ABSTRACT

Title of Dissertation: THE ENDOGENOUS REGULATION OF THE HUMAN MACROPHAGE ACTIVATION RESPONSE

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Macrophages are innate immune cells that participate in host defense to invading pathogens. They are powerful producers of cytokines and inflammatory mediators due to their efficient recognition of pathogen associated molecular patterns (PAMPs) via toll like receptors (TLRs). We and others have shown that the activation response to PAMPs is transient. In the present work, we demonstrate that stimulated macrophages produce adenosine and prostaglandin E2, which function as regulators of the macrophage activation response. Macrophages also upregulate receptors for these regulators to terminate inflammation and promote wound healing. We performed high throughput RNA sequencing to characterize the transcriptomes of human monocyte-derived macrophages in response to stimulation with LPS + Adenosine or LPS + PGE_2. These cells exhibited a decrease in inflammatory transcripts and an increase in transcripts associated with cell growth and repair when compared to cells stimulated in the absence of these regulators.

Macrophages can be generated from precursor cells in response to two different growth factors; M-CSF (macrophage colony stimulating factor) and GM-CSF (granulocyte-macrophage colony stimulating factor). M-CSF is expressed constitutively in a variety of tissues, while GM-CSF is expressed primarily in the lung, but can be

induced in other tissues under inflammatory conditions. We demonstrate that human macrophages differentiated in M-CSF readily adopt an anti-inflammatory, growth promoting phenotype in response to LPS + Adenosine or LPS + PGE_2, while macrophages differentiated in GM-CSF do not. This observation suggests that M-CSF derived human macrophages may be better able to alter their activation state in response to surrounding signals in order to maintain homeostasis. GM-CSF derived macrophages, in contrast, may undergo a more prominent activation response that is associated with inflammation and tissue destruction due to their inability to efficiently respond to resolving molecules.

THE ENDOGENOUS REGULATION OF THE HUMAN MACROPHAGE ACTIVATION RESPONSE

by

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Dedication

I would like to dedicate this dissertation to my parents,

Babak and Stacey Hamidzadeh.

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List of Abbreviations

Ado	adenosine
ATP	adenosine triphosphate
BMDM	bone marrow derived macrophage
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
COX	cyclooxygenase
DEG	differentially expressed gene
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein activated by cAMP
GM-CSF	granulocyte macrophage colony stimulating factor
GO	gene ontology
GPCR	g-protein coupled receptor
HUVEC	human umbilical vein endothelial cell
IFN	interferon
IFNAR	interferon alpha receptor
IRF	interferon regulatory factor
IL-6	interleukin-6
IL-10	interleukin-10
IL-12p40	interleukin-12p40
JAK	Janus kinase
KO	knockout
Log_2FC	log_2 fold change

LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
mRNA	messenger ribonucleic acid
NK	natural killer
PAMP	pathogen associated molecular pattern
PAP	pulmonary alveolar proteinosis
PGE_2	prostaglandin E2
PKA	protein kinase A
PCA	principal component analysis
PLAUR	plasminogen activator, urokinase receptor
RT-PCR	reverse transcriptase polymerase chain reaction
RNA-seq	RNA sequencing
SLE	systemic lupus erythematosus
STAT	signal transducing
THBS1	thrombospondin-1
TGFβ	transforming growth factor beta
TLR	toll like receptor
TNF	tumor necrosis factor
TYK	tyrosine kinase
UMAP	uniform manifold approximation and projection
VEGFa	vascular endothelial growth factor alpha
WT	wild type

1 Introduction

Parts of this chapter are adapted from published works:

Hamidzadeh, K., S.M. Christensen, E. Dalby, P. Chandrasekaran, and D.M. Mosser. 2017. Macrophages and the Recovery from Acute and Chronic Inflammation. *Annu. Rev. Physiol.* 79:567–592. doi:10.1146/annurev-physiol-022516-034348.

Hamidzadeh, K., and D.M. Mosser. 2016. Purinergic Signaling to Terminate TLR Responses in Macrophages. *Front. Immunol.* 7:74. doi:10.3389/fimmu.2016.00074.

1.1 Macrophages and Innate Immunity

Macrophages reside in almost all tissues of the body and engage in inflammatory processes in order to protect the host from invading pathogens and to fight infection. They are part of the innate immune system, which is the host's first line of defense once an immunogen has bypassed external barriers such as the skin. Macrophages are efficient phagocytes and are able to rapidly clear debris, dead cells, and microbes at the site of infection. In addition to phagocytosis, macrophages release important inflammatory mediators including cytokines in order to initiate systemic immune responses. These inflammatory macrophages are a vital component of host defense. However, the transition to an anti-inflammatory state is crucial during the resolution of infection in order to prevent damage to host tissue. The failure to resolve inflammation can result in autoimmunity.

Macrophages originate from two different sources: the embryonic yolk sac, and hematopoietic stem cells in the bone marrow[1]. During inflammation, tissue macrophages can be derived from circulating blood monocytes. These cells migrate to the tissue, or

sites of inflammation, where they encounter signals such as macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) that promote their differentiation into macrophages. It has been demonstrated that during chronic infection, macrophages from embryonic origin largely disappear, and are replaced with macrophages of hematopoietic origin in the lung, liver, and peritoneum among other tissues[2,3]. Macrophages residing in close proximity to a variety of signals exhibit dramatically different phenotypes.

1.2 Macrophage polarization

Macrophages represent a heterogeneous population of immune cells. Because they are present in so many tissues, they are exposed to a wide variety of microenvironments and must be able to respond to a wide range of stimuli. Macrophages express the family of surface receptors known as Toll-like receptors (TLRs), which are able to recognize pathogen associated molecular patterns (PAMPs). These are conserved patterns present on pathogens that the cells have evolved to recognize efficiently to mount an inflammatory immune response. In addition to PAMP signals, macrophages also recognize small molecules in their surroundings that allow them to alter their phenotype. This is termed macrophage "plasticity" and it is important that macrophage phenotypes can be reversed. Due to the plasticity of these cells, macrophages with an infinite number of diverse phenotypes may exist[4]. However, it is generally accepted that 3 main populations of macrophages may represent the polar extremes of macrophage phenotypes. These include classically activated (M1), alternatively activated (M2), and "regulatory" macrophages. While these 3 groups have been initially defined in the

mouse[5], it is proposed that macrophage activation falls along a spectrum as they exist within diverse environments within the human host.

M1 Macrophages

M1 macrophages are defined by stimulation with interferon gamma (IFNγ) + lipopolysaccharide (LPS). Early on in infection the type II interferon, IFNγ, is produced by innate and adaptive immune cells. Macrophages exposed to IFNγ are primed for secretion of large amounts of inflammatory cytokines, and reactive oxygen and nitrogen species[6]. This secretion is typically triggered by a second stimulus. Classically activated macrophages receive a secondary signal through TNF receptor or TLR stimulation. Stimulation of TLRs results in the activation of NFκB transcription factors, which induce the transcription of pro-inflammatory cytokines such as IL-12 and TNF, and many cytokines and mediators through interferon response elements. Our lab has contributed to the body of knowledge surrounding classically activated mouse macrophages by demonstrating that IFNγ primed bone marrow-derived macrophages (BMDMs) do not downregulate their own activation state[7]. This occurs due to the failure to upregulate the A2br receptor, which renders these macrophages insensitive to the endogenous anti-inflammatory effects of adenosine.

M2 Macrophages

M2 macrophages are typically induced in response to IL-4 or IL-13. These macrophages produce precursors important for collagen production. These same precursors may lead to tissue fibrosis and Th2 pathology. While IL-4 alone does not induce cytokine production in these macrophages, it does promote the induction of arginase (Arg1)[6]. IL-4 receptor stimulation also results in the activation of STAT6[8].

Alternatively activated macrophages are considered to be more susceptible to microbial infection and intracellular pathogens and this is thought to be through the metabolism of L-arginine to polyamines[9]. In this way, arginase induction diverts arginine away from NO production and provides microorganisms with necessary nutrients for survival. Surprisingly, M2 or alternatively activated macrophages have not sufficiently been characterized in humans. Preliminary, unpublished, transcriptomic data from our lab indicates that IL-4 treatment of monocyte-derived macrophages has a more limited effect on their phenotype, with little overlap with the effects of IL-4 treatment of mouse macrophages.

Regulatory Macrophages

Regulatory macrophages (R-Mϕ) were first defined in our laboratory in mouse bone marrow derived macrophages[10,11]. They require a combination of an inflammatory signal, such as TLR stimulation and a secondary signal, which can include immune complexes, adenosine or prostaglandin E_2[5,12]. The hallmark of regulatory macrophages is the reciprocal change of IL-10 and IL-12 cytokine production. In mouse, R-Mϕ produce high levels of IL-10 compared to LPS stimulation alone. IL-10 is an anti-inflammatory cytokine, which can act to suppress macrophage activation and it is critical for the resolution of inflammation. R-Mϕ stimulated with adenosine and PGE_2 produce decreased amounts of IL-12 despite the inflammatory TLR signal. IL-12 is involved in the initiation of cell-mediated immune responses. Our lab has identified a number of other highly upregulated genes in regulatory macrophages, including growth and angiogenic factors, and we were able to demonstrate that these macrophages are distinct from classically and alternatively activated macrophages. From RNA sequencing data, it

is hypothesized that regulatory macrophages are not only involved in decreasing inflammation but also involved in promoting homeostasis.

1.3 Toll like receptors:

The innate immune system is our first line of defense against foreign antigens. It is critical that the innate immune response acts quickly and efficiently in order to destroy invading pathogens. Through the evolutionary process, mammalian innate immune cells became equipped with a number of cell surface and intracellular receptors, known as Toll-like receptors (TLRs), that are able to recognize highly conserved and repetitive sequences known as pathogen associated molecular patterns (PAMPs)[13]. In this way, TLRs can rapidly trigger an immune response to different pathogens.

TLR signaling

TLRs are type I transmembrane glycoproteins. The extracellular portion of TLRs is composed of between 16 and 28 leucine rich repeats that contain conserved amino acid motifs[14]. TLR signaling originates with dimerization of the receptor and subsequent recruitment of adaptor proteins to its intracellular TIR domain[15]. There are five adaptors that can initiate TLR signaling: MyD88, MAL, TRIF, TRAM, and SARM[16]. The adaptor MyD88 is critical for the production of inflammatory cytokines by all TLRs. MyD88 recruits IRAK-4 to the receptors, which then activates IRAK-1 and IRAK2[17]. Activated IRAKs associate with TRAF6, and this complex can then activate two pathways[18]. The first is the MAPK pathway leading to AP-1 transcription factor activation. The second is the phosphorylation of IκB kinase, which dissociates from the IKK complex in order to activate the NF-κB transcription factor. These transcription factors lead to the production of many inflammatory genes.

Lipopolysaccharide and TLR4

One of the most widely used tools to activate macrophages is lipopolysaccharide (LPS), a TLR ligand. LPS is a glycolipid present in the outer membrane of gram-negative bacteria including *E. coli*. The structure of LPS consists of a hydrophobic lipid A component, hydrophilic core of polysaccharides, and O-antigen[19]. Trace amounts of LPS in certain tissues, such as blood, can lead to a fatal disease called sepsis due to uncontrollable amounts of inflammation and this toxicity is primarily due to the lipid A component[20]. LPS is recognized by TLR4, which forms a heterodimer with myeloid differentiation factor 2 (MD-2) that aids in LPS binding[21,22]. Two additional proteins are also required for LPS recognition, LPS binding protein (LBP) and CD14[23]. LBP facilitates the association of LPS with CD14[23]. CD14 is expressed on myeloid cells and binds to LPS, subsequently presenting it to the TLR4/MD-2 complex[24]. CD14 is also required for endocytosis of TLR4, which is part of the signal transduction process[25]. TLR4 and its interaction with LPS is one of the most studied and well-characterized processes in innate immunity.

1.4 Adenosine in the Immune System:

Adenosine is a purine nucleoside circulating at low levels in the blood and in tissue. Adenosine concentrations surrounding cells can increase via nucleoside transport proteins in the cell membrane, or via ATP catabolism (Illustration 1). ATP and adenosine can be released in local environments by platelets, dead and dying cells, tumors, and from endothelial cells, among other sources. It is proposed that all immune cells can contribute to adenosine concentrations due to the fact that ATP is produced by

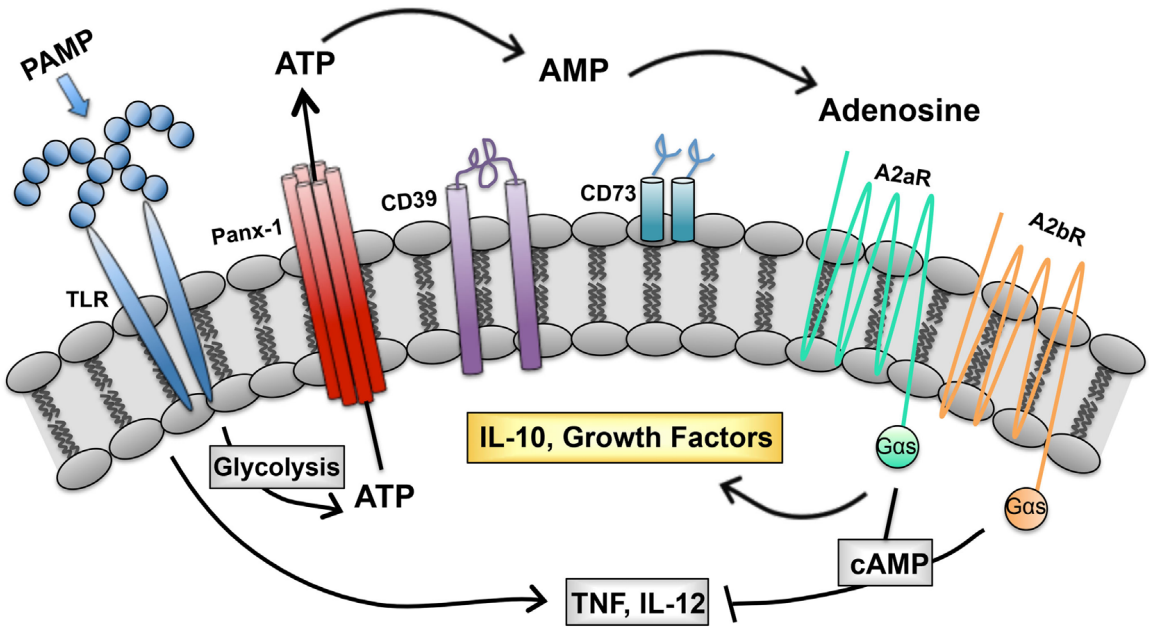

Illustration 1. ATP catabolism at the macrophage surface. ATP is converted to adenosine via the action of two ecto-enzymes, CD39 and CD73. Adenosine signals through the A2a and A2b receptors which are coupled to Gαs proteins. This leads to an increase in intracellular cAMP levels.

Figure from:
Hamidzadeh, K., and D.M. Mosser. 2016. Purinergic Signaling to Terminate TLR Responses in Macrophages. Front. Immunol. 7:74. doi:10.3389/fimmu.2016.00074.

glycolysis and immune cells express the ecto-enzymes required to convert this ATP to adenosine.

ATP Release and Hydrolysis

ATP release from resting macrophages is low, but ATP release is significantly increased following TLR activation[26]. Some of the cytosolic ATP generated following TLR stimulation is released into the extracellular milieu through pannexin-1 channels. This ATP is catabolized by macrophages in a coordinated two-step process. ATP is first hydrolyzed to AMP by the surface ecto-enzyme CD39 (E-NTPDase1) in a Ca^{2+} and Mg^{2+} dependent process[27]. Next, AMP is rapidly converted to adenosine by CD73 (Ecto5'NTase)[28]. These two enzymes and their expression level on the macrophage surface can greatly affect the concentration of adenosine in the extracellular environment directly adjacent to the cell.

Adenosine Receptor Signaling

Macrophages respond to adenosine via signaling through the P1 class of seven transmembrane G-protein coupled receptors (GPCR), which includes: A1R, A2aR, A2bR, and A3R[29]. The A1 and A3 receptors are coupled to the G_i family of proteins, which act to decrease cAMP levels. A2a receptors are $G\alpha_s$-coupled receptors which act to increase intracellular cAMP, and they are high affinity for adenosine[30,31]. Similarly, A2b receptors can signal through $G\alpha_s$ or G_q proteins, also leading to increased cAMP, but these receptors are low affinity for adenosine[30,32]. In combination with TLR stimulation, adenosine drives the transition from a pro-inflammatory to a regulatory macrophage[4]. Adenosine is immuno-suppressive in mouse macrophages as it leads to increased IL-10 release and decreased TNF and IL-12 release[26]. High-throughput RNA-sequencing data

from our lab indicated that macrophages stimulated with LPS in combination with adenosine upregulated 501 transcripts and downregulated 610 transcripts relative to LPS exposure alone[5]. Furthermore a number of the upregulated transcripts were involved in cell growth, while many of the downregulated transcripts were involved in inflammation[33].

While signaling through GPCRs controls the levels of intracellular cAMP, the role of the cAMP/PKA pathway in terms of inflammatory cytokine inhibition by adenosine receptor signaling is not fully understood. Some researchers have proposed that the inhibition of macrophage TNF production by adenosine is due to a cAMP/PKA-independent pathway, and is rather a pathway dependent on phosphatases[34]. However, others have shown that cAMP/PKA levels are linked to TNF production in an inverse manner[35]. It has also been shown that the A2bR interacts with and inhibits NFκB, and that A2bR knockout macrophages produce less IL-10 and more IL-12 and TNF[36]. Thus, adenosine may regulate macrophage phenotypes by mechanisms that have not yet been fully elucidated.

Adenosine in disease

Adenosine receptors have been associated with a variety of diseases. These receptors are expressed in many tissues including the brain, heart, spleen, muscle and lung[37,38]. This ubiquitous pattern of expression is one of the obstacles of developing therapeutics that are able to specifically target the receptors. Studies have been done that reveal a role for A2aR and A2bR in diabetes due to the fact that they are involved in the regulation of glucose as a result of increased cAMP[39–41]. There is potential for A2ar agonists to be anti-inflammatory in ischemia reperfusion injury[42]. A2aR and A2bR have

both been implicated in reducing foam cell formation, which is a feature of atherosclerosis[43,44]. However, it has been demonstrated that knocking out the A2ar has a protective outcome in a mouse model of hypercholesterolemia largely due to the fact that macrophages in these mice are pro-inflammatory and therefore reduce atherosclerotic lesions[45]. Furthermore, adenosine receptors contribute to wound healing and modulate cytokine production by macrophages of patients with chronic obstructive pulmonary disease[29,46].

1.5 Prostaglandin in the immune system:

Prostaglandins are bioactive lipids present in many tissues that are implicated in processes including proliferation, angiogenesis, and inflammation. They are part of the prostanoid family of lipids, which are synthesized step-wise from fatty acids. There are numerous prostaglandins that can be synthesized, including PGI_2, PGD_2, and PGF_2, but the most studied and widely acting prostaglandin is PGE_2. PGE_2 is typically seen as a perpetuator of inflammation, which is why it is the target of non-steroidal anti-inflammatory drugs (NSAIDs). This is true for many cell types including T and B cells. However, the opposite is true for macrophages, as PGE_2 promotes the production of anti-inflammatory molecules and the shutting off of cytokine production.

PGE_2 synthesis and secretion

First, phospholipases hydrolyze membrane phospholipids, releasing arachidonic acid[47]. Next, arachidonic acid is oxidized into PGG_2 and reduced to PGH_2 by the cyclooxygenase enzymes COX-1 and COX-2[47]. These two enzymes are highly upregulated throughout the immune system in response to pro-inflammatory signals[48,49]. Lastly, PGH_2 is converted into PGE_2 via three synthases: mPGES-1, mPGES-2, and

cPGES[49]. PGE_2 levels are regulated by both its synthesis and by its degradation. 15-PGDH and 13-PGR are catabolic enzymes that rapidly remove PGE_2 from the cellular environment[50].

PGE_2 receptor signaling

Macrophages respond to PGE_2 via four transmembrane G-protein coupled receptors: EP1-4. EP2 and EP4 are coupled to Gαs proteins, which stimulate intracellular cAMP production[51]. In combination with TLR stimulation, PGE_2 inhibits IL-12 and TNF and partially decreases IL-6 production by macrophages[52–54]. At the same time, PGE_2 enhances IL-10 release from mouse macrophages in a PKA-dependent manner[55]. PGE_2 inhibits inflammasome activation in macrophages via signaling through the EP4 receptor, dampening the production of IL-1β[56]. Additionally, IL-17 production is increased in the presence of PGE_2 therefore promoting M2 macrophage microenvironments[57]. Our lab previously showed that mouse macrophages stimulated with LPS and PGE_2 exhibit an immuno-regulatory phenotype[58].

PGE_2 and the immune response

PGE_2 helps to regulate the activation of many cells of the innate immune system[59]. The involvement of PGE_2 in acute inflammation has been well-documented[60] but, in contrast, PGE_2 has also been demonstrated to also play a significant role in immunosuppression[60,61]. Inflammation in the lung in response to allergens and pollutants, as well as colonic inflammation, is dampened by PGE_2 through the EP4 receptor signaling on macrophages[62,63]. PGE_2 also downregulates MHC class II expression on dendritic in lymphoid organs in order to decrease antigen presentation[64]. It has been demonstrated that PGE_2 inhibits the phagocytosis of bacterial pathogens and

bacterial killing by alveolar macrophages in a dose-dependent manner[65,66]. Along with this, many bacteria and intracellular parasites, including *L. donovani*, have developed mechanisms to stimulate PGE_2 production by macrophages in order to suppress inflammation and promote survival inside the host[59,67,68].

Cyclooxygenase (COX) inhibitors are commonly used NSAID drugs that inhibit inflammatory responses. However, it has been shown that chronic inhibition of the COX enzymes in macrophages drives them towards a pro-inflammatory phenotype, partly due to lower synthesis of PGE_2[69]. Furthermore, it has been proposed that these classic inhibitors of prostaglandin synthesis may act to prolong chronic inflammation when taken during the resolving phase[70]. Macrophages are well-known producers of PGE_2[71]. Exposure of macrophages to LPS increases arachidonic acid metabolism, leading to greater PGE_2 secretion[72]. Since macrophages synthesize endogenous PGE_2, we recognize the profound effects of this molecule on the regulation of macrophage activation.

1.6 Interferons and innate immunity:

Type I Interferon

Interferons (IFNs) are cytokines that are best known for their involvement in the immune response to viral pathogens. However, IFNs also affect the response to microbial pathogens, the stimulation of antigen presentation, cell proliferation and apoptosis[73]. Type I IFNs include IFNα and IFNβ and signal through the heterodimeric IFNα receptor (IFNAR). Signaling through IFNAR activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), followed by activation of signal transducer and activator of transcription (STAT) transcription factors, STAT1 and STAT2 as well as IFN regulatory factor 9 (IRF9)[74,75]. Type I IFNs can be produced by many cells and in macrophages they

can be produced following stimulation of TLR4, TLR3, TLR7 and TLR9[18]. The effects of Type I IFN in response to bacterial infection are complicated and not fully understood. Often times they can contribute to host resistance to bacteria but sometimes they can promote bacterial survival via suppression of the innate immune response[76]. In mouse models of sepsis, Type I IFN deficiency led to a reduction in endotoxin lethality despite no change in inflammatory cytokine levels[77]. Type I IFNs also have contradicting roles in autoimmune disorders. Patients with systemic lupus erythematosus (SLE) have increased type I interferon in circulation that is thought to promote disease[78]. On the other hand, IFNβ administration is an effective treatment for multiple sclerosis[79]. The activity of Type I IFNs appears to be highly context dependent.

Type II Interferon

The Type II IFN family consists solely of IFNγ, which signals through a heterodimeric IFNγ receptor to activate JAK1 and JAK2, which subsequently activate STAT1[80]. Further downstream, IRF1 is strongly induced by IFNγ which promotes Th1 responses[73,81]. IFNγ is highly efficient at priming macrophages for enhanced bacterial killing and inflammation[82]. Its ability to enhance bacterial killing is largely mediated through nitric oxide and superoxide production[83,84]. IFNγ is used as effective treatment for patients with chronic granulomatous disease, in which phagocytes are defective in superoxide and hydrogen peroxide production[85]. Priming also acts in coordination with TLR signaling to stabilize mRNAs that encode inflammatory genes leading to more efficiency in both transcription and translation of these proteins[86,87]. Type II IFN is mainly produced by natural killer (NK) cells and Th1 cells in response to infection[88]. While it is highly effective at stimulating innate immune cells to control pathogens, it can

have negative consequences for the host. For example, IFNγ contributes to the pathogenesis of rheumatoid arthritis and SLE[89,90]. Overall, IFNγ is effective at potentiating inflammatory immune responses.

1.7 Colony-stimulating factors:

M-CSF and GM-CSF are both colony-stimulating factors that are important in myeloid cell differentiation and immune modulation. Many studies have addressed the role of these two growth factors in terms of cell survival, as they prolong the life of macrophages and are regulators of hematopoiesis. A few gene expression studies have been done comparing macrophages differentiated in each growth factor in both mouse and human[33,91–93]. However, extensive research has not been done on the inflammatory response of both M-CSF and GM-CSF macrophage populations and their sensitivity to endogenous mediators.

M-CSF

M-CSF signals through the C-FMS receptor or CSF-1R. This receptor is a single pass type I membrane protein and has a tyrosine kinase domain[94]. Upon binding of M-CSF, receptors dimerize. Downstream of this receptor is PI3K, Src family kinases, Ras, ERK1/2, Akt, and PLCγ2 signaling[94–96]. SHIP2 tyrosine phosphorylation following M-CSF stimulation leads to reduced Akt activation and inhibition of NFκB gene transcription independently of a functional SH2 domain, unlike SHIP1[97]. Macrophages have been shown to regulate the levels of M-CSF in circulation by CSF-1R mediated endocytosis[98].

M-CSF is produced ubiquitously by many cells types including endothelial cells, lymphocytes, fibroblasts and monocytes[99]. CSF1R is critical for the maintenance of

monocyte and macrophage populations. Mice lacking CSF1R are deficient in several tissue macrophage populations including Kupffer cells, microglia and skin macrophages[100]. The addition of recombinant human M-CSF in mice resulted in a significant increase in blood monocytes in circulation and an expansion of resident macrophage populations[101]. Increased levels of M-CSF have been reported in numerous diseases including arthritis, pulmonary fibrosis, inflammatory bowel disease, and cancer[102]. A number of monoclonal antibodies and small molecules targeting the CSF-1R have been in clinical trials for targeting solid tumors[102].

GM-CSF

GM-CSF signals via the βc receptor Type I cytokine receptor family, similarly to IL-3 and IL-5. A ternary complex between the βc receptor, the GM-CSF receptor specific alpha chain and the GM-CSF molecule is required for signaling[103]. Downstream of the GM-CSF receptor complex is JAK2/STAT5 activation, MAPK, and PI3 kinase/Akt pathway activation[104,105]. GM-CSF also activates NFκB by interaction of the alpha chain of the GM-CSF receptor and IκB kinase[106]. Further downstream, GM-CSF activates IRF5 transcription factor, which shapes macrophage polarization[107]. It has recently been shown to also activate IRF4 in order to drive the CCL17.

GM-CSF, like M-CSF, can also be produced by endothelial cells, epithelial cells, and fibroblasts that are activated, but is mainly produced by TH_{17} T cells and innate lymphoid cells in response to infection or trauma[99]. The inflammatory cytokine, IL-1 appears to be particularly important in the induction of GM-CSF from a number of cell types[108–111]. There are a multitude of diseases in which the circulating levels of GM-CSF are increased including encephalomyelitis, rheumatoid arthritis, systemic

inflammation[112], and even allergic responses[113]. Multiple clinical trials for monoclonal antibodies to GM-CSF have been undertaken in the context of rheumatoid arthritis, asthma and multiple sclerosis[102]. The main tissue in which GM-CSF is constitutively expressed is the lung. Humans with a point mutation in the common beta chain of the GM-CSF receptor develop a condition called pulmonary alveolar proteinosis (PAP)[114]. Mice lacking GM-CSF develop lung abnormalities and also have symptoms mirroring PAP[115]. Additionally, these GM-CSF deficient mice are more susceptible to local infections, in the lung particularly[115]. For example, neutralization of GM-CSF in mice led to a reduction in protective immunity to histoplasma infection, indicating that this cytokine plays a role in host defense[116].

1.8 Macrophage activation during disease or injury

Sepsis

Macrophages are critical drivers of inflammation due to their potent cytokine producing capabilities. In the case of sepsis, this high level of cytokine production, known as a "cytokine storm", by macrophages in the early phases of infection is what leads to a high mortality rate for the host. Sepsis is a serious condition that affects over 30 million people per year, and results in over 5 million deaths. The blockade of inflammatory cytokines such as TNF and IL-1 have proven to be inefficacious in the reversal of sepsis. However, the anti-inflammatory cytokine IL-10 contributes significantly to the progression of the disease. Cecal ligation and puncture model (CLP) in IL-10 knockout mice was associated with 15-fold higher serum levels of TNF, but treatment with recombinant IL-10 led to improved survival and a longer therapeutic window for rescue surgery[117]. Regulatory macrophages are partly defined by their ability

to secrete increased amounts of IL-10. A previous graduate student in our lab demonstrated in mice, that macrophages stimulated with LPS + Adenosine and LPS + PGE_2 had a protective effect on mouse survival when injected into the peritoneum of the mice in an endotoxemia model[118]. Additionally, earlier work in our lab demonstrated that FcγR ligation promotes IL-10 production in mice, leading to protection in sepsis models[11]. Therefore, multiple methods of generating these regulatory macrophages can have a potentially therapeutic effect in sepsis.

Tumor associated macrophages

Macrophages are one of the main immune cells residing in the tumor microenvironment. These macrophages are termed tumor associated macrophages (TAMs) and generally are not activated against the tumor. The production of pro-inflammatory cytokines by macrophages in the tumor environment is critical for the activation of cytotoxic T cell responses[119]. A few treatment strategies such as checkpoint inhibitors and CAR T cell therapies targeting tumor antigens have proven successful in many cancer patients. However, it has been demonstrated that in those patients in which these immunotherapies are unsuccessful, there can be an abundance of tumor associated myeloid cell infiltrates, including macrophages[120]. These macrophages display an immunosuppressive, and pro-angiogenic phenotype. Tumors secrete immunosuppressive molecules, including both adenosine and PGE_2, which we believe shapes the macrophage phenotype to one that is anti-inflammatory and angiogenic in order to support the tumor's growth[121,122]. Therefore, methods of reversing or preventing this macrophage phenotype could prove beneficial in cancer patients.

Intracellular Parasitic Infections

Macrophages are host to numerous parasitic and bacterial pathogens. These pathogens include *Leishmania, Mycobacteria, Toxoplasma*, and *Trypanosoma*, among others[123]. These pathogens reside intracellularly and have evolved numerous mechanisms to evade the intense macrophage anti-pathogen response. The anti-pathogen response includes the formation of reactive nitrogen and oxygen species, protease activation, programmed cell death, and cytokine production[124]. The phenotype of macrophages largely affects the survival of these pathogens within them. For example, many studies have demonstrated that *L. major* have decreased cell proliferation and increased death in macrophages that produce nitric oxide and super oxide in mice[125–128]. A number of molecules can stimulate macrophages to produce these oxygen radicals including zymosan, LPS and IFNγ. GM-CSF also changes the macrophage phenotype leading to poor *L. major* and *L. tropicana* survival[129,130]. Additionally, GM-CSF has been shown to increase hydrogen peroxide release by mouse peritoneal macrophages and GM-CSF cultured microglia restricted the intracellular multiplication of *T. gondii* via the synthesis of reactive nitrogen intermediates[131,132]. GM-CSF also leads to greater control of *T. cruzi* by inhibiting its replication via increased IL-12, NO and IFNγ production[133,134]. Conversely, *M. tuberculosis* seem to have increased survival in GM-CSF macrophages compared to M-CSF macrophages[135]. One study showed that adding M-CSF to restore homeostatic levels in the lungs of *M. tuberculosis* infected mice, led to greater activation of the adaptive immune response to the pathogen and led to decreased survival[136]. Overall, manipulation of the macrophage phenotype has clear consequences for the survival of intracellular pathogens.

Wound Healing

Macrophages play a critical role in the response to injury in muscle, skeleton, skin and other organs. While macrophages initially mount an inflammatory immune response following injury, they also are active in the return to homeostasis[137]. An early study in mice found that the depletion of macrophages in the wound sites led to delayed debridement and also slowed fibroblast recruitment and proliferation affecting the wound closure time[138]. The wound healing process is largely orchestrated by the macrophage secretome. The secretome includes molecules like TGFβ, VEGF and EGF, which have been shown to be essential for angiogenesis. One early study indicated that recombinant human M-CSF accelerated wound healing in non-ischemic wounds in rabbits by significantly increasing the levels of TGFβ [139]. The timing of the conversion of inflammatory, TNF producing macrophages to growth promoting, TGFβ secreting macrophages is particularly important in muscle regeneration[140,141]. Other molecules secreted by macrophages, such as anti-inflammatory cytokines IL-10, IL-4 and IL-13 along with lipid mediators like lipoxins and resolvins can contribute to the initiation of tissue repair. In muscle, infiltrating blood monocytes that differentiate into macrophages convert to pro-regenerative macrophages that promote the growth of new myofibers once phagocytosis of debris is complete[142]. Macrophages also indirectly contribute to wound healing by recruiting other cell types such as fibroblasts, mesenchymal cells and mesoangioblasts[143]. Subsequently, macrophages promote the proliferation of these cells at the wound site in order to instruct the tissue repair mechanism and initiate angiogenesis[144]. If macrophages do not function properly during wound healing, this can lead to fibrosis.

1.9 Scope and Limitations

The research in this dissertation was performed on human monocyte-derived macrophages. These cells represent a subset of macrophages in the host that develop once monocytes migrate into different tissues in response to inflammatory signals, and subsequently encounter colony-stimulating factors. We studied these macrophages due to the feasibility of their collection from human blood. Although these macrophages are separate in origin from yolk sac or fetal liver-derived tissue macrophages, we believe that all macrophages exhibit plasticity and can respond to molecules in their microenvironment in order to alter their phenotype.

2 Materials and Methods

2.1 Mouse BMDM preparation

Bone marrow hematopoietic stem cells were flushed from the femurs of 6-8 week old C57/bl6J mice (Cat# 000664, Jackson Laboratory, Bar Harbor, ME) in saline containing 1% penicillin/streptomycin[145]. Cells were plated on petri dishes in DMEM/F-12 + Glutamax media (Cat# 10565018, Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Cat# S11550, Atlanta Biologicals, Flowery Branch, GA) supplemented with 20% conditioned media obtained from the culture of the L-929 mouse fibroblast cell line (Cat# ATCC). New media was added on day 4. On day 7, differentiated macrophages were removed from petri dishes using Cell stripper (Corning). Other strains of mouse macrophages were also used including $A2br^{-/-}$[146], $A2ar^{-/-}$ and $STAT1^{-/-}$ mice (Cat#s 010685, 012606, Jackson Laboratory).

2.2 Mouse BMDM stimulation

Mouse macrophages were stimulated with the following reagents: 10 ng/mL ultra pure LPS from Escherichia coli K12, IFNγ 10000 U/mL, IFNβ 10000 U/mL, and IFNλ 10000 U/mL. They were also stimulated with various TLR-ligands (Cat# tlrl-kit1mw, Invivogen).

2.3 Human macrophage differentiation

Whole blood was isolated from healthy donors under University of Maryland, IRB approved protocols. Human monocytes were isolated via density gradient centrifugation followed by negative isolation using immunomagnetic beads (Cat# 130-096-537, Miltenyi Biotec, San Diego, CA). Monocytes were cultured for 7 days in X-VIVO 15 serum-free media (Cat# 04-744Q, Lonza, Walkersville, MD) containing 1% penicillin-

streptomycin, 1% L-glutamine (Cat# 25-005-CI, Gibco, Gaithersburg, MD), and supplemented with either 30 ng/mL recombinant human M-CSF or 20 ng/mL recombinant human GM-CSF (Cat# AF-300-25, Cat# 300-03 respectively, Peprotech, Rocky Hill, NJ). Media containing either growth factor was replenished on day 4 following initial culture. Prior to stimulation on day 7, media containing growth factor was replaced with X-VIVO 15 media containing 5% heat-inactivated fetal bovine serum (Cat # S11550, Atlanta Biologicals, Flowery Branch, GA).

2.4 Human macrophage stimulation

LPS stimulated macrophages were generated by the addition of 10ng/mL ultra pure LPS from Escherichia coli K12 (Invivogen, San Diego, CA). LPS + Adenosine macrophages were generated by the addition of 10 ng/mL LPS and 50 μM adenosine (Cat# A4036, Sigma-Aldrich, St. Louis, MO). LPS + PGE_2 macrophages were generated by the addition of 10 ng/mL LPS and 50 nM PGE_2 (Cat# 2296, Tocris, Bristol, UK). Pharmacological inhibitors, ONO AE3 208, PF 04418948, SCH 442416, were used to inhibit EP4, EP2 and A2aR, respectively (Cat#s 3565, 4818, 2463, Tocris). TLR agonists, FSL-1, HKLM, Loxoribine, and Poly I:C were added to macrophages for 4 hours (Cat#s tlrl-fsl, tlrl-hklm, tlrl-lox, tlrl-pic Invivogen). Cell permeable cAMP analogs, 8-Bromo-cAMP, specific for PKA, and 8-pCPT-2-O-Me-cAMP-AM, specific for Epac, were added to stimulated macrophages for 24 hours (Cat#s 1140 and 4853, respectively, Tocris).

2.5 RNA sequencing sample and library preparation

Total RNA was isolated from macrophages using the Trizol reagent. RNA cleanup was done using RNeasy Mini Kit columns (Cat# 74106, Qiagen, Hilden, Germany). RNA

quality was determined using an Agilent 2100 bioanalyzer. Poly(A)+-enriched cDNA libraries were generated using the Illumina TruSeq sample preparation kit (Cat#s 16027084, 15027387, 1502062, Illumina, San Diego, CA) and quality of the cDNA was determined again with the bioanalyzer. Paired end reads (100bp) were obtained from an Illumina HiSeq 1500. Reads were aligned to the human genome (Homo_sapiens.GrCh38.79) obtained from the UCSC genome browser (http://genome.ucsc.edu) using Kallisto[147]. Count tables were restricted to protein-coding genes (34,425) and on-expressed or weakly expressed genes (< 1 read per million in n=5 samples) were removed prior to subsequent analyses, resulting in 12,857 genes analyzed. Quantile normalization and $\log_2$-transformation was done on all samples.

2.6 RNA sequencing data assessment, visualization and differential expression analysis

Limma, a Bioconductor package, was used to perform differential expression analysis. The voom module was used to transform the data based on observational level weights derived from the mean-variance relationship prior to statistical modeling. Experimental batch effects were adjusted for by including experimental batch as a covariate in our statistical model. Differentially expressed genes were defined as genes with a $\log_2$ fold-change > 1 and a Benjamini-Hochberg (BH) multiple-testing adjusted p. value < 0.05. All components of the statistical pipeline, named cbcbSEQ, can be accessed on GitHub (https://github.com/kokrah/cbcbSEQ/).

2.7 Single cell RNA-sequencing sample and library preparation

Monocyte derived macrophages generated in M-CSF from a single donor were stimulated for 4 hours with nothing, LPS, LPS + Adenosine and LPS + PGE_2 and processed

according to the 10x library preparation method. Briefly, Gel Beads-in-emulsion (GEMs) were generated by combining Single Cell 3' v3 Gel Beads, master mix containing cells, and partitioning oil onto Chromium Chip B. Following GEM generation, the Gel Beads were dissolved and mixed with primers containing the 10x Barcode, a TruSeq sequencing primer, a unique molecular identifier and a poly(dT) sequence, in order to produce full-length, barcoded cDNA. This barcoded cDNA was amplified by PCR in order to construct libraries. The above components were included in a library construction kit (Cat# PN-1000075, 10x Genomics, San Francisco, CA). Libraries were sequenced using paired-end Illumina sequencing.

2.8 Single Cell RNA-sequencing analysis

The samples were sequenced and processed with cell ranger 3.0.1 at the Johns Hopkins Genetics Resources Core Facility. The resulting outputs were passed to Seurat 3.1.0, merged by sample, and filtered to remove cells with high mitochondrial content (> 15%) and few features (< 200). The remaining data was passed through the default Seurat pipeline. The analysis was done on 17306 cells, with an average of 4327 cells per sample. The analysis entailed normalization, variable feature selection, data scaling, neighbor and cluster searches, the accompanying visualizations, and differential expression of markers across conditions and samples.

2.9 ELISA

Cytokine and growth factor levels were measure in the supernatants of 24 hour stimulated macrophages. IL-12p40 and TNF were measured using paired antibody ELISA kits (Cat# BMS2013MST, Invitrogen, Vienna, Austria and Cat# 555212, BD Biosciences,

San Diego, CA). GM-CSF, VEGF, and THBS1 were measured using DuoSet ELISA kits (Cat#s DY215, DY293B, DY3074, respectively, R&D, Minneapolis, MN).

IL-6 and IL-10 were also measured using OptEIA ELISA sets (Cat#s 555220 and 555157, respectively, BD Biosciences).

2.10 Quantitative real-time PCR

RNA was isolated from cells using the Trizol reagent. cDNA was synthesized using Superscript VILO cDNA synthesis kit. Relative quantitation of transcript levels was performed using SYBR-Green. Samples were analyzed in a Roche Light Cycler 480. Expression levels were calculated using the ΔCt method relative to the geometric mean of GAPDH and RAB7 as internal control genes. The primer sequences used to measure transcripts are listed in Table 1.

Table 1. Primer Sequences

Gene	Accession number	Sequence
GAPDH		
Forward	NM_002046.7	5'-ATAAATTGAGCCCGCAGCC-3'
Reverse		5'-CATGTAAACCATGTAGTTGAGGTC-3'
RAB7		
Forward	NM_177403.5	5'-GGTTCCAGTCTCTCGGTGTG-3'
Reverse		5'-CGCTTTGTGGCCACTTGTC-3'
THBS1		
Forward	NM_003246.4	5'-GAAGGACTCTGACGGCGATG-3'
Reverse		5'-GATGTCCCTTTGGGGTCCAG-3'
CD300E		
Forward	NM_181449.3	5'-GTTTCCCCAGCAATTACAACCC-3'
Reverse		5'-CAGAAGACAGCACCCAGCAT-3'
AREG		
Forward	NM_001657.4	5'-TGTCGCTCTTGATACTCGGC-3'
Reverse		5'-GGCATTTCACTCACAGGGGA-3'
VEGFA		
Forward	NM_001171623.1	5'-CATGCCAAGTGGTCCCAGG-3'
Reverse		5'-GCTGGCTTTGGTGAGGTTTG-3'
CD93		
Forward	NM_012072.4	5'-TGGAGAACCAGTACAGTCCG-3'
Reverse		5'-GAGTCACGAAATCCCCACCG-3'
CXCL13		
Forward	NM_006419.2	5'-TCTCTCCAGTCCAAGGTGTTC-3'
Reverse		5'-AGCTTGAGGGTCCACACAC-3'
MMP10		
Forward	NM_002425.3	5'-CAGGCATTTGGATTTTTCTACTTCT-3'
Reverse		5'-CTGTCTTCCCCCTATCTCGC-3'
RGS2		
Forward	NM_002923.4	5'-AAGAGCGAGGAGAAGCGAG-3'
Reverse		5'-GCAAGACCATATTTGCTGGCT-3'
TGFA		
Forward	NM_003236.4	5'-CCTGTTCGCTCTGGGTATTGT-3'
Reverse		5'-GGTGATGGCCTGCTTCTTCT-3'
ADORA2A		
Forward	NM_001278497	5'-CATCCCGCTCCGGTACAATG-3'
Reverse		5'-TGGTTCTTGCCCTCCTTTGG-3'
ADORA2B		
Forward	NM_000676.2	5'-GACGCCCACCAACTACTTCC-3'
Reverse		5'-TTTATACCTGAGCGGGACACA-3'
PTGER2		
Forward	NM_000956.4	5'-GCTCCTTGCCTTTCACGATTT-3'
Reverse		5'-AGGATGGCAAAGACCCAAGG-3'
PTGER4		
Forward	NM_000958.3	5'-CCGCTCGTGGTGCGAGTATT-3'

Reverse		5’-GGCCTGACATGGCAGAAGAT-3’
COX1		
Forward	NM_000962.4	5’-TGGTTCTTGCTGTTCCTGCT-3’
Reverse		5’-CACAGGCCAGGGATGGTG-3’
COX2		
Forward	NM_000963.4	5’-CATCCCCTTCTGCCTGACAC-3’
Reverse		5’-TCCTACCACCAGCAACCCTG-3’
MPGES1		
Forward	NM_004878.5	5’-GAAGTGGCTGATGGGAACCA-3’
Reverse		5’-GGAGGGAGAGGGAGTGATGT-3’
Mouse A2AR		
Forward	NM_009630.3	5’-CCATTCGCCATCACCATCAG-3’
Reverse		5’-CCCGTCACCAAGCCATTGTA-3’
Mouse A2BR		
Forward	NM_007413.4	5’-GACTCTTCGCCATCCCCTTT -3’
Reverse		5’-ACAGCAATGATCCCTCTCGC-3’

2.11 Measurement of PGE_2 production

Prostaglandin E2 levels in the supernatants of 24 hour LPS stimulated M-CSF and GM-CSF macrophages were measured using a monoclonal antibody competitive ELISA kit (Cat # 514010, Cayman Chemical, Ann Arbor, MI).

2.12 Measurement of ATP degradation

M-CSF and GM-CSF macrophages were given a spike of 20 μM ATP (Cat# A6419, Sigma-Aldrich). Supernatants were collected at 2 hours, 1 hour, 30 minutes and 15 minutes following this spike and ATP was measured using the ATPlite reagent (Cat# 6016941, Perkin Elmer, Waltham, MA). Levels of ATP were normalized to the amount of protein in the wells using the Pierce BCA Protein Assay Kit for protein quantification (Cat# 23227, ThermoScientific, Rockford, IL). Luminescence was read in the dark-adapted plate using a luminometer.

2.13 HUVEC tube formation assay

Primary human endothelial cells (HUVECs) were obtained and cultured in EGM-2 media (Cat# CC-3162, Lonza) on tissue culture treated plates coated with 1% gelatin from porcine skin (Cat# G1890, Sigma). For the assay, HUVEC cells were distributed at a concentration of 40,000 cells in each well of a 48-well plate. These wells contained growth factor reduced and phenol red-free Matrigel (Cat# 356231, Corning). Supernatants collected from M-CSF and GM-CSF macrophages that were unstimulated, or stimulated with LPS, LPS + Adenosine, and LPS + PGE_2 for 24 hours were added to the HUVEC cells on the Matrigel and allowed to incubate for 24 hours. Images of the HUVEC cells were captured in brightfield on an inverted Nikon ECLIPSE Ti2 Microscope at 20x total magnification. Images were converted to high contrast using the

"Find edges" function in ImageJ in order to see the cells. Tube length and number of nodes were assessed manually using the ImageJ software.

2.14 Flow cytometry

CD300E and PLAUR surface expression was measured on macrophages stimulated for 24 hours and 8 hours, respectively, using APC conjugated antibodies (Cat# 17-3007-42 and Cat# 17-3879-42, Invitrogen, Carlsbad, CA). Fc block was used to reduce nonspecific binding (Cat# 130-059-901, Miltenyi Biotec). Debris and doublets were removed using gating analysis in FlowJo version X. Surface expression is expressed as median fluorescence intensity (MFI).

3 The effects of interferons on purinergic signaling in mouse macrophages

Parts of this chapter are adapted from published work:

Cohen HB, Ward A, Hamidzadeh K, Ravid K, Mosser DM. IFN-γ Prevents Adenosine Receptor (A2bR) Upregulation To Sustain the Macrophage Activation Response. *J Immunol*. 2015;195(8):3828-3837. doi:10.4049/jimmunol.1501139

3.1 Introduction

It has previously been demonstrated in mouse macrophages that purinergic signaling dampens inflammatory responses to LPS[29,148,149]. Specifically, inflammatory cytokines including TNF and IL-12p40 are significantly reduced in activated macrophages in the presence of ATP or adenosine[26,150]. Purinergic signaling is thought to be a mechanism to control the level and duration of macrophage inflammation in the host. However, there are situations in which a prolonged inflammatory response is desirable, such as with severe infections. There are a number of signals that are associated with modulating the severity of an immune response, including interferons.

Interferons come in different varieties, called Type I, II and III. IFN beta (IFNβ) and IFN alpha (IFNα) are Type I, IFN gamma (IFNγ) is Type II and IFN lambda (IFNλ) is Type III. M1 macrophages, which are generated by stimulation with IFNγ + LPS exhibit severe inflammation[151]. Our lab established a connection between interferons and the purinergic system in the mouse, by demonstrating that IFNγ priming of macrophages prevented the upregulation of the A2b receptor (A2br) following LPS stimulation[7]. We extended this research to further explore the effects of IFNγ on adenosine signaling in

macrophages, and we also examined the role of Type II interferon, IFNβ, in the mouse macrophage response to LPS.

3.2 Results

Type II interferon modulation of macrophage activation. The action of IFNγ priming of LPS stimulated mouse macrophages has been shown to significantly augment the levels of inflammatory cytokine production. It was demonstrated in our lab that this heightened inflammation was due to IFNγ priming preventing the upregulation of the A2b receptor upon LPS stimulation[7]. As part of this work, we examined the effects of other TLR ligands on A2BR and A2AR mRNA expression (Figure 1). All of the TLR ligands tested (LPS-EK, Pam3Csk4, HKLM, Poly(I:C) LMW, Poly(I:C) HMW, ST-FLA, FSL-1 and ssRNA) which activate TLRs 1-9 upregulated mRNA levels for A2BR. In all stimulations except ssRNA, IFNγ priming significantly downregulated expression of the A2BR (Figure 1A). As for A2AR mRNA levels, there was no significant difference between unprimed and IFNγ primed macrophages (Figure 1B). In order to further validate the effect of IFNγ priming on mouse macrophages, we looked at purinergic receptor expression in STAT1 -/- mouse BMDMs since IFNγ is known to signal through STAT1[152]. The prevention of the upregulation of A2BR mRNA by IFNγ priming was not observed in STAT1 -/- macrophages (Figure 2A). Additionally, STAT1 -/- macrophages did not significantly differ in mRNA expression of A2AR (Figure 2B). As expected, the lack of STAT1 abrogated the effect of IFNγ priming on TNF production following LPS stimulation and also allowed adenosine to function in decreasing the amount of TNF produced by LPS stimulated macrophages, presumably through the A2b receptor (Figure 3A)[7]. Preliminary data also suggested that the lack of STAT1

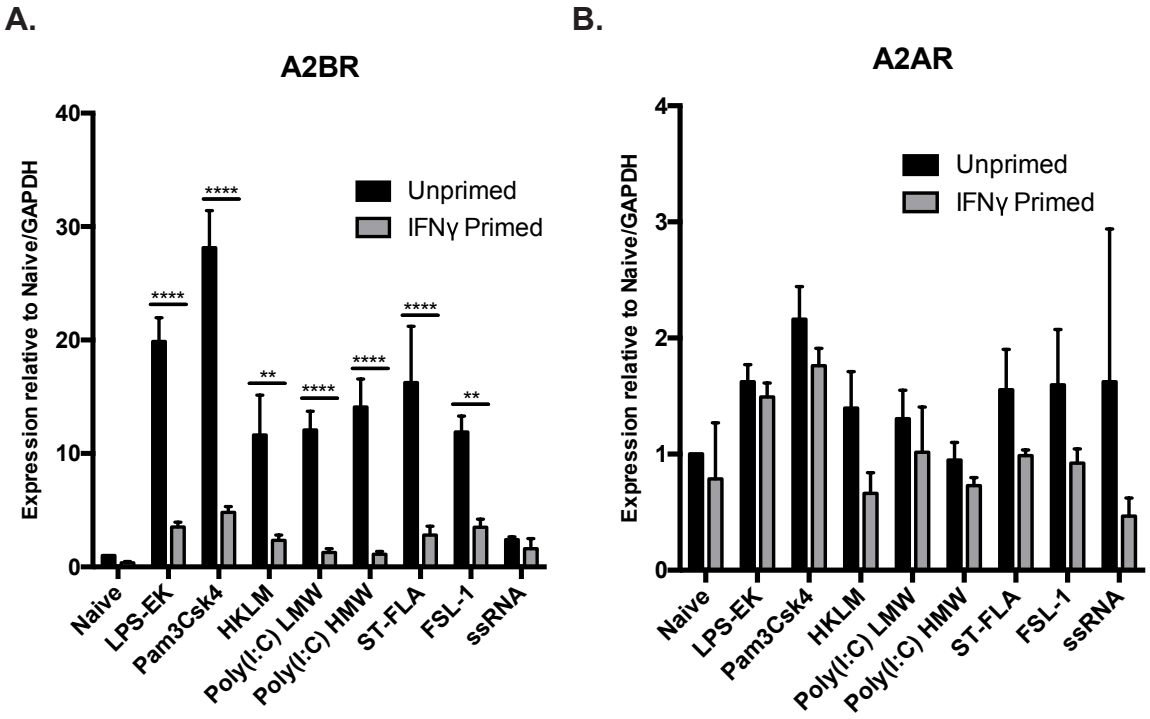

Figure 1. Adenosine receptor expression following IFNγ priming and stimulation with various TLR ligands. (A) A2BR and **(B)** A2AR mRNA expression was measured by qPCR following 16 hours of IFNγ priming and 4 hours of stimulation with TLR ligands (n=3, ** *P*-value < 0.01, **** *P*-value < 0.0001, error bars represent SEM).

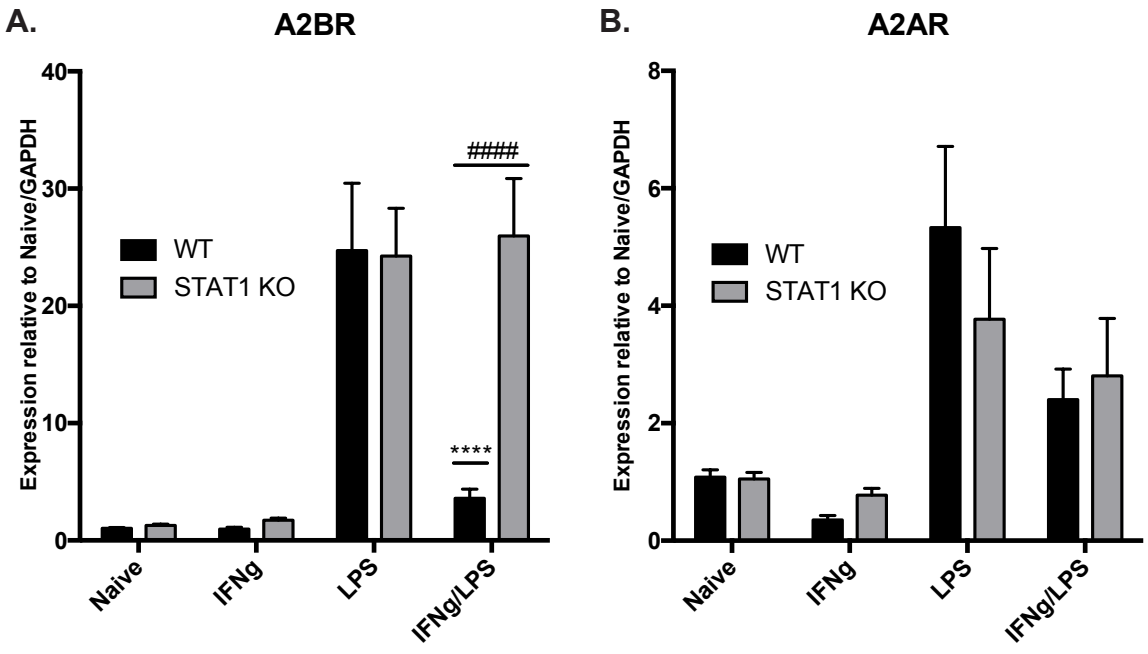

Figure 2. Adenosine receptor expression in STAT1 knockout BMDMs. (A) A2BR and **(B)** A2AR mRNA expression was measured in WT (black) and STAT1 KO (grey) macrophages following 16 hours of IFNγ priming and 4 hours of LPS stimulation (n=3, **** *P*-value < 0.0001 relative to WT LPS alone, #### *P*-value < 0.0001 between WT and KO, error bars represent SEM).

Figure generated with Dr. Heather B. Cohen

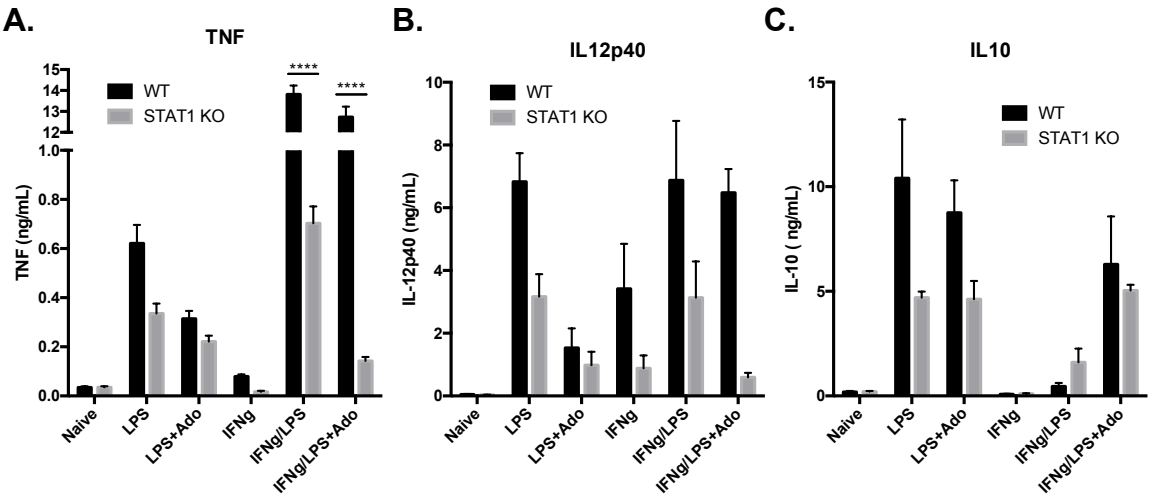

Figure 3. Cytokine levels in WT and STAT1 knockout BMDMs. Macrophages were unprimed or primed for 16 hours with IFNγ followed by LPS stimulation for 8 hours at which point supernatants were collected and assayed for **(A)** TNF by ELISA (n=3, **** *P*-value < 0.0001, error bars represent SEM). Preliminary data is shown for **(B)** IL-12p40 and **(C)** IL-10 levels assayed by ELISA (n=2, error bars represent SD).

Figure generated with Dr. Heather B. Cohen

diminished the inhibitory effect of IFNγ priming on adenosine signaling in terms of IL-12p40 (Figure 3B) and IL-10 production (Figure 3C). Next we examined the effect of A2br -/- on inflammatory cytokine production and demonstrated that the downregulation of IL-12p40 by adenosine is mediated largely through the A2b receptor for both unprimed and IFNγ primed macrophages (Figure 4A). IFNγ primed macrophages stimulated with LPS + Adenosine also significantly differed in terms of TNF production in WT and A2br -/- mice (Figure 4B). A time course experiment indicated that TNF levels were significantly higher in A2br -/- macrophages between 0-2 hours in the presence of LPS, however this difference was not sustained over time in the proceeding absence of LPS (Figure 4C).

Type I interferon modulation of macrophage activation. We wondered whether priming with Type I or III interferons had any effects on the purinergic pathway. While IFNγ lowers mRNA expression for A2BR, IFNβ significantly increased mRNA expression for both A2BR (Figure 5A) and A2AR (Figure 5B) while IFNλ had no significant effects. Therefore, we continued to investigate the effect of IFNβ by examining adenosine receptor expression in IFNAR -/- mice (Figure 6). mRNA expression of A2BR was higher in LPS stimulated IFNAR -/- BMDMs, but there was no difference in its expression in IFNβ primed WT and IFNAR-/- BMDMs (Figure 6A). The upregulation of A2AR mRNA expression by IFNβ priming was abolished in IFNAR -/- BMDMs (Figure 6B). IFNβ priming did not significantly change the levels of IL-12p40 or TNF in WT BMDMs but increased the levels of IL-10 (Figure 7). A2AR -/- BMDMs also did not significantly differ in IL-12p40 (Figure 7A) or TNF levels (Figure 7B) relative to WT BMDMs with the exception of IFNγ priming which led to lower

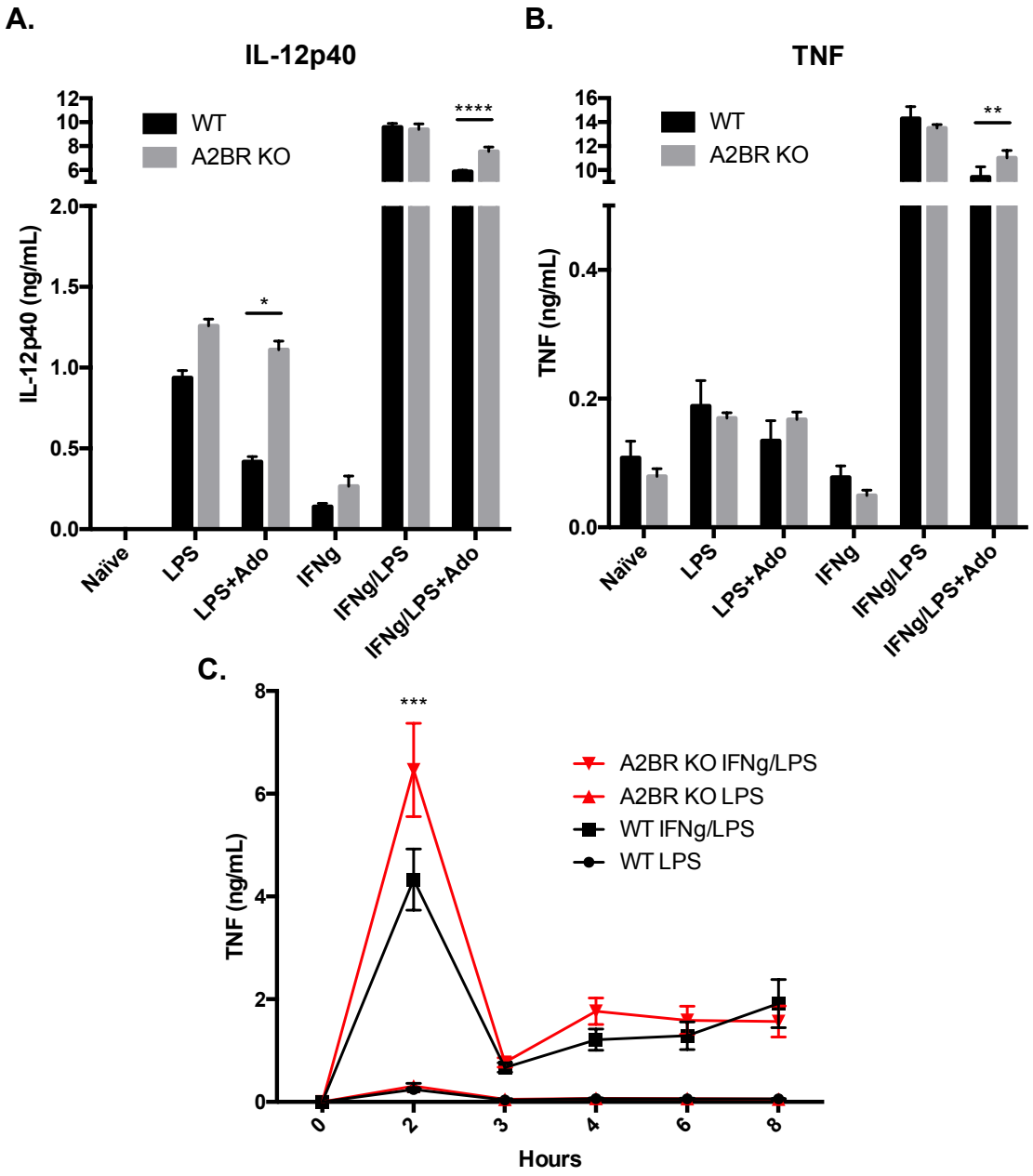

Figure 4. Cytokine levels in WT and A2B receptor knockout BMDMs. Macrophages were unprimed or primed for 16 hours with IFNγ followed by LPS stimulation for 8 hours at which point supernatants were collected and assayed for **(A)** IL-12p40 and **(B)** TNF (n=3, * *P*-value < 0.05, ** *P*-value < 0.01, **** *P*-value < 0.0001, error bars represent SEM). **(C)** Unprimed and IFNg primed macrophages were stimulated with LPS for 2 hours followed by a wash. Supernatants were collected at subsequent incubation timepoints following the wash and assayed for TNF levels (n=4, *** *P*-value < 0.001, error bars represent SEM).

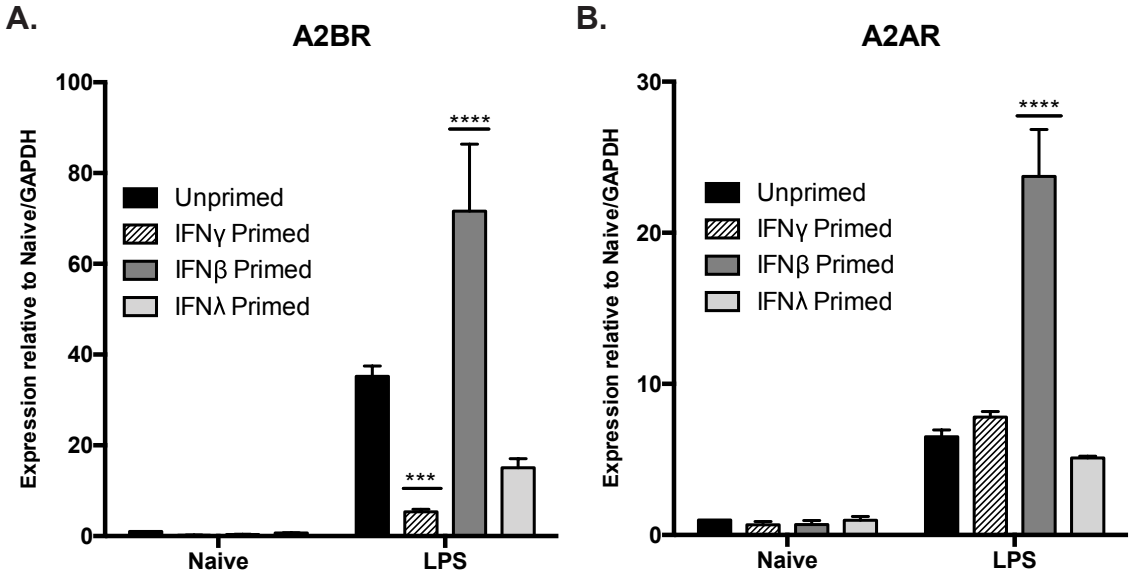

Figure 5. Adenosine receptor expression following priming with Type I, II and III IFNs. mRNA expression of **(A)** A2BR and **(B)** A2AR was measured by RT-PCR following 16 hours of priming with IFNγ , IFNβ, and IFNλ and subsequent stimulation for 4 hours with LPS (n=3, *** *P*-value < 0.001, **** *P*-value < 0.0001, error bars represent SEM).

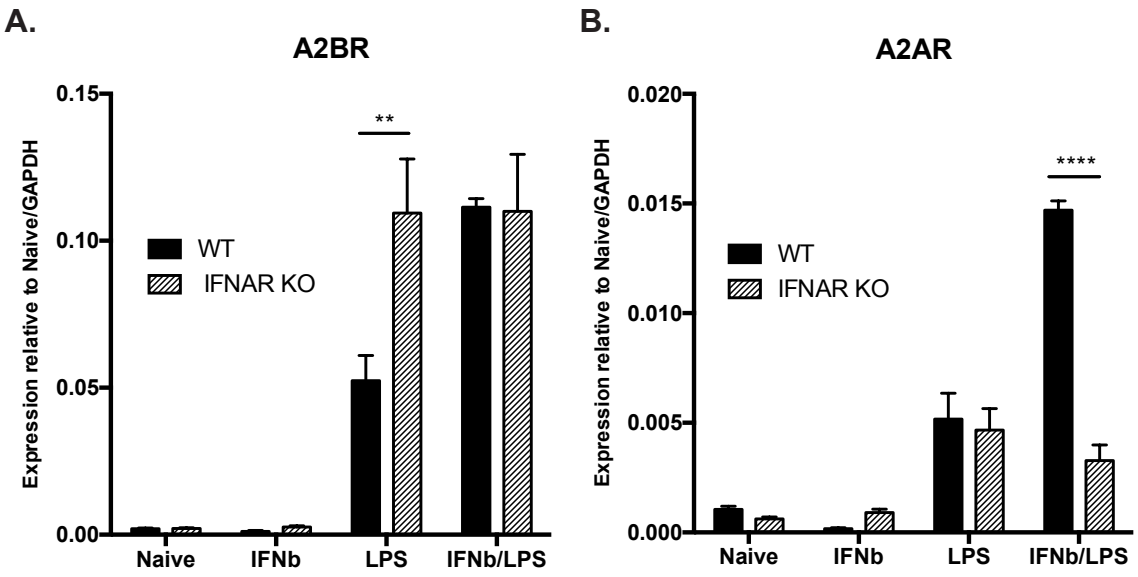

Figure 6. Adenosine receptor expession in IFNAR knockout BMDMs. (A) A2BR and **(B)** A2AR mRNA expression was measured in WT (black) and IFNAR KO (hatched) macrophages following 16 hours of priming with IFNβ and 4 hours of LPS stimulation (n=3, ** *P*-value < 0.01, **** *P*-value < 0.0001, error bars represent SEM).

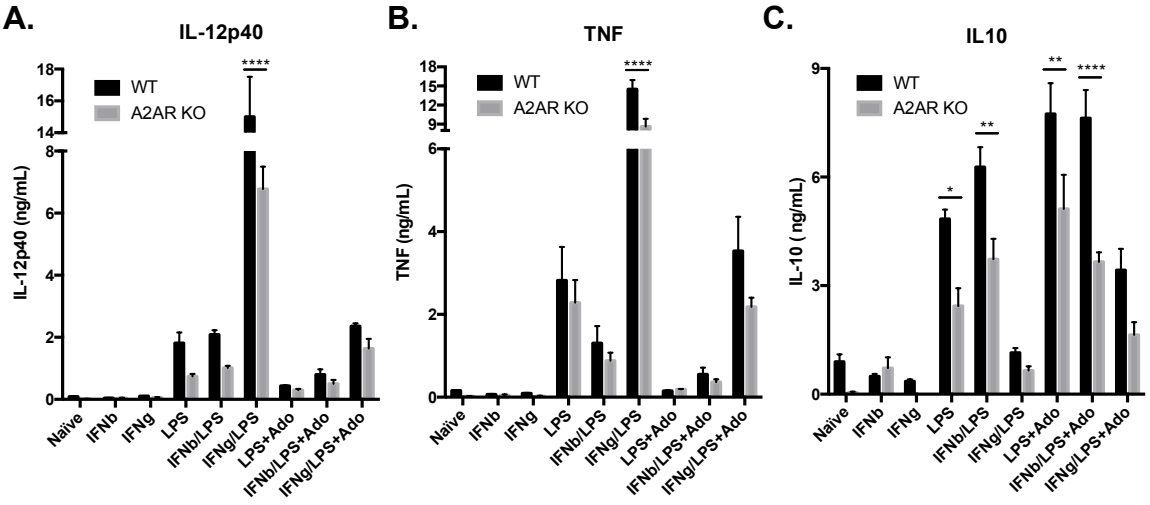

Figure 7. Cytokine levels in WT and A2A receptor knockout BMDMs. Macrophages were unprimed or primed for 16 hours with IFNγ or IFNβ followed by LPS, and LPS + Ado stimulation for 8 hours at which point supernatants were collected and assayed for **(A)** IL-12p40, **(B)** TNF and **(C)** IL-10 secretion (n=3, error bars represent SEM).

levels of these inflammatory cytokines relative to WT. In contrast, A2AR -/- BMDMs produced significantly lower levels of IL-10 following LPS and LPS + Adenosine stimulation in unprimed and IFNβ primed macrophages (Figure 7C). Based on our results we concluded that priming with Type I and Type II interferons had different effects on the purinergic pathway in mouse macrophages.

3.4 Discussion

It is widely accepted that IFNγ treatment of macrophages renders them hyper-inflammatory. We demonstrated that this is partly due to the prevention of the upregulation of the A2B receptor and that this mechanism is universal for a number of pathogenic components that activate different TLR ligands in macrophages[7]. We also demonstrated that the adenosine A2A receptor is not implicated in the IFNγ primed macrophage response. We verified that the effects of IFNγ on A2B receptor expression and TNF production were mediated through the STAT1 signaling pathway as its effects were diminished in STAT1 knockout macrophages. This was no surprise since IFNγ is known to signal through STAT1, so it should not be able to function if STAT1 is not present. We demonstrated using knockout macrophages that the A2b receptor is critical for the immunosuppressive effects of adenosine on IL-12p40 and TNF production by macrophages, but that its effects are not sustained in the absence of inflammatory stimulus.

In the literature, there are conflicting reports of the effects of IFNβ treatment on inflammation, including cytokine production[76]. We observed that IFNβ increased transcription of both the A2BR and A2AR in macrophages, which suggests greater susceptibility to a spontaneous reversion to homeostasis. This is in contrast to the IFNγ

downregulation of the A2B receptor, which we believe indicates that IFNβ instructs a milder and more controlled inflammatory response in macrophages. Studies in IFNAR knockout macrophages indicated that the upregulation of A2A receptor expression was mediated through IFNβ and not IFNα. However, A2B receptor expression was significantly higher in LPS stimulated IFNAR knockout macrophages compared to WT, at levels roughly equal to the level induced by IFNβ priming. This suggested that IFNα may play a role in suppressing A2BR expression in WT cells, as this IFN also signals through the IFNAR receptor, and that perhaps IFNβ may oppose this effect. The A2a receptor contributed to the production of increased IL-10 in IFNβ primed macrophages and their sensitivity to adenosine, as knockout macrophages produced lower levels of IL-10 compared to wild type. However, the A2a knockout macrophages did not produce notably different levels of TNF or IL-12p40. Altogether, the data in this chapter implicates a role for adenosine and its receptors in the potentiation of inflammation by IFNγ and IFNβ in mouse macrophages.

4 Characterization of the transcriptome of human M-CSF derived macrophages stimulated with LPS + Adenosine and LPS + PGE_2

4.1 Introduction

The study of human monocyte derived macrophages is critical, considering the importance of these cells in a number of organs. Monocytes infiltrate tissues and differentiate into macrophages following exposure to a constitutively expressed growth factor, M-CSF. They do so in response to infection, or to replenish the native populations of tissue macrophages that may have become depleted. It has been shown that monocyte derived macrophages are particularly important in the intestine, skin, and peritoneum as well as in disturbances such as atherosclerosis, muscle injury and inflammation[153].

In this research, we attempt to describe the phenotype of human monocyte derived macrophages stimulated by TLR ligands, such as LPS in the presence of adenosine (LPS + Adenosine) or prostaglandin E2 (LPS + PGE_2), using RNA sequencing. We believe that our samples and stimulation conditions mimic physiological environments that macrophages can potentially encounter in the body. For example, the tumor microenvironment contains high levels of purinergic signaling molecules as well as PGE_2. These molecules are also produced during inflammatory immune responses by macrophages and other cell types. We use RNA sequencing because it gives us a snapshot of the entire transcriptome at our chosen timepoints. This can allow us to characterize the nature and function of macrophages under different physiological contexts.

4.2 Results

RNA-sequencing analysis. RNA-seq was performed on unstimulated, LPS stimulated, LPS + Adenosine and LPS + PGE_2 stimulated macrophages from 5 blood donors.

Differential expression analysis allowed us to determine the effects of adenosine and PGE_2 on LPS stimulation (Figure 8). Our analysis revealed that adenosine and PGE_2 have similar effects on the stimulated macrophage transcriptome. 4 of the 10 most highly upregulated genes and 5 of the 10 most downregulated genes in LPS + Adenosine (Figure 8A, starred) and LPS + PGE_2 (Figure 8B, starred) versus LPS alone were shared. Venn diagrams of the number of significant differentially expressed genes further highlight the degree of similarity between adenosine and PGE_2 stimulation relative to LPS stimulation alone (Figure 9A). 101 of the 259 upregulated genes and 91 of the 294 downregulated genes were shared by LPS + Adenosine and LPS + PGE_2 stimulation. For comparison, LPS stimulation alone versus control leads to 1350 significantly differentially expressed genes (data not shown). This highlights the fact that the transcriptomic changes made by the addition of adenosine and PGE_2 to TLR stimulated macrophages are quite limited. However, at the same time, the addition of adenosine and PGE_2 to TLR stimulated macrophages leads to a similar transcriptomic phenotype as indicated by spearman correlation analysis yielding a correlation coefficient of R= 0.772 (Figure 9B). Single cell RNA sequencing of M-CSF macrophages from 1 donor allowed us to compare the trancriptomic signature of a multitude of individual cells in order to see the variable expression of genes within a population. UMAP analysis was performed as a dimension reduction technique to cluster cells based on the variability of gene expression (Figure 10). LPS, LPS + PGE_2 and LPS + Adenosine stimulated macrophages cluster separately from unstimulated cells indicating relatively homogeneous stimulation throughout the macrophage population. There was some overlap between the LPS + PGE_2 and LPS +

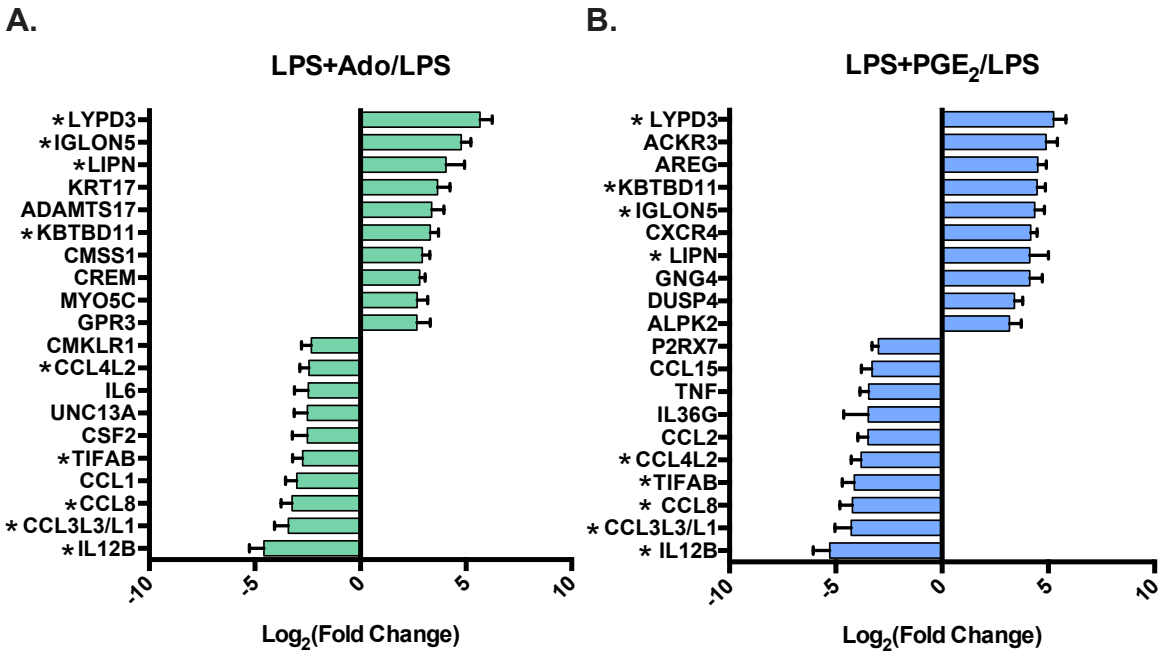

Figure 8. Most highly upregulated and downregulated genes by adenosine and PGE_2 relative to LPS stimulated macrophages alone. Bulk RNA-seq was done on M-CSF macrphages and differential expression analysis was performed for **(A)** LPS + PGE_2 versus LPS and **(B)** LPS + Adenosine versus LPS. The top and bottom 10 genes are listed for each comparison. N=5 individuals, *P*-value < 0.05, $\log_2$FC > 2, error bars represent SEM. (*) indicates genes in common between LPS+PGE_2/LPS and LPS+Adenosine/LPS.

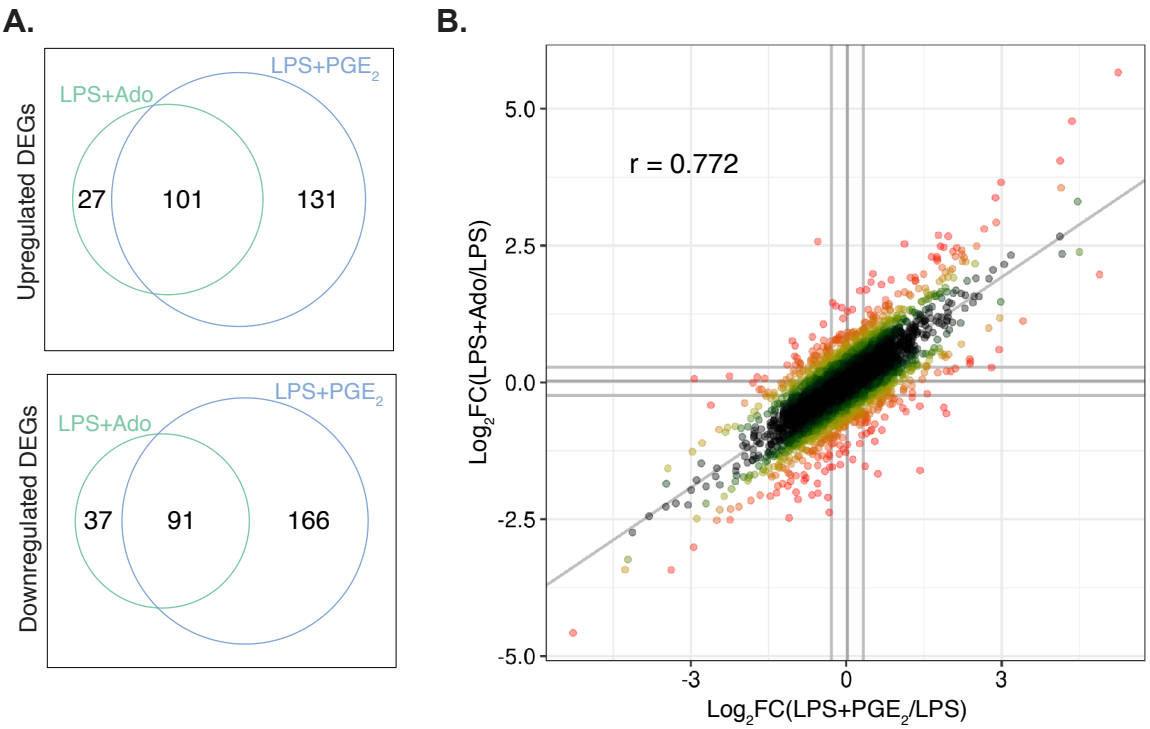

Figure 9. Degree of transcriptional similarity between LPS + Adenosine and LPS + PGE_2 stimulated macrophages. (A) The number of unique and shared DEGs of LPS + Adenosine and LPS + PGE_2 relative to LPS alone are depicted as venn diagrams. **(B)** The log_2FC of the DEGs of LPS + Adenosine versus LPS + PGE_2 relative to LPS alone are depicted on a scatter plot. Spearman correlation was performed to obtain a correlation coefficient (r) printed in the top left quadrant (*P*-value < 2.2^{-16}).

Figure 9B generated with Dr. Ashton Trey Belew

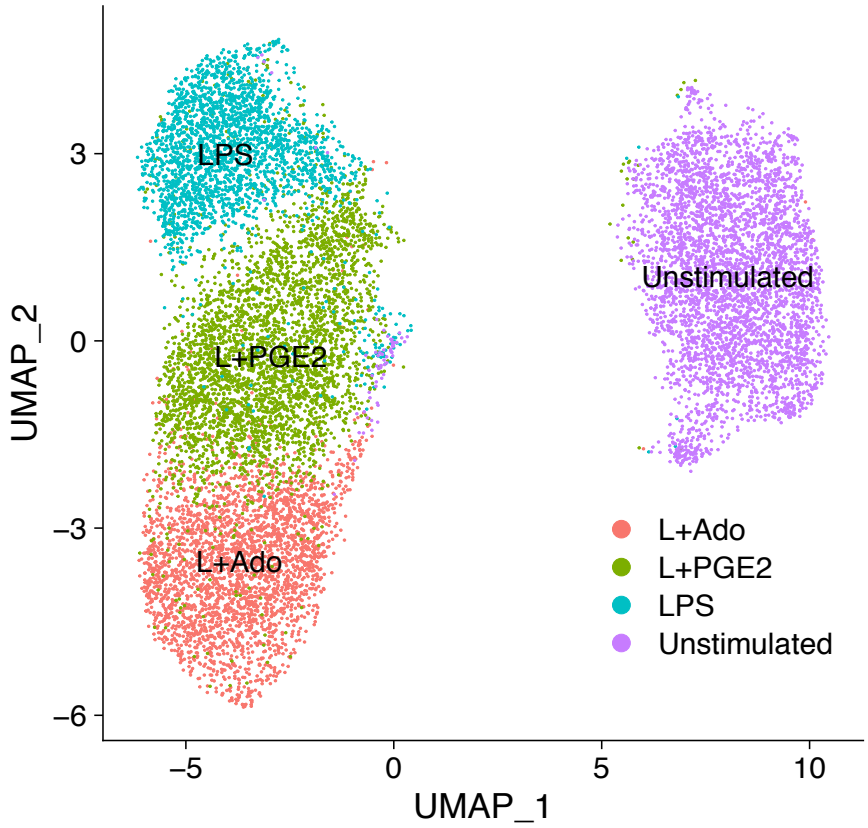

Figure 10. Cluster visualisation of M-CSF macrophage single cell transcriptomes. Uniform manifold approximation and projection (UMAP) was used to arrange points (representing cells) based on their relative transcriptomes. Single cells clustered based on their stimulation conditions: unstimulated, LPS, LPS + Ado and LPS + PGE2.

Figure generated with Dr. Ashton Trey Belew

Adenosine clusters, further indicating that a number of cells in these stimulation conditions are transcriptionally similar.

Pathway Analysis. In order to explore the functional roles of macrophages stimulated with adenosine and $PGE_{2,}$ we took a closer look at the genes modulated by these conditions. Of the most highly upregulated genes in common between LPS + Adenosine and LPS + PGE_2 versus LPS alone, 10 of the top 20 genes have published growth promoting or anti-inflammatory roles (Figure 11A, purple). LYPD3, or C4.4A, deficiency has been implicated in delayed wound healing, and its expression levels correlate with cell invasiveness in numerous cancers[154–158]. LIPN has been shown to be involved in proper formation of the skin barrier as a 2bp mutation in this gene led to ichthyosis[159]. AREG is well known for its role in tissue restoration and is an activator of transforming growth factor beta[160]. ACKR3, also known as CXCR7, is involved in angiogenesis and cell migration, as well as cancer cell invasiveness[161,162]. KRT17 has recently been found to be overexpressed in a number of cancers and contributes to tumor cell invasiveness[163,164]. Its primary role is in wound healing, but it also regulates skin inflammation[165,166]. CXCR4 is a receptor for CXCL12, which has been shown to recruit macrophages to tumor environments and promote M2 phenotypes[167]. THBS1 is involved in wound healing, the maintenance of homeostasis in the lung, and is an activator of latent TGFbeta[168–170]. FFAR3 is a short-chain fatty acid receptor and stimulation of this receptor results in decreased inflammatory cytokine production by human monocytes[171]. CRISPLD2 has been shown to modulate proliferation of fetal lung fibroblasts and is involved in the regulation of extracellular matrix genes during wound healing[172]. Of the most highly downregulated genes in common between LPS + Adenosine and LPS +

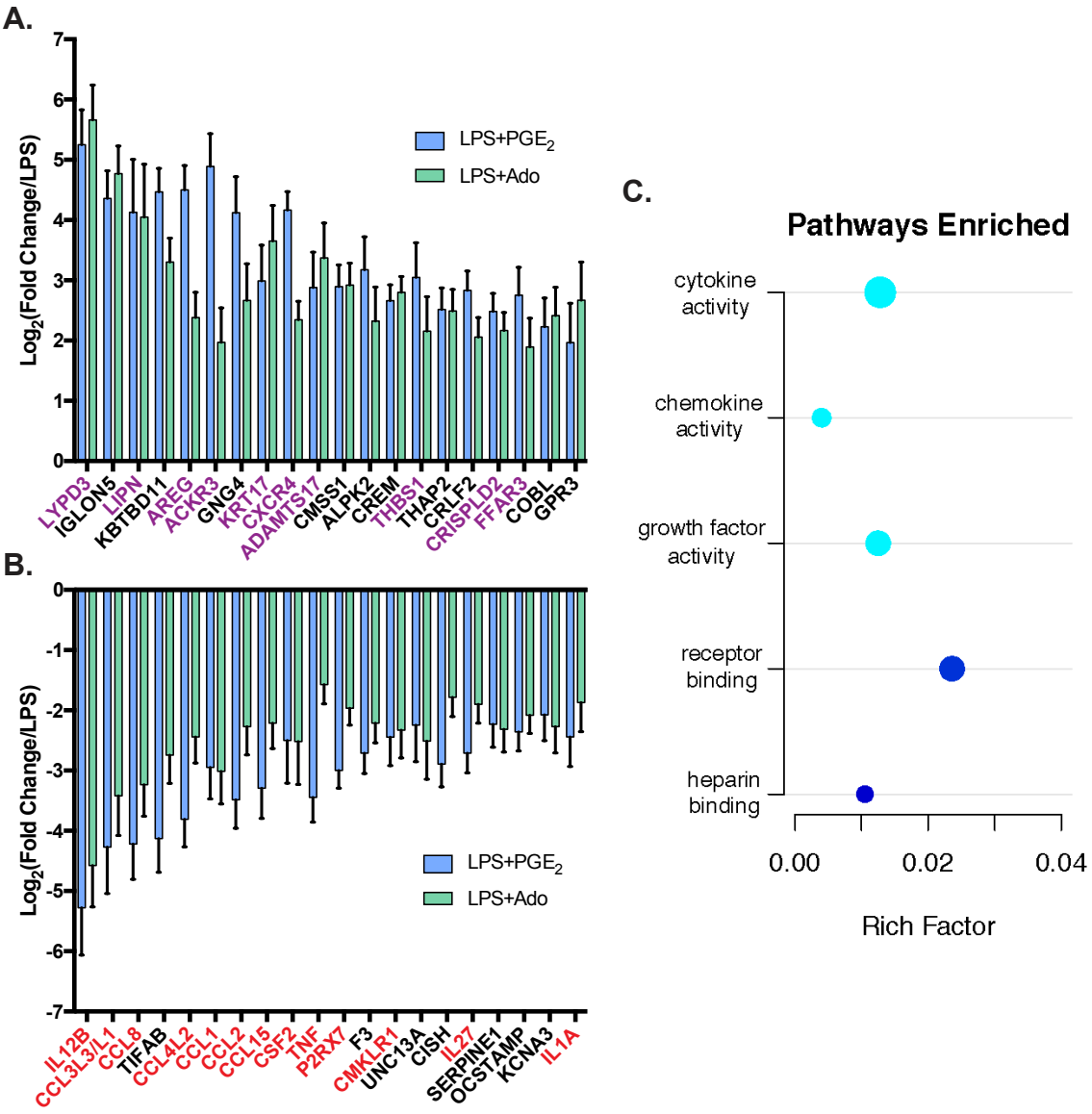

Figure 11. Functional characteristics of shared DEGs between LPS + Adenosine and LPS+PGE2 relative to LPS stimulation. Differential expression analysis was performed and the list of shared **(A)** most highly upregulated genes and **(B)** most highly downregulated genes are depicted as the Log_2FC relative to LPS stimulation alone. The individual Log_2FC of each gene for LPS + Adenosine (green bars) and LPS + PGE_2 (blue bars) stimulations are shown. The genes colored in purple have roles in growth, proliferation and angiogenesis. The genes colored in red have roles in inflammation. N=5 individuals, *P*-value < 0.05, $log_2FC > 1$. **(C)** GO term analysis for molecular function was performed and the top 5 GO terms are plotted. Point size indicates # of DEGs in the GO term category. Point color indicates *P*-value. Rich factor is the ratio of the # of DEGs per # of genes in the GO term category.

PGE_2 versus LPS alone, 13 out of the bottom 20 genes have published roles in inflammation (Figure 11B, red). IL12B is an inflammatory cytokine that promotes the development of Th1 $CD4^+$ T cells[173]. CCL3L3/L1 are sequence variants of the same gene (CCL3), which is a chemokine that recruits CCR5 expressing cells[174]. CCL8, CCL4L2, CCL1, CCL2, and CCL15 are also chemokines, which participate in the recruitment of immune cells to sites of inflammation[175–177]. CSF2 encodes GM-CSF, which can function as a cytokine to promote inflammation[178]. TNF is a cytokine that mediates inflammation, anti-microbial immunity, and was named after its[179] cytotoxicity towards tumors. P2RX7 is a receptor for ATP, which plays a role in the activation of the NLRP3 inflammasome[180]. CMKLR1 encodes the receptor for chemerin, a potent macrophage chemoattractant[181]. IL27 is a member of the IL-12 family of cytokines and promotes expansion and IFNγ production by $CD4^+$ T cells[182]. IL1A encodes an inflammatory cytokine that can function as an 'alarmin' and stimulates the production of chemokines[183]. Our transcriptomic analysis suggests that the M-CSF macrophage response to adenosine and PGE_2 during inflammation is highly similar and overlapping. Gene ontology (GO) analysis of the list of shared differentially expressed genes by LPS + Adenosine and LPS+PGE_2 relative to LPS alone, revealed a number of processes that were significantly enriched. These included cytokine activity, chemokine activity, and growth factor activity among the top 5 most significantly enriched categories (Figure 11C). Using the pathway analysis software, Cytoscape, the differentially expressed genes shared by LPS + Adenosine and LPS + PGE_2 were separated into 5 predicted network clusters based on annotated signaling pathways (Figure 12). The first cluster includes the upregulation of a few growth promoting genes and the downregulation of cytokine genes,

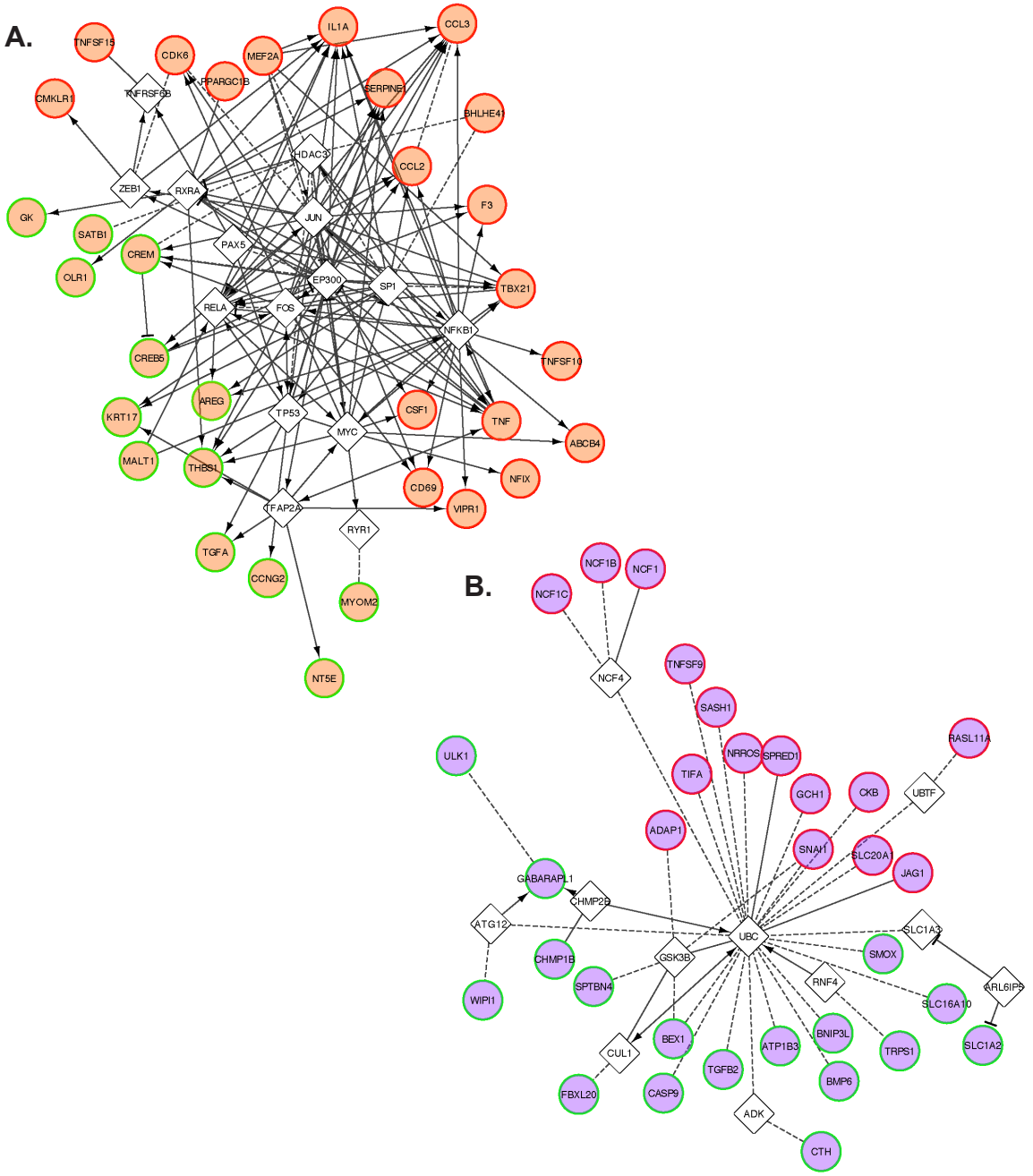

Figure 12. Functional interaction groups in shared DEGs between LPS + PGE_2 and LPS + Ado relative to LPS stimulation. Clusters comprised of 47 and 43 genes (A and B, respectively) exhibit functional interactions depicted by edges/arrows. Genes from the shared DEGs in each cluster are depicted by colored circular nodes. The direction of differential expression is depicted by node border color: green represents upregulated and red represents downregulated genes. White diamond nodes represent predicted regulators. **(A)** Network consisting of genes involved in cytokine signaling and growth factor activity including TNF, IL1A, THBS1, TGFA and AREG. Regulators include AP-1 (Jus/Fos), NFKB1, and EP300. **(B)** Network consisting of genes involved in tissue repair including TGFB2 and BMP6. Regulators include UBC and GSK3B.

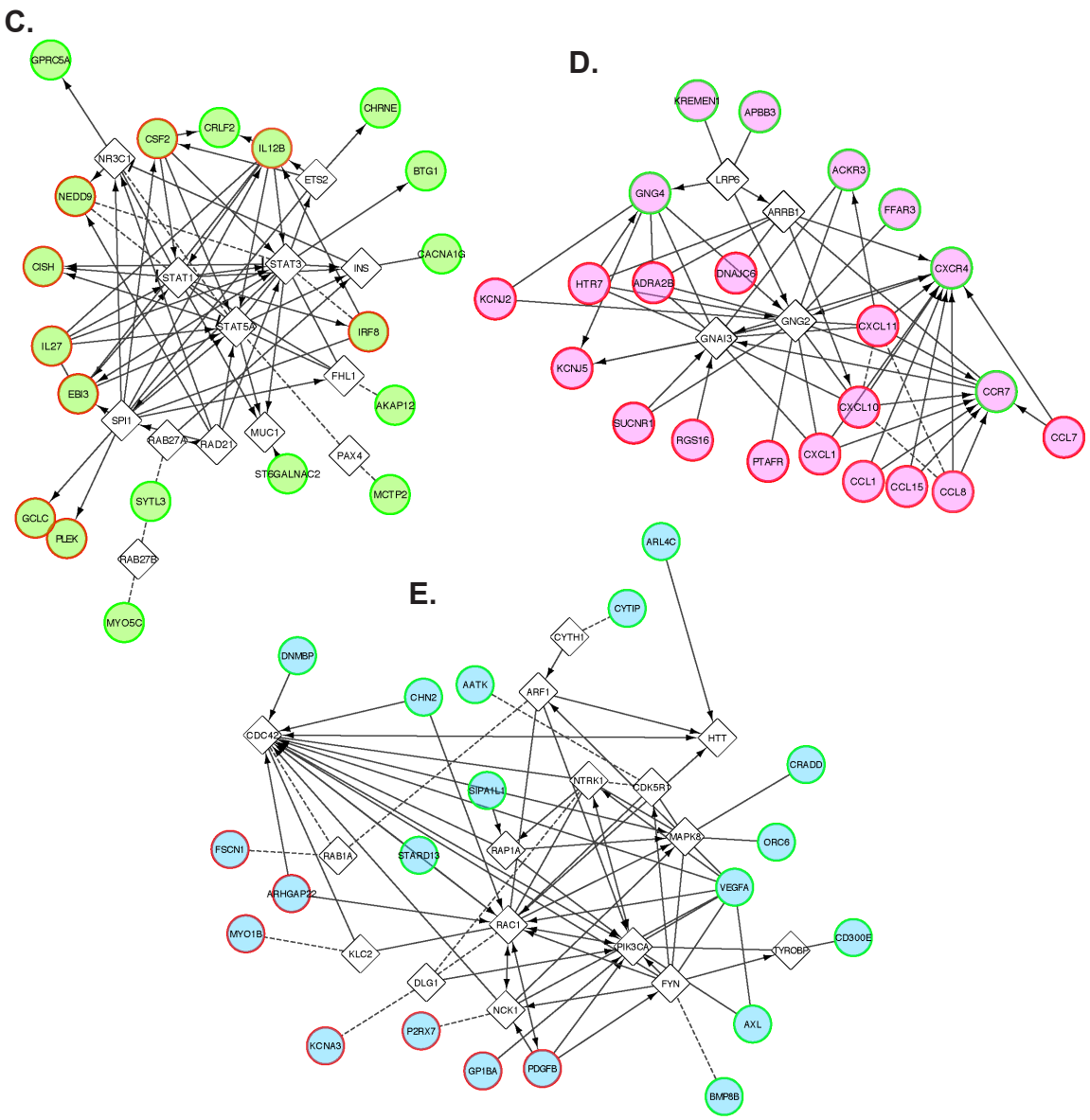

Figure 12 continued. Clusters comprised of 32, 26 and 35 genes (C, D, E respectively) exhibit functional interactions depicted by edges/arrows. **(C)** Network consisting of genes involved in inflammation including IL12B, CSF2, and IL27. Regulators include STAT1, STAT3 and STAT5A. **(D)** Network consisting of genes involved in chemokine activity including CCL1, CCL15, CCL8, CXCL10, CXCL11, CCR7 and CXCR4. Regulators include GNG2 and ARRB1. **(E)** Network consisting of growth promoting genes including VEGFA, CD300E, and PDGFB. REgulators include MAPK8, FYN and PIK3CA.

and predicts that NFκB, RelA, and AP-1 (fos, jun), among other transcription factors are involved in the regulation of these genes (Figure 12A). The second network consists of genes involved in tissue repair including TGFB2 and BMP6 and implicates UBC and GSK3B as regulators (Figure 12B). The third network includes two growth-promoting genes of interest, VEGFA and CD300E, and implicates the tyrosine kinases MAPK8 and FYN (Figure 12C). The fourth network predicts that STAT1, STAT3 and STAT5A are involved in the regulation of this group of genes including inflammatory CSF2 and IL12B (Figure 12D). The last network includes a number of differentially expressed chemokine genes, which are predicted to be regulated by GNG4, GNG2 and ARRB1 (Figure 12E).

Single Cell RNA-Sequencing Analysis. Single cell sequencing was performed in order to enhance our search for marker genes for regulatory macrophages. It allowed us to not only look for expression levels of genes, similar to bulk RNA-sequencing, but also to look at cell numbers expressing select genes. Lists of the most differentially expressed genes by LPS + Adenosine and LPS + PGE_2 stimulated cells versus LPS alone as determined by single cell sequencing are available in Table II and Table III, respectively. From these lists we chose a number of candidate marker genes, mainly encoding cell surface or secreted proteins, to examine their expression on a per cell basis for each sample, depicted in violin plots, which allowed us to look at the distribution of gene expression between cells of each stimulation (Figure 13) as well as feature plots which allowed us to see which specific cells had high and low expression of each gene (Figure 14). We hypothesized that some of these genes could serve as potential transcript biomarkers for LPS + Adenosine and LPS + PGE_2 macrophages, including THBS1,

Table II. Single cell RNA sequencing markers for LPS + PGE_2 versus LPS

	p_val	avg_logFC	pct.1	pct.2	p_val_adj
THBS1	0.000000e+00	1.5116250	0.712	0.181	0.000000e+00
FTH1	0.000000e+00	0.3905140	1.000	1.000	0.000000e+00
CCL3	0.000000e+00	-1.0605658	0.938	0.995	0.000000e+00
CCL4	0.000000e+00	-1.0990854	0.959	0.997	0.000000e+00
TNF	2.071387e-289	-1.4675118	0.088	0.493	6.781307e-285
G0S2	2.484608e-283	1.2760579	0.744	0.315	8.134109e-279
IL8	2.055254e-252	-0.5784006	0.996	0.999	6.728490e-248
FTL	4.241731e-217	0.3095936	1.000	1.000	1.388658e-212
SLAMF7	1.679642e-212	-0.6433449	0.762	0.941	5.498813e-208
NBN	3.915597e-197	-0.6210506	0.691	0.899	1.281888e-192
PRDX1	2.295205e-191	0.4720393	0.978	0.930	7.514041e-187
TIMP1	1.439140e-176	0.7002907	0.905	0.745	4.711456e-172
PLEK	1.671320e-175	-0.7291354	0.392	0.708	5.471567e-171
EREG	2.040622e-173	0.5781449	0.984	0.968	6.680588e-169
CYBA	2.862073e-172	0.4637345	0.953	0.857	9.369853e-168
PTX3	1.791745e-171	-1.1279416	0.287	0.620	5.865813e-167
RPS2	1.041126e-164	0.4048864	0.977	0.924	3.408439e-160
FNIP2	1.924515e-162	-0.6647760	0.511	0.783	6.300478e-158
MIR155HG	1.777701e-150	-0.6838640	0.358	0.668	5.819838e-146
CD300E	7.292776e-144	0.8285652	0.466	0.160	2.387509e-139

Table III. Single cell RNA sequencing markers for LPS + Ado versus LPS

	p_val	avg_logFC	pct.1	pct.2	p_val_adj
THBS1	0.000000e+00	2.2714325	0.889	0.181	0.000000e+00
G0S2	0.000000e+00	1.9417623	0.839	0.315	0.000000e+00
CREM	0.000000e+00	1.8509289	0.892	0.200	0.000000e+00
INHBA	0.000000e+00	1.5476170	0.866	0.366	0.000000e+00
ATP1B3	0.000000e+00	1.4491966	0.926	0.569	0.000000e+00
CD300E	0.000000e+00	1.3897212	0.700	0.160	0.000000e+00
TIMP1	0.000000e+00	1.3845081	0.945	0.745	0.000000e+00
ISG15	0.000000e+00	1.3525049	0.995	0.956	0.000000e+00
PLAUR	0.000000e+00	1.1641703	0.936	0.675	0.000000e+00
EREG	0.000000e+00	1.0786691	0.997	0.968	0.000000e+00
SAMSN1	0.000000e+00	1.0551717	0.832	0.369	0.000000e+00
BTG1	0.000000e+00	0.8130690	0.980	0.866	0.000000e+00
PRDX1	0.000000e+00	0.7539932	0.981	0.930	0.000000e+00
FTH1	0.000000e+00	0.4473626	1.000	1.000	0.000000e+00
NCF2	0.000000e+00	-0.9195243	0.644	0.933	0.000000e+00
CYP1B1	0.000000e+00	-0.9656822	0.892	0.991	0.000000e+00
CCL2	0.000000e+00	-1.5773813	0.523	0.923	0.000000e+00
BHLHE41	3.781501e-292	-1.1658486	0.061	0.495	1.237988e-287
NBN	1.461365e-275	-0.8256825	0.602	0.899	4.784218e-271
REL	2.825889e-268	0.8714018	0.919	0.750	9.251397e-264

Tables II and III generated with Dr. Ashton Trey Belew

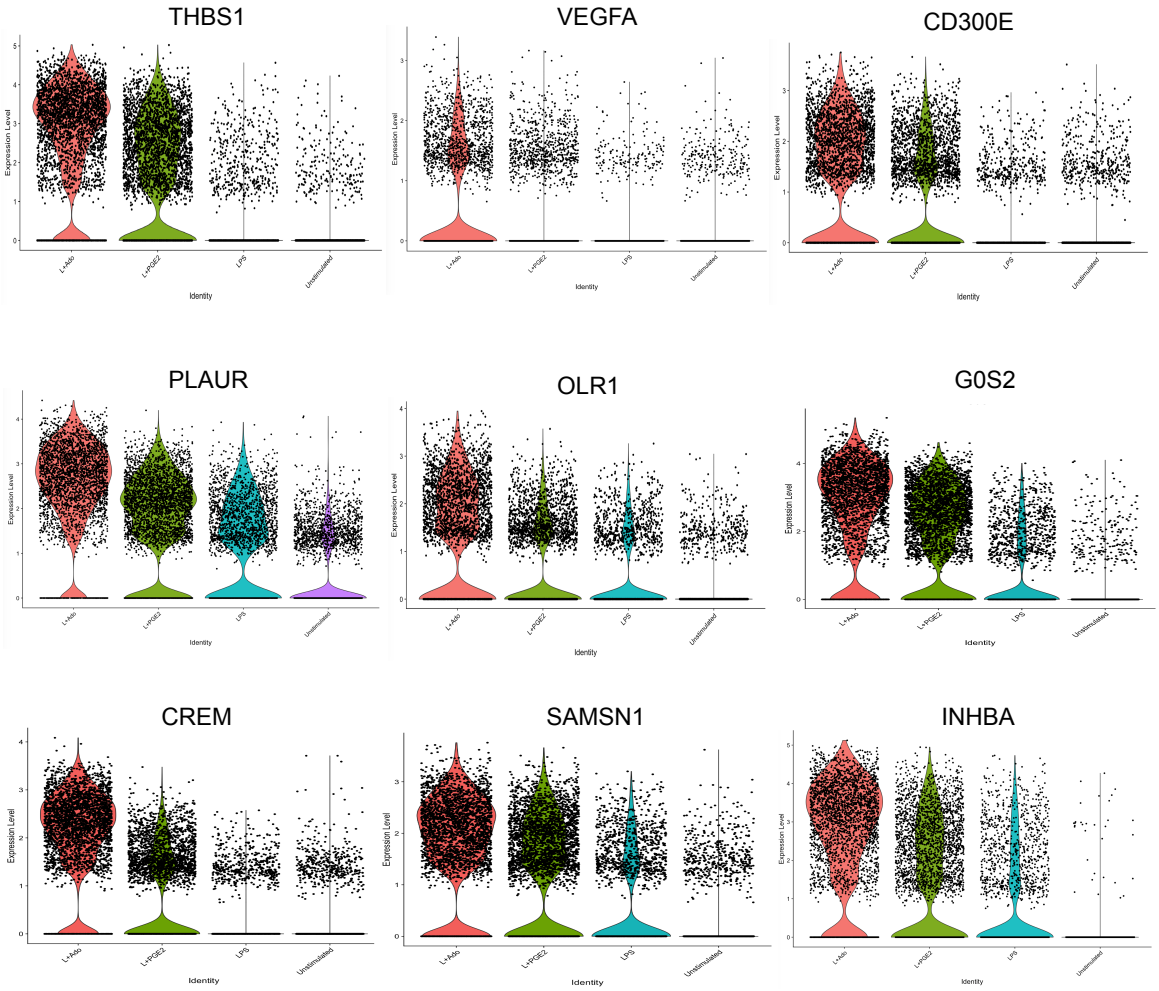

Figure 13. Violin plots of candidate RNA markers for LPS+Adenosine and LPS+PGE$_2$ stimulated M-CSF macrophages. Single cell RNA-seq was performed on M-CSF macrophages and differential expression analysis was done between sample stimulation groups: LPS + Ado (Red), LPS + PGE2 (Green), LPS (Teal), Unstimulated (Purple). From this analysis, 9 genes **(A)** THBS1, **(B)** VEGFA, **(C)** CD300E, **(D)** PLAUR, **(E)** OLR1, **(F)** G0S2, **(G)** CREM, **(H)** SAMSN1, and **(I)** INHBA, were selected based on their similar expression pattern between LPS + PGE$_2$ and LPS + Adenosine. These plots indicate the distribution of individual cells based on their expression level for these genes.

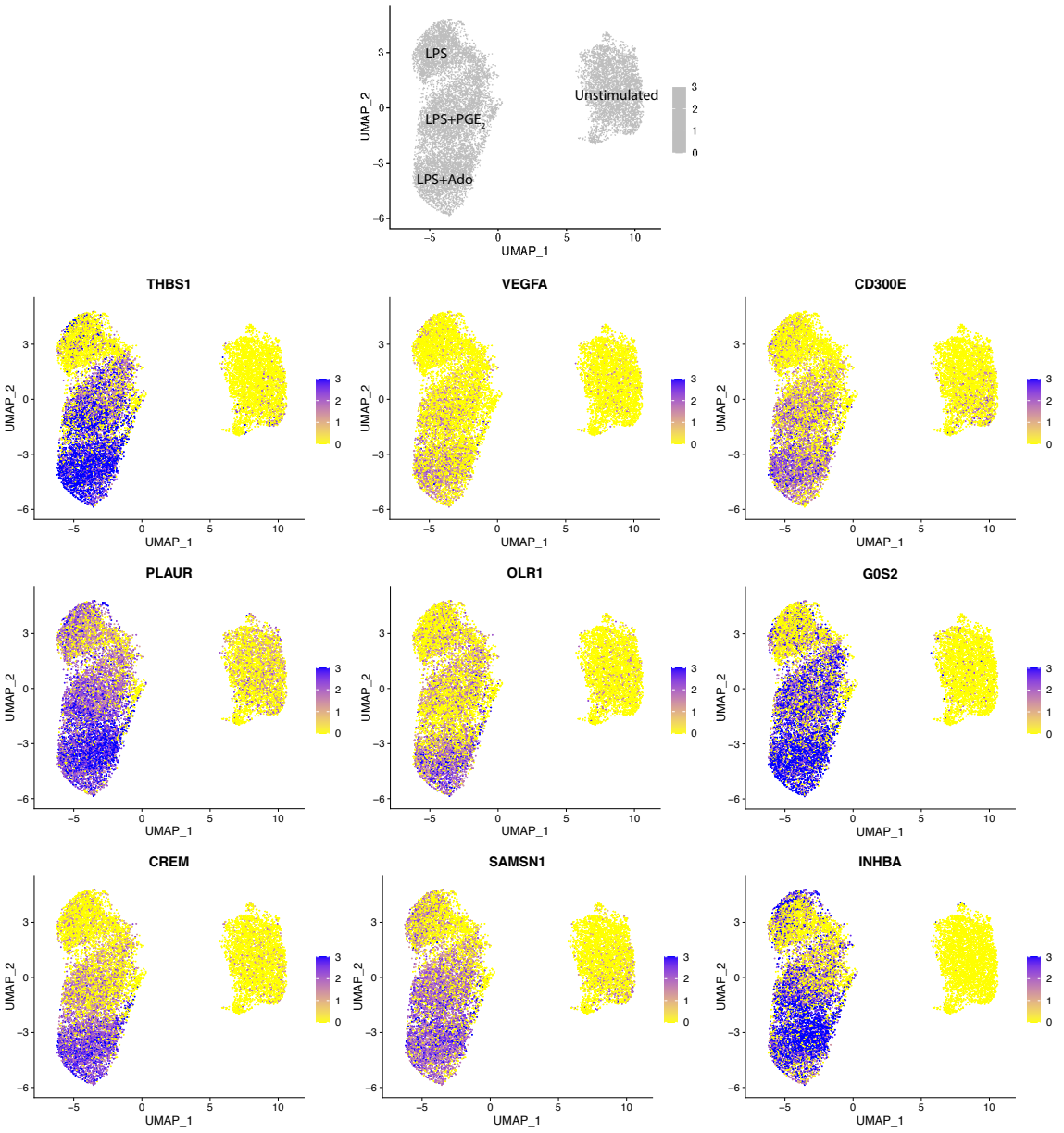

Figure 14. Feature plots of candidate RNA markers for LPS + Adenosine and LPS + PGE_2 stimulated M-CSF macrophages. Single cell RNA-seq was performed on M-CSF macrophages and differential expression analysis was done between sample stimulation groups. From this analysis, 9 genes **(A)** THBS1, **(B)** VEGFA, **(C)** CD300E, **(D)** PLAUR, **(E)** OLR1, **(F)** G0S2, **(G)** CREM, **(H)** SAMSN1, and **(I)** INHBA, were selected based on their similar expression pattern between LPS + PGE_2 and LPS + Adenosine. These plots indicate which cells are expressing these genes relative to one another on a scale of 0-3 (yellow-blue). The grey plot indicates the identity of the clusters based on stimulation condition.

VEGFA, CD300E, PLAUR, OLR1, G0S2, CREM, SAMSN1 and INHBA. A plot of the most variable genes between all 4 samples highlighted a number of genes that we believe comprise a part of the M1 macrophage signature, or the response to LPS, because these should theoretically be most different from both unstimulated and LPS + Adenosine or LPS + PGE_2 samples (Figure 15A). The top 30 genes are labeled and include CXCL10, CXCL11, TNF, IL23A, CCL3, CCL4 and a number of other chemokine genes. The genes colored in orange are those that were also found in the top 100 most highly upregulated genes by LPS stimulation versus unstimulated in our conventional RNA-sequencing data. Conventional RNA-sequencing also demonstrated that the majority of the top 25 most highly upregulated genes by LPS relative to unstimulated macrophages were significantly downregulated by either adenosine, PGE_2 or both (Figure 15B). This suggests that while the transcriptomic changes by adenosine and PGE_2 are limited, they have important consequences in the host.

4.3 Discussion

In this chapter we explored the effects of two molecules, adenosine and PGE_2, which have previously been shown to modulate the aspects of the inflammatory response induced by LPS. We performed conventional RNA sequencing on macrophages stimulated with LPS in the presence of adenosine and PGE_2, and found that their transcriptional program was highly similar using thorough differential expression analysis. This degree of similarity was observed in the number of shared DEGs between the two stimulation conditions. It was also observed in the direction and extent of the changes in gene expression, two parameters that were factored into the Spearman correlation analysis. Single-cell RNA sequencing also supported the likeness between

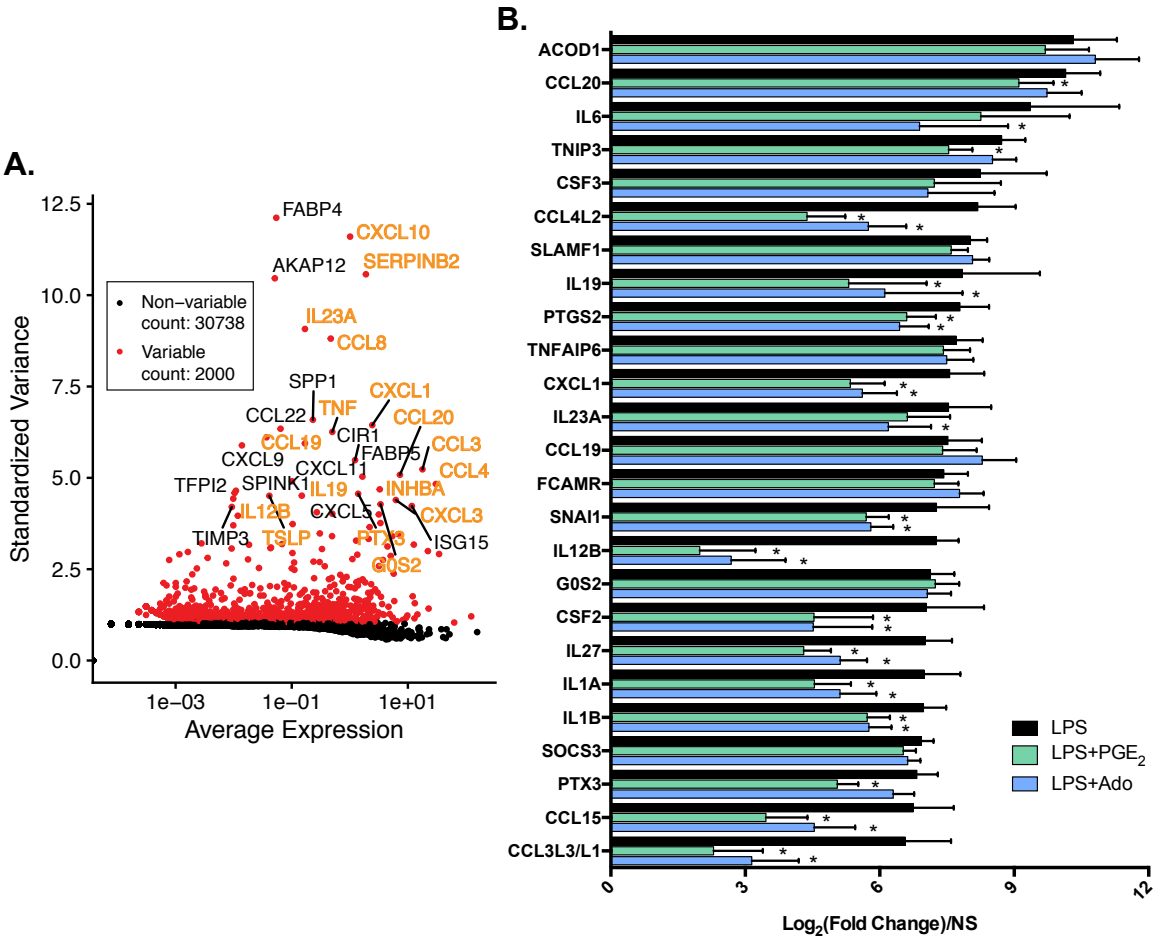

Figure 15. M1 genes in M-CSF macrophages. (A) The most significant variable genes among all samples (unstimulated, LPS, LPS + Ado and LPS + PGE_2) are indicated with red dots. The top 30 most variable genes are labeled. The genes labeled in orange are also found in the top 100 most differentially expressed genes by LPS versus unstimulated in bulk RNA-seq analysis. **(B)** The top 25 most highly upregulated genes by LPS (black) versus unstimulated (NS) macrophages and their log_2FC are depicted. The corresponding log_2FC of LPS + Ado (blue) and LPS + PGE_2 (green) versus NS is also depicted. (n=5, * indicates genes with a $log_2FC > 1$ and *P*-value < 0.05 relative to LPS as determined by differential expression analysis, error bars represent SEM).

Figure 15A generated with Dr. Ashton Trey Belew

adenosine and PGE_2 stimulated samples. We believe that this high degree of similarity could be attributed to signaling through g-protein coupled receptors (GPCRs) which leads to the intracellular release of cAMP, and downstream activation of transcription factors that control numerous genes involved in immune responses. While similar, PGE_2 appeared to have a more pronounced effect on LPS stimulated macrophages than adenosine, due to a higher number of uniquely perturbed transcripts. However, this could be concentration dependent and perhaps it requires more adenosine to achieve even further overlap in transcriptomic phenotype with PGE_2.

Pathway analysis, including GO, and careful inspection of individual gene changes by LPS + Adenosine and LPS + PGE_2 relative to LPS alone led us to conclude that these stimuli lead to what our lab refers to as a "regulatory" phenotype. This regulatory phenotype was one characterized by the induction of growth promoting genes and the suppression of inflammatory genes. Single-cell RNA sequencing was performed in order to aid in our identification of potential biomarkers for LPS + adenosine and LPS + PGE_2 stimulated macrophages. This technique was powerful because it allowed us to look at the gene expression patterns of a large number of cells (roughly 4000 cells per sample). It also indicated to us that in vitro stimulation of macrophages is fairly homogeneous, since individual samples were stimulated in separate wells, but when all the sample data was combined during analysis, the cells clustered together according to their stimulation condition. We identified numerous transcripts that could be used as RNA biomarkers to find regulatory macrophages in tissues including THBS1, VEGFA, CD300E, PLAUR, OLR1, SAMSN1, and G0S2. RNA biomarkers also have the

potential to be used to scan transcriptomic data generated from different disease or immune conditions for the likely presence of regulatory macrophages.

Single-cell sequencing led us to identify the most variable genes in our data set between all stimulations including unstimulated, LPS, LPS + Adenosine and LPS + PGE_2. We propose that these genes represented mainly the M1 phenotype, because M1 genes should be the most changed in the presence of adenosine and PGE_2 and relative to no stimulation. This was supported by the fact that 18 out of the 30 most variable genes were also present in the top 100 most highly differentially expressed genes by LPS versus unstimulated macrophages found by conventional RNA sequencing. We then demonstrated with conventional RNA sequencing that the majority of the top 25 most highly upregulated genes by LPS stimulation over resting cells were significantly downregulated by combination with adenosine, PGE_2, or both. Many of these genes encode inflammatory cytokines and chemokines. These data imply that while the global transcriptomic changes induced by adenosine and PGE_2 are relatively limited in number, they are highly specific and target some of the most important genes that comprise the LPS inflammatory M1 signature. Overall, the work in this chapter describes two highly similar populations of M-CSF macrophages that are characterized by increased expression of tissue repair genes and decreased expression of cytokine and chemokine genes.

5 Comparison of human GM-CSF derived macrophages to M-CSF derived macrophages both stimulated with LPS + Adenosine and LPS + PGE_2

5.1 Introduction

While M-CSF is the growth factor constitutively expressed in a number of tissues that gives rise to macrophages from infiltrating monocytes, there is a second growth factor, GM-CSF, which also leads to differentiated macrophages. GM-CSF is constitutively produced in the lung, but is also induced during inflammatory responses. We wanted to know if macrophages differentiated in GM-CSF are equally as capable of being programmed into "regulatory" macrophages, as are M-CSF macrophages, in response to LPS + Adenosine and LPS + PGE_2 stimulation. This information would be useful as GM-CSF could potentially be used to modulate the macrophage activation response.

To explore this question we performed RNA-sequencing on donor matched GM-CSF derived macrophages stimulated with LPS, LPS + Adenosine, and LPS + PGE_2 and compared the transcriptomic data to our analysis in the previous chapter for M-CSF derived macrophages. We also did in vitro assays of M-CSF and GM-CSF macrophages side by side to further validate the results from our M-CSF RNA-sequencing analysis, and to compare the in vitro phenotypes of GM-CSF and M-CSF macrophages. Before we began, we examined the expression of macrophage marker CD68 (Figure 16A), dendritic cell marker CD1a (Figure 16B) and macrophage marker CD11b (Figure 16C) to confirm that following 7 days of differentiation in M-CSF and GM-CSF, our blood monocyte samples did fully mature into macrophages and not dendritic cells.

5.2 Results

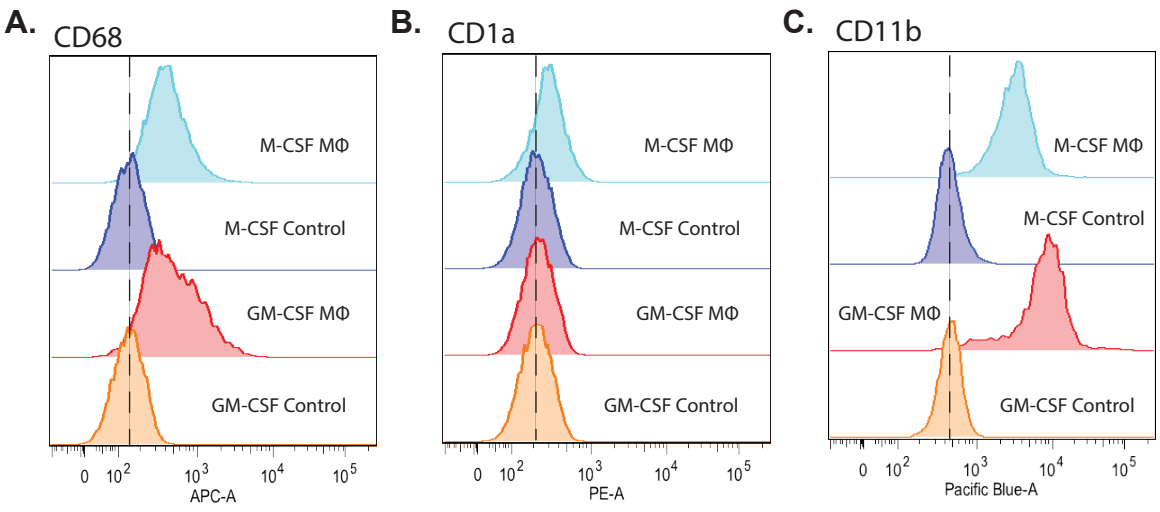

Figure 16. Flow cytometry validation of M-CSF and GM-CSF derived macrophages. Markers for **(A)** macrophage CD68, **(B)** dendritic cell CD1a and **(C)** macrophage CD11b were detected on macrophages differentiated in M-CSF and GM-CSF for 7 days to confirm that our working cells in both differentiation conditions are in fact mature macrophages. Relevant isotype controls are depicted for each antibody.

Adenosine and PGE_2 sensing in human macrophages. It has recently been published that GM-CSF macrophages degrade less ATP in vitro, and produce less adenosine when stimulated, compared to M-CSF macrophages[184]. We confirmed these results in our M-CSF and GM-CSF macrophages by examining the kinetics of ATP degradation over a period of 2 hours (Figure 17A). This important finding fits in line with our hypothesis that M-CSF macrophages produce more ATP and convert it to adenosine via CD39 and CD73, which can subsequently act in an autocrine fashion to suppress macrophage inflammation[26]. Similarly, at 24 hours post-LPS stimulation, supernatants from M-CSF macrophages contained higher levels of PGE_2 while the levels of PGE_2 in supernatants from GM-CSF macrophages were unchanged with LPS stimulation (Figure 17B). The expression of a number of purinergic and prostaglandin receptor genes was measured by RT-PCR (Figure 18). M-CSF macrophages stimulated with LPS upregulated the expression of the A2a receptor (Figure 18B), EP2 receptor (Figure 18G) and EP4 receptor (Figure 18H) while GM-CSF macrophages did not upregulate any of these receptors to the same extent, and exhibited lower expression levels for these receptors overall. mRNA expression of the A2b receptor (Figure 18C) did not change with LPS stimulation in either M-CSF or GM-CSF, though its expression was higher in M-CSF macrophages overall. The expression of the A1 and A3 receptors decreased following LPS stimulation in both M-CSF and GM-CSF macrophages (Figures 18A and 18D, respectively). These two receptors are coupled to the G_i family of signaling proteins, which typically decrease cAMP release when activated[185]. The mRNA expression of the two ecto-enzymes involved in the hydrolysis of ATP, CD39 and CD73, did not change with LPS stimulation and were not significantly different between M-CSF and GM-CSF

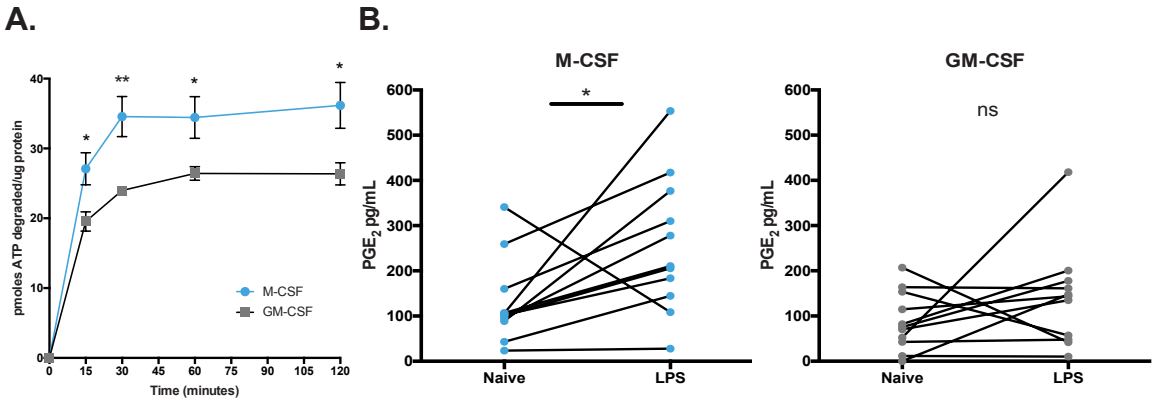

Figure 17. ATP degradation and PGE_2 production by human macrophages. (A) The degradation of 20μM ATP was measured over time and expressed per μg of protein per sample (n=3 donors, * *P*-value < 0.05, ** *P*-value < 0.01, error bars represent SEM). **(B)** PGE_2 was measured in the supernatants of macrophages stimulated with LPS for 24 hours (n=10 donors, * *P*-value < 0.05, lines connect samples from the same individual).

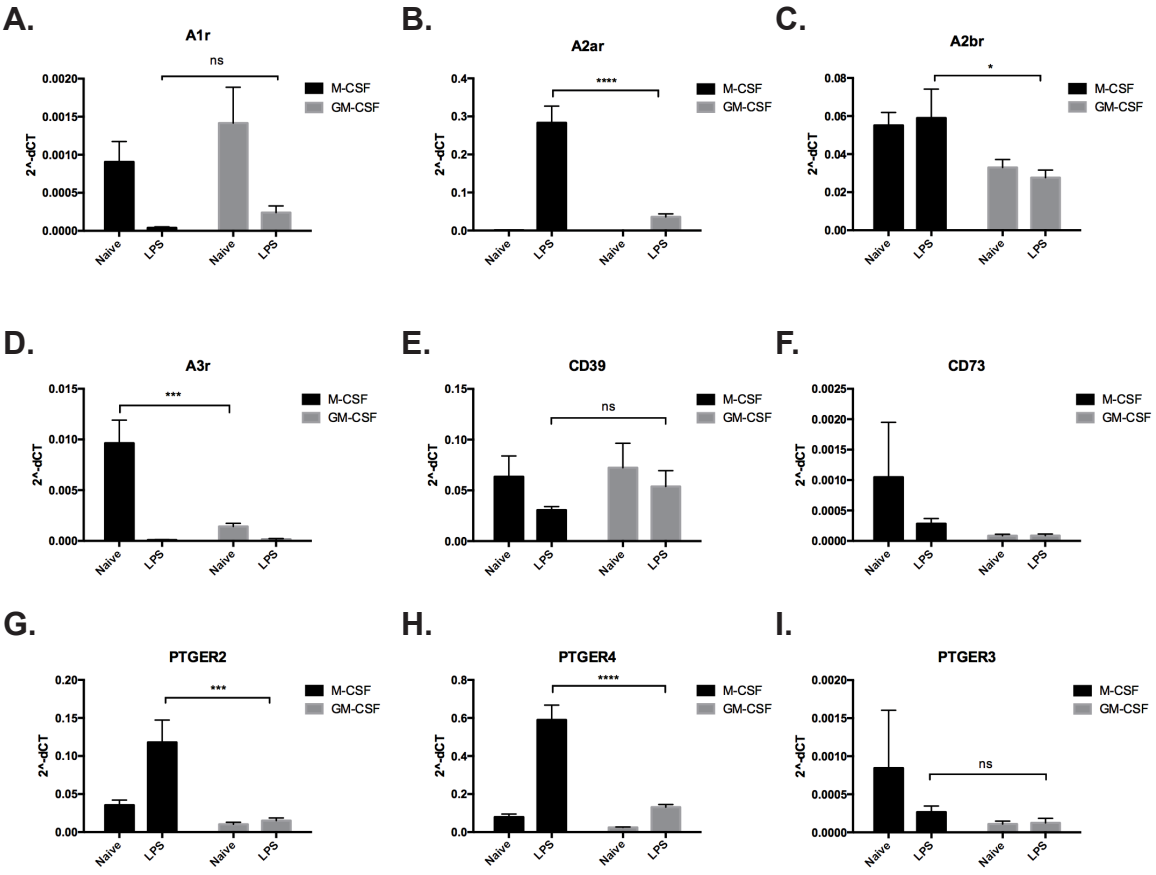

Figure 18. mRNA expression of purinergic pathway and PGE2 receptor genes in M-CSF and GM-CSF macrophages. Quantitative RT-PCR was used to measure transcript levels of **(A)** A1R, **(B)** A2AR, **(C)** A2BR, **(D)** A3R, **(E)** CD39, **(F)** CD73, **(G)** PTGER2, **(H)** PTGER4, and **(I)** PTGER3 following stimulation with LPS for 4 hours (n=5 donors, * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001, **** *P*-value < 0.0001, error bars represent SEM).

macrophages (Figures 18E and 18F, respectively. Lastly, the third receptor for PGE_2, PTGER3, did not change at the transcript level following LPS stimulation and was expressed similarly at low levels in M-CSF and GM-CSF macrophages (Figure 18I). The mRNA level of constitutively expressed COX1 did not change with LPS stimulation, nor was it different in M-CSF and GM-CSF macrophages (Figure 19A). However, the increase in PGE_2 levels in the supernatants of LPS stimulated M-CSF macrophages correlated with an increase in the expression of COX2 mRNA, which is inducible (Figure 19B). The expression of the prostaglandin synthase gene, MPGES1, seemed to increase slightly in LPS stimulated M-CSF macrophages, but this observation was not found to be significant (Figure 19C). The expression pattern of adenosine and PGE_2 pathway genes in M-CSF macrophages was not exclusive to LPS stimulation. We tested various TLR ligands including, FSL-1 (TLR2/6), HKLM (TLR2), Loxoribine (TLR 7), and Poly I:C (TLR 3). We noticed that A2AR expression was significantly different between M-CSF and GM-CSF macrophages (Figure 20A). A2BR expression was not significantly changed (Figure 20B). mRNA expression for PTGER2 (Figure 20C), PTGER4 (Figure 20D), MPGES1 (Figure 20E), COX1 (Figure 20F) and COX2 (Figure 20G) was higher in M-CSF macrophages than in GM-CSF macrophages for most of the TLR ligands with the exception of COX1, which was expected.

Transcriptome comparison of M-CSF and GM-CSF macrophages. Principal component analysis (PCA) revealed that M-CSF and GM-CSF macrophages are notably different, even following stimulation, and they separated along principal component 1 (PC1), which explains approximately 54% of the variance among samples (Figure 21). LPS + Adenosine, LPS + PGE_2 and LPS stimulated samples differed from each other as

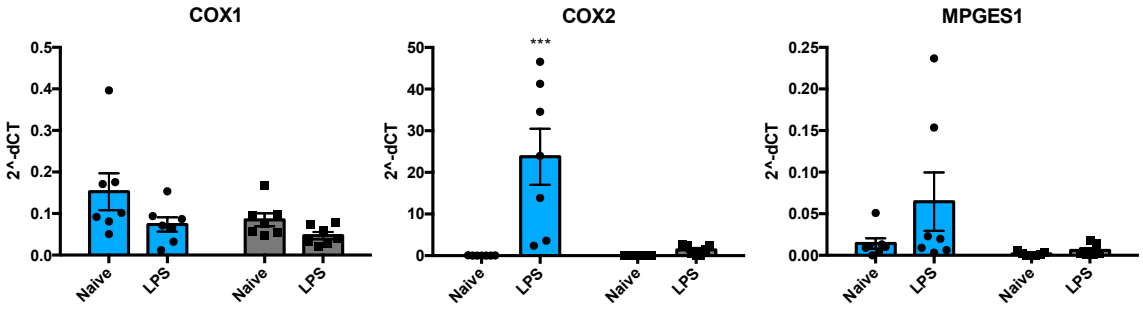

Figure 19. mRNA expression of PGE_2 synthesis pathway genes. M-CSF (blue) and GM-CSF (grey) macrophages were stimulated with LPS for 4 hours. **(A)** COX1, **(B)** COX2 and **(C)** MPGES1 mRNA levels were measured by RT-PCR (n=7, *** *P*-value < 0.001, error bars represent SEM).

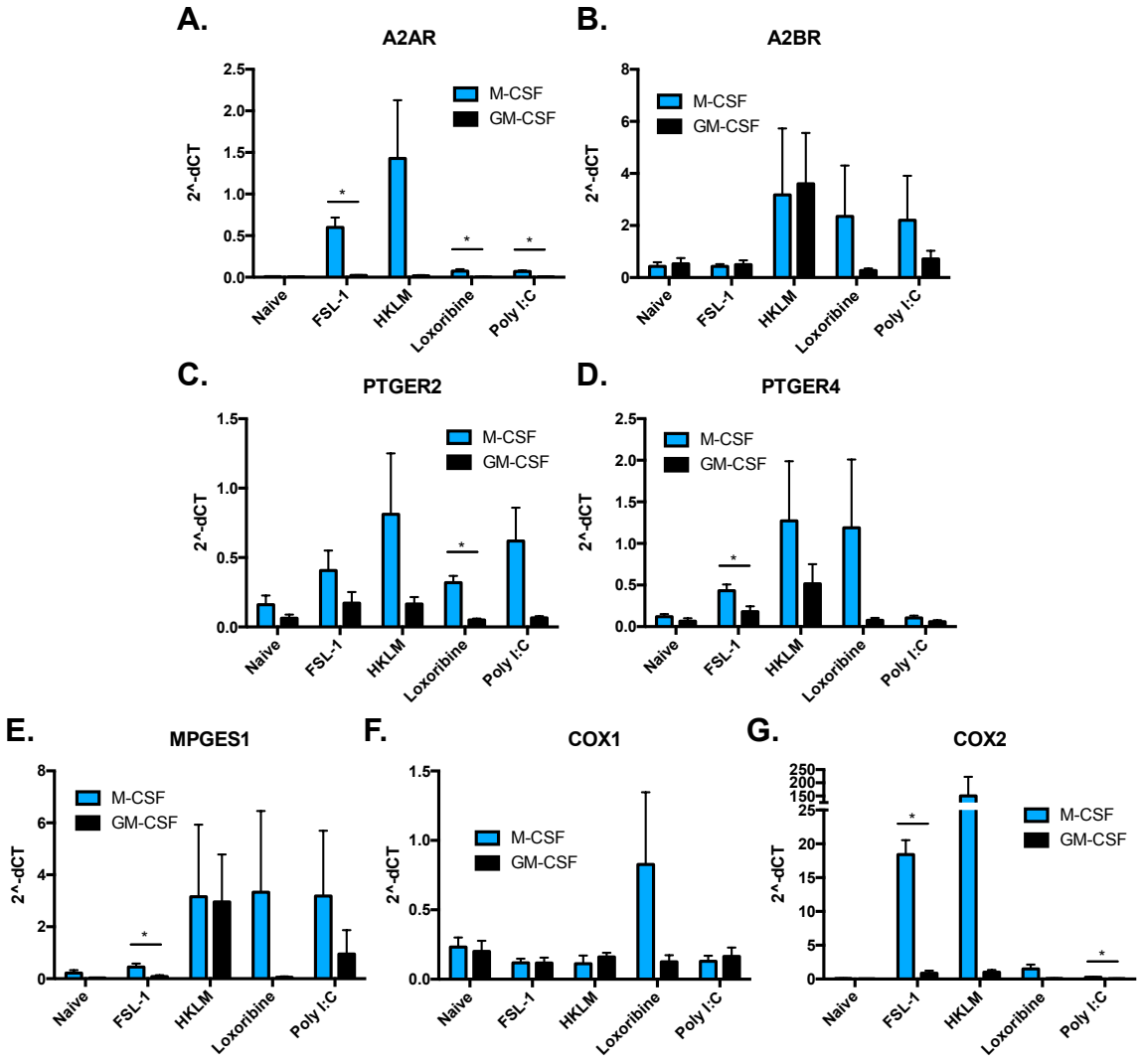

Figure 20. Purinergic and PGE_2 pathway gene expression following stimulation with various TLR ligands. mRNA expression for **(A)** A2AR, **(B)** A2BR, **(C)** PTGER2, **(D)** PTGER4, **(E)** MPGES1, **(F)** COX1 and **(G)** COX2 following 4 hours stimulation with FSL-1, HKLM, Loxoribine, and Poly I:C (n=3, * *P*-value < 0.05, error bars represent SEM).

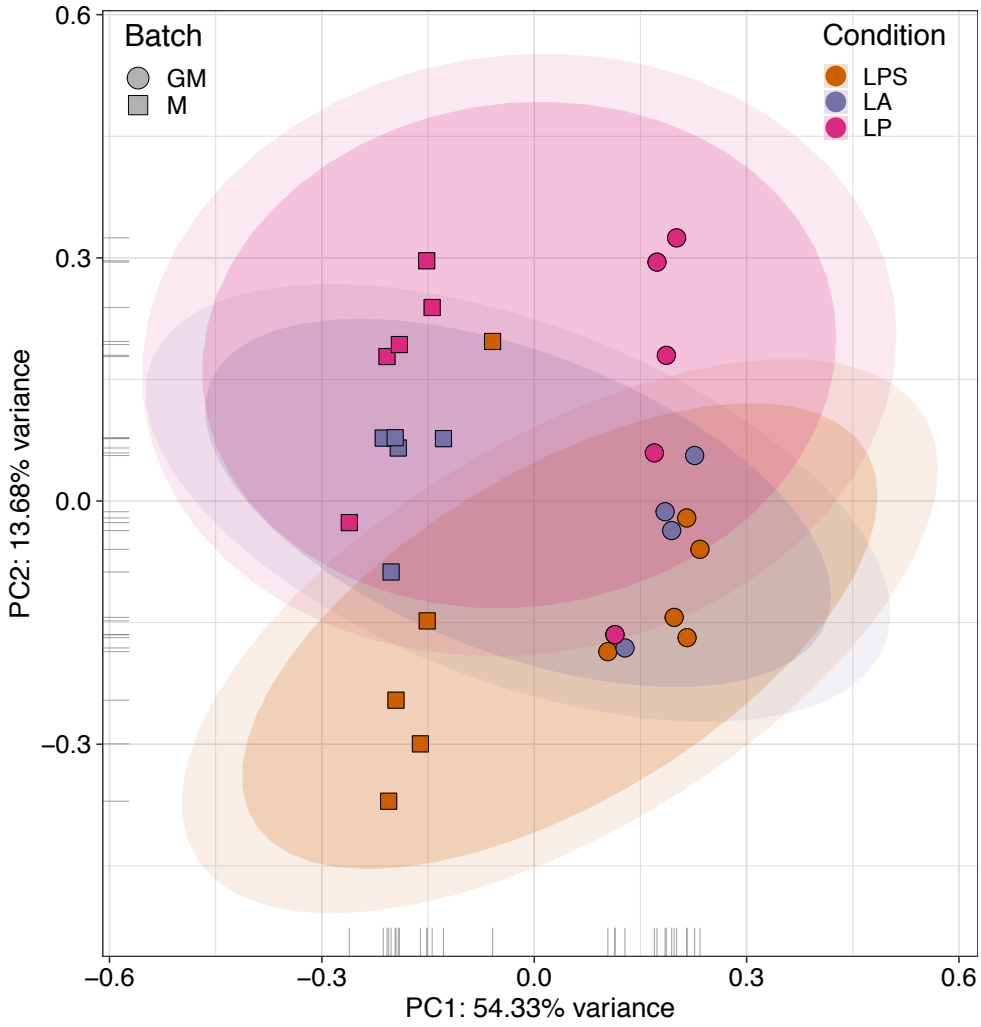

Figure 21. PCA plot of stimulated human macrophage samples. M-CSF (squares) and GM-CSF (circles) macrophages were stimulated with LPS (orange), LPS + Ado (purple) and LPS + PGE_2 (pink) for 4 hours and bulk RNA-seq was performed. Principal component analysis (PCA) indicating variance among samples is visualized above.

Figure generated with Dr. Ashton Trey Belew

seen on principal component 2 (PC2), which describes approximately 14% of the variance between samples. Also on PC2, we observed a larger spread between LPS, LPS + Adenosine, and LPS + PGE_2 samples, indicating greater variance between M-CSF samples than we observed in GM-CSF samples. Volcano plots of all measured transcripts indicated that LPS + Adenosine and LPS + PGE_2 stimulation of GM-CSF macrophages resulted in only 7 and 126 differentially expressed genes (DEGs), respectively, compared to LPS alone (Figure 22). This is significantly lower than the 256 and 489 DEGs by LPS + Adenosine and LPS + PGE_2, respectively, relative to LPS alone in M-CSF macrophages. We selected the top 20 differentially expressed genes by M-CSF macrophages stimulated with LPS + PGE_2 (Figure 23A) and LPS + Adenosine (Figure 23B) versus LPS alone, and compared their fold changes with the corresponding stimulation conditions in GM-CSF macrophages. Many of these transcripts were not as highly upregulated in GM-CSF samples. Similarly, none of the most highly downregulated transcripts by LPS + PGE_2 (Figure 23C) and LPS + Adenosine (Figure 23D) versus LPS alone in M-CSF macrophages were as highly downregulated by adenosine and PGE_2 in GM-CSF macrophages. The expression of several genes of interest, based on their involvement in cell growth and tissue remodeling, was measured by RT-PCR in M-CSF and GM-CSF macrophages in order to supplement RNA-sequencing data. These genes, including: THBS1, CD93, AREG, VEGFA, CD300E, CXCL13, MMP10, and RGS2, were all significantly upregulated by LPS + Adenosine and LPS + PGE_2 stimulation compared to LPS alone in M-CSF macrophages (Figure 24). With the single exception of TGFA, an upregulation of these regulatory transcripts was not observed in GM-CSF macrophages (Figure 24). Altogether, we believe that this

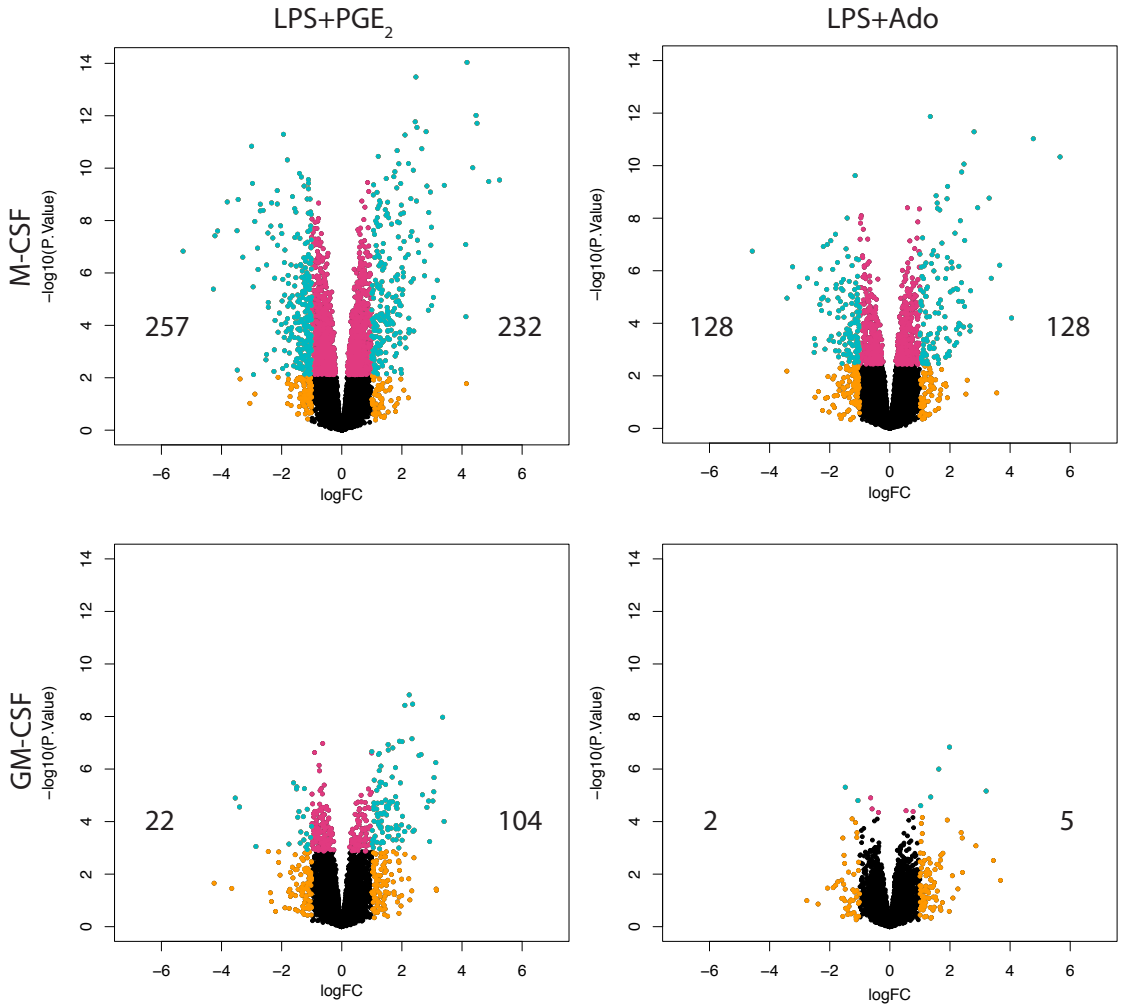

Figure 22. Volcano plot visualization of DEGs from LPS + Ado and LPS + PGE_2 stimulated macrophages relative to LPS alone. Bulk RNA-seq of M-CSF (top) and GM-CSF (bottom) macrophages stimulated with LPS + PGE_2 (left) and LPS + Ado (right) was analyzed for differential expression of genes relative to LPS alone. Green points represent significantly changed genes (*P*-value < 0.05, $\log_2$FC > 1). The numbers of significantly upregulated and downregulated genes are indicated in the plot area. Pink points represent genes with a *P*-value < 0.05 and $\log_2$FC < 1. Yellow points represent genes with a *P*-value > 0.05 and $\log_2$FC > 1. Black points represent genes with a *P*-value > 0.05 and $\log_2$FC < 1.

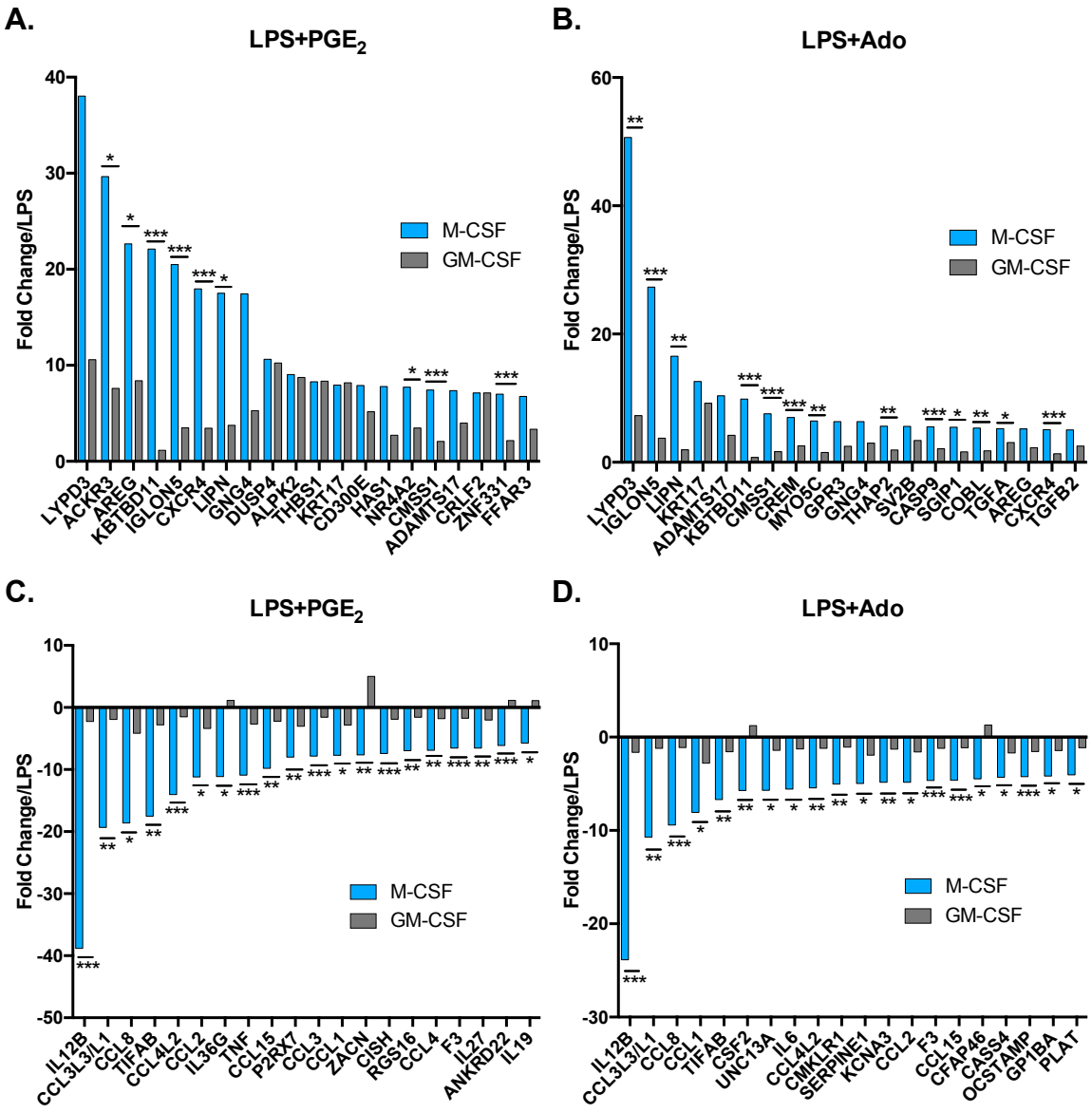

Figure 23. Comparison of expression of DEGs by LPS + Adenosine and LPS + PGE_2 relative to LPS alone in M-CSF and GM-CSF macrophages. The 20 most upregulated differentially expressed genes in M-CSF (blue) **(A)** LPS + PGE_2 and **(B)** LPS + Ado stimulated macrophages relative to LPS alone are listed on the x-axis. Their corresponding fold changes in GM-CSF (grey) LPS+ PGE_2 and LPS + Adenosine macrophages relative to LPS are plotted (n=5, **P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001). The 20 most downregulated differentially expressed genes in M-CSF **(C)** LPS + PGE_2 and **(D)** LPS+Adenosine stimulated macrophages relative to LPS alone are listed on the x-axis. Their corresponding fold changes in GM-CSF LPS + PGE_2 and LPS + Ado macrophages relative to LPS are plotted (n=5, **P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001).

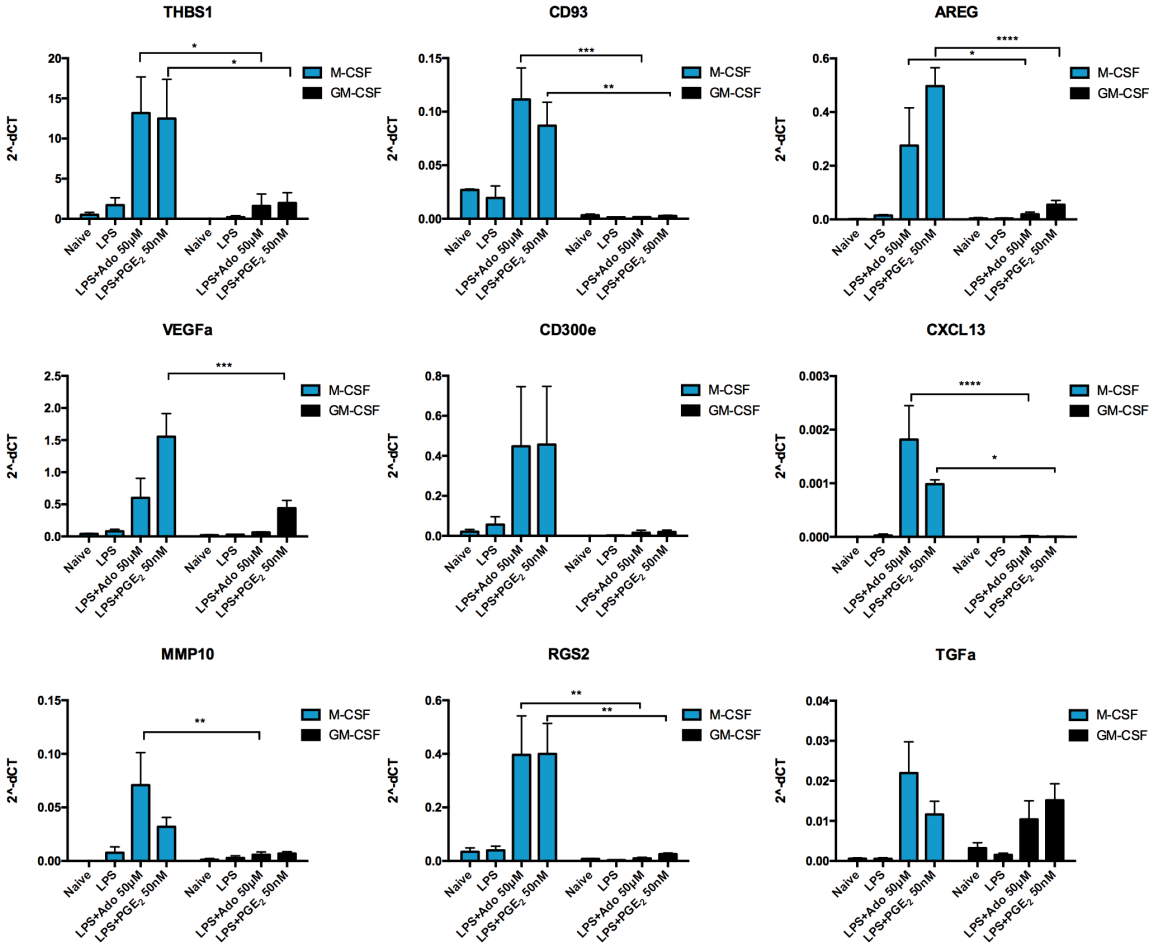

Figure 24. mRNA expression of genes of interest in M-CSF and GM-CSF macrophages. Macrophages were stimulated with LPS, LPS + Adenosine and LPS + PGE_2 for 4 hours and the expression for genes THBS1, CD93, AREG, VEGFA, CD300E, CXCL13, MMP10, RGS2, and TGFA was measured by RT-PCR (n=5, * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001, **** *P*-value < 0.0001, error bars represent SEM).

transcriptomic data indicates a defect in adenosine and PGE_2 sensing by GM-CSF macrophages. We hypothesize that this lack of sensing contributes to GM-CSF macrophages being hyper-inflammatory.

Functional assays of human macrophages. Human umbilical vein endothelial (HUVEC) cells cultured in conditioned media for 24 hours from stimulated M-CSF and GM-CSF macrophages exhibited tube formation on a Matrigel surface (Figure 25A). HUVEC cells cultured in conditioned media from M-CSF macrophages stimulated with LPS + Adenosine and LPS + PGE_2 exhibited the highest levels of tube formation based on tube length (Figure 25B) and the number of nodes between tubes (Figure 25C). HUVEC cells cultured in conditioned media from GM-CSF macrophages stimulated with LPS + Adenosine and LPS + PGE_2 showed no increase in tube formation relative to HUVECs cultured in supernatants of LPS stimulated macrophages. In fact, HUVEC cells cultured in media from stimulated GM-CSF macrophages exhibited defects in tube formation relative to those grown in media from unstimulated GM-CSF macrophages and relative to those grown in media from M-CSF macrophages.

One of the transcripts that emerged from both our bulk RNA-sequencing and single cell RNA-sequencing analysis as being highly differentially expressed in LPS + Adenosine and LPS + PGE_2 stimulated M-CSF macrophages relative to LPS alone was CD300E. CD300E was expressed on the surface of a higher percentage of cells in LPS+ Adenosine and LPS + PGE_2 stimulated M-CSF macrophages compared to LPS stimulated M-CSF macrophages (Figure 26A). The level of CD300E expression (median fluorescence intensity) was also increased with LPS + Adenosine and LPS + PGE_2 stimulation in M-CSF macrophages, but its expression did not increase beyond baseline

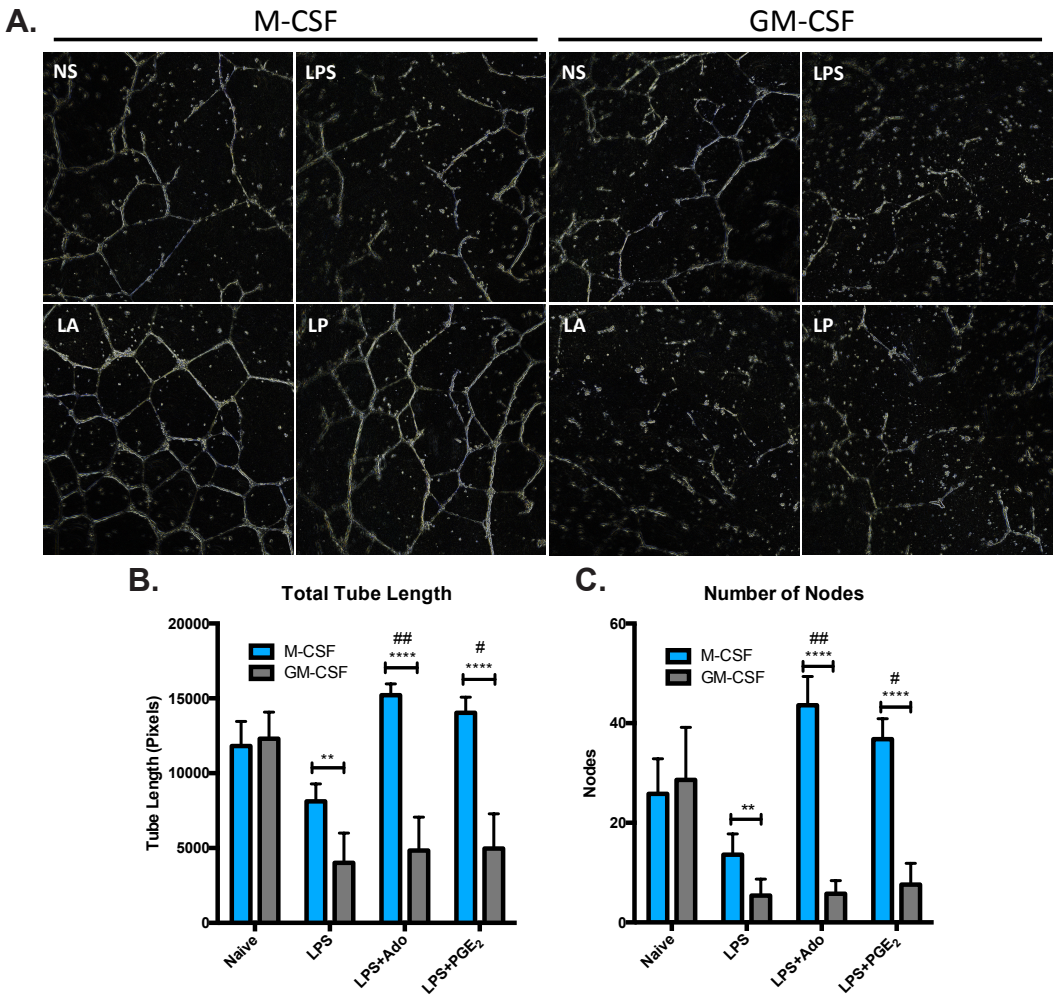

Figure 25. HUVEC cell tube formation in the presence of macrophage conditioned media. Human monocytes were cultured in M-CSF or GM-CSF for 7 days then left unstimulated or stimulated with LPS, LPS + Ado and LPS + PGE_2 for 24 hours at which point supernatants were collected for further studies. **(A)** HUVEC cell tube formation was observed after 24 hour exposure to supernatants harvested from stimulated M-CSF and GM-CSF macrophages from one representative donor (n=3 donors total). Representative images were captured by brightfield microscopy with "find edges" contrast applied in ImageJ in order to be able to see the tubes. **(B)** Total tube length was measured in pixels manually using ImageJ software on various images of HUVEC cells exposed to supernatants of macrophages from multiple donors (n=3 donors, ** *P*-value <0.01, **** *P*-value <0.0001 between M-CSF and corresponding GM-CSF samples; # *P*-value <0.05, ## *P*-value <0.01 for M-CSF samples relative to NS supernatants; error bars represent SEM). **(C)** The number of nodes, defined as 3 or more tubes originating from one point, was counted manually using ImageJ software on various images of HUVEC cells exposed to supernatants of macrophages from multiple donors (n=3 donors, ** *P*-value <0.01, **** *P*-value <0.0001 between M-CSF and corresponding GM-CSF samples; # *P*-value <0.05, ## *P*-value <0.01 for M-CSF samples relative to NS supernatants; error bars represent SEM).

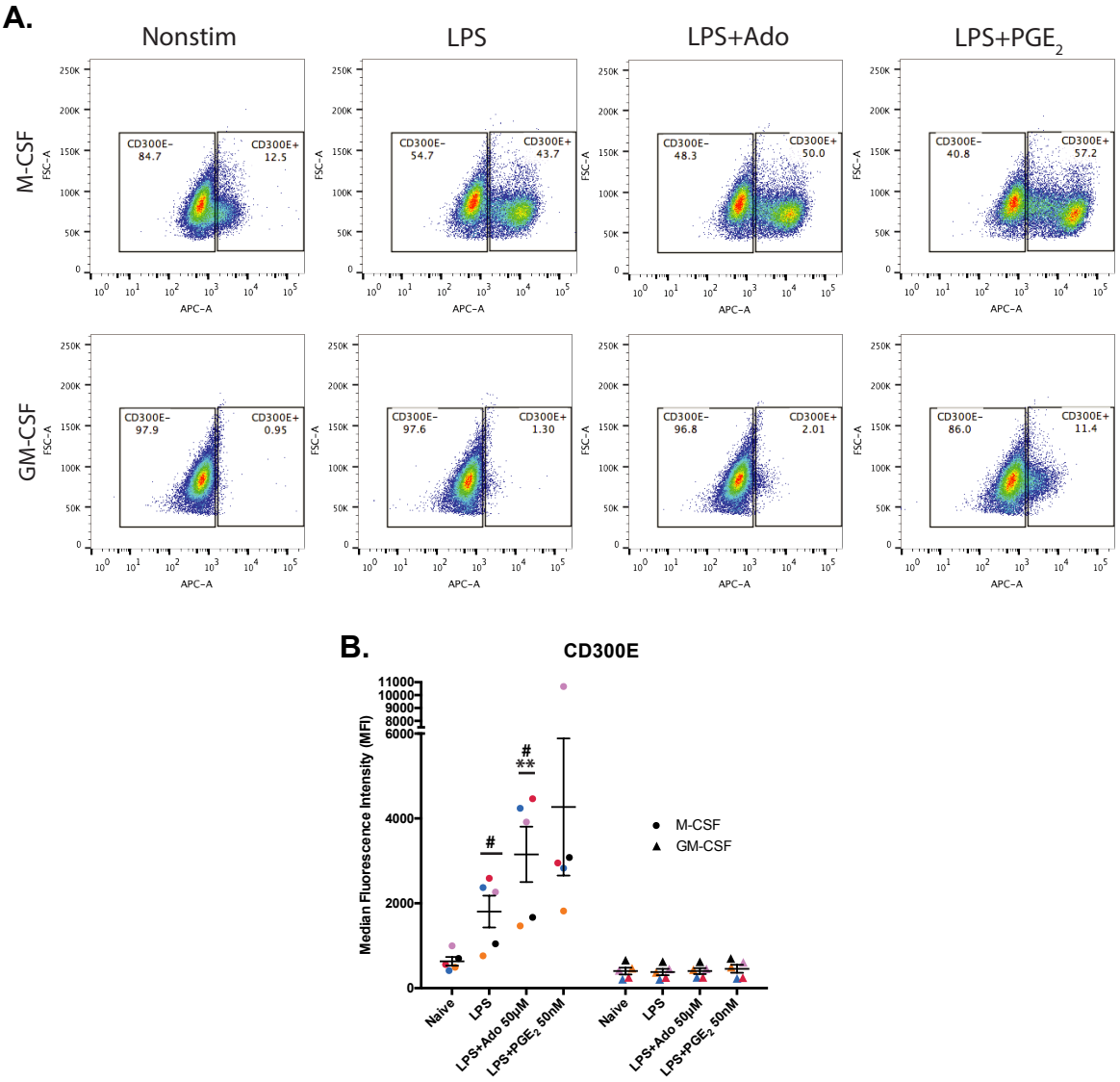

Figure 26. Flow cytometry of CD300E surface expression. Macrophages were untreated or stimulated with LPS, LPS + Ado and LPS + PGE_2 for 24 hours. **(A)** Dot plots of CD300E expression from 1 representative donor are shown and gates indicate the percentage of cells that are $CD300E^+$ and $CD300E^-$. **(B)** Median fluorescence intensity of CD300E levels on stimulated macrophages was calculated (n=5, ** *P*-value < 0.01 versus LPS stimulated samples alone; # *P*-value < 0.05, ## *P*-value < 0.01 between M-CSF and GM-CSF samples, error bars represent SEM).

in stimulated GM-CSF macrophages (Figure 26B). This lead us to conclude that CD300E is a suitable biomarker unique to M-CSF macrophages stimulated with LPS + Adenosine and LPS + PGE_2. PLAUR was another one of the transcripts that was highly differentially expressed in our single cell RNA-sequencing analysis. We measured the surface expression of PLAUR by flow cytometry and found that its expression was indeed increased by LPS + Adenosine and LPS + PGE_2 stimulation in M-CSF macrophages (Figure 27). We believe that PLAUR could be used in combination with CD300E as a secondary marker for these regulatory cells; however, it is also expressed in GM-CSF macrophages so it would not be a suitable marker to use on its own. We also looked at the levels of secreted, soluble proteins from M-CSF and GM-CSF macrophages. Thrombospondin-1 (Figure 28A) and VEGFα (Figure 28B), both growth-promoting proteins, were secreted at higher levels by M-CSF macrophages than by GM-CSF macrophages. The cytokine IL-6 was significantly decreased with adenosine and PGE_2 stimulation in M-CSF macrophages but not in GM-CSF macrophages (Figure 28C). To our surprise, anti-inflammatory IL-10 levels were not increased by adenosine and PGE_2 in M-CSF cells, and were actually decreased by PGE_2, which is contrary to the behavior of mouse macrophages (Figure 28D). However, the levels of IL-10 were significantly higher in M-CSF macrophages than in GM-CSF macrophages. Preliminary data indicated that stimulation of M-CSF macrophages with LPS in combination with a cell permeable cAMP analog specific for protein kinase A (PKA) activation led to increased levels of both THBS1 and VEGFα (Figure 28E). However, stimulation with a cell permeable cAMP analog specific for exchange protein activated by cAMP (Epac) did not affect the levels of these two cytokines.

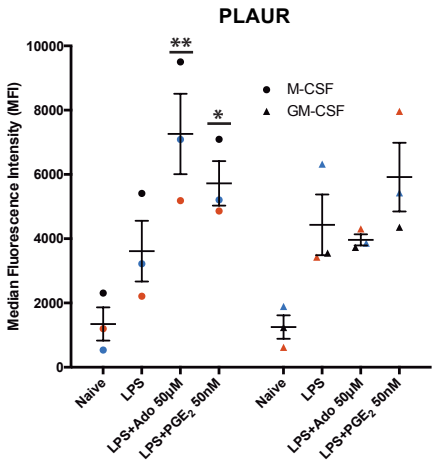

Figure 27. Flow cytometry of PLAUR surface expression. Macrophages were untreated or stimulated with LPS, LPS + Ado and LPS + PGE_2 for 8 hours. The median fluorescence intensity of PLAUR levels on stimulated macrophages was calculated (n=3, * *P*-value < 0.05, ** *P*-value < 0.01 versus LPS stimulated samples alone, error bars represent SEM).

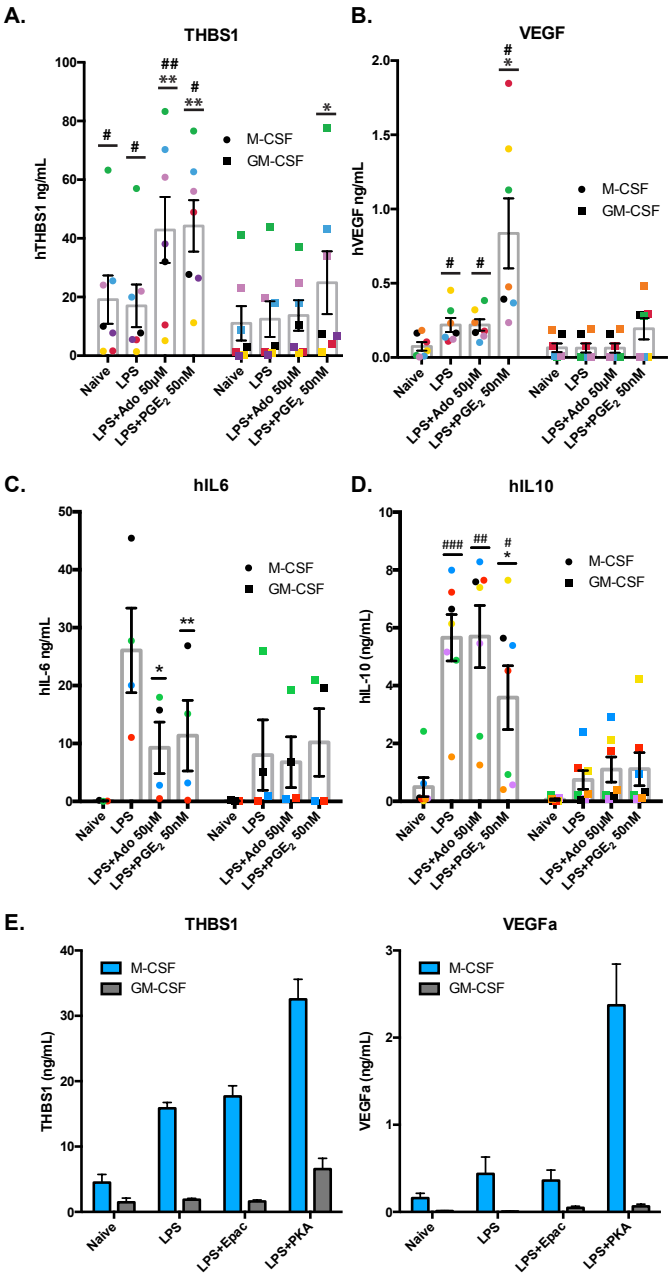

Figure 28. Growth promoting, anti-inflammatory cytokine secretion by M-CSF and GM-CSF macrophages. Macrophages were differentiated for 7 days in M-CSF or GM-CSF and then stimulated with LPS, LPS + Adenosine, and LPS + PGE_2 for 24 hours. **(A)** THBS1, **(B)** VEGFa, **(C)** IL-6 and **(D)** IL-10 levels were measured in the supernatants by ELISA (n=4-7; * *P*-value < 0.05, ** *P*-value < 0.01 relative to LPS stimulation alone; # *P*-value < 0.05, ## *P*-value < 0.01, ### *P*-value < 0.001 between M-CSF and GM-CSF samples; error bars represent SEM; points are color coded by donor). **(E)** Preliminary data depicting THBS1 and VEGFa levels in macrophages stimulated with LPS coupled to cell permeable Epac selective (8-pCPT-2-O-Me-cAMP-AM) cAMP analog or PKA selective (8-Br-cAMP) cAMP analog (n=2, error bars represent SD).

The levels of inflammatory cytokines TNF (Figure 29A), IL-12p40 (Figure 29B) and GM-CSF (Figure 29C) were higher in supernatants collected from stimulated GM-CSF derived macrophages than in supernatants from M-CSF macrophages. IL-12p40 and GM-CSF levels were unchanged by LPS + Adenosine and LPS + PGE_2 relative to LPS stimulation alone in GM-CSF derived macrophages, indicating a resistance of these macrophages to regulatory stimuli. To further explore the extent of GM-CSF resistance to adenosine and PGE_2, we use IL-12p40 production as a readout in response to increasing concentrations of adenosine and PGE_2 coupled to LPS stimulation (Figure 30). M-CSF macrophages responded with a dose dependent decrease in IL-12p40 production with a significant decrease with just 25 μM of adenosine and 1 nM of PGE_2, while GM-CSF macrophages did not have a significant decrease in IL-12p40 production in the presence of concentrations as high as 50 μM adenosine and 50 nM PGE_2.

Kinetics and modulation of cytokine production. We sought to gain insight regarding the kinetics of responses for both M-CSF and GM-CSF macrophages in response to LPS stimulation and also to examine the effects of different priming conditions on cytokine production. First we investigated endotoxin tolerance, a mechanism by which innate immune cells limit their inflammation[186–188]. We demonstrated that LPS priming of M-CSF and GM-CSF macrophages both led to the tolerance of a second LPS exposure in terms of TNF production and that there was no difference in tolerance between the two populations of macrophages (Figure 31A). Next, we examined whether GM-CSF priming had any effect on the cytokine production of M-CSF macrophages. Priming M-CSF derived macrophages with GM-CSF led to an increase in TNF (Figure 31B) and IL-12p40 (Figure 31C) production that correlated with the length of priming time. However,

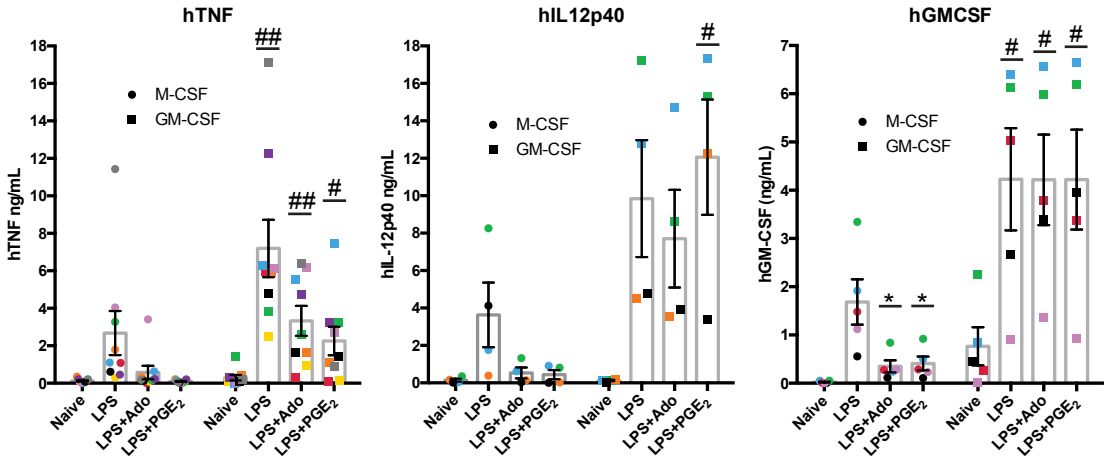

Figure 29. Inflammatory cytokine secretion by M-CSF and GM-CSF macrophages. Macrophages were differentiated for 7 days in M-CSF or GM-CSF and then stimulated with LPS, LPS + Adenosine, and LPS + PGE_2 for 24 hours. **(A)** TNF, **(B)** IL-12p40 and **(C)** GM-CSF levels were measured in the supernatants by ELISA (n=5-9, * *P*-value < 0.05 relative to LPS stimulation alone; # *P*-value < 0.05, ## *P*-value < 0.01 between M-CSF and GM-CSF samples, error bars represent SEM, points are color coded by donor).

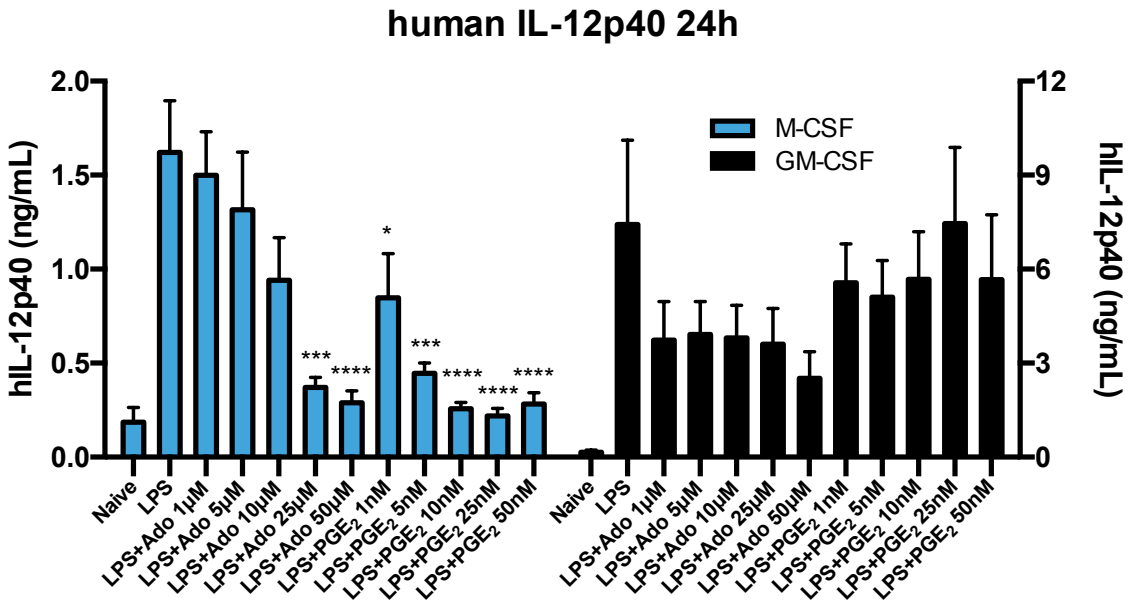

Figure 30. Modulation of macrophage IL-12p40 secretion by increasing concentrations of adenosine and PGE_2. M-CSF (blue) and GM-CSF (black) macrophages were stimulated with LPS coupled with various concentrations of adenosine and PGE_2, indicated on the x-axis, for 24 hours and IL-12p40 levels were measured in the supernatants by ELISA (n=5 M-CSF, n=4 GM-CSF, * *P*-value < 0.05, *** *P*-value < 0.001, **** *P*-value < 0.0001, error bars represent SEM).

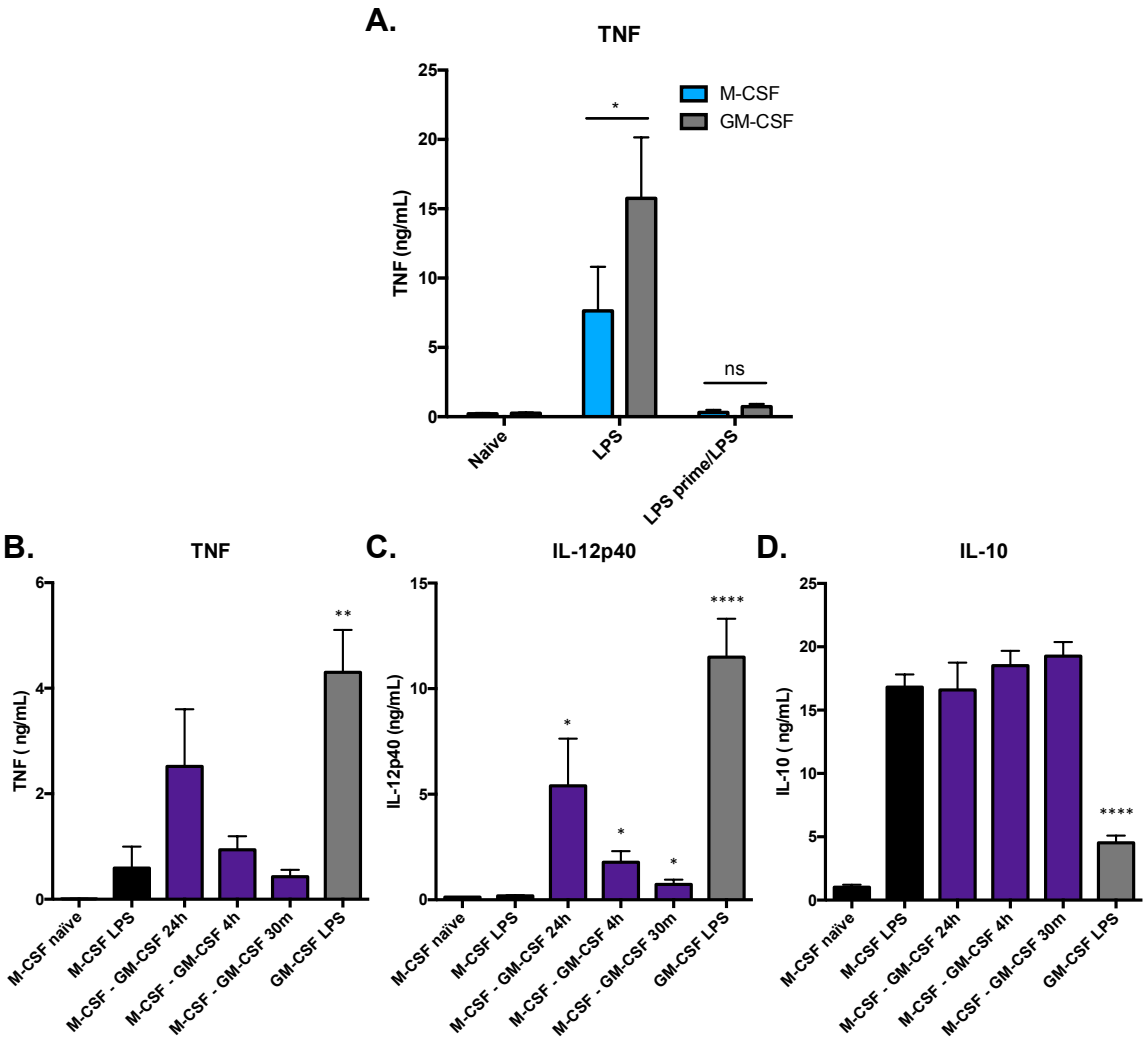

Figure 31. Priming in M-CSF and GM-CSF macrophages. (A) M-CSF (blue) and GM-CSF (grey) macrophages were unprimed or primed for 24 hours with LPS followed by a wash and subsequent LPS stimulation for an additional 24 hours. Supernatants were collected and assayed for TNF levels by ELISA (n=3, * *P*-value < 0.05, error bars represent SEM). Macrophages were grown for 7 days in M-CSF and then unprimed (black) or primed with GM-CSF for different lengths of time as indicated on the x-axis (purple) or grown in GM-CSF alone for 7 days (grey). Following a wash, macrophages were stimulated with LPS for 24 hours and supernatants were collected to assay for **(B)** TNF, **(C)** IL-12p40 and **(D)** IL-10 by ELISA (n=3, * *P*-value < 0.05, ** *P*-value < 0.01, **** *P*-value < 0.0001 relative to M-CSF LPS stimulation alone, error bars represent SEM).

the levels of TNF and IL-12p40 in GM-CSF primed macrophages were not as high as in macrophages that were derived in GM-CSF alone for 7 days. IL-10 production by M-CSF macrophages was not affected by various lengths of GM-CSF priming time (Figure 31D). Altogether, this data indicates that although GM-CSF is able to alter the M-CSF macrophage response, it is most effective at macrophage polarization when present as a growth factor during the monocyte to macrophage differentiation process. We next observed that TNF production was sustained longer in LPS stimulated GM-CSF macrophages compared to M-CSF macrophages following removal of the LPS stimulus (Figure 32A). Additionally, the accumulated levels of TNF (Figure 32B) and IL-12p40 (Figure 32C) were higher over the collected timepoints in LPS stimulated GM-CSF macrophages compared with M-CSF macrophages. In order to examine the role of endogenously produced adenosine and PGE_2 during inflammatory contexts, we made use of pharmacological inhibitors of adenosine and PGE_2 receptors. Preliminary data suggested that simultaneous pharmacological blockade of the A2A receptor, EP2 receptor and EP4 receptor appeared to prevent the upregulation of transcript levels of three growth promoting and candidate marker genes for regulatory macrophages, CD300E (Figure 33A), VEGFA (Figure 33B) and THBS1 (Figure 33C) at later (12 hour) but not earlier (4 hour) time points. Pharmacological blockade of the even the EP4 receptor alone led to a significant increase in inflammatory TNF levels in LPS stimulated M-CSF macrophage supernatants after 24 hours (Figure 33D). Conversely, the EP4 antagonist had virtually no effect on LPS stimulated GM-CSF macrophages. This suggests that M-CSF macrophages are sensitive to endogenously produced PGE_2 in their environment, but GM-CSF macrophages are not.

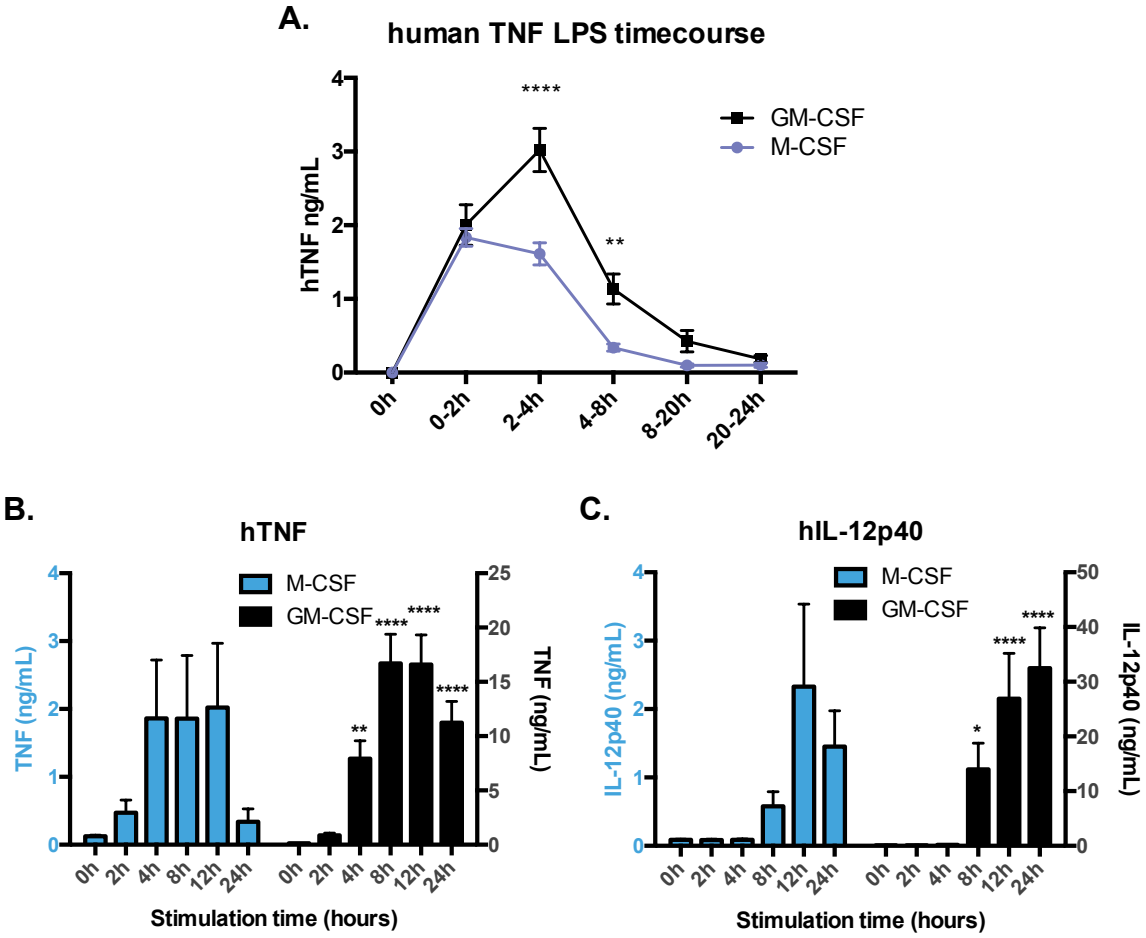

Figure 32. Kinetics of inflammatory TNF and IL-12p40 cytokine secretion by M-CSF and GM-CSF macrophages. (A) Human macrophages were stimulated for 2 hours with LPS and supernatants were collected and assayed for TNF levels by ELISA. Macrophages were washed and media was replaced at each timepoint following supernatant collection (n=7, ** *P*-value < 0.01, **** *P*-value < 0.0001, error bars represent SEM). The accumulation of **(B)** TNF and **(C)** IL-12p40 was measured by ELISA over a period of 24 hours (n=7, * *P*-value < 0.05, ** *P*-value < 0.01, **** *P*-value < 0.0001, error bars represent SEM).

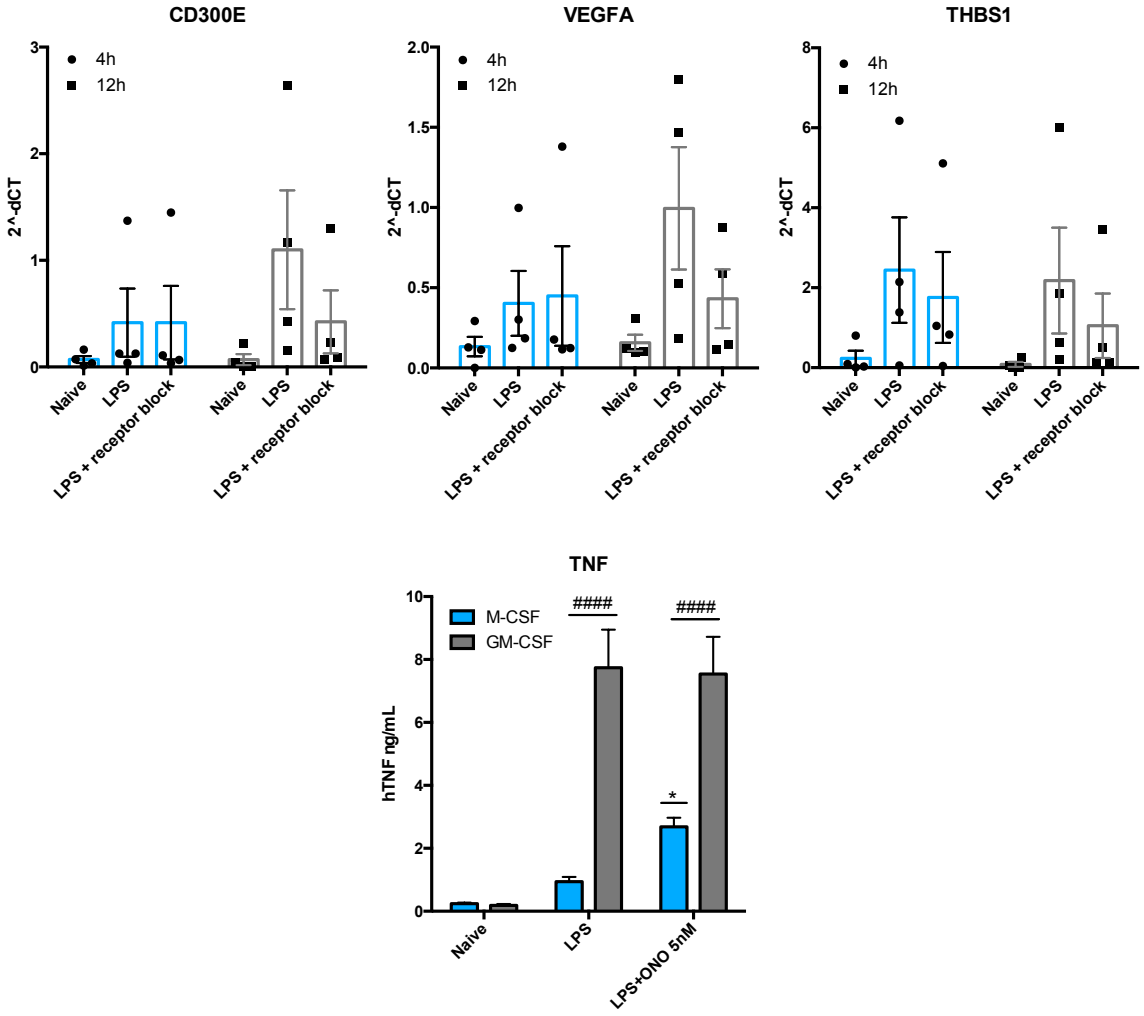

Figure 33. Pharmacological inhibition of adenosine and PGE_2 receptors. M-CSF macrophages were stimulated with LPS, and LPS + a cocktail of small molecule antagonists (against A2a, A2b, EP2, and EP4 receptors) for 4 and 12 hours. **(A)** CD300E, **(B)** VEGFA and **(C)** THBS1 mRNA was measured (n=4, error bars represent SEM). **(D)** M-CSF and GM-CSF macrophages were stimulated with LPS and LPS + ONO AE3 208 5nm (an EP4 receptor antagonist) for 24 hours and TNF levels in the supernatants were measured by ELISA (n=7, * *P*-value < 0.05 relative to LPS stimulation alone; #### *P*-value < 0.0001 between M-CSF and GM-CSF samples, error bars represent SEM).

5.3 Discussion

In this chapter, we elaborated on the findings pertaining to the human M-CSF macrophages described in chapter 4. We also made side-by-side comparisons of M-CSF and GM-CSF macrophages in order to explore whether these two growth factors lead to macrophages that adopt similar phenotypes upon stimulation. It is common to use either M-CSF or GM-CSF to generate monocyte-derived macrophages for further study. However, the combination of GM-CSF with IL-4 is used to obtain monocyte derived dendritic cells[189], which may lead to questioning of whether our differentiation protocol resulted in mature macrophage populations. The expression of CD68 and CD11b on both M-CSF and GM-CSF derived cells and the lack of expression of CD1a led us to believe that our working cells were in fact macrophages. This is in line with results from other labs[190,191].

We demonstrated that M-CSF and GM-CSF macrophages differed with respect to the purinergic and prostaglandin E2 pathways. We hypothesized that M-CSF macrophages may produce more ATP upon TLR stimulation than GM-CSF macrophages, but ATP secretion is difficult to measure due to its rapid conversion to adenosine by enzymes on the macrophage surface. Therefore, we measured the degradation of high concentrations of exogenously added ATP by M-CSF and GM-CSF macrophages to find that M-CSF macrophages degraded higher levels of ATP over the chosen timepoints. This supported similar results found by another group who demonstrated that M-CSF macrophages degraded more ATP than GM-CSF macrophages in 30 minutes regardless of stimulation condition[184]. Increased ATP degradation suggests that there is more endogenous, immunosuppressive adenosine available adjacent to the

macrophage surface to signal through the adenosine receptors to promote a transition to a regulatory phenotype. We also demonstrated that LPS stimulated M-CSF macrophages secrete more PGE_2, which we believe can act in an autocrine fashion in order to promote the transition to a regulatory phenotype in M-CSF but to a lesser extent in GM-CSF macrophages. This was further supported by mRNA expression of COX2, which was significantly induced in LPS stimulated M-CSF macrophages but not in GM-CSF macrophages. Not only were these molecules produced at lower levels in GM-CSF macrophages, but the receptors for sensing them, A2a receptor, A2b receptor, EP2 receptor and EP4 receptor, were also transcribed at significantly lower levels following TLR stimulation by LPS and various other TLR ligands. Notably, these 4 receptors mediate the immunosuppressive effects of adenosine and PGE_2[56,192,193]. This also suggests that the differential regulation of the purinergic and PGE_2 pathway genes by M-CSF and GM-CSF macrophages is consistent in different contexts of pathogenic infection.

Transcriptomic data allowed us to compare global genetic changes between stimulated M-CSF and GM-CSF macrophages. The PCA analysis alone indicated to us that M-CSF and GM-CSF macrophages are transcriptionally different even under similar stimulation conditions. Stimulation with LPS, LPS + Adenosine, and LPS + PGE_2 exhibited more variance among samples in M-CSF than in GM-CSF macrophages suggesting that GM-CSF macrophages are less susceptible to phenotypic modulation by adenosine and PGE_2. This was further supported by the number of DEGs by LPS + Adenosine and LPS + PGE_2 relative to LPS alone, which was significantly higher in M-CSF macrophages than GM-CSF macrophages. Many of the upregulated and

downregulated genes by LPS + Adenosine and LPS + PGE_2 relative to LPS alone in M-CSF macrophages were not up- or downregulated to the same extent in GM-CSF macrophages subject to the same conditions. Many of these upregulated genes had growth promoting functions, demonstrating that GM-CSF macrophages are not easily programmed to promote tissue repair. This was highlighted by the fact that M-CSF macrophages stimulated with adenosine and PGE_2 secreted factors that promoted tube formation by HUVEC cells, while stimulated GM-CSF macrophages secreted factors the inhibited tube formation. Tube formation has been proposed to be a reliable in vitro assay for angiogenesis as it involves adhesion, migration and tubule formation all in one experiment[194].

Surface proteins CD300E and PLAUR were found to have increased expression on adenosine and PGE_2 stimulated macrophages. CD300E in particular was not expressed above baseline on GM-CSF macrophages, making it a more suitable biomarker for M-CSF regulatory macrophages. Secreted proteins THBS1 and VEGFα were also higher in adenosine and PGE_2 stimulated M-CSF macrophages, suggesting that they could also be used as biomarkers for M-CSF regulatory macrophages. Both of these proteins are well known contributors to the wound healing process[195,168]. On the other hand, levels of inflammatory cytokines, TNF, IL-12p40 and GM-CSF were secreted at higher levels in LPS stimulated GM-CSF macrophages, and the addition of adenosine and PGE_2 did not dampen their secretion. This implies that GM-CSF macrophages are programmed to resist phenotypic change in response to resolving molecules in the inflammatory milieu in order to maintain higher levels of activation. Because macrophages are highly plastic in nature, we wanted to know if adding GM-CSF to M-

CSF differentiated macrophages could skew their phenotype. This had previously been shown to be true by another group who demonstrated that the M-CSF and GM-CSF differentiation of macrophages was not end stage, and that a subsequent period of 6 days in the opposite growth factor could reverse original phenotypes[196]. We demonstrated that this was the case for the secretion of inflammatory cytokines TNF and IL-12p40, and the extent of skewing correlated with the length of GM-CSF priming time. However, GM-CSF priming did not negatively affect the levels of IL-10 secretion. Additionally, GM-CSF priming did not restore TNF and IL-12p40 levels to the levels seen in GM-CSF differentiated macrophages. Therefore, it appears that exposure longer than 24 hours to GM-CSF is most effective in programming macrophages to reach a more inflammatory potential.

While total amounts of inflammatory cytokine secretion contribute to immunopathology, the duration of cytokine secretion also has an important role in immune responses. GM-CSF macrophages secreted TNF at higher levels than M-CSF macrophages for up to 18 hours following the removal of stimulus. GM-CSF macrophages also had significantly higher steady state levels of TNF and IL-12p40 in their supernatants at different time points up to 24 hours. Together these data suggest that M-CSF macrophages terminate their activation more effectively and faster than their GM-CSF counterparts and transition to a resolving phenotype. The expression of marker gene candidates CD300E, VEGFA and THBS1 were measured at early (4 hour) and later (12 hour) time points following LPS stimulation in the presence of pharmacological inhibitors of the A2ar, EP2 and EP4 receptors. This preliminary data suggested to us that pharmacological blockade of these receptors may prevent the upregulation of these

transcripts at later time points, but may not have an effect on the upregulation of these transcripts at early time points. We hypothesize that this may be due to the fact that the ligands for these receptors need some time to be produced endogenously in order for them to act in an autocrine manner. The pharmacological blockade of the EP4 receptor alone led to more than double an increase in TNF production by M-CSF macrophages in response to LPS, suggesting that under normal conditions, endogenous PGE_2 helps to limit inflammatory TNF. The pharmacological blockade of the EP4 receptor had no effect on the high levels of TNF produced by GM-CSF macrophages, which is not surprising, due to the presumed lack of EP4 receptor expression by GM-CSF macrophages based on mRNA data.

Overall, the data in this chapter combined with the data in chapter 4 led us to propose a model for both the regulation of M-CSF macrophage activation and the lack of regulation in GM-CSF macrophages (Figure 34). We hypothesize that M-CSF macrophages are better equipped to turn off inflammation and initiate a program of tissue repair due to the production and increased sensitivity to the resolving molecules, adenosine and PGE_2. We demonstrate that a number of components proposed in our model were lacking or present at lower levels in GM-CSF macrophages, which we believe helps to explain why GM-CSF macrophages are known to be hyper-inflammatory. In M-CSF but not GM-CSF macrophages, the expression of A2ar, A2br, EP2 and EP4 receptors is upregulated following TLR stimulation, making them more ready to sense adenosine and PGE_2 in their environment. The degradation of ATP and production of PGE_2 by GM-CSF macrophages is also lower than in M-CSF macrophages. We propose that together these differences in the purinergic and PGE_2 pathways, between

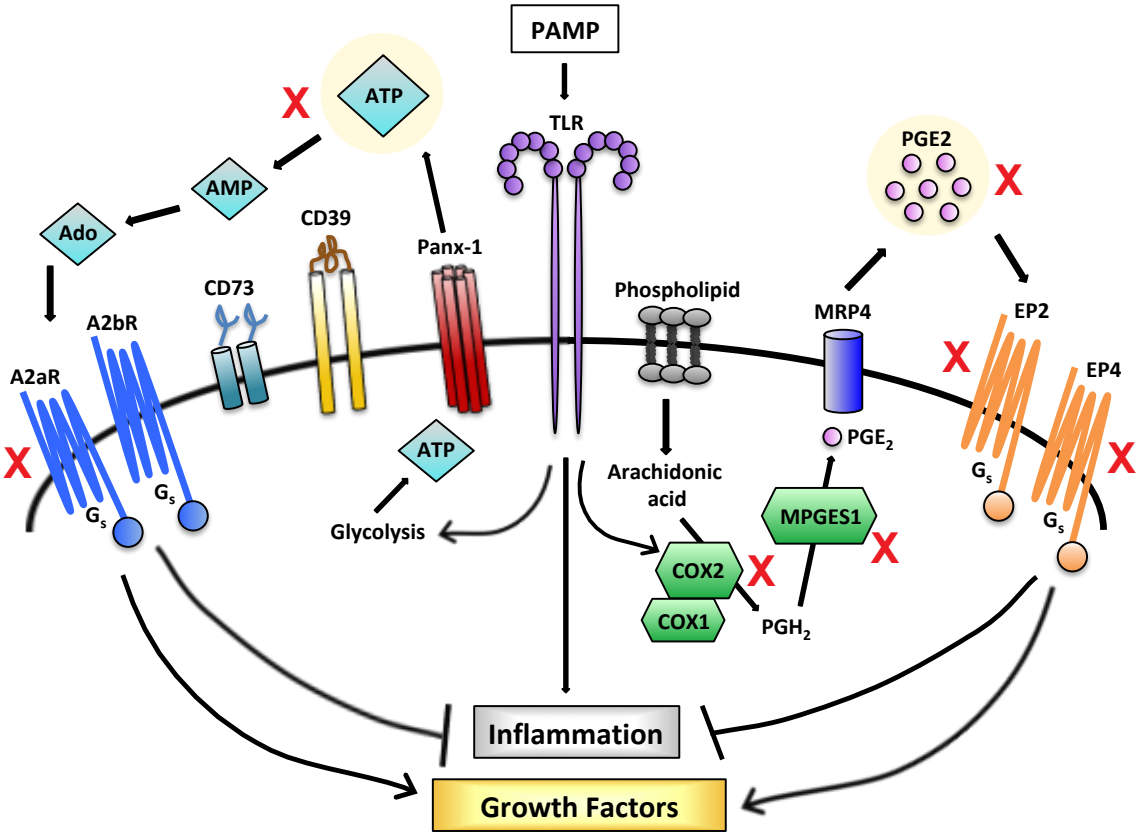

Figure 34. Proposed pathway for endogenous regulation of M-CSF macrophage activation and lack of regulation of GM-CSF macrophages. TLR activation by PAMPs leads to the transcription and production of inflammatory mediators including the cytokines and chemokines. However, TLR activation also leads to the production of ATP which is degraded to adenosine. Adenosine signals through A2aR and A2bR to inhibit inflammation and initiate production of growth promoting proteins. Similarly, TLR activation leads to the production of PGE_2,via COX and MPGES proteins, which then signals through EP2 and EP4 receptors to inhibit inflammation and initiate production of growth promoting proteins. We propose that these two molecules contribute to the resolution and control of M-CSF macrophage activation in response to pathogens. Components marked with a red (X) are those that are inhibited in macrophages differentiated in GM-CSF. Therefore, we propose that GM-CSF macrophages are unable to respond to adenosine and PGE2 in order to limit their activation or contribute to tissue homeostasis.

M-CSF and GM-CSF macrophages, are contributing factors to the propensity of these cells to either perpetuate inflammation or promote tissue repair.

6 Conclusions and Future Directions

Macrophages are highly responsive to their tissue environments. In this research, we explore the effects of a number of different stimuli that macrophages may encounter in the host under different contexts. We demonstrate that Type I and Type II IFNs modulate the expression of adenosine receptors in mouse macrophages. We have also characterized a population of human M-CSF macrophages with growth promoting and pro-angiogenic activity that we believe arises following the termination of every immune response as a mechanism to restore tissue homeostasis. We demonstrate that GM-CSF human macrophages exhibit prolonged inflammatory responses because they are defective in this transition. These observations have several potential implications for influencing immunity and inflammatory responses. First, they predict that IFN inflammatory responses are partially regulated in macrophages by the purinergic system. Second, they suggest that M-CSF macrophages are poised to promote tissue repair and that a lack of this growth factor has the potential to lead to chronic inflammatory conditions. Third, they suggest that GM-CSF may prolong immunity and delay immune resolution not only by increasing inflammation but also by delaying its resolution and preventing the upregulation of genes critical for tissue repair. The failure of GM-CSF macrophages to transition to a growth-promoting phenotype could explain the mechanism of action of this protein in disease. Tissue GM-CSF levels are elevated in numerous autoimmune/inflammatory conditions including multiple sclerosis[197], rheumatoid arthritis[198], systemic inflammation[112], and allergic responses[113]. A multitude of clinical trials for monoclonal antibodies to GM-CSF have been undertaken in the context of these diseases[102,199].

Exploring macrophage activation on a spectrum by examining responses to molecules aside from the usual M1 and M2 stimuli, furthers our understanding of macrophage function in numerous physiological and disease environments. For example, our results are relevant in the context of certain cancers, as adenosine and PGE_2 are present at high levels in the tumor microenvironment, known to harbor numerous tumor associated macrophages[47,57,200–202]. Because macrophages are highly sensitive to small changes in their surroundings, it is important to continue to investigate macrophage responses to a wide range and combination of stimuli.

The findings presented in this work illuminate the similarity of the macrophage response to adenosine and PGE_2 during inflammation. We believe that this could be attributed to the signaling of these molecules through g-protein coupled receptors (GPCRs) and downstream cAMP production. It is possible that this macrophage phenotype extends to stimulation with numerous other GPCR ligands, which should be further explored. GPCRs make up the largest class of receptors for approved membrane drug targets[203]. Therefore, it is possible that there are existing drugs that can be used in contexts that we have not yet discovered.

Much of the research on human monocyte-derived macrophages is highly variable as different labs have multitudes of protocols for generating macrophages from monocytes. This work shows that using GM-CSF alone as a differentiation factor can skew or bias the resulting macrophages to be inflammatory and resistant to the transition to a growth promoting phenotype. We propose that M-CSF is the growth factor most "neutral" to generate human macrophages.

Our observation that stimulated GM-CSF macrophages are resistant to the anti-inflammatory effects of adenosine and PGE_2 illuminates the potential use of GM-CSF in contexts in which it would be beneficial for macrophages to be hyper-inflammatory. One example would be the use of GM-CSF as a vaccine adjuvant. There are recent clinical trials testing GM-CSF as an adjuvant in cancer vaccines due to its anti-tumor properties[204,205]. GM-CSF could also potentially be used in parasitic diseases such as leishmaniasis in which cell mediated immune responses are needed for pathogen killing[130]. In fact, topical application of GM-CSF to lesions has been demonstrated to reduce the healing time in cutaneous leishmaniasis patients, due to increased parasite killing[206].

We identified promising protein biomarkers for regulatory macrophages including, THBS1, VEGFA, CD300E and PLAUR which could potentially be used in combination with cell specific markers such as CD68 and CD11b to identify growth promoting macrophages in histological samples or in vivo. Exploring where these macrophages are located in the host can help us better understand their functional roles and allow us to target them in different diseases. Along with this, another potential use for these biomarkers is during therapeutic testing in order to determine if certain drugs are effective in producing the intended phenotype in macrophages.

This work raises new questions that would benefit from further research. Pathway analysis led to the prediction of many transcriptional regulators of the genes modulated by regulatory macrophages. It would be beneficial to identify specific transcriptional regulators using pharmacological tools or protein interaction studies. This way, regulatory macrophage phenotypes could be mimicked with the modulation of a few key

proteins. Additionally, further exploration of the mechanism of M-CSF and GM-CSF control of the purinergic and PGE_2 pathways is needed, including how the expression of the receptors for these molecules is regulated. We highlighted the pro-angiogenic nature of LPS + Adenosine and LPS + PGE_2 macrophages, which has implications for macrophage actions in the tumor microenvironment as well as in wound healing or tissue repair. It would be interesting to compare the transcriptomic data generated in this work with transcriptomic data generated by other researchers on macrophages in known disease environments. This could help us to determine whether regulatory macrophages are participating in specific disease situations. Additionally, it would be useful to compare the transcriptomic data in this research to datasets from primary human tissue-resident macrophages, such as alveolar macrophages from the lung in order to explore the similarity of the macrophages we studied, to those in different tissues. Ultimately, the hope is that any of the molecular pathways discussed in this work could be targeted to ameliorate different disease pathologies.

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