP-2 action, I treated C2C12s with SNAP and BMP-2 in regular culture media (Figure 10B). The addition of SNAP inhibited BMP-2 directed C2C12

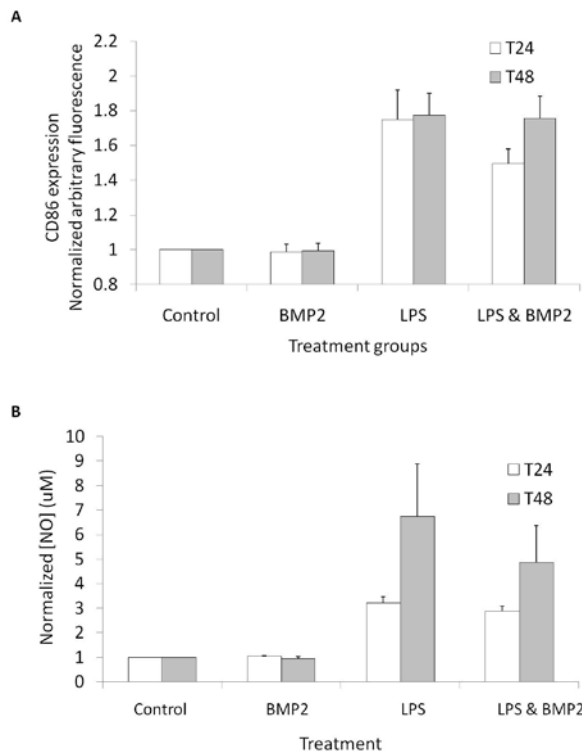
osteogenesis, and the addition of IL-10, as expected, was unable to rescue BMP-2 directed differentiation. SNAP treated cells indicate a lack ALP expression only in the middle of the well; this could be due to poor mixing technique during the treatment. Additionally, a closer look reveals that SNAP treated C2C12s lacking ALP expression display a paucity in numbers, suggesting apoptosis induced by the NO radical (Figure 10C). Subsequently, I pre-treated FSDCs with and without 10 $\mu$ M 1400W 30 minutes prior to LPS stimulation and collected conditioned media 24 hours later. C2C12s treated with 1400W and LPS CM stained positive for ALP, indicating that the inhibition of iNOS via 1400W pretreatment rescued BMP-2-induced osteoblastogenesis in C2C12s to levels comparable to C2C12s treated with control CM. As expected, LPS CM treated C2C12s displayed very minimal ALP expression (Figure 10D). Finally, as a last determinant of the effect of NO radicals on the differentiation process, I tested cPTIO, a nitric oxide radical scavenger. LPS CM treated C2C12s displayed minimal ALP expression while C2C12s treated with FSDC 24 hour LPS CM with 25 mM of cPTIO displayed BMP-2-induced ALP expression. Additionally, cPTIO itself will not inhibit BMP-2 action or cause osteoblastogenesis. Thus, the cPTIO is able to inhibit the action of NO on BMP-2 directed osteogenesis. Together, these three separate experiments described the effects of NO on BMP-2's osteogenic actions.

### **3.3.5 BMP 2 activation of FSDCs**

Bioengineered tissue implants targeted to encourage bone formation often include BMP-2 as a growth factor. The surgery to implant such tissue engineered constructs causes inflammation, and inherent properties in the tissue engineered construct may or may not cause inflammation. If in addition to these factors, the very growth factor utilized for target tissue regeneration can cause additional inflammation, the regenerative process will be further delayed.

As resident tissue dendritic cells will be among the first few cells to contact the tissue engineered construct and growth factor, it is important to determine if BMP-2 will activate dendritic cells.

To test if BMP-2 causes dendritic maturation, I exposed FSDCs to BMP-2 (100ng/ml) and LPS (100ng/ml) for 24 or 48 hours and assayed the level of nitric oxide secreted as well as CD86 expression as indicators of activation and maturation. Figure 11A indicates that BMP-2 does not increase CD86 expression in FSDCs at 24 or 48 hours, and it does not have any



**Figure 11:** Exposure to BMP-2 does not activate FSDCs. **A.** CD86 expression is not upregulated with 24 or 48 hours of BMP-2 treatment. **B.** NO secretion is not upregulated with 24 or 48 hours of BMP-2 treatment. [LPS] = 100ng/ml, [BMP-2] = 100ng/ml.

synergistic effects with LPS with regards to CD86 expression. Figure 11B shows that BMP-2 does not enhance NO secretion by FSDCs alone or in concert with LPS. Thus, BMP-2 does not result in dendritic cell maturation, and will not exacerbate the inflammatory response during infection, or cause further delays in the regenerative process.

### 3.4 Discussion

The dendritic cell connects the innate and acquired immune system, hence its role is an important one.

Foreign pathogenic invasion results in

the activation of dendritic cell with bacterial components, such as LPS. During the maturation process, the secretion of chemokines (such as KC) and cytokines (such as TNF- $\alpha$ ) will alter the microenvironment by attracting and activating neutrophils, macrophages and other cells of innate

immune system, as well as affect stem cell survival and differentiation. Finally, these activated dendritic cells will migrate to the draining lymph nodes to activate T helper cells, triggering the acquired immune system. While this inflammatory process is a crucial part of the body's defense mechanism, it can also delay the regenerative process.

To prevent delay in tissue regeneration, I proposed the use of IL-10 as an anti-inflammatory cytokine. I demonstrated the use of IL-10 to dampen the inflammatory response via dendritic cells. Dendritic cells exposed to LPS and IL-10 in culture and immobilized on DermaMatrix displayed decreased amounts of nitric oxide secretion as well as decreased CD86 expression as compared to LPS treated cells. Moreover, IL-10 is said to cause dendritic cells to activate  $T_H0$  cells towards immune tolerant  $T_{reg}$ ; this bodes well for tissue engineered construct as it would encourage acceptance over rejection [27]. The ability of immobilized IL-10 to combat LPS induced inflammation also bodes well for local therapies, avoiding the unwanted side effects of systemic manipulations. Currently, the maximum level of endotoxin for intravenous applications of pharmaceutical and biologic product is 5 endotoxin units (EU) (biological activity of an endotoxin) per kilogram of body weight per hour [28]. While this threshold provides safety for the public, it poses a challenge for biotechnology and pharmaceutical companies, for example, 120 pg of endotoxin from *Escherichia coli* O111:B4 has an activity of 1 EU [29]. As decreasing the endotoxin content of implants below this level is difficult, IL-10 might provide an alternative solution to combat unwanted effects due to residual endotoxin.

I showed that FSDCs were able to adapt to changing environments with changes in CD86 expression and NO secretion. However, Steinbrink *et. al.* determined that IL-10 had no effects on mature human dendritic cells isolated from peripheral progenitors [25]. As my experiments were

conducted on mouse dendritic cells also isolated from peripheral progenitors, my experimental results may not be directly comparable. To gain a better understanding of dendritic cell plasticity, I should test human dendritic cells as well. Nonetheless, the ability of FSDCs to respond to changing environments indicates that IL-10 treatment will be able to target dendritic precursors as well as activated dendritic cells, thus covering both ends of the developmental spectrum.

Without the use of IL-10 to abrogate inflammation, LPS activated FSDCs inhibited BMP-2-induced C2C12 osteoblastogenesis. Conditioned media harvested from LPS treated FSDCs at 24 and 48 hours inhibited BMP-2 action, but IL-10 and LPS CM rescued ALP expression. Examination of LPS CM components revealed significant increases in expression of TNF- $\alpha$ , IL-6, IL-1 $\alpha$ , GCSF, KC, LIX, MIP-2 and RANTES. IL-6 is secreted by T cells and macrophages in response to situations such as trauma, infections or burns [30]. The presence of TNF- $\alpha$ , a proinflammatory cytokine has been linked to diseases ranging from periodontal disease [31] to inflammatory bowel disease [32]. IL-6 plays dual roles; as a pro-inflammatory cytokine, it stimulates osteoclast development, yet as an anti-inflammatory cytokine, IL-6 can inhibit TNF- $\alpha$  effects. Additionally, IL-6 expression has been linked with multiple chronic inflammatory diseases [33-35]. IL-1 $\alpha$  is released in response to injury, and can induce apoptosis in an inflammatory environment [36]. Additionally, IL-1 $\alpha$  is said to have a synergistic effect with TNF- $\alpha$  as well as inducing further secretion of TNF- $\alpha$  [37]. GCSF stimulates the maturation of granulocytes from the bone marrow [38], causing neutrophil mobilization and migration in response to tissue insult [39, 40]. KC (IL-8) is a chemoattractant for leukocytes. Expression of KC causes monocyte adherence, extravasation and migration [41]. LIX, also known as CXCL5, is part of CXC family of chemokines, and is upregulated during TNF- $\alpha$  and IL-1 induced inflammation, is involved in neutrophil chemotaxis [42]. MIP-2 is a chemotactic factor for

granulocytes and hematopoietic stem cells [43]. Last but not least, RANTES, is a chemoattractant for monocytes and memory T cells [44]. These secreted cytokines and chemokines not only help to attract more neutrophils, macrophages and T cells, they work to extend the inflammatory duration. Fortunately, IL-10 and LPS CM showed significant decreases in expression of the above mentioned cytokines and chemokines, thus providing an option as to how expression of these inflammatory proteins could be controlled to the point where inflammation is not eliminated but reduced, so that tissue regeneration can take place, speeding up the healing process.

Osteoinductive implants such as Medtronic's INFUSE® come preloaded with BMP-2. My studies indicate that this should not be an issue as BMP-2 does not upregulate CD86 expression or NO secretion from dendritic cells, thus this bodes well for implant acceptance. However, Martinez *et. al.* determined that BMP-4 activated human dendritic cells isolated from buffy coats [45], stated that BMP-4 activated dendritic cells upregulated CD83 expression, exhibited increased T cell stimulatory capacity, and secreted increased levels of inflammatory cytokines such as TNF- $\alpha$ . While this finding is relevant, BMP-4 and BMP-2 are different growth factors despite the fact that both are capable of stimulating osteogenesis. It is not uncommon for the pair of growth factors to exhibit different behaviours despite their similarities, for example, BMP-2 and 4 play opposing role in hypoxic pulmonary hypertension, where BMP-2's protective effects are mediated by increasing endothelial nitric oxide synthase expression, and BMP-4 promotes pulmonary hypertension by increasing vascular smooth muscle cell proliferation and vascular remodeling [46].

Human dendritic cells isolated from the blood possess different properties from peripheral murine dendritic cells. Nonetheless, dendritic cells from both sources play an

important role in inflammatory process and are able to affect stem cell differentiation. In conclusion, the above experiments offer a better understanding of how dendritic cells can affect the differentiation process, and the methods that can be employed to abrogate inflammation. The use of IL-10 is able to decrease LPS mediated secretions in dendritic cells, providing a hospitable environment for BMP-2 mediated C2C12 differentiation. Together, these data provide more insight into the role that dendritic cells play in regulating the microenvironment, thus helping to connect separate pieces of knowledge temporally and molecularly in the field of osteoimmunology.

### 3.5 Acknowledgements

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## Chapter 4: Effects of macrophage and dendritic cell conditioned media on different types of stem cells.

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## 4.1 Introduction

Stem cells are highly influenced by local chemokines, growth factors and cytokines. Chemokines such as stromal cell derived factor 1 alpha attract stem cells to leave stem cell niches [1], such as the bone marrow, and migrate towards the site of injury, where regeneration must take place. At the wound site, these cells will then encounter growth factors such as bone morphogenetic protein 2 that direct them down a differentiation pathway towards a specific lineage. However, the cells will also encounter cytokines such as tumor necrosis alpha that can inhibit the actions of the growth factor, or free radicals like nitric oxide that result in toxicity and DNA damage [2].

To study the various processes described above, many laboratories use a variety of stem cells, for example, MC3T3 and C2C12 cell lines are both used to study osteoblastogenesis. However, MC3T3 is mainly a preosteoblastic cell line, while C2C12s can differentiate towards skeletal muscle or bone depending on the culture conditions and growth factors [3, 4]. Thus, different stem cell lines possess different properties and within each line, there may be much variability [5]; different cell lines could yield very different results for the same experiment. In this chapter, I investigate whether lipopolysaccharide -induced inflammatory environments established by macrophage and dendritic cell secretions will produce similar or different results in the following cell lines: MC3T3s, a pre-osteoblastic cell line, muscle derived stem cells, and human mesenchymal stem cells. Additionally, I attempt to determine how C2C12, a mouse myoblast cell line, responds to direct additions of LPS under different conditions.

MC3T3s have been widely used as a model to study osteoblastogenesis [6], however as pre-osteoblastic cells, they could be described as possessing the least amount of multipotency as compared to the other cell lines tested. Thus, these cells will provide us with an understanding of

how pre-osteoblastic cells near the wound site will be affected by the inflammatory milieu. MC3T3s have been reported to be more responsive to TNF- $\alpha$  treatment than LPS with regards to the upregulation of RANKL expression [7], suggesting that MC3T3s may be more responsive to the inflammatory secretions than to the stimulant itself. However, Xing *et. al.* report that 10 $\mu$ g/ml of *P. gingivalis* lipopolysaccharide is able to inhibit mineralization in MC3T3s cells stimulated with 10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/ml ascorbic acid, and 10 nM dexamethasone (instead of BMP-2) [8]. Wang *et. al.* report that low concentrations (500 ng/ml) of *E. coli* LPS results in proliferation of MC3T3 cells [9]; Thammasitboon *et. al.* confirm that 100 ng/ml of *E. coli* LPS does not affect MC3T3 viability, but conditioned media generated with RAW264.7 cells (a human macrophage cell line) induces MC3T3 apoptosis [10]. I aim to determine whether MC3T3 will respond as C2C12s did in the previous chapters, when stimulated with BMP-2, LPS or conditioned media as described.

MDSCs are a multipotent population of stem cells isolated from murine skeletal muscle using a pour plate method [11] that are able to differentiate into muscle, tendon, and bone with the right cues [12, 13]. Jackson *et. al.* found that mesenchymal stem cells isolated from traumatically injured muscle tissue could differentiate into osteoblasts, adipocytes and chondrocytes, but demonstrated limited lineage commitment as compared to bone-marrow derived mesenchymal stem cells [14]. However, mesenchymal stem cells harvested from traumatically injured muscle are slightly different than MDSCs; they are rapidly adherent and present in substantial numbers during the harvesting procedure, as opposed to the slow adherence and rare occurrence of MDSCs [15, 16]. Apart from these differences, these two cell types are thought to possess similar properties [15]. So stem cells isolated from injured tissue still possess

the capability to differentiate into various lineages; I investigate whether these stem cells respond to LPS, and are able to differentiate into bone in an inflammatory environment.

Harvested and cultured from normal human bone marrow, hMSCs are considered to be the most multipotent stem cells of the cell lines I tested. These cells are known for their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages. In addition to their multipotentiality, hMSCs are said to be immunomodulatory; hMSCs altered the cytokine secretion profile of dendritic cells, T cells, and natural killer cells, decreasing TNF- $\alpha$  secretion, and increasing IL-10 secretion, thus inducing an increased anti-inflammatory phenotype [17]. hMSCs are also said to secrete factors that are angiogenic, anti-apoptotic, and regenerative. Moreover, intravenous delivery of allogeneic hMSCs has been linked with the suppression of graft vs. host disease as well as regenerative events in stroke, spinal cord injury, meniscus regeneration, tendinitis, acute renal failure, and heart disease [18]. Thus, it will be interesting to determine if hMSCs will react similarly to C2C12s or are responsive to BMP-2 in the face of a LPS-induced inflammatory environment.

Finally, I demonstrated that C2C12s failed to differentiate in the presence of BMP-2 when cultured in an inflammatory environment (LPS conditioned media), but were not responsive to BMP-2 with direct treatments of LPS. However, Frost *et. al.* determined that C2C12s were responsive to LPS stimulation, which resulted in an increase in IL-6 protein expression [19]. I therefore attempted to determine whether C2C12s would be responsive to LPS if I varied culture conditions.

Together these three different stem lineages will provide a range of multipotency, allowing us to determine if the potency of a cell line would affect its ability to respond to LPS and inflammatory environments. Additionally, I will also be able to determine if the C2C12

response to LPS and inflammatory environments is specific to the cell line, or is representative of stem cells in general.

## **4.2 Methods and materials**

*Escherichia coli* lipopolysaccharide was purchased from Sigma (St Louis, MO), *Porphyromonas gingivalis* lipopolysaccharide (PGLPS) was purchased from Invivogen (San Diego, CA). Bone morphogenetic protein 2 was purchased from Medtronic (Minneapolis, MN). Interleukin 10 was a gift from Dr. Xin Xiao Zheng. Phosphate buffered saline was purchased from Fisher Scientific (Pittsburgh, PA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. All experiments were performed three times in triplicate unless noted otherwise.

### **4.2.1. Cell culture**

#### **4.2.1.a MC3T3-E1 subclone 4**

MC3T3-E1 subclone 4 cells (ATCC, Manassas, VA) were grown in Alpha-Mem (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (ATCC, Manassas, VA), 1% penicillin-streptomycin (Invitrogen Corp., Carlsbad, CA)). For experiments, cells were seeded at a density of 26315 cells per centimeter squared or 50, 000 cells per well (close to 90% confluency) in 24 well plates.

#### **4.2.1.b Muscle derived stem cells**

Female derived muscle derived stem cells, isolated from primary mouse gastrocnemius muscle biopsies following a modified preplate technique were grown in DMEM (high glucose; (Invitrogen, Carlsbad, CA), 10% FBS, 10% heat inactivated horse serum, 0.5% chick embryo

extract (Accurate Chemical Co, Westbury, NY), 1% PS as previously described [11, 20]. For experiments, cells were seeded at a density of 63000 cells per cm squared or 120, 000 cells per well (close to 90% confluency) in 24 well plates.

#### **4.2.1.c Human mesenchymal stem cells**

Female human mesenchymal stem cells (BioWhittaker Inc, Walkersville, MD) were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM™, Lonza Walkersville Inc., Walkersville, MD) according to the manufacturer's instructions. For experiments, cells were seeded at a density of 63000 cells per cm squared or 120,000 cells per well (close to 90% confluency) in 24 well plates.

#### **4.2.1.d C2C12**

Mouse C2C12 cells (ATTC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Media, 10% fetal bovine serum and 1% penicillin-streptomycin, and were kept at 37°C, 5% CO<sub>2</sub> in a humidified incubator. For experiments, 200,000 cells per well were cultured in a 12 well plate.

#### **4.2.2 Conditioned media isolation**

For FSDC conditioned media, 10<sup>6</sup> FSDC cells were treated with the following concentrations for 24 or 48 hours: 100 ng/ml LPS, 100 ng/ml IL-10, 100 ng/ml each of IL-10 and LPS. For J774a.1 conditioned media, 10<sup>6</sup> J774a.1 cells were treated with the same concentrations for 24 or 48 hours. Conditioned media were harvested 24 or 48 hours post treatment and kept frozen prior to use.

### **4.2.3 Conditioned media experiments**

$12 \times 10^4$  C2C12 cells/well or  $15 \times 10^4$  MC3T3-E1 subclone 4 cells/well (12 well plate) were treated with conditioned media, with or without 100 ng/ml of BMP-2. The conditioned media were refreshed at day 2 of culture and cells were stained for alkaline phosphatase activity at day 4 of culture.  $12 \times 10^4$  MDSCs (24 well plate) were treated identically, except that conditioned media were refreshed every 2 days in culture and MDSCs were stained for alkaline phosphatase activity at day 8 of culture. For control experiments, C2C12s, MC3T3s, MDSCs and hMSCs were treated using 100ng/ml of LPS orPGLPS, 100ng/ml of IL-10, or 100ng/ml of LPS plus IL-10 directly, with or without 100ng/ml of BMP-2. In additional experiments, C2C12s received pretreatments of 10 ng/ml of Interferon gamma (Isokine, Kopavogur, Iceland), or 100 ng/ml of BMP-2. Cells were cultured for 4, 8 or 10 days (depending on cell type and experiment), with media refreshed every 2 days, before ALP staining.

### **4.2.4 Alkaline phosphatase staining**

Alkaline phosphatase activity (SIGMAFAST) was detected according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Brightfield images of the cells were captured using a 10X and 20X objective on a Leica DMIL LED microscope (Buffalo Grove, IL).

### **4.2.5 Quantification of alkaline phosphatase staining**

Quantification of alkaline phosphatase staining was done by blind spectral unmixing by non-negative matrix factorization. Simple linear unmixing is defined by:  $V = W \times H$ , where  $V$  is the source image, an  $(m \times n)$ -by- $c$  matrix (the number of colors,  $c$ , is 3 for RGB images),  $W$  is the color-bases matrix, a  $c$ -by- $r$  matrix (where  $r$  is the number of sources to be separated), and  $H$  is the unmixed image of the same size as the source image. Because of experimental variation, the spectra of immunocytochemical dyes are often not consistent across every image, making simple

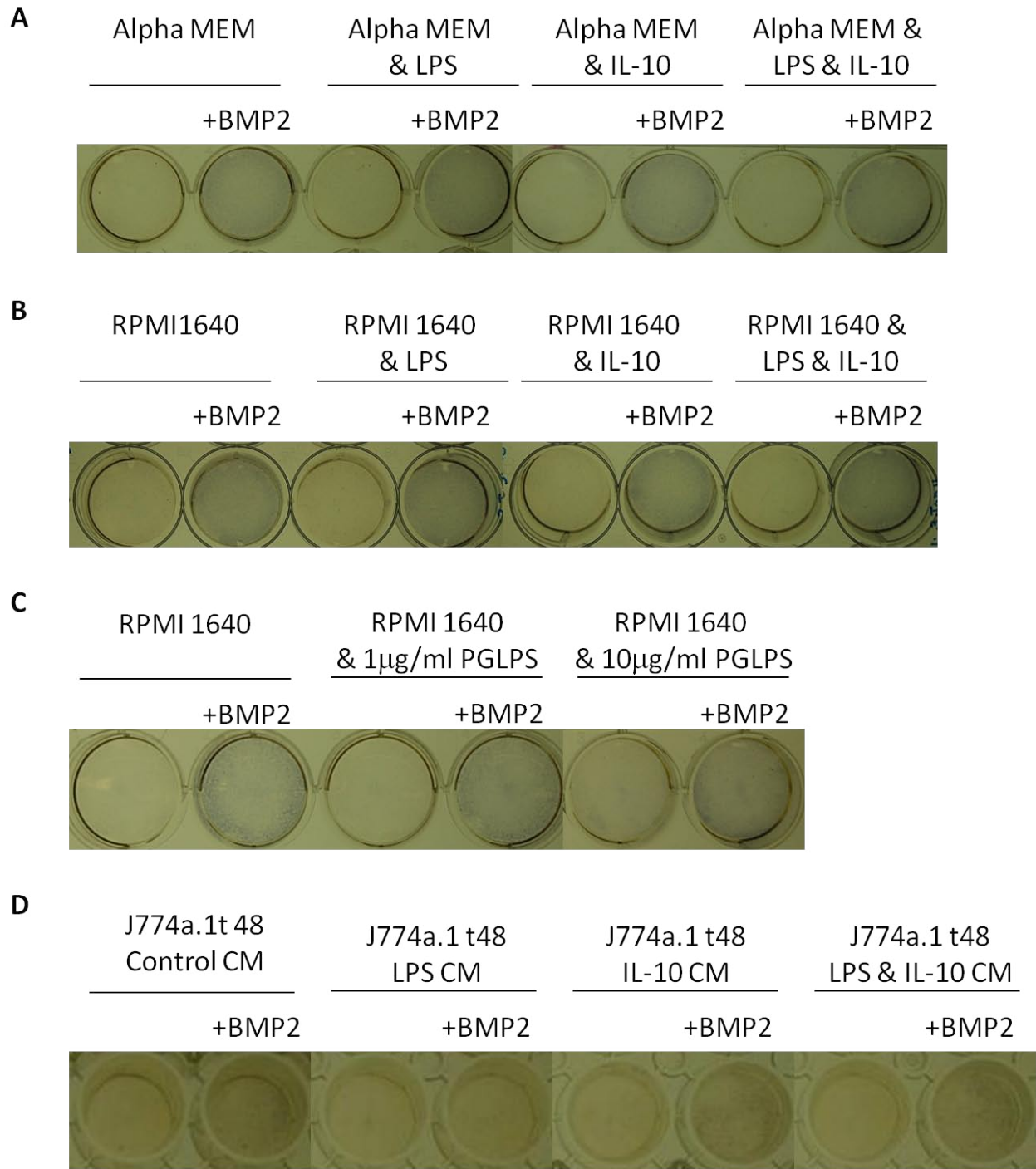
linear unmixing inappropriate. Thus, I used a method demonstrated by Newberg *et. al.* [21] that uses non-negative matrix factorization to blindly unmix the images. Briefly, NMF assumes that each stain contributes non-negatively to the overall image intensity. This method has been shown to be effective in unmixing brightfield images [21]. Blind spectral unmixing by NMF uses a different color matrix,  $W$ , for each image.  $H$  is randomly initialized and NMF is used to solve for the non-negative matrix factors  $W$  and  $H$  by iteratively minimizing the distance between  $V$  and  $W \times H$ . The post-processing of  $H$  into a new unmixed image was similar to that of linear unmixing, in which each channel was scaled and then remapped into the image data channels. The unmixed image was summed to determine the amount of ALP staining.

## **4.3 Results**

### **4.3.1 MC3T3 cells**

MC3T3s, as pre-osteoblastic cells, may react similarly to C2C12s when treated with an inflammatory environment. Depending on the extent of the differentiated state of MC3T3s, secreted inflammatory cytokines such as  $TNF-\alpha$  might interrupt BMP-2 mediated osteoblastogenesis. Conversely, MC3T3s may be influenced directly by LPS treatment as suggested by the literature.

To determine if MC3T3 cells are susceptible to LPS, these cells were stimulated with 100ng/ml of LPS and/or 100ng/ml of IL-10, with and without BMP-2 and stained for ALP expression. Figure 1A and 1B indicate that BMP-2 mediated osteoblastogenesis by MC3T3s is not affected by LPS treatment in either their native medium (alpha MEM) or RPMI 1640 (medium of conditioned media).

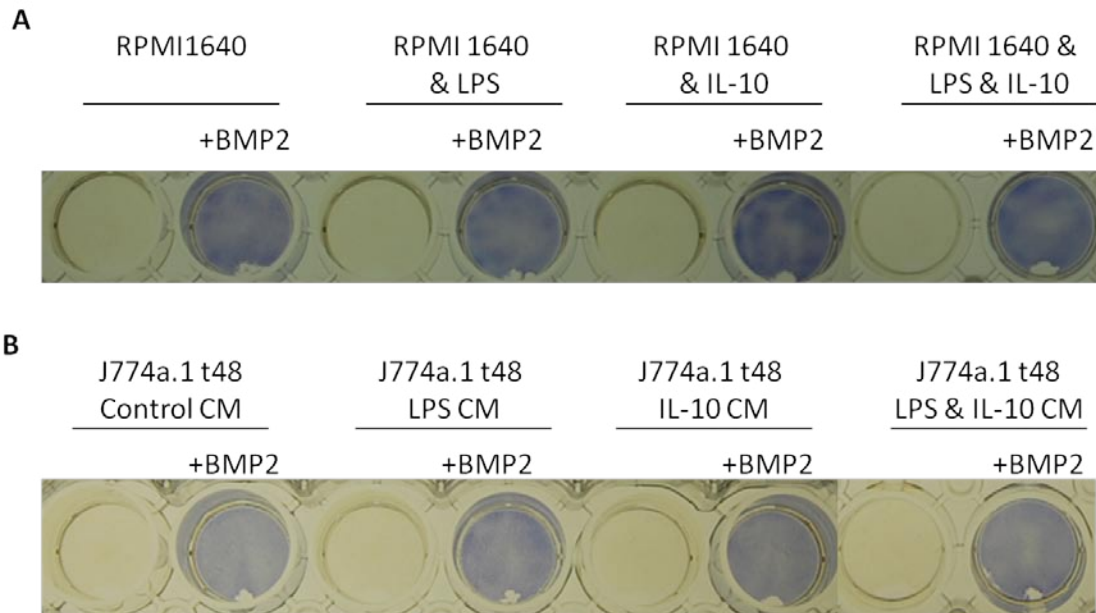


**Figure 1:** ALP expression of BMP-2 stimulated MC3T3 cells cultured with LPS, PGLPS and J774a.1 48 hour conditioned media. **A.** Direct treatment of 100 ng/ml of LPS, 100 ng/ml IL-10 and 100 ng/ml of LPS and IL-10 in Alpha MEM media. **B.** Direct treatment of 100 ng/ml of LPS, 100 ng/ml IL-10 and 100 ng/ml of LPS and IL-10 in RPMI 1640 media. **C.** Direct treatment of 1 μg/ml and 10μg/ml of PGLPS in RPMI 1640 media. **D.** MC3T3s cultured in 48 hour control CM, LPS CM, IL-10 CM and LPS and IL-10 CM.

Hence, any residual LPS present in the conditioned media did not alter the results. Xing *et. al.* reported that MC3T3 mineralization was compromised by PGLPS treatment [8], so I tested the effects of PGLPS on ALP expression. Figure 1C demonstrates that PGLPS has no effect on BMP-2 mediated osteoblastogenesis at 1µg/ml or at 10µg/ml (as used in [8]); ALP expression at 1µg/ml and 10µg/ml of PGLPS with BMP-2 was robust. While BMP-2-mediated osteoblastogenesis in MC3T3s may not be affected by direct treatments using LPS and PG-LPS, BMP-2's osteogenic effects might be inhibited by conditioned media treatment. Figure 1D indicates, despite the weak ALP staining, that MC3T3s cultured in BMP-2 and 48 hour J774a.1 LPS conditioned media have no ALP expression, while MC3T3s cultured in BMP-2 and 48 hour J774a.1 LPS and IL-10 conditioned media have weak ALP expression. Thus, LPS CM's inhibition of BMP-2 function mimics the inhibition seen in the CM obtained from RAW 264.7 cells [10] and LPS plus IL-10 conditioned media can rescue BMP-2's osteogenic function.

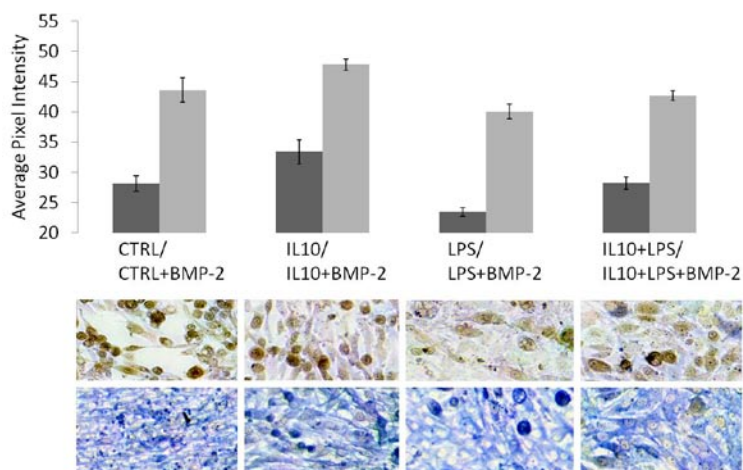
#### **4.3.2 Muscle derived stem cells**

I tested MDSCs under similar conditions and found surprising results. Like C2C12s, BMP-2 mediated osteoblastogenesis was not inhibited by a 100ng/ml LPS treatment (Figure 2A), as evidenced by ALP staining. Unlike C2C12s, BMP-2 mediated osteoblastogenesis was not inhibited by LPS conditioned media at 24 or 48 hours. Figure 2B indicates representative data of MDSCs treated with or without 100ng/ml of BMP-2, cultured in J774a.1 48 hour conditioned media. ALP expression is clearly not inhibited by LPS CM. Conditioned media from J774a.1 (24 hour) and FSDC (24 hour) yielded similar results.



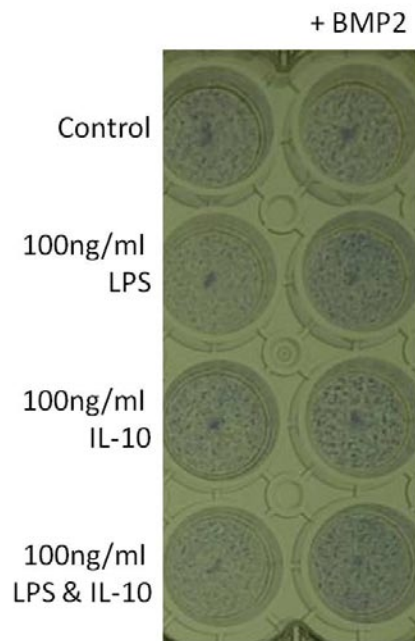
**Figure 3:** Effect of direct treatment of LPS and conditioned media on BMP-2's osteogenic effects on MDSCs. **A.** Direct treatment of 100 ng/ml of LPS, 100 ng/ml IL-10 and 100 ng/ml of LPS and IL-10 in RPMI 1640 media. **B.** MDSCs cultured in 48 hour J774a.1 control CM, LPS CM, IL-10 CM and LPS and IL-10 CM.

Quantification of ALP expression captured in brightfield images of MDSCs treated with 48 hour J774a.1 CM confirmed qualitative estimates (Figure 3). Apoptosis and morphological changes observed with C2C12 cultured in LPS CM were absent, cells appeared to have



**Figure 2:** Quantitation of brightfield images captured at 10X of MDSCs treated with J774a.1 48 hour conditioned media.

proliferated and ALP staining was positive. To determine that these results were valid, I tested all the batches of conditioned media on C2C12s. Results indicated that BMP-2 mediated osteoblastogenesis was indeed inhibited by LPS CM tested on C2C12s, while IL-10 plus LPS



**Figure 4:** Effect of direct treatments of LPS and IL-10 on BMP-2 mediated osteoblastogenesis of hMSCs.

CM rescued BMP-2 mediated osteoblastogenesis. Thus, these results are not due to a lack of LPS-stimulated macrophage secretions in the harvested CM, but rather that MDSCs are able to withstand the effects of LPS CM components, such as  $\text{TNF-}\alpha$ , IL-6, and NO to differentiate towards bone in the presence of BMP-2.

#### 4.3.3 Human mesenchymal stem cells

Having tested MDSCs and received surprising results, I was eager to determine if hMSCs would yield similar results. However, I have not been able to complete the conditioned media set, and so I present only the data from direct treatments using 100ng/ml of LPS, 100ng/ml of IL-10, and LPS plus IL-10(100ng/ml each; Figure 4).

Non-BMP-2 treated as well as BMP-2 treated cells stained positive for ALP; this indicates that hMSCs either spontaneously differentiate towards bone, or normally express a fairly high amount of ALP. ALP is used as a stemness marker for some stem cell lines but these are mostly embryonic stem cells and not hMSCs [22]. However, these results do show that 100ng/ml of BMP-2 did not increase ALP expression, and 100ng/ml of LPS was unable to decrease ALP expression. Hence, we cannot conclude if hMSCs behave similarly to C2C12s, MC3T3s and MDSCs as none of the other cell types displayed similar levels of ALP expression with or without BMP-2 treatment. It will be interesting to determine if hMSCs respond to the components of LPS CM.

#### 4.3.4 C2C12 cells

I failed to observe LPS mediated inhibition of BMP-2 osteogenesis in C2C12s under my standard conditions, so I varied my treatment conditions, treatment times, and type of LPS to

| Media    | Culture Conditions                                       | BMP-2 | ALP stain | Notes                                 |
|----------|--|-------|-----------|---------------------------------------|
| RPMI1640 | 24hour BMP-2 pretreatment                                | yes   | ++++      |                                       |
| RPMI1640 | 24hour BMP-2 pretreatment, then LPS                      | yes   | ++++      |                                       |
| RPMI1640 | 24hour BMP-2 pretreatment, then IL-10                    | yes   | ++++      |                                       |
| RPMI1640 | 24hour BMP-2 pretreatment, then LPS and IL-10            | yes   | ++++      |                                       |
| RPMI1640 | 48hour 10ng/ml IFN- $\gamma$ pretreatment                | yes   | ++++      |                                       |
| RPMI1640 | 48hour 10ng/ml IFN- $\gamma$ pretreatment, LPS           | yes   | ++++      |                                       |
| RPMI1640 | 48hour 10ng/ml IFN- $\gamma$ pretreatment, IL-10         | yes   | ++++      |                                       |
| RPMI1640 | 48hour 10ng/ml IFN- $\gamma$ pretreatment, IL-10 and LPS | yes   | ++++      |                                       |
| RPMI1640 | none   | yes   | ++++      |                                       |
| RPMI1640 | 1 $\mu$ g/ml LPS   | yes   | ++++      |                                       |
| RPMI1640 | 10 $\mu$ g/ml LPS  | yes   | ++++      |                                       |
| RPMI1640 | none   | yes   | ++++      | Experiment was conducted over 10 days |
| RPMI1640 | LPS  | yes   | ++++      | Experiment was conducted over 10 days |
| RPMI1640 | IL-10  | yes   | ++++      | Experiment was conducted over 10 days |
| RPMI1640 | LPS and IL-10  | yes   | ++++      | Experiment was conducted over 10 days |
| RPMI1640 | none   | yes   | ++++      |                                       |
| RPMI1640 | 100ng/ml PG-LPS  | yes   | ++++      |                                       |
| RPMI1640 | 1 $\mu$ g/ml PGLPS                                       | yes   | ++++      |                                       |
| RPMI1640 | 10 $\mu$ g/ml PGLPS                                      | yes   | ++++      |                                       |
| RPMI1640 | 100ng/ml PGLPS and IL-10                                 | yes   | ++++      |                                       |
| RPMI1640 | 1 $\mu$ g/ml PGLPS and IL-10                             | yes   | ++++      |                                       |

**Table 1:** Experimental conditions of LPS and BMP-2 treated C2C12s. [LPS] = 100ng/ml, [IL-10] = 100ng/ml, [BMP-2] = 100ng/ml.

determine if these changes would result in LPS mediated inhibition of BMP-2 action in C2C12s (Table 1). The results indicate that I was unable to inhibit BMP-2 mediated osteoblastogenesis in C2C12s under any of these conditions, suggesting that LPS may not affect C2C12 differentiation.

#### 4.4 Discussion

The responses of MC3T3s to direct treatments of LPS and conditioned media are similar to those of C2C12, suggesting that cells of differentiative capacity similar to C2C12 and cells further along the differentiation pathway may respond similarly to such conditions. I was puzzled by our inability to show that PGLPS inhibited BMP-2-mediated ALP expression. Xing et. al. used osteogenic media (10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/ml ascorbic acid, and 10 nM dexamethasone) rather than BMP-2, to stimulate bone formation. Thus, our dissimilar results are probably due to the difference in stimulation method.

MDSCs are not responsive to LPS or LPS CM from J774a.1 or FSDCs. These results are different from those obtained with C2C12s. However, MDSCs are primary cells isolated from mouse gastrocnemius muscle, and all data obtained for MDSC experiments in this project were from one round of cell isolation. These primary

| Treatment groups                 | ALP expression |        |       |       |
|----------------------------------|----------------|--------|-------|-------|
|                                  | C2C12s         | MC3T3s | MDSCs | hMSCs |
| Control                          | -              | -      | -     | +     |
| BMP-2                            | +              | +      | +     | +     |
| LPS + BMP-2                      | +              | +      | +     | +     |
| LPS + IL-10 + BMP-2              | +              | +      | +     | +     |
| 100ng/ml PGLPS + BMP-2           | +              | n.a.   | n.a.  | n.a.  |
| 1 $\mu$ g/ml PGLPS + BMP-2       | +              | +      | n.a.  | n.a.  |
| 10 $\mu$ g/ml PGLPS + BMP-2      | +              | +      | n.a.  | n.a.  |
| J774a.1 control CM               | -              | -      | -     | n.a.  |
| J774a.1 control CM + BMP-2       | +              | +      | +     | n.a.  |
| J774a.1 LPS CM + BMP-2           | -              | -      | +     | n.a.  |
| J774a.1 (LPS + IL-10) CM + BMP-2 | +              | +      | +     | n.a.  |
| FSDC control CM                  | -              | n.a.   | -     | n.a.  |
| FSDC control CM + BMP-2          | +              | n.a.   | +     | n.a.  |
| FSDC LPS CM + BMP-2              | -              | n.a.   | +     | n.a.  |
| FSDC (LPS + IL-10) CM + BMP-2    | +              | n.a.   | +     | n.a.  |

**Table 2:** Summary of experimental results for C2C12, MC3T3, MDSC and hMSC. [BMP-2] = 100ng/ml, [LPS]= 100ng/ml, [IL-10] = 100ng/ml unless otherwise noted. Experiments that have not been carried out are marked as non applicable (n.a). + denotes positive ALP staining and – denotes negative ALP staining.

cells respond differently when isolated from female versus male mice [5], and are known to have much variability between animal isolates. Hence it is important to repeat these experiments with at least 2 more different isolates to verify that BMP-2 mediated osteoblastogenesis in MDSCs is not affected by LPS and LPS CM. However, if BMP-2 actions in MDSCs are unaffected, it could be due to the increased ability of MDSCs to withstand oxidative stress [23]. The ability to withstand oxidative stress in an inflammatory situation could be the body's answer to tissue remodeling despite inhospitable microenvironments, however, further experiments must be carried out before this can be determined.

The final stem cell type tested, hMSCs, also provided interesting results. Should this cell line behave similarly to the MDSCs, it could suggest that i) coincidentally, these two stem cell lines just happen to be particularly resistant to inflammatory conditions or ii) stem cells lines that are less committed towards a specific lineage have additional alternatives to cope with inflammatory conditions, so that they are able to respond to BMP-2 action. However, further experiments must be carried out before any conclusions can be drawn about the multipotency of stem cells and their ability to withstand inflammatory conditions. Additionally, as hMSCs appear to express high levels of ALP in the presence and absence of BMP-2, alternative methods to evaluate the response of hMSCs towards LPS CM must be developed.

C2C12s may be said to respond to LPS treatment but I have been unable to have LPS inhibit BMP-2 mediated osteoblastogenesis in C2C12s, despite trying PG-LPS, increased LPS exposure and other changes in experimental conditions. Together, these data indicate that different cell lines respond very differently to the same experiment *in vitro* (Table 2). Thus it is important to verify experimental results with several cell lines.

While it is interesting to note how stem cells respond to LPS *in vitro*, it is unlikely that stem cells will ever encounter LPS *in vivo* without the presence of the innate and acquired immune system. It is also unlikely that the effect of LPS on stem cells would be greater than the secretions, elicited by LPS treatment from the innate and acquired immune system on stem cells. Thus, even though LPS may affect stem cell proliferation [9], attention should be directed to the inflammatory milieu that alters stem cell differentiation and delays regeneration.

While data from previous chapter indicate that an inflammatory milieu inhibits C2C12 differentiation, data from this chapter indicates that these results cannot be extrapolated to all stem cell lines currently utilized by researchers studying bone formation. Nonetheless, this chapter serves to highlight the differences between the stem cell lines. Further experiments, *in vitro* and *in vivo* must be conducted to determine the effect of an inflammatory microenvironment on stem cell differentiation and survival.

## **4.5 Acknowledgements**

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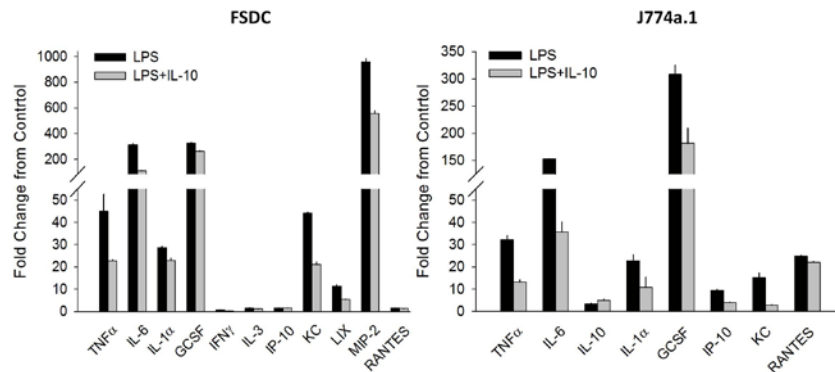
## Chapter 5: Conclusions and appendices

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## 5.1 Conclusions and further work

My thesis work provides a link between osteoblastogenesis and inflammation. The studies presented in the previous chapters focused on the effect of activated macrophages and dendritic cells on BMP-2-mediated stem cell differentiation. I also tested different stem cell lines to determine if this effect was representative of all stem cell lines. I demonstrated that LPS activates J774a.1s toward a classic inflammatory phenotype (M1), and that I could decrease CD86 (M1 marker) expression with the use of IL-10 plus LPS. IL-10 treatment alone upregulated CD163

expression (M2c marker). Likewise, I demonstrated that LPS caused an increased secretion of NO and an upregulation of CD86 in dendritic cells, while LPS plus IL-10



**Figure 1:** List of chemokines and cytokines whose secretion is upregulated by LPS stimulation, and down regulated by LPS plus IL-10 stimulation in FSDCs and J774a.1s.

treatment decreased NO secretion and CD86 expression. In addition to these markers, I determined that LPS stimulation of FSDCs and J774a.1s also upregulated a select group of pro-inflammatory cytokines and chemokines. While dendritic cells and macrophages upregulated many similar pro-inflammatory mediators (e.g. TNF- $\alpha$  and IL-6), there were also differences. Figure 1 is a comparison of the list of secreted cytokines and chemokines whose expression is upregulated with LPS stimulation of FSDCs and J774a.1s and down regulated by LPS plus IL-10

treatment. IL-10 treatment of FSDCs and J774a.1s did not increase expression of these cytokines and chemokines.

Subsequently, I demonstrated that media conditioned by LPS-activated dendritic and macrophage cells inhibited BMP-2 mediated osteoblastogenesis in C2C12s. However, LPS plus IL-10 treated macrophage and dendritic cell conditioned media was permissive for stem cell differentiation. I then proceeded to test other cells types, MC3T3s, MDSCs and hMSCs, to determine if different stem cells of varying multipotentialities would respond different to inflammatory environments. As I have not completed testing of hMSCs, I am unable to provide any definitive conclusions. However, should the hMSCs respond similarly to the MDSCs, this would suggest that stem cells with greater multipotentialities are able to respond to BMP-2 despite the inflammatory environment. If this is true, it would be interesting to determine why and how these multipotent cells are able to respond in inflammatory environments, as well as how this affects and impacts implant remodeling and diseases such as, heterotopic ossification.

My studies using mouse dendritic cells have yielded slightly different results from data of studies in the literature using human dendritic cells. To resolve these differences, I should test human dendritic cells to determine whether the differences stem from species variability or experimental methodology. Additionally, I should further refine the interactions between dendritic cells, macrophages and T cells with stem cells. I have demonstrated the ability to modulate the inflammatory phenotype of dendritic cells and macrophages early on using IL-10, however, the neutrophil is the first cell type to respond to a wound site. Hence, if IL-10 is able to affect the neutrophil similarly, then this suggests that the use of IL-10 will help to control a wound microenvironment by targeting cells of the innate immune system from the start.

Throughout my studies, I have used ALP as an indicator of stem cell osteoblastogenesis. While this is an accepted and suitable marker, I should include other markers as well, such as quantitative PCR of other markers of differentiation such as Osterix and Runx2, and mineralization assays. Subsequently, I should also test the efficacy of IL-10 and BMP-2 *in vivo* in an inflammatory environment. *In vitro* experiments have indicated that the presence of IL-10 permits BMP-2-mediated osteoblastogenesis despite LPS stimulation, but more definitive answers regarding the capabilities of IL-10 can be obtained with *in vivo* experiments. However, it will be crucial to determine the dynamics of IL-10's binding capacity (so as to determine if IL-10 can be delivered locally versus systemically) and concentration of IL-10 required for a successful *in vivo* experiment. Last but not least, I would like to extend my model to other tissue types apart to determine if inflammatory environments affect cartilage, tendon and muscle formation in a similar manner. Answers to these potential projects will help to refine the interactions between the fields of immunology and regenerative medicine.

My data has suggested that I am able to modulate inflammatory responses from dendritic cells and macrophages using IL-10, thus creating a permissive microenvironment for stem cell differentiation. As dendritic cells and macrophages respond quickly to any wound environment, these are the cells that will be responsible for establishing the microenvironment. Thus, if I am able to modify the behavior of these cells, I can establish a hospitable microenvironment from the beginning, thus minimizing the need for excessive amounts of growth factors, such as BMP-2 in grafts. Additionally, being able to deliver IL-10 locally and not systemically will result in a local modulation of the immune response, minimizing unwanted side effects to the rest of the body. These data help to link vital pieces of information regarding bone regeneration and

inflammation, and hopefully, these data will encourage the clinical application of IL-10 to control inflammation.

## **5.2 Appendices**

### **5.2.1 J774a.1 cell culture**

I have made a few modifications to the culture conditions of J774a.1. ATCC recommends DMEM with 10% FBS and 1% PS, however, J774a.1 cultured in RPMI 1640 with 10% FBS and 1% PS increases macrophage responsiveness, making it easier to determine if J774a.1 cells have been activated. To passage the cells, J774a.1 cells must be scraped gently as J774a.1s do not respond to trypsin. To scrape the cells properly, check that the entire edge of the scraper is in contact with the plate (not just the tip), as this will prevent undue pressure on the cells when scraping, minimizing cell death. Centrifuge at 110G for 5 minutes at 4°C before resuspending gently via pipetting to obtain a homogenous single cell suspension.

Unactivated cells are round, while moving unactivated cells will appear polar. Activated cells will have multiple cellular projections, appear vesiculated, and under higher magnification, a ruffled membrane. These cells are easily activated, e.g. low CO<sub>2</sub> concentration, non-sterile technique, and centrifugation at higher speeds. In a field of view under 10X magnification, there should be no more than 1 to 2 activated cells. J774a.1s should be cultured for no longer than 2 months before thawing a new vial of cells.

### **5.2.2 FSDC cell culture**

I have made a few modifications to the culture conditions of FSDC. ATCC recommends RPMI 1640 with 10% FBS and 1% PS. I noticed that LPS stimulated NO production decreased

with passage number. This was confirmed per personal discussion with the Lopes lab (Universidade de Coimbra, Coimbra, Portugal), and they recommended having at least 10mM of Glutamax in the media. As RPMI 1640 contains 2mM glutamine, I added 9mM of Glutamax to the media to make it a total of 10mM. FSDCs respond to trypsin, but these cells must be incubated in trypsin for a short period time. Hence, with these cells, it is important that the trypsin is warmed to 37°C, and ~ 4 ml of trypsin should be added to a 100mm petri dish for no longer than 1 minute for the cells to detach. The petri dish can be tilted from side to side, or tapped VERY gently to dislodge the cells. FSDCs should only be cultured to 70% confluency.

Unactivated cells have one or two short projections with a ruffled membrane (looks like a frilly skirt), while activated cells will have multiple long projections. These cells are easily activated, e.g. low CO<sub>2</sub> concentration, non-sterile technique, and centrifugation at higher speeds. In a field of view under 10X magnification, there should be no more than 1 to 2 activated cells. FSDCs should be cultured for no longer than 1 month before thawing a new vial of cells. It is important to freeze at least 2 vials of cells ( $1 \times 10^6$  cells/vial) for every vial thawed. Freeze down media is complete FSDC media with 5% dimethylsulfoxide.

### **5.2.3 Conditioned media storage**

Conditioned media should be frozen on dry ice and then transferred immediately to the -80°C freezer. Ideally, conditioned media should be used no later than 1 month after harvest, and should not be frozen and thawed repeatedly. Conditioned media must always be tested with C2C12s as an internal control, in addition to observing morphological changes in the LPS-treated macrophages and dendritic cells. It is critical to observe cell morphology (especially FSDCs) prior to treatment with LPS or IL-10 for conditioned media harvest. Activated FSDCs will not respond to LPS treatment, resulting in a nullified batch of CM. Poor mixing of LPS and IL-10

can also result in poor batches of CM. To ensure good mixing, use a pipette to mix LPS and(or) IL-10 with cell culture media. DO NOT attempt to achieve good mixing by swirling the media in the plate.

#### 5.2.4 LPS and IL-10 concentrations

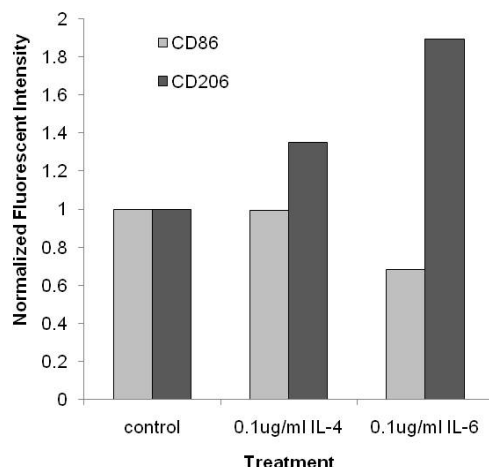
To determine the appropriate dose of LPS and IL-10 to use, I treated the macrophages and dendritic cells with a variety of doses. I determined that 100ng/ml of LPS resulted in a suitable increase in CD86 expression and NO secretions, allowing us to determine that the cells were activated. Additionally, 100ng/ml of LPS treatment resulted in ample secretions that it inhibited C2C12 differentiation. Additionally, I found that 100ng/ml of LPS treatment did not saturate the response, allowed for modulation.

Co-treatment of IL-10 with LPS indicated that increased concentrations of IL-10 (e.g.200ng to 500ng/ml) did not decrease LPS stimulated increases in CD86 expression significantly in J774a.1. Hence, I decided that equal treatment of IL-10 along with LPS was

sufficient to provide a permissive environment for BMP-2's osteogenic effects.

#### 5.2.5 Tested cytokines and antibodies

I tested a series of antibodies and cytokines and found that these did not work as well as the cytokines and antibodies used in the previous chapters of this thesis. For example, treatment of IL-6 did not cause an increase in



**Figure 2:** Flow cytometry analysis of CD86 and CD206 expression of J774a.1s treated with IL-4 and IL-6 for 24 hours.

CD86 (M1 marker) expression in J774a.1s, but instead caused an almost 2 fold increase in CD206 (thought to be a M2a or M2c marker). IL-4, described in the literature as an M2a marker, did not result in a significant increase in CD206, but as expected, did not increase CD86 expression (Figure 2). I treated J774a.1s with IFN- $\gamma$ , IL-12 and IL-1 $\beta$  and found that these did not increase CD86 expression as much as LPS. Additionally, I tested TGF- $\beta$ , IL-23, IL-4 and IL-13 in addition to IL-10. TGF- $\beta$  was not able to decrease LPS-mediated NO secretions or CD86 expression. IL-23 increased CD86 expression but not significantly, and did not increase CD163 expression. IL-4 and IL-13 were unable to increase CD163 expression as much as IL-10 and hence, IL-10 was chosen. Additionally, IL-4 and IL-13 are described to be M2a stimulators in the literature. As I was more interested in M2c macrophages, it made sense to pursue IL-10 over these 2 cytokines. IL-6 is known to be an inflammatory or anti-inflammatory cytokine depending on the microenvironment, as Figure 2 indicates, IL-6 appears to play more of an anti-inflammatory role when treated with J774a.1s alone, however as its function is highly dependant on the microenvironment. In *in vivo* experiments, I would not be able to control the microenvironment, thus I would not be able to predict IL-6 function, thus I chose IL-10 over IL-6, as IL-10 can be relied upon to function as an anti-inflammatory.

#### **5.2.6 Flow cytometry with FSDCs and J774a.1s**

FSDCs are particularly sensitive towards the preparation protocol and the process for flow cytometry. As I have not been able to obtain live FSDCs post-sort, most flow cytometric work regarding FSDCs have been analytical in nature. J774a.1 cells are not as sensitive as FSDCs and can survive a FACS procedure, however, do not keep J774a.1 cells out of media for more than 5 hours. Keep J774a.1 cells at 4°C as often as possible.