The FIIND domain of Nlrp1b promotes oligomerization and pro-caspase-1 activation in response to lethal toxin of *Bacillus anthracis*

by

Vineet Joag

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Masters of Science

Laboratory Medicine and Pathobiology
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Abstract

Lethal toxin (LeTx) of *Bacillus anthracis* kills murine macrophages in a caspase-1 and Nod-like-receptor-protein 1b (Nlrp1b)-dependent manner. Nlrp1b detects intoxication, and self-associates to form a macromolecular complex called the inflammasome, which activates the pro-caspase-1 zymogen. I heterologously reconstituted the Nlrp1b inflammasome in human fibroblasts to characterize the role of the FIIND domain of Nlrp1b in pro-caspase-1 activation. Amino-terminal truncation analysis of Nlrp1b revealed that Nlrp1b\textsubscript{1100-1233}, containing the CARD domain and amino-terminal 42 amino acids within the FIIND domain was the minimal region that self-associated and activated pro-caspase-1. Residues \textsubscript{1100}EIKLQIK\textsubscript{1106} within the FIIND domain were critical for self-association and pro-caspase-1 activation potential of Nlrp1b\textsubscript{1100-1233}, but not for binding to pro-caspase-1. Furthermore, residues \textsubscript{1100}EIKLQIK\textsubscript{1106} were critical for cell death and pro-caspase-1 activation potential of full-length Nlrp1b upon intoxication. These data suggest that after Nlrp1b senses intoxication, the FIIND domain promotes self-association of Nlrp1b, which activates pro-caspase-1 zymogen due to induced pro-caspase-1 proximity.
Acknowledgements

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<tbody>
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<td>ANTXR1</td>
<td>Anthrax toxin receptor 1</td>
</tr>
<tr>
<td>ANTXR2</td>
<td>Anthrax toxin receptor 2</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis associated Speck-like-protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone Marrow Derived Macrophages</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase Activation and Recruitment Domain</td>
</tr>
<tr>
<td>Casp1</td>
<td>Caspase-1</td>
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<tr>
<td>Casp5</td>
<td>Caspase-5</td>
</tr>
<tr>
<td>CBP</td>
<td>Calmodulin Binding Peptide</td>
</tr>
<tr>
<td>CMG2</td>
<td>Capillary Morphogenesis Protein 2</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP responsive element binding protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent resistant membrane</td>
</tr>
<tr>
<td>EdTx</td>
<td>Edema Toxin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Edema Factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-Signal Regulated Kinase</td>
</tr>
<tr>
<td>FIIND</td>
<td>Function Unidentified Domain</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LD</td>
<td>Lethal Dose</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LeTx</td>
<td>Lethal Toxin</td>
</tr>
<tr>
<td>LF</td>
<td>Lethal Factor</td>
</tr>
<tr>
<td>LMP</td>
<td>Lysosomal Membrane Permeabilization</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich-repeat</td>
</tr>
<tr>
<td>Ltxs</td>
<td>Lethal-toxin sensitive</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-Associated-Molecular-Pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MDP</td>
<td>Murymyl Dipeptide</td>
</tr>
<tr>
<td>MEK1</td>
<td>MAPK/ERK kinase 1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>NACHT</td>
<td>NAIP CIITA HET-E TP1</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nod-like receptor protein</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding and oligomerization domain</td>
</tr>
<tr>
<td>PA</td>
<td>Protective Antigen</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition-receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetra-ethyl ammonium acetate</td>
</tr>
<tr>
<td>TEM8</td>
<td>Tumor Endothelial Marker 8</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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</tbody>
</table>
1.1 Anthrax

Study of the bacterium that causes anthrax has played an important role in microbiology. Robert Koch first identified the microbe which caused anthrax disease in 1875, and this was the first demonstration that established a causal relationship between microbes and disease. Based on this work, Koch developed a set of guidelines called the Koch’s Postulates which continues to inform the approach to microbiologic diagnosis even today. Following Koch’s discovery, Louis Pasteur successfully tested the first anti-anthrax vaccine in domesticated animals (Turnbull, 2002). The study of anthrax has led to many scientific advances in the field of microbiology and vaccinology. However, the development and misuse of anthrax as a biological weapon, exemplified by the accidental release of weapons-grade anthrax in Sverdlovsk, Russia and the terrorists attacks in September 2001 in the United States have caused serious concern in the international community.

*Bacillus anthracis*, a rod shaped, Gram-positive, spore-forming bacterium is the causative agent of anthrax. As a spore the bacterium does not replicate, and can survive adverse environmental conditions (Gould, 1977). Domestic livestock and wild herbivores are especially vulnerable to the disease and transmission to humans usually occurs by contact with infected animals or animal products, consumption of contaminated meat, or inhalation of airborne spores (Turnbull, 1991).

The most common route of infection of *B. anthracis* spores in humans is through abrasions in the skin. This form of anthrax disease is known as cutaneous anthrax and has a
fatality rate of less than 1% after antibiotic treatment (Brook, 2002; Friedlander, 1999).

Cutaneous infection is usually localized and characterized by a black eschar surrounded by massive edema and inflammatory infiltrates (Dixon et al., 1999). Gastrointestinal anthrax is usually caused by ingestion of contaminated meat, and is extremely rare in developed countries with an associated mortality rate between 25 and 60 percent. The most lethal form of anthrax disease is inhalation anthrax. Early detection of disease is difficult because initial symptoms are similar to a viral upper respiratory tract infection and are characterized by fever, non-productive cough, myalgia, and malaise. Within 2 or 3 days there is a sudden onset of severe respiratory distress, and death ensues from respiratory failure, sepsis and shock (Turnbull and Kramer, 1995). Inhalation anthrax is usually fatal even with aggressive anti-microbial therapy. It is believed that antibiotic treatment can clear the bacteraemia, but high levels of toxins circulating in the blood cause death (Mourez, 2004).

In each form of anthrax infection, spores are phagocytosed by macrophages and transported to regional lymph nodes that drain the inoculation site. Inside the macrophage, dormant spores germinate into vegetative bacteria, which are capable of replication (Guidi-Rontani et al., 1999). The vegetative bacilli lyse the macrophage, continue to divide in the lymphatics and disseminate into the bloodstream causing bacteraemia and toxaemia, resulting in death in nearly 100 percent of all untreated cases (Young and Collier, 2007).

Vegetative bacilli produce two major virulence factors: a tri-partite exotoxin and an anti-phagocytic poly-D-glutamic acid capsule. The genes that encode the tripartite toxin are encoded on a large plasmid, pXO1 (Okinaka et al., 1999), while components required for synthesis, transport, attachment and degradation of the capsule are encoded on pXO2 (Green et al., 1985; Mourez, 2004). Strains of *B. anthracis* that lack either pXO1 or pXO2 have attenuated virulence.
1.2. Poly-D-glutamic acid capsule

The capsule of *B. anthracis* is composed of extensive polymers of over 215 kDa which contain D-glutamic acid residues that are $\gamma$-linked. The capsule has non-immunogenic properties as evident by the absence of an immune response when the capsule is injected into rabbits (Goodman and Nitecki, 1967). While non-encapsulated bacteria undergo phagocytosis by cultured macrophages, encapsulated bacteria are rarely phagocytosed (Drysdale et al., 2005). Addition of CapD, a capsule depolymerizing enzyme renders bacilli susceptible to phagocytosis by macrophages and killing by neutrophils (Scorpio et al., 2007). Thus, by preventing phagocytosis, the capsule allows the vegetative bacillus to evade immune recognition, thereby facilitating persistence in the host.

1.3 Anthrax Toxins

1.3.1 Toxin assembly and internalization

Anthrax toxin belongs to the AB family of toxins. AB toxins are characterized by an active (A) moiety that exerts its effect in the host cytosol and a binding (B) moiety that binds to cell surface receptors and allows entry of the A moiety into the cytosol. Anthrax toxin is comprised of two A moieties, lethal factor (LF) and edema factor (EF) and a single B moiety called protective antigen (PA). Secreted anthrax toxin proteins form two binary toxins, edema toxin (EdTx= EF+PA) and lethal toxin (LeTx= LF+PA) (Baldari et al., 2006).

PA is released by *B. anthracis* as a 83 kDa protein, which binds to one of two ubiquitously expressed cell surface receptors ANTXR1/2 (Bradley et al., 2001; Scobie et al., 2003) (Fig. 1). Following binding of PA$_{83}$ to either cell-surface receptor, PA$_{83}$ is cleaved by a
furin-family protease into two fragments, PA_{20} and PA_{63} (Klimpel et al., 1992; Molloy et al., 1992). PA_{20} is released into the surrounding medium, and plays no further role in the intoxication process. Receptor-bound PA_{63} spontaneously oligomerizes into a heptameric structure called the prepore (Milne et al., 1994). The PA_{63} heptamer can bind up to 3 molecules of EF and/or LF (Mogridge et al., 2002). PA_{63} oligomerization also triggers clustering into detergent-resistant microdomains (DRMs or lipid rafts) (Abrami et al., 2003). Within the rafts, the cytosolic domain of the receptor is ubiquitinated, leading to internalization of the toxin-receptor complex by clathrin-mediated endocytosis into early endosomes (Abrami et al., 2006). The toxin complex is then sorted into nascent intraluminal vesicles (Abrami et al., 2004). The low pH environment of the early endosome triggers a conformational change in PA_{63}, allowing its dissociation from the receptors and insertion into the vesicular membrane (Milne and Collier, 1993). The acidic environment also facilitates unfolding of EF and LF and translocation through the PA pore in an N- to C-terminal direction (Krantz et al., 2004). The toxin-containing multivesicular bodies then traffic into late endosomes where PA_{63} is degraded while EF and LF are protected in the lumen from degradative enzymes (Abrami et al., 2004). Intraluminal vesicles then back-fuse with the limiting membrane of the late endosome, thereby releasing EF and LF into the cytosol. Once in the cytosol EF and LF exert catalytic effects.
PA$_{83}$ binds to cell-surface receptors ANTXR1/2 and is cleaved by a furin-like protease releasing PA$_{20}$. Receptor-bound PA$_{63}$ forms a heptamer (PA$_{63}$)$_7$ which can bind up to 3 molecules of LF or EF. PA$_{63}$ heptamer formation is also accompanied by receptor clustering into lipid rafts triggering receptor-mediated endocytosis into early endosomes. Toxin-receptor complexes end up in intraluminal vesicles that are formed by invagination of the early endosomal limiting membrane. The acidic environment of late endosomes triggers a conformational change in heptameric PA$_{63}$ from a prepore to a pore which inserts into the endosomal membrane and allows translocation of LF and EF into the lumen of intraluminal vesicles. Intraluminal vesicles then traffic to late endosomes where back-fusion of toxin-containing vesicles with the limiting membrane causes release of LF and EF into the cytosol. (Reprinted with permission, from the Annual Review of Biochemistry, Volume 76 © 2007 by Annual Reviews www.annualreviews.org).
1.3.2 Edema Toxin

EF, an 89 kDa protein is a calcium and calmodulin-dependent adenylate cyclase. The N-terminal 250 amino acids of EF share significant sequence and structural similarity to the N-terminus of LF, and this region binds to heptameric PA$_{63}$. The C-terminal region of EF binds to calmodulin, which exposes an adenine triphosphate (ATP) binding site (Drum et al., 2002). The central catalytic core of EF converts ATP to cyclic adenosine monophosphate (cAMP) at a rate 1000 times higher than mammalian calmodulin-activated adenylate cyclase (Leppla, 1982).

The contribution of EdTx to systemic anthrax is debated as EF knockout $B.~anthracis$ is attenuated only 10-fold (Pezard et al., 1991; Pezard et al., 1993). However, EdTx causes lethality in mice at doses similar to those used for LeTx. Symptoms include hemorrhagic lesions in many organs, hypotension, and bradycardia (Firoved et al., 2005). The cause of lethality due to EdTx is not known.

Studies show that EdTx dampens the innate immune response through its effects on various cell types. Neutrophils are highly chemotactic phagocytes that kill bacteria and are an important component of the early innate immune response. EdTx causes cAMP production and inhibition of chemotaxis, phagocytosis and oxidative burst by human polymorphonuclear neutrophils (PMNs) in vitro (O'Brien et al., 1985). Oxidative burst is critical for phagocytes to degrade internalized bacteria. In vivo, mice infected with a $B.~anthracis$ strain lacking EF had elevated neutrophil infiltration and decreased levels of necrosis in the spleen compared to those infected with wildtype $B.~anthracis$ (Heninger et al., 2006). This suggests that loss of EdTx activity results in an increase in neutrophilic response, and greater clearance of bacteria, which lessens tissue damage.
EdTx inhibits the production of inflammatory cytokines and chemokines, thereby inhibiting the migratory and bactericidal effects of immune cells. In human monocytes EdTx-dependent cAMP release resulted in inhibition of LPS-stimulated TNF production; however, IL-6 levels were slightly elevated (Hoover et al., 1994). TNF helps the immune system fight bacterial infection while IL-6 has pro- and anti-inflammatory properties. In its anti-inflammatory role IL-6 can inhibit the effect of TNF (Hoover et al., 1994). These results suggest that cAMP produced by EdTx causes aberrant production of cytokines, which may help *B. anthracis* evade the immune response.

cAMP is a cellular messenger involved in many signalling pathways. cAMP activates protein kinase A (PKA) which can activate various transcription factors including CREB. CREB binds to cAMP response elements (CREs), which regulate transcription of cAMP responsive genes. CREs are found upstream of anthrax toxin receptor genes, and treatment with EdTx increases transcription of ANTXR genes in monocyte-derived cells in a PKA-dependent manner. Interestingly, EdTx increased cell-surface expression of ANTXR1/2, which allowed more LF to enter the cytosol, leading to increased toxin sensitivity (Maldonado-Arocho et al., 2006). Kim et al. (2008) found that EdTx induced the activation of CREB via PKA, which increased expression of CREB-responsive genes syndecan-1 and VEGF. Syndecan-1 promotes the migration of macrophages infected with *B. anthracis* (Kim et al., 2008) while VEGF promotes lymphangiogenesis and edema, which facilitates spreading of infected macrophages. Thus, EdTx manipulates the host immune system by promoting migration and spreading of infected macrophages.
1.3.3 Lethal Toxin

1.3.3.1 Lethal Factor

LF is a 90 kDa protein that has an N-terminal PA binding domain, a large central domain that binds substrates, and a C-terminal catalytic domain (Pannifer et al., 2001). The C-terminal domain contains the sequence HEXXH that is common to metalloproteinases and harbours residues that are required for catalysis and zinc ion co-ordination. Catalytic activity of LF is absolutely required for *in vitro* and *in vivo* toxicity. The only known substrates of LF are Mitogen Activated Protein Kinase Kinase (MKK) proteins (Chopra et al., 2003; Duesbery and Vande Woude, 1999). MAP kinase proteins have an N-terminal docking-site (D-site) and a C-terminal catalytic domain. The substrate of each MKK binds to the D-site on the MKK and is activated by phosphorylation. MKKs activate downstream MAPKs which regulate targets involved in cell division, transcription, differentiation, inflammation and apoptosis. Of the seven known MKKs, LeTx cleaves MKK1 (also known as MEK1 for MAPK/ERK kinase 1), MKK2 (MEK2), MKK3, MKK4, MKK6 and MKK7 (Duesbery and Vande Woude, 1999; Turk, 2007). MEK1/2 phosphorylates and activates ERK1/2 (extracellular-signal-regulated kinase), MKK3/6 activates p38, while MKK4/7 activates c-Jun amino-terminal kinase (JNK). LF cleaves at the D-site of MKKs, preventing docking of MAPKs, and consequently, their activation (Chopra et al., 2003; Mourez, 2004). Thus, LF is able to shut down the ERK, p38, and JNK signalling pathways.
1.3.3.2 LeTx and impairment of the immune response

Inhibition of the MAPK signalling pathway by LeTx affects various cell types such as neutrophils, macrophages, endothelial cells, dendritic cells (DCs) and lymphocytes resulting in dampening of both innate and adaptive immune responses.

Bacterial components induce superoxide production and chemotaxis of neutrophils, which is impaired by LeTx. Inhibition of chemotaxis occurs by LeTx-mediated reduction in filamentous actin assembly and cell polarity (During et al., 2005). LeTx action is likely mediated by impairing the p38 pathway, because neutrophil chemotaxis is inhibited by p38 inhibitors in vitro and in vivo (Cara et al., 2001; Coxon et al., 2003; Zu et al., 1998). Chemokine production by neutrophils is inhibited by pharmacological inhibition of p38 and probably is also impaired by LeTx treatment (Nick et al., 2002). Along with neutrophils, macrophages constitute an important initial line of defence against an invading pathogen before the onset of adaptive immunity. Macrophages respond to various microbial products such as lipopolysaccharide (LPS) and peptidoglycan (PG) by inducing production of pro-inflammatory cytokines through the NFκB and MAPK signalling pathways. LF targets the MAPK signalling pathway to dampen the production of pro-inflammatory cytokines. LeTx inhibits TNF production at the transcriptional level by targeting the ERK pathway (Comer et al., 2005; Guha et al., 2001; Shi et al., 2002); while inhibition occurs at the translational level by targeting the p38 and JNK pathways (Kotlyarov et al., 1999; Neininger et al., 2002; Rousseau et al., 2002). LeTx also inhibits secretion of IL-6, IL-1β, and IL-10 from macrophages (Cleret et al., 2006; Popov et al., 2002a). Endothelial cells secrete cytokines and chemokines to alert neutrophils and monocytes to exit from the bloodstream and target sites of infection. Endothelial cell production of IL-8, a potent neutrophil chemoattractant is inhibited by LeTx. LeTx induces destabilization of IL-8 mRNA
transcript through the inhibition of p38, JNK and ERK pathways (Batty et al., 2006). Thus, LeTx acts on endothelial cells, neutrophils and macrophages to interfere with cytokine and chemokine production which impairs the innate immune response and promotes bacterial survival.

In addition to its dampening effect on the innate immune response, LeTx also interferes with adaptive immunity. DCs are critical for the initiation of the adaptive immune response as they present antigens on their cell surface to T cells. DCs are normally found in the bloodstream and are in an immature state. Stimulation of Toll-like receptors (TLRs) on DCs by bacterial products causes DC maturation and migration to secondary lymphoid organs. Mature DC express co-stimulatory molecules CD40, CD80 and CD86 and MHC Class II, which promote T-cell activation. Agrawal et al. (2003) showed that LeTx blocked LPS-stimulated surface expression of co-stimulatory molecules on DCs, production of pro-inflammatory cytokines and T-cell priming. Combined pharmacological inhibition of p38 and ERK pathways mimicked the effect of LeTx and inhibited cytokine production by DCs. However, death of DCs was not reported in this study. Alileche et al. (2005) found that LeTx killed human DCs in vitro and murine DCs in vitro and in vivo. The discrepancy between the two reports regarding cell death of DCs from C57BL/6 mice probably results from the different methods used to monitor cell death. The former measured plasma membrane perturbation while the later study measured cell viability. Nevertheless, these reports demonstrate that LeTx impairs the ability of DCs to mount an immune response and also causes DC death.

1.3.3.3 LeTx and macrophage death

The discovery that macrophages from certain strains of mice undergo rapid cell death due to LeTx while macrophages from other strains are resistant to toxin led to the study of this cell
type as a target of LeTx (Friedlander, 1986; Friedlander et al., 1993). Hanna et al. (1993) suggested that macrophage lysis and release of cytokines TNF and IL-1β caused death in susceptible mice. However, Moayeri et al. (2003; 2004) showed that LeTx killed mice harbouring susceptible and resistant macrophages as well as mice lacking macrophages. A rapid transitory IL-1β and cytokine burst occurred only in mice harbouring sensitive macrophages and exacerbated LeTx-induced vascular collapse and lethality, but only in some strains. All strains of mice died through a TNF-independent, non-inflammatory mechanism involving vascular collapse and hypoxic tissue injury (Moayeri et al., 2003). Since these pathological symptoms leading to death were evident in mice with sensitive as well as resistant macrophages, lethality of mice from LeTx is believed to be independent of macrophage lysis and cytokine release.

Mice harbouring LeTx-susceptible and -resistant macrophages respond differently to *B. anthracis* infection and lethal doses of LeTx. The LD₅₀ of A/J and DBA/2J mice harbouring LeTx-resistant macrophages was 10⁳ spores of unencapsulated Sterne strain while various mouse strains harbouring LeTx-susceptible macrophages had an LD₅₀ of 10⁶ spores (Welkos et al., 1986). Thus, the mouse strains that have LeTx-susceptible macrophages are more resistant to bacillus challenge and vice versa. Consistent with this observation, *in vivo* challenge with *B. anthracis* Sterne strain caused a faster and greater migratory response by macrophages to the site of infection in mice harbouring toxin-susceptible macrophages (Welkos et al., 1989). The cause for the paradoxical inverse relationship between mouse macrophage susceptibility to LeTx and *B. anthracis* is not understood. However, it makes the effect of LeTx and *B. anthracis* on mouse macrophages an attractive avenue for study. An understanding of the cellular and molecular mechanisms by which LeTx mediates macrophage death and cytokine secretion will further our understanding of the role of macrophages during *B. anthracis* infection.
Cell death can be classified as occurring through apoptosis, necrosis or pyroptosis (Fink and Cookson, 2005). Apoptosis is a highly regulated non-inflammatory, active form of cell death that involves cytoplasmic and chromatin condensation, chromatin cleavage, membrane blebbing, formation of apoptotic bodies and maintenance of plasma membrane integrity. At the molecular level, apoptosis involves the activation of a family of proteases called caspases. Apoptotic caspases target various substrates that facilitate the disintegration of the cell, ultimately leading to cell death. In contrast to apoptosis, necrosis is not a process of cell death but a term used by pathologists to designate the presence of inflammatory cells, dead cells and dead tissue. Unlike the slow apoptotic cell death, pyroptosis is a rapid form of programmed cell death. By definition, pyroptosis is mediated by caspase-1 and does not depend on activation of pro-apoptotic caspases. Infection by intracellular pathogens, such as *Salmonella typhimurium* and *Shigella flexneri*, can trigger pyroptotic cell death. Pyroptotic cell death involves plasma membrane breakdown, water influx, cellular swelling, osmotic lysis, and release of pro-inflammatory cellular content (Fink and Cookson, 2005).

Currently, the understanding of mechanisms by which LeTx causes death of human macrophages is very limited. Monocytes circulate in the bloodstream and then differentiate into macrophages in response to inflammatory signals. Kassam et al. (2005) reported that LeTx did not cause death of monocytic cell lines (U-937, HL-60 and THP-1), but inhibited their proliferation and differentiation into macrophages. However, activation of monocytes by PMA-induced differentiation into macrophages rendered these cells susceptible to LeTx. Of the three cell lines tested, differentiated HL-60 was most susceptible to toxin-induced death. Apoptotic death was caspase-independent. Inactivation of MAPK signalling by LeTx probably did not cause death because decreased phosphorylation of p38, ERK1/2 and JNK was observed in
undifferentiated and differentiated HL-60 cells. The susceptibility of an activated monocyte to die in response to LeTx is not limited to monocytic cell lines, as human peripheral blood mononuclear cells activated with IFNγ also undergo apoptosis following inhibition of cell division (Popov et al., 2002a). The ability of LeTx to inhibit proliferation and differentiation of monocytic cells and to cause apoptosis of activated macrophages would reduce killing of bacilli by macrophages and prolong bacterial persistence in the host.

In comparison to our understanding of death of human macrophages, the cellular and molecular mechanisms underlying the death of murine macrophages are better understood. Toxin-sensitive macrophages undergo pyroptosis within 90 min while resistant macrophages succumb to a slower apoptotic form of death over 16-72 h (Muehlbauer et al., 2007; Park et al., 2002). The apoptotic response is caused by LeTx- mediated inhibition of p38 MAPK activity (Park et al., 2002). The slow apoptotic response is also observed in sensitive macrophages treated with sub-lytic doses of LeTx (Popov et al., 2002b). Therefore, the apoptotic response to LeTx seems to be present in all macrophages, however, it is masked by the faster pyroptotic cell death in sensitive macrophages.

The rapid pyroptotic death of murine macrophages from certain inbred strains of mice in response to LeTx requires several seemingly unrelated cellular events. Firstly, the activity of the proteasome, a large protein complex that degrades unnecessary or damaged proteins is required for cell death as treatment with MG-132, a proteasome inhibitor protects LeTx-susceptible RAW 264.7 macrophages (Tang and Leppla, 1999). Secondly, ion fluxes, especially the efflux of potassium is an early event required for LeTx-induced pyroptosis, as susceptible macrophages grown in medium containing ~110 mM KCl were resistant to toxin-induced pyroptosis (Fink et al., 2008). Thirdly, the catalytic activity of LeTx is required for cell death, suggesting that
cleavage of a cellular substrate is necessary. However, MEK cleavage was observed in LeTx-susceptible and -resistant macrophages and various other cell types that did not die in response to LeTx (Muehlbauer et al., 2007; Pellizzari et al., 1999). This suggests that inactivation of MAPK signalling is insufficient to cause pyroptotic cell death and that LF may cleave yet unidentified cytosolic targets.

1.4 Nlrp1b

To understand how LeTx causes pyroptosis of murine macrophages a search for the genetic determinants that underlie macrophage susceptibility was performed. Nlrp1b, a gene at the Ltxs1 locus on chromosome 11 was identified as the determinant of murine macrophage susceptibility to LeTx (Boyden and Dietrich, 2006). Nlrp1b is a highly polymorphic gene that has five alleles. Toxin-susceptible macrophages have allele 1 or 5, while resistant macrophages have alleles 2, 3 or 4. Expression of a functional Nlrp1b allele1 in resistant macrophages renders them susceptible to LeTx, and knockdown of Nlrp1b induces partial resistance to LeTx in susceptible mice (Boyden and Dietrich, 2006). LeTx-mediated macrophage death is dependent on caspase-1, a cellular protease which becomes active after LeTx treatment only in toxin-susceptible macrophages. Subsequent research demonstrated that in response to LeTx, Nlrp1b activates pro-caspase-1, which causes pyroptosis of macrophages as well as maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18 (Muehlbauer et al., 2007; Wickliffe et al., 2008).

Until very recently, the prevailing belief among anthrax researchers was that rapid pyroptotic death of macrophages caused by LeTx dealt a crucial blow to the innate immune response, which promoted bacillus survival. However, a recent study by Terra et al. (2010) has
challenged this perception. The authors compared C57BL/6 mice harbouring LeTx-resistant allele 2 of Nlrp1b and transgenic C57BL/6 mice (C57B6\textsuperscript{Nlrp1b(129S1)}) containing toxin-sensitive Nlrp1b allele 1 from the 129S1 strain and found that the transgenic mice had an early IL-1β and inflammatory response to LeTx, and increased resistance to \textit{B. anthracis} infection. The finding that allelic variation of Nlrp1b controls mouse susceptibility to \textit{B. anthracis} infection supports a model where LeTx sensing by Nlrp1b and subsequent lysis of macrophages does not promote bacterial survival, but is a protective, host-mediated innate immune response.

1.4.1 Nod-like-receptors

Nlrp1b belongs to a family of proteins called Nod-like receptors (NLRs). NLRs are germline-encoded pattern recognition receptors (PRRs) that sense host danger signals and conserved microbial structures often referred to as microbe-associate-molecular-patterns (MAMPs). PRRs respond to danger signals by alerting the immune system. MAMPs are often critical for microbial survival and include LPS and peptidoglycan, major components of bacterial cell walls, flagellin, important for motility, and nucleic acids. The presence of multiple PRRs capable of recognizing different MAMPs on the surface of a microbe ensures that an immune response is generated even when a particular signalling pathway is disrupted. TLRs and NLRs are important classes of PRRs (Schroder and Tschopp, 2010). TLRs are expressed on the cell surface where they sense microbial products such as LPS (TLR4), peptidoglycan (TLR2), and flagellin (TLR5). Some TLRs, such as TLR3 which recognizes double stranded RNA are found in endosomes. In contrast to TLRs, NLRs are intracellular proteins that recognize cytosolic danger signals (Yu and Finlay, 2008).
To date, 23 NLR family members have been identified in humans and 34 NLR genes described in mice (Franchi et al., 2009). NLR family members have a shared domain organization. Most NLRs have an N-terminal effector region containing a Caspase Recruitment Domain (CARD) or pyrin (PYD) domain, centrally located nucleotide-binding and oligomerization domain (NOD) also referred to as the NACHT domain, and C-terminal leucine-rich repeats (LRRs) (Fig 2). The LRR domain is thought to fold back onto the NACHT domain to maintain auto-inhibition until ligand sensing occurs through the LRR domain (Hu et al., 1998). The NACHT domain is the only domain common to all NLRs and mediates downstream signalling via ATP-dependent oligomerization. CARD and PYD domains mediate homotypic protein-protein interactions with downstream effectors (Yu and Finlay, 2008).
FIG. 2. Domain organization of various Nod-like receptors (NLRs), CARDINAL, ASC, and pro-caspase-1. NLRs generally have an amino-terminal effector domain that is either a PYD domain or CARD domain. The NACHT domain is common to all NLRs and is followed by leucine rich repeats (LRRs). NLRP1 is unique among NLRs as it has two effector domains, an N-terminal PYD and a C-terminal CARD domain. Nlrp1b lacks an N-terminal effector domain but has a CARD domain at the C-terminus. The FIIND (function unidentified) domain is found in NLRP1, Nlrp1b, and CARDINAL.

For many NLRs the activating signal and downstream targets are not known. NOD1 and NOD2 were the first characterized NLRs and recognize bacterial peptidoglycan fragments mesodiaminopimelic acid and murymyl dipeptide (MDP), respectively. Among other NLRs whose activators are known NLRC4, Nlrp1b, and NLRP1 (the human orthologue of murine Nlrp1b) sense flagellin, catalytic activity of LeTx, and MDP respectively (Boyden and Dietrich,
2006; Faustin et al., 2007; Franchi et al., 2006; Miao et al., 2006). NLRP3 is unique in the NLR family in that it senses a plethora of danger signals including MAMPs (LPS, MDP, bacterial and viral RNA), host danger signals (ATP, uric acid), and environmental irritants (silica, asbestos, UVB radiation). The variety and structural diversity of NLRP3 activators suggests that NLRs may sense endogenous indicators of cellular danger or stress, rather than conserved molecular patterns. Additionally, there are no reports of a direct interaction between a NLR protein and its activator, suggesting that activation occurs through indirect mechanisms involving endogenous signals.

Upon activation, many NLRs such as NLRP3, Nlrp1b, NLRP1 and NLRC4 assemble into a macromolecular complex called the inflammasome which contains the effector protease caspase-1. Martinon et al. (2002) identified the first inflammasome and coined the term after the macromolecular complex apoptosome which initiates apoptosis after receiving a cell death stimulus. Caspase-1 inflammasomes are mediators of cell death and pro-inflammatory cytokine release.

1.4.2 Caspase-1 Inflammasomes

Caspases are cysteiny1-aspartate-specific proteinases with essential roles in apoptosis and inflammation. They are synthesized as inactive zymogens containing a pro-domain of variable length followed by a large and a small catalytic subunit. Caspases are activated by proteolytic cleavage at Asp(P1)-X(P1'), which releases their pro-domain. Further cleavage at the boundary of the large and small subunits allows formation of an active heterotetramer containing two large and two small subunits. Caspases are classified as either apoptotic or inflammatory caspases.
Caspase-2, 3, 6, 7, 8 and 9 are apoptotic caspases. The pro-inflammatory caspases are comprised of caspase-1, 4, and 5 in humans and caspase-1, 11, and 12 in the mouse (Martinon et al., 2002).

Caspase-1 is the central effector of the inflammasome. Pro-caspase-1 consists of an N-terminal CARD domain followed by the large (p20) and small (p10) catalytic subunits (Fig. 2). Caspase-1 causes proteolytic processing of pro-inflammatory cytokines IL-1β, IL-18 and IL-33. IL-1β and IL-18 are synthesized as biologically inactive precursors and after processing by caspase-1, the mature cytokines can exert their biological effects. IL-1β is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. IL-18 helps recruit inflammatory cells to sites of infection (Li et al., 1995; Martinon et al., 2002) while IL-33 promotes T helper cell type 2 (T\textsubscript{H}2) responses (Arend et al., 2008).

Although the induction of pro-inflammatory cytokines by caspase-1 inflammasomes is critical for the innate immune response, aberrant activation of caspase-1 by NLRs can cause inflammatory disease. Mutations in NLRs can cause constitutive activation of pro-caspase-1, which leads to excessive production of pro-inflammatory cytokines. For instance, gain-of-function mutations of NLRP3 are associated with hereditary periodic-fever syndromes: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and neonatal-onset multisystem inflammatory disease. Monocytes from these patients secrete IL-1β and IL-18 even in the absence of a stimulus suggesting that certain mutations in NLRP3 render the protein constitutively active (Gattorno et al., 2007). A locus in NLRP1 is responsible for familial vitiligo, a chronic disorder associated with depigmentation of the skin and involving immune destruction of melanocytes (Jin et al., 2007).
1.4.3 Inflammasome assembly and pro-caspase-1 activation

Several reports suggest that stimulation of an NLR with its activator causes recruitment of inflammasome components pro-caspase-1 and ASC to the NLR protein. Martinon et al. (2002) showed that when cell lysates of THP-1 monocytes were stimulated with heat shock at 30°C, pro-caspase-1 was activated in a NLRP1- and ASC-dependent manner. Unstimulated and stimulated cell extracts of THP-1 cells were subjected to size exclusion chromatography followed by immunoblotting of eluted fractions. Compared to unstimulated cell extracts, analysis of stimulated cell extracts demonstrated a shift of pro-caspase-1 and ASC into a high molecular weight fraction containing NLRP1 (Martinon et al., 2002). Similarly, gel filtration performed on cell extracts of unstimulated J774.1 cells (LeTx-susceptible murine macrophage-like cells) showed a pro-caspase-1 complex at ~ 200 kDa, ASC at ~ 350 kDa, and a Nlrlp1b complex at ~ 800 kDa. In LeTx-treated cells, pro-caspase-1 shifted to the higher molecular weight complex of ~ 800 kDa, however ASC remained at ~ 350 kDa (Nour et al., 2009). These results suggest that when an NLR senses its stimulus, pro-caspase-1, and in some cases, ASC are recruited to the NLR forming a large multiprotein complex.

ASC is usually required for pro-caspase-1 to bind to the NLR protein if the NLR contains a PYD domain. ASC contains a PYD and a CARD domain and its PYD domain interacts with the PYD domain of the NLR while the CARD domain can bind to the CARD domain of pro-caspase-1 (Fig. 2 and 3). NLRP1-14 contain N-terminal PYD domains, so interaction between these NLRPs and pro-caspase-1 is presumably mediated by ASC. For instance, ASC knockout macrophages are unable to activate pro-caspase-1 in response to various NLRP3 activators such as monosodium urate (MSU) crystals, bacterial RNA, and various TLR agonists plus ATP (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006). This demonstrates that
ASC is required for activation of the NLRP3 inflammasome. Among NLRP1-14, NLRP1 is an exception as it contains two effector domains, an N-terminal PYD domain and a C-terminal CARD domain (Fig. 2). Martinon et al. (2002) proposed that the N-terminal PYD domain of NLRP1 interacts with pro-caspase-1 via ASC while pro-caspase-5 binds to the C-terminal CARD domain of NLRP1 (Fig. 3). Sequestering ASC in cell lysates using anti-ASC antibody prevented activation of pro-caspase-1 in extracts from THP-1 cells stimulated with 30°C heat shock. However, Faustin et al. (2007) reconstituted the NLRP1 inflammasome using purified proteins and showed that ASC was dispensable for pro-caspase-1 activation in response to the NLRP1 activator MDP and co-factor ATP. Pro-caspase-1 presumably interacted with the C-terminal CARD domain (Fig. 3). I speculate that the high concentration of proteins present in the in vitro NLRP1 assay using purified proteins allowed pro-caspase-1 activation to be measured using sensitive fluorometric substrate Ac-WEHD-AMC. However, in cell lysates, where concentrations of proteins are lower, ASC may be required for pro-caspase-1 activation to be detected by immunoblotting for either the p10 fragment of caspase-1 or mature IL-1β. The evidence to date suggests that maximum pro-caspase-1 activation by NLRP1 is dependent on ASC. Nlrp1b contains a CARD domain but no PYD domain, so pro-caspase-1 interacts directly with Nlrp1b through homotypic CARD-CARD interactions (Fig. 3). LeTx-sensitive RAW 264.7 cells do not express ASC, and transient transfection of Nlrp1b and pro-caspase-1 is both necessary and sufficient to render HT1080 human fibroblasts susceptible to cell death following LeTx treatment (Liao and Mogridge, 2009; Pelegri et al., 2008). These results demonstrate that Nlrp1b, and pro-caspase-1 but not ASC, form the minimal components of the Nlrp1b inflammasome.
Recruitment of pro-caspase-1 to the NLR is followed by pro-caspase-1 activation, which is best modeled by the induced-proximity model. Several investigators tested this model for activation of pro-caspase-1 (MacCorkle et al., 1998), pro-caspase-9 (Srinivasula et al., 1998), and the lone caspase in *C. elegans* ced-3 (Yang et al., 1998). Proenzymes of these caspases were
fused to a heterologous inducible dimerization motif. Induction of homo-dimerization triggered protease activation and cell death. This suggests that as monomers pro-caspase molecules have low intrinsic proteolytic activity; however, when brought into proximity, \textit{trans} proteolytic activation occurs. In NLRs, the NACHT domain acts as the oligomerization domain which brings bound pro-caspase-1 molecules into close proximity. The role of the NACHT domain as an oligomerization domain has been demonstrated for various NLRs as well as Apaf-1 which recruits pro-caspase-9 to form the apoptosome in response to a cell death stimulus. The NACHT domain of NALP3, and the equivalent NOD domain of NOD1 and Apaf-1, contain a conserved lysine residue within the Walker A motif (P-loop) (Danot et al., 2009). The lysine binds to ATP and substitution mutation of the lysine abolishes self-association of NALP3 (Duncan et al., 2007), NOD1 (Inohara et al., 1999) and Apaf-1 (Hu et al., 1998) as well as activation of downstream effectors pro-caspase-1, NF-κB, and pro-caspase-9, respectively.

1.4.4. Key cellular events involved in intoxication, Nlrp1b activation, and cell death

The discovery of Nlrp1b as a determinant of LeTx-induced rapid pyroptosis led to increased efforts to understand the mechanism by which LeTx causes Nlrp1b-mediated pro-caspase-1 activation. It is not known how catalytic activity of LF causes activation of the Nlrp1b inflammasome (Liao and Mogridge, 2009). The only known substrates of LF, the MAPKKs, are cleaved in LeTx-sensitive as well as LeTx-resistant macrophages (Muehlbauer et al., 2007; Pellizzari et al., 1999). Hence, MEK cleavage is not sufficient for LeTx-mediated pyroptosis; however, the role of MEK cleavage in inflammasome activation is not clear. LF may either cleave other unidentified substrates that inhibit inflammasome activation or directly cleave Nlrp1b, although there are no reports of either of these activities.
Proteasome activity is required as an early upstream event in Nlrp1b activation, as proteasome inhibitors prevent LeTx-induced pro-caspase-1 activation and cell death in toxin-sensitive macrophages (Fink et al., 2008; Squires et al., 2007; Tang and Leppla, 1999). However, pro-caspase-1 activation and cell death mediated by the NLRC4 inflammasome in response to S. typhimurium and by the NLRP3 inflammasome in response to LPS/nigericin are not inhibited by proteasome inhibition (Fink et al., 2008; Squires et al., 2007). These results demonstrate that proteasomal inhibition blocks an event upstream of Nlrp1b activation that is not common for activation of other caspase-1 inflammasomes. It has been proposed that the proteasome may degrade a negative regulator of Nlrp1b, although such an inhibitor has not been found.

Nlrp1b shares a similarity with NLRP3 as both NLRs require the efflux of potassium ions in order to activate pro-caspase-1. Petrilli et al. (2007) monitored pro-caspase-1 activation in BALB/cJ peritoneal macrophages as the appearance of mature form of IL-1β by immunoblotting. Exogenous 130mM KCl in culture medium prevented the appearance of mature IL-1β in the supernatant in response to LeTx and various NLRP3 agonists (Petrilli et al., 2007). To measure inflammasome activation Fink et al. (2008) used FAM-YVAD-FMK, a fluorescent probe that specifically recognizes active caspase-1. Fluorescent signal was detected in LeTx-treated BALB/cJ bone marrow-derived macrophages (BMDMs) cultured in exogenous 110 mM NaCl, but not KCl. This supports the idea that activation of pro-caspase-1 by Nlrp1b requires potassium efflux. Wickliffe et al. (2008) found that addition of 130 mM KCl, or potassium channel inhibitors quinidine or tetra-ethyl ammonium acetate (TEA) to LeTx treated BALB/cJ BMDMs prevented the appearance of the p10 fragment of active caspase-1 and of mature IL-18 in cell lysates that were analyzed by immunoblotting. These findings suggest that efflux of potassium is required for Nlrp1b inflammasome activation.
Another early event following LeTx intoxication is mitochondrial dysfunction, which can be measured by a drop in mitochondrial membrane potential. Alileche et al. (2006) observed a drop in mitochondrial membrane potential ~ 30 min post LeTx treatment which continued as toxin-susceptible J774.1 macrophages died over a course of 2 h. Targeting of the pro-apoptotic protein Bnip3 to the outer mitochondrial membrane leads to a drop in mitochondrial membrane potential, early plasma membrane and mitochondrial damage, and caspase-independent necrotic death (Ray et al., 2000; Vande Velde et al., 2000). Interestingly, knockdown of Bnip3 and Bnip3-like-protein (Bnip3L) renders RAW 264.7 cells resistant to LeTx (Ha et al., 2007). It would be interesting to test whether LeTx causes localization of Bnip3 to the mitochondria, resulting in mitochondrial dysfunction.

Lysosomal membrane permeabilization (LMP) was recently implicated in LeTx-induced pyroptosis. Lysosomes are acidic vesicles that contain various degradative enzymes such as proteases, lipases, and nucleases which function optimally under acidic conditions. Lysosomes degrade endocytosed receptors, phagocytosed bacteria, unwanted cellular proteins, and whole cellular organelles. LMP is the loss of lysosomal acidity and the leakage of lysosomal compartments into the cytosol, resulting in apoptosis and necrosis (Boya and Kroemer, 2008; Guicciardi et al., 2004). In severe LMP lysosomal membranes become unstable which leads to the rapid loss of lysosomal contents. This is usually an end stage of LMP. Slow LMP can alter intracellular signalling and cause both caspase-dependent and caspase-independent apoptosis-like cell death (Boya and Kroemer, 2008; Brunk et al., 1997; Cirman et al., 2004). Lysosomal contents mainly implicated in cell death are cathepsin B, D, and L as these proteases can function at cytosolic pH. Averette et al. (2009) demonstrated that macrophages from transgenic C57BL/6 mice (C57B6^Nlpr1b(129S1)), which contain toxin-sensitive Nlrp1b allele 1 from 129S1 mice,
underwent LMP when treated with LeTx. However, macrophages from toxin-resistant C57BL/6 mice did not undergo LMP. This suggests that LMP is downstream of Nlrp1b-mediated procaspase-1 activation. LeTx-induced LMP was associated with release of cathepsin B into the cytosol and the cathepsin B inhibitor CA-074Me protected (C57BL/6Nlrp1b(129S1)) BMDMs and RAW 264.7 cells from LeTx. However, CA-074Me probably inhibited other proteases in addition to cathepsin B as efficient knockdown of cathepsin B using siRNA in RAW 264.7 cells does not prevent LeTx-cytotoxicity (Newman et al., 2009). The likely explanation is that in addition to cathepsin B, other cathepsins that are released into the cytosol during LMP cause induction of pyroptosis.

1.5 Characterization of Nlrp1b

Since Nlrp1b is central to LeTx-mediated murine macrophage death, characterization of Nlrp1b is of significant research interest. To study various aspects of Nlrp1b inflammasome activation, another member of my laboratory reconstituted the Nlrp1b inflammasome in HT1080 human fibroblasts (Liao and Mogridge, 2009). Unless otherwise specified, I will refer to the LeTx-responsive allele 1 of Nlrp1b as simply Nlrp1b. To reconstitute the Nlrp1b inflammasome, HT1080 fibroblasts were transiently transfected with Nlrp1b, pro-caspase-1, and pro-IL-1β for 24 h followed by treatment with LeTx (10^{-8}M LF and 10^{-8}M PA) for 3 h. LeTx-induced procaspase-1 activation was measured by decrease of pro-IL-1β in cell lysates, appearance of mature IL-1β in the supernatant as well as cell death (Liao and Mogridge, 2009). Cell death was measured by the level of lactate dehydrogenase (LDH) in supernatants since LDH is a cytosolic enzyme that is released from cells when plasma membrane integrity is compromised during pyroptosis (Fink and Cookson, 2005). Compared to untreated cells, cells treated with LeTx
showed less pro-IL-1β in cell lysates while mature ~ 17 kDa IL-1β appeared in the supernatant. This response was dependent on the catalytic activity of LeTx (Liao and Mogridge, 2009). LeTx-induced processing of pro-IL-1β in cell lysates and appearance of mature IL-1β in the supernatant was prevented in cells treated with MG-132 and also in cells transfected with Nlrp1b allele 3. The requirement of proteasome activity and allele specific ability of Nlrp1b to promote pro-caspase-1 activation agrees with previous findings and validates the heterologous, reconstituted Nlrp1b inflammasome in fibroblasts (Boyden and Dietrich, 2006; Tang and Leplla, 1999). The heterologous reconstituted Nlrp1b inflammasome system in HT1080 fibroblasts has allowed my laboratory to demonstrate that Nlrp1b and pro-caspase-1 are necessary and sufficient to render a non-myeloid cell-type susceptible to pyroptosis in response to LeTx (Liao and Mogridge, 2009). The system has also allowed for structure-function analysis of Nlrp1b to understand the role of various domains of Nlrp1b in inflammasome assembly and pro-caspase-1 activation (Liao and Mogridge, 2009).

Nlrp1b contains an N-terminal NACHT domain, central LRRs, a FIIND domain whose function is not known, and a C-terminal CARD domain (Fig. 2). Deletion of the CARD domain abolished pro-caspase-1 activation potential of Nlrp1b in response to LeTx, presumably because the CARD domain is required for pro-caspase-1 binding. Transfection of certain Nlrp1b mutants along with pro-caspase-1 and pro-IL-1β caused mature IL-1β to be secreted into the supernatant in the absence of LeTx (Liao and Mogridge, 2009). I refer to these mutants either as constitutively active or as having potential to activate pro-caspase-1. Deletion of the LRRs is known to cause various NLRs to activate downstream effectors in the absence of their activating stimulus, presumably because auto-inhibition between the LRR and NACHT domains is
relieved. Consistently, deletion of the LRRs of Nlrp1b renders the protein constitutively active (Liao and Mogridge, 2009).

1.5.1 Rationale of project

Various N-terminal truncation constructs of Nlrp1b were made by Liao and Mogridge (2009). The nomenclature that I have used to define these mutants and the Nlrp1b mutants that I made are presented in Table 1. Truncation mutants of Nlrp1b are abbreviated by the letter N. Numbering of amino acid denotes its position according to allele 1 of Nlrp1b. Amino acids are denoted by single letter codes (Table 1). Deletion of the NACHT domain (N436) rendered Nlrp1b constitutively active. This suggested that in the absence of the NACHT domain, self-association of Nlrp1b could occur through another region of Nlrp1b. N1086 containing the CARD domain and N-terminal 56 amino acids in the FIIND domain was constitutively active, but N1142 containing the CARD domain did not activate pro-caspase-1. N1142 binds pro-caspase-1, but only N1086 was able to self-associate. Thus, the FIIND domain promotes oligomerization of N1086, thereby inducing pro-caspase-1 molecules into close proximity, facilitating proteolytic trans activation.

The aim of my project was to further characterize the FIIND domain of Nlrp1b and to understand its role in Nlrp1b inflammasome activation in response to LeTx.
Table 1. Nomenclature for N-terminal truncation mutants of Nlrp1b and for alanine substitution mutants of Nlrp1b1100-1233.

<table>
<thead>
<tr>
<th>Abbreviation of mutant</th>
<th>Nlrp1b N-terminal truncation mutant</th>
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<tbody>
<tr>
<td>N436</td>
<td>Nlrp1b436-1233</td>
</tr>
<tr>
<td>N720</td>
<td>Nlrp1b720-1233</td>
</tr>
<tr>
<td>N1086</td>
<td>Nlrp1b1086-1233</td>
</tr>
<tr>
<td>N1100</td>
<td>Nlrp1b1100-1233</td>
</tr>
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<td>N1102</td>
<td>Nlrp1b1102-1233</td>
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<tr>
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<td>Nlrp1b1142-1233</td>
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<tr>
<td>N1100-E0A,I1A</td>
<td>Nlrp1b1100-1233 E1100A,I1101A</td>
</tr>
<tr>
<td>N1100-K2A,L3A</td>
<td>Nlrp1b1100-1233 K1102A,L1103A</td>
</tr>
<tr>
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</tr>
<tr>
<td>N1100-7A</td>
<td>Nlrp1b1100-1233 1100EIKLQIK1106→AAAAAAA</td>
</tr>
</tbody>
</table>

1.5.2 Hypothesis

I hypothesize that the FIIND domain of Nlrp1b promotes self-association and pro-caspase-1 activation in response to LeTx of B. anthracis.

1.5.3 Summary of project

I identified N1100 as the smallest region that could self-associate and activate pro-caspase-1 in the absence of LeTx. Substitution of residues 1100EIKLQIK1106 with alanine in N1100 abolished self-association and pro-caspase-1-activating potential of N1100 without impairing binding to catalytically inactive pro-caspase-1-C284A. This suggests that residues 1100EIKLQIK1106 promote the ability of N1100 to activate pro-caspase-1 by inducing self-association of N1100. Substitution of residues 1100EIKLQIK1106 with alanine in (full-length) Nlrp1b prevented cell death and processing of pro-IL-1β into its mature form in response to LeTx. These data support the induced-proximity model of pro-caspase-1 activation whereby the FIIND domain of Nlrp1b promotes pro-caspase-1 activation by bringing pro-caspase-1 molecules into close proximity, which allows proteolytic trans activation.
Chapter 2

Results

2.1 $N_{1100}$ is the minimal region of Nlrp1b that constitutively activates pro-caspase-1 and self-associates

Previous studies had identified $N_{1086}$ as having the ability to activate pro-caspase-1 in the absence of LeTx (Liao and Mogridge, 2009). To identify the smallest constitutively active mutant of Nlrp1b, I tested various Nlrp1b truncation mutants (Fig. 4A) for their potential to activate pro-caspase-1 by reconstituting the Nlrp1b inflammasome system in HT1080 fibroblast cells, as previously described (Liao and Mogridge, 2009). Various Nlrp1b truncation mutants (Fig. 4A) were transfected into HT1080 cells along with pro-caspase-1 and pro-IL-1β, and 24 h after transfection, the medium was probed for HA tagged IL-1β by immunoblotting, and cell lysates were immunoblotted to test expression of Nlrp1b constructs. Appearance of ~17 kDa mature IL-1β-HA in the medium served as an indicator of pro-caspase-1 activation. In agreement with previous findings (Liao and Mogridge, 2009), IL-1β was detected in the medium of cells expressing $N_{1086}$ but not $N_{1142}$ (Fig. 4B). By testing several truncation mutants of Nlrp1b, $N_{1100}$ was determined to be the minimal region that induced IL-1β secretion while further truncation of two amino acids - $N_{1102}$ did not induce IL-1β secretion (Fig. 4B). Expression levels of Nlrp1b truncation mutants were similar. These results demonstrate that $N_{1100}$ is the smallest region of Nlrp1b that has potential to activate pro-caspase-1.
FIG. 4. \(N_{1100}\) is the minimal region of Nlrp1b that activates pro-caspase-1 and self-associates in the absence of LeTx. (A) Cartoon representations of various Nlrp1b truncation constructs. (B) Nlrp1b truncation constructs were cotransfected with plasmids pcDNA3-pro-caspase-1-FLAG (1 µg) and pcDNA3-pro-IL-1β-HA (1 µg) into HT1080 cells. After 24 h, cells were lysed and lysates were probed for T7-tagged Nlrp1b truncation mutants and β-actin by immunoblotting (IB); supernatants were immunoprecipitated (IP) with anti-HA antibodies and then probed for HA-tagged IL-1β by immunoblotting. (C) HT1080 cells were transfected with pcDNA3 His6-Nlrp1b-HA and pcDNA3 His6-Nlrp1b-T7 tagged Nlrp1b truncation constructs as indicated. Cells were lysed 24 h after transfection, and proteins were immunoprecipitated (IP) using anti-HA antibody, followed by immunoblotting with anti-T7 antibody and anti-HA antibody. (D) HT1080 cells were transfected with pcDNA3 His6-N\(_{1100}\)-HA and pcDNA3 His6-N\(_{1100}\)-T7. Cells were lysed 24 h after transfection, and proteins were immunoprecipitated using anti-HA antibody, or non-specific anti-Enhanced Green Fluorescent Protein (anti-EGFP) antibody followed by immunoblotting with anti-T7 antibody and anti-HA antibody. Blots are representative of three independent experiments.
According to the induced-proximity model of caspase activation, the NLR protein can self-associate and bind pro-caspase-1 thereby bringing pro-caspase-1 molecules into proximity. This interaction facilitates proteolytic \textit{trans} activation of pro-caspase-1. According to the induced-proximity model, the difference in the ability of N\textsubscript{1100} and N\textsubscript{1102} to activate pro-caspase-1 should correlate with the difference in ability to self-associate. Therefore, I predicted that N\textsubscript{1100} could self-associate while the ability of N\textsubscript{1102} to self-associate would be diminished. Co-immunoprecipitation experiments were performed to test self-association of various Nlpr1b truncation mutants. HT1080 cells were transfected with His\textsubscript{6}-Nlrp1b-HA and His\textsubscript{6}-Nlrp1b-T7 vectors containing truncation mutants N\textsubscript{1086}, N\textsubscript{1100}, N\textsubscript{1102}, and N\textsubscript{1142}. HA-tagged protein was immunoprecipitated and the associated T7-tagged protein was analyzed by immunoblotting (Fig. 4C and D). T7-tagged N\textsubscript{1086} and N\textsubscript{1100} immunoprecipitated with their HA-tagged versions, but self-association of N\textsubscript{1102} and of N\textsubscript{1142} was not detectable (Fig. 4C and D). These data suggest that N\textsubscript{1100} is the smallest region of Nlpr1b that self-associates.

2.2 Residues \textsubscript{1100}EIKLQIK\textsubscript{1106} are critical for pro-caspase-1 activation potential of N\textsubscript{1100} and for self-association

Analysis of the 56 amino acid region of Nlpr1b from residues 1086 to the beginning of the CARD domain at 1142 using secondary structure prediction program Jpred (http://www.compbio.dundee.ac.uk/www-jpred/) revealed a putative 6 amino acid beta strand at \textsubscript{1101}IKLQIK\textsubscript{1106} (Fig. 5A) (Cole et al., 2008). I speculated that the reduction in pro-caspase-1 activation potential and self-association between N\textsubscript{1100} and N\textsubscript{1102} may be due to disruption of the beta strand at \textsubscript{1101}IKLQIK\textsubscript{1106} in N\textsubscript{1102} (Fig. 4B and C).
FIG. 5. Residues 1100EIKLQIK1106 are critical for pro-caspase-1 activation by N1100
(A) Analysis of Nlrp1b between amino acid residues 1086 and 1141 by secondary structure prediction program Jpred. Residues highlighted in red denote alpha helix and residues in green denote beta strand. (B and C) Wildtype pNTAP-N1100-T7 or pNTAP-N1100-T7 with various alanine substitutions as indicated were cotransfected with plasmids pcDNA3-pro-caspase-1-FLAG (1 µg) and pcDNA3-pro-IL-1β-HA (1 µg) into HT1080 cells. Cells were lysed 24 h after transfection. Cell lysates were probed for T7-tagged Nlrp1b constructs and for β-actin by immunoblotting (IB); supernatants were immunoprecipitated (IP) with anti-HA antibodies and then probed for HA-tagged IL-1β by immunoblotting. Alanine substitution mutation of 1100EIKLQIK1106 is denoted by 7A. Blots are representative of three independent experiments.

I hypothesized that residues 1100EIKLQIK1106 would be critical for self-association and pro-caspase-1 activation potential of N1100 (Fig. 5A). I substituted residues 1100EIKLQIK1106 with alanine residues (7A mutation) in N1100 and tested whether the N1100-7A mutant had reduced pro-caspase-1 activation potential and lower levels of self-association. HT1080 cells were transfected
with either wildtype N\textsubscript{1100} or alanine-substitution mutants of N\textsubscript{1100} along with pro-caspase-1 and pro-IL-1\textbeta. Release of mature IL-1\textbeta was assessed in the medium by immunoblotting. Cells transfected with N\textsubscript{1100}-7A did not secrete mature IL-1\textbeta into the medium. However, IL-1\textbeta was present in the medium of cells transfected with N\textsubscript{1100} and N\textsubscript{1100}-N7A,K8A,K9A which contained alanine substitution of residues \textsubscript{1107}NKK\textsubscript{1109} that lie outside of the putative beta strand (Table 1 and Fig. 5 A and B). This suggests that residues \textsubscript{1100}EIKLQIK\textsubscript{1106} are important for pro-caspase-1 activation potential of N\textsubscript{1100}. Expression of Nlrp1b-7A was higher than expression of N\textsubscript{1100}, while N\textsubscript{1100}-N7A,K8A,K9A expression was undetectable in cell lysates (Fig. 5B). I have observed that Nlrp1b truncation mutants that constitutively activate pro-caspase-1 have a tendency to precipitate in the cell pellet following centrifugation of cell lysates. Immunoblot analysis is performed on the soluble fraction of cell lysates following centrifugation, which may explain the lower expression levels of N\textsubscript{1100} and N\textsubscript{1100}-N7A,K8A,K9A compared to N\textsubscript{1100}-7A.

In comparison to N\textsubscript{1100}, specific alanine substitutions of \textsubscript{1102}KL\textsubscript{1103} and \textsubscript{1104}QIK\textsubscript{1106} in N\textsubscript{1100} led to complete loss of mature IL-1\textbeta in the medium, and only a small amount of IL-1\textbeta was detected in the medium of cells transfected with N\textsubscript{1100}-E0A,I1A (Fig. 5C). These results demonstrate that residues \textsubscript{1100}EIKLQIK\textsubscript{1106} within the FIIND domain of N\textsubscript{1100} are critical in promoting pro-caspase-1 activation.

To test whether residues \textsubscript{1100}EIKLQIK\textsubscript{1106} are important for self-association of N\textsubscript{1100}, I made alanine substitutions in the \textsubscript{1100}EIKLQIK\textsubscript{1106} region of N\textsubscript{1100} and tested the mutants in a co-immunoprecipitation assay. HT1080 cells were transfected with His\textsubscript{6}-Nlrp1b-HA and His\textsubscript{6}-Nlrp1b-T7 pcDNA3 vectors containing either wildtype or alanine substitution mutants of N\textsubscript{1100}. T7-tagged N\textsubscript{1100}-7A did not co-immunoprecipitate with HA-tagged N\textsubscript{1100}-7A (Fig. 6A). However, T7-tagged N\textsubscript{1100} and N\textsubscript{1100}-N7A,K8A,K9A co-immunoprecipitated with HA-tagged
N_{1100} and N_{1100}-N7A,K8A,K9A, respectively (Fig. 6A). Alanine substitution of \textit{KL}, and \textit{QIK} in \textit{N}_{1100} abolished self-association (Fig. 6B). Similar to an incomplete loss of pro-caspase-1 activation by \textit{N}_{1100}-E0A,I1A compared to \textit{N}_{1100} (Fig. 4B), self-association of \textit{N}_{1100}-E0A,I1A showed a modest reduction compared to \textit{N}_{1100} (Fig. 6B). These data demonstrate that residues \textit{EIKLQIK} are critical for self-association of \textit{N}_{1100}. The ability of \textit{N}_{1100} alanine substitution mutants to self-associate correlates with their ability to activate pro-caspase-1. This supports the induced-proximity model of pro-caspase-1 activation by Nlrp1b.
FIG. 6. Substitution of residues \(1100^{\text{EIKLQIK}}_{1106}\) with alanine abolishes self-association of \(N_{1100}\) but does not affect its binding to pro-caspase-1. (A and B) HT1080 cells were transfected with pcDNA3 His\(_6\)-Nlrp1b-HA and pcDNA3 His\(_6\)-Nlrp1b-T7 vectors containing either wildtype \(N_{1100}\) or \(N_{1100}^{-\text{7A}}\) with indicated alanine substitution mutations. Cells were lysed 24 h after transfection, and proteins were immunoprecipitated (IP) using anti-HA antibody, followed by immunoblotting with anti-T7 antibody. Alanine substitution mutations of \(1100^{\text{EIKLQIK}}_{1106}\) is denoted by 7A. (C) HT1080 cells were transfected with pcDNA3 pro-caspase-1-T7-C284A and indicated Nlrp1b truncation mutants in pcDNA3 His\(_6\)-Nlrp1b-HA vector. Cells were lysed 24 h after transfection, and proteins were immunoprecipitated using anti-T7 antibody or a non-specific anti-EGFP antibody, followed by immunoblotting with anti-HA antibody and anti-pro-caspase-1 antibody (C, left panel). Lysates were immunoblotted with pro-caspase-1 antibody and anti-HA antibody to test expression levels of pro-caspase-1-C284A and Nlrp1b truncation constructs respectively. Lysates were also immunoblotted for β-actin (C, right panel). Casp-1, pro-caspase-1. * indicates His\(_6\)-N\(_{1142}\)-HA. EGFP, Enhanced Green Fluorescent Protein. Blots are representative of three independent experiments.
2.3 The 7A mutation does not impair binding of N\textsubscript{1100} to pro-caspase-1-C284A

I next tested whether the inability of N\textsubscript{1100-7A} to activate pro-caspase-1 was due to impairment in binding pro-caspase-1. I used catalytically inactive pro-caspase-1-C284A because co-expression of wildtype pro-caspase-1 with wildtype N\textsubscript{1100} causes pro-caspase-1 activation, as evident by presence of mature IL-1\textbeta in the medium (Fig. 4B). HT1080 cells were transfected with pro-caspase-1-T7-C284A along with HA-tagged N\textsubscript{1100} or N\textsubscript{1100-7A} or N\textsubscript{1142}, and 24 h after transfection, lysates were divided equally for immunoprecipitation with control anti-Enhanced Green Fluorescent Protein (anti-EGFP) or specific anti-T7 antibodies (Fig. 6C, left panel). Immunoprecipitated proteins were analyzed by immunoblotting for HA-tagged Nlrip1b proteins. Similar amounts of HA-tagged N\textsubscript{1100} and N\textsubscript{1100-7A} immunoprecipitated along with T7-tagged pro-caspase-1-C284A (Fig. 6C, left panel). The level of co-immunoprecipitation with T7 antibody was greater than non-specific pulldown with anti-EGFP antibody, demonstrating the specificity of the interactions. This data demonstrates that the inability of N\textsubscript{1100-7A} to activate pro-caspase-1 was not due to impaired binding to pro-caspase-1. Expression of various Nlrip1b truncation constructs and of pro-caspase-1-C284A is shown in Figure 6C, right panel. N\textsubscript{1142} did not bind pro-caspase-1-C284A, suggesting that residues 1100-1141 within the FIIND domain are required to bind pro-caspase-1.

2.4 Residues \textsubscript{1100}EIKLQIK\textsubscript{1106} of Nlrip1b are critical for Nlrip1b mediated pro-caspase-1 activation in response to LeTx.

I next tested the effect of the 7A mutation in full-length Nlrip1b on pro-caspase-1 activation in the presence of LeTx. To reconstitute the Nlrip1b inflammasome, HT1080
fibroblasts were transfected with Nlrp1b, pro-caspase-1 and pro-IL-1β for 24 h followed by treatment with LeTx (10^{-8} M LF and 10^{-8} M PA) for 3 h. As described previously (Fink and Cookson, 2005; Liao and Mogridge, 2009), LeTx-induced pro-caspase-1 activation can be measured by the processing of pro-IL-1β in cell lysates, appearance of mature IL-1β in the medium, and cell death as percent LDH present in the medium. In the absence of LeTx, HT1080 cells transfected with wildtype Nlrp1b or Nlrp1b-7A expressed similar levels of Nlrp1b, pro-caspase-1, and pro-IL-1β in cell lysates, and mature IL-1β was not detected in the medium (Fig. 7A and B). The appearance of wildtype Nlrp1b as a doublet and Nlrp1b-7A as a singlet is discussed in Chapter 3.2. Upon treatment with LeTx, cells transfected with wildtype Nlrp1b, but not Nlrp1b-7A, showed processing of pro-IL-1β in cell lysates and appearance of mature IL-1β in the medium (Fig. 7A). Cells transfected with wildtype Nlrp1b released 2% LDH while toxin-treated cells released 22% LDH; however, LDH release remained at 2% following toxin treatment of cells transfected with Nlrp1b-7A (Fig. 7C). These results demonstrate that substitution of residues EIKLQIK_{1106} with alanine in full-length Nlrp1b prevents cell death and pro-caspase-1 activation in response to LeTx.
FIG. 7. Substitution of residues 1100EIKLQIK1106 with alanine in full-length Nlrp1b inhibits LeTx-induced pro-caspase-1 activation and cell death. Combinations of wildtype pNTAP-Nlrp1b (1 µg) or pNTAP-Nlrp1b-7A and pcDNA3-pro-caspase-1-T7 (1 µg), and pcDNA3-pro-IL-1β-HA (1 µg) were transfected into HT1080 cells. Alanine substitution mutation of 1100EIKLQIK1106 is denoted by 7A. Approximately 24 h after transfection, cells were treated with LeTx (10^{-8} M LF and 10^{-8} M PA) for 3 h and supernatants were immunoprecipitated (IP) with anti-HA antibodies and then probed for HA-tagged IL-1β by immunoblotting (A). Cell lysates were also probed for HA-tagged pro-IL-1β (A) and pro-caspase-1 (B) and β-actin (A and B) by immunoblotting (IB); TAP-tagged Nlrp1b proteins were precipitated from cell lysates using streptavidin resin and immunoblotted using an antibody against calmodulin binding peptide (CBP) to detect the TAP-tagged Nlrp1b (B). Casp-1, pro-caspase-1. (C) Supernatants were assayed for LDH activity. Blots are representative of three independent experiments.
Chapter 3
Discussion

3.1 The FIIND domain promotes self-association of Nlrp1b and activation of pro-caspase-1

Previous studies have proposed that in comparison to N_{1086} the inability of the CARD domain to activate pro-caspase-1 is due to impaired self-association and not due to impaired pro-caspase-1 binding. This conclusion is based on the observation that pro-caspase-1-C284A bound to GST-N_{1142}, albeit less so than to GST-N_{1086} (Liao and Mogridge, 2009). I found that pro-caspase-1-C284A did not associate with N_{1142} in a co-immunoprecipitation assay (Fig. 6C). In previous studies, the presence of purified GST-N_{1142} at high concentration (~0.4mg) coupled to glutathione sepharose beads allowed the detection of bound pro-caspase-1-C284A (Liao and Mogridge, 2009). These findings collectively suggest that in comparison to N_{1086} and N_{1100}, N_{1142} associates weakly with pro-caspase-1. Therefore, the weak affinity between N_{1142} and pro-caspase-1-C284A and the inability of N_{1142} to self-associate explains why N_{1142} does not induce pro-caspase-1 activation (Fig. 4B). Since the CARD domain requires the N-terminal 42 amino acids within the FIIND domain to interact with pro-caspase-1-C284A (Fig. 6C), the FIIND domain may directly bind pro-caspase-1 or may alter the structure of the CARD domain thereby exposing pro-caspase-1 binding sites.

Liao and Mogridge (2009) suggested that, contrary to previous belief, the NACHT domain of Nlrp1b is not necessary for activation of pro-caspase-1. N_{1086} could self-associate and promote pro-caspase-1 activation suggesting that the FIIND domain promotes oligomerization of Nlrp1b. In the present study, I further characterized the FIIND domain of Nlrp1b.
I found that \( N_{1100} \) is the smallest fragment of Nlrp1b that can self-associate and promote pro-caspase-1 activation (Fig. 4). Alanine substitution of residues \( 1100EIKLQIK_{1106} \) in \( N_{1100} \) abolished self-association and pro-caspase-1 activation. However, \( N_{1100-7A} \) and \( N_{1100} \) bound similar amounts of pro-caspase-1-C284A (Fig. 5 and 6). These results suggest that the inability of \( N_{1100-7A} \) to promote pro-caspase-1 activation was not due to impaired binding to pro-caspase-1, but due to impaired self-association. These data support the induced-proximity model of pro-caspase-1 activation where residues 1100-1141 within the FIIND domain of Nlrp1b promote self-association. Self-association of Nlrp1b brings pro-caspase-1 molecules into close proximity, which promotes proteolytic \textit{trans} activation. Residues \( 1100EIKLQIK_{1106} \) are critical for promoting self-association of \( N_{1100} \). However, due to the absence of structural information about the FIIND domain, the specific intermolecular interactions that are promoted by residues \( 1100EIKLQIK_{1106} \) cannot be determined. It is important to note that in comparison to \( N_{1100-7A} \), oligomerization-proficient wildtype \( N_{1100} \) did not bind more pro-caspase-1-C284A (Fig 6C). This suggests that Nlrp1b oligomerization does not enhance pro-caspase-1 binding, but induces proximity of bound pro-caspase-1 molecules.

It is unclear whether \( N_{1100} \) self-associates because of FIIND-FIIND interactions or whether residues 1100-1141 of Nlrp1b promote CARD-CARD interactions by altering the conformation of the CARD domain. Data on human CARDINAL protein suggests that the FIIND domain may be an oligomerization domain. CARDINAL contains a FIIND domain and a CARD domain and can interact with CARDINAL-FIIND presumably through FIIND-FIIND interactions (Agostini et al., 2004). CARDINAL also causes pro-caspase-1 activation, suggesting that the FIIND domain may be the oligomerization domain that brings pro-caspase-1 molecules into close proximity. CARDINAL-FIIND and Nlrp1b-FIIND share 48% identity and 63% similarity.
suggesting that Nlpr1b_{FIIND} may also be an oligomerization domain. Future experiments should address the relative contribution of the FIIND domain and the CARD domain in mediating self-association of N_{1100}.

NLRP1 contains a PYD domain and NACHT domain at the N-terminus and a FIIND domain and CARD domain at the C-terminus (Fig. 2). It is conceivable that NLRP1 has two separate oligomerization domains for activation of different downstream effectors. In light of my findings that the FIIND domain promotes oligomerization of Nlrp1b, it is of interest to assess the contribution of the NACHT domain and FIIND domain towards activation of NLRP1.

Oligomerization through the NACHT domain may cause ASC recruitment and pro-caspase-1 activation by NLRP1, while oligomerization through the FIIND domain may cause pro-caspase-1 or pro-caspase-5 recruitment to the CARD domain followed by caspase activation. I speculate that residues 1331-1378 in NLRP1_{FIIND} domain, which have 55% identity and 67% similarity to residues 1100-1141 in Nlrp1b_{FIIND} domain, will promote oligomerization at the C-terminus of NLRP1 and cause pro-caspase-1 and/or pro-caspase-5 activation. Residues that correspond to $^{1100}$EIKLQIK$^{1106}$ of Nlrp1b are similar in NLRP1, $^{1331}$GIRLQVK$^{1337}$, so I expect that residues $^{1331}$GIRLQVK$^{1337}$ will promote proximity-induced activation of pro-caspase-1 and/or pro-caspase-5 by NLRP1.

Alanine substitution of residues $^{1100}$EIKLQIK$^{1106}$ in full-length Nlrp1b abolished pro-caspase-1 activation and cell death in response to LeTx. This demonstrates the importance of oligomerization through the FIIND domain in Nlrp1b activation. It also shows that when oligomerization through the FIIND domain is disabled by the 7A mutation, the NACHT domain cannot substitute as an oligomerization domain to promote pro-caspase-1 activation.

Oligomerization through the NACHT domain is required for downstream effector activation by
NOD1 (Inohara et al., 1999), NLRP3 (Duncan et al., 2007) and Apaf-1 (Hu et al., 1998). The NACHT domain is adjacent to the effector domains in NOD1, NLRP3, and Apaf-1. Therefore, the requirement for oligomerization through the FIIND domain rather than the NACHT domain for Nlrp1b activation may reflect a general requirement for the oligomerization domain of NLRs to be adjacent to the effector (CARD or PYD) domain.

3.2 Cleavage of Nlrp1b in the FIIND domain correlates with potential of Nlrp1b to promote pro-caspase-1 activation

In the following discussion subscript 1 and 3 are used for allele 1 and allele 3 of full-length Nlrp1b, respectively. Comparison of wildtype Nlrp1b1 and Nlrp1b1-7A in the IL-1β assay revealed that N-terminally TAP-tagged wildtype Nlrp1b1 migrated as a double band at ~135 kDa and ~115 kDa while Nlrp1b1-7A migrated as a single band at ~135 kDa (Fig. 4). The appearance of these bands was independent of LeTx treatment or pro-caspase-1 transfection (data not shown), so I suspect that a cellular protease cleaves Nlrp1b1. Despite the absence of a cleavage product in cells transfected with Nlrp1b1-7A, cleavage probably does not occur within the region 1100EIKLQIK1106 because Nlrp1b3 has residues 1039EIKLQIK1106 in the FIIND domain, yet it migrates as a single band at ~130 kDa (Liao and Mogridge, 2009). Based on the estimated molecular weight difference of the wildtype Nlrp1b1 bands, I predict that cleavage occurs within the FIIND domain ~20 kDa from the C-terminus of Nlrp1b1. It is intriguing to note that wildtype Nlrp1b1 is cleaved and can activate pro-caspase-1; whereas Nlrp1b1-7A and Nlrp1b3 are not cleaved and cannot activate pro-caspase-1 (Boyden and Dietrich, 2006; Liao and Mogridge, 2009). The significance of the correlation between susceptibility of Nlrp1b to cleavage and its potential to activate pro-caspase-1 is not known. In Chapter 3.3 I have outlined the approaches.
that can be taken to identify the cleavage site and test whether cleavage of Nlrp1b1 is required for pro-caspase-1 activation. After identification of the residue that is required for cleavage, the residue can be mutated in Nlrp1b1 and this cleavage deficient mutant can be tested for ability to promote pro-caspase-1 activation in the IL-1β assay.

If Nlrp1b1 activation occurs in cleavage-deficient mutants, then cleavage is not required for Nlrp1b1 activation. Cleavage of Nlrp1b1 and inflammasome activation would be independent events that occur due to a potentially common requirement of oligomerization through the FIIND domain. Oligomerization through the FIIND domain may expose a protease-sensitive site in Nlrp1b, hence, wildtype Nlrp1b1 may be cleaved at the exposed site while Nlrp1b1-7A cannot oligomerize through the FIIND domain rendering its cleavage site inaccessible to the cellular protease. I cannot extend the hypothesis of oligomerization-dependent cleavage to Nlrp1b3 because it is not known whether Nlrp1b3 oligomerizes through the FIIND domain.

Cleavage-deficient Nlrp1b1 mutant may not be able to activate pro-caspase-1 in the presence of LeTx, indicating that cleavage is required for inflammasome activation. After sensing of LeTx activity by Nlrp1b which relieves autoinhibition, cleaved Nlrp1b may have more freedom of movement to assemble a functional caspase-1 inflammasome. Alternatively, the C-terminal cleavage product of Nlrp1b1 may be released from its N-terminal counterpart and activate pro-caspase-1. Future experiments have been described that test this hypothesis.

3.3 Future Directions

To test whether there is a causal link between susceptibility of Nlrp1b to cleavage and its potential to activate pro-caspase-1, the cleavage site in Nlrp1b1 should be identified. A novel Nlrp1b mutant constructed in the laboratory has helped me predict putative cleavage sites. The
mutant is a fusion protein of residues 1-921 of Nlrp1b3 and residues 983-1233 of Nlrp1b1 (Fig. 8B). This Nlrp1b allele 3/1 (A3/1) mutant appears as a doublet at ~130 kDa and ~110 kDa and has pro-caspase-1 activation potential in the absence of LeTx (Liao, unpublished data). The A3/1 mutant is equivalent to Nlrp1b3 except for six single amino acid substitutions to their allele 1 residues (Fig. 8A), I hypothesize that one of the six amino acid differences confers susceptibility of A3/1 to cleavage and possibly with the potential to activate pro-caspase-1. The corollary to this hypothesis is that in the Nlrp1b allele 1 background, cleavage occurs at one of the following residues V988, A996, V1012, Q1014, N1026 or K1112 (Fig. 8A). To test the latter hypothesis, single amino acid substitutions of the aforementioned residues in Nlrp1b1 can be made to the equivalent Nlrp1b allele 3 residues. These Nlrp1b1 mutant proteins can be tested for expression as single bands or double bands in HT1080 cells. If a particular mutant is resistant to cleavage, its potential to activate pro-caspase-1 can be tested in the IL-1β assay.
**FIG. 8.** The A3/1 mutant differs from Nlrp1b allele 3 by 6 amino acids in the FIIND domain. (A) Alignment of Nlrp1b allele 1 and allele 3 from the beginning of the FIIND domain to the end of Nlrp1b performed using ClustalW multiple sequence alignment tool. Grey highlighting denotes the FIIND domain and green denotes the CARD domain. Allelic differences are marked by colon. Allelic differences between residues S984 and K1112 of allele 1 and the corresponding allele 3 residues are highlighted in red. The six residues highlighted in red comprise the difference between Nlrp1b allele 3 and the A3/1 mutant. Figure adapted from Clustal W alignment output. (B) Cartoon illustrates the A3/1 mutant made by fusion of residues 1-921 of Nlrp1b allele 3 and residues 988-1233 of Nlrp1b allele 1 respectively. Numbering of amino acids in blue and red correspond to the numbering in allele 1 and allele 3, respectively.
There remains a possibility that cleavage does not occur at one of the six predicted residues in Nlrp1b1. A different protease sensitive site may be exposed in wildtype Nlrp1b1, but not in Nlrp1b1-7A and Nlrp1b3 due to differences in the three dimensional folding of these proteins. Therefore, in addition to the targeted mutagenesis approach, an unbiased approach could be employed to identify the cleavage site. Nlrp1b1 will be cloned with a C-terminal TAP tag in pCTAP vector. Nlrp1b1-TAP would be expressed in HT1080 cells, and cleavage confirmed by immunoblotting using anti-CBP antibody. Nlrp1b1-TAP expressed in HT1080 cell extracts could be analyzed by SDS-PAGE followed by Coomassie Blue staining. The band of interest at ~28 kDa, including the 8 kDa TAP tag, would be excised and analyzed by Edman sequencing to obtain the N-terminal amino acid sequence. The protease that cleaves Nlrp1b1 could recognize an amino acid that is N- or C-terminal to the identified cleavage site; hence, both amino acids should be singly mutated in TAP-Nlrp1b1 to their Nlrp1b3 counterparts. Mutants should then be tested for cleavage and potential to activate pro-caspase-1, as previously described.

The C-terminal cleavage product presumably will have potential to activate pro-caspase-1, because the predicted cleavage site is between residues 988 and 1112 of Nlrp1b1 (see Chapter 3.3) and truncation mutants N720 (Liao and Mogridge, 2009) and N1100 are constitutively active (Fig. 4A). However, in the absence of LeTx, Nlrp1b1 is cleaved, but cannot activate pro-caspase-1 suggesting that the cleavage products do not dissociate. Whether the cleavage product is released upon LeTx sensing can be tested by expressing TAP-Nlrp1b1-T7 in the IL-1β assay. TAP-tagged proteins from LeTx treated and untreated cells can be precipitated from cell lysates and immunoblotted with anti-T7 antibody. A decrease in T7 signal in toxin-treated cells,
compared to untreated cells, would indicate that the T7-tagged C-terminal truncation product
dissociates from its N-terminal counterpart after LeTx sensing.

Another potential avenue for further investigation is to assess the relative contribution of
FIIND-FIIND and CARD-CARD interactions in N_{1100}. This can be done by comparing binding
of N_{1100-7} to N_{1100-1141}-EGFP-HA, N_{1100-1141-7A}-EGFP-HA and N_{1142-EGFP-HA} in the co-
immunoprecipitation assay.
Chapter 4

Materials and Methods

4.1 Cell lines

HT1080 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. PA and LF were purified as described previously (Go et al., 2009) and applied to cells at a final concentration of $10^{-8}$M.

4.2 Plasmid construction and site-directed mutagenesis

Construction of plasmids pcDNA3-T7, pcDNA3-His$_6$-HA, pcDNA3-His$_6$-T7, pN-TAP$_A$ Nlrp1b allele 1, pcDNA3 pro-caspase-1-T7, pcDNA3 IL-1β-HA and introduction of C284A mutation in pcDNA3-pro-caspase-1-T7 were described previously (Liao and Mogridge, 2009). pcDNA3-HA was constructed as described previously (Go et al., 2009). To construct pcDNA3-pro-caspase-1-FLAG, pcDNA3-pro-caspase-1-T7 was cut with ApaI and NheI to remove the T7 tag. FLAG-tag oligonucleotide was constructed by annealing forward oligonucleotide 5’-CATGGA CTA CAA GGA CGA CGA TGA CAA GG-3’ and reverse oligonucleotide 5’-CTA GCC TTG TCA TCG TCC TTG TAG TCC ATG GGC C-3’. The resulting annealed oligonucleotide was ligated at ApaI and NheI restriction sites of the restriction digested pcDNA3-pro-caspase-1-T7 vector.

pN-TAP$_A$-T7 plasmid was constructed by annealing T7-tag forward oligonucleotide 5’-TCG AGA TGG CTA GCA TGA CTG GA-3’ and reverse oligonucleotide 5’-CTA GGA CGA CGA TGA CAA GG-3’.
reverse oligonucleotide 5’-CCT AAC CCA TTT GCT GTC CAC CAG TCA TGC TAG CCA TC-3’. The resulting annealed T7 oligonucleotide was ligated at Xho1 and Apa1 restriction sites of pN-TAPB. Synthesized oligonucleotides were purchased from Integrated DNA Technologies.

Nlrp1b truncation plasmids were constructed by amplifying fragments from pN-TAPB - Nlrp1b allele 1. The reverse primer 5’-CGC CTC GAG TGA TCC CAA AGA GAC CCC ACC TG-3’ was used with the following forward primers to amplify the designated fragments: for N1086, 5’-CGC GGA TCC TT CCA ACT CTT CTC TGA GAT CTA C-3’; for N1100, 5’-CGC GGA TCC GAG ATT AAG CTG CAA ATC AAA AAC; for N1102, 5’-CGC GGA TCC AAG CTG CAA ATC AAA AAC; and for N1142, 5’-CGC GGA TCC CTG CAA ATC AAA AAC-3’ for N1142. The PCR products were digested with restriction enzymes BamHI and XhoI, and the resulting products were ligated into vectors pN-TAPB-T7, pcDNA3-His6-HA and pcDNA3-His6-T7.

To make the N1100 construct with alanine substitution of residues 1100EIKLQIK1106, the following PCR primers were used: forward primer 5’-CGC GGA TCC GCG GCG GCG GCG GCG GCG GCG AAC AAA AAG CAC ATG AAA CTC ATA T-3’ and reverse primer 5’-CGC CTC GAG TCC CAA AGA GAC CCC ACC TG-3’. The forward primer contained seven GCG sequences, coding for seven alanine residues. This sequence was followed by the nucleotide sequence starting at amino acid 1107 of Nlrp1b. This allowed substitution of 7 alanine residues at position 1100-1106 of N1100. The PCR product was digested with restriction enzymes BamHI and XhoI and the resulting products were ligated into vectors pN-TAPB-T7, pcDNA3-His6-HA and pcDNA3-His6-T7.
QuikChange site-directed mutagenesis (Stratagene) was performed according to the manufacturer's instructions. Alanine substitutions were made at residues $^{1100}$EI$^{1101}$, $^{1102}$KL$^{1103}$, $^{1104}$QIK$^{1106}$, and $^{1107}$NKK$^{1109}$ in pN-TAP$_B$-N$_{1100}$ using the following primer pairs: for $^{1100}$EI$^{1101}$ to alanine mutation, forward primer 5'-CAT CAC CAT GGA TCC GCC GCC AAG CTG CAA ATC AAA AAC-3' and reverse primer 5'-GTT TTT GAT TTG CAG CTT GGC GGC GGA TCC ATG GTG ATG-3'; for $^{1102}$KL$^{1103}$ to alanine mutation, forward primer 5'-GGA TCC GAG ATT GCG GCG CAA ATC AAA AAC AAA AAG C-3' and reverse primer 5'-GCT TTT TGT TTT TGA TTT GCG CCG CAA ATC C-3'; for $^{1104}$QIK$^{1106}$ to alanine mutation, forward primer 5'-CCG AGA TTA AGC TGG CCG CCG CCA ACA AAA AGC ACA TG-3' and reverse primer 5'-CAT GTG CTT TTT GTT GGC GGC GGC CAG CTT AAT CTC GG-3'; for $^{1107}$NKK$^{1109}$ to alanine mutation, forward primer 5'-CTG CAA ATC AAA GCC GCC GCG CAC ATG AAA CTC ATA TG-3' and reverse primer 5'-CAT ATG AGT TTC ATG TGC GCG GCG GCT TTG ATT TGC AG-3'.

The $^{1100}$EI$^{1101}$K$^{1102}$L$^{1103}$QIK$^{1106}$ to alanine substitution in pN-TAP$_A$-Nlrp1b was performed in three sequential mutagenesis reactions on the product of the previous reaction: $^{1100}$EI$^{1101}$, then $^{1102}$KL$^{1103}$ and then $^{1104}$QIK$^{1106}$ to alanine substitutions were made using the following primer pairs: $^{1100}$EI$^{1101}$ to alanine mutation forward primer 5'-GAG ATC TAC GTT GGA AAC ATG GGT TCA GCG GCG GAG CTG CAA ATC AAA AAC AAA AAG-3' and reverse primer 5'-CTT TTT GTT GAT TTG CAG CTT CGC CGC TGA ACC CAT GTT TCC AAC GTA GAT CTC-3'; $^{1102}$KL$^{1103}$ to alanine mutation forward primer 5'-GGA AAC ATG GGT TCA GCG GCG GCG CAA ATC AAA AAC AAA AAG-3' and reverse primer 5'-CAT GTG CTT TTT GTT GAT TTG CGC CGC CGC TGA ACC CAT GTT TCC-3'; $^{1104}$QIK$^{1106}$ to alanine mutation forward primer 5'-GGG TTC AGC GGC GGC GGC GGC...
GGC GGC GAA CAA AAA GCA CAT GAA ACT CAT ATG GGA AGC-3’ and reverse primer 5’- GCT TCC CAT ATG AGT TTC ATG TGC TTT TTG TTC GCC GCC GCC GCC GCC GCC GCC GCT GAA CCC-3’.

4.3 IL-1β and LDH release assays

One million HT1080 cells were seeded on a 10-cm dish the day before transfection. On the day of transfection, 1 µg each of pN-TAP\textsubscript{B}-Nlrp1b-T7, pcDNA3-pro-caspase-1-FLAG, and pcDNA3-pro-IL-1β-HA was transfected using 9 µl of 1 mg/ml polyethylenimine, pH 7.2. Approximately 24 h after transfection, cells were treated with LF (10\textsuperscript{-8} M) and PA (10\textsuperscript{-8}M) for 3 h. The cell supernatant was mixed with 1 µl of antihemagglutinin (anti-HA) antibody (Sigma-Aldrich H9658) overnight, followed by the addition of 100 µl of BSA-blocked protein A Sepharose beads (GE Healthcare) and a 2-h incubation. Proteins were eluted from the protein A Sepharose beads with sodium dodecyl sulfate (SDS) loading dye and subjected to immunoblotting using a polyclonal HA antibody (Santa Cruz sc805). Cells from each 10-cm plate were scraped into 300 µl of EBC lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.5% [vol/vol] NP-40, 1 mM phenylmethylsulfonyl fluoride). Each sample was sonicated 3 times, each for 10 seconds followed by 10 second incubation on ice. Lysates were clarified by centrifugation, and protein concentrations determined using the Bradford assay. Equivalent amounts of cell lysate protein (~40 µg) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-HA (Santa Cruz sc805), anti-T7 (Sigma 69522) and anti-β-actin (Sigma-Aldrich A5441) antibodies. Release of cytoplasmic LDH into the cell supernatant was measured by a CytoTox 96 nonradioactive cytotoxicity assay (Promega G-1780) in accordance
with the manufacturer’s instructions. The percentage of LDH released was calculated as 
\[(\text{experimental LDH} - \text{spontaneous LDH})/(\text{maximum LDH} - \text{spontaneous LDH})\] *100%.

4.4 Detection of TAP-tagged proteins

Three 10-cm dishes of HT1080 cells were transfected with 1µg of each of pcDNA3 pro-
caspase-1-T7, and pcDNA3 pro-IL1β-HA and wildtype pN-TAP\_A-Nlrp1b or pN-TAP\_A-Nlrp1b-7A. 24 h after transfection, cells were harvested and cell pellets from each plate lysed with 300 
µl EBC buffer by sonication. Cell lysates were clarified by centrifugation. Equivalent amount of 
cell lysate protein from three plates was incubated with 25 µl streptavidin agarose resin (Thermo 
Scientific 20349) for ~2 h. Beads were washed three times with 1 ml EBC buffer. Proteins were 
eluted with SDS and analyzed by immunoblotting with anti-Nlrp1b antibody.

4.5 Co-immunoprecipitation assay - self-association of Nlrp1b truncation mutants

To test self-association of Nlrp1b truncation mutants, two plates of HT1080 cells were 
transfected with pcDNA3-His\_6-Nlrp1b-HA and pcDNA3-His\_6-Nlrp1b-T7 vectors containing 
N\textsubscript{1086}, N\textsubscript{1100}, N\textsubscript{1102} or N\textsubscript{1142}.

To test the effect of alanine substitution of residues 1100EIKLQIK\textsubscript{1106}, 1100EI\textsubscript{1101}, 
1102KL\textsubscript{1103}, 1104QIK\textsubscript{1106}, and 1107NKK\textsubscript{1109} on self-association of N\textsubscript{1100}, two plates of HT1080 cells 
were transfected with pcDNA3-His\_6-N\textsubscript{1100}-HA and pcDNA3-His\_6-N\textsubscript{1100}-T7 vectors containing 
either wildtype or each alanine substitution mutant of Nlrp1b.
After transfection of cells for 24 h, cells were lysed in 300 µl EBC buffer by sonication, and lysates were clarified by centrifugation. Equal amount of proteins in lysates was incubated overnight with 1 µl of anti-HA antibody (Sigma-Aldrich H9658) or 1 µl of control anti-Enhanced Green Fluorescent Protein (anti-EGFP) antibody (Covance MMS-118R), followed by the addition of 50 µl of BSA-blocked protein A Sepharose beads (GE Healthcare) and a 2 h incubation at 4C. Complexes were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted using an anti-T7 antibody (Novagen 69522).

4.6 Co-immunoprecipitation assay - association between pro-caspase-1-C284A and N$_{1100}$, and N$_{1100}$-7A

Two plates of HT1080 cells were transfected with pcDNA3-pro-caspase-1-T7-C284A and pcDNA3-His$_6$-N$_{1100}$-HA or His$_6$-N$_{1100}$-7A-HA. Cells were lysed in 600 µl EBC buffer by sonication, and the lysates were clarified by centrifugation. Lysates were incubated with 1 µl of anti-T7 antibody (Novagen-Aldrich 69522-3) or 1 µl of control anti-EGFP antibody (Covance MMS-118R) overnight, followed by the addition of 50 µl of protein A Sepharose (GE Healthcare) for 2 h. Complexes were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted using an anti-HA antibody (Santa Cruz sc-805). Nitrocellulose membranes were stripped using Pierce Restore Western Blot Stripping Buffer (Pierce 21059) and immunoblotted using anti-caspase-1 p-10 antibody.
4.7 Antibodies

Rabbit antibody was raised against an N-terminal epitope of Nlrp1b-MEESPPKQKSNTKVAQHE. Membranes were probed with the following antibodies: anti-Nlrp1b polyclonal antibody (1:5000), anti-caspase-1 p10 (M20) polyclonal antibody (1:500, Santa Cruz Biotechnologies sc-514, anti-HA polyclonal antibody, (1:1000, Santa Cruz Biotechnologies sc-805), anti-T7-tag monoclonal antibody (Novagen 69522), and anti-β-actin monoclonal antibody (1:10000, Sigma Aldrich A-5441).

For immunoprecipitation, anti-T7-tag monoclonal antibody (Novagen 69522), and anti-HA monoclonal antibody H9658 (Sigma) were used.
References


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