FLUORESCENCE MICROSCOPY OF NLR INFLAMMASOME INTERACTIONS

A Major Qualifying Project Report submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the Degree of Bachelor of Science

in

Biology and Biotechnology

by

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November 1, 2012

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Abstract

Inflammation is initiated by the innate immune system to remove harmful stimuli and heal infections. Essential to this operation are multiprotein complexes, called inflammasomes, which process important inflammatory cytokines such as interleukin (IL)-1β and IL-18 for optimal host response to invasion. Inflammasomes are typically comprised of one or more nod-like receptor (NLR), a pro-caspase protease, and an adaptor molecule. NLRP3 and NLRP12 are NLRs with particular specificities towards certain pathogens and vaccines; however, many aspects of their activation remain unclear. To investigate a possible mode of assembly, we generated fluorescently tagged nod-like receptor pyrin domain-containing protein (NLRP) plasmids for transformation into cells. Expression of NLRP3 and NLRP12 inflammasome components was successfully observed by fluorescence microscopy.

Acknowledgements

I would like to express my upmost gratitude towards Dr. Egil Lien for allowing me opportunities to work in his lab and funding the tools necessary for this project. Likewise his advice has been vital for bringing the assignment to completion. I would also like to thank Gregory Valdimer for his aid throughout the experimental phase of the project. I am deeply indebted to him for the massive amount of help he imparted with experimental procedures and troubleshooting problems in addition to supplying the NLRP12-mCherry plasmid. Without his guidance the project could not have been completed. Greg has been an invaluable mentor to me for several years, and it was a pleasure working with him on this particular project. I would like to recognize Professor Destin Heilman for his assistance advising this project and editing the paper. As always, his sponsorship and counseling is greatly appreciated and has been instrumental in developing the final report. Finally, I would like to thank all other members who have provided me with advice and support through this process.
Introduction

Elimination of invasive pathogens is crucial for the survival of multicellular organisms. Accordingly, eukaryotic hosts have evolved various immune defense systems to sense and destroy invading microbes. Mammals employ two means to ensure protection: innate and adaptive responses. The innate immune system, which is conserved from plants to mammals, responds as the first line of defense. At the infection site non-specific stimuli are recognized to trigger inflammation, remove foreign substances, and activate the adaptive immune system. Through a series of highly specified processes adaptive immunity ultimately results in the formation of pathogen recognition and defense memories for future assaults.

Inflammation works to create a physical barrier against the spread of contaminants as well as heal injured tissue. Cardinal signs of inflammation occur immediately within minutes to hours of infection and include pain, heat, redness, and swelling possibly in conjunction with dysfunction of infected areas. Although inflammation is necessary to protect the host, overwhelming damage or chronic inflammation can in fact be detrimental. Numerous disorders have been associated with persistent inflammatory stimulation including several autoimmune and autoinflammatory diseases. This suggests close regulation of inflammatory responses is an essential component of proper host function.

The innate immune system relies on germline-encoded receptors, or pattern-recognition receptors (PRRs), to recognize preserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMP) of cellular stress. Several families of PRRs have thus far been identified: toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs). TLRs and CLRs can be found on the plasma membrane, while RLRs and NLRs reside in the cytosol of macrophages, monocytes, dendritic cells, neutrophils, and epithelial cells. Induction of inflammation is dependent on the TLR and NLR families.

TLR members, in addition to those of a larger superfamily that includes interleukin-1 receptors (IL-1Rs), are the main mammalian PRR sensors. Humans maintain ten TLRs (Table 1) each of which are structured with amino (N)-terminal leuine-rich repeats (LRRs) and up to two cysteine-rich regions (CRRs). In TLR units LRRs form a horseshoe shape for sensing and autoregulation. The CRRs facilitate ligand binding to a broad variety of conserved bacterial, virus, fungi, and protozoa markers. Along the same construct a short transmembrane region
extends from the CRR to a Toll/Interleukin (IL)-1R (TIR) cytoplasmic tail that regulates protein-protein interactions and signal transduction. TLRs operate either individually or as heterodimerers to recruit various TIR domain-containing adaptors upon stimulation.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
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</table>
| TLR1     | Triacyl lipopeptides (Pam$_3$CSK$_4$)  
          | Soluble factors  
          | OspA  
          | Porin PorB |
| TLR2     | Lipoprotein/lipopeptides (Pam$_3$CSK$_4$, MALP2SK$_4$)  
          | Peptidoglycan  
          | Lipoteichoic acid  
          | Lipoarabinomannan  
          | Phenol-soluble modulin  
          | Glycoinositolphospholipids  
          | Glycolipids  
          | Porins  
          | Atypical lipopolysaccharide (LPS)  
          | Zymosin  
          | Hsp70  
          | Hyaluronan  
          | Hemagglutinin |
| TLR3     | Poly (I-C) double-stranded RNA |
| TLR4     | LPS  
          | Taxol  
          | Fusion protein  
          | Envelope protein  
          | Flavolipin  
          | Hsp60  
          | Hsp70  
          | Type III repeat extra domain A of fibronectin  
          | Oligosaccharides of hyaluronic acid  
          | Polysaccharide fragments of heparin sulfate  
          | Fibrogen  
          | αA crystalline and HSPB8 |
| TLR5     | Flagellin |
| TLR6     | Diacyl lipopeptides |
| TLR7     | Imidazoquinolines (imiquimod, R-848)  
          | Bropirimine  
          | Guanosine analogs |
| TLR8     | R-848 |
| TLR9     | Unmethylated CpG DNA  
          | Chromatin-IgG complexes |
| TLR10    | Unknown |

Table 1: Human Toll-Like Receptors and Ligands | The human toll-like receptor (TLR) family consists of ten receptors: TLR 1-10. A broad array of bacterial, viral, fungal, plant, insect, host, and synthetic stimuli activate the various TLRs. Bacterial components are recognized by TLR1, TLR2, TLR4-6, and TLR9. TLRs 2-4 and 9 can ligate to viral components. TLR2, 4, and 9 are furthermore activated by fungi, plant, and insect factors, respectively. These three TLRs also recognize host components. Synthetic compounds have been found to simulate TLR2, TLR7, and TLR8. No stimuli of TLR10 have been found yet. Other TLR ligands may exist or may be under research. Adapted from: Gay and Gangloff, 2007.
These adaptors are crucial for TLR-mediated signaling, which results in nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) transcription proteins. Four proteins facilitate signal transduction: myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP) also known as MyD88-adaptor-like protein (MAL), TIR-domain-containing adaptor protein including interferon beta (IFN-β) (TRIF), and TRIF-related adaptor molecule (TRAM). TIRAP and TRAM provide specificity for TLR3- and TLR4-mediated signaling.

Typically, TLRs signal via MyD88-dependent pathways with the exception of TLR3, which exclusively operates by TRIF-dependent/MyD88-independent pathways. MyD88 utilizes death domain (DD) interactions to transmit activation signals through two major signal pathways. DDs are part of the death-fold domain family, which also includes the death effector domain (DED), caspase recruitment domain (CARD) and pryin domain (PYD). These domains are capable of forming dimers or multimers with other members of the same subfamily to mediate apoptosis signals.

One MyD88 signal pathway for NF-κB activation involves family members p65 and p105 successor molecule p50. p65 and p50 form a heterodimer inactive molecule directly attached to a nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) protein complex. Upon MyD88 signaling phosphorylation of IκB by multiprotein IκB kinase (IKK) allows NF-κB to move into the nucleus. The second pathway, for activation of AP-1, relies on mitogen-activated protein kinases (MAPKs). MAPKs rapidly respond within the cell to a diverse array of events to regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. When phosphorylated by mitogen-activated kinase kinases (MKKs) MAPK members c-jun N-terminal kinase (JNK) and p38 freely translocate to the nucleus where they in turn phosphorylate AP-1 residues to form a mature dimeric structure. Within the nucleus both NF-κB and AP-1 transcribe and release pro-inflammatory cytokines interleukin (IL)-1β and interleukin-18 (IL-18) to the cytosol.

Interleukin-1 beta (IL-1β) and Interleukin-18 (IL-18) are especially important pro-inflammatory cytokines that recruit phagocytic lymphocytes and endothelial cells to the infection site. Mature IL-1β has a strong ability to penetrate injured areas and adhere intercellular and endothelial molecules. IL-18 stimulates production of another cytokine with similar function called interferon-gamma (IFN-γ). Further, it enhances cytolytic activity of natural killer cells
NK), which attack invading, infected, or transformed cells as well as secrete toxic proteins to propagate immune response.\textsuperscript{140} These two cytokines have been linked to afflictions including fever syndromes, vitiligo, Crohn’s disease, gout, asbestosis, silicosis, Alzheimer’s disease, septic shock, and several other autoinflammatory and autoimmune disorders.\textsuperscript{49,131} Moreover, IL-1\(\beta\) related syndromes have responded to interleukin-1 receptor antagonist (IL-Ra) therapies.\textsuperscript{36,51} While the exact mechanisms leading to secretion are not fully understood several models proposed involve secretion by exosome vesicles and shedding of vesicles from or directly through the plasma membrane by unidentified transporters.\textsuperscript{10,18,102,108} Release of IL-1\(\beta\) and IL-18 is dependent on the conversion from inactive precursors pro-IL-1\(\beta\) and pro-IL-18, respectively, to their mature active form. Caspase-1 is fundamental for production of IL-1\(\beta\) and IL-18, although other independent mechanisms have been acknowledged.\textsuperscript{161}

Caspase proteases initiate protein catabolism and can be classified into subgroups based on whether their function is related to inflammation, group I, or apoptosis, group II or III.\textsuperscript{96} The human inflammatory caspases include caspase-1, caspase-4, caspase-5, and caspase-11.\textsuperscript{81,110} Caspase-1, previously known as IL-1\(\beta\) converting enzyme (ICE), is involved in cleavage of inactive interleukin pro-forms IL-1\(\beta\), IL-18, and IL-33 as well as pro-caspase-7. On top of its role in apoptosis activation, this inflammatory mediator facilitates pyroptotic cell death via cell lysis when triggered by DNA fragmentation or pore formation.\textsuperscript{47,92} Somewhat similar to mature IL-1\(\beta\) and IL-18, formation of caspase-1 involves the activation of zymogen pro-caspase-1.\textsuperscript{24,156} Pro-caspase-1 contains an N-terminal CARD followed by 10-kilodalton (p10) and 20-kilodalton (p20) domains. Upon activation apoptosis-associated speck-like protein containing CARD (ASC) recruits and autoprocesses pro-caspase-1 to a p10-p20 heterodimer structure with two active sites.\textsuperscript{24,44,108,171} ASC is an adaptor protein, made of PYD and CARD, that is necessary for caspase-1 activation.\textsuperscript{13,44,46,71,76,104,106,109,144} NLR members also function with ASC to process caspase-1.

The NLR family is comprised of twenty-three human genes primarily expressed by macrophages and dendritic cells. Its central nucleotide-binding oligomerization domain (NACHT/NOD) is structurally conserved and oligomerizes for signaling. NACHT is commonly flanked by carboxy (C)-terminal LRRs and an N-terminal effector domain.\textsuperscript{145} N-terminal domains include CARD, PYD, the baculoviral inhibitor of apoptosis repeat (BIR), and transactivator domain (AD).\textsuperscript{145} There exist four NLR subfamilies which can be classified based
on their respective N-terminus: NLRAs (CIITA), NLRBs (NAIP), NLRCs (NOD1-2, NLRC3-5, NLRX1), and NLRPs (NLRP1-14) (Figure I). NLRs with a CARD N-terminal recruit CARD-containing molecules to initiate transcription of pro-inflammatory genes, such as cytokines, whereas those with PYDs recruit other PYDs to bridge protein processing of pro-inflammatory caspases.\textsuperscript{152,153} Like other inflammatory components, the NLRs have extensively been associated with multiple disorders (Table II).

**Figure I: Human Nod-Like Receptor Subfamilies, Functions, and Associated Disorders** | Nod-like receptors (NLRs) are organized by into four subfamilies based on their N-terminal domain, which plays a role in signal transduction and protein-protein interactions. NLRA receptor class II, major histocompatibility complex, transactivator (CIITA) contains transactivator domain (AD); NLRB receptor neuronal apoptosis inhibitory protein (NAIP) holds baculoviral inhibitor of apoptosis repeat (BIR); NLRCs nucleotide-binding oligomerization domain-containing proteins (NOD) 1-2, and NLRC3-4 have caspase recruitment domain (CARD); and NLRPs NLRP1-14 contain pyrin domain (PYD). Additionally all NLRs have a nucleotide-binding oligomerization (NACHT) domain, that can oligomerize for signaling, and leuine-rich repeats (LRRs), which sense and bind ligands. NLR10 does not contain LRRs. Adapted from: Conforti-Andreoni, Ricciardi-Castagnoli, & Mortellaro, 2011 and *Nod-Like Receptors Review*, 2012.
<table>
<thead>
<tr>
<th>Function</th>
<th>Associated Disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIITA</td>
<td>Tans activates class II major histocompatibility complex</td>
</tr>
<tr>
<td>NAIP</td>
<td>Prevents motor-neural apoptosis</td>
</tr>
<tr>
<td>NOD1</td>
<td>Caspase-9-mediated apoptosis, Increases proliferation and effector function of T-cells</td>
</tr>
<tr>
<td>NOD2</td>
<td>Bacterial LPS recognition</td>
</tr>
<tr>
<td>NLRC3</td>
<td>T-cell activation</td>
</tr>
<tr>
<td>NLRC4</td>
<td>Apoptosis, Negative regulator of NF-κB</td>
</tr>
<tr>
<td>NLRC5</td>
<td>Regulates NF-κB, Type I &amp; II interferon signal pathways</td>
</tr>
<tr>
<td>NLRX1</td>
<td>Regulates MAVS-mediated response, NF-κB signaling</td>
</tr>
<tr>
<td>NLRP1</td>
<td>Caspase-9 and caspase-3 mediated apoptosis, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP2</td>
<td>Suppresses NF-κB, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Apoptosis, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP4</td>
<td>Regulates type I interferon signal pathway</td>
</tr>
<tr>
<td>NLRP5</td>
<td>Progression of zygote beyond primary embryonic cell divisions</td>
</tr>
<tr>
<td>NLRP6</td>
<td>Colon inflammatory response, Negative regulator of NF-κB, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP7</td>
<td>IL-1β secretion, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP8</td>
<td>Inflammation potential</td>
</tr>
<tr>
<td>NLRP9</td>
<td>Inflammation potential</td>
</tr>
<tr>
<td>NLRP10</td>
<td>IL-1β secretion, PYD-mediated apoptosis</td>
</tr>
<tr>
<td>NLRP11</td>
<td>Inflammation potential</td>
</tr>
<tr>
<td>NLRP12</td>
<td>NF-κB activation, Inflammasome assembly</td>
</tr>
<tr>
<td>NLRP13</td>
<td>Inflammation potential</td>
</tr>
<tr>
<td>NLRP14</td>
<td>Inflammation potential, Spermatogenesis potential</td>
</tr>
</tbody>
</table>

Table II: Functions and Associated Disorders of NLRs

Functions of all human NLRs are related to cell apoptosis, inflammation, or production of inflammatory mediators.
Multiple autoinflammatory and autoimmune disorders have been associated with malfunction of these receptors.

Several NLR or pyrin and HIN200 domain-containing protein (PYHIN) members along with a pro-caspase and, generally, ASC assemble to form multiprotein inflammasome complexes that initiate the processes of inflammation. Currently nine PRRs are known to form caspase-1 containing inflammasomes: PYHIN subfamilies absent in melanoma 2 (AIM2) and interferon-induced protein 16 (IFI16), and NLR constituents NLRC4, NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, and NLRP12 (Figure II).\(^4,6,8,3,13,9,14\) Only one homooligermized inflammasome per cell has been observed to form to date.\(^46\)

![Figure II: Caspase I Activating Inflammasomes and Activators](image-url)

*Figure II: Caspase I Activating Inflammasomes and Activators* | Nine inflammasomes are known to process pro-caspase-1 to mature caspase-1 upon assembly: absent in melanoma 2 (AIM2), interferon-induced protein 15 (IFI16), and nod-like
receptors NLRC4, NLRP1-3, 7, 6, and 12. Their homooligomerized structures with pro-caspase and apoptosis-associated speck-like protein containing CARD (ASC) adaptor protein are illustrated. Pyrin and HIN200 domain containing protein (PYHIN) subfamilies AIM2 and IFI16 are activated by double-stranded DNA. Particularly, IFI16 is assemblies in the presence of nuclear double-stranded DNA. NLR members NLRC4, NLRP1, NLRP2, NLRP7 and NLRP12 are stimulated by bacterial components. NLRP12 has only been found to respond to *Yersinia pestis*, the causative agent of plague. NLRP6 is activated by microflora. In addition to intracellular stimuli, NLRP3 recognizes a broad variety of pathogen and danger signals.

Two signals have been suggested as a requirement for inflammasome oligomerization. First, a priming signal via TLR pathways results in transcript of pro-IL-1β and pro-IL-18. Next a second signal leads to indirect stimulation of the NACHT domain, which self-oligomerizes to recruit ASC via its CARD or PYD. In turn, ASC uses CARD to autocleave pro-caspase-1 into caspase-1 for subsequent maturation and secretion of pro-IL-1β and pro-IL-18 cytokines or pyroptosis. Detailed mechanisms of inflammasome assembly and signaling remain unknown. Based on the vast number of inflammatory and immune related diseases and unclear understanding of inflammation processing it is obvious that the role of NLRs in inflammation and homeostasis begs for further inspection. This study will focus on the NLRP3 and NLRP12 inflammasomes.

NLRP3, also known as cyopyrin, NALP3, or PYPAF1, is the most extensively characterized inflammasome. It is activated by a number of structurally diverse ligands through sense of general cellular stress as well as direct and indirect recognition of activation signals. Stimulators include antibacterial, viral, fungal and parasitic microbial stimuli; crystalline or accumulated particulate substances such as asbestos, silica, uric acid, Abeta peptides, etc; pore-forming toxins, low intracellular potassium amounts, and/or calcium influx; extracellular adenosine triphosphate (ATP) or necrotic cell components; and reactive oxygen species (ROS). Three main models for activation of the NLRP3 inflammasome have been offered (Figure III). One mechanism suggests ATP acts as a DAMP released at sites of cellular injury and necrosis. Through the purinergic P2X7 receptor, an ATP-gated ion channel, this stimulates rapid potassium (K+) efflux, which in turn triggers recruitment and pore formation of pannexin-1 hemichannel. To counteract membrane depolarization by K+ efflux, calcium (Ca^{2+}) influx may be promoted. Either sense of low intracellular potassium, high intercellular calcium, breakdown of membrane integrity, or pore formation leads to NLRP3 inflammasome assembly. A second model proposes that incomplete clearance of phagocytosed particulates or crystalline material leads to membrane disruption. Phagosomal
destabilization then releases proteinase cathepsin B into the cytosol activating the inflammasome.\textsuperscript{38,125} The exact role of cathepsin B for activation is still under examination.\textsuperscript{38,125} All activators, including the two aforementioned means, induce ROS. Normally, ROS is produced through cellular metabolism in resting cells as a compound with thioredoxin-interacting protein (TXNIP) bound to oxidoreductase thioredoxin (TRX). Although ROS is not sufficient, a third model for NLRP3 activation believes cellular infection or stress increases ROS levels causing dissociation of TXNIP from TRX and increase in oxidative stress.\textsuperscript{22,30,38,133,175} Dissociation of TXNIP allows for ROS interaction and assembly of the inflammasome.\textsuperscript{175} Additionally, upregulation of NLRP3 is seen to positively affect activity of the inflammasome.\textsuperscript{13} A number of autoinflammatory cryopyrin-associated periodic syndromes (CAPS) are associated with this inflammasome’s activity including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome, neonatal-onset multisystemic inflammatory disease (NOMID), chroming infantile neurological cutaneous and articular syndrome (CINCA), and familial Mediterranean fever.\textsuperscript{45,49,93,134,157} The inflammasome also plays a role in gout pathogenesis and tumor surveillance.\textsuperscript{85}

Figure III: NLRP3 Inflammasome Activation | Assembly of the NLRP3 inflammasome requires two signals. (1) Pro-interleukin (IL)-1β and pro-IL-18 must first be transcribed and secreted to the cytosol. Two toll-like receptor (TLR) and interleukin-1
receptor (IL-1R) stimulated pathways involving toll/interleukin-1R (TIR) containing adaptor proteins such as myeloid differentiation primary-response protein 88 (MyD88) or TIR-domain-containing adaptor protein including interferon beta (TRIF) are activated for pro-cytokine transcription by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1) transcription proteins. TIR-domain-containing adaptor protein/MyD88-adaptor-like protein (TIRAP/MAL) and TRIF-related adaptor molecule (TRAM) provide specificity some TLR-mediated signaling. NF-κB translocates to the nucleus after nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) to phosphorylate IκB from NF-κB family member p65 and successor p50. Similarly, AP-1 forms in the nucleus after phosphorylation of c-jun N-terminal kinase (JNK) and p38 by mitogen-activated kinase kinases (MKKs). (2) A variety of secondary signals initiate inflammasome oligomerization with ASC and pro-caspase-1. Signals include danger-associated molecular pattern (DAMP) adenosine triphosphate (ATP) that stimulates rapid potassium (K+) efflux through the purinergic P2X7 receptor, which in turn triggers pore formation of pannexin-1 hemichannel. K+ efflux may promote calcium (Ca2+) influx. Additionally, incomplete clearance of phagocytosed particulates or crystalline materials can trigger phagosomal membrane destabilization and release of cathepsin B to the cytosol. These activators induce reactive oxygen species (ROS) to release thioredoxin-interacting protein (TXNIP) from oxidoreductase thioredoxin (TRX). TXNIP interacts with NLRP3 during inflammasome assembly. Upon assembly pro-caspase-1 is rapidly autoprocessed to caspase-1, which then either begins pyroptosis or cleavage of pro-IL-1β and pro-IL-18 to mature cytokines IL-1β and IL-18 and secretion of active cytokine forms.

Adapted from: R&D Systems, 2010.

Monarch-1, also known as NLRP12, has not extensively been studied. Notably, it was the first NLR to show interaction with ASC for formation of an IL-1β mature inflammasome by biochemical assay. In addition to caspase-1-dependent cytokine processing, NLRP12 plays a role in regulation of NF-κB and hypersensitivity. Curiously, NLRP12 expression differs in various diseases where it may exhibit either inflammatory or inhibitory functions. In some cases, such as hereditary period fever (HPF), mutations in the domain are associated with gain-of-function, similar to that of cyopyrin-associated periodic syndromes caused by mutations in NLRP3. However while the NLRP3 associated diseases result from single nucleotide missense mutations, additional NLRP12 periodic fever mutants have been identified as splice variants that show loss-of-function. Furthermore, this inflammasome is linked to collagen fibril degradation, tumors, complete x-linked congenital stationary night blindness, and resistance to Yersinia pestis.

Survival of Y. pestis within the host strongly relies on avoiding activation of the innate immune defenses. Interestingly, in addition to NLRP12, the bacterium has been found to stimulate other inflammasomes such as NLRP3, NLRC4, but not AIM2. Moreover, NLRP12 deficiency was not sufficient for complete reduction of mature cytokine secretion after its exposure. This, as well as structural similarities between NLRP3 and NLPR12, their link to
several inflammatory and immune diseases, and that both interact by ASC and pro-caspase-1 association suggests several inflammasomes may work together for optimal host response to pathogens.

Here we sought to complete expression studies by fluorescence microscopy of NLRP3 and NLRP12 for future analysis of a possible mode of inflammasome assembly. Cerulean and mCherry dyes were selected to generate NLRP3 and NLPR12 fluorescent tagged plasmids, respectively. To purify the product and decrease vector background the cerulean vector was treated with calf-intestinal alkaline phosphatase (CIP) prior to ligation with the NLRP3 insert. Plasmids were transfected into human macrophages at several overexpressed concentrations alone and together, but failed to show NLRP expression. Due to difficulties with macrophage transformation this cell type was abandoned. Following transfection of the plasmids into human embryonic kidney T-cells (HEK293-T) expression of NLRP3 and NLRP12 inflammasome components was successfully observed.
Materials and Methods

Preparation of NLRP Color Tagged Constructs

Vector pRZ-LmCitrz-FLAG was dephosphorylated with 10μL calf-intestinal alkaline phosphatase (CIP) for 60 min at room temperature and purified with MP Biomedicals Geneclean Spin Kit (cat. #1101-400). To create plasmid pRZ-mNLRP3-l-c-HA (NLRP3-cerulean), a 20μL total reaction with NLRP3 insert was ligated to the vector (1:6μL) using a New England BioLabs T4 DNA ligase (cat. #M0202S) and buffer (cat. #B0202S) for 15 min at room temperature. The mNLRP12huo-mCherpypRB (NLRP12-mCherry) plasmid was previously ligated (NLRP12 inserted in pRZ-LmmCherry-HA) by and obtained from Gregory Vladimer.

Plasmid Identification

NLRP3-cerulean was digested with New England BioLabs restriction endonuclease XhoI (cat. #R0146S) for 60 min at 37°C. Electrophoresis of plasmid was run on 3% agarose gel with a 1kb ladder. NLRP12-mCherry was digested with restriction enzymes XhoI and New England BioLabs BsiWI (cat. #R0553S). A 1% agarose gel with 100bp ladder was used for electrophoresis of plasmid. NLRP12-mCherry was sequenced by GENEWIZ.

Plasmid Growth, Replication, and Purification

Plasmid NLRP3-cerulean was transformed into Invitrogen Max Efficiency DH10 Bac chemically competent cells (cat. #10361-012) (1:50μL) and grown on lysogney broth (LB) ampicillin (AMP) plates overnight at 37°C. Single colonies were selected for mini culture growth in 3mL LB with 3μL AMP. Cultures shook at 37°C overnight and a total of 4.5mL was used for maxi culture growth. 200mL LB broth enriched with 200μL ampicillin and cell culture was allowed to shake at 37°C overnight. Qiagen Endofree Plasmid Maxi Kit (cat. #12362) was used for cell purification. Concentrations of plasmids were found to be 788.4ng/μL (1.89/2.27) NLRP3-cerulean and 644.3ng/μL (1.90/2.34) NLRP12-mCherry using a NanoDrop Spectrophotometer.
**Cell Culture**

Human embryonic kidney (HEK293) T-cells were grown in Lonza Biowhittaker™ Dulbecco’s Modified Eagle Medium (DMEM) with 4.5g/L glucose and L-glutamine (cat. #12-604F). 5% Fetal calf serum (FCS) and 10μg/mL ciprofloxacin (CIPRO) were supplemented into media. Cells grew to confluence at 37⁰C in 5% CO₂.

**Cell Plating**

First, media was removed from cells before washed with 3mL phosphate buffered saline (PBS). PBS was removed and 500μL trypsin was used to detach cells. Cells were spun at 2000 RPM for five minutes and pellet resuspended in 2mL DMEM+FCS+CIPRO media. A 3x3 grid hemocytometer was used to count cells. Cells were diluted to 8 x 10⁵ cells/well with the DMEM+FCS+CIPRO media. 250μL of cell media were plated into the center four square Lab-Tek® II Chamber Slide™ System glass chambers (cat. #154534). Cells incubated at 37⁰C in 5% CO₂ overnight.

**Cell Transfection**

Working stocks of plasmids were diluted to 10ng/μL with PBS. 25μL of Invitrogen GIBCO® DMEM high glucose 1x media with 4.5g/L D-glucose and L-glutamine but no sodium pyruvate (cat. #11965) was added to the determined amount of plasmid DNA in a 96 well plate and allowed to sit for 5 minutes. 5μL of a FCS-free DMEM and 4μL Novagen GeneJuice transfection reagent (cat. #70767 M00066223) mixture was added to the plates. After 15 min 50μL of plated cells was removed from the glass chambers and the transfection mixture was added. NLRP-color plasmids were transfected at various concentrations: NLRP3 – 10, 50ng/μL; NLRP12 – 10, 50, 100ng/μL; and NLRP3:NLRP12 – 10:10, 50:50, 10:50, 50:10, 100:100ng/μL. Plates were incubated at 37⁰C in 5% CO₂ for 24-48 hrs.
Cell Expression Analysis by Confocal Microscopy
To fix cells for imaging media was first removed and cells washed with 250μL PBS. PBS was then removed and cells fixed with 50μL PBS and 50μL USB paraformaldehyde solution in 4% PBS (cat. #11943) for 5 min. Fix solution was discarded and cells washed with 200μL PBS before chamber walls were removed from the glass slide. Leica TCS SP2 spectral confocal and multiphoton system was used for fluorescence microscopy analysis.

Figures and Images
Figures were created using Microsoft PowerPoint and Microsoft Paint. Confocal images were visually enhanced using Adobe Photoshop.
Results

Some members of the nod-like receptor (NLR) family assemble to create protein complexes capable of processing pro-caspase-1 to active caspase-1 for the subsequent maturation and secretion IL-1β and IL-18. Protease caspase-1 and cytokines IL-1β and IL-18 play especially important roles in inflammatory response of the innate immune system. Several autoinflammatory and infectious diseases have been associated with chronic and overwhelming inflammasome activity. Although inflammation is essential for the body and its malfunction can be devastating, relatively little is known about activation and assembly of inflammasomes. Recent studies have found only one inflammasome structure forms per cell; however some pathogens such as the plague agent, *Yersinia pestis*, can stimulate multiple inflammasomes. NLRP3 and NLRP12 are two inflammasomes activated by *Y.pestis*. Here we created fluorescent color tagged NLRP plasmids for use in expression studies by confocal microscopy and future experiments to understand possible mechanisms of inflammasome assembly.

First, appropriate fluorescent color tags needed to be attached to NLRP cDNA. Previously NLRP12 was ligated to vector pRZ-LmmCherry-HA. After digestion with restriction enzymes XhoI and BsiWI the NLRP12-mCherry plasmid was run on a 1% agarose gel with a 100bp ladder. Photography of gel (Figure IA) shows correct approximate band sizes of the vector at 1200bp and the NLRP12 insert at 1000bp. Additionally, to verify both color tag and cDNA were correctly ligated, the plasmid was sequenced by GENEWIZ (Figure IB). Excitation of the mCherry dye overlaps the emission of fluorescent dye cerulean; thus, it was chosen as the color tag for NLRP3. pRZ-LmCitz-FLAG was ligated to NLRP3 cDNA to make the pRZ-mNLRP3-l-c-HA plasmid. The NLRP3-cerulean plasmid was digested with XhoI and ligation was verified by gel electrophoresis on 3% agarose gel with a 1kb ladder (Figure IC). The NLRP3 fragment is seen between 7kb and 8kb. Due to lack of resources, NLRP3-cerulean ligation could not be verified by double digestion. Furthermore, although attempts to sequence the plasmid were made, sequencing results failed. A NanoDrop Spectrophotometer was utilized to find the plasmid concentrations to be 788.4ng/μL of NLRP3-cerulean and 644.3ng/μL NLRP12-mcherry.

Next, the plasmids needed to be transformed into cells for analysis. The NLRP plasmids were diluted to 10ng/μL working stocks with phosphate buffered saline (PBS). These stocks were used with a DMEM high glucose 1x media with 4.5g/L D-glucose and L-glutamine but no
sodium pyruvate and GeneJuice mix for the transformation. Plasmids were transfected into human embryonic kidney (HEK293) T-cells at various concentrations: 10 and 50ng/μL NLRP3-cerulean alone; 10, 50, and 100ng/μL of NLRP12-mCherry alone; and 10:10, 50:50, 10:50, 50:10, and 100:100ng/μL NLRP3-cerulean:NLRP12-mCherry. Transfected cells incubated between 24-48 hours at 37°C in 5% CO₂ prior to confocal viewing.

To view the cells by microscopy they were fixed to the glass slides with paraformaldehyde solution. The confocal system was adjusted to excite NLRP3-cerulean and NLRP12-mCherry at 450nm and 590nm, and produce emissions at 500nm and 630nm respectively. Areas with single layer cells are necessary for viewing fluorescence. Images of NLRP3 (Figure IIA) and NLRP12 (Figure IIC) transformed cells in single layer growth were photographed. The fluorescence displays at these points were adjusted to capture two images showing possible inflammasome assembly. NLRP3 inflammasome component assembly (Figure IIIB) at 50ng/μL overexpression alone and NLRP12 inflammasome component assembly (Figure IID) at the same concentration alone were observed. Both obtained fluorescent images showed background noise as indicated by the dispersed and weak fluorescence signaling. Somewhat steady signaling was seen in the areas of fluorescence grouping. The fluorescence images were overlapped on their corresponding single layer cell images to further examine the signals. The proposed NLRP3 expression (Figure IIE) and NLRP12 expression (Figure IIF) was seen to reside within the cells. Foremost this indicates that the pRZ-mNLRP3-l-c-HA plasmid was a successful ligation of NLRP3 cDNA into the cerulean color vector. Additionally, both plasmids show capability of exhibiting inflammasome component expression when transformed into HEK293 T-cells.
Discussion

Inflammation is a necessary attempt of the innate immune system to remove harmful stimuli and heal the host. However chronic or overwhelming inflammatory response can be detrimental to the host. Our current understanding of inflammation mechanisms and activation remains incomplete. The prevalence of inflammatory dysfunction attributed diseases confirms the need to further investigate this innate response.

Multiprotein complexes called inflammasomes have been found to initiate inflammation. They are constructed with members of the nod-like receptor (NLR) or pyrin and HIN200 domain-containing protein (PYHIN) family as well as a pro-caspase protease and apoptosis-associated speck-like protein containing CARD (ASC) adaptor protein. Inflammasomes involved in autoprocessing of mature caspase-1 are of particular importance because they lead to the cleavage of certain pro-inflammatory cytokines, which play a large role in inflammation initiation. Secretion of mature interleukin (IL)-1β and IL-18 are the result of caspase-1 processing inflammasomes. NLRP3 and NLRP12 are two subfamily NLR members capable of forming inflammasomes that lead to caspase-1 and subsequently IL-1β and IL-18. So far only one inflammasome per cell has been observed to form.\textsuperscript{153} Interestingly, some pathogens such as \textit{Yersinia pestis}, which is responsible for plague, stimulate multiple inflammasomes.\textsuperscript{162} In particular the NLRP3 and NLPR12 inflammasomes are two complexes that are activated. This raises several questions about the assembly of inflammasomes.

Previously, antibody microscopy studies of protein interactions have caused problems due to weak sensitivity. Instead of this traditional approach we considered fluorescence microscopy. Here we created fluorescent tagged NLRP3 and NLPR12 plasmids for expression studies. First, NLRP cDNA was ligated to a vector tagged with cerulean dye to produce plasmid pRZ-mNLRP3-l-c-HA. The mNLRP12huo-mCherrypRB plasmid was previously created and obtained for these experiments. Use of retroviral plasmids is a future option. NLRP12-mCherry was double digested and run on an agarose gel to roughly determine if the ligation was successful. Both the vector and insert fragments were seen on the gel. Additionally, the plasmid was sequenced to verify proper ligation. The NLRP3-cerulean plasmid was single digested and when run on an agarose gel showed the NLRP3 insert fragment. Sequencing of the plasmid failed despite several attempts, so the gel confirmation was determined to be sufficient to proceed. Nevertheless, it is recommended this plasmid is sequenced for verification purposes.
Both plasmids were then transfected into human embryonic kidney (HEK293) T-cells at various concentrations alone and mixed together. HEK293 T-cells are easily transfected; however more natural immune cells, such as macrophages, exist. Studies of expression in macrophages or other natural cells may aid in better understanding of pathogen activation of inflammasomes. The transfected cells were viewed using confocal microscopy. Inflammasome component expression of NLRP3 and NLRP12 alone at 50ng/µL concentration resulted, but the amount of background and weak signaling must also be considered. Foremost the results indicate the NLRP3-cerulean plasmid was successfully created. Mixed inflammasome component expression was not seen in any NLRP3-NLRP12 double transfected cells.

Cerulean and mCherry color dyes were ideal selections for fluorescence studies because the excitation wavelength of mCherry overlaps the emission wavelength of cerulean. Thus, the plasmids created for study of inflammasome expression in this project can be of use in future fluorescence resonance energy transfer (FRET) studies. FRET is a radiationless tool used to investigate molecular interactions. It relies on distance-dependent transfer of energy from donor to acceptor molecule to produce a resonant fluorescence. When the donor molecule, a dye or chromophore, absorbs the initial energy and transfers it to the acceptor molecule. This transfer of energy results in reduction of donor fluorescence intensity and excited state lifetime, and increases the acceptor’s emission intensity.\textsuperscript{60} Proper FRET analysis is dependent on certain primary conditions such as distance, fluorescence spectral overlap, and sufficient concentrations of both molecules. Since we know the fluorescent tagged plasmids are able to express the inflammasomes and appropriate color dyes were selected, favorable transformation concentrations of the plasmids should be found for mixed expression studies by FRET. Once the concentrations are determined this may be used to investigate whether several NLR family members homooligermize or assemble as heteroligomerized protein complexes for optimal host innate immune response.

Additionally, cells may need to be stimulated with inflammasome activators such as lipopolysaccharide (LPS). The results here, and with all overexpression studies, should be taken with caution. Overexpression of DNA may lead to spontaneous aggregation without ligand stimulation. Furthermore, overexpression of the protein tests on higher than normal protein levels, which is suboptimal for understanding real protein interactions. Overall, further studies are suggested to help pave way for infectious disease and inflammatory syndrome drug targets.
**Figure 1: NLRP cDNA Ligation to Color Tags** | NLRP cDNA was ligated to distinct color tags for fluorescence imaging. Plasmids were then digested and photographed after gel electrophoresis. (A) The NLRP12-mCherry plasmid was double digested and run on a 1% gel. NLRP12 and vector fragments are seen close to each other around 1,000bp. (B) Additionally, the plasmid was sequenced by GENEWIZ. Sequencing shows the mCherry fluorescence gene ligated prior to NLRP12. (C) The second plasmid was a NLRP3-cerulean ligation. NLRP3-cerulean was run on a 3% gel after single digestion and subsequently photographed. The gel shows the NLRP3 insert fragment between 7-8kb.
Figure II: Inflammasome Assembly | (A) NLRP3 transformed cells were photographed at single layer cell growth. (B) Expression of NLRP3 inflammasome components is represented by blue fluorescence color. Areas of clumped coloring is possible inflammasomes expression at 50ng/μL. (C) Similarly, single layer cells of NLRP12
transformed cells were photographed. (D) Fluorescent NLRP12 inflammasome component expression is seen as red coloring at concentration 50ng/μL. (E) Cells and NLRP3 fluorescence images were overlayed to show the full inflammasome component expression map. Fluorescence is seen to reside within the cells, supporting the belief of suspected inflammasome component expression. (F) The same was done for NLRP12 cells and fluorescent imaging, also showing fluorescence located within the cell.
References


23 Cebailos-Oivera, I, and M. Sahoo, and MA. Miller, and L. Del Barrio, and F. Re. (2011). Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1Beta is deleterious. *Public Library of Science Pathology*, 7 (12), pp. e1002452.


41 Duncan, JA, and X. Gao, and MT. Huang, and BP. O’Connor, and CE. Thomas, and SB. Willingham, and DT. Bergstrahl, and GA. Jarvis, and PF. Sparling, and JP. Ting. (2009).
Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. The Journal of Immunology, 182 (10), pp. 6460-6469.


Receptor-Mediated Activation in CD8 T Cells. *Public Library of Science Open for Discovery, 7* (7), pp. e42170.


143 Schneider, M, and AG. Zimmermann, and RA. Roberts, and L. Zhang, and KV. Swanson, and H. Wen, and BK. Davis, and IC. Allen, and EK. Holl, and Z. Ye, and AH. Rahman, and BJ. Conti, and TK. Eitas, and BH. Koller, and JP. Ting. (2012). The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling
adaptor TRAF6 and transcription factor NF-κB. Nature Immunology, 13 (9), PP. 823-831.


