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Interleukin-1 Receptor / Toll-like Receptor Signaling: A Therapeutic Target for Pulmonary Inflammatory Disease

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Karen Laguren Saroca

Committee in charge:

Professor Timothy D. Bigby, Co-Chair
Professor Michael David, Co-Chair
Professor Stephen M. Hedrick

2008

The Thesis of Karen Laguren Saroca is approved and it is acceptable in quality and form for publication on microfilm:

Co-Chair

Co-Chair

University of California, San Diego

2008

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LIST OF ABBREVIATIONS

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
CBT	core body temperature
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
Eos	eosinophils
EtOH	ethanol
FITC	fluorescein isothiocyanate
H&E	hemotoxylin & eosin
IFN-β	interferon beta
Ig	immunoglobulin
IκB	inhibitor of κB
IKK	inhibitor of κB kinase
IL-1β	interleukin-1 beta
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IL-1RAcP	interleukin-1 receptor accessory protein
IP	intraperitoneal
IRAK	interleukin-1 receptor associated kinase
IRF3	interferon regulatory factor 3
IT	intratracheal
IV	intravenous
LPS	lipopolysaccharide
Lymphs	lymphocytes
MØ	macrophages
MAL	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
MyD88	myeloid differentiation factor 88
Neutros	neutrophils
NFκB	nuclear factor κB
Ova	ovalbumin
PAS	periodic acid Schiff
PBS	phospho-buffered saline
SARM	sterile α and armadillo-motif-containing protein
SNS	sterile normal saline
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TAK	transforming growth factor activator kinase
Th2	T helper 2
TICAM	TIR-domain-containing adaptor protein inducing interferon-β
TIR	Toll/IL-1R signaling domain
TIRAP	TIR adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
Ub	ubiquitin

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ABSTRACT OF THE THESIS

Interleukin-1 Receptor / Toll-like Receptor Signaling:
A Therapeutic Target for Pulmonary Inflammatory Disease

by

Karen Laguren Saroca
Master of Science in Biology
University of California, San Diego, 2008
Professor Timothy D. Bigby, Co-Chair
Professor Michael David, Co-Chair

Numerous studies have shown the importance of interleukin-1β (IL-1β) and IL-1 receptor (IL-1R) / Toll-like receptor (TLR) signaling in the development of pulmonary inflammation, including acute lung injury (ALI) and asthma. Thus, inhibition of IL-1β receptor signaling is a potential therapeutic target in the treatment of ALI and asthma. EM-163 is a small organic molecular mimic synthesized by the Rebek laboratory of the Scripps Research Institute based on molecular modeling of the crystal structure of the IL-1β receptor with its adaptor protein. The Rebek laboratory has shown that this compound inhibits IL-1β signaling, *in vitro*.

In vivo studies were done in C57BL/6 or BALB/c mice using a LPS model of ALI and an ovalbumin model of asthma. Inhibition of IL-1 signaling in these models

was done to determine the efficacy of EM-163 in the development of ALI and modulating allergic inflammation in an asthma model. *In vitro* studies are being conducted to determine specificity of EM-163 for targeting IL-1R signaling.

In the ALI model, EM-163 showed attenuation of LPS-induced neutrophilic inflammation, capillary leak, and loss of lung compliance. In the ovalbumin model of asthma, IL-1β inhibition attenuated airway hyperresponsiveness in C57BL/6 mice, while effects on inflammation were minimal. In BALB/c mice, airway inflammation was reduced.

These results suggest EM-163 will be effective in treatment of ALI and asthma, supporting the targeting of IL-1R signaling in the treatment of inflammatory disease.

Introduction

Immunity: Innate and Adaptive

Immune responses can be characterized as either innate or adaptive. Adaptive immunity utilizes a diverse set of antigen receptors that are created by gene rearrangement or somatic hypermutation. Antigen recognition sites on antibodies and T cell receptors in adaptive immunity are thus created specifically for each new antigen encountered. In addition, immunological "memory" is characterized as a component of adaptive immunity, with a more powerful and rapid response upon re-exposure to the same antigen. Adaptive immune responses are slower, but last longer, while innate immune responses are rapid and transient. Though distinguished by significant differences, the innate and adaptive arms of the immune system are integrated as a single defense. The interaction of innate and adaptive immunity has recently gained more interest, as adaptive responses can be regulated by the innate system (Kanzler, et al. 2007). The integration of the two systems is further seen in that adaptive immune responses are dependent upon antigen presentation and cytokine production by cells of the innate system (Beutler, 2004).

Innate immunity has both cellular and humoral elements, which each include an afferent sensor arm and an efferent effector arm (Beutler, 2004). Cell-based immunity relies on phagocytic myeloid cells, which include macrophages, dendritic cells, neutrophils, basophils, and eosinophils. Mast cells are also myeloid and mediate allergic responses. Sensing by these cells is done by a limited number of receptors which recognize specific, individual molecules with conserved structures.

A well-characterized example of these molecules is lipopolysaccharide (LPS), a component of gram-negative bacterial wall which is recognized by Toll-like receptor 4 (TLR4), an innate immune receptor, and causes the release of cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6). This cytokine release leads to various responses which result in "fever, hypotension, inadequate tissue perfusion, metabolic acidosis, and organ failure" (Beutler, 2004). An increased understanding of innate immunity can be applied toward therapeutics for disease (Kanzler, et al. 2007). One such therapeutic target in the innate immune system are the Toll-like receptors (TLRs).

Toll-like Receptors

Toll-like receptors were discovered first in *Drosophila*, whose toll gene is crucial for antifungal immunity. Other species have a number of homologous TLRs. The toll-like family of receptors function in microbial sensing, with each TLR sensing a separate set of ligands. The use of a single family of receptors in microbial sensing among divergent species is remarkable. (Beutler, 2004). Vertebrates have ten to twelve TLRs, while sea urchins have more than 200. TLRs are membrane proteins characterized by a cytoplasmic Toll/interleukin-1 receptor homology signaling domain, or TIR domain, as well as an antigen recognition domain. In humans, the ten functional TLRs are divided according to their location in the cell. Cell surface TLRs that are activated and then migrate to phagosomes include TLR 1, 2, 4, 5, 6 and 10. TLRs 3, 7, 8, and 9 are located in intracellular compartments such

as endosomes and the endoplasmic reticulum, with antigen recognition domains sampling the internal compartment (Kanzler, et al. 2007).

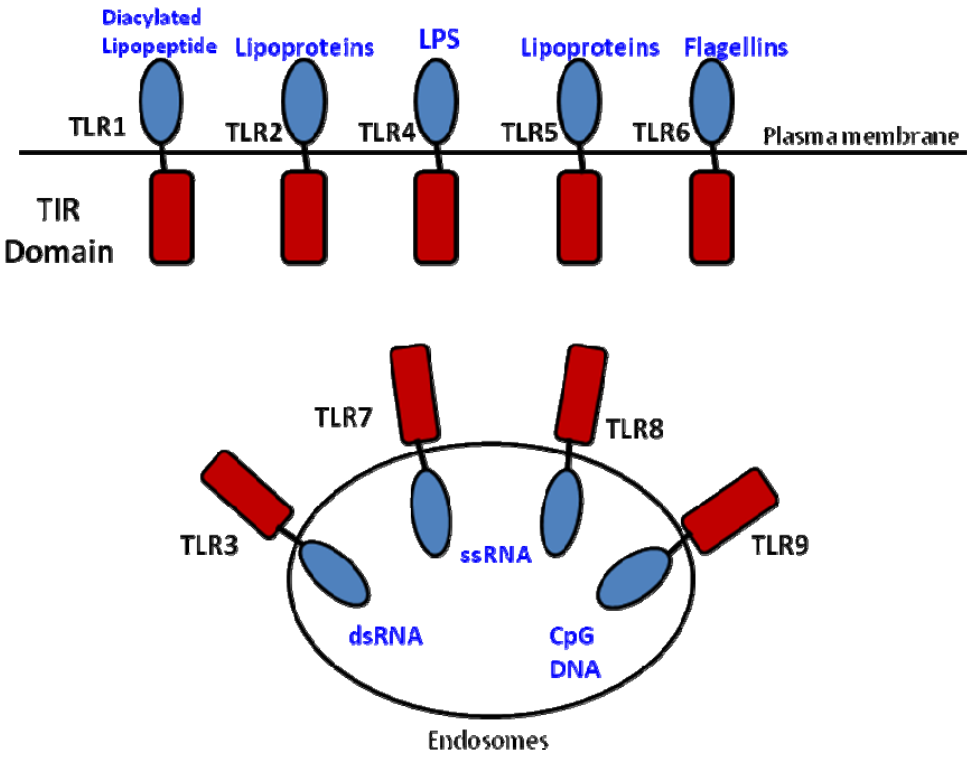


Figure 1. Toll-like receptor ligands and cellular location. TLRs 1, 2, 4, 5, and 6 are located on the plasma membrane, while TLRs 3, 7, 8, and 9 are located in intracellular compartments such as endosomes. Each TLR recognizes a discrete set of ligands. (Figure modified from Krishnan, et al. 2007).

Toll-like receptors are potent stimulators of immune responses such as inflammation; stimulation of TLRs can result in disease. Thus, TLR signaling must be tightly controlled. This can be seen in the initiation of TLR signaling, in which TLRs function as dimers. These dimers are thought to exist in a low-affinity complex until the ligand binds. TLR4, TLR9, TLR3, and TLR5 homodimerize to recognize LPS, unmethylated CpG-containing DNA, dsRNA, and bacterial flagellin, respectively. Heterodimerization occurs between TLR1 and TLR2 to sense bacterial

triacylated lipopeptides. TLR2 also dimerizes with TLR6 to recognize bacterial diacylated lipopeptides. TLR8 dimerizes with TLR7 to sense ssRNA. Additionally, TLR8 dimerizes with TLR9. TLR7 and TLR9 signaling are antagonized by TLR8, while TLR9 interaction with TLR7 also antagonizes TLR7 signaling. These interactions highlight the complexity of dimerization function among TLRs (O'Neill and Bowie, 2007).

Following ligand binding, conformational change brings the two cytosolic TIR domains of the TLRs closer together in preparation for signaling. There are five human adapter proteins containing TIR domains involved in TLR signaling. These are MyD88, MAL (MyD88-adapter-like, also TIRAP), TRIF (TIR-domain-containing adaptor protein inducing interferon-β, or TICAM1), TRAM (TRIF-related adaptor molecule, or TICAM2), and SARM (sterile α- and armadillo-motif-containing protein), which interferes with TRIF function. These adaptor proteins have TIR domains which interact with the TIR domain on TLRs (O'Neill and Bowie, 2007). This TIR:TIR interaction leads to the activation of a signaling cascade which leads to cellular activation. A "core" signaling pathway involving TLRs begins with MyD88 recruitment of IRAK-4 (interleukin-1 receptor associated kinase 4), which then phosphorylates IRAK-1 and IRAK-2 to possibly act as scaffolds. This then results in recruitment of TRAF-6 (TNF receptor associated factor 6) to activate TAK1 (transforming growth factor activator kinase 1), followed by IKK-γ (IκB kinase) phosphorylation. This activation of IKK-γ leads to phosphorylation and

degradation of IκB (inhibitor of NFκB), resulting in free NFκB for nuclear translocation and transcriptional activation of cytokine genes (Beutler, 2004).

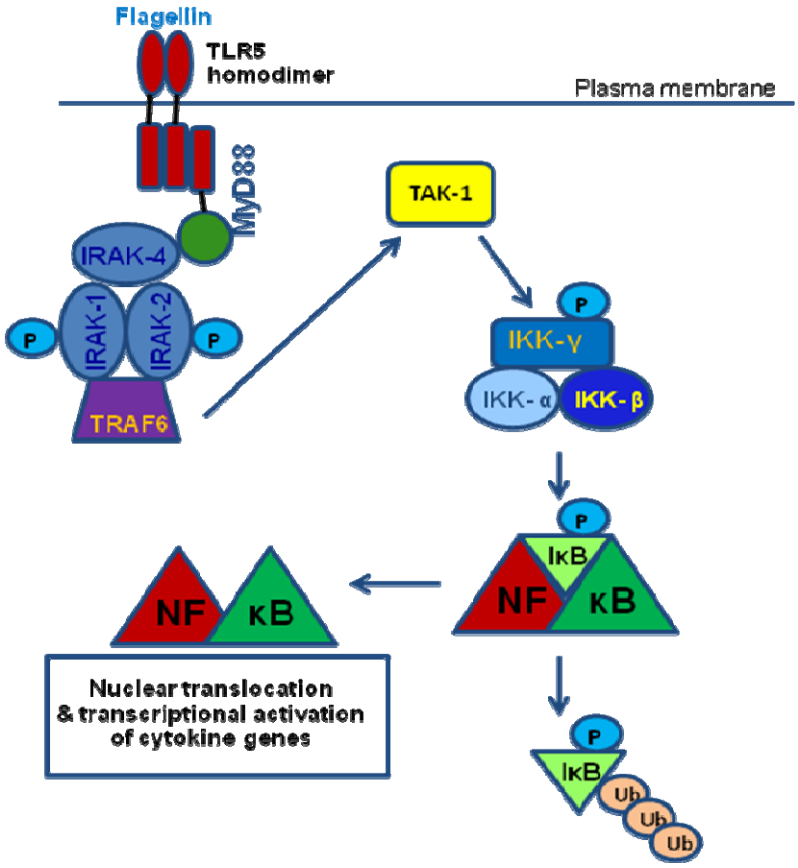


Figure 2. A "Core" Signaling Pathway in Mammalian Toll-like Receptors. MyD88-dependent signaling in several mammalian TLRs acts through activation of IRAKs, TRAF-6, and TAK-1 to activate the IKK signalosome. This promotes IκB ubiquitin-mediated degradation, releasing NFκB for nuclear translocation and transcriptional activation of cytokine genes. (Figure modified from Krishnan, et al. 2007).

Most mammalian TLRs use this "core" signaling pathway. However, there are exceptions in which signaling pathways are dependent on TRIF rather than MyD88. Such signaling leads to IRF-3 (interferon regulatory factor 3) activation and up-regulation of interferon-β (IFN-β) production and response to antigen. IFN-β can induce further effects through the STAT pathway. Examples of TRIF-dependent

signaling are TLR3 signaling, which is entirely independent of MyD88, and one MyD88-independent arm of TLR4 signaling. Another modification is the use of MAL with MyD88 signaling done by TLRs 2 and 4 (Beutler, 2004).

Interleukin-1 Receptor Signaling

A later evolutionary event resulted in a "modified TLR." Rather than leucine-rich repeats in the receptor ectodomain characteristic of TLRs, related receptors contain Ig motifs, the basis of the IL-1 receptor. The IL-1 receptor (IL-1R) is a member of the TLR/IL-1 family and has a cytoplasmic Toll/IL-1 Receptor (TIR) domain. IL-1β is a pro-inflammatory cytokine that plays a predominant role in inflammatory responses (Goodman, et al. 2003). When IL-1β binds the IL-1R, it is thought that a heterodimer of IL-1R and the IL-1 receptor accessory protein (IL-1RAcP) is formed at their cytoplasmic TIR domains (Li, et al. 2005). This dimerization leads to recruitment of a complex of proteins including myeloid differentiation primary-response protein 88 (MyD88), which has a TIR domain interacting with the receptor and a death domain interacting with other proteins. The death domain activates Interleukin Receptor-Associated Kinases (IRAK) 1-4 (Frobose, et al. 2006). IRAK1-P then dissociates from MyD88 and associates with Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6). A series of further signal transduction molecules are then activated, including Transforming Growth Factor Activated Kinase (TAK1) and the Inhibitor of nuclear factor κB kinase (IκB) complex. These signaling events lead to activation of mitogen-activated protein

(MAP) kinase p38 phosphorylation and activation of the transcription factor nuclear factor κB (NFκB), which regulate several inflammatory response pathways.

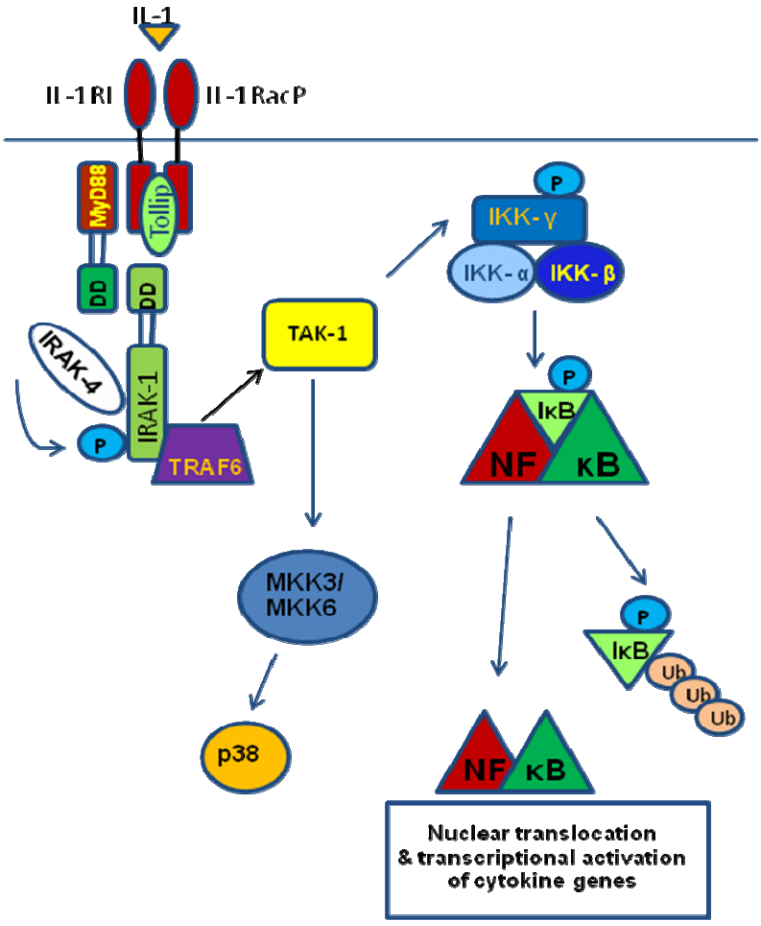


Figure 3. IL-1R Signaling Pathway. Binding of MyD88 and IL-1R at the cytoplasmic TIR domains initiates a signaling cascade which triggers IRAKs and TRAF6 for TAK1 activation, ultimately leading to the p38 MAP kinase or NFκB pathways (Figure redrawn and modified from Frobose, et al. 2006 and O'Neill and Bowie, 2007).

Numerous studies have shown the importance of interleukin-1β (IL-1β) and IL-1R/TLR signaling and the common adaptor protein, MyD88, in the development of pulmonary inflammation. Such studies have focused on acute lung injury and asthma in particular.

IL-1R/TLR Signaling and Acute Lung Injury

Acute lung injury is characterized by neutrophilic inflammation, endothelial and epithelial injury at the alveolar level. This injury is present at the alveolar-capillary interface, and is associated with the leak of edema fluid into the alveolus. The characteristic neutrophilic inflammation is initiated by a variety of cytokines, in which IL-1β is prominent (Goodman, et al. 2003) because it stimulates production of an array of mediators in response to injury. In addition to the IL-1 receptor I (IL-1RI), IL-1β is regulated by an IL-1 receptor antagonist (IL-1Ra) and IL-1 receptor II (IL-1RII). Both IL-1β and IL-1Ra have been identified in the lung lining fluid of ARDS patients (Goodman, et al. 1996; Park, et al, 2001; Donnelly, et al, 1996). Normally, IL-1β is present at a 1:1 stoichiometry with IL-1Ra (Arend, 1993). Mao and colleagues argue that imbalance of the IL-1Ra/IL-1β ratio is a key characteristic of asthmatic inflammation in the airway (Mao, et al. 2000). This imbalance is also present in patients with ARDS, with IL-1β and IL-1Ra at an approximate 10:1 stoichiometric ratio. This identifies a possible role for IL-1β in persistent inflammation in ARDS patients (Goodman, et al. 1996). This imbalance is further observed in ARDS patients who have high IL-1RII concentrations in bronchoalveolar lavage fluid (Park, et al. 2001), as the inhibitory activity of IL-1Ra is enhanced when IL-1RII binds IL-1β (Burger, et al., 1995). Also, the inhibitory activity of IL-1Ra can be hindered under inflammatory conditions when IL-1RI maintains binding to IL-1Ra (Burger, et al. 1995). The interaction between IL-1β and its antagonists is further complicated by the required binding of the IL-1 receptor

accessory protein (IL-1RAcP) to the IL-1 receptor for successful signal transduction (Jensen, et al. 2000). In the lung lining fluid of patients with ARDS, a net pro-inflammatory activity results from IL-1β interacting with its antagonists and other proteins (Park, et al. 2001). These studies show that IL-1β plays a key role in acute lung injury. Inhibition of downstream IL-1β signaling is a promising target for treatment of this disease. As the variety of therapies attempted to treat acute lung injury have proven largely unsuccessful, new effective therapies are required.

IL-1R/TLR Signaling and Asthma

Asthma is a chronic inflammatory disease which is common worldwide. An elevated production of Th2 cytokines, infiltration of inflammatory cells, and airway hyperresponsiveness are characteristics of this disease (Wang, et al. 2006). Following accumulation of inflammatory cells, mediators are released which are involved in tissue damage and dysfunction of airway smooth muscle. Highly inflammatory cytokines IL-1β and IL-33 are involved in asthma, and when their expression is not regulated, can lead to severe pathological effects. Clinical studies have shown that increased IL-1β is associated with asthma and chronic obstructive pulmonary disease (Chung, 2001; Rusznack, et al. 2000; Joos, et al. 2001), and that prevention of IL-1β signaling attenuates airway inflammation and hyperresponsiveness in a murine model of asthma (Johnson, et al. 2005). These studies point to the importance of IL-1β signaling as a therapeutic target. Previous and current treatments for asthma are limited by numerous side effects, or are ineffective for many individuals.

Targeting the TIR Domain

IL-1R/TLR signaling is an important therapeutic target in pulmonary inflammatory diseases such acute lung injury and asthma. A point mutation in the TIR domain of the TLR4 lipopolysaccharide receptor prevents recruitment of adaptor proteins, causing mutant mice to be resistant to endotoxin (Poltorak, et al. 1998). This study highlights the importance of the TIR domain in signaling. By blocking the TIR domain association of IL-1R and MyD88, signaling is disrupted and consequently, NFκB remains in its inactive form. This will lower the secretion of associated cytokines and adhesion molecules in asthmatics.

Structural Inhibition of TIR:TIR Domain Interactions

The X-ray structure of the human TLR2 TIR domain reveals a structure consisting of five-stranded parallel β-sheets and five surrounding α helices connected by loops (Xu, et al. 2000). In addition to IL-1R/TLRs, adaptor proteins contain TIR domains with distinct surface properties. It is thought that electrostatic complementarity between the TIR domains of adaptor proteins and TLRs allows for their specific interactions. Two loop regions are present in each TIR domain: the BB loop and DD loop, and are required for TIR:TIR interactions (O'Neill and Bowie, 2007). A key surface in TIR domain protein-protein interactions, the BB loop with (F/Y)-(V/L/I)-(P/G) amino acid consensus sequences is conserved in different TLRs, IL-1R and MyD88. Bartfai and colleagues have synthesized a mimetic based on these protruding amino acids of the MyD88 BB loop which interferes with MyD88/IL-1R interaction, thus disrupting IL-1β signaling.

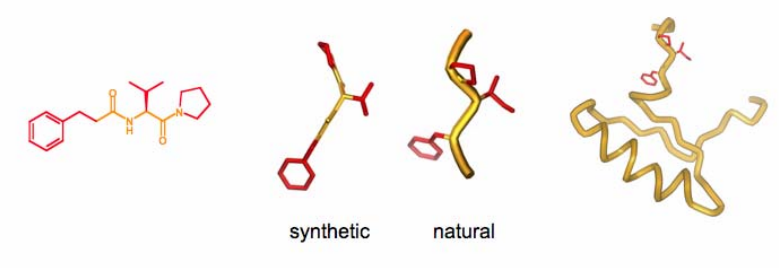


Figure 4. β barrel loop mimetic and the β barrel loop of the TIR domain.
(Rebek, J. The Scripps Research Institute.)

In vitro testing of the activity of small molecule BB loop mimetics to inhibit MyD88/IL-1R association and block IL-1β signaling have been done by Bartfai and colleagues (Bartfai, et al. 2003). In this study, the small molecule inhibitor used was the compound AS-1. The phosphorylation of p38 MAPK is a target of IL-1β signaling, and was used to determine the efficacy of AS-1 in EL-4 murine thymoma cells and murine lymphocytes, as well as human THP-1 cells. AS-1 was shown to inhibit p38 MAPK activation mediated by IL-1β in EL4 cells (Figure 5A). Specificity for targeting IL-1R signaling was demonstrated by co-immunoprecipitation and sandwich ELISA assays detecting protein-protein interactions between MyD88 and IL-1R (Figure 5B, Figure 6).

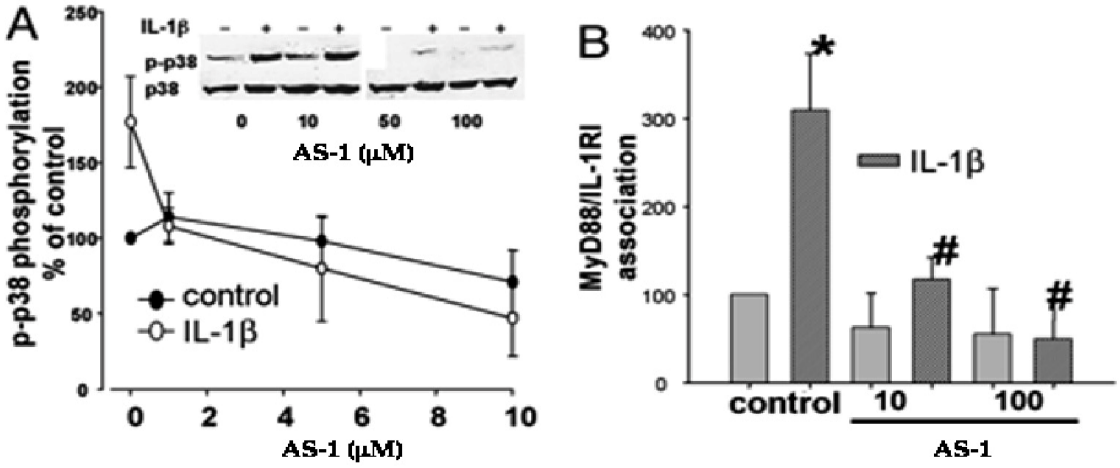


Figure 5. BB loop mimetic AS-1 inhibits IL-1β-mediated p38 MAPK phosphorylation and IL-1β-mediated MyD88/IL-1R association in murine EL4 cells. A) EL4 cells incubated with or without AS-1 were also exposed to IL-1β or water (control). Western blotting showed greater inhibition of p38 phosphorylation with increasing AS-1 concentration. B) Cells treated same as in (A), and analyzed by sandwich ELISA for MyD88/IL-1R association. Preincubation with AS-1 prevented IL-1β mediated MyD88/IL-1R association. # = statistical significance with respect to IL-1β at $P < 0.05$ by ANOVA followed by Tukey test. (Bartfai, et al. 2003)

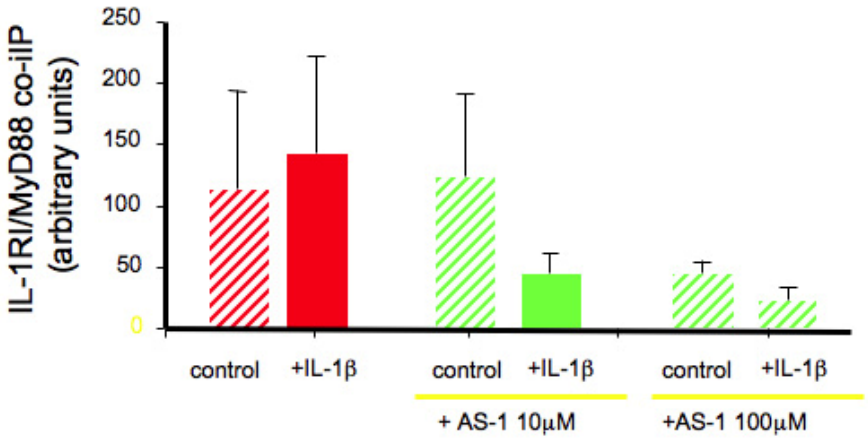


Figure 6. AS-1 inhibits co-immunoprecipitation of MyD88/IL-1R. Assay was performed using antibody specific for MyD88 followed by Western blots with IL-1R antibody. (Rebek, J. The Scripps Research Institute.)

EM-163 is another mimetic which has been developed to specifically inhibit IL-1β signaling and expression of inflammatory genes by preventing TIR domain interaction between IL-1R and MyD88 (Bartfai, et al. 2003; Davis, et al. 2005). *In vitro* results have shown EM-163 to block IL-1β-mediated p38 MAPK phosphorylation at a lower concentration than AS-1, indicating more promising potential as a mimetic compound (Figure 7).

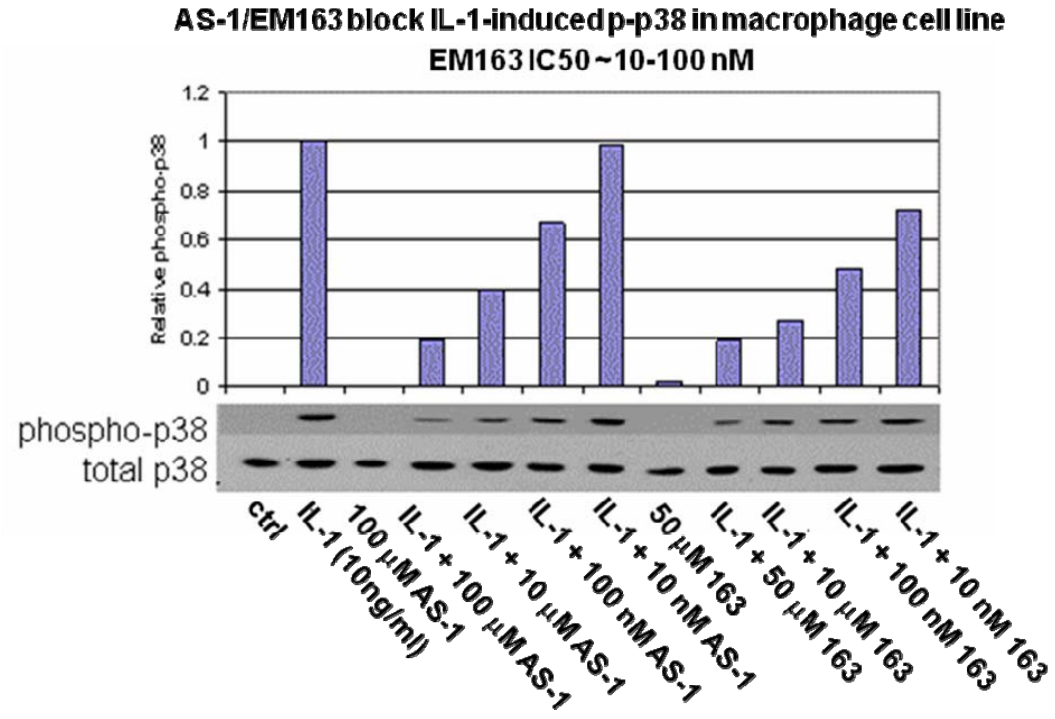


Figure 7. AS-1 and EM-163 inhibit IL-1β-mediated p38 phosphorylation in murine macrophages. Bar graph shows average of two independent experiments corresponding to Western blots. (Rebek, J. The Scripps Research Institute.)

Additionally, Bartfai and colleagues have conducted *in vivo* studies of the efficacy of BB loop mimetics in an animal model of fever, an IL-1β-induced response. In this model, intraperitoneal injection of IL-1β induces fever which peaks

after two hours. A telemetry device was implanted into the peritoneal cavity and used to measure core body temperature (CBT). Following one hour baseline recording of CBT, pre-treatment with AS-1, EM-163, or vehicle was done (P) and a second treatment (0) was administered after 20 minutes. Animal handling increased CBT, but this effect was independent of IL-1β and unaffected by AS-1 or EM-163. Using this model, it was shown that EM-163 is effective in inhibiting fever induced by IL-1β (Figure 8).

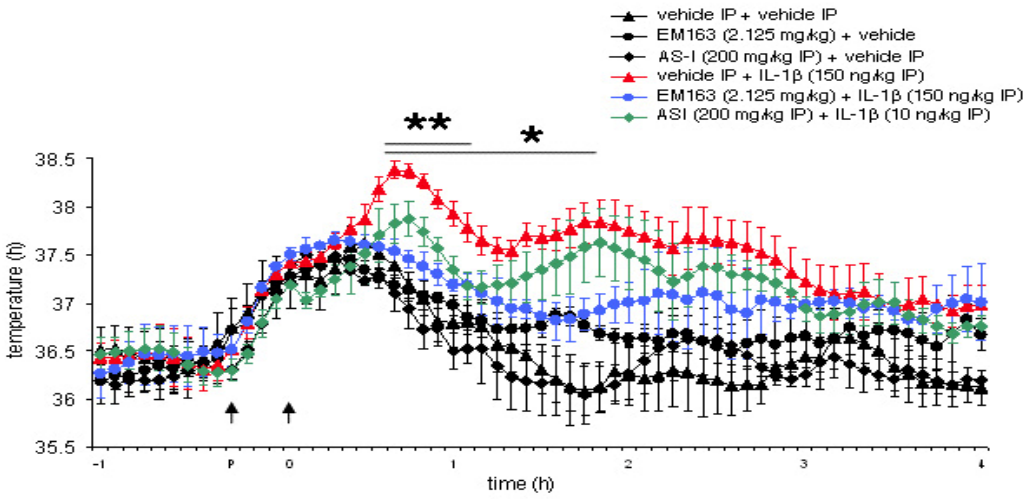


Figure 8. EM-163 inhibits fever response induced by IL-1β. Vehicle (saline) + IL-1β treatment results in elevation of CBT for 3 hours (red). AS-1 pretreatment (green) significantly decreased fever, while pretreatment with EM-163 (blue) improves this inhibition of IL-1β-induced fever response. (Rebek, J. The Scripps Research Institute.)

The efficacy of EM-163 is tested further in this following paper through *in vivo* studies using murine models of pulmonary inflammatory disease. It is hypothesized that inhibition of IL-1R signaling through targeting TIR:TIR domain interaction using EM-163 will attenuate inflammation characteristic of acute lung injury and asthma. The aims of this study are to determine, *in vivo*, whether EM-163

treatment alleviates inflammation, capillary leak in the lung, and loss of static lung compliance induced by LPS in a murine acute lung injury model and whether EM-163 treatment reduces airway hyperresponsiveness and eosinophilia in a murine ovalbumin model of asthma.

Materials and Methods

I. Mice

Six-week old male C57BL/6 or BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed standard Teklad 7001 diet and were studied in accordance with NIH Guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committees of the University of California, San Diego and the VA San Diego Healthcare System.

II. LPS-Induced Acute Lung Injury

Intratracheal instillation of 100 μg of RE595 Salmonella Minnesota LPS was done in BALB/c or C57BL/6 mice using the laryngoscopy technique by Hastings (Hastings, et al. 1999).

A. Measurement of Static Compliance of the Lung

Six hours after LPS instillation, mice were induced with 3% Isoflurane followed by injection of a ketamine/xylazine (20 mg/kg / 20 mg/kg) cocktail. Mice were maintained at 2% Isoflurane, intubated (Hastings, et al., 1999), placed on a computer-controlled small animal ventilator (Flexivent, SCIREQ, Montreal, Canada) and paralyzed with 100 μl of 100mg/ml pancuronium bromide. Two total lung capacity maneuvers were performed, followed by calculation of quasi-static compliance and quasi-static elastance using Flexivent 5.1.1 software. Compliance measurements reflect the pressure of lung recoil associated with a given volume,

while elastance is the reciprocal of compliance. These parameters were used to measure stiffness of the lung induced by acute lung injury.

B. Collection of Cells, BALF, and Serum

Following measurement of lung function, the animal was then exsanguinated by collection of a blood sample from the heart, cannulation of the right heart and perfusion with PBS until clear. Lungs were lavaged three times with 0.5 ml of PBS. Cells from bronchoalveolar lavage fluid were counted, cytocentrifuged, and then stained with May-Grunwald and Giemsa stains to determine cell differentials. Cells were centrifuged to form a pellet and bronchoalveolar lavage fluid was collected for analysis.

C. Histology

Lungs were fixed and preserved with 10% formalin, embedded, sectioned, and stained with hemotoxylin and eosin (H&E).

D. Measurement of Capillary Permeability in the Lung

Capillary leak in the lung was measured using a modification from a previous study (Guery, et al. 1999) by injecting 100 μg of 40,000 MW FITC-dextran into the tail vein two hours following LPS intratracheal instillation. Serum and bronchoalveolar lavage fluid (BALF) was collected after sacrifice at hour six. The ratio of fluorescence of FITC-dextran in BALF compared to serum were measured using a fluorometer set at 435/535 nm for 1 second. This ratio was multiplied by 100 to give the lung leak index.

E. Inhibition of IL-1R signaling in Acute Lung Injury Model

EM-163 was delivered via intraperitoneal injection fifteen minutes prior to LPS instillation and at the third hour of the time course.

F. *In Vitro* Studies

RAW264.7 cells (#TIB-71, ATCC, Manassas, Virginia) were grown in RPMI with 10% fetal calf serum, penicillin and streptomycin and allowed to become confluent before passage. A vector containing an NFκB promoter fused to luciferase (NF-kB (1) Luciferase Reporter; Panomics; Freemont, CA) was transiently transfected into RAW264.7 cells using Fugene HD transfection reagent (Roche Applied Science, Indianapolis, Indiana). Forty-eight hours later, cells were stimulated with 10 ng/ml LPS, LPS with 20% ethanol, or LPS with EM-163 for six hours and compared to untreated controls and cells treated with 20% ethanol alone.

III. Ovalbumin Model of Asthma

A. Immunization and Sensitization

A twenty-one day immunization and sensitization model using ovalbumin was modified from a previous study (Velasco, et al. 2005). C57BL/6 or BALB/c animals were immunized by intraperitoneal injection of 50 µg ovalbumin and 1 mg alum in phosphate-buffered saline (PBS) on day 0 and day 7. On days 17, 18, 19, and 20 animals were challenged by intratracheal instillation of 20 µg ovalbumin in 50 µl PBS. This was done using a direct laryngoscopy technique (Hastings, et al., 1999), in which animals were exposed to 3% Isoflurane for 1 minute with

supplemental oxygen, then held upright and the solution was instilled with a gel-loading pipette under visualization with a pediatric otoscope speculum.

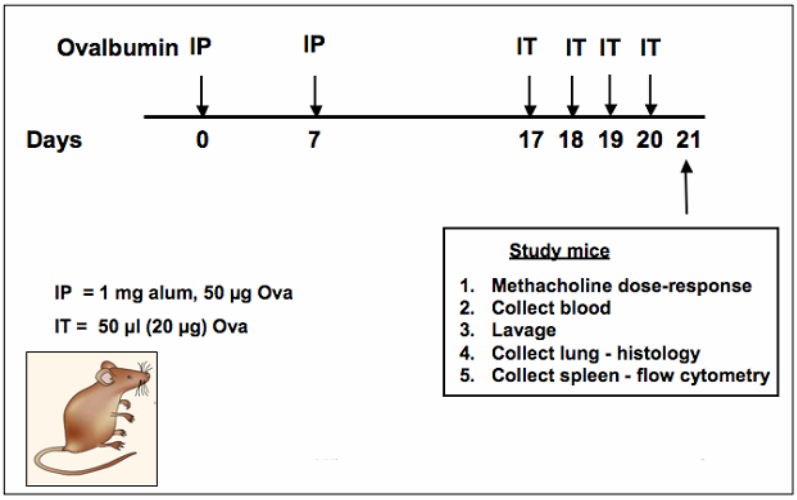


Figure 9. Ovalbumin model protocol. Immunization on days 0 and 7 were done by intraperitoneal injection of 50 µg ovalbumin and 1 mg alum. Challenge with 20 µg ovalbumin by direct intratracheal instillation was performed on days 17-20. Mice were studied on day 21.

Measurement of Airway Resistance

On day 21, animals were anesthetized with a ketamine/xylazine (20 mg/kg / 20 mg/kg) cocktail, intubated, placed on a computer-controlled small animal ventilator (Flexivent, SCIREQ, Montreal, Canada), and paralyzed with 100 µl of 100 mg/ml pancuronium bromide. After a standard volume history and two total lung capacity maneuvers were performed, animals were challenged with an aerosol of PBS containing 0, 3, 6, 12, and 24 mg/ml of methacholine for 10 seconds each. For 5 minute intervals, small volume amplitude oscillations at a frequency of 0.9 Hz were applied at a constant volume to the airway opening for 16 seconds and

respiratory resistance (Rrs) were calculated every 30 seconds. The peak Rrs with each dose were used to create a dose response curve.

C. Collection of Cells, BALF, and Serum

The animal was then exsanguinated by collection of a blood sample from the heart, cannulation of the right heart and perfusion with PBS until clear. Bronchoalveolar lavage was performed; cells were counted, cytocentrifuged, and stained with May-Grunwald Giemsa stain for cell differentials.

D. Histology

Lungs were fixed and preserved with 10% formalin, embedded, sectioned, and stained with hemotoxylin and eosin (H & E) and periodic acid Schiff (PAS).

E. Inhibition of IL-1R Signaling in Ovalbumin Model of Asthma

C57BL/6 mice were treated with 350 μg neutralizing IL-1β antibody or isotype control via intraperitoneal injection once a week in the ovalbumin model. The ovalbumin model was also studied in IL-1R knockout mice bred on a C57BL/6 background. Another method was intraperitoneal treatment with AS-1 or EM-163 on days 17, 18, 19, and 20, thirty minutes prior to ovalbumin intratracheal instillation. In addition, studies were conducted with continuous delivery of EM-163 via osmotic pump. BALB/c mice were also studied using the ovalbumin model and were treated with EM-163 via intraperitoneal injection on days 17, 18, 19, and 20, thirty minutes prior to ovalbumin intratracheal instillation.

Results

Acute Lung Injury Model

A lipopolysaccharide (LPS) model of acute lung injury was developed in BALB/c mice in order to study the effects of inhibiting IL-1β signaling on acute lung inflammation. After testing different models of LPS-induced acute lung injury, a six hour model was preferred over a twenty-four hour model (Figure 10). In the six hour model, a greater inflammatory response was seen through total cells and differentials from bronchoalveolar lavage than in the 24 hour model (Figure 11). In addition, the lung leak assay in the 24 hour model resulted in a loss of fluorescence signal from the FITC-dextran in BALF and serum, most likely indicating the dextran had left the system during the long time course (Figure 11). Therefore, a six hour model using a four hour rather than two hour FITC-dextran incubation was used, which gave a higher fluorescence signal.

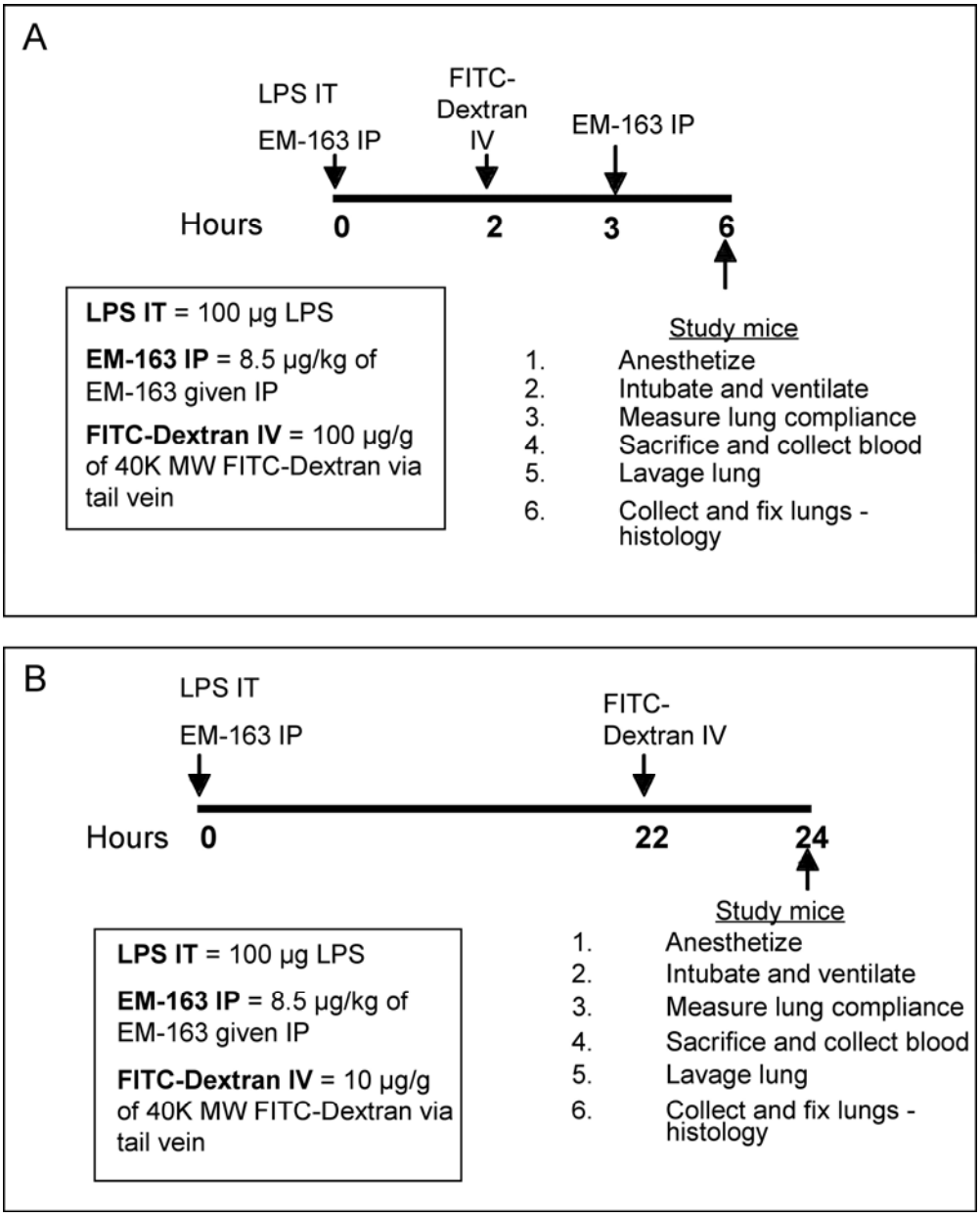


Figure 10. Six hour and twenty-four hour LPS acute lung injury models. A) Mice were given LPS intratracheally and EM-163 intraperitoneally at time 0. Two hours later, FITC-dextran was injected into the tail vein. At hour 6, mice were studied and BALF, serum, and lung collected. B) Mice were given LPS intratracheally and EM-163 intraperitoneally at time 0. FITC-dextran was injected into the tail vein two hours prior to study.

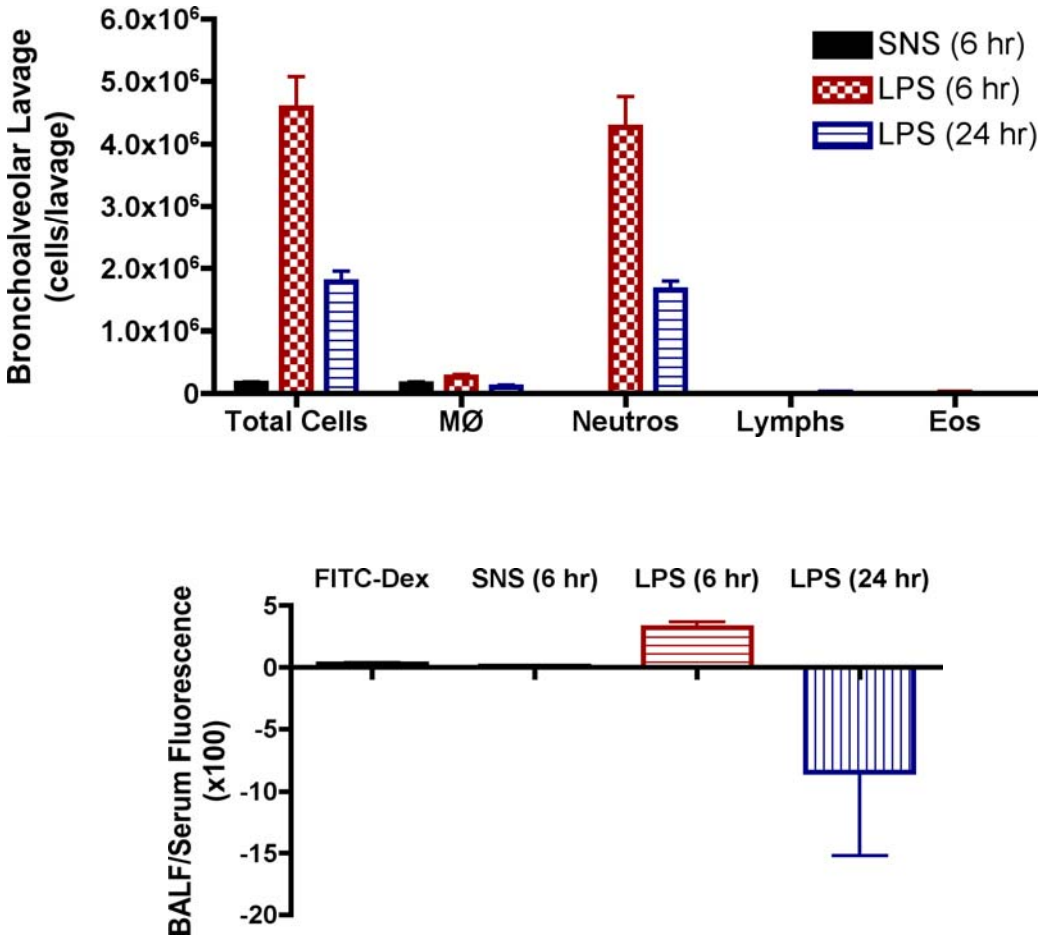


Figure 11. Acute lung injury model in BALB/c mice over a 6 hour versus a 24 hour time course. A) Total cell counts and cell differentials in mice given 100 µg LPS intratracheally and sacrificed after 6 or 24 hours. SNS = sterile normal saline. B) Ratio of BALF to serum fluorescence of FITC-dextran injected into the tail vein in mice given 100 µg LPS intratracheally and sacrificed after 6 or 24 hours.

Lung compliance can be calculated as the change in volume over the change in pressure, and refers to the ability of the lung to stretch in response to a given pressure. For the purpose of this study, lung compliance was used to measure the stiffness of the lung associated with the leak of edema fluid into the alveolus as a result of acute lung injury. LPS treatment induced inflammation and loss of lung

compliance in both C57BL/6 and BALB/c mice (Figure 12 & 13). However, LPS treatment in the acute lung injury model was not sufficient to induce lung leak in C57BL/6 mice (Figure 13), while BALB/c mice showed lung leak due to LPS treatment (Figure 12). Thus, further testing of the model was done in BALB/c mice, which exhibited lung leak with a high BALF to serum fluorescence ratio (Figure 12).

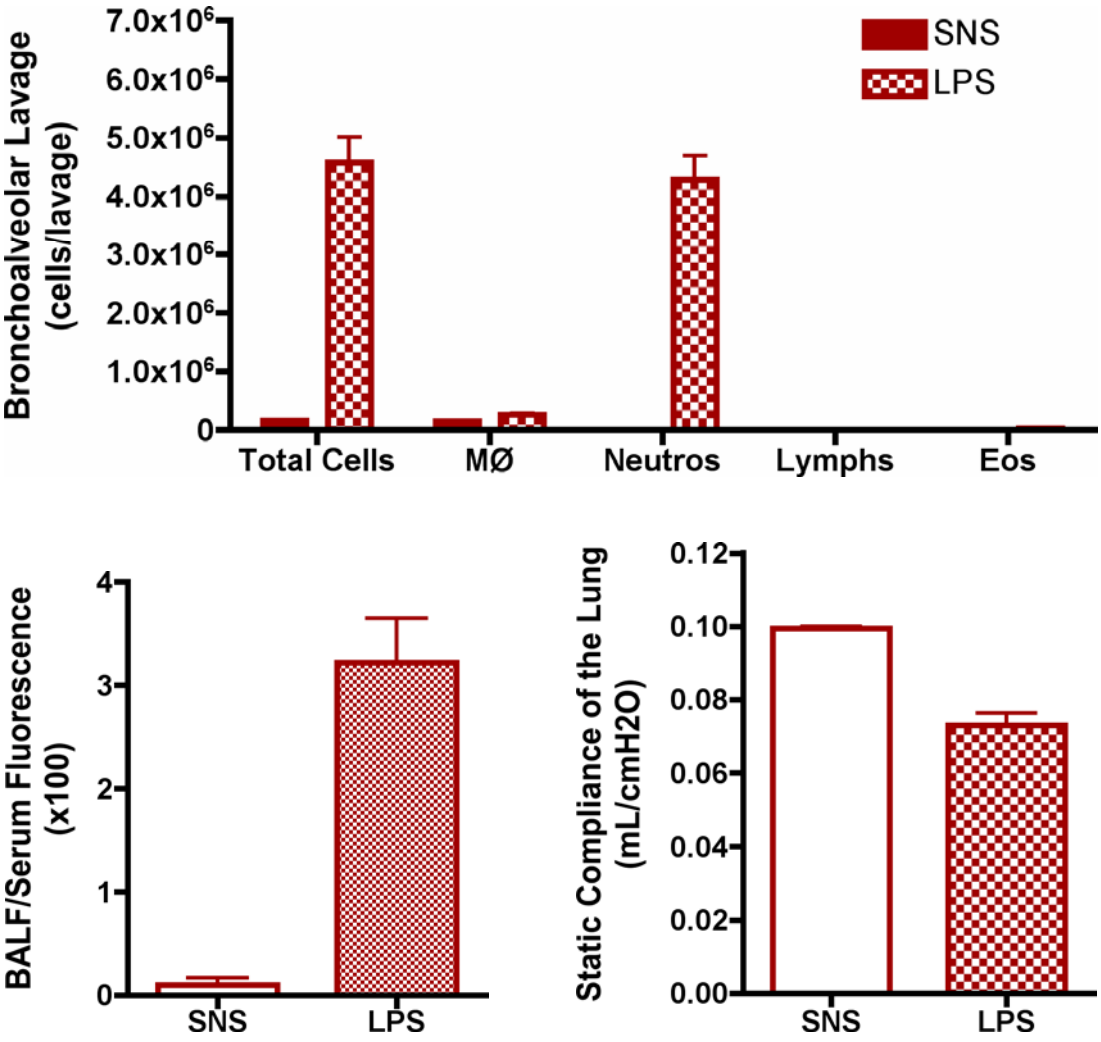


Figure 12. Six hour LPS acute lung injury model in BALB/c mice. A) Total cell counts and cell differentials from bronchoalveolar lavage show increased total cells and neutrophilic influx with LPS treatment. B) Ratio of fluorescence in BALF to serum from FITC-dextran injected into the tail vein increased with LPS treatment. C) Reduced static compliance of the lung with LPS treatment.

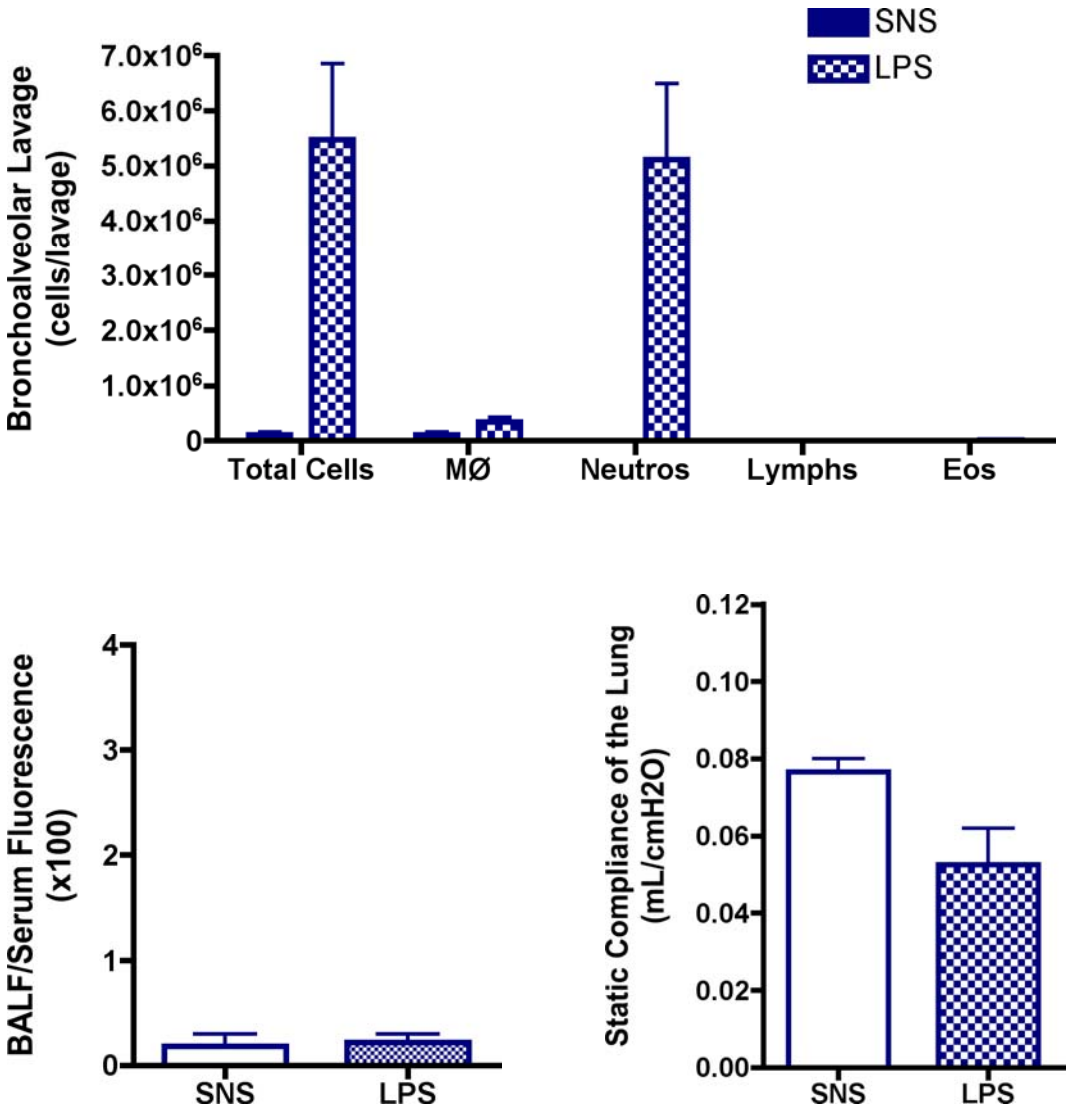


Figure 13. Six hour LPS acute lung injury model in C57BL/6 mice. A) Total cell counts and cell differentials from bronchoalveolar lavage show increased total cells and neutrophilic influx with LPS treatment. B) Ratio of fluorescence in BALF to serum from FITC-dextran injected into the tail vein similar to controls. C) Reduced static compliance of the lung with LPS treatment.

To test whether inhibition of IL-1R signaling attenuates LPS-induced inflammation in this model, EM-163 was given intraperitoneally at time of LPS treatment and at hour 3 (Figure 10A). Dimethyl sulfoxide (100% DMSO) and ethanol (50% or 20% EtOH) were tested as potential vehicles for EM-163. The total

volume of DMSO administered was 0.8% of animal body weight. While intraperitoneal injection of DMSO seemed to attenuate inflammatory cell influx and lung leak induced by LPS, DMSO treatment with LPS caused greater loss of lung compliance than LPS treatment alone (Figure 14). In addition to the variability of its effects, DMSO is an unsuitable vehicle because EM-163 is not soluble in DMSO.

Greater solubility of EM-163 was achieved in 50% ethanol. However, intraperitoneal injection of 50% ethanol alone showed a significant reduction of LPS-induced inflammation and lung leak, and also restored static lung compliance (Figure 15). The total volume of ethanol administered was 0.4% of animal body weight, a significant amount. Reduction to 20% ethanol, with total ethanol volume administered being 0.16% of animal body weight, resulted in less of an effect due to ethanol. Further attenuation of inflammation, static compliance of the lung, and lung leak were seen with EM-163 treatment than with 20% ethanol alone (Figure 16). Histology revealed significant neutrophilic inflammation in lungs of LPS treated mice, and less neutrophil influx with EM-163 treatment (Figure 17). These results are promising; however, solubility remains an issue and studies must be continued to improve methods for delivery in order to properly study the effects of EM-163.

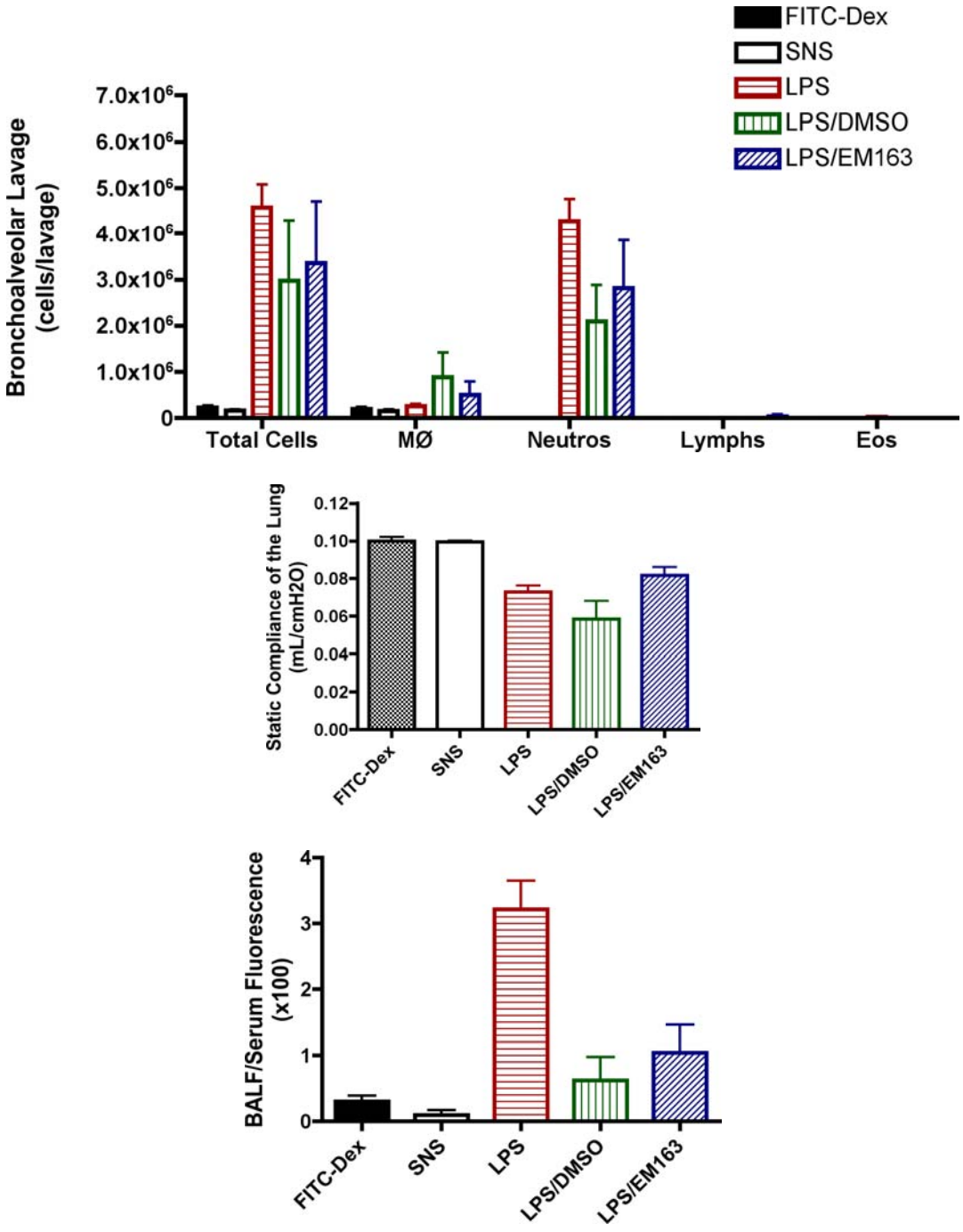


Figure 14. DMSO has variable, independent effects as a vehicle for EM-163 in a 6 hour LPS acute lung injury model in BALB/c mice. A) Total cell counts and differentials from bronchoalveolar lavage showed some attenuation by DMSO. B) Static compliance of the lung decreased with DMSO. C) Ratio of BALF to serum fluorescence of tail vein-injected FITC dextran, multiplied by 100. Leak was reduced with DMSO, but still higher than controls.

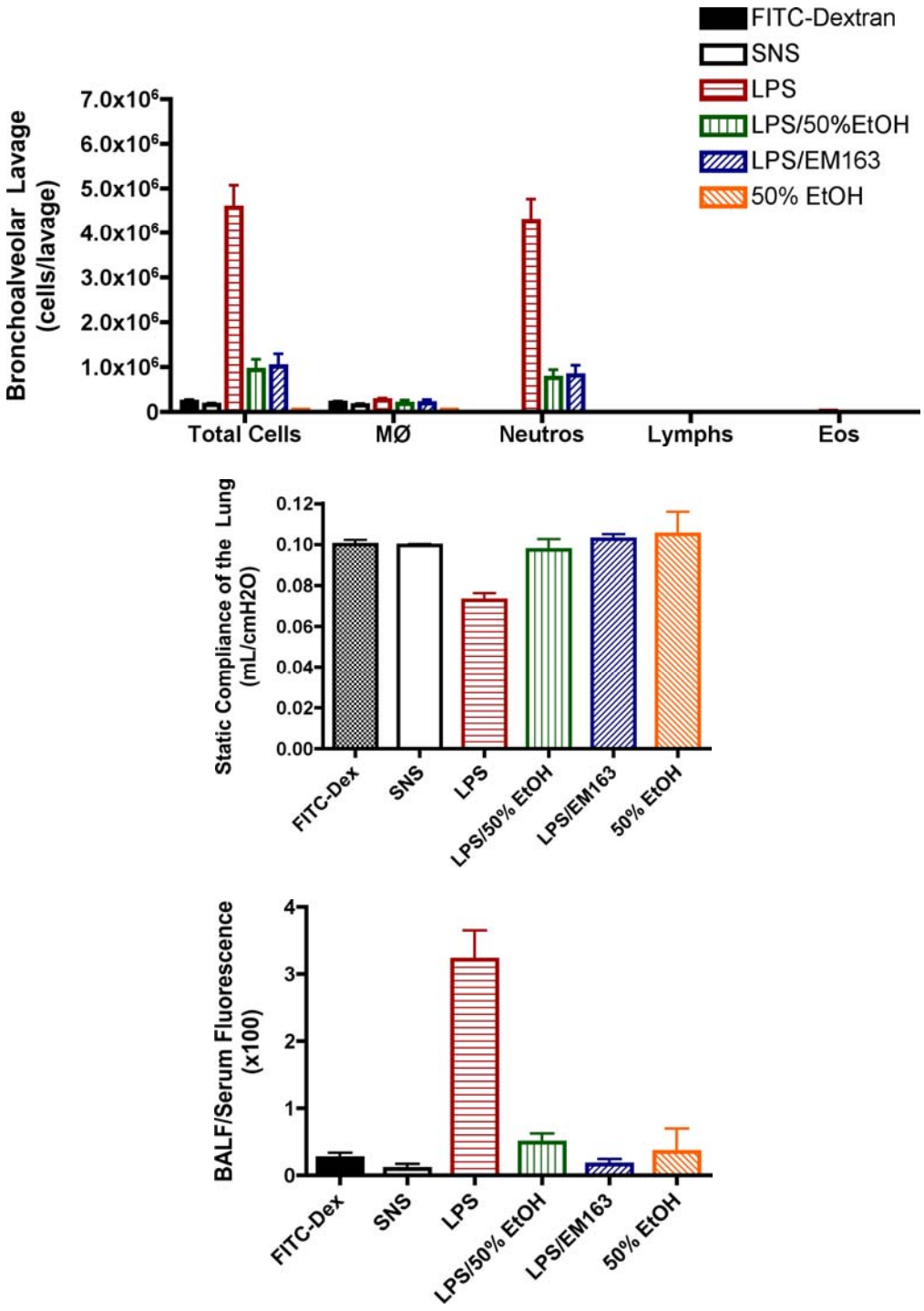


Figure 15. Independent diminishing effects of 50% ethanol as vehicle for delivery of EM-163 in 6 hour LPS acute lung injury model in BALB/c mice. A) Significant attenuation by 50% ethanol of total cell counts and neutrophils from bronchoalveolar lavage. B) Static compliance of the lung restored to baseline with 50% ethanol. C) Ratio of BALF to serum fluorescence of tail vein-injected FITC dextran, multiplied by 100. Leak was significantly reduced with 50% ethanol.

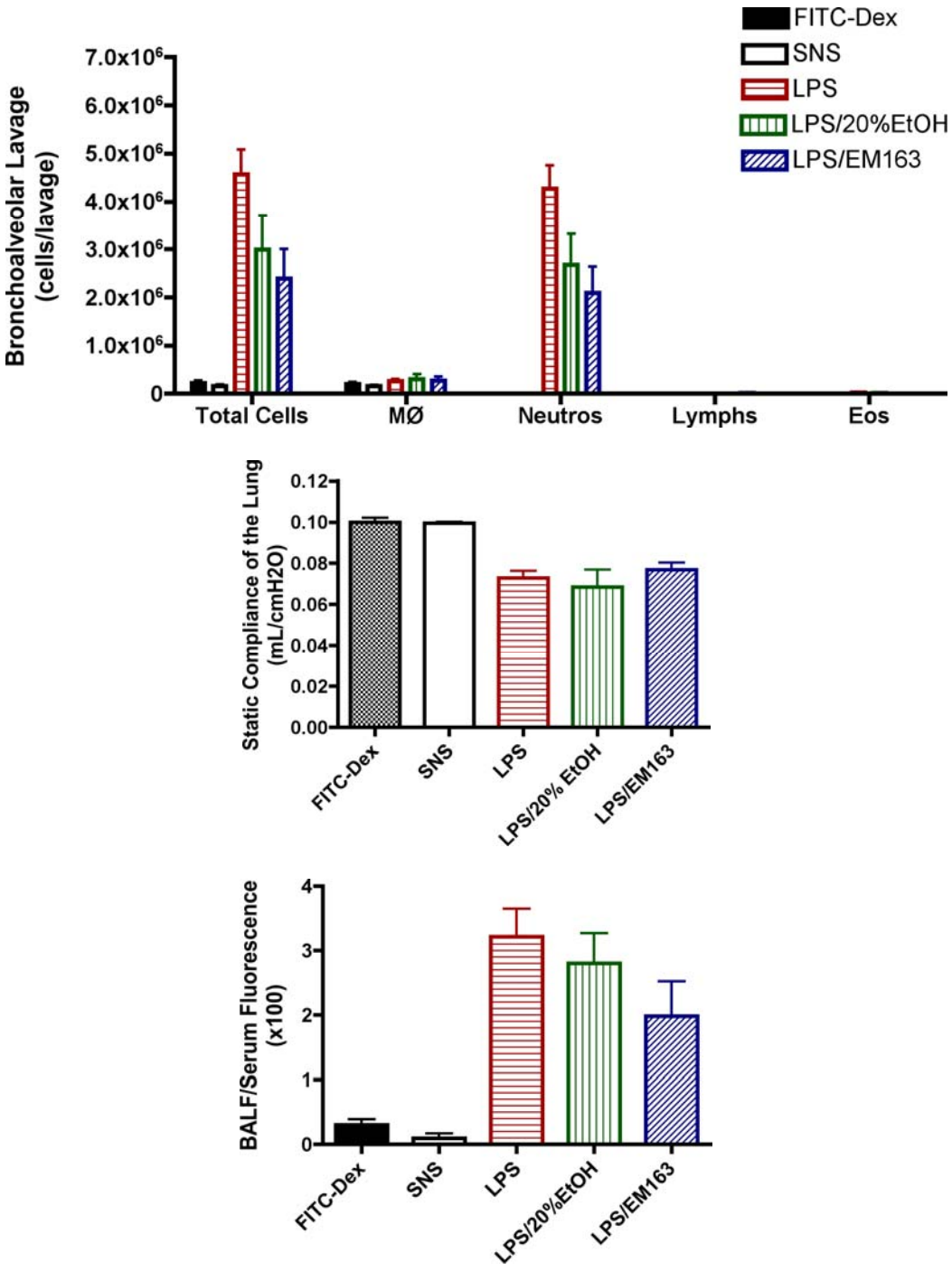


Figure 16. Reduction of independent ethanol effect using 20% ethanol as a vehicle for delivery of EM-163 in 6 hour LPS acute lung injury model in BALB/c mice. A) Total cell counts and differentials from bronchoalveolar lavage. B) Static compliance of the lung with LPS treatment is unaffected by 20% ethanol. C) Ratio of BALF to serum fluorescence of tail vein-injected FITC dextran, multiplied by 100. Greatest attenuation of leak shown with EM-163 treatment.

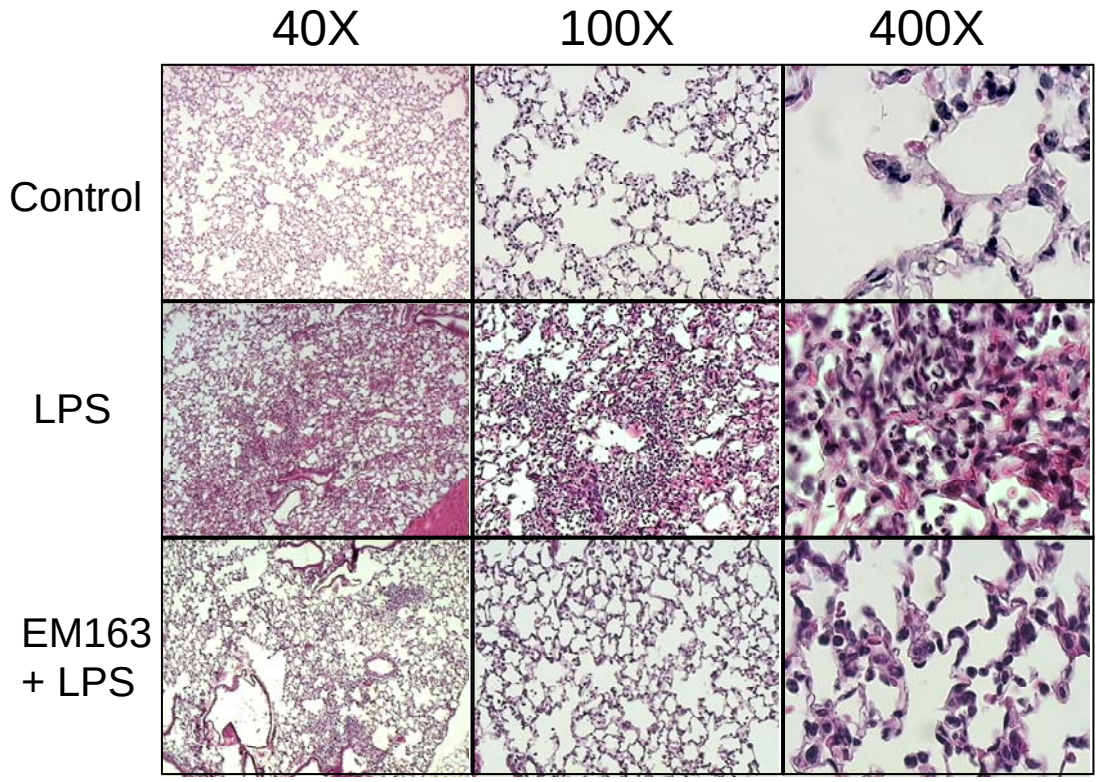


Figure 17. Reduction of inflammatory cell influx with EM-163 treatment in a 6 hour model of LPS-induced lung injury in BALB/c mice. Lungs were fixed in 10% formalin, embedded, sectioned, and stained with H&E. Images were taken at 40X, 100X, and 400X magnification.

To test the specificity of EM-163 for inhibiting MyD88 interactions with IL-1R via TIR domains, *in vitro* studies were done by transiently transfecting a vector containing an NFκB promoter fused to luciferase into RAW264.7 murine macrophage cell line. Forty-eight hours later, cells were stimulated for six hours with LPS, LPS with EM-163, and appropriate controls. After six hours, luciferase activity was assayed. Ethanol seemed to have a slight independent effect, while

EM-163 did not significantly inhibit NFκB activation (Figure 18). Studies will continue to examine effects of EM-163 specifically in response to IL-1β stimulation.

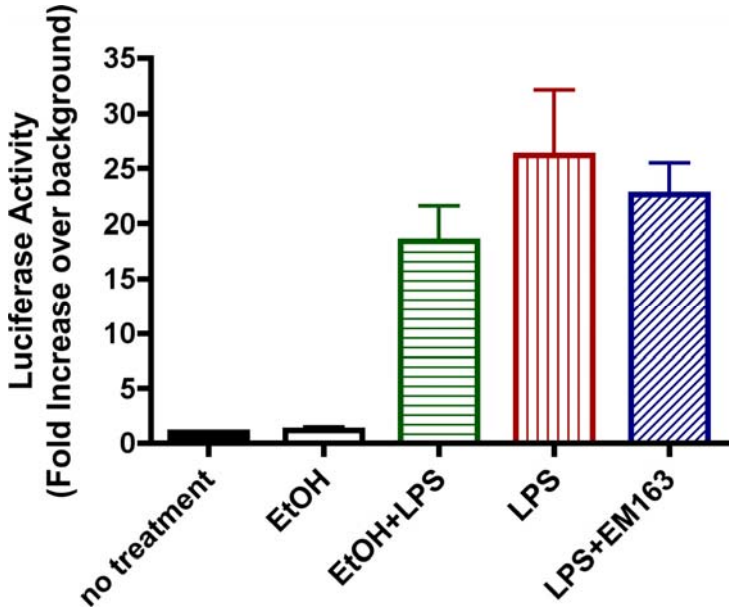


Figure 18. NFκB activation reported by luciferase activity in response to treatment with LPS and EM-163, compared to controls. Cells were stimulated with 20% ethanol, LPS with 20% ethanol, LPS alone, and LPS with EM-163 and luciferase activity assayed. Luciferase activity was compared to that in untreated cells.

Ovalbumin Model of Asthma

A twenty-one day protocol of immunization with ovalbumin followed by airway challenge was developed in C57BL/6 or BALB/c mice (Figure 9). On day 21, mice were anesthetized, placed on a mechanical ventilator, paralyzed, and airway resistance measured using the forced oscillation technique. An aerosol of increasing doses of methacholine was delivered by nebulization, and airway measurements taken. Bronchoalveolar lavage was then performed, cell counts and differentials taken, cytokine measurements and lung histology performed. Mice immunized and

challenged with ovalbumin showed airway hyperresponsiveness in response to methacholine compared to controls (Figure 19A). These mice also showed significant increases in total cells from lavage and over 70% eosinophils compared to none in controls (Figure 19B). Also, these mice had increased serum IgE and increased IL-4 and IL-1β in lavage fluid (Figure 19C). Histology revealed eosinophilic inflammation in the airways and increased goblet cell hyperplasia compared to controls (Figure 19D).

Using this model, IL-1β signaling was inhibited by treatment with neutralizing antibodies to IL-1β or isotype control once a week in C57BL/6 mice. Significant reduction in airway hyperresponsiveness compared to controls was seen with this inhibition (Figure 20A). IL-1R knockout mice (C57BL/6 background) were also studied using the ovalbumin model, and airway hyperresponsiveness was also attenuated (Figure 20B). Treatment with AS-1, a β-barrel loop mimetic, as well as EM-163 treatment via osmotic pump or intraperitoneal injection resulted in decreased airway hyperresponsiveness as IL-1R knockouts (Figure 20C, D).

Lung inflammation was not significantly lessened as represented by bronchoalveolar lavage cells seen in C57BL/6 mice. Mucin production by goblet cells was decreased in airways, as observed through histology (Figure 21). However, a reduction of inflammation, particularly eosinophils, was observed in bronchoalveolar lavage cells when BALB/c mice were treated with EM-163 (Figure 22).

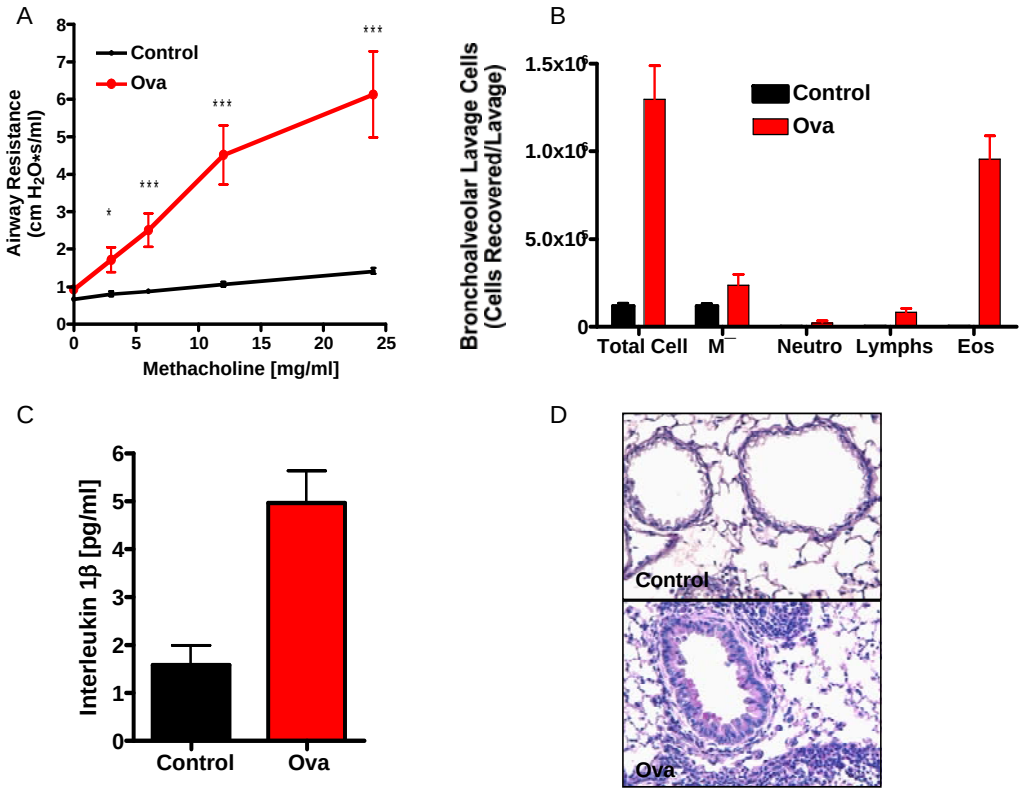


Figure 19. Ovalbumin model of asthma in C57BL/6 mice. A) Airway hyperresponsiveness in response to increasing doses of methacholine in mice immunized and challenged with ovalbumin compared to controls. B) Total cell counts and differentials in ovalbumin treated mice compared to controls. C) Levels of IL-1β with ovalbumin treatment compared to controls. D) PAS staining of airways with and without ovalbumin treatment.

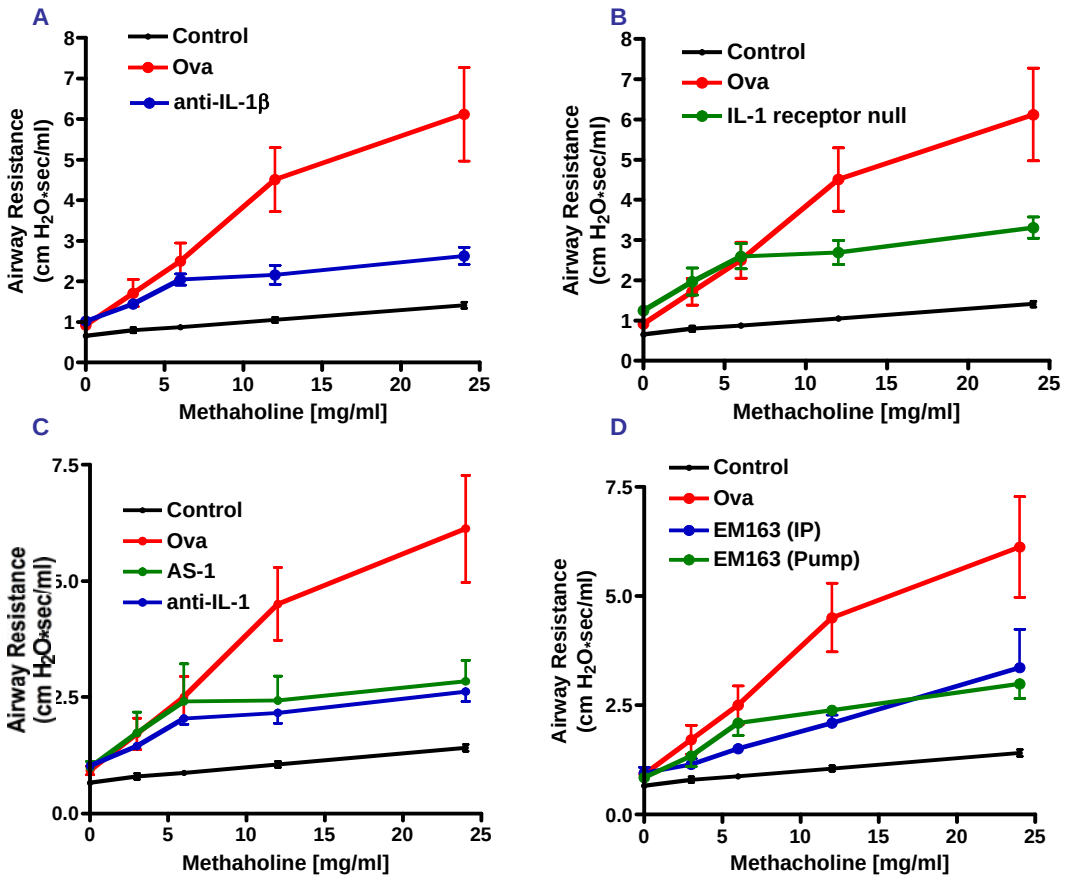


Figure 20. EM-163 treatment attenuates airway hyperresponsiveness and airway inflammation in an ovalbumin model of asthma in C57BL/6. A) Reduction of airway hyperresponsiveness in C57BL/6 mice with IP injection of neutralizing antibody to IL-1β. B) Attenuation of airway hyperresponsiveness in IL-1R knockout mice (C57BL/6 background). C) AS-1 inhibits airway hyperresponsiveness in C57BL/6 mice. D) EM-163 is effective via IP injection or continuous delivery by osmotic pump in C57BL/6 mice.

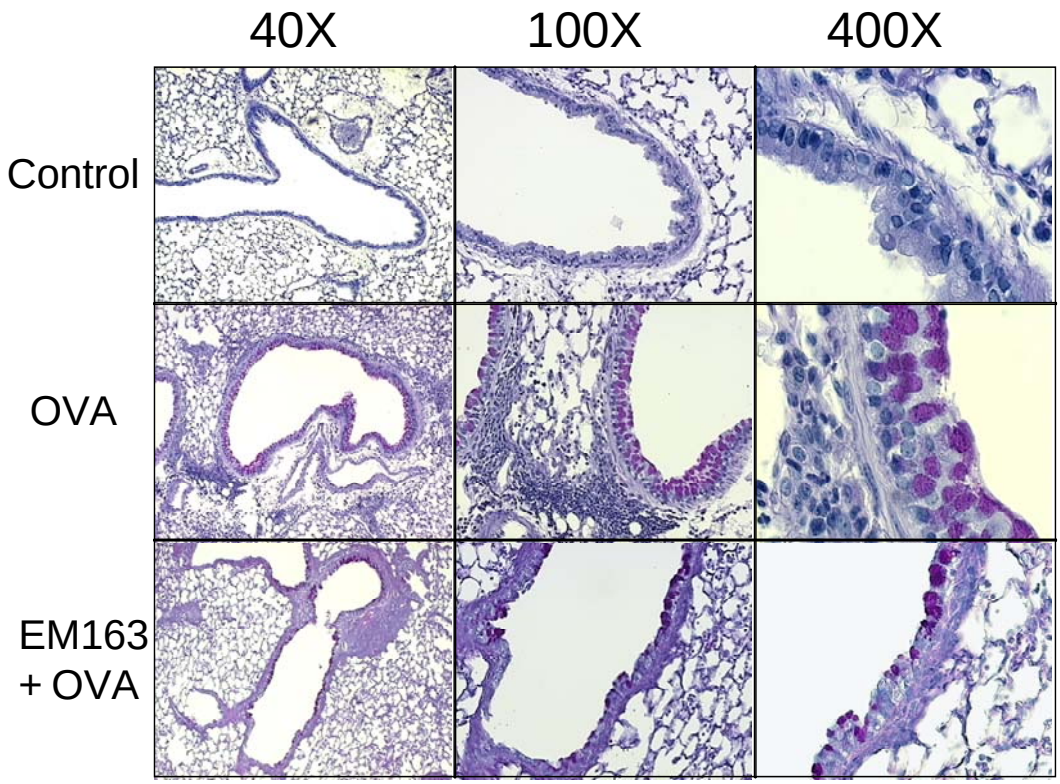


Figure 21. Reduction of airway goblet cell hyperplasia with EM-163 treatment in an ovalbumin model of asthma in C57BL/6 mice. Lungs were fixed in 10% formalin, embedded, sectioned, and stained with PAS. Images were taken at 40X, 100X, and 400X magnification.

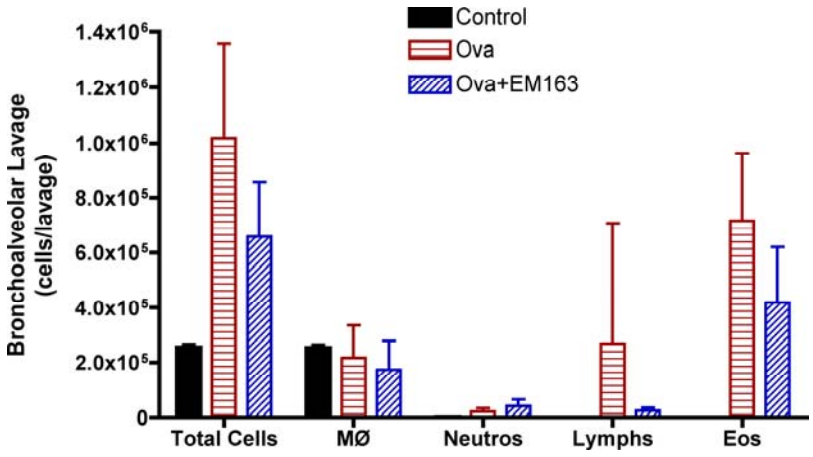


Figure 22. EM-163 attenuates inflammation in BALB/c mice in an ovalbumin model of asthma. Total cell counts and differentials show decreased total cells and eosinophils with EM-163 treatment in ovalbumin model compared to ovalbumin immunization and challenge alone.

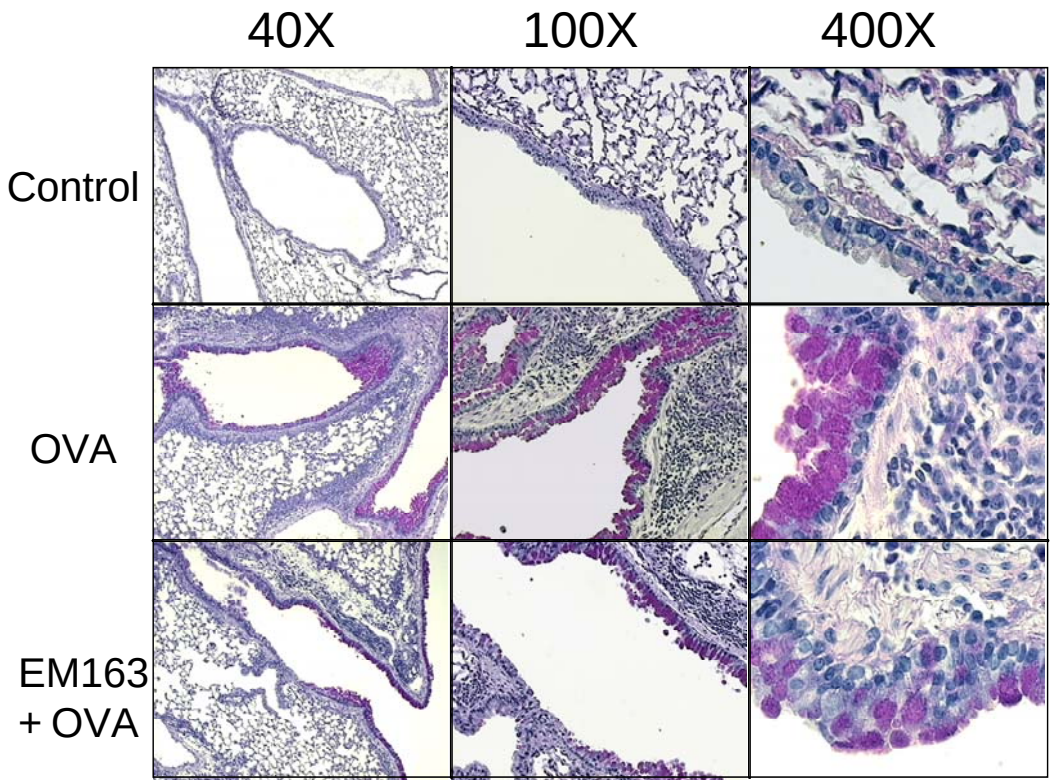


Figure 23. Reduction of airway goblet cell hyperplasia with EM-163 treatment in an ovalbumin model of asthma in BALBc mice. Lungs were fixed in 10% formalin, embedded, sectioned, and stained with PAS. Images were taken at 40X, 100X, and 400X magnification.

Discussion

Previous work *in vitro* had been conducted in the Rebek laboratory at The Scripps Research Institute in which inhibitory activity and specificity for IL-1R signaling of EM-163, a BB loop mimetic which blocks TIR:TIR interactions between MyD88 and IL-1R, was analyzed. In EL-4 mouse thymoma cells, the BB loop mimetic AS-1 was shown to inhibit IL-1β-mediated p38 phosphorylation (Figure 5A), and the specificity of this mimetic for targeting IL-1R/MyD88 interactions was demonstrated (Figure 5B, Figure 6). EM-163 was shown to be a more potent mimic through inhibition of p38 phosphorylation at a lower concentration than AS-1 (Figure 7). The *in vivo* fever model showed greater inhibition of IL-1β-induced fever using EM-163 (Figure 8), supporting the *in vitro* observation of EM-163 as a more potent mimic than AS-1.

As such, it was hypothesized that EM-163 would also inhibit inflammation induced by IL-1β, specifically inflammation characteristic of acute lung injury and asthma. A six hour *in vivo* model of LPS-induced acute lung injury was developed in BALB/c mice (Figure 10A), and was shown to be effective in inducing inflammation, capillary leak in the lung, and loss of static compliance in the lung (Figure 12). To test the effects of IL-1R signaling inhibition at the level of MyD88 interaction, EM-163 was administered. DMSO and 50% ethanol were shown to have independent effects and problems with solubility, and thus were not proper vehicles for EM-163 delivery in this model (Figure 14, Figure 15). The animal body mass and total blood volume should be considered in vehicle administration. Total administered DMSO was 0.8% of animal body mass and 20% of blood volume.

When diluted to 50%, total administered ethanol was 0.4% of animal body mass and 10% of blood volume. Total administration of 20% ethanol was 0.16% of animal body mass and 4% of blood volume. The total amount of vehicle given per animal was a significant portion of animal body mass and blood volume, and therefore the independent effects observed are not surprising. Inhibitory effects of EM-163 in response to LPS treatment were shown when delivered in 20% ethanol (Figure 16). However, solubility issues remain to be addressed, either by finding a suitable solvent or in improving the properties of the compound for use in aqueous solvents.

In addition to solubility, the potency of EM-163 in IL-1R inhibition was not seen in the *in vivo* acute lung injury model to the same degree as in the *in vivo* fever model discussed previously or in previous *in vitro* studies. Thus, it was decided that additional *in vitro* work should be conducted. It is expected that EM-163 treatment *in vitro* will not significantly attenuate LPS-induced inflammation, which was observed in RAW264.7 murine macrophages stimulated with LPS and treated with EM-163 (Figure 18). These results imply that EM-163 does not have inhibitory effects among a broad range of receptors, as expected due to its structural design which is specific for IL-1R inhibition. However, there was a slight decrease, on average, of LPS-induced NFκB activation with EM-163 treatment, which could be due to possible IL-1β production and subsequent inhibition of IL-1R signaling by EM-163. More studies will be done to determine specificity of EM-163 in response to IL-1β, including stimulation with IL-1β and other cytokines such as TNF. Significant inhibition of inflammatory response should be seen with EM-163

treatment with IL-1β stimulation, as EM-163 is specific for IL-1R interaction with MyD88. EM-163 should not inhibit NFκB activation induced by TNF stimulation. EM-163 should not target TNFR signaling, which uses a different adaptor protein, TNF Receptor-associated via death domain (TRADD), to mediate NFκB activation (Hsu, et al. 1995).

However, targeting of IL-1R signaling was successful *in vivo* in attenuation of airway hyperresponsiveness and airway inflammation in an ovalbumin model of asthma (Figure 20, Figure 22). Moreover, EM-163 was shown to be highly effective as a molecular inhibitor of IL-1R signaling in this model, both through delivery by intraperitoneal injection as well as continuous delivery by osmotic pump (Figure 21, 22, 23). This verifies the targeting of IL-1R signaling as a potential therapeutic route for treating pulmonary inflammation. Though, physiological measurements should be repeated using BALB/c mice in the ovalbumin model with inhibition of IL-1R signaling.

Greater attention to the innate immune system has led to better understanding of how the innate branch regulates adaptive responses. This increased understanding of the innate system has generated interest in how that system can be manipulated in the treatment of disease (Kanzler, et al. 2007), which has been applied to IL-1R signaling in this discussion. Signaling via IL-1β occurs on the cytoplasmic TIR domain of the IL-1R, which interacts with the TIR domain of the adaptor protein MyD88. The TIR domains present in TLRs are homologous to that in IL-1R; though distinct, these receptors form the IL-1R/TLR superfamily of receptors. Like the IL-

1R, TLRs also signal through TIR domain interactions with MyD88 (O'Neill and Bowie, 2007).

An essential component of the innate system in anti-microbial defense, TLRs activate innate immune responses. Another discovered function of TLRs is inducing apoptosis (Salaun, et al. 2007), which has been demonstrated *in vitro* as an effective anti-tumor mechanism in TLR-positive tumors (Kanzler, et al. 2007). TLRs have been a key target in therapeutic development, and agonists for TLRs are being developed for treatments for cancer, viral disease, allergy, infectious disease, and cancer vaccines. TLR ligands trigger innate responses which will ultimately lead to acquired immunity; use of TLR ligands as vaccine adjuvants has potential for enhancing CD8+ T cell responses and generating responses to tumor-associated antigens similar to self-antigens. TLR agonists in cancer vaccines have thus far been successful in preclinical studies, reporting use of CpG-ODN, a TLR9 ligand, as an adjuvant in an antitumor peptide vaccine in humans and confirmed in mouse tumor models. Potential for agonists for TLRs 3, 4, 7, and 8 in cancer and chronic viral infections has also been reported in preclinical studies (Kanzler, et al. 2007).

Infectious diseases, especially viral infections, can potentially be treated by agonists for TLRs 3, 7, 8, and 9. Currently, an approved treatment for genital warts resulting from human papilloma virus uses the TLR7 ligand imiquimod. Antiviral mechanisms include induction of type I interferons and enhancement of natural killer cell activity and T cells. Clinical development of TLR3 agonists are being focused on antiviral treatment for human immunodeficiency virus (HIV), while CpG-ODN

may be usful in protection against viral and bacterial pathogens and possibly agents of biological warfare (Kanzler, et al. 2007).

While TLR agonists have shown promise in the treatment of diseases discussed previously, TLR antagonists also have potential applications in treatment of autoimmune and inflammatory disease. Autoimmune disease arises from immune challenges in distinguishing self from non-self antigens. It is evident that TLRs can recognize self-antigens, which contributes to autoimmunity. Elevated levels of these self-antigens can be released as TLR ligands by damaged tissues or apoptotic cells, which can lead to chronic activation of TLRs. Viral infection or nucleic acid recognition can stimulate TLR7 or TLR9, inducing IFN-α. Excess levels of IFN-α and its inducible genes have been seen in patients with systemic lupus erythematosus (SLE). A possible therapeutic route is through inhibition of both TLR7 and TLR9, and the use of synthetic TLR9 antagonists has been reported in animal models (Kanzler, et al. 2007).

Further work on TLR antagonists in therapeutic development includes lipid A analogs being studied in clinical trials for treatment of sepsis, as inflammation caused by microbial infection can be targeted through antagonistic inhibition of the LPS receptor TLR4 (Kanzler, et al. 2007). Contributing to the inflammatory response induced by LPS binding to TLR4 is downstream production of IL-1β, a pro-inflammatory cytokine which signals through the IL-1R. Thus, in addition to the TLRs, the IL-1 receptor is also a potential target for the treatment of inflammatory disease.

Another means of approaching this target is through an examination of its signaling pathways. Frobose and colleagues have uncovered suppressor of cytokine signaling 3 (SOCS3) as an inhibitor of IL-1R signaling through ubiquitin-mediated targeting of TRAF6 (Frobose, et al. 2006), revealing a downstream target for inhibition of IL-1 signaling. Furthermore, Rothlin and colleagues have shown that SOCS3 and SOCS1 have potential to inhibit TLR signaling through targeting the adaptor proteins TRAF3, TRAF6, and Mal. This is achieved through interferon-stimulated STAT1 induction of TAM (Tyro/Axl/Mer tyrosine kinase) receptors (Rothlin, et al. 2007). TAM receptors and their ligands are yet another target for TLR signaling inhibition. These studies reveal negative feedback control of signaling, highlighting another approach that can be taken in the manipulation of innate immune receptors for treatment of disease.

While signaling is controlled downstream of the receptor, synthesis of organic TIR domain mimetics can target signaling at the level of the receptor itself (Bartfai, et al. 2003), which represents a novel therapeutic method targeting IL-1β-mediated inflammation. Use of mimetics are advantageous in their capacity for structure-based specificity in targeting protein-protein interactions, as well as control of half-life and potency. This paper has described mimetics that specifically block IL-1R signaling and show potential for use in pulmonary inflammatory disease. However, more work must be done to determine the efficacy of mimetics in blocking inflammation through targeting protein-protein interactions. Specificity of mimetics can be used to target specific receptors other than the IL-1R; this can potentially lead

to an ability to block signaling induced by other molecules and possibly differentiate between signaling pathways. Complexity in TLR signaling exists in the variety of ligands that can stimulate TLRs and several combinations that occur with dimerization. In addition, signal transduction in general is complex in its numerous pathways, crosstalk between those pathways, as well as regulation mechanisms (Basak and Hoffman, 2008). This paper discussed the prominence of IL-1R signaling in acute lung injury and asthma and use of TIR domain mimetics to attenuate inflammation, which is a highly specific target. As discussed, there are a number of diseases in which TLR signaling is involved. Given the complexity and scope of TLR signaling, it is clear that beyond the prospective function of blocking inflammatory responses, mimetics also have the potential to target specific receptors and thus block specific pathways. This study has touched upon this specificity, but more work must be done for this as well as for possible development of other mimetics.

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