Proinflammatory responses triggered by ricin toxin require macrophages and

IL-1β signaling through the NALP3 inflammasome

by

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

ARDS   Acute respiratory distress syndrome

ASC   Apoptosis-associated specklike protein containing a CARD

BAL   Bronchoalveolar lavage

BMDM   Bone marrow derived macrophages

CARD   Caspase-1 recruitment domain

CCL2   Chemokine (C-C motif) ligand 2 (also MCP-1)

COP   CARD-only domain protein

CXCL1   Chemokine (C-X-C motif) ligand 1 (also Gro-α)

DAMP   Danger or damage-associated molecular pattern

dsRNA   Double-stranded RNA

eIF2α Eukaryotic initiation factor 2 alpha

Gapdh   Glyceraldehyde 3-phosphate dehydrogenase

Gro-α Cotton rat growth regulated protein alpha (also CXCL1)

ICP-MS   Inductively coupled plasma mass spectroscopy

IL-1   Interleukin-1

IL-1ra   Interleukin-1 receptor antagonist

IL-6   Interleukin-6

JNK   c-Jun kinase

LPS   Lipopolysaccharide

LRR   Leucine rich repeats

MAFIA   Macrophage fas-induced apoptosis

MAPK   Mitogen activated protein kinase

MCP-1   Monocyte chemotactic protein-1

NAC   N-acetyl cysteine

NACHT   domain present in NAIP, CIITA, HET-E and TP1

NALP3   NACHT-, LRR- and PYD- domains containing 3 protein (also NLRP3)

NLR	Nod-like receptor
PAMP	Pathogen associated molecular pattern
PERK	PKR-like ER localized eIF2α kinase
PKR	Protein kinase RNA-activated
POP	PYD-only domain protein
PRR	Pattern recognition receptor
PYD	Pyrin domain
ROS	Reactive oxygen species
SAPK	Stress activated protein kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TXNIP	Thioredoxin interacting protein
ZAK	MLK7/Leucine zipper- and sterile alpha motif-containing kinase

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THESIS ABSTRACT

Ricin toxin is a ribotoxic stressor that exhibits well-characterized actions on the ribosomes of cells leading to the inhibition of protein synthesis and the phosphorylation of stress activated protein kinases (SAPKs). In addition, ricin triggers a robust inflammatory response *in vivo*, but the underlying mechanisms for ricin's inflammatory effects are incompletely understood.   Due to its ease of delivery to human populations by aerosol in the event of bioterrorism, it is important to understand the pathological consequences of ricin exposure in the pulmonary system.   In this thesis, I explore the molecular mechanisms of the ricin-mediated inflammatory response in the lungs of mice. Here I report that expression of inflammatory genes, edema, increased microvascular permeability, and in particular, neutrophilia, are important characteristics of ricin-mediated lung damage in mice. I found that depletion of macrophages and genetic deficiency of interleukin-1β (IL-1β) signaling suppress ricin-mediated pulmonary inflammation and improve survival of animals. Importantly, a single dose of IL-1 receptor antagonist was protective against inflammatory signs in wild-type animals exposed to ricin. *In vitro*, I found that bone-marrow derived macrophages release significant amounts of active IL-1β in response to ricin treatment, and that ricin-mediated IL-1β release is dependent on the NALP3 inflammasome, a scaffolding complex that mediates pro-IL-1β cleavage to active IL-1β by the cysteine protease caspase-1. In order to determine if ricin-induced IL-1β release from macrophages was dependent the activation of SAPKs, I employed inhibitors of p38 MAPK and JNK. I found that ricin-mediated release of IL-1β was enhanced, rather than suppressed, by inhibition of SAPK phosphorylation, suggesting that inflammasome activation and IL-1β release in response to ricin occurs via a

distinct mechanism. To determine if ricin-mediated inhibition of protein synthesis triggers activation of pro-IL-1β processing, I examined the ability of a panel of well-known inhibitors of protein synthesis (cycloheximide, emetine, puromycin, pactamycin, and anisomycin) to elicit IL-1β release from bone-marrow derived macrophages. I found that inhibition of protein translation was sufficient to induce IL-1β release in a manner dependent on the NALP inflammasome. I hypothesized that the disappearance of a labile protein might explain the ability of inhibitors of protein synthesis to activate NALP3 inflammasome-dependent IL-1β release from macrophages. To test this, I examined the effect of co-treatment with proteasome inhibitors MG-132 and Bortezomib on the release of IL-1β from macrophages.   Proteasome inhibitors completely blocked the release of IL-1β from cells exposed to inhibitors of protein synthesis.   Next, I examined the behavior of a known NALP3-binding protein, TXNIP, whose levels are regulated by the proteasome, after treatment of cells with cycloheximide. I found that TXNIP is a labile protein whose half-life was extended in cells co-treated with cycloheximide and the proteasome inhibitor MG-132. Taken together, these data suggest that ricin-mediated translational inhibition itself, by fostering the disappearance of labile protein(s) that normally suppress NALP3 inflammasome formation, may constitute the mechanism underlying IL-1-dependent inflammatory signaling by ricin.   These data support the hypothesis that protein synthesis inhibition acts as a danger signal that triggers innate immunity. Furthermore, data presented in this thesis suggest that exposure to any agent that inhibits protein synthesis could lead to IL-1β-dependent inflammation.

Background and Introduction

I. Innate immunity and the pulmonary system

The innate immune system is a highly conserved defense system composed of cellular components including macrophages, neutrophils, and epithelial cells that express extracellular and cytosolic sensors that detect and respond to pathogens and cell damage (Bals et al., 1999; Janeway and Medzhitov, 2002). In contrast to adaptive immunity, which relies on specific antibody-mediated defenses against pathogens and requires several days to take effect (Janeway, 2004) innate immunity relies on the detection of molecular patterns associated with pathogens or cell damage to initiate an immediate host immune response (Palsson-McDermott, 2007; Janeway, 2004). Pathogen associated molecular patterns are exogenous signals derived from microbes that include the bacterial endotoxin, lipopolysaccharide (Thieblemont et al., 1998), bacterial flagellin (Hayashi et al., 2001), and viral dsRNA (Balachandran et al., 2000). In contrast, damage or danger associated molecular patterns are endogenous proteins and other signals released from damaged cells and tissues to the extracellular space, such as hyaluronan (Scheibner et al., 2006), ATP (Bours et al., 2006) and uric acid (Shi et al., 2003). While much of the research surrounding innate immunity has focused on understanding the responses of cells to microbes, only recently have the mechanisms governing detection and response to non-microbial (or "sterile") danger signals been elucidated.

Inflammation, the main defense of the innate immune system against infection, is the culmination of both local and systemic events that result in increased blood flow, elevated cellular metabolism, vasodilation, leakage of fluid, cellular influx, and release of

soluble mediators like cytokines (Nathan, 2002; Janeway, 2004). Inflammatory injury to local tissue by host immune cells is viewed as collateral damage in an infection (Janeway, 2004; Male, 2006). However, in the case of inflammation induced by nonmicrobial toxins or tissue trauma, in which there is no pathogen to clear, tissue damage occurs without benefit to the host. Importantly, understanding how to suppress innate immune responses can help prevent or reduce inflammatory injury.

The respiratory system, and the lung in particular, serves as a major barrier between host and the external environment, and represents an important site of innate immune defense. The respiratory system is composed of the upper respiratory tract (nasopharynx, oral cavity and throat) and the lower respiratory tract (trachea, bronchi and lungs). The lung is a complex organ with three distinct compartments, including the airways and airspaces, interstitium, and vasculature (Drake, 2010). During breathing, air is drawn in via the nasal passages, through the conducting airways of the lung, and into the millions of tiny alveoli, which constitute the delicate epithelial/endothelial barrier across which gas exchange takes place (Figure 1.1) (Van de Graff, 2002; Drake, 2010). Given the potentially large number of toxic or allergic particles able to reach this region during breathing, it is not surprising that the respiratory system possesses multiple defenses against inhalants. Epithelial cells lining the nasal cavity and goblet cells in the lower respiratory tract secrete mucus to filter the air by trapping foreign particles; cilia on cells in the nasal cavity and on respiratory epithelial cells in the lower respiratory tract mechanically remove trapped particles by transporting them to the upper respiratory tact where they are expelled in saliva and nasal secretions (Van de Graff, 2002; Drake, 2010). Inhalants that do reach the lung are normally cleared quickly by resident macrophages (Janeway, 2004). Highly infectious or toxic particles that cannot be cleared by alveolar macrophages engage the pulmonary innate immune system,

mediated by airway and alveolar epithelial cells, alveolar endothelial cells, resident macrophages and recruited leukocytes (Janeway, 2004). In this thesis, I discuss the roles of alveolar macrophages and recruited neutrophils in the inflammatory response to toxins in the lung.

Alveolar macrophages are the resident phagocytes of the pulmonary system, and their primary response to an inhaled foreign particle is to process and clear the inhalant *without* triggering an amplified inflammatory response (Janeway, 2004; Male, 2006). By phagocytosis and secretion of reactive oxygen species, antimicrobial peptides and proteases, alveolar macrophages eliminate most of the dust and toxins from typical microbes that humans are exposed to on a daily basis through breathing (Sibille and Reynolds, 1990). When faced with more virulent microbes or larger numbers of toxic particles, alveolar macrophages synthesize and secrete a wide array of cytokines (including interleukins-1, -6, and tumor necrosis factor-α) and chemokines (including interleukin-8 in humans/CXCL-1 in mice) that initiate a pulmonary inflammatory cascade and recruit leukocytes from the bloodstream (Rinaldo et al., 1985). Neutrophils, in particular, accumulate in the airways in large numbers in response to chemotactic signals released by alveolar macrophages through interactions with adhesion molecules on alveolar endothelium (Kang et al., 1993; Rinaldo et al., 1985).

Neutrophils are polymorphonuclear leukocytes, first described by Paul Ehrlich in the late 1800s when fixation and staining techniques made it possible to identify them by their lobulated nucleus and granules that stain neutral by a mixture of basic and acidic dyes (Ehrlich, 1900; reprinted in Himmelweit 1956). Neutrophils are phagocytic cells that contain two main types of granules: specific granules, characterized as secretory granules that play important roles in initiation inflammatory responses (Ringel et al., 1984) and azurophil granules, which contain myeloperoxidase and are considered to be

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lysosomes that digest phagocytosed material (Baggiolini, 1972; Spitznagel et al., 1974). Through phagocytosis, degranulation, and the release of reactive oxygen species, neutrophils that migrate into the lung during infection are well equipped to kill most microbes and clear damaged epithelium from the airways (Hyde et al., 1999; Kobayashi et al., 2005). In the healthy lung it is rare to find neutrophils in the airways, and neutrophils that reach the alveolar surface are rapidly cleared (Cox et al., 1995). Closely regulated neutrophil migration allows neutrophils to conduct surveillance functions while preventing an inflammatory cascade from being inappropriately initiated at the alveolar epithelial/endothelial surface where gas exchange occurs. Indeed, much of the collateral tissue damage that occurs as a result of the inflammatory process in the lung is due to the release of anti-microbial proteases from neutrophils (Abraham, 2003; Till et al., 1982). In Chapter 2 of this thesis, I describe the role that recruitment of neutrophils plays in the inflammatory response to ricin toxin in the lung.

II. Ricin toxin and ricin-mediated inflammatory responses

Ricin, a plant toxin derived from the castor bean plant *Ricinus communis*, is one of the most potent and lethal poisons known (Endo and Tsurugi, 1987; Smallshaw et al., 2002). A single molecule of ricin is sufficient to kill a cell; in humans, 1 to 10 µg per kg of body weight is a lethal dose (Smallshaw et al., 2002). Ricin belongs to a family of toxins called ribotoxic stressors that include bacterial and fungal small molecular metabolites (anisomycin, trichothecene, mycotoxins) as well as various bacterial (Shiga toxin, alpha-sarcin) and plant-derived ribosome inactivating proteins (ricin, saporin) (Iordanov et al., 1997; Stirpe and Battelli, 2006). Human populations are exposed to them through accidental inhalation or ingestion or as agents of bioterrorism (Crompton and Gall, 1980; Kifner, 1995; Mayor, 2003; Shar, 1995). In the case of ricin, aerosol

inhalation represents the most plausible and dangerous scenario in the event it is used as an agent of warfare (Greenfield et al., 2002).

Ricin is a 62 kD protein that consists of two peptide chains, the ricin A- and B-chain. The B chain binds to mannose receptors on the cell surface and permits internalization of the toxin via endocytic uptake (Barbieri et al., 1993; Lord et al., 1994). The A chain is a glycosidase that depurinates a single adenine in the highly conserved sarcin/ricin loop of 28S ribosomal RNA, which prevents the binding of elongation factor 2 and renders the ribosome incapable of protein synthesis (Carrasco et al., 1975). Concomitant with inhibition of protein translation, through interactions with the 3' end of the ribosome, ricin and all ribotoxic stressors trigger phosphorylation of stress activated protein kinases p38 MAPK and JNK (Iordanov et al., 2002; Iordanov and Magun, 1999; Iordanov et al., 1997; Iordanov et al., 1998).

Although the ribotoxic effects of ricin have been well established, evidence suggests that ribosome inactivation by ricin may not completely account for ricin's toxicity to cells and organisms. Ricin elicits a potent inflammatory response in both rodents and humans (Brown and White, 1997; Greenfield et al., 2002; Wong et al., 2007a) and stimulates cytokine and chemokine expression in primary macrophages and pulmonary epithelial cells *in vitro* (Korcheva et al., 2007; Wong et al., 2007b). Aerosol delivery of ricin to animals results in an inflammatory response typical of acute lung injury and acute respiratory distress syndrome (Greenfield et al., 2002; Wong et al., 2007a) (Figure 1.2). Acute respiratory distress syndrome is characterized by inflammation and increased permeability of the alveolar epithelial/endothelial barrier (Bernard et al., 1994; Shanley et al., 1995), and is a leading cause of morbidity and mortality in clinical settings (Goss et al., 2003; Hudson et al., 1995). When administered to the lungs of animals, ricin induces a robust inflitration of inflammatory cells,

predominantly neutrophils, and causes apoptosis of alveolar macrophages (Brown and White, 1997; Korcheva et al.).   Subsequent effects of ricin exposure include pulmonary edema as well as apoptosis and necrosis of the alveolar epithelium and endothelium (Wilhelmsen and Pitt, 1996).   Indeed, ricin delivered to the lungs causes lesions in ribosomal RNA in cells of several other tissues including the kidney, liver, and spleen, likely due to deterioration of the lung barrier and the dissemination of ricin through the bloodstream (Korcheva et al., 2005). Primary human airway epithelial cells and primary murine macrophages respond to ricin *in vitro* (Korcheva et al., 2007; Wong et al., 2007b), but the specific cell type(s) responsible for ricin's lethal inflammatory effects *in vivo* remain unclear. In Chapter 2 of this thesis, I will describe the role of macrophages in the inflammatory response to ricin delivered to the pulmonary system of mice.

The pulmonary innate immune response is activated not only by pathogens but also by a wide variety of xenobiotic agents (Glazer and Newman, 2004; Khalil et al., 2007; Kim et al., 2000), and alveolar macrophages represent the first line of defense (Fels and Cohn, 1986).   Previous work demonstrated that macrophages respond robustly to ricin *in vitro.* In primary bone-marrow derived macrophages and alveolar macrophages, exposure to ricin causes phosphorylation of stress-activated protein kinases p38 MAPK and JNK, triggering the expression of so-called early response cytokines, TNF-α and IL-1 (Korcheva et al., 2007; Wong et al., 2007b), which are linked to the generation of inflammatory cascades in multiple lung pathologies (Barnes, 2004; Mukhopadhyay et al., 2006). While it is unknown whether macrophage-derived TNF-α and IL-1 are required for ricin-induced inflammation in vivo, when administered to the lungs of animals, ricin triggers apoptosis in alveolar macrophages (Brown and White, 1997; Korcheva et al., 2005).   Importantly, macrophages undergoing apoptosis are known to release inflammatory cytokines that can initiate acute inflammatory responses

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and tissue injury (Hohlbaum et al., 2001; Park et al., 2003). Taken together, these data suggest that macrophages may be essential mediators of ricin-induced pulmonary inflammation.   In Chapter 2 of this thesis, I address the requirement for macrophages in the pulmonary response to ricin; in Chapters 3 and 4, I discuss the molecular mechanisms governing ricin-mediated IL-1β release from macrophages.

III. Interleukin-1, a critical mediator of inflammation

Interleukin-1 (IL-1), is known to be a potent mediator of inflammatory disease. IL-1 was first identified as an endogenous pyrogen in the mid 1980s, in a search for the molecule that was causing fever in experimental animals (Dinarello and Kent, 1985). Soon after, two human cDNAs encoding 31 kDa leaderless precursor proteins were cloned and named IL-1α and IL-1β (March et al., 1985). The third member of the IL-1 family, IL-1 receptor antagonist (IL-1ra) was described soon after, and was found to inhibit both IL-1α and IL-1β activity (Eisenberg et al., 1991; Eisenberg et al., 1990). IL-1ra is a specific antagonist of IL-1 receptors that has an affinity for IL-1 receptors similar to either IL-1α or IL-1β but doesn't transduce a signal itself (Arend et al., 1989). During infection, all three IL-1 family members may be expressed and compete for receptor binding; in this way the ratio of expression levels of IL-1 family members determines the cellular response to stimuli of IL-1 gene expression (Arend, 2002; Dinarello, 2004). IL-1 is not constitutively expressed in cells and requires stimulation by lipopolysaccharide, IL-1 itself or other Toll-like receptor agonists for transcription and synthesis of the precursor form (Dinarello, 2004). Unlike pro-IL-1α, which is active as a precursor and remains cytosolic (Kurt-Jones et al., 1985), pro-IL-1β is inactive and requires processing by IL-1 converting enzyme (now called caspase-1) prior to secretion of its active form, IL-1β (Cerretti et al., 1992; Thornberry et al., 1992). One focus of this thesis is the role of IL-1β in ricin-mediated inflammatory signaling.

IL-1β is considered an apical cytokine that initiates and amplifies a variety of innate immune defenses to microbial infection and tissue injury (Dinarello, 2004). IL-1β is thought to be a major participant in the pulmonary inflammatory cascade in acute respiratory distress syndrome; IL1-β, rather than TNF-α, was found to be the major inflammatory mediator in bronchoalveolar lavage fluid of patients (Pugin et al., 1996). IL-1 signaling mediates inflammatory lung injury by a variety of stimuli including endotoxemia (Calkins et al., 2002), subacute ozone exposure (Johnston et al., 2007), thermal injury (Chen et al., 2007), bleomycin administration (Gasse et al., 2007), and experimental ventilation (Frank et al., 2008). Furthermore, administration of IL-1 is sufficient to induce chemokine expression and recruitment of neutrophils to the airways (Calkins et al., 2002), which accumulate in the airways in acute respiratory distress syndrome and contribute to injury and loss of epithelial integrity (Abraham, 2003).

Perhaps due to its potent biological effects, IL-1β secretion is a tightly regulated process involving the detection of at least two distinct signals (Faustin et al., 2007; Perregaux and Gabel, 1994). Cells first require priming by Toll-like receptor ligands (such as lipopolysaccharide) or by cytokines (such as TNF-α or IL-1β itself) to induce the expression and accumulation of the inactive pro-form, pro-IL-1β. Next, a second signal is required for the assembly of cytosolic molecular scaffolds, called inflammasomes, to stimulate caspase-1 activity, leading to cleavage of the precursor and secretion of active IL-1β (Martinon et al., 2002; Tschopp et al., 2003). In recent years, much research has focused on identifying stimuli which provide this second signal that promotes assembly of inflammasomes. In Chapters 3 and 4 of this thesis, I describe the requirement for one inflammasome in particular, the NLRP/NALP3 inflammasome, in ricin-mediated IL-1β processing and release.

IV. NLRP/NALP3, a pattern recognition receptor involved in detection of toxins

Critical to the efficacy of the innate immune system is the proper detection of pathogen associated molecular patterns and danger associated molecular patterns by macrophages expressing pattern recognition receptors in the cytosol and at the cell surface. Three main types of pattern recognition receptors survey the extracellular and intracellular milieu: 1) Toll-like receptors at the cell surface and in endosomal compartments (Lien and Ingalls, 2002); 2) retinoic-acid inducible gene-I like RNA helicases in the cytosol (Yoneyama and Fujita, 2007) and 3) nucleotide binding and oligonucleotide domain-like (Nod-like) receptors, also present in the cytosol (Martinon, 2005). Pattern recognition receptors are expressed by many cell types including macrophages, monocytes, dendritic cells, neutrophils, and epithelial cells, and they are important for early detection of pathogens, toxins, and sterile inflammatory signals (Imaeda et al., 2009; Iyer et al., 2009; Licastro et al., 2005; Nathan, 2002).

The NLRP/NALP subfamily of receptors, encompassing at least 14 members in humans, is the most extensively studied of the intracellular Nod-like receptors (Martinon and Tschopp, 2007). NLRPs are Nod-like receptor proteins containing nucleotide and oligonucleotide binding domains, leucine-rich repeats, and protein interaction (pyrin) domains (Tschopp et al., 2003). Most NLRPs form active molecular scaffolds—inflammasomes—by homodimerizing and binding via pyrin/pyrin interactions to pyrin-domain containing adaptor proteins that recruit pro-caspase-1 (Agostini et al., 2004; Faustin et al., 2007; Grenier et al., 2002; Martinon et al., 2002; Srinivasula et al., 2002). NLRPs exhibit distinct expression profiles in human tissues (Kummer et al., 2007; Zhang et al., 2008). A large repertoire and distinct expression of NLRPs confers flexibility to host cells and the potential to respond distinctly to a variety of signals that might be encountered in various tissues (Petrilli and Martinon, 2007; Watanabe et al., 2007).

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NALP1, NALP3 and NALP4 are the best studied, and they are expressed in T cells, B cells, dendritic cells, monocytes, granulocytes and macrophages (Kummer et al.; Martinon, 2007). NALP1 is an important mediator of cell death due to anthrax lethal toxin (Boyden and Dietrich, 2006) and NALP4 is activated by numerous gram-negative bacteria (Mariathasan et al., 2004; Sutterwala et al., 2007). The focus of this thesis is NALP3.

NALP3 is activated by a diverse array of agents including exogenous danger signals such as bacterial RNA, Candida albicans, and influenza (Hise et al., 2009; Ichinohe et al., 2009; Kanneganti et al., 2006), environmental stressors such as silica and asbestos (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008), and endogenous danger signals such as ATP, uric acid and amyloid β (Halle et al., 2008; Mariathasan et al., 2006; Martinon et al., 2006). In every case, the assembly of NALP3 inflammasomes triggers the conversion of pro-IL-1β to IL-1β; however the mechanism(s) by which NALP3 receptors detect these disparate agents remains unclear. In the absence of evidence for direct binding, one scenario seems likely: that NALP3 detects a common downstream signal produced by every activator. Interestingly, the best characterized NALP3 activators, the bacterial toxin nigericin and the nucleotide ATP, both trigger the inhibition of protein synthesis in cells with the same kinetics as NALP3-dependent IL-1β release (Alonso and Carrasco, 1981; Perregaux and Gabel, 1994). In Chapters 3 and 4 I examine the role of the NALP3 inflammasome in ricin-mediated IL-1β secretion from macrophages, and address the possibility that protein synthesis inhibition by ricin might provoke the assembly of the NALP3 inflammasome after ricin treatment.

V. Protein synthesis inhibition as a mechanism of innate immune activation

Protein synthesis (or translation) is a fundamental biological process that represents the final and irreversible step in the conversion of genetic information (RNA)

into the functional macromolecules of the cell, proteins (Lodish, 2008). As such, protein translation is carefully regulated. Cellular stressors such as changes in metabolic state or nutrient deprivation are important physiological regulators of protein translation (Kimball and Jefferson, 2002; Proud, 2002, 2004). In addition, mammalian cells have evolved to detect and respond to the presence of pathogens by suppressing their own protein synthesis.

Transient inhibition of cellular translation occurs in a variety of circumstances in nature, triggered by exposure to pathogens trying to co-opt host cell machinery or to sterile inflammatory signals released from damaged tissues (Rock et al., 2010; Sharp et al., 1993). Multiple types of cellular stresses activate pathways that lead to the phosphorylation of the translation initiation factor eIF2α, which results in a global inhibition of protein synthesis (Muaddi et al., 2010; Preedy et al., 1985; Sharp et al., 1993). Inhibition of protein synthesis via phosphorylation of eIF2α occurs after exposure of cells to viral dsRNA through activation of protein kinase R (PKR), by hypoxia through the PRK-like endoplasmic reticulum kinase (PERK), and by glucose deprivation through activation of both PKR and PERK (Muaddi et al., 2010; Preedy et al., 1985; Sharp et al., 1993). Importantly, viral dsRNA and hypoxia also elicit IL-1β release from cells that is dependent on the presence of the NALP3 inflammasome (Iyer et al., 2009; Kanneganti et al., 2006). Furthermore, it has long been known that the bacterial toxin nigericin, a potassium ionophore and one of the more potent activators of the NALP3 inflammasome (Perregaux and Gabel, 1994), inhibits protein synthesis concomitant with loss of intracellular potassium (Alonso, 1981; Cahn, 1978; Panet, 1979). Interestingly, it does so at the same doses and kinetics as it triggers IL-1β secretion (Alonso, 1981; Perregaux and Gabel, 1994). Taken together, these data suggest the possibility that the inhibition of protein synthesis might constitute a danger signal that triggers innate

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immune activation through stimulation of inflammasomes. In Chapter 4 of this thesis, I examine the ability of protein synthesis inhibition to elicit IL-1β release from macrophages, and discuss the role of the NALP3 inflammasome in this process.

VI. Hypotheses and Rationale

In summary, ricin delivered by aerosol to the lungs of mice triggers, at low doses, acute inflammatory disease limited to the pulmonary system and, at high doses, systemic inflammation leading to multiple organ failure and death (Brown and White, 1997; Wong et al., 2007a). Pulmonary macrophages, bone-marrow derived macrophages and alveolar epithelial cells all respond robustly to ricin *in vitro*, displaying phosphorylation of stress-activation protein kinases and the transcription of inflammatory cytokines such as TNF-α and IL-1 (Korcheva et al., 2007; Wong et al., 2007b). In the lung, overactive IL-1β signaling has been associated with multiple pathologies (Barnes, 2004; Dostert et al., 2008; Gasse et al., 2009; Mukhopadhyay et al., 2006). Furthermore, IL-1β production and release by cells has been implicated in responses to other known inhibitors of protein synthesis, both microbial (nigericin, viral dsRNA) and sterile (extracellular ATP, hypoxia), via the NALP3 inflammasome (Shi, 2003; Alonso, 1981; Kanneganti, 2006; Perregaux, 1994; Iyer, 2009). However, the precise cell type(s) that are required for the pulmonary response to ricin and the pathways linking inhibition of protein synthesis, kinase activation, and ricin-mediated inflammatory responses are incompletely understood (Figure 1.3).

I hypothesize that the inflammatory consequences of ricin exposure are deleterious and that modulation of the inflammatory response to ricin may improve survival and reduce tissue injury in animals exposed to ricin. I hypothesize that

macrophages are an important target of ricin *in vivo*, and that depletion of macrophages or the major cytokines they produce (TNF-α and IL-1) will reduce ricin-mediated inflammatory responses. Finally, I hypothesize that the inhibition of protein synthesis by ricin constitutes a danger signal that triggers IL-1β release from macrophages, and that ricin-mediated IL-1β processing requires the NALP3 inflammasome.

To test these hypotheses, this thesis addresses the following aims:

1. Identify and the cell type(s) responsible for the inflammatory consequences of pulmonary exposure to ricin toxin and the cytokines critical for initiating the proinflammatory cascade.

2. Examine the mechanism(s) by which ricin-mediated inhibition of protein synthesis contributes to inflammatory signaling.

3. Examine the ability of protein synthesis inhibitors to trigger inflammatory signaling and identify the critical pathways involved in this process.
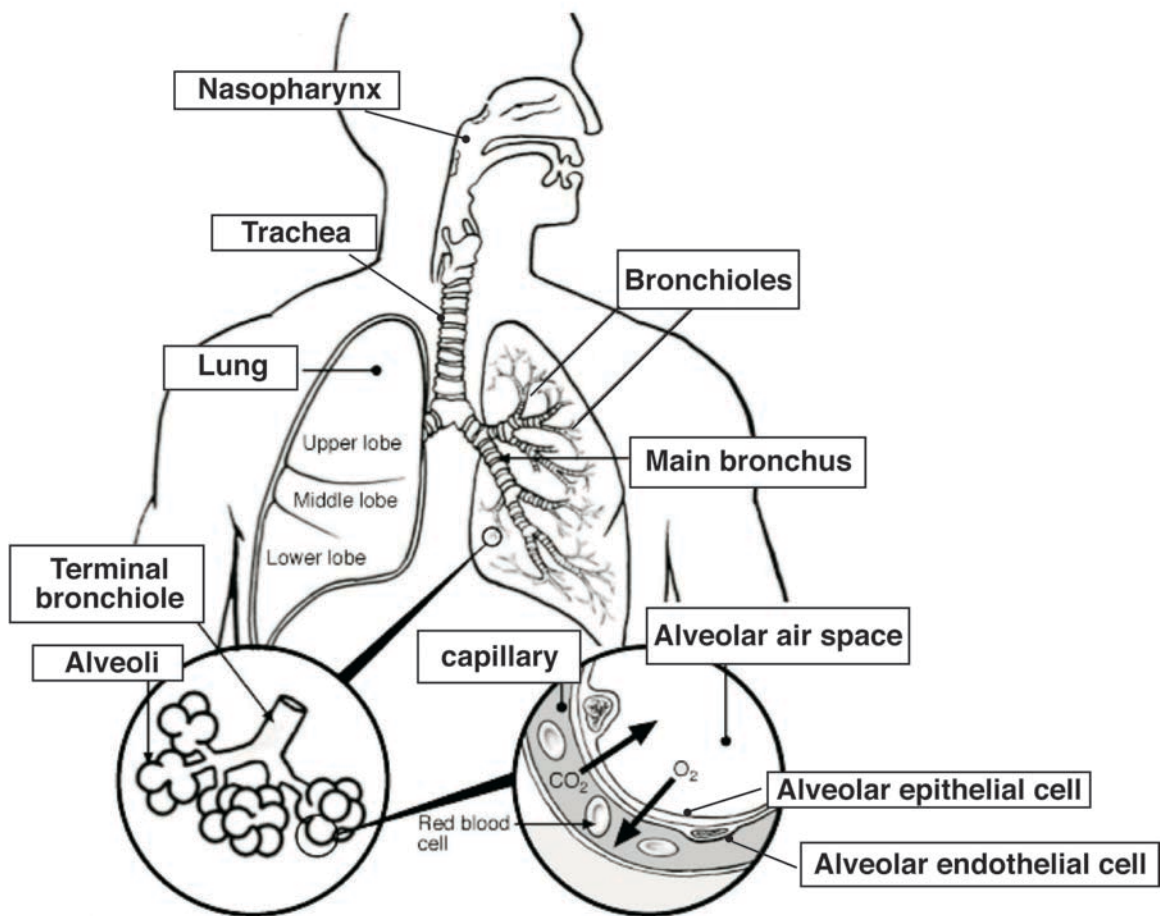
Figure 1.1 The human pulmonary system is composed of the upper respiratory tract—the oral cavity, throat, and nasopharynx—and the lower respiratory tract— the trachea, bronchi and lungs. Gas exchange takes place across the delicate alveolar epithelial/endothelial cell border, the barrier between the environment and the bloodstream of the host.   The lung contains multiple defenses against inhalants. The nasal cavity is lined with epithelial tissue that secretes mucus to filter the air. Ciliated respiratory epithelium and mucus-producing goblet cells in the lower respiratory tract mechanically clear inhalants by trapping and transporting them to the upper respiratory tract, where they are expelled in saliva and nasal secretions. Inhalants that do reach the lung are either cleared quickly by pulmonary macrophages or engage the pulmonary innate immune system, coordinated by resident macrophages, alveolar epithelial and endothelial cells, and recruited leukocytes.   Image adapted from http://www.patient.co.uk/health/Lungs-Respiratory-Tract-and-Breathing.htm.

Wild-type mouse lung treated with aerosolized saline
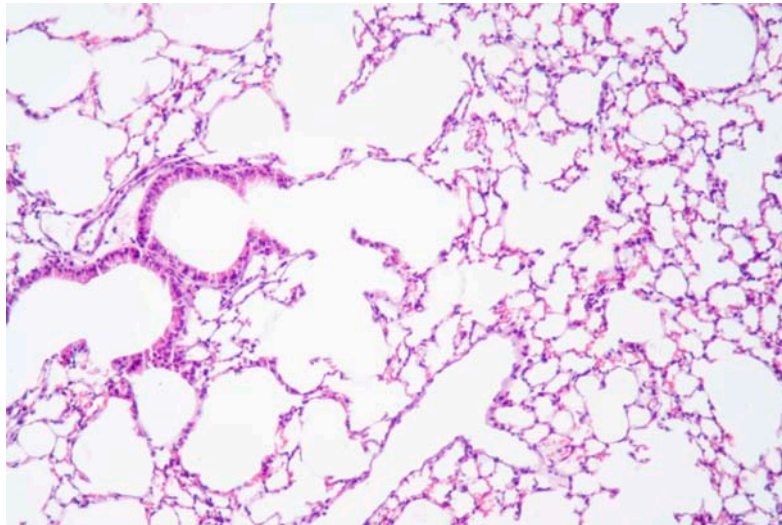
Wild-type mouse lung treated with aerosolized ricin
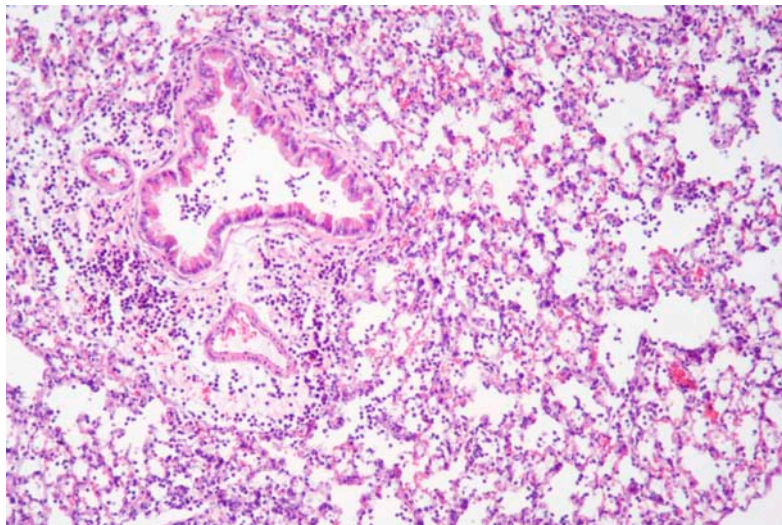
Figure 1.2 Low-dose (60 μg ricin/ 20 g body weight) ricin delivered by aerosol to the lungs of mice results in inflammatory lung injury after 48 hours. Lung tissues were fixed in paraformaldehyde and then paraffin-embedded, followed by sectioning (5 μm) and staining with hematoxylin and eosin for examination of tissue morphology.
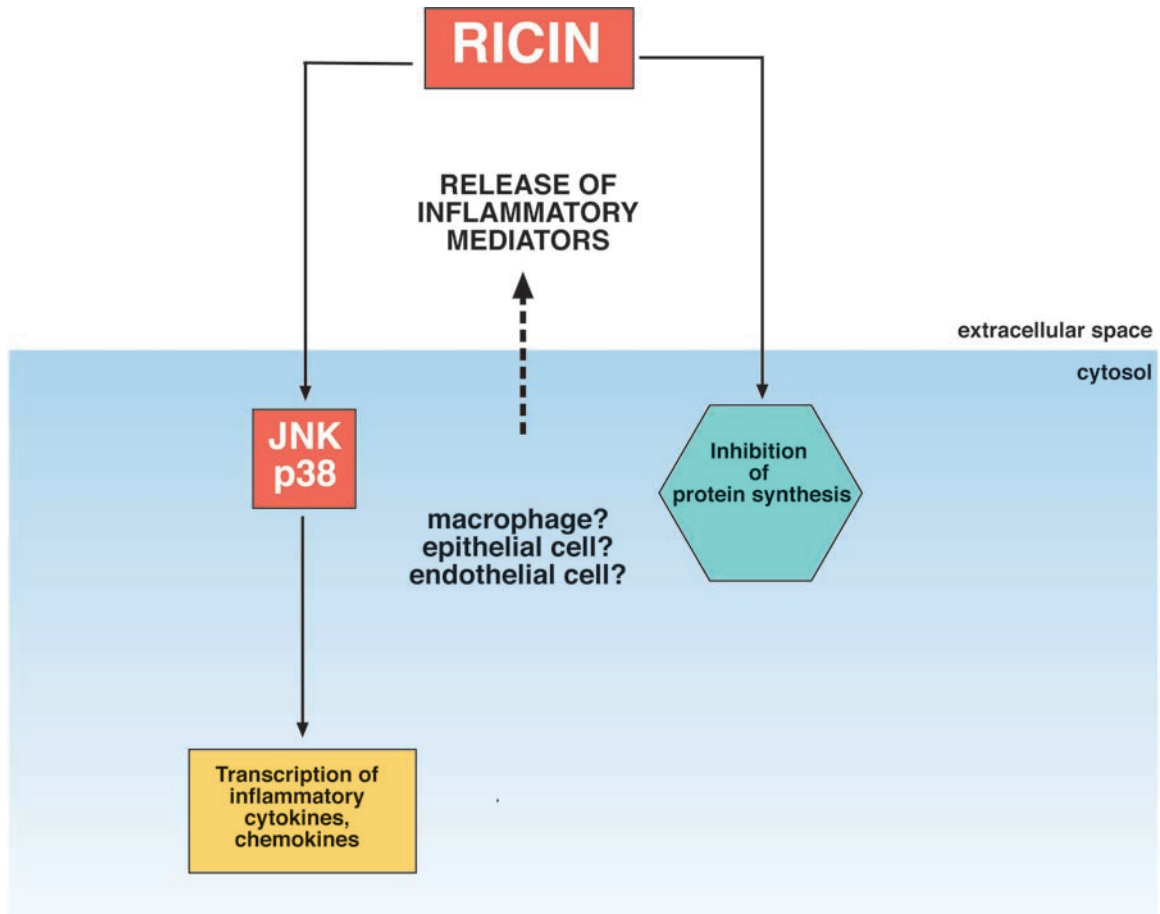
Figure 1.3   Ricin is known to elicit two main responses in cells, both requiring interactions with the ribosome: the inhibition of protein synthesis, through the depuration of A4324 on the 3' end of 28S ribosomal RNA, and phosphorylation of stress-activated protein kinases JNK and p38 MAPK, leading to the transcription of inflammatory cytokines and chemokines including TNF-α, IL-1, IL-6, and CXCL-1. Ricin also triggers a robust inflammatory response in animals after delivery of ricin to the pulmonary system. However, it is not known which cells in the lung are responsible for the generation of this response, and it is unclear how the inhibition of protein synthesis and the activation of protein kinases are connected with the release of inflammatory mediators that initiate ricin-induced inflammatory injury.

Pulmonary inflammation triggered by ricin toxin requires macrophages and IL-1 signaling

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Dr. John Wong performed tracheal intubation of mice for experiments requiring pulmonary delivery of ricin and the immunohistochemistry in Figure 2.4A. Dr. Yoichiro Iwakura provided the IL-1 α/β-deficient mouse strain.

ABSTRACT

Ricin is a potent ribotoxin considered to be a potentially dangerous bioterrorist agent due to its wide availability and the possibility of aerosol delivery to human populations. Studies in rodents and non-human primates have demonstrated that ricin delivered to the pulmonary system leads to acute lung injury and symptoms resembling acute respiratory distress syndrome (ARDS). Increasing evidence suggests that the inflammatory effects triggered by ricin are responsible for its lethality. We demonstrated previously that ricin administered to the lungs of mice causes death of pulmonary macrophages and the release of proinflammatory cytokines, suggesting macrophages may be a primary target of ricin. Here we examined the requirement for macrophages in the development of ricin-mediated pulmonary inflammation by employing transgenic (MAFIA) mice that express an inducible gene driven by the *c-fms* promoter for Fas-mediated apoptosis of macrophages upon injection of a synthetic dimerizer, AP20187. Administration of aerosolized ricin to macrophage-depleted mice led to reduced inflammatory responses including recruitment of neutrophils, expression of proinflammatory transcripts, and microvascular permeability. When compared to control mice treated with ricin, macrophage-depleted mice treated with ricin displayed a reduction in pulmonary IL-1β. Employing mice deficient in IL-1, we found that ricin-induced inflammatory responses were suppressed, including neutrophilia. Neutrophilia could be restored by co-administering ricin and exogenous IL-1β to IL-1$\alpha/\beta^{-/-}$ mice. Furthermore, IL1Ra/Anakinra co-treatment inhibited ricin-mediated inflammatory responses, including recruitment of neutrophils, expression of proinflammatory genes, and histopathology. These data suggest a central role for macrophages and IL-1 signaling in the inflammatory process triggered by ricin.

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INTRODUCTION

The US Chemical Warfare Service began studying ricin as a weapon of war near the end of WWII. In recent years, ricin has become a tool of extremist groups in the US and abroad, as a result of ricin's ease of production and high toxicity (Shar, 1995; Kifner, 1995; Zilinskas, 1997; Mayor, 2003). The toxicity of ricin is approximately 1000 fold greater by aerosol delivery to the respiratory system than by oral ingestion (Franz, 1997), suggesting that delivery of ricin in aerosol form would constitute an effective means of delivery to human populations by terrorist groups. Studies in rodents and non-human primates have demonstrated that ricin delivered into the pulmonary system leads to acute lung injury and symptoms resembling acute respiratory distress syndrome (ARDS) (Greenfield, 2002). ARDS is characterized by inflammation and increased permeability of the lung epithelial barrier (Bernard, 1994), and remains a leading cause of morbidity and mortality in clinical settings (Hudson, 1995; Goss, 2003). When administered to the lungs of animals, ricin induces a rapid massive migration of inflammatory cells, predominantly neutrophils, and causes apoptosis of alveolar macrophages (Brown, 1997). Subsequent effects of ricin exposure include pulmonary edema as well as apoptosis and necrosis of the endothelium and epithelium that constitute the lung surface barrier (Wilhelmsen, 1996). In addition to direct effects of ricin on the pulmonary system, we have reported that intratracheal delivery of ricin to the lungs produces systemic consequences that produce inflammatory changes in multiple organs of the body, including the kidney, liver, and spleen (Wong, 2007).

Ricin is a 62 kDa protein consisting of two glycoprotein chains linked by a disulfide bridge. The 34 kDa B chain, a lectin that binds primarily to galactose-containing surface proteins, facilitates the internalization of the 32 kDa A chain, the toxic moiety. Following endocytosis and retrograde transfer through the Golgi apparatus, the

A subunit of ricin enters the cytosol where it depurinates a single adenine (A4256 in mouse) in the 28S rRNA ribosomes. The depurination of the A4256 is directly responsible both for the inhibition of protein translation (Sandvig, 2002; Saxena, 1989; Endo, 1987) and the initiation of upstream events that lead to inflammatory responses (Korcheva, 2005). We reported the mechanism connecting the inhibition of protein synthesis and the activation of proinflammatory phenomena by demonstrating that ricin mediates activation of stress-activated protein kinases (SAPKs) by producing lesions in the peptidyl transferase center of 28S rRNA (Iordanov, 1997). The two classes of SAPKs in mammalian cells are the JNKs and the p38 MAPKs, both of which are activated by upstream kinases called SAPKKs and SAPKKKs (Derijard, 1994; Galcheva-Gargova, 1994; Han, 1994; Lee, 1994). SAPKs belong to the family of MAPKs, which also include ERKs, and are important mediators through which stress signals are transduced to modulate expression of proinflammatory genes (Kyriakis, 2001; Karin, 1997; Waskiewicz, 1995). Recent studies have identified ZAK/MLK7 as the MAP3K whose activation by ricin and related toxins leads ultimately to the phosphorylation and activation of the SAPKs (Jandhyala, 2008). We demonstrated that delivery of ricin to both pulmonary and extrapulmonary tissues mediated the activation of JNK and p38. Another early consequence of ricin exposure is the activation of NF−κB, a rapid-acting primary transcription factor that induces expression of genes encoding several proinflammatory cytokines and chemokines (Korcheva, 2005; Korcheva, 2007; Wong, 2007). Primary human airway epithelial cells and primary murine macrophages respond to ricin *in vitro* through activation of both MAPK and NF−κB (Korcheva, 2007; Wong, 2007), but the specific cell type(s) responsible for ricin's lethal inflammatory effects *in vivo* remain unclear.

The pulmonary innate immune response is activated not only by pathogens but also by a wide variety of xenobiotic agents (Kim, 2000; Khalil, 2007; Glazer, 2004), and alveolar macrophages represent the first line of defense. Evidence from our laboratory suggests that macrophages play a role in mediating the proinflammatory effects of ricin in the lungs. Intratracheal administration of ricin leads to almost complete elimination of macrophages in the lungs (Brown, 1997) and intravascular administration of ricin leads to the death of macrophages in multiple organs (Roche, 2008; Zenilman, 1988), suggesting that macrophages may serve as primary targets of ricin. Exposure of primary bone marrow-derived and alveolar murine macrophages to ricin *in vitro* leads to activation of SAPKs, increased mRNA transcripts encoding proinflammatory genes, and the increased production and release of TNF$-\alpha$, suggesting the possibility that macrophages may mediate the early proinflammatory effects of ricin *in vivo* (Korcheva, 2007).

In the present study we investigated the role of pulmonary macrophages in ricin-mediated lung injury by employing a transgenic mouse line that harbors an inducible suicide gene in macrophages and their precursors (Burnett, 2004). Using this model, we were able to achieve depletion of macrophages in the lungs of mice treated with the inducer. Compared with non-depleted mice, macrophage-depleted mice displayed markedly decreased inflammatory signs in response to ricin, including decreased recruitment of neutrophils, decreased expression of proinflammatory mediators and reduced microvascular permeability. Employing mice deficient in IL-1α and IL-1β or wild-type mice co-treated with IL-1Ra, a recombinant human form of the IL-1 receptor antagonist, we determined that ricin-mediated inflammation was dependent on IL-1 signaling. Taken together, our data suggests that the proinflammatory effects of aerosolized ricin are dependent on IL-1 signaling and the presence of pulmonary

macrophages, either as the source of IL-1 or as the target cells upon which ricin-induced IL-1 acts to amplify the inflammatory cascade.

RESULTS AND DISCUSSION

Exposure of primary murine macrophages to ricin *in vitro* causes activation of SAPKs in a dose-dependent manner and triggers the expression of key cytokines, chemokines and cell-surface recognition molecules involved in proinflammatory signaling (Korcheva, 2007). To determine whether alveolar macrophages are required for ricin-mediated expression of proinflammatory transcripts *in vivo*, we compared the responses of wild-type mice with mice depleted of macrophages just prior to ricin treatment. Transgenic MAFIA mice were treated with AP20187 for 5 days, at which time apoptotic macrophages were detected in the lung tissue and in Cytospin™ preparations of the cells from bronchoalveolar lavage (BAL) fluid (data not shown). Apoptosis was confirmed both by morphologic features and immunocytochemical detection of activated caspase-3, and, consistent with results of previous studies characterizing the MAFIA mouse model, we achieved 90 percent ablation of macrophages using this method (Burnett, 2004). Control and macrophage-depleted MAFIA mice were exposed to either aerosolized saline or 0.3 mg/100 g aerosolized ricin, a lethal dose that consistently caused death in 100% of wild-type mice within 48-96 hours. Forty-eight hours later, lysates of lung tissue were examined by quantitative real-time RT-PCR (qRT-PCR) for expression of mRNA transcripts that encode a variety of proinflammatory genes. When compared with MAFIA mice receiving ethanol vehicle, MAFIA mice receiving AP20187 to deplete macrophages exhibited significantly reduced expression (p<0.01) of several

proinflammatory cytokines (IL-1β, TNF$-\alpha$, IL-6), chemokines (CXCL-1/Gro$-\alpha$ and CCL-2/MCP-1) and the cell adhesion molecule E-selectin (Figure 2.1). These data indicate that the presence of macrophages was indeed required for the ricin-induced expression of several proinflammatory mediators *in vivo.*

Recruitment of neutrophils to the lung parenchyma and alveolar spaces is a hallmark of acute pulmonary inflammation (Abraham, 2003). In response to ricin, neutrophils accumulate in the BAL fluid and lung tissue of mice 48 hours after administration of ricin (Wong, 2007). To determine whether macrophages were required for ricin to induce the recruitment of neutrophils to the lungs, lung sections and Cytospin[TM] preparations of BAL fluid were examined 48 hours after administration of ricin. Compared with non-depleted mice, animals depleted of macrophages exhibited dramatically reduced numbers of neutrophils in both the lung parenchyma (Fig. 2.2A) and the BAL fluid (Fig. 2.2B), suggesting that the presence of macrophages was required for ricin-mediated neutrophil recruitment to the lungs.

Another hallmark of acute lung injury is enhancement of pulmonary capillary permeability to proteins and fluid across the endothelial cell barrier (Shanley, 1995). Evans blue dye (EBD) has been used as a marker of extravascular protein leakage due to its high affinity for binding albumin when injected into the bloodstream (Baluk, 1999). In order to determine the role of alveolar macrophages in ricin-mediated leakage of extravascular proteins, MAFIA mice treated with ricin were injected i.v. with 50 mg/kg EBD one hour prior to sacrifice. In lungs of MAFIA mice depleted of macrophages, the concentration of extravasated EBD after ricin exposure was reduced by 60 percent compared with MAFIA mice that had only received ethanol vehicle prior to ricin (Fig. 2.2C). This result suggests that ricin-induced vascular permeability to proteins across

the lung endothelial barrier is at least partially dependent on the presence of macrophages.

We showed previously that ricin exposure triggers the production and release of early response cytokines TNF−α and IL-1β (Korcheva, 2007), which are linked to the generation of inflammatory cascades in multiple lung pathologies (Mukhopadhyay, 2006; Barnes, 2004). TNF−α and IL-1 are considered to be initiator cytokines, inasmuch as their release from macrophages has been shown to orchestrate inflammatory responses. In view of the increased expression of mRNA transcripts encoding TNF−α and IL-1β in response to aerosolized ricin in MAFIA mice (Fig. 2.1), we sought to determine whether these cytokines were required for ricin to elicit inflammatory responses. To approach this question, we first examined TNF−α and IL-1β protein levels in lung homogenates of MAFIA mice treated with ricin. Both groups of MAFIA mice treated with AP20187 exhibited slightly higher basal levels of IL-1β in the lungs compared to non-depleted mice. However, MAFIA mice whose macrophages had not been depleted displayed a 3-4-fold increase in IL-1β after exposure to ricin, while no ricin-mediated induction was observed in IL-1β levels of macrophage-depleted animals (Fig. 2.3). Meanwhile, TNF−α measured from lung homogenates did not change significantly between groups (data not shown), suggesting that IL-1β may play a larger role in the response to ricin in MAFIA mice.

To further probe the roles of IL-1 and TNF−α in ricin-mediated inflammation, we compared responses of mice harboring null mutations in IL-1α and IL-1β (IL-1α/$\beta^{-/-}$) and TNF−α (TNF−$\alpha^{-/-}$). Lung tissue sections prepared from IL-1α/$\beta^{-/-}$ at 48 hours after ricin treatment showed markedly reduced vascular congestion, destruction of alveoli, and accumulation of neutrophils in the airways compared to wild-type tissue sections (Fig.

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2.4A). Furthermore, IL-1 deficient animals had reduced neutrophil counts in the BAL

fluid (Fig. 2.4B), improved survival compared to wild-type (Fig. 2.4C) and diminished

pulmonary edema as measured by lung tissue wet/dry ratios (Table I). TNF$-\alpha^{-/-}$ mice,

however, failed to show improvement over wild-type animals in inflammatory responses

after ricin treatment (data not shown). Taken together, the data in Figure 2.3 and Table I

indicate that IL-1 was required for ricin to mediate its proinflammatory effects in the

pulmonary system and, furthermore, that TNF$-\alpha$ was dispensable for this response.

If the provoked release of IL-1 from alveolar macrophages were responsible for

the proinflammatory effects of ricin, then the co-administration of exogenous IL-1 and

ricin to IL-1$\alpha/\beta^{-/-}$ mice should restore the inflammatory responses in these animals. To

test this possibility, we administered 15 ng IL-1β in the presence or absence of ricin, and

measured the accumulation of neutrophils in the BAL fluid. Whereas IL-1β alone failed

to induce recruitment of neutrophils to the BAL fluid of IL-1$\alpha/\beta^{-/-}$mice, the co-

administration of ricin and IL-1β to these mice resulted in the accumulation of BAL

neutrophils to levels observed in wild-type mice treated with ricin alone (Compare Figs.

2.5A and 2.2B). Taken together with the data shown in Figures 2.3 and 2.4, these

results support the conclusion that ricin-mediated pulmonary inflammation is dependent

on the presence of IL-1.

To explore the possibility that reduced IL-1β production was a contributing factor

in the suppressed inflammatory response observed in macrophage-depleted MAFIA

mice, we examined the ability of macrophage-depleted animals to recruit neutrophils to

the lungs when exposed to aerosolized ricin in conjunction with exogenous IL-1β. Co-

administration of ricin and 15 ng IL-1β to macrophage-depleted MAFIA mice resulted in a

significant ($p > 0.01$) 10-fold increase in the number of neutrophils in the BAL fluid when

compared to mice treated with either IL-1β or ricin alone (Fig. 2.5B). The data in Figure 5 demonstrate that IL-1β can contribute to ricin-mediated recruitment of neutrophils in both IL-1α/$\beta^{-/-}$ and macrophage-depleted mice.

Experimental studies show that recombinant human IL-1 receptor antagonist (IL-1Ra), a competitive inhibitor that interferes with binding of IL-1α and IL-1β to the IL-1R1, may limit the release of cytokines and the development of neutrophilia in animal models of lung inflammation (Frank, 2008; Abraham, 1994; Calkins, 2002; Gasse, 2007). In order to determine the ability of IL-Ra to block ricin-induced pathology, we administered IL-1Ra to wild-type mice at the time of ricin exposure and examined lung tissue sections after 48 hours. Animals receiving IL-1Ra exhibited reduced vascular congestion, destruction of alveoli, and accumulation of neutrophils in the airways compared to animals receiving ricin alone (Fig. 2.6A). Lungs from IL-1Ra-treated mice had significantly lower expression of proinflammatory transcripts (p<0.01; Fig. 2.6B) and reduced appearance of neutrophils in the BAL fluid (Fig. 2.6C). These data confirm the central role of IL-1 signaling in the inflammatory response to ricin, and suggest the possibility that blockade of the IL-1 pathway could be employed to suppress ricin-mediated inflammation.

Here we show that acute inflammatory lung injury induced by aerosolized ricin was characterized by increased mRNA expression of proinflammatory genes, production of IL-1β, recruitment of neutrophils to the lungs, and elevated microvascular permeability, and that these responses were dependent on the presence of pulmonary macrophages and IL-1 signaling. Indeed, lung pathology was significantly reduced in animals depleted of macrophages or genetically deficient in IL-1α/β, as well as in wild-type animals injected with IL-1Ra to block IL-1 signaling at the time of ricin exposure. Furthermore, at a dose insufficient to produce inflammatory effects on its own,

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exogenous IL-1β, when co-administered with ricin to mice deficient in IL-1, recapitulated neutrophil recruitment to the BAL fluid in numbers consistently observed in wild-type animals exposed to ricin alone. Taken together, these results demonstrate that pulmonary IL-1 signaling is essential for the acute inflammatory response to aerosolized ricin.

There is increasing evidence that the inflammatory effects triggered by ricin are responsible for ricin's lethality (Wong, 2007; Mabley, 2009). Since aerosolized ricin represents the most plausible means of exposure for human populations in the event of a bioterrorist attack, understanding the pulmonary immune response to inhaled ricin toxin is critical for developing therapies aimed at managing symptoms and reducing mortality in case ricin is used as an agent of warfare. In the lung, immune responses to inhaled substances are orchestrated by resident macrophages, which initiate inflammatory cascades through the production and release of immunomodulatory mediators like early response cytokines TNF$-\alpha$ and IL-1 (Fels, 1986). We previously demonstrated that ricin triggers the enhanced expression of genes encoding TNF$-\alpha$ and IL-1 (Korcheva, 2005), and the release of TNF$-\alpha$ from primary murine macrophages in culture (Korcheva, 2007). During microbial invasion, the release of cytokines and chemokines by macrophages serves to recruit and activate other leukocytes, mainly neutrophils, to help clear pathogens and resolve the infection. Inflammatory injury to local tissue by host immune cells is viewed as collateral damage in an infection (Male, 2006). However in the case of ricin exposure, in which there is no pathogen to clear, tissue damage occurs without benefit to the host. In view of this, we hypothesized that suppression of the innate immune response to ricin would serve to reduce inflammatory lung injury and lethality caused by ricin.

Depletion of macrophages resulted in diminished ricin-mediated expression of proinflammatory transcripts (Fig. 2.1), accumulation of neutrophils (Fig. 2.2A and 2.2B), microvascular barrier permeability (Fig. 2.2C), and tissue IL-1β levels (Fig. 2.3), indicating that macrophages are required for these inflammatory processes. Not surprisingly, since macrophages are important for pulmonary homeostasis and constitute the cell type primarily responsible for the clearance of cell debris (Gordon, 2007), the depletion of macrophages from mice caused some inflammation and weight loss that was independent of ricin treatment, consistent with studies published previously on MAFIA mice (Burnett, 2004). We established 20% weight loss as a criterion for mortality and, to conform with ethical guidelines established by OHSU IACUC, sacrificed the animals that had reached this endpoint. For this reason, the weight loss consistently observed in MAFIA mice injected with AP20187 alone (10-15%) confounded our ability to perform survival studies. Both groups of macrophage-depleted animals, saline- and ricin-treated, exhibited some alveolitis (Fig 2.2A) and increased IL-1β levels (Fig. 2.3) over control animals, and these effects were observed only in MAFIA and not AP20187-treated wild-type C57BL/6 mice (data not shown), indicating that the effect was specific to the induction of Fas-mediated apoptosis of macrophages in the transgenic animals. It is known that ligation of Fas on tissue macrophages induces proinflammatory cytokine release that can initiate acute inflammatory responses and tissue injury (Park, 2003; Hohlbaum, 2001), which may explain the higher level of "basal" inflammation in macrophage-depleted animals. Despite these limitations, inflammatory responses were drastically induced after ricin treatment, and we were able to glean from these experiments that macrophages play an essential role in the development of ricin-mediated inflammatory disease.

Since the depletion of macrophages prior to ricin treatment resulted in an attenuation of inflammatory symptoms, we investigated the requirements for TNF-α and IL-1, the major proinflammatory mediators produced by macrophages. Early response cytokines such as TNF-α and IL-1 act on a variety of cells via cell membrane-bound receptors (Dinarello, 2000; Dinarello, 2002; Cohen, 2002) to initiate a proinflammatory cascade resulting in chemokine production, upregulation of adhesion molecules, transmigration of neutrophils into alveolar compartment and lung interstitium, and the release of proteases and reactive oxygen radicals which are linked to tissue damage (Holmes, 2002; Shanley, 1995). Mice deficient in both TNF-α and IL-1 signaling exhibit impaired neutrophilic inflammation in response to *Streptococcus pneumoniae* (Jones, 2005). In order to determine the requirement for these cytokines in the response to ricin, we obtained animals genetically deficient in TNF-α and IL-1α/β and administered aerosolized ricin. Notably, mice deficient in TNF-α showed no improvement over wild-type animals (data not shown), indicating that TNF-α, although induced by ricin (Fig. 2.1), is dispensable for this response. In contrast, animals lacking IL-1α/β had significant protection from ricin-mediated inflammatory indices (Figs. 2.4A, 2.4B and Table 1) and improved survival over wild-type animals (Fig. 2.4C), although they all eventually succumbed after several days. Furthermore, a 10-fold higher dose of ricin administered by aerosol to the pulmonary system led to death of all animals within 48 hours regardless of genotype (data not shown). Because ricin ultimately leads to apoptosis of pulmonary epithelium (Wong, 2007), the death observed in IL-1 deficient animals in the absence of pulmonary inflammation may have resulted from the loss of barrier function and the subsequent entry of ricin into the vascular system.

IL-1β is thought to be a major participant in the pulmonary inflammatory cascade in ARDS; IL1-β, rather than TNF-α, was found to be the major inflammatory mediator in

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BAL fluid of patients with ARDS (Pugin, 1996). IL-1 signaling mediates inflammatory lung injury induced by a variety of stimuli including endotoxemia (Calkins, 2002), subacute ozone exposure (Johnston, 2007), thermal injury (Chen, 2007), bleomycin administration (Gasse, 2007), and experimental ventilation (Frank, 2008). Administration of IL-1 is sufficient to induce chemokine expression and recruitment of neutrophils to the airways (Calkins, 2002). Neutrophils responding to cytokine and chemokine gradients accumulate in the airways and contribute to injury and loss of epithelial integrity that characterize ARDS/ALI (Abraham, 2003). Indeed, in the present study, mice lacking IL-1 or pulmonary macrophages (and thus the major producers of IL-1) showed drastically reduced numbers of neutrophils in the BAL fluid following ricin exposure (Fig 2.4B; Fig 2.2B), and this reduction in neutrophils coincided with reduced edema in IL-1$\alpha/\beta^{-/-}$ mice (Table 1) and diminished microvascular permeability in macrophage-depleted MAFIA mice (Fig. 2.2C). In addition, mice treated with IL-1Ra to block IL-1R1 signaling at the time of ricin exposure exhibited reduced expression of proinflammatory transcripts (Fig 2.6C) and accumulation of neutrophils in the BAL fluid (Fig 2.6B), supporting a primary role for IL-1 signaling in ricin-mediated inflammation.

Precisely which cells respond to ricin-induced secretion of IL-1 remains unknown. Potential targets of IL-1 include pulmonary macrophages acting in an autocrine or paracrine fashion, microvascular endothelial cells, and alveolar epithelial cells. Type I (TI) and Type II (TII) alveolar epithelial cells are known to be capable of responding directly to inflammatory stimuli as well as through inflammatory mediators produced by first-responders. For example, alveolar epithelial cells secrete chemokines MIP-2 (CINC-3/CXCL3) and MCP-1 (CCL2) in response to IL-1β and LPS (Manzer, 2006) but require stimulation from macrophage-derived IL-1α to elicit chemokine secretion in response to ozone exposure (Manzer, 2008). Similarly, human lung microvascular

endothelial cells up-regulate the expression of adhesion molecules and release MCP-1, IL-8 and Gro−α/CXCL-1) in response to IL-1 (Beck, 1999), suggesting that these cells may also play an active role in the propagation of inflammation initiated by ricin.

The finding that IL-1Ra suppressed the transcription of other genes following ricin exposure (Fig. 2.6C) suggests that IL-1 signaling is situated upstream of other events in the inflammatory process. Regulation of IL-1 responsive genes is mediated in large part through activation of the ubiquitously expressed transcription factor, NF−κB (Ray, 1993). Nuclear NF−κB activity is rapidly induced in the presence of IL-1 in human lung epithelial cells, and this activation is inhibited by the addition of IL-1 receptor antagonist (Ray, 1993). Activation of NF−κB in airway epithelial cells has been implicated in the development of neutrophilic inflammation by multiple stimuli (Poynter, 2003; Jones, 2005; Haegens, 2007), including ricin (Wong, 2007). We reported previously that NF−κB is activated in human epithelial cells after exposure to ricin, and that targeted inhibition of NF−κB by siRNA results in an inhibition of the expression of proinflammatory genes (Wong, 2007). The data presented here suggest that ricin-mediated IL-1 signaling may contribute to the activation of NF−κB in the airways to promote the development of acute inflammatory disease.

Although many investigators have elucidated mechanisms of lung inflammation and injury following administration of pathogens and microbial products such as LPS, the mechanisms by which environmental xenobiotics induce lung inflammatory responses are not well understood. In addition to providing insight for development of therapeutic approaches to counteract the use of aerosolized ricin as a bioterrorist agent, an increased understanding of mechanisms underlying ricin's toxicity could serve to provide greater understanding of how inhaled toxins can cause inflammation, injury, and death.

MATERIALS AND METHODS

Animals. C57BL/6J, MAFIA and TNF$-\alpha^{-/-}$ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. MAFIA mice are catalogued with the Jackson Laboratory as strain C57BL/6J-Tg(Csf1r-GFP, NGFR/FKBP12)2Bck/J. IL-1α/$\beta^{-/-}$ were developed as described (Horai, 1998). Male mice 8 to 10 weeks of age and weighing 18 to 24 g were used throughout the experiments. Mice were housed under 12-hour light-dark cycle and fed with standard diet ad libitum. All studies were conducted with approval by the Institutional Animal Care and Use Committee at Oregon Health & Science University. For both treatment and euthanasia, mice were anesthetized with an i.p. injection of a cocktail containing 0.75 mg ketamine, 0.15 mg xylazine and 0.03 mg acepromazine. For treatment, a PennCentury^{TM} (Philadelphia, PA) 23-gauge microsprayer (model IA-1C) was inserted into the opening of the trachea with the aid of a laryngoscope. A volume of 50 μl saline or 0.3 mg/100 g ricin diluted in saline was delivered by aerosol to the animal. The dose of ricin used was determined to be the lethal dose at which wild-type animals succumbed within 48-96 hours. Exogenous recombinant IL-1β (EMD Chemicals, Gibbstown, NJ) and IL-1Ra/Anakinra (Kineret^{TM}, Amgen Inc, Thousand Oaks, CA) were delivered in the same manner at indicated concentrations. For survival experiments, animals were euthanized when they became moribund according to the criteria of lack of response to stimuli or lack of righting reflex.

Reagents and antibodies. Ricin was purchased from Vector Laboratories (Burlingame, CA). AP20187 was provided by ARIAD pharmaceuticals, Inc (Cambridge, MA). BD OptEIA^{TM} mouse IL$-\beta$ ELISA set was purchased from BD Biosciences (San Diego, CA). Anti-Gr-1/Ly6G (no. 550291) was purchased from BD Bioscience Pharmingen (San Jose, CA) and anti-cleaved caspase-3 (no. 9664) was purchased from Cell Signaling Technology (Danvers, MA).

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In vivo depletion of macrophages. AP20187 was a gift from Ariad Pharmaceuticals, Inc, Cambridge, MA (www.ariad.com/regulationkits). Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 62.5 mg/ml stock solution and was stored at −20°C. As recommended by Ariad Pharmaceuticals, injection solutions consisted of 4% ethanol, 10% PEG-400 and 2% Tween-80 in water. All injections were administered i.p. within 30 minutes after preparation. The volume of injection solution was adjusted according to the average mouse body weight to deliver a dose of 10 mg/kg AP20187 per mouse, in an average volume of 100 µl. Mice were injected daily for 5 days prior to ricin treatment.

Bronchoalveolar lavage (BAL) and neutrophil counts. At 48 hours after treatment with ricin, lungs were lavaged four times with 1 ml of cold 0.7x PBS. After lavage, the population of cells from the BAL fluid was examined using Cytospin™ (Thermo Fisher Scientific Inc., Waltham, MA) slide preparations and staining by Hema 3 stain set (Fisher Healthcare, Houston, TX). Polymorphonuclear neutrophils (PMNs) were identified morphologically and were counted. BAL slides from at least five animals from each treatment group were analyzed to ensure reproducibility.

RNA isolation. Lung tissues were dissected and were immediately frozen and ground in liquid nitrogen. RNA was extracted using TRIzol reagent in accordance with the manufacturer's instructions and was further digested with DNase. Both reagents were purchased from Invitrogen Life Technologies, Carlsbad, CA.

Real-time PCR analysis. Two µg RNA were reverse-transcribed in the presence of SuperScript II and oligo-dT primer (both reagents were purchased from Invitrogen Life Technologies). The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in the

presence of the commercially available SYBR Green PCR Master Mix (Applied Biosystems) and 20 µmol/L of the corresponding sense and anti-sense RT-PCR primers for 120-bp amplicons in a 40-cycle PCR. Fold induction in gene expression was measured using absolute quantitation of a standard curve in arbitrary units and using levels of GAPDH for normalization. The nucleotide sequences of the primers used in this study have been previously published (Korcheva, 2005). The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, respectively. RNA from 5 animals per group were analyzed by real-time PCR.

Histology and immunohistochemical analysis. Animals were sacrificed at 48 hours unless otherwise indicated. After dissection, lungs were fixed in 4% paraformaldehyde solution for 24 hours, at which time tissues were dehydrated and embedded in paraffin. For histology, 5 µm sections were mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin (H&E) following standard procedures. For immunohistochemical analysis of activated caspase-3, antigen retrieval was performed by placing the deparaffinized slides in 10 mmol/L sodium citrate (pH 6) in a microwave oven for 10 minutes. Immunohistochemical detection of Gr-1/Ly6G did not require pretreatment for antigen retrieval. After blocking in serum, the slides were incubated with primary antibodies overnight at 4°C at appropriate dilutions. Slides were further processed using the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations using 3,3'-diaminobenzidine as substrate. Immunohistochemical analysis was performed on specimens from at least five animals in each group to verify reproducibility. For examination of embedded lungs, one lung from each animal was sliced transversely into 2 mm slices. To ensure thorough microscopic examination of the entire lung, all slices from each lung were

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processed, embedded, sectioned by microtomy, and mounted on a single glass slide for examination either by H&E or by immunohistochemistry.

Evans blue dye assay. Forty-eight hours after ricin treatment, animals were anesthetized and injected with 50 mg/kg Evans blue dye (EBD) diluted in saline to a total volume of 200 μl through the retro-orbital vein. The dye was allowed to circulate for 30 minutes, at which time the vasculature was perfused with 5 mL saline through the right ventricle to remove residual EBD from the systemic circulation prior to dissection of the lungs. Lungs were removed, weighed, and homogenized; the dye was extracted in 1.5 mL formamide. The optical density of each sample was determined spectrophotometrically (absorbance 620 nm) and EBD concentration was calculated using a standard curve of known EBD dilutions. Values are expressed in fold induction over saline-control after being normalized for the dry weight of each tissue, and represent the average of 3 animals per group.

ELISA. Lung tissue was homogenized in lysis buffer in PBS containing 2% NP-40 and Complete[TM] protease inhibitor cocktail (Roche, Indianapolis, IN) and centrifuged at 10,000 x *g* for 20 minutes at 4°C. The resulting supernatant was analyzed for IL-1β levels using the BD OptEIA[TM] mouse IL-1β ELISA set from BD Biosciences (San Diego, CA) per the manufacturer's instructions. The detection limit for IL-1β was 4 pg/mL. Absorbance of standards and samples were determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of the standard curve. Results are expressed as pg/mL IL-1β and represent the average of 3 animals per group.

Lung wet/dry weight ratio. Left lungs were excised and rapidly weighed for wet weight. Samples were oven dried at 65 degrees for 72 hours to a stable dry lung weight.

Data are presented as the ratio of lung wet to dry weight 48 hours after either saline or ricin treatment. Five animals from each treatment group were analyzed for wet/dry weight ratios.

Statistical Analysis. Individual groups were compared using unpaired t-test analysis. To estimate P values, all statistical analyses were interpreted in a two-tailed manner. P values <0.05 were considered to be statistically significant. Kaplan-Meier analysis was performed for survival curves. One-way ANOVA analysis was performed for EBD and wet/dry weight ratio experiments. All data are presented as means ± SEM.
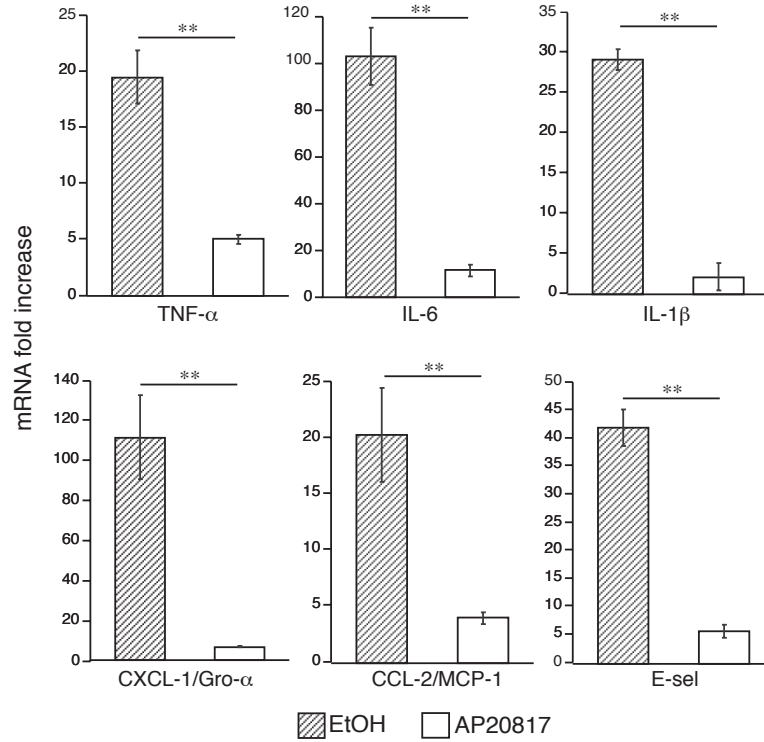
FIGURE 2.1 Macrophage depletion reduces ricin-mediated expression of proinflammatory RNA transcripts. Mice pretreated for 5 days with ethanol vehicle (hatched bars) or AP20187 (white bars) were administered either saline or 0.3 µg/100 g ricin. Total RNA was purified from lung tissue harvested 48 h later and processed for real-time PCR. Values displayed represent fold induction over respective saline control, normalized to GAPDH ($n = 5$; **, $p < 0.01$). Error bars represent SEM.
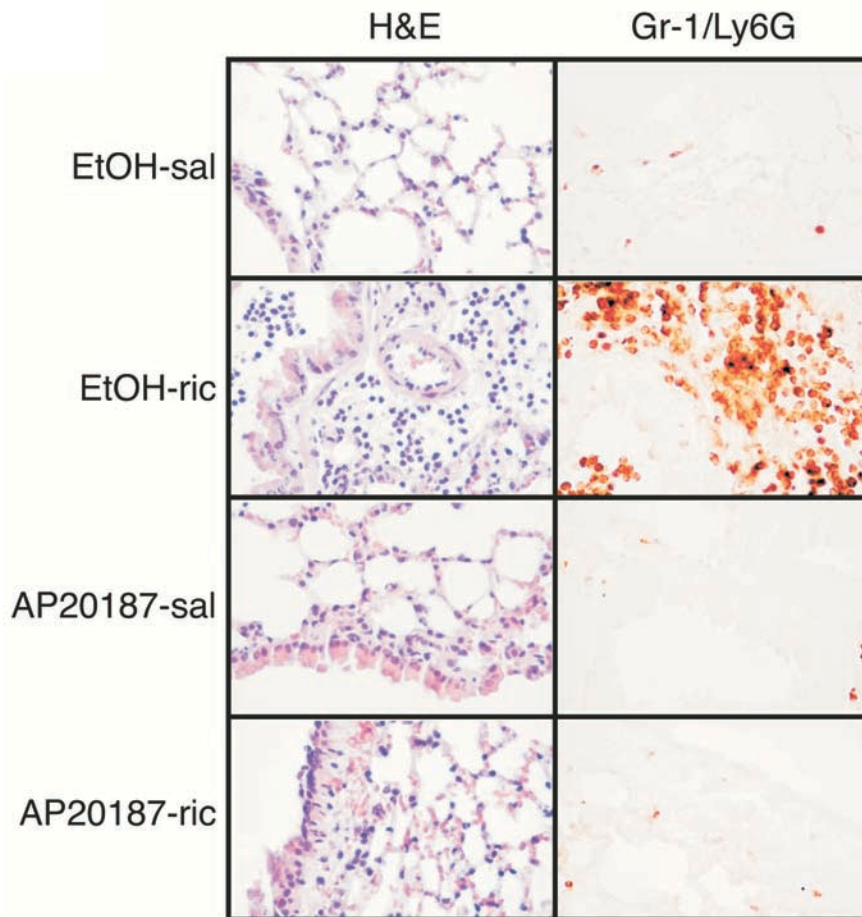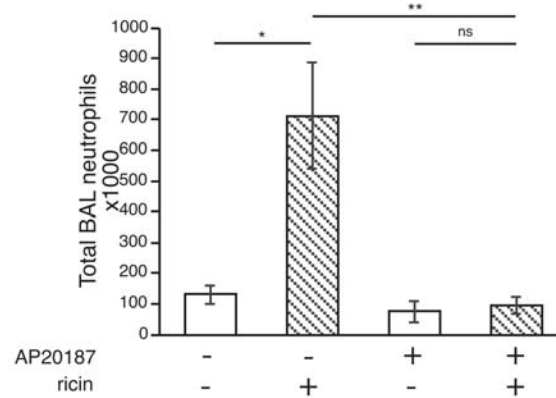
A.

FIGURE 2.2 Macrophage depletion results in reduced ricin-mediated neutrophil accumulation and barrier permeability. Mice pretreated for 5 days with ethanol vehicle or AP20187 were administered either saline or 0.3 µg/100 g ricin and euthanized 48 h later. A) Lung tissue sections (40x) stained with H&E and processed for immunohistochemical detection of Gr-1/Ly6G show ricin-mediated damage to alveolar epithelium and accumulation of Gr-1/Ly6G-positive cells in alveoli and airways (n = 5). B) Total neutrophils counted in the BAL fluid (n = 5; $p < 0.05$, **, $p < 0.01$). C) Microvascular permeability as measured by Evans blue dye (EBD) permeability assay and expressed as fold induction over respective saline controls (n = 5; **, $p < 0.01$). Error bars represent SEM.
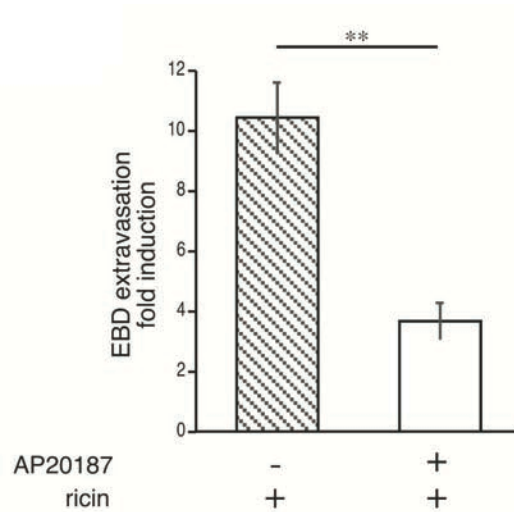
B.

C.

FIGURE 2.2 Macrophage depletion results in reduced ricin-mediated neutrophil accumulation and barrier permeability. Mice pretreated for 5 days with ethanol vehicle or AP20187 were administered either saline or 0.3 µg/100 g ricin and euthanized 48 h later. A) Lung tissue sections (40x) stained with H&E and processed for immunohistochemical detection of Gr-1/Ly6G show ricin-mediated damage to alveolar epithelium and accumulation of Gr-1/Ly6G-positive cells in alveoli and airways (n = 5). B) Total neutrophils counted in the BAL fluid (n = 5; p < 0.05, **, p < 0.01). C) Microvascular permeability as measured by Evans blue dye (EBD) permeability assay and expressed as fold induction over respective saline controls (n = 5; **, p < 0.01). Error bars represent SEM.
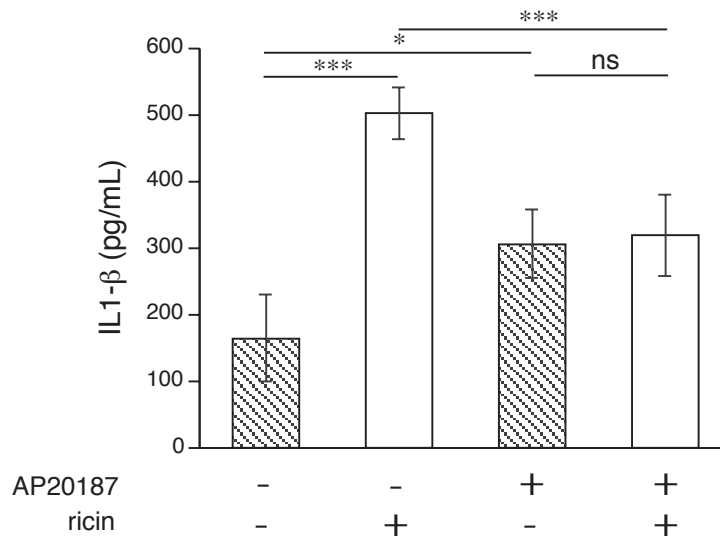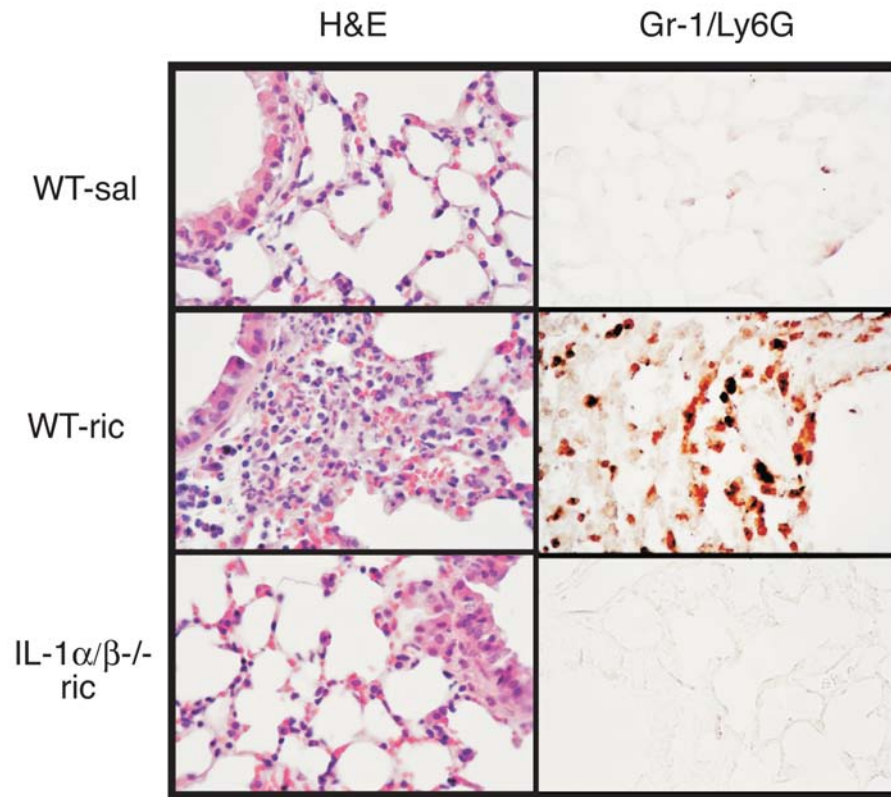
FIGURE 2.3 IL-1β protein is increased in lung tissue after ricin treatment in control but not macrophage-depleted mice. Protein levels were measured by ELISA from lung homogenates collected 48 hours after ricin treatment ($n = 5$; *, $p < 0.05$; ***, $p < 0.001$; ns, not significant). Error bars represent SEM.

A.

Dr. John Wong performed the histology for this experiment. The data analyses
were performed by ML.

FIGURE 2.4 IL-1α/$\beta^{-/-}$ mice have markedly reduced neutrophilia, and improved survival
compared with wild-type mice. IL-1α/$\beta^{-/-}$ and wild-type animals were administered ricin
and euthanized 48 h later. A) Representative photomicrographs of lung sections stained
with H&E and labeled with anti-Gr-1/Ly6G. B) Total neutrophils counted in the BAL fluid
($n = 5$; **, $p < 0.01$). C) Survival curves comparing IL-1α/$\beta^{-/-}$ (circles) and wild-type
(squares) survival percentages over 9 days following ricin treatment ($n = 10$; $p = 0.002$).
Error bars represent SEM.

B.
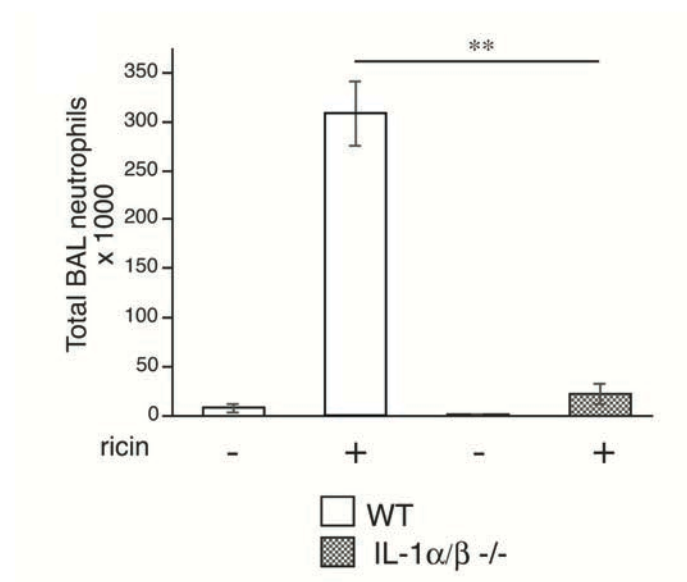
C.
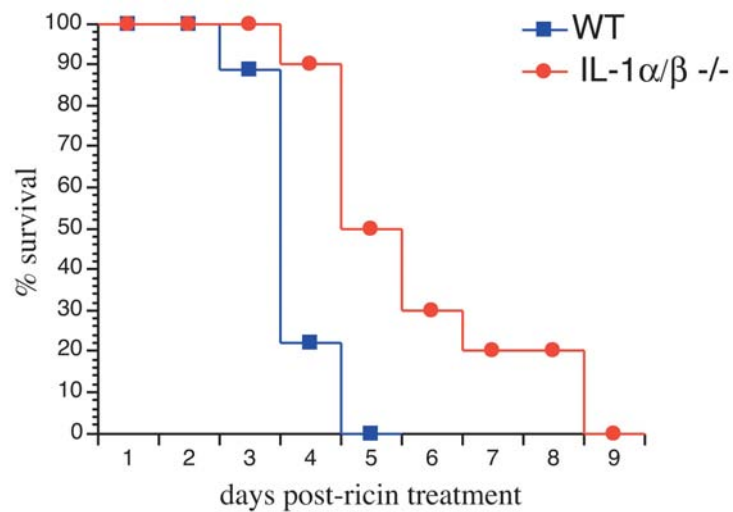
FIGURE 2.4 IL-1α/β$^{-/-}$ mice have markedly reduced neutrophilia and improved survival compared with wild-type mice. IL-1α/β$^{-/-}$ and wild-type animals were administered ricin and euthanized 48 h later. A) Representative photomicrographs of lung sections stained with H&E and labeled with anti-Gr-1/Ly6G. B) Total neutrophils counted in the BAL fluid ($n = 5$; **, $p < 0.01$). C) Survival curves comparing IL-1α/β$^{-/-}$ (circles) and wild-type (squares) survival percentages over 9 days following ricin treatment ($n = 10$; $p = 0.002$). Error bars represent SEM.

Table 1. Lung wet/dry ratios

Treatment	Wild type		IL-1α/β-/-	
	Mean	SEM	Mean	SEM
saline	4.58	0.17	4.80	0.09
ricin	5.72	0.08[a]	5.05	0.29[b]

a: P<0.05 vs. saline
b: P=0.63 (ns) vs. saline, P<0.05 vs. wild type

A.
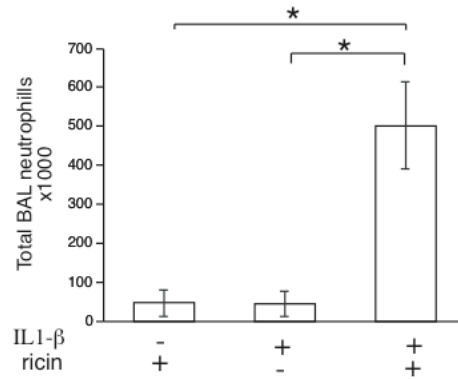
B.
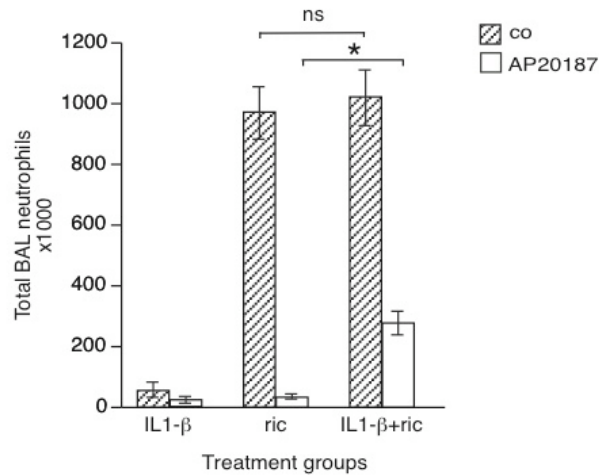
FIGURE 2.5 Exogenous IL-1β administered in combination with ricin restores ricin-mediated recruitment of neutrophils to the BAL fluid in both IL-1α/β$^{-/-}$ and macrophage-depleted MAFIA mice. Animals were treated with ricin and euthanized 48 h later. A) Total neutrophils counted in the BAL fluid of IL-1α/β$^{-/-}$ mice treated with ricin, 15 ng of IL-1β or ricin, and 15 ng of IL-1β ($n = 3$; *, $p < 0.05$). B) Total neutrophils counted in the BAL fluid of MAFIA mice pretreated with ethanol vehicle (hatched bars) or AP20187 (white bars) for 5 days followed by administration of either 15 ng of IL-1β or ricin, or 15 ng of IL-1β and ricin ($n = 3$; *, $p < 0.05$; ns, not significant). Error bars represent SEM.
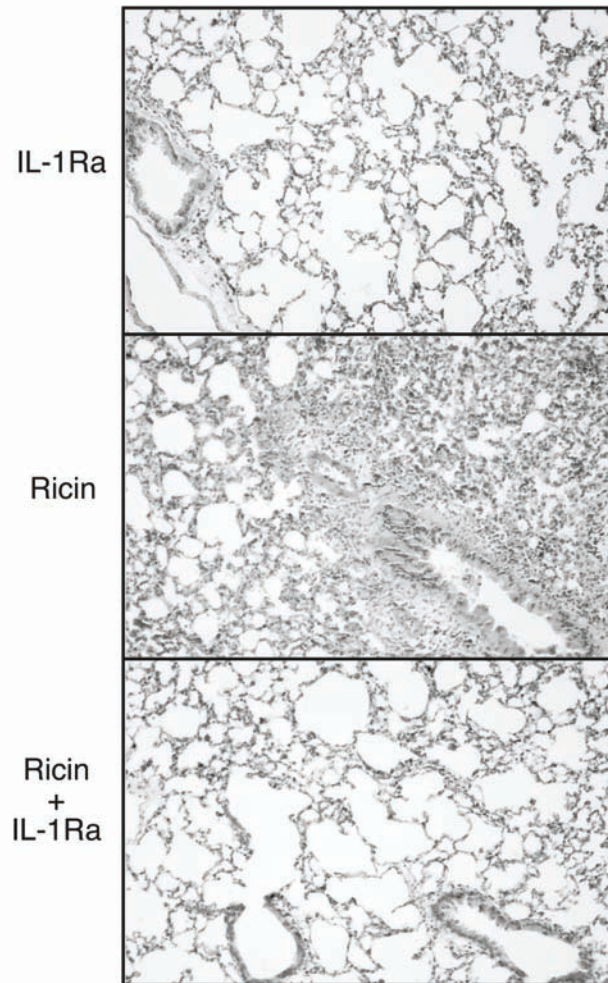
A.

FIGURE 2.6 Coadministration of IL-1Ra with ricin to wild-type mice prevents ricin-mediated inflammatory responses. Wild-type mice received ricin, 30 mg/kg IL-1Ra, or ricin plus 30 mg/kg IL-1Ra and were euthanized 48 h later. A) Representative photomicrographs of lung tissue sections stained with H&E at 10x magnification. B) Total neutrophils counted in the BAL fluid (n = 3; p < 0.05). C) mRNA transcript levels of proinflammatory genes measured from lung homogenates by real-time PCR (*n* = 3; *p* < 0.01). Error bars represent SEM.

B.
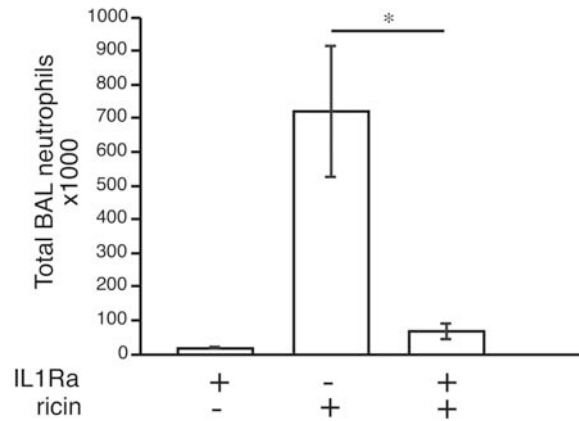
C.

FIGURE 2.6 Coadministration of IL-1Ra with ricin to wild-type mice prevents ricin-mediated inflammatory responses. Wild-type mice received ricin, 30 mg/kg IL-1Ra, or ricin plus 30 mg/kg IL-1Ra and were euthanized 48 h later. A) Representative photomicrographs of lung tissue sections stained with H&E at 10x magnification. B) Total neutrophils counted in the BAL fluid (n = 3; $p < 0.05$). C) mRNA transcript levels of proinflammatory genes measured from lung homogenates by real-time PCR ($n = 3$; $p < 0.01$). Error bars represent SEM.

Ricin toxin activates the NALP3 inflammasome

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Dr. John Wong performed the western blot and ELISA in Figure 3.5 as well as preliminary experiments that determined the doses and time courses used in experiments throughout the manuscript.

ABSTRACT

Ricin exhibits well characterized ribotoxic actions that lead to the inhibition of protein synthesis and the phosphorylation of stress activated protein kinases (SAPKs). Proinflammatory effects of ricin are thought to be caused by upregulation of genes encoding proinflammatory transcripts as a result of the activation of c-Jun N-terminal kinase (JNK) and p38 MAPK. We reported previously that macrophages and interleukin-1β (IL-1β) signaling are required for murine host immune responses to ricin delivered to the lungs. Here we report that ricin-mediated IL-1β release from bone-marrow derived macrophages is dependent on the NALP3 inflammasome, a scaffolding complex that mediates pro-IL-1β cleavage to active IL-1β by caspase-1. Release of IL-1β from macrophages was suppressed by the reactive oxygen species (ROS) scavenger N-acetyl cysteine (NAC) and high extracellular K+, which are two agents known to inhibit NALP3/cryopyrin/CIAS1 inflammasome formation. By employing inhibitors of p38 MAPK and JNK, we demonstrated that ricin-mediated release of IL-1β was enhanced, rather than suppressed, by inhibition of SAPK phosphorylation. In contrast, proteasomal inhibitors bortezomib and MG-132 completely suppressed ricin-induced IL-1β release from macrophages. These data suggest that ricin-mediated translational inhibition itself, by fostering the disappearance of labile protein(s) that normally suppress inflammasome formation, may constitute the mechanism underlying IL-1-dependent inflammatory signaling by ricin.

INTRODUCTION

Ricin is a potent inhibitor of protein synthesis that triggers a robust proinflammatory response when administered to mice and non-human primates (Greenfield, 2002). Mounting evidence points to the macrophage as the specific cell type responsible for ricin's lethal effects in vivo (Brown, 1997; Korcheva, 2007; Zenilman, 1988). In primary bone marrow derived- (BMDM) and alveolar macrophages, exposure to ricin causes the phosphorylation of stress activated protein kinases (SAPKs) p38 MAPK and JNK, and triggers the transcriptional upregulation of genes encoding proinflammatory cytokines and chemokines (Korcheva, 2007; Wong, 2007). In vivo, depletion of macrophages prior to delivery of ricin to the pulmonary system of mice results in diminished inflammatory signs, including reduced neutrophilia and pulmonary edema (Lindauer, 2009).

IL-1β signaling is an essential component of ricin-mediated inflammation. Mice deficient in IL-1α/β or IL-1R display enhanced survival as well as decreased neutrophilia and pulmonary edema compared to wild-type mice after exposure to ricin (Lindauer, 2009). Ricin's ability to induce neutrophilia can be restored in IL-1α/β-deficient animals by the co-administration of exogenous IL-1β with ricin. Furthermore, the IL-1 receptor antagonist anakinra (Kineret®) provides protection against ricin-mediated inflammatory effects in wild-type mice, confirming a central role of IL-1 in ricin toxicity.

IL-1β secretion by macrophages is a tightly regulated process involving at least two distinct signals (Perregaux, 1994; Faustin, 2007). Macrophages first require priming by Toll-like receptor (TLR) ligands (such as LPS) or by cytokines (such as TNFα or IL-1β itself) in order to induce the expression of pro-IL-1β. The pro-form of IL-1β is inactive and requires cleavage by the cysteine protease caspase-1 for maturation and secretion

(Thornberry, 1992). Molecular platforms called inflammasomes stimulate caspase-1 activity and are necessary for IL-1β processing (Martinon, 2002; Tschopp, 2003). One of the best-characterized inflammasome complexes is the NALP3 (or NLRP3/cyropyrin) inflammasome. This multiprotein complex includes the NOD-like receptor (NLR) family member NALP3, the cysteine protease caspase-1, and the adaptor protein apoptosis-associated specklike protein (ASC), which facilitates interaction of caspase-1 with NALP3 (Srinivasula, 2002; Sutterwala, 2006).

NLRs such as NALP3 are intracellular sensors of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Martinon, 2005). Unlike AIM2, an NLR family member that senses dsRNA through direct ligand binding, NALP3 is thought to be a more general sensor of cellular stress through a mechanism that is not fully understood (Stutz, 2009). NALP3 inflammasomes are activated by a diverse array of agents including exogenous danger signals such as bacterial RNA, Candida albicans, and influenza (Kanneganti, 2006; Hise, 2009; Ichinohe, 2009), environmental stressors such as silica and asbestos (Hornung, 2008; Cassel, 2008; Dostert, 2008), and endogenous danger signals such as ATP, uric acid and amyloid β (Mariathasan, 2006; Martinon, 2006; Halle, 2008).

While a singular mechanism cannot yet explain the activation of the NALP3 inflammasome by its wide array of activators, two events that may be common to all known activators are the generation of reactive oxygen species (ROS) and the efflux of potassium (Tschopp, 2010; Dostert, 2008; Petrilli, 2007; Cassel, 2008). ROS generation by NALP3 agonists is believed to indirectly cause activation of the inflammasome via the ROS-sensitive TXNIP protein (Zhou, 2010). Studies show that inhibitors of ROS can reduce the amount of mature IL-1β released by cells after stimulation with ATP, asbestos, and silica (Cruz, 2007; Dostert, 2008). However, work on human monocytes

lacking functional NADPH oxidase did not show a connection between ROS and NALP3 inflammasome activation (van de Veerdonk, 2009), suggesting that ROS generation may not always be required. In regards to the role of potassium, it is thought that the intracellular assembly of NALP3 inflammasomes requires a low potassium environment (Saleh, 2007). Activation of caspase-1 is suppressed by normal levels of intracellular potassium (Walev, 1995), and two laboratories have shown that the efflux of K+ can directly promote the oligomerization of ASC and its association with caspase-1 (Fernandes-Alnemri, 2007; Petrilli, 2007).

Here we report that ricin toxin is an activator of the NALP3 inflammasome. We found that ricin-mediated IL-1β release from primary bone marrow-derived macrophages not only required the expression of NALP3, ASC and caspase-1, but also was inhibited by co-treatment of cells with the ROS scavenger N-acetyl-cysteine (NAC) or high extracellular potassium. In addition, we found that the activation of JNK and p38 MAPK by ricin was not inhibited by either NAC or elevated extracellular potassium, suggesting that ricin mediates IL-1β release from cells in a manner that is independent of its ability to activate kinases.

Proinflammatory consequences of ricin are thought to be initiated by phosphorylation of ZAK, a MAP3K that transduces the signal through downstream kinases p38 MAPK and JNK and leads to proinflammatory gene expression (Jandhyala, 2008). We found that treatment of cells with inhibitors reported to bind to ZAK (Manley, 2009; Karaman, 2008) (Nilotinib and Sorafenib) led to inhibition of p38 and JNK activity but failed to block ricin-mediated release of IL-1β from macrophages. We hypothesized that inhibition of protein synthesis might be the event that triggers activation of the NALP3 inflammasome by contributing to the disappearance of important repressor proteins from the cell. By employing proteasome inhibitors to suppress proteasomal

degradation of cellular proteins, we found that ricin-induced IL-1β release from macrophages required active proteasomes. Together these data describe a novel mechanism governing ricin-mediated inflammatory signaling.

RESULTS AND DISCUSSION

Studies *in vivo* and *in vitro* have demonstrated that macrophages constitute primary targets of ricin (Korcheva, 2007; Wong, 2007; Lindauer, 2009). Wild-type (WT) alveolar and bone-marrow derived macrophages respond similarly to ricin in that they both display phosphorylation of p38 MAPK and JNK in dose-dependent manner inversely proportional to the ricin-mediated decrease in levels of protein translation (Korcheva, 2007). In order to determine if macrophages release IL-1β in response to these same doses, wild-type BMDM were first primed for 4 hr with LPS (50 ng/mL) to induce pro-IL-1β expression, after which cells were rinsed and exposed to varying concentrations of ricin for 4 hr (Figure 3.1). Whole cell lysates (WCL) and media supernatants were subjected to immunoblotting and ELISA for detection of IL-1β. Ricin alone did not induce expression of pro-IL-1β. Priming with LPS led to an increase in levels of 37 kDa pro-IL-1β in cell lysates, and subsequent exposure to ricin led to the appearance of processed 17 kDa IL-1β in the media. Processed IL-1β was detected in media supernatants for all doses tested. We chose to use the lowest dose, 0.01 µg/mL, for all subsequent experiments. The B subunit of ricin is responsible for binding to cell surfaces, but lacks the N-glycosidase activity of the A subunit that is responsible for depurination of 28S rRNA. Exposure of LPS-primed macrophages to purified ricin B subunit failed to elicit appearance of p17 IL-1β in the culture medium (data not shown), suggesting that interactions between ricin holotoxin and surface molecules do not elicit signals that elicit processing of pro-IL-1β.

Secretion of processed IL-1β from cells requires discrete events, including the accumulation of pro-IL-1β through a priming step and the assembly of the inflammasome, a multiprotein scaffolding complex that both activates caspase-1 and brings caspase-1 into close proximity with pro-IL-1β (Perregaux, 1994; Martinon, 2002). The best-studied inflammasome complex is the NALP3 inflammasome, in which NALP3 associates with caspase-1 through the adaptor protein ASC (Srinivasula, 2002; Sutterwala, 2006). The NALP3 inflammasome has been shown to mediate cellular responses to several danger-associated molecular patterns: exogenous danger signals such as silica and asbestos (Hornung, 2008; Dostert, 2008), and endogenous danger signals such as ATP and uric acid (Mariathasan, 2006; Martinon, 2006; Gasse, 2009). To determine if macrophages sense and respond to ricin through the NALP3 inflammasome, we compared the ricin-mediated responses of WT cells to responses of cells deficient in NALP3, ASC, or caspase-1 (Figure 3.2). BMDM from each mouse strain were primed with LPS for 4 hr prior to ricin treatment. After 4 hr exposure to ricin, cells were harvested and processed for immunoblotting (Figure 3.2A) to examine expression levels of inflammasome components and pro-IL-1β. Media supernatants were subjected to detection of IL-1β by immunoblotting and ELISA (Figure 3.2B). Although pro-IL-1β was similarly induced in the LPS-primed cells of each strain, the processing of pro-IL-1β to active IL-1β was significantly decreased in cells deficient in either NALP3, ASC or caspase-1. These results suggest that the NALP3 inflammasome is required for ricin-mediated IL-1β processing and release from BMDM.

If NALP3 mediates the release of IL-1β from macrophages exposed to ricin, then agents shown to inhibit the activity of the NALP3 inflammasome should block the appearance of IL-1β in the medium after exposure to ricin. To address this question we employed two inhibitors of the NALP3 inflammasome: increased extracellular potassium

(K$^+$) and the ROS scavenger, N-acetyl-cysteine. High extracellular K$^+$ blocks IL-1β release caused by a variety of danger signals that activate NALP3 including asbestos, silica, and ATP (Martinon, 2009). *In vitro* studies of inflammasome activation suggest that NALP3 inflammasome assembly requires a low K$^+$ intracellular environment (Petrilli, 2007). In addition, activation of NALP3 is reportedly blocked by ROS inhibitors like NAC through a mechanism that is not well understood (Tschopp, 2008). Wild-type BMDM were primed with LPS for 4 hr, after which cells were co-treated with increased extracellular potassium or NAC and ricin. Four hours later, cells and media supernatants were harvested and processed for immunoblot analysis and ELISA. Media collected from cells co-treated with ricin and either NAC or elevated extracellular K$^+$ contained 50% and 75% less IL-1β respectively, than cells treated with ricin alone. Untreated cells and cell exposed to NAC or elevated K$^+$ expressed equivalent amounts of pro-IL-1β (Figure 3.3). Interestingly, exposure of cells to NAC or elevated K$^+$ did not diminish ricin-mediated phosphorylation of p38 MAPK or the p38 MAPK target, MAPKAP2. Furthermore, NAC by itself led to phosphorylation of p38 MAPK and MAPKAP2 while diminishing the release of IL-1β from ricin-treated cells, suggesting that ricin-mediated inflammasome activation and SAPK phosphorylation are not necessarily linked.

If ricin-mediated phosphorylation of SAPKs is an upstream event leading to NALP3 activation, then blocking the phosphorylation of these kinases should prevent the appearance of IL-1β in the media of ricin-treated cells. To address this question we employed SB203580, an inhibitor of p38 MAPK, and SP600125, an inhibitor of JNK. We also employed two inhibitors (Nilotinib and Sorafenib) that have been reported to have very high affinity for the ATP-binding site of ZAK (Manley, 2009; Karaman, 2008), the upstream MAP3K that is phosphorylated by ricin and other ribotoxic stressors

(Jandhyala, 2008). LPS-primed cells were treated with SB203580 (SB), SP600125 (SP), Sorafenib, Nilotinib or ricin, alone and in combination for 4 hr, at which time cell lysates and media supernatants were collected (Figure 3.4). Phosphorylated SAPKs and pro-IL-1β levels were examined in cell lysates by immunoblotting. Processed IL-1β from media supernatants was detected using both immunoblotting and ELISA. Inhibitors exhibited varying degrees of effectiveness in suppressing SAPKs. JNK activation was decreased marginally by SP and significantly by Sorafenib; p38 MAPK activation was diminished significantly by Nilotinib and Sorafenib; and the activation of MAPKAP2, a target of p38 MAPK, was significantly decreased by SB, Nilotinib, and Sorafenib. Surprisingly, SB stimulated IL-1β release from macrophages on its own, and cells co-treated with kinase inhibitors and ricin secreted similar or greater amounts of IL-1β as cells treated only with ricin, despite diminished levels of phosphorylated p38 MAPK and JNK in these cells. These data suggest that SAPK activation by ricin is not a requirement for the release of IL-1β from ricin-treated cells and that suppression of ricin-mediated ZAK activation did not reduce ricin-induced activation of the NALP3 inflammasome.

Importantly, the dose of ricin used in this study (0.01 µg/mL) elicits a 50% inhibition of protein synthesis by 3 hr in addition to its effects on SAPKs and the release of IL-1β (Korcheva, 2007). We hypothesized that inhibition of protein synthesis per se could lead to activation of NALP3 by contributing to the disappearance of labile proteins which may regulate the availability of NALP3 to participate in formation of the inflammasome. In such case, proteasomal activity may be required for ricin-induced activation of NALP3 and release of IL-1β from macrophages. To test this hypothesis, we employed the specific and potent proteasome inhibitors bortezomib and MG-132. Although cells exposed to bortezomib or MG-132 expressed similar levels of pro-IL-1β to cells exposed only to LPS or ricin, cells exposed to bortezomib or MG-132 exhibited

complete suppression of ricin-mediated secretion of IL-1β (Figure 3.5). These data are consistent with the notion that translational inhibition itself may be a mechanism by which ricin triggers IL-1β dependent inflammatory signaling.

Previously we demonstrated that, in addition to inhibiting protein synthesis, ricin triggers the phosphorylation of JNK and p38 MAPK. Activation of these kinases is required for ricin-mediated expression of mRNAs encoding inflammatory cytokines and chemokines (Iordanov, 1997; Korcheva, 2005). Furthermore, we showed that ricin administration to the lungs results in a neutrophilic inflammation *in vivo* that is dependent on IL-1β and on the presence of macrophages (Lindauer, 2009). Experiments in BMDM and alveolar macrophages demonstrated that macrophages constitute primary targets for ricin, and are a likely source of ricin-induced production of IL-1β (Korcheva, 2007; Wong, 2007; Lindauer, 2009). However, the mechanism of ricin-mediated processing and release of IL-1β was unclear.

Here we report that ricin triggers IL-1β release from macrophages in a NALP3-, ASC- and caspase-1-dependent manner, and that ricin-mediated IL-1β release, but not ricin-mediated SAPK phosphorylation, may be blocked by treatment of cells with high extracellular potassium or the ROS scavenger, NAC. In addition, we found that IL-1β release is not dependent upon p38 or JNK activation, since inhibitors that target the p38 MAPK and JNK pathways failed to block IL-1β release and indeed, even enhanced IL-1β secretion from primed cells. Furthermore, treatment of macrophages with proteasome inhibitors in combination with ricin demonstrated that ricin-mediated IL-1β release was dependent upon the proteasomal degradation of cellular proteins. Taken together, these data suggest a novel mechanism for ricin-mediated inflammatory signaling, and helps to

explain why inhibitors of IL-1 signaling are capable of diminishing ricin's inflammatory effects.

The major finding of this study is that macrophages require components of the NALP3 inflammasome (NALP3, ASC, and caspase-1) in order to process and release active IL-1β after ricin exposure (Figure 3.2). NALP3 is a Nod-like receptor protein containing a pyrin-domain (PYD) through which it interacts with the PYD-containing adaptor protein, ASC, upon oligomerization (Mariathasan, 2007). ASC binds caspase-1 through its caspase-1 recruitment domain (CARD) and stimulates its activity, so that the IL-1β precursor may be cleaved into its active form, p17 IL-1β, and secreted by the cell in response to danger signals. Although primed WT BMDM expressed similar levels of pro-IL-1β as the NALP3-, ASC-, and caspase-1-deficient BMDM (Figure 3.2A), the mutant BMDM all failed to release IL-1β into the medium after ricin treatment. These results suggest that the NALP3 inflammasome is required for ricin-mediated processing of IL-1β.

As a sensor of a myriad of microbial and non-microbial danger signals and the culprit of pyrin-associated autoinflammatory diseases caused by mutations in the *nlrp3* gene (Hoffman, 2001; Agostini, 2004), the NALP3 inflammasome is well-studied but still poorly understood. Unlike TLRs, which bind directly to their ligands (Takeda, 2003), studies suggest that NALP3 may instead sense an intermediate molecule produced by its activators. While the list of activators grows longer each year, the mechanism through which dissimilar signals trigger NALP3 activity remains unclear.

Although insufficient to activate the NALP3 inflammasome on their own, potassium efflux and ROS production are common events that occur during NALP3 activation by all known activators (Tschopp, 2008). Evidence suggests that ASC

oligomerization and caspase-1 activation require a low potassium environment (Fernandes-Alnemri, 2007; Petrilli, 2007). Indeed, high extracellular potassium applied to macrophages to prevent ricin-mediated potassium efflux resulted in significantly diminished IL-1β secretion in ricin-treated cells (Figure 3), despite expressing normal levels of pro-IL-1β after priming. Furthermore, the ROS-scavenger NAC was able to reduce IL-1β release by 50% in ricin-treated BMDM (Figure 3.3), consistent with the notion that intracellular ROS may be permissive for activation of the NALP3 inflammasome.

In order to determine whether SAPK phosphorylation was required for ricin-mediated inflammasome activation, we employed inhibitors known to have high affinity for ZAK (Nilotinib and Sorafenib) as well as inhibitors specific to the p38 MAPK (SB203580) and JNK (SP600125). Surprisingly, we found that inhibiting ricin-mediated SAPK activation enhanced, rather than suppressed, IL-1β release from primed macrophages (Figure 3.4). These data suggest that SAPK phosphorylation by ricin may suppress the NALP3 inflammasome rather than contribute to its activation. The partial suppression of SAPKs by kinase inhibitors failed to diminish NALP3 activation, suggesting that NALP3 activation by ricin occurs by a mechanism that does not involve ZAK. However, the possibility that ZAK contributes to ricin-mediated activation of the NALP3 inflammasome cannot be excluded at this time.

Another possibility is that ricin induces activity of the NALP3 inflammasome through the inhibition of cellular protein translation per se by depleting cells of proteins that suppress formation of inflammasome complexes under normal conditions. The existence of a family of small proteins that have emerged as important inflammasome regulators lends merit to this idea. These proteins contain either pyrin-only domains (POPs) or CARD-only domains (COPs) and act as endogenous dominant-negative

modulators of inflammasome activity (Stehlik, 2007). One might imagine a scenario in which their disappearance from the cell could trigger activation of the inflammasome as a result of ricin-induced inhibition of protein synthesis combined with normal protein turnover.

To explore this possibility, we treated WT BMDM with two different proteasome inhibitors, bortezomib and MG-132, and examined ricin-mediated IL-1β release under conditions in which proteasomal degradation of cellular proteins was blocked. Our experiments demonstrated that both bortezomib and MG-132 signficantly reduced IL-1β secretion from WT cells exposed to ricin, supporting the hypothesis that ricin-mediated translational inhibition itself may lead to activation of the NALP3 inflammasome by fostering the disappearance of labile protein(s). It would be interesting to examine whether other inhibitors of protein translation similarly activate inflammatory signaling through NALP3. Since host immune responses triggered by toxins are nonproductive and often deleterious, investigation into mechanisms underlying inflammatory signaling by toxins is warranted so that we might better understand and manage toxin-induced pathologies.

MATERIALS AND METHODS

Reagents and antibodies. Ricin was purchased from Vector Laboratories (Burlingame, CA). LPS (L-2630), N-acetyl cysteine (NAC), and bovine-pancreas insulin (I6634) were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Pittsburgh, PA). Nilotinib, Sorafenib and Bortezomib were obtained from LC Laboratories (Woburn, MA). MG-132, the p38-specific inhibitor SB203580, and the JNK inhibitor SP600125 were purchased from EMD Biosciences (Gibbstown, NJ). The mouse IL-1β enzyme-linked immunosorbent assay (ELISA)

Ready-Set-Go was purchased from eBioscience (San Diego, CA). Anti-IL-1β was purchased from Abcam (Cambridge, MA), anti-ASC was purchased from Enzo Life Sciences (Lausen, Switzerland), and anti-p38, anti-cryopyrin (H-66)/NALP3, and anti-caspase-1 (M-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-SAPK/JNK (9251S), anti-phospho-p38 MAPK (9211S), anti-phospho-MAPKAP-2 (3041S) were purchased from Cell Signaling Technologies (Danvers, MA).

Animals and animal procedures. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University, Portland, Oregon. C57BL/6J and Caspase-1 deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). ASC-deficient and NALP3-deficient mice were kindly provided by V. Dixit (Genentech, San Francisco, CA). Male mice, 8–10 weeks of age, were used throughout the experiments. Before experimental procedures, mice were anesthetized intraperitoneally with 80 mg/kg of ketamine and 10 mg/kg of xylazine.

Isolation and treatment of bone marrow-derived macrophages. Bone marrow-derived macrophages were prepared from C57BL/6J, ASC$^{-/-}$, Caspase-1$^{-/-}$, and NALP3$^{-/-}$ mice. Marrow was flushed from femurs and tibias with PBS and cultured in a-Minimum Essential Medium (α-MEM, Cellgro, Herndon, VA), supplied with 10% Fetal Bovine Serum (FBS, Cellgro, Herndon, VA), 50 µg/mL gentamicin, and 100 ng/mL recombinant mouse Colony Stimulating Factor 1 (CSF-1, R&D Systems, Minneapolis, MN) for 72 hr on non-tissue culture treated 10-cm petri dishes. Cells were passaged and cultured for an additional 72hr. Cells from one confluent 10-cm dish were plated into one 6-well tissue culture plate (Sarstedt, Newton, NC) for an additional 24 hr before experiments. Cells were serum-deprived in a-MEM for 30 min followed by treatment with 50 ng/mL

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LPS for 4 hr. Cells were rinsed with fresh media and exposed to 0.01 µg/mL ricin in serum-free media for an additional 4 hr prior to harvesting. In experiments involving co-treatments with ricin plus N-acetyl-cysteine (30 mM), high potassium (130 mM), Nilotinib (1 µM), Sorafenib (1 µM), SB203580 (10 µM), SP600125 (20 µM), bortezomib (0.5 μM) or MG-132 (10 μM), cells were exposed to indicated concentrations of inhibitors. In experiments involving high potassium, potassium was substituted for sodium in the medium in order to maintain equivalent ion concentrations.

Immunoblotting and ELISA. BMDM cells were lysed in 2X ESB lysis buffer in preparation for immunoblotting. Equal volumes of the cell lysates were separated on a 10% denaturing polyacrylamide gel in the presence of sodium dodecyl sulfate and were transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Proteins from BMDM media supernatants were precipitated using TCA and run on 13% gels. Briefly, samples were incubated at 4°C overnight with 200 µg insulin carrier and 400 µl ice-cold 100% TCA prior to centrifugation at 12,000 rpm for 5 min. Pellets were air dried and resuspended in 2X ESB lysis buffer followed by separation on a 13% denaturing polyacrylamide gel. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies; signals were detected using enhanced chemiluminescence. Media supernatants were analyzed in triplicate using IL-1β ELISA (eBioscience) according to the manufacturer's protocol.

Statistical analysis. Individual groups were compared using unpaired *t* test analysis and were interpreted in a two-tailed manner.
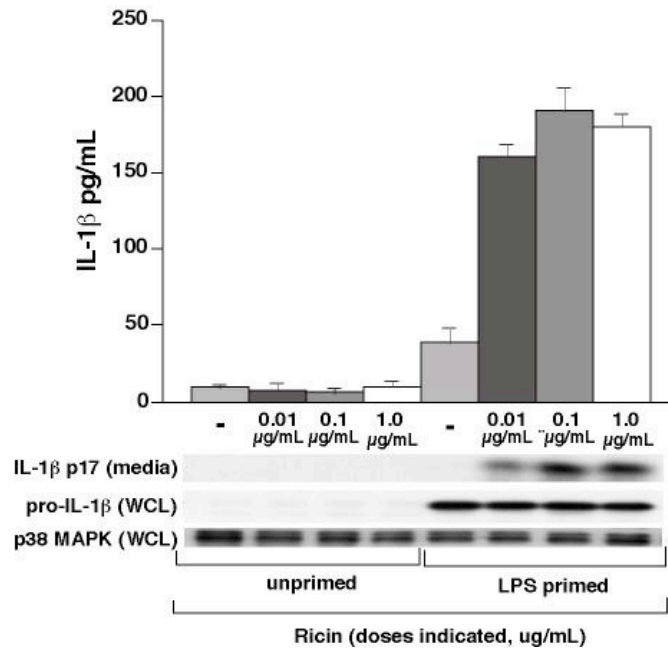
Figure 3.1. Ricin stimulates IL-1β release from WT BMDM. Secreted IL-1β was measured by ELISA analysis of media supernatants collected from LPS-primed WT BMDM after 4 hr ricin exposure. Bars represent the mean ± s.d. of triplicate wells. Results are representative of three individual experiments. Media supernatants were also precipitated and subjected to immunoblot analysis for detection of IL-1β p17. WCLs were subjected to immunoblot analysis for detection of pro-IL-1β and p38 MAPK as a loading control.
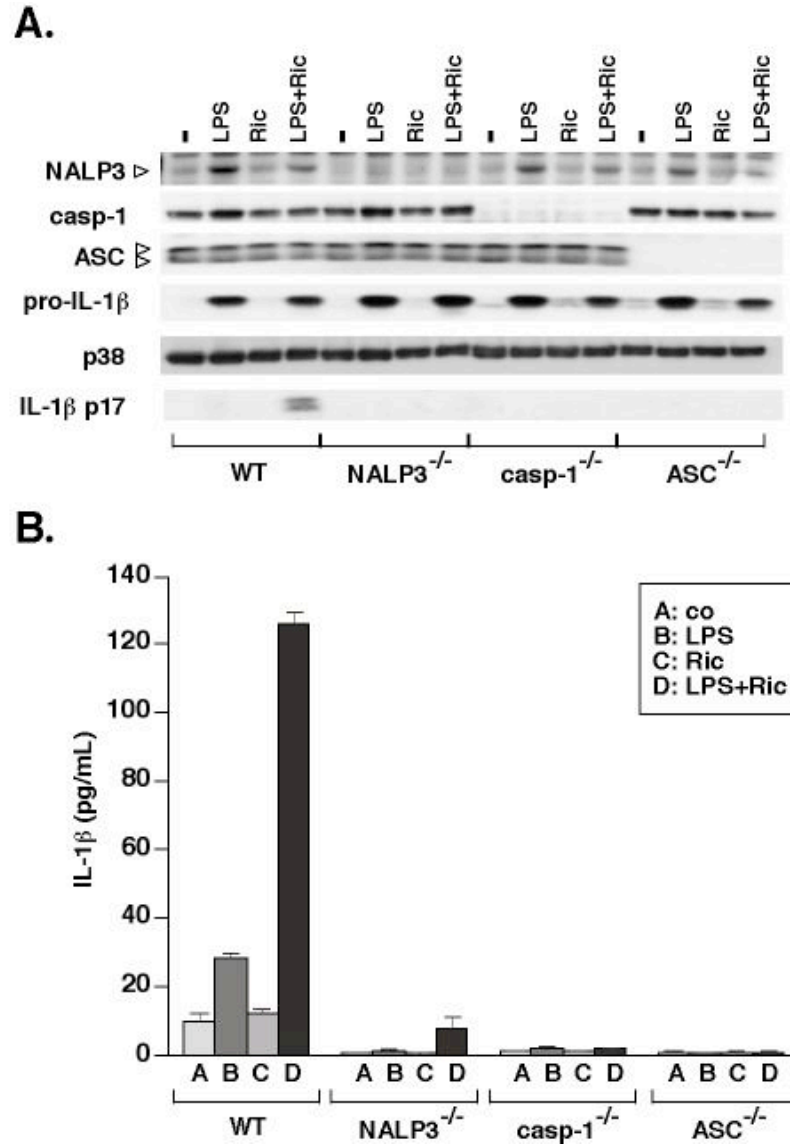
Figure 3.2. Ricin-mediated release of IL-1β requires NALP3, ASC, and caspase-1. Primed and unprimed WT, NALP3$^{-/-}$, casp-1$^{-/-}$, and ASC$^{-/-}$ BMDM were treated with ± 0.01 μg/mL ricin or vehicle alone (co) for 4 hr. WCLs were subjected to immunoblotting for NALP3 inflammasome components, pro-IL-1β and p38 MAPK. Media supernatants were either precipitated for immunoblot analysis (A) or subjected to ELISA for measurement of secreted IL-1β (B). Bars represent the mean ± s.d. of triplicate wells.

A.

B.
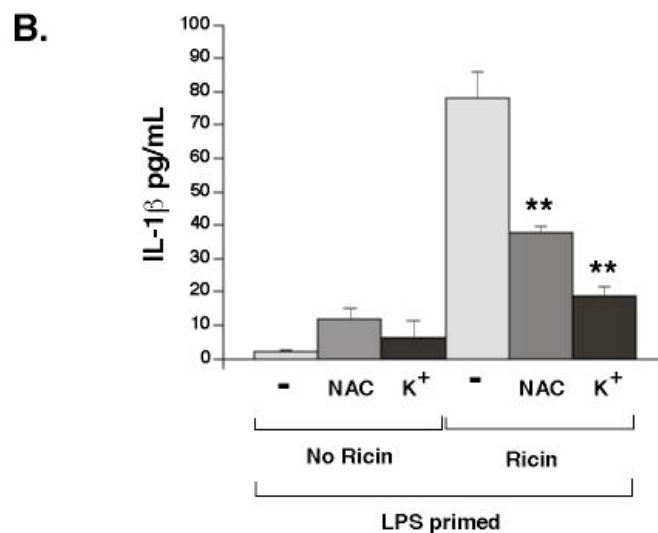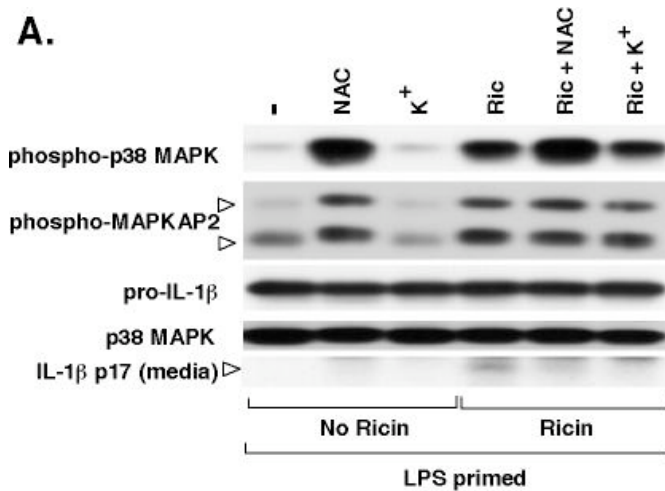
Figure 3.3. Elevated extracellular K^+ and NAC prevent ricin-mediated secretion of IL-1β from WT BMDM. Primed WT cells were treated ± 0.01 μg/mL ricin in combination with either NAC or K^+ for 4 hr. A) WCLs were subjected to immunoblotting for phospho-p38 MAPK, phospho-MAPKAP2, pro-IL-1β and p38 MAPK as a loading control. Media supernatants were either precipitated for immunoblotting (A) or subjected to ELISA for determination of secreted IL-1β (B). Bars represent the mean ± s.d. of triplicate wells (**, p < 0.01).
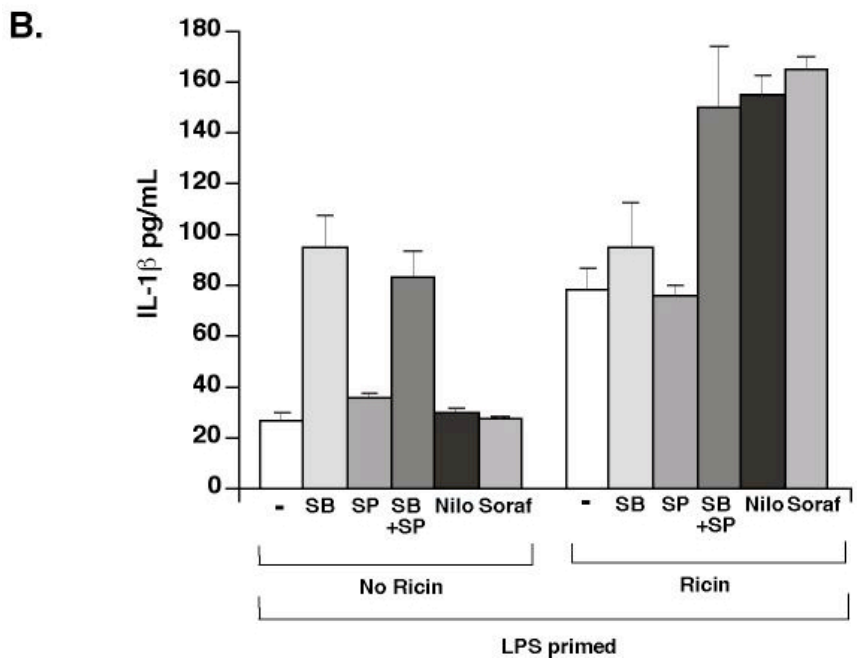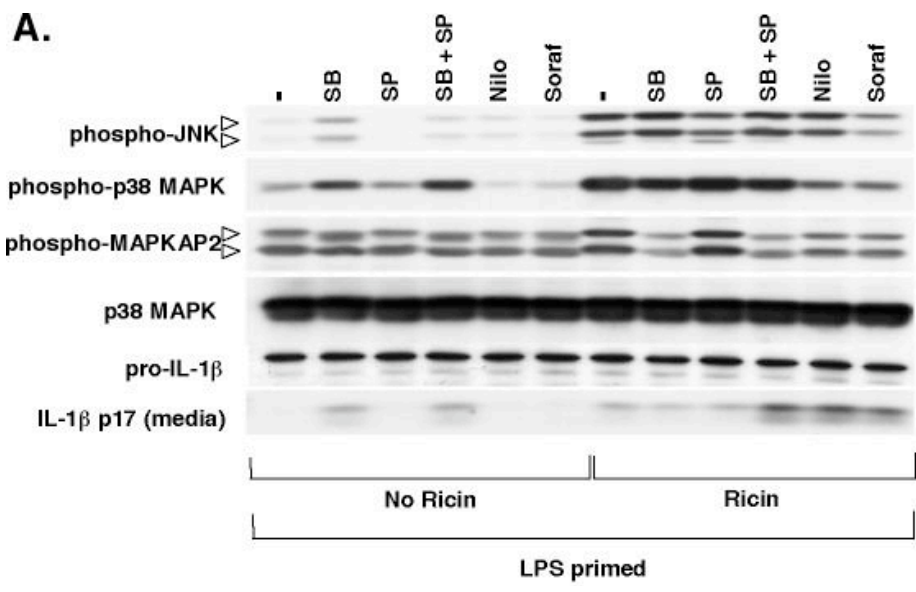
Figure 3.4. Ricin-mediated phosphorylation of p38 MAPK and JNK is not required for ricin-mediated IL-1β secretion. Primed WT cells were treated ± 0.01 μg/mL ricin in combination with either SB203580, SP600125, SB+SP, Nilotinib or Sorafenib for 4 hr. A) WCLs were subjected to immunoblotting for phospho-JNK, phospho-p38 MAPK, phospho-MAPKAP2, pro-IL-1β and p38 MAPK as a loading control. Media supernatants were either precipitated and subjected to immunoblotting (A) or analyzed for IL-1β by ELISA (B). Bars represent the mean ± s.d. of triplicate wells.
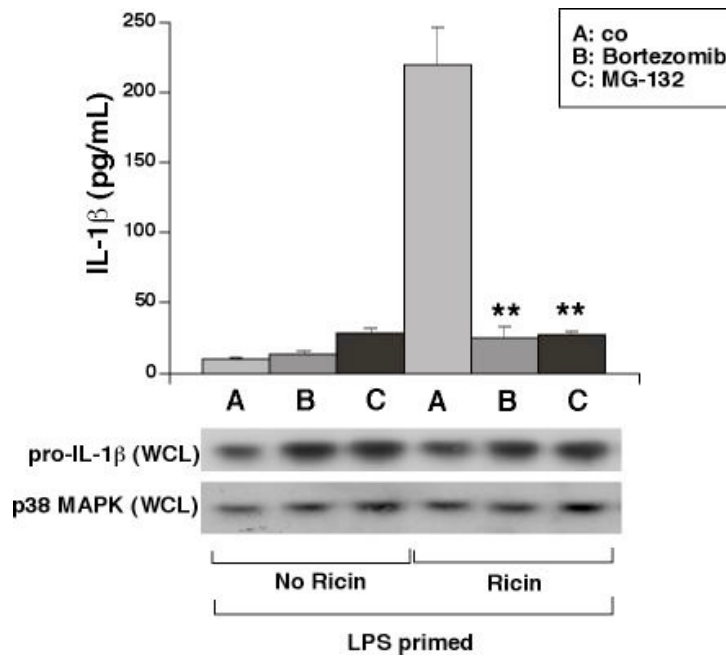
Dr. John Wong performed this experiment.

Figure 3.5. Proteasome inhibitors block ricin-mediated release of IL-1β from WT BMDM. Primed cells were treated ± 0.01 µg/mL ricin and either bortezomib, MG-132, or vehicle alone (co) for 4 hr. Secreted IL-1β was measured by ELISA from media supernatants. Bars represent the mean ± s.d. of triplicate wells (** $p < 0.01$). WCLs were subjected to immunoblotting for detection of pro-IL-1β and p38 MAPK as a loading control.

The inhibition of protein synthesis is sufficient to activate the NALP3 inflammasome

Dr. Martina Ralle processed samples for ICP-MS measurements of intracellular potassium.

ABSTRACT

Potassium efflux is triggered by various stimuli that activate the NALP3 inflammasome, and is thought to be required for NALP3-dependent IL-1β processing. However, the mechanism by which intracellular potassium is linked to NALP3 activity remains unclear. The best-described NALP3 stimuli, the bacterial toxin nigericin and extracellular ATP, are known to elicit rapid inhibition of protein translation with similar kinetics to potassium leakage from cells. Here we show that inhibition of translation is sufficient to stimulate NALP3-dependent IL-1β release from bone-marrow derived macrophages, without apparent intracellular potassium loss. Activation of the NALP3 inflammasome by inhibitors of translation required active proteasomes. The NALP3-binding protein, TXNIP, was rapidly degraded in cells exposed to inhibitors of translation and was stabilized by co-treatment with the proteasome inhibitor MG-132. Cellular levels of TXNIP inversely correlated with IL-1β release. Our findings suggest that potassium efflux is not a requirement for NALP3 inflammasome activation. In addition, these data support the inhibition of protein synthesis as a novel mechanism of stimulating IL-1β release via NALP3 inflammasomes, possibly through the destabilization of endogenous NALP3 inhibitors like TXNIP.

INTRODUCTION

Aberrant interleukin-1β (IL-1β) signaling has been implicated in a variety of inflammatory diseases ranging from arthritis to diabetes, making the manipulation of the IL-1 pathway an attractive therapeutic option for a growing number of pathologies stemming from innate immune activation (Dinarello, 2010; Gabay, 2010). Critical to the efficacy of the innate immune system is the proper detection of invading microbes and toxic substances by macrophages expressing pattern recognition receptors (PRRs) in the cytosol and at the cell surface (Martinon, 2005;Nathan, 2002; Licastro, 2005; Imaeda, 2009; Iyer, 2009). The Nod-like receptor (NLR) family member, NALP3, is a cytosolic PRR that is activated by a large array of pathogen- and danger-associated molecular patterns to stimulate IL-1β processing and release (Palsson-McDermott, 2007; Perregaux, 1994; Faustin, 2007). Through binding of TLR-4 ligands like LPS, inactive pro-IL-1β and NALP3 are expressed and accumulate in cells. Upon stimulation by a second danger signal, NALP3 molecules form a scaffolding complexes called inflammasomes that recruit pro-caspase-1 via binding of the adaptor protein, ASC, which leads to activation of caspase-1 and the conversion of pro-IL-1β to active IL-1β (Thornberry, 1992; Cerretti, Kozlosky, 1992;Srinivasula, 2002). ATP, bacterial pore-forming toxins, viruses, asbestos and uric acid crystals all stimulate IL-1β processing via NALP3 inflammasomes, but the mechanism(s) by which disparate activators are detected by NALP3 to trigger inflammasome activation is incompletely understood (Lamkanfi, 2009).

Intracellular potassium concentration has emerged as an important feature of NALP3 inflammasome regulation based on the robust NALP3-dependent release of IL-

1β by cells exposed to the potassium ionophore nigericin or to the nucleotide ATP (Perregaux, 1994). Indeed, preventing loss of intracellular potassium by bathing cells in high-potassium medium is sufficient to block IL-1β release by cells exposed to these and other agents (Perregaux, 1994; Petrilli, 2007), but how intracellular potassium concentration is linked with the detection of danger signals by NALP3 remains obscure.

Interestingly, potassium efflux from cells also leads to inhibition of cellular protein synthesis (Cahn, 1978; Panet, 1979). It has long been known that potassium ionophores like nigericin trigger rapid translational inhibition concomitant with efflux of cellular potassium, and that application of high extracellular potassium rescues protein synthesis activity in nigericin-treated cells (Alonso, 1981). Recently, we reported that ricin toxin, a well-defined inhibitor of protein synthesis, leads to IL-1β- and macrophage-dependent inflammation when delivered to the lungs of mice (Lindauer, 2009). By examining ricin-mediated IL-1β release from primary bone marrow-derived macrophages (BMDM), we found that ricin-mediated IL-1β release from BMDM requires the known components of the NALP3 inflammasome: NALP3, caspase-1, and ASC (Lindauer, 2010). This discovery led us to hypothesize that translational inhibition itself may activate the NALP3-inflammasome by contributing to the disappearance of a labile protein regulator from the cell.

RESULTS AND DISCUSSION

In this study we examined the ability of known inhibitors of translation to stimulate IL-1β release from bone marrow derived macrophages. We tested a panel of agents including cycloheximide, emetine, puromycin, pactamycin and anisomycin and found that at doses sufficient to suppress 90% protein synthesis, every inhibitor tested led to IL-1β release from LPS-primed BMDM (Fig. 4.1). To gain insight into the mechanism of

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IL-1β release triggered by translational inhibitors, we exposed bone marrow derived macrophages from NALP3-, ASC- and caspase-1-deficient mice to translational inhibitors and compared their responses to responses of WT cells. All three mutant cell lines secreted markedly reduced IL-1β compared to WT in response to protein synthesis inhibitors (Fig. 4.2), suggesting that release of IL-1β triggered by the inhibition of protein synthesis requires activation of the NALP3 inflammasome.

NALP3 inflammasomes respond to a number of disparate stimuli including ATP, bacterial toxins, urate crystals, alum and silica (Mariathasan, 2006; Gurcel, 2006; Martinon, 2006; Hornung, 2008; Cassel, 2008; Dostert, 2008). However, the precise mechanism(s) governing activation of NALP3 inflammasomes are poorly understood. Without evidence for direct ligand binding, it is thought that NALP3 detects signals of cell stress, including the presence of reactive oxygen species and reduced intracellular potassium (Ogura, 2006; Martinon, 2010). Previous studies have described a requirement for potassium efflux by every agent reported to activate NALP3 (Petrilli, 2007). Indeed, application of 130 mM extracellular potassium (exchanged for 130 mM sodium in the medium) is sufficient to completely suppress the release of processed IL-1β by cells treated with activators of NALP3, and these results have led to the conclusion that NALP3 stimulation requires a drop in intracellular potassium (Petrilli, 2007). To determine if translational inhibitors trigger potassium efflux in BMDM, we exposed LPS-primed BMDM to our panel of inhibitors as well as nigericin and ATP and measured intracellular potassium over time by ICP-MS. Nigericin and ATP produced a 50% drop in intracellular potassium by 15 mins and 45 mins respectively (Fig. 4.3B). In contrast, cellular levels of intracellular potassium were maintained in untreated macrophages as well as in macrophages exposed to ricin, cycloheximide, emetine, puromycin, pactamycin and anisomycin over 60 min (Fig. 4.3B). These data demonstrate that

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potassium leakage from cells is not required for IL-1β processing and release through the NALP3 inflammasome.

The activity of the bacterial toxin nigericin was first characterized in the 1980s when it was shown to elicit translational inhibition in cells at the same doses and kinetics as potassium efflux (Alonso, 1981). It has been well established that the rate of protein synthesis is directly dependent on the concentration of intracellular potassium (Cahn, 1978; Panet, 1979). The data presented here suggest that inhibition of protein synthesis may be a common mechanism of NALP3 inflammasome activation.

Transient translational inhibition occurs in a variety of circumstances in nature, triggered by exposure to toxins, to pathogens trying to co-opt host cell machinery, and to sterile inflammatory signals released from damaged tissues. Several forms of stress induce pathways leading to the phosphorylation of the translation initiation factor eIF2α, which results in a global inhibition of protein synthesis (Sharp, 1993). Viral dsRNA, through activation of protein kinase R (PKR); hypoxia, through the PRK-like endoplasmic reticulum kinase (PERK); and glucose deprivation, through activation of both PKR and PERK, all induce global inhibition of protein synthesis that is protective to cells over short periods of time (Sharp, 1993; Preedy, 1985; Muaddi, 2010). In light of our data showing that translational inhibition by itself stimulates NALP3-dependent IL-1β release, we hypothesized that translational inhibition mediates inflammatory signaling by contributing to the disappearance of labile protein regulator(s) critical for suppressing NALP3 activation under steady-state conditions.

If the disappearance of a labile protein were responsible for the activation of NALP3 inflammasomes by inhibitors of protein synthesis, then blocking the degradation of that protein would be expected to prevent the activation of NALP3 by these agents.

Active proteasomes are required for ATP- and ricin-mediated NALP3 inflammasome activation (Qu, 2007; Lindauer, 2010) as well as for activation of NALP1 inflammasomes by anthrax lethal toxin (Squires, 2007). In order to determine if proteasomes control NALP3 inflammasome activation by translational inhibitors, we tested proteasome inhibitors MG-132 and Bortezomib and found that, in every case, IL-1β release mediated by inhibitors of protein synthesis was blocked in BMDM co-treated with either MG-132 or Bortezomib (Fig. 4.5A). These data are consistent with the involvement of a labile repressor of NALP3 that is suppressed in cells exposed to inhibitors of protein synthesis, and whose cellular levels are controlled through the ubiquitin-proteasome degradation pathway.

One labile protein that is known to bind NALP3 and is regulated by ubiquitin-dependent proteasomal degradation is TXNIP, or thioredoxin interacting protein (Zhou, 2009; Zhang, 2010). TXNIP is a ROS-sensitive protein known to play important roles in glucose metabolism, tumor growth, and, it was recently reported, NALP3 activation by MSU and ATP (Zhou, 2009). To test whether proteasomal inhibition, which prevents NALP3 activation by translational inhibitors, also leads to stabilization of TXNIP in bone marrow derived macrophages, we measured TXNIP levels in cells treated with translational inhibitors in the presence of MG-132. Interestingly, MG-132 reduced the disappearance of TXNIP in cells treated with translational inhibitors for 4h (Fig. 4.5A), and led to greater expression of intracellular pro-IL-1β that correlated with markedly reduced cleaved IL-1β in the media (compare Figs. 4.5A and 4.4). To determine the half-life of TXNIP, primed bone marrow derived macrophages were treated with cycloheximide and TXNIP expression was examined over time. TXNIP was rapidly turned over in macrophages with a half-life of about 30 min (Fig. 4.5B). Perhaps due to a stress response induced by medium exchange at t=0, TXNIP levels declined in the first

30 min in control cells before recovering to baseline levels by 120 mins. In contrast, cycloheximide-treated cells displayed complete loss of measurable TXNIP by 60 minutes. These data imply that TXNIP is particularly sensitive to short-term translational inhibition, and supports the idea that perturbations in this known NALP3 binding protein may result in the inflammasome activation observed by these agents.

Zhou et al demonstrated that the binding of TXNIP to NALP3 is required for activation of the inflammasome in THP-1 (Human acute monocytic leukemia) cells, and that TXNIP-deficient bone marrow derived macrophages fail to respond NALP3 stimuli (Zhou, 2009). Given the kinetics of TXNIP degradation in bone marrow derived macrophages, our results do not preclude this possibility as a mechanism for NALP3 activation by inhibitors of translation. TXNIP could bind NALP3 within the first hour of exposure to inhibitors of protein synthesis, perhaps to localize NALP3 or aid in complex formation. Further investigation is required to understand the role of TXNIP in inflammasome activation by inhibitors of translation. In addition, it is likely that multiple pathways are targeted by inhibitors of protein synthesis, and other short-lived proteins known to be involved in NALP3 regulation, like the endogenous inhibitor Pyrin (Stehlik, 2007), should be examined as potential mediators.

The mechanism(s) by which NALP3 inflammasomes sense disparate stimuli have remained unclear, although evidence suggests that a common event such as intracellular potassium loss may account for activation of NALP3 by dissimilar activators (Tschopp, 2010; Dostert, 2008; Petrilli, 2007; Cassel, 2008). This study demonstrates that potassium efflux is *not r*equired for activation of NALP3, and suggests that inhibition of protein synthesis may be a trigger by which seemingly dissimilar activators stimulate NALP3 inflammasomes. Furthermore, these data suggest that perturbations in TXNIP may be a important for NALP3 activation by inhibitors of protein translation.

MATERIALS AND METHODS

Reagents and antibodies. Ricin was purchased from Vector Laboratories (Burlingame, CA). Cycloheximide, emetine, puromycin, anisomycin, nigericin, and ATP, LPS (L-2630), and bovine-pancreas insulin (I6634) were all purchased from Sigma-Aldrich (St. Louis, MO). Pactamycin was a generous gift from The Upjohn Co (now Pfizer, New London, CT). Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Pittsburgh, PA). Bortezomib were obtained from LC Laboratories (Woburn, MA). MG-132 was purchased from EMD Biosciences (Gibbstown, NJ). The mouse IL-1β enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go was purchased from eBioscience (San Diego, CA). Anti-IL-1β was purchased from Abcam (Cambridge, MA), anti-ASC (AL177) was purchased from Enzo Life Sciences (Lausen, Switzerland); anti-p38, anti-cryopyrin (H-66)/NALP3, and anti-caspase-1 (M-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TXNIP was purchased from MBL International (Woburn, MA).

Isolation and treatment of bone marrow-derived macrophages. Bone marrow-derived macrophages were prepared from C57BL/6J, ASC$^{-/-}$, Caspase-1$^{-/-}$, and NALP3$^{-/-}$ mice. Marrow was flushed from femurs and tibias with PBS and cultured in α-Minimum Essential Medium (Cellgro, Herndon, VA), supplied with 10% Fetal Bovine Serum (Cellgro, Herndon, VA), 50 μg/mL gentamicin, and 100 ng/mL recombinant mouse Colony Stimulating Factor 1 (CSF-1, R&D Systems, Minneapolis, MN) for 72h on non-tissue culture treated 10-cm petri dishes. Cells were passaged and cultured for an additional 72h before being plated into 6-well tissue culture plate (Sarstedt, Newton, NC) for an additional 24h and grown to confluency before experiments. BMDM were serum deprived in α-MEM for 30 min followed by treatment with 50 ng/mL LPS for 4h. Cells were rinsed and exposed to 0.01 μg/mL ricin, 25 μg/mL cycloheximide, 10 μg/mL

emetine, 75 µg/mL puromycin, 0.2 µg/mL pactamycin, or 10 µg/mL anisomycin in serum-free media for an additional 4h prior to harvesting. In experiments involving treatments with inhibitors of protein synthesis in the presence of bortezomib (0.5 µM) or MG-132 (10 µM), cells were exposed to indicated concentrations of inhibitors 15 min prior to protein synthesis inhibitors.

Immunoblotting and ELISA.   BMDM cells were lysed in 2X ESB lysis buffer in preparation for immunoblotting. Equal volumes of the cell lysates were separated on a 10% denaturing polyacrylamide gel and were transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Proteins from BMDM media supernatants were precipitated using TCA and run on 13% gels. Briefly, 1 mL media samples were incubated at 4°C overnight with 100 µg insulin carrier and 400 µl ice-cold 100% TCA prior to centrifugation at 12,000 rpm for 5 min. Pellets were air dried and resuspended in 2X ESB lysis buffer followed by separation on a 13% denaturing polyacrylamide gel. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies; signals were detected using enhanced chemiluminescence. Media supernatants were also analyzed in triplicate using IL-1β ELISA (eBioscience) according to the manufacturer's protocol.

ICP-MS.   Primed BMDM were exposed to inhibitors of protein synthesis for indicated times up to 1h and cells were digested overnight in 1 mL 10% HNO_3.   Inductively coupled plasma mass spectrometry (ICP MS) analysis was performed by Martina Ralle using an Agilent 7700x system equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon flow rate of 15 L/min, carrier gas flow rate of 1.04 L/min and helium gas flow rate of 4.3 ml/min (only in He mode). Data were quantified using a 7-point (0, 1, 10, 100, 1000, 2000, 5000 ng/g) calibration curve with external standards for K^{+}. For the analysis, 30 µl of each sample was diluted

in 1.5 ml 1% HNO3 (Metals grade, Fisher) and measured in He mode to remove interferences. For each sample data were acquired in triplicates and averaged. To prevent protein deposition and minimize nebulizer clogging as well as cross contamination the sample probe was rinsed with doubly deionized water for 10 seconds followed by 3 x with 1% HNO_3 for 30 seconds. Internal standards introduced with the samples were used to correct for plasma instabilities, NIST standard reference material (SRM) was used to ensure elemental recovery of >90 % (SRM # 1598a, animal serum, and SRM # 1577c, bovine liver) (M. Ralle, personal communication).

Statistical analysis. Individual groups were compared using unpaired *t* test analysis and were interpreted in a two-tailed manner.
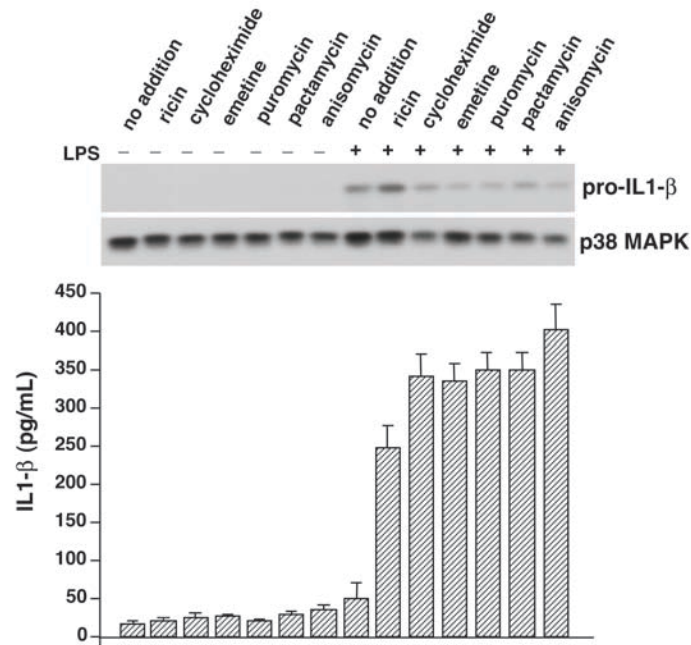
Figure 4.1. Inhibition of protein synthesis triggers IL-1β secretion from mouse bone marrow derived macrophages. WT BMDM were stimulated ± LPS for 4h followed by exposure to 10 ng/mL ricin, 25 μg/mL cycloheximide, 10 μg/mL emetine, 75 μg/mL puromycin, 0.2 μg/mL pactamycin, or 10 μg/mL anisomycin for 4h prior to harvesting. Cell extracts were analysed by immunoblotting for pro-IL-1β and p38 (loading control). Media supernatants were analyzed by ELISA for released IL-1β. Values are represented as mean ± SEM.
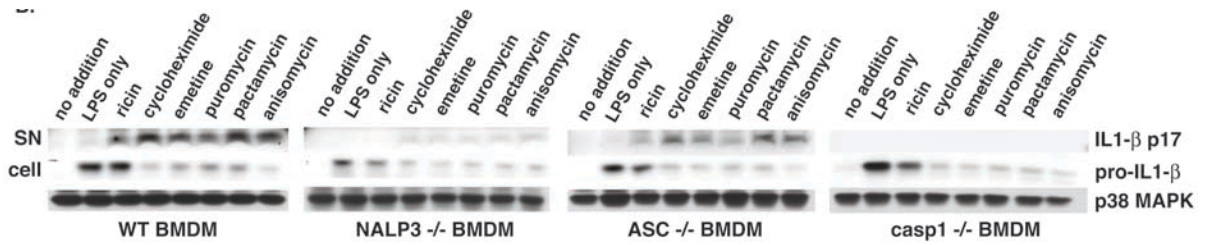
Figure 4.2 IL-1β release triggered by inhibitors of protein translation is dependent on the NALP3 inflammasome. Bone marrow derived macrophages derived from WT, NALP3$^{-/-}$, ASC$^{-/-}$, and casp-1$^{-/-}$ mice were subjected to LPS for 4h followed by exposure to inhibitors of protein synthesis for 4h. Cell extracts were analyzed by immunoblotting for pro-IL-1β and p38 MAPK (loading control). Proteins from media supernatants were precipitated and analysed by immunoblotting with IL-1β.
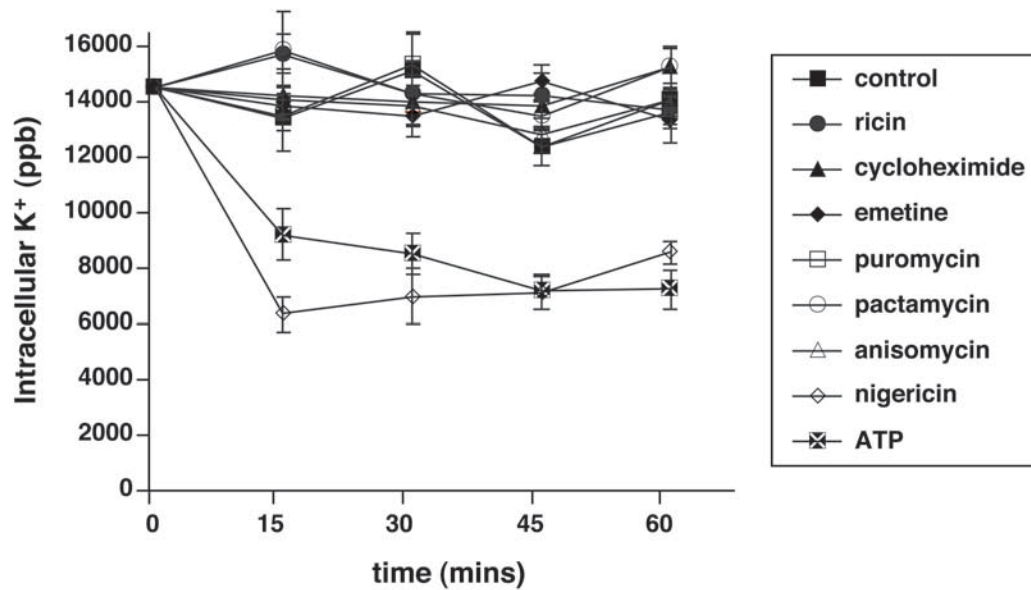
Figure 4.3 Inhibitors of protein synthesis do not elicit potassium leakage from cells, unlike nigericin and ATP. Intracellular potassium was analyzed by ICP-MS by Dr. Martina Ralle from samples of WT bone marrow derived macrophages that were primed with LPS for 4h and exposed to inhibitors of protein synthesis for indicated times (n=3). Data represented as mean ± SEM.
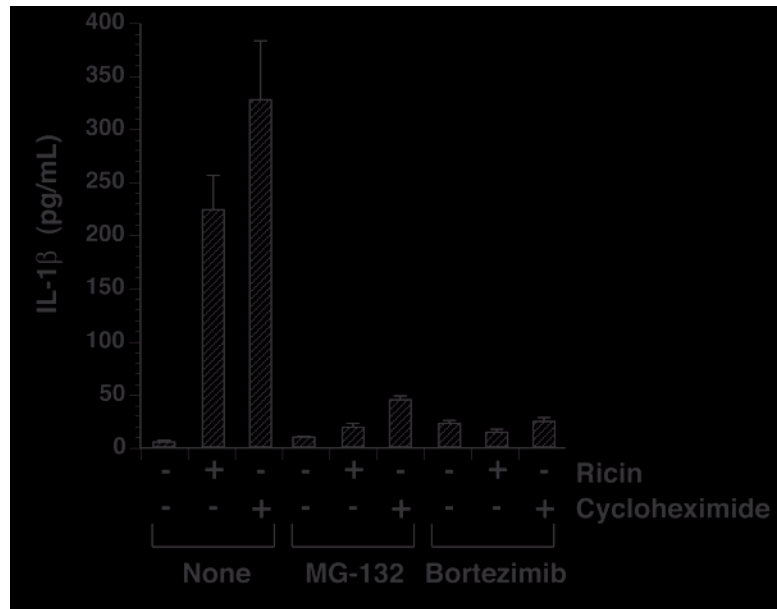
Figure 4.4 IL-1β release triggered by inhibitors of protein synthesis requires active proteasomes. Media supernatants from primed bone marrow derived macrophages stimulated with ricin or cycloheximide for 4h in the presence of control media versus media containing MG-132 or Bortezomib. Data are represented as mean ± SEM.

A.
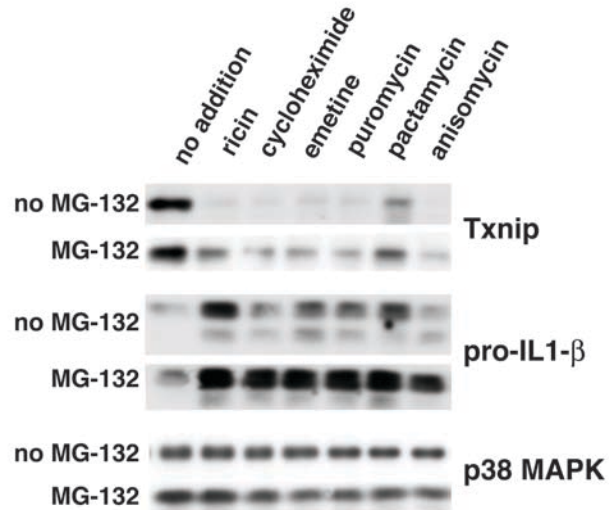
B.
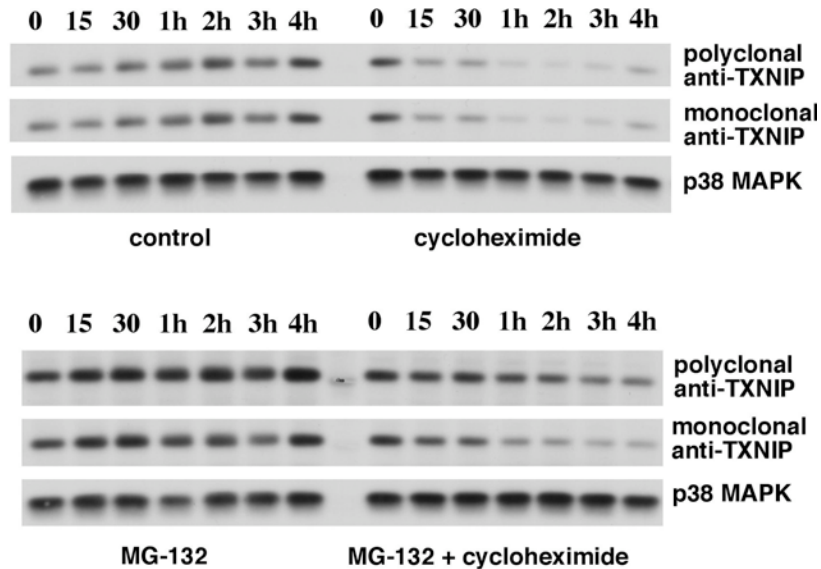
Figure 4.5   The NALP3 binding protein, TXNIP, is rapidly degraded in cells stimulated with inhibitors of protein synthesis, and is stabilized in cells co-treated with MG-132. A) Primed bone marrow derived macrophages were stimulated with inhibitors of protein translation ± MG132 for 4h. Cell extracts were analyzed by immunoblotting for TXNIP and pro-IL-1β. B) Primed BMDM were stimulated ± cycloheximide for indicated times in the presence of control media or MG-132. Cell extracts were analyzed using both a polyclonal and a monoclonal antibody against TXNIP, and p38 MAPK (loading control).

Summary and Conclusions

Since exposure to aerosolized ricin represents the most plausible means of delivery to human populations in the event of a bioterrorist attack (Franz and Jaax, 1997), it is important to understand the development of pulmonary immune responses to inhaled ricin toxin. Ricin elicits a potent inflammatory response in both rodents and humans (Brown and White, 1997; Greenfield et al., 2002; Wong et al., 2007a) and results in symptoms typical of acute lung injury and acute respiratory distress syndrome (Greenfield et al., 2002; Wong et al., 2007a) (Figure 1.2). Previous work suggests that the inflammatory effects of ricin exposure contribute to its lethality (Korcheva et al., 2005; Wong et al., 2007a) and that macrophages respond robustly to ricin in culture (Korcheva et al., 2007). However, the cell types responsible for ricin's inflammatory effects *in vivo*, and the signaling pathways involved in the development of ricin-mediated inflammation have been unclear (Figure 1.3).

In this thesis, I report that aerosolized ricin triggers pulmonary inflammation in experimental animals that is characterized by the recruitment of neutrophils to the airways, and is dependent on the presence of macrophages or IL-1 signaling. Lung pathology and expression of inflammatory genes is significantly reduced in animals depleted of macrophages prior to ricin administration (Figures 2.1 and 2.2) or genetically deficient in IL-1α/β (Figure 2.4). Exogenous IL-1β, when co-administered with ricin to mice deficient in IL-1, recapitulates neutrophil recruitment to the bronchoalveolar lavage fluid (Figure 2.5). Furthermore, wild-type animals are protected from ricin when administered IL-1 receptor antagonist to block IL-1 signaling at the time of ricin exposure

(Figure 2.6). I concluded from the *in vivo* model of ricin exposure that macrophages and IL-1 signaling are essential components of ricin-mediated inflammatory responses in the lung. Importantly, upon investigation of ricin-induced release of IL-1 from macrophages in culture, I found that the production and release of IL-1 by ricin-treated cells depends on a pathway not previously known to be involved in ricin responses.

Previously we knew that ricin, like other ribotoxic stressors, causes the inhibition of protein synthesis and phosphorylation of SAPKs (Iordanov et al., 1997). However, the connections between reduced protein synthesis, SAPK phosphorylation and the initiation of an inflammatory cascade by ricin were unclear (Figure 1.3). The inflammatory effects of ricin exposure were generally attributed to the activation of SAPK pathways p38 MAPK and JNK (Jandhyala et al., 2008; Korcheva et al., 2005; Korcheva et al., 2007; Wang et al., 2005). However, data presented here argues against previous notions that SAPK activation is the only mechanism underlying ricin-mediated inflammatory processes.

This thesis demonstrates that p38 MAPK and JNK may be unnecessary for the IL-1 dependent inflammation that defines acute lung injury after ricin administration to the lungs of mice. Ribotoxic stressors such as ricin mediate the activation of JNK and p38 MAPK through the leucine _Z_ipper- and sterile _A_lpha motif-containing _K_inase (ZAK), a MAP3K whose activation leads to the phosphorylation of JNK and p38 MAPK in response to cellular stress (Jandhyala et al., 2008; Wang et al., 2005). These so-called stress-activated protein kinases play a central role in mediating inflammatory processes, for instance in mediating responses to bacterial lipopolysaccharide (Han et al., 1994; Lee et al., 2000). Upon phosphorylation, JNK and p38 MAPK translocate to the nucleus where they phosphorylate transcription factors that activate the transcription of a number of inflammatory cytokines including IL-1 and TNF-α. Small molecule inhibitors of JNK and p38 MAPK (sorafenib and nilotinib) were shown to be highly effective in bone

marrow derived macrophages and other cell types in suppressing ricin-induced activation of JNK and p38 MAPK and the expression of proinflammatory cytokines and chemokines (Korcheva et al., 2005; Wong et al., 2007b), suggesting that blockade of kinase activation by ricin might prove useful in preventing ricin-mediated inflammatory responses in animals. In this thesis, I demonstrate that a critical event in ricin-mediated lung injury is an increase in IL-1β in the lungs of mice after ricin exposure that is responsible for the recruitment of neutrophils to the airways (Figures 2.3-2.6). Rather surprisingly, I found that small molecule inhibitors of JNK and p38 MAPK boost, rather than suppress, IL-1β release from cultured macrophages in response to ricin (Figure 3.4). This result suggests that while SAPK phosphorylation may prime cells by activating transcription of pro-IL-1β in ricin-treated cells, the processing and release of IL-1β occurs via a distinct mechanism that does not require p38 MAPK and JNK. Since LPS was used to prime cells in my *in vitro* experiments, SAPK activation was unnecessary for production of pro-IL-1β. *In vivo*, where it is unclear how and when cells are primed, it cannot be ruled out that SAPK activation plays an important role in the development of ricin-mediated inflammatory signaling through IL-1. Future work should focus on understanding the role of p38 and MAPK phosphorylation in contributing to IL-1 dependent inflammation by ricin in the pulmonary system.

The data presented in this thesis support a new action of ricin. In Chapter 3 I describe the activation of a novel pathway involved in ricin responses that leads to the processing and release of IL-1β from macrophages through the activity of the NALP3 inflammasome. IL-1 is known to mediate inflammatory lung injury by a variety of stimuli including endotoxemia (Calkins et al., 2002), subacute ozone exposure (Johnston et al., 2007), thermal injury (Chen et al., 2007), bleomycin administration (Gasse et al., 2007), and experimental ventilation (Frank et al., 2008). Furthermore, it has been reported that administration of IL-1 is sufficient to induce chemokine expression and recruitment of

neutrophils to the airways (Calkins et al., 2002), which accumulate in the airways in acute respiratory distress syndrome and contribute to injury and loss of epithelial integrity (Abraham, 2003). Indeed, I found that the release of IL-1 from cells in response to ricin was critical to the inflammation and mortality observed in ricin-treated mice; genetic deficiency of IL-1β or the administration of IL-1 receptor antagonist to the lungs effectively blocked neutrophilia and improved mortality after ricin exposure (Figures 2.4-2.6). In an attempt to understand the regulation of ricin-mediated IL-1β release from cells, I examined the requirement for one common mechanism of pro-IL-1β processing: the NALP3 inflammasome.

IL-1β secretion is a tightly regulated process involving the detection of at least two distinct signals (Faustin et al., 2007; Perregaux and Gabel, 1994). Cells first require priming by Toll-like receptor ligands (such as lipopolysaccharide) or by cytokines (such as TNF-α or IL-1β itself) to induce the expression and accumulation of the inactive pro-form, pro-IL-1β. Next, a second signal is required for the assembly of cytosolic molecular scaffolds, called inflammasomes, to stimulate caspase-1 activity, leading to cleavage of the precursor and secretion of active IL-1β (Martinon et al., 2002; Tschopp et al., 2003). This thesis suggests that ricin provides this second signal to cells and promotes the assembly of inflammasome complexes containing the pattern recognition receptor, NALP3 (Figure 5.1). I found that primed bone marrow derived macrophages deficient in the components of the NALP3 inflammasome—NALP3, the adaptor protein, ASC, and IL-1 converting enzyme (also known as caspase-1)—displayed impaired IL-1β secretion to the media after ricin exposure (Figure 3.2). In addition, since small molecule inhibitors of p38 and MAPK were unable to suppress ricin-mediated IL-1β release (Figure 3.4), it became clear that another mechanism must be responsible for the activation of NALP3 inflammasomes by ricin. This result led me to hypothesize that

protein synthesis inhibition *per se* may be the mechanism underlying NALP3 inflammasome activation by ricin. I tested the ability of a panel of protein synthesis inhibitors, including one that does activate SAPKs (anisomycin) and four that do not (cycloheximide, emetine, puromycin and pactamycin) (Iordanov and Magun, 1999; Iordanov et al., 1997), to elicit IL-1β release from bone marrow derived macrophages. Interestingly, I found that all inhibitors of protein synthesis triggered IL-1β release in a manner that was dependent on the expression NALP3, ASC and caspase-1, suggesting that they, too, worked through the NALP3 inflammasome (Figure 4.1-4.2). This result also confirmed that SAPK activation was not required for IL-1β release through the NALP3 inflammasome, as the only action these agents had in common was their ability to inhibit protein synthesis.

NALP3 inflammasomes are activated by a diverse array of agents including exogenous danger signals such as bacterial RNA, Candida albicans, and influenza (Hise et al., 2009; Ichinohe et al., 2009; Kanneganti et al., 2006), environmental stressors such as silica and asbestos (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008), and endogenous danger signals such as ATP, uric acid and amyloid β (Halle et al., 2008; Mariathasan et al., 2006; Martinon et al., 2006). In every case, the assembly of NALP3 inflammasomes triggers the conversion of pro-IL-1β to IL-1β. However, the mechanism(s) by which NALP3 receptors detect these disparate agents to elicit inflammasome formation is not understood. It is thought that a common downstream event—a decrease in intracellular potassium—occurs after exposure to all NALP3 activators, and that potassium efflux from cells triggers assembly of inflammasomes and processing of IL-1β {Petrilli, 2007}. My results demonstrate that NALP3 inflammasome activation can occur without the efflux of cellular potassium (Figure 4.3). Furthermore, my data suggest a new mechanism of activating NALP3 inflammasomes, through the

inhibition of protein synthesis.

Interestingly, the phenomena of protein synthesis inhibition and potassium efflux from cells may be closely related. It has long been known that the bacterial toxin nigericin, a potassium ionophore and one of the more potent activators of the NALP3 inflammasome (Perregaux and Gabel, 1994), inhibits protein synthesis concomitant with loss of intracellular potassium (Alonso and Carrasco, 1981; Cahn and Lubin, 1978; Panet and Atlan, 1979). Indeed, potassium loss from cells is tightly coupled with a reduction in the rate of protein synthesis (Alonso and Carrasco, 1981; Cahn and Lubin, 1978), suggesting that any agent that induces potassium loss from cells also inhibits protein synthesis. Taken together, these data suggest that the inhibition of protein synthesis might constitute a common mechanism of NALP3 inflammasome activation, regardless of whether the inhibition occurs via ribotoxic stress or as a result of decreased intracellular potassium concentration. Furthermore, the observation that other protein synthesis inhibitors in addition to ricin elicit IL-1β release from cultured macrophages suggests that any agent that causes a reduction in protein synthesis could potentially trigger IL-1 dependent inflammation.

Fortunately, the manipulation of the IL-1 pathway using exogenous IL-1 receptor antagonist (anakinra) holds promise for the treatment of IL-1 dependent inflammatory diseases, and is already used successfully in patients for the treatment of several autoinflammatory syndromes (Hawkins et al., 2003) and gout (So et al., 2007). In mouse models of bleomycin and silica-induced pulmonary fibrosis, treatment with IL-1ra is reported to reduce lung damage (Piguet et al., 1993). My data suggest that ricin poisoning may also be treated successfully using IL-1ra, because wild-type animals co-treated with IL-1ra and ricin displayed reduced pulmonary inflammation and improved mortality compared with wild-type animals treated with ricin alone (Figure 2.6). Considering that IL-1R blockade was sufficient to block neutrophilia in ricin-treated mice,

my data supports the idea that any detrimental pulmonary inflammatory condition characterized by the accumulation of neutrophils in the airways (including ricin, silica, and asbestos-mediated pulmonary inflammation) might be treated by blocking the IL-1 pathway using IL-1ra (Dostert et al., 2008).

Alternatively, proteasome inhibition may be a potential therapeutic avenue in the case of ricin exposure. Proteasome inhibition has been shown to prevent pulmonary inflammation in response to anthrax lethal toxin (Squires et al., 2007). My data from Chapters 3 and 4 demonstrates that inhibition of the proteasome using proteasome inhibitors Bortezomib or MG-132 is sufficient to block IL-1β release from cells treated with ricin and other protein synthesis inhibitors in culture. Although the mechanism remains obscure, I hypothesize that proteasome inhibitors prevent the degradation of important NALP3 regulators in the cell that are no longer translated after a reduction in protein synthesis by ricin (Figure 5.1). Proteasome inhibitors like Bortezomib and MG-132 are currently used successfully in the clinic for the treatment of cancers such as multiple myeloma {San Miguel, 2008}. Future experiments should focus on testing the efficacy of proteasome inhibitors in blocking ricin-mediated inflammation *in vivo*.

In addition to providing insight for the development of therapeutic approaches to counteract the use of aerosolized ricin as a bioterrorist agent, a better understanding of mechanisms underlying ricin's toxicity may provide understanding of how other inhaled toxins cause inflammation, injury and death. Furthermore, it has been recently demonstrated that endogenous signals from damaged tissues can trigger the same signaling pathways as exogenous toxins like ricin. For example, ATP, uric acid, and necrotic cells all elicit IL-1β release from cells that is dependent on the NALP3 inflammasome (Bours et al., 2006; Iyer et al., 2009; Shi et al., 2003). These reports suggest that understanding mechanisms underlying inflammatory responses to ricin—a toxin not very relevant to everyday life—may provide important contributions to human

health by contributing to the understanding of how endogenous danger signals initiate IL-1 dependent inflammation that can lead to the development of chronic inflammation and autoimmune disease. By developing therapeutic approaches to block ricin-mediated inflammatory signaling, we may discover new avenues for treating inflammatory conditions (such as diabetes, arthritis and gout) that are more commonly encountered in the clinic.
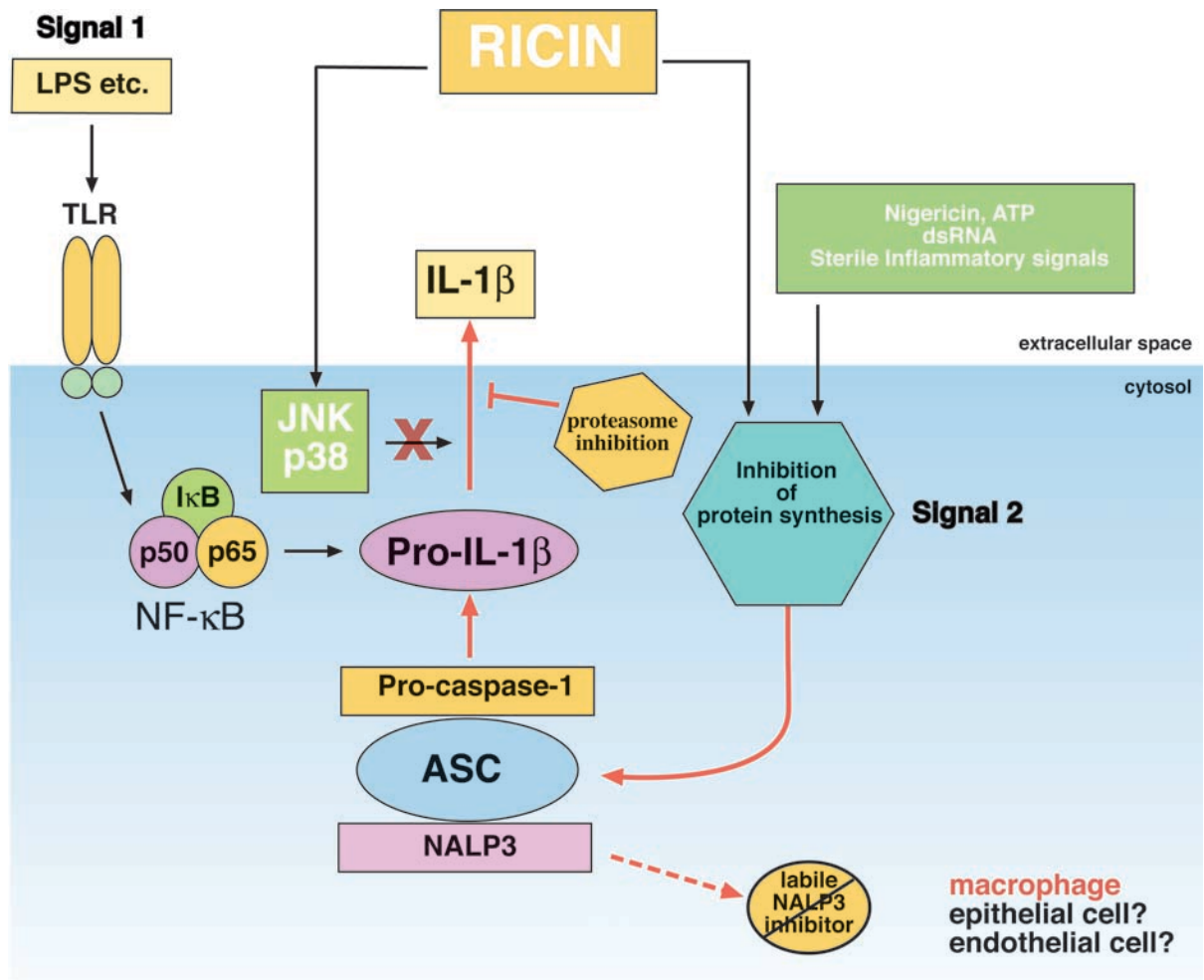
Figure 5.1 Proposed mechanism of ricin-mediated IL-1β signaling in macrophages. Areas highlighted in red represent work presented in this thesis. Ricin exhibits two well-described actions in cells: the inhibition of protein synthesis and the phosphorylation of stress-activated protein kinases, p38 MAPK and JNK. Data from this thesis demonstrate that ricin elicits IL-1β release from macrophages via the activation of the NALP3 inflammasome. Ricin-mediated activation of the NALP3 inflammasome is not dependent on the phosphorylation of JNK and p38, but requires active proteasomes. Furthermore, inhibition of protein synthesis elicited by other agents triggers activation of the NALP3 inflammasome, supporting the hypothesis that ricin stimulates NALP3 by inhibiting protein synthesis, possibly through contributing to the disappearance of important labile protein regulator(s) from the cell. These data suggest that ricin, and protein synthesis inhibitors in general, provide the second signal to primed cells that is required for the processing of pro-IL-1β to IL-1β by caspase-1 via the NALP3 inflammasome.

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Chapter 1

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