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Manipulation of Host Signaling by Vector-Borne and Non-Vector-Borne Pathogens

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ABSTRACT OF THE DISSERTATION

Manipulation of Host Signaling by Vector-Borne and Non-Vector-Borne Pathogens

by

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Infectious diseases affect individuals all over the world. Both vector-borne and non-vector pathogens that cause these illnesses have developed strategies to subvert immune recognition. Since the innate immune system is the first line of defense against potentially noxious substances, the host is also able to adjust innate immune signaling in order to perpetuate its survival. This host-pathogen interaction is highly conserved, ranging from plants to mammals. Unfortunately, there remains quite a disparity between the plethora of pathogens and what is known about host signaling in response to their recognition. Deciphering the modes by which vector-borne and non-vector-borne pathogens influence host signaling will ultimately provide a clearer understanding of targets for protective measures against these life-threatening diseases.

The nod-like receptors (NLRs) are crucial components for host protection from a wide array of pathogen and danger associated molecular patterns. Whether they act alone or as a protein platform, NLRs effectively initiate key innate immune signaling cascades in order to promote the expression and/or secretion of pro-inflammatory genes and cytokines. Additionally, the inflammasome, a protein scaffold formed by NLRs/AIM2, the ASC adaptor molecule, and caspase-1 can elicit a potent form of cell death triggered by inflammation called pyroptosis. As a result, NLRs are very attractive to both the host and the pathogen: (1) for the host, it provides a method by which it can protect itself from intracellular detection of pathogens, while (2) for the pathogen, it is a target for manipulation so that it may propagate. This dissertation will provide an overview and insight regarding vector-borne and non-vector-borne pathogens and their effects on NLR signaling pathways.

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CHAPTER ONE

Introduction

1.1 Abstract

Nod-like receptors (NLR) are innate immune pattern recognition receptors involved in sensing microbial molecules and danger signals. Nod1 and Nod2 signaling result in nuclear factor (NF)-κB and MAP kinase (MAPK) activation, while caspase-1 associated NLRs regulate the inflammasome – an important protein scaffold that governs the maturation of interleukin (IL)-1β and IL-18. NLRs have been shown to recognize numerous intracellular pathogen-associated-molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Recently, several vector-borne pathogens have been shown to induce NLR activation. However, whether and how arthropod saliva counters NLR sensing, thus, inhibiting inflammation and facilitating pathogen transmission to the mammalian host remains elusive. Here, we provide a brief overview of NLR signaling and discuss clinically relevant vector-borne and non-vector-borne pathogens recognized by NLR pathways (Table 1.1 and 1.2). We also elaborate on NLR regulatory mechanisms and possible anti-inflammatory effects of arthropod saliva on NLR signaling and microbial pathogenesis.

1.2 Introduction

1.2.1 Summary

Vector-borne diseases affect individuals worldwide and, with their frequencies increasing, they are becoming a crucial public health problem in need of attention [1].

Table 1.1: Examples of vector-borne pathogens recognized by NLRs that affect human health.

Disease	Pathogen	Vector	Nod-like receptor	References
Malaria	*Plasmodium* spp.	*Anopheles gambiae*	NLRP3 Nod1 Nod2	[2 (a),3–8]
Dengue fever	Dengue virus	*Aedes aegypti*, *Ae. albopictus*	NLRP3	[9 (d),10 (b),11]
West Nile neuroinvasive disease	West Nile virus	*Culex quinquefasciatus*	NLRP3	[12 (e),13 (c),14–16]
Leishmaniasis	*Leishmania* spp.	*Lutzomyia longipalpis* *Phlebotomus papatasi*	NLRP3? NLRC4?	[17 (f),18]
Chagas disease	*Trypanosoma cruzi*	*Rhodnius prolixus*	Nod1	[19 (d),20,21]
Lyme Borreliosis	*Borrelia burgdorferi*	*Ixodes spp.*	Nod2	[22,23,24 (g),25]
Plague	*Yersinia pestis*	*Xenopsylla cheopis*	NLRP12 NLRP3 NLRC4	[26 (g),27–30]
Human granulocytic anaplasmosis	*A. phagocytophilum*	*Ixodes spp.*	NLRC4	[31 (h),32]
Tularemia	*Francisella tularensis*	*Dermacentor* spp., *Amblyomma americanum*	AIM2 NLRP3	[33 (i),34–38]
Yellow fever	Yellow fever virus	*Ae. aegypti*	NLRP1? NLRP3?	[39 (e),40 (j),41]
Lymphatic filariasis	*Wuchereria bancrofti* *Brugia* spp.	*Culex spp.*, *Anopheles spp.*, *Aedes spp.*, *Mansonia spp.*	Nod1 Nod2	[42,43 (k)]

? Potential association, needs further confirmation

Table 1.2: Examples of non-vector-borne pathogens recognized by NLRs that affect human health.

Pathogen	Nod-like receptor	References
Clostridium difficile	Nod1, NLRP3	[44,45]
Escherichia coli	Nod1, Nod2, NLRP3, NLRC4	[46–51]
Helicobacter pylori	Nod1	[52,53]
Listeria monocytogenes	Nod1, Nod2, NLRP3, NLRP6?, NLRC4, AIM2	[54–56]
Mycobacterium tuberculosis	Nod2, NLRP3	[57–62]
Streptococcus pneumoniae	Nod2, NLRP3	[63–65]
Toxoplasma gondii	Nod2, NLRP1	[66,67]
Bacillus anthracis	NLRP1	[68,69]
Candida albicans	NLRP3, NLRC4	[70–73]
Saccharomyces cerevisiae	NLRP3	[74]
Influenza virus	NLRP3	[75–77]
Salmonella typhimurium	NLRP3, NLRC4	[48,78]
Legionella pneumophila	NLRC4	[79–82]
Pseudomonas aeruginosa	NLRC4	[83,84]
Shigella flexneri	NLRP3, NLRC4	[48,85,86]

? Potential association, needs further confirmation

With more than 200 million affected individuals, malaria is spreading rampant in tropical and subtropical regions and dengue fever is following close behind. The spread of these illnesses, as well as other vector- borne diseases, has been attributed to rapid globalization, environmental changes, and the lack of effective vaccines [87]. These maladies have been combated by preventive care and therapeutics [88,89]. In order to develop novel treatments, scientists are continuously attempting to elucidate the mechanism of transmission of these dire organisms and aspects of the immune system that are being targeted by these pathogens [90]. Considering the variability between pathogens being passed from arthopod vector to host, one can imagine why the development of a vaccine has been an arduous task. However, vaccine development has taken a new route towards a common factor that all disease-transmitting vectors share: saliva [88]. To promote feeding, hematophagous arthropods rely on salivary proteins to not only impart anti-hemostatic capabilities but also anti-inflammatory properties [91]. The relationship between saliva and components of the immune system, such as Toll-like receptors (TLR), have been studied. However, one crucial element of the innate immune system that still remains vague, with regards to vector-borne diseases, are NLRs. NLRs are an evolutionarily conserved mechanism for pathogen recognition found in both plants and mammals [92]. Since their discovery, numerous groups have identified the role of NLRs in the recognition of self derived danger associated molecular pattern, such as ATP, and pathogen associated molecular patterns, such as those from fungi, bacteria, and viruses [93]. Only recently have researchers turned to the detection of these pathogens by NLRs; even more ambiguous is the connection between salivary proteins from these

vectors and NLRs. I will address the recognition of pathogens by NLRs, highlighting vector-borne pathogens, and discuss potential mechanisms by which saliva may modulate this interaction. Though not all-encompassing, my focus is on acknowledging major examples by which saliva can modify immunity during infection.

1.2.2 Arthropod saliva

Hematophagous arthropods have developed ways to promote the extraction of blood from their hosts while evading detection. The penetration of an arthropod mouthpart not only allows for the acquisition of a blood meal but also the release of saliva which contains proteins to assist in the process. Though some components of saliva are ubiquitous to all arthropods, some possess proteins found only in specific organisms [94]. For over a hundred years, researchers have identified and dissected the components of saliva and found it to contain anti-hemostatic and anti-inflammatory properties [95]. In order to maintain a fluid supply of blood, salivary proteins act as vasodilators, inhibitors of platelet activity, and anti-coagulants [96]. To avoid recognition by the host, saliva modulates the inflammatory response. Effects of tick saliva can been seen in a range of immune cell types, such as macrophages, neutrophils, T cells, and B cells [97–99]. As of recent, the specific pathways targeted by salivary proteins have started to emerge. I will summarize this by discussing the effects of salivary proteins in the context of mammalian immunity.

1.2.3 Nod-like receptors

Approximately two decades ago, a group of sensors were added to the pattern recognition receptor family, expanding what was known about intracellular recognition of endogenous and exogenous molecules [100]. Appropriately named nod-like receptors due to their characteristic nucleotide binding and oligomerization domain (NOD), NLRs may also contain leucine-rich repeats (LRR) at their N-terminus and a variable effector domain at their C-terminal end, all of which play a role in the recognition and response of the aforementioned molecules [101]. Although 22 human and 30 mouse NLRs have been discovered, to stay within our scope, I will only address Nods and NLRs that are able to form inflammasomes [101,102].

1.2.4 Nod1 and Nod2

Nod1 and Nod2 are crucial for the recognition of peptidoglycan components (Figure 1.1). Signaling through Nod1 and Nod2 begins with the initiation of Nod1 by D-glutamyl-meso-diaminopimelic acid (DAP) and/or Nod2 by muramyl dipeptide (MDP) [103,104]. While the NOD portion acts as a receiver in the presence of these pathogenic molecules, the effector CARD domain(s) of Nod1 and Nod2 transduces the signal by interacting with RIP2/RICK [105]. Classically, RIP2/RICK is polyubiquitinated by TRAF6, this signal is required for the recruitment of the adaptor molecules TAB2 and TAB3 and activation of TAK1 [106]. Together this forms the TAK1 complex that promotes the degradation of IκB, inhibitor of NF-κB, thereby allowing the translocation

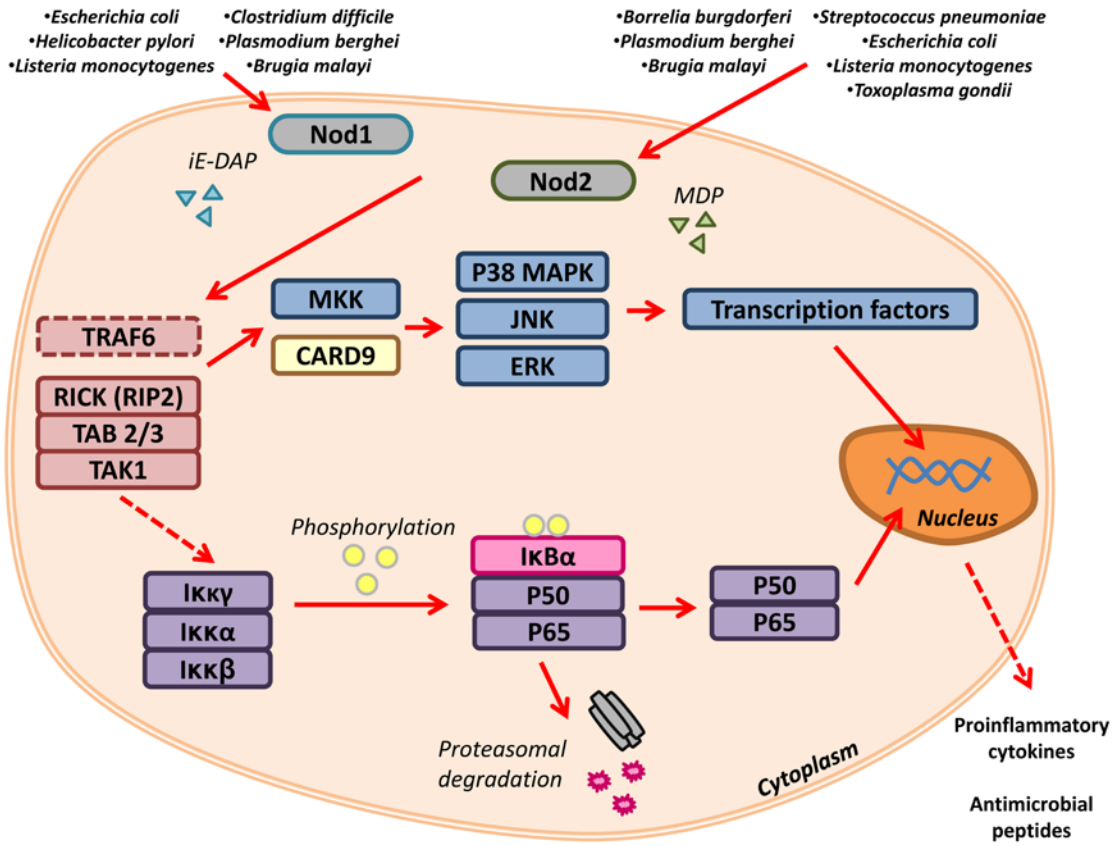

Figure 1.1: Nod1 and Nod2 signaling.

Nod1 and Nod2 are activated by the peptidoglycan components D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. Downstream of recognition, Nod1 and Nod2 signal for the recruitment of TRAF6, RICK/RIP2, TAB2/3, and TAK1. As the cascade progresses, two key pathways may be initiated: (1) MAPK and (2) NF-κB. MKK and CARD9 are key proteins for Nod-dependent MAPK signaling. The MAPKs P38, JNK, and ERK then signal to transcription factors to promote the expression of genes associated with cellular maturation, inflammation, regulation. Alternatively, the NF-κB pathway involves the sequestration of the NF-κB heterodimer (in this case, RelA/p65 and p50) in the cytosol. When Nod1 and Nod2 activate this pathway, the IκB kinase phosphorylates the inhibitor of NF-κB (IκBα). This modification signals the inhibitor for proteasomal degradation and reveals the nuclear localization signal of the NF-κB components. The dimer can then translocate to the nucleus to upregulate genes for pro-inflammatory cytokines and antimicrobial peptides.

of NF-κB into the nucleus. This is only one signaling cascade that is activated by the Nods, the MAPK pathway is another branch that can be driven by these NLRs. Nod1 and Nod2 can activate three key MAPK: ERK, JNK, and p38. The latter two can also be signaled by Nod2 through the adaptor CARD9 [107]. The activation of each

pathway results in the expression of pro-inflammatory mediators, such as cytokines and antimicrobial peptides. Nod1 and Nod2 can be regulated by A20-mediated ubiquitination and caspase-12 inhibition of RIPK2-TRAF6 complex formation [108,109].

1.2.5 Inflammasome

The inflammasome is a potent innate immune structure characterized by its ability to activate pro-caspase-1 in response to a microbe associated molecular patterns (MAMP) or DAMP (Figure 1.2). The inflammasome scaffold is created by the oligomerization and recruitment of several proteins. The receptor defines the inflammasome; it can originate from the NLR family or contain the HIN-200 domain [110]. Depending upon the receptor type, the adaptor molecule ASC may or may not be implicated. Since ASC possesses both a pyrin and CARD domain, it facilitates the association between the CARD-containing pro-caspase-1 and a receptor lacking the CARD domain [102]. Common to all inflammasomes is the presence of the enzyme pro-caspase-1. Caspase-1 is responsible for the maturation of the pro-inflammatory cytokines IL-1β and IL-18 and the inflammation-related cell death process termed pyroptosis [111]. Classically, inflammasome-mediated cytokine secretion is the product of a two-tiered signaling system (Figure 1.2) [112]. The first signal concerns the activation of the NF-κB pathway in order to promote the gene expression of IL-1β and IL-18 and other pro-inflammatory genes, such as *Nlrp3*. The second signal involves the assembly of the inflammasome, which results in the secretion of the previously mentioned cytokines.

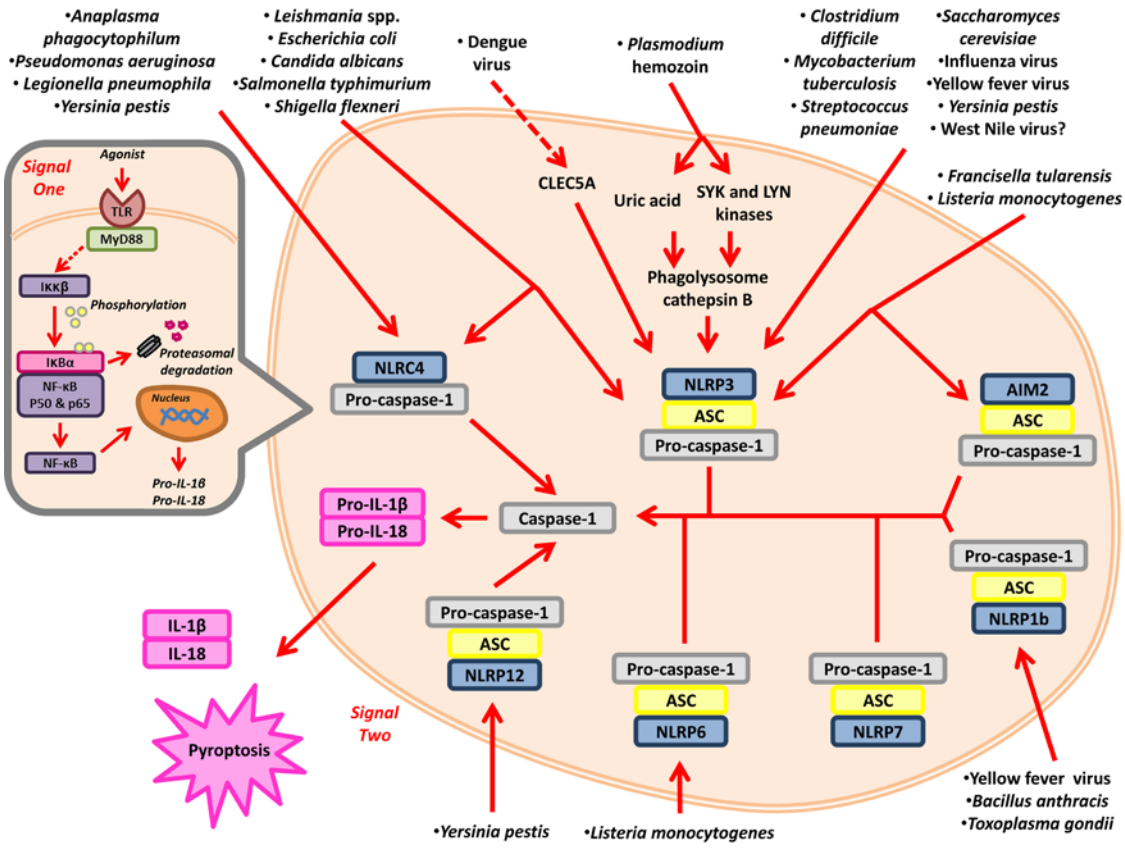

Figure 1.2: Inflammasome signaling.

Inflammasome signaling generally requires a two step activation process. The first step involves the initiation of the NF-κB pathway to increase the levels of *pro-IL-1β*, *pro-IL-18*, and *Nlrp3* transcripts. Activation by a TLR agonist is used as an example. The adaptor molecule MyD88 signals down through the cascade and triggers the phosphorylation of IκBα. This leads to the ubiquitination and proteasomal degradation of the inhibitor and release of the p50 and p65 NF-κB complex from isolation in the cytoplasm. The NF-κB heterodimer is then free to translocate to the nucleus. The second signal is the actual activation of the inflammasome. After activation of the inflammasome, signaling converges on the maturation of pro-caspase-1 to caspase-1 by autocatalytic cleavage. Caspase-1 then cleaves pro-IL-1β and pro-IL-18. These cytokines can be secreted in order to fuel inflammation. Inflammasome activation can also lead to inflammation-related cell death, pyroptosis.

Caspases other than caspase-1 have also been shown to be involved in the inflammasome signaling pathway. Caspase-11 was recently discovered to modulate caspase-1 in response to certain Gram-negative bacteria, such as *Citrobacter rodentium* [113,114]. Another non-canonical inflammasome involves caspase-8. Caspase-8 can be

a negative regulator of pro-inflammatory NLRP3 inflammasome activity [115]. During macrophage infection with *Francisella tularensis* subspecies *novicida*, caspase-8 can form a complex with AIM2 and ASC [116]. Caspase-8 associates with dectin-1 in the presence of fungi and mycobacteria [117]. Caspase-5 can also bind with an inflammasome, specifically NLRP1 [118]. Not only can caspases bind to the inflammasome, they can also be cleaved by the caspase-1 component of the protein scaffold, like IL-1β. This phenomenon is seen in caspase-7 activation by caspase-1, downstream of recognition by NLRC4, during *Legionella pneumophila* infection [119]. Multiple checkpoints are crucial for inflammasome regulation due to its strength as a pro-inflammatory initiator.

1.3 Recognition of pathogens by NLRs

Medically relevant vector-borne and non-vector borne pathogens have plagued the health of individuals all over the globe. Even more concerning is the rate at which these diseases are escalating and claiming the lives of thousands of people [120,121 (f)]. The relationship between these daunting pathogens and recognition by NLRs is not fully understood.

1.3.1 Nod1 and Nod2

Being one of the first NLRs discovered, many studies have been done to identify the role of Nods in the context of bacterial pathogenesis [103,122,123]. Research involving the sensing of bacteria in the intracellular compartment of a wide range of cell

types has dominated the Nod field. Since Nod1 and Nod2 recognize such conserved components found in Gram negative and Gram positive bacteria, researchers have been able to link a vast number of bacteria to Nods. To mention but a few, Nod1 is important for the production of inflammatory mediators in response to *Clostridium difficile*, *Escherichia coli*, *Helicobacter pylori*, and *Listeria monocytogenes* [44,46,52,54]. The host relies heavily on Nod1 during *C. difficile* infection. Nod1 deficiency results in decreased neutrophil recruitment and consequently increases mortality [44]. Colonic cells expressing dominant negative Nod1 are not able to induce NF-κB activation during infection with *E.coli* [46]. *H. pylori* recognition by Nod1 initiates type 1 IFN, which guides cytokine responses to limit the effects of *H. pylori* [52]. Activation of Nod1 is also important for MAPK and NF-κB- dependent IL-8 production after exposure to *L. monocytogenes* [54]. Nod2 is activated during, for example, *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* infection [57,58,63]. Recently, it was discovered that Nod2 mediates TNF-α and IL-1β secretion and the growth of *M. tuberculosis* in human cells. In addition, Nod2 signals the NF-κB pathway after recognition of *S. pneumoniae* [63]. I refer you to a more comprehensive review regarding Nod1 and Nod2 by Moreira et al. [101]. Recent developments have identified a new role for Nod1 and Nod2 in immunomodulation and the recognition of pathogens lacking peptidoglycan. Studies have reported that the Nod2 protein can respond to protozoan parasites, like *Toxoplasma gondii* [66]. Surprisingly, Nod2 has been shown to respond to single-stranded RNA [124]. The activation of Nod2 in this case is dependent upon the mitochondrial antiviral signaling protein MAVS and results in the facilitation of IRF3 mediated IFN gene

expression. Another protective measure that Nod1 and Nod2 are involved in is the induction of ATG16L1-dependent autophagy in response to bacterial invasion, such is the case with *Listeria monocytogenes* [55]. Most commonly acknowledged as a sensor for peptidoglycan molecules, there is also debate that Nod1 and Nod2 may possess regulatory abilities [125]. Nod1 and Nod2 are gradually revealing their complex nature. Studies regarding Nod1 and Nod2 function are continuously being assessed in order to develop a comprehensive understanding of these key proteins.

The Nods also play a role in the detection of many vector-borne-pathogens. Silva et al. were able to report that Nod1 is a crucial component for the resistance to the parasite *Trypanosoma cruzi* [21]. *T. cruzi* is transmitted by the kissing bug, *Rhodnius prolixus*, primarily in Latin American countries. It is the causative agent of Chagas disease, which can be characterized by fever, edema, or inflammation in the heart and/or brain [126 (a)]. Through the use of *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice, Silva et al. found that IL-12 and TNF-α levels were reduced after infection. Since nitric oxide is a key factor for *T. cruzi* containment, IFN-γ was used to treat *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ bone marrow-derived macrophages. This resulted in a high load for the *Nod1*$^{-/-}$ macrophage, highlighting the specificity of Nod1, not Nod2, for *T. cruzi* infection.

Borrelia burgdorferi is a spirochete transmitted by *Ixodes* spp. Infection *by B. burgdorferi* can cause Lyme disease, the most common vector-borne disease north of the equator [22,25,127]. Lyme disease can manifest into a three stage infection: (1) erythema migrans is characterized by localized infection, (2) early disseminated infection results in inflamed joints and CNS, and (3) persistent infection consists of chronic inflammation of

joints and the CNS and sensory polyneuropathy [25]. It has been established that TLR2 plays an important role in the recognition of *B. burgdorferi*. Recent evidence points to Nod2 as an important factor in the sensing of this pathogenic spirochete. Nod2 is upregulated in mouse microglia and individuals with mutated Nod2 were not able to mount an efficient cytokine response after infection with *B. burgdorferi* [128,129].

The Nods also seem to possess redundancy in that they are able to detect similar arthropod-borne pathogens. Individuals who encountered an antigenic component from the *Brugia malayi* adult demonstrated an increase in Nod1 and Nod2 expression [42]. *Wuchereria bancrofti* and *Brugia* species can cause lymphatic filariasis which can manifest as elephantiasis, lymphedema, and hydrocele. Independently, the obligate intracellular pathogen *Anaplasma phagocytophilum*, transmitted by *Ixodes* spp., is involved in the increased expression of Rip2, a critical molecule in Nod1 and Nod2 signaling [130]. More importantly, the ability for *Rip2*$^{-/-}$ mice to control and clear *A. phagocytophilum* was severely hindered. The *Plasmodium* parasite is also detected by Nod proteins [6]. Certain instances result in upregulation of Nods in the presence of *Plasmodium* sporozoites, while in other cases Nod1 and Nod2 confer changes in cytokines but do not promote survival after infection [7,8].

1.3.2 NLRP1 inflammasome

The NLRP1 inflammasome was the first to be characterized [118]. NLRP1 has been shown to recognize the *Bacillus anthracis* lethal toxin and, like Nod2, MDP [68,131]. The activation of pro-caspase-1 activity elicited by these bacterial components

is distinct. Cleavage of the NLRP1 inflammasome by the lethal toxin is required as mutation of the cleavage site by amino acid substitution resulted in desensitization [132]. On the other hand, MDP activation of NLRP1 requires the presence of MDP and ribonucleoside triphosphates [131]. It was observed that a cohort given a yellow fever vaccine showed upregulation of caspase-1 and caspase-5. These two caspases are present in the NLRP1 inflammasome. This indicates that the NLRP1 inflammasome may be activated by the yellow fever virus. This virus is transmitted by *Aedes aegypti*. Inoculation of yellow fever virus by a mosquito can lead to mild reactions, such as fever, ache, and nausea, or more serious ones, such as organ failure (Centers for disease control and prevention (b)).More studies need to be done in order to clarify what components trigger a NLRP1 inflammasome response to the yellow fever virus.

1.3.3 NLRP3 inflammasome

Of all NLRs, NLRP3, currently, has the most known associations with vector-borne diseases. It is well known that NLRP3 is triggered by: (1) potassium efflux, (2) phagolysosomal disruption, and (3) ROS production [102]. As of late, mitochondrial DNA, prokaryotic mRNA, and calcium levels were suggested to be other activators of the NLRP3 inflammasome [50,133,134]. A wide range of agonists initiate the NLRP3 inflammasome including, but not limited to: endogenous and exogenous particulate matter, ATP, *Candida albicans, Saccharomyces cerevisiae*, *Listeria monocytogenes*, influenza virus, and pore-forming toxins [135]. The number of NLRP3 inflammasome activators is immense and continues to grow. Therefore, it is unlikely that all these bind

directly to NLRP3; many studies have classified the mode of NLRP3 inflammasome activation based upon the three key mechanisms described above. For more information, please refer to these elaborate reviews by Vladimer et al. and Bauernfeind et al. [136,137]. Only recently has vector-borne-pathogen recognition by NLRP3 been described. The *Anopheles gambiae* transmitted *Plasmodium* parasite that causes malaria that is associated with fevers, anemia, and organ failure, has demonstrated the ability to activate the NLRP3 inflammasome through the crystalline particle hemozoin [3–5]. Monosodium urate (uric acid), together with hemozoin, has also been reported to result in pro-inflammatory reactions through the MAPK signaling pathway [4,5]. Hemozoin is a byproduct of heme detoxification by *Plasmodium*. The phagocytosis of hemozoin initiates signals through Syk and Lyn, tyrosine kinases, in order to initiate the NLRP3 inflammasome [4]. Another mosquito-borne pathogen, the dengue virus, is transmitted by *Ae. aegypti* or *Ae. albopictus*. Dengue virus can cause dengue fever or dengue shock syndrome. Wu et al. elucidated that, in human macrophages dengue virus can signal through Syk-coupled C-type lectin 5A (CLEC5A) to induce NLRP3-mediated cytokine secretion and pyroptosis [11]. Though not much is known about yellow fever virus and the inflammasome, one study shows that vaccination with a live attenuated yellow fever vaccine is able to increase the expression of caspase-1 associated with the NLRP3 inflammasome [41]. For the mouse model of West Nile virus infection, IL-1β is crucial for the protection of the CNS from West Nile neuroinvasive disease [16]. Specifically, it was shown that this is specific for NLRP3 inflammasome mediated IL-1β secretion. Additionally, IL-1β combined with type I IFN results in the reduction of West Nile virus

infection. Non-mosquito-borne pathogens also influence NLRP3. Infection by *Leishmania* spp., transmitted by *Lutzomyia longipalpis*, can result in skin, organ, and/or mucosal complications [17 (c)]. In murine macrophages, Sani et al. found that the expression of *Nlrp3* is increased after exposure to *Leishmania major* [18]. Another non-mosquito-borne pathogen is *Francisella tularensis*, which is commonly transmitted by ticks. Tularemia can cause sores and respiratory complications. Uniquely in human leukemia cell line (THP-1) but not mouse cells, *Francisella* is capable of activating the NLRP3 inflammasome [36]. Supporting this, the use of NLRP3 inflammasome inhibitors and *Nlrp3* siRNA revealed that the IL-1β secretion in response to *Francisella* was lessened. The type III secretion system (T3SS) from the plague causing *Yersinia pestis* is able to activate the NLRP3 inflammasome *in vitro* [29,30]. With the addition of KCl, the NLRP3 inflammasome activity was nullified. However, other inflammasomes are also involved in the detection of *Yersinia* as well.

1.3.4 NLRC4 inflammasome

The CARD-containing NLRC4 inflammasome mediates pro-inflammatory responses to the recognition of flagellin and type III/IV secretion systems [102]. NLRC4, previously called IPAF, inflammasomes confer protection against bacteria, such as *Salmonella typhimurium, L. pneumophila,* and *Pseudomonas aeruginosa* [79,83,84]. It is also able to directly and indirectly associate with pro-caspase-1, via its CARD domain or the adaptor molecule ASC, respectively. Additionally, another level of specificity is added by the NLRC4 interaction with NAIP5/6 or NAIP2, which modifies NLRC4

activation in response to flagellin and the rod portion of the type III secretion system, respectively [79,80]. NLRC4 has been implicated in the vector-borne illnesses human granulocytic anaplasmosis and leishmaniasis. *Nlrc4*$^{-/-}$ mice showed heightened susceptibility to *A. phagocytophilum* and decreased levels of IL-18 relative to the wildtype [138]. Sani et al. found that *Nlrc4* expression increased after exposing macrophages to *L. major* [18]. As was previously mentioned, *Y. pestis* is able to activate several inflammasomes, and it is also able to combat this recognition with effector proteins [29]. The NLRC4 inflammasome is another protein complex involved in the recognition of *Y. pestis*, via its T3SS [29].

1.3.5 NLRP12 inflammasome

The NLRP12 inflammasome is a member of the NLR family that has been suggested to reduce and potentiate inflammatory cytokine secretion [139–143]. Currently, NLRP12 has been shown to play a role in hereditary period fever syndromes; but with respect to pathogen detection, little is known [28]. Vladimer et al. discovered that NLRP12 regulates IL-1β and IL-18 secretion in response to *Yersinia pestis* [28]. After infection of *Nlrp12*$^{-/-}$ mice with *Y. pestis*, they observed an increase in bacterial load and death which was associated with decreased levels of IL-18 and IL-1β.

1.3.6 Non-NLR inflammasome

The AIM2 (absent in melanoma 2) inflammasome does not contain the typical NLR domain as do other inflammasomes, rather it possesses the HIN-200 domain [144].

The formation of the AIM2 inflammasome consists of the AIM2 receptor, ASC, and pro-caspase-1. In particular, AIM2 is known for sensing double stranded DNA in the cytosol [135]. This is demonstrated by the detection of viruses and intracellular bacteria, such as cytomegalovirus and *L. monocytogenes*. Mouse cytomegalovirus, a DNA virus, elicited IL-1β secretion which was highly dependent upon AIM2 recognition [145]. The release of *L. monocytogenes* DNA during escape into the cytoplasm is detected by AIM2 [146]. Upon recognition of cytoplasmic DNA, AIM2 is able to coordinate pyroptosis and the release of IL-1β and IL-18 via pro-caspase-1 maturation [111]. Of the vector-borne pathogens discussed here, AIM2 is able to recognize *F. tularensis* in mouse macrophages. Moreover, IRF3 is needed for a type 1 interferon response to help effectively activate AIM2 after *F. tularensis* infection [37].

1.3.7 NLRP6 and NLRP7 inflammasome

NLRP6 and NLRP7 are not as well characterized as their other pyrin-containing family member NLRP3. NLRP6 has been shown to be important for gastrointestinal health. NLRP6 deficiency results in decreased IL-18 in epithelial cells from the colon, as well as an increased risk for colitis [147]. Aside from its protective role, NLRP6 seems to be detrimental to the host during *L. monocytogenes*, *S. typhimurium*, and *E. coli* infection due to the increased number of circulating immune cells and decreased MAPK and NF-κB activation [148].

The NLRP7 inflammasome is distinctly found in humans but not mice. It was recently shown to recognize the PAMP acylated lipopeptide and causes the release of

pro-inflammatory inflammasome-mediated cytokines and pyroptosis [149]. Clearly, more needs to be done in this area in order to further define the signaling mechanisms.

1.4 NLRs and saliva

As previously mentioned, vector saliva is a tool used to promote successful acquisition of a blood meal. Inadvertently, it is also able to facilitate the transmission of vector-borne pathogens by manipulating the innate immune system during feeding (Figure 1.3). Overall, arthropod saliva is able to decrease activation, proliferation, differentiation, and maturation of immune cells. As a result, intra- and inter- cell signaling becomes skewed to favor host immune evasion and anti-inflammatory responses.

1.4.1 Salivary proteins

More specifically, arthropod saliva is composed of a plethora of salivary proteins that possess unique immunomodulatory functions (Table 1.3). Salivary proteins from numerous arthropods have been identified, such as those from: *Rhodnius prolixus, Rhipicephalus appendiculatus, Lutzomyia longipalpis, Ae. aegypti*, and *A. gambiae*, have been discovered. These proteins do not simply target one immune constituent but rather they span the gamut of cellular and molecular immunity. Evasins manipulate signaling by binding key chemokines thus inhibiting the production of cytokines [184,185]. The tick proteins ISL929, ISL1373, sialostatin L, IRS-2, Ir-LBP, and TSLP1 all target neutrophils, usually the first immune cell to respond to a pathogen [150,157–

159,166,169,171]. Antigen presenting cells are the focus of the following salivary proteins: sialostatin L, PGE_2, IRIS, Salp15, Ado, and maxadilan [25,89,98,157–159,161,162,168,172–174,188–190]. Histamine release factor (HRF) and histamine binding proteins (HBP) both act on granule releasing cells [163,164,175–177]. The complement cascade is a crucial factor involved in directing inflammatory responses through the formation of complexes on the pathogen surface, opsonization, and membrane-attack complex (MAC). ISAC, Salp20, IRAC I/II, TSLP1, and Salp15 can all inhibit the complement system [25,151,155,167,172–174,189]. Salivary proteins not only aim for the innate immune system, but it also acts on the adaptive immune system as well. Salivary components may act on T cells, B cells, or antibodies, as is the case with IL-2 binding protein, IsSMase, IRIS, BIP, Salp15, IgG-BP, and maxadilan [89,153,160,168,170,179–181].

Although some of these proteins have overlapping cellular targets, their activity at the molecular level demonstrate some variability. For instance, ISAC, Salp20, IRAC I/II, TSLP1, and Salp15 inhibit complement though through different mechanisms. ISAC, Salp20, and IRACI/II dissociates the crucial complement convertase molecule C3 [151,155,156,167,171,173–177,185,188,191,192]. However, TSLP1 and Salp15 target the complement pathway by inhibiting mannose-binding lectin and MAC, respectively [189]. Even within the same organism, salivary proteins can influence T cells in different ways. IL-2 binding, does as its name implies, blocks IL-2 while IsSMase affects T cells by increasing IL-4 [153,160]. In summary, immune regulation by arthropod vectors discussed here consists of: (1) impediment of attachment, (2) reduction of oxidants, (3)

decrease of pro-inflammatory enzymatic activity, (4) modification of cytokine levels, (5) attenuation of co-receptor binding, and (6) sequestration of pro-inflammatory mediators from binding to their receptors (Table 1.3).

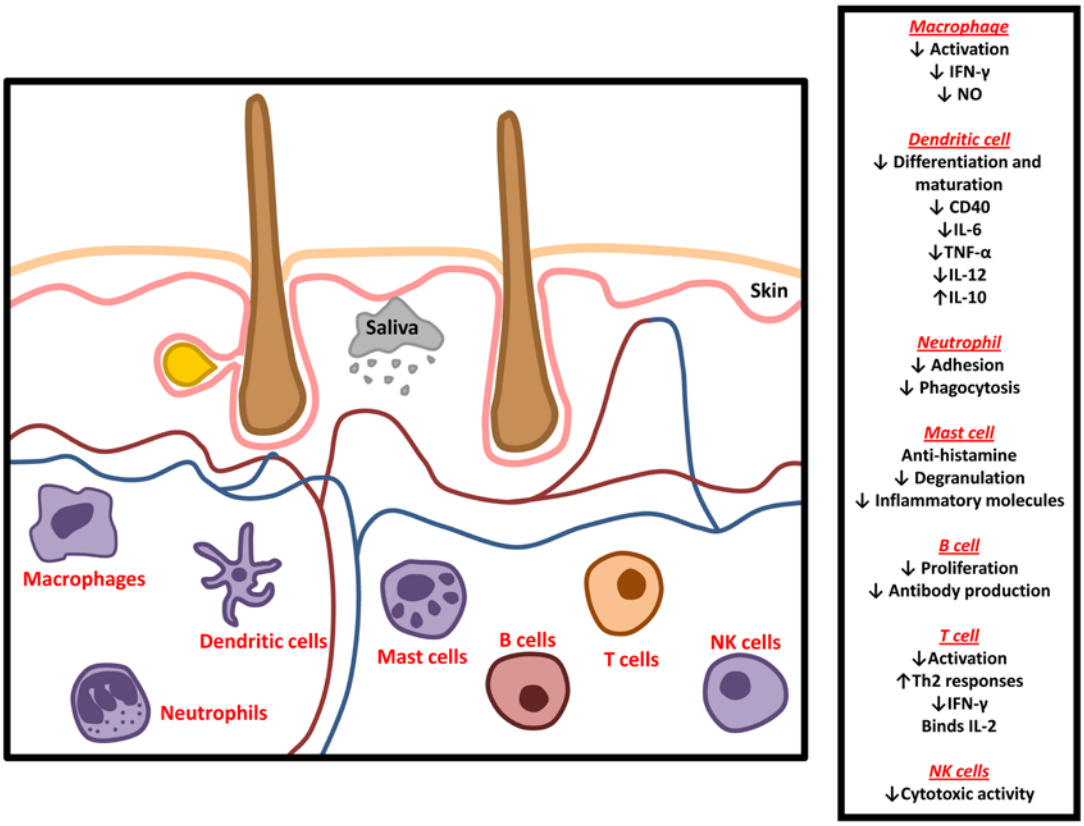

Figure 1.3: Tick saliva and its effects on immunity.

A variety of immune cells can be affected by arthropod saliva. In this schematic we have depicted macrophages, dendritic cells, neutrophils, mast cells, B cells, T cells, and NK cells. Cells have been placed arbitrarily below the skin in order to demonstrate that saliva influences cellular activity. The skin is delineated by the peach and pink lines. Glands are in yellow while brown is indicative of hair. Red and blue lines correspond to arteries and veins, respectively. Cells of the innate immune system are in purple while adaptive immune cells are not. A summary of the impact of saliva on immune cells is listed in the figure.

Table 1.3: Salivary components and immunity.

Protein component	Vector	Cellular	Molecular	Reference
ISL929 ISL1373	*I. scapularis*	Neutrophils	↓ Superoxide production ↓ β2-integrins	[150]
ISAC	*I. scapularis*	Complement	Dissociates C3 convertase	[151,152]
IL-2 binding protein	*I. scapularis*	T cells	Binds IL-2	[153]
Salp 25D	*I. scapularis*		Catalyzes the reduction of hydrogen peroxide with glutathione and glutathione reductase (antioxidant)	[154]
Salp20	*I. scapularis*	Complement	Dissociates C3 convertase	[155,156]
Sialostatin L	*I. scapularis*	Neutrophils, Dendritic cells	↓ Neutrophil influx, CD80/86, IL-12p70, TNF-α, MHC II, cathepsin L, IFN-γ, IL-17, T cell proliferation	[157–159]
IsSMase	*I. scapularis*	T cells	↑ IL-4	[160]
PGE_2	*I. scapularis*	Dendritic cells	↓ IL-12, TNF-α, CD40, inhibitor of differentiation Induces cAMP-PKA signaling	[161,162]
Histamine release factor (HRF)	*I. scapularis* *D. variabilis*	Basophils, Mast cells	Release of histamine	[163,164]
DAP-36	*D. andersoni*	T cells		[165]
IRS-2	*I. ricinus*	Neutrophils	Inhibits cathepsin G and chymase	[166]
IRAC I and II	*I. ricinus*	Complement	Dissociates C3 convertase	[167]
IRIS	*I. ricinus*	Monocytes, Macrophages, T cells	↓ TNF-α and IFN-γ	[89,168]
Ir-LBP	I. ricinus	Neutrophils (chemotaxis)	Binds leukotriene B4	[169]
BIP	*I. ricinus*	B cells	Inhibits B cell activation	[170]
TSLPI	*Ixodes* spp.	Complement, Neutrophils	Inhibits mannose-binding lectin	[171]
Salp15	*Ixodes* spp.	Dendritic cells, T cells, Complement	Raf-1/ MEK activation ↓ IL-6, TNF-α, and IL-12p35 CD4 binding ↓ T cell activation and IL-2 ↓ Membrane attack complex	[25,172–174]
Histamine binding proteins (HBP) Lipocalins	*Ixodes* spp. *Rh. prolixus*	Basophils, Mast cells	Binds histamine	[175–177]
Nitrophorins	*Rh. prolixus*		Binds histamine	[178]
IgG-BP	*Ixodes spp.* *R. appendiculatus*	IgG	Binds IgG	[179–181]
Maxadilan	*L. longipalpis*	T cells, Macrophages	↓ Nitric oxide, TNF- α ↑ Prostaglandin E2, IL-10, IL-6	[98]
Adenosine and Adenosine monophosphate	*P. papatasi* *R. sanguineus*	T cells, Macrophages, NK cells?, Dendritic cells	↓ Nitric oxide and IFN-γ	[162,182,183]
Evasin-1, -3, -4	*R. sanguineus* Tick spp.		Binds chemokines (Evasin-1:CCL3, CCL4, CCL18) (Evasin-3: CXCL8, CXCL1) (Evasin-4: CCL5, CCL11)	[184,185]
Ado	*R. sanguineus*	Dendritic cells	Induce cAMP-PKA to reduce cytokine production	[162]
D7 Proteins	*Ae. aegypti* *A. gambiae*		Binds histamine	[186]
Sialokinins	*Ae. aegypti*	T cell		[187]

1.5 Conclusion/Discussion

The importance of NLRs and vector saliva has been demonstrated through numerous elaborate studies by our colleagues. Further research in this area has the potential to reveal even more relationships between NLRs and vector-borne pathogens, as well as the salivary proteins that can modulate these interactions. I highlighted NLRs and salivary components in vector-borne diseases. Due to the vast amount of literature available in the field of sialomic studies, I have focused on those pertinent to the vectors discussed here. Elucidating the mechanisms behind NLR recognition and salivary modulation of vector-borne pathogenic agents may shed light on the fundamental basis of pathogen-vector-host interaction. Additionally, it may provide potential targets for therapeutic intervention of these devastating diseases, since this is an area in which we lack necessary vaccines.

Based on our current knowledge, arthropod saliva may regulate NLR inflammasome activity during transmission or after infection. Vector saliva has been shown to minimize reactive oxygen species (ROS) [150]. ROS has been identified as an agonist for the inflammasome, therefore salivary proteins can potentially reduce ROS to decrease activation. Another mechanism by which arthropod saliva can hinder the inflammasome is by acting on caspase-1. Caspase-1, the key enzymatic component of the inflammasome, is a member of the cysteine protease family. Salivary proteins have demonstrated the ability to target cysteine proteases, such as sialostatin L inhibition of cathepsin L [157]. Therefore, it is plausible that proteins of this nature block caspase-1 activation and subsequent IL-1β and IL-18 secretion. These are only two potential ways

for saliva to exhibit anti-inflammatory effects but many more are waiting to be discovered. A better understanding of salivary components regulating vector-borne pathogens and NLR interaction may allow us to gain a foothold on controlling these diseases.

CHAPTER TWO

A. phagocytophilum dihydrolipoamide dehydrogenase 1 (LPDA1) mitigation of macrophage cytokine secretion

2.1 Abstract

A. phagocytophilum, the causative agent of human granulocytic anaplasmosis, is an obligate intracellular pathogen transmitted by the *I. scapularis* tick. Human granulocytic anaplasmosis occurs irrespectively of pathogen load and results instead from host-derived immunopathology. Thus, characterizing *A. phagocytophilum* genes that affect the inflammatory process is critical for understanding disease etiology. By using an *A. phagocytophilum Himar1* transposon mutant library, a single transposon insertion into the *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 gene (*lpda1 - APH_0065*) affects inflammation during infection. LPDA1 correlated with enhanced reactive oxygen species from NADPH oxidase and nuclear factor (NF)-κB signaling in macrophages. These findings suggest that signaling pathways in macrophages are crucial for innate immune responses during *A. phagocytophilum* invasion and highlight the importance of LPDA1 as an immunopathological molecule.

2.2 Introduction

A. phagocytophilum is a tick-borne rickettsial pathogen that replicates inside myeloid and non-myeloid cells causing human granulocytic anaplasmosis (HGA) – an important tick-borne disease in the United States and Europe [193,194]. HGA clinical and laboratory findings are fever, myalgia, headache, malaise, thrombocytopenia, leukopenia, anemia, mild hepatic injury and splenomegaly. Symptoms vary from asymptomatic to mortality and may include septic shock-like syndrome, acute respiratory distress and opportunistic infections [194]. Infection results in hospitalization for 36% of

patients, whereas 7% of clinical cases lead to intensive care unit admission and 0.6% death [194].

A. phagocytophilum inflammatory response is induced by host innate immune mechanisms and does not directly correlate with pathogen load [195–198]. The importance of interferon (IFN)-γ for *A. phagocytophilum* immunity is well documented in IFN-γ-deficient mice. Hence, IFN-γ produced by Natural Killer (NK) and NKT cells contribute to pathogen defense [199,200] and mice deficient in IFN-γ are more susceptible to *A. phagocytophilum* [197,201]. During *A. phagocytophilum* infection, elevated IFN-γ levels are observed in the peripheral blood of severely ill patients [202]. Reactive nitrogen species causes damaging inflammatory histopathology [195] and commonly observed pathological features are decreased bone marrow function and changes in hematopoietic progenitor cells in the spleen most likely due to aberrant CXCL12/CXCR4 signaling [197,203,204]. IL-12/23p40, IL-18 and $CD4^+$ T cells are critical for pathogen elimination from the host [138,205]. Conversely, mice deficient in TLR2, TLR4, inducible nitric oxide synthase (iNOS), myeloid differentiation primary response gene 88 (MyD88), tumor necrosis factor (TNF), NADPH oxidase, perforin and Fas/FasL are all capable of clearing *A. phagocytophilum* infection but these molecules play a role in host-derived immunopathology [198,199].

The primary site of *A. phagocytophilum* infection in the mammalian host is neutrophils [32,193]. However, increasing evidence suggests that neutrophils do not seem to play a major role in *A. phagocytophilum* innate immunity. Neutrophils are significantly decreased during *A. phagocytophilum* infection and do not efficiently clear *A.*

phagocytophilum from the blood [194]. Furthermore, *A. phagocytophilum* colonization of neutrophils leads to impaired signaling and polarization [206], reduced binding to endothelial cells and IFN-γ signaling [207], transmigration [208,209], lowered phagocytosis and shedding of cell surface adhesion molecule receptors [210]. Macrophages are not a site of infection; however, these cells are important for combating *A. phagocytophilum* infection. *A. phagocytophilum* triggers pro-inflammatory responses in macrophages via NF-κB signaling through TLR2 [211] and animal models show increased macrophage infiltration and hemophagocytosis in tissues infected with *A. phagocytophilum* [212,213]. HGA clinical and histopathological features in patients also suggest macrophage activation [202].

This dichotomy between pathogen eradication and inflammation suggests that different immune cells regulate cytokine secretion or pathogen survival during *A. phagocytophilum* infection. These data also argue for the presence of molecules that regulate inflammation but not bacterial infection. In this study, I show that the *A. phagocytophilum* molecule LPDA1 affects inflammation in mice. LPDA1-derived immunopathology positively correlated with macrophage activation. These findings suggest that LPDA1 acts as an immunopathological molecule during *A. phagocytophilum* infection.

2.3 Materials and methods

Ethics statement

Animals were housed in the Animal Resources Facility according to the guidelines described under the federal Animal Welfare Regulations Act. Food and water were provided *ad libitum* and all animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California-Riverside. Mouse strains *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$ (from Millenium Pharmaceuticals) and caspase-1$^{-/-}$ (from Richard Flavell at Yale University) were previously described [214]. C57BL/6, *Nox2* (*gp91*^{phox})$^{-/-}$ and *il1r1*$^{-/-}$ mice were purchased from Jackson Laboratories. I used mice at 6-10 weeks of age.

Bacterial strains

The University of California-Riverside approved the use of *A. phagocytophilum* strains. The *A. phagocytophilum* wildtype HZ and the mutant *lpda1::*TnHimar1 strains were grown in HL-60 cells (ATCC CCL-240). Cells were maintained in Iscove's Modified Dulbecco's Media (IMDM) with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) (Thermo Scientific), 20% heat-inactivated fetal bovine serum (FBS) in 5% CO_2 and humidified air at 37°C. Cell lines were transformed to express mCherry and spectinomycin resistance under the control of the *amtr* promoter and flanked by the transposase recognition sequences using the Himar 1 transposon system. Construction of the transposase expression plasmid, bacterial transformation, selection and rescue cloning assays were performed as previously described [215]. A

single transposon insertion into the *A. phagocytophilum lpda1* sequence was detected by inverse PCR. Sequence annotation was determined by using the genome browser software Artemis [216].

Generation of bone-marrow derived cells

The generation of bone marrow derived macrophages (BMDMs) and bone marrow derived dendritic cells (BMDDCs) has been previously described [217,218]. For BMDMs, femurs and tibias of mice were flushed out with a 25G needle and DMEM (Thermo scientific) and then spun for 10 minutes at 4°C at 1500 revolutions per minute (rpm). Pellets were re-suspended in DMEM supplemented with 30% L929 cell-conditioned media and 10% FBS. Cells were then cultured in 10 cm petri dishes at 37 °C in a 5% CO_2 tissue culture incubator for 5–6 days, with fresh media added on day 3.

Confocal microscopy

Cells were stimulated with *A. phagocytophilum* strains. After washing twice with PBS, cells were stained with 1 μg/ml cholera toxin B (Molecular Probes) at 4°C for 10 minutes and fixed with methanol. Cells were blocked with 5% bovine serum albumin (BSA) in PBS and stained for 60 minutes with primary polyclonal antibodies – either *A. phagocytophilum* (1:100) (raised in rabbits at Yale University) or the nuclear localization signal of the p65 subunit of NF-κB (1:100) (Millipore). Cells were then washed with PBS and stained with fluorescence-conjugated secondary antibodies for 30 minutes at room temperature. Cells were mounted with Vectashield mounting media containing 4',6-

diamidino-2-phenylindole (DAPI). Confocal microscopy was done by using a Leica SP2 microscope.

Reactive oxygen species detection

Rotenone, antimycin and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma. I assessed reactive oxygen species (ROS) using the ROS-specific fluorescence probe 2'7'-dichlorofluorescin diacetate (H2DCFDA) (Invitrogen), as described [219]. Fluorescence was recorded in 96-well plates over time with a Perkin Elmer Victor 2 1420 multi-label counter using the fluorescein isothiocyanate (FITC) filter (excitation 485 nm, emission 538 nm). Mitochondria-associated ROS levels were measured by staining cells with MitoSOX (2.5 μM) (Molecular Probes) for 30 minutes at 37^{o}C. Cells were washed with Fluorescence Activated Cell Sorter (FACS) buffer and resuspended for analysis. Cells were analyzed using the BD FACSCanto II flow cytometer and the FCS Express analysis software (BD Biosciences).

Immunoblot analysis

Proteins were extracted in Radioimmunoprecipitation assay (RIPA) buffer with complete protease and phosphatase inhibitor cocktails from Roche. Protein concentration was measured using the protein assay kit from Bio-Rad. Total cell lysates were separated using sodium dodecylsulphate-polyacrylamide gel for electrophoresis and transferred onto PVDF membrane (Bio-Rad). Membranes were blocked and incubated with primary and secondary antibodies. Development was made by the enhanced chemiluminescence

(ECL) western blot analysis system from Pierce. Rabbit anti-β-actin antibody was purchased from Thermo Scientific. Rabbit anti-mouse IκB-α and mouse anti-mouse p-IκB-α was purchased from Cell Signaling. Densitometry was performed with the ImageJ open-source software (National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA)

Mouse Tumor Necrosis factor (TNF)-α, IL-1β and IL-6 were measured with the BD OptEIA Set from BD Biosciences. Mouse IFN-γ, macrophage inflammatory protein 2 (MIP-2) and IL-12p40 were measured with capture and detection antibodies from eBioscience.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Gaussian distribution was determined by the D'Agostino and Pearson normality test. For data points that followed a Gaussian distribution, the following parametric analyses were used: unpaired Student's t test (two-group comparisons); Two-way ANOVA (three or more group comparisons - two variables); Three-way ANOVA (three or more group comparisons - three variables). Kruskal-Wallis ANOVA was used for data points that did not follow a Gaussian distribution. Bonferroni (parametric). All statistical calculations were performed by using GraphPad Prism Version 5.04 and Minitab 16 (Three-way ANOVA). Graphs were made by using GraphPad Prism Version 5.04. $P<0.05$ was considered statistically significant.

2.4 Results

A. phagocytophilum LPDA1 modulates macrophage NF-κB-driven cytokine secretion

A. phagocytophilum-induced immunopathology is associated with macrophage activation [202,209,212,213]. Therefore, I investigated whether LPDA1 had any effect on BMDM stimulation. The mutant *lpda1::*TnHimar1 caused increased secretion of TNF-α and IL-12p40 when compared to the wildtype strain HZ (Figure 2.1A-B). Complementation studies for *A. phagocytophilum* are not available [193]. Therefore, I could not rescue the wildtype phenotype using classical bacterial genetics studies. However, the effect observed for the *A. phagocytophilum* mutant *lpda1::*TnHimar1 was not due to a transposon artifact. First, infection of the *A. phagocytophilum* harboring a Himar1 insertion in an irrelevant intergenic region did not affect secretion of NF-κB cytokines (*data not shown*). Second, secretion of IL-1β - a cytokine that is regulated by the inflammasome [220] - was not affected by the *Himar1* transposon when the *A. phagocytophilum* mutant *lpda1::*TnHimar1 was compared to the wildtype strain (Figure 2.1 C), suggesting that the cytokine effect observed in macrophages was specific for NF-κB signaling. Third, the mutant strain *A. phagocytophilum lpda1::*TnHimar phenocopied activation of the inflammasome during macrophage stimulation (Figure 2.1D-F; *data not shown*) [138]. *A. phagocytophilum lpda1::*TnHimar1 triggered reduced IL-1β secretion during pathogen stimulation of *asc*- and *caspase-1*-deficient macrophages when

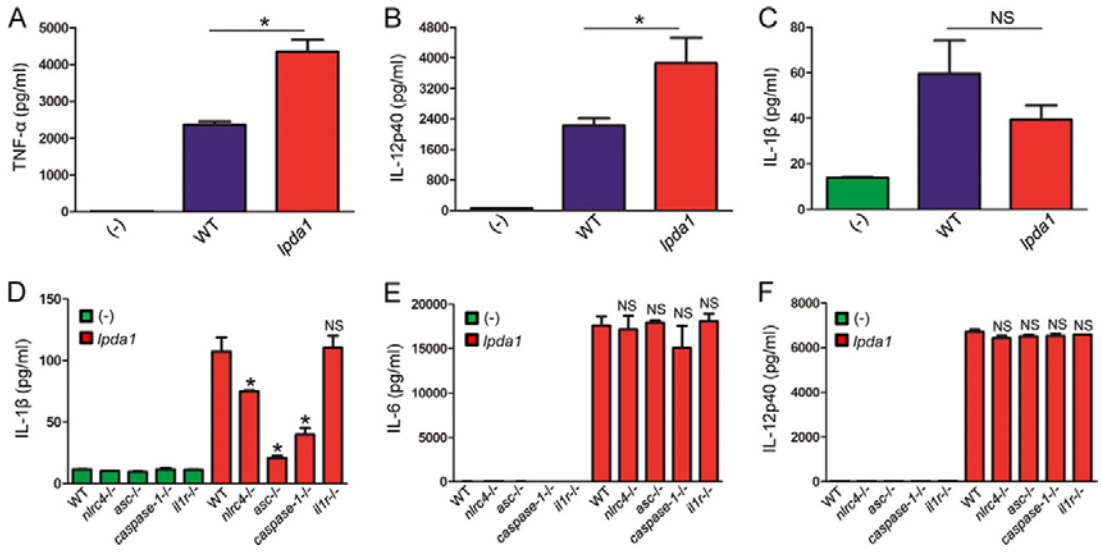

Figure 2.1: *A. phagocytophilum* LPDA1 inhibits NF-κB-mediated cytokine secretion in macrophages.

(A to C) BMDMs($1x10^6$) from C57BL/6 mice were stimulated with the wild-type HZ (WT) and *lpda1*::TnHimar1 (*lpda1*) *A. phagocytophilum* strains (MOI, 50) for 18 h. Supernatants were collected, and (A) TNF-α, (B) IL-12p40, and (C) IL-1β were measured by ELISA. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means ± SEM. (D to F) BMDMs ($8x10^5$) from wild-type, *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$, caspase-1$^{-/-}$, and *Il1r1*$^{-/-}$ mice were stimulated with the *lpda1 A. phagocytophilum* strain at an MOI 50 for 18 h. (D) IL-1β, (E) IL-6, and (F) IL-12p40 were measured by ELISA. Experiments were repeated at least twice. Comparisons were performed by one-way ANOVA—Bonferroni (knockout cells, WT); data are presented as means ± SEM. *, $P < 0.05$.

compared to wildtype cells (Figure 2.1D). NLRC4 was only partially required for IL-1β secretion by macrophages during *A. phagocytophilum lpda1::*TnHimar1 stimulation, as shown for *Nlrc4*-deficient macrophages (Figure 2.1D). The IL-1β receptor did not play a major role during *A. phagocytophilum* stimulation of macrophages, as mice deficient in the IL-1β receptor (*il1r1*) did not show any differences in IL-1β secretion when compared to wildtype cells during pathogen stimulation (Figure 2.1D). The effect of the inflammasome is restricted to IL-1β secretion [221]. Indeed, the secretion of IL-6 and IL-12p40 was not affected during infection of macrophages deficient in components of the inflammasome (Figure 2.1E-F).

A. phagocytophilum LPDA1 inhibits NF-κB activation in macrophages via reactive oxygen species (ROS) from NADPH oxidase

The regulatory effect of *A. phagocytophilum* LPDA1 on NF-κB in macrophages was confirmed by western blot and confocal microscopy. Time course series showed increased and more rapid NF-κB activation of BMDMs (as judged by faster phosphorylation, degradation of the inhibitor protein IκB-α and p65 translocation) when macrophages were stimulated with the *lpda1::*TnHimar1 mutant and contrasted to the wildtype *A. phagocytophilum* HZ strain (Figure 2.2A-D). Phosphorylation of IκB during stimulation with the *lpda1::*TnHimar1 mutant strain was twice the amount when compared to non-stimulated BMDMs at 6 hours (Figure 2.2C). However, phosphorylation of IκB increased only 50% when the wildtype *A. phagocytophilum* HZ strain stimulated BMDMs at its peak (Figure 2.2C). The increased effect of the mutant *lpda1::*TnHimar1 *A. phagocytophilum* on macrophage signaling also correlated with IκB degradation. The presence of IκB was reduced to 50% at its lowest level when the mutant *lpda1::*TnHimar1 strain stimulated macrophages, whereas there was only a 25% reduction in IκB degradation for BMDMs stimulated with the wildtype *A. phagocytophilum* HZ strain (Figure 2.2B and D). Enhanced NF-κB activation in macrophages during stimulation with the *lpda1::*TnHimar1 mutant correlated well with increasing numbers of the *lpda1::*TnHimar1 mutant inside the cells at 2 and 6 hours post-stimulation (Figure 2.2E). These results suggested that the *A. phagocytophilum* *lpda1::*TnHimar1 mutant was eliminated faster by BMDMs when compared to the

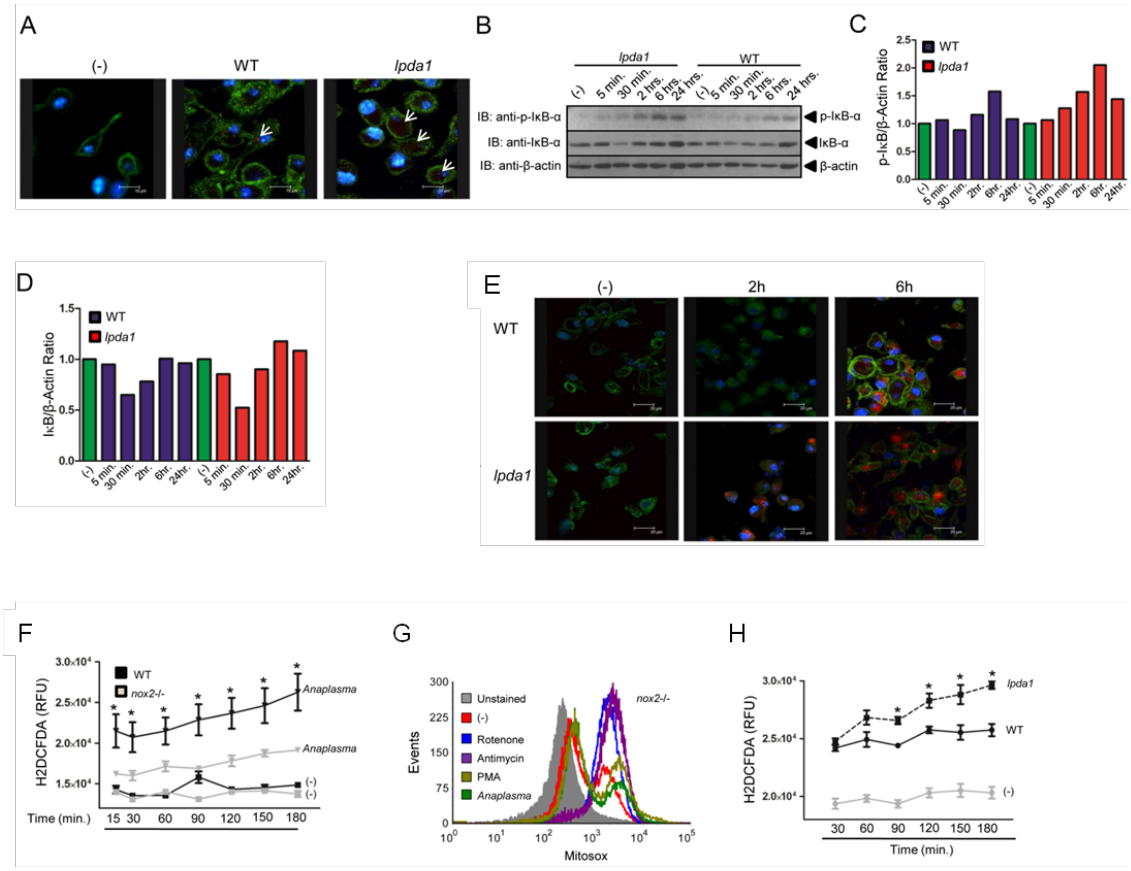

Figure 2.2: LPDA1 inhibits NF-κB nuclear translocation in macrophages via NADPH ROS.

(A) BMDMs ($1x10^6$) from C57BL/6 mice were stimulated for 6 hours with either the wildtype HZ (WT) or the *lpda1*::TnHimar1 (*lpda1*) *A. phagocytophilum* strains (MOI 50) and stained with cholera toxin subunit B (green), DAPI (blue) and an antibody against the nuclear localization signal of the p65 subunit of NF-κB (red; white arrows). Bar = 10 μm. (B) Cell lysates were collected and immunoblotted (IB) with p-IκB-α and IκB-α antibodies. β-actin was used as a normalizing control. Densitometry was performed for (C) p-IκB-α and (D) IκB-α. (E) BMDMs ($1x10^6$) from C57BL/6 mice were stimulated with the WT or the *lpda1 A. phagocytophilum* strains (MOI 50) and stained with cholera toxin subunit B (green), DAPI (blue) and an antibody against *A. phagocytophilum* (red) for confocal microscopy. Bar = 20 μm. (F) BMDMs ($2x\ 10^5$) from WT and *nox2*$^{-/-}$ mice were stimulated with the WT *A. phagocytophilum* strain (MOI 50). ROS production was monitored using the fluorescent probe H2DCFDA at the indicated time points. (G) BMDMs ($1x\ 10^6$) from *Nox2*$^{-/-}$ mice were stimulated for 30 minutes with the WT *A. phagocytophilum* (MOI 100), mitochondrial oxidants rotenone (40 μM) and antimycin (40 μg/ml) or the NOX-dependent respiratory burst chemical PMA (5 μM). ROS generation in the mitochondria was measured by flow cytometry using the fluorescent probe MitoSOX (2.5 μM). (H) BMDMs ($2x10^5$) from WT mice were stimulated with the WT or the *lpda1 A. phagocytophilum* strains (MOI 10) at the indicated time points. Comparisons were performed a two-way ANOVA-Bonferroni (*lpda1*, WT). Experiments were repeated at least twice. Data are presented as mean ± SEM. Student's t test; *P < .05; (-) non-stimulated cells.

wildtype *A. phagocytophilum* HZ strain. At 24 hours most if not all *A. phagocytophilum* bacteria were degraded by macrophages (*data not shown*).

Next, I tested the ability of *A. phagocytophilum* to induce ROS during macrophage stimulation. ROS is widely linked to inflammation and is important for NF-κB activation [222] . Contrary to neutrophils, where *A. phagocytophilum* suppresses ROS, BMDMs induced ROS in response to *A. phagocytophilum* (Figure 2.2F). Among the many sources of ROS inside the cells, mitochondria and NADPH oxidases are the most common [222]. To determine the source of ROS during *A. phagocytophilum* stimulation of BMDMs, I used *Nox2* (*gp91*^{phox})-deficient mice. *Nox2*-deficient mice do not produce ROS from NADPH oxidase, which can be used as a mechanism of defense against microbial infection. Although ROS in *Nox2*-deficient mice was not completely abolished during pathogen stimulation, wildtype *A. phagocytophilum* stimulation produced significantly less ROS in *Nox2*-deficient cells when compared to wildtype macrophages (Figure 2.2F).

Our observation was further confirmed by measuring ROS in mitochondria. Contrary to rotenone and antimycin (two compounds that produce ROS by inhibiting complex I and III from the mitochondria), *A. phagocytophilum* did not induce any detectable ROS in *Nox2*-deficient BMDMs (Figure 2.2G). Phorbol-12-myristate-13-acetate (PMA), which induces cells to undergo the NADPH oxidase-dependent respiratory burst, was used as a negative control for *Nox2*-deficient cells and did not increase ROS levels. To determine whether the *lpda1::*TnHimar1 *A. phagocytophilum* mutant produces more ROS when compared to wildtype *A. phagocytophilum* during BMDM stimulation, I measured total ROS. I noticed a consistent and statistically significant increase in the ROS levels

during BMDM stimulation with the *lpda1::*TnHimar1 mutant when compared to the wildtype *A. phagocytophilum* HZ strain (Figure 2.2H). Taken together, our data show that LPDA1 inhibits BMDM ROS production by NADPH oxidase leading to reduced NF-κB activation and cytokine production during stimulation with *A. phagocytophilum*.

2.5 Discussion

A. phagocytophilum studies have defined the contribution of innate and adaptive immunity during infection. On the one hand, infection-induced immunopathology is due to innate immunity [195–197,212,223–225]. On the other hand, pathogen eradication is obtained by adaptive immunity [138,198,199,226]. This clear-cut dichotomy suggests that some genes in the *A. phagocytophilum* genome may contribute to pathogen colonization while others may be directly associated with the disease state in mammals. The latter are clinically relevant because cytopenias and splenomegaly - common HGA symptoms - are largely due to host inflammation [203,204,227,228]. In this study, I characterized the molecule LPDA1 during *A. phagocytophilum* colonization of the mammalian host.

One intriguing result from our study is that signaling in neutrophils and macrophages during *A. phagocytophilum* infection were not alike. Neutrophils were refractory to *lpda1::*TnHimar1 mutant infection and triggered a much lower NF-κB response when compared to the wildtype strain (*data not known*). On the contrary, macrophages stimulated with the *A. phagocytophilum lpda1::*TnHimar1 mutant had higher levels of NF-κB signaling and were fully capable of binding and internalizing the

lpda1::TnHimar1 mutant. These results suggest that LPDA1 may play a functional role in phagocytosis of *A. phagocytophilum*. Independent groups have determined that tetrasaccharide sialyl Lewis (sLe^x) present on the protein P-selectin glycoprotein ligand-1 (PSGL-1) by *A. phagocytophilum* is required for human neutrophil infection [229,230]. On the other hand, *A. phagocytophilum* uses α-1,3-fucosylation but not PSGL-1 for infection of murine neutrophils [231] and it is known that some *A. phagocytophilum* strains may use PSGL-1-dependent and -independent routes of cell invasion [232,233]. Future experiments comparing and contrasting the NF-κB pathway in neutrophils and macrophages during *A. phagocytophilum* infection should uncover signaling differences between these cell types. Emphasis should be given towards the synergistic effect of neutrophil-macrophage interaction and the contribution of *A. phagocytophilum* LPDA1 in the immunological synapse.

In summary, the results provided here demonstrate a role for *A. phagocytophilum* LPDA1 in infection-induced immunopathology. Further investigation examining how LPDA1 contributes to *A. phagocytophilum* pathogenesis and immunobiology will be critical for understanding HGA etiology.

CHAPTER THREE

Inhibitory activities of tick saliva and sialostatin L2

3.1 Abstract

Inflammasomes are protein scaffolds comprised of innate immune receptors that activate caspase-1 via the adaptor molecule ASC. During microbial infection, inflammasome assembly may occur and maturation of cytokines, such as IL-1β and IL-18, leads to the inflammatory process. While it is acknowledged that several vector-borne pathogens trigger inflammasome function during infection, it remains unknown whether the anti-inflammatory properties of arthropod saliva modulate inflammasome activity. Here, I demonstrate that tick saliva and the salivary protein sialostatin L2 reduces pro-inflammatory responses. *I. scapularis* saliva inhibits inflammatory cytokine secretion by macrophages during stimulation of TLR and NLR receptor signaling pathways. Caspase-1 inhibition by sialostatin L2 was independent of NF-κB. However, ROS from NADPH oxidase were seemingly important for the regulatory process. Diminished caspase-1 function during *A. phagocytophilum* stimulation appeared pathogen-specific because IL-1β secretion remained unaltered when macrophages were stimulated by known NLRP3, NLRC4 and AIM2 agonists in the presence of sialostatin L2. Taken together, our results establish that interference with innate immune signaling is not only restricted to endogenous regulators or microbial molecules. Disease vectors, such as ticks, may also alter inflammasome activity; thus, affecting inflammation during pathogen transmission.

3.2 Introduction

The inflammasome is critical for the inflammatory process [93,234,235]. Inflammasome pathway signaling requires two key steps. The first is the initiation of the

NF-κB pathway, for example by a TLR agonist. Second, the canonical paradigm in inflammasome activation establishes that NLR or absent in melanoma 2 (AIM2) recruit the adaptor molecule apoptosis-associated speck-like protein (ASC) to activate the enzyme caspase-1[93,234,235]. Caspase-1 then cleaves inactive cytokine precursors, such as pro-IL-1β and pro-IL-18, leading to IL-1β and IL-18 maturation. Four classical inflammasomes have been described; NLRP3 is the best studied inflammasome and is activated by a wide-range of stimuli with diverse physicochemical structures [236]. The NLRC4 inflammasome is mainly activated in response to cytosolic flagellin or bacterial type III and IV secretion systems from Gram-negative bacteria [237]. The AIM2 inflammasome directly binds viral and bacterial cytosolic DNA, whereas the NLRP1 inflammasome was the first scaffold to be described and confers susceptibility to the *Bacillus anthracis* lethal toxin [93,234,235].

Surprisingly, how disease vectors inhibit inflammasome activation during pathogen transmission remains elusive. Saliva of blood-feeding arthropods facilitates the establishment of vector-borne pathogens [238]. Salivary gland secretion is among the most common physiological and biochemical adaptation in hematophagous arthropods and salivary proteins from blood-feeding organisms ensue defense against host homeostasis and inflammation [89,163,238–243]. Combating inflammation is particularly problematic for ixodid ticks because these arthropods have to feed for a prolonged period of time and are exposed to a wide range of immune cells [97,244]**.** Pioneering studies have characterized the physiology of tick salivary glands [245] and raised the importance of saliva as an instrumental force for immune evasion [246–249]. Several groups have

demonstrated that both proteinaceous and non-proteinaceous components of tick saliva impair immune function [151,162,170–174,243,250–252]. From these studies it also became apparent that pathogens take advantage of the immunomodulatory properties of vector saliva to colonize the host.

This phenomenon was first observed during infection by *Leishmania* parasites [253], and subsequent studies demonstrated that enhanced pathogen transmission by saliva is universal among blood-feeding arthropods [89,238]. For instance, mosquito saliva augments the transmission of malaria parasites [254], West Nile [255], La Crosse [256] and Cache Valley [257] viruses. Similarly, tick saliva counteracts host-derived inflammation [89,238] by impairing the complement system [243], the function of macrophages, dendritic cells and T cells [258], and inhibiting cytokine secretion [89]. More specifically, the salivary protein Salp15 from the tick *Ixodes scapularis* binds *Borrelia burgdorferi* outer surface protein C (OspC) to shield the Lyme disease agent, protecting this pathogen from antibody-mediated killing [259] and dendritic cell function [172]. Sialostatin L2, an *I. scapularis* cystatin protein also facilitates the growth of the Lyme disease agent *B. burgdorferi* [260]. In addition, *I. ricinus* saliva inhibits interferon and TLR signaling during cell stimulation with *B. afzelli* [261,262]. Finally, infection by the tick-borne encephalitis virus can be prevented by immunizing animals against a truncated recombinant form of a tick salivary protein named 64P [263]. The implication of tick saliva on the macrophage, a key immune cell, remains mostly unknown. Recently, our group uncovered that *I. scapularis* saliva inhibits inflammatory cytokine secretion by macrophages during stimulation of TLR and NLR signaling pathways [264].

Additionally, I show that the salivary protein sialostatin L2 from the tick *I. scapularis* inhibits inflammasome activity during *A. phagocytophilum* stimulation. Inhibition by sialostatin L2 was independent of nuclear factor (NF)-κB but correlated with ROS from NADPH oxidase. These findings expand previous scientific knowledge on the immunomodulatory properties of tick saliva and tick salivary proteins at the vector-host interface.

3.3 Materials and methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC number: A-20110030BE, 1104066 and 03759) and Biological Use Authorization (BUA numbers: 20120020, 130047, 20120020 and 130047) Committeesat the University of California, Riverside. I used C57BL/6 (database number 000664) and *Nox*$2^{-/-}$ (database number 002365) mice purchased from Jackson Laboratories. *A. phagocytophilum* was grown in HL-60 cells (ATCC CCL-240). HL-60 cells were maintained in Iscove's Modified Dulbecco's Media (IMDM) with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) (Thermo Scientific), 20% heat-inactivated fetal bovine serum (FBS) (Sigma) in 5% CO_2 and humidified air at 37°C, as previously described [99]. *Francisella tularensis* subsp. holarctica LVS was obtained from ATCC (ATCC 29684). *Francisella tularensis* LVS was grown on DIFCO cysteine heart agar supplemented with 9% sheep red blood cells (SRBC) for 48 h at 37°C, as previously described [265]. *P. aeruginosa* PAK was obtained from J. Mattick (University

of Queensland, Australia) and cultured in Luria–Bertani (LB) broth overnight, as described [84].

Reagents

Lipopolysaccharide (LPS), Pam3CSK4, Zymosan, *Porphyromonas gingivalis* (PG)-LPS and muramyl dipeptide (MDP) were obtained from Invivogen. DOTAP was obtained from Roche.

Cell culture generation

The generation of bone marrow-derived macrophages (BMDMs) has been previously described [266]. Briefly, mouse femurs were flushed with a 25 gauge needle and bone marrow cells were differentiated in complete Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 30% L929 cell-conditioned media, 10% FBS and 1% PSA (100 U/mL penicillin, 100 mg/ml streptomycin, and 0.25μg/ml amphotericin) (Thermo Scientific). Cells were cultured at 37°C in a 5% CO_2 tissue culture incubator for 5–6 days, with media changed on day 3.

Sialostatin L2

Sialostatin L2 was produced, as previously described [158,260,267]. Briefly, sialostatin L2 cDNA was PCR-amplified and subcloned into the pET17b bacterial expression vector. The expression vector was placed into the *Escherichia coli* strain BL21(DE3)pLysS for expression. Cultures were grown and induced by adding isopropyl

1-thio-β-d-galactopyranoside (IPTG). Inclusion bodies were dissolved in 6 M guanidine hydrochloride, 20 mm Tris-HCl, pH 8.0, and reduced with 10 mM dithiothreitol (DTT). Sialostatin L2 was refolded in a large volume of 20 mM Tris HCl, pH 8.0, 300 mM NaCl, and stirred overnight at 4 °C. The refolded protein was concentrated with a tangential flow filtration device and purified by gel filtration chromatography on Sephacryl S-100 followed by anion exchange chromatography on Q-Sepharose. Dialysis followed against 20 mM Tris-HCl, pH 7.4, 150 mm NaCl. LPS contamination was removed by using the detergent-based method from Arvys Proteins. Endotoxin presence was estimated by using a sensitive fluorescence-based endotoxin assay from Lonza Biologics.

Tick saliva collection

The collection of tick saliva has been previously described [99,217,268]. *I. scapularis* saliva was collected 4–5 days after feeding because studies suggest that transmission of *A. phagocytophilum* initiates slowly between 24 and 48 hours and is enhanced during rapid feeding to repletion around 72 h–96 h post tick attachment [198,240,269]. Therefore, saliva from *I. scapularis* would reflect actual conditions during *A. phagocytophilum* transmission at the vector-host interface. In addition, *I. scapularis* saliva collection at 24–48 hours is technically very challenging. The alternative would be using salivary glands. However, salivary glands bring a technical artifact to the system because this organ in ticks is rich in intracellular proteins and other immune effectors such as nucleotides, which may skew cytokine response in immune cells. To isolate vector saliva, *I. scapularis* ticks were allowed to feed on New Zealand white rabbits. A

restraining collar was placed around the neck of each rabbit, and their ears were covered prior to tick exposure. Ticks were permitted to engorge for 4–5 days on the ear of a rabbit. Upon harvesting, ticks were rinsed in distilled water and were immediately fixed to glass slides with double-sided tape. A sterile glass micropipette was placed around the hypostome to collect saliva. Salivation was induced by the application of pilocarpine to the scutum of the tick. Saliva was pooled and stored at −80°C for use.

Immune cell stimulation

BMDMs from C57BL/6 mice were stimulated with the TLR agonists LPS (500 ng/ml), Pam3CSK4 (1 μg/ml), Zymosan (10 μg/ml) and PG-LPS (500 ng/ml), the Nod2 stimulant MDP (10 μg/ml) or *A. phagocytophilum* (multiplicity of infection (MOI) 10 and 50) at indicated dilutions of tick saliva. Pro-inflammatory cytokines such as TNF-α, interleukin (IL)-12p40, IL-6 and IL-1β were measured by ELISA.

ELISA

Mouse TNF-α, IL-1β, andIL-6 were measured with the BD OptEIA Set from BD Biosciences. Mouse IL-12p40 was measured with capture and detection antibodies from eBiosciences. For the ELISA assays, wells were coated with recommended capture antibody dilutions in freshly prepared coating buffer (0.1 M sodium carbonate, pH 9.5). Plates were sealed and incubated overnight at 4°C followed by aspiration. Wells were then washed 3 times with ≥ 300 μL/well of freshly prepared wash buffer (phosphate buffered saline (PBS) with 0.05% Tween-20). After the last wash, plates were inverted

and blotted on absorbent paper to remove any residual buffer. Wells were then blocked with ≥ 200 μL/well of assay diluent (PBS with 10% FBS, pH 7.0) and incubated at room temperature for 1 hour. Plates were washed 3 times with wash buffer. Then, standards and sample dilutions were prepared in assay diluent, as recommended (BD Biosciences). 100 μL of samples and standards were pipetted into the wells, incubated for 2 hours at room temperature followed by 5 washes. 100 μL of detection antibodies were diluted in assay diluent and added to each well. Plates were sealed and incubated for 1 hour at room temperature. Plates were washed 5 times. Then, 100 μL of enzyme reagent (BD OptEIA Set from BD Biosciences) were diluted in assay diluent, pipetted into each well and incubated for 30 minutes at room temperature. Wells were aspirated and washed 7 times with wash buffer. 100 μL of substrate solution (BD OptEIA Set from BD Biosciences) were added to each well and incubated for 30 minutes (without plate sealer) at room temperature in the dark. 50 μL of 2 N H_2SO_4 was added to each well. Absorbance was read in the ELISA plate reader (Bio-Rad) at 450 nm within 30 minutes. Background was corrected by reading the subtract absorbance at 570 nm.

Cell death assay

Cell death was assayed by measuring lactate dehydrogenase (LDH), as recommended by the manufacturer (Takara). Briefly, 100 μL of each sample was placed into a well. Then, a catalyst solution (Takara) was added to the samples and controls. Samples were incubated for 10–30 minutes, at room temperature, protected from light. Reactions were stopped at the end of the incubation period by adding 50 μL of 1 N HCl.

Absorbance was measured at 490 nm. The iMark microplate absorbance reader (Bio-Rad) was used according to the manufacturer's instruction.

Immunoblotting

Cell lysates were extracted using radioimmunoprecipitation (RIPA) lysis buffer (Boston Bioproducts) with Complete Mini Protease Inhibitor Cocktail and PhosSTOP, both from Roche Applied Science. Protein concentration was determined via the Bradford protein assay method, using protein assay dye reagent concentrate and iMark reader, both from Bio-Rad. SDS polyacrylamide gel was made and ran at 200 volts for 1 hour. Transfer was done in wet conditions with polyvinylidene fluoride (PVDF) membranes for 60 minutes at 100 volts. Membranes were blocked in 5% non-fat dry milk (LabScientific, Inc.). Western blot antibodies for β-actin (Neomarker-Thermo Scientific) (1:500-1:1000) (Catalogue 497 number - RB-9421p), IκB-α (Cell Signaling) (1:1000) (Catalogue number - 4814), p-IκB-α (Cell Signaling) (1:1000) (Catalogue number – 9246S), IL-1β (R&D systems) (1:1000) (Catalogue number - AF-401-NA), caspase-1 (Millipore) (1:1000) (Catalogue number – 06-503), (Santa Cruz) (1:100-1:1000) (Catalogue number – SC-514), IL-18 (MBL) (0.5-4 μg/ml) (Catalogue number – JM-5180-100), anti-goat horseradish peroxidase (HRP) (Santa Cruz) (1:7500-1:10000) (Catalogue number SC-2352), anti-rabbit HRP (Santa Cruz) (1:7500-1:10000) (Catalogue number SC-2374), anti-mouse HRP (Santa Cruz) (1:7500-1:10000) (Catalogue number SC-2375) were used. In some experiments, supernatants were concentrated with centrifugal filter units (3K) (Amicon) (Catalogue number – UFC500324) and caspase-1

immunoblots were performed. Enhanced chemiluminescence (ECL) western blotting substrate and super signal West Pico Chemiluminescent substrate were used (Pierce Thermo Scientific).

ROS and Fluorescent Labeled Inhibitor of Caspase-1 (FLICA)

I detected ROS using the fluorescence probe 2′7′-dichlorofluorescin diacetate (H2DCFDA) (Invitrogen), as described previously [99]. Fluorescence was recorded in 96-well plates over time with a Spectra MAX Gemini EM microplate reader (Molecular Devices) using a fluorescein isothiocyanate (FITC) filter (excitation, 485 nm; emission, 538 nm). Green FLICA Assay Kit (Immunochemistry) (Catalogue number 98) was used to detect active caspase-1 in macrophages. Measuring was done with excitation at 490 nm and emission at 520 wavelengths.

Statistical analysis

Data were expressed as means ± standard errors of the means (SEM). Gaussian distribution was determined by the D'Agostino and Pearson normality test. The following parametric analyses were used: unpaired Student's *t* test (two-group comparisons); one-way analysis of variance (ANOVA) (comparisons of three or more groups). Bonferroni post hoc multiple comparison tests were used following ANOVA. All statistical calculations and graphs were made by using GraphPad Prism version 5.04. $P < 0.05$ was considered statistically significant.

3.4 Result

I. scapularis saliva diminishes inflammatory cytokine secretion by murine macrophages

Tick saliva has immunomodulatory properties [89,238]. To determine whether tick saliva inhibits macrophage function, I first stimulated mouse BMDMs with LPS in the presence or absence of different dilutions of *I. scapularis* saliva. Cytokine levels were not altered during BMDM stimulation with tick saliva alone, suggesting that the saliva does not carry any contaminants, PAMPs, or DAMPS. As expected, LPS induced high levels of cytokine secretion in murine BMDMs (Figures 3.1 and 3.2). However,

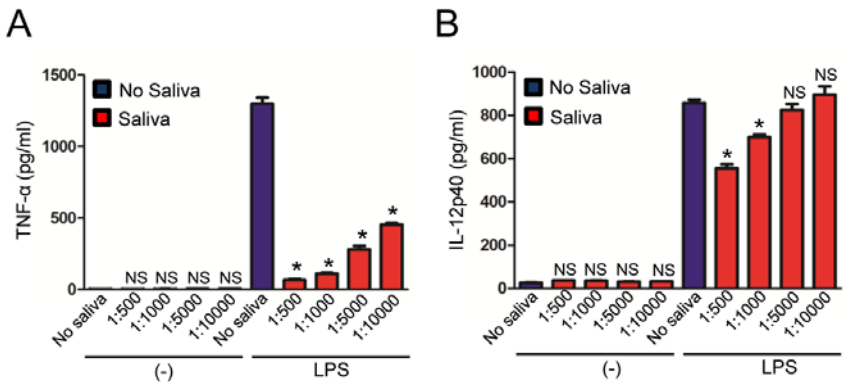

Figure 3.1: *I. scapularis* saliva mitigates LPS- mediated cytokine secretion by murine macrophages.

BMDMs (8×10^5) from C57BL/6 mice were stimulated with LPS (500 ng/ml) for 18 hours, in the presence or absence of indicated dilutions of tick saliva. (A) TNF-α and (B) IL-12p40 were measured by ELISA. Tick saliva was added 2 hours before stimulation. Responses were measured in triplicate and presented as mean ± SEM within the representative experiment. Experiments were repeated three times. *P < .05, One-way ANOVA, post-hoc Bonferroni; (−) non-stimulated cells. NS – not significant.

I. scapularis saliva inhibited secretion of both TNF-α and IL-12p40 by BMDMs after stimulation with LPS (Figure 3.1). This effect was more pronounced for TNF-α, as a tick saliva dilution of 1:10000 (v/v) still affected cytokine secretion by BMDMs during LPS

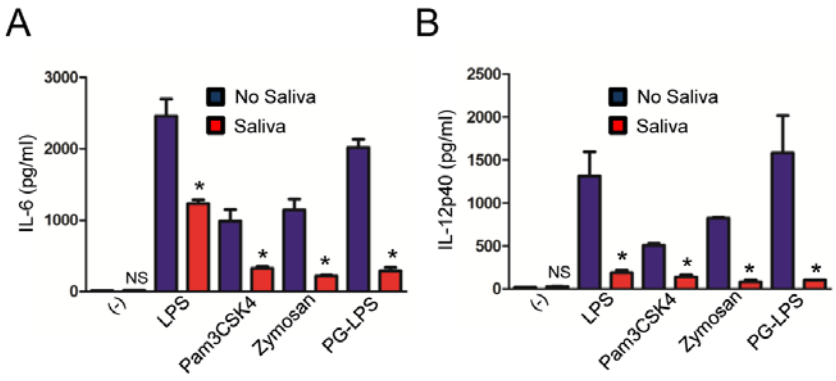

Figure 3.2: *I. scapularis* saliva inhibits TLR-mediated cytokine secretion by macrophages.

BMDMs (8×10^5) from C57BL/6 mice were stimulated with LPS (500 ng/ml), Pam3CSK4 (1 μg/ml), Zymosan (10 μg/ml) and PG-LPS (500 ng/ml) for 18 hours in the presence or absence of tick saliva (1:500 dilution). Tick saliva was added 2 hours before stimulation. (A) IL-6 and (B) IL-12p40 were measured by ELISA. Responses were measured in triplicate and presented as mean ± SEM within the representative experiment. Experiments were repeated three times. *P < .05, Student's t test. (−) non-stimulated cells. NS – not significant.

stimulation (Figure 3.1A). A reduction in IL-12p40 secretion by macrophages after LPS stimulation was only observed for a tick saliva dilution of 1:1000 (v/v) and below (Figure 3.1B). I then stimulated mouse BMDMs with a wide range of TLR ligands and measured IL-6 and IL-12p40 secretion in the presence of *I. scapularis* saliva. Tick saliva 1:500 (v/v) inhibited IL-6 and IL-12p40 secretion by BMDMs when stimulated with TLR agonists, such as LPS, Pam3CSK4, Zymosan and PG-LPS (Figure 3.2). Next, I stimulated BMDMs with Nod1 and Nod2 agonists to determine whether the effect of *I. scapularis* saliva on cytokine secretion was restricted to TLRs. Nod1 and Nod2 are considered cytosolic receptors and are part of the NLR protein family[270]. The Nod1 and Nod2 agonists iE-DAP and MDP did not induce cytokine production in BMDMs during extracellular stimulation (Figure 3.3; data not shown). However, transfection of MDP to the cytosol using the cationic lipid DOTAP led to secretion of both IL-6 and IL-

12p40 by BMDMs (Figure 3.3). Further, *I. scapularis* saliva inhibited IL-6 and IL-12p40 secretion mediated by the transfected Nod2 agonist MDP at the saliva dilution of 1:500 (v/v). Taken together, I report that *I. scapularis* saliva mitigates cytokine secretion by BMDMs during extracellular and cytosolic stimulation of TLR and NLR pathways.

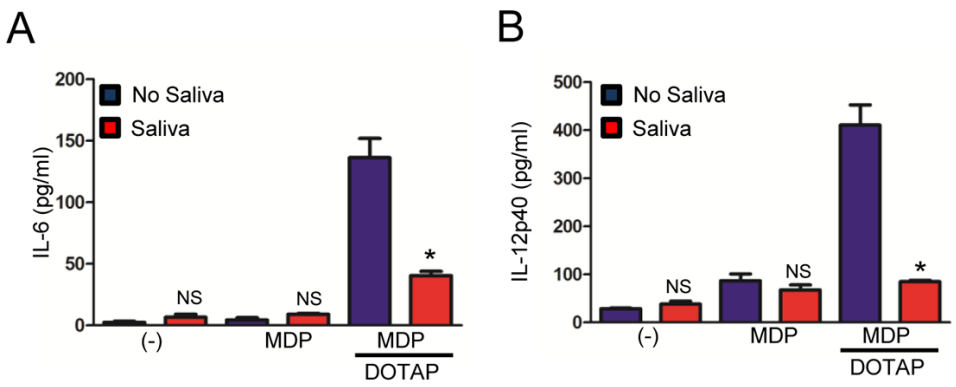

Figure 3.3: *I. scapularis* saliva impairs Nod2-mediated cytokine secretion by murine macrophages.

BMDMs (1×10^6) from C57BL/6 mice were stimulated with MDP (10 μg/ml) or DOTAP (10 μg/ml) + MDP (10 μg/ml) for 20 hours, in the presence or absence of tick saliva (1:500). The secretion of (A) IL-6 and (B) IL-12p40 were measured by ELISA. Responses were measured in triplicate and presented as mean ± SEM within the representative experiment. Experiments were repeated three times. *P < .05, Student's t test. (−) non-stimulated cells. NS – not significant.

I. scapularis saliva lessens inflammatory cytokine secretion by murine macrophages during *A. phagocytophilum* stimulation

To determine whether the effect of tick saliva was restricted to TLR and NLR ligands, I stimulated BMDMs with the *I. scapularis* rickettsial pathogen *A. phagocytophilum*. *A. phagocytophilum* induced the secretion of large amounts of cytokines by murine macrophages (Figures 3.4). Nonetheless, tick saliva was also efficient in reducing cytokines by BMDMs, such as IL-6, IL-12p40 and TNF-α during *A. phagocytophilum* stimulation (Figure 3.4). Similar to BMDM stimulation with LPS, the

effect of tick saliva was more pronounced on TNF-α secretion by BMDMs during pathogen stimulation. TNF-α secretion was completely abolished when BMDMs were stimulated with *A. phagocytophilum* in the presence of tick saliva (Figure 3.4C). Overall, the inhibitory effect of tick saliva on cytokine secretion by BMDMs during *A. phagocytophilum* stimulation was best observed at a 1:150 dilution (v/v). Experiments performed with tick saliva at a 1:300 dilution (v/v) also showed reduction of cytokine secretion by BMDMs during *A. phagocytophilum* stimulation (Figure 3.4). However, the effect was milder (although statistically significant) for IL-6 and IL-12p40 (Figure 3.4A

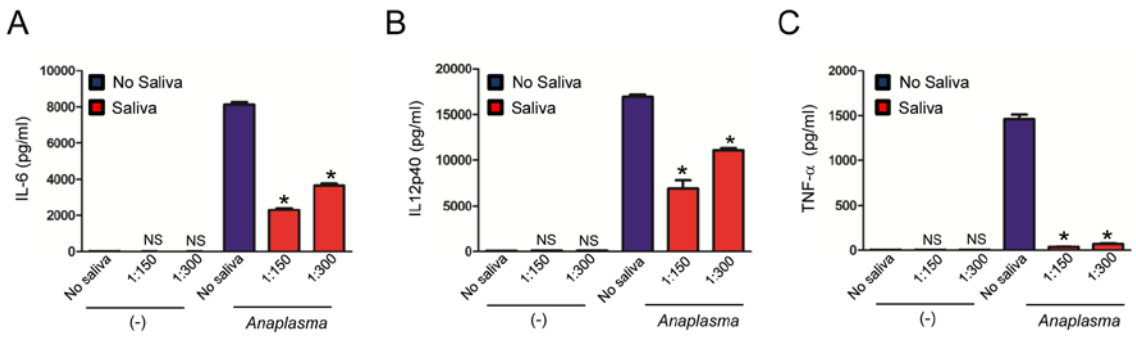

Figure 3.4: *I. scapularis* saliva mitigates cytokine secretion by macrophages during *A. phagocytophilum* stimulation in a dose-dependent manner.

BMDMs (1×10^6) from C57BL/6 mice were stimulated with the wild-type *A. phagocytophilum* HZ strain (MOI 50) for 18 hours in the presence or absence of tick saliva (1:150 and 1:300 dilution). (A) IL-6, (B) IL-12p40 and (C) TNF-α were measured by ELISA. Responses were measured in triplicate and presented as mean ± SEM within the representative experiment. *P < .05, One-way ANOVA, post-hoc Bonferroni; (-) non-stimulated cells. NS – not significant.

and B). I did not detect a dilution effect on TNF-α secretion by BMDMs at a 1:300 dilution (v/v) (Figure 3.4C). These results suggest that the inhibition threshold for tick saliva on TNF-α secretion by BMDMs during *A. phagocytophilum* stimulation is greater than 1:300 (v/v) compared to 1:150 dilution (v/v). It is unclear why more concentrated

tick saliva is required to inhibit cytokine secretion during *A. phagocytophilum* stimulation when compared to individual TLR or NLR agonists. I reasoned that the presence of multiple PAMPs in a pathogen, such as *A. phagocytophilum*, may require a stronger dose of tick saliva to mitigate cytokine secretion by BMDMs.

Sialostatin L2 inhibits caspase-1 maturation, IL-1β and IL-18 secretion by macrophages during A. phagocytophilum stimulation

It was previously shown that sialostatin L2 facilitates the growth of *B. burgdorferi* in the mouse skin, and vaccination against sialostatin L2 decreases the feeding ability of *I. scapularis* nymphs [260,267] - suggesting that this molecule may play a protective role against host immune responses. To ascertain whether this protein could affect cytokine secretion, I first stimulated macrophages with a panel of TLR, Nod1 and Nod2 agonists in the presence or absence of sialostatin L2. Sialostatin L2 did not inhibit TLR, Nod1 Nod2 signaling in macrophages after PAMP stimulation, as judged by measurements of IL-6 and IL-12p40 (Figure 3.5A-C; *data not shown*). In non-stimulated cells, IκB sequesters NF-κB dimers in the cytoplasm by masking the nuclear localization signals (NLS) of the NF-κB protein p65. Activation of NF-κB is initiated by phosphorylation and subsequent degradation of IκBs, which leads to the translocation of the NF-κB complex to the nucleus and expression of inflammatory genes [271]. Using a time course experiment, I visualized p-IκB-α and IκB-α degradation during LPS stimulation of macrophages. I observed similar levels of NF-κB activation in the presence or absence of sialostatin L2 during LPS stimulation (Figure 3.5D). In both treatments, phosphorylation

of IκB-α initiated at 5 minutes post-challenge, while degradation of IκB-α occurred at 10 minutes and returned to background levels after 30 minutes.

I then investigated whether sialostatin L2 could affect the inflammasome cytokines IL-1β and IL-18. Pro-IL-1β and pro-IL-18 are induced by pattern recognition receptors or pro-inflammatory cytokines via NF-κB. This signal is referred to as priming. The second signal is mediated by caspase-1 activation, which cleaves IL-1β and IL-18

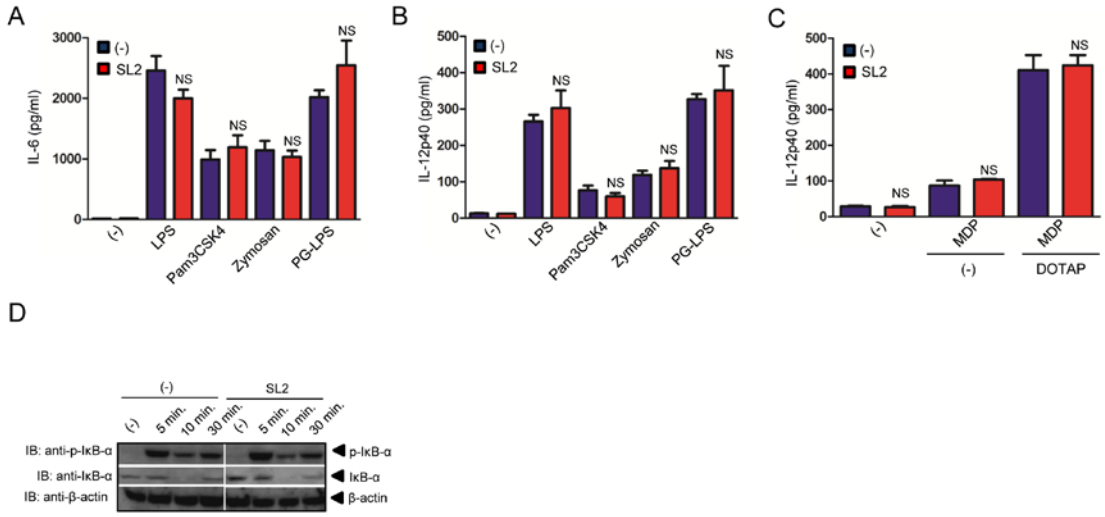

Figure 3.5: Sialostatin L2 does not inhibit TLR or Nod2 signaling in macrophages.

BMDMs (8x 10^5) were stimulated with LPS (0.5 μg/ml), Pam3CSK4 (1μg/ml), Zymosan (10 μg/ml) and PG-LPS (0.5 μg/ml) for 18 hours in the presence or absence of sialostatin L2 (SL2 - 3 μM). SL2 was added 2 hours before stimulation. (A) IL-6 and (B) IL-12p40 were measured by ELISA. (C) BMDMs (1x 10^6) were stimulated with MDP (10 μg/ml) or with DOTAP (10 μg/ml) + MDP (10μg/ml) for 20 hours in the presence or absence of SL2 (3 μM). The secretion of IL-12p40 was measured by ELISA. (D) BMDMs (1x 10^6) were stimulated with LPS (0.5 μg/ml) in the presence or absence of SL2 (3 μM). Cell lysates were collected at the indicated time points and immunoblotted (IB) with p-IκB-α and IκB antibodies. Actin was used as loading control. Cytokine measurements were taken in triplicate and presented as mean ± SEM. These experiments were repeated twice. Student's t test. (-) non-stimulated cells. NS – not significant.

into its mature forms [93,234,235]. As previously observed, our studies showed that IL-1β and IL-18 secretion by macrophages during *A. phagocytophilum* stimulation did not

require lipopolysaccharide (LPS) priming and did not affect cell death (Figure 3.6A) [264]. These results were consistent with the lack of genes for LPS synthesis in the *A. phagocytophilum* genome [272] and the *A. phagocytophilum* anti-apoptotic properties [273]. *A. phagocytophilum* induced both IL-1β and IL-18 secretion by macrophages, and

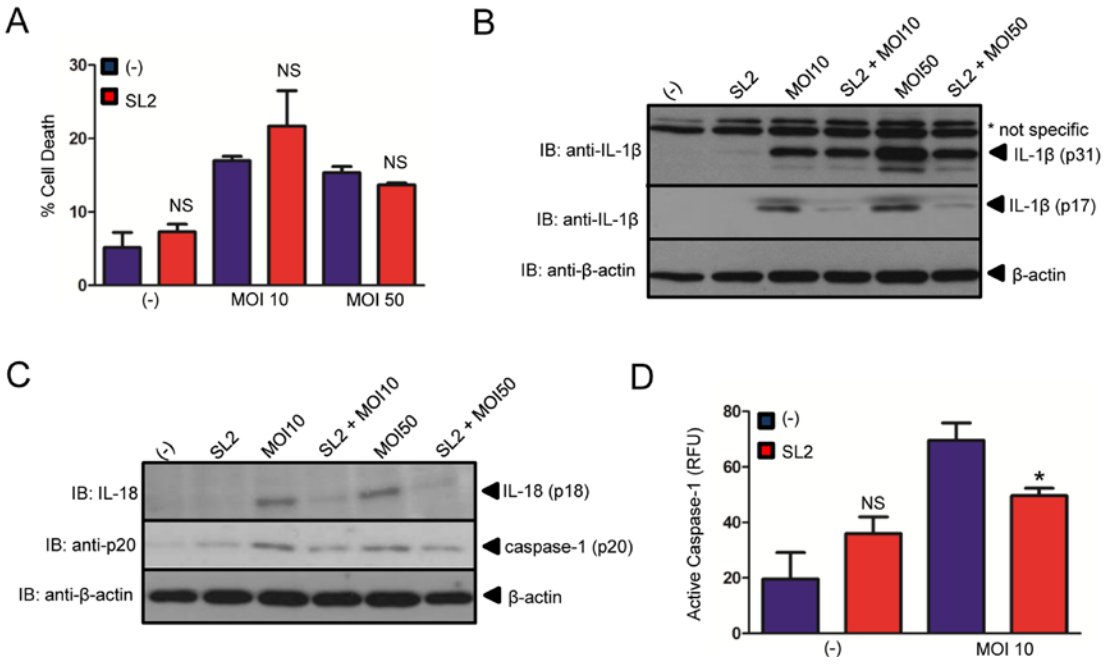

Figure 3.6: Sialostatin L2 inhibits *A. phagocytophilum*-induced caspase-1-mediated cytokine secretion.

(A-D) BMDMs (1x 10^6) were stimulated with *A. phagocytophilum* (MOIs 10 and 50) in the presence or absence of sialostatin L2 (SL2) for 18 hours. (A) Cell death was measured by using the LDH assay. (B) IL-1β (p31 and p17) was measured by western blots (IB). β-actin was used as a loading control. (C) IL-18 (p18) and mature caspase-1 (p20) mature forms were measured by western blots (IB). (D) BMDMs (2x 10^5) were stimulated with *A. phagocytophilum* (MOI 10) for 18 hours in the presence or absence of SL2. The fluorescent inhibitor probe FAM-YVAD-FMK was used to label active caspase-1 in macrophages. The signal is shown as a function of relative fluorescence units (RFU). A and D (SL2 - 3 μM) are shown in triplicate and presented as mean ± SEM. SL2 (10 μM) was used in B and C. These experiments were repeated at least twice. *P < .05, Student's t test. (-) non-stimulated cells. NS – not significant.

this effect was dependent on caspase-1 activation (Figure 3.6B-D). Surprisingly, sialostatin L2 inhibited maturation of both IL-1β and IL-18 (here depicted as the mature forms p17 and p18, respectively) (Figure 3.6B and C). Consistent with sialostatin L2

regulating signaling upstream of IL-1β and IL-18 secretion, I observed an effect on caspase-1 activation (Figure 3.6C-D).

To demonstrate that the results obtained with caspase-1 were not an antibody artifact, I measured caspase-1 by using a fluorescence assay (FLICA) [274]. Our results also showed that sialostatin L2 inhibited caspase-1 activation during *A. phagocytophilum* stimulation of macrophages (Figure 3.6D). The effect of sialostatin L2 on IL-1β secretion appeared specific for *A. phagocytophilum* because IL-1β secretion remained unaltered when macrophages were stimulated by known NLRP3, NLRC4 and AIM2 agonists (Figure 3.7). Sialostatin L2 did not inhibit IL-1β secretion by macrophages before (NF-κB activation) or after priming (inflammasome activation). On the contrary, I noticed a slight increase of IL-1β secretion by macrophages when sialostatin L2 was added to cells prior to LPS priming and *F. tularensis* stimulation (Fig. 3.7C).

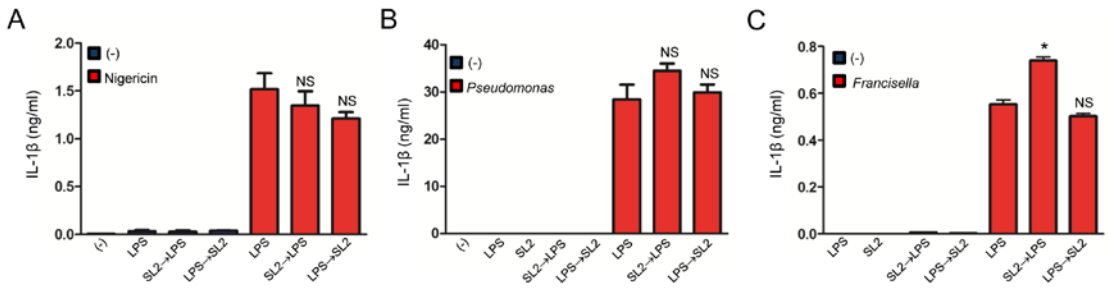

Figure 3.7: Sialostatin L2 does not hinder IL-1β secretion mediated by known NLRP3, NLRC4 and AIM2 agonists.

BMDMs (8x 10^5) were stimulated with (A) nigericin (10 μM) (B) *P.aeruginosa* PAK (MOI 10) or (C) *F. tularensis* LVS (MOI 50) for 9 hours in the presence or absence of sialostatin L2 (SL2 - 3 μM) for 30 minutes either prior to or immediately after priming with LPS (50 ηg/ml). IL-1β secretion in the supernatants was assessed by ELISA. Experiments represent mean ± SEM performed in triplicate and were repeated twice. *P < .05, ANOVA (post-hoc Bonferroni). (-) non-stimulated cells. NS – not significant.

Sialostatin L2 inhibits caspase-1 maturation, IL-1β and IL-18 secretion by macrophages via NADPH ROS during A. phagocytophilum stimulation

I then investigated the mechanism by which sialostatin L2 inhibits caspase-1 activation. I used the *Nox2*$^{-/-}$ mice, which do not produce ROS from NADPH oxidase [99], to confirm our findings. As previously observed, *A. phagocytophilum* induced ROS production in macrophages (Figure 3.8A) [99]. This was contrary to neutrophils, where *A. phagocytophilum* actively suppresses NADPH oxidase assembly and ROS production [193,273]. Although not completely abrogated, I observed that *Nox2*$^{-/-}$ macrophages produced less ROS when compared to wildtype cells. Furthermore, sialostatin L2 completely inhibited ROS production by wildtype macrophages during *A. phagocytophilum* stimulation. These results implicated the regulation of ROS by sialostatin L2 via NADPH-dependent and possibly independent pathways. The effect of NADPH-dependent ROS or sialostatin L2 on IL-1β secretion was not due to differential IL-1β translation (p31), because *Nox*2$^{-/-}$ macrophages produced similar amounts of pro-IL-1β in the presence or absence of sialostatin L2 when compared to wildtype macrophages (Figure 3.8B). Conversely, I noticed a dose-dependent effect for sialostatin L2 and NADPH-mediated ROS production on IL-1β secretion by macrophages during *A. phagocytophilum* stimulation (Figure 3.8C). This phenomenon appeared specific for IL-1β secretion because IL-6 was not affected (Figure 3.8D). Corroborating with our findings, caspase-1 activation was inhibited by sialostatin L2 and NADPH-mediated ROS production during *A. phagocytophilum* stimulation of macrophages (Figure 3.8E). Taken together, our findings showed that sialostatin L2 inhibited NADPH-mediated ROS

production in macrophages, which correlated with decreased caspase-1 activation and IL-1β and IL-18 secretion during *A. phagocytophilum* stimulation.

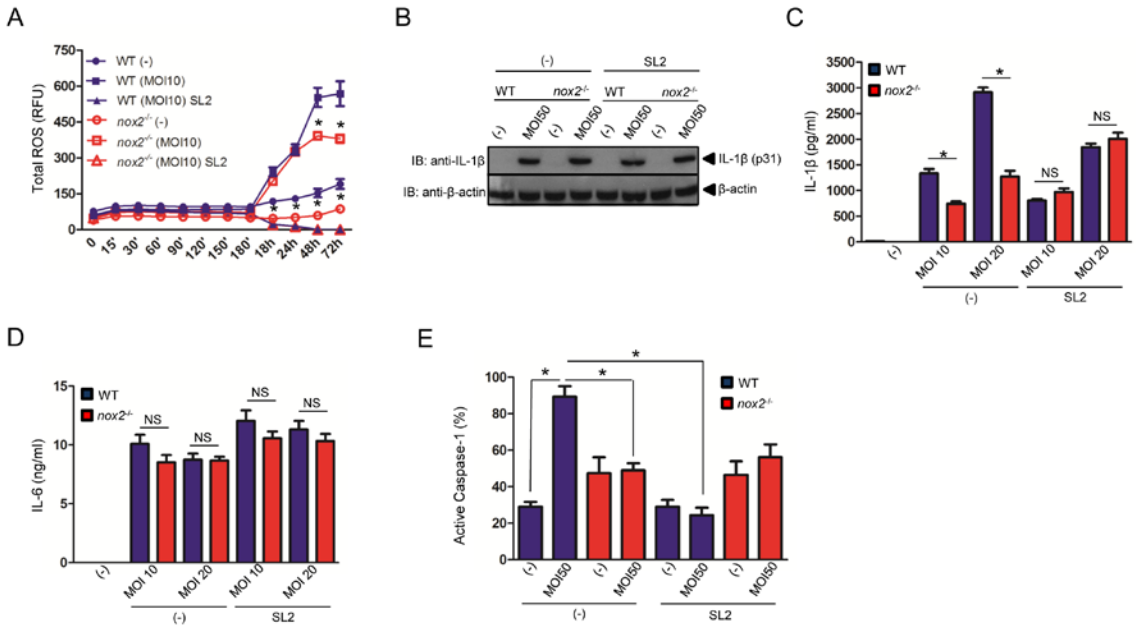

Figure 3.8: Sialostatin L2 inhibits IL-1β secretion via NADPH ROS during A. phagocytophilum stimulation of macrophages.

(A) BMDMs ($2x10^5$) from wildtype (WT) and *Nox2*$^{-/-}$ mice were stimulated with *A. phagocytophilum* (MOI 10) in the presence or absence of sialostatin L2 (SL2– 3 μM). ROS production was monitored using the fluorescent probe H2DCFDA at the indicated time points. (B) BMDMs ($1x10^6$) from wildtype and *Nox2*$^{-/-}$ mice were stimulated with *A. phagocytophilum* (MOI 50) in the presence or absence of SL2 (3 μM) for 18 hours. pro-IL-1β (p31) was measured by western blot (IB). β-actin was used as a loading control. (C-D) BMDMs ($1x10^6$) from wildtype (WT) and *Nox2*$^{-/-}$ mice were stimulated with *A. phagocytophilum* (MOIs 10 and 20) in the presence or absence of SL2 (3 μM) for 18 hours. (C) IL-1β and (D) IL-6 were measured by ELISA. (E) BMDMs ($2x\ 10^5$) from wildtype (WT) and *nox2*$^{-/-}$ mice were stimulated with *A. phagocytophilum* (MOI 50) for 18 hours in the presence or absence of SL2 (3 μM). The fluorescent inhibitor probe FAM-YVAD-FMK was used to label active caspase-1 in macrophages. The signal was corrected for the background and the percentage of caspase-1 activation in macrophages is shown as a function of relative fluorescence units. Cytokine measurements were taken in triplicate and presented as mean ± SEM. A, C and D were repeated twice, whereas B and E were repeated five times. **P* < .05, (A) ANOVA (post-hoc Bonferroni). (C-E) Student's t test. (-) non-stimulated cells. NS – not significant.

3.5 Discussion

Inflammation is characterized by complex interactions between innate and adaptive immunity [275]. Pro-inflammatory cytokines and chemokines recruit immune cells to the site of tick feeding. Tick salivary proteins then mitigate the secretion of cytokines by immune cells, thereby, diminishing inflammation [89,238,240]. Despite significant progress in the past, how ectoparasites, such as ticks, regulate host innate immune signaling remains mostly elusive. In this study, I demonstrate that *I. scapularis* saliva has the ability to inhibit cytokine secretion by murine immune cells. These findings are supported by our results showing that extracellular and cytosolic stimulation of macrophages with PAMPs can be inhibited by *I. scapularis* saliva. I also performed experiments with *A. phagocytophilum* and show that similar mitigation effects occur in macrophages. To our knowledge, I describe for the first time that secretion of IL-6 and IL-12p40 after stimulation with the Nod2 ligand MDP was diminished in macrophages during treatment with tick saliva. Nod2 has emerged as a critical regulator for immunity and inflammation since it activates canonical and non-canonical NF-κB signaling, mitogen activated protein kinases, cytokines, chemokines and antimicrobial reactive oxygen species [270].

Previously, it was shown that *A. phagocytophilum* is partially recognized by the NLRC4 inflammasome [138], a protein scaffold that regulates the secretion of IL-1β and IL-18 [93]. Mice deficient in caspase-1 and *Asc*, essential components of the inflammasome, were more susceptible than wild-type animals to *A. phagocytophilum* infection. These findings were due to the absence of IL-18 secretion and reduced

interferon (IFN)-γ levels in the peripheral blood. It is unclear how *I. scapularis* saliva regulates IL-1β secretion by macrophages during *A. phagocytophilum* stimulation. In this study, I demonstrate that the tick salivary protein sialostatin L2 inhibits inflammasome-mediated inflammation during stimulation with the rickettsial pathogen *A. phagocytophilum*. The sialostatin L2 effect on caspase-1 activation and IL-1β secretion appeared specific for *A. phagocytophilum* because stimulation of macrophages with either *P. aeruginosa* (a non-vector borne pathogen) or *F. tularensis* (a non-*I. scapularis* tick pathogen) did not affect caspase-1 . This is not entirely surprising because the intricate relationship between the tick vector and *A. phagocytophilum* is molded by evolutionary selection [89]. It is possible that multiple salivary proteins regulate IL-1β secretion during hematophagy. Biologically active proteins in the tick saliva are commonly used as a strategy for immune evasion during feeding and it is estimated that hematophagy has evolved independently in more than 14,000 arthropod species [89,238]. Ticks have large genomes and carry many gene paralogs [276]. These gene paralogs may act redundantly to provide inhibition of immune protein scaffolds in the mammalian host. Two earlier articles provided experimental support for this hypothesis. Ramachandra and Wikel showed that salivary gland extracts from the tick *D. andersoni* reduced IL-1 levels during the early phases of tick feeding [277], whereas Fuchsberger et al., determined that human IL-1β secretion was mitigated when treated with LPS and salivary gland extracts from partially fed adult female *R. appendiculatus* [278]. In addition, *A. phagocytophilum* may need redundant mechanisms of innate immune recognition to trigger IL-1β secretion. Secretion of IL-1β requires NF-κB activation to generate pro-IL-1β [111,112]. Dumler

and colleagues demonstrated that *A. phagocytophilum* triggers TLR2 activation during immune cell stimulation [211]. TLR activation is known to initiate NF-κB signaling in immune cells [102]. More recently, our group participated in a study showing that receptor interacting protein-2 (RIP2) affects *A. phagocytophilum* infection in mice [130]. RIP2 is an adaptor molecule for the innate immune receptors Nod1 and Nod2, which also regulates NF-κB signaling [279]. Finally, assembly of a multi-protein complex coined "inflammasome" is critical for IL-1β secretion [111,112]. It was previously demonstrated that the inflammasome is critical for immunity against *A. phagocytophilum* infection [138]. Taken together, our findings reinforce the notion that *A. phagocytophilum* immunity is multi-factorial, and suggests a holistic inhibitory effect of tick saliva on innate immunity. This is important because a pathogen such as *A. phagocytophilum* may need multiple layers of immune evasion during transmission. Therefore, the properties of tick saliva may be a major strategy of host immune evasion during pathogen transmission. Clearly, further studies are necessary to determine the contribution of salivary proteins to *A. phagocytophilum* pathogenesis and immunity.

Further characterization of tick salivary proteins, such as sialostatin L2, is not a trivial task. First, for example, RNAi silencing and vaccination against sialostatin L2 impairs the feeding ability of *I. scapularis* nymphs [260,267]. Therefore, a reliable comparison of pathogen transmission in control and RNAi-silenced ticks; or, alternatively immunized and control groups is not possible. Second, mice are the natural hosts of ticks; thus, they do not typically develop immunity against salivary proteins [238,240]. Third, many sialostatin L2 paralogues are present in the *I. scapularis* genome.

Hence, these molecules may cross-react with antibodies, questioning the validity of any assay that measures sialostatin L2 concentration in the tick saliva. Due to these shortcomings, and the fact that potential host receptors (or cell mediators) in immune cells need saturation to reveal a noticeable phenotype, our approach of 'decomposing' tick saliva by studying individual molecules may be regarded as a conceptual advance for the understanding of inflammasome biology and vector-borne diseases. The technical challenges of tick salivary protein experimentation has truly limited this field of research.

The role of ROS contributing to caspase-1 activation during infection remains highly controversial. Earlier studies demonstrated that ROS originating from NADPH oxidase were deemed important for inflammasome activation [219]. However, subsequent work showed mitochondria as a requirement for caspase-1 activation [280]. Mitochondria is likely not involved in caspase-1-mediated inflammation during *A. phagocytophilum* infection because our results did not show any effect on mitochondrial ROS [99]. Conversely, NADPH oxidase appears important for IL-1β secretion (Figure 3.8) and NF-κB signaling [99]. I observed that *Nox2*$^{-/-}$ macrophages have decreased but not abrogated ROS when macrophages are stimulated with *A. phagocytophilum* [99]. Therefore, caspase-1 signaling in the *A. phagocytophilum* model may include non-canonical ROS sources, such as: β-oxidation of peroxisomes, prostaglandin synthesis and detoxification reactions by cytochrome P450s [281]. Second, ROS studies have been mostly done by using the NLRP3 protein [93,234,235]. In the NLRC4 model, inflammasome function seems independent of mitochondrial involvement [282]. Third, the baseline activation level of the redox system, which determines caspase-1 activation

and IL-1β secretion, can be considered a complicating factor when comparing infectious systems [283]. Finally, the production of IFN-γ, a cytokine that controls *A. phagocytophilum* immunity [273], regulates NADPH oxidase and ROS production [284]. Thus, I posit that an integrated view of ROS production may explain seemingly contrasting findings.

Currently, vaccines for arthropod-borne diseases are only available for the yellow fever virus, Japanese encephalitis virus, Rift valley fever virus and the tick-borne encephalitis virus [89]. The association of traditional pathogen- and vector-based vaccines could improve protection against vector-borne diseases. This rationale is further supported by work showing that previous exposure of mice to salivary gland extracts of sandflies, mosquitoes and ticks reduce pathogen load and vector fitness during transmission [89,238]. The effective use of salivary gland molecules that target the pro-inflammatory pathways as vaccine candidates could be, in theory, used to reduce morbidity and mortality associated with major vector-borne diseases. In conclusion, our studies show that *I. scapularis* tick saliva and sialostatin L2 inhibit pro-inflammatory signaling pathways.

CHAPTER FOUR

NSD1 mitigates caspase-1 activation by listeriolysin O in macrophages

4.1 Abstract

Mammals and plants share pathogen-sensing systems named nod-like receptors (NLRs). Some NLRs form the inflammasome, a protein scaffold that regulates the secretion of IL-1β and IL-18 by cleaving catalytically inactive substrates into mature cytokines. Here, I show an immune conservation between plant and mammalian NLRs and demonstrate that the murine nuclear receptor binding SET domain protein 1 (NSD1), a protein that bears similarity to the NLR regulator enhanced downy mildew 2 (EDM2) in *Arabidopsis*, diminishes caspase-1 activity during extracellular stimulation with *Listeria monocytogenes* listeriolysin O (LLO). I observed that NSD1 neither affects nuclear factor (NF)-κB signaling nor regulates NLRP3 inflammasome gene expression at the chromatin, transcriptional or translational level during LLO stimulation of macrophages. Silencing of NSD1 followed by LLO stimulation led to increased caspase-1 activation, enhanced post-translational maturation of IL-1β and IL-18 and elevated pyroptosis, a form of cell death associated with inflammation. Furthermore, treatment of macrophages with LLO^{W492A}, which lacks hemolytic activity due to a tryptophan to alanine substitution in the undecapeptide motif, indicates the importance of functional LLO for NSD1 regulation of the NLRP3 inflammasome. Taken together, our results indicate that plant NLRs may be used as a platform for gene discovery in mammalian NLR signaling, and NSD1 modulates an immune response against *L. monocytogenes* LLO.

4.2 Introduction

The inflammasome is a critical component of the innate immune system that provides immediate protection against an infectious insult or cellular damage [135]. The canonical protein scaffold is formed by NLRs or AIM2, ASC and caspase-1. Inflammasome activation leads to the release of IL-1β and IL-18 and occurs as a two-tier system [111]. The first signal (priming) involves the activation of the NF-κB pathway, which induces the transcription and translation of pro-inflammatory cytokines and other genes. Following this, inflammasome activation results in the maturation of IL-1β and IL-18 by the enzyme caspase-1 [102,285]. Compared to the classical NLRC4, NLRP1 and AIM2 inflammasomes [286], NLRP3 is uniquely activated by innumerable stimulants, ranging from danger signals to bacterial structures and pore-forming toxins [221].

Owing to the importance of inflammasomes in immune recognition and response to pathogen infection, numerous groups have examined their activity during exposure to the model pathogen *Listeria monocytogenes* [146,287–289]. Initially studied for its ability to escape vacuoles as a means to promote its dissemination, it has been shown that AIM2, NLRC4, NLRP7 and NLRP3 inflammasomes can recognize *L. monocytogenes* [56,149]. The intracellular role of the virulence factor listeriolysin O (LLO), encoded by the gene *hly,* has also been well characterized in terms of phagosomal evasion; however, its extracellular activities remain mostly unclear [290]. Though several studies have confirmed that LLO activity is sensitive to an acidic pH, a fraction of LLO is functional at the neutral pH found in the extracellular space [291]. Outside the cell, LLO has

demonstrated its role in the initiation of bacterium internalization and autophagy, as well as its ability to manipulate histone and post-translational modifications [290]. It was also recently revealed that potassium (K^+) efflux induced by pores formed by external LLO activates caspase-1[292]. Nonetheless, the components of this pathway continue to be elusive.

I drew upon the similarities between plant and mammalian pathogen-sensing systems to address the void in our knowledge regarding the connection between extracellular LLO and inflammasome activity. NLRs are functionally conserved between plants and mammals and they contain the characteristic nucleotide-binding leucine-rich repeat domains. Previously, it was demonstrated that the *Arabidopsis* protein enhanced downy mildew 2 (EDM2) regulates a NLR gene named *Recognition of Peronospora Parasitica 7* (*RPP7*) during oomycete infection in plants [293]. EDM2 shares similarity with the nuclear receptor-binding SET domain protein 1 (NSD1) in mice and humans[293]. NSD1 plays a role in several pathologies, including but not limited to: Sotos and Weaver syndromes, acute myeloid leukemia, breast cancer, neuroblastoma and glioblastoma formation [294–298]. NSD1 haploinsufficiency, hypermethylation, and fusion with NUP98 are associated with these diseases. NSD1 was described as a cofactor that interacts with nuclear receptors, acting both as a co-activator or co-repressor depending on the presence or absence of a ligand [299,300]. NSD1 can also alter transcription by interacting with the protein NSD1-interacting zinc finger protein 1 (NIZP1) [301] and may act as a methytransferase that preferentially methylates histone 3 and 4 on lysines 36 and 20, respectively[302,303].

In this study, I examined the relationship between NSD1 and the NLRP3 inflammasome during extracellular exposure to the cholesterol-dependent cytolysin LLO. Here, I show that the NSD1 regulation of caspase-1 activation during LLO stimulation of macrophages does not influence NF-κB signaling, chromatin dynamics or transcription and translation of inflammasome genes. NSD1 affects the maturation of caspase-1, which in turn modulates IL-1β, IL-18 secretion and a specialized form of cell death referred to as pyroptosis.

4.3 Materials and methods

Bioinformatics

Amino acid sequences of PHD fingers were analyzed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [304]. Protein schematic was obtained from SMART: Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) [305]. Nucleosome prediction for primer design was done using the NuPoP: Nucleosome Positioning Prediction Engine (http://nucleosome.stats.northwestern.edu/) [306]. Prediction of methylated lysines was performed using MeMo: Methylation Modification Prediction Server 2.0 (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo.html) [307]. The caspase-1 gene map was created using Ensembl (http://uswest.ensembl .org/index.html) [308].

Ethics statement

All animal breeding and experiments were performed in strict compliance with guidelines set forth by the National Institutes of Health (Office of Laboratory Animal Welfare (OLAW) - Assurance number A3439-01). All animal and biosafety procedures were approved by the Institutional Animal Care and Use (IACUC number: A-20110030BE) and Biological Use Authorization (BUA number: 20120020) Committees at the University of California, Riverside. C57BL/6 mice were purchased from Jackson Laboratories. *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice were obtained from Millennium Pharmaceuticals.

Cell culture generation

I used male mice 12-20 weeks of age. Bone marrow-derived macrophages (BMDMs) were generated as previously described with minor modifications [266]. Briefly, femurs and tibias were removed from C57BL/6, *Nlrp*3$^{-/-}$ and *Nlrc*4$^{-/-}$ mice and kept in phosphate buffered saline (PBS) + 1% Penicillin-Streptomycin-Amphotericin (PSA) (ThermoScientific). Muscle was removed from femurs and tibias using scissors and razor blades. The ends were cut and marrow was flushed from the bone using cold Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with a 27 gauge needle. BMDMs were grown on 10 cm petri dishes in 10 ml of DMEM media supplemented with 10% fetal calf serum (FCS) (Invitrogen), 30% L929 cell conditioning medium, and 1% PSA. BMDMs were grown in a humidified incubator at 37°C with 5% CO_2 for 6 days prior to stimulation. On the 3rd day, 10 ml of DMEM + 10 % FCS + 30% L929 cell conditioning medium + 1% PSA was added to each dish. BMDMs were plated on 24-

well culture plates at 1 x 10^6 cells per well, unless otherwise stated, in 500 µl of DMEM + 10% FCS + 1% PSA.

Macrophage silencing, stimulation and infection

Nsd1 was silenced with 100 nM of Ambion Silencer Negative Control siRNA #1 (Ambion AM 4635) or Ambion Silencer Pre-designed siRNA for *Nsd1* (Ambion AM 16706) using a 1:1 ratio of siRNA to Lipofectamine 2000 (Invitrogen). 48 hours after siRNA transfections, BMDMs were primed with 0.1 µg/ml of LPS (Invivogen) for 2 hours. BMDMs were stimulated with 500 ng/ml or 8 µg/ml of LLO for 30 minutes or 1 hour, respectively. Treatment with 500 µg/ml of Imject alum (Thermo Scientific) or 10 µM of nigericin (Sigma) was done for 6 hours. Stationary phase *Pseudomonas aeruginosa* PAO1 was used to infect BMDMs at a multiplicity of infection (MOI) of 50 for 1 hour. Wild-type (WT) (10403S), Δ*hly* (DP-L2161), and *L. pneumophila* flagellin (*L.p*. FlaA) (DP-L5964) expressed by *L. monocytogenes* were grown in BD Bioscience Bacto Brain Heart Infusion media overnight at 30°C while kept stationary. Absorbance at OD 595 nm was measured and values between 1.2-1.4 were used. Cultures were diluted (1:10) with sterile PBS (Thermo). BMDMs were infected using MOI 10. 30 minutes after infection, media was replaced with 50 µg/ml Gentamicin/Amphotericin B (Cascade Biologics) + DMEM.

Recombinant LLO and LLO^{W492A} expression

E. coli strain BL21 (DE3) carrying plasmid pET29:6xHis-LLO or pET29b-LLO W492A-His6 was used to express recombinant LLO and LLO^{W492A}, respectively. Expression and purification was done as described with minor modifications [309]. *E. coli* was grown with agitation, at 37°C overnight, in 10 ml of LB broth (Teknova) supplemented with 50 μg/ml of kanamycin (Sigma). The following day, 100 ml of Luria Bertani (LB) broth was added and 50 μg/ml of kanamycin was supplemented. Expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) from Sigma. Cultures continued to grow at 30°C for 18 hours with agitation. *E. coli* was pelleted (4,000 x g, 15 minutes, 4°C). The pellet was resuspended in 1 ml of lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM imidazole). The pelleted expression culture was sonicated 4 times (20% power, 15 second pulses, 1 minute rests on ice) (VWR Scientific Branson Sonifier 450). Purification was done using a Qiagen Ni-NTA spin column. Purification was done as recommended by Qiagen. Lysates were centrifuged in columns for 5 minutes at 270 x g. Spin columns were washed with wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 1 mM PMSF, 20 mM imidazole). A 16% glycerol wash (16% glycerol and wash buffer) and a high NaCl wash (700 mM NaCl and wash buffer) were performed. A rinse with wash buffer was done after each wash. Proteins were eluted twice into an elution buffer (0.136 g of imidazole and wash buffer). All washes and elution were centrifuged at 700 x g for 2 minutes at 4°C. Eluate was concentrated with Millipore Amicon Ultra 3000 MWCO filter unit. Recombinant LLO was kept at -80°C in a storage buffer (10 mM 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid (HEPES), 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)). Several batches of recombinant LLO were expressed and purified. Variability between lots resulted in the adjustment of concentrations used.

Immunofluorescence microscopy

BMDMs were cultured as described above. $2x10^6$ cells were grown on 18 mm glass coverslips in 6 well plates. Cells were stimulated with tumor necrosis factor (TNF)-α (50 ng/ml) and LLO (500 ng/ml). Cells were washed twice with PBS and fixed with methanol. A 1:200 dilution of a custom made NSD1 antibody (Fisher Scientific) was used. Coverslips were incubated in the primary antibody for 1 hour at room temperature. Slips were washed and incubated in an anti-rabbit fluorescence-conjugated secondary antibody (Millipore) at room temperature for 30 minutes. Slips were mounted onto a slide using Vectashield mounting media with 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopy was done with a Leica SP2. Original magnification was 63x with an enlargement of 4x.

Quantitative real-time RT-PCR (qPCR)

RNA extraction was done with TRIzol (Invitrogen). First strand cDNA was synthesized using Verso cDNA kit purchased from Thermo Scientific. qPCR was done with iQ SYBR Green Supermix, on either a Bio-Rad iQ5 or MyiQ real-time PCR detection system, and data was processed by iQ5 software from Bio-Rad. Data was

analyzed by the $\Delta\Delta C_T$ method [310]. β-actin was used as the normalizing control. Primer sequences were as follows: for β-actin-F (5'-CGCATCCTCTTCCTCCCT-3') and β-actin-R (5'-TGGAATCCTGTGGCATCC-3'); caspase-1 a-F (5'-CAACCATTCCTTGGTCCACT-3') and caspase-1 a-R (5'-ATTGATGTGGGGGAAAGGTT-3'); caspase-1 b-F (5'-TACCTGGCAGGAATTCTGGA-3') and caspase-1 b-R (5'-GCAGAGCCACAGACACAAAA-3'); caspase-1 c-F (5'-CCTACCAGCATTTCAGGCATA-3) and caspase-1 c-R (5'-TGTTGGCTGTAGGTGTGGAA-3'); *Nlrp3* (a)- F (5'-TTATGTTGGACTGGGCACTG-3') and *Nlrp3* (a)-R (5'-ATCAAAGCCATCCATGAGGA-3'); *Nlrp3* (b)-F (5'-CCCCATTACCTAACCCCATC-3') and *Nlrp3* (b)-R (5'-GGAAATTCTGATGTACCTG AACAC-3'); *Asc*-F (5'-TGTCAGGGGATGAACTCAAA-3') and *Asc*-R (5'-CAGCTCCTG TAAGCCCATGT-3'); *Nsd1*-F (5'-ACCTGACAGAGCCTCTCCAA-3') and *Nsd1*-R (5'-GCTGGAGTTTTCTCCACTGC-3'); *caspase-11*- F (5'-ACGATGTGGTGGTGAAAGAGGAGC- 3') and *caspase-11*- R (5'-TGTCTCGGTAGGACAAGTGATGTGG-3'). β-actin, *Asc*, *Nlrp3* (a), *Nsd1*, and caspase-1 c primers were used for qPCR. All caspase-1 primers and *Nlrp3* (b) were used for chromatin immunoprecipitation (ChIP)-qPCR. Primers were designed using Life Technologies OligoPerfect Designer (http://tools.invitrogen.com).

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed for the detection of IL-1β, IL-18, and IL-6 using BD OptEIA kits from BD Biosciences. Supernatants used were collected from cells cultured and stimulated in all experiments. Absorbance was measured using Bio-Rad iMark at 450 nm with a 595 nm correction.

Immunoblotting

Total cell lysates from 24 well plates cultured and stimulated, as described previously, were extracted using RIPA lysis buffer (Boston Bioproducts) with Complete Mini Protease Inhibitor Cocktail and PhosSTOP, both from Roche Applied Science. Protein concentration was determined via the Bradford protein assay method, using protein assay dye reagent concentrate and iMark reader, both from Bio-Rad. Either an 8% or 15% SDS polyacrylamide gel was made and ran at 200 volts for 1 hour. Transfer was done in wet conditions with polyvinylidene fluoride (PVDF) membranes for 60-90 minutes at 100 volts. Membranes were blocked in 5% non-fat dry milk (LabScientific, Inc.). Western blot antibodies for NSD1 (Santa Cruz and Custom made Fisher Scientific) (1:500 and 1:625), β-actin (Neomarker-Thermo Scientific) (1:500 and 1:1000), caspase-1 (Millipore) (1:500), lamin B1 (Abcam) (1:100), p-IκB-α (Cell Signaling) (1:250), NLRP3 (Abcam) (1:500), ASC (Enzo) (1:250), pro-IL-1β (R&D) (1:1000) and caspase-11 (Sigma) (1:500). Enhanced chemiluminescence (ECL) western blotting substrate and super signal West Pico Chemiluminescent substrate were used to image the blots (Pierce Thermo Scientific).

Nuclear protein extraction

$5x10^6$ BMDMs were silenced and stimulated as indicated above. Nuclear protein was extracted using the G-Biosciences: Nuclear and Cytoplasmic Extraction Kit (Catalog# 786-182). The protocol was scaled down appropriately and extraction was done according to the protocol provided. Complete Mini Protease Inhibitor Cocktail (Roche Applied Science) was used.

Transcription and degradation inhibition

Transcription and degradation inhibition was done as previously described [67]. Inhibition of transcription was accomplished using 5 μg/ml of actinomycin D (Sigma-Aldrich). Proteasomal degradation inhibition was done using 1 μM of the reversible proteasome inhibitor MG-132 (Calbiochem).

Fluorescent labeled inhibitor of caspases (FLICA)

Cells were cultured and stimulated as stated above. Green FLICA Caspase-1 Assay Kit (Catalog #98) was from Immunochemistry and the provided protocol was used. Cell counting was done with BD Biosciences FACSCanto Flow Cytometer. Data was processed using BD FACSDiva Software (BD Biosciences).

ChIP assay

BMDMs ($45x10^6$) were seeded onto a 15 cm dish. Cells were silenced and primed as previously described. Cells were stimulated with 8 μg/ml of recombinant LLO. After 1

hour of stimulation, the Active Motif ChIP-IT Express kit (Catalog# 53008) was used to prepare chromatin. Briefly, cells were fixed with a fixation solution (37% Sigma formaldehyde and DMEM). Fixation was stopped with a glycine stop-fix solution. Cells were dounced on ice with 20 strokes of rod A and 20 strokes of rod B prior to sonication. The cell lysate was sonicated (Fisher Scientific Sonic Dismembrator Model 100) with 5 pulses at power 6 for 15 seconds in 700 µl of shearing buffer. DNA was cleaned up, as suggested, in order to assess shearing efficiency. 3 µg of NSD1 (Custom made Fisher Scientific), histone 3 (H3), histone 3 lysine 36 dimethylation (H3K36me2), histone 3 lysine 36 trimethylation (H3K36me3) and the negative control goat anti-rabbit HRP antibodies (all from Abcam) were used per ChIP. Reactions were incubated at 4°C for 4 hours. Magnetic bead-antibody complexes were washed twice in 800 µl of ChIP Buffer 1 and three times with 800 µl of ChIP Buffer 2. After chromatin elution, reverse cross-linking and protein degradation was done. The Qiaquick PCR Purification kit from Qiagen (Catalog# 28104) was used to purify samples. qPCR was used to analyze enrichment as stated above. Fold change and standard errors were determined using the following $\Delta\Delta C_T$ protocol: http://www.protocol-online.org/biology-forums/posts/29733.html.

Hemolytic assay

10% sheep red blood cells (RBC) were obtained from Lampire Biological Laboratory. A suspension of 0.2% RBC was made by washing RBC three times by centrifuging the suspension at 1500 rpm for 15 minutes at 4°C. 1% Triton X was used as

a positive control and PBS was used as a negative control. A serial dilution of the toxin was created, added to the RBC and incubated for 5 minutes on ice. Samples were then incubated for 30 minutes at 37°C and the absorbance was measured at 450 nm.

LDH assay

$1x10^6$ BMDMs (WT or *Nlrp3*$^{-/-}$) were transfected with control silencer or *Nsd1* siRNA. BMDMs were primed and untreated or treated with 8 μg/ml WT LLO or LLO^{W492A} for 1 hour. Medium alone was used as the negative control and 1% Triton-X was used as the positive control. Supernatant from controls and samples were reserved for the assay. Percent of LDH release was assayed using the Takara LDH cytotoxicity detection kit. Assay was conducted as recommended. Absorbance was measured using Bio-Rad iMark at 450 nm.

Statistical analysis

All graphs were generated in GraphPad Prism 5 and *P* values were calculated using Student's t test. $P < 0.05$ was considered statistically significant.

4.4 Results

LLO stimulation of macrophages upregulates NSD1

Since *L. monocytogenes* LLO is important for bacterial virulence [304,305] and LLO extracellular activities remain mostly unclear [290,311], I decided to investigate how host signaling recognizes this bacterial toxin. Owing to the conservation of NLR

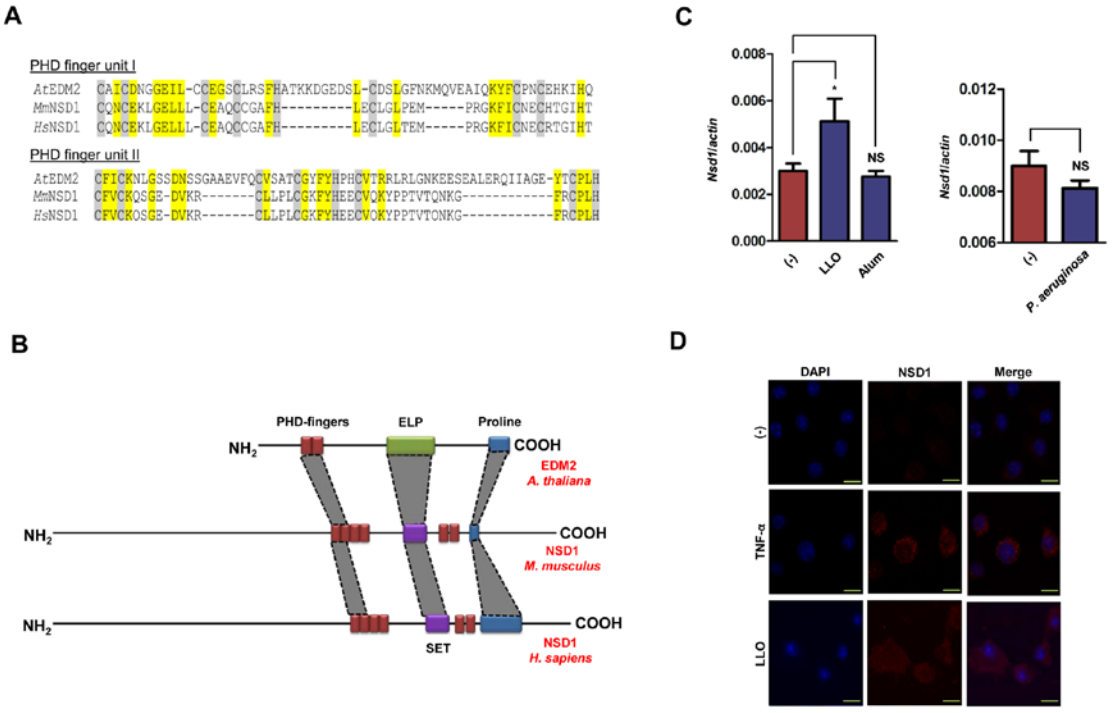

Figure 4.1: *Nsd1* is upregulated during LLO stimulation of macrophages.

(A) Displayed peptide stretches are directly adjacent to each other and cover two repeated PHD finger units. Consensus sequences of both PHD finger units based on similarities between *Arabidopsis* (*At*) EDM2 and NSD1 in mice (*Mm*) and humans (*Hs*). Cys or His residues of the conserved zinc coordinating C4HC3 pattern of PHD fingers are highlighted in grey. The last C of the C-terminal PHD finger unit is replaced by H in EDM2/NSD1-type proteins. Other residues conserved between EDM2 and NSD1 are highlighted in yellow. (B) PHD fingers from EDM2 and NSD1 are shown in red. Methyltransferase domains in EDM2 (ELP) and NSD1 (SET) are shown in green and purple, respectively. A proline rich region in both NSD1 and EDM2 is shown in blue. (C) BMDMs ($1x10^6$) from C57BL/6 mice were stimulated with (C, *left*) LLO (500 ng/ml) (n=4) for 30 minutes and alum (500 μg/ml) (n=4) for 6 hours and (C, *right*) *P. aeruginosa* (MOI 50) (n=6) for 1 hour. *Nsd1* transcription was evaluated by qPCR and analyzed by the $\Delta\Delta C_T$ method. β-actin was used as a normalizing control. Student's t test; (*) $P < 0.05$ compared to (-) non-stimulated cells. NS – not significant. (D) Confocal microscopy of BMDMs from C57BL/6 mice untreated or treated with TNF-α (100 ng/ml) or LLO (500 ng/ml). BMDMs were stained with DAPI (blue) and anti-NSD1 (red). Original magnification 63x with an enlargement of 4x. Scale bar = 10 μm. Experiments were repeated at least three times.

pathways in plants and mammals, I drew upon the similarities between these two eukaryotic kingdoms for our study. Previously, EDM2 in *Arabidopsis* was identified as a regulator of RPP7, a NLR that mediates recognition of a plant pathogen [293]. EDM2

bears similarity to the mammalian protein NSD1 (Figure 4.1A and B). An *in silico* analysis of EDM2 and NSD1 revealed that they possess PHD finger domains that have many conserved residues (Figure 4.1A). PHD fingers are involved in nuclear protein-protein interaction. Typically, zinc coordinating sites within PHD fingers carry a featured C4HC3 pattern [312,313]. The EDM2 and NSD1 C-terminal PHD finger units are characterized by a conserved C4HC2H structure (Figure 4.1A). In addition to the PHD fingers, EDM2 and NSD1 share other domains, such as a C-terminal proline-rich region which is thought to play a role in protein-protein interactions and/or transcriptional activation (Figure 4.1B) [314]. Furthermore, while NSD1 bears a Su(Var)3-9, Enhancer-of-zeste, Trithorax (SET) methyltransferase domain, EDM2 features an EDM2-like protein (ELP) domain which is likely to have methyltransferase activity [293,315].

I then proceeded to establish a relationship between NSD1 and caspase-1 activation, since it is known that EDM2 plays a role in mediating the activities of an *Arabidopsis* NLR-containing protein named RPP7 [293] and LLO is recognized by the NLRP3 inflammasome [146] . I stimulated BMDMs with LLO, alum, and *P. aeruginosa*, which are agonists for the NLRP3 and the NLRC4 inflammasomes, and evaluated transcription levels of *Nsd1* by qPCR. I noticed an increase in *Nsd1* transcript levels when cells were exposed to LLO, an NLRP3 stimulant that induces inflammasome activation via K^+ efflux [292] (Figure 4.1C, *left*). This effect was not apparent with alum, a particulate agonist of the NLRP3 inflammasome that stimulates caspase-1 via lysosomal disruption and cathepsin B release [234] (Figure 4.1C, *left*). Similarly, I did not detect an effect on *Nsd1* transcription during macrophage stimulation with *P.*

aeruginosa, a NLRC4 agonist [234] (Figure 4.1C, *right*). I then used confocal microscopy to elucidate the impact of LLO on NSD1 protein levels. Similar to our results observed with *Nsd1* transcription, the levels of NSD1 also increased with TNF-α, a positive control, and LLO treatment compared to untreated cells (Figure 4.1D). With these experiments, I concluded that *Nsd1* expression increases when mouse macrophages are exposed to the NLRP3 agonist LLO.

LLO activates the NLRP3 inflammasome and promotes IL-1β secretion

LLO is known for its key cytolysin feature [316]. It possesses efficient hemolytic abilities (Figure 4.2A). Cellular lysis is frequently linked with cell death and here I observed that LLO induces macrophage cell death [317] (Figure 4.2B). Moreover, recombinant LLO is a clear inducer of IL-1β secretion (Figure 4.2C). To examine the effect of *L. monocytogenes*-derived LLO on IL-1β secretion and inflammasome activity in macrophages, I infected bone marrow-derived macrophages (BMDMs) with WT and a *L. monocytogenes* strain lacking the virulence factor LLO (here described as Δ*hly L. monocytogenes*) [56]. As a positive control, I used a genetically engineered strain of *L. monocytogenes* that expresses *Legionella pneumophila* flagellin (*L.p*. FlaA), a strong inducer of the NLRC4 inflammasome [318]. In the absence of priming, minimal levels of IL-1β were secreted by WT, *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ macrophages during infection with WT and Δ*hly L. monocytogenes* (Figure 4.2D and F). However, unprimed WT macrophages stimulated with the *L.p*. FlaA strain were able to secrete moderate levels of IL-1β, most likely due to the effects of flagellin on TLR5 and NLRC4 recognition. Although

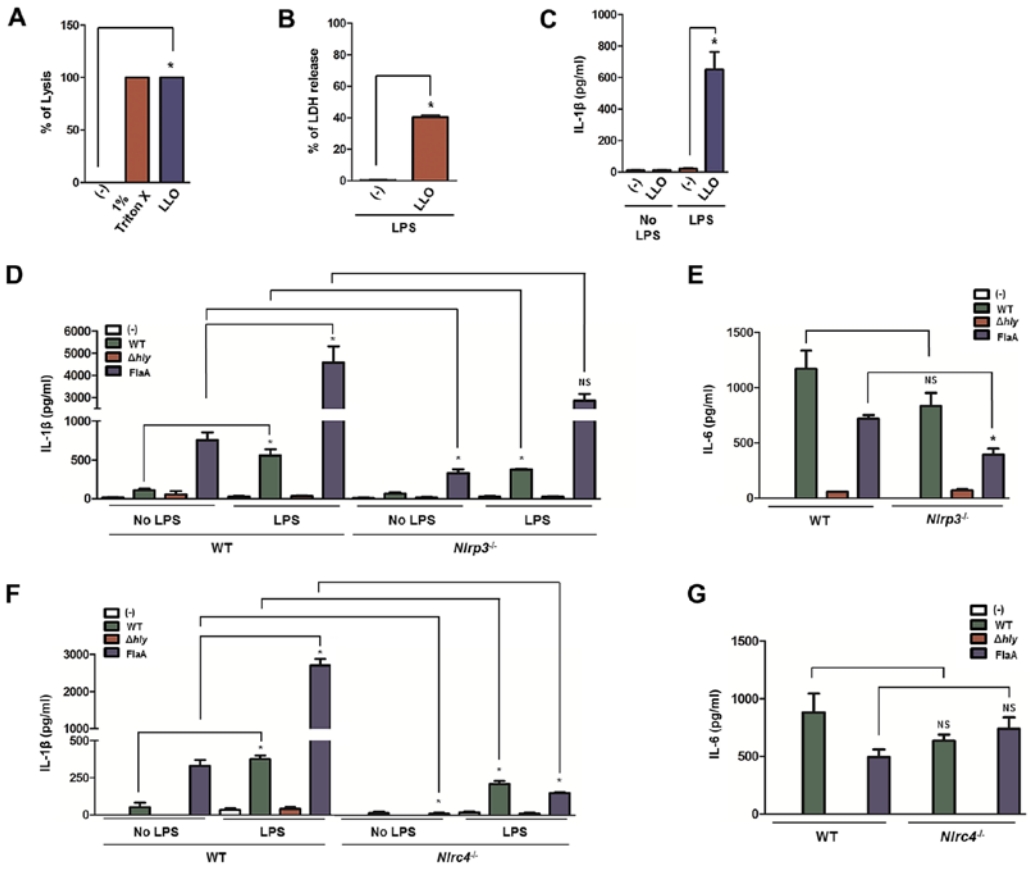

Figure 4.2: Listeriolysin O is recognized by the NLRP3 inflammasome.

$1x10^6$ BMDMs were primed with LPS (100 ng/ml) for 2 hours. BMDMs were treated with recombinant LLO (8 μg/ml for hemolysis and LDH or 500 ng/ml for IL-1β) or infected with the *L. monocytogenes* WT, Δ*hly*, or *L.p.*FlaA strains MOI 10 for 6 hours after gentamicin (50 μg/ml) medium replacement at 30 minutes. (A) Hemolysis, (B) LDH, and (C) IL-1β were measured for recombinant LLO. (D and F) IL-1β secretion by WT (n=4), *Nlrp3*$^{-/-}$ (n=4) and *Nlrc4* $^{-/-}$ macrophages (n=4) was analyzed by ELISA. (E and G) Stimulation was repeated, as previously stated, without priming and IL-6 secretion by WT (n=4), *Nlrp3*$^{-/-}$ (n=4) and *Nlrc4* $^{-/-}$ macrophages (n=4) was determined. Student's t test; (*) P <0.05, WT compared to either unprimed or knockout. NS – not significant. Experiments were repeated at least twice.

TLR5 and NLRC4 both recognize flagellin, TLR5 distinctly identifies the D1 region of flagellin on the cell surface [319], while NLRC4 detects the C-terminus of the D0 region of this bacterial component intracellularly [81,234]. Hence, *L. pneumophila* flagellin may

act to prime the production of immature IL-1β via TLR5 and induce maturation via caspase-1 activation.

After priming, WT macrophages secreted IL-1β during WT *L. monocytogenes* infection and, more so, with the *L.p.* FlaA strain (Figure 4.2D and F). Confirming functionality, *Nlrc4*$^{-/-}$ macrophages demonstrated a deficiency in IL-1β secretion when infected with the *L.p.* FlaA strain (Figure 4.2F). Even though less pronounced, *Nlrc4*$^{-/-}$ macrophages also secreted lower levels of IL-1β during infection with WT *L. monocytogenes*, most likely due to the lack of endogenous *L. monocytogenes* flagellin recognition by NLRC4. In all instances, treatment with *L. monocytogenes* lacking *hly*, the gene that codes for LLO, resulted in negligible amounts of IL-1β secretion (Figure 4.2D and F). When contrasting primed WT and *Nlrp3*$^{-/-}$ macrophages, I observed a statistically significant decrease in IL-1β secretion in cells exposed to WT but not the *L.p.* FlaA strain (Figure 4.2D). The decrease in IL-1β secretion from *Nlrp3*$^{-/-}$ cells treated with the WT *L. monocytogenes* strain could potentially be due to the absence of LLO detection by NLRP3.

IL-6 was measured to determine the effect of *Nlrp3* or *Nlrc4* deficiency on caspase-1 independent signaling pathways. WT and *L.p.* FlaA *L. monocytogenes* elicited IL-6 from WT macrophages, whereas *L. monocytogenes* Δ*hly* was unable to induce IL-6 secretion (Figure 4.2E and G). The absence of *Nlrc4* or *Nlrp3* did not affect IL-6 secretion by macrophages during stimulation with WT *L. monocytogenes*. On the other hand, *Nlrp3*$^{-/-}$ macrophages slightly altered IL-6 levels after infection with the *L.p.* FlaA strain (Figure 4.2E). It is unclear why IL-6 levels were moderately affected by the *L.p.*

FlaA strain in *Nlrp3*$^{-/-}$ macrophages. However, it has been shown that IL-6 signaling may be affected by IL-1β secretion downstream of caspase-1-dependent signaling pathways [320,321] . Taken together, our results suggest that: (1) WT *L. monocytogenes* elicits low levels of IL-1β secretion; and (2) flagellin and LLO are seemingly important for NLRC4 and NLRP3 recognition in mouse macrophages.

NSD1 restricts NLRP3 inflammasome-mediated cytokine secretion during LLO stimulation of macrophages

Due to the embryonic lethality of *Nsd1* knockout mice [303] , I used siRNA-mediated silencing to study the effects of NSD1 on innate immunity. I tested both 50 and 100 nM of siRNA in our experimental design and discovered that these concentrations resulted in substantial reduction of *Nsd1* expression in BMDMs at 48 hours post-transfection (Figure 4.3A, *left*). This decrease was confirmed by immunoblotting, which revealed a 50% decrease of NSD1 in the cells given *Nsd1* siRNA (Figure 4.3A, *right*). IL-1β and IL-18 were analyzed to determine if NSD1 influences the maturation of these pro-inflammatory cytokines (Figure 4.3B-C). Secretion of IL-1β and IL-18 increased with *Nsd1* reduction and LLO stimulation of macrophages. This effect seemed specific for LLO because alum, another NLRP3 inflammasome stimulant, did not affect IL-1β release when NSD1 was silenced in macrophages (Figure 4.3B). Furthermore, I observed that NLRP3 is crucial during LLO stimulation because *Nlrp3*$^{-/-}$ macrophages exhibited abolishment of IL-1β secretion (Figure 4.3D). As a negative control, NLRC4-mediated IL-1β secretion was measured and observed to be unaffected when *P. aeruginosa* was

used to activate the inflammasome in the presence of normal and reduced levels of NSD1 (Figure 4.3E). Taken together, our findings suggest the specificity of NSD1 regulation of the NLRP3 inflammasome in the presence of LLO.

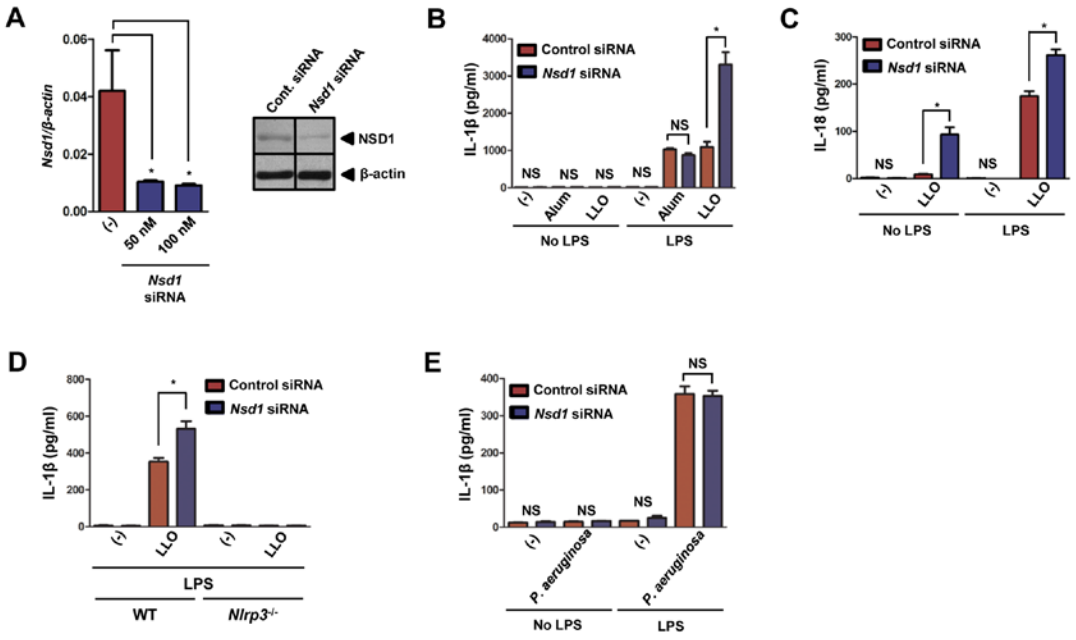

Figure 4.3: NSD1 inhibits LLO-mediated secretion of IL-1β and IL-18 by macrophages.

(A, *left*) 50 and 100 nM of *Nsd1* siRNA successfully reduced *Nsd1* transcription at 48 hours (n=6). (A, *right*) 100 nM of *Nsd1* or control silencer siRNA was transfected into BMDMs ($1x10^6$) by using Lipofectamine 2000. Cell lysate was immunoblotted for NSD1. β-actin was used to verify equal loading. $1x10^6$ BMDMs were primed with LPS (100 ng/ml) and treated with control silencer or *Nsd1* siRNA. Also, BMDMs were untreated or stimulated with LLO (500 ng/ml) for 30 minutes. (B) IL-1β (n=6) and (C) IL-18 (n=6) were measured by ELISA after NLRP3 (500 ng/ml LLO or 500 μg/ml alum) stimulation. (D) After priming with LPS and stimulation with 8 μg/ml recombinant LLO, IL-1β levels secreted by BMDMs from WT (n=3) and *Nlrp3*$^{-/-}$ (n=4) mice were measured by ELISA. (E) IL-1β was measured by ELISA after NLRC4 stimulation (*P. aeruginosa* MOI 50) (n=4). Student's t test; (*) $P <$ .05 compared to cells transfected with control siRNA. NS – not significant. Experiments were repeated at least twice.

NSD1 does not affect NF-κB signaling in response to LLO stimulation of macrophages

Since the NF-κB pathway regulates transcription of inflammatory genes [322] and LLO stimulates the NLRP3 inflammasome [289], I measured the amount of IL-6 for LPS and LLO treated cells after siRNA transfection. Diminished levels of NSD1 did not influence the secretion of LPS-induced IL-6 secretion from macrophages (Figure 4.4A). A negligible amount of IL-6 was released from LLO-stimulated cells (*data not shown*).

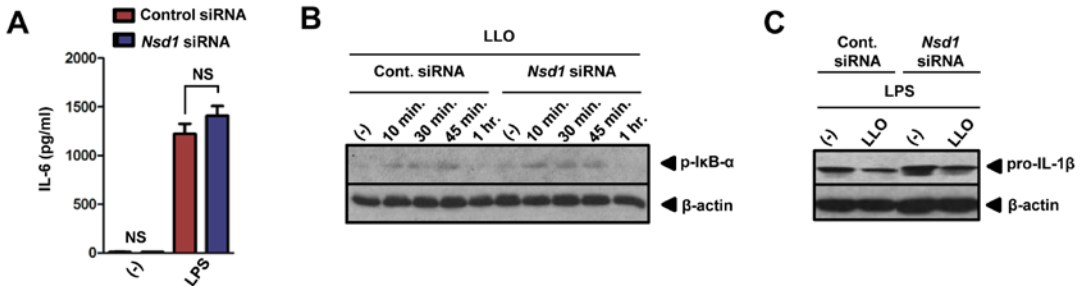

Figure 4.4: NSD1 does not affect the NF-κB signaling pathway in macrophages.

$1x10^6$ BMDMs were transfected with control silencer or *Nsd1* siRNA. After 48 hours, BMDMs were untreated (-) or treated overnight with LPS (500 ng/ml). (A) IL-6 was measured by ELISA (n=8). Student's t-test; (*) $P < 0.05$ compared to cells given control siRNA. NS – not significant. (B) BMDMs ($1x10^6$) were untreated or stimulated with LLO (500 ng/ml). Cell lysates were collected at the indicated time points. Immunoblotting was performed to determine levels of p-IκB-α. β-actin was used to determine equivalent loading. (C) $1x10^6$ BMDMs were primed with LPS (100 ng/ml) and treated with control silencer or *Nsd1* siRNA. Also, BMDMs were untreated or stimulated with LLO (500 ng/ml) for 30 minutes. Cell lysates were immunoblotted for pro-IL-1β. β-actin was used to determine equal loading. Experiments were repeated at least twice.

Further analysis, using a time course experiment visualizing p-IκB-α, a read-out for NF-κB activation [43], showed similar levels of NF-κB activation in non-silenced and *Nsd1* silenced cells given LLO (Figure 4.4B). In both treatments, phosphorylation of IκB-α initiated 10 minutes after stimulation with LLO and returned to pre-treatment levels after 45 minutes. Further confirming that NSD1 regulatory activity is independent of signal 1,

pro-IL-1β was measured by western blot. After LPS priming, pro-IL-1β levels remained relatively constant in silenced and non-silenced cells (Figure 4.4C). These results suggested that the NSD1 role during LLO stimulation of macrophages was independent of the NF-κB pathway.

NSD1 does not restrict NLRP3 inflammasome activation at the chromatin level

Earlier work demonstrated the ability of NSD1 to act as a histone methyltransferase [323] and has indicated that NSD1 targets the 5' end of genes [303]. Therefore, I investigated whether any NSD1-mediated chromatin modifications were associated with NLRP3 inflammasome regulation during LLO stimulation of macrophages. First, I used a prediction engine named NuPoP to determine nucleosome positioning in the NLRP3 inflammasome genes. I then silenced *Nsd1* and observed, by western blot, a 50% reduction of nuclear NSD1 during LLO stimulation of macrophages (Figure 4.5A). Utilizing chromatin immunoprecipitation (ChIP), I observed that areas corresponding to the region -1000 bp to +2000 bp from the transcription start site (TSS) of caspase-1 were not differentially occupied by NSD1 during stimulation with LLO (Figure 4.5B-C). Initially, our experiments included the analysis of the TSS; however, NSD1 binding at the +1 site was highly variable and could not be represented. Physical association of NSD1 with *Nlrp3* and *Asc* was also quantified and did not show enrichment after treatment with LLO in *Nsd1*-silenced versus non-silenced cells (Figure 4.5C, *data not shown*).

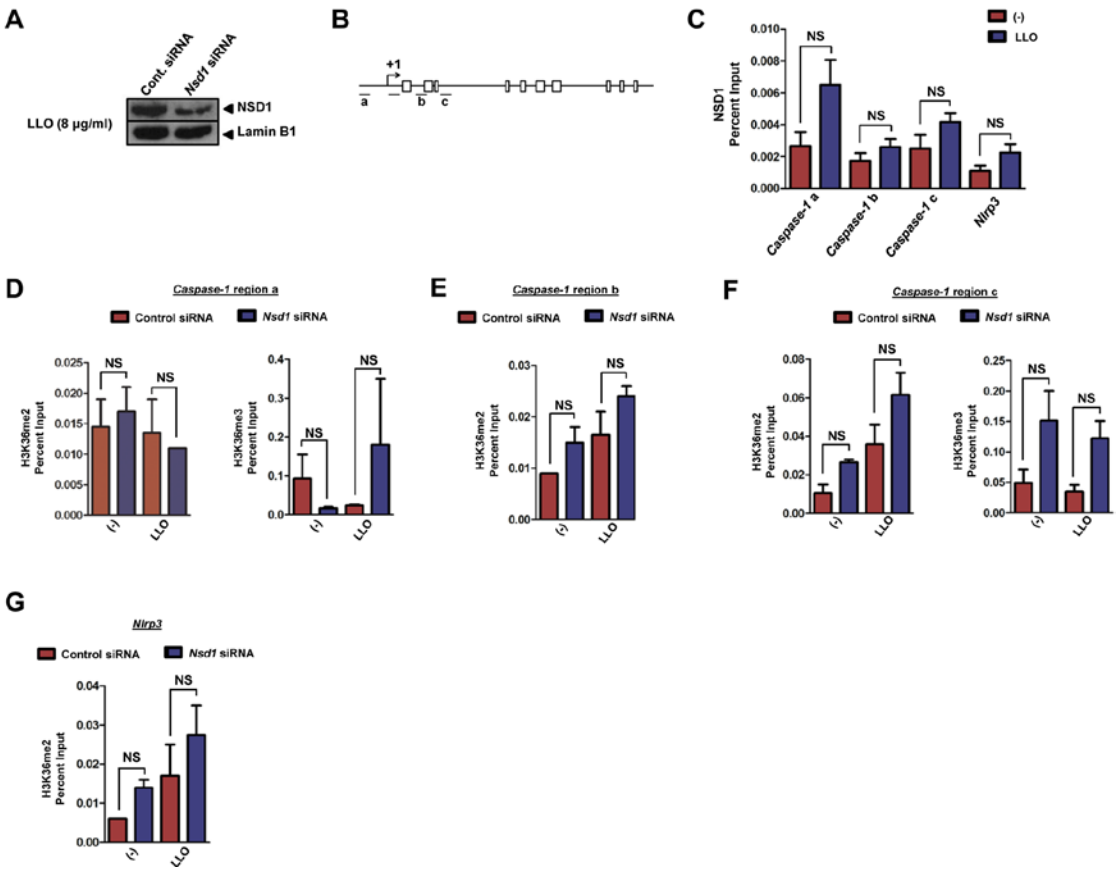

Figure 4.5: NSD1 does not impart chromatin modifications at the 5' end of caspase-1.

BMDMs ($5x10^6$) were transfected with 100 mM of *Nsd1* or control silencer siRNA using Lipofectamine 2000 (n=6). Following 48 hours, BMDMs were primed with LPS (100 ng/ml) for 2 hours and treated with 8 µg/ml of LLO for 1 hour. Nuclear proteins were isolated from whole cell lysates and an (A) immunoblot was performed for NSD1. Lamin B1 was used to determine equal loading. BMDMs ($45\ x10^6$) were treated as previously described in (A). (B) Regions of caspase-*1* which were analyzed are as follows: (a) -1000 bp upstream of the transcription start site (TSS), (b) +1000 bp downstream of the TSS, and (c) +2000 bp downstream of the TSS. Boxes indicate exons. (C) ChIP was performed for NSD1 followed by qPCR for the indicated regions of caspase-*1* and *Nlrp3*. *Nlrp3* was measured as a negative control. ChIP-qPCR data is represented as mean + SE. A graph was chosen to be representative of two experiments. Additionally, BMDMs ($45\ x10^6$) were transfected with 100 mM of *Nsd1* or control silencer siRNA using Lipofectamine 2000. Following 48 hours, BMDMs were primed with LPS (100 ng/ml) for 2 hours and treated with 8 µg/ml of LLO for 1 hour. H3K36me2 was analyzed for each region of caspase-*1* by ChIP-qPCR (n=2): (D, *left*) region a, (E) region b, and (F, *left*) region c. H3K36me3 was also measured for (D, *right*) region a and (F, *right*) region c. (G) H3K36me2 of *Nlrp3* was determined as a negative control. ChIP-qPCR data is represented as mean $\pm$ SE. Student's t test; $P < 0.05$ compared to cells transfected with control siRNA. NS – not significant.

To further elaborate our findings, I investigated whether the histone marks associated with three regions previously analyzed (-1000 bp, +1000 bp, and +2000 bp from the caspase-1 TSS) would be altered upon stimulation. NSD1 regulates the methylation status of histone 3 lysine 36 (H3K36) and histone 4 lysine 20 (H4K20). However, increased specificity has been reported for H3K36[295,302,315]. Our experiments showed dimethylation (me2) and trimethylation (me3) of histone 3 lysine 36 (H3K36) at sites along the caspase-1 locus (Figure 4.5D-F). Overall, a difference in enrichment between cells transfected with control or *Nsd1* siRNA was not observed. Dimethylation of *Nlrp3* was used as a negative control (Figure 4.5G). Trimethylation of the caspase-1 locus region b and *Nlrp3* were not represented because of the lack of consensus between ChIP-qPCR experiments. Taken together, our observations support the findings that NSD1 does not regulate the NLRP3 inflammasome genes at the chromatin level.

The NLRP3 inflammasome gene expression in macrophages was not influenced by NSD1 during stimulation with LLO

I then examined the transcriptional and translational levels of NLRP3, ASC and caspase-1. I first used the transcription inhibitor actinomycin D to determine whether NSD1 affected the mRNA stability of *Nlrp3* and *Asc*. *Asc* and *Nlrp3* transcript levels were measured by qPCR after transfection with control and *Nsd1* siRNA. The half-life of mRNA from both genes was found to be similar at approximately 2 hours (Figure 4.6A). Overall, the percentage of *Asc* and *Nlrp3* mRNA remaining from silenced and non-

silenced cells stayed consistent after the addition of the transcriptional inhibitor. Translation of ASC, NLRP3, and the effector enzyme precursor pro-caspase-1 was analyzed by immunoblot in the presence or absence of 1 μM of the proteasomal degradation inhibitor MG-132 (Figure 4.6B). At each time point, there were similar levels of ASC in the control and *Nsd1* siRNA transfected cells when the proteasomal inhibitor MG-132 was used. However, moderate protein levels of ASC were observed when NSD1 was silenced and compared to control siRNA at 1 and 4 hours in the absence of MG-132. Next, I observed increased protein levels of NLRP3 for 6 hours. Neither *Nsd1* silencing nor proteasomal degradation affected NLRP3 translation. Pro-caspase-1 was also not affected by any conditions used in our analysis (Figure 4.6B).

Asc, *Nlrp3*, and caspase-1 transcription and translation were also measured after stimulation with LLO. With the exception of a slight increase of *Nlrp3* transcript levels and reduction in the caspase-1 transcript levels in the presence of LLO, *Nlrp3*, *Asc* and caspase-1 did not exhibit significant transcriptional changes regardless of the addition of *Nsd1* siRNA (Figure 4.6C). Our observation with NSD1 silencing was supported by the analysis of ASC, NLRP3, and pro-caspase-1 proteins, which remained consistent despite treatment and *Nsd1* reduction (Figure 4.6D). Overall, any potential effects of NSD1 on NLRP3 inflammasome genes were most likely not through transcription and translation.

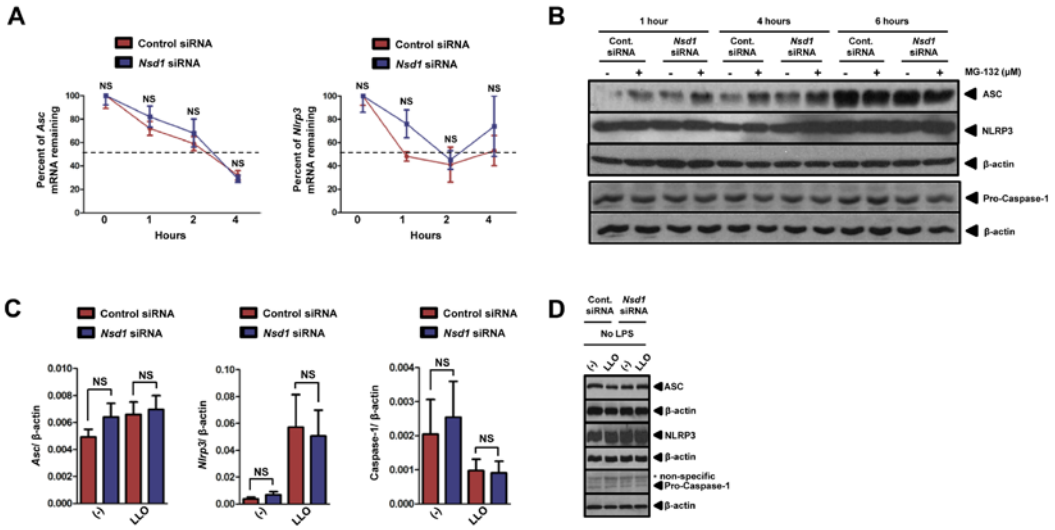

Figure 4.6: *Nsd1* silencing does not alter *Asc, Nlrp3,* and caspase-1 gene expression.

BMDMs ($1x10^6$) were transfected with 100 nM of *Nsd1* or control silencer siRNA using Lipofectamine 2000. BMDMs (n=5) were treated with actinomycin D (5 μg/ml) followed by qPCR analysis of remaining mRNA for (A, *left*) *Asc* and (A, *right*) *Nlrp3*. Dashed line indicates 50% of mRNA remaining. (B) Silenced and non-silenced BMDMs were either untreated (-) or treated (+) with 1 μM of MG-132, a reversible proteasomal inhibitor. ASC, NLRP3, and pro-caspase-1 levels were determined by immunoblotting. β-actin was used to confirm equal loading. (C) 48 hours after silencing, BMDMs were stimulated with LLO (500 ng/ml) for 30 minutes (n=8). (C, *left*) *Asc*, (C, *center*) *Nlrp3*, and (C, *right)* caspase-1 transcription was evaluated by qPCR. qPCR was analyzed using the $\Delta\Delta C_T$ method. Student's t test; $P < 0.05$ compared to cells transfected with control siRNA. NS – not significant. (D) Silenced and non-silenced BMDMs were either non-treated or stimulated with LLO (500 ng/ml) for 30 minutes. Immunoblot was performed for ASC, NLRP3, pro-caspase-1. Equal loading was determined using β-actin. * – non-specific bands. Experiments were repeated at least twice.

LLO pore formation is necessary for NSD1 restriction of caspase-1 activation in macrophages

I was prompted to investigate the involvement of NSD1 in the mediation of LLO-dependent NLRP3 inflammasome post-translational activity. An increase of active caspase-1 in macrophages treated with LLO relative to non-treated cells was detected (Figure 4.7A). Furthermore, post-translational activation of caspase-1 was enhanced with *Nsd1* silencing in macrophages stimulated with LLO, as judged by flow cytometry

(Figure 4.7A). Recently, it was demonstrated that stimulation of macrophages with pathogenic bacteria, such as *Citrobacter rodentium* and *Vibrio cholera*, led to the activation of caspase-11 via TLR4-Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF) signaling. This pathway then activates caspase-1 and enhances IL-1β secretion [113,114,324]. Arguing against the regulation of caspase-11 by NSD1, Kayagaki and collaborators have shown that caspase-1 activation in macrophages occurs independently of caspase-11 during LLO stimulation of macrophages [113]. Our data also supported this finding. The measurement of caspase-11 pre- or post-*Nsd1* reduction did not reveal differential transcription in the presence or absence of LLO (Figure 4.7B, *left*). Correspondingly, differences in the caspase-11 protein were not observed despite stimulation with LLO (Figure 4.7B, *right*). To evaluate the importance of LLO pore formation on the NSD1 regulation of caspase-1, I compared caspase-1 activation during exposure to LLO and LLO^{W492A}. This mutant lacks hemolytic activity due to a tryptophan to alanine substitution in the undecapeptide motif [325]. To assess the lytic activity of LLO and LLO^{W492A}, hemolytic assays were performed (Figure 4.7C). There was a dose-dependent increase in the percentage of lysis by LLO; a concentration as low as 8 ng/ml was able to promote membrane disruption. LLO^{W492A} demonstrated complete abrogation of red blood cell lysis. Additionally, LLO has been shown to trigger cell death [292]. To elucidate the role of NSD1 in the regulation of pyroptosis (caspase-1 mediated cell death), LDH assays were performed after macrophage stimulation with LLO and LLO^{W492A} (Figure 4.7D). Stimulation of WT macrophages with LLO and LLO^{W492A} resulted in about 40% and 0% LDH release, respectively. After transfection of WT

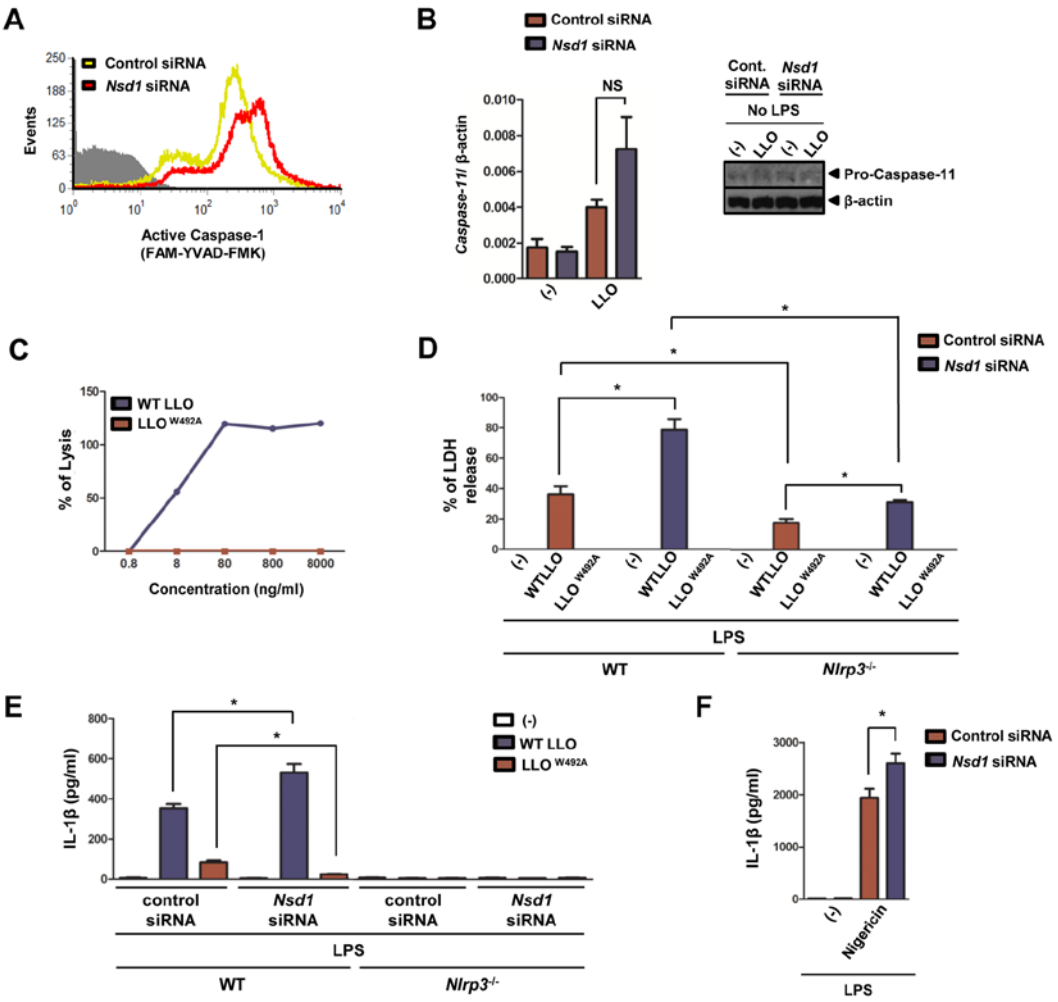

Figure 4.7: NSD1 inhibits LLO-mediated caspase-1 activation and requires functional LLO for the regulation of IL-1β secretion.

$1x10^6$ BMDMs were primed with LPS (100 ng/ml) and treated with control silencer or *Nsd1* siRNA. Also, BMDMs were untreated or stimulated with LLO (500 ng/ml) for 30 minutes. (A) Caspase-1 activity in BMDMs ($1x10^6$) transfected with *Nsd1* or control silencer siRNA was determined after LLO stimulation by flow cytometry using the fluorescent inhibitor probe FAM-YVAD-FMK (FLICA). Control cells are shown in gray, while cells treated with *Nsd1* or control siRNA are shown in red and yellow, respectively. Additionally, silenced $1x10^6$ BMDMs were primed with LPS (100 ng/ml) and were untreated or stimulated with LLO (8 μg/ml) for 1 hour. (B, *left*) RNA was collected and caspase-11 transcription was analyzed by qPCR (n=4). (B, *right*) Protein was also harvested and pro-caspase-11 translation was measured by immunoblot. β-actin was used to normalize for qPCR and determine equal loading for immunoblot. (C) Hemolytic assay was performed on sheep RBC using varying concentrations of recombinant WT LLO or LLO^{W492A}. (D-E) $1x10^6$ WT BMDMs were primed with LPS (100 ng/ml) and treated with WT LLO or LLO^{W492A} (8 μg/ml) for 1 hour. (D) LDH assay was performed to measure cell death induction after treatment. (E) IL-1β levels secreted by BMDMs from WT (n=3) and *Nlrp3*$^{-/-}$ (n=4) mice were measured by ELISA. (F) IL-1β was measured by ELISA after nigericin (10 μM) stimulation (n=4). Student's t test; (*) $P < .05$ compared to cells transfected with control siRNA or knockout. NS – not significant. Experiments were repeated at least twice.

macrophages with *Nsd1* siRNA, supernatant LDH increased for WT LLO treated cells to 80%. However, cells treated with LLO^{W492A} still did not induce LDH release. This result implied that NSD1 plays a role in the inhibition of cell death during stimulation with functional LLO. In *Nlrp3*$^{-/-}$ macrophages, after control siRNA transfection, LLO released about 20% LDH, whereas the extracellular LDH level was about 30% for LLO-treated cells post-*Nsd1* silencing (Figure 4.7D). In primed WT macrophages, LLO was able to induce IL-1β secretion after being transfected with control siRNA and levels of IL-1β increased after silencing with *Nsd1* siRNA (Figure 4.7E). On the other hand, IL-1β secretion triggered by LLO^{W492A} was not mediated by NSD1 in the same manner as WT LLO. IL-1β release when *Nlrp3*$^{-/-}$ macrophages were stimulated with LLO and LLO^{W492A} was not observed (Figure 4.7E). Finally, *Nsd1* silencing also affected IL-1β secretion during nigericin stimulation (Figure 4.7F). Nigericin is a molecule that is similar to LLO in that it can lead to pore formation in the plasma membrane of macrophages [135]. Taken together, our findings suggest that alteration of the plasma membrane by pore-forming agents may be a factor for NSD1 mediated regulation of IL-1β secretion.

4.5 Discussion

Discoveries made in plants have facilitated the understanding of innate immunity in mammals. Yet, several elegant studies are not translated into human health. I took advantage of the NLR functionality in plants and mammals and discovered NSD1 as a possible regulator of the NLRP3 inflammasome (Figure 4.8). EDM2 was previously identified in a genetic screen for suppressors of the NLR gene RPP7 that provides

resistance against the oomycete *H. parasitica* in plants [293]. EDM2 mutations phenocopied the RPP7 observation in *Arabidopsis*, and the defense mechanism was highly specific for *H. parasitica*.

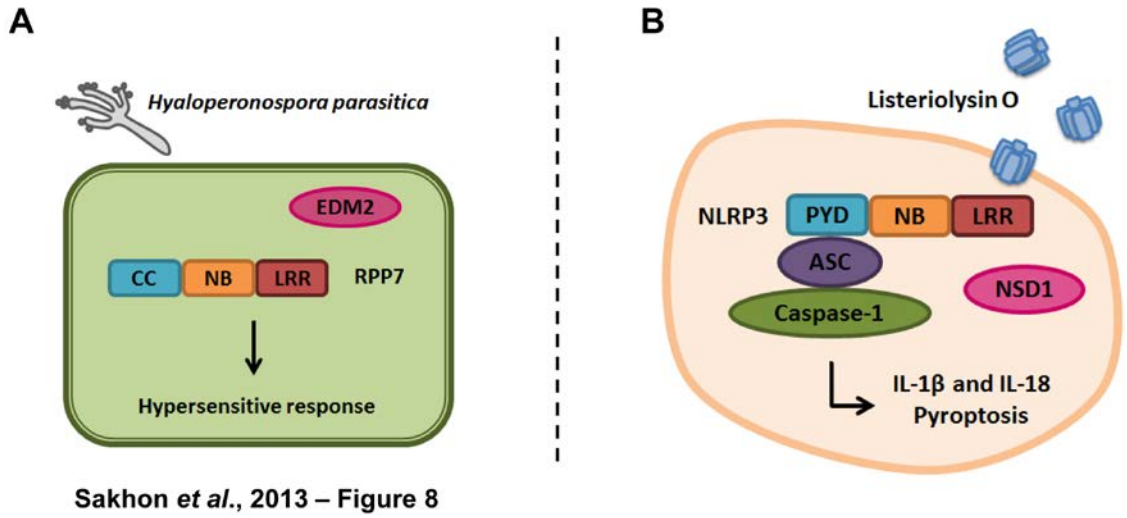

Figure 4.8: Comparative model for NLR regulation in plants and mammals.

(A) *Arabidopsis* is infected with the pathogenic oomycete *Hyaloperonospora parasitica*. EDM2 enhances RPP7-mediated resistance to *H. parasitica* and a hypersensitive response (cell death) ensues in order to contain the infection. (B) Mouse macrophages are exposed to the cholesterol-dependent pore-forming toxin LLO. LLO activates the NLRP3 inflammasome and initiates caspase-1 mediated IL-1β and IL-18 maturation and pyroptosis. NSD1 regulation results in the decrease in the pro-inflammatory response prompted by caspase-1 activation. Coiled-coil domain (CC); nucleotide binding domain (NB); leucine-rich repeat (LRR); pyrin domain (PYD); enhanced downy mildew 2 (EDM2), apoptosis-associated speck-like protein containing a CARD (ASC); and nuclear receptor binding SET domain protein 1 (NSD1).

Similar to the phenotype observed for EDM2 in *Arabidopsis*, I observed specificity for our NSD1 results in macrophages. NSD1 silencing affected the NLRP3 inflammasome when LLO stimulation of macrophages occurred. However, I did not notice any effect on alum, a NLRP3 stimulant via phagolysosomal instability, and *P. aeruginosa*, a NLRC4 stimulant [135]. These results may underscore the importance of pore formation for NSD1 regulation of the NLRP3 inflammasome. Pore-forming toxins

are virulence proteins utilized by numerous bacteria in order to damage cell membranes [326]. Analysis of cholesterol-dependent cytolysins, perfringolysin O and intermedilysin, revealed that these pore-forming toxins have conserved structures and mechanisms of action [290]. Reiterating this hypothesis is the fact that *Nsd1* silencing also affects IL-1β secretion during stimulation of macrophages with the pore-forming agent nigericin. Future studies involving additional pore-forming toxins could reveal a broader application of NSD1 regulation on the NLRP3 inflammasome.

I tested the hypothesis that NSD1 could likely be a candidate for chromatin-based NLRP3 inflammasome regulation. However, after ChIP analysis, I was unable to identify a region with uniquely increased NSD1 enrichment. Effects of histone methylation were also not evident for inflammasome-related genes. This observation is not entirely surprising because a previous study observed that LLO-mediated histone modifications and inflammasome activation are independent pathways [292]. On the other hand, I focused our study on a commonly analyzed time point during inflammasome stimulation with LLO. Thus, although unlikely, our analysis may not entirely exclude the possibility that histone methylation by NSD1 could still be observed after macrophages are exposed to LLO, resulting in alteration of caspase-1 activation.

I speculate that methylation of the NLRP3 protein scaffold catalyzed by NSD1 may regulate IL-1β and IL-18 secretion. Four lines of evidence support this hypothesis. First, it was previously demonstrated that, contrary to our findings in macrophages, NSD1 increases NF-κB activity in cancer cell lines by methylating the p65 subunit [327]. This provides strong evidence that NSD1 can methylate proteins other than histones.

Second, I determined that methylation of lysines in NLRP3 and ASC proteins are likely by using the prediction server MeMo: Methylation Modification. In fact, ASC was first named Target of Methylation-Induced Silencing-1 (TMS1) [328]. Third, experiments in our laboratory indicate a possible interaction between NSD1 and NLRP3 during co-transfection of plasmids in 293T cells. Fourth, post-translational modifications such as deubiquitination of NLRP3 in macrophages, initiated by LPS priming and perpetuated by ATP treatment, activate the NLRP3 inflammasome [329,330]. Along those lines, ubiquitination of ASC also regulates inflammasome function [331,332]. Furthermore, phosphorylation of serine 533 of NLRC4 in macrophages stimulated with *Salmonella typhimurium* was recently suggested as a requirement for NLRC4 inflammasome activation [333]. These findings posit that inflammasome regulation by post-translational modifications, such as methylation, may mediate inflammasome activity.

Our study focused on the interaction between NSD1 and caspase-1 because this inflammatory caspase is considered a canonical regulatory enzyme for IL-1β and IL-18 secretion. I do not exclude the possibility that NSD1 could target other caspases upstream of caspase-1, thereby, modulating IL-1β and IL-18 secretion. The measurement of caspase-11 pre- or post-*Nsd1* reduction did not reveal differential regulation in the presence or absence of LLO. A potential caspase of interest, caspase-7, has been shown to respond to *L. monocytogenes* and may act as a protective mechanism against membrane damage [309]. However, caspase-1 activation during *L. monocytogenes* infection is independent of caspase-7, as caspase-1-deficient mice did not show a defect in caspase-7 activation [309] . Another caspase known to form a non-canonical

inflammasome is caspase-8. A relationship between caspase-8 and LLO has not been formally elucidated and, hence, I am not able to exclude any possible effects of NSD1 on caspase-8 activity during LLO stimulation of macrophages. Presently, caspase-8 has been known to associate with the AIM2-ASC complex during *Francisella tularensis* subspecies *novicida* stimulation of macrophages and dectin-1 recognition of fungi and *Mycobacteria* [116,117].

Clearly, additional studies encompassing NSD1 and the regulation of the canonical and non-canonical inflammasome components are necessary. However, our study potentially unveils a novel function for NSD1 during LLO stimulation of macrophages. Because the mechanism of action for many pore-forming toxins is evolutionarily conserved, our observation may also uncover a widely applicable regulatory innate immune mechanism in the mammalian host.

CHAPTER FIVE

Concluding remarks

The innate immune system is a powerful tool used by an organism to protect itself against danger. It plays a crucial role in the initial defense against a disease-causing pathogen as well as the subsequent commencement of an adaptive immune response to protect against future infections. Due to the effectiveness of this system, it is evolutionarily beneficial for pathogens, vectors, as well as the host themselves to develop ways to modulate immune responses. This dissertation highlights the importance of proteins that direct the activities that occur in the complex web of innate immune signaling in the macrophage.

Successful infection by a vector-borne or non-vector borne pathogen and reliable safeguard against these threats by a host is a well coordinated exchange. The pathogen is able to subvert the host immune system by inhibiting recognition, pro-inflammatory responses, and fundamental cellular function, as well as release factors that can promote anti-inflammatory reactions [334]. Additionally, they are also able to remain hidden within cellular compartments or eliminate immune cells. Our findings support the notion that pathogens possess numerous evasion strategies, but moreover, new tactics can be discovered in proteins that did not previously demonstrate the capacity to promote immune evasion. The concept termed moonlighting signifies that a protein carries out more than one function [335]. This seems to be the case with LPDA1; it has been shown to play a role in metabolic processes (pyruvate dehydrogenase complex, peroxynitrite reductase/peroxidase, and branched-chain keto acid dehydrogenase) but now it can also act as an immunopathological molecule [99,336]. These findings emphasize the need to

reanalyze proteins for alternative functions, as this method may reveal more information about pathogen evasion strategies.

Not only do vector-borne pathogens possess endogenous evasion molecules, they are also able to use exogenous proteins to their benefit. One essential tool pathogens take advantage of is vector saliva. Blood-feeding vector saliva is composed of an extensive number of proteins that aid in establishing and encouraging a blood meal. Anti-inflammatory molecules are crucial during this period. These factors not only aid the vector in avoiding detection but also facilitate the transmission of a pathogen from vector to host. Due the importance of arthropod vector saliva and salivary proteins in the spread of disease-causing agents, novel therapeutics have begun to utilize methods targeting vector saliva instead of pathogen-centric molecules [88]. Work in this area may lead to the design of much needed vaccines against devastating vector-borne pathogens that affect millions of people all around the world.

Mammals utilize the immune system as a way to counter the attack by virulence factors in pathogens and anti-inflammatory molecules in arthropod vectors. The regulation of innate immunity is crucial for an effective battle against invading pathogens. Multiple levels of regulation exist in order to coordinate an appropriate response. Cytokines, chemokines, inhibitory proteins (such as the inhibitor of κB), post-translational modifications, adaptive immune cells are able to impart signals to the innate immune system, thereby allowing the suitable pro-inflammatory or anti-inflammatory action to occur [275]. I demonstrated that plant innate immunity may be used as a platform for the study of mammalian innate immunity. Through this method, I was able

to identify NSD1 as a regulator of the caspase-1 component of the NLRP3 inflammasome. NSD1 negatively regulates caspase-1 activity in the presence of the pore-forming toxin LLO. Though I was able to exclude regulation at the following levels: (1) NF-κB, and (2) NLRP3 chromatin and expression; the NSD1 mechanism of caspase-1 regulation still remains to be determined.

The number of pathogens that affect humans is colossal. Additionally as jarring is the variability amongst pathogens (bacteria, virus, fungi, parasites, etc.) that lends to the difficulty of the development of preventive and/or protective therapeutics. Whether it be vector-borne or non-vector borne pathogens, there are numerous facets that must be taken into account when decrypting innate immune signaling pathways. I focused on one crucial branch of innate immunity in this dissertation, the NLR signaling pathway. Like many others, in reality NLR signaling is intertwined with other components of immune signaling. Therefore, we must remain cautious when deciphering mammalian innate immune pathways and at what point pathogen-, vector-, and endogenously-derived components come into play. Continuous study of the manipulation of host innate immunity will pave the way for the advancement of protective measures against pathogens that threaten human health.

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