Defining stress-induced systemic sterile inflammatory responses: Network, signals, and pathways

by

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Abstract

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Defining stress-induced systemic sterile inflammatory responses: Network, signals, and pathways

Thesis directed by Professor Monika Fleshner, Ph.D.

Systemic sterile inflammatory responses (SSIRs) are characterized by an increase in concentrations of blood and tissue cytokines, chemokines and other inflammatory proteins. SSIRs are commonly evoked by trauma or exposure to severe stressors and may result in negative consequences including multi-organ failure and death. Current attempts to treat SSIRs are often ineffective, reflecting the need for additional characterization of the signals and pathways that drive these processes. Specifically, it is important to explore recent advances in our understanding of the underlying signaling pathways and inflammatory proteins involved in SSIRs such as those that follow stressor exposure. The goals of this dissertation, therefore, are the following: (1) characterize the network of diverse cytokines, chemokines, inflammatory proteins and their receptors involved in SSIRs; (2) discuss factors impacted by the stress response including danger- and microbe associated molecular patterns (DAMPs and MAMPs) that may contribute to signaling processes to stimulate SSIRs; and (3) highlight a recently discovered signaling complex, the inflammasome, and its role in stress-induced SSIRs. We propose that systemic sterile inflammatory processes are initiated by a wide variety of molecules and involve the release of a plethora of cytokines, chemokines, and other immunomodulatory proteins. Importantly, we present evidence that this cytokine storm, although initiated by many

diverse signals, may converge on the inflammasome. Additional research is necessary to determine whether the inflammasome is a suitable pharmacological target for SSIRs.

Dedication

To Moni Fleshner. I showed up in your immunology class with no voice and a note asking for a job in your lab. You gave me an amazing opportunity and always challenged me to make the most of it. Thank you for always believing that I could do better.

To my committee. I'm lucky to have a group that is so good at exposing the flaws in my work and yet, equally good at letting me feel like I could fix them. Thank you.

To my co-workers. My dissertation is built entirely on the long hours we've worked together. Thank you for singing along in Transgenics.

To Mom and Dad. You pushed me to be my best and supported me along the way. Thank you for making me feel like I could never fail. Thank you for never asking when I'll find a "real job".

To John, Jeff, and Liz. Thank you for telling me I was the smart one and not focusing on the fact I'm the short, curly haired kid that probably got switched out at the birthing center.

To my wife Alex. You've listened to me when I talk about everything that's gone into this… every day… for the past 5 years. I almost feel like your dedication should read like an apology; you've been with me on this one every step of the way. Thank you for listening. Thank you for all your love and support.

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Chapter I

Introduction

Sterile inflammation, as generally discussed, is characterized by the release of inflammatory proteins such as cytokines and chemokines in response to a non-infectious stimulus such as tissue damage or stress. Under normal circumstances, sterile inflammatory responses are beneficial in that they accelerate wound healing following tissue damage, and enhance innate immune responses in tissues like the skin, spleen, and liver; however, intense stimuli including trauma or severe stressors may evoke global production of inflammatory proteins. Under these conditions, known as a systemic sterile inflammatory response (SSIR), inflammatory processes overwhelm their benefit to the host and drive severe complications including multi-organ failure and death. Current treatments designed to treat SSIRs range from non-steroidal anti-inflammatory drugs to interleukin (IL)-1 receptor antagonists. These treatments, however, are often ineffective which reflects the need for a better characterization of the mechanisms that drive SSIRs.

The purpose of this dissertation, therefore, is to provide new information about the SSIR that is evoked upon exposure to an intense acute stressor beginning with an overall description of the response and ending with identification of novel therapeutic targets. The first chapter, which is a review of the literature, serves to frame the content of this dissertation within the context of our current understanding of stress-evoked SSIRs. In the second chapter, the impact of stress on a large number of cytokines, chemokines, and other inflammatory proteins is examined in the spleen to provide a better characterization of the network of stress-responsive inflammatory proteins involved in the SSIR. The third chapter examines the role of microbial signals known

as microbe associated molecular patterns (MAMPs) in the stress-evoked SSIR. The role of commensal bacteria is also considered in this section as they represent the primary source of MAMPs in the absence of infection. The fourth and final chapter of this dissertation examines the signaling pathway underlying SSIRs with a focus on a newly discovered signaling complex, the inflammasome. This chapter also examines another family of signals that could play a role in stress-evoked inflammatory protein production called danger associated molecular patterns (DAMPs).

The results of the studies presented herein describe a large network of cytokines, chemokines, and other inflammatory proteins that are impacted by stressor exposure highlighting the difficulties faced by researchers and clinicians attempting to treat SSIRs. Importantly, in spite of the diversity of signals involved in the stress-evoked cytokine storm, the response appears to converge upon the inflammasome which may represent a suitable pharmacological target for treating SSIRs.

Chapter II

Defining stress-induced systemic sterile inflammatory responses: Network, signals, and pathways

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Abstract

Systemic sterile inflammatory responses (SSIRs) are characterized by an increase in concentrations of blood and tissue cytokines, chemokines and other inflammatory proteins. SSIRs are commonly evoked by trauma or exposure to severe stressors and may result in negative consequences including multi-organ failure and death. Current attempts to treat SSIRs are often ineffective, reflecting the need for additional characterization of the signals and pathways that drive these processes. Specifically, it is important to explore recent advances in our understanding of the underlying signaling pathways and inflammatory proteins involved in SSIRs such as those that follow stressor exposure. The goals of the current review, therefore, are the following: (1) characterize the network of diverse cytokines, chemokines, inflammatory proteins and their receptors involved in SSIRs; (2) discuss factors impacted by the stress response including danger- and microbe associated molecular patterns (DAMPs and MAMPs) that may contribute to signaling processes to stimulate SSIRs; and (3) highlight a recently discovered signaling complex, the inflammasome, and its role in stress-induced SSIRs. We propose that systemic sterile inflammatory processes are initiated by a wide variety of molecules and involve the release of a plethora of cytokines, chemokines, and other immunomodulatory proteins. Importantly, we present evidence that this cytokine storm, although initiated by many diverse signals, may converge on the inflammasome. Additional research is necessary to determine whether the inflammasome is a suitable pharmacological target for SSIRs.

Introduction

The inflammatory response is characterized by the release of inflammatory proteins such as cytokines and chemokines and is driven by a number of diverse stimuli. Infection with pathogenic viruses, bacteria, or other microorganisms is a trigger of the inflammatory response. Pathogens are not, however, the only stimuli that will evoke inflammation. Endogenous (sterile) stimuli like cell death [1–3] or activation of the stress response [4–7] may also stimulate the release of potent immunostimulatory molecules and evoke cytokine and chemokine responses. Local sterile inflammatory responses are important for tissue repair [8,9] and other innate immune activity [10–12].

Localized sterile inflammatory responses can, however, escape local containment barriers resulting in a systemic sterile inflammatory response (SSIR). Systemic sterile inflammatory responses commonly follow trauma and exposure to severe stressors. Rather than being beneficial to the host, these responses can cause pathology. Like sepsis, which is the result of systemic infectious stimuli, SSIRs [13] present similar complications including multi-organ failure and death [14,15]. Current attempts to treat SSIRs are often ineffective; therefore the signals and pathways that drive these processes require better characterization.

The purpose of this review is to bring together results from recent investigations of the stress-evoked systemic sterile, cytokine and chemokine production and enhance our mechanistic understanding of the inflammatory proteins and signaling pathways involved in SSIRs. This review will focus primarily on the following three areas of research: (1) characterize the network of diverse cytokines, chemokines, inflammatory proteins and their receptors that comprise the SSIR; (2) elucidate the factors impacted by the stress response which contribute to signaling that drives inflammatory protein production; and (3) highlight a recently discovered

signaling complex, the inflammasome, and its role in stress-induced inflammatory responses. Collectively, the results from these studies will create a more complete view of what is currently known about systemic sterile inflammation and may support the development of more effective therapeutics to appropriately contain these responses.

The network of diverse cytokines and chemokines that comprise the stress-evoked SSIR

Previous investigations of the impact of stress on immune function often focus on the impact of stressor exposure on an immune response to a subsequent challenge. These studies demonstrate that adaptive immune responses like antigen specific antibody titers [16–18] and T-cell responses to viral challenges (reviewed in [19]) were reduced in subjects previously exposed to various stressors. Conversely, these studies also describe primed innate immune responses including enhanced macrophage function [20,21] and improved bacterial killing and clearance [12,22] in subjects that underwent prior stressor exposure.

In the absence of a subsequent immune challenge, the stress response can stimulate an immune response on its own. Stress-evoked sterile inflammatory responses are characterized by increases in interleukin-1 family cytokines, including IL-1β [23] and IL-1α [24] and IL-18 [25]. Other cytokines such as IL-6 [26] and IL-10 [27] are similarly stress responsive despite different synthesis and release pathways. Stress also impacts chemokine responses including monocyte-chemotactic-protein (MCP)-1 [5] and CXCL-5 [11]. To our knowledge, however, relatively few studies consider networks of cytokines in their examinations of stress-evoked immune activity [28]. Instead, the majority of research has focused on the effects of stress on a small number of inflammatory proteins or even a single cytokine. Results from the preponderance of studies that focus on a small number of cytokines and chemokines, lends support to the importance of a single, stress-responsive, immunomodulatory pathway. On the contrary, the reactivity of IL-1 family cytokines, other cytokines such as IL-6, and chemokines including MCP-1 suggests that multiple immunological signals are involved in the mechanism of stress-evoked sterile inflammatory responses.

Cytokines and chemokines rarely function in isolation. They operate in the context of larger networks [29–32]. The cumulative impact of the network of inflammatory proteins drives an immune response. Thus one inflammatory protein is likely insufficient to evoke stress-evoked systemic sterile inflammation. The characterization of stress-evoked SSIRs, therefore, is evolving and clearly demonstrates the broad impact of stress on a large network of cytokines and chemokines. As a result, the number of cytokines, chemokines, other immune messengers and receptors displaying systemic reactivity to stress has greatly expanded (**Table 1**). These inflammatory proteins increase in tissues including the spleen, liver, lungs, brain, muscles, kidneys, adrenals, adipose, skin, and gut, and can spill over into the circulation (measured in plasma) following stressor exposure (**Table 1**).

Given that cytokines and chemokines work in networks to achieve immunological effects, the breadth of cytokines and chemokines impacted by the stress-induced sterile inflammatory response highlights the difficulties inherent in designing treatments to constrain and regulate this cytokine storm. Blocking a single cytokine, for instance, is unlikely to impact the outcome of a sterile inflammatory response as other inflammatory proteins likely play biologically redundant roles [11,29]. It is, therefore, necessary to elucidate targets upstream of multiple cytokines and chemokines to develop therapeutics that are effective at controlling sterile systemic inflammation.

Table 1: Recent Reports of Stress Induced Cytokine Release									
Species	**Stressor**	**Cytokine Source**	**Additional Stimuli**	**Cytokines Affected (Direction)**	**Reference**	***In Vivo***	***In Vitro***	***Ex Vivo***	**mRNA or Proteins?**
Rat	Chronic Cold	Plasma	LPS	IL-1β (+), IL-6 (+), MCP-1 (+), CXCL1 (+)	73	X			Protein
Rat	Tail shock	Spleen	(none)	Tnf(-), LTA (-), Ccl12 (-), Il1r2 (+), Il1a (+), Il10 (+), Ccl20 (+), Cxcl5 (+), Cd86 (+)	11	X			mRNA
Rat	Tail shock	Spleen	(none)	IL-1β (+), IL-1α (+)	11	X			Protein
Rat	Chronic Mild Stress	Spleen	(none)	IL-1β (0), IL-18 (-), TNFα (+), IL-6 (+), IL-10 (0), TGFβ (-), IL-4 (0)	74	X			mRNA
Rat	Tail shock	Adipose Tissue	(none)	Il-1β, IL-6, Il-10	75	X			Protein
Rat	Chronic Mild Stress	Hypothalamus	(none)	IL-1β (0), IL-18 (0), TNFα (0), IL-6 (+), IL-10 (0), TGFβ (0), IL-4 (0)	74	X			mRNA
Rat	Chronic Cold	Hypothalamus	Acute Foot shock	IL-1β (+)	73	X			Protein
Rat	Ischemia	Hippocampus	(none)	IL-1α (+), IL-1β (+), IL-4 (+), IL-6 (+), GM-CSF (+), IFN-γ (+), TNFα (+)	76	X			Protein
Rat	Chronic Mild Stress	Cortex	(none)	IL-1β (+), IL-18 (0), TNFα (+), IL-6 (0), IL-10 (-), TGFβ (0), IL-4 (+)	74	X			mRNA
Rat	Chronic Mild Stress	Hippocampus	(none)	IL-1β (0), IL-18 (+), TNFα (+), IL-6 (0), IL-10 (0), TGFβ (-), IL-4 (+)	74	X			mRNA
Mouse	Exhaust Inhalation	Lung	(none)	Tnfα (+), IL-6 (+), IL-8	77	X			mRNA
Mouse	Social Defeat	Microglia	LPS	Il-1β (+), IL-6(+), TNFα (+), MCP-1 (+)	78	X			Protein
Mouse	Chronic Cold	Peritoneal Macrophages	(none)	Tnfα (+), IL-6 (+), IL-10 (0)	79			X	mRNA
Mouse	Communication Box	Adrenal	DSS (Colitis)	IL-18 (+), Tnfα (+), IL-6 (+)	4	X			mRNA
Mouse	Exercise	Muscle	(none)	IL-1β (+), Tnfα (-), IL-6 (+)	80	X			mRNA
Mouse	Communication Box	Colon	DSS (Colitis)	IL-18 (+), Tnfα (+), IL-6 (+)	4	X			mRNA
Mouse	Alcohol	Liver	(none)	MCP-1 (+), IL-1β (+), TNFα (+), IL-6 (+)	81	X			mRNA
Mouse	Chronic Hypergravity	Serum	(none)	MCP-1 (-), IP-10 (-), IFNγ (-), IL-2 (-), IL-4 (0), IL-5 (0), IL-10 (0)	82	X			Protein
Mouse	Metal Particulate Inhalation	Serum	(none)	CCl7 (+), IL-1β (+)	83	X			Protein
Human	Spaceflight	Plasma	(none)	IL-10 (+)	84	X			Protein
Human	Surgery	Plasma	(none)	IL-6 (+), IL-8 (+), IL-10 (+), TNFα (0)	85	X			Protein
Human	Exercise	Serum	(none)	CRP (+), IL-6 (+), IL-10 (+), TNFα (+)	86	X			Protein
Human	Ischemia (stroke)	Plasma	(none)	IL-1β (+), Tnfα (+), Ifnγ (+), IL-10 (-)	87	X			Protein
Human	Sleep Disruption	Plasma	Dialysis	CRP (+), IL-18 (+)	88	X			Protein
Human	Exercise	Serum	(none)	Tnfα (+), IL-6 (+), IL-1ra (+)	89	X			Protein
Human	Acute Psychological Stress	Whole Blood	LPS	IL-6 (+), Tnfα (+)	90			X	mRNA
Human	UV irradiation	Skin (Organ Culture)	(none)	IL-1α (+), IL-6 (+), IL-8 (+), IL-10 (+), TNFα (+)	91		X		Protein
Human	Metabolic Stress	Kidney HRCC's	(none)	IL-6 (+), IL-8 (+), TNF-α (+), RANTES (+), MCP-1 (+)	92		X		mRNA

Table 1: Recent publications characterize the network of diverse cytokines, chemokines, and other inflammatory proteins known to comprise the stress-evoked systemic sterile inflammatory response. The table presents a summary of these publications and includes the species and stressor used, the cytokines and chemokines measured, the source from which they were

measured, and the type of measurements taken (protein or mRNA), along with bibliographic information.

Upstream factors contributing to stress-induced SSIRs

DAMPs

In general, sterile inflammatory responses are evoked by endogenous molecular signals released in response to danger or tissue damage called danger associated molecular patterns (DAMPs). Exposure to an acute stressor stimulates systemic elevations in DAMPs which may be released either actively [33,34] or following cell death [2,35]. Stress-induced increases in peripheral norepinephrine are upstream in the signaling cascade and likely drive these responses via α-1 adrenergic receptors [33,36].

Several DAMPs, including Hsp72 [7,33,37] and uric acid [7], may underlie stress-evoked SSIRs. These DAMPs are released immediately following stressor exposure and are linked to stress-induced immune modulation. Using a stepwise multiple regression, Maslanik et. al., [7] recently reported that circulating concentrations of Hsp72 and uric acid are predictive of plasma levels of several stress-responsive inflammatory proteins including IL-1β, IL-18, IL-6, and IL-10. Thus, stress-evoked increases in Hsp72 and uric acid may contribute to stress-evoked SSIRs, although the precise receptor-mediated mechanisms remain unknown. Other DAMPs exhibit delayed release following acute stressors such as high mobility group box (HMGB)-1 [38] and could be involved in persistent or delayed stress-evoked immune activity. Of course, additional factors besides DAMPs also have been identified to play a role in the stress-evoked SSIR.

The Commensal Flora

The commensal flora is a population of symbiotic bacteria and other microorganisms that inhabit the skin and mucosal surfaces of an organism. Even in the absence of infection, endogenous bacteria and other microorganisms that compose the commensal flora outnumber the body's own cells by a factor of 10 [39]. Following a brief developmental period, ecological

secessions culminate in a relatively stable community of commensal bacteria [40]. Regular interactions between the mucosal immune system and these stable bacterial communities are critical for proper regulation of physiological function including mucosal, as well as systemic, immune function [41–43].

Consequently, commensal bacteria are an important factor in stress-evoked sterile inflammatory responses. Both acute and chronic stress impact the composition of the commensal flora [5,40]. Such stress-induced decreases in anti-inflammatory genre of bacteria or stress-evoked shifts in alpha diversity are thought to contribute to stress-evoked cytokine and chemokine production. Alpha diversity is a measure of the diversity of species from a single site such as in fecal samples. It takes into account both the total number of species present in a given sample as well as the relatedness of these species, or their distance on a phylogenetic tree. The idea that stress-evoked shifts in the composition of commensal bacteria play a role in stress-evoked SSIRs is supported by data demonstrating that reduction of the commensal flora using antibiotics attenuates stress-evoked IL-1β and IL-18 responses [5]. Interestingly, antibiotic treatment does not impact stress-induced levels of other inflammatory proteins including IL-6, IL-10, and MCP-1.

MAMPs

The link between the commensal flora and stress-induced SSIRs also implicates another family of immunostimulatory molecules known as microbe associated molecular patterns (MAMPs). MAMPs, such as lipopolysaccharide (LPS), are components of bacterial or other microorganisms' cell walls or membranes and increase in the circulation following stressor exposure. Elevated plasma LPS concentrations have been observed following a single bout of

tail shock [5,44]. Similarly, circulating peptidoglycans are elevated following repeated exposure to the social defeat paradigm [21].

Recent data confirm that MAMPs may play a role in stress-evoked SSIRs. Similarly to oral antibiotic treatment, neutralization of LPS, using an endotoxin inhibitor (EI), also attenuates stress-evoked IL-1β and IL-18 without impacting levels of IL-6, IL-10, or MCP-1 [5]. These results demonstrate that MAMPs play a role in stress-evoked SSIRs. More importantly, they may reveal important information about the pathways stimulated in stress-evoked inflammatory protein production.

IL-1β and IL-18 are synthesized as inactive precursors [45,46]. Post-translational processing, therefore, is required in the complete synthesis and release pathway for IL-1β and IL-18. Intracellularly, this post-translational processing is predominately mediated by caspase-1, an enzyme activated upon the assembly of a signaling complex called the inflammasome [47–49]. Given that antibiotics and EI selectively affect IL-1β and IL-18, these results suggest that the inflammasome is involved in stress-induced SSIRs.

The inflammasomal pathway of stress-evoked SSIRs

The inflammasome is a signaling complex consisting of a receptor bound to inactive proCaspase-1 by an intermediate apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC). Activation of the inflammasome requires two distinct signaling events [50–52]. The first results in the synthesis of pro-IL-1β, pro-IL-18, and components of the inflammasome [53,54]. The first signal also evokes the synthesis of inflammasome-independent inflammatory proteins such as IL-6, IL-10, and MCP-1 [55] which do not require post translational cleavage [46,56]. The second signal stimulates assembly of the inflammasome, resulting in caspase-1 activation, and cleavage of pro-IL-1β and pro-Il-18 into the active cytokines [47,57]. ProIL-1β and proIL-18 may also be cleaved by matrix metalloproteinases, serine proteases, mast cell chymases [58], or other enzymes like PR3 [59] if proIL-1β or proIL-18 are released by necrotic cell death.

Several molecules are candidate signals for inflammasomal pathways. Endogenous, DAMPs [2,35,60,61], including Hsp72 [62] and uric acid, [14,63,64] as well as MAMPs such as LPS, [65] all potently stimulate inflammasome activity [21,22,34,36,44,66]. In fact, co-administration of a DAMP and MAMP is often necessary to activate the inflammasome; a DAMP [67] or MAMP [50–52,57] alone is not sufficient. In the context of stress-evoked SSIRs it appears that MAMPs from the commensal bacteria provide the second signal necessary for inflammasome activation because they were only necessary for stress-induced synthesis of inflammasome-dependent cytokines. On the contrary, DAMPs can be linked levels of both inflammasome-independent and inflammasome-dependent cytokines in the stress-evoked SSIR and, thus, probably act as the first signal in stress-evoked cytokine and chemokine production.

Collectively, the results describing the role of MAMPs and DAMPs in stress-evoked cytokine and chemokine responses suggest that the inflammasome would be an important component of the signaling pathway underlying stress-evoked SSIRs. Thus, a recent investigation examined whether inflammasome associated processes contributed to stress-evoked cytokine and chemokine responses [7]. In this study, administration of the caspase-1 inhibitor ac-YVAD-cmk attenuated stress-induced cytokine and chemokine production supporting a role for the inflammasome. Interestingly, while the authors hypothesized reduction of stress-induced IL-1β and IL-18 responses by the caspase-1 inhibitor, the drug also unexpectedly influenced stress-induced inflammasome-independent IL-6 and MCP-1 responses. These data suggest that the inflammasome may play a role in sterile inflammatory processes that reach beyond its traditional role in IL-1β and IL-18 synthesis.

The role of the caspase-1 inhibitor on inflammasome-independent inflammatory proteins may be due to a secondary or indirect effect of the drug on other participants in the stress-evoked network of cytokines, chemokines, and other immunostimulatory molecules. For example, if inflammasome-dependent cytokines such as IL-1β provide the signal for an IL-6 response; then inhibition of caspase-1 would also attenuate production of IL-6. The notion of a role for IL-1β in IL-6 release is supported by the literature [68]. Furthermore, a characterization of the stress-evoked SSIR over time reveals that IL-1β appears in the circulation prior to IL-6, suggesting, at least temporally, that IL-1β could signal IL-6 production [7]. Finally, based on stepwise multiple regression analyses these studies reveal that circulating levels of IL-1β are significantly predictive of circulating levels of IL-6 thus substantiating a role for IL-1β in IL-6 production [7]. The impact of the caspase-1 inhibitor on other stress-responsive immunostimulatory molecules such as Hsp72 also could be linked to a reduction of IL-6 by the drug [69]. A stepwise multiple

regression revealed that circulating Hsp72 is also predictive of circulating IL-6 [7]; hence a reduction of Hsp72 by the caspase-1 inhibitor could also contribute to decreased IL-6. Similar logic may explain changes in MCP-1 concentrations evoked by the caspase-1 inhibitor. Thus the impact of the caspase-1 inhibitor on inflammasome-independent cytokines may be due to effects on multiple factors in the cascading cytokine network.

In addition to having a direct influence on cytokines such as IL-1β and IL-18, the inflammasome appears to control relationships between inflammatory proteins in the network of cytokines and chemokines produced by a stress-evoked SSIR. Given the apparently different signaling requirements for each inflammatory protein in an SSIR, the importance of the network wide influence of the inflammasome on a stress-evoked SSIR cannot be understated. Not only do several signals converge upon the inflammasome, but the inflammasome also appears to contribute to the downstream synthesis of multiple cytokines and chemokines. The inflammasome, thus, may represent a therapeutic target that could effectively exert broad control of SSIRs.

Summary

As depicted in **Figure 1**, we propose that DAMP and MAMP signaling activate the inflammasome to evoke a diverse set of cytokines and chemokines that function in a network during SSIRs. Because MAMPs from the commensal bacteria were only necessary for stress-induced synthesis of inflammasome-dependent cytokines, MAMPs likely provide the second signal necessary for inflammasome activation. DAMPs, on the other hand, can be linked levels of both families of cytokines in the stress-evoked SSIR and, thus, likely act as the first signal in stress-evoked cytokine and chemokine production.

Current limitations in characterizations of systemic sterile inflammation have resulted in the development of treatments that are often ineffective. Therapeutic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) are often used. These drugs, however, target downstream tissue inflammation [70] and can exacerbate ongoing cytokine and chemokine production resulting in worsened patient outcomes [71]. Several drugs also have been developed to target the cytokine and chemokine responses themselves. Unfortunately, past tendencies to implicate single cytokines in systemic inflammatory responses have supported the development of therapies that are ineffective at reducing many other cytokines and chemokines present in systemic sterile networks of inflammatory proteins. Thus, drugs, such as IL-1ras or NF-κB inhibitors, may fail to contain systemic inflammatory responses because neither factor acts as a central hub [72] for inflammatory proteins in these networks.

Limiting inflammasome activity via caspase-1 inhibition is effective, however, at containing the stress-induced production of pro-inflammatory cytokines. Thus, caspase-1 or other inflammasomal components may be promising targets for future therapeutics to treat systemic sterile inflammation. Additional pre-clinical research is required to optimize the dose or

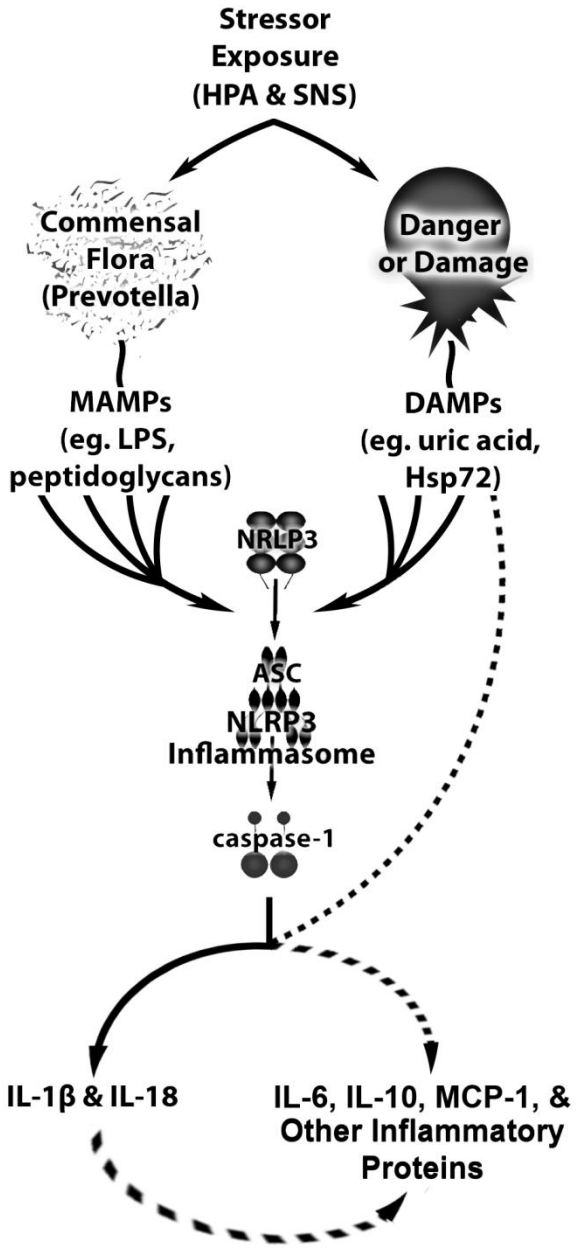

Figure 1: Exposure to a stressor activates the hypothalamic-pituitary-adrenal axis and sympathetic nervous system resulting in changes to the commensal flora (including a decrease in *Prevotella*) and the release of microbe associated molecular patterns (MAMPs) as well as the release of danger associated molecular patterns (DAMPs) either actively or via cell death. DAMP and MAMP signals then converge upon the inflammasome to yield IL-1β and IL-18 production. DAMPs may also act to drive responses from inflammasome-independent inflammatory proteins including IL-6, IL-10, and MCP-1. Solid lines originating at caspase-1 represent direct effects of the inflammasome, while dashed lines represent indirect, network effects of the drug.

structure of the caspase-1 inhibitor required to block SSIRs while minimizing cell death. Furthermore, it is unknown whether or not a caspase-1 inhibitor would be effective at preventing cytokine and chemokine increases that occur in response to other sterile inflammatory stimuli such as trauma. Examining whether or not caspase-1 inhibition is effective at reducing an ongoing response is also necessary. Nonetheless, the data presented in the review suggest that the inflammasome acts as a central mediator of systemic sterile inflammation and is a suitable target for reducing the broad network of cytokines and chemokines produced by sterile inflammatory responses.

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Chapter III

The Impact of Acute-Stressor Exposure on Splenic Innate Immunity: A Gene Expression Analysis

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Abstract

Exposure to intense, acute-stressors modulates immune function. We have previously reported, for example, that exposure to a single session of inescapable tail shock suppresses acquired and potentiates innate immune responses mediated by the spleen. The mechanisms for these changes remain unknown, however, they likely involve stress-induced modulation of cytokines. Cytokines operate in coordinated networks that include other immunoregulatory factors. Broad-scoped analyses are required to gain an understanding of the net-impact of stress on these immunoregulatory factors and the immune system. The goal of this study, therefore, is to examine the impact of acute-stressor exposure on network-wide changes in splenic immunoregulatory factor expression. 161 genes linked to innate immune responses were quantified in the spleen following exposure to tail shock using an RT-PCR based gene array. Expression changes in 17 of the measured genes were confirmed using individual RT-PCR reactions. Further assessment of the expression changes using Exploratory Gene Association Networks (EGAN) identified important ontologies, processes and pathways that are indicative of a broader impact of stress on the immune system. Interestingly, EGAN identified several linkages between immunoregulatory factors that may be important in explaining previous results concerning the functional consequences of stress on splenic immunity. Additional processes, some of which are novel to this study, were also uncovered that may be important in directing future studies examining the impact of stress on the immune system. In this way, these analyses provide a better understanding of how acute stressor exposure modulates splenic immunity and may function as predictive tool for future related studies.

Introduction

Exposure to an acute intense stressor modulates splenic host defense and immune responses. Acute stressor exposure, for example, can increase bacterial clearance (Bailey et al., 2007; Bailey et al., 2006), proliferation of splenic macrophages (Bailey et al., 2007; Salman et al., 1999), and increase antimicrobial responses from splenocytes (Bailey et al., 2004; Campisi et al., 2002), decrease viral defense (Semenov et al., 1998), decrease antibody production (Kennedy et al., 2005) and decrease T-cell proliferation (Gazda et al., 2003; Salman et al., 1999). The immunological mechanisms for these effects likely involve stress-altered cytokine responses (Elenkov and Chrousos, 2006; Johnson et al., 2005). Several potent cytokines including interleukin-1 beta (IL-1β) (Steptoe et al., 2007), interleukin-1 alpha (IL-1α) (Tarantini et al., 2001), interleukin-6 (IL-6) (Ando et al., 1998), interleukin-10 (IL-10) (Connor et al., 2005), and transforming growth factor beta (TGFβ) (Lee et al., 2000) all respond to acute stress. To our knowledge, however, only a few studies have examined the cytokine milieu as a whole (Engler et al., 2005). Instead the majority of publications focus on the effect of stress on a few immune effectors or even a single cytokine.

Our current understanding of cytokine responses suggests that cytokines rarely function in isolation but rather operate in carefully orchestrated networks (Brennan and Feldman, 2000; Frankenstein et al., 2006; Sheu et al., 2008; Tieri et al., 2005). These networks include immunoregulatory factors comprised of not only cytokines, but also chemokines, co-stimulatory molecules and their respective receptors. Rather than acting by themselves, the immunoregulatory factors in these networks operate in a coordinated or interacting manner to determine the net effect of an immune response. This dependence of immunoregulatory factors on one another necessitates studying cytokines and other immunoregulatory factors within the

context of broad networks (Brennan and Feldman, 2000; Kim and Maes, 2003). Proinflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNFα, for example, can all function synergistically to stimulate or enhance host defense. Increases in these cytokines which are classically immunostimulatory may not necessarily stimulate any type of host defense as their role in an immune response may be heavily opposed by similar increases in the strongly immunosuppressive regulatory factors including TGFβ, IL-10, and several decoy receptors such as the type 2 interleukin-1 receptor (IL1R2) (Akoum et al., 2007). Furthermore, IL-6 can have an anti-inflammatory influence in the absence of other proinflammatory cytokines, particularly TNFα and IL-1β (Bruunsgaard, 2005; Petersen, 2005). Changes in any immunoregulatory factor, therefore, are more meaningful if they are measured and reported within networks of other related immune effectors. The goal of the current study is to provide a better understanding of the network of immunoregulatory factors affected by the stress response. We examined data generated from an RT-PCR based gene array using a newly available bioinformatics tool to uncover network-wide changes in immunoregulatory factor production in response to stress and to interpret the functional consequences of these alterations.

The bioinformatics tool, Exploratory Gene Association Networks (EGAN), was used to analyze changes in gene expression and identify important ontologies, processes, and pathways (GO, KEGG, MeSH) common to this stress-responsive immunoregulatory factor gene network. This analysis is important in that it may add to an understanding of the mechanisms underlying many of the previously reported stress-induced alterations of splenic host defense. To identify stress responsive immunoregulatory factors in the spleen for these analyses, the effect of tailshock was examined on a number of immunoregulatory factors important early in the immune

response using RT-PCR based gene arrays. Tailshock was selected because it is a well characterized intense acute stressor previously reported to impact splenic immunity and host defense including T-cell proliferation (Gazda et al., 2003), proinflammatory responses to LPS (O'Connor et al., 2003) and antigen specific antibody production (Gazda et al., 2003; Kennedy et al., 2005). We assessed gene expression changes immediately after stressor termination because previous work demonstrated changes in splenic immune processes are disrupted at this time (Kennedy et al., 2005). The use of an RT-PCR array enabled the assessment of 161 cytokines, chemokines, co-stimulatory molecules, and associated receptors. This approach has several advantages over "whole genome" arrays. The approach, for example, minimizes the analytical complexities of gene chip-Affymetrix type analyses that measure a much larger number of genes most of which are not relevant to immune function. In addition, the array analysis is both broad-scoped (161 genes) and focused on innate or early immune processes that are likely rapidly impacted by stressor exposure. To account for false positives and provide the most accurate possible list of splenic immunoregulatory factor gene changes in response to stress, significant changes in gene expression identified in the array were confirmed using individual quantitative RT-PCR reactions.

Materials and Methods

Sample collection and animal treatment

Subjects

Adult male Fischer 344 rats (200-230 g) were maintained on a 12:12-h light-dark cycle (lights on from 0700 to 1900) in a specific pathogen free environment. Animals were allowed 1 week to acclimate to the colony room prior to any experimental manipulation, during which time rats were handled briefly each day. All animals were housed in Plexiglas Nalgene cages and allowed access to food (Harlan Laboratories, Denver, CO) and water *ad libitum*. Colony room temperature was maintained at 23°C. The care and treatment of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Stressor Protocol

On the day of the experiment, animals either remained in their home cages as controls (Control) or were exposed to 100, 1.5mA, 5-second, intermittent, (average inter-trial interval = 60 seconds +/- 25 seconds) inescapable tail shocks (Stress) as previously described (Campisi and Fleshner, 2003; Kennedy et al., 2005). This stressor was selected because of its established role in modulation of the immune system (Campisi and Fleshner, 2003; Deak et al., 1999; Johnson et al., 2005; Kennedy et al., 2005). During the stress procedure rats were placed in a Plexiglas restraining tube (23.4 cm long, 7 cm diameter). Electrodes were then placed across the tail that was protruding from the back of the shock tube. The shocks were administered by an automated shock system (Precision Calculated *Animal Shocker***;** *Colbourne* Instruments). Stress occurred

between 0900 and 1200. Immediately after termination of stress, all animals were sacrificed via rapid decapitation.

Sample collection

Immediately following decapitation spleens were aseptically dissected, sectioned into equal halves and transferred to 1.5ml polypropylene tubes and frozen on dry ice. The spleens were frozen at -80.0°C until further processing. All gene expression analysis procedures measured changes in immunoregulatory factor expression in cross-sections of spleens from the same animals. Confirmatory protein measurement was performed in a separate set of spleens taken from age matched male F344 rats after exposure to the identical tailshock stressor previously described (n=16, number of groups (j)=2).

Quantitative RT-PCR gene arrays

RNA Isolation and Reverse Transcription

RNA was isolated from frozen spleens (~90.0 mg; n = 4 per group; j = 2) using TRI Reagent (Sigma Aldrich, St. Louis, MO) per included instructions. Briefly, samples were homogenized, chloroform added and RNA isolated with isopropanol. The resultant RNA pellet was washed, dried and resuspended in RNase-free water. RNA was further purified using the RNeasy Kit (Qiagen, Valencia, CA) and stored at -80ºC.

Reverse transcription of total spleen RNA (1.0 µg) was achieved using the QuantiTect Reverse-Transcription Kit (Qiagen, Germantown, Md). Briefly, RNA in RNase-free water was

incubated with genomic-DNA wipeout buffer at 42^{o}C for two minutes to eliminate any contaminating DNA. Samples were then reverse-transcribed in a 20.0 µl volume containing oligo(dT) and random primers, Quantiscript Reverse Transcriptase containing RNase Inhibitor, and Quantiscript RT buffer containing dNTPs. The reaction was carried out for 45 min at 42.0^{o}C and the resultant cDNA used in real-time PCR analysis.

Real-Time PCR Arrays

Differences in gene expression were determined using RT^{2}Profiler^{TM} arrays (SuperArray Biosciences Fredrick, Md). Differences in splenic gene expression between stress and control animals were compared using toll-like receptor signaling (**Table 1**) and inflammatory cytokines and receptors (**Table 2**) arrays that contained 84 unique target genes and 12 negative and positive control wells. A master mix containing cDNA, RT^{2} SYBR Green with Fluorescein Universal PCR Master Mix (Super Array Biosciences, Fredrick, Md), and RNase free water was aliquoted into 25.0 µl reactions. Real-time PCR reactions were first denatured for 10 min at 95^{o}C followed by 40 cycles of 15 seconds at 95^{o}C (denature) and 60.0 sec of 60.0^{o}C (anneal and amplification). Real-time PCR reactions were performed using a Bio-Rad iCycler (Hercules, CA). To ensure specificity of primer combinations dissociation curves were run with each array and PCR products from randomly chosen arrays were separated on a 1.0% agarose gel revealing the presence of a single band for each PCR product.

The threshold cycle (Ct) value for each gene was defined as the PCR cycle at which the emitted fluorescence rose above a background level of fluorescence. Results of real-time PCR

Table 1: Toll-Like Receptor Gene Array							
Btk	*Fadd*	*Il1a*	*Loc299827*	*Mapk8ip3*	*Eif2ak2*	*Tlr4*	*Rplp1*
Casp8	*Fos*	*Il1b*	*Tlr7*	*Mapk9*	*Ptgs2*	*Tlr5*	*Hprt*
Ccl2	*Hmgb1*	*Il1r1*	*Lta*	*Myd88*	*Rel*	*Tlr6*	*Rpl13a*
Cd14	*Hras*	*Il2*	*Cd180*	*Nfkb1*	*Rela*	*Tlr9*	*Ldha*
Cd80	*Hspa1a*	*Il6*	*Ly96*	*Nfkbia*	*Nfkb2*	*Tnf*	*Actb*
Cd86	*Hspd1*	*Il6ra*	*Mal*	*Nfkbib*	*Ripk2*	*Tnfrsf1a*	
Cebpb	*Ifna1*	*Irak1*	*Map2k3*	*Nfkbil1*	*Rnf138*	*Tnip2*	
Chuk	*Ifnb1*	*Irak2*	*Map2k4*	*Nfrkb*	*Sarm1*	*Tollip*	
Clecsf9	*Ifng*	*Irf1*	*Map3k1*	*Nr2c2*	*Ticam2*	*Tradd*	
Csf2	*Ikbkb*	*Irf3*	*Map3k7*	*Peli1*	*Tlr1*	*Traf6*	
Csf3	*Il10*	*Jun*	*Map4k4*	*Pglyrp1*	*Tlr2*	*Ube2n*	
Cxcl10	*Il12a*	*Kcnh8*	*Mapk8*	*Ppara*	*Tlr3*	*Ube2v1*	

Table 1. Genes measured for relative expression levels in the RT^{2}Profiler^{TM} rat toll-like receptor signaling pathway PCR Array (Catalog no. APRN-018A; Lot. 20061114; SuperArray).

Table 2: Inflammatory Cytokine Gene Array							
Abcf1	*Ccl21b*	*Ccr3*	*Cxcl11*	*il13ra1*	*Il2rg*	*Ltb*	*Rplp1*
Bcl6	*Ccl22*	*Ccr4*	*Cxcl12*	*il15*	*Il3*	*Mif*	*Hprt*
Blr1	*Ccl24*	*Ccr5*	*Cxcl2*	*il16*	*Il4*	*Cxcl4*	*Rpl13a*
C3	*Ccl25*	*Ccr6*	*Cxcl5*	*il17b*	*Il5*	*Scye1*	*Ldha*
Rgd1561905	*Ccl3*	*Ccr7*	*Cxcl9*	*il18*	*Il5ra*	*Spp1*	*Actb*
Casp1	*Ccl4*	*Ccr8*	*Cxcr3*	*il1a*	*Il6ra*	*Tgfb1*	
Ccl11	*Ccl5*	*Ccr9*	*Gpr2*	*il1b*	*Il6st*	*Tnfa*	
Ccl12	*Ccl6*	*Crp*	*Ifng*	*il1f5*	*Il8ra*	*Tnfrsf1a*	
Ccl17	*Ccl7*	*Cx3cl1*	*Il10*	*il1f6*	*Il8rb*	*Tnfrsf1b*	
Ccl19	*Ccl9*	*Cx3cr1*	*Il10ra*	*il1r1*	*Itgam*	*Cd40lg*	
Ccl2	*Ccr1*	*Cxcl1*	*Il11*	*il1r2*	*Itgb2*	*Tollip*	
Ccl20	*Ccr2*	*Cxcl10*	*Il13*	*il2rb*	*Lta*	*Xcr1*	

Table 2. Genes measured for relative expression levels in the RT^{2}Profiler^{TM} rat inflammatory cytokines and receptors PCR Array (Catalog no. APRN-011A; Lot 20061114; SuperArray)

experiments between stress and control animals were determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak, 2008). Data were first normalized by determining differences in Ct values between the target gene of interest and the average of the housekeeping genes run on the same array, defined as ΔCt (Ct of target gene – Ct of housekeeping gene average). The fold change was calculated as $2^{(SAvg\Delta Ct - CAvg\Delta Ct)}$ where SAvgΔCt -CAvgΔCt is the difference between the stress sample ΔCt and the control sample ΔCt. Appropriate housekeeping genes were identified by selecting a combination of housekeeping genes (Rplp1, Hprt, Rpl13a, Ldha, Actb) with average Ct values that were less than one cycle different.

Quantitative real-time PCR

RNA extraction

RNA was isolated from frozen spleens (200 mg; n=10, j=2) using Trizol (catalog no. 15596-028; Invitrogen) per included instructions. Briefly, samples were homogenized, chloroform added and RNA isolated with isopropanol. The resultant RNA pellet was washed, dried and resuspended in RNase-free water. RNA was further purified using the RNeasy Kit (Qiagen, Valencia, CA) and stored at -80ºC. Each reconstituted sample was diluted 1:300 and split into 20.0 µl aliquots which were frozen at -80.0°C. A standard curve created using additional dilutions (1:10, 1:100, 1:1000, 1:10000) of RNA from a control animal.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was used to verify significant expression differences for individual genes selected from the gene arrays using QuantiTect SYBR Green RT-PCR (catalog no. 204243; Qiagen) in accordance with manufacturer's instructions. Aliquots of frozen RNA were thawed, gently agitated, and briefly centrifuged. 5.0 µl of each aliquot was added to an optical 96-well reaction plate (catalog number N801-0560; Applied Biosystems) on ice to which 20.0 µl of SYBR Green Mastermix (750.0 µl 2x mix, 15 µl reverse transcriptase, 350.0 µl RNase free H_2O, and 30.0 µl forward and reverse primers) was added. The reaction plate was covered with an optical adhesive cover and subjected to thermal cycling using an Applied Biosystems 7500 Real Time PCR system in accordance with manufacturer's instructions. Data were normalized by determining differences in Ct values between the target gene of interest and the average of *Actin*, a housekeeping gene, defined as ΔCt (Ct of target gene – Ct of *Actin*). Fold change was then calculated as described above. A dissociation curve was run at the end of 41 cycles to confirm amplification of a single product.

Gene Specific Primers

The gene specific primer sequences for *Actin*, *Il1a*, *Il1r1*, *Il1r2*, *Il6*, *Il6ra*, *Il10*, *Mip2*, *Tnf*, *Lta*, *Tnfrsf1b*, *Ccl12*, *Ccl20*, *Cxcl5*, *Ccr3*, *Ccr9*, *Tlr3*, *Tlr7*, *Hspa1a*, *CD14*, *CD180*, and *CD86* were custom synthesized (Custom oligos; Invitrogen) from sequences designed using Primer3 and verified using a sequence viewer and ePCR (NIH). When possible, primers were designed to span an exon-exon boundary. The primer sequences used in this analysis are shown on **Table 3**.

Table 3: RT-PCR Primer Sequences		
Gene	**Forward Primer**	**Reverse Primer**
Actin	5' GACCCAGATCATGTTTGAGACC 3'	5' TAACCCTCATAGATGGGCAGAG 3'
Ccl12	5' AATGTCGCTAAGCAGAAGATCC 3'	5' CATGGAGTCCTTAACCCACTTC 3'
Ccl20	5' CTGCCTCACGTACACAAAGAAC 3'	5' CACACGGATCTTTTCGACTTC 3'
Ccr3	5' CCAACGAAGAGGAACTCAAGAC 3'	5' CTGATGCTGACTTTTTCACAGG 3'
Ccr9	5' ATGTAAGGCAGTTTGCGAGTC 3'	5' TTGTGCAGTACCAGTAGACAAGG 3'
Cd14	5' CTCTGCCCTTGAAGAGGCTTAC 3'	5' CCTCGAGGTTTTCAAGAGTCAG 3'
Cd180	5' TAATGCTATCCATCACCTGTCG 3'	5' CGGGCTCTATTCCTGTAATGTC 3'
Cd86	5' CAGGCTCTACGACTTCACAATG 3'	5' TCTGAGCCTCCTCTATTTCAGG 3'
Cxcl5	5' CCACAAGCTCCGTTGATAAAG 3'	5' CTGATCTGACCAGTGCAAGTG 3'
Hspa1a	5' CAAGGTGCAGGTGAACTACAAG 3'	5' GCGATCTCCTTCATCTTGGTC 3'
Il10	5' CCCCTGTGAGAATAAAAGCAAG 3'	5' TCATTCATGGCCTTGTAGACAC 3'
Il1a	5' GCCCTTTACTGAAGATGACCTG 3'	5' TCATGATGAACTCCTGCTTGAC 3'
Il1r1	5' CGAAGTCTTGTGTGCCCTTATC 3'	5' GAATCCAAAGAAGTTTCCATCG 3'
Il1r2	5' CCCTGACCTGAAAGAGTTCATC 3'	5' CTTGCCCTCGTAGGTAAATGTC 3'
Il6	5' CTAAGGACCAAGACCATCCAAC 3'	5' TTTCTGACCACAGTGAGGAATG 3'
Lta	5' CCAGCAAACAGAATTCACTGC 3'	5' GAGAAAACCACCTGGGAGTAGAC 3'
Mip2	5' CTACCAAGGGTTGACTTCAAGAAC 3'	5' GCCTTTGTTCAGTATCTTTTGGAC 3'
Tlr3	5' CACAAGCATCCAGAATCTCTCTC 3'	5' GAGGCTGTTGTAGGAAAGATCAAG 3'
Tlr7	5' CTCTACCTTGTGATGTCTCTCTCG 3'	5' AGGGTAAGGTTGGTGGTATTAGTG 3'
Tnfa	5' TCTCAAAACTCGAGTGACAAGC 3'	5' TACCACCAGTTGGTTGTCTTTG 3'
Tnfrsf1b	5' AGGTCTGGAACCATCTGCATAC 3'	5' GAATGCAATTTCAAGGCACAG 3'

Table 3. Primers used in verifying the previously measured significant changes from the array results. All primers were designed using primer3 and verified using sequence viewer and ePCR. When possible, primers span exon-exon boundaries.

Bioinformatics Analysis

EGAN examines a list of genes for significant changes within the context of all genes measured to expose both functional linkages, as well as enriched biological themes by looking for the common occurrence of GO, MeSH, and KEGG terms using a standard one-tailed Fisher exact (hypergeometric) test for enrichment calculations (Paquette and Tokuyasu, 2010). The program then outputs a diagram portraying the network of genes and their functional or pathway-based relationship to one another. For this analysis all genes examined in the array were entered into the software and all significant gene changes, as well as genes exhibiting only significant positive or negative fold changes were selected for use in constructing networks. Non-overlapping terms from EGAN were selected for inclusion in the final diagram.

Protein Quantification

Sample Preparation

Approximately 200 mg of each spleen (n=24, j=3) was homogenized in extraction buffer (Enzo Biosciences) containing 1x concentration of Complete EDTA free protease inhibitor (Roche) using an automated, bead based homogenizer (Precellys). Homogenates were aliquoted and then frozen at -80 °C until further analysis could occur.

Protein Measurement

Splenic immunoregulatory factor protein levels (IL-1α, IL-10, and Hsp72) as well as total protein concentration were measured from plasma in 96-well microtiter plates using commercially available ELISAs or other colormetric assays in accordance with manufacturer's instructions. IL-1α and IL-10 were measured in ELISAs from R&D Systems. Hsp72 were measured in ELISAs from Enzo Biosciences. Splenic total protein was measured via

bicinchoninic acid protein assay (BCA) from Thermo Scientific. All splenic immunoregulatory factor levels were normalized to total protein for the purpose of graphs and analyses.

Statistical Analysis

An independent T-test was performed on linear Ct values between groups. Significant changes in Ct, as identified by statistical analysis, were converted to fold change (Livaka et al., 2001) and presented for the arrays. The level of significance was set at $p < 0.05$. A multiple analysis of variance was performed to determine the presence of an overall effect of stress on all genes measured in individual RT-PCR reactions. Multiple comparisons using analyses of variance and a Bonferonni correction were performed in order to examine the effect of stress on each individual gene. Outliers, as identified by the Grubbs' Test for Outliers with $p<0.05$, were removed from all datasets (Grubbs, 1969). Significance was determined based upon $p<0.05$. All statistical analyses were performed using SPSS Statistics v. 17.0. Figures 1 and 2 show gene expression changes measured in each array visualized using a volcano plot. Volcano plots facilitate the selection of significant gene changes based upon both statistical significance (p-value) and biological importance (magnitude of expression change). Figures 3 and 4 show group means with error bars representing 1 standard error.

Results

TLR Signaling Pathway PCR Gene Array

Of the 84 genes measured in the TLR signaling pathway gene array, 10 were expressed at significantly different levels ($p<0.05$) after acute stress and warranted further investigation in order to confirm observed changes. **Figure 1** shows the relative fold change of each gene's expression level and the corresponding level of significance. Genes appearing outside the vertical lines (representing a 2 fold or greater change) and above the horizontal line (representing $p=0.05$) were selected to be included in further RT-PCR analysis. Those genes selected for further examination, in order of the magnitude of the fold difference from control, are as follows: *Hspa1a* (+32.00), *Il1a* (+3.19), *Cd14* (+2.69), *Il10* (+2.51), *Cebpb* (+2.42), *Cd86* (+2.38), *Tnfa* (-2.22), *Cd180* (-2.14), *Il6* (-2.07), and *Lta* (-2.00). In addition to these genes with significant 2 fold expression differences, 1 other gene, *Tlr3* (+1.97), was also chosen for further analysis. A significant effect of acute stress on *Tlr3* gene expression was observed.

Inflammatory Cytokine and Chemokine PCR Gene Array

Of the 84 genes measured in the inflammatory cytokine and chemokine gene array, 7 were expressed at significantly different levels ($p<0.05$) after acute stress and warranted further investigation in order to confirm observed changes. **Figure 2** is a volcano plot that depicts the relative fold change of each gene's expression level and the corresponding level of statistical significance (p-value). Genes appearing outside the vertical lines (representing a 2 fold or greater change) and above the horizontal line (representing $p=0.05$) were selected to be included in further RT-PCR analysis. Those genes selected for further examination, in order of the magnitude of the fold difference from control, are as follows: *Ccl12* (-11.51), *Il1a* (+6.06),

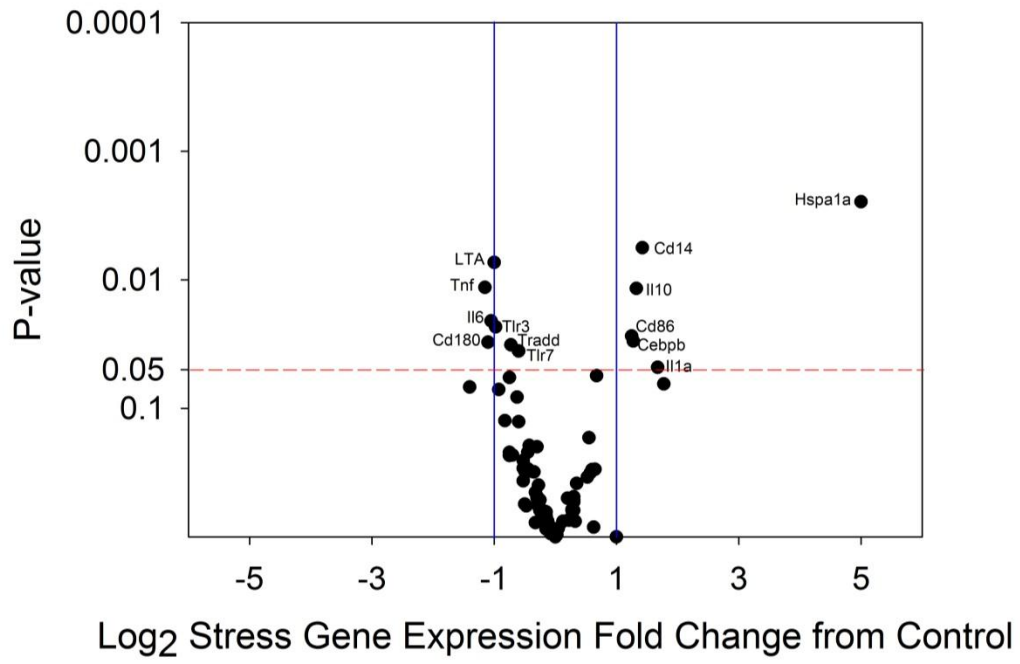

Figure 1. Log_2 Fold change in gene expression following stressor exposure vs. p-value as measured by the RT^{2}Profiler^{TM} rat toll-like receptor signaling pathway PCR Array. Genes occurring outside the solid vertical lines represent genes that experienced a 2 fold or greater change in expression. Genes present above the dashed line represent significant changes (p<0.05). As a general rule, those genes outside of the blue lines and above the red line were selected for further analysis via quantitative real time RT-PCR.

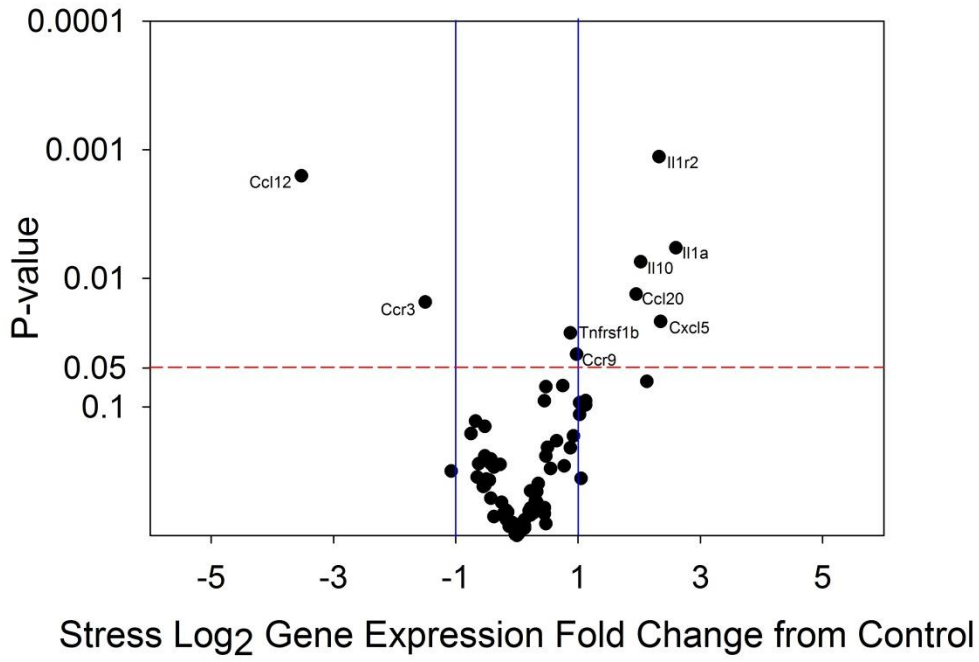

Figure 2. Log_2 Fold change in gene expression following stressor exposure vs. p-value as measured by $RT^2Profiler^{TM}$ rat inflammatory cytokines and receptors PCR Array. Genes occurring outside the solid vertical lines represent genes that experienced a 2 fold or greater change in expression. Genes present above the dashed line represent significant changes ($p<0.05$). As a general rule, those genes outside of the blue lines and above the red line were selected for further analysis via quantitative real time RT-PCR.

Cxcl5 (+5.10), *Il1r2* (+5.01), *Il10* (+4.07), *Ccl20* (+3.86), and *Ccr3* (-2.83). In addition to these genes with significant 2 fold expression differences, 2 other genes, *Ccr9* (+1.97) and *Tnfrsf1b* (+1.83), were also chosen for further analysis. These genes can be seen to also have significant differences in gene expression after acute stress in the array data.

Quantitative Real-Time RT-PCR

Of the 20 gene expression changes re-examined using RT-PCR, 17 of the changes remained significantly different ($p<0.05$), replicating the data gleaned in the arrays (**Figure 3**). 3 genes, *Ccl20, Il6*, and *Tnfa* exhibited no significant difference in expression between stressed and control samples. *Ccl20* expression trended ($p=0.06$) upward after stress as it did in the array. Of the 3 genes measured that displayed fold changes of less than 2 (*Ccr9, Tnfrsf1b,* and *Tlr3*), *Ccr9* and *Tlr3* had similarly small but significant fold changes, while *Tnfrsf1b* increased by greater than 2-fold upon verification with RT-PCR. Fold change in expression levels of *Hspa1a* from control samples to stressed samples was much larger than any other significant change observed. This is in agreement with what was observed in the array. *Il1a* had a large significant increase in expression under stress, as does the IL-1 decoy receptor *Il1r2*. Overall, *Ccr9, Cd14, Cd86, Cxcl5, Hspa1a, Il1a, Il1r2, Il10,* and *Tnfrsf1b* expression levels all increased significantly following stress, while *Ccl12, Ccr3, Cd180, Lta,* and *Tlr3* expression levels all decreased significantly following stress.

Protein analysis

Protein was measured for three genes, Il1a, Hspa1a, and Il10. These genes were selected from the list of stress-responsive immunoregulatory factors to confirm the relevance of the gene

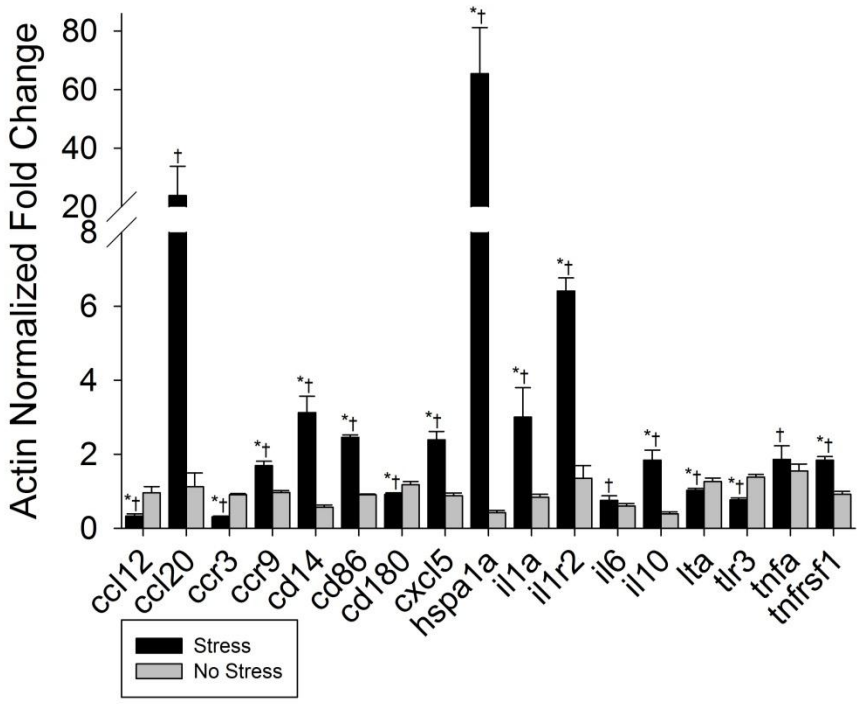

Figure 3. Individual quantitative RT-PCR reactions confirm 17 of 20 gene changes measured in the arrays display altered levels of expression in response to stress. *= significant change was measured in the RT-PCR reaction ($p<0.05$), †= sign of change is the same as measured in the array.

changes. IL-1α (**Figure 4a**) and Hsp72 (**Figure 4b)** each changed in response to stress ($p<0.05$) just as measured in the RT-PCR arrays and individual RT-PCR reactions. Of interest, IL-1β, which was not transcriptionally affected by stress (**Figure 4c**), also increased in response to stress suggesting the presence of other downstream regulatory mechanisms that are important to stress-induced immunoregulatory factor production. No changes in total protein were observed.

EGAN Analysis

Using the genes exhibiting significant expression changes, EGAN identified multiple processes, ontologies, and pathways common to a number of genes in the network (**Figure 5**). The gene network was then broken down into genes exhibiting positive expression changes and genes exhibiting negative expression changes. In doing so, some processes or pathways can be seen to be specifically up or down regulated by stress. Double-stranded RNA binding, for instance, is associated with negative expression changes suggesting that this process may be inhibited by stress. Conversely, hematopoietic cells, the MAPK signaling pathway, cell proliferation, and the intestinal immune network for IgA production are all associated with positive expression changes suggesting that these processes may be stimulated by the stress response.

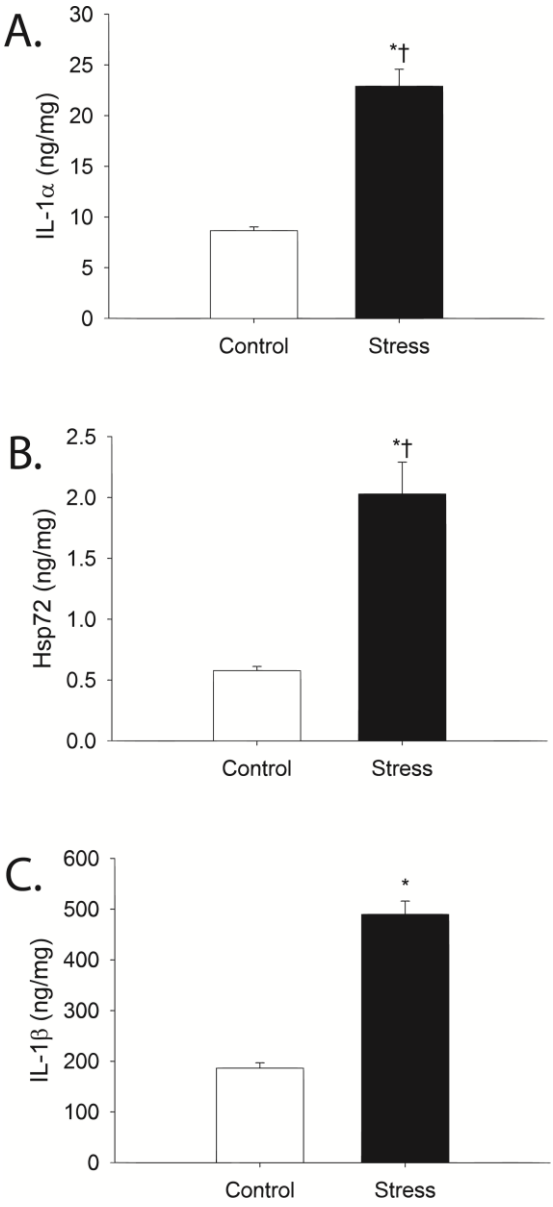

Figure 4. Protein changes for several immunoregulatory factors measured via RT-PCR were quantified to examine the relevance of the gene changes. (**Figures A and B**) Two of the proteins exhibited similar changes to those measured in the array and individual RT-PCR reactions. (**Figure C**) However, IL-1β protein levels changed despite any effect of stress on Il1b mRNA levels. *= significant change was measured in the RT-PCR reaction ($p<0.05$), †= sign of change is the same as measured in the array.

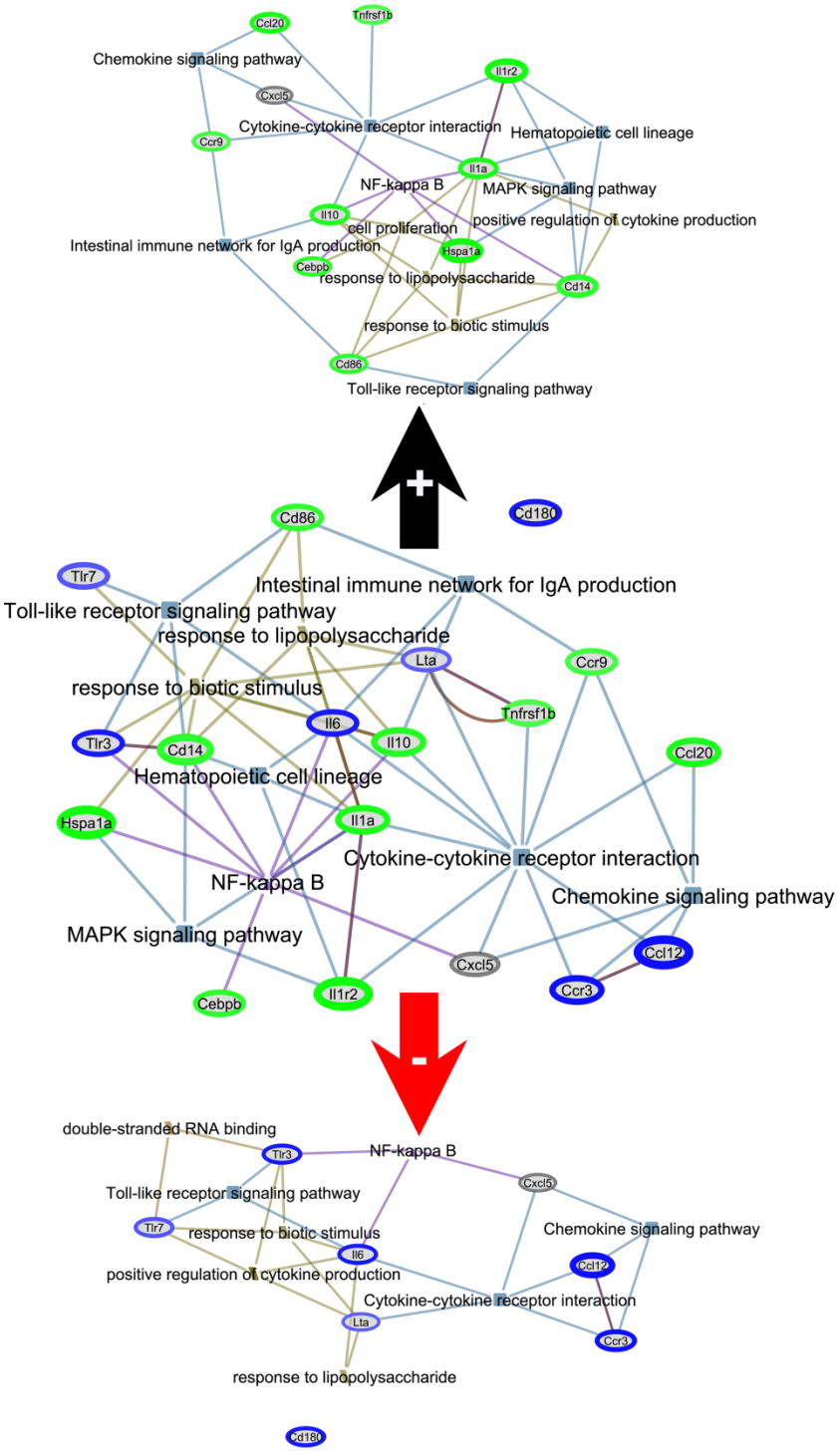

Figure 5. EGAN identified multiple processes and pathways linking the genes that responded to stress in the spleen. These linkages were established using GO, MESH, and KEGG terms. The genes were then broken down into those with upregulated or downregulated transcription in order to better visualize which processes or pathways were stimulated by stress, and which were inhibited. All colors used in the diagram represent source of information as defined in the default settings of EGAN (Paquette and Tokuyasu, 2010).

Discussion

The profound impact of the stress response on the immune system is well established. Stress-induced changes in immunoregulatory factor production potentially underlie the impact of stress on splenic host defense including increased bacterial clearance (Bailey et al., 2007), increased antimicrobial responses (Bailey et al., 2007), decreased antibody production (Fleshner, 2000; Fleshner et al., 1989; Kennedy et al., 2005), and decreased viral clearance (Semenov et al., 1998). The present study confirmed that exposure to a well characterized acute stressor can affect the expression of a number of immunoregulatory factors that operate in splenic host defense. Additionally, this study confirmed the relevance of these gene expression changes by measuring similar effects at the level of several of the encoded proteins. These data are also consistent with gene array work in the lung that found several similarly stress responsive immunoregulatory factors including ccl12 and il1r2 (Engler et al., 2005). Interestingly, the current investigation identified a number of splenic immunoregulatory factors that, to our knowledge, have not been previously identified as stress-responsive.

The immune focused broad scope of this study also provided a sufficient number of inputs to detect many functional and mechanistic targets of the splenic stress-immunoregulatory factor network using the EGAN bioinformatics tool. This analysis of the gene expression data using EGAN identified several links between the stress-responsive immunoregulatory factors and immune processes, pathways, and other ontologies that adds to our mechanistic understanding of previously reported stress-induced alterations in host defense. Furthermore, this analysis reveals future targets for stress-immune studies.

Using EGAN, we have identified several linkages that offer mechanistic insight into orchestration of facilitated antimicrobial immunity and improved bacterial clearance reported after acute stressor exposure. Acute stress can improve subcutaneous and splenic bacterial clearance (Bailey et al., 2007; Campisi et al., 2002; Deak et al., 1999; Dhabhar and McEwen, 1999; Fleshner et al., 2009) and there are several different immune mechanisms including IL-1β (Bailey et al., 2009), TNF-α (Bailey et al., 2009), CD14 (Muthu et al., 2010), and Hsp72 (Campisi and Fleshner, 2003; Fleshner et al., 2009; Fleshner and Johnson, 2005) that have been proposed. These earlier studies, however, focused on single cytokines in signaling mechanisms. As such, they likely underestimated the complexity of cytokines and chemokine modulation that could contribute to changes in host defense. The bioinformatics analyses from the current study can provide an explanation for how each of these factors could operate within a network of other immunoregulatory factors that could facilitate antimicrobial immunity and improve bacterial clearance. In doing so, this analysis offers a more sophisticated understanding of the mechanism underlying stress-enhanced bacterial clearance than previous investigations were capable of providing.

The diagram constructed for the current study using EGAN identifies interactions between several stress responsive immunoregulatory factors including Tnfa and Hspa1a (which encodes Hspa72) that along with Cd14, as well as Cd86, and Il1a could enhance the recognition of lipopolysaccharides or other biotic stimuli, a critical step in antimicrobial host defense. In addition, signaling pathways common to several of these stress-responsive immunoregulatory factors also provide important information. MAPK was only significantly linked to the network of immunoregulatory factors that were increased by stress; suggesting that antimicrobial

immunity is enhanced by stress through a mechanism involving MAPK. Of course, there are many immune responses that have been identified as stress responsive in addition to facilitated antimicrobial immunity and enhanced bacterial clearance. Similar scrutiny of the diagrams produced by these analyses could be used analogously to explain these other stress-induced alterations of splenic immune responses such as modulation of viral immunity or decreased antibody production.

In addition to using the network diagram produced in the current study to explain previous observations concerning stress-immune interactions, this network of stress-immunoregulatory factors can also be used as a predictive tool for future studies. The immune mechanism behind the development of allergic diseases, for example, is not well understood. However, the spleen is known to play a role in proper immune regulation critical to preventing allergic sensitization (Nagatani et al., 2006). Furthermore, a role for the stress response has been uncovered in the development of these diseases (Höglund et al., 2006; Tao Liu, 2006). Taken together, these observations may suggest that knowledge of a network of cytokines that are both affected by stress and involved in immune processes inherent to the onset of allergic sensitization could be a suitable starting place for the formulation of a hypothesis to explain the development of allergy. Thus, the current results could be used to form a hypothesis that identifies several immunoregulatory factors may be important to the development of allergy. In addition to allergy, these networks could be used as an initial means of investigating other similar processes of unknown etiology that are affected by stress via in order to make more informed hypotheses concerning an underlying mechanism.

While investigating changes in gene expression afforded the examination of a large number of mediators of immunity, understanding how the observed transcription changes affect protein levels of these immunoregulatory factors is also important. The present investigation measured several of these proteins and found changes that both mirrored and did not mirror the changes in gene expression. Increases in both gene expression and protein for IL-1α and Hsp72 were observed. In contrast, IL-1β gene expression was not notably increased, yet protein levels were increased, in the spleen in response to stress. It is clear that the mechanism by which immunoregulatory factor synthesis takes place is different depending upon the immune messenger. IL-1β for instance may be impacted at the level of post-transcriptional mechanisms such as post-translational processing by caspase-1 (Iyer et al., 2009) or stress-mediated increases of components of the NALP3 inflammasome, also known to play an integral role in IL-1β production (Iyer et al., 2009; Schroder, 2010). Thus further studies that investigate the impact of stressor exposure on the translation of each of these immune messengers are warranted.

The current study is just the beginning. This type of work also should be extended beyond the spleen to include different compartments of the immune system, as it could shed light on the roles of these networks in stress-induced alterations of other specific immune responses. In addition, investigating expression changes at other time points, such as 24 or 48 hours post stress could also be important. Both the later changes resulting from compensatory mechanisms or as part of other slower responses, as well as the timecourse of a return to baseline of the immediately effected immunoregulatory factors, could be very informative. Finally, additional studies are needed to identify the neuroendocrine mechanism(s) responsible for the observed changes in gene expression within the spleen. For example, changes in mRNA could be

indicative of changes in gene expression per cell or could be due to migratory changes in splenic cellular populations.

The current study is important as an initial focused immune array based investigation of the consequences of acute stressor exposure on splenic immunity and has for the first time used bioinformatics tools to describe a splenic stress-immunoregulatory factor network. These data demonstrate the importance of a network-based approach to fully understand the net effect of stress or any similar process on immunoregulatory factor production. The use of these networks could reveal novel pathways involved in stress altered immune responses.

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Chapter IV

Commensal bacteria and MAMPs are necessary for stress-induced increases in IL-1β and IL-18 but not IL-6, IL-10 or MCP-1

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Abstract

Regular interactions between commensal bacteria and the enteric mucosal immune environment are necessary for normal immunity. Alterations of the commensal bacterial communities or mucosal barrier can disrupt immune function. Chronic stress interferes with bacterial community structure (specifically, α-diversity) and the integrity of the intestinal barrier. These interferences can contribute to chronic stress-induced increases in systemic IL-6 and TNF-α. Chronic stress, however, produces many physiological changes that could indirectly influence immune activity. In addition to IL-6 and TNF-α, exposure to acute stressors upregulates a plethora of inflammatory proteins, each having unique synthesis and release mechanisms. We therefore tested the hypothesis that acute stress-induced inflammatory protein responses are dependent on the commensal bacteria, and more specifically, lipopolysaccharide (LPS) shed from Gram-negative intestinal commensal bacteria. We present evidence that both reducing commensal bacteria using antibiotics and neutralizing LPS using endotoxin inhibitor (EI) attenuates increases in some (inflammasome dependent, IL-1 and IL-18), but not all (inflammasome independent, IL-6, IL-10, and MCP-1) inflammatory proteins in the blood of male F344 rats exposed to an acute tail shock stressor. Acute stress did not impact α- or β-diversity measured using 16S rRNA diversity analyses, but selectively reduced the relative abundance of *Prevotella.* These findings indicate that commensal bacteria contribute to acute stress-induced inflammatory protein responses, and support the presence of LPS-mediated signaling in stress-evoked cytokine and chemokine production. The selectivity of the commensal bacteria in stress-evoked IL-1β and IL-18 responses may implicate the inflammasome in this response.

Introduction

The enteric mucosal immune system is a unique immunological site that must maintain a balance between responding to harmful pathogens and avoiding inappropriate immune responses to food or symbiotic bacteria. During a brief developmental period, ecological secession culminates in a relatively stable community of commensal bacteria [1]. Regular interactions between the mucosal immune system and these bacteria are critical for proper regulation of mucosal as well as systemic immune function [2–4]. Moreover, disruptions to the mucosal environment such as changes in barrier function or microbial composition can lead to severely dysregulated immunity [5,6].

Several diverse factors may impact the mucosal barrier or the composition of the commensal bacteria including antibiotic use [7,8], changes to diet or hygiene [7,8], and activation of the stress response [9–14]. Dense sympathetic innervation of the intestine [15], and stress-inducible, localized mast cell degranulation [5], could facilitate stress-evoked changes to both the composition of the commensal bacteria [9,11,16,17] and the integrity of the intestinal barrier [13,18,19]. Importantly, stress-induced changes to the intestinal barrier or the composition of the commensal bacteria appear to drive some aspects of stress-evoked mucosal and even systemic immune activity. Stress-induced disruptions to the mucosal barrier, for example, are linked to increased serum cytokine levels including tumor necrosis factor α (TNFα) [20]. Similarly, reducing the commensal bacteria via antibiotic administration attenuates chronic or repeated stress-induced enhancements in splenic macrophage activity [18] and circulating levels of the cytokine interleukin-6 (IL-6) [1].

Exposure to stressors, however, evokes a broad cytokine and chemokine response beyond the few cytokines that manipulations to the mucosal environment have been shown to modulate. Stress, for example, increases circulating concentrations of several inflammatory proteins including not only TNFα and IL-6, but also interleukin-1β (IL-1β) [21–23], interleukin-18 (IL-18) [21], interleukin-10 (IL-10) [24], and monocyte chemotactic protein-1 (MCP-1) [25–27]. Importantly, these and other cytokines operate in networks with other inflammatory proteins to achieve immunological effects [24]. Moreover, activation, synthesis, release, and mechanisms of various stress-responsive cytokines and chemokines are different and could vary in their modulation by the intestinal bacteria. Multiple stress-responsive cytokines must therefore be considered when investigating the role of intestinal bacteria in stress-induced alterations in immune activation.

Furthermore, previous studies implicating changes to the enteric mucosal immune system in stress-evoked immune activity focus on chronic or repeated stressors such as social defeat or repeated restraint [1]. These stressors not only activate the stress response, but can produce long-term changes to metabolic processes [28], feeding [29], and grooming behavior [30], which could themselves influence immune function or the role of intestinal bacteria in stress-evoked immune activation. Stress-evoked cytokine and chemokine secretion occurs in response to acute stressors. Thus the acute stress response itself might affect the production of these cytokines independent of other stress-evoked long-term adaptations. Understanding the role of commensal bacteria in the acute stress-induced production of a broad range of inflammatory proteins could provide important new information about how stress affects specific immunological pathways.

We therefore tested the hypothesis that acute stress-induced immune modulation depends on commensal bacteria. We reduced commensal bacteria using antibiotics, exposed rats to an acute

tail shock stressor, and measured cytokine and chemokine production. Alterations in gut microbiota composition can influence immune function. A second goal was to test if exposure to an acute stressor would produce changes in microbiota diversity measured using 16S rRNA diversity analyses. Finally, the mechanism by which the commensal bacteria communicate with the immune system during stressor exposure, including acute stressor exposure, remains unknown. LPS, a microbe-associated molecular pattern (MAMP), is found in the cell membrane of some commensal bacteria and can increase in the circulation [19] following intestinal barrier disruption as occurs with chronic stress. Thus a third goal was to determine whether LPS is an important signaling molecule for communication between commensal bacteria and the immune system. We administered endotoxin inhibitor (EI) to block LPS, and measured circulating cytokines and chemokines after acute stress. Our results make several novel contributions to the literature in that they reveal an important role for intestinal bacteria in acute stress-induced immune activation, and support the presence of LPS-mediated signaling from the commensal bacteria in stress-induced cytokine and chemokine production. Furthermore, the results may reveal details of the signaling pathway underlying stress-evoked cytokine and chemokine production and could support the future development of therapeutics designed to manipulate stress-induced immune activity.

Methods

Subjects and Housing

Adult male Fischer 344 rats (240-260 g) were divided equally into four groups crossing stress and antibiotic (N=64) or stress and EI administration (N=32). The Fischer Rat is a highly stress responsive inbred rat, and was chosen for these experiments as the stress response is robust and consistent across animals allowing us to use fewer animals per group. To characterize the impact of stress on the commensal bacteria, rats (N=25) were divided into 3 groups to examine the effect of stress both immediately and 24 hours following stressor termination. All rats were maintained on a 12:12-h light-dark cycle (lights on from 0700 to 1900) in a specific pathogen free environment. Animals were allowed two weeks to acclimate to the colony room prior to any experimental manipulation. Rats were handled briefly each day for 1 week before the start of the study. All animals were housed in Plexiglas Nalgene cages and allowed *ad libitum* access to food (Harlan Laboratories, Denver, CO) and water. Colony room temperature was maintained at 23°C. The care and treatment of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Stress

On the day of the experiment, animals either remained in their home cages (*Control*) or were exposed to 100, 1.5mA, 5-second, intermittent, (average trial interval = 60 seconds +/- 25 seconds) inescapable tail shocks (*Stress*) as previously described [24,35–37]. During the stress procedure, rats were placed in a Plexiglas restraining tube (23.4 cm long, 7 cm diameter). Electrodes were then placed across the tail that protruded from the back of the shock tube. The shocks were administered by an automated shock system (Precision Calculated Animal Shocker**;**

Colbourne Instruments). This tail shock procedure is a well-established model of acute stress that has been thoroughly characterized in terms of both the stress response and the immune response. To examine the role of the commensal flora in an immune response to an acute stressor we selected this model of stress for its robust impact on immune function [24,31–34]. *Stress* occurred between 0730 and 1130 to avoid differences in cytokine and chemokine production due to circadian rhythms. Immediately after termination of stress, all animals were sacrificed via rapid decapitation unless otherwise noted.

Quantification of the stress response

Because the duration, intensity, and chronicity of a stressor determines the immunological consequences of a stressor exposure [35,38,39], and because the absence of the commensal bacteria in germ-free rodents modulates HPA responses after stress [40], we measured two important markers of activation of the stress response to provide a characterization of tail shock. Corticosterone and spleen weights were measured to demonstrate activation of the stress response. Corticosterone is a measure of hypothalamic-pituitary-adrenal axis (HPA) output, and reductions in spleen weight are directly proportional to sympathetic nervous system activity. Corticosterone was measured in 96-well microtiter plates using commercially available ELISAs in accordance with manufacturer's instructions (Enzo Life Sciences). Optical densities were measured using a SpectraMax Plus 354 plate reader (Molecular Devices) and concentrations were analyzed using a four-parameter curve fitting software (SoftMax 5.4.1). Spleens were harvested aseptically and weighed immediately.

Antibiotic Administration

For 4 days prior to *Stress* rats received either drinking water plus 4.0 mg/ml streptomycin and 2.0 mg/ml penicillin g (antibiotic) or drinking water alone (water) *ad libitum* as previously

described [41,42]. Antibiotics were administered in the drinking water to avoid the potential stress-response associated with other delivery methods such as oral gavage [43,44]. The current antibiotic regimen was selected for its broad-spectrum antibacterial effects and because it is consumed by the rats without the addition of any flavoring or sweetener. Each morning, antibiotic solution was replaced because penicillin G has a short half-life at room temperature. Water bottles were weighed daily to estimate water intake of all rats and ensure equivalent doses between animals. Body weights were recorded and fecal matter was examined to monitor sickness or diarrhea in rats receiving antibiotics.

Endotoxin Inhibitor Administration

On the day of *Stress,* EI was prepared by dissolving 1.0mg/ml of EI into sterile PBS, which was stored on ice in the dark until use. Fifteen minutes prior to *Stress,* rats received an intraperitoneal injection (i.p.) of either 1.0 mg/kg EI (Bachem) or PBS alone. This dose was adapted from previous investigations demonstrating that this concentration of EI was sufficient to reduce LPS activity [45,46]. The time between the injection of EI and the start of *Stress* was necessary to achieve maximal efficacy of the drug based upon the short half-life of EI.

Sample Collection

Immediately following sacrifice, whole blood was collected in EDTA coated vacutainers using a polypropylene funnel and centrifuged at 3000xg for 15 minutes at 4°C to obtain plasma samples. Fecal samples were collected from each animal immediately prior to the beginning of *stress* in sterile, media free, dual culture swabs (Becton Dickinson). Additional samples were taken immediately following termination of *stress* and from animals 24 hours following the termination of *stress* in the same manner. Following collection, all samples were frozen at -80°C.

Quantification of Bacteria

In order to confirm the efficacy of our antibiotic regimen, fresh fecal samples were collected from a subset of rats immediately prior to the beginning of *stress*. These samples were homogenized in 2.0 ml PBS and plated at several dilutions on nutrient agar. Plated samples were allowed to incubate at 37.0°C for 48 hours. Following incubation, colony forming units (CFU) of bacteria were counted, and dilution-corrected averages were recorded. Although many anaerobic bacteria will not grow on nutrient agar, this media was selected because it grows both Gram-positive and Gram-negative bacteria. Because the selected antibiotic regimen is broad spectrum, and targets both aerobic and anaerobic bacteria, reduced CFU counted on nutrient agar confirms effective reduction of commensal bacteria by the antibiotic regimen.

Endotoxin Measurement

In a separate experiment, whole blood was collected from *Stress* and *Control* rats in endotoxin free tubes. After 1 hr at room temperature, these samples were centrifuged at 3000xg for 15 min at 4.0 °C to separate serum. LPS was quantified in serum using a Limulus amebocyte lysate (LAL) assay per manufacturer's instructions (Lonza).

16S rRNA microbial community analysis

Fecal samples and cecal contents were collected and prepared for sequencing using previously established protocols [47,48]. Briefly, the MoBio 96 htp PCR clean up kit was used to triplicate, combine, and clean each sample. The samples underwent the PCR reaction with both forward and reverse primers (F515/R806) to target the V4 variable region of the 16S rRNA. The reverse primer contained an error-correcting 12-base Golay code allowing correct demultiplexing of ~1,500 samples even when sequencing introduces errors in the barcode region. After gel purification and ethanol precipitation to remove PCR artifacts, a composite sample containing

equimolar ratios of the amplicons were sequenced with the Illumina HiSeq 2000 (average sequences per sample 34,664 ± 13,577 standard deviation (SD)). The open-source software package QIIME 1.3.0 [49] was used to process the sequences and conduct statistical analysis. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using Uclust [50]. Taxonomy was assigned to OTUs using the Ribosomal Database Project classifier [51] against the GreenGenes 16S rRNA database [52].

Alpha diversity of samples was assessed with the Phlyogenetic Diversity metric, and results confirmed with Chao1 and observed species metrics (data not shown). Each sample was randomly subsampled 10 times, without replacement, at sequence depths from 2,000 to 30,000 sequences per sample at steps of 2,000 sequences per sample. The error bars on **Figure 5c** indicate the range of alpha diversity values at each sampling depth. ANOVA was used to determine whether any bacterial taxa significantly changed in abundance as a result of stress, with a p-value < 0.05 following false discovery rate (FDR) correction.

Cytokine measurement

Circulating concentrations of cytokines (IL-1β, IL-6, IL-10, MCP-1, IL-18, IL-15) were measured from plasma in 96-well microtiter plates using commercially available ELISAs in accordance with the manufacturer's instructions. IL-1β, IL-6, and MCP-1 were measured in ELISAs from R&D Systems. IL-10 and IL-18 were measured in ELISAs from Invitrogen. Optical densities were measured using a SpectraMax Plus 354 plate reader (Molecular Devices) and concentrations were analyzed using a four-parameter curve fitting software (SoftMax 5.4.1).

Statistical Analyses

A two-tailed independent t-test was used to determine whether antibiotic affected numbers of colony forming units of commensal gut bacteria. Two-way repeated measures analyses of variance (ANOVA) were used to test for differences body weight between all groups of rats. Two-way ANOVAs were run to analyze the effect of stress or antibiotic on individual cytokines and chemokines. Data points were treated as outliers if they failed Grubb's test for outliers [53] and were also recorded as affected by experimental procedures by the experimenter. Data are presented as means ± the standard error of the mean. $P<0.05$ was considered statistically significant.

Results

Single exposure to tail shock activated the stress response

Consistent with prior work using this stressor [54–56], exposure to acute tail shock stress resulted in increased plasma corticosterone over control levels ($p<0.001$) (**Figure 1**). Reduced spleen weight, indicating sympathetic nervous system activity [57,58], was observed following stress independent of antibiotic or EI treatment ($p<0.001$) (**Figure 2**). These values represent large changes from baseline and are indicative of a severe acute stressor.

Antibiotic administration effectively reduced commensal bacterial load

The number of colony forming units measured in the fecal samples of rats receiving antibiotics was significantly lower than control rats ($p<0.001$) (**Figure 3A**). In fact, in all but one rat, the number of CFU observed in rats receiving antibiotics was below the detectable limit. This decrease in CFU count suggests that antibiotic administration significantly reduced the commensal bacteria, as expected. No decrease in body weight was observed, suggesting that antibiotics did not create other gross physiological changes that could confound the interpretation of the results (**Figure 3B**).

Stress increased circulating LPS

Levels of LPS in the circulation are quite low at baseline reflecting adequate barrier function of the mucosal surfaces that contain the commensal flora. Exposure to acute stress increases the concentration of LPS measured in plasma ($p<0.01$) (**Figure 4**). Levels of LPS measured in the circulation changed from 0.2708 ± 0.0361 EU to 0.7084 ± 0.1700 EU possibly reflecting changes to the mucosal environment in response to stress.

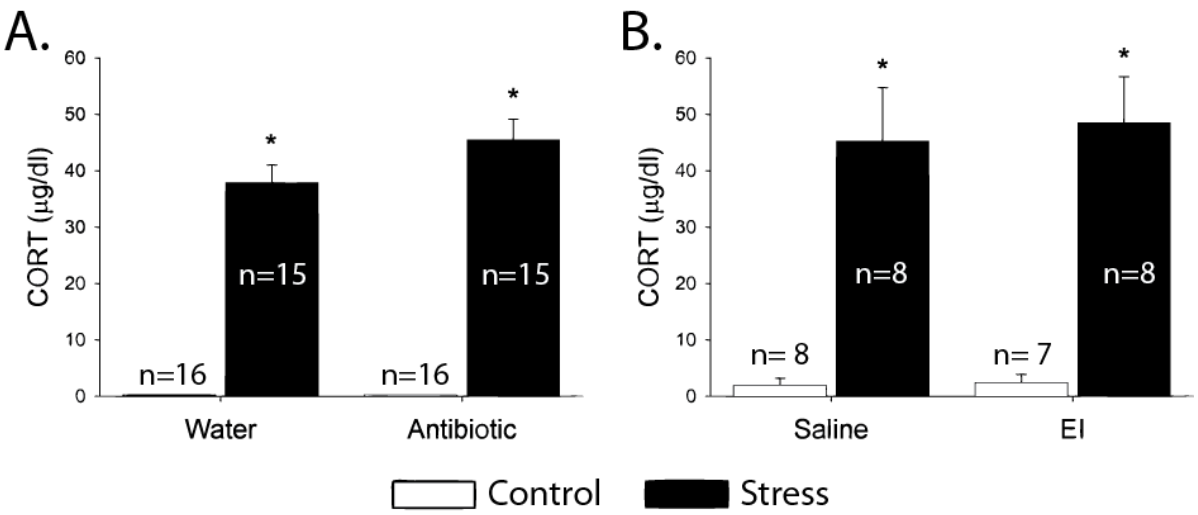

Figure 1: Exposure to an acute stressor significantly increases circulating corticosterone. The increase in circulating corticosterone is typical of acute activation of the hypothalamic-pituitary-adrenal axis such as that which occurs as part of activation of the stress response. Neither antibiotics (**A**) nor endotoxin inhibitor (**B**) impacted the corticosterone response to tail shock. (*$p<0.05$).

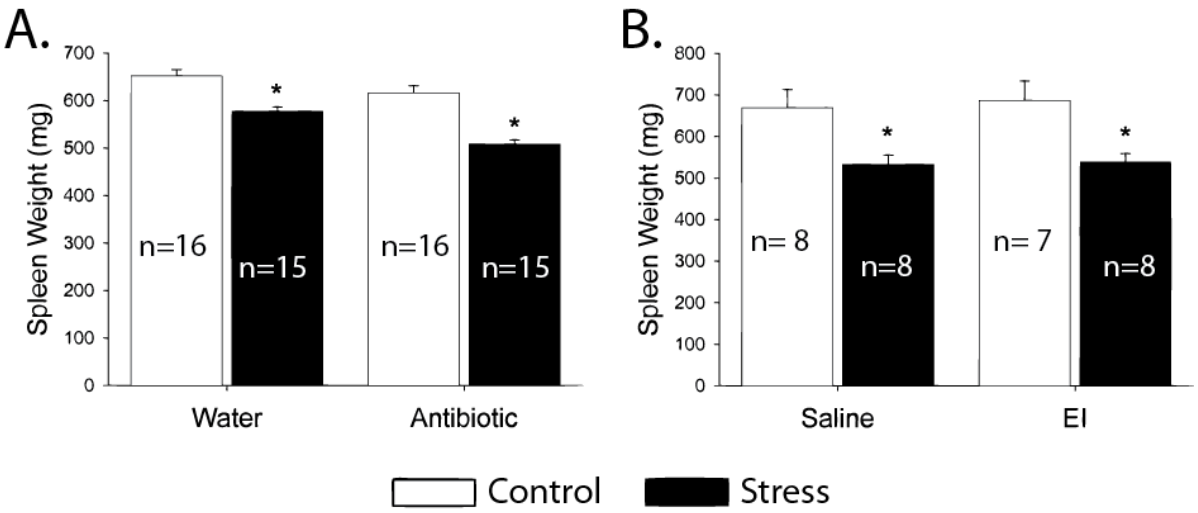

Figure 2: Exposure to an acute stressor causes significant splenic atrophy. This increase is not impacted by either antibiotics (**A**) or endotoxin inhibitor (**B**). Splenic atrophy is typical of acute activation of the sympathetic nervous system such as that which occurs as part of activation of the stress response. (*$p<0.05$).

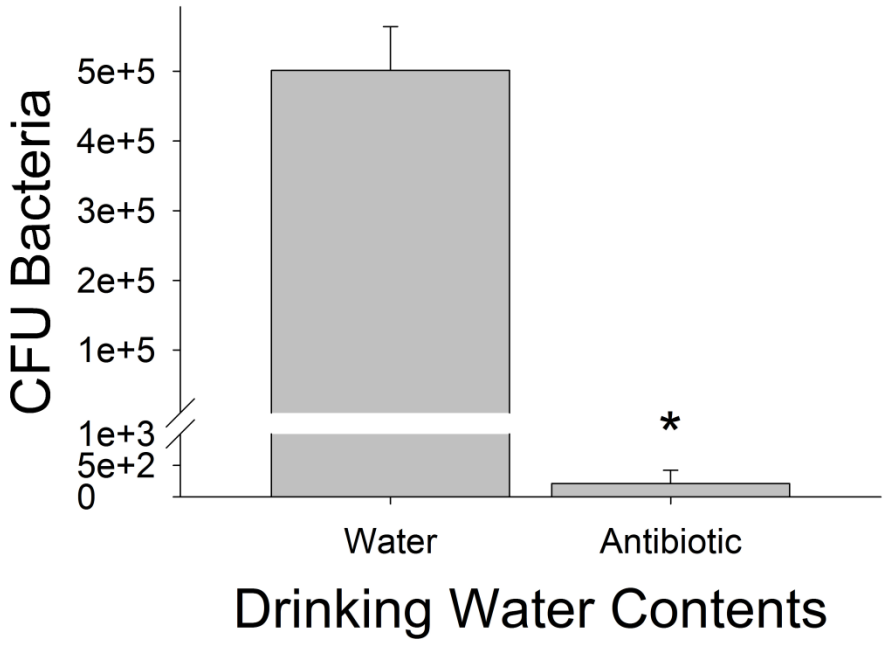

Figure 3A: Colony forming units of bacteria cultured on nutrient agar were significantly reduced in rats receiving antibiotic treatment. Although many species of commensal bacteria cannot be cultured on nutrient agar, the reduction produced by antibiotics is indicative of successful depletion of commensal bacteria by antibiotics, as neither the agar or the antibiotic regimen are specific for any particular group of bacteria. (*$p<0.05$).

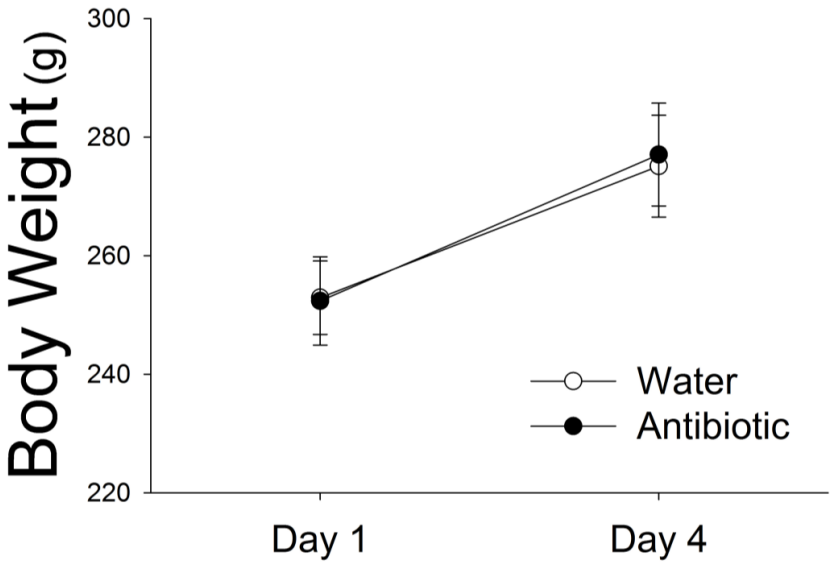

Figure 3B: Body weight changes across antibiotic treatment regimen. Body weights increased in all groups across time and were unaffected by antibiotic treatment. (*$p<0.05$).

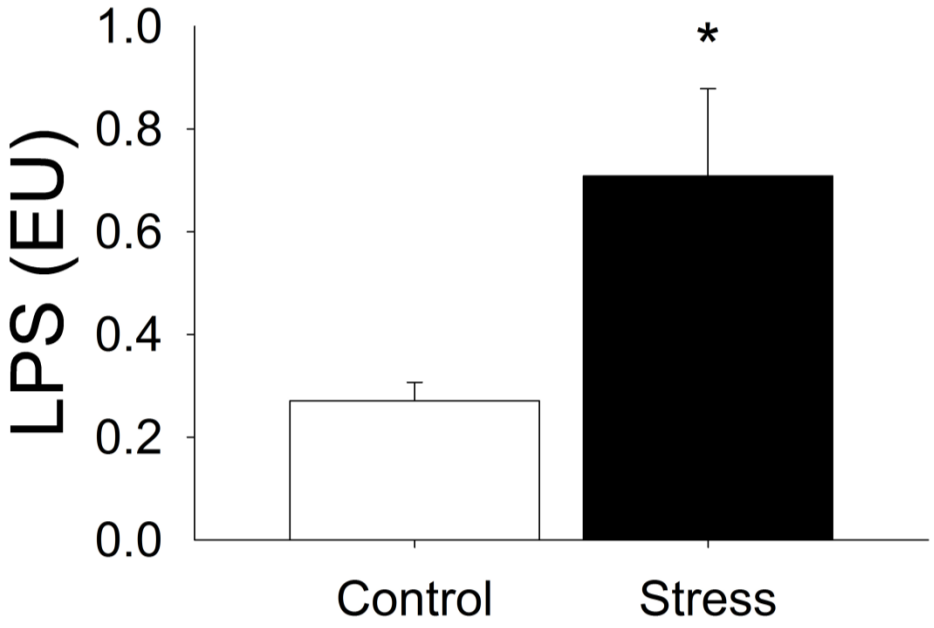

Figure 4: Stress evokes a significant increase in circulating concentrations of LPS. The systemic or circulating increased concentration of LPS is indicative of leakage of the commensal bacteria and their byproducts. (*$p<0.05$)**.**

Acute-stress alters the relative abundance of *Prevotella* but does not impact overall diversity

16S rRNA analysis can reveal the relative abundance of all genera in the microbiome (including genus-level clusters of DNA sequences that have not yet been formally described). Stress caused a decrease in the relative abundance of a single genus, *Prevotella.* The decrease was detectable immediately following stress ($p<0.05$), and persisted for 24 hours after stressor termination ($p<0.01$), in fecal samples taken from the colon (**Figure 5a**). The relative abundance of *Prevotella* also decreased in cecal samples ($p<0.01$), although this decrease was only statistically significant 24hrs after stressor termination (**Figure 5b**). There was no change α-diversity (the mean species diversity on a local scale, such as within a fecal sample) created by acute tail shock stress (**Figure 5c**). Similarly, stress did not impact β-diversity (differentiation in mean species diversity between collection sites).

Antibiotic administration attenuated the production of some cytokines

Stress increased circulating levels of IL-1β ($p<0.001$), IL-6 ($p<0.001$), IL-10 ($p<0.001$), IL-18 ($p<0.001$), and MCP-1 ($p<0.001$) (**Figure 6**). Administration of antibiotics attenuated the stress-induced production of IL-1β ($p<0.01$) and IL-18 ($p<0.05$) (**Figure 6**). However, administration of antibiotics failed to attenuate the stress-induced production of IL-6, IL-10, and MCP-1 (**Figure 6**). Interestingly, although antibiotics reduced the impact of stress on some cytokines, they increased circulating levels of IL-6 following stress ($p<0.05$).

EI administration attenuated the production of the same cytokines as antibiotics

Stress again increased circulating levels of IL-1β ($p<0.001$), IL-6 ($p<0.001$), IL-10 ($p<0.001$, IL-18 ($p<0.001$), and MCP-1 ($p<0.001$) (**Figure 7**). As with antibiotic administration, administration of EI attenuated the stress induced production of IL-1β ($p<0.01$) and IL-18

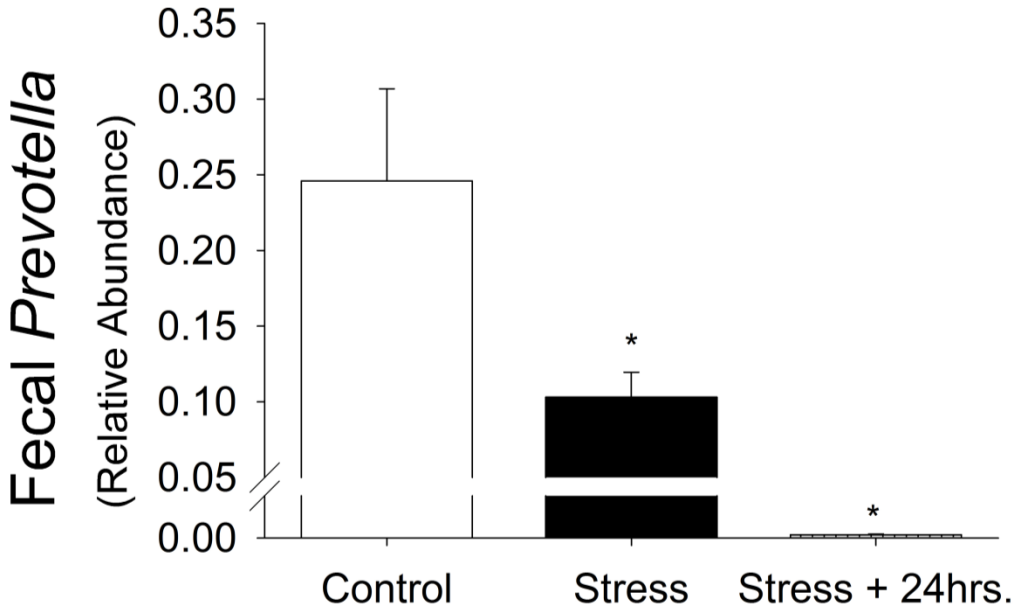

Figure 5A: The relative abundance of *Prevotella* decreased in fecal samples immediately following termination of the stressor. These changes persisted for at least 24 hours after the rats were returned to their home cages. (*$p<0.05$).

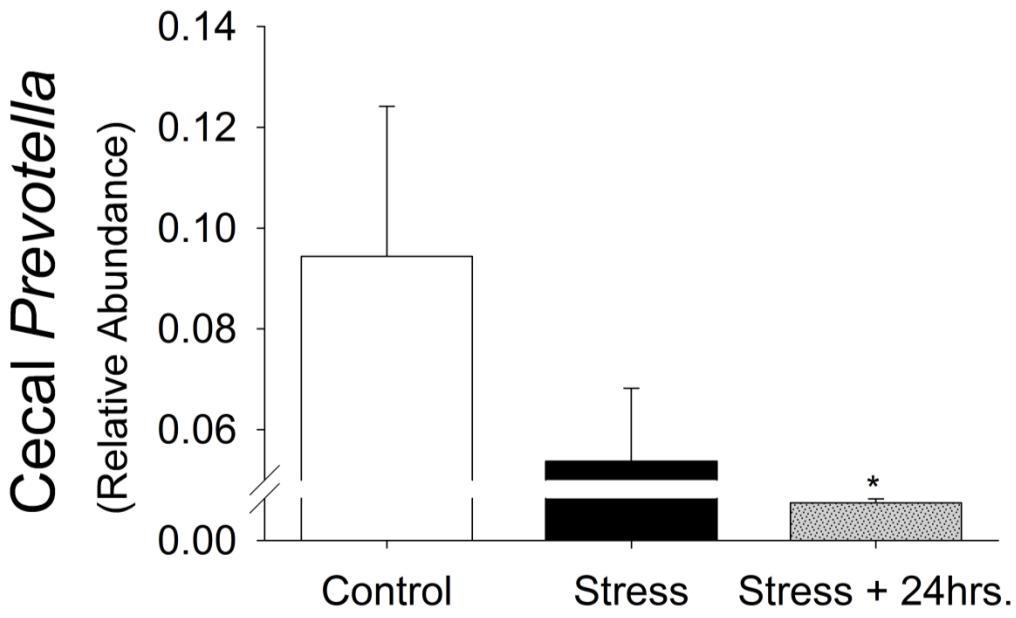

Figure 5B: The relative abundance of *Prevotella* decreased in cecal content samples. Although there was an immediate trend following the termination of the stressor, the difference in the relative abundance of *Prevotella* was only significant 24 hours later (*$p<0.05$).

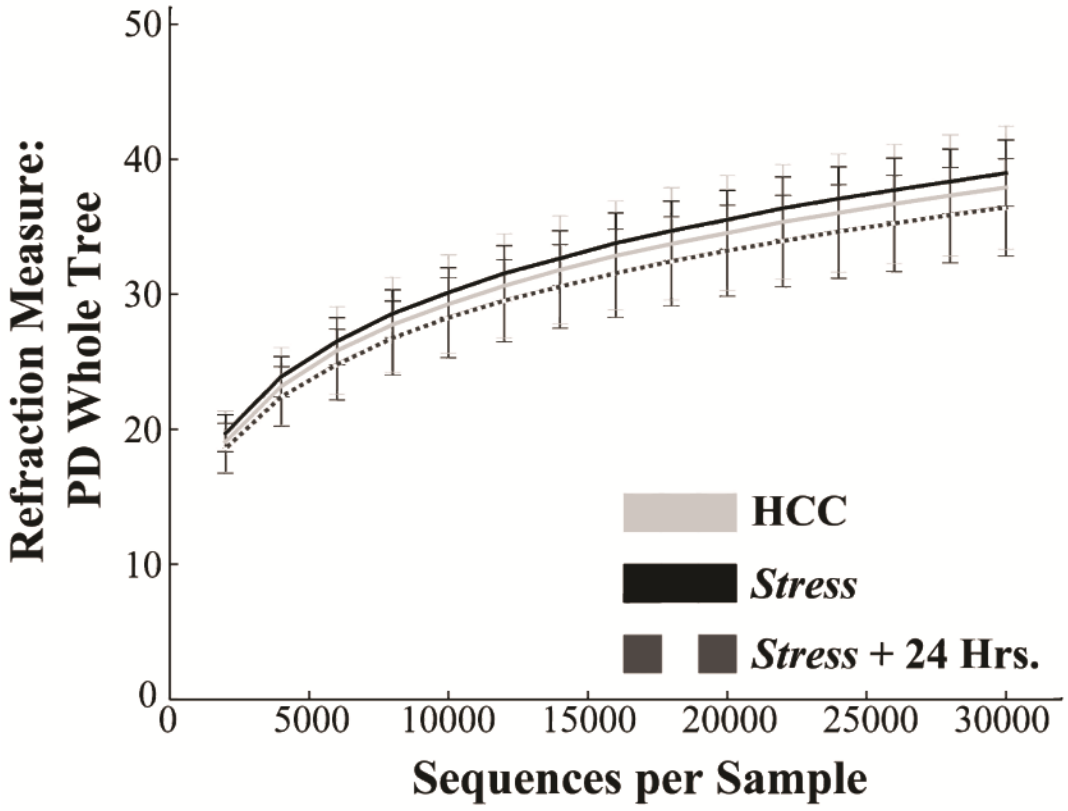

Figure 5C: The alpha diversity plot of fecal contents from rats reveals no effect of stress on overall diversity. Cecal samples similarly showed no changes in overall diversity attributable to stress (data not shown).

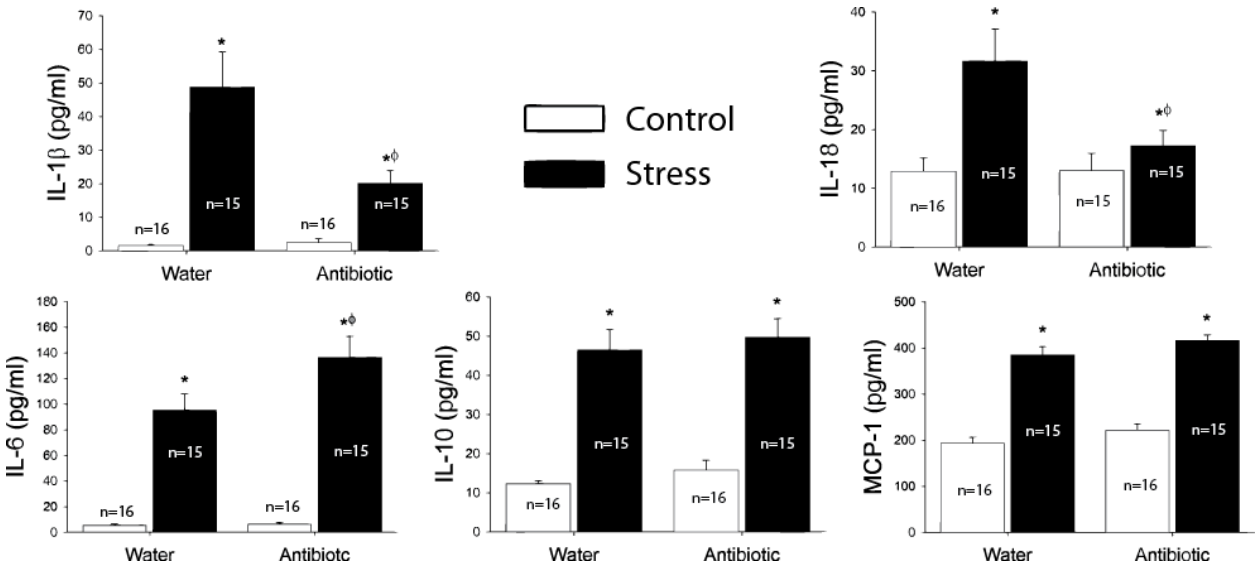

Figure 6: Stress evokes a significant increase in circulating IL-1β, IL-6, IL-10, IL-18, and MCP-1. Administration of antibiotics significantly attenuated the impact of stress on IL-1β and IL-18. Antibiotics, however, did not attenuate IL-10 or MCP-1, and actually increased levels of circulating IL-6. (*$p<0.05$ vs. control, water; Φ $p<0.05$ vs. stress, water).

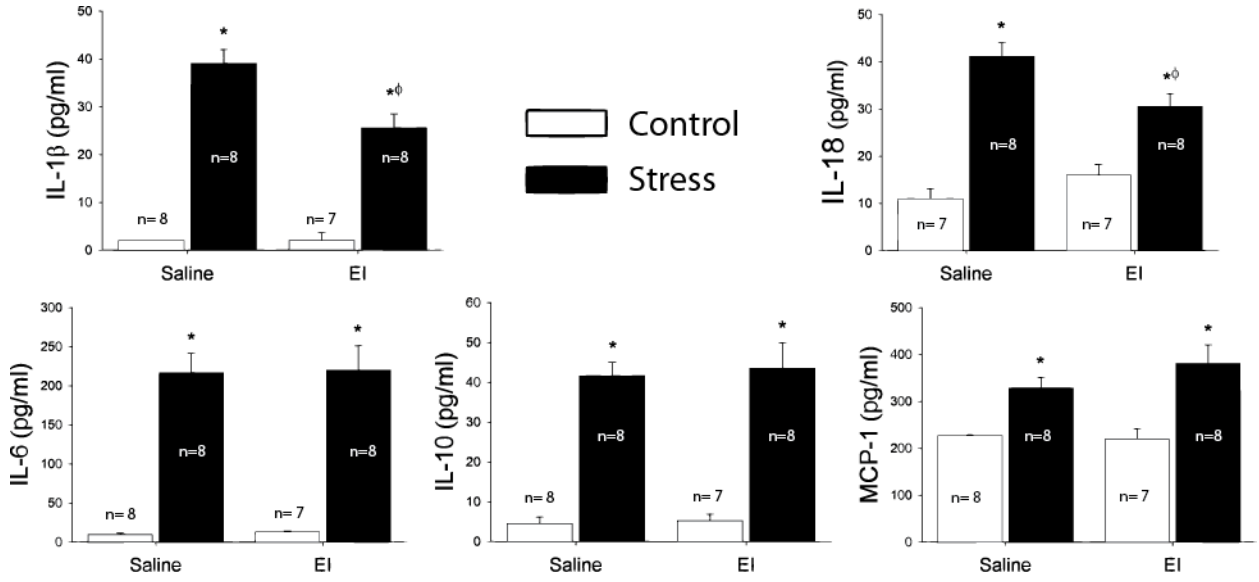

Figure 7: Stress evokes a significant increase in circulating IL-1β, IL-6, IL-10, IL-18, and MCP-1. Administration of endotoxin inhibitor significantly attenuated the impact of stress on IL-1β and IL-18. Endotoxin inhibitor, however, did not attenuate IL-6, IL-10, or MCP-1. (*$p<0.05$ vs. control, water; Φ $p<0.05$ vs. stress, water).

($p<0.05$) (**Figure 7**). Administration of EI also failed to attenuate the stress-induced production of IL-6, IL-10, and MCP-1 (**Figure 7**).

Discussion

The results support the hypothesis that commensal bacteria and LPS release contribute to stress-evoked increase in cytokines and chemokines in the blood. Adult male rats exposed to acute tail shock displayed robust elevations in plasma concentrations of several cytokines and chemokines including IL-1β, IL-6, IL-10, IL-18, and MCP-1. The administration of oral antibiotics or endotoxin inhibitor reduced the stress-induced elevation of IL-1β and IL-18, but interestingly, not IL-6, IL-10, or MCP-1. Thus, another signal beyond the commensal bacteria or released LPS is sufficient for synthesis and release of cytokines and chemokines such as IL-6, IL-10, and MCP-1 following stressor exposure.

Chronic stressors can create shifts in commensal bacterial diversity [9], and this change can impact peripheral immunity, as well as render the intestine vulnerable to pathogenic bacteria infection. Our results suggest that although intact commensal bacteria are necessary for stress-evoked increases in IL-1β and IL-18, these increases are not correlated with decreases in overall bacterial community diversity. Exposure to acute tail shock did not impact overall diversity (either α-diversity or β-diversity) in either fecal or cecal samples. Tail shock did, however, reduce the relative abundance of *Prevotella. Prevotella* is a highly prevalent genus of Gram-negative bacteria in the normal commensal bacterial community and has been reported to drive the overall composition of the flora [59]. Changes in the relative abundance of this genus may have broad physiological and immunological consequences. Reduced abundance of *Prevotella* can impact immunological inflammatory disease states such as inflammatory bowel disease [60], eczema [61], and rheumatoid arthritis patients [62]. Thus the stress-induced reduction in *Prevotella* may reduce anti-inflammatory status of the mucosal immune system and potentially contribute to the pro-inflammatory state produced by stress. Alternatively, the stress-induced

reduction in *Prevotella* may result from death of these bacteria and which would result in the release of pro-inflammatory MAMPs, such as LPS as observed following stressor exposure.

Another goal of the current studies was to determine the specific role of LPS as a signaling molecule important for communication between commensal bacteria and the immune system. The current data support a signaling pathway involving LPS, released from the commensal bacteria in stress-induced cytokine and chemokine responses. Because stress increased circulating concentrations of LPS, leakage of LPS from the commensal bacteria may be important in stress-induced cytokine and chemokine production. Inhibiting LPS by administering EI produced the same effects as antibiotic administration, reducing stress-induced increases in IL-1β and IL-18 but not attenuating IL-6, IL-10, or MCP-1. Although the magnitude of the EI induced attenuation of stress induced IL-1β and IL-18 production was smaller than that observed with antibiotics, EI only inhibits the signaling action of LPS, whereas antibiotic treatment should also reduce other MAMPs such as peptidoglycans [63]. As in the antibiotic study, another signal was sufficient for stress-induced synthesis and release of IL-6, IL-10, and MCP-1.

The present study goes beyond prior work in several important respects. First, it examined a greater number of cytokines and chemokines than previous investigations, providing a broader view of the immune response. Second, it demonstrated that the commensal bacteria have a role in stress-induced inflammatory protein production following a single exposure to an acute stressor. Third, it described no effect of acute stressor exposure on intestinal diversity and a selective reduction in *Prevotella.* And finally, for the first time, it directly examined the mechanism by which the commensal bacteria (via LPS release) influence stress-induced cytokine and chemokine production. Our results thus reveal a novel role for the gut commensal bacteria

in selective modulation of inflammatory proteins. This selectivity of the flora to impact IL-1β and IL-18 may help to fully reveal the mechanisms by which stress and the commensal flora impact immune function.

Recent studies highlight several features unique to the synthesis and release of IL-1β and IL-18 that are not necessary for the production of other cytokines and chemokines such as IL-6, IL-10, or MCP-1 [64]. The unique features in the synthesis pathways of these proteins result in different signaling requirements for these two families of inflammatory proteins [65] and could, thus, explain the selectivity of the antibiotic or EI induced attenuation in the stress-induced cytokine and chemokine response. Of particular importance, while IL-6, IL-10, MCP-1, and the majority of other cytokines and chemokines are synthesized in their releasable form, IL-1β and IL-18 are synthesized as inactive precursors [64,66]. Processing, therefore, is required in the complete synthesis and release pathway for IL-1β and IL-18. This post-translational processing is predominately mediated by caspase-1, an enzyme activated upon the assembly of a multimeric signaling complex called the inflammasome [67–69]. Recent data suggests that the inflammasome is involved in stress-evoked cytokine and chemokine production [70]. Given that antibiotics and EI selectively affect IL-1β and IL-18, these data suggest an interaction between the stress response, the commensal bacteria, and the inflammasome in stress-induced cytokine and chemokine production.

Investigations examining the inflammasome have highlighted the necessity of two signals for inflammasome assembly or activation. The first signal leads to synthesis of components of the inflammasome, as well as pro-IL-1β, and pro-IL-18 [71,72]. Inflammasome independent cytokines are also completely synthesized in response to a single signal [73]. The second signal leads to final inflammasome assembly and caspase-1 activation [67,74]. *In vitro,* the

requirement for two signals has been demonstrated using a MAMP and a danger associated molecular pattern (DAMP) such as ATP, Hsp72, Uric Acid, or even elevated concentrations of glucose [65,71,75]. Administration of a MAMP [65,76,77] or DAMP [78] alone is not capable of activating the inflammasome, however, co-administration of both ligands is sufficient for inflammasome and cytokine production.

Although the exact nature of the requirement for the combination MAMPs and DAMPs is unknown, neutralizing only a single MAMP was sufficient to selectively suppress the inflammasome dependent cytokines *in vivo*. Because MAMPs from the commensal bacteria were only necessary for stress-induced synthesis of inflammasome dependent cytokines, MAMPs likely provide the second signal necessary for inflammasome activation. Although speculative, it seems probable that *in vivo* after exposure to an acute stressor, DAMPs likely act as the first signal in stress-evoked cytokine and chemokine production. DAMPs such as Hsp72 and uric acid are known to increase in response to many stressors [79–81] including tail shock [55,56,70,82–84]. Furthermore, DAMPs, as well as stress-evoked IL-6, IL-10, or MCP-1 responses are not impacted by either antibiotic or EI treatment. Thus, DAMPs may underlie the stress-induced release of the inflammasome independent cytokines and chemokines and hence, act as the first signal in the inflammasomal pathway of stress-evoked cytokine and chemokine production. Other secretions of the stress response such as catecholamines may also act as the first signal in stress-evoked cytokine and chemokine production [85,86].

The present study is the first to demonstrate that release of stress-inducible inflammasome dependent inflammatory proteins depend on commensal bacteria. It is also the first to establish that commensal bacteria mediate acute stress-induced immune activity, and to directly demonstrate a role for MAMPs in this process. Each of these findings provides a novel

mechanistic description of how exposure to stressors elevates blood concentrations of cytokines and chemokines. Furthermore, the aggregate of these findings alludes to a novel, inflammasomal pathway for stress-induced cytokine and chemokine production as summarized in **Figure 8**. Further examination of the interplay between the commensal bacteria and the inflammasome is important and may result in the development of therapeutic candidates that can suppress the cytokine storm evoked by severe stressors or trauma [70,87,88].

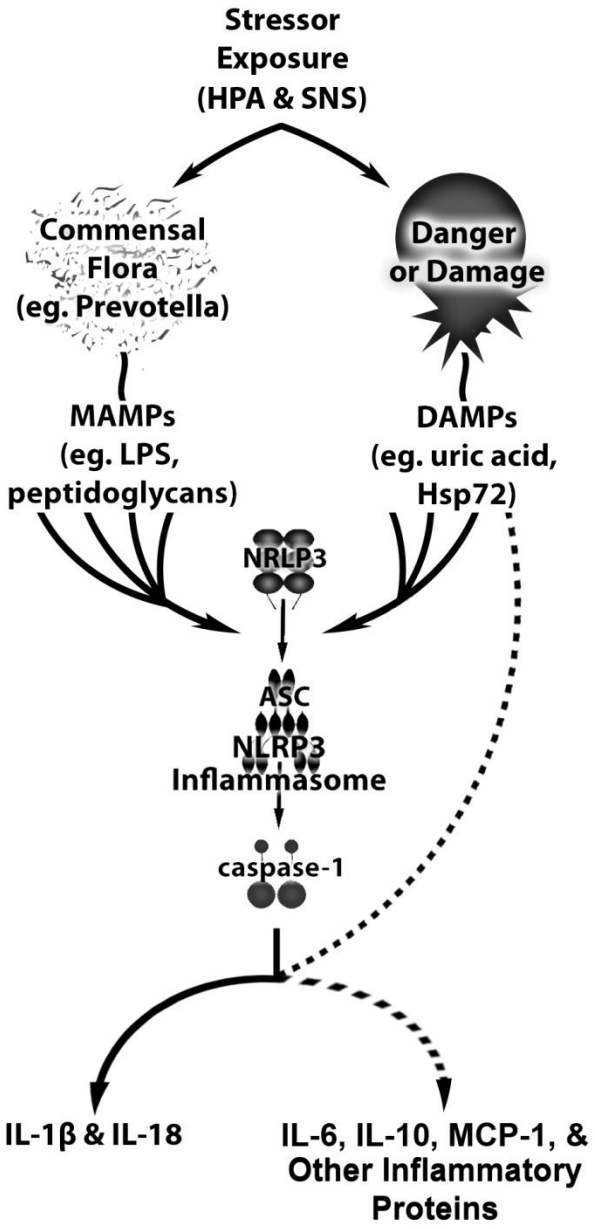

Figure 8: Exposure to a stressor activates the hypothalamic-pituitary-adrenal axis and sympathetic nervous system resulting in changes to the commensal flora (including a decrease in prevotella) and the release of microbe associated molecular patterns (MAMPs) as well as the the release of danger associated molecular patterns (DAMPs) either actively or via cell death. DAMP and MAMP signals then converge upon the inflammasome to yield IL-1β and IL-18 production. DAMPs may also act to drive responses from inflammasome independent inflammatory proteins including IL-6, IL-10, and MCP-1.

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Chapter V

The inflammasome and Danger Associated Molecular Patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure

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Abstract

Exposure to stressors or trauma in the absence of pathogenic challenge can stimulate a systemic sterile inflammatory response characterized by high concentrations of blood and tissue cytokines, chemokines, and danger associated molecular patterns (DAMPs) such as heat shock protein-72 (Hsp72), and uric acid. The signaling pathways responsible for these responses remain unclear, however, the inflammasome may play a role. *In vitro*, DAMPs are known to stimulate the inflammasome in the presence of LPS to activate caspase-1 which cleaves immature precursors of interleukin (IL)-1β and IL-18 into their mature releasable forms. Furthermore, *in vivo* neutralization of the LPS selectively attenuates the stress-induced increase in the inflammasome-dependent cytokines IL-1β and IL-18. Thus, the current experiments tested the hypothesis that inflammasome-mediated processes are necessary for a systemic stress-induced inflammatory response to an acute stressor. The data presented 1) establish that male F344 rats exposed to an acute severe stressor (100 tail shocks) have elevated plasma concentrations of inflammatory proteins (IL-1β, IL-18, IL-6, IL-10, and monocyte chemotactic protein (MCP)-1), and DAMPs (uric acid and Hsp72); 2) demonstrate that inhibiting caspase-1 *in vivo*, using the caspase-1 inhibitor ac-YVAD-cmk, attenuates stress-induced production of IL-1β, IL-18, and IL-6 in both the circulation and peripheral tissues; and 3) implicates the DAMPs uric acid and Hsp72 as important signals contributing to inflammasome-dependent inflammatory responses using a stepwise multiple regression. The results increase our mechanistic understanding of systemic sterile inflammatory responses, and provide novel evidence that the inflammasome may be an important pharmacological target for treatment of these conditions.

Introduction

The inflammatory response is an essential part of successful host defense and is triggered in response to pathogenic and non-pathogenic challenges. Pathogenic inflammation occurs in response to exposure to bacteria, viruses, and other microorganisms and involves the release of cytokines and chemokines that work in concert to combat an infection. The inflammatory response that follows non-pathogenic challenges (sterile inflammation) is stimulated by a variety of signals that originate from dying cells following tissue damage (Kono & Rock, 2008; Rock et al., 2010) or that are released in response to danger or stress. Like their pathogen-driven cousin, sterile inflammatory processes are also beneficial because they enhance wound healing (Piccini & Midwood, 2010; Werner & Grose, 2003) and other innate immune responses (Deak et al., 1999; Johnson et al., 2008; Maslanik et al., 2012a).

In general, the adaptive consequences of inflammatory responses are limited to instances of localized inflammation. When inflammatory responses break local containment barriers and inflammation becomes systemic, inflammatory processes can become maladaptive and pose serious, often life threatening challenges. Exposure to severe stressors or trauma, for instance, can evoke a systemic increase in cytokines and chemokines that in some circumstances may result in multi-organ failure and death (Frink et al., 2007; Namas et al., 2009). Currently available therapeutics are often ineffective at treating systemic sterile elevations in inflammatory proteins (Bernard et al., 1997; Souza et al., 2003); thus, a better mechanistic understanding of systemic sterile inflammatory responses could lead to improved treatment options.

The inflammasome, a recently characterized protein complex, may provide a link between stressor exposure and sterile inflammatory protein production. The inflammasome is a complex consisting of a receptor linked to inactive proCaspase-1 by an intermediate apoptosis-associated

speck-like protein containing a caspase activation and recruitment domain (ASC). Activation of the inflammasome occurs upon ligation of an inflammasomally associated receptor, such as Nod-like receptor family, pyrin domain containing 3 (NLRP3), and results in activation of caspase-1. Caspase-1 is responsible for cleaving proIL-1β and proIL-18 into the active inflammasome-dependent cytokines IL-1β and IL-18 (Menu & Vince, 2011; Menzel et al., 2011; Tschopp & Schroder, 2010). ProIL-1β and proIL-18 may also be cleaved by matrix metalloproteinases, serine proteases, mast cell chymases (Kono et al., 2012), or other enzymes such as PR3 (Sugawara et al., 2001) if proIL-1β or proIL-18 are released intact following necrotic cell death. Other inflammasome-independent cytokines and chemokines, such as IL-6 and IL-10, and MCP-1 do not require post-translational cleavage by caspase-1 (Menzel et al., 2011).

Activation of the inflammasome occurs in response to a variety of signals. Danger associated molecular patterns (DAMPs) (Gasse et al., 2009; Hoffman & Wanderer, 2010; Kono & Rock, 2008; Shi et al., 2003), including Hsp72 (Pittet et al., 2002) and uric acid (Jeevanandam et al., 1991; Namas et al., 2009; Tayag et al., 1996), and microbe associated molecular patterns (MAMPs) such as LPS (Ganz et al., 2011) all potently activate the inflammasome and are released in response to stressor exposure (Allen et al., 2012; Campisi et al., 2012; Fleshner et al., 2010, 2007; Johnson & Fleshner, 2006; Williams & Ireland, 2008). Importantly, *in vivo* neutralization of LPS that is shed from commensal bacteria during stress selectively inhibits the production of inflammasome-dependent cytokines without impacting inflammasome-independent inflammatory proteins (Maslanik et al., 2012b), suggesting that stress-induced release of MAMPs may signal the inflammasome, *in vivo*.

Thus, in light of previous results that implicate a role for the inflammasome; the current experiments tested the hypothesis that inflammasome-mediated processes are necessary for a

systemic stress-induced inflammatory response. We present the results from a series of studies that 1) establish the impact of stress on several cytokines, chemokines, and DAMPs across time during exposure to an acute stressor; 2) test the impact of inhibiting the enzyme caspase-1, *in vivo,* using the irreversible inhibitor ac-YVAD-cmk, on stress-induced inflammasome-dependent and -independent inflammatory proteins and DAMPs in the circulation and peripheral tissues; and 3) implicate the DAMPs Hsp72 and uric acid as important signals contributing to inflammasome dependent inflammatory responses.

Methods

Animals and Housing

Adult male Fischer 344 rats (240-260 g) (N=60) were used in all experiments. Rats were maintained on a 12:12-h light-dark cycle (lights on from 0700 to 1900) in a specific pathogen free environment. Animals were allowed two weeks to acclimate to the colony room prior to any experimental manipulation. Rats were handled briefly each day for 1 week prior to the start of the study. All animals were housed in Plexiglas Nalgene cages and allowed *ad libitum* access to food (Harlan Laboratories, Denver, CO) and water. Colony room temperature was maintained at 23°C. The care and treatment of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Stress

On the day of the experiment, animals either remained in their home cages (*Control*) or were exposed to 1.5mA, 5-second, intermittent, (average trial interval = 60 seconds +/- 25 seconds) inescapable tail shocks as previously described (Campisi & Fleshner, 2003; Fleshner et al., 1995; Maslanik et al., 2012a; Moraska et al., 2002) (*Stress*). During the stress procedure, rats were placed in a Plexiglas restraining tube (23.4 cm long, 7 cm diameter). Electrodes were then placed across the tail that protruded from the back of the shock tube. The shocks were administered by an automated shock system (Precision Calculated Animal Shocker**;** Colbourn Instruments). Tail shocks occurred between 0830 and 1130 to avoid differences in cytokine and chemokine production due to circadian rhythms. Immediately following the end of a session of shocks, all animals were sacrificed via rapid decapitation.

Caspase-1 inhibition

Caspase-1, which is activated by the inflammasome, was inhibited using the irreversible Caspase-1 inhibitor ac-YVAD-cmk (Cayman Chemical). Caspase-1 inhibitor was prepared at 1.5 mg/ml in saline the morning of the experiment. Thirty minutes prior to the onset of tail shock, rats received either 3.0 mg/kg caspase-1 inhibitor in sterile saline or saline alone via intraperitoneal injection as previously described (Kumar et al., 2009; Suzuki et al., 2009).

Experimental Design

Stressor Characterization

Rats were randomized into 4 groups of 7 animals per group (N=28) and received either 0, 10, 50, or 100 tail shocks. Given that these shocks were spaced approximately 1 minute apart, animals were, thus, sacrificed at approximately 0 (Control), 10 (10 shocks), 50 (50 shocks), or 100 (100 shocks) minutes into the tail shock procedure. The onset of tail shock for each group was staggered to ensure that sacrifices of all groups occurred within 1 hour.

The Impact of Caspase-1 Inhibition

Upon arrival rats were randomized into 4 groups of 8 animals per group (N=32) in a balanced design crossing *stress* (control or stress) and caspase-1 inhibitor (caspase-1 inhibitor or saline). Following completion of the tail shock procedure (100 shocks), animals were immediately sacrificed. Sacrifice times were alternated between groups to ensure that sacrifices of all groups occurred over the same period of time.

Sample collection

Following sacrifice, whole blood was collected in EDTA coated vacutainers and immediately centrifuged at 3000 x g for 15 minutes at 4°C to obtain plasma samples. Spleens and the left medial lobe of the livers nearest to the portal vasculature were aseptically dissected, collected in

polypropylene tubes, and snap frozen in liquid nitrogen. All samples were frozen and stored at -80°C unless otherwise noted.

Spleen and liver homogenizations

Spleens and livers (150 mg) were placed into a 2.0 ml CK28 microtube (Precelly's) with 0.5 ml homogenization buffer (84 mg Sodium Fluoride, 90 mg Sodium Pyrophosphate, 185 mg Sodium Orthovanadate, 1 ml RIPA buffer, 200 µl phenylmethanesulfonylfluoride (PMSF), 100 µl phosphatase inhibitor cocktail and ¼ tablet complete protease inhibitor cocktail (Roche) diluted to 10 ml total in distilled water). Each tube was then placed into a Precelly's 24 bench top homogenizer. The samples were homogenized two times at 5000 rpm for 20 seconds with 10 seconds between each cycle. The homogenates were then centrifuged at 4°C for 15 minutes at 21000 x g. Supernatants were divided into aliquots which were then stored at -80°C. Prior to refreezing, one aliquot of liver homogenate was assayed for caspase-1 activity.

DAMP measurement

DAMPs (Hsp72 and uric acid) were measured from plasma using commercially available ELISAs or other colorimetric assays in accordance with manufacturer's instructions. Other DAMPs (HMGB1, S100) do not appear elevated immediately following exposure to 100 inescapable tail shocks (data not shown). Hsp72 was measured in an ELISA from Enzo Biosciences. Uric acid was measured in a colorimetric assay from AbCam. Blood glucose was also measured in whole blood using an Accu-Chek Compact blood glucose monitoring system immediately following sacrifice. Elevated blood glucose is also reported to drive inflammasome activation and inflammatory protein production (Schroder et al., 2010). LDH release, a marker of cell death, was quantified using a colorimetric assay from Bioo Scientific to provide information about the nature of DAMP release.

Inflammatory protein measurement

Concentrations of inflammatory proteins (IL-1β, IL-18, IL-6, IL-10, and MCP-1) in the plasma, livers, and spleens were measured using ELISAs. Plasma samples were all run neat. Spleen and liver homogenates were diluted 1:8 for IL-1β and IL-10, and neat for IL-6 and MCP-1, and 1:100 for IL-18. IL-1β, IL-6, and IL-10 were measured in ELISAs from R&D Systems. MCP-1 was measured in an ELISA from Invitrogen. IL-18 was measured in an ELISA from Immuno-Biological Laboratories, Inc. (IBL). Optical densities were measured using a SpectraMax Plus 354 plate reader (Molecular Devices) and analyzed using four-parameter curve fitting software (SoftMax 5.4.1).

Caspase-1 activity measurement

To confirm efficacy of caspase-1 inhibitor administration, caspase-1 activity was measured in liver homogenates prior to refreezing. Pilot data demonstrated that splenic homogenates were not compatible with the available assay for these measurements. Using a colorimetric assay (AbCam), caspase-1 activity was measured in samples diluted 1:5 in lysis buffer according to manufacturer's instructions. Active caspase-1 (AbCam) was included in each assay as a positive control. Data is collected from the assay as relative O.D.'s so caspase-1 activity values are presented as percent of saline control.

Statistical Analyses

A one-way analysis of variance (ANOVA) was used to analyze the effect of *Stress* on each cytokine, chemokine, and DAMP. Fisher's protected least significant difference (PLSD) post-hoc tests were used to examine the impact of each time-point on cytokine, chemokine and DAMP. Two-way ANOVAs were run to analyze the effect of stress and caspase-1 inhibitor on

the cytokines, chemokines and DAMPs. The predictive power of each DAMP, inflammatory protein, and two-way interaction between a DAMP and inflammatory protein on the other inflammatory proteins was also analyzed using a hierarchical stepwise multiple regression to determine which signals could contribute to stress induced elevations of each cytokine or chemokine. Cytokines that were significantly increased early on in the stress-induced inflammatory response (10 shocks), as well as stress-responsive DAMPs, were input as predictive variables. Individual variables were input into block 1 and additional variance explained by interaction terms was examined in block 2. Data points were treated as outliers if they failed Grubbs' test for outliers (Grubbs, 1969) and were also recorded as affected by experimental procedures by the experimenter. Data are presented as means ± the standard error of the mean. $p<0.05$ was considered statistically significant.

Results

Characterizing the impact of stress on DAMPs during stressor exposure

Stress impacted circulating levels of uric acid ($F(3,24)=58.490$, $p<0.001$) (**Figure 1A**), Hsp72 ($F(3,24)=16.984$, $p<0.001$) (**Figure 1B**) and blood glucose ($F(3,24)=90.217$, $p<0.001$) (**Figure 1C**). LDH was also stress responsive ($F(3,22)=5.274$, $p<0.01$) (**Figure 1D**) indicating that exposure to tail shock produced significant cell death. Uric acid and blood glucose increased in the circulation the most rapidly following the onset of the stressor while Hsp72 and LDH exhibited slower, but similar stress-evoked responses.

Characterizing the impact of stress on cytokines and chemokines during stressor exposure

The concentration of several inflammatory proteins increased as part of the stress-evoked sterile inflammatory response (**Figure 2**). Circulating levels of IL-1β ($F(3,24)=74.348$, $p<0.001$) (**Figure 2A**), IL-18 ($F(3,22)=4.593$, $p<0.05$) (**Figure 2B**), IL-6 ($F(3,24)= 113.588$, $p<0.001$) (**Figure 2C**), IL-10 ($F(3,22)=19.233$, $p<0.001$) (**Figure 2D**), and MCP-1 ($F(3,24)=4.704$, $p<0.01$) (**Figure 2E**) all increased in response to stress. IL-1β and IL-18 were the most rapidly secreted cytokines. Plasma IL-6 and IL-10 were elevated shortly after stress-evoked increases in IL-1β and IL-18 while plasma levels of MCP-1 were the slowest to increase in response to stress.

Circulating levels of each cytokine or chemokine are related to unique combinations of DAMPs and other potential synthesis or release signals

Hierarchical stepwise multiple regressions were used to examine relationships between cytokines, chemokines, and DAMPs in the circulation. A stepwise regression was used to identify variables that are uniquely predictive of each inflammatory protein in order to exclude variables that are simply related to the other DAMPs and cytokines used as predictors. The results from the regression analyses, including a summary of factors identified as significantly

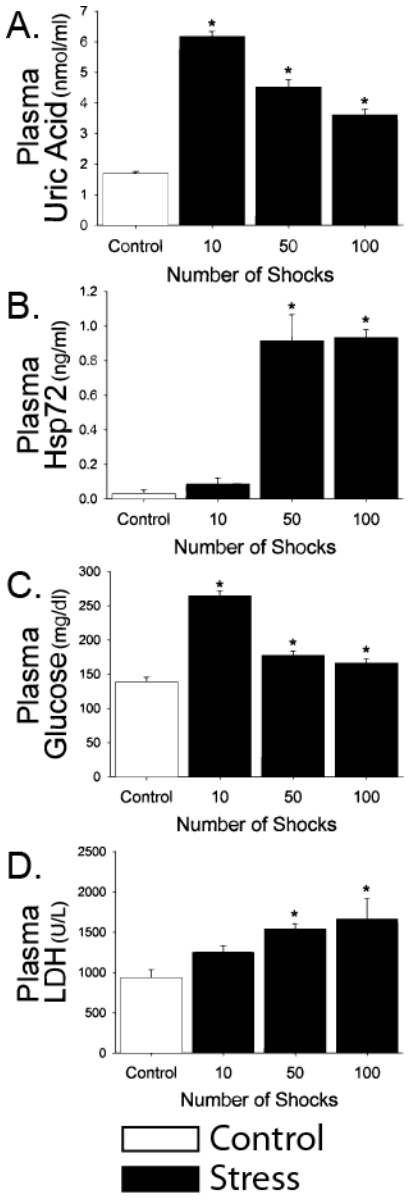

Figure 1: The time course of the impact of acute stressor exposure on circulating DAMPs uric acid (A) and Hsp72 (B), blood glucose (C), and LDH (D). LDH is included as a marker of cell death as cell death may contribute to the release of danger signals. Active release of DAMPs may explain increases occurring prior to LDH increase. Error bars represent ± 1 SEM. *$p<0.05$ from control.

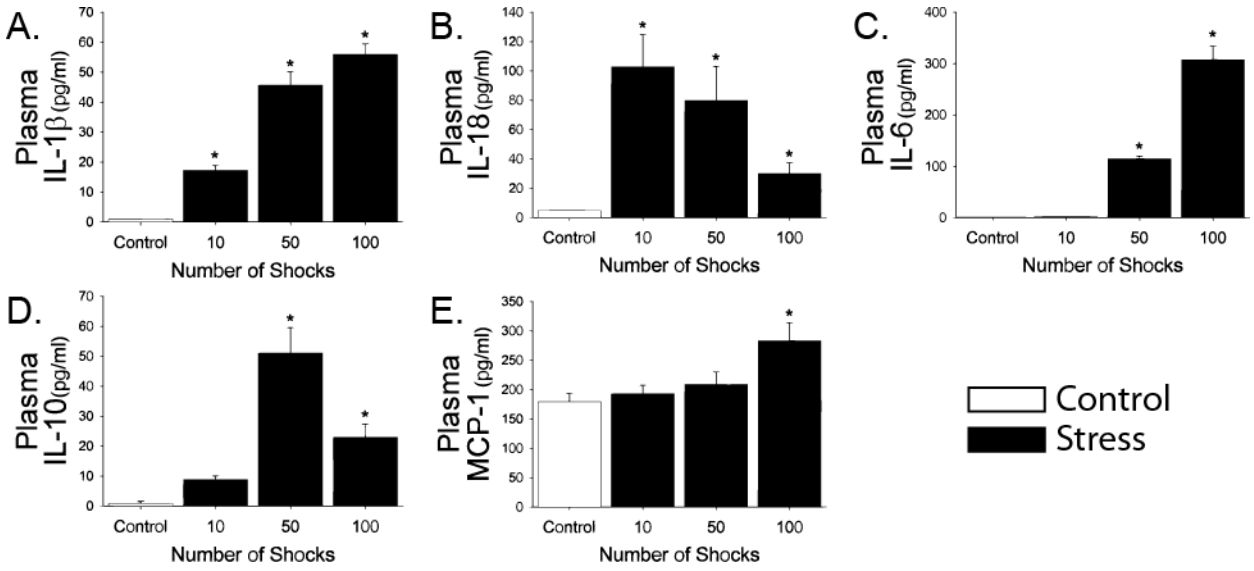

Figure 2: The time course of the impact of acute stressor exposure on circulating IL-1β (A), IL-18 (B), IL-6 (C), IL-10 (D), and MCP-1 (E). Error bars represent ± 1 SEM. *$p<0.05$ from control.

predictive of levels of circulating inflammatory proteins, is presented in **Table 1**. Circulating levels of IL-1β were predicted by Hsp72 (β=2.748, $p<0.05$) and LDH (β=0.480, $p<0.01$). Further variability was predicted by an Hsp72 x glucose interaction (β=-2.318, $p<0.05$) indicating that an interaction with glucose negatively impacts, or reduces the strength of the relationship between Hsp72 and IL-1β. Circulating levels of IL-18 were predicted by uric acid (β=0.603, $p<0.001$). IL-1β (β=2.085, $p<0.001$) was significantly predictive of IL-6. Furthermore, IL-1β x uric acid (β=-1.498, $p<0.01$) and IL-1β x IL-18 (β=.283, $p<0.05$) interactions also added significant predictive power indicating that uric acid and IL-18 influence the relationship between IL-1β and IL-6. Only an IL-1β x uric acid interaction (β=0.428, $p<0.05$) was reliably predictive of IL-10. Finally, LDH (β=0.588, $p<0.01$) was the only variable that was significantly predictive of MCP-1. In summary, the results demonstrate unique combinations of DAMPs, LDH, and early released cytokines are predictive for stress-induced circulating levels of each inflammatory protein.

The effect of Caspase-1 inhibitor on stress-induced DAMPs, blood glucose, and cell death

Replicating the previous results, *stress* increased circulating levels of uric acid ($F(1,26)=9.542$, $p<0.001$) (**Figure 3A**), Hsp72 ($F(1,27)=100.658$, $p<0.001$) (**Figure 3B**), and blood glucose ($F(1,27)=43.773$, $p<0.001$) (**Figure 3C**). Increases in DAMPs were, again, accompanied by a stress-induced increase in LDH indicating stress-induced cell death ($F(1,27)=19.510$, $p<0.001$) (**Figure 3D**). Administration of the caspase-1 inhibitor attenuated the stress-induced increase in Hsp72 ($F(1,27)=12.584$, $p<0.001$) (**Figure 3B**). The caspase-1 inhibitor also reduced uric acid ($F(1,26)=7.943$, $p<0.05$) (**Figure 3A**), but in both stress and control rats. Conversely, LDH ($F(1,27)=6.439$, $p<0.05$) (**Figure 3D**) was increased by caspase-1

Dependent Variable: IL-1β		
Predictor	β	p-value
LDH	0.480	0.001
Hsp72	2.748	0.016
Hsp72xGlucose	-2.318	0.035

Dependent Variable: IL-18		
Predictor	β	p-value
Uric Acid	0.603	0.001

Dependent Variable: IL-6		
Predictor	β	p-value
IL-1β	2.085	<0.001
IL-1βxUric Acid	-1.498	0.003
IL-1βxIL-18	0.283	0.016

Dependent Variable: IL-10		
Predictor	β	p-value
IL-1βxUric Acid	0.429	0.046

Dependent Variable: MCP-1		
Predictor	β	p-value
LDH	0.588	0.002

Table 1: The results of hierarchical stepwise multiple regressions reveal unique predictors of each cytokine and chemokine. Separate regressions were run for each inflammatory protein, as shown, and significantly predictive variables ($p<0.05$) along with β for each significant variable is reported in the table for each regression.

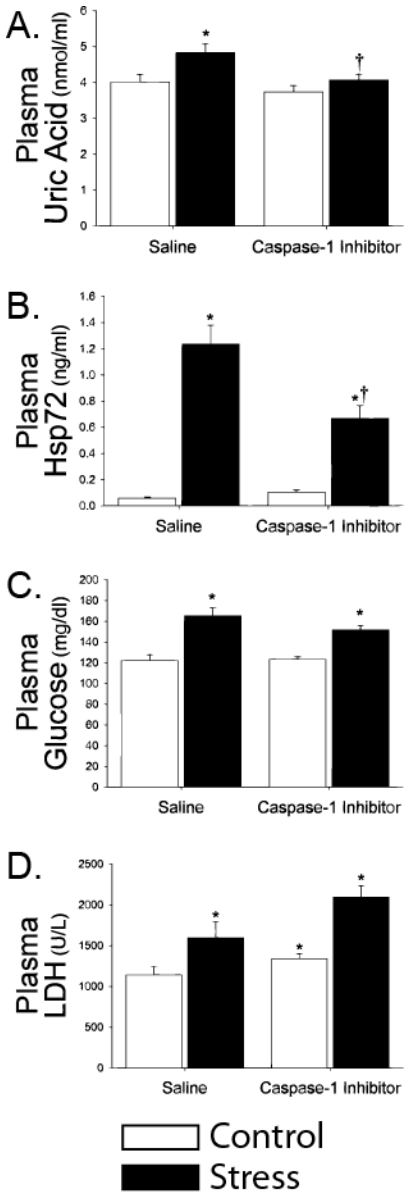

Figure 3: The impact of stress and caspase-1 inhibitor on circulating DAMPs uric acid (A) and Hsp72 (B), blood glucose (C), and cell death as measured by LDH (D). Error bars represent ± 1 SEM. *$p<0.05$ from saline control, †$p<0.05$ stress x drug interaction.

inhibition in both stress and control rats. Blood glucose was not reliably impacted by caspase-1 inhibition.

Caspase-1 inhibitor attenuated the stress-induced circulating cytokine and chemokine responses

Stress once again increased circulating levels of IL-1β ($F(1,27)=54.007$, $p<0.001$) (**Figure 4A**), IL-18 ($F(1,26)=54.731$, $p<0.001$) (**Figure 4B**), IL-6 ($F(1,27)=48.952$, $p<0.001$) (**Figure 4C**), IL-10 ($F(1,27)=85.406$, $p<0.001$) (**Figure 4D**), and MCP-1 ($F(1,27)=7.430$, $p<0.05$) (**Figure 4E**). Administration of the caspase-1 inhibitor attenuated stress-induced increases in circulating IL-1β ($F(1,27)=11.403$, $p<0.01$) (**Figure 4A**), IL-18 ($F(1,26)=5.438$, $p<0.05$) (**Figure 4B**), and IL-6 ($F(1,27)=8.781$, $p<0.01$) (**Figure 4C**). In contrast, the caspase-1 inhibitor increased MCP-1 ($F(1,27)=29.053$, $p<0.001$) levels in both the control and stress groups (**Figure 4E**) and did not significantly impact circulating IL-10 concentrations.

Caspase-1 inhibitor attenuated the stress-induced tissue cytokine and chemokine responses

In the liver, *Stress* increased levels of IL-1β ($F(1,26)=33.822$, $p<0.001$) (**Figure 5A**), IL-18 ($F(1,26)= 17.467$, $p<0.001$) (**Figure 5B**), IL-6 ($F(1,26)=33.440$, $p<0.001$) (**Figure 5C**), IL-10 ($F(1,26)=7.929$, $p<0.01$) (**Figure 5D**), and MCP-1 ($F(1,26)=8.021$, $p<0.01$) (**Figure 5E**). Similar to the effect found in plasma, administration of the caspase-1 inhibitor attenuated stress-induced production of IL-1β ($F(1,26)=4.476$, $p<0.05$) (**Figure 5A**), IL-18 ($F(1,26)=6.965$, $p<0.05$) (**Figure 5B**), and IL-6 ($F(1,26)=9.202$, $p<0.01$) (**Figure 5C**). Stress-evoked levels of MCP-1 in the liver were also decreased by caspase-1 inhibition ($F(1,26)=4.895$, $p<0.05$) (**Figure 5E**). Stress induced IL-10 was not impacted by the caspase-1 inhibitor in the liver, however, levels of IL-10 increased in control rats receiving the drug ($F(1,26)=4.721$, $p<0.05$) (**Figure 5D**).

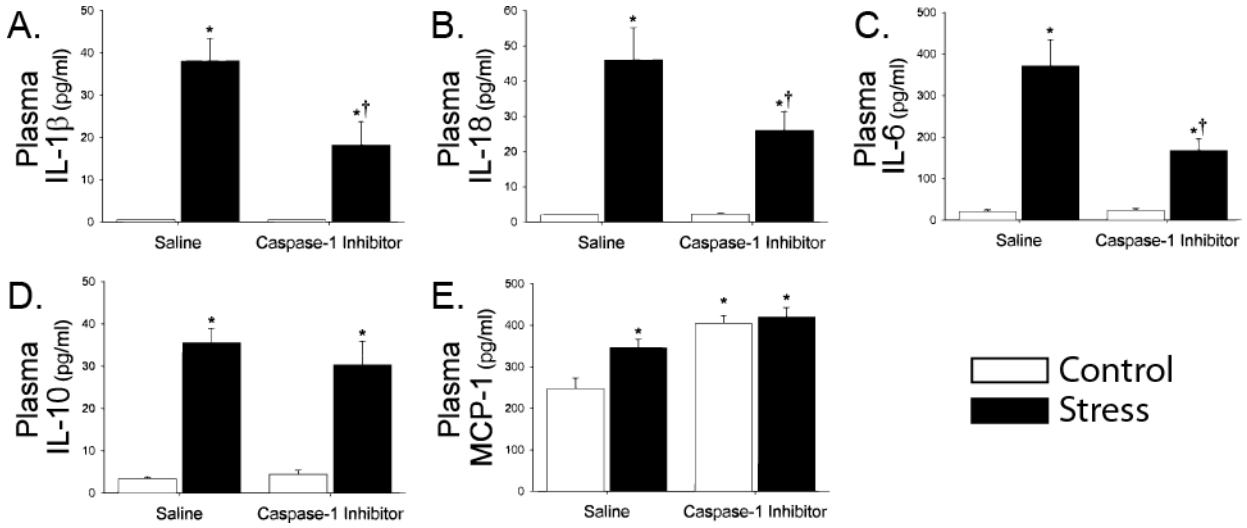

Figure 4: The impact of stress and caspase-1 inhibitor on circulating IL-1β (A), IL-18 (B), IL-6 (C), IL-10 (D), and MCP-1 (E). Error bars represent ± 1 SEM. *$p<0.05$ from saline control, †$p<0.05$ stress x drug interaction.

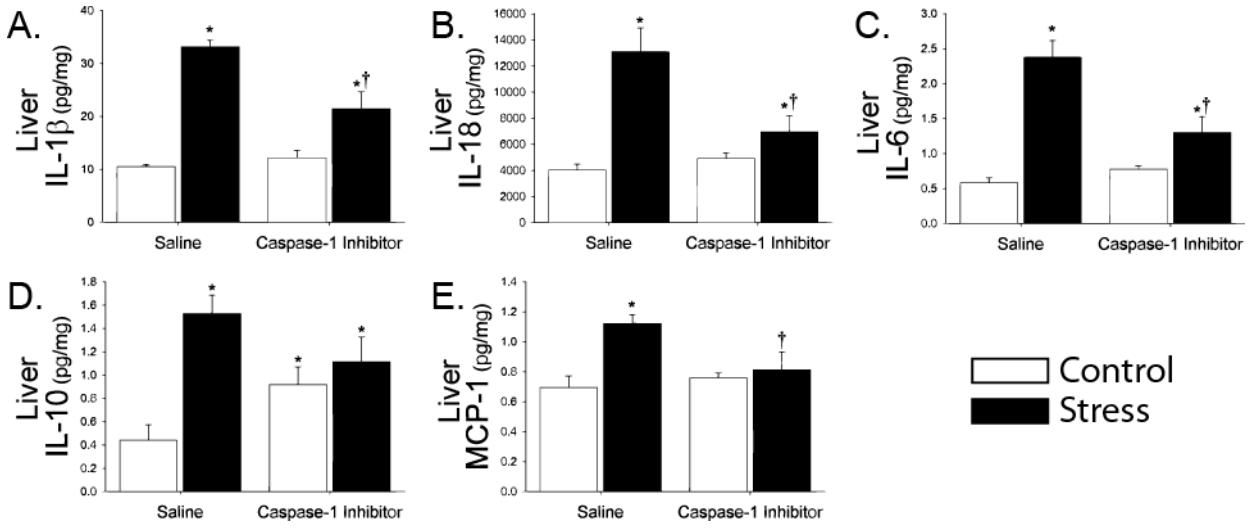

Figure 5: The impact of stress and caspase-1 inhibitor on liver IL-1β (A), IL-18 (B), IL-6 (C), IL-10 (D), and MCP-1 (E). Error bars represent ± 1 SEM. *p<0.05 from saline control, †p<0.05 stress x drug interaction.

In the spleen, *stress* also increased levels of IL-1β ($F(1,26)=67.379$, $p<0.001$) (**Figure 6A**), IL-6 ($F(1,26)=5.515$, $p<0.05$) (**Figure 6C**), and MCP-1 ($F(1,26)=36.369$, $p<0.001$) (**Figure 6E**). Unlike the plasma or liver, splenic IL-10 decreased in response to stress ($F(1,26)=6.693$, $p<0.05$) (**Figure 6D**). Splenic levels of IL-18 (**Figure 6B**) were not significantly impacted by stress; however, given the time course, splenic IL-18 may have peaked earlier than 100 shocks. Stress-induced increases in splenic MCP-1 were attenuated by the caspase-1 inhibitor ($F(1,26)=4.297$, $p<0.05$) (**Figure 6E**). Splenic IL-1β ($F(1,26=5.222$, $p<0.05$) (**Figure 6A**) and IL-18 ($F(1,26)=6.470$, $p<0.05$) (**Figure 6B**) were also reduced by caspase-1 inhibitor administration, but in both stress and control animals. The caspase-1 inhibitor did not impact either IL-6 or IL-10 in the spleen.

Caspase-1 inhibitor administration effectively attenuated Caspase-1 activity

Stress reliably increased caspase-1 activity measured in livers ($p<0.01$). Caspase-1 inhibitor administration attenuated the stress-induced increase in caspase-1 activity ($p<0.05$) indicating that the drug was effective (**Figure 7**). Levels of caspase-1 activity in saline treated, stressed rats were 59% higher than in saline treated controls. The caspase-1 inhibitor reduced stress-induced caspase-1 activity to 13% higher than controls.

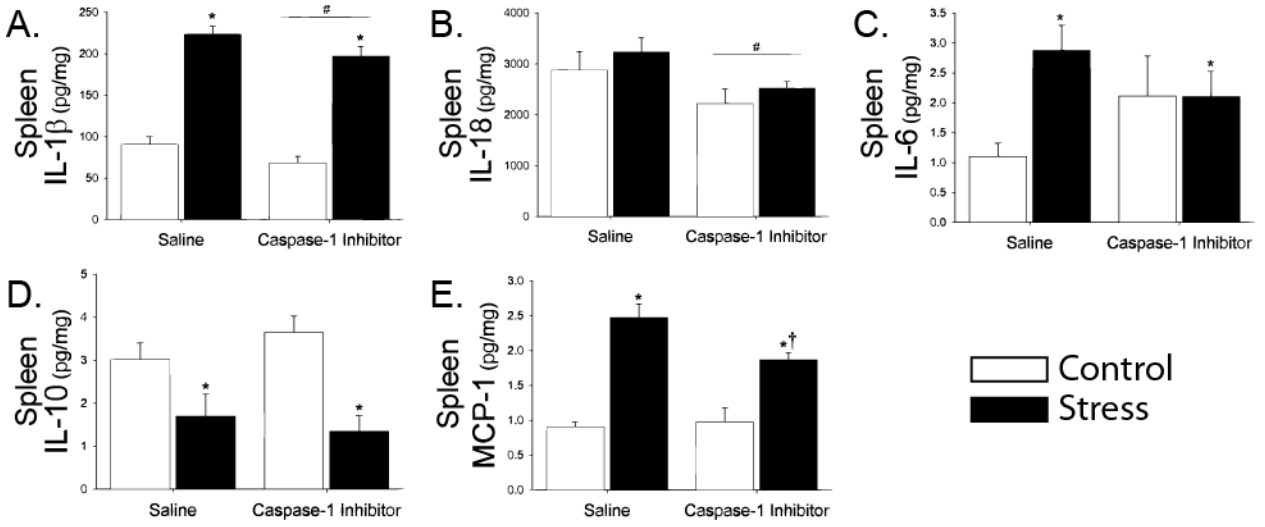

Figure 6: The impact of stress and caspase-1 inhibitor on splenic IL-1β (A), IL-18 (B), IL-6 (C), IL-10 (D), and MCP-1 (E). Error bars represent ± 1 SEM. *p<0.05 from saline control, †p<0.05 stress x drug interaction, #p<0.05 main effect of drug.

Figure 7: Caspase-1 activity measured in the liver presented as percent of control. Error bars represent ± 1 SEM. *p<0.05 from saline control, †p<0.05 stress x drug interaction.

Discussion

The results support the hypothesis that the inflammasome plays a role in evoking a systemic sterile stress-evoked inflammatory response. Adult male rats exposed to an acute tail shock stressor displayed robust elevations in concentrations of several cytokines and chemokines measured in the plasma, liver, and spleen. Interestingly, administration of a caspase-1 inhibitor reduced both stress and control levels of IL-1β and IL-18 in the spleen. These data may suggest that inflammasomal pathways drive low levels of basal inflammatory activity in certain tissues. Alternatively, other cleavage pathways involving matrix metalloproteinases, serine proteases, or mast cell chymases (Kono et al., 2012), may also play a role in stress-evoked cytokine and chemokine responses in the spleen. Nonetheless, the caspase-1 inhibitor selectively impacted stressor evoked inflammatory proteins and not basal levels in the liver and plasma indicating that caspase-1 is critical in stress-evoked systemic sterile inflammatory responses.

IL-1β and IL-18 are both synthesized as inactive proIL-1β and proIL-18. These pro-cytokines are cleaved by caspase-1 to yield active IL-1β and IL-18. The attenuation of stress-induced IL-1β and IL-18 responses therefore, was anticipated in response to caspase-1 inhibition. Reduction of inflammasome-independent inflammatory proteins by the caspase-1 inhibitor, however, was initially surprising. For instance, IL-6 is not cleaved by caspase-1 (Khare et al., 2012), but was reduced in this experiment by treatment with a caspase-1 inhibitor. Further investigation, however, reveals that in some instances inflammasome-dependent cytokines such as IL-1β may provide the signal for an IL-6 response. The notion of a role for IL-1β in IL-6 release is supported by the literature (Shio et al., 2009). Furthermore, the observation that IL-1β appears in the circulation prior to IL-6 supports the idea that IL-1β could signal IL-6 production. Finally, the stepwise multiple regression analysis revealed that circulating levels of IL-1β are

significantly predictive of circulating levels of IL-6, which also points to a role for IL-1β in stress-induced IL-6 secretion.

The stepwise multiple regression may also help explain why administration of a caspase-1 inhibitor decreases MCP-1 in tissues but increases MCP-1 in the circulation following stressor exposure. Unlike the other cytokines that responded to the caspase-1 inhibitor, MCP-1 does not appear to be related to any of the stress-evoked DAMPs or the inflammasome-dependent cytokines. Levels of MCP-1, instead, were only predicted by cell death as measured by LDH. Furthermore, LDH was detectable in the circulation prior to MCP-1 release. Since administration of the caspase-1 inhibitor resulted in elevated LDH, the decrease of MCP-1 in tissues and concomitant increase in the circulation could result from dying cells in tissues releasing MCP-1 into the circulation. Of course, it is possible that the relationship between MCP-1 and cell death may arise from cell death mediated by MCP-1. A role for MCP-1 in cell death, however, is unlikely because MCP-1 does not induce cell death *in vitro* (Hinojosa et al., 2011).

A complete examination of the stepwise multiple regressions for each cytokine and chemokine reveals that no two inflammatory proteins appear to be predicted by the same combination of signals. These data, thus, suggest unique signaling pathways for the stress-induced release of a given cytokine or chemokine. Of course, further research is needed to confirm the role of each signal or interaction in the synthesis of each inflammatory protein, as regression models are not sufficient to demonstrate causality. Additional stress-inducible signals, such as catecholamines which can stimulate IL-10 production (Riese et al., 2000), must also be examined.

Importantly, the unique combination of signals that predict levels of any one cytokine or chemokine highlights the difficulties faced by researchers and clinicians attempting to mitigate similar sterile inflammatory responses. No single upstream signal appears to be a suitable pharmacological target for blocking the network of inflammatory proteins stimulated during systemic sterile inflammatory responses. Furthermore, with cytokines such as IL-6 and IL-10, the predictive ability of multiple signals and interactions between signals suggests biologically redundant pathways. Previous research shows that a single signal, such as from uric acid, is sufficient to stimulate the production of these cytokines (Martin et al., 2010). Finally, because *in vitro* data demonstrates that inflammasomal pathways often require signaling from both a DAMP and a MAMP, the role of MAMPs also must be considered. For instance, the release of LPS from the commensal flora also may be necessary for IL-1β and IL-18 synthesis via the inflammasome (Maslanik et al., 2012b). IL-1β and IL-18 require two signals; one for synthesis of the inactive pro-cytokines and a second for processing of the pro-cytokines into active IL-1β and IL-18. The DAMPs discussed herein may only represent the first signal in stress-evoked IL-1β and IL-18 production.

In light of these data, it would appear that targeting downstream mediators of inflammation might be more effective at suppressing systemic sterile inflammatory responses. Classical anti-inflammatory treatments such as NSAIDs have already been investigated. NSAIDs target downstream mediators of inflammation like cyclo-oxygenase (COX)-2 (Paiotti et al., 2012). These treatments, however, fail to improve morbidity and mortality rates related to systemic inflammation (Bernard et al., 1997) possibly because they paradoxically increase inflammatory cytokine production in the tissues and circulation (Kim et al., 2008; Page et al., 2010; Peebles et

al., 2002). The cytokines and chemokines themselves appear to be principally responsible for multi-organ failure following systemic inflammatory responses.

For the first time, however, these studies demonstrate that a signaling complex, the inflammasome, may represent a suitable pharmacological target for constraining systemic sterile inflammatory responses. Restricting caspase-1 activity through the administration of a caspase-1 inhibitor attenuated the production of multiple proinflammatory cytokines in response to stress. The broad impact of caspase-1 inhibition on the stress-evoked cytokine and chemokine response suggests that the pathways modulating sterile stress-evoked cytokine and chemokine production converge on the inflammasome.

Interestingly, the caspase-1 inhibitor also effectively suppressed stress-evoked levels of DAMPs. Stress-evoked DAMP release may occur following cell death which is increased in response to stress and caspase-1 inhibitor administration. It may also occur via an active release pathway which is signaled by catecholamines binding α1 adrenergic receptors (Johnson & Fleshner, 2006; Johnson et al., 2005). The dual modality of DAMP release, possibly coupled with different metabolism pathways for the break-down of each DAMP, is supported by the differing kinetics of the DAMP response. Thus, caspase-1 inhibitor mediated decreases in stress-evoked DAMPs may be due to inhibition of caspase-1 dependent pyroptotic cell death (Lu et al., 2012) occurring in response to stress-evoked increases in caspase-1 activity (Fink & Cookson, 2005). The caspase-1 inhibitor may also interfere with other active release pathways for DAMPs. Finally, the caspase-1 inhibitor may stimulate metabolism pathways for DAMPs like Hsp72 and uric acid.

In summary, the complexities underlying systemic sterile inflammatory responses elucidated in this study may explain the inefficacy of current treatments targeting single cytokines or DAMPs. Constraining the inflammasome via inhibiting caspase-1 is sufficient, however, to reduce systemic sterile stress-induced production of several inflammatory proteins. Whether or not these findings translate to clinical manifestations of systemic sterile inflammation requires further investigation. Additional research is also required to optimize the dose of the caspase-1 inhibitor required to completely block systemic sterile inflammatory responses and to extend these results from stress to other sterile inflammatory stimuli. Examining whether or not caspase-1 inhibition is effective at reducing an ongoing response is also necessary. Nonetheless, by demonstrating efficacy of a caspase-1 inhibitor in such a response, the current data provide novel experimental evidence that the inflammasome may be an important pharmacological target for systemic sterile elevations in inflammatory proteins, which have severe, life-threatening consequences.

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Chapter VI

General Discussion

The results presented in this dissertation represent a significant advancement in our understanding of systemic sterile inflammatory responses (SSIRs). In the first and second chapters, we examined what is currently known about SSIRs. Evidence in the literature suggests that these responses are more complicated than previously appreciated, highlighting the need for further characterization of SSIRs. In chapter 3 we explored the network of cytokines, chemokines, and other inflammatory proteins that compose a stress-evoked SSIR. Consistent with the collective results from recent research, we reported that the network of inflammatory proteins involved in an SSIR is large and involves a very diverse number of cytokines and chemokines. Chapter 4 began our investigation of the factors that underlie SSIRs. Beginning with an observation that the commensal bacteria contribute to stress-evoked cytokine and chemokine production, we subsequently narrowed our focus to a family of microbe associated molecular patterns (MAMPs) that were necessary for the synthesis of IL-1 family cytokines. Intracellularly, these cytokines often require inflammasome-mediated post-translational cleavage. Thus, Chapter 5 examined the inflammasome mediated pathway of a stress-evoked SSIR and further identified a second family of signals involved in this response known as danger associated molecular patterns (DAMPs).

Given the severity of complications associated with SSIRs and the inefficacy of available treatments, the current characterization of the response could be instrumental in developing improved therapeutics to combat the cytokine storm that occurs following trauma or severe stress. Of course, the results of these studies are not sufficient to demonstrate that the

inflammasome is a suitable therapeutic target to treat systemic sterile inflammation. For instance, the current studies measured the impact of inhibiting the inflammasome on a representative number of cytokines and chemokines. Others inflammatory proteins, including those identified in Chapter 3, must also be examined to determine if they are responsive to caspase-1 blockade. Although other stimuli such as trauma evoke a very similar pattern of cytokine and chemokine release to a stress-evoked SSIR, whether or not these models of sterile systemic inflammation converge upon the inflammasome also must be investigated. Additionally, the effectiveness of a caspase-1 inhibitor at preventing clinical complications that are frequently associated with SSIRs, including multi-organ failure, is also an important future consideration. Other preclinical studies such as dose and structural optimization of a compound targeting caspase-1 or another component of the inflammasome also will be necessary.

Nonetheless, the results presented in this dissertation do provide a rich framework for future investigations into the mechanism of SSIRs. Importantly, in spite of the diverse number of factors underlying stress-evoked cytokine storms, our characterization of the network, signals, and pathways involved in SSIRs suggests that this response may converge upon the inflammasome.

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