Title of Document: **THE IDENTIFICATION AND CHARACTERIZATION OF AN INTRINSIC CD39/A2R-based REGULATORY MECHANISM THAT GOVERNS MACROPHAGE ACTIVATION RESPONSES.**

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Abstract

Macrophages are acutely sensitive to changes within their environment and can readily develop into a variety of activation states important for both the progression and resolution of inflammation. In response to immunological threats, macrophages must be able to effectively clear infections without sacrificing the integrity of the affected tissue. Thus, these cells must successfully balance their activation responses in order to preserve tissue function and the overall health of the host. The failure to properly regulate macrophage activation responses often manifests in the clinic in a variety of disease scenarios including sepsis, chronic inflammatory disorders, and cutaneous Leishmaniasis. While many factors that drive the initiation of macrophage activation are known, it remains unclear what governs the transition to an immunosuppressive state.

This study reveals that macrophages can control their own activation status through the coordination between the ecto-ATPase, CD39, and the adenosine 2a and 2b receptors (A2Rs). The first part of this work shows that soon after toll-like receptor (TLR) stimulation, macrophages secrete and convert

ATP into immunosuppressive adenosine via CD39. Moreover, we show that CD39 on macrophages is necessary to induce regulatory macrophage development and prevent severe immunopathology in a mouse model of septic shock. The next sets of data demonstrate that TLR activation also enhances A2bR expression, thus completing the CD39-initiated autoregulatory circuit to limit inflammatory macrophage responses. The second part of this work demonstrates that the chronic inflammatory disease-asociated cytokine, IFNγ, prevents TLR-induced A2bR expression and consequently promotes the hyper-production of inflammatory cytokines by macrophages thereby revealing a novel mechanism by which IFNγ maintains overactive macrophages. The final chapter illustrates that while the A2bR is the dominant adenosine receptor mediating the inhibition of inflammatory cytokine production, A2aR signaling inhibits nitric oxide generation and that its expression may be hijacked by intracellular parasites to evade innate host defense mechanisms.

Thus, this study demonstrates that inflammatory macrophage activation is inherently transient and that macrophages can reprogram themselves. These results culminate in the discovery of a novel immunomodulatory mechanism reliant on macrophage purinergic signaling and offer new targets and strategies to more effectively treat myriad inflammatory and infectious diseases.

THE IDENTIFICATION AND CHARACTERIZATION OF AN INTRINSIC CD39/A2R-BASED REGULATORY MECHANISM THAT GOVERNS MACROPHAGE ACTIVATION RESPONSES

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Dedication

The culmination of my graduate studies is dedicated to my grandmothers

Janice Bloom
And
Esther Kalin

Who have taught me

The importance of depending on others
Whilst finding the courage and strength to depend on yourself.

I am continuously humbled by their encouragement, love, and support.

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"I tried to warn you about graduate school…"

I could easily fill another 100 pages detailing all the reasons why I'm grateful for person who has remained by my side since my transition into graduate school. But because he is just as eager as I am for me to submit this dissertation, I'll spare the pages and simply thank Tom McCaughtry for all his patience and love throughout my graduate career. He has enouraged and cheered me on throughout every marathon I have run and completing my Ph.D. has been no exception. I am one lucky woman.

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List of Abbreviations

A1R; adenosine 1 receptor

A2Rs; adenosine 2a and 2b receptors

A2aR; adenosine 2a receptor

A2bR; adenosine 2b receptor

A3R; adenosine 3 receptor

ADA; Adenosine deaminase

ADP; adenosine diphosphate

Ado; adenosine

AMP; adenosine monophosphate

Arg; Arginase

ARs; adenosine receptors

ATP; adenosine triphosphate

BMDM; bone marrow-derived macrophage

cAMP; cyclic adenosine monophosphate

CD39; cluster of differentiation 39 (ENTPDase1)

CD73; cluster of differentiation 73 (ecto-5'-nucleotidase)

CREB; cAMP response element binding

DAMPs; danger associated molecular patterns

eATP; extracellular adenosine triphosphate

ENTPDase; ecto-nucleoside triphosphate diphosphohydralse

$G\alpha_i$; G-protein alpha inhibitory subunit

$G\alpha s$; G-protein alpha stimulatory subunit

GAS; gamma (γ)-activated sequences

GFP; green flourescent protein

GPCR; G-protein coupled receptor

HB-EGF; heparin binding-epidermal growth factor

IFNγ; interferon gamma

IL-10; interleukin-10

IL-10; interluekin-10

IL-12/23p40; interleukin-12/23, p40 subunit

IL-13; interleukin-13

IL-1β; interleukin-1 beta

IL-33; interluekin-33

IL-4; interleukin-4

Inducible nitric oxide synthase; iNOS

L.major; *Leishmania major*

LPS; lipopolysaccharide

M1; inflammatory macrophage

NFkB; Necrosis factor kappa binding

Nitric oxide; NO

NLRP3; nod-like receptor family, pyrin domain containing-3

NMR; nuclear magnetic resonance spectroscopy

P2X7; P2X purinoceptor 7

P_i; free inorganic phosphate

PAMPs; pathogen associated molecular patters

Panx1; pannexin 1 channel

SCID; Severe combined immunodeficiency

Sp1; Specificity protein 1

Sphk1; sphingosine kinase

TGFβ; transforming growth factor beta

T_h1; T helper cell 1

T_h2; T helper cell 2

TLR; toll-like receptor

TNFα; tumor necrosis factor alpha

Chapter 1: Introduction

Macrophage Activation States

On a daily basis, humans encounter myriad microorganisms, some of which can cause detrimental damage to the host if not cleared efficiently. The innate component of the host immune system is responsible for reacting immediately to microbial intrusion. Effective protection of the host from infectious disease requires immune cells that must be able to 1) quickly recognize modulations within their microenvironment and 2) proficiently alert neighboring cells of infection. The innate immune cells present at the site of infection are responsible for initiating the host immune response to ultimately resolve the infection and return tissue to normal homeostatic conditions. There are a number of mechanisms by which the innate immune system operates to provide the first line of defense against pathogens including: surface epithelia, complement activation, and phagocytosis by leukocytes at the site of infection.

Macrophages are present in virtually all tissues of the body and are among the first resident cells to encounter invading pathogens. Circulating monocytes that emigrate from the bloodstream, develop into tissue-resident macrophages. A major function of macrophages is to rid the tissue of dead cells and cellular debris via phagocytosis. However, phagocytosis alone does not induce sufficient levls of intracellular signaling that result in macrophage activation [1]. Macrophages are also potent cytokine producers and rapidly respond to changes in their microenvironment. Thus, the stimuli present in their extracellular environment are responsible for mediating macrophage activation responses including: cytokine production, anti-microbial activity, and antigen

presentation. Macrophages have the unique ability to alter their function and phenotype depending on the stimuli they encounter in tissue.

Inflammatory macrophages

Toll-like receptor (TLR) stimulation by pathogen-associated molecules such as lipopolysaccharide (LPS) elicits a pro-inflammatory activation state in macrophages characterized by their secretion of high amounts of inflammatory cytokines including IL-12 and TNFα. Inflammatory macrophages are the prototypical macrophage that develops at the onset of bacterial infection, inflammatory insult, and tissue damage.

Activation of natural killer (NK), iNKT cells, and T_h1 cells leads to the release of the type II interferon, IFNγ. There are a number of IFNγ responsive genes including: inducible nitric oxide synthase (iNOS), class II major histocompatibility complex II (MHCII) and CD40, thus IFNγ is a major cytokine that instructs naïve macrophage to become potent anti-microbial cells. Furthermore, IFNγ primes macrophages to be hyper-sensitive to TLR activating stimuli and intensifies TLR-induced inflammation. TLR signaling culminates in the activation of the nuclear transcription factor, NFκB, which induces the transcription of pro-inflammatory cytokines such as IL-12 and TNFα and further upregulates IFNγ-primed genes. Thus, upon exposure to TLR ligands and IFNγ, macrophages elevate secretion of TNFα, IL-12 as well as reactive nitrogen species, which inhibit the growth of intracellular pathogens [2]. Such pro-inflammatory mediators act to bias $CD4^+$ T cells to become T_h1 effectors that

respond by producing high levels of IFNγ, which in turn amplify inflammatory macrophage activation responses, further recruits cells to the site of infection, and aids in the clearance of infection [3].

Regulatory Macrophages

Regulatory macrophages were first identified by our laboratory as being responsive to immune complexes in the presence of LPS [4]. The hallmark regulatory macrophages is their ability to secrete increased levels of the potent anti-inflammatory cytokine, IL-10 [5]. These cells produce high levels of IL-10, but little IL-12; making these macrophages distinct from inflammatory macrophages. In addition, regulatory macrophages upregulate genes, which are different from those induced by the combination of IFNγ and LPS. We have recently shown that in addition to IL-10, regulatory macrophages preferentially express heparin-binding epidermal growth factor-like growth factor (HB-EGF), sphingosine kinase (SphK1), and IL-33 [6, 7]. Since the initial discovery that immune complexes and LPS yields an anti-inflammatory activation state, other regulatory-inducing stimuli have emerged including prostaglandins (PGE_2), apoptotic cells, cAMP, and IL-10 itself. Previous work demonstrated these stimuli enhanced ERK activation in macrophages, which subsequently led to the phosphorylation of histone H3 at regulatory macrophage-associated loci. ERK-mediated chromatin remodeling was important for increased promoter access by LPS-induced Sp1 resulting in enhanced transcription of macrophage-derived immunomodulatory mediators including IL-10 and HB-EGF [8]. Indeed, we previously demonstrated that treating macrophages with cAMP could promote the development of regulatory

macrophages via ERK activation [7]. Furthermore, regulatory macrophage-derived IL-10 is an important suppressor of inflammation and may also act to inhibit macrophage function and contribute to uncontrolled parasitic infection [9].

Alternative Activation and Wound Healing Macrophages

The T_h2 cytokines, IL-4 and IL-13, polarize macrophages to assume a so-called 'alternative activation state' [86-88]. This alternatively activated macrophage (AAM) nomenclature stems from observations that in response to the IL-4/IL-13, macrophages are not potent cytokine producers [86-88]. Thus, this activation state represents a stark contrast to any other previously identified characterizations of macrophages. However, the AAM terminology assumes that all non-inflammatory macrophage states are the same. Moreover, this simplistic classification was largely based on in vitro observations and thus undermines the spectrum of immunomodulatory macrophage phenotypes observed throughout the pathogenesis of a disease in vivo. Alternatively activated macrophages uniquely express elevated levels of the mannose receptor, *Fizz1,* and *Ym1*, which permit the identification of IL-4/IL-13-induced activation [86-88]. Thus, IL-4/IL-13 treatment yields a state of activation distinct from regulatory and inflammatory macrophages. Interestingly, the major macrophage function induced by IL-4/IL-13 in has been enabling a tissue-remodeling program in macrophages. Both IL-4 and IL-13 signal to activate STAT6, which can drive expression of arginase. Arginase uses L-arginine as a substrate to produce L-ornithine, which goes on to serve as a precursor for collagen, thereby contributing to the maintenance of the extracellular matrix and resolution of tissue damage [1]. Thus, we have recently

proposed that their wound healing function be used in referring to IL-4/IL-13 treated macrophages; in order to adequately distinguish between various macrophage activation states refer these cells. Moreover, it is known that the presence of arginase promotes the survival of intracellular pathogens by producing polyamines and thereby providing microorganisms with nutrients that they cannot produce de novo [41, 42, 43]. Arginase also competes with iNOS for available L-arginine present in tissue, which can lead to ineffective production of anti-microbial NO [10].

Summary

These activation states vary dramatically in regards to mode of activation, anti-microbial activity, cytokine production, and antigen presentation. Inflammatory macrophages (also known as M1) are potent cytokine producers that contribute to the T_h1 and T_h17 responses and eradication of pathogens. In contrast, wound healing and regulatory macrophages (collectively referred to as M2) are more permissive to bacterial and parasitic infections and are associated with an anti-inflammatory T_h2 response. It is important to note, that despite distinct functional differences between these three activation states, there is considerable overlap-particularly with regard to the genetic landscape each activation state exhibits, Furthermore, these macrophage phenotypes may be rapidly reversible. It is believed that the phenotypic changes that macrophages undergo play an important role in determining the outcome of immune responses. It is believed that macrophages have the ability to alter their function and phenotype depending on the stimuli they encounter in tissue. Collectively,

the ability for macrophages to readily alter their activation status in response to their changing microenvironment is a property referred to as “macrophage plasticity”. Moreover, it is plausible that depending on the combination of and exposure kinetics of stimuli sensed, macrophages may exhibit hybrid activation states and thus expressing biomarkers associated with more than one of these most well-characterized activation states. Thus there may be a “spectrum of macrophage activation states” (Figure 1).

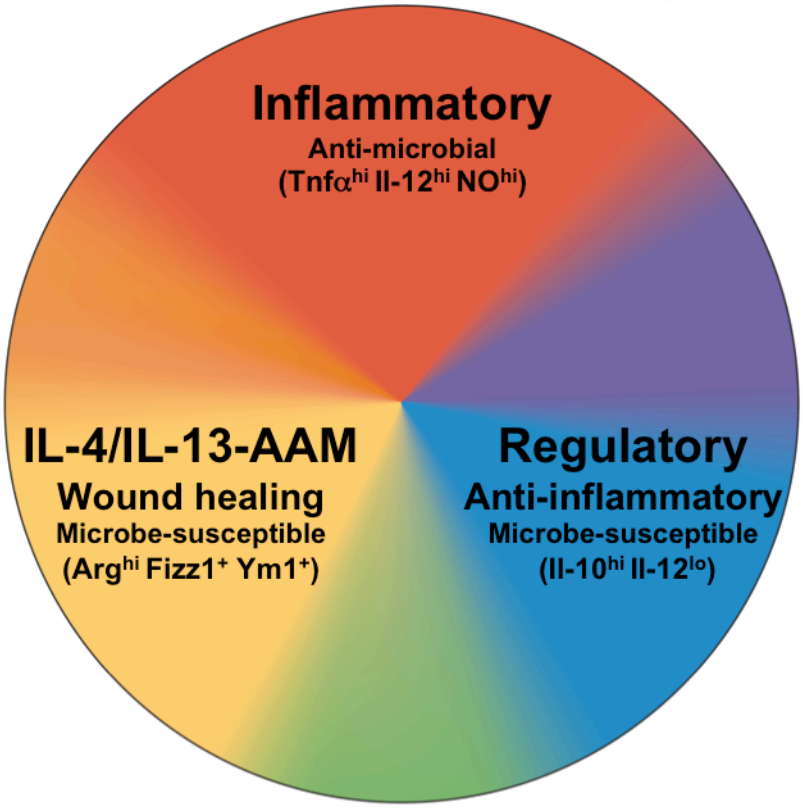

Figure 1. A schematic representing the myriad macrophage activation states. IFNγ and pathogen-associated molecules such as LPS induce inflammatory macrophages (red), which are potent producers of TNFα, IL-12 and anti-microbial nitric oxide. In contrast, regulatory macrophages secrete high levels of IL-10 and arise in response to TLR stimulation in the presence of immunosuppressive signals (blue). In response to IL-4/IL-13 macrophages that are pro-angiogenic and suceptible to infection develop (yelllow). Although at least three distinct activation states have been identified, macrophages exhibit functional plasticity and hybrid activation states may exist as they transition in response to changes sensed in their tissue environment. (Color wheel adapted from Mosser, DM and Edwards, JP *Nat. Immun. Reviews*. 2008). [14]

Importantly, macrophages must act quickly and appropriately to clear an infection before it spreads to the rest of the body, but these cells are also tasked with the challenge of controlling inflammation and repair damaged tissue once the pathogen is eradicated. Failure to properly balance between anti-microbial activity and tissue repair therefore may result in chronic infection or tissue damage. Therefore, it is believed that the phenotypic and functional changes macrophages undergo, play a vital role in determining the outcome of infectious diseases. Thus, the body's immune system relies on the ability to maintain properly regulated macrophages in order to effectively tune its response to physiological threats and ultimately to maintain tissue homeostasis.

Macrophage plasticity during disease

[*] Macrophages are major cellular contributors to the homeostasis of the host. They can both respond to infection to enhance immune responses, and conversely contribute to the repair of tissue in the aftermath of an immune response. It has been appreciated for many decades that exposure of macrophages to bacterial products can induce a profound phenotypic change, resulting in the secretion of many inflammatory cytokines and mediators. Under different circumstances, however, these same macrophages can produce anti-inflammatory molecules, thus macrophages make important contributions to both the initiation and resolution of inflammation. These dramatic changes in their physiology are vital to maintaining homeostasis and protecting the host from severe immunopathology. A variety of inflammatory disease scenarios suggest that macrophages equally exhibit the capacity to uniquely alter their responses to changing tissue environments, an ability referred to as 'functional plasticity' (Figure 2). Despite these observations, however there remains a considerable dearth of understanding about how this transition from pro- to anti-inflammatory activation occurs in macrophages. The inability to maintain the temporal reprogramming of macrophage responses is evident in a variety of inflammatory and infectious disease scenarios.

[*] Paragraph adapted from: Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. *J Leukoc Biol.* Nov 2013;94(5):913-919. [11]

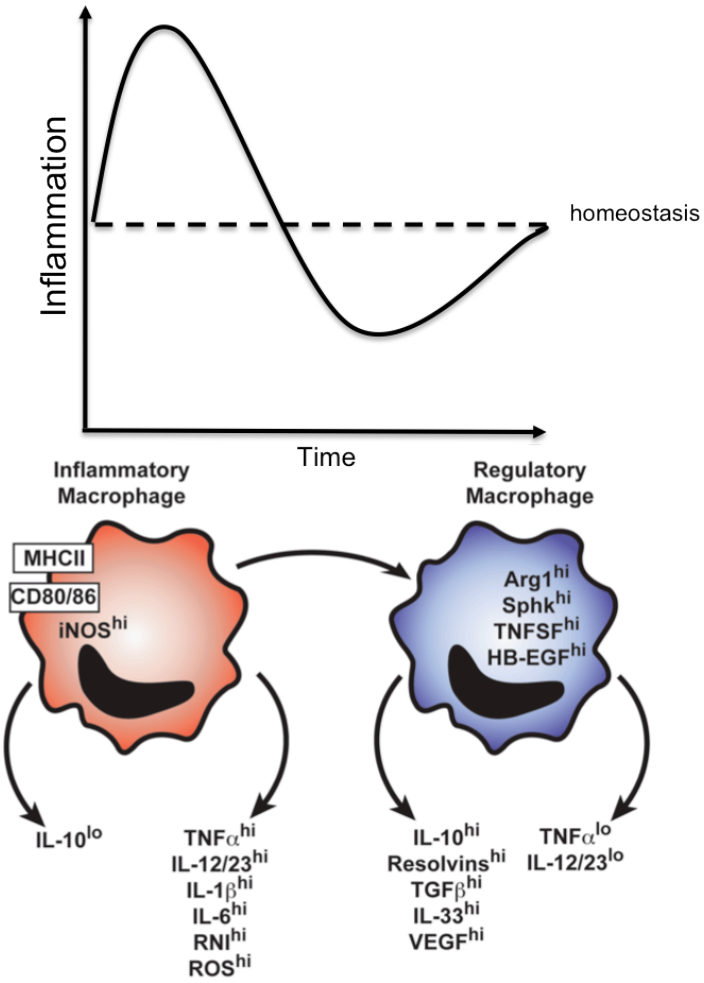

Figure 2. Schematic representation of phenotypic markers of macrophages at different stages of disease. Upon insult to tissue, the initial macrophage activation state is characterized by the expression of biomarkers associated with inflammation. At later stages of tissue injury or infection, macrophages switch to a regulatory activation state by expressing phenotypic markers that suppress inflammation and promote tissue repair. (Adapted from Cohen and Mosser, *JLB*, 2013). [11]

Sepsis

[*] More than 200,000 people in the US annually die from sepsis [12], a multifaceted disease that exemplifies the importance of intact regulated macrophage inflammatory responses. The initial phase of sepsis manifests as a systemic inflammatory response syndrome (SIRS) that is triggered by inflammatory responses to toll-like receptor (TLR) agonists, such as bacterial lipopolysaccharides (LPS) [13]. The severe pathology associated with SIRS occurs in response to the hyper-production of macrophage-derived inflammatory cytokines including TNFα, IL-12, and IL-1β which coalesce in a 'cytokine storm' that can lead to tissue destruction, vascular damage, septic shock, multiple organ failure, and death [13]. Compellingly, individuals who survive the initial pro-inflammatory response, display a compensatory anti-inflammatory response develops that is accompanied by the appearance of monocytes/macrophages exhibiting a regulatory phenotype [14, 15]. Thus, the pathogenesis of sepsis is a temporally regulated process initiated by hyper-inflammatory macrophage response followed by a state of profound immunosuppression.

[*] In fact, sepsis represents one of clearest examples of macrophages transitioning from a highly inflammatory population of cells to a population with immunoregulatory properties. This transition has been referred to as "endotoxin tolerance", a term that incorrectly implies that macrophages become non-responsive to re-stimulation by bacterial products. This, of course, is not the

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case because these macrophages actually undergo a transcriptional reprogramming resulting in dramatic shifts, both up and down, in transcript levels. There are many in vitro and in vivo models of endotoxin tolerance, and many of these models produce macrophages with subtle differences in their physiology, leading to some confusion about the specifics of tolerance. Perhaps the most straightforward illustration of this phenomenon comes from an analysis of human monocytes taken from post septic patients and stimulated ex vivo with LPS. These monocytes produce substantially lower amounts of inflammatory cytokines, such as TNFα and IL-6, and they increase their production of immunoregulatory molecules, including IL-1RA and IL-10 [16]. It is thought that this modulation in cytokine production is necessary to protect the host from immunopathology associated with prolonged macrophage activation. In fact, in an in vivo mouse model of endotoxin tolerance, pre-exposure of mice to low levels of LPS protected mice from lethal doses of endotoxin [17]. There have been many reports identifying different potential molecular mechanisms behind tolerance [16] and it is likely that multiple molecular mechanisms may be responsible for this transition to a regulatory phenotype. Despite differences of opinion regarding the mechanisms behind tolerance, there is general consensus that this phenomenon is real and that it may actually predispose post-septic patients to subsequent infections. Thus, a mechanism to spare the host of inflammatory tissue damage may present an opportunity that infectious microbes can exploit thereby highlighting the essential role for tightly regulated macrophage responses in order to fully protect the host. Moreover, it is believed

that the inability to effectively control inflammatory macrophage activation responses is what drives this severe immunopathology and elucidating the molecular mechanisms mediating the macrophage plasticity will likely expand the currently limited arsenal of effective treatments for sepsis patients.

Chronic inflammatory diseases

[*] Chronic diseases that are instigated by inflammation exemplify the importance of the temporal regulation of macrophage activation in response to tissue damage. The destruction of tissue can lead to the release danger associated molecular patterns (DAMPs) from dying cells and an alteration in the extracellular matrix. Neighboring monocytes/macrophages interpret this debris as 'danger' leading to their production of inflammatory cytokines. High levels of ATP and HMGB1 that usually reside within cells, are released by dying cells into the extracellular tissue milieu to enhance the recruitment of migrating monocytes and activate macrophages to release the pro-inflammatory cytokine IL-1β [18-20]. These events trigger an initial pro-inflammatory phase that is required to recruit innate immune cells and develop inflammatory macrophages at the site of tissue damage/injury to help fight potential microbial infection. Inflammatory macrophages originally develop at sites of tissue damage to initiate the wound healing process by phagocytosing dead cells and tissue debris and participate in host defense against microbes that may occupy the wound site. Thus, early injury-associated macrophages typically release chemokines, lipid mediators,

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and a number of inflammatory cytokines, including (but not limited to) TNFα, IL-1β, and IL-12, [21, 22].

However, there are a variety of pathologies that develop in response to uncontrolled macrophage responses. Unlike acute inflammatory diseases such as sepsis in which there is a distinct phase of inflammation followed by a compensatory immunosuppressive phase, chronic inflammatory diseases persist and display macrophages that fail to regulate their activation status. There is an expansive list of diseases that exhibit chronic inflammation including Alzheimer's, atherosclerosis, obesity, arthritis and inflammatory bowel disease[23-26]. Thus, regardless of the initiating insult, the innate immune responses remain in a state of profound hyper-activation. One explanation for this is the continuous presence of inflammatory-causing stimuli such as beta-amyloid, elevated levels of low-density lipoprotein, or persistent low-grade infections. For all these diseases, damage to the tissue is apparent, thus leading to the constant exposure of inflammatory danger signals to resident macrophages. Interestingly, elevated levels of IFNγ has been observed in rheumatoid arthritis and lupus individuals, suggesting a pathogenic role for IFNγ [25, 26]. To date, the most common treatments for many chronic inflammatory diseases are anti-IFNγ/TNFα therapies, which do not actively regulate inflammation, but rather merely inhibit pro-inflammatory responses. This strategy, thus commonly results in substantially enhanced susceptibility to infections, as IFNγ and TNFα are crucial in host defense. Thus, the need to investigate alternative mechanisms by which inflammation is sustained is eminent.

[*] Conversely, fibrosis develops as a result of over-active wound healing responses. In genetically manipulated mice that lack macrophages, wound healing and re-epithelialization are substantially delayed [27]. Such observations indicate that the ensuing regenerative phase of wound healing requires the transition of macrophages from an inflammatory to a wound-healing phenotype [21]. Once the inflammatory environment has subsided due to the release of potent anti-inflammatory mediators including IL-10 and TGF-β, macrophages present in tissue can successfully participate in the regeneration of tissue by producing tissue growth factors and promote collagen synthesis. IL-10 is particular important in coordinating tissue repair within damaged muscle. Although IL-10 makes important contributions to the later (regulatory) stages of muscle development, it is actually detrimental to the early (inflammatory) stages of muscle development [28, 29]. Thus, the blocking of late IL-10 production can interfere with muscle regeneration, just as the addition of exogenous IL-10 early in the muscle repair process can disrupt regeneration. This differential response to IL-10 (early versus late) illustrates how carefully the macrophage transition from inflammatory to regulatory must be controlled, and how important this transition is to proper muscle development.

[*] Moreover, the activation status of macrophages at all stages of inflammation and wound healing appears to be tightly regulated. If the early inflammatory response does not transition to an immunoregulatory response,

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[*] Paragraph adapted from: Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. *J Leukoc Biol.* Nov 2013;94(5):913-919. [11]

then inflammatory immunopathology will result in sustained inflammation and cause further tissue damage. If the later regenerative phase is not properly regulated, then the wound healing response will progress to an obstructive fibrotic response that is associated with the formation of scar tissue as well as helminthic worm infections [30, 31]. In addition, the magnitude of these responses must also be carefully controlled. Too much early inflammation, for example by injecting granulocyte-macrophage colony stimulating factor (GM-CSF), can exacerbate joint swelling [32], whereas too much late immunoregulation, for example by ablating arginase (*Arg1*), can lead to fibrosis [33]. Therefore, macrophages must be able to regulate their activation responses to prevent detriment to their local tissue environment, suggesting that the functional plasticity of macrophages is vital to maintaining tissue homeostasis.

Cutaneous Leishmaniasis

Leishmania are flagellated trypanosomes that cause a disease that plagues 1-2 million individuals each year throughout the world [34]. There are several forms of the Leishmaniasis including: cutaneous, mucocutaneous and visceral, with cutaneous Leishmaniasis being the most prevalent form of the disease in humans. Individuals infected with *L. major* typically produce ulcers at the site of infection. In most cases the lesions slowly self-healing, but in immuno-compromised individuals ulcers do not heal. Such individuals often suffer from debilitating lesions at the infection site that arise when the body fails to clear parasites before repairing the infected tissue scars. Cutaneous Leishmaniasis is a vector-borne disease that is transmitted by the sand fly, *Phlebotomus papatasi,*

which lives in endemic areas to North and West Africa and the Middle East. In these areas, individuals may develop up to hundreds of lesions that often lead to severe debilitation [34-36].

Female sand flies are hematophagous and require a blood meal once a week for egg development. To initiate its blood meal, the *P. papatasi* insert its mouthparts deep into the skin, reaching below the epidermis into the dermis, where it can reach blood vessels. As the sand fly feeds, it secretes saliva to facilitate probing and feeding, resulting in the development of a small hematoma at the bite site and also regurgitates components within its gut into the open wound [37]. It is at this point, that infected sand flies transmit *L.major* parasites into the mammalian host. The parasite continues to be transmitted when the sand fly acquires its next blood meal from an infected mammal.

The life cycle of the parasite is complex and begins after the sand fly acquires blood from an infected individual. Metacyclic promastigotes represent the infectious stage during the *L. major* lifecycle because this is the form of the parasite that is transmitted from the sand fly into the mammalian blood stream (Figure 3). Promastigotes are efficiently opsonized by host serum factors including complement and reactive IgG and due to their high expression of complement and Fc receptors, their phagocytic capacity, and residence in virtually every tissue of the body, macrophages are the primary host cell infected by Leishmania spp. [35, 38, 39]

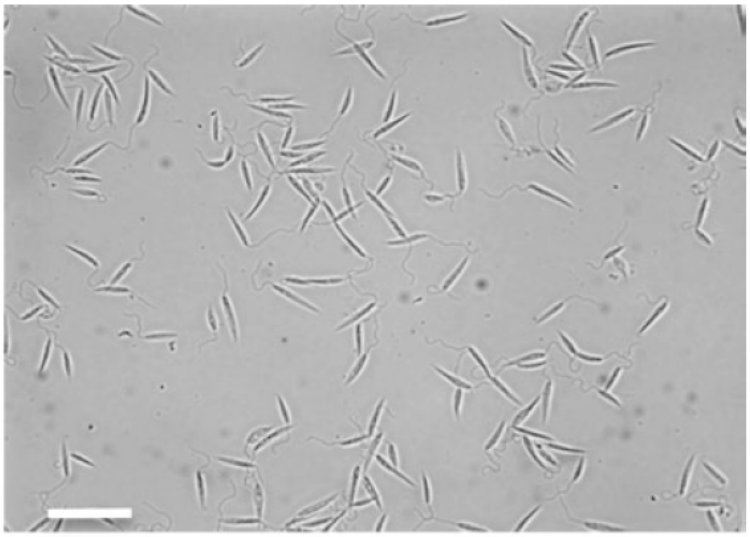

Figure 3. *L. major* metacyclic promastigotes as seen with phase-contrast microscopy. Leishmaniasis is vector-borne infectious disease that is caused by *Leishmania spp*. This image captures the morphology of these flagellated trypanasomes at the stage of their life-cycle when they are transmitted from the sand fly into the blood of a mammalian host. *L. major* metacyclic promastigotes primarily infect macrophages where they differentiate into a non-flagellated amastigote form that thrives intracellularly and can establish severe lesions at the site of infection in susceptible hosts. Scale bar, 50mM. (Image taken by Cohen, HB and from Goncalves, R., Zhang, X., Cohen, H., Debrabant, A., and Mosser, DM. *JEM*. 2011). [40]

The use of mouse strains has established our foundational understanding of the pathogenesis of this disease caused by *L.major*. Interestingly, regardless of genetic background, both resistant and susceptible strains produce elevated levels of IL-4 early in infection. Thus, at the onset of infection, macrophages exhibit an immunoregulatory activation status. Additionally, increases in parasite burden in both susceptible and resistant mice are associated with the induction of arginase in macrophages, which generates polyamines that support parasite growth [41, 42]. Furthermore, arginase utilizes the same sustrate as iNOS, L-arginine and can thus interfere with the ability to effectively thwart intracellular pathogens [43].

While the initial wound healing response is common between mouse strains, the ultimate fate is determined at later stages of infection. In susceptible strains, parasite-specific antibodies begin to rise in the serum and can opsonize extracellular parasites, thus enhancing infection. Furthermore, IgG-opsonized parasites promote IL-10 secretion from macrophages, thus sustaining the T_h2 environment and enhanced resistance in IL-10-deficient mice evidences the importance of IL-10 production during infection [9]. It is unclear how *L. major* inhibits classical macrophage function and evades the host innate immune response. Moreover, the hyper-production of IL-10 has been associated with chronic cutaneous lesions in human patients [35, 44]. Susceptible strains, thus exhibit long-lasting T_h2 responses rich in IL-4 and IL-10 that support parasite survival, infection-induced tissue damage, and the development of non-healing lesions at the site of infection. In contrast, resistant strains ultimately control the

infection by eventually developing a T_h1 cytokine environment, dominated by IFNγ and IL-12 [35, 45]. Thus, macrophages isolated from infected resistant strains exhibit an activation state consistent with inflammatory macrophages and are able to adequately clear control the infection. It is important to note that in resistant mice, the site of infection eventually heals thereby indicating that macrophages alter their physiology again to assume a homeostatic activation state. Thus, susceptibility to *L. major* infection is positively associated with chronic immunoregulatory macrophage activation and the failure to transition to an inflammatory activation state.

Summary

Macrophages readily alter their physiology in response to changes sensed within their local extracellular milieu. Because macrophages are central to the development of both the progression and resolution of inflammation, it is imperative that we work towards improving our understanding of how macrophage plasticity is regulated.

In myriad inflammatory and infectious diseases, the clinical outcome is often dictated by the ability of macrophages to maintain their capacity to readily alter their physiology. Failure to retain functional plasticity and regulation between activation states can consequently lead to chronic diseases that can result in either severe tissue destruction or enhanced susceptibility to infections. In each setting, exogenous factors such as apoptotic cells, prostaglandin derivatives, cytokines, and growth factors accumulate over time that promote the development of regulatory macrophages. Thus, the switch from inflammatory to

regulatory macrophages is believed to play an integral role in returning damaged tissue to homeostatic conditions thereby protecting the host from further detriment. It is further believed that the inability to effectively control the kinetics of inflammatory macrophage activation responses is what drives severe immunopathology. Studying the intrinsic ability of macrophages to transition to a regulatory state may provide some clues about the temporal regulation of macrophage activation during these diseases.

It is important to recall that a wound caused by either needle inoculation or sand fly bite initiates the infection, thus a wound healing response may naturally develop to repair the damaged tissue. Tissue-resident macrophages in the presence of infectious agents, however, must be properly educated by their local microenvironment to promote host defense rather than tissue remodeling. While, the role of cytokines including IFNγ, IL-12, IL-4 and IL-10 in directing the course of these diseases is well-documented, the role of other factors present in tissue during infection remain elusive.

Purinergic signaling

Despite its simplistic structure, composed of the purinergic nucleoside, adenosine bonded to a trio of phosphate groups, adenosine triphosphate (ATP) is essential for virtually all aspects of cellular function. The vital role of ATP within cells is very well established as many cellular mechanisms depend on ATP including receptor signaling, transcriptional activation and DNA synthesis. For these reasons, ATP is the most abundant nucleotide in cells existing at relatively high concentrations of 1-5mM within cells [46].

Paradoxically, many mammalian cells are equipped with a variety of receptors and ecto-enzymes that recognize extracellular ATP. Thus, ATP must gain access to the extracellular space in order to activate these membrane-bound molecules. In response to infection and tissue injury, damaged cells alert the immune system of a physiological breach by releasing danger associated molecular patterns (DAMPs). Over 1mM ATP is within mammalian cells and when they lose membrane integrity, they release their cellular contents thus releasing high levels of ATP into the extracellular space. Infiltrating and resident innate immune cells express the P2X7 receptor, a membrane bound ion channel that has low affinity for ATP. Thus in the presence of 1-5mM ATP, the P2X7 becomes activated causing an efflux of potassium and activation of the intracellular NOD-like receptor protein 3 (NLRP3)-inflammasome, which ultimately leads to the secretion of the pro-inflammatory cytokine IL-1β. If cell death persists and extracellular ATP levels remain elevated, severe tissue destruction caused by an overactive inflammatory response can occur.

Importantly, levels of extracellular ATP within the millimolar range are thought to occur only under pathological conditions. Therefore, in order to protect the host from such detriment, the ability to control extracellular ATP availability is critical. Indeed, a complex catabolic and signaling cascade exists to regulate the bioavailability of extracellular ATP, collectively referred to as “the purinergic signaling system” (Figure 4).

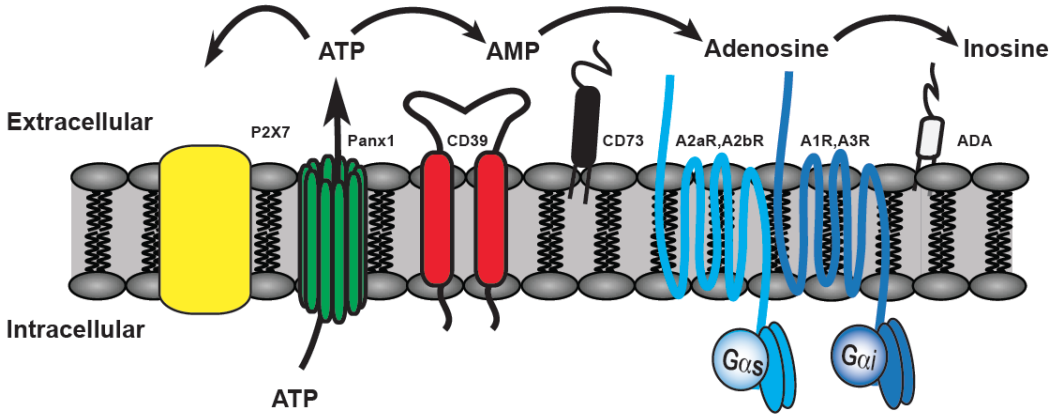

Figure 4. Components of the purinergic signaling system. In response to injury or infection, extracellular ATP levels can increase. eATP may be released through pannexin channels (Panx1, green) from intact, activated cells as well. eATP is metabolized by ecto-enzymes including CD39 (red) and CD73 (black) to generate extracellular adenosine. There are four adenosine receptors that are all GPCRs and include the A2aR and A2bRs (light blue), which couple to the Gαs subunit, and the A1R and A3R (dark blue), which couple to the Gαi subunit. Finally, extracelllular adenosine levels can be further regulated its conversion to inoside by ADA (gray). High amounts of eATP (>1mM) are released from dying and dead cells as they lose membrane integrity and can stimulate the P2X7 receptor (yellow).

Mechanisms of ATP release

With regard to the immune system, much attention has been directed toward the consequence of immune cells recognizing high levels of ATP passively released from damaged and dying cells in response to immunological threats. However, under homeostatic conditions, many intact healthy cells are able to actively release low levels (<1mM) ATP into their surrounding microenvironment. Decades before its role in inflammation was appreciated, the active release of low levels (<1mM) ATP was first observed in activated neurons. Moreover, similar levels of ATP released from healthy muscle cells and platelets were also observed. That discovery first suggested the idea that intact, healthy cells could possess the ability to respond to external cues in the form of ATP release, and importantly, use secreted ATP as a form of cellular communication in both paracrine and autocrine manners.

ATP release from intact cells was first shown to occur via vesicular transport to release vesicle-stored ATP into the cleft of neurochemical synapses. Some non-neuronal cell types such as platelets exhibit a similar vesicle-based ATP release mechanism, however other cells may release ATP via cell membrane channels composed of either connexin or pannexin molecules. Originally identified for their role in gap junctions between adjacent cells, connexin and pannexin channels on individual, non-adjoining cells can act as a pore that controls the release of cellular ATP into the extracellular space. The pannexin family consists of three members. Of particular interest is pannexin 1 (PANX1), which is the most abundant pannexin protein expressed in immune

tissues. Recently, it was demonstrated that activated red blood cells and human neutrophils release ATP via pannexin-1 and that pannexin-1 channels also facilitate the release of ATP from activated T cells in response to T cell receptor and CD28 co-stimulation. Thus, pannexin-1 channels been identified as a common component of ATP release from intact immune cells. It is important to note that in these conditions of immune cell activation mentioned above, the pannexin-dependent release of ATP resulted in the release of relatively low levels (nanomolar-micromolar) of ATP into the extracellular space.

Notably, although the pro-inflammatory effects of high levels of extracellular ATP released as a consequence of cell death is well documented, these relatively low levels of ATP released by intact cells are below the documented threshold of ATP required to activate the P2X7 receptor, therefore it remains unclear the immunological role for the release of low levels of ATP. Because excessive levels of ATP can contribute to severe tissue damage and inflammation, the immune system must be equipped with the ability to properly respond to and regulate the availability of secreted ATP in order to effectively support tissue homeostasis and overall health of the host.

Extracellular ATP metabolism

Ecto-enzymes are enzymes localized to the plasma membrane, in which their active site faces the extracellular space. Four families of ectonucleotidases, which are capable of hydrolyzing extracellular ATP, have been identified in mammalian cells. Ectonucleoside triphosphate diphosphohydrolases (ENTPDases) have affinity for both extracellular tri- and diphosphates (ATP and

ADP) and can hydrolyze both to generate AMP and inorganic phosphate. This gene family is comprised of seven isoforms (ENTPD1-6,8), with ENTPD1-3 and ENTPD8 being extracellularly expressed. The first ENTPDase identified, and the best studied is ENTPD1, CD39 was originally cloned and sequenced as a B cell activation marker with unknown function in the mid-1990s [62, 63]. At the same time, a group of studies were performed that elucidated the function of CD39. First, soluble ATP diphosphohydrolyase (apyrase) from potato tubers was purified, cloned and found to have high sequence similarity to human CD39. Second, a dominant vascular ectonucleotidase was identified to possess potent thromboregulatory activity was cloned and found to be identical to CD39 as well. Since then, the role of CD39 in vascular and platelet biology has been extensively studied. The protein topology of CD39 reveals that the majority of the protein exists as a large extracellular catalytic domain flanked by two transmembrane domains and short cytoplasmic tails [62, 63]. Although, no direct intracellular signaling capability has been shown for CD39, it is believed to affect physiological conditions entirely by its ability to rapidly control bioavailability of extracellular ATP/ADP.

More recently, with the development of CD39-deficient mice and CD39-specific pharmacological inhibitors, the immunological consequence of CD39 has begun to be revealed. Under a variety of disease models including inflammatory bowel disease, contact hypersensitivity and lung injury CD39-defecient mice developed exacerbated inflammation compared to their wild-type counterparts [47]. Additionally, cells may express other ecto-enzymes capable of hydrolyzing

extracellular ATP including ectonucleotide pyrophosphatases (ENPPs), which hydrolyze ATP directly to AMP and pyrophosphate.

The final adenine metabolite generated from ATP hydrolysis is adenosine, in which the single phosphate group of AMP is removed. This is achieved by either the sole ecto-5'-nucleotidase, CD73, or by non-specific alkaline phosphatases. Mice lacking CD73 exhibit augmented inflammatory responses similar to CD39-deficient mice suggesting that the concerted effort of these ecto-enzymes to completely hydrolyze extracellular ATP into adenosine is a vital immunoregulatory mechanism for the ultimate protection of the host. It is important to note that although these mouse models have established a role for CD39 and CD73 in the control of inflammation, it remains elusive which cell types are responsible for mediating these observations as these mice were not engineered as cell or tissue-specific conditional knockouts.

Adenosine receptor signaling

Extracellular adenosine levels are less than 1μM during normal physiological states; however it has been shown in various models that adenosine levels increase 10-100 fold during inflammatory pathological conditions. In patients with sepsis, systemic adenosine concentrations were determined to be 4-10μM [48] and in the synovial fluid of rheumatoid arthritis patients, adenosine is in the 10-100μM range [49]. Therefore, elevated extracellular adenosine may arise in response to immunological threats. Interestingly, is believed that the rise of extracellular adenosine levels is largely due to the extracellular metabolism of adenine nucleotides into adenosine. In contrast to excessive levels of

extracellular ATP, uncontrollable levels of extracellular adenosine can be detrimental to the host. Adenosine deaminase (ADA) irreversibly deaminates adenosine into inosine, thereby modulating adenosine levels. Individuals who are genetically deficient for ADA develop severe combined immunodeficiency (SCID) and exhibit significantly higher levels of adenosine in tissue and sera [50]. Although ADA was originally described as a strictly cytosolic enzyme, it has recently been shown that cells can release ADA into the extracellular milieu, thereby altering extracellular adenosine levels as well [47]. The notion that consistently high extracellular adenosine levels are associated with SCID underscores the concept of adenosine as an important modulator of the immune response. Furthermore, it has been observed that human newborn serum exhibits significantly more adenosine compared to adults. In the same study, the authors suggest that adenosine may act to dampen neonatal T_h1 responses to protect the developing fetus as evidenced by the lower TNFα present in newborn serum compared to the adult counterparts [51]. However, the investigators did not evaluate the mechanism by which adenosine modulates TNFα production, thus it is believed that ATP and adenosine play essential, yet opposing roles in determining the outcome of disease.

Adenosine receptors are guanine nucleotide-binding protein (G-protein) coupled receptors (GPCRs). GPCRs consist of seven transmembrane domains with an extracellular amino terminus and a cytosolic carboxy-terminus that is coupled to a heterotrimeric G-protein signaling complex to induce intracellular signaling. The four adenosine receptors are expressed across species in human

and mice and exhibit differences with regard to G-alpha protein association and affinity to their ligand, adenosine. Both A1 and A3 receptors are coupled to $G\alpha_i$ proteins, which inhibit adenylyl cyclase activation and intracellular cAMP formation. In contrast, both A2 receptors couple to the stimulatory G protein, $G\alpha_s$, which stimulate adenylyl cyclase activity and enhances intracellular cAMP levels [52]. In addition, the A1R and A2aR demonstrate greater affinity for adenosine than the A2bR and A3R receptors; however it is important to highlight that these demonstrations were established under conditions in which each receptor was separately, ectopically expressed in a cell-line culture system. Thus, the relative activation threshold for these receptors under physiological conditions remains elusive. Moreover, there remains a lack of understanding regarding how each of these receptors is regulated with regard to macrophage biology.

Through the generation of genetic knockout mice, a greater understanding of the role of adenosine receptors in immunological responses has been established. Interestingly, however, the A2b receptor appears to be upregulated during pathological conditions, consistent with the notion that as extracellular adenosine levels increase in response to perilous conditions, the A2bR may be required to mediate appropriate adenosine-dependent responses.

Summary

The purinergic signaling system represents a highly regulated mechanism capable of dictating the course of action in response to not only homeostatic conditions such as muscle contraction and nerve function, but also in response to pathological settings such as infection and inflammatory disease. For these reasons, a variety of purinergic signaling-specific drugs are currently in clinical trials for the treatment of diabetic ulcers, chronic pulmonary obstruction disease (COPD) and aspirin-induced gastric injury [53]. However, it is imperative that we understand the consequence of purinergic signaling on specific cell types and under various homeostatic and pathological conditions in order to design effective treatment strategies without compromising the patient's overall health and ability to fight infections. Although, a greater understanding of the role each member of this purinergic system has been achieved through the development of genetic knockout mice, the bulk of these studies obscure the specific contribution of individual cell types in the development of the pathology observed, since the use of whole body knockout mice were used in which the gene has been deleted from all cells in the body. Importantly, however, there remains a significant under-appreciation and demonstration for the specific contribution these molecules have in regulating macrophage physiology. Because macrophages represent an integral part of both the progression and attenuation of inflammation, improving our understanding of how macrophage activation is regulated is important in the development of novel treatments for pathologies in which macrophages play an essential role.

Chapter 2: TLR stimulation initiates a CD39 based autoregulatory mechanism that limits macrophage inflammatory responses

Adapted From: Cohen, HB, Briggs, KT, Marino, JP, Ravid, K, Robson, SC and Mosser, DM. “TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses”. *Blood*. 2013. [54]

Introduction

Failure to control inflammatory macrophage activation responses can lead to pathological diseases, best exemplified by sepsis. Despite our growing understanding of its pathogenesis, sepsis continues to affect more than 200,000 people annually in the US with a mortality rate as high as 50% [12, 55]. The severe pathology associated with sepsis occurs in response to the hyper-production of macrophage-derived inflammatory cytokines, which can lead to vascular and tissue destruction, multiple organ failure, shock, and death [13]. Intriguingly though, macrophages isolated from late-stage septic individuals exhibit the phenotype of immunosuppressive, regulatory macrophages, expressing high levels of the anti-inflammatory cytokine IL-10, and low levels of TNFα and IL-12 [14, 15, 56]. These observations suggest the transition from inflammatory to immunosuppressive macrophages may be critical to control initial inflammatory responses and prevent lethal septic shock [14, 57, 58]. However, the molecular mechanism by which this transition is achieved remains poorly understood.

In the present work, we examine the role that endogenous CD39 and ATP play in regulating the macrophage inflammatory response. Recently, extracellular ATP (eATP) has been characterized as a 'danger signal' that can promote inflammation through P2X7-dependent activation of the NLRP3 inflammasome leading to IL-1β production [19, 59-61]. Importantly, however, such results were shown to be dependent on the addition of millimolar levels of exogenous ATP in vitro. These high levels of eATP have yet to be discovered in vivo, and the concentration of eATP is carefully controlled by the ecto-nucleoside

triphosphate diphosphohydralse (ENTPDase1/CD39) [62, 63]. It was recently shown macrophages express CD39, and that it played a role in modulating the P2X7-dependent production of IL-1β [64] in the presence of exogenously added ATP. Several issues remain incompletely understood regarding how ATP and CD39 contribute to the regulation of macrophage activation. First, the potential for macrophages to be an endogenous source of ATP has not been fully addressed. Unlike most cells, macrophages primarily generate ATP through glycolysis [65, 66]. Moreover, macrophages increase the rate of glycolysis in response to TLR stimulation [67]. Previous attempts to measure ATP release from macrophages have yielded inconsistent results [68-70] that were likely complicated by endogenous CD39 activity. Second, if macrophages are indeed a source of ATP, the mechanism by which they release ATP into the extracellular milieu has not been previously defined. Third, there is currently a paucity of studies investigating how ATP and CD39 influence inflammasome-independent responses, such as TNFα production [14, 58, 71]. Finally, the specific contribution of CD39 on macrophages in directing the course of inflammatory disease remains unknown.

In the present study, we demonstrate that in response to a variety of TLR ligands, macrophages synthesize, secrete, and hydrolyze ATP to control their own activation status. Surprisingly, the ATP that is generated does not stimulate IL-1β release but rather suppresses the production of pro-inflammatory cytokines like TNFα and IL-12. Mechanistically, we show that macrophages hydrolyze self-released ATP via CD39, to generate immunosuppressive adenosine. CD39-

mediated adenosine generation results in a dramatic reprogramming of the macrophage activation response, to enhance not only IL-10 production, but also many newly identified regulatory macrophage-specific transcripts [1, 6, 7, 72]. Finally, we demonstrate that macrophage-specific expression of CD39 is critical in preventing lethal hyper-inflammatory responses to LPS in vivo. Taken together, our data indicate that CD39 acts as a "molecular switch" that controls the balance between inflammatory and regulatory macrophage differentiation.

Materials and Methods

Mice and macrophage isolation. BALB/c and C57BL/6 mice were purchased from the Charles River Laboratories. These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. Bone marrow-derived macrophages were prepared from 6-8 week old female Balb/c, C57BL/6, *Cd39*$^{-/-}$ [73]; and *A2br*$^{-/-}$ [74] mice, as previously described [75] and differentiated in 20% L929 (LC14) conditioned media unless otherwise noted. Human monocytes were obtained from healthy volunteers with their informed consent and IRB approval. Monocytes were purified for immediate use or differentiated in the presence of 50ng/mL M-CSF (CSF-1) for 7 days as previously described [76]. Murine peritoneal macrophages were isolated as previously described [77]. RAW264.7 cells (TIB-71™) were obtained from ATCC (Manassas, VA).

Reagents. ATP, ADP, AMP, adenosine, clodronate, pyrophosphate, phosphate, ATPγs, apyrase, adenosine deaminase, 2-deoxy-glucose, CBX, FFA and LPS *E. coli* K-235 were purchased from Sigma-Aldrich (St. Louis, MO). Pam3CSK4, Heat-killed *L. monocytogenes* (HKLM), Poly(I:C) LMW, and Ultra Pure LPS *E. coli* K12 were purchased from InvivoGen (San Diego, CA). POM-1 and ^{10}Panx were purchased from Tocris Bioscience (R&D Systems; Minneapolis, MN).

Stimulation conditions. All in vitro experiments were performed at 1-2x10^{6}c/mL in tissue culture treated plates. Mouse macrophages studies were performed in

DMEM/F12+GlutaMax (Gibco, Life Technologies;, Grand Island, NY) supplemented with 10% FBS, 1% pen/strep, and 1% glutamine (Gibco, Life Technologies;, Grand Island, NY) unless otherwise indicated. Human monocyte/macrophage studies were performed in RPMI1640 (Gibco, Life Technologies; Grand Island, NY) supplemented with 10% FBS, 1% pen/strep, and 1% glutamine (Gibco, Life Technologies;, Grand Island, NY). For all experiments, monocytes/macrophages were co-stimulated in the presence of 100mM ATP and 10^8cells/mL heat-killed *L. monocytogenes*, 1μg/mL Pam3CSK4, 10μg/mL Poly(I:C), or 10ng/mL Ultra Pure LPS unless indicated otherwise.

Adoptive Transfer and in vivo LPS challenge. $1x10^6$ BMDMs from *Cd39*$^{-/-}$ or wild-type littermate controls were injected i.p. into 6-8 week old female C57BL/6 mice and challenged with 300μg LPS *E. coli* K-235 i.p. For survival experiments, mice were monitored twice daily for survival for 5 days.

Gene Expression Analysis. Total RNA was isolated by using TriZol (Invitrogen, Life Technologies;, Grand Island, NY) and converted to cDNA using the ThermoScript Kit (Invitrogen, , Life Technologies) according to the manufacture's protocol. qPCR analysis was performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics Corporation-Roche Applied Science;, Indianapolis, IN) and GoTaq qPCR Master Mix (Promega;, Madison, WI). Primer pairs used to amplify specific gene products are listed in Table 1. Relative expression levels

were calculated using the ΔΔCt method [6]. *Gapdh* was used as the housekeeping gene for normalization.

Flow Cytometry Antibodies and Analysis. CD39 expression on peritoneal macrophages was determined after gating on $F4/80^{+}CD11b^{+}$ cells. CD39 expression on human monocytes/macrophages was determined after gating on $CD14^{+}$ cells. Anti-mouse CD16/32 was purchased from AnaSpec (Freemont, CA). Human Fc blocking reagent was purchased from Miltenyi Biotech Inc. (Auburn, CA). Anti-mouse CD39-AF647 (24DMS1) was purchased from eBioscience (San Diego, CA). Anti-mouse F4/80-pacific blue (BM8) and anti-human CD39-PE (A1) were purchased from BioLegend (San Diego, CA). Anti-mouse CD11b-FITC (M1/70) and anti-human CD14-PerCP (MϕP9) were purchased from BD Biosciences (San Jose, CA). Samples were analyzed on a BD FACSCanto (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Secreted Cytokine Detection. Cytokine production was determined by a sandwich ELISA using specific capture and biotinylated detection antibody pairs: anti-mouse TNF-purified (G281-2626), TNF-biotin (MP6-XT3), IL-12/23p40-purified (C15.6), IL-12/23p40-biotin (C17.8), IL-10-purified (JES5-2A5), IL-10-biotin (JES5-16E3), and anti-human TNF OptEIA kit were purchased from BD Biosciences (San Diego, CA) [6], anti-human IL-12/23p40 module set and anti-human IL-10 Ready-Set-Go! Kit were purchased from eBioscience (San Diego, CA)

ATP Release Assay. eATP release was performed in performed in 200μL serum-free media. Luminescence was detected using a Centro XS3 LB 960 Microplate Luminometer (Berthold technologies; Bad Wildbad, Germany).

Inorganic Phosphate Release Assay. Phosphate release was performed in 500μL nucleotidase buffer (154mM NaCl, 2mM KCl, 4mM $MgCl_2$, 18mM $NaHCO_3$, 25mM HEPES pH7.4 and 10mM D-glucose).

Thin Layer Chromatography (TLC). Experiments were performed in 200μL nucleotidase buffer containing 0.02mCi ^{14}C-ADP (PerkinElmer; Waltham, MA). Supernatants were spotted onto polyethylenimine cellulose TLC plates (Sigma-Aldrich;St. Louis, MO) and placed in a humidifying chamber containing filter paper and solvent containing 4M ammonium sulfate and 1.5M KH_2PO_4 in water at 1:1.5. Luminescence was detected using a FLA7100 Fugifilm Life Science Phoshorimager following a 24 hr exposure to phosphorimager film (GE Healthcare Biosciences; Pittsburg, PA).

Nuclear Magnetic Resonance (NMR) spectroscopy. Samples were prepared in 500μL nucleotidase buffer and supernatants were stored at -80ºC until NMR analysis. Immediately prior to NMR, samples were further supplemented with 10% Deuterium Oxide (D_2O) (Cambridge Isotope Laboratories, Inc.; Andover, MA) to serve as the lock signal. The ^{31}P spectra were collected at 37ºC using a Bruker

Avance-600MHz spectrometer, with a Bruker broadband SMART probe (Bruker BioSpin Corporation; Billerica, MA). NMR data were collected using Topspin Version 2.1 and Icon Automation NMR software and NMR Case^{TM} multi-sample carousel. ^{31}P spectra were acquired with an offset of -20 ppm, sweep width of 24.271 Hz (~100 ppm), 65536 complex points and 1024 scans. All spectra were processed using Topspin Version 1.3.

Statistical Analysis. Data analysis was performed using GraphPad Prism software (GraphPad Software Inc.; La Jolla, CA) and analyzed using the Student's *t* test. Kaplan-Meier survival curves were determined using the log-rank test. The statistical differences between groups, with the p-values are indicated in the related graphs as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

TLR stimulation results in ATP release from macrophages

The potential for macrophages to be a source of eATP remains controversial. To address this, macrophages were stimulated with TLR agonists and the release of ATP was measured. All TLR agonists tested were capable of inducing significant ATP release from the macrophage-like cell line RAW264.7 (Figure 1A). TLR-induced ATP release was glycolysis-dependent as treatment with 2-deoxyglucose, a competitive inhibitor of glycolysis [78], inhibited ATP release (Figure 1B). Pannexin-1 is a plasma membrane channel that was recently shown to be the major mediator of ATP release from red blood cells [79] and activated T cells [80]. To investigate whether pannexin-1 channels were important for ATP release from macrophages, RAW264.7 cells were stimulated with LPS in the presence of ^{10}Panx, a pannexin-1 specific inhibitor [60], and the release of ATP was measured. The results revealed that ATP secreted by macrophages was strongly dependent on pannexin-1 (Figure 1C). The blockade of pannexin-1-mediated ATP release was also recapitulated when macrophages were stimulated with LPS in the presence of carbenoxolone (CBX), but not in the presence of flufenamic acid (FFA), which preferentially inhibits pannexin and related connexin channels, respectively [81] (Figure 1C). Thus, these results suggest that pannexin-1 channels are indeed the major conduits from which ATP is released from TLR-stimulated macrophages.

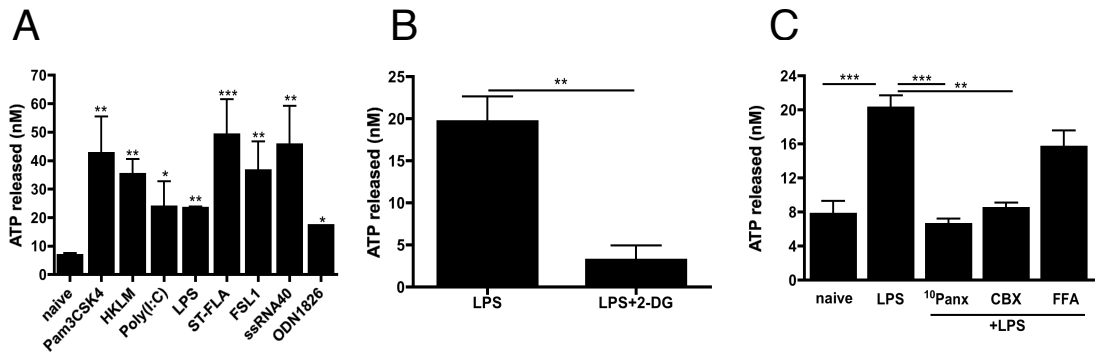

Figure 1. TLR stimulation results in ATP release from macrophages. (A) RAW264.7 cells were stimulated with the indicated TLR stimuli or left untreated. After 3 hrs, ATP released was measured. The means +/- SEM of 3 independent experiments are shown and statistical analysis is relative to naïve samples. **(B)** RAW264.7 cells were stimulated with LPS in absence or presence of 200μg/mL 2-DG and levels of ATP released was measured 3 hrs later. The means +/-SD of triplicate determinations are shown and are representative of 2 independent experiments. **(C)** RAW264.7 cells left untreated or stimulated with LPS alone or in the presence of 50μM ^{10}Panx, 10μM CBX, or 10μM FFA. After 3 hrs ATP released was measured. The means of 3 independent experiments +/- SEM are shown.

Differential CD39 expression on macrophage cell types

Surprisingly, very little eATP was detected from LPS-stimulated bone marrow-derived murine macrophages (BMDMs) relative to LPS-stimulated RAW264.7 cells (Figure 2A), despite similar expression levels of pannexin-1 both at the mRNA and the protein level (Figure 2B,C). We hypothesized that BMDMs may control the bioavailability of self-released eATP and express higher levels of CD39, thereby decreasing eATP detection. Indeed, *Cd39* transcript levels were below detection in RAW264.7 cells, but present in BMDMs (Figure 2B). CD39 expression on the surface of RAW264.7 cells was 10-fold less than on BMDMs (Figure 2D). We further evaluated whether culture conditions used to develop BMDMs influenced CD39 expression and observed that CD39 levels were significantly higher on L929 and M-CSF differentiated BMDMs compared to GM-CSF-induced BMDMs (Figure 2E. Furthermore, L929/M-CSF differentiated BMDMs most closely resembled peritoneal-resident macrophages with regard to CD39 expression. These observations are consistent with previous work showing that in vitro cultivation of BMDMs in the presence of M-CSF is a more representative model of tissue resident macrophages than GM-CSF derived BMDMs [82]. Human monocytes and macrophages also expressed high levels of CD39 (Figure 2F).

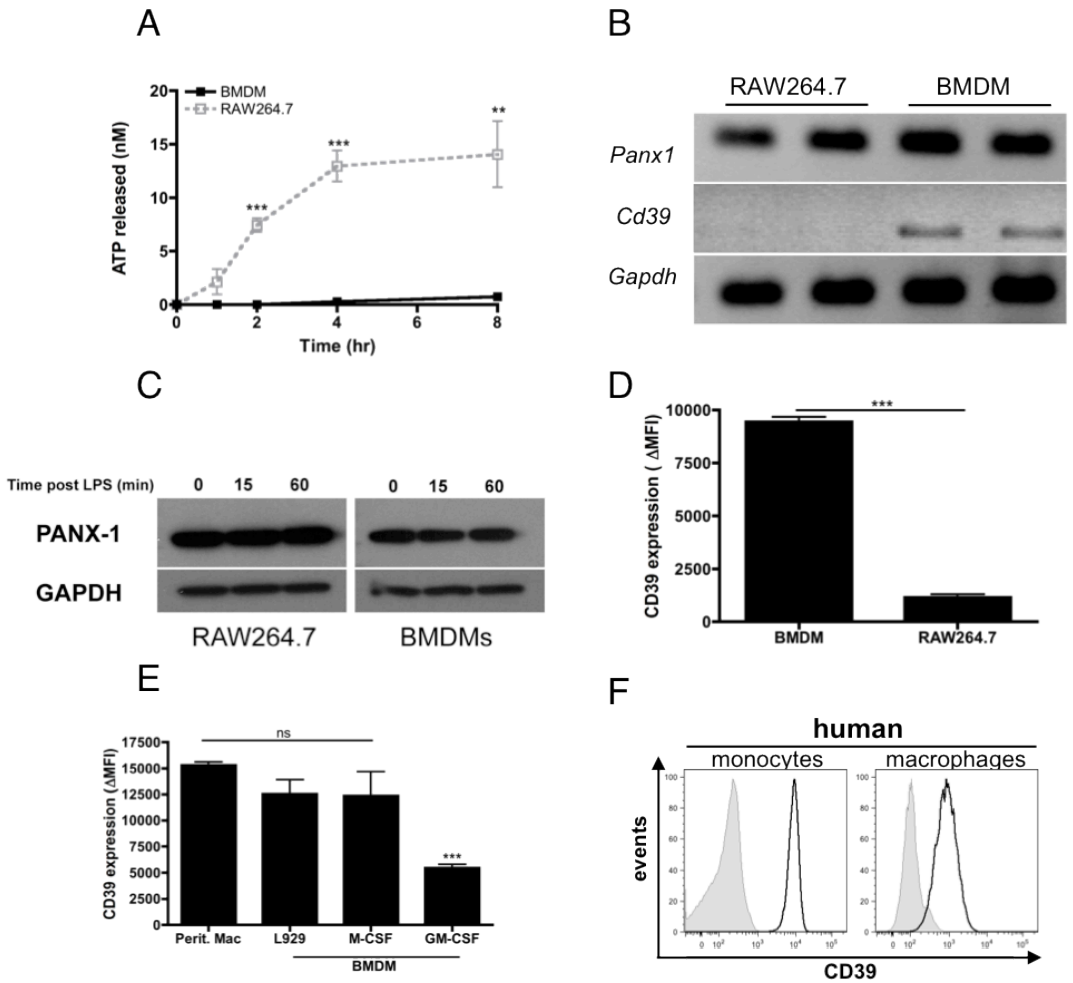

Figure 2. CD39 is differentially expressed on macrophage cell types. (A) Levels of ATP released from LPS-stimulated RAW264.7 cells (open squares; dashed line) or BMDMs (closed squares; solid line) was measured over time. The means +/- SEM of 3 independent experiments are shown. **(B)** The mRNA expression of *Panx1* and *Cd39* in RAW264.7 cells and BMDMs. Data are representative of 3 independent experiments. **(C)** The PANX1 expression was detected by Western Blot in RAW264.7 cells and BMDMs. GAPDH was probed for loading control, Data are representative of 2 independent experiments. **(D)** The surface expression of CD39 on RAW264.7 cells and BMDMs was determined by FACS and expressed as ΔMFI, compared to the isotype control for each group. The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. **(E)** CD39 expressed on peritoneal macrophages and BMDMs differentiated in the presence of L929 conditioned media, M-CSF or GM-CSF for 7 days was determined by flow cytometry and expressed as ΔMFI, compared to isotype control for each group. The mean +/- SD of triplicate determinations is shown and is representative of 2 independent experiments. **(F)** Analysis of CD39 surface expression (black histograms) on human monocytes (left) and macrophages (right) compared to isotype controls (gray histograms). Data are representative of 3 independent experiments.

CD39 controls eATP levels released by macrophages

To address whether CD39 was directly responsible for regulating levels of macrophage-derived eATP, we examined the levels of ATP released from LPS-stimulated *Cd39*$^{-/-}$ macrophages. In contrast to wild-type macrophages, eATP was detectable following LPS stimulation, and sustained for up to 24 hours post activation in *Cd39*$^{-/-}$ macrophages (Figure 3A). These data suggest that TLR-stimulated wild-type macrophages, secrete ATP but rapidly hydrolyze it, therefore preventing eATP detection. Furthermore, similar to CD39^{lo} RAW264.7 cells, ATP secretion by *Cd39*$^{-/-}$ macrophages was critically dependent on pannexin-1 (Figure 3B). Taken together, these data demonstrate that TLR stimulation induces intrinsic ATP release from macrophages via pannexin-1 channels, and under normal conditions macrophages utilize CD39 to control eATP bioavailability.

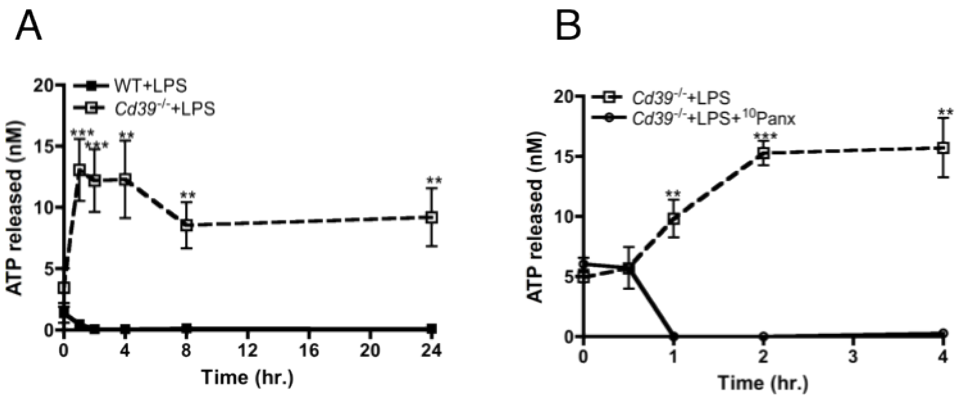

Figure 3. CD39 controls eATP levels released by macrophages. (A) Levels of ATP released from LPS-stimulated *Cd39*$^{-/-}$ (open squares; dashed line) and wild-type (closed squares; solid line) BMDMs were measured over time. The means +/- SEM of 3 independent experiments are shown. **(B)** The levels of ATP produced by *Cd39*$^{-/-}$ BMDMs stimulated with LPS in the absence (open squares; dashed line) or presence (closed circles; solid line) of 50μM ^{10}Panx was measured over time. The means of 3 independent experiments +/- SEM are shown.

Extracellular ATP is rapidly hydrolyzed by macrophage ecto-enzymes

Having demonstrated that CD39 is important for regulating the levels of macrophage-derived eATP, we next characterized the CD39-specific e-NTPDase activity in macrophages. ATP hydrolysis yields the production of adenine metabolites and inorganic phosphates (P_i), therefore we first examined levels of P_i released over time by RAW264.7 cells and BMDMs in the presence of eATP. Consistent with the CD39 expression data, $CD39^{lo}$ RAW264.7 cells exhibited substantially impaired eATP hydrolysis compared to $CD39^{+/+}$ BMDMs (Figure 4A). eATP hydrolysis by macrophages was also dependent on CD39, as treatment with the CD39 inhibitor POM-1 [83] as well as CD39-deficient macrophages exhibited, substantially impaired ATP hydrolysis (Figures 4B and 4C). The defects in purine nucleotide hydrolysis were specific to ATP and ADP, but not AMP (Figure 4D)[62]. We next investigated whether genetic ablation of *Cd39* could be rescued by the presence of apyrase, a soluble e-NTPDase [84]. Indeed, apyrase fully complemented the loss of endogenous CD39, achieving hydrolysis rates similar to $Cd39^{+/+}$ macrophages (Figure 4D). The low level of ATP hydrolysis by CD39-deficient macrophages may be due to the presence of low affinity non-specific phosphatases. To address this, we compared hydrolysis over time from macrophages exposed to lower levels of ATP and demonstrate that wild-type macrophages completely hydrolyze 5μM ATP within 15 minutes, compared to 30% of 500μM ATP hydrolyzed within the same time (Figure 4E). Importantly, macrophages lacking CD39 exhibited substantially reduced hydrolysis rates regardless of the amount of eATP present.

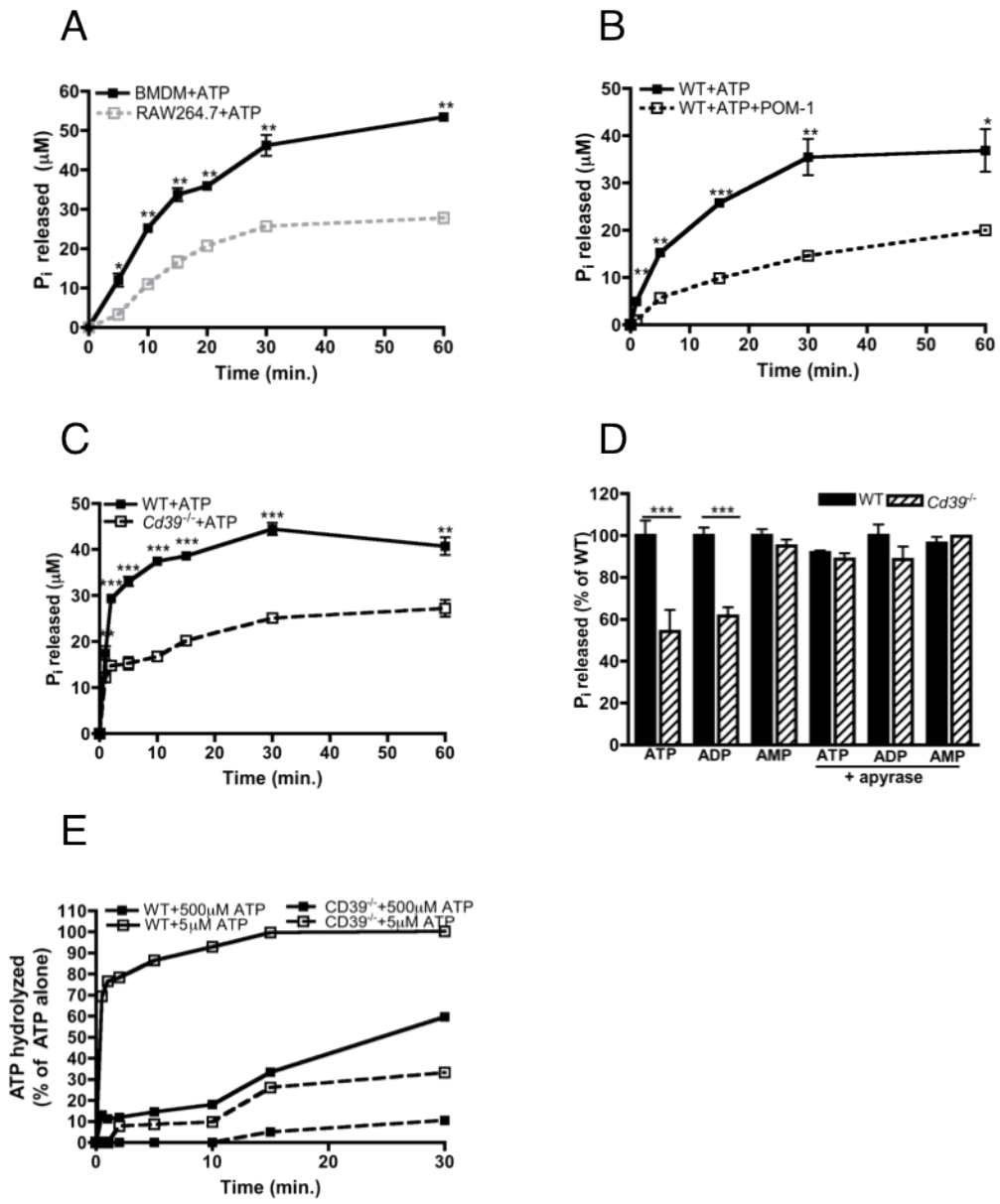

Figure 4. Kinetics and substrate specificity macrophage-CD39. (A-C) The hydrolysis of 500μM ATP by wild-type BMDMs (closed squares; solid black line) over time compared to RAW264.7 cells (open squares; gray, dashed line) (A), in the presence of 10mM POM-1 (open squares; black, dashed line) (B), or *Cd39*$^{-/-}$ (open squares; black, dashed line) BMDMs (C) and was measured by the inorganic phosphate (P_i) release assay. **(D)** The percent of adenine nucleotides hydrolyzed within 30 min. as measured by inorganic phosphate (P_i) released by wild-type BMDMs (solid bars) and *Cd39*$^{-/-}$ (striped bars) BMDMs in the absence (left) or presence of apyrase (right). **(A-D)** The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. **(E)** The percent of 5μM (open squares) or 500μM (closed squares) ATP hydrolyzed over time by wild-type BMDMs (solid lines) and *Cd39*$^{-/-}$ (dashed lines) BMDMs. Percent of hydrolysis was compared to levels of ATP detected in the absence of cells and determined by the ATPlite assay. The data are representative of 3 independent experiments.

To further elucidate the CD39-specific e-NTPDase activity in macrophages, we utilized ^{31}P NMR spectroscopy, which enables the identification of each phosphorus-containing intermediate metabolite generated via eATP hydrolysis. NMR revealed that ATP remained intact and stable in macrophage-free culture medium for several hours (Figure 5A); however, eATP stability was drastically reduced in the presence of $CD39^{+/+}$ macrophages as evidenced by the generation of ADP and AMP from ATP. By 4 hours, ATP was completely hydrolyzed (Figure 5B). In contrast, a substantial fraction of ATP remained intact, even after 8 hours of exposure to *Cd39*$^{-/-}$ macrophages (Figure 5C). While some ATP/ADP was hydrolyzed by CD39-deficient macrophages, which is likely due to the compensatory non-specific phosphatases, the rate of conversion of eATP to ADP and then AMP was dramatically reduced. Collectively, these data suggest that CD39 is the major, high-affinity ecto-ATPase utilized by macrophages to hydrolyze eATP.

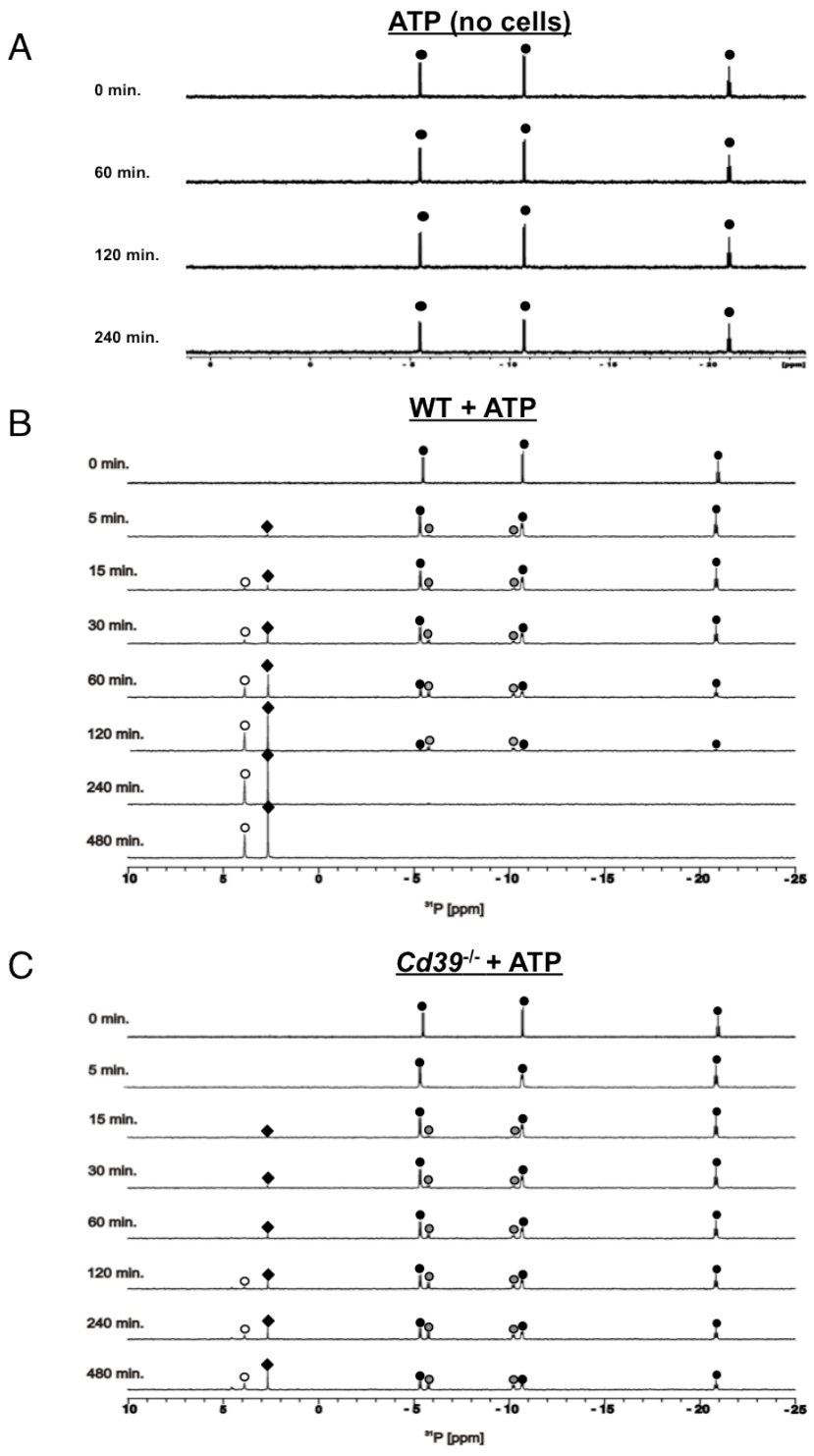

Figure 5. ^{31}P NMR analysis of eATP hydrolysis kinetics by macrophages. (A-C) The ^{31}P NMR spectra of 500μM ATP alone (A) or in the presence of by wild-type (B) or *Cd39*$^{-/-}$ BMDMs (C) are shown over time. The location of the α,β,γ phosphorous peaks of ATP (black circles), α,β phosphorous peaks of ADP (gray circles), phosphorous peaks of AMP (white circles), inorganic phosphate (black diamonds) was based on reference ^{31}P NMR spectra. NMR spectra are representative of at least 3 independent experiments.

eATP promotes regulatory macrophage development following TLR stimulation

Although the inflammatory effect of millimolar levels of eATP is well documented [19, 59, 60, 85], the role of sub-millimolar levels of eATP in influencing macrophage physiology remains unresolved. To address this, macrophages were stimulated with LPS in the presence or absence of 100 μM ATP, and genetic biomarkers associated with inflammatory and regulatory macrophage activation states were assessed. Macrophages stimulated with LPS alone upregulated the expression of mRNA encoding the inflammatory cytokines TNFα (Figure 6A) and IL-12/23p40 (Figure 6B), as expected. Macrophages stimulated in the presence of low levels of ATP, suppressed *Tnfα* and *Il-12/23p40* transcripts by ~80% and 60%, respectively (Figure 6A,B). Additionally, a significant upregulation of many transcripts expressed by regulatory macrophages was observed including *Il-10*, *Il-33*, *Hb-egf,* and *Sphk1* [1, 6, 7, 72]. Although eATP enhanced LPS-induced *Arg1* levels, it did not induce expression of *Fizz1*, *Ym1, Mrc1, Msr1, or Fabp4,* which are biomarkers of alternatively activated (IL-4/IL-13 treated) macrophages (AAMs) [86-88] (Figure 6C). Thus, eATP did not merely inhibit inflammatory macrophage activation, but rather actively promoted the development of a regulatory macrophage phenotype.

The addition of ATP to macrophages also attenuated TNFα (Figure 6D) and IL-12/23p40 (Figure 6E) protein secretion and increased IL-10 production (Figure 6F). These cytokine modulations were dose dependent and were achievable within the range of ATP released intrinsically from TLR-activated

macrophages (Figures 6G). Furthermore, the decrease of inflammatory cytokine production and the induction of IL-10 release were not specific to LPS stimulation, since eATP also promoted regulatory macrophage differentiation in response to a variety of other TLR agonists (Figure 6H). We further tested whether CD39 contributed to the development of ATP-induced regulatory macrophages by activating macrophages in the presence of POM-1, which resulted in complete reversal of ATP-mediated immunosuppression and blocked the induction of a regulatory activation state in mouse (data not shown) and human macrophages (Figure 6I). Together, these results demonstrate that macrophages utilize CD39 and eATP to regulate their activation status towards a regulatory phenotype.

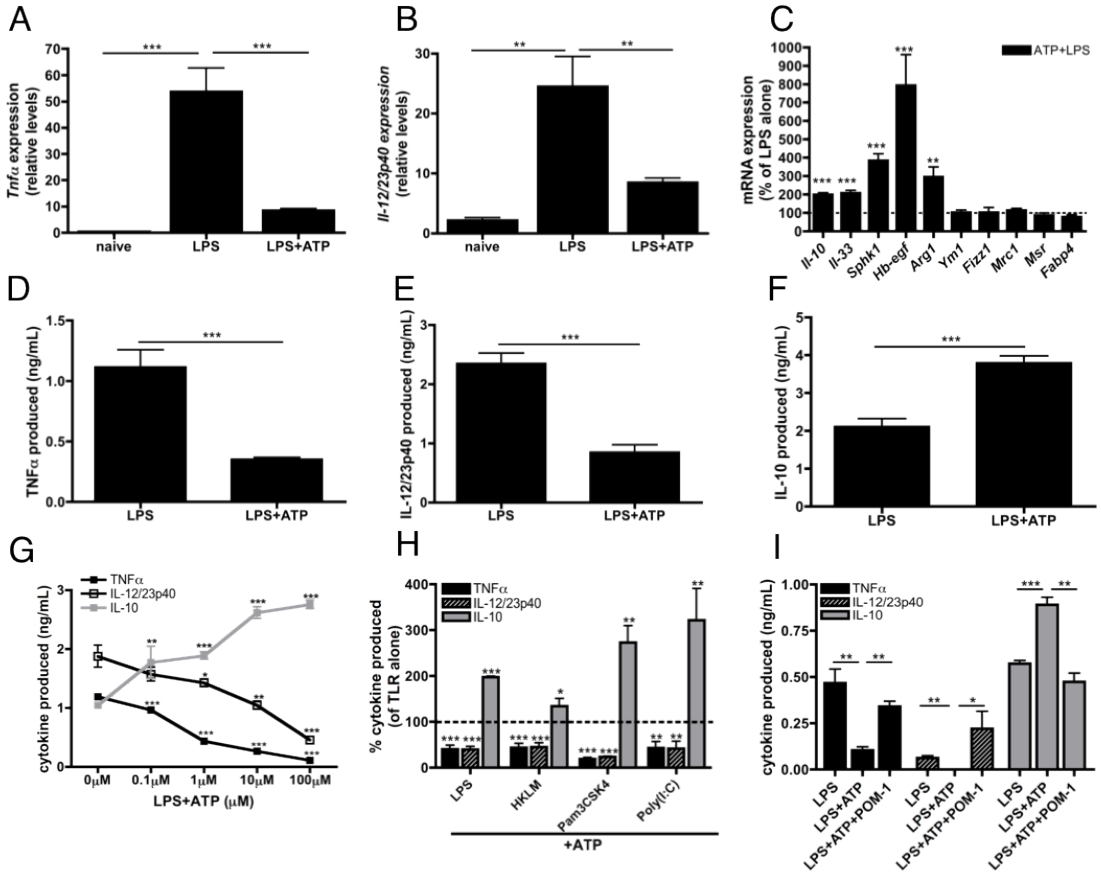

Figure 6. eATP promotes regulatory macrophage development. (A-B) *Tnfα* (A) and *Il-12/23p40* (B) mRNA expression in naïve wild-type BMDMs or BMDMs stimulated with LPS in the absence or presence of 100μM ATP was determined after 4 hrs of stimulation. The means of 3 independent experiments +/- SEM are shown. (**C)** The percent increase of regulatory macrophage-associated transcripts in wild-type BMDMs in the presence of LPS and 100mM ATP are shown at 4 hrs post-stimulation by qRT-PCR. The dashed line represents the expression of regulatory transcripts induced by LPS alone. The means of at least 3 independent experiments +/- SEM are shown. (**D-F)** Secreted levels of TNFα (D), IL-12/23p40 (E) and IL-10 (F) by wild-type BMDMs stimulated with LPS in the absence or presence of 100μM ATP at 8 hrs post-stimulation. The means of 5 independent experiments +/- SEM are shown. **(G)** TNFα (closed squares; black line), IL-12/23p40 (open squares; black line) and IL-10 (closed squares; gray line) secreted by wild-type BMDMs stimulated with LPS in the presence of increasing concentrations of ATP. Cytokine levels were determined after 8 hrs by ELISA. The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. **(H)** The percent of TLR-induced cytokines produced in the presence of 100 μM ATP. Cytokine levels were determined after 8 hrs by ELISA and are relative to TLR stimulation alone (dashed line). The means of TNFα (black bars), IL-12/23p40 (striped bars) and IL-10 (gray bars) production +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. Statistical analysis is relative to samples stimulated with TLR stimuli alone. **(I)** LPS-induced TNFα (black bars), IL-12/23p40 (striped bars) and IL-10 (gray bars) production by human macrophages exposed to 100μM ATP alone or in the presence of 10μM POM-1 was determined by ELISA at 8 hrs post-stimulation. The means +/- SD of triplicate determinations are shown and are representative of 2 independent experiments.

ATP-derived adenosine modulates inflammatory macrophage activation

Having demonstrated that low levels of eATP have a previously unrecognized influence in directing regulatory macrophage development, we next sought to determine the molecular mechanism underlying this phenomenon. To address whether eATP hydrolysis contributed to regulatory macrophage induction by eATP, TNFα, IL-12/23p40 and IL-10 production by macrophages stimulated in the presence of the non-hydrolyzable ATP analog, ATPγs were measured. In contrast to ATP, ATPγs did not inhibit LPS-induced TNFα or IL-12/23p40 production, nor did it enhance IL-10 secretion (Figure 7A). These data therefore suggest that hydrolysis of eATP was required for the modulation of TNFα and IL-12 production. To investigate which ATP metabolite(s) was responsible for attenuating TLR-induced inflammatory macrophage responses, we exposed macrophages to LPS in the presence of ADP, AMP, and adenosine and observed that each adenine metabolite suppressed TLR-induced TNFα and IL-12/23p40 production, while enhancing IL-10 production (Figure 7B). Although pyrophosphate has been shown to influence IL-1β production from macrophages [89], phosphate, pyrophosphate nor its non-hydrolyzable analog clodronate altered TNFα, IL-12/23p40, or induce IL-10 production from macrophages (Figure 7B). Because adenosine has been associated with immunosuppression [90, 91], we examined whether macrophages were capable of generating adenosine from adenine nucleotides by TLC. Macrophages produced AMP within 15 minutes of exposure to ADP, and by one hour, substantial adenosine generation was

evident by TLC (Figure 7C). Within 2 hours, virtually all the ADP provided to the cells was converted to AMP and adenosine. Thus, the TLC data are consistent with the NMR results (above) and indicate that macrophages convert extracellular adenine nucleotides to adenosine.

To further implicate macrophage-generated adenosine as a key immunomodulatory mediator, we assessed cytokine production by macrophages stimulated in the presence of adenosine deaminase (ADA), which rapidly converts adenosine into inosine thereby effectively removing adenosine from the culture. The addition of ADA to macrophages prevented the ability of adenosine or ATP to inhibit LPS-induced TNFα (Figure 4D) and IL-12/23p40 production (Figure 7E). ADA also blocked adenosine and ATP-enhancement of IL-10 production (Figure 7F). Thus, induction of the regulatory macrophage phenotype by eATP requires macrophage hydrolysis of ATP to adenosine.

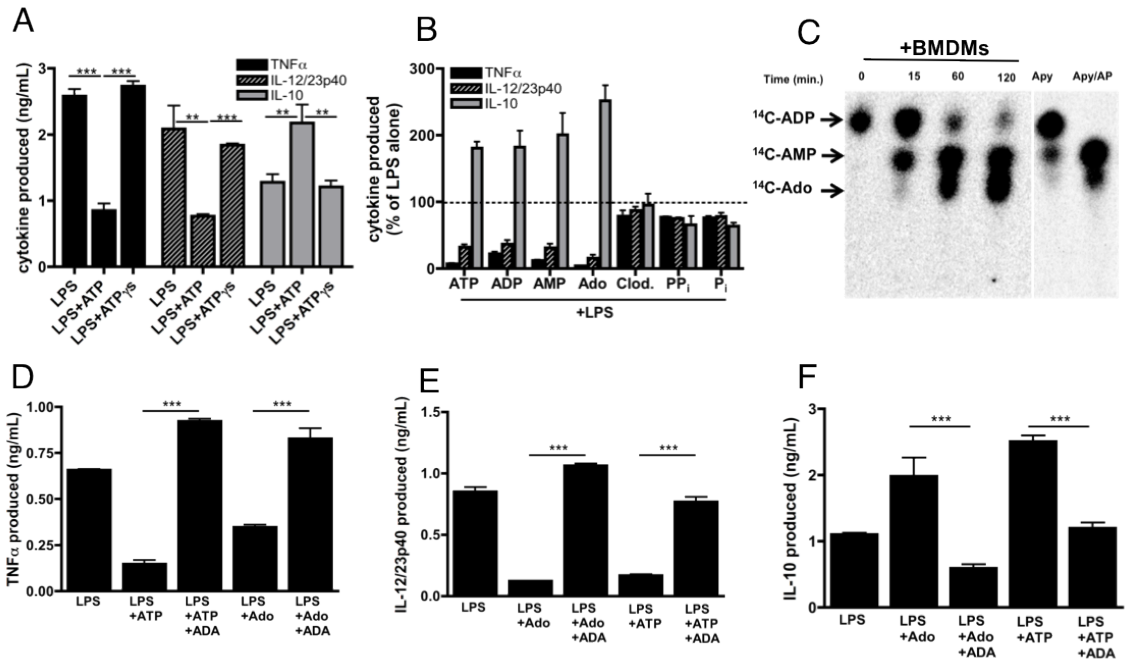

Figure 7. ATP-derived adenosine modulates inflammatory macrophage activation. (A) Wild-type BMDMs were stimulated with LPS in the absence or presence of 100 μM ATP or non-hydrolyzable ATPγs and TNFα (black bars), IL-12/23p40 (striped bars), and IL-10 (gray bars) production was measured by ELISA 8 hrs later. The means of 3 independent experiments +/- SEM are shown. **(B)** Levels of TNFα (black bars), IL-12/23p40 (striped bars), and IL-10 (gray bars) produced by wild-type BMDMs stimulated with LPS in the absence or presence of 100μM ATP, ADP, AMP, adenosine, clodronate, pyrophosphate, or phosphate. Cytokine levels were measured by ELISA after 8 hrs. The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. Percent of cytokine produced is relative to samples stimulated with LPS alone. **(C)** The kinetics of ^{14}C-ADP hydrolysis by resting macrophages over time (left) was determined by TLC. Hydrolysis controls consist of apyrase alone for 5 min (Apy), or apyrase in the presence of alkaline phosphatase (Apy/AP) and ^{14}C-ADP (right). Data are representative of at least 3 independent experiments. **(D-F)** Levels of TNFα (D), IL-12/23p40 (E), and IL-10 (F) produced by wild-type BMDMs stimulated with LPS in the absence or presence of 100mM ATP or adenosine in the absence or presence of 1 U/ml adenosine deaminase (ADA). Cytokines were measured 8 hrs after stimulation by ELISA. The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments.

ATP-derived adenosine attenuates inflammatory cytokine production via A2bR signaling

There are four known receptors for adenosine: A1R, A2aR, A2bR, and A3R. qRT-PCR indicated that macrophages express low levels of transcripts for all four adenosine receptors (Figure 8A), and analysis of their expression over time revealed that adenosine receptor expression was dynamically regulated during macrophage activation (Figure 8B). Naïve macrophages express A2aR most highly (Figure 8A), whereas the A2bR increased in response to LPS. Transcript levels of the other adenosine receptors declined over time (Figures 8A and 8B). These data suggest that TLR stimulation selectively upregulates A2bR expression to increase the sensitivity of the macrophage to available adenosine within the extracellular milieu.

To further study the role of the A2bR in mediating regulatory macrophage development, we examined *A2br*$^{-/-}$ macrophages following stimulation with LPS and ATP. *A2br*$^{-/-}$ macrophages expressed significantly more inflammatory transcripts compared to wild-type macrophages (Figure 8C), and LPS-stimulated *A2br*$^{-/-}$ macrophages were functionally more inflammatory than their wild-type counterparts, secreting higher amounts of TNFα and IL-12/23p40 (Figure 8D). These data indicate that the A2bR was involved in the modulation of inflammatory cytokines. The A2bR was not essential for the regulation of IL-10 as the expression of *Il-10* transcripts was not significantly different between *A2br*$^{-/-}$ and wild-type macrophages (Figure 8C). Interestingly, we observed an increase in *A2ar* expression in *A2br*$^{-/-}$ macrophages upon activation (Figure 8E)

suggesting macrophages may compensate for the absence of A2bR signaling by preferentially upregulating the A2aR. Together, these results demonstrate that in response to LPS, macrophages upregulate the A2bR to respond to (ATP-derived) adenosine to limit inflammatory macrophage responses.

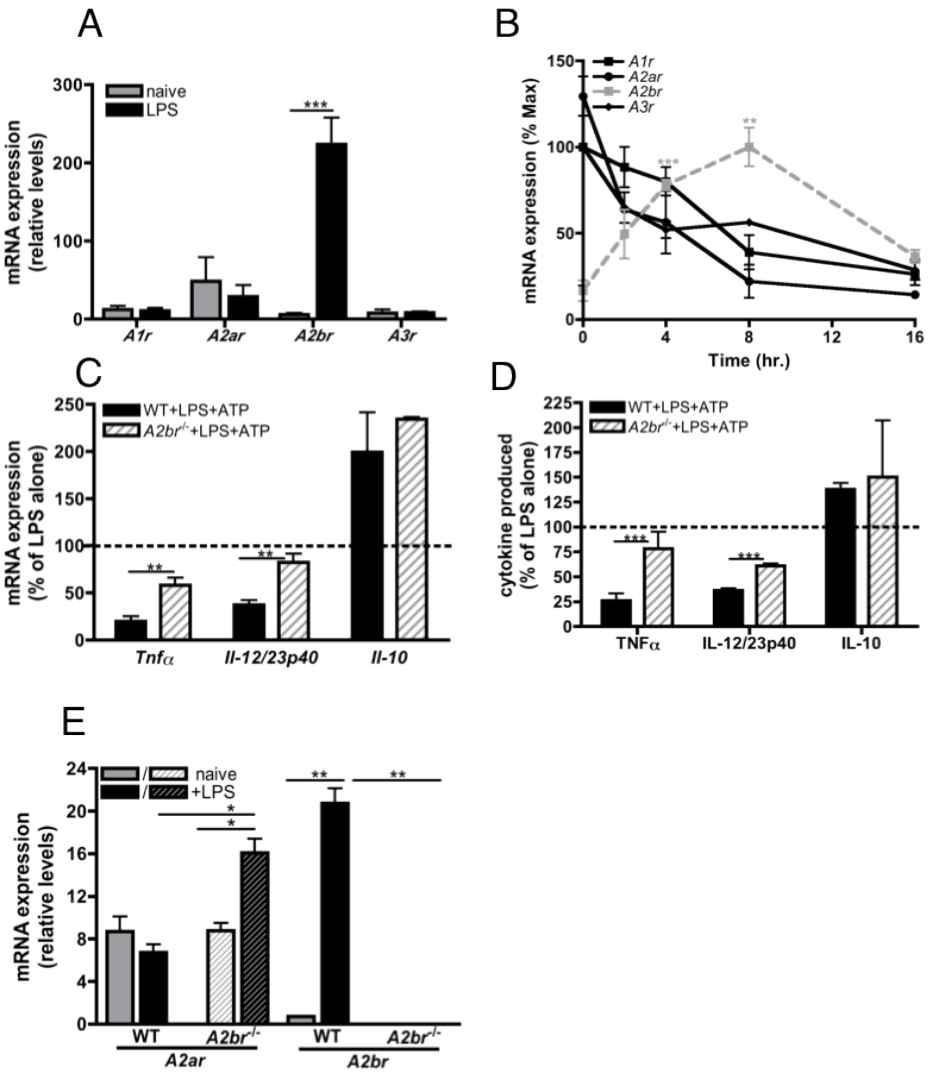

Figure 8. ATP-derived adenosine attenuates inflammatory cytokine production via A2bR signaling. (A) Adenosine receptor mRNA expression in naïve (gray bars) or LPS stimulated (black bars) BMDMs at 4 hrs post-stimulation. The means +/- SD of duplicates are shown and are representative of 3 independent experiments. **(B)** mRNA expression of *A1r* (black, closed squares; black line), *A2ar* (black, closed circles; black line), *A2br* (gray closed squares; gray, dashed line), and *A3r* (black, closed diamonds; black line) in BMDMs stimulated with LPS over time. The means +/- SD of duplicates are shown and are representative of 3 independent experiments. **(C)** The percent of LPS-induced cytokine transcript expression modulated by 100μM ATP in wild-type (solid bars) or *A2br*$^{-/-}$ (striped bars) BMDMs 4 hrs post-stimulation. The dashed line represents mRNA expression in BMDMs stimulated with LPS alone. The means of 3 independent experiments +/- SEM are shown. **(D)** The percent of LPS-induced cytokine produced in the presence of 100μM ATP in wild-type (solid bars) or *A2br*$^{-/-}$ (striped bars) BMDMs was determined by ELISA at 16 hrs post-stimulation. The dashed line represents cytokine production induced by LPS alone. The means +/- SD of triplicates are shown and are representative of 2 independent experiments. **(E)** *A2ar* and *A2br* mRNA expression in wild-type (solid bars) and *A2br*$^{-/-}$ (striped bars) BMDMs unstimulated (gray bars) or stimulated with LPS (black bars) was determined by qRT-PCR at 4 hrs post stimulation. The means +/- SD of duplicates are shown and are representative of 3 independent experiments.

CD39 blockade renders macrophages hyper-inflammatory

Because CD39 initiates the hydrolysis cascade necessary to generate adenosine, we hypothesized that CD39 may function as an intrinsic regulator of the macrophage inflammatory response. To address the functional significance of ATP release by macrophages on their own activation state, we examined the expression of cytokine mRNA from TLR-activated macrophages in the presence of POM-1. Blocking ATP hydrolysis with POM-1 augmented LPS-mediated inflammatory cytokine expression, while significantly decreasing the production of *Il-10* (Figure 9A-C). We also evaluated the kinetics of cytokine production by macrophages stimulated with LPS in the presence of POM-1, and found they secreted significantly more TNFα and IL-12/23p40 and less IL-10 over time compared to macrophages activated in the absence of POM-1 (Figures 9D-F). It is important to highlight that these data were observed in the absence of exogenous ATP, thus revealing that CD39 blockade results in the failure to convert macrophage-released ATP into adenosine, thereby enhancing macrophage inflammatory responses.

We next examined whether CD39 activity could also influence the duration of inflammatory macrophage activation. To do this, macrophages were briefly stimulated with LPS in the presence or absence of POM-1. The LPS was then removed and the macrophages were re-cultured in fresh LPS-free media and cytokine release was measured over time. In the absence of POM-1, LPS-stimulated macrophages decreased TNFα and IL-12/23p40 production shortly after the removal of LPS (Figures 9G and 9H). In contrast, POM-1 treatment

resulted in the continued production of TNFα and IL-12/23p40, despite the removal of LPS from macrophage cultures (Figures 9G and 9H). IL-10 secretion was not similarly affected, suggesting that LPS-induced IL-10 transcripts are inherently unstable (Figure 6I).

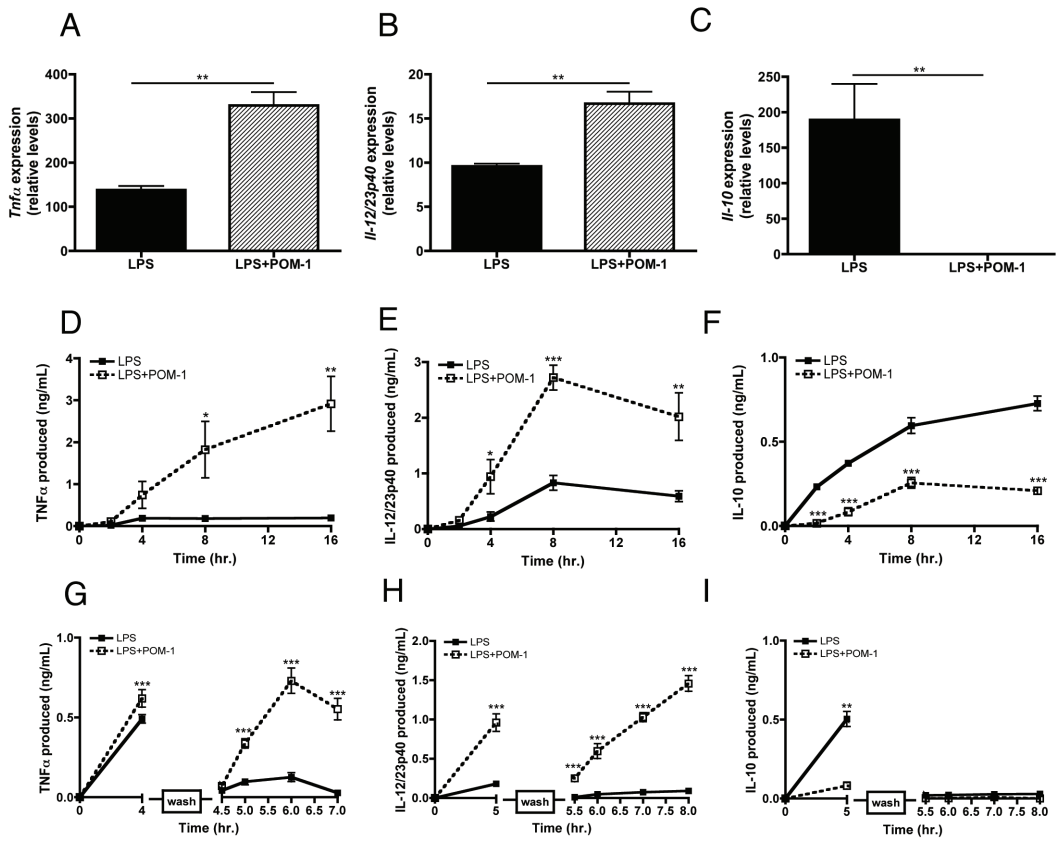

Figure 9. CD39 blockade renders macrophages hyper-inflammatory. (A-C) *Tnfα* (A), *Il-12/23p40* (B) and *Il-10* (C) mRNA expression at 4 hrs post- stimulation in wild-type BMDMs stimulated with LPS in the absence (solid bars) or presence of 10μM POM-1 (striped bars). The means +/- SD of triplicate determinations are shown and are representative of 3 independent experiments. **(D-F)** Cytokine production by BMDMs stimulated with LPS in the absence (closed squares; solid line) or presence (open squares; dashed line) of 10μM POM-1. TNFα (A), IL-12/23p40 (B) and IL-10 (C) levels were detected by ELISA. The means of at least 3 independent experiments +/- SEM are shown. **(G-I)** BMDMs were stimulated with LPS in the absence (closed squares; solid line) or presence (open squares; dashed line) of 10μM POM-1 then washed with PBS (break in y-axis) and replaced with LPS-free media and monitored over the next 5 hours for TNFα (D), IL-12/23p40 (E), and IL-10 (F) production. Protein levels was measured by ELISA. The means +/- SD of triplicates are shown and are representative of 3 independent experiments.

CD39-deficient macrophages exhibit impaired regulatory macrophage induction

The function of macrophage-CD39 during inflammatory diseases remains poorly understood. Therefore, inflammatory responses by *Cd39*$^{-/-}$ macrophages were compared to wild-type macrophages. Similar to POM-1-treated macrophages, CD39-deficient macrophages produced significantly more TNFα and IL-12/23p40 and less IL-10 than their wild-type counterparts when exposed to LPS in vitro (Figure 10A). To investigate the role of macrophage-specific expression of CD39 in orchestrating a pro-inflammatory response in vivo, we adoptively transferred naïve *Cd39*$^{-/-}$ macrophages into the peritoneum of wild-type mice. These mice were then challenged with low levels of LPS and their survival was monitored over the next 5 days. 75% of mice that received *Cd39*$^{+/+}$ macrophages survived, whereas only 25% of the mice that received *Cd39*$^{-/-}$ macrophages survived the endotoxin challenge (Figure 10B). Regulatory macrophage-specific biomarkers were also assessed in peritoneal cells from wild-type mice following adoptive transfer of either wild-type or CD39$^{-/-}$ macrophages after endotoxin challenge. Consistent with the results generated in vitro (Figure 10A-C), CD39-deficient macrophages promoted significant enhancement of *Tnf*α and *Il-12/23p40* expression in vivo (Figure 10C), while the induction of genes associated with regulatory macrophages including *Il-10*, *Il-33*, and *Arg1* was significantly impaired (Figure 1C). These results therefore suggest that normal transition from an inflammatory to regulatory macrophage activation state is important in preventing lethal septic pathology. Taken together, these data demonstrate that CD39 expressed on macrophages is necessary to limit

endotoxin-induced lethality in vivo, and that the presence of relatively few *Cd39*$^{-/-}$ macrophages are capable of dysregulating the course of an inflammatory response.

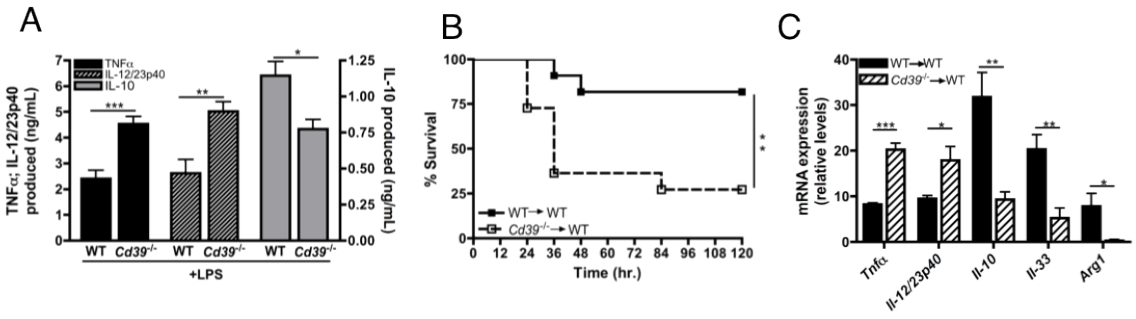

Figure 10. CD39-deficient macrophages exhibit impaired regulatory macrophage induction and enhance LPS toxicity in vivo. (A) Levels of TNFα (black bars), IL-12/23p40 (striped bars), and IL-10 (gray bars) produced by wild-type and *Cd39*$^{-/-}$ BMDMs stimulated with LPS for 8 hrs. Cytokine production was determined by ELISA. The means +/- SEM are shown. **(B)** Kaplan-Meier survival curves of wild-type mice challenged with 300μg LPS (i.p.) 3 hrs after adoptive transfer (i.p) of wild-type (closed squares; solid line) or *Cd39*$^{-/-}$ BMDMs (open squares; dashed line). Data represent the means of 10 mice per group. **(C)** The expression of cytokine transcripts from ex vivo peritoneal cells was determined 1 hr post LPS challenge from wild-type mice receiving 1 x 10^6 naïve wild-type (solid bars) or *Cd39*$^{-/-}$ (striped bars) BMDMs. The means of samples from 4 mice per group +/- SEM are shown.

Discussion

In response to infection macrophages produce inflammatory mediators that, if not appropriately regulated, have the potential to harm the host. This is often manifested during sepsis [13, 15, 92], where a switch from inflammatory to immunosuppressive macrophages has been suggested to play a critical role in protecting the host from lethal septic shock [14, 57, 58]. However, the molecular mechanisms governing this transition remain poorly understood. In this study, we investigated the potential for macrophages to self-regulate their activation state during inflammation. We demonstrate that the induction of inflammatory macrophage responses is accompanied by intrinsic purinergic regulatory mechanisms to limit the development of a hyper-inflammatory activation state. First, TLR-induced glycolysis results in the generation of ATP, a fraction of which is released through pannexin-1 membrane channels. Second, following its release into the extracellular milieu, eATP is rapidly hydrolyzed via CD39 on macrophages. Third, over the next 4 hours, TLR-activated macrophages convert ATP to adenosine and selectively upregulate endogenous A2bR expression, thus enhancing their sensitivity to ATP-derived adenosine. Thus, these results illustrate a new model of macrophage activation that is centered upon their ability to inherently transition from an inflammatory to a regulatory activation state (Figure 11).

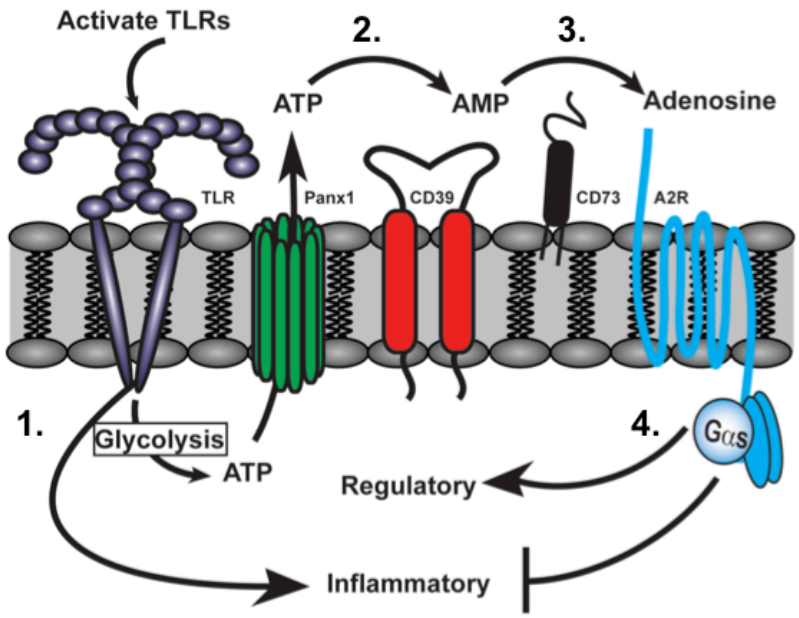

Figure 11. A model depicting macrophage-intrinsic regulation of the activation response. Upon stimulation with LPS or other TLR ligands, macrophages assume an inflammatory activation status. **(1.)** Initially, they produce inflammatory mediators including TNFα and IL-12 while at the same time releasing ATP into their local extracellular environment through Panx1 channels (green) **(2.)** In the presence of CD39 (red), macrophages hydrolyze self-produced extracellular ATP to AMP **(3.)** Within the next 4 hours, macrophages further dephosphorylate AMP to generate adenosine while at the same time upregulating A2bAR expression (blue) **(4.)** Adenosine signals through primarily the A2bAR receptor to inhibit the production of inflammatory cytokines, and together with A2aAR-mediated signaling enhances immunoregulatory mediators (including IL-10, IL-33, HB-EGF and SphK1). In the absence of CD39, activated macrophages are unable to convert self-produced ATP to immunosuppressive adenosine and as a consequence are pushed to a hyper-inflammatory state of activation.

Therefore, in contrast to IL-1β-dependent inflammation driven by high levels of exogenous ATP [19, 59, 60, 85], our data demonstrate that endogenous ATP can negatively regulate macrophage activation. The novelty of this work is the demonstration that TLR-mediated macrophage activation is normally transient, and that blockade of any steps of this newly defined macrophage-intrinsic regulatory cascade results in hyper-inflammatory macrophage responses.

This work reveals for the first time, an essential role of macrophage-specific CD39 in directing the course of inflammatory disease progression in vivo. To date, there are no animal models in which macrophages are the only cell type deficient in CD39; therefore the specific contribution of macrophage-CD39 to disease pathology has been unclear. As demonstrated here, the transfer of small numbers of *Cd39*$^{-/-}$ macrophages into wild-type mice resulted in increased lethality in a murine model of septic shock. Furthermore, CD39-deficient macrophages maintained a hyper-inflammatory state and failed to transition into regulatory macrophages in vitro or in vivo. These data support previous reports suggesting that the ability of inflammatory macrophages to transition to an anti-inflammatory state is vital to the survival of septic individuals [14, 56, 57] and reveals a novel role for macrophage-CD39 in mediating this transition.

Although it has been well documented that macrophages exposed to exogenous adenosine exhibit immunomodulatory responses [52, 91], the present work expands this idea and demonstrates: 1) that TLR-activated macrophages, themselves represent an important source of eATP, 2) that macrophages rapidly and efficiently convert eATP to adenosine, 3) that this conversion is dependent

on CD39, and 4) that macrophages respond to eATP-derived adenosine by assuming a transcriptional profile that is characteristic of regulatory macrophages. Our data are consistent with previous reports suggesting a role for A2bR-mediated immunosuppression [93-95] and implicating the A2aR as the major adenosine receptor involved in IL-10 induction in macrophages [96]. Interestingly, both the A2aR and A2bR are G-protein coupled receptors (GPCRs) associated with the Gαs subunit and are capable of activating adenylate cyclase and enhancing intracellular cAMP levels [90]. We previously demonstrated that stimulating macrophages in the presence of cAMP led to the development of the regulatory phenotype [7] and the activation of GPCRs resulted in ERK-dependent IL-10 hyper-induction [8, 97]. Indeed, LPS-stimulated macrophages exposed to low levels of ATP/adenosine resulted in ERK activation and ERK-dependent IL-10 production (data not shown), thereby supporting a role for ERK in driving adenosine-induced regulatory macrophage development.

Importantly, in addition to IL-10, macrophages exposed to ATP upregulate a number of transcripts that are preferentially upregulated in regulatory macrophages compared to classically (M1) including IL-33, Sphk, and HB-EGF [1, 6, 7, 72]. Some of these regulatory macrophage-specific transcripts have been associated with lung inflammation, however their expression is likely to be tissue-dependent. Indeed, the negative correlation between LPS toxicity and expression of these regulatory macrophage biomarkers in mice strongly suggests that these molecules promote immunosuppression. Although *Arg1* was upregulated in response to ATP, *Arg1* may not be a reliable AAM-specific

biomarker, because it can be induced through IL-4/IL-13-independent mechanisms. *Arg1* expression via STAT3-dependent [98] and cAMP-mediated pathways [99, 100] have been previously reported. Furthermore, although it was recently reported that adenosine augments IL-4-induced alternative activation in macrophages [101], we did not observe enhanced expression of any AAM-specific biomarkers in TLR-activated macrophages exposed to ATP. Together, these observations indicate that TLR-activated macrophages exposed to eATP represent a state of activation distinct from AAMs.

Homeostatic concentrations of eATP within tissues have been estimated to be within the nM-μM range [46, 102]; however these values likely underestimate the level of eATP within local, pericullular environment. Furthermore, eATP levels have been shown to increase 10-100-fold during pathophysiological states [102, 103]. Therefore, it will be interesting to understand the molecular control of ATP release from macrophages. We did not observe any change in pannexin-1 expression over time in macrophages suggesting that the induction of pannexin-1 expression is not a point of regulating ATP release from macrophages. Recently, caspases 3 and 7 have been implicated in controlling pannexin-1 function in apoptotic cells [81], however the macrophages used in this study were not apoptotic. Thus, ATP release is likely not due to caspase activation in these cells. Interestingly, however we observed Hif1α expression and intracellular ATP levels increased substantially over the first 1-2 hours post stimulation (data not shown). Therefore, we predict that the activation of glycolysis needed to

synthesize intracellular stores of ATP prior to secretion may represent an important checkpoint governing the kinetics of ATP release from macrophages.

There are currently a number of adenosine-directed therapeutics under development to treat various inflammatory diseases [53], however none that specifically target macrophages. Our data reveal that the release and subsequent hydrolysis of ATP is a previously unrecognized mechanism controlling the magnitude and duration of macrophage activation. The ability for CD39-mediated ATP hydrolysis to direct regulatory macrophage development was shown to also be applicable to human monocyte and macrophages, thus highlighting the translational potential of this work. The results of this study therefore broaden the spectrum of therapeutics that could be designed to treat diseases in which macrophage activation plays a contributing role [14].

In summary, we have identified a novel macrophage-intrinsic regulatory pathway that may provide a mechanism to explain how macrophages are capable of playing disparate roles in the progression and the resolution of inflammation. This work further demonstrates that TLR-induced macrophage activation is a transient state that can be efficiently reversed, and that CD39 serves a central role in orchestrating the transition from a pro-inflammatory to an immunoregulatory state of activation in macrophages.

Chapter 3: IFNγ-mediated inhibition of the adenosine 2b receptor renders macrophages hyper-inflammatory

From: Cohen, HB, Ward, A, Chandraselaran, P, Ravid, K and Mosser, DM "IFNγ-mediated inhibition of the adenosine 2b receptor renders macrophages hyper-inflammatory". *Manuscript in preparation for submission*. 2014.

Introduction

Although inflammatory macrophage activation is necessary to combat invading microbes, as resident protectors of host tissue, macrophages are additionally challenged to regulate themselves in order to fight infections without destroying their residing tissue and compromising the overall health of the host. Tissue destruction is a consequence of the persistent release of many macrophage-derived noxious molecules including nitric oxide (NO) and tumor necrosis factor α (TNFα). Moreover, severe tissue pathology and inflammatory macrophages are shared characteristics between myriad diseases including type-2 diabetes, obesity, Alzheimer's and autoimmune disorders[14, 23, 24, 104]. However, the underlying mechanisms sustaining chronic inflammation remain elusive.

Due to their natural plasticity, macrophages possess the unique ability to actively participate in both the maintenance of tissue integrity and protection from pathogens. Thus, once infection is controlled, inflammation must be resolved in order to retain homeostatic tissue function. The exposure of macrophages to microbial toll-like receptor (TLR) stimuli initiates the production of inflammatory molecules, however such stimuli may concomitantly activate negative feedback mechanisms to restrict macrophage activation leading to the development of acute, short-lived inflammation. We recently demonstrated that upon TLR stimulation, macrophages are glycolytically activated and release low levels of endogenous adenosine triphosphate (ATP) into their local extracellular milieu. Over the next 2-4 hours, macrophages actively convert this ATP into

immunosuppressive adenosine via CD39-mediated hydrolysis and transition towards an anti-inflammatory activation state. Moreover, deficiency of CD39 specifically on macrophages resulted in severe immunopathology in a mouse model of septic shock due to the inability of these cells to develop into regulatory macrophages. Thus, inflammation is limited as a consequence of adenosine generation and its signaling to prevent overactive inflammatory macrophage responses.

The presence of high levels of interferon γ (IFNγ) represents an important differing quality between acute and chronic inflammatory environments. IFNγ is secreted by activated natural killer (NK) cells, iNKT, and T_h1 cells and primes macrophages to become highly sensitive to TLR stimuli and increases their production of TNFα and interleukin-12 (IL-12). Thus, IFNγ plays vital roles in cell-mediated immunity and host defense against intracellular pathogens. Interestingly, elevated levels of IFNγ and macrophage exhibiting an IFN signature are observed in rheumatoid arthritis and lupus individuals, suggesting a pathogenic role for IFNγ-dependent chronic macrophage activation[25, 26]. Although, the ability for IFNγ to enhance the inflammatory potential of TLR-activated macrophages is a well-known phenomenon, how IFNγ affects the intrinsic regulation of macrophage activation remains to be determined.

Here, we investigated the effect IFNγ has on TLR-activated macrophages and reveal that IFNγ sustains inflammatory macrophage activation by attenuating their sensitivity to extracellular adenosine. Specifically, we demonstrate that IFNγ treatment selectively blocks the induction of the adenosine 2b receptor (A2bR).

We further show that IFNγ inhibition of LPS-induced A2bR prevents completion of an important negative feedback loop, thus rendering macrophages hyper-inflammatory. Finally, we reveal the therapeutic potential of enhancing A2bR expression in macrophages to treat inflammatory disorders. In summation, our data illustrate the therapeutic potential of targeting macrophage-specific A2bR to reduce chronic inflammation.

Materials and Methods

Mice and macrophage isolation. C57BL/6 mice were purchased from the Charles River Laboratories. These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. Bone marrow-derived macrophages were prepared from 6-8 week old female C57BL/6 as previously described [75] and differentiated in 20% L929 (LC14) conditioned media unless otherwise noted. RAW264.7 cells (TIB-71™) were obtained from ATCC (Manassas, VA).

Reagents. ATP and adenosine were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IFNγ was purchased from R&D systems (Minneapolis, MN) and reconstituted according to the manufacture's directions. Ultra Pure LPS *E. coli* K12 were purchased from InvivoGen (San Diego, CA). MRS1754, CGS21680, N-ethyl carboxamidoadenosine (NECA), and BAY 60-6583 were purchased from Tocris Bioscience (R&D Systems; Minneapolis, MN).

Stimulation conditions. All in vitro experiments were performed at 1-2x10^6c/mL in tissue culture treated plates. Mouse macrophages studies were performed in DMEM/F12+GlutaMax (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% FBS, 1% pen/strep, and 1% glutamine (Gibco, Life Technologies;, Grand Island, NY) unless otherwise indicated. For all experiments, macrophages were primed overnight with 100U/mL IFNγ followed by addition of 10ng/mL Ultra Pure LPS with or without 10μM ATP or adenosine unless indicated otherwise.

Gene Expression Analysis. Total RNA was isolated by using TriZol (Invitrogen, Life Technologies;, Grand Island, NY) and converted to cDNA using the ThermoScript Kit (Invitrogen, , Life Technologies) according to the manufacture's protocol. qPCR analysis was performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics Corporation-Roche Applied Science;, Indianapolis, IN) and GoTaq qPCR Master Mix (Promega;, Madison, WI). Primer pairs used to amplify specific gene products are listed in Table 1. Relative expression levels were calculated using the $\Delta\Delta$Ct method [6]. *Gapdh* was used as the housekeeping gene for normalization.

Flow Cytometry Antibodies and Analysis. Anti-mouse CD16/32 was purchased from AnaSpec (Freemont, CA). Anti-mouse CD39-AF647 (24DMS1) was purchased from eBioscience (San Diego, CA). Anti-mouse F4/80-pacific blue (BM8) was purchased from BioLegend (San Diego, CA). Anti-mouse CD11b-FITC (M1/70) was purchased from BD Biosciences (San Jose, CA). Samples were analyzed on a BD FACSCanto (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Secreted Cytokine Detection. Cytokine production was determined by a sandwich ELISA using specific capture and biotinylated detection antibody pairs: anti-mouse TNF-purified (G281-2626), TNF-biotin (MP6-XT3), IL-12/23p40-purified

(C15.6), IL-12/23p40-biotin (C17.8), IL-10-purified (JES5-2A5), IL-10-biotin (JES5-16E3).

ATP Hydrolysis Assay. eATP hydrolysis was detected using the ATPlite Luminescence ATP Detection Assay System (PerkinElmer; Waltham, MA) according to the manufacture's protocol omitting the cell-lysis step.

Inorganic Phosphate Release Assay. Adenine nucleotide hydrolysis was detected using the BIOMOL Green Reagent (Enzo Life Sciences; Farmingdale, NY) according to the manufacture's protocol.

Primary Macrophage Transfection. BMDMs were transfected by nucleofection using the Amaxa Mouse Macrophage Nucleofector Kit (Lonza Cologne AG, Cologne, Germany) according to manufacture's protocol. Briefly, 1×10^6 BMDMs were resuspended in 100µl of nucleofector solution (Lonza Cologne AG, Cologne, Germany) and nucleofection performed using the Nucleofector I Device, program Y-01. Cells were transfected with 3µg of pmaxGFP (Lonza Cologne AG, Cologne, Germany) alone or with pcDNA4/V5-His-A2b [105] (provided by Ravid, K.). The cytomegalovirus (CMV) promoter drives the expression of both cDNAs. 500µL of pre-warmed media containing was immediately added to the cuvette and cells transferred to tissue culture plates. Cells were allowed to recover overnight and media was replaced prior to stimulation.

Statistical Analysis. Data analysis was performed using GraphPad Prism software (GraphPad Software Inc.; La Jolla, CA) and analyzed using the Student's *t* test. The statistical differences between groups, with the p-values are indicated in the related graphs as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

IFNγ sustains inflammatory macrophage responses

The prototypical T_h1-associated cytokine, IFNγ is known to promote classical macrophage activation. To test whether IFNγ can influence the magnitude and duration of inflammatory cytokine production by TLR-activated macrophages, we exposed bone marrow-derived macrophages (BMDMs) to IFNγ overnight followed by subsequent exposure to the TLR4 ligand, LPS over time (Figure 1A). Indeed, we found that macrophages treated with IFNγ significantly increased the total amount of TNFα produced by LPS-activated macrophages throughout the time of LPS exposure compared to macrophages stimulated with LPS alone. We then examined whether IFNγ modulated the stability of an induced activated state in macrophages. To do this, we stimulated unprimed or IFNγ primed macrophages with LPS. After 2 hours, the LPS was removed and the macrophages were re-cultured in fresh LPS-free media and cytokine production was assessed 2 hours later.

As we previously demonstrated [54], unprimed, LPS-stimulated macrophages significantly reduce TNFα production in response to the loss of activating stimuli from their culture environment (Figure 1B). In contrast, IFNγ primed macrophages continued to secrete TNFα, with little reduction in the amount of TNFα produced, even once the LPS had been removed the macrophage cultures (Figure 1B). Similar results were achieved with regard to IL-12p40 production (Figure 1C). Collectively, these results suggest that TLR

stimulation promotes a short-lived inflammatory activation status in macrophages and that IFNγ blocks this inherent ability to self-regulate, thus rendering macrophages chronically inflammatory.

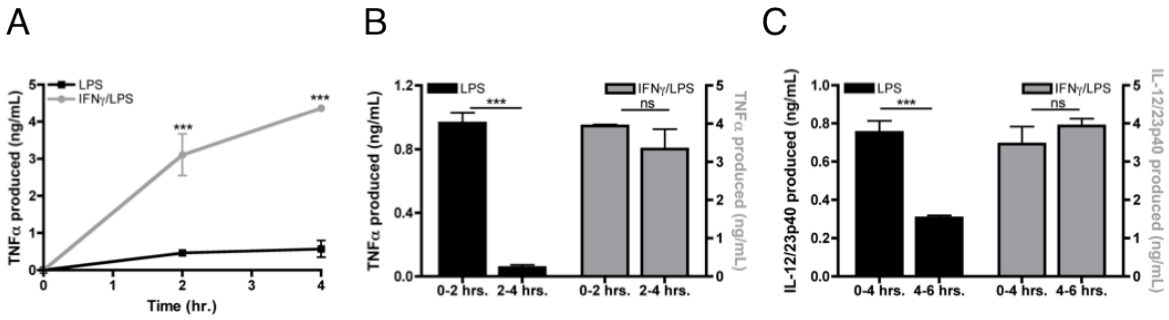

Figure 1. IFNγ prevents transient inflammatory activation, rendering macrophages hyper-inflammatory. (A) Cytokine produced by LPS stimulated unprimed (black squares, solid line or IFNγ primed (gray circles, solid line) over time. TNFα was measured by ELISA. The means +/- SD of triplicates are shown and are representative of 3 independent experiments. **(B,C)** Unprimed (black bars) and IFNγ primed (gray bars) BMDMs were stimulated with LPS for 2-4 hr, then washed with PBS and replaced with LPS-free media and monitored over the next 2 hours for TNFα (B), IL-12/23p40 (C). Protein levels were measured by ELISA. The means +/- SD of triplicates are shown and are representative of 2 independent experiments.

LPS-induced ATP release and hydrolysis is intact in the presence of IFNγ

We recently demonstrated that in response to TLR stimulation, macrophages actively transition from an inflammatory to a regulatory activation status by actively releasing and converting extracellular ATP into anti-inflammatory adenosine via CD39 [54]. Because IFNγ treatment prevents the intrinsic development of regulatory macrophages upon exposure to TLR stimuli, we postulated that IFNγ might interfere an important component of this newly identified CD39-based autoregulatory mechanism in macrophages. This signaling cascade is initiated by the release of intracellular stores of ATP by TLR-activated macrophages into the extracellular space. Accordingly, we assessed whether IFNγ treatment may modulate TLR-induced ATP release and observed negligible levels of eATP in the culture media of LPS stimulated BMDMs in the absence and presence of IFNγ (data not shown). These data were consistent with the prior observations that BMDMs express high levels of CD39, thus limiting the detection of eATP [54]. To more closely examine the effect IFNγ may have on eATP release, we stimulated RAW264.7 cells, which express low levels of CD39, thus permitting the ability to detect eATP released by macrophages [54]. Although we could detect the presence of ATP released into the culture medium upon LPS stimulation, it was unchanged in the presence of IFNγ (Figure 2A). These data suggest that IFNγ does not substantially modulate CD39 levels in macrophages.

We next compared the level of CD39 expressed on the surface of macrophages exposed to LPS alone or following IFNγ treatment. CD39 remained highly expressed by macrophages regardless of culture conditions and was actually upregulated by IFNγ (Figure 2B). Next, we examined the effect of IFNγ on ecto-ATPase activity and observed that similar rates of eATP hydrolysis between unprimed and IFNγ primed macrophages (Figure 2C).

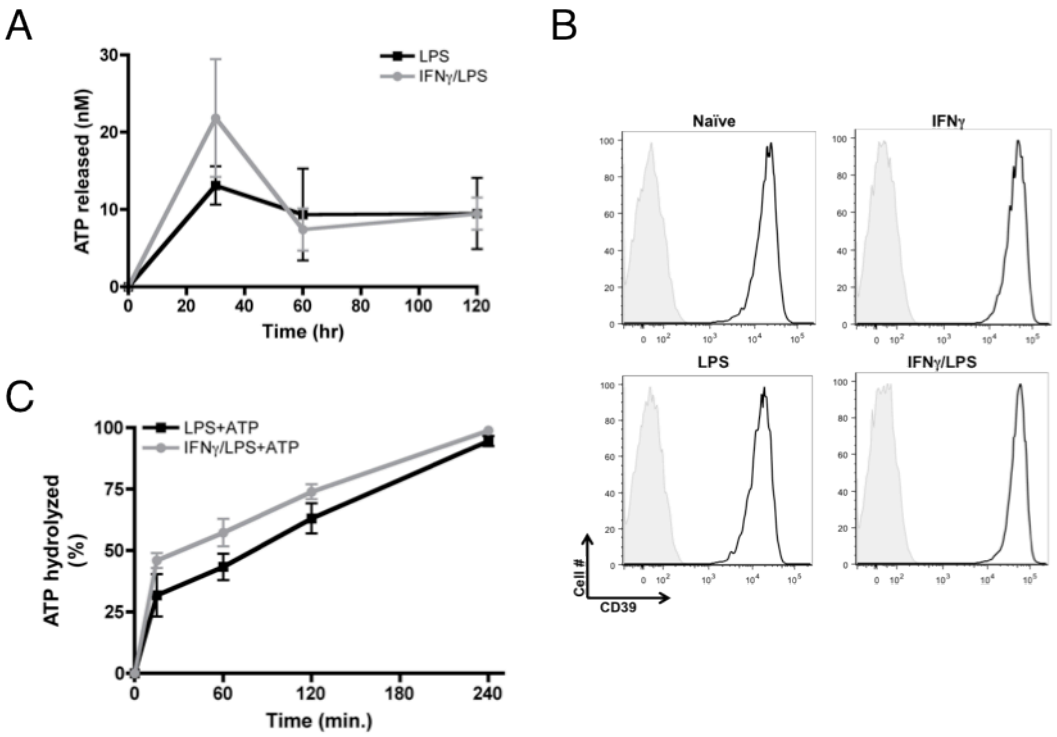

Figure 2. LPS-induced ATP release and hydrolysis is intact in the presence of IFNγ. (A) Levels of ATP released from LPS-stimulated unprimed (black line) and IFNγ primed (gray line) RAW264.7 cells was measured over time. The means +/- SEM of 2 independent experiments are shown. **(B)** Analysis of CD39 surface expression (black histograms) on unprimed (left) and IFNγ primed (right) BMDMs compared to isotype controls (gray histograms). Data are representative of 2 independent experiments. **(C)** The hydrolysis of 500μM ATP by unprimed BMDMs (black squares, solid line) over time compared to IFNγ primed BMDMs (gray circles, solid line). Percent of hydrolysis was compared to levels of ATP detected in the absence of cells and determined by the ATPlite assay. The means +/-SEM from 3 independent experiments is shown.

The functional consequence of CD39 activity is adenosine-mediated immunosuppression [54]; therefore we next investigated if IFNγ make macrophages refractive to eATP-induced immunosuppression. As we have previously shown, low levels of eATP potently suppresses LPS-induced TNFα and IL-12/23p40 production by unprimed macrophages (Figure 3A,B). However, the attenuation of inflammatory cytokine secretion was completely abolished in the presence of IFNγ, thus indicating that IFNγ primed macrophages fail to readily transition to an immunoregulatory activation state in the presence of eATP (Figure 3A,B). Having shown that CD39 expression and activity are in tact in the presence of IFNγ, we next addressed whether IFNγ modulates macrophage responses to adenosine itself. Indeed, adenosine significantly reduced the amount of TNFα and IL-12/23p40 secreted by macrophages stimulated with LPS alone and IFNγ treatment negated the immunosuppressive effect of adenosine, thus recapitulating the results observed by macrophages exposed to eATP (Figure 3C,D). However, adenosine-mediated IL-10 production was unaffected by IFNγ treatment (Figure 3E). Together, these data indicate that the inability of IFNγ primed inflammatory macrophages to readily transition to an anti-inflammatory state must occur downstream of eATP hydrolysis and is caused by a decreased ability to respond to adenosine.

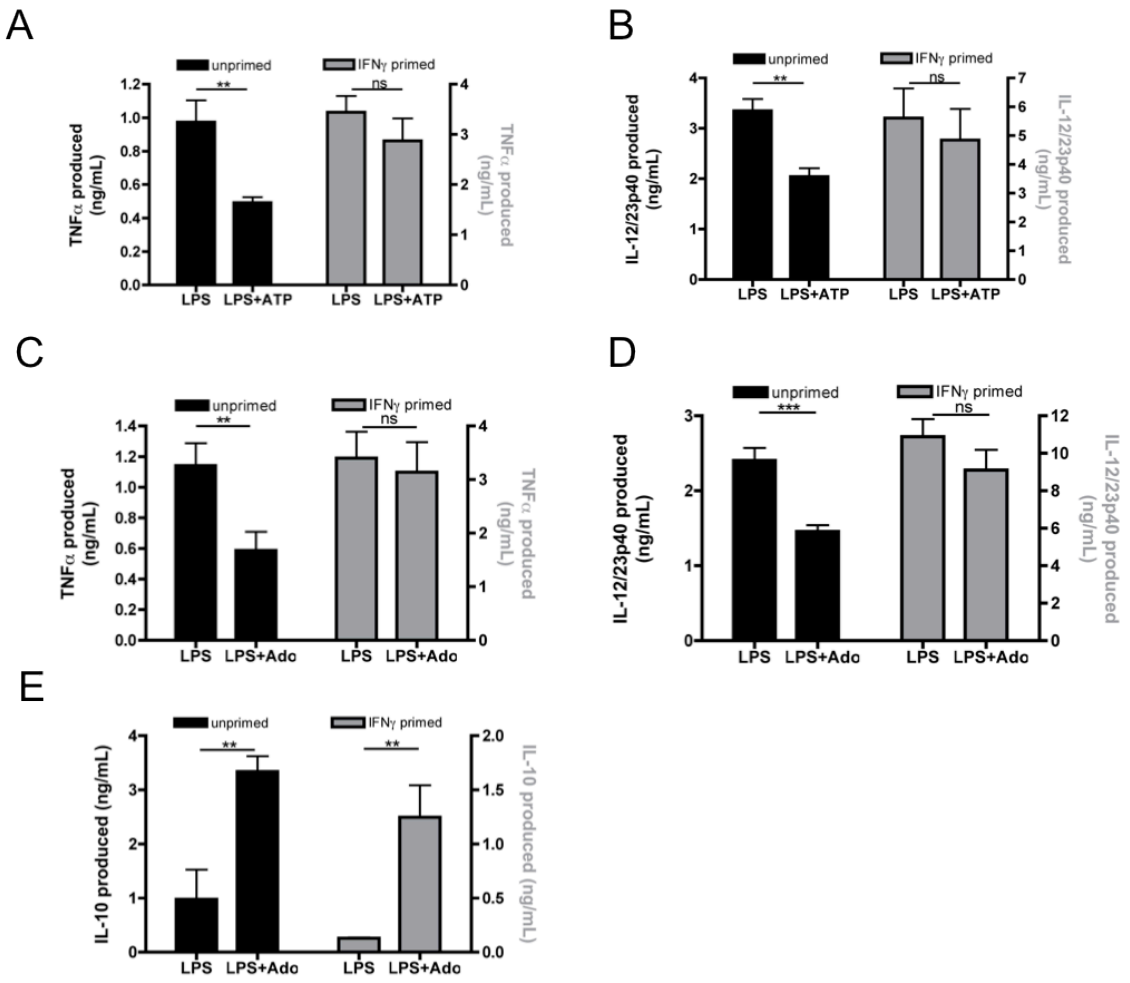

Figure 3. IFNγ blocks ATP-induced immunosuppression downstream of eATP hydrolysis. **(A,B)** Unprimed (black bars) and IFNγ primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 10μM ATP for 8 hrs. TNFα (A) and IL-12/23p40 (B) production was measured by ELISA. The means +/- SEM from three independent experiments are shown. **(C-E)** Unprimed (black bars) and IFNγ primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 10μM adenosine for 8 hrs. TNFα (C), IL-12/23p40 (D), and IL-10 (E) production was measured by ELISA. The means +/- SEM from three independent experiments are shown.

IFNγ inhibits LPS-induced A2bR expression in macrophages

The plasticity of inflammatory macrophages relies on their ability to respond to eATP-derived adenosine; however it remains elusive whether IFNγ modulates the adenosine receptor expression profile of inflammatory macrophages. Therefore we compared adenosine receptor expression of LPS activated macrophages under primed and IFNγ primed conditions. In the absence of IFNγ, LPS dramatically upregulated A2bR transcript levels compared to naïve macrophages (Figure 4A). Moreover, expression of the other adenosine receptors decreased upon exposure to LPS. Interestingly however, the induction of the A2bR by LPS was severely inhibited by IFNγ (Figure 4A). In addition, IFNγ appears to specifically limit the A2bR expression in inflammatory macrophages, as it was the only adenosine receptor to be downregulated by IFNγ treatment. Moreover, IFNγ completely inhibited the induction of the A2bR for the first hour of LPS stimulation, while unprimed macrophages significantly increased transcript levels of the A2bR (Figure 4B). Furthermore, IFNγ primed macrophages continuously expressed low levels of A2bR transcript and protein regardless of time exposed to LPS (Figure 4B,C). Collectively, these data indicate that IFNγ stably decreases the ability for macrophages to sense extracellular adenosine.

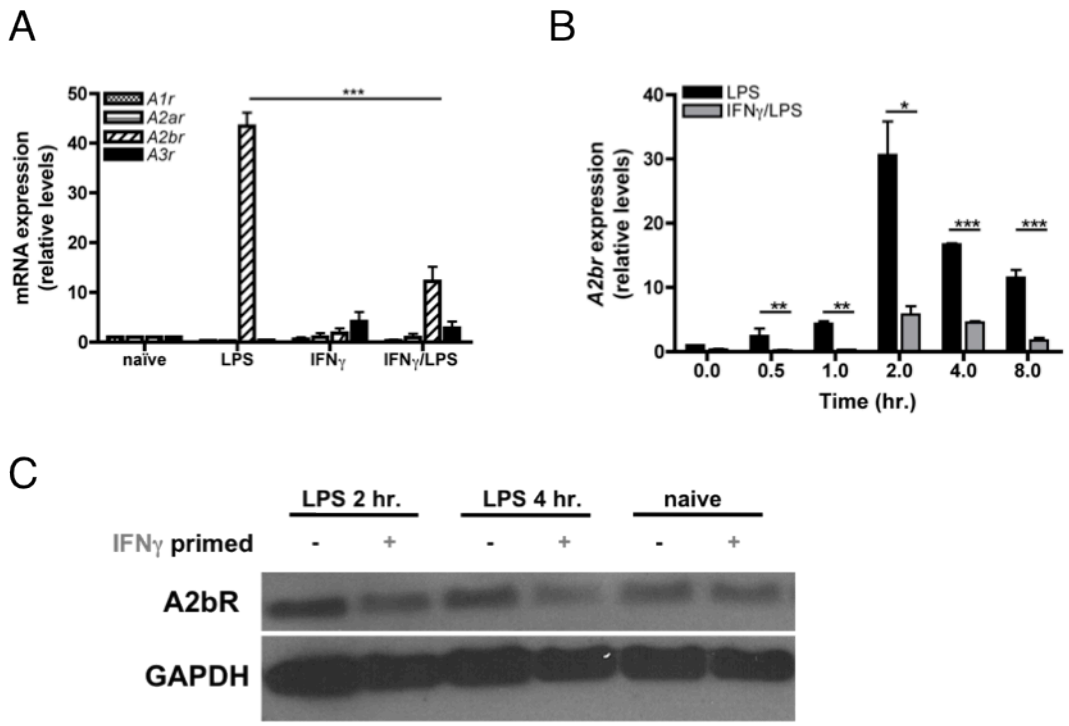

Figure 4. IFNγ inhibits LPS-induced A2bR expression in macrophages (A) mRNA expression of *A1r* (dotted bars), *A2ar* (horizontally striped bars), *A2br* (diagonally striped bars), and *A3r* (solid black bars) in unprimed and IFNγ primed BMDMs stimulated with LPS over time. The means +/- SD of duplicates are shown and are representative of 3 independent experiments. **(B)** Unprimed BMDMs (black bars) and IFNγ primed BMDMs (gray bars) were stimulated with LPS over time. A2bR mRNA levels were determined by qPCR. The means +/-SD of triplicate determinations are shown and are representative of 2 independent experiments. **(C)** Protein expression of the A2bR was determined by Western Blot. Unprimed (-) and IFNγ primed (+) BMDMs were stimulated over time with LPS. GAPDH was probed for loading control. Data are representative of 2 independent experiments.

IFNγ inhibits A2bR-dependent immunosuppression

Because LPS-induced adenosine generation represents an important negative feedback loop controlling inflammatory activation responses in macrophages, we hypothesized that IFNγ may specifically inhibit the induction of the A2bR in order to maintain an inflammatory activation state. To address the functional significance of the A2bR during inflammatory macrophage activation we primed macrophages with an A2bR-specific antagonist, MRS1754, and then exposed the cells to LPS in the presence or absence of adenosine. Unprimed macrophages were highly sensitive to adenosine and exhibited marked reduction in LPS-induced TNFα and IL-12/23p40 production compared to macrophages stimulated with LPS alone (Figure 5A,B). The effect of adenosine was completely dependent on the A2bR as macrophages primed with MRS1754, an A2bR-specific antagonist, demonstrated no significant difference in TNFα or IL-12/23p40 levels regardless of adenosine being present in their extracellular milieu (Figure 5A,B). Thus, these results suggest that specific A2bR antagonism recapitulates the inhibitory effects of IFNγ treatment. Adenosine potently suppressed LPS-induced TNFα in a dose-dependent manner in unprimed cells, indicating that in the absence of priming macrophages are highly sensitive to low levels of extracellular adenosine (Figure 5C). MRS1754 treatment completely recapitulated the inhibitory effect induced by IFNγ, thus further demonstrating that IFNγ impairs adenosine-mediated immunosuppression via blocking A2bR signaling.

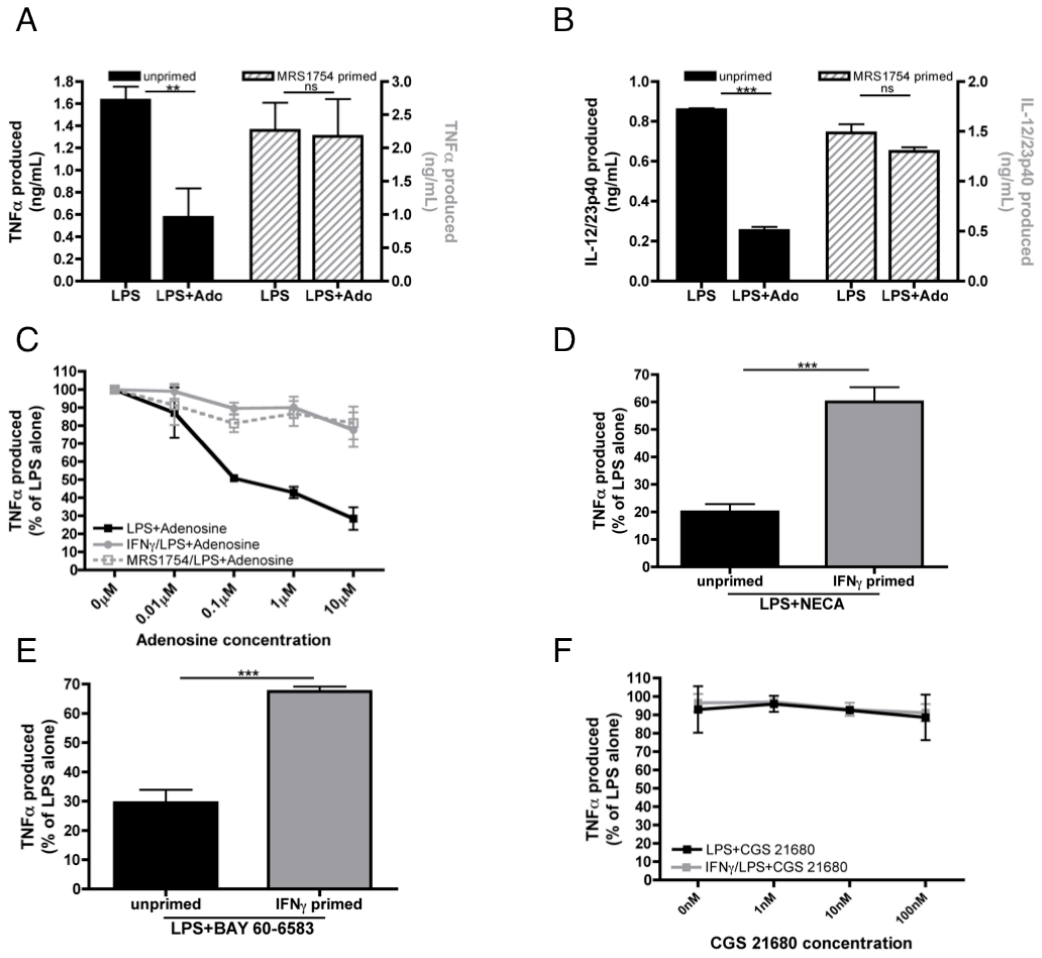

Figure 5. IFNγ inhibits A2bR-dependent immunosuppression in macrophages. (A,B) Unprimed (black bars) and MRS1754 primed (gray striped bars) BMDMs were stimulated with LPS in the absence or presence of 10mM adenosine for 8 hrs. TNFα (A), IL-12/23p40 (B) production was measured by ELISA. The means +/- SEM from at least three independent experiments are shown. **(C)** Unprimed (black squares, solid line), IFNγ primed (gray circles,solid line), and MRS1754 primed (gray open squares, dotted line) BMDMs were stimulated with LPS in the presence or absence of adenosine at various concentrations. 8 hrs. post stimulation, TNFα levels were detected by ELISA. Percent of TNFα produced is based on TNFα levels produced by LPS alone for each condition. The means +/-SEM from at least 3 independent experiments are shown. **(D,E)** Unprimed (black bars) and IFNγ primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 1nM of the A2bR-specific agonist NECA (D) or BAY 60-6583 (E). TNFα production was measured 8 hrs. after stimulation by ELISA. Percent of TNFα produced is based on TNFα levels produced by LPS alone for each condition. The means +/- SEM from three independent experiments are shown. **(F)** Unprimed (black line) and IFNγ primed (gray line) BMDMs were stimulated with LPS in the absence or presence of the A2aR-specific agonist, CGS 21680 at various concentrations. TNFα levels were detected 8 hrs. after stimulation by ELISA. Percent of TNFα produced is based on TNFα levels produced by LPS alone for each condition. The means +/- SD from triplicate determinations are shown and is representative of 2 independent experiments.

Having shown that IFNγ prevents the induction of LPS-induced A2bR expression, we next addressed whether IFNγ blockade of A2bR was responsible for sustaining inflammatory macrophage responses in the presence of adenosine. Macrophages can express all four known adenosine receptors to some degree, thus the specific contribution of the A2bR in regulating macrophage responses may be masked since adenosine is a ligand for these four receptors. Therefore, we further examined the role of A2bR-dependent signaling in inflammatory macrophages exposed to the A2bR-specific agonists, NECA or BAY 60-6583. Unprimed macrophages stimulated with LPS and either A2bR agonist resulted in a 70-80% reduction in TNFα secreted, compared to macrophages exposed to LPS alone (Figure 5D,E). Macrophages treated with IFNγ; however, largely retained their ability to secrete high levels of TNFα even in the presence of NECA or BAY 60-6583 compared to their unprimed counterparts. Moreover, we investigated whether the A2bR is the dominant adenosine receptor that suppresses inflammatory cytokine production by exposing macrophages to LPS in the presence of the closely related adenosine mimetic, CGS 21680, which preferentially stimulates the A2aR. Interestingly, we observed that TNFα levels remained unchanged upon addition of CGS21680 and that IFNγ treatment did not modulate A2aR-specific responses (Figure 5F), thereby suggesting that the A2aR is not a major adenosine receptor involved in the suppression of TNFα. Together, these data indicate that IFNγ specifically hinders the induction of the A2bR, the major adenosine receptor responsible for auto-regulating inflammatory cytokine production in TLR-stimulated macrophages.

A2bR overexpression permits the development of immunosuppressive macrophage in the presence of IFNγ

Having identified that IFNγ specifically prevents the induction of A2bR expression to maintain a hyper-inflammatory activation status in macrophages, we wished to explore whether IFNγ-dependent inhibition of A2bR expression was directly responsible for halting the development of immunoregulatory macrophages. To do this, we overexpressed the A2bR in BMDMs by transfection along with a GFP-containing plasmid to readily track transfection efficiency. After 24 hours, we assessed transfection efficiency and confirmed that the A2bR was overexpressed in co-transfected BMDMs (Figure 6A,B). We then exposed these cells to IFNγ followed by stimulation with LPS in the presence or absence of adenosine and monitored TNFα production. Despite the presence of IFNγ, macrophages over-expressing the A2bR secreted 20% less TNFα compared to macrophage transfected with the GFP containing plasmid alone (Figure 6C). These results preliminarily demonstrate that IFNγ renders macrophages hyper-inflammatory by specifically downregulating the A2bR. Together, these data reveal that IFNγ interferes with the autoregulation of macrophages following TLR activation by specifically attenuating A2bR-mediated responses, thus resulting in exacerbated inflammatory macrophage responses.

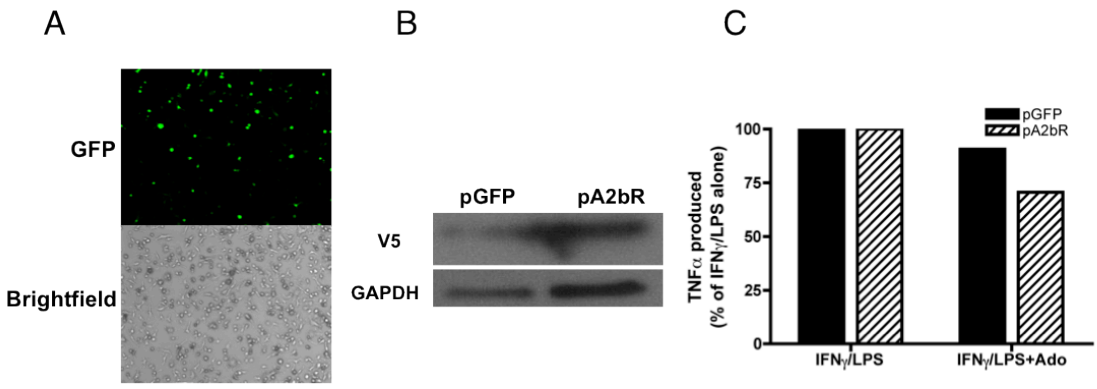

Figure 6. A2bR overexpression permits the development of immunosuppressive macrophages in the presence of IFNγ. (A,B) BMDMs were co-transfected with plasmids containing GFP and A2bR cDNA. After 24 hrs., transfection efficiency were assessed by immunofluorescence microscopy (A) and probed for A2bR-V5 expression by Western Blot (B). Data are representative of 2 independent experiments. **(C)** BMDMs trasnfected with pGFP alone (black bars) or co-transfected with pA2bR (black striped bars) were primed with IFNγ and stimulated with LPS in the presence or absence of 10μM adenosine. TNFα production was measured 8 hrs. after stimulation by ELISA. Percent of TNFα produced is based on TNFα levels produced by LPS alone for each condition. Data are preliminary and from 1 determination per condition.

Discussion

Although inflammatory macrophage activation is a necessary component of host defense, if left uncontrolled, it can lead to severe tissue damage, organ failure and even death. Indeed, previous observations suggest that transient macrophage activation is integral to survival of the host[14, 54, 106]. The inability for macrophages to control their activation state has been suggested to support the pathogenesis of an extensive list of chronic inflammatory diseases including inflammatory bowel disease, lupus, and rheumatoid arthritis [25, 107]. However, the molecular mechanisms preventing transient inflammatory macrophage activation under these conditions remain incompletely understood. Herein, we reveal a novel mechanism supporting IFNγ-mediated induction of chronic inflammatory macrophages and demonstrate that IFNγ actively desensitizes macrophages to immunosuppressive adenosine thereby sustaining inflammatory responses.

The novelty of this work is the demonstration that IFNγ represents the first known physiological inhibitor of the purinergic-dependent auto-regulation of inflammatory macrophages. We previously demonstrated that pharmacological inhibition or genetic deletion of the ecto-ATPase, CD39 renders macrophage hyper-inflammatory due to the inability to convert extracellular ATP into adenosine. Here, we reveal that blockade of adenosine-mediated effects can occur in response to IFNγ, which blocks the inherent ability for inflammatory macrophages to transition towards an adenosine-dependent immunosuppressive state by preventing A2bR signaling (Figure 7).

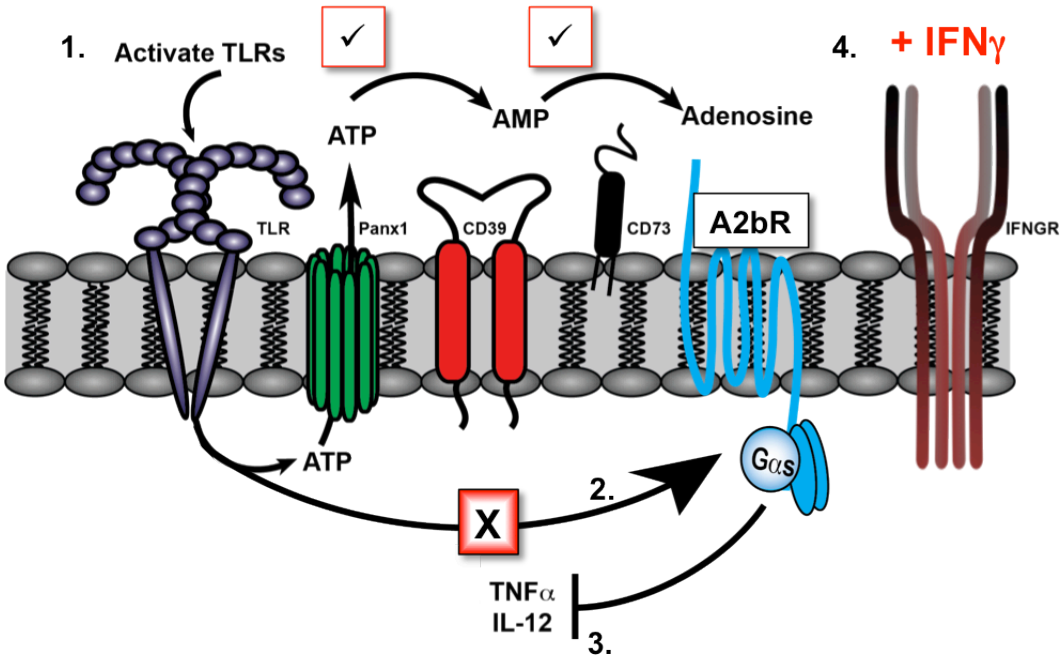

Figure 7. A new model of IFNγ-mediated chronic inflammatory activation in macrophages. (1.) In response to LPS activation alone, inflammatory cytokine production is transient and dependent on the inherent ability for macrophages to convert extracellular ATP into immunosuppressive adenosine**. (2.)** Within the first hours of exposure to LPS, macrophages selectively upregulate the A2bR (blue) to increase sensitivity to newly generated adenosine levels rising within their extracellular space. **(3.**) The A2bR potently inhibits LPS-induced TNFα; IL-12 production, thereby transitioning the macrophages towards an immunoregulatory state. **(4.)** Despite this auto-regulatory mechanism, chronic inflammatory macrophages can persist. This model illustrates that while IFNγ treatment does not interfere with CD39 (red) and CD73 (black) activity (boxed check marks), IFNγ severely inhibits the induction of the LPS-induced A2bR (boxed 'X'). Thus, IFNγ is able to sustain TLR-induced TNFα;IL-12 production promoting hyper-inflammatory macrophage responses, which are prevalent in a variety of chronic diseases.

Although the anti-inflammatory effect of adenosine on macrophages is well known, the intracellular mechanisms supporting its action remains unclear. Here, we demonstrate that the A2bR is the dominant adenosine receptor employed by TLR-activated macrophages to limit TNFα and IL-12/23p40 production. The elegant work by Ivashkiv and colleagues has demonstrated a role for STAT3-dependent inhibition of STAT1 indicating that IL-10-induced STAT3 may repress IFN/STAT1 activity [26, 108, 109]. However, in our studies ATP/adenosine-induced IL-10 production was minimally affected by IFNγ treatment. Thus, while it is known that IFNγ can antagonize IL-10-depedent suppression of TLR-induced TNFα and IL-12/23p40, the failure for IFNγ to suppress these inflammatory cytokines suggests a mechanism independent of IL-10 signaling that is largely responsible for induction of a hyper-inflammatory macrophage activation status.

All adenosine receptors are GPCRs, however only the A2aR and A2bR are coupled to Gαs, thus inducing the generation of intracellular cAMP upon stimulation. We have previously demonstrated that cAMP can drive the development of anti-inflammatory macrophages[7]. Because of the relatively lower affinity of the A2bR to adenosine compared to the A2aR, it has also been proposed that the A2bR may be preferentially employed by cells during pathophysiological diseases when extracellular adenosine levels increase 10-100 fold above homeostatic conditions. Indeed, our work demonstrating the selective upregulation of the A2bR by LPS is consistent with other reports showing that inflammatory TNFα and hypoxia induce the A2bR [93, 105, 110], thus these data

collectively support a particular role for the A2bR in mediating a negative feedback loop to prevent overactive inflammation.

Despite our observations regarding adenosine receptor induction in a variety of inflammatory scenarios, how the A2bR is regulated at the transcriptional level remains poorly understood. Our current study expands our understanding of A2bR regulation under inflammatory conditions and shows for the first time that IFNγ directly inhibits the development of regulatory macrophages following TLR activation via inhibiting the transcription of A2bR. It is interesting that we observed increased *A2br* levels in macrophages exposed to LPS and ATP or adenosine, suggesting that adenosine signaling itself may positively regulate the expression of the A2bR. Hif1α has been implicated in the transcriptional regulation of the A2bR in endothelial cells under hypoxic conditions; however, we found that macrophages stimulated with LPS in the presence of a HIF1 stabilizer (DMOG)[111] did not enhance A2br levels (data not shown). Thus, the transcriptional regulation of the A2br may be differentially regulated depending on the tissue environment and the presence of TLR ligands. Interestingly, we found putative cAMP response element binding (CREB), γ-activated sequence (GAS), and specificity protein 1 (Sp1) binding sites in close proximity to the transcriptional start site of *A2br* (Figure 8). Both CREB and Sp1 have been shown to play roles in the induction of many regulatory macrophage-associated genes including, HB-EGF and IL-10 [7, 109]. Thus, it is interesting to speculate that the A2bR may be included as a novel genetic marker of immunosuppressive macrophages.

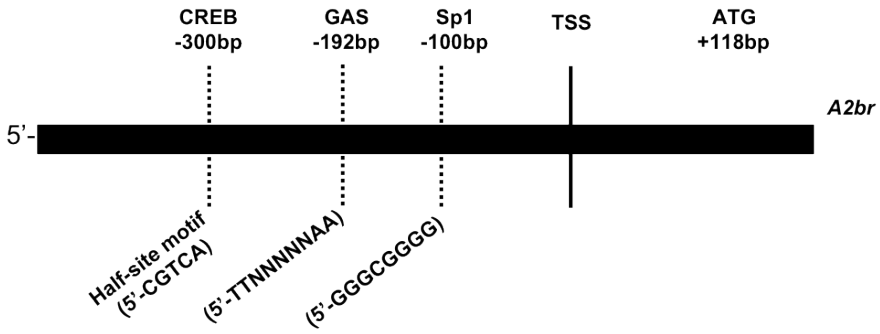

Figure 8. Schematic representation of transcription factor binding sites within the promoter region of mouse *A2br*. Location of putative transcription factor DNA binding sites was determined manually based on established consensus core sequences recognized by transcription factors. Of the major transcription factors implicated in macrophage responses, putative binding sites for CREB, GAS, and Sp1 were found within 300bp of the transcriptional start site (TSS).

This work suggests a therapeutic potential of over-expressing the A2bR in macrophages to circumvent IFNγ pro-inflammatory signaling. These data suggest for the first time that targeting the A2bR specifically on macrophages may represent a novel strategy to treat chronic inflammatory diseases. It will be valuable to assess the impact of macrophage-A2bR over-expression in vivo. High TNFα and IFNγ levels often accompany chronic inflammatory disorders[112]. Moreover, extracellular adenosine levels have been demonstrated to increase 100 fold during chronic inflammatory conditions such as rheumatoid arthritis[49]. Thus, our results showing that CD39 activity is intact in the presence of IFNγ, is consistent with these observations. Here, we show that IFNγ potentiates the release of these inflammatory cytokines from macrophages by blocking induction of the anti-inflammatory receptor, A2bR. In this study we demonstrate that preserving or over-expressing the A2bR in macrophages may rescue the anti-inflammatory phenotype of macrophages exposed to IFNγ. Thus targeting A2bRs on macrophages may enhance our current arsenal of treatment options for chronic inflammatory diseases.

Chapter 4: Exploitation of the adenosine 2a receptor promotes *L.major* survival within classically activated macrophages

From: Cohen, HB, and Mosser, DM. “Exploitation of the adenosine 2a receptor promotes *L.major* survival within macrophages”. *Manuscript in preparation for submission*. 2014.

Introduction

Cutaneous leishmaniasis is a parasitic disease that exemplifies the importance of properly regulated macrophage responses. Infected individuals often suffer from debilitating lesions at the infection site that arise when the body fails to clear parasites before repairing the infected tissue. The World Health Organization estimates that 1-2 million individuals annually contract Leishmaniasis and there are few treatment options available to alleviate the disease[34].

Individuals contract the disease when an infected sand fly takes a blood meal, whereby the promastigote form of *Leishmania major* (*L.major*) is transmitted from the sand fly into the blood of the host. Promastigotes are rapidly taken up by professional phagocytic cells, and transform into the intracellular amastigote form. Macrophages are the primary host cells in which these reside. Thus, understanding how macrophage anti-microbial activity is regulated during infection is key to enhancing our understanding of the pathogenesis of cutaneous Leishmaniasis and the development of more effective therapeutics for this disease.

Successful host defense against this intracellular parasite involves the generation of IFNγ. This results in the generation of classically activated macrophages, which are defined by their ability to kill this and other intracellular pathogens [1]. Classically activated macrophages (also known as M1) develop within environments rich in T_h1-associated cytokines such as IFNγ and upon sensing pathogenic molecules via toll-like receptors (TLRs). Upon IFNγ/TLR stimulation, macrophages produce high amounts of inflammatory cytokines

including TNFα and IL-12, which enhance the recruitment of immune cells to the site of infection to help clear the pathogen from the host. In addition, macrophages can produce nitric oxide (NO), which is toxic to intracellular pathogens. Alternatively, macrophages can also play an important role in maintaining tissue homeostasis by assuming a regulatory phenotype. The production of pro-inflammatory mediators and anti-microbial activity is reduced and the secretion of anti-inflammatory IL-10 is increased in regulatory macrophages. *L. major* infection persists in part due to the inability of macrophages to maintain an anti-microbial activation status, thus immunosuppressive macrophages ensue to the detriment of the host [35]. Despite the evidence to suggest roles for regulatory macrophages and IL-10 in this disease [9, 39], there are significant gaps in our understanding of how *L.major* inhibits classical macrophage activation and evades the host innate immune response.

Extracellular adenosine triphosphate (eATP) and its degradation product, adenosine, have recently emerged as important modulators of innate immune responses [53]. High levels of eATP (millimolar) acts as a 'danger signal' to warn the body of infection largely by activating the P2X7 receptor, which stimulates the coordination of an active NLRP3 inflammsome complex and subsequent secretion of IL-1β, which further potentiates neutrophil recruitment and warns the body of infection. Paradoxically, we recently revealed that low levels of eATP (sub-millimolar) can render macrophages immunosuppressive due to their inherent ability to readily generate anti-inflammatory adenosine [54]. Macrophages

are equipped with the ecto-enzymes necessary to convert eATP into adenosine as well as the adenosine receptors required to mediate the transition from an inflammatory to regulatory macrophage. Thus, the balance between eATP and adenosine appears to be an important regulator of the inflammatory process. Although, stimulating adenosine receptors has been shown to improve non-infectious inflammatory diseases and wound healing responses [53, 74, 90, 113], our understanding of how eATP hydrolysis and adenosine signaling is regulated during infection remains poorly understood. In this study, we demonstrate that adenosine potently inhibits nitric oxide production by macrophages during *L. major* infection and that these cells also exhibit enhanced susceptibility to intracellular parasite growth. We further show that during infection, macrophages preferentially upregulate the adenosine 2a receptor (A2aR) and that this adenosine receptor is the dominant adenosine receptor suppressing anti-microbial activity in macrophages. The results from this study suggest that macrophage-A2aR may be a promising new target to effectively treat Leishmaniasis.

Materials and Methods

Mice and macrophage isolation. Balb/c mice were purchased from the Charles River Laboratories. These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. Bone marrow-derived macrophages were prepared from 6-8 week old female Balb/c mice as previously described [75] and differentiated in 20% L929 (LC14) conditioned media unless otherwise noted.

Parasites. *L. major* (WHO MHOM/IL/80/Friedlin) were isolated from infected BALB/c mice and grown as previously described. [8] Briefly parasites were grown in medium containing equal parts Schneider's insect medium (Sigma-Aldrich, St. Louis, MO) and M-199 (Invitrogen, Rockville, MD) supplemented with 20% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM glutamine. Metacyclic promastigotes were obtained as previously described [114] and used for all killing experiments. Briefly, stationary phase parasites were washed three times with DPBS at 3500 RPM for 15 minutes. The pellet was resuspended in PBS and counted via hemacytometer.

Reagents. ATP, adenosine, and *N*6,2-*O*-dibutyryladenosine 3,5-cyclic adenosine monophosphate (dbcAMP) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IFNγ and IL-1β was purchased from R&D systems (Minneapolis, MN) and reconstituted according to the manufacture's directions. Ultra Pure LPS *E. coli* K12 were purchased from InvivoGen (San Diego, CA).

POM-1 and CGS21680 were purchased from Tocris Bioscience (R&D Systems; Minneapolis, MN).

Stimulation conditions. All in vitro experiments were performed at 1-2x10^6 cells/mL in tissue culture treated plates. Mouse macrophages studies were performed in DMEM/F12+GlutaMax (Gibco, Life Technologies;, Grand Island, NY) supplemented with 10% FBS, 1% pen/strep, and 1% glutamine (Gibco, Life Technologies;, Grand Island, NY) unless otherwise indicated. For all experiments, macrophages were primed overnight with 100U/mL IFNγ followed by addition of 10ng/mL Ultra Pure LPS with or without 100μM ATP or adenosine unless indicated otherwise. All *L.major* infections were performed on glass coverslips within wells of a 48-well culture plate at a multiplicity of infection (MOI) of 5:1 in the presence of 3% C5d-deficient serum. Infected cells were cultured for 2 hours at 37ºC+5%CO_2, washed thoroughly to remove any unbound parasites and then re-cultured in the absence of free parasites for 24-48 hours.

Gene Expression Analysis. Stimulations were performed in 1mL. Total RNA was isolated by using TriZol (Invitrogen, Life Technologies;, Grand Island, NY) and converted to cDNA using the ThermoScript Kit (Invitrogen, , Life Technologies) according to the manufacture's protocol. qPCR analysis was performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics Corporation-Roche Applied Science;, Indianapolis, IN) and GoTaq qPCR Master Mix (Promega;, Madison, WI). Primer pairs used to amplify specific gene products are listed in

Table 1. Relative expression levels were calculated using the ΔΔCt method [6]. *Gapdh* was used as the housekeeping gene for normalization.

Flow Cytometry Antibodies and Analysis. Anti-mouse CD16/32 was purchased from AnaSpec (Freemont, CA). Anti-mouse CD39-AF647 (24DMS1) was purchased from eBioscience (San Diego, CA). Anti-mouse F4/80-pacific blue (BM8) was purchased from BioLegend (San Diego, CA). Anti-mouse CD73-PE was purchased from BD Biosciences (San Jose, CA). Samples were analyzed on a BD FACSCanto (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Inorganic Phosphate Release Assay. Adenine nucleotide hydrolysis was detected using the BIOMOL Green Reagent (Enzo Life Sciences; Farmingdale, NY) according to the manufacture's protocol.

Microscopy and Immunofluorescence. To stain parasites by immunofluorescence, 5% anti-*L. major* immune serum from BALB/c mice was added to the monolayers for 20 min followed by FITC goat anti-mouse IgG (eBioscience) for 30 min. The nuclei were counterstained for 1 min with DAPI. Slides were examined using a Carl Zeiss Inverted Fluorescence Microscope (Axio Observer Inverted Microscope, Carl Zeiss SMT AG, Germany). Acquired images were used to count the number of parasites per cells and the number of infected cells.

Nitrite Detection Assay. NO production was estimated from the accumulation of NO_2^- in the medium after 24 h of macrophage activation using the Greiss reagent, as described previously [6]. Briefly, equal volumes of culture supernatant and Greiss reagent were mixed for 10 min at room temperature. Absorbance at 540nm was measured with a Labsystems Multiscan Ascent assay plate reader. A solution of Na NO_2^- was used to construct a standard curve.

Statistical Analysis. Data analysis was performed using GraphPad Prism software (GraphPad Software Inc.; La Jolla, CA) and analyzed using the Student's *t* test. The statistical differences between groups, with the p-values are indicated in the related graphs as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

eATP-induced IL-1β minimally influences macrophage NO production

The role of eATP on macrophages during infectious disease is elusive and further complicated due to its differing role depending on its level of bioavailability. Inflammatory macrophages that briefly encounter high levels of ATP (>1mM) secrete elevated amounts of IL-1β (Figure 1A). We recently demonstrated that macrophages highly express the ecto-ATPase, CD39, and can rapidly hydrolyze eATP [54]. Indeed, exposing inflammatory macrophages to eATP in the presence of the CD39-specific inhibitor, POM-1, permitted the production of IL-1β at sub-millimolar concentrations of eATP (Figure 1B). These data support previous reports suggesting a role for CD39 in the control of P2X7-induced IL-1β responses [64]. The role of IL-1β in promoting inflammation, neutrophil recruitment and T_h17 differentiation is well-documented [61, 115]; however the role of eATP-induced IL-1β in regulating macrophage-intrinsic host defense mechanisms is unclear.

The production of reactive nitrogen species (RNS) is toxic to intracellular pathogens and plays an integral part in limiting parasite survival within macrophages. To investigate whether IL-1β affects NO production, we performed a Griess assay on supernatants collected from macrophages primed with IFNγ overnight, followed by a 24-hour stimulation with LPS in the absence or presence of rIL-1β. We observed a significant, but minimal effect of rIL-1β on nitrite production compared to IFNγ treatment alone; however rIL-1β did not further

enhance NO production by macrophages stimulated in the presence of LPS (Figure 1C). Moreover, the ability for macrophages to control intracellular *L.major* was unchanged in the presence of rIL-1β (Figure 1D), thus suggesting that ATP-induced IL-1β does not act in an autocrine manner to further modulate anti-microbial responses in macrophages. Thus, high levels of eATP may play a more impactful role orchestrating cellular immunity than regulating macrophage-intrsinic anti-microbial programs. Together, these observations further strengthen previous work indicating that under normal conditions, macrophages are equipped to readily metabolize eATP to limit inflammation [54]. However, whether low levels of eATP contribute to the susceptibility of macrophages to intracellular pathogens have not been thoroughly explored.

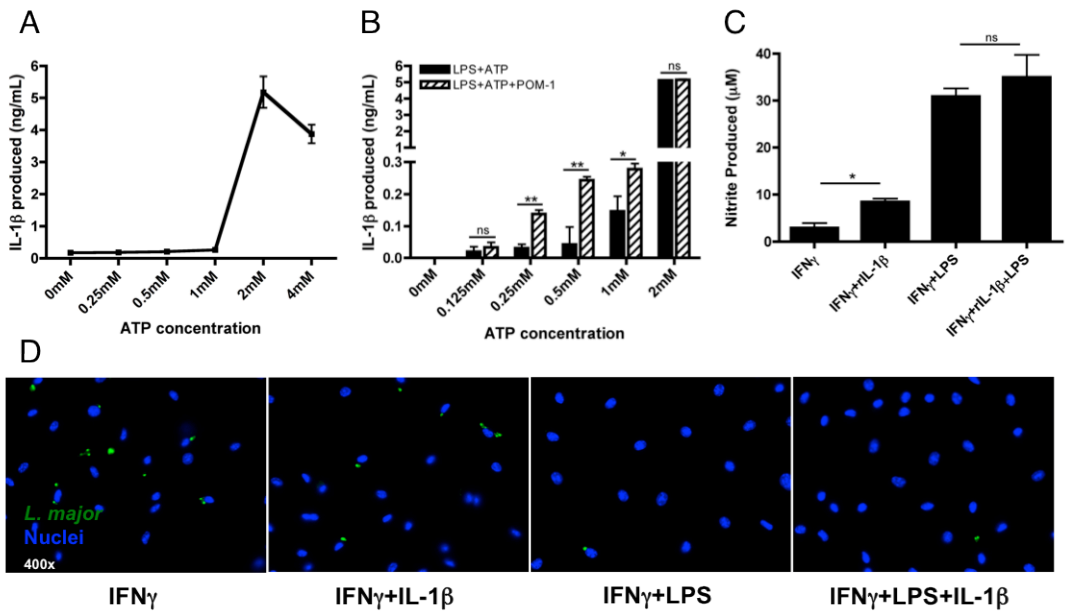

Figure 1. eATP-induced IL-1β minimally influences macrophage NO production. (A,B) LPS primed BMDMs were stimulated for with various concentrations of ATP in the absence (A) or presence (B) of 10μM POM-1. IL-1β production was detected by ELISA. The means +/- SD of triplicates are shown and are representative of 3 independent experiments. **(C)** BMDMs were primed with IFNγ alone or stimulated with 5ng/mL rIL-1β and/or LPS for 24 hrs. Nitrite production was determined by Griess assay. The means +/- SD of triplicates are shown and are representative of 2 independent experiments. **(D)** BMDMs were primed with IFNγ alone or stimulated with 5ng/mL rIL-1β and/or LPS at the time of *L. major* infection. Representative images are shown of parasites (green) within macrophages (blue) 48 hrs post infection.

CD39 activity attenuates nitric oxide production by macrophages

We next examined the potential for IFNγ/LPS-stimulated macrophages to secrete nitrite in the presence of low levels of eATP (100μM) by Griess assay. We observed ~40% decrease in nitrite production from macrophages exposed to eATP compared to IFNγ/LPS-stimulated macrophages alone (Figure 2A). Moreover, this attenuation was completely reversed in the presence of POM-1 suggesting that CD39-mediated eATP hydrolysis negatively influences NO production by macrophages. To investigate the functional consequence of this, we primed macrophages with IFNγ overnight and then exposed macrophages to LPS and *L.major* in the presence or absence of 100μM ATP for 48 hours. The survival of intracellular parasites positively correlated with the NO data indicating that inhibition of NO by eATP can prevent effective anti-microbial responses in macrophages. Importantly, the ability for inflammatory macrophages to kill *L.major* was restored in the presence of POM-1 (Figure 2B). Together, these data suggest that in addition to its role in limiting inflammatory cytokine production, CD39-mediated ATP hydrolysis promotes *L.major* survival within macrophages by blocking NO production.

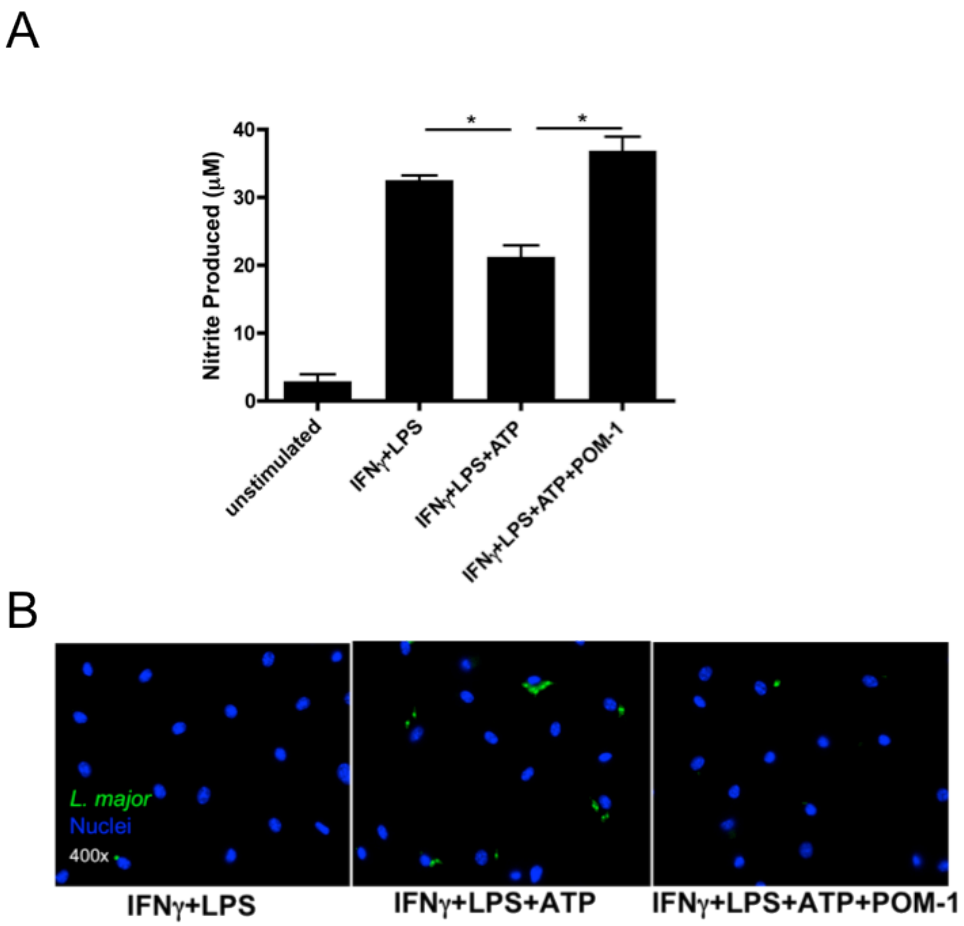

Figure 2. CD39 activity inhibits NO production and enhances *L.major* survival in macrophages. **(A)** BMDMs were primed with IFNγ and stimulated with LPS in the absence or presence of 100μM ATP and/or 10μM POM-1 for 24 hrs. Nitrite production was determined by Griess assay. The means +/- SD of triplicates are shown and are representative of 2 independent experiments. **(B)** BMDMs were primed with IFNγ and stimulated with LPS in the presence or absence of 100μM ATP and/or 10μM POM-1 at the time of *L.major* infection. Representative images are shown of parasites (green) within macrophages (blue) 48 hrs post infection.

Macrophage ecto-enzyme activity is intact during infection

Leishmania are obligate intracellular pathogens that lack the ability to synthesize adenosine de novo. Therefore, the parasite must acquire adenosine from the mammalian environment for its survival. Therefore, we investigated whether *L.major* modulated the ability of macrophages to convert eATP into adenosine by stimulating macrophages with IFNγ/LPS in the absence or presence of *L.major.* eATP hydrolysis is controlled by CD39, which dephosphorylates ATP and ADP into AMP and CD73 is an AMP-specific 5'-ectonucleotidase that removes the phosphate group to yield adenosine. At 24 hours post infection, we evaluated CD39 and CD73 expression and activity to investigate whether *L.major* modulated the macrophage-mediated eATP hydrolysis. Macrophage CD39 expression and activity remained unchanged (Figure 3A,B). Similarly, CD73 activity was not affected by infection, despite the slight downregulation of CD73 expressed on the surface of macrophages during infection (Figure 3B,C). *L.major* infection resulted in no significant modulation in eATPase or eAMPase activity (Figure 3B,D). Together, these data indicate that although CD39-mediated ATP hydrolysis renders macrophages susceptible to *L.major* infection, CD39 and CD73 are not specifically targeted by the parasite as a means to evade host anti-microbial responses.

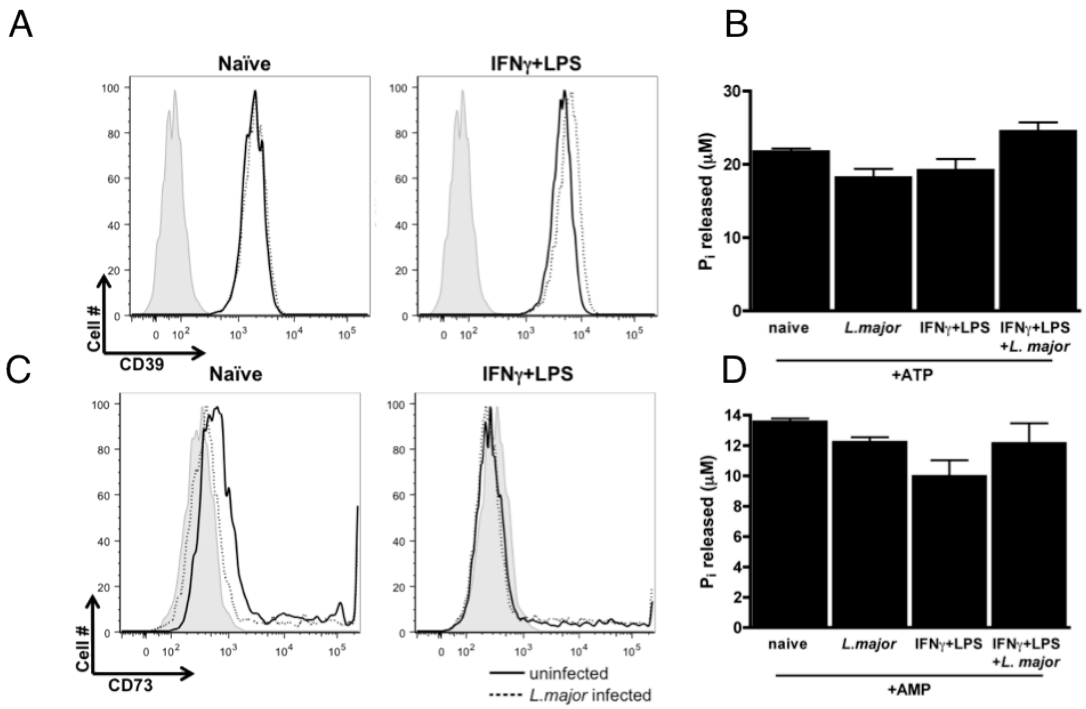

Figure 3. Macrophage CD39 and CD73 activity are intact during *L.major* infection. (A,C) Analysis of CD39 (A) and CD73 (C) surface expression on naïve (left) and IFNγ/LPS treated BMDMS (right) in the absence (solid black histograms) or presence of *L.major* (dotted black histograms) compared to isotype controls (gray histograms). Data are representative of 2 experiments. **(B,D)** The hydrolysis of 500μM ATP (B) or AMP (D) by BMDMs in treated in the presence or absence of IFNγ/LPS and/or during *L.major* infection. Hydrolysis was measured 1 hr after exposure to extracellular nucleotides and was measured by the inorganic phosphate (P_i) release assay. The mean +/-SD from triplicate determinations is shown and is representative of 2 independent experiments.

Extracellular adenosine inhibits NO production in classically activated macrophages

Having shown that CD39-mediated eATP hydrolysis renders macrophages susceptible to *L.major* infection without any substantial change in CD39 expression or activity during infection, we then hypothesized that *L.major* may alter the sensitivity of macrophages to respond to ATP-derived adenosine. We previously identified that adenosine was the major metabolite generated from ATP hydrolysis, which mediated the immunosuppressive effects observed from inflammatory macrophages exposed to low levels of eATP [54]. To demonstrate whether adenosine affects NO production, we stimulated IFNγ primed macrophages with LPS and increasing concentrations of adenosine. Indeed, adenosine inhibited nitrite production in a dose-dependent manner (Figure 4A). Interestingly, adenosine decreased nitrite levels by ~40%, thus recapitulating results observed with inflammatory macrophages exposed to ATP (Figure 2). IFNγ primes macrophages to effectively thwart intracellular pathogens and upon TLR stimulation are subsequently able to produce copious amount of NO. Indeed, macrophages treated with IFNγ/LPS effectively kill intracellular parasites compared to naïve macrophages as evidenced by the lack of parasites found within these macrophages 48 hours post infection (Figure 4B,C). However, anti-microbial activity by IFNγ/LPS treated macrophages was largely blocked by the addition of extracellular adenosine at the time of infection (Figure 4B,C). These results suggest that extracellular adenosine promotes *L.major* intracellular growth by inhibiting NO production in macrophages.

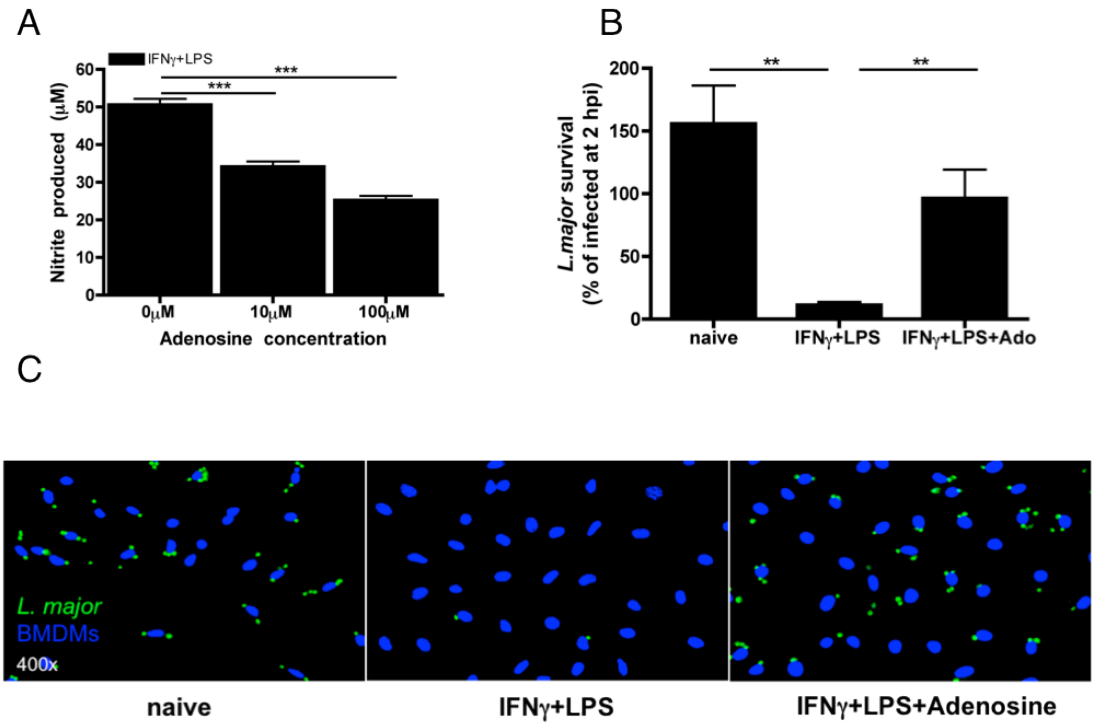

Figure 4. Extracellular adenosine inhibits NO production in classically activated macrophages. (A) BMDMs were primed with IFNγ and stimulated with LPS in the absence or presence of various adenosine concentrations for 24 hrs. Nitrite production was determined by Griess assay. The means +/- SD of triplicates are shown and are representative of 2 independent experiments. **(B,C)** BMDMs were infected with *L.major* in the absence (naïve) or presence of IFNγ/LPS +/- 100μM adenosine. 48 hrs later, parasite survival was quantified within 100 BMDMs via fluorescence microscopy (B). The means +/- SD of triplicates are shown and are representative of 2 independent experiments. Representative images are shown of parasites (green) within macrophages (blue) (C).

A2aR expression is upregulated during L.major *infection in macrophages*

There are four known adenosine receptors: A1R, A2aR, A2bR, and A3R. All of which can be expressed by macrophages, with the A2aR being most highly expressed on naïve cells (Figure 5A). While it has been demonstrated that inflammatory stimuli such as LPS and TNFα preferentially upregulates the A2bR to enhance sensitivity to immunosuppressive adenosine [54, 93], it remains unknown whether the expression of these receptors are influenced during parasitic infection. In fact, *L.major* significantly enhanced the expression of the *A2ar*, *A2br* and *A3r* transcripts compared to naïve, uninfected cells (Figure 5B). It is interesting to note, *L.major* alone was able to enhance expression of the *A2br*. However, *A2br* levels were diminished in infected macrophages in the presence of IFNγ (Figure 5C). Surprisingly, however, the upregulation of the A2aR by *L.major* remained elevated even in the presence of IFNγ (Figure 5C). Similar results were observed at the protein level such that macrophages infected with *L.major* expressed greater levels of the A2aR on their surface (Figure 5D). Thus, these results suggest that in the presence of *L.major*, macrophages selectively augment adenosine receptor expression to enhance sensitivity to available adenosine within the local extracellular environment.

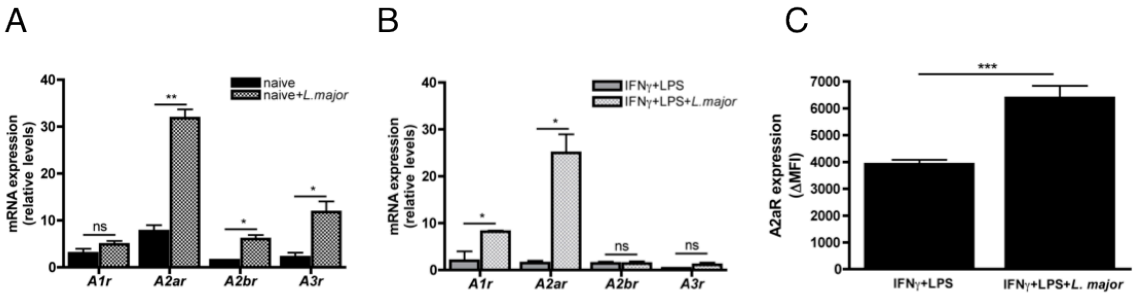

Figure 5. *L.major* upregulates A2aR expression in macrophages. (A,B) Adenosine receptor mRNA expression in naïve (A) and IFNγ/LPS (B) stimulated BMDMs in the absence (dark bars) or presence (light bars) of *L.major* at 24 hrs post-infection. Transcript levels were determined by qPCR and normalized to *Gapdh* expression for each condition. The means +/- SD of triplicates are shown and are representative of 3 independent experiments. **(C)** Analysis of A2aR surface expression on IFNγ/LPS stimulated cells alone or infected with *L.major*. 48 hrs post stimulation, A2aR levels were measured by FACS on F4/80+ cells. The means +/-SD are shown and are representative of 3 independent experiments.

A2aR signaling attenuates NO production in activated macrophages

All adenosine receptors are G-protein coupled receptors (GPCRs); however only the A2aR and A2bRs are coupled to the Gαs and capable of increasing intracellular cAMP levels. We previously showed that macrophages exposed to cAMP are skewed towards a regulatory activation status, thus suggesting that cAMP contributes to the attenuation of classical macrophage responses [7]. We also observed that cAMP inhibited nitrite production from classically activated macrophages to a similar extent as adenosine (Figure 6A). To investigate the role of A2aR-dependent signaling on NO production, we infected macrophages in the presence or absence of the A2aR-specific agonist, CGS21680. CGS21680 inhibited nitrite production by infected IFNγ/LPS treated macrophages by 40-50% (Figure 6B). We next addressed the functional consequence of A2aR stimulation during *L.major* infection in macrophages. Indeed, CGS21680 promoted the survival of intracellular parasites compared to infected IFNγ/LPS-treated macrophages cultured in the absence of adenosine or CGS21680 (Figure C,D). Moreover, we saw that CGS21680 completely recapitulated results with adenosine with regard to both inhibition of NO and parasite survival (Figures 6B-D), thereby suggesting that the A2aR is the dominant adenosine receptor that inhibits nitric oxide production upon stimulation. All together, we demonstrate that the A2aR is preferentially upregulated in macrophages during *L.major* infection and that the stimulation of the A2aR by low levels of ATP/adenosine inhibits the ability of macrophages to effectively clear Leishmania infection.

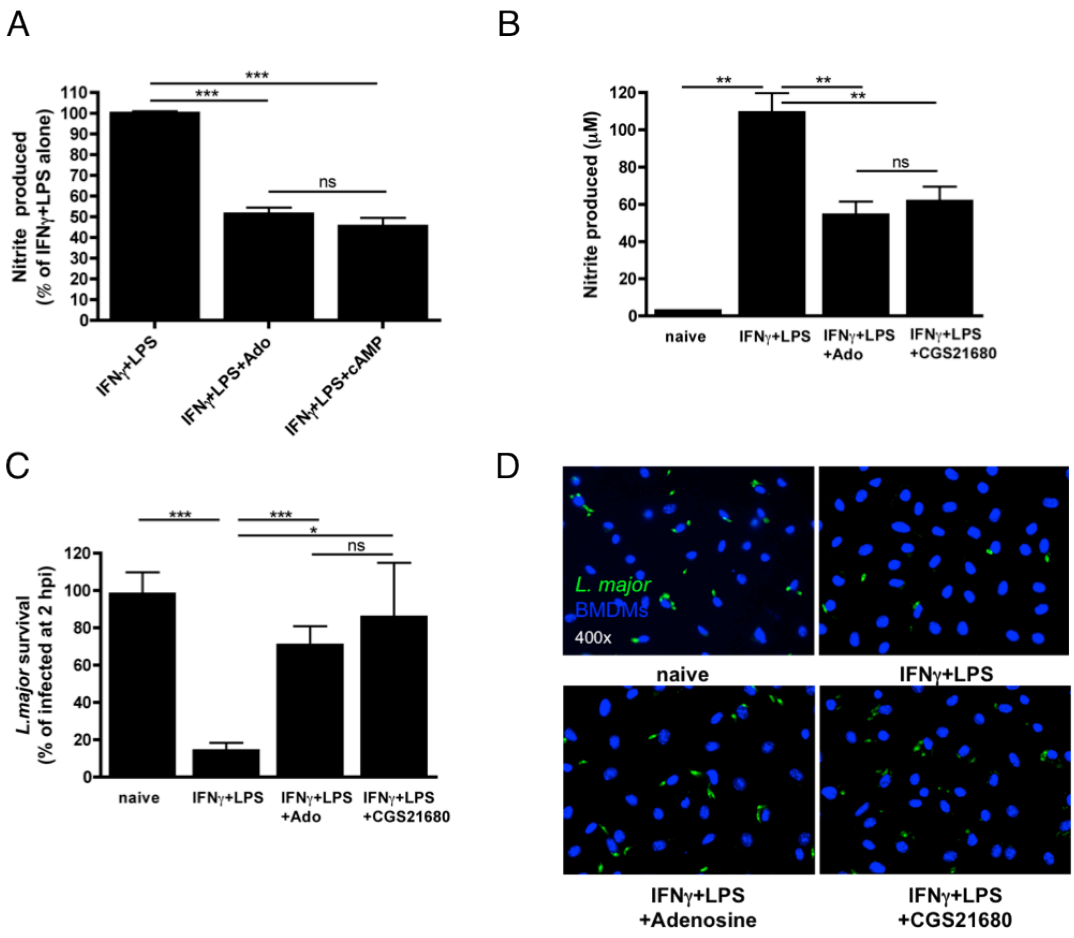

Figure 6. A2aR signaling attenuates NO production in activated macrophages. (A) BMDMs were primed with IFNγ and stimulated with LPS in the absence or presence of 100μM adenosine or dbcAMP for 24 hrs. Nitrite production was determined by Griess assay. The means +/- SD of triplicates are shown and are representative of 2 independent experiments. **(B-D)** BMDMs were infected with *L.major* in the absence (naïve) or presence of IFNγ/LPS +/- 100μM adenosine or 10μM CGS21680. 48 hrs later, nitrite production (B) and parasite survival was quantified within 100 BMDMs via fluorescence microscopy (C). The means +/- SD of triplicates are shown and are representative of 2 independent experiments. Representative images are shown of parasites (green) within macrophages (blue) (D).

Discussion

Leishmaniasis is a potentially life-threatening disease that is caused by parasites that preferentially infect tissue-resident macrophages. In immunocompetent humans and mice, the immune response eventually polarizes and a dominant cell-mediated response emerges to ultimately clear the infection. In contrast, susceptible individuals develop non-healing lesions at the site infection occur in response to the maintenance of T_h2 conditions. While it has been shown that IgG-opsonized parasites promote the T_h2 environment to the detriment of the host via FcR ligation [8, 9, 39], it remains elusive what factors present in tissue early in infection render macrophage susceptible to parasites, regardless of the production of IFNγ. Here, we reveal that extracellular ATP can inhibit NO production in classically activated macrophages via conversion to adenosine, which subsequently sustains survival of *L.major* parasites within macrophages. Specifically, this study provides exciting new insight regarding the roles of the adenosine receptors on macrophages including 1) the A2aR is the major adenosine receptor responsible for the inhibition of nitric oxide in macrophages, 2) A2aR-dependent signaling promotes intracellular survival of pathogenic parasites and 3) *L.major* itself may specifically target the upregulation of the A2aR to evade innate host defenses.

These results are consistent with reports indicating that excessive adenosine levels present in serum and tissue are a hallmark of individuals deficient in adenosine deaminase (ADA), which represents nearly 10% of total severe combined immunodeficiency disease (SCID) patients and are highly

susceptible to a variety of microbial infections [50]. In addition, it has been proposed that high extracellular adenosine concentrations present in newborn serum may play a role in the inefficient clearance of microbial infections in newborns and children, compared to adults [51]. However, while it has been known that adenosine can interfere with host defenses, the specific contribution of each adenosine receptor has remained unknown.

The A2Rs, A2aR and A2bR, exhibit great immunomodulatory activity and are the only adenosine receptors coupled to the Gαs subunit. Much of their anti-inflammatory activity is attributed to the induction of cAMP and blockade of NFκB pathways [116]. Although, the anti-inflammatory potential of adenosine via A2Rs is well documented, the role of adenosine-mediated signaling in regulating anti-microbial responses remains elusive. Recently, Alfonso and colleagues have demonstrated that administration of adenosine at the time of infection with Leishmania parasites decreases the size of the lesion at the site of infection in mice and that these results were reversed upon addition of the A2bR-specific antagonist, MRS1754 suggesting that A2bR signaling may be important for mediating the severity of the lesion [117]. Importantly, the modulation in lesion size appears to be predominantly due to changes in the number of immune cells infiltrating to the site of infection. However, it is important to note that administration of adenosine receptor antagonists in vivo will negatively affect adenosine receptors on every cell type and that the A2bR is expressed on immune cells and structural cells including smooth muscle cells and endothelial cells [74]. Indeed, A2bR-deficient mice exhibit decreased leukocyte migration on

account of increased integrin expression and binding [74]. Therefore, it will be important to develop macrophage-specific adenosine receptor-deficient mice to address the specific contribution of adenosine signaling on macrophages in vivo. Moreover, it was demonstrated that blockade of the A2bR improves polymicrobial sepsis survival in mice. This effect was largely attributed enhancement of phagocytosis capacity, thus limiting the severity of bacteremia [95]. Although these results highlight a role for the A2bR in mediating phagocytosis and inflammatory cell recruitment, the study presented here is the first to identify a role for the A2aR in mediating host defense. Moreover, our study indicates that pathogens may specifically target the upregulation of the A2aR as a virulence mechanism to evade macrophage killing. However, it remains unclear how the expression of A2Rs is regulated, thus it will be interesting to further investigate the molecular mechanisms governing transcriptional induction of *A2ar* by *L.major*.

Interestingly, a growing list of pathogens has been shown to modulate the host purinergic signaling system. *Myobacterium tuberculosis*, for example, enhances ATP secretion from infected macrophages leading to the P2X7/NLRP3-inflammasome activation[118]. While we did see a marginal improvement of NO production with the addition of rIL-1β to IFNγ treatment, compared to IFNγ treatment alone, we did not observe a significant increase in NO or decrease in *L.major* survival in the presence of LPS, suggesting that autocrine IL-1β signaling overlaps considerably with LPS-induced signaling. This is likely due to common intracellular domains and association with MyD88. Importantly, the level of ATP required to elicit IL-1β release is relatively high and

under normal conditions, can only be achieved with >1mM eATP. While such levels of eATP have yet to be discovered in vivo, it is estimated that 1-5mM ATP resides within healthy cells. Thus, under pathological conditions in which cells lose their membrane integrity due to stress, damage, or death, intracellular stores of ATP can be released into the extracellular space. As such, it is conceivable that this IL-1β-dependent control of parasites may be enlisted later in the disease when the infected tissue becomes necrotic. Additionally, ATP release and IL-1β may further augment tissue destruction causing greater harm to the host.

We recently discovered that TLR ligands induce the release of ATP from macrophages; however the level of ATP released under these conditions is relatively low (nano-micromolar range). Subsequently, macrophages rapidly hydrolyze this self-derived eATP into adenosine, thus representing an autonomous regulatory mechanism to control TLR-induced inflammatory responses [54]. It will be interesting to assess whether *L.major* induce a similar mechanism upon infecting macrophages, whereby low levels of ATP are released to enhance their pathogenicity. Indeed, *Shigella flexneri* has been shown to actively induce ATP release from host epithelial cells leading to increased dissemination [119] and recently low levels of ATP released form HIV-infected cells was shown to be important in enhancing envelope fusion and viral entry into the targeted T cells [120]. Therefore, our data support the notion that host-derived eATP may be a virulence factor utilized by pathogens. Collectively, the results from this study illustrate a novel A2aR-dependent mechanism

employed during *L.major* infection that suppresses macrophage anti-microbial responses (Figure 7).

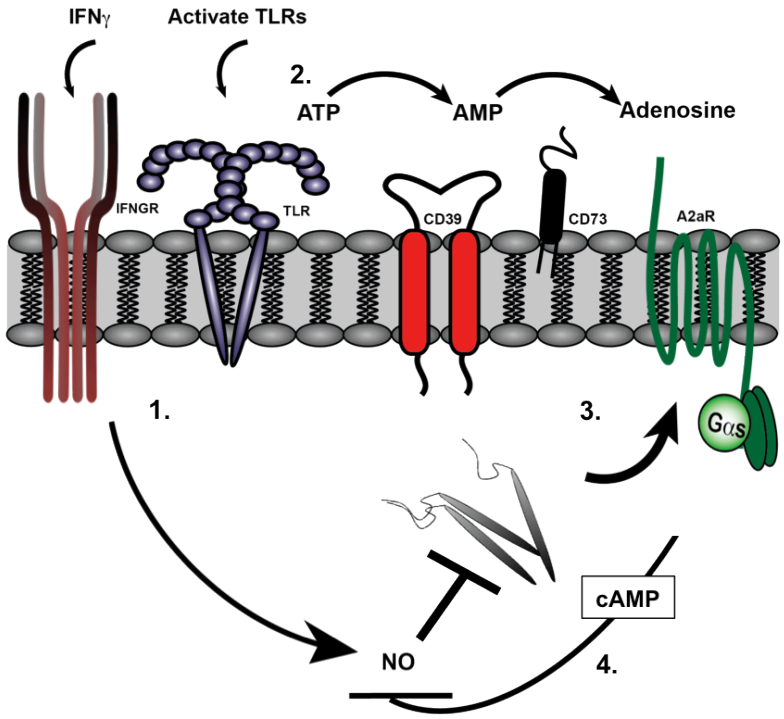

Figure 7. A model illustrating a novel A2aR-dependent immune evasion mechanism elicited by *L.major*. In response to IFNγ/LPS stimulation, macrophages exhibit a classical activation state hallmarked by the ability to produce high levels of nitric oxide. **(1.)** Thus, upon infection, classically activated macrophages readily kill intracellular pathogens such as *L.major*. **(2.)** However, extracellular ATP levels likely rise at the site of infection due to immune cell activation and stress. **(3.)** The results of this study reveal that while CD39 (red) and CD73 (black) activity are intact, macrophages upregulate the A2aR (blue) in response to infection. **(4.)** Upon A2aR stimulation, IFNγ/LPS-induced NO decreases likely through a cAMP-dependent pathway. Thus, in the presence of low levels of eATP/adenosine, classically activated macrophages are rendered susceptible to *L.major* infection.

Chapter 5: Conclusions and Final Thoughts

In the clinic and in animal models of inflammatory and infectious diseases, the switch between macrophage activation states has been well documented. However, the signals that direct macrophage plasticity remained enigmatic. The major discovery of the work presented here, is the identification and characterization of a complex macrophage-intrinsic regulatory mechanism that is initiated early in response to inflammatory stimuli and directs the transition to an anti-inflammatory, regulatory activation state. These observations suggest that inflammatory macrophage activation is inherently transient and that a switch in macrophage activation states may be critical to maintaining homeostasis. Results collected throughout this study challenge our understanding of regulatory macrophage development and thus illustrates a new model of macrophage activation such that macrophages, themselves, are capable of controlling their own activation state. They do so by releasing and converting ATP and by increasing adenosine (A2) receptor expression and sensitivity to newly generated adenosine within their local microenvironment.

[*] The novelty of the work presented in Chapter 2 was the demonstration that macrophages actively secrete low levels of ATP into their local extracellular environment upon sensing pathogen-associated molecules. While, it has been known that unlike most mammalian cells, tissue macrophages are primarily dependent on glycolysis for cellular metabolism [65], we revealed for the first time that following toll-like receptor (TLR) ligation, macrophages actively release a

[*] Paragraph adapted from: Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. *J Leukoc Biol.* Nov 2013;94(5):913-919. [11]

fraction of glycolysis-generated ATP into the extracellular milieu via pannexin-1 channels [121]. We then monitored the fate of macrophage-derived eATP and found that macrophages rapidly hydrolyze eATP by employing the ecto-nucleoside triphosphate diphosphohydralse (ENTPDase1/CD39). Within hours of stimulation, macrophages completely convert released eATP into adenosine. We further show that low (sub-millimolar) levels of eATP drive a switch in macrophage polarization from an inflammatory to a regulatory activation status. In the time it takes to generate, release, and hydrolyze ATP, TLR-activated macrophages are simultaneously undergoing a dramatic transcriptional reprogramming, culminating in the development of a regulatory activation status. CD39-mediated generation of adenosine from eATP was integral to this transition, as it is required to initiate the hydrolysis of eATP into eAMP, which then is selectively dephosphorylated by the ecto-AMPase, CD73 to yield adenosine.

[*] We then observed that adenosine, derived from eATP potently suppressed TNFα and IL-12 synthesis, while simultaneously enhancing the expression IL-10, the hallmark of regulatory macrophage activation. Therefore, we also provided a detailed analysis of the genetic signature of ATP/adenosine-treated macrophages. Importantly, we observed that in addition to IL-10, ATP-derived adenosine also upregulated an array of immunomodulatory molecules including heparin-binding epidermal growth factor (HB-EGF), sphingosine kinase (Sphk1), and IL-33 [1, 6, 72]. Interestingly, no upregulation of Fizz1, Ym1, or

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Mannose Receptor was observed indicating that ATP/adenosine selectively promote the development of regulatory macrophages and are not representative of IL-4-induced alternatively activated macrophages (AAMs). Arg1 was also upregulated on these macrophages. Although, Arg1 has historically been thought of as a distinct AAM marker, it has recently been demonstrated that Arg1 can be induced via IL-4/STAT6-independent mechanisms including via STAT3 [98] and GPCR/cAMP pathways [99, 100], thus indicating that Arg1 may not be a stable, reliable marker of AAMs, and thus may be expressed more broadly by macrophages. The biomarker signature of TLR-stimulated macrophages in the presence of low levels of ATP/adenosine is therefore consistent with the phenotype associated with regulatory macrophages.

[*] CD39 expressed on macrophages was integral in this reprogramming process, and macrophages from mice lacking CD39 were unable to transition to a regulatory phenotype. To demonstrate the biological relevance of these observations, we examined a mouse model of septic shock and demonstrated that the transfer of CD39-deficient macrophages into wild-type mice challenged with a sub-lethal dose of endotoxin conferred lethality (<25% survival). This was in stark contrast to mice receiving wild-type ($CD39^{+/+}$) macrophages, which largely survived (>75%) the challenge. Furthermore, macrophages isolated from mice receiving wild-type macrophages exhibited elevated levels of IL-10, Arg1, HB-EGF, and IL-33 and low levels of TNFα and IL-12, whereas macrophages from mice that received CD39-deficient macrophages continued to exhibit an

[*] Paragraph adapted from: Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. *J Leukoc Biol.* Nov 2013;94(5):913-919. [11]

inflammatory phenotype. This was the first study to specifically address the role of CD39 on macrophages in vivo. This observation supports the theory that the development of regulatory macrophages is associated with increased survival of the host.

We additionally extended these observations to human monocyte and macrophages. Human blood-derived monocytes and monocyte-derived macrophages exhibited a similar regulatory profile in the presence of low levels of ATP/adenosine and also express high levels of CD39, suggesting that the natural ability to switch activation states in response to TLR activation is conserved among these species. Monocytes and macrophages play vital roles in the progression of inflammation and their inability to properly transition to a regulatory activation status to aid in the resolution of inflammatory responses often lead to severe tissue damage, organ failure and even death. While, the literature regarding the role of CD39 in regulating inflammatory diseases such as diabetes, obesity, and cancer is growing[122-125], the results presented here are the first to demonstrate the role of CD39 specifically on macrophages during inflammation. It will be important to develop macrophage-specific CD39-deficient mice to further interrogate the role of CD39 on macrophages during these diseases. In addition, genome wide association studies (GWAS) within the human population should be performed to assess the potential correlation between CD39 and disease susceptibility or resistance. Thus, results from this study emphasize the potential of macrophage-CD39 as a currently un-tapped

target for the development of more effective therapeutics for inflammatory disease.

Throughout the course of this study, we also better characterized the mechanism by which ATP-derived adenosine promotes regulatory macrophage activation. Interestingly, we observed that TLR-activated macrophages selectively increase their expression of the adenosine 2b receptor (A2bR). Thus, these data indicate that upon activation, macrophages not only release and hydrolyze self-derived ATP, but also actively upregulate the A2bR to enhance their sensitivity to extracellular adenosine accumulating in their local extracellular environment. We also reveal that in response to TLR activation, macrophages primarily employ the A2bR to self-regulate the production of inflammatory cytokines, thereby completing their CD39-initiated auto-regulatory circuit. Interestingly, in endothelial and aortic smooth muscle cells, CD39 and A2bR co-associate with caveolae, suggesting that these molecules are strategically localized near each other on the surface of cells [126]. It will be interesting to examine whether this is true for macrophages, thus supporting the hypothesis that A2bRs reside in close proximity to CD39 to readily respond to newly generated macrophage-derived adenosine. Macrophages isolated from A2bR-deficient mice expressed and secreted higher levels of TNFα and IL-12 compared to their wild-type counterparts and exhibited substantial delays in the transition into a regulatory phenotype. However, A2bR-deficient macrophages showed no defect in IL-10 production, thus indicating that the A2bR is not the major adenosine receptor mediating ATP/adenosine-induced IL-10. Indeed, this

is consistent with previous work demonstrating that macrophages exposed to LPS or *E.coli* preferentially secrete IL-10 via an A2aR-dependent mechanism [96]. Therefore, we identified a novel negative feedback mechanism controlling inflammatory cytokine production by macrophages that relies on the expression of the A2bR following TLR stimulation.

Functional plasticity is at the root of mounting an effective macrophage response to immunological threats without causing severe detriment to the host. While we showed that TLR activation normally results in a transient state of activation, IFNγ is a well-known sustainer of macrophage inflammatory responses, thus presenting us with a paradoxical situation: how can chronic inflammation persist in spite of self-limiting mechanisms that are mobilized to prevent overactive macrophage responses? In Chapter 3, we propose an underlying mechanism that may answer this question, wherein IFNγ blocks the induction of TLR-induced A2bR expression leading to the chronic production of high levels of TNFα and IL-12.

Similarly, it remains unknown the factors involved in regulating A2bR expression. Our results show that naïve macrophages express low levels of the A2bR and that LPS-induced A2bR is temporally expressed, peaking at between 2-8 hrs post stimulation. Furthermore, the inhibition of A2bR expression by IFNγ persists throughout the exposure to LPS, indicating that IFNγ may interfere at the transcriptional level. Future studies should be done to investigate whether IFNγ inhibits A2bR expression and signaling in human macrophages as well to establish that this phenomenon is central to the maintenance of hyperactive

macrophages across species. It is important to note that although Hif1α has been shown to drive hypoxia-induced A2bR expression in human endothelial cells [110], our study indicates that it does not appear to be involved in mediating the induction of *A2br* by LPS in macrophages. Thus, A2bR expression may be differentially regulated depending on the cell type and tissue environment. We further showed Sp1 and CREB may have a role in regulating *A2br* levels. These transcription factors have also been implicated in the regulation of other regulatory macrophage associated genes including IL-10 and HB-EGF [7, 109], therefore it is compelling to speculate that A2bR expression may be part of the regulatory macrophage gene signature and be used as a potential diagnostic tool to track the presence of macrophages transitioning into a regulatory activation state. We are currently investigating the relevance of each of these binding sites in macrophage-A2bR expression upon TLR activation. It is tempting to assume that IFNγ prevents the induction of the A2bR by blocking the activation of an LPS-induced transcription factor responsible for increasing A2bR expression. However, IFNγ stimulation is a strong activator of STAT1, which itself can bind DNA and regulate transcription [26]. Indeed, we also discovered a putative GAS binding site, which can recruit STAT1, positioned between the Sp1 and CREB sites upstream of the *A2br* promoter. Therefore, IFNγ-induced STAT1 may physically block the ability of LPS-induced transcription factors to bind DNA, thereby preventing the expression of the A2bR. Alternatively, IFNγ may inhibit A2bR expression via post-transcriptional mechanism or epigenetic modifications. Because of the paucity of knowledge regarding the mechanism(s) by which the

A2bR is regulated, particularly in macrophages, these points will be important to address in order to improve our comprehensive understanding immune regulation.

We also proposed the therapeutic application of engineered macrophages that over-express the A2bR as a potential treatment for chronic inflammatory diseases, in which high levels of IFNγ persist. IFNγ intensifies macrophage inflammatory responses and is associated with the pathogenesis of chronic inflammatory bowel disease, lupus, and rheumatoid arthritis [25, 26]. It is important to re-iterate that although adenosine agonists may be viable treatment options, these molecules do not currently possess any specificity towards particular cell types. Besides modulating inflammatory cytokine production, adenosine also affects the vasculature, muscle function and motor neuron activity. Thus, for inflammatory diseases in which macrophages have a major role, it is imperative to develop adenosine-modulating therapies to specifically target macrophages in order to effectively resolve inflammation with minimal negative side effects. Our preliminary studies highlighted in Chapter 3, reveal the ability to circumvent IFNγ-induced hyper-inflammation by over-expressing the A2bR in macrophages. In the future, it will be important to create macrophage cell lines, which stably express abundant A2bR levels and to test the efficacy for this treatment in in vivo models of chronic inflammatory disease. It will also be important to assess whether immunomodulatory molecules such as IL-4, synergize with adenosine to promote severe immunosuppression or fibrosis.

IFNγ is also important for priming macrophages for effective host defense,

thus we were interested in investigating the consequence of extracellular ATP on adenosine signaling during infection. To address this, we infected macrophages with *Leishmania major*, a virulent parasite that targets macrophages for their intracellular survival. The survival of *L.major* within macrophages is critically dependent on the presence of IFNγ, which drives the production of microbicidal NO. Surprisingly, CD39 and CD73 activity appeared to remain unchanged during infection; however extracellular ATP/adenosine potently inhibited NO production and enhanced the survival of parasites despite the presence of IFNγ. While the A2bR expression remained lower in IFNγ primed macrophages compared to unprimed cells, which was consistent with our findings in chapter 3, the A2aR was the adenosine receptor most greatly upregulated in macrophages during *L.major* infection. Intriguingly, upregulation of the A2aR persisted in the presence of IFNγ, further corroborating our finding that IFNγ specifically interferes with A2bR expression. Subsequent use of A2aR-specific agonists demonstrated that A2aR signaling is the major adenosine receptor enabling the survival of parasites within activated macrophages. The results from this study suggest that *L. major* may induce an increase in host A2aR expression as a virulence strategy to evade innate host defense mechanisms. However, it will be important to determine whether *L.major* is directly responsible for increasing A2aR levels.

Currently, there is no cure for Leishmaniasis and few treatment options are available for the 1-2 million people infected annually [34]. Interestingly, infected individuals displaying non-healing lesions who do not receive antibiotic therapy, exhibit enhanced susceptibility to bacterial infections [127]. Thus, individuals

infected with *Leishmania spp*. are likely to be simultaneously exposed to pathogenic bacteria. The most common treatment for Leishmaniasis is the administration of pentavalent antimonials, which is painful because they are injected directly into the lesion and also requires multiple doses with various dosing intervals. More recent therapies have been made available such as SNAP cream (S-nitroso-N-acetylpenicillamine), which enhances NO production and is applied topically to the lesion as well as oral anti-fungal azoles that limit parasite growth [36]. However, all existing treatments do not specifically target any particular cell type, and thus have multiple unfavorable side-effects including reactivation of latent viruses, enhanced susceptibility to bacterial infections, and even toxicity issues leading to pancreatitis or liver failure [127,128]. Thus, this study proposes that blocking A2aR-dependent signaling on macrophages may represent a novel way to more effectively therapeutically combat this infection.

Perhaps one of the most unique findings was the apparent differential use of adenosine receptors by macrophages to mediate their diverse functional repertoire. Throughout this study, we provided novel evidence that distinguishes these A2Rs on macrophages based on their induction of expression and the regulation of adenosine-mediated responses. We report that TLR activation elicits the selective upregulation of the A2bR on macrophages, while *L.major* infection preferentially induces the A2aR. Moreover, the A2bR is the primary adenosine receptor that inhibits TLR-induced TNFα and IL-12 production, while stimulation of the A2aR is the dominant adenosine receptor mediating NO attenuation and IL-10 production in macrophages. It will be important to

delineate the molecular mechanisms regulating the differential expression and elucidate how these two closely related receptors can instill different outcomes upon activation. Importantly, IL-10 production remains intact even in the presence of IFNγ as well as in A2bR-deficient macrophages. Thus, indicating that A2bR-mediated suppression of TNFα and IL-12 is independent of autocrine IL-10 signaling and that ATP/adenosine directly modulates macrophage activation.

The A2aR and A2bR (A2Rs) are GPCRs coupled to the Gαs subunit. Other ligands that stimulate GPCR-induced cAMP production such as prostaglandins, sphingosine phosphate, and C5a have also been shown to possess immunomodulatory behavior [116]. Previous work demonstrated that treating macrophages with cAMP could promote the development of regulatory macrophages via ERK activation [7]. We also observed adenosine treatment prolongs LPS-induced ERK activation (data not shown) and therefore we hypothesize that adenosine signaling through the A2R may similarly influence macrophage physiology. A2aR and A2bR activation results in different downstream effects, however the molecular mechanisms governing this remain elusive. One hypothesis is that GPCR-induced cAMP functions differently depending on the stimulus, thereby yielding various downstream effects. Additionally, it will be important to investigate whether over-expression of the A2bR can modulate anti-microbial activity or mask A2aR signaling. Elucidating the molecular mechanism governing the differential effects mediated by adenosine will likely lead to the production of a novel class of therapeutics that

can substantially improve the way we treat inflammatory and infectious diseases.

A major hurdle of treating inflammatory diseases with inflammatory blockers (anti-TNFα for example) is that although they may effectively attenuate the inflammatory responses, they often significantly impair the ability to clear infections. The results discussed in the work presented here, demonstrate that CD39 and A2Rs on macrophages represent unique targets to treat an array of ailments. For example, results from this work proposes the following scenarios in the future:

- Treating sepsis by modulating macrophage-CD39 activity according to the progression of the disease, whereby activating CD39 on macrophages during the initial phase of sepsis (SIRS) to prevent septic shock. At later stages of the disease, a dampening CD39 activity once the inflammatory response subsides to prevent enhanced susceptibility to infection. Moreover, A2bR expression on macrophages could be used to monitor the transition of inflammatory to immunosuppressive macrophages in sepsis patients as a prognostic tool to better tailor their treatment.

- Managing rheumatoid arthritis by injecting A2bR-engineered macrophages into the inflamed joint, which will dampen the inflammation and aid in the resolution of the disease without negating innate anti-microbial activity, thus restoring the ability of patient's ability to fight infections.

The culmination of this work is the identification and characterization of a novel autoregulatory mechanism governing inflammatory macrophage plasticity. Prior to these studies, eATP was predominantly regarded as a pro-inflammatory stimulus. However, based on the work presented here we propose that eATP, derived from TLR-stimulated macrophages can be anti-inflammatory and drive the development of regulatory macrophages. A major discovery presented throughout this thesis was that inflammatory macrophage activation is normally transient and that CD39-mediated regulation is critical in maintaining functional plasticity in macrophages. Thus, this model proposes that the resolution of inflammation is actively induced and does not arise by passive exhaustion of the pro-inflammatory response or is merely quiescent. Rather immunosuppressive macrophages actively participate in resolving inflammation and maintaining homeostatic tissue conditions. This work further reveals that this complex auto-regulatory mechanism can be dynamically altered during the course of a disease, thus re-directing the functional destiny of an activated macrophage to either become chronically inflammatory or severely susceptible to infection. Results from the studies presented here offer many promising new targets and novel strategies that may improve the diagnosis and clinical outcome for myriad inflammatory and infectious diseases in which the switch from inflammatory to regulatory macrophages is important.

Appendix

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
Gapdh	AAGGTCGGTGTGAACGGATTT	AATTTGCCGTGAGTGGAGTCATAC
Tnfα	AAAGGGATGAGAAGTTCCCAAAT	GTCTTTGAGATCCATGCGGTTG
Il-12/23p40	AAGACGTTTATGTTGTAGAGGTGG	ACTGGCCAGTATCTAGAAACTCTTT
Il-10	GACTTTAAGTACTTGGGTTGC	TCTTATTTTCACAGGGGAGAAATCGAA
Il-33	ATGGGAAGAAGCTGATGGTG	CCGAGGACTTTTTGTGAAGG
Arg1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTGACC
Sphk1	GGCAGTCATGTCCGGTGATG	ACAGCAGTGTGCAGTTGATGA
Hb-egf	CAGGACTTGGAAGGGACAGA	GGCATTTGCAAGAGGGAGTA
Fizz1	GGTCCCAGTGCATATGGATGAGAC	CACCTCTTCACTGCAGGGACAGTT
Ym1	CACAGGTCTGGCAATTCTTCT	GTACACAGGCAGGGGTCAAT
Mrc1	CCATTGCAGTTTGAGGGAAG	TGAGTCCAATCCAGAGTCCC
Msr1	AATAGATGCTGAATGCCCTGT	CAAAAGAAGATGCCCAAAATTC
Fabp4	CATCAGCGTAAATGGGGATT	TCGACTTTCCATCCCACTTG
Cd39	ATCGGTGCGTACCTGGCCGA	GGAGGCTCCGCCGAGATCCA
Panx1	GAGCGAGTCTGGAAACCTCC	GCGCGGTTGTAGACTTTGTC
A1r	ACAAAAACCAGTGGTGGAGTGA	TCTGTCCCCTCCCCTTGTC
A2ar	TGAAGGCGAAGGGCATCA	GCAGAAGCCCAAGCTGATG
A2br	ACGTGGCCGTGGGACTC	GCAGAAGCCCAAGCTGATG
A3r	GAGACCTGCATCCTCCAGGTT	GGCCTGTTACAGGACCATCAA

Table 1. Primer sequences used in this study.

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