Catecholamines induce a regulatory macrophage phenotype and confer protection during acute lung injury and endotoxemia via activation of the β_2 adrenergic receptor

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Abstract

Macrophages are predominant drivers of inflammation during an acute inflammatory response. Pro-inflammatory mediator production induced by pathogen-associated molecular patterns (PAMPs) can have profound physiological consequences manifesting disease states such as acute lung injury (ALI) and sepsis. Macrophages can assume two distinct phenotypes defined by differential expression patterns and immune functions [1]. An M1 phenotype activated by lipopolysaccharide (LPS) is characterized by the expression and secretion of pro-inflammatory cytokines, whereas an M2 phenotype is more anti-inflammatory and associated with wound healing [1]. While the role of catecholamines in regulating various physiological functions has been extensively studied, there is accumulating evidence suggesting that they may also play a pivotal role in regulating immune responses. In this study, we describe the ability of catecholamines to skew LPS-induced M1 macrophages to an M2 phenotype through the modulation of TLR4 signaling and regulation of phagocytosis. This occurred via a non-canonical β_2 adrenergic receptor signaling pathway and the downstream activation of phosphoinositide 3-kinase (PI3K). Mice which received a β_2 adrenergic receptor antagonist prior to the induction of ALI or sepsis demonstrated more M1-like responses, and their survival was severely attenuated. Altogether, our results suggest a role for the engagement of catecholamines with the β_2 adrenergic receptor to induce an M2 phenotype during inflammation in vivo.

Introduction

The scientific discipline of immunology can be unambiguously divided into two distinct yet intrinsically connected categories, innate and adaptive immunity. These two immunological arms work in coordination to provide both immediate and long-term immunity to various pathogens. Although adaptive responses, mediated predominately by B and T lymphocytes, provide a specific and sustained response to antigens through the secretion of antibodies and the employment of apoptosis-inducing mechanisms, effective responses require approximately 5 days following primary lymphocyte activation. Thus, the immune responses demonstrated in the early stages of pathogenic challenge are dependent upon innate inflammatory cells, most commonly neutrophils, macrophages, and dendritic cells. While different pathogens elicit different responses, macrophages are the main drivers of inflammation when activated by virulent motifs (pathogen-associated molecular patterns, PAMPs) specific to bacterial endotoxins and viral RNA, among other molecules. Upon stimulation, macrophages coordinate subsequent immune responses through the secretion of cytokines and chemokines, the phagocytosis of foreign cells and immune complexes, and the presentation of antigen to T lymphocytes.

The cytokines and chemokines secreted by macrophages and other immune cells serve a variety of functions. In the context of an infection, pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β) act as endogenous pyrogens to induce fever while acting on the vascular endothelium to increase the rate of immune cell extravasation. Systemic infections (i.e., sepsis) generate robust amounts of circulating TNF-α and IL-1β, and in consequence, they can

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induce fever, severe hypotension, and multi-organ failure. Conversely, anti-inflammatory cytokines (e.g., IL-10) suppress these pro-inflammatory pathways to limit physiological harm. In addition, smaller secreted ligands known as chemokines can be released from macrophages to induce the chemotaxis of additional immune cells towards sites of infection in a concentration-dependent fashion. While these pathways are all characteristic of an immediate reaction to pathogens, another important function of the cytokines released during the acute phase of an infection is to coordinate a subsequent adaptive immune response, in conjunction with the presentation of antigen to helper T lymphocytes.

The interface between innate and adaptive immunity is critical in mounting an appropriate and effective response to foreign specimens. While the clearance of some pathogens may require cell-mediated, inflammatory pathways, others may be best combated through the employment of humoral strategies that are characterized by a lack of inflammation. The two predominant cell types responsible for the immune system committing to one pathway or the other are Th1 and Th2 helper T lymphocytes. More specifically, Th1 inflammatory T cells serve as facilitators of inflammation through the activation of cytotoxic T lymphocytes, natural killer cells, macrophages, and neutrophils, whereas Th2 helper T cells promote B lymphocyte activation leading to the production and secretion of antibodies used to neutralize and target pathogenic antigens for immune uptake and destruction. Upon the activation of helper T cells by antigen presentation, the surrounding cytokine environment is pivotal for determining which of these two cell types will dominate in the ensuing response. Specifically, it has been well documented that IL-10, for example, is known to promote Th2 T cell

differentiation while inhibiting Th1 T cell differentiation [1]. Conversely, IL-12 has been shown to promote a Th1 T cell phenotype while inhibiting Th2 T cell differentiation [1]. Thus, the cytokines produced by macrophages during acute inflammation largely influence the nature of subsequent adaptive responses.

Central to the crosstalk between innate and adaptive immunity are the pattern recognition receptors (PRRs), which are expressed on cells of the innate immune system. This family of receptors includes the Toll-like receptors, NOD-like receptors, RIG-I-like RNA helicases, and C-type lectin receptors. Unlike T and B cell receptors, which specifically recognize and bind to protein epitopes, PRRs become activated by particular motifs inherent to various pathogens. One PRR that has been extensively studied in the context of gram-negative bacterial infections is the Toll-like receptor 4 (TLR4). As a transmembrane protein receptor, TLR4 binds to an endotoxin, lipopolysaccharide (LPS), which is present on the outer cell wall of gram-negative bacteria. This ligand-receptor interaction activates an intracellular signaling cascade leading to the downstream nuclear translocation of a transcription factor, NF-κB, which is responsible for the expression of pro-inflammatory cytokines. How various extracellular mediators regulate this pathway has been under extensive investigation in recent years.

Activated macrophages can be broadly classified as exhibiting either an M1 or M2 phenotype. Whereas M2 macrophages are known to be functionally more anti-inflammatory, M1 macrophages have been shown to readily produce pro-inflammatory cytokines (e.g., IL-12$_{(p40)}$ and TNF-α), anti-microbial reactive oxygen species, and nitrogen intermediates [1,2]. More specifically, the synthesis of nitric oxide by iNOS is a

well-documented pathway characteristic of M1 macrophages [3]. As a result, the M1 macrophage phenotype is a potent inducer of Th1 inflammatory T cell responses and tissue damage [1]. Referred to as being classically activated, the M1 phenotype can be elicited through the activation of TLR4 by LPS [1].

Conversely, M2 macrophages are comprised of a collection of phenotypes that can be non-classically activated via stimulation by type 2 cytokines, such as IL-4, IL-10, and IL-13, and glucocorticoid hormones [4]. The M2 phenotype is characterized by the suppressed production of pro-inflammatory cytokines and chemokines, and thus, these macrophages are known to promote Th2-mediated, anti-inflammatory responses [4]. In addition, while M1 macrophages express high levels of iNOS, M2 macrophages are known to highly express arginase-1 [5]. Both enzymes compete for the same substrate, L-arginine, and as a result, their reciprocal regulation can be used to effectively define the M1 and M2 phenotypes [5]. The arginase-1-mediated pathway confers M2 macrophages with wound-healing capacities through the induction of angiogenesis and fibrosis [6]. Whereas all M2 macrophages possess these general characteristics, recent work has further classified the M2 phenotype based on disparities in the etiology of activation and subsequent functional differences [1]. Identifiable by specific surface markers, M2a (alternatively activated) macrophages can be induced through their exposure to IL-4 and IL-13 [7,8]. Another M2 phenotype, the regulatory macrophage, can be elicited by exposure to IgG immune complexes (M2b subtype) or IL-10 (M2c subtype) [1,9]. Regulatory macrophages differ from alternatively activated macrophages by their enhanced production of IL-10 and augmented phagocytic activity [9]. Given the vast differences in the function of M1 and M2 macrophages during

infection, understanding how various environmental factors influence their plasticity is of great importance in science and medicine.

While neuroendocrine hormones (e.g., catecholamines) have been extensively studied in the context of various physiological systems, there is emerging evidence for their role in regulating both acute and chronic immune responses. Specifically, it has been shown that catecholamines are upregulated in vivo during inflammatory conditions [10]. Various β adrenergic receptor agonists have also been shown to modulate cytokine production pathways in vitro [11-14]. Our lab recently published a study illustrating that in addition to their bronchodilating effects, synthetic β_2 adrenergic receptor agonists are able to suppress macrophage pro-inflammatory cytokine production both in culture and during experimental acute lung injury (13). Given these findings, we hypothesized that, like their synthetic analogs, catecholamines may engage with the β_2 adrenergic receptor to modulate TLR4 signaling in macrophages not only to suppress an M1 phenotype, but also to induce M2-like responses in vivo during acute inflammation.

In this study, we report that epinephrine and norepinephrine activate the β_2 adrenergic receptor both in vitro and in vivo to modulate TLR4-mediated macrophage cytokine and phenotype marker expression. While LPS-stimulated M1 macrophages demonstrate strong pro-inflammatory responses, macrophages stimulated with LPS in the co-presence of catecholamines exhibit an M2 regulatory macrophage phenotype with enhanced IL-10 and arginase-1 expression and augmented phagocytic activity. In vivo, mice administered a β_2 adrenergic receptor antagonist had severely exacerbated acute lung injury characterized by increased pro-inflammatory cytokine production in the

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lung. In addition, the survival of endotoxemic mice administered the same inhibitor was significantly compromised. Importantly, M2 macrophages polarized by catecholamines in vitro were protective when adoptively transferred during experimental endotoxemia. Altogether, these results suggest a protective role for catecholamines in vivo in promoting an M2, anti-inflammatory macrophage phenotype.

Methods

Animals

Procedures performed in this study were all in accordance with the U.S. National Institutes of Health guidelines and were approved by the University of Michigan Committee on the Use and Care of Animals. All experiments were performed in male, age-matched C57BL/6 mice (10-12 weeks old) purchased from Jackson Laboratories (Bar Harbor, ME, USA) housed in pathogen-free conditions. $TRIF^{-/-}$ and $MyD88^{-/-}$ mice on the C57BL/6 background were also purchased from Jackson Laboratories.

Isolation of Peritoneal Macrophages

Elicited peritoneal macrophages were harvested 4 days following the intraperitoneal (i.p.) injection of 2.4% thioglycollate (Life Technologies, Grand Island, NY, USA). Cells were cultured in RPMI 1640 media (Life Technologies) containing 100 U/ml penicillin-streptomycin and 0.1% bovine serum albumin, and purification was achieved via culture plate adherence. Cell-free supernatants were stored at -80°C until later use. In vitro experiments contained triplicate samples (1 x10^6 cells per sample) replicated over at least 2 independent experiments.

Enzyme-linked immunosorbent assays (ELISA)

Albumin ELISAs were supplied by Bethyl Laboratories (Montgomery, TX, USA).

Detection of mouse IL-10, TNF-α, and IL-12$_{(p40)}$ was achieved with sandwich ELISAs

(kits were purchased from R&D Systems – Minneapolis, MN, USA). All protocols were

performed according to the manufacturer's recommendations. Cytokine concentrations

were determined from log-transformed standard concentrations plotted on a standard

curve.

Real-Time PCR

Total RNA was isolated from cultured cells using TRIzol (Sigma-Aldrich, St. Louis, MO,

USA). Following purification, DNAse (Life Technologies) was used to remove any

genomic DNA. cDNA was generated using the reverse transcription kit provided by Life

Technologies and RT-PCR (SYBR, Life Technologies) was performed on a 7500 real-

time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers

were used:

GAPDH Fwd: 5' CTTCAACAGCAACTCCCACTCTTCC 3'

GAPDH Rev: 5' GGTGGTCCAGGGTTTCTTACTCC 3'

IL-10 Fwd: 5' CCAGTTTTACCTGGTAGAAGTGATGCC 3'

IL-10 Rev: 5' GTCTAGGTCCTGGAGTCCAGCAGAC 3'

TNF-α Fwd: 5' CTGAACTTCGGGGTGATCGGTCC 3'

TNF-α Rev: 5' GTATGAGATAGCAAATCGGCTGACG 3'

Arginase-1 Fwd: 5' ATGGAAGAGACCTTCAGCTACCTGC 3'

Arginase-1 Rev: 5' GCTGTCTTCCCAAGAGTTGGG 3'

iNOS Fwd: 5' ACATCGACCCGTCCACAGTAT 3'

iNOS Rev: 5' CAGAGGGGTAGGCTTGTCTCTGG 3'

Relative expression levels were calculated using 2^{-ddCt} method and were normalized to GAPDH.

Phagocytosis Assays

Peritoneal-elicited macrophages (unstimulated, LPS-treated, or LPS and epinephrine co-treated) were incubated with pHrodo Green *E. Coli* Bioparticles® (1 mg/ml; Life Technologies). Reactions proceeded for 1 hour. Flow cytometric analysis was carried out on a BD LSR-II flow cytometer equipped with FACSDiva software (both from BD Biosciences, San Jose, CA, USA). More than 5 x10^4 cells were analyzed from each sample and data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA).

Acute Lung Injury

ALI was induced via the intratracheal (i.t.) injection of LPS as previously described [13]. Following a medial thoracic incision performed under anesthesia with ketamine, mice received 60 μg of LPS from *E. coli* (o111:B4; Sigma-Aldrich) in a volume of 30 μl saline solution. Sham control mice received an equivalent volume of sterile saline. Bronchoalveolar lavage fluids were collected via the instillation and retraction of sterile PBS into the lung. For studies involving ICI 118, 551 (Sigma-Aldrich), the small molecule inhibitor was administered at 25 mg/kg i.p. 1 hour prior to LPS administration

and 6 ng i.t. during the induction of ALI. Vehicle controls received equivalent volumes of sterile saline solution.

Endotoxemia

Mice received either 10 mg/kg or 20 mg/kg body weight i.p. injection of lipopolysaccharide (LPS) from *E. coli* (o111:B4; Sigma-Aldrich). Plasma was harvested by bleeding from the retro-orbital venous plexus under isoflurane anesthesia. 500 μg of ICI 118,551 (Sigma-Aldrich) was administered i.p. to achieve β_2 adrenergic receptor blockade. For adoptive transfer experiments, thioglycollate-elicited peritoneal macrophages were treated in vitro with LPS in the presence or absence of epinephrine for 1 hour prior to administration. Treated or untreated macrophages were harvested, washed, and injected i.p. at 1 $\times 10^6$ cells in 200 μl of PBS with the induction of endotoxemia (20 mg/kg) occurring 4 hours later. For survival studies, mice were monitored at least every 12 hours for 7 days.

Statistical Analysis

Data in this study (values expressed as mean ± SEM) were analyzed using GraphPad Prism 6 graphing and statistical analysis software (GraphPad Inc., La Jolla, CA, USA). Significance between sample means was determined by an unpaired, two-tailed Student's *t* test. Survival study data were analyzed by Log-rank (Mantel-Cox) tests. Differences were considered significant when the p value was less than 0.05.

Reagents

Epinephrine, norepinephrine, ICI 118,551, U0126, SP600125 (all from Sigma, St. Louis, MO, USA), 2', 5'-dideoxyadenosine (2', 5'-DDA; Santa Cruz Biotech, Santa Cruz, CA, USA), SQ22536 (Santa Cruz Biotech), PKA inhibitor 14-22 amide (EMD Millipore, Billerica, MA, USA), wortmannin (Santa Cruz Biotech), were all used at concentrations indicated in each figure. LPS (*E. coli* o111:B4) was purchased from Sigma. Optimal concentrations for these reagents were determined in preliminary experiments.

Results

Catecholamines modulate TLR4-induced macrophage cytokine production via activation of the β_2 adrenergic receptor

To illustrate whether catecholamines could regulate cytokine production pathways in vitro, peritoneal-elicited macrophages from wild-type mice were incubated for 4 hours with LPS alone or LPS in the co-presence of epinephrine or norepinephrine. Results showed that both adrenergic receptor agonists influenced cytokine production in elicited cells. Specifically, macrophages stimulated with either catecholamine in the co-presence of LPS demonstrated attenuated production of the pro-inflammatory cytokine, TNF-α, and enhanced production of the anti-inflammatory cytokine and Th2 T cell activator, IL-10 (Fig. 1A and 1B). Epinephrine and norepinephrine modulated the production of these two cytokines in a dose-dependent fashion with IC50s of approximately 5 nM and 700 nM respectively. Additionally, RT-PCR was used to illustrate the regulation of IL-10 and TNF-α at the transcriptional level following the co-activation of TLR4 and the adrenergic receptors via stimulation with catecholamines.

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Specifically, decreases in supernatant TNF-α protein levels were accompanied by a

suppression of TNF-α mRNA expression under the same conditions (Fig. 1C).

Similarly, increases in supernatant IL-10 protein levels following activation of these

receptors were accompanied by enhanced IL-10 mRNA expression within the cell (Fig.

1C). Altogether, these data demonstrated that epinephrine/norepinephrine are able to

regulate cytokine production pathways in macrophages via transcriptional control.
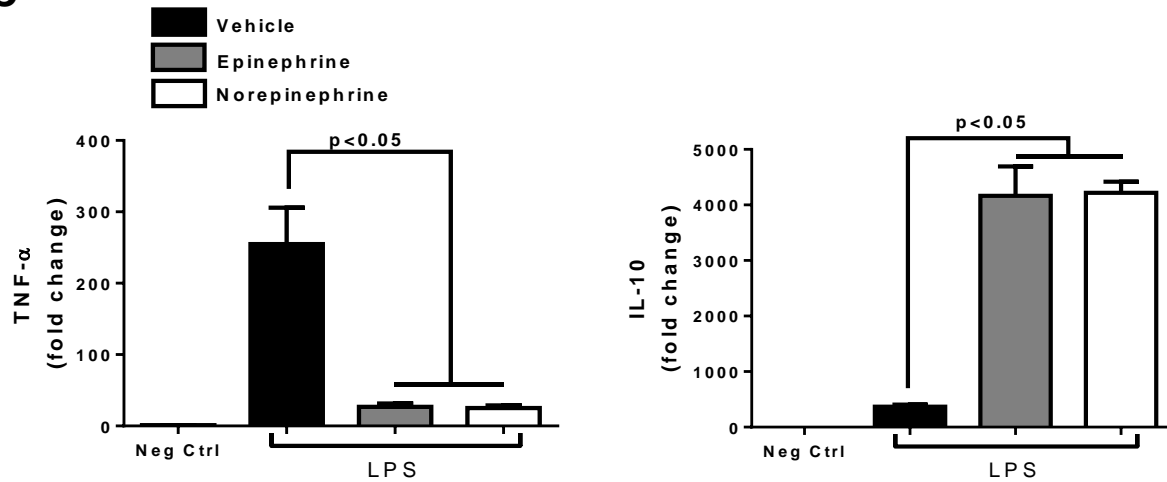
A

B
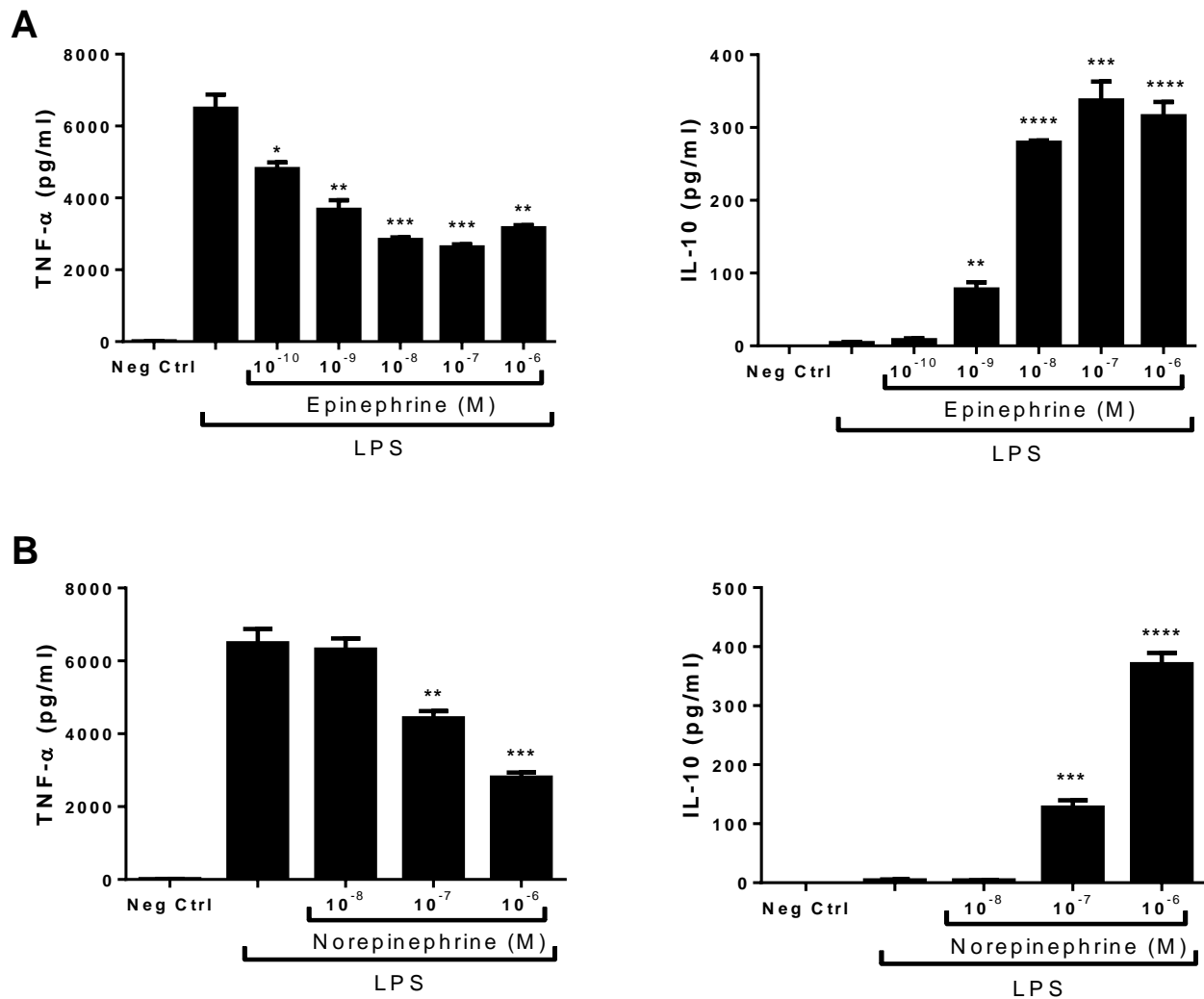
C

Figure 1. **Catecholamines modulate TLR4-mediated cytokine expression and secretion**. A-B) Mouse peritoneal macrophages were stimulated with LPS (100 ng/ml) in the absence or co-presence of varying concentrations of epinephrine or norepinephrine for 4 hours. Culture supernatant cytokine concentrations were determined by ELISA. A) TNF-α (left panel) and IL-10 (right panel) dose response curves for epinephrine. B) TNF-α (left panel) and IL-10 (right panel) dose response curves for norepinephrine. C) Relative expression of mRNA transcripts for TNF-α (left panel) and IL-10 (right panel) (RT-PCR). Mouse peritoneal macrophages were stimulated with LPS (100 ng/ml) in the absence or co-presence of epinephrine or norepinephrine (10^{-6} M) for 18 hours. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

It is widely accepted that, upon TLR4 activation by LPS in macrophages, both the TRIF-dependent and MyD88-dependent intracellular signaling cascades are initiated. To illustrate the effect of endogenous catecholamines on both pathways, peritoneal elicited macrophages from TRIF$^{-/-}$ and MyD88$^{-/-}$ mice were stimulated with LPS in the

absence or co-presence of epinephrine. Our data indicated that epinephrine modulates

both the TRIF-dependent and MyD88-dependent signaling pathways (Fig. 2A and 2B).

Specifically, in a similar fashion to wild-type macrophages (see Fig. 1A), TRIF$^{-/-}$

peritoneal macrophages treated with both LPS and epinephrine displayed significantly

reduced levels of TNF-α and increased levels of IL-10 when compared to knockout

macrophages treated solely with LPS (Fig. 2A). Similarly, LPS-induced TNF-α

production by MyD88$^{-/-}$ peritoneal macrophages was significantly vitiated upon treatment

of these cells with epinephrine (Fig. 2B). Under similar conditions, IL-10 protein levels

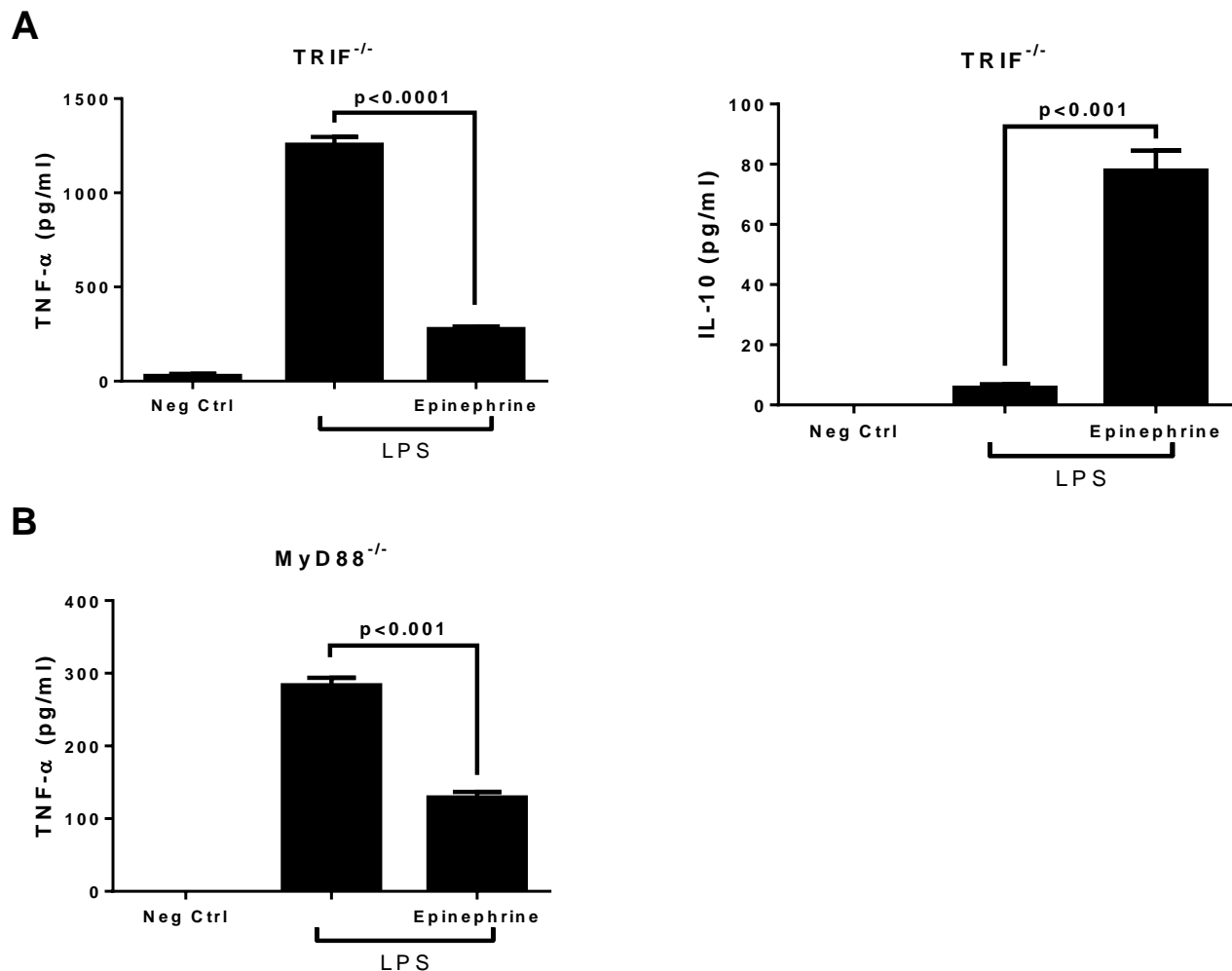in the supernatants of the MyD88$^{-/-}$ macrophages were undetectable (data not shown).

A

B

Figure 2. **Catecholamines regulate both the TRIF and MyD88 signaling pathways**.

A-B) Mouse peritoneal macrophages from TRIF$^{-/-}$ or MyD88$^{-/-}$ mice were stimulated with

LPS (100 ng/ml) in the absence or co-presence of epinephrine (10^{-6} M) for 4 hours. A)

TNF-α (left panel) and IL-10 (right panel) supernatant concentrations from TRIF$^{-/-}$

macrophages quantified by ELISA. B) TNF-α supernatant concentrations from MyD88$^{-/-}$

macrophages as determined by ELISA.

      To confirm the importance of β_2 adrenergic receptor signaling in this effect, use of

the selective β_2 adrenergic receptor antagonist, ICI 118,551, was employed. Our data

showed that blockade of the β_2 adrenergic receptor with the small-molecule inhibitor

negated the effects of the endogenous catecholamines on both IL-10 and TNF-α

production (Fig. 3A). Additional data illustrated that the regulation of these cytokines

due to the activation of β_2 adrenergic receptors was also dependent upon the

engagement of TLR4 by LPS (Fig. 3B). The presence of either endogenous

catecholamine alone did not influence cytokine expression, but rather, simultaneous

activation of TLR4 and the β_2 adrenergic receptor was required for altered IL-10 and

TNF-α production in macrophages. Taken together, these data indicated that the

signaling mechanism resulting from β_2 adrenergic receptor activation influences LPS-

induced cytokine release via modulation of both the TRIF-dependent and MyD88-
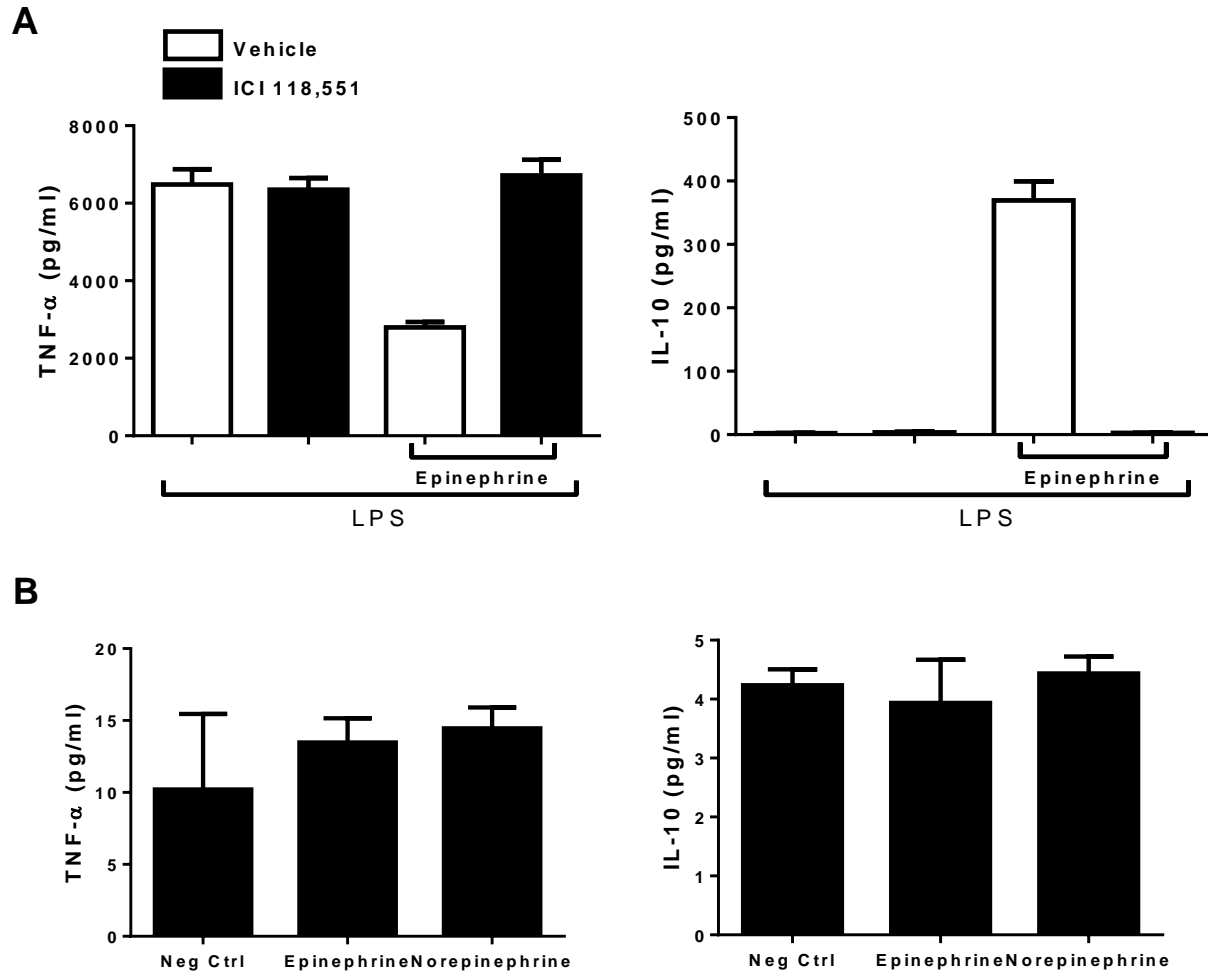
dependent intracellular signaling pathways.

A

B

Figure 3. **Modulation of TLR4-mediated cytokine production is dependent upon the simultaneous activation of TLR4 and the β_2 adrenergic receptor**. A) Peritoneal macrophages were stimulated in the absence or presence of LPS (100 ng/ml), epinephrine (10^{-6} M), and/or ICI 118,551 (β_2 adrenergic receptor antagonist) for 4 hours. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations quantified by ELISA. B) Peritoneal macrophages were simulated in the presence or absence of epinephrine or norepinephrine (10^{-6} M) for 4 hours. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations as determined by ELISA.

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β_2 adrenergic receptor signaling regulates TLR4-mediated cytokine production independent of the canonical pathway with dependence on phosphoinositide 3-kinase

Historically, it has been widely accepted that β_2 adrenergic receptors initiate signal transduction pathways via coupling to heterotrimeric G-proteins and subsequent interaction of the Gαs subunit with adenylate cyclase leading to increased levels of cAMP and the activation of protein kinase A (PKA). Interestingly, we have found that the regulation of macrophage cytokine production by catecholamines is independent of this classical pathway and our data have suggested dependence on the $\beta\gamma$ subunits. Incubation of peritoneal macrophages with the adenylate cyclase inhibitors, 2',5'-DDA and SQ22536, for 1 hour prior to stimulation with LPS and epinephrine had no effect on β_2 adrenergic receptor signaling and its regulation of the TLR4 pathway (Fig. 4A). Specifically, our data illustrated that blockade of adenylate cyclase did not significantly affect the modulation of IL-10 or TNF-α production by epinephrine. Additionally, inhibition of PKA was used to further bolster these findings. Macrophages pre-incubated with the PKA inhibitor for 1 hour prior to co-stimulation with LPS and epinephrine produced IL-10 and TNF-α levels comparable to stimulated samples without PKA inhibitor (Fig. 4B). These findings suggested that the regulation of macrophage cytokine production by β_2 adrenergic receptor signaling was achieved independent of the canonical pathway.
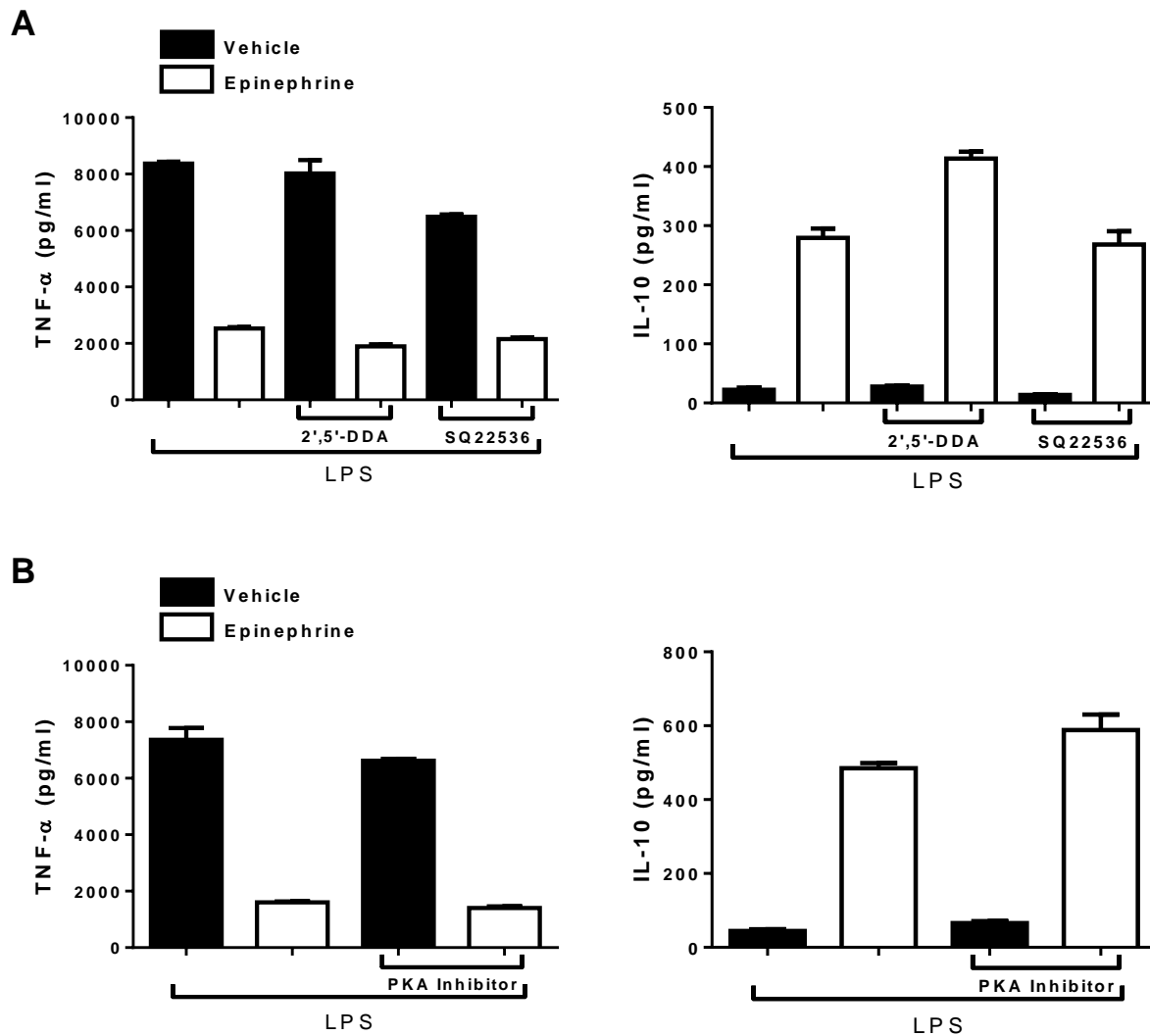
A

B

Figure 4. β_2 **adrenergic receptor signaling regulates macrophage cytokine production via a non-canonical pathway**. A-B) Peritoneal macrophages were incubated with LPS (100 ng/ml) in the absence or co-presence of epinephrine (10^{-6} M) for 4 hours. A) Macrophages were pretreated with the adenylate cyclase inhibitors, 2',5'-DDA (20 μM) or SQ22536 (10 μM), or a vehicle control 1 hour prior to stimulation. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations as determined by ELISA. B) PKA activity was inhibited with a PKA inhibitor 14-22 amide (5 μM) added 1 hour prior to stimulation. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations quantified by ELISA.

The TLR4-activated MyD88-dependent signaling cascade includes the mitogen-activated protein kinases (MAPKs) (p38, MEK1/2, Erk1/2, and Jnk1/2) as well as PI3K and Akt among other intracellular mediators. To better understand the mechanism underlying changes in TLR4 signaling via activation of the β_2 adrenergic receptor, peritoneal-elicited macrophages were stimulated with LPS and epinephrine following a 1-hour pre-incubation with specific inhibitors of Jnk1/2 (SP600125), MEK1/2 (U0126), and PI3K (wortmannin). IL-10 and TNF-α protein levels in the supernatants of these samples were compared to levels quantified from samples stimulated according to the same conditions, but lacking pre-incubation with the inhibitors. As illustrated in Figures 5A and 5B, blockade of Jnk1/2 and MEK1/2, via inhibition with SP600125 and U0126 respectively, did not affect epinephrine-mediated regulation of macrophage IL-10 and TNF-α production. Specifically, the magnitude of enhanced IL-10 and suppressed TNF-α production by epinephrine remained consistent regardless of the inhibitor used. Altogether, these data suggested that regulation of TLR4 signaling by endogenous catecholamines was largely independent of MAPK signaling.
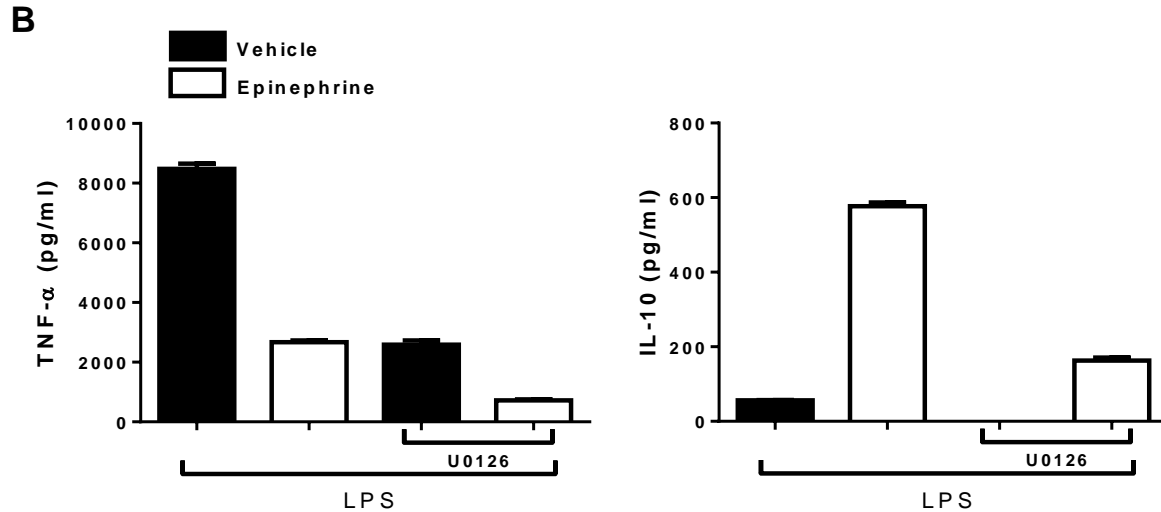
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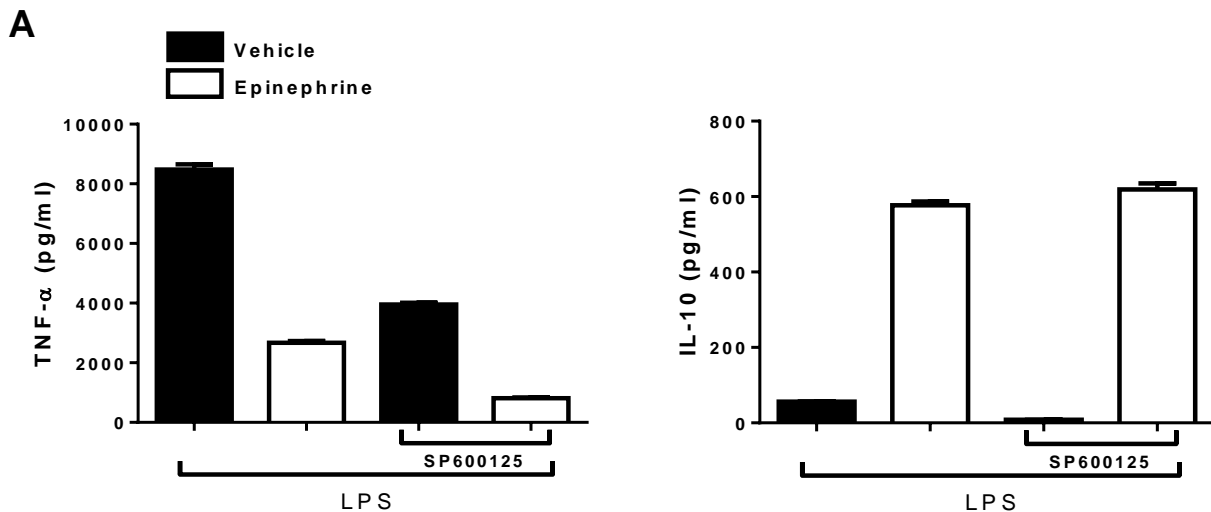
Figure 5. **Catecholamines regulate TLR4 signaling independent of the MAP Kinases**. A-B) Peritoneal macrophages were incubated with LPS (100 ng/ml) in the absence or co-presence of epinephrine (10^{-6} M) for 4 hours. A) Macrophages were pretreated with the Jnk1/2 inhibitor, SP600125 (50 μM) 1 hour prior to stimulation. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations as determined by ELISA. B) MEK1/2 activity was inhibited with U0126 (50 μM) added 1 hour prior to stimulation. TNF-α (left panel) and IL-10 (right panel) supernatant concentrations quantified by ELISA.

To examine the role of PI3K in this effect, macrophages were incubated with a specific inhibitor of this enzyme, wortmannin, prior to stimulation with LPS in the co-presence of epinephrine. Interestingly, we demonstrated that inhibition of PI3K with 5 μM wortmannin negated the effects of β_2 adrenergic receptor signaling on modulating TNF-α and IL-10 production (Fig. 6A). This effect became less marked with decreasing concentrations of inhibitor. Although the impact of the inhibitor on IL-10 production was

less drastic, there was still a noticeable decline (~50%) in IL-10 production from LPS

and epinephrine co-stimulated macrophages incubated with wortmannin in comparison

to non-inhibited macrophages (Fig. 6A). These data strongly suggested that

macrophage cytokine production is mediated by endogenous catecholamines via the
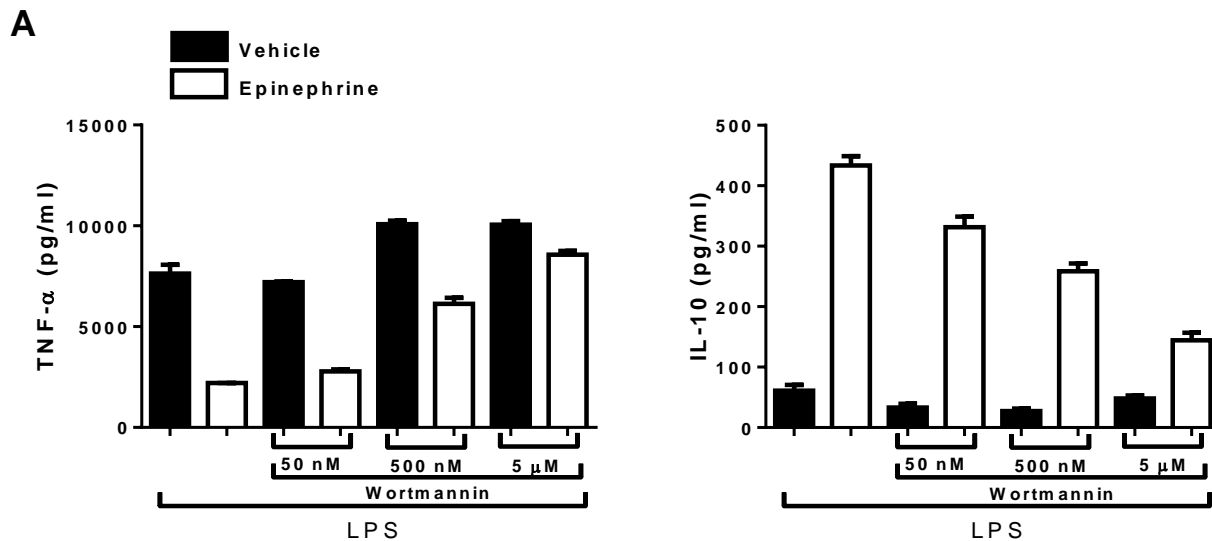
downstream activation PI3K.

A

Figure 6. **Modulation of TLR4 signaling by the β_2 adrenergic receptor requires**

downstream activation of PI3K. A) Peritoneal macrophages were stimulated in the

absence or presence of LPS (100 ng/ml), epinephrine (10^{-6} M), and/or varying

concentrations of wortmannin (PI3K inhibitor) for 4 hours. Cells were pre-treated with

the inhibitor for 1 hour. TNF-α (left panel) and IL-10 (right panel) supernatant

concentrations as determined by ELISA.

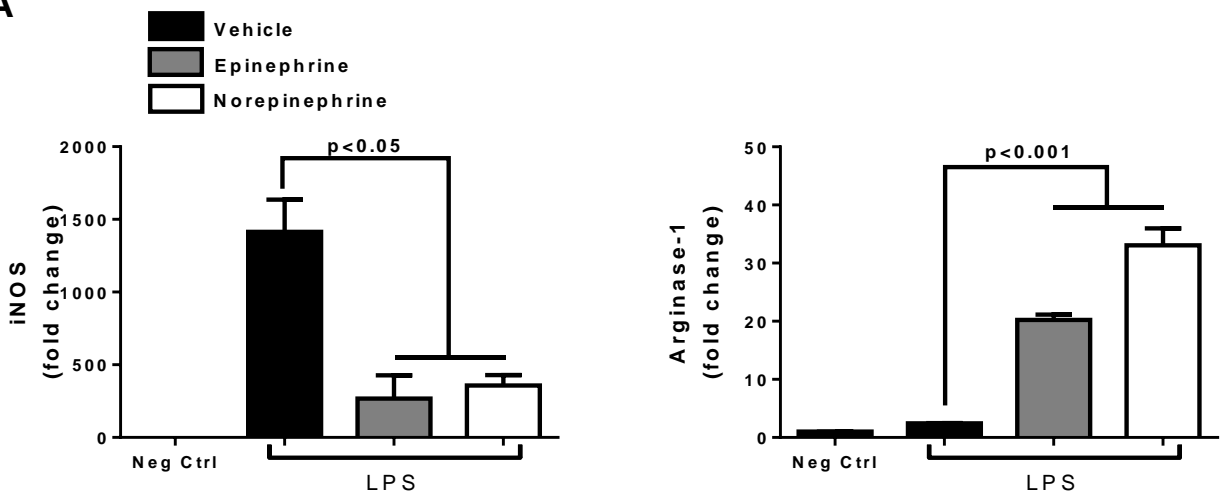*Catecholamines induce a regulatory macrophage phenotype*

Macrophages can assume two distinct phenotypes (M1 and M2) characterized by

variegated cytokine and phenotypic marker expression profiles [1]. Because these

specific phenotypes bring about different functional responses, we were interested in illustrating the effect of endogenous catecholamines on macrophage polarization. Specifically, it is known that each of these phenotypes is characterized by specific cellular markers, including arginase-1, an M2 macrophage marker, and iNOS, an M1 macrophage marker [3,5]. We first studied the effect of epinephrine and norepinephrine on macrophage polarization by looking at the transcriptional control of these two genes following stimulation with LPS in the co-presence of catecholamines. As shown in Figure 7A, activation of the β_2 adrenergic receptor by treatment of macrophages with epinephrine or norepinephrine in the co-presence of LPS enhanced arginase-1 expression and suppressed iNOS expression when compared to LPS treatment alone. These data suggested that catecholamines could promote a shift in macrophage phenotype from M1 to M2 and may subsequently serve as an immunoprotective agent during the inflammatory response. To bolster our conclusions, we also illustrated the effect of epinephrine and norepinephrine on regulating the production of a well-defined M1 cytokine, IL-12$_{(p40)}$, which is a potent activator of Th1 inflammatory T cells. As shown in Figure 7B, the production of this LPS-induced cytokine was significantly downregulated in the presence of epinephrine and norepinephrine, again suggesting that these endogenous ligands act to promote an anti-inflammatory response in macrophages upon activation by gram-negative endotoxins.

   To further characterize this switch in phenotype, we wanted to better illustrate the functional changes associated with an anti-inflammatory response induced by catecholamines. Importantly, M2 regulatory macrophages, a subtype of M2 macrophages, not only release copious amounts of IL-10 but are also known to be

veraciously phagocytic [9]. To demonstrate the effect of epinephrine on macrophage phagocytosis, we measured the uptake of fluorescently labeled, heat killed *E. coli* by flow cytometric analysis. Specifically, co-stimulation of macrophages with LPS and epinephrine enhanced the ability of macrophages to phagocytose surrounding *E. coli* cells (Fig. 7C). These data, in conjunction with our cytokine and phenotypic marker expression analyses, strongly suggest that catecholamines skew macrophages from an LPS-induced M1 phenotype to an M2 regulatory macrophage phenotype through the regulation of cytokine expression and phagocytosis.
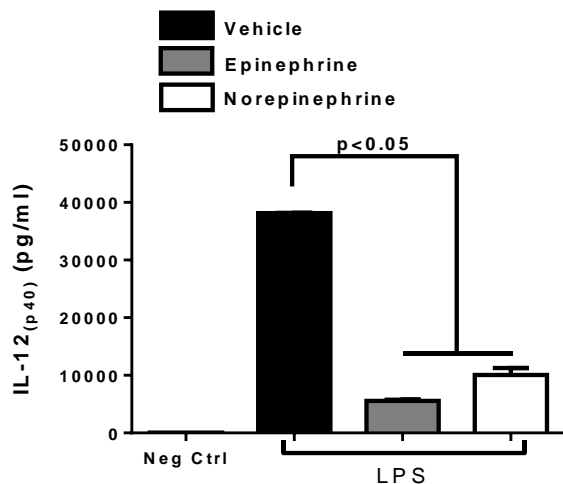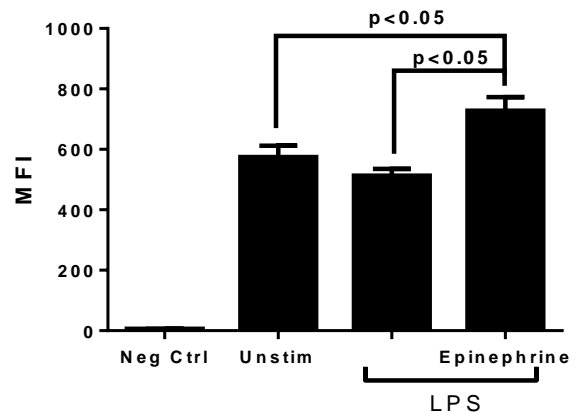
Figure 7. **Catecholamines induce an M2 regulatory macrophage activation phenotype**. A-B) Mouse peritoneal macrophages were incubated with LPS (100 ng/ml) in the absence or co-presence of epinephrine or norepinephrine (10^{-6} M) for 18 hours. A) RT-PCR was used to determine the relative mRNA transcript expression levels for iNOS (left panel) and arginase-1 (right panel). B) Culture supernatant levels of IL-12$_{(p40)}$ quantified by ELISA. C) MFI, mean fluorescence intensity, was determined by flow cytometric analysis of fluorescent *E. Coli* Bioparticle phagocytosis.

Because IL-10 has been shown to act as a mediator for paracrine/autocrine signaling in macrophages, and our previous data showed that catecholamines enhanced IL-10 production by peritoneal macrophages, we were interested to see whether IL-10 was acting as an intermediate to regulate arginase-1 and iNOS expression. Using an IL-10 neutralizing antibody, our data indicated some level of reliance on the presence of IL-10 for catecholamine-mediated regulation of arginase-1 and iNOS expression (Fig. 8). Specifically, macrophages incubated with a neutralizing antibody to IL-10, and stimulated with both LPS and epinephrine in tandem, displayed increased levels of iNOS expression and decreased levels of arginase-1 expression when compared to macrophages that were stimulated according to the same conditions, but lacked treatment with the IL-10 neutralizing antibody. In both cases, the anti-IL-10 antibody impaired the ability of epinephrine to modulate iNOS and arginase-1 expression levels, thus suggesting that IL-10 is an essential mediator in the M1 to M2 regulatory macrophage phenotype shift induced by catecholamines.
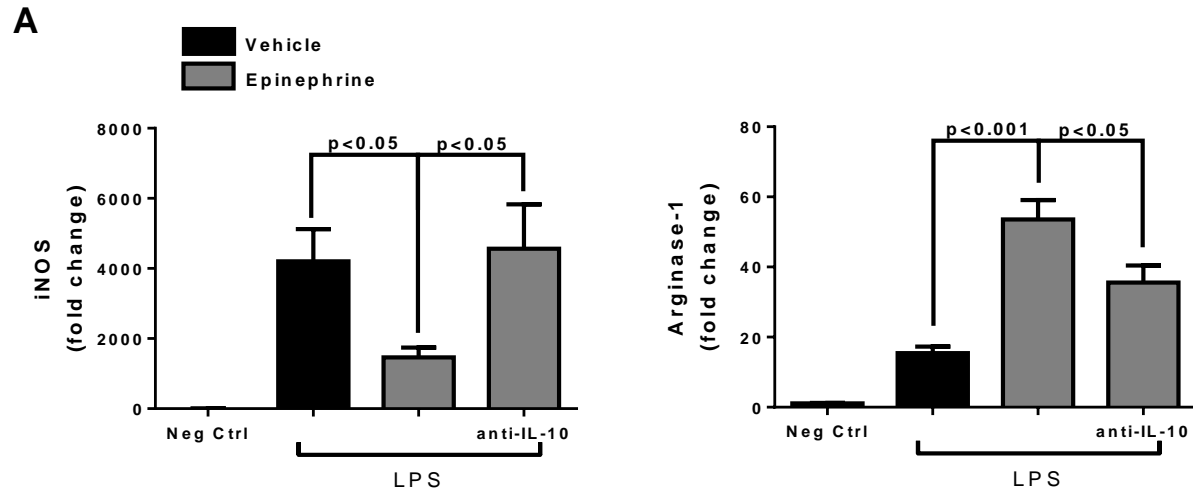
A

Figure 8. **IL-10 acts as a mediator for catecholamines to regulate iNOS and arginase-1 expression**. Mouse peritoneal macrophages were incubated in the presence or absence of LPS (100 ng/ml), epinephrine (10^{-6} M), and/or anti-IL-10 for 18 hours. Relative expression levels for iNOS (left panel) and arginase-1 (right panel) were quantified by RT-PCR.

β_2 adrenergic receptor activation confers protection during acute lung injury (ALI) and experimental endotoxemia

Given the immune modulating effects clearly demonstrated of epinephrine and norepinephrine, we next focused on examining the immunoprotective behavior of β_2 adrenergic receptor activation during ALI by using our well-established in vivo LPS-induced ALI injury model. Specifically, we compared the levels of TNF-α, IL-10, and serum albumin found in the bronchoalveolar lavage fluids (BALF) of wild-type mice administered either the β_2 receptor antagonist, ICI 118,551, or sterile saline solution (vehicle control). Albumin leak into the alveolar space indicates damage to the alveolar epithelium and pulmonary vasculature, and as result, it is an effective method for

quantifying injury in the lung. Mice were administered the ICI 118,551 via an i.p.

injection 1 hour prior to surgery and an i.t. injection of the inhibitor upon the onset of

ALI. In these experiments, ALI was induced via the i.t. injection of LPS and the BALF

were collected after 8 hours. As shown in Figure 9A, mice having undergone β_2

blockade produced significantly more TNF-α in the lung during ALI when compared to

the vehicle control mice, though surprisingly, no changes in IL-10 levels were observed

(Fig. 9B). Additionally, mice receiving the β_2 adrenergic receptor antagonist had

significantly exacerbated ALI as indicated by the increase in albumin leak (Fig. 9C).

While it is not entirely clear as to why an increase in injury level was not accompanied

by definitive changes in cytokine production, these data do suggest a protective role for

the catecholamines in activating the β_2 adrenergic receptor during ALI. Although this

unexpected result could be due simply to the pharmacokinetics of the β_2 adrenergic

receptor antagonist, another plausible explanation may involve the role of other

neurendocrine targets in controlling injury severity independent of the mechanism

provided above. The confirmation of an alternative pathway will demand further
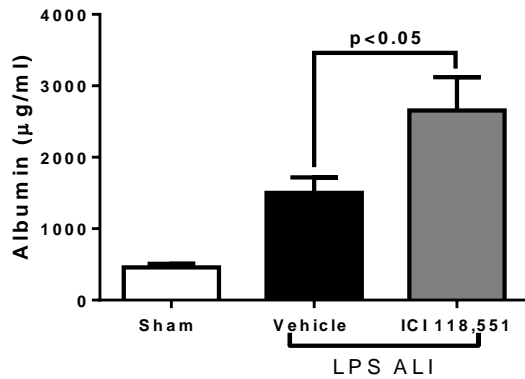
investigation.

A

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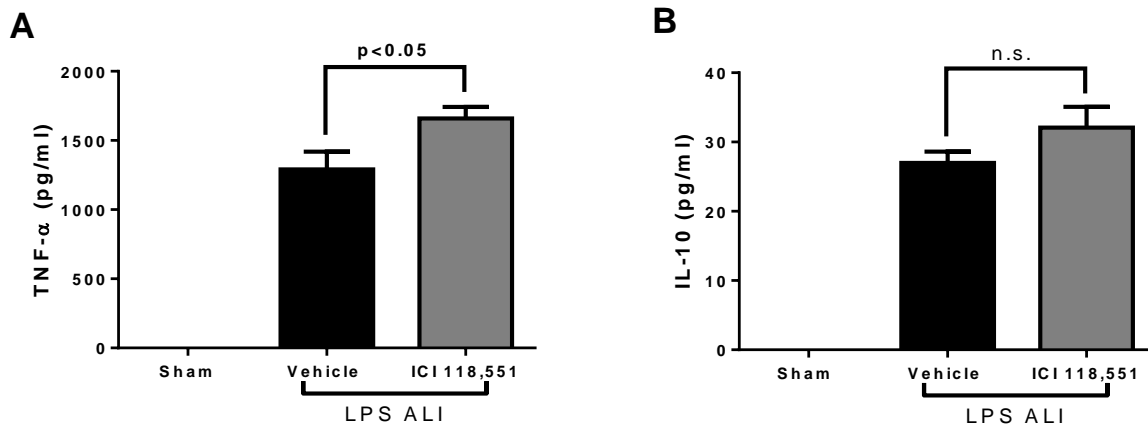
C

Figure 9. **Activation of the β_2 adrenergic receptor is protective during acute lung injury**. A-C) ALI was induced by 60 μg of LPS i.t. and BALF was harvested after 8 hours (n $\geq$ 9 mice per group). Administration of ICI 118,551 (25 mg/kg i.p. and 6 ng i.t.) was used to block the β_2 adrenergic receptor. A) TNF-α, B) IL-10, and C) albumin levels were determined by ELISA.

To add credence to our mechanism provided concerning the essential role of catecholamines in mediating acute inflammation, we decided to broaden our in vivo studies to include an experimental endotoxemia model. For this model, LPS is injected directly into the peritoneum to induce a systemic inflammatory response and doses can be adjusted to test for lethality. Because IL-10 is an essential cytokine in negatively regulating macrophage and Th1 cell pro-inflammatory responses during sepsis, we tested for the effect of β_2 adrenergic receptor blockade on IL-10 production during experimental endotoxemia. As shown in Figure 10A, injection of LPS i.p. upregulated the levels of IL-10 detected in mouse plasma. This concentration spiked at 2 hours following endotoxin administration and gradually declined over time with a 50%

reduction observed after approximately 8 hours (Fig. 10A). Importantly, administration of the ICI 118,551 inhibitor i.p. 1 hour prior to the induction of endotoxemia significantly lessened the levels of IL-10 quantified in serum after 1.5 hours with modest reduction still observed after 3 hours (Fig. 10B). These data suggest a role for catecholamines in depressing the inflammatory response through the upregulation of IL-10 during sepsis.

To substantiate these in vivo findings, we carried out two survival studies illustrating the protective role of catecholamines and their activation of the β_2 adrenergic receptor during endotoxemia. As shown in Figure 10C, administration of ICI 118,551 greatly attenuated survival in septic mice when compared to those receiving the vehicle control. While the inhibitor induced a marked effect, it is important to acknowledge its non-specific targets. More specifically, it has been widely documented that the β_2 adrenergic receptor is involved in regulating a number of physiological processes, such as cardiac function, vascular permeability, and metabolism, among others. To circumvent these issues, we performed a highly controlled experiment involving the adoptive transfer of macrophages during endotoxemia. Wild-type mice were administered previously elicited peritoneal macrophages (unstimulated, LPS pre-treated, or those stimulated with LPS in the co-presence of epinephrine in vitro) prior to the i.p. injection of LPS, and percent survival was measured. This experimental design allowed us to specifically illustrate the effects of catecholamines on inducing a shift in macrophage phenotype and their resulting physiological consequences during sepsis. As shown in Figure 10D, the adoptive transfer of macrophages pre-treated with LPS in the co-presence of epinephrine prior to the onset of endotoxemia greatly enhanced the survival of these mice when compared to mice receiving either the LPS-treated or

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untreated macrophages. Altogether, this elegant experiment effectively illustrated the

protective role of catecholamines in skewing macrophages towards an M2, anti-

inflammatory phenotype during acute inflammation induced by the administration of

gram-negative endotoxins. This effect is presumably achieved via the induction of a

M2/Th2-dominated response characterized by a lack of inflammation and subsequent
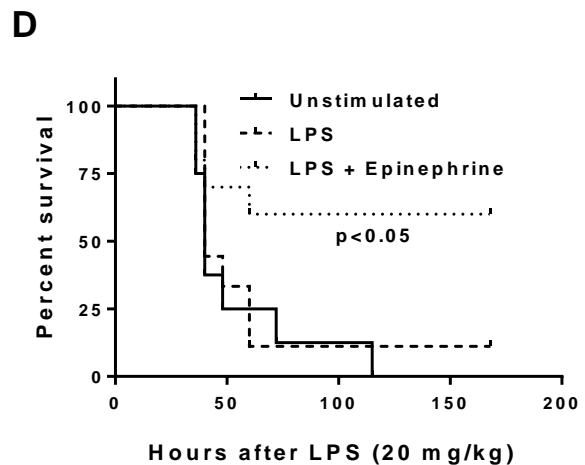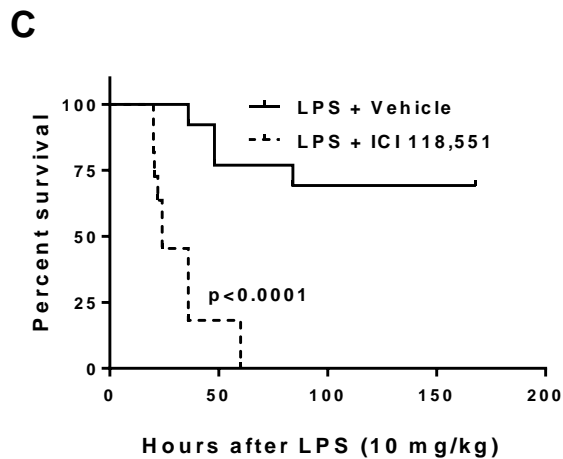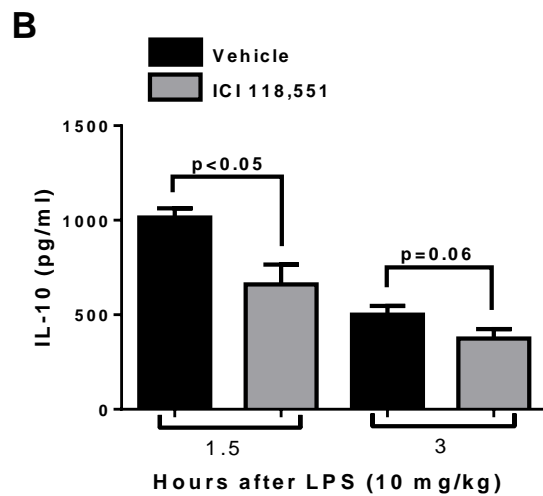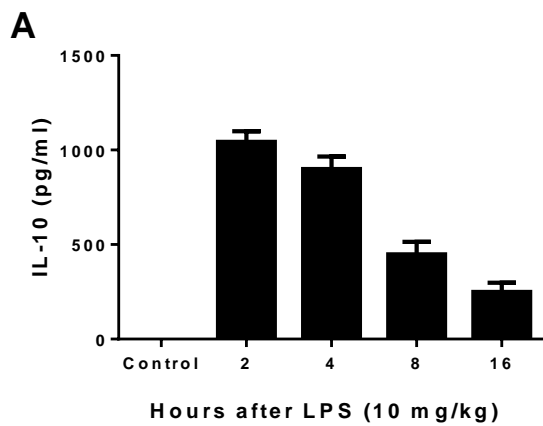
tissue damage.

Figure 10. **The M2 regulatory macrophage phenotype induced by catecholamines is protective during endotoxemia**. A) Serum IL-10 levels quantified by ELISA following the i.p. administration of 10 mg/kg body weight LPS (n = 4 mice per group). B) Endotoxemia induced as described in A. ICI 118,551 administered at 500 μg per mouse. Serum IL-10 levels as determined by ELISA. C) Survival curve following the administration of LPS as described in A with or without ICI 118,551 as described in B (n ≥ 11 mice per group). D) Macrophages were incubated in vitro with or without LPS (100 ng/ml) and epinephrine (10^{-6} M) for 1 hour. Cultured cells were harvested, washed, and transferred to mice. After 4 hours, endotoxemia was induced using 20 mg/kg body weight LPS (n ≥ 8 mice per group).

Discussion

It has been shown in previous studies that catecholamines can suppress the production of pro-inflammatory cytokines in human blood models [15-17]. The presence of epinephrine and norepinephrine at high concentrations specifically inhibited endotoxin-induced TNF-α, IL-6, and IL-1β production. In addition, our lab recently showed that synthetic β_2 adrenergic receptor agonists elicited similar expression profiles in macrophages in the context of LPS-induced acute lung injury [13]. In this report, we expanded upon these findings to show that while catecholamines do indeed suppress pro-inflammatory mediator production, their activation of the β_2 adrenergic receptor also plays a pivotal role in regulating macrophage phenotype during LPS-induced inflammation. Whereas LPS-stimulated macrophages elicited M1-type responses, macrophages stimulated in the co-presence of catecholamines assumed an anti-

inflammatory phenotype characterized by the production of potent Th2 T cell activators. Specifically, co-stimulated macrophages released suppressed levels of IL-12$_{(p40)}$ and TNF-α along with enhanced levels of IL-10. In addition, these macrophages displayed an M2 regulatory macrophage phenotype defined by the attenuated expression of iNOS, potentiated expression of arginase-1, and augmented phagocytic activity. Importantly, the enhanced expression of arginase-1 orients its substrate, L-arginine, towards the production of cell growth- and fibrosis-promoting mediators [6]. Thus, the M2 regulatory macrophage phenotype elicited by catecholamines is more anti-inflammatory in function with wound-healing capacities. Altogether, our data provide a novel mechanism by which epinephrine and norepinephrine activate the β_2 adrenergic receptor to exert regulation over macrophage polarization both in vitro and in vivo during acute inflammation (summarized in Fig. 11).
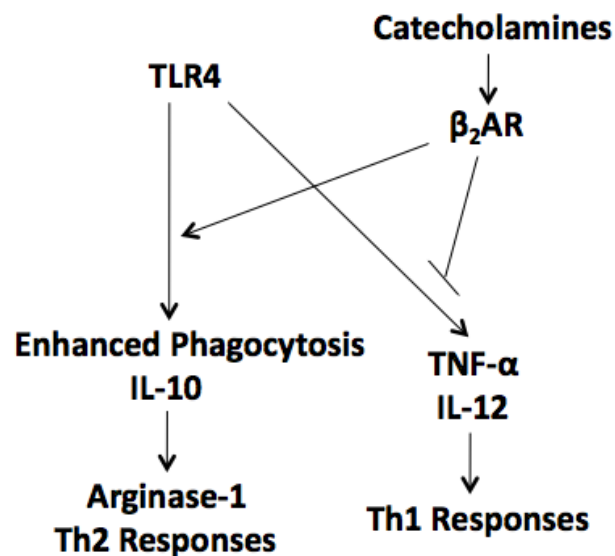

Figure 11. **Summary of the effects of catecholamines on promoting an M2, anti-inflammatory macrophage phenotype during TLR4-mediated inflammation**.

Interestingly, the regulation over intracellular TLR4 signaling demonstrated by catecholamines did not occur via the activation of a canonical β_2 adrenergic receptor signaling pathway. Rather, our data indicated that this modulatory effect was carried out independently of adenylate cyclase and PKA activation, thus indicating some reliance on the G protein $\beta\gamma$ or alternative Gα subunits. Building upon these results to fully elucidate the underlying intracellular mechanism could provide additional therapeutic options for manipulating these pathways in a clinical setting. Nonetheless, our studies illustrating the essential downstream role for PI3K in mediating a catecholamine-induced M2 macrophage phenotype are in agreement with previous reports illustrating its function in similar contexts [18,19].

An important mediator involved in the skewing of macrophages towards the M2 phenotype is IL-10 [1]. We have shown that neutralization of this cytokine negated the effects of catecholamines on influencing macrophage polarization in vitro. Importantly, our work also illustrated that the induction of macrophage IL-10 production by epinephrine/norepinephrine could not proceed without the concomitant activation of TLR4. This requirement for the simultaneous activation of a TLR and a signaling modulator has been demonstrated elsewhere in the context of prostaglandin e2 [20]. Thus, it is important to note that catecholamines do not directly exert transcriptional control over macrophage phenotype determinants, but rather, this effect involves the production and secretion of an autocrine/paracrine factor. While we conclude that the catecholamines exert their regulation to limit pro-inflammatory pathways active during acute inflammation, we must also carefully consider the context in which these hormones are acting. Although the secretion of catecholamines is enhanced upon an

inflammatory stimulus [10], this is also true of many known macrophage phenotype regulators. Thus, understanding how catecholamines fine-tune the immune response to limit inflammation will be pivotal in characterizing the immunoregulatory roles of these neuroendocrine factors in vivo.

To illustrate the protective effects of catecholamines alluded to above, we performed in vivo ALI and endotoxemia experiments in the presence and absence of the β_2 adrenergic receptor inhibitor, ICI 118,551. Mice receiving the antagonist demonstrated severely exacerbated injury levels presumably characterized by distinct pathways. For the LPS-induced ALI model, β_2 blockade was accompanied by increased epithelial and vascular damage and TNF-α production, whereas IL-10 secretion was not significantly altered. Conversely, endotoxemic mice receiving the receptor antagonist demonstrated significantly reduced serum IL-10 levels. While the bases for these disparities are unclear, they could be manifested if catecholamines are to differentially modulate peritoneal and alveolar macrophage biology in vivo. Another plausible explanation concerns the presence of an alternative pathway by which catecholamines could regulate lung injury severity. Consequently, the characterization of additional neuroendocrine targets (e.g., vascular endothelial cells) may provide for novel therapeutic developments in the treatment of diseases elicited by pulmonary inflammation. Nonetheless, we have provided substantive evidence for the protective role of catecholamines in regulating macrophage phenotype during sepsis. Specifically, we demonstrated that endotoxemic mice adoptively receiving peritoneal macrophages pre-treated with LPS and epinephrine in vitro exhibited impressive survival rates. Thus,

it is clear that the induction of an M2-dominated response during sepsis circumvents issues arising from excessive inflammation and the resulting tissue damage.

Although these experiments suggest a protective role for catecholamines in regulating inflammation during the acute phase of sepsis, an interesting clinical question arises from their effect in the long term. Specifically, while septic patients are often treated with catecholamines upon the disease's onset to bolster cardiovascular function, mortality often occurs at later time points due to immunosuppression, and in consequence, contraction of opportunistic infections. It remains to be seen whether the induction of an M2 phenotype through the administration of β_2 adrenergic receptor agonists could render septic patients immunocompromised, but with a mortality rate ranging from 20% to 30% and a prevalence of about 600,000 cases occurring annually in the United States [21,22], it is clear that answering this question could profoundly impact clinical survival rates. Another finding that demands a mechanistic explanation comes from van der Poll's early studies illustrating that catecholamines could inhibit the LPS-induced production of IL-1β in human whole blood samples [17]. In a dose-dependent fashion, increased levels of intracellular cAMP decreased IL-1β production from human monocytes, though this was not specifically attributed to an effect brought about by catecholamines [17]. It is now known that a large contributor of circulating IL-1β during endotoxemia comes from an intracellular protein complex called the NLRP3 inflammasome [23]. This complex becomes activated by microbial endotoxins (among other molecules) and cleaves a precursor protein to generate mature IL-1β [23]. While it has recently been shown that intracellular cAMP can act to negatively regulate the activation of this complex [24], no work has been done to illustrate the effects of

catecholamines on its activity during bacterial infections. These studies could also provide an additional seminal discovery concerning the role of catecholamines in modulating acute inflammation.

In summary, we report an important function for the β_2 adrenergic receptor in regulating macrophage phenotype. Specifically, simultaneous activation of the β_2 adrenergic receptor and TLR4 skews macrophages from an LPS-induced M1 phenotype towards an M2 regulatory macrophage phenotype. This effect occurs through a non-canonical β_2 adrenergic receptor signaling pathway with downstream dependence on PI3K. Catecholamines regulate LPS-induced cytokine and phenotype marker expression via this signaling mechanism to bring about enhanced IL-10 production, potentiated arginase-1 expression, and augmented phagocytic activity. Consequently, these neuroendocrine factors confer protection during acute inflammation by inducing a subsequent Th2-dominated, anti-inflammatory immune response. Much of the data presented herein is included in a report entitled "Induction of M2 Regulatory Macrophages through the β_2-Adrenergic Receptor with Protection during Endotoxemia and Acute Lung Injury" that was recently accepted for publication by the Journal of Innate Immunity.

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scientist.