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Dissecting the Role of a lncRNA and Involvement of *Plasmodium* Infections in the Innate Immune Response: A Dissertation

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Dissecting the role of a lncRNA and involvement of *Plasmodium* infections in the innate immune response

A Dissertation Presented

By

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Dissecting the role of a lncRNA and involvement of Plasmodium infections in the innate immune response

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Abstract

The innate immune system is a multicomponent response governed by intricate mechanisms of induction, regulation and resolution to elicit antimicrobial defenses. In recent years, the complexity of eukaryotic transcriptomes has become the subject of intense scrutiny and curiosity. It has been established, that RNA polymerase II (RNAPII) transcribes hundreds to thousands of long noncoding RNAs (lncRNAs), often in a stimulus and cell-type specific manner. However, the functional significance of these transcripts has been particularly controversial. While the number of identified lncRNAs is growing, our understanding of how lncRNAs themselves regulate other genes is quite limited. In chapter 2, a novel lncRNA is identified, more specifically, a natural antisense transcript, that mediates the transcription of the pro-inflammatory cytokine IL-1α. Through loss-of-function studies, I report the necessity of this transcript in mediating IL-1α mRNA expression by affecting RNAPII binding to the IL-1α promoter after toll-like receptor signaling. For the first time, I show that IL-1α is regulated at the transcriptional level.

As a second independent component of this thesis, we explore the role of the innate immune response after infection by the malaria-causing parasite, *Plasmodium berghei ANKA* (PbA), and how innate immune components are both beneficial and detrimental to the host depending on when and where inflammation is triggered during infection. We attempt to identify the “malarial toxin” responsible for aberrations in the immune response that is detrimental for

disease outcomes and the innate signaling pathways that are involved. Many pathogens induce pathological inflammatory conditions that lead to irreparable homeostatic imbalances and become fatal to the host. Here, type I Interferon signaling is required to dampen parasite load during liver-stage infections, but leads to host mobidity if these pathways are activated in the erythrocytic phase of infection. Together, this thesis provides new insights on how components of the innate immune system are regulated, and how dysregulation of immunity can potentially lead to adverse effects during active infections.

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Copyright Information

Material subject to copyright is listed in the Preface of each chapter. Schematic model slides adapted from publications have been used to clarify concepts and may have been published/submitted prior to this thesis. All copyrighted information has been referenced.

LIST OF ABBREVIATIONS

2ME	β-mercaptoethanol
AIM2	Absent in melanoma-2
ASC	Apoptosis-associated speck-like protein containing CARD
BBB	Blood brain barrier
BLASTN	Basic local alignment search tool for nucleotides
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
CARD	Caspase activation and recruitment domain
CBP	cAMP response element-binding (CREB)-binding protein
CDC/mDC	Conventional/myeloid DC
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CM	Cerebral malaria
CS	Circumsporozoite protein
CTCF	CCCTC-binding factor
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Danger-associated molecular pattern
DBD	DNA binding domain
DC	Dendritic cell
DMXAA	5,6-Dimethylxanthenone-4-acetic acid aka Vadimezan
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
dsRNA	Double strand RNA
ECM	Experimental cerebral malaria
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ENCODE	Encyclopedia of the DNA element
GENCODE	Gene and gene variants consortium within the ENCODE project
HAVANA	Human and Vertebrate Analysis and Annotation
HCV	Hepatitis C Virus
HEK	Human embryonic kidney
HMPV	Human metapneumovirus
hnRNP	heterogenous ribonucleoprotein
HSV	Herpes simplex virus
HTT	Huntington gene
Hz	Hemozoin
IAD	IRF-associated domain
IAV	Influenza A virus
ISG15	Interferon stimulatory gene 15
IFI44	Interferon inducible gene 44
IFIT1	interferon-induced protein with tetrapeptide 1

IFN	Interferon
IFNAR	Interferon-α/β receptor
imac	immortalized macrophage
IL	Interleukin
IP	Intraperitoneal
IPAF	ICE Protease-Activating Factor
IPC	IFN-producing cell
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
iRBCs	Infected red blood cells
ISD	Interferon Stimulatory DNA
ISG	Interferon Stimulated Gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knock-out
LGP2	Laboratory of genetics and physiology 2
Lm	*Listeria monocytogenes*
LNA	Locked nucleic acid
lncRNA	long noncoding RNA
lincRNA	long intergenic noncoding RNA
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
Mal	MyD88-adapter-like
MAP	Mitogen-activated protein
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated gene-5
MEFs	Mouse Embryonic Fibroblasts
Mtb	*Mycobaterium tuberculosis*
MyD88	Myeloid differentiation primary response gene 88
ncRNA	noncoding RNA
NAT	Natural antisense transcript
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NeST	nettoie Salmonella pas Theiler's
NI	Non-infected
NK	Natural killer
NLR	NOD like receptors
NLRP	NACHT, LRR and PYD domain-containing protein
NOD	Nucleotide-binding oligomerization domain containing
ODNs	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PbA	Plasmodium berghei ANKA

PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
Pf	Plasmodium falciparum
PGN	Peptidoglycan
PKR	dsRNA-dependent protein kinase
PolyIC	Polyriboinosinic polyribocytidylic acid
PRC2	Polycomb repressive complex 2
PRRs	Pattern recognition receptor
PTPN	Protein tyrosine phosphatase N-terminal
PYD	Pyrin domain
RBC	Red blood cell
RIG-I	Retinoic acid-inducible gene-I
RIG-IC	RIG-I helicase domain only
RIP	Receptor-interacting serine-threonine kinase
RLR	RIG-I like receptor
RNAPI	RNA polymerase 1
RNAPII	RNA polymerase 2
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SHP-1	Src homology region 2 domain-containing phosphtase 1
shRNA	short hairpin RNA
SLE	Systemic Lupus Erythematosus
spz	sporozoite
ssRNA	Single strand RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN gene
SeV	Sendai virus
Sg	Salivary gland
TALENS	Transcription activator-like effector nucleases
TBK1	TANK-binding kinase 1 TIR Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	Tyrosine kinase 2
UBP18	Ubl carboxyl-terminal hydrolase 18
USP18	ubiquitin-specific protease 18
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WNV	West Nile virus
WT	Wild type

Preface to Chapter 1

This chapter contains content (lncRNA introduction) that is in submission for the following publications/manuscripts:

Jennie Chan and Katherine A. Fitzgerald. 2015. Long noncoding RNAs in regulating the innate immune response. European Journal of Immunology.

CHAPTER 1: Introduction

Overview of the innate immune system

Given the large number and variety of microorganisms in our environment, we are frequently threatened with infection and injury and must have a system in place to defend ourselves against harmful pathogens. The mammalian immune system is divided into two categories. (1) The innate immune response is the first line of defense against pathogens and is coordinated by a specialized group of cells consisting of monocytes, macrophages, dendritic cells, mast cells, Eosinophils, Basophils, neutrophils and Natural Killer cells (Hato and Dagher, 2014). When infectious agents breach the skin and mucosal membranes, these cells are called upon to defend the organism. Activation of these cells occurs by triggering germline-encoded pattern recognition receptors (PRRs) to initiate an inducible program of inflammatory gene expression that elicits an antimicrobial environment. The molecular effectors of an inflammatory response tend to be quiescent, either not-expressed, or inducible, but can then be rapidly mobilized to generate a defensive and alarmed environment. The alarmed environment is what we call inflammation. Indicators of inflammation include swelling, heat, fever, and pain (Medzhitov, 2008). The quality of signals produced by the innate immune effectors generates the blue print for the signals that initiate the appropriate (2) adaptive immune response to mobilize B and T cells to undergo class switch recombination and somatic hypermutation for highly antigen-specific recognition receptors that are essential for long-term antigen specific immunity.

These cells have much slower kinetics, but produce memory and long-lived effector cells that self-renew and can be called upon quickly if there's a second exposure to the same antigen for rapid elimination and minimal tissue damage.

Not all microorganisms are detrimental to our health and some can be symbiotically beneficial, helping us digest nutrients or out-compete other populations of dangerous microbes. The immune system contains a sophisticated set of mechanisms that can distinguish between self versus non-self, but also harmful versus harmless agents. Distinguishing among these differences are critical for survival. Hypo- or hyper- responses toward pathogens can lead to serious consequences that range from incomplete pathogen clearance to excessive pathology and irreparable tissue damage. Furthermore, misidentification of innocuous molecules from pollen or food byproducts as threats can lead to allergies and hypersensitivies; misidentification of self as non-self can lead to autoimmunity or autoinflammatory diseases. Ultimately, this powerful system is the basis for our survival, but must always be stringently regulated.

Transcriptional regulation of the inflammatory mediators

The innate immune response consists of PRRs that recognize conserved molecular moieties called Pattern-associated molecular patterns (PAMPs) that are common to many pathogens. Once these receptors are triggered, signals transmit to the nucleus through transcription factors, resulting in the activation of numerous genes via both transcriptional and post-transcriptional mechanisms.

Mainly in the form of cytokines, chemokines, or type I IFNs, these proteins carry out diverse physiological functions that can either target infectious organisms by directly interfering with function or replication or by activating and recruiting other innate immune cells to the site of infection to neutralize the target (Medzhitov, 2009). Detailed studies of specific transcription factors and the chromatin organization of proinflammatory genes have revealed remarkable diversity in the range of mechanisms employed for transcriptional activation. This diversity extends beyond the binding of distinct sets of inducible transcription factors to different promoters and enhancers and the selective regulation of these genes occur at the chromatin level.

Many of these genes are divided into two broad categories, deemed primary and secondary response genes (Smale et al., 2012). Primary response genes are usually the most rapidly induced without *de novo* protein synthesis. The transcription factors required for activation of these genes must be expressed in unstimulated cells and must either be constitutively active or activated via posttranslational mechanisms after cell stimulation. These transcription factors include NF-kB, IRFs, AP1 and cAMP-responsive-element-binding protein 1 (CREB1) families (Medzhitov and Horng, 2009). The signaling pathways that control the activation of these transcription factors are described below (Sasai and Yamamoto, 2013).

Secondary response genes are induced more slowly and require new protein synthesis. The transcription of secondary genes can depend on *de novo*

synthesis of transcription factors, signaling molecules needed for the activation of transcription factors, or cytokines that can act in an autocrine fashion to activate additional signaling pathways and transcription factors (Amit et al., 2011). Factors that are required for primary response genes can also contribute to the transcription of secondary response genes. The inflammatory program must accurately scale its response to avoid the consequences of excessive pathology-inducing inflammation, but also to be robust enough to fight off an infection.

lncRNAs in the genome

The increasing accessibility of techniques that allow researchers to interrogate biological phenomena at the genome-wide scale has resulted in an explosion of information. Now, we can investigate the full genome in the context of a given biological phenomenon of interest. Studies examining the transcriptomes of eukaryotic cells have revealed a bewilderingly complex array of RNA transcripts. Indeed, current estimates indicate that roughly 1-2% of the genome has protein coding potential whereas 85% of the genome is transcribed and belong to a class of RNA polymerase II (RNAPII) transcripts referred to as lncRNAs (Consortium et al., 2012; Dinger et al., 2009; Kowalczyk et al., 2012; Li and Ramchandran, 2010).

These observations are provocative, and have been met with considerable skepticism (Hongay et al., 2006) mainly due to the following: 1) improved sequencing technologies can detect low abundance transcripts and blur the line

between *bone fide* transcripts and technical noise, 2) lncRNAs generally lack sequence conservation which has been an established means for identifying functional genes, and 3) currently few mutations in lncRNAs have been documented as the cause of genetic diseases. Eventually, all of these issues will need to be addressed by the lncRNA community.

High-throughput sequencing technologies have made it possible to interrogate the full complement of RNA transcripts in the cell at an unprecedented depth. It is now possible to detect transcripts with an abundance of less than one copy per cell (Mercer et al., 2009; 2012). This raises the question as to whether many of these pervasive transcripts are inconsequential transcriptional byproducts or unspecific hybridization (Kowalcyzk 2012). Early studies using tiling arrays, which are prone to false positive detection, when in actuality, by RNA sequencing, a technique which has been shown to be more accurate, revealed that pervasive transcription made up a much smaller fraction of the poly(A)+ transcriptomes (~11% in humans, 4% in murine) (Tian, 2005; van Bakel et al., 2010). This study was, however, able to identify several thousand distinct, low abundance transcripts, representing 1% of all polyadenylated transcripts. What remains to be determined is whether lncRNA expressed at ultra low levels are indeed functional. This criticism can only be addressed by the functional validation of individual lncRNAs. While thousands of lncRNA transcripts have been annotated in eukaryotic cells, few have been functionally characterized. In-depth functional characterization for the majority of lncRNAs is

lacking, but the observation that many lncRNAs are expressed with cell-type specificity perhaps argues that their expression may be highly regulated (Dinger et al., 2009). Such regulation may not be anticipated for transcriptional noise, but that remains to be proven.

Currently, it is unclear whether the majority of pervasive transcription contributes to functional genome organization or is merely the result of unanticipated and promiscuous RNAPII activity. Evolutionary selection often denotes that a particular sequence is useful to an organism, and thus, sequence conversation is used as a means of identifying functional motifs and domains within the genome. mRNAs, and particularly the open reading frames (ORFs) of mRNA transcripts, display a high degree of conservation (Guttman and Rinn, 2012). In comparison to mRNAs, lncRNAs display very little sequence conservation (Pang et al., 2006). Those skeptical of the functional significance of pervasive transcription argue that if a lncRNA was functional, then throughout evolution there would be some selective pressure on conserving its sequence. However, studies identifying novel lncRNAs based on the chromatin signature of active transcription in intergenic regions, have revealed lncRNAs that do not show sequence conservation (Guttman et al., 2009; Zhao et al., 2008). Additionally, it might not be fair to evaluate lncRNAs with the same parameters of sequence conservation as mRNAs. The constraint on an mRNA to maintain coding potential mandates sequences that code for amino acids, thereby have stringent selective pressures, whereas lncRNAs are not under the same

constraints to maintain coding potential and potentially have the freedom to evolve faster (Beltran et al., 2008; Pang et al., 2006).

Despite these considerations, efforts to identify conserved lncRNAs between species have been attempted via sequence homology and synteny alignments. The hope, despite contrary evidence, is that nucleotide conservation implies functionality and significance. Mutations that would alter sequence would alter function, and the more critical the mutation, the less it would be observed in a viable organism or inherited in viable offspring. Initial evolutionary studies concluded that noncoding RNA sequences appear as poorly conserved as other intergenic sequences that exhibit no indications of transcription (Okazaki et al., 2002; Pang et al., 2006) but further studies described noncoding RNAs that exhibited signatures of functionality more closely related to protein-coding genes. More specifically, these noncoding RNAs displayed fewer nucleotide substitutions, insertions or deletions both within their predicted promoter regions and within their transcribed regions, and additional conservation at their splice sites than random (Guttman et al., 2009; Ponjavic et al., 2007) and therefore, may be exhibiting evolutionary constraint afterall. All this suggests that although sequence conservation may not be necessary in dictating functional importance, it may be sufficient and may be used as an initial screen in identifying biologically critical noncoding RNA species. Syntenic genome alignments utilizing *in silico* comparative genomic mapping may help identify species orthologs and nucleotide basic local alignment search tools (BLASTN) would be useful in the

preliminary searches for evolutionarily conserved lncRNAs. However, this can be difficult as the absence of lncRNA annotation in many organisms preclude proper comparative analyses (Necsulea et al., 2014). Additionally, there are natural assumptions that “functional” sequences are maintained by the genome and therefore, the opposite must also be true—that “junk” DNA would undergo negative selection. Because the genome is always part of the evolutionary process, genomic regions can contain both functional and nonfunctional parts. Nonfunctional DNA may later on acquire functions and become part of the gene-repertoire and vice versa (Doolittle, 2013). Therefore, in the early phases of lncRNA discovery, applying the initial criteria for defining genes may be useful for identification purposes, but can also lead to ambiguities.

Strong conservation in promoter sequences (Carninci et al., 2006) and weaker conservation in the sequences of their transcripts (Ponjavic et al., 2007), suggest that the act of transcription itself may have a greater biological consequence than the transcript sequence. In these situations, the regulation of nearby protein-coding gene expression in *cis*, may represent a more prominent function for lncRNAs. Identifying active transcription nearby, but not at the direct site of the protein-coding gene chromatin location, may reveal orthologous lncRNAs.

Another criticism that is commonly used to downplay the biological importance of lncRNAs is that not only have few lncRNAs have been identified in genetic screens but also few natural mutations in lncRNA genes are the main

cause of genetic disorders. While this criticism is generally true of monogenic traits and diseases, where a single allele can account for the phenotype, there is growing evidence that mutations in non-protein coding regions contribute to a phenotype (Manolio, 2009; Mattick, 2009). Genome-wide association studies cataloguing trait associated single nucleotide polymorphisms suggest that over 80% of associated variants occur in noncoding regions (Hindorff et al., 2009). Additionally, evidence for the involvement of lncRNAs in disease states come from the finding that lncRNAs are dysregulated in a number of cancers and neurological disorders (Niland et al., 2012). Also, it is conceivable that genetic screens, which yielded hits in putative lncRNAs, would have been ignored, as many lncRNAs are found in intronic or intergenic regions and would have been considered inconsequential. While it is currently unclear whether all pervasive transcripts will turn out to have important functions in the cell, it is clear that the burgeoning lncRNA field is compelling us to re-evaluate the content of the genome.

RNA-dependent processes permeate many of the fundamental aspects of gene expression. Despite the recent flurry of interest into the ncRNA field, specific classes of ncRNAs have been known for decades to be critical components of important cellular machines. ncRNAs of the small nuclear variety (snRNAs) play important roles in the catalysis of pre-mRNA splicing. The synthesis of proteins from an mRNA template is carried out by the coordinated action of ribosomal RNAs (rRNAs), which catalyze peptide bond formation, and

transfer RNAs (tRNAs), which deliver the appropriate amino acids to the ribosome. These ncRNA genes were thought to be a small subset of the cases and that the majority of distinct RNA transcripts in the cell were messengers as templates for amino acids in protein synthesis, with the major functional output of the genome being proteins (Wilusz et al., 2009).

Whether or not proteins were the only functional mediators of the genome began to be questioned heavily in the middle of the last decade when researchers studying diverse model organisms found that not only was RNAPII detected in unexpected genomic locations (Kim et al., 2005; Steinmetz et al., 2006), but that the genome was pervasively transcribed (ENCODE Project Consortium et al., 2007). The vast majority of these transcripts were inferred to contain minimal coding capacity and thus belonged to an exploding class of lncRNAs. Most lncRNAs are thought to be RNAPII transcripts and similar to their mRNA cousins, have been demonstrated to bear distinctive features at their extremities (ie. A 5' cap and 3' poly(A) tail) (Berretta and Morillon, 2009; Guttman et al., 2009; Khalil et al., 2009). Because of these resemblances, lncRNAs are often described as mRNA-like. For mRNA transcripts, the 5' 7-methylguanosine cap structure and the 3' polyadenosine tail protect the body of the transcript from degradation and the rates of removal for these features dictate how long the mRNA transcript persists in the cell. lncRNAs are typically modified in similar manners to mRNAs. While the functional consequence of the presence of these end modifications is currently unknown, they may also play analogous protective

roles for lncRNAs.

lncRNAs are further classified based on their genomic orientation relative to protein coding genes (**Figure 1.1**). For example, long intergenic noncoding RNAs (lincRNAs) originate from intergenic regions of the genome (Guttman et al., 2009), whereas natural antisense transcripts (NATs) are produced from the opposite strand as a coding transcript (Li and Ramchandran, 2010) Divergent transcription occurs when a lncRNA is transcribed in the opposite direction as a coding transcript (Preker 2008). This list is not exhaustive, and as efforts improve to describe and refine the full complement of lncRNA transcripts continues, more subtypes and categories will likely emerge.

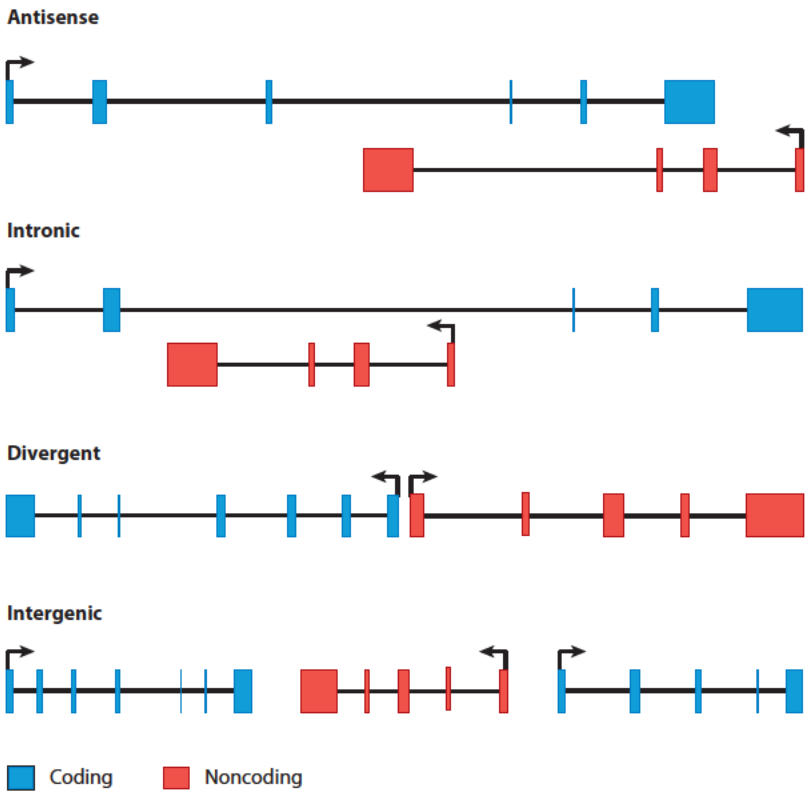


Figure 1.1. The various classifications of lncRNAs lncRNAs have been defined by their relative location to protein-coding genes. Antisense lncRNAs or natural antisense transcripts (NATs) are transcribed in the opposite orientation from nearby protein-coding genes and may overlap at an exon Intronic lncRNAs are transcribed within the intron of another protein-coding gene, but does not overlap with that gene. Divergent lncRNAs are transcribed in the opposite direction as the neighboring lncRNA and may share a promoter. Intergenic lncRNAs (lincRNAs) do not overlap or share a transcriptional unit with other protein-coding genes (as described in (Rinn and Chang, 2012)).

lncRNAs in inflammation and microbial defense

Despite efforts to functionally characterize individual lncRNAs are lagging, there is now a growing number of *bone fide* lncRNAs with physiological relevance. Research efforts have focused heavily on the capacity of lncRNAs to regulate the expression of protein coding genes. These transcripts execute their fuctions via lncRNA:protein interactions, lncRNA:mRNA interactions or lncRNA:DNA interactions. (as shown in **Figure 1.2**).

In some cases, it has been suggested that polymerase movement through the genomic locus of a lncRNA can influence expression of nearby genes in cis through transcriptional interference (Hirota et al., 2008; Lefevre et al., 2008). Regulation of transcriptional interference implies that transcription itself, rather than the RNA transcript produced, accounts for the functional output of the pervasively transcribed locus. Compelling evidence for transcriptional interference by lncRNAs has been demonstrated at a number of loci in various organisms with slight variations in the exact mechanism employed. Transcription of ncDNA can influence nucleosome positioning and/or transcription factor binding to either repress or activate nearby genes (Hainer et al., 2011). lncRNAs are thought to increase the binding of transcriptional activators by remodeling the nucleosomes at the promoter (Hirota et al., 2008). Activation by nucleosome remodeling can occur by binding of transcriptional activators, but can also inhibit the binding of a transcriptional repressor like CTCF (Lefevre et al., 2008).

Chromatin can also be influenced by the transcription of ncDNA through

post-translational modification of histones. It has been demonstrated that spurious transcription within protein-coding genes is repressed by the co-transcriptional recruitment of histone modifying enzymes to the RNAPII C-terminal domain (Carrozza 2005). The NRON lncRNA indirectly represses genes regulated by the nuclear factor of activated T-cells (NFAT) transcription factor by inhibiting NFAT's nucleo-cytoplasmic shuttling (**Figure 1.2**). Specifically, NFAT is localized to the cytoplasm and is imported into the nucleus in response to calcium-dependent signals. NRON inhibits the nuclear accumulation of NFAT by binding to nuclear transport factors (Willingham et al., 2005).

Additionally, lncRNAs have been implicated in influencing chromatin states via more direct RNA-dependent cis and trans-acting mechanisms. Xist is a quintessential cis-acting lncRNA involved in the silencing of the inactive X chromosome in females. Xist is a 17 kb lncRNA transcribed from the inactive X chromosome and accumulates on the inactive X chromosome and create a repressive nuclear compartment that exclude RNAPII and transcription factors (Chaumeil et al., 2006). In recent years, a number of lncRNAs have been described to function in concert with Xist during X inactivation. Of these, RepA, a 1.6kb lncRNA generated from within the Xist locus, has been shown to directly bind to the polycomb-repressive complex (PRC2) to recruit PRC2 in cis to its own genomic locus (Zhao et al., 2008).

More examples of *bone fide* lncRNAs in the innate immune response

The emerging role of ncRNAs in promoting, fine-tuning and dampening innate immune gene expression is gaining attention. Our current understanding of the role of lncRNAs in health and disease is limited to a few examples thus far. An elegant paper by Gomez et al. 2013 utilized comparative genomics to identify disease susceptibility loci in two mouse strains that differed in their susceptibility to Theiller's virus infection. Polymorphism differences between the SJL/J and B10.S mice identified a susceptibility locus that was mapped to a conserved lncRNA called NeST (nettoie Salmonella pas Theiler's) (Gomez et al., 2013). NeST was shown to be a non-coding RNA transcribed from the IFN-γ locus, which in turn enhances IFN-γ expression in $CD8^+$ T cells (Gomez et al., 2013). Because NeST is transcribed from the same locus as IFN-γ, it is considered to act in cis as an enhancer RNA by binding to WDR5, a component of the histone 3 lysine 4 (H3K4) methyltransferase complex, and altering histone 3 methylation at the locus to allow inducible IFN-γ expression, thereby conferring mice expressing NeST with higher resistance against Theiller's virus infection (Gomez et al., 2013).

lncRNAs of the NAT variety have been shown to influence the splicing patterns of mRNA transcripts encoded on the sense strand at the N-myc and Zeb2 loci in mammalian cells (Beltran et al., 2008). In these cases, the NAT production is complimentary to the affected region. It has been suggested that NATs from these loci form RNA-RNA duplex interactions between sense and

antisense strands which then inhibit splicing (Krystal 1990, Monroe and Lazar 1991). Translation of Zeb2 relies on an IRES, and the NAT inhibits splicing of an IRES containing intron, therefore, expression of the NAT facilitates expression of the Zeb2 protein (Beltran et al., 2008).

microRNAs have been shown to dampen the expression level of specific cytokines (Ambros, 2004; Matsui et al., 2008). A number of examples have recently been discovered that define important roles for lncRNAs as regulators of cytokines and other immune mediators, in particular acting in *cis*. One example is a lncRNA that has been shown to run antisense to IL-1β in murine RAW 264.7 macrophages, thereby dampening IL-1β expression (Lu et al., 2013). This report showed that LPS signaling induces the IL-1β antisense, and that overexpression of the antisense lncRNA decreases H3K4me3 marks at the IL-1β promoter. This decrease in trimethylation at the promoter leads to decreased RNA polymerase II (RNAPII) occupancy and therefore dampens transcription of IL-1β.

While our knowledge of the mechanisms and scope of lncRNA-mediated regulation is growing, our understanding of how lncRNAs themselves are regulated is still quite limited. Regulating lncRNA expression would be expected to be an important cellular consideration given that lncRNAs have been implicated in regulating a variety of processes in eukaryotes including imprinting, dosage compensation, cell cycle regulation, pluripotency, retrotransposon silencing, meiotic entry, and telomere length (Guttman et al., 2009; Hongay et al., 2006; Loewer et al., 2010; Luke et al., 2008; Ponting et al., 2009). Moreover,

altered expression of lncRNAs has been implicated in disease states and antimicrobial defenses. Furthermore, lncRNAs often display exquisite tissue-specificity and achieving such specificity necessitates extensive regulation (Dinger et al., 2009; Gomez et al., 2013; Khaitovich et al., 2006).

At the transcriptional level, parallels have been drawn between lncRNA and mRNA transcriptional regulation. For example both mRNA and lncRNA chromosomal loci have been shown to exhibit the active chromatin signatures consisting of histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 36 trimethylation (H3K36me3) in the promoter region as active transcriptional markers. This chromatin signature has been used extensively to identify thousands of lncRNA genomic loci on a genome-wide scale in mammals (Arthur and Ley, 2013; Guttman et al., 2009; Khalil et al., 2009; Ouyang et al., 2014; Peng et al., 2010). In yeast the *GAL10* lncRNA was identified by a similar strategy in that the same signature of an actively transcribed gene was detected at the DNA locus. Thereafter, it was confirmed that a lncRNA was produced (Houseley et al., 2008; Vilaysane and Muruve, 2009). Additionally, transcription of a few lncRNAs, much like mRNA transcription, has been shown to be negatively regulated by DNA methylation at CpG dinucleotides within lncRNA promoter regions (Gazzinelli et al., 2004; Lujambio et al., 2010). Thus it appears that at least at some level, lncRNAs are subject to similar types of epigenetic regulation as mRNAs.

Many immune cells develop from the bone marrow (through hematopoesis

and myelopoesis) and circulate as non-differentiated peripheral blood mononuclear cells until they are triggered to differentiate by various differentiation factors; lncRNAs have recently been added to this list of differentiation triggers. For example, human granulocyte differentiation has been recently shown to be partly mediated by HOX antisense intergenic RNA myeloid 1 (HOTAIRM1), an antisense lncRNA on the HOXA gene locus (Khor et al., 2007; Mockenhaupt et al., 2006; Zhang et al., 2009). Knockdown of HOTAIRM1 abrogated retinoic acid-dependent activation of HOXA1/A2 and CD11b and CD18 (Mac-1), two Beta2 integrin transcripts that are hallmark myeloid maturation-associated genes (Mayadas and Cullere, 2005; Zhu et al., 2005).

Dendritic cell (DC) differentiation has also been shown to depend on a lncRNA. An RNA sequencing (RNA-seq) profiling study of various stages in the differentiation of human peripheral blood mononuclear cells (PBMCs) (Carpenter et al., 2013; Morse et al., 1997; Wu et al., 2010), identified lncDC as having expression highly correlated with DC maturation. This lncRNA was shown to be highly specific to DCs, not being expressed in monocytes but expressed highly in $Lin^{-}MHCII^{+}CD11c+$ conventional DCs as determined by cell sorting, and was shown to promote DC maturation by regulating STAT3 activity. lncDC acts on STAT3 by preventing its dephosphorylation by the protein phosphatase SHP-1, thereby maintaining STAT3 in an active conformation to promote STAT3 target gene expression (**Figure 1.2**). In this situation, lncDC is acting post-translationally in the cytoplasm. Together, these studies demonstrate that the

mechanisms by which lncRNAs regulate immune cell differentiation are diverse, but also that the underlying premise that lncRNAs are key players in cellular differentiation is gaining more support.

lncRNA expression itself is regulated as part of the inducible inflammatory program, i.e. lncRNAs are only expressed after cells are activated. However, a subset is basally expressed and when TLRs are activated, the lncRNA expression becomes abrogated (Carpenter et al., 2013). As mentioned above, many immune cells must undergo various levels of cellular differentiation to be activated. Likewise, the gene expression programming of the differentiated cells is only turned on downstream of an inflammatory signal. One of the strongest triggers of the innate inflammatory response system is the Gram-negative bacterial cell wall component lipopolysaccharide (LPS). LPS activates the pathogen recognition receptor (PRR) Toll-like receptor 4 (TLR4), and initiates a signaling cascade that ultimately leads to the activation of transcription factors nuclear factor of kappa b (NF-kB) and interferon regulatory factors (IRFs). lncRNAs are being identified as signaling mediators downstream of these stimulation events, and one such lncRNA is lincRNA-Cox2 (long intervening non-coding RNA)-Cox2.

LincRNA-Cox2 was identified via RNA-sequencing of bone marrow-derived murine macrophages activated by LPS via TLR4, and subsequently named due to its genomic organization proximal to the protein-coding gene Ptgs2, which encodes for Cox-2. Further analysis of lincRNA-Cox2 indicated that

another PRR trigger, the TLR1/TLR2 ligand Pam_3CSK_4, also induces its expression, which requires the activation of the adaptor protein MyD88 and NF-kB (Carpenter et al., 2013). lincRNA-Cox2 in turn broadly regulates innate immune gene expression; and this regulation was shown to be both positive and negative. The negative regulatory activity of this lincRNA occurs through its interactions with hnRNPA2/B1 and hnRNP-A/B to dampen the expression of chemokines and interferon stimulatory genes (ISGs) such as Ccl5, IFN-α and IFN-β, in the murine macrophages (**Figure 1.2**). lincRNA-Cox2 was also shown to positively influence the expression of the pro-inflammatory cytokine, IL-6, as well as hundreds of additional inducible immune genes, via as-yet-undefined mechanisms.

Like lincRNA-Cox2, THRIL is another inducible lncRNA shown to function in part via its interaction with hnRNP proteins (Li et al., 2013). This lncRNA was identified in the THP-1 human monocyte cell line and was amongst 159 lncRNAs differentially expressed upon activation with Pam_3CSK_4 treatment. THRIL loss-of-function (shRNA) studies revealed that THRIL contributes to the inducible expression of the pro-inflammatory cytokine mediators TNF-a and IL-6 upon Pam_3CSK_4 stimulation. To support this, chromatin immunoprecipitation (ChIP) experiments indicate that THRIL and heterogenous ribonucleoprotein (hnRNP)-L both localize to the TNF-α promoter upon Pam_3CSK_4 stimulation (**Figure 1.2**). RNA-seq performed on THRIL-knockdown THP-1 cells revealed 319 genes that

were attenuated in their expression, indicating a broad range of candidate THRIL-regulated genes.

Another lncRNA, nuclear enriched abundant transcript 1 (NEAT1), is associated with the expression of IL-8 in human cell lines after HSV-1, influenza A (IAV) or dsRNA stimulation and promotes re-localization of HIV1 mRNA from the nucleus to the cytosol (Lu et al., 2013; Zhang et al., 2013). .

Yet another group compared four strains of mice infected with SARS-CoV or Influenza virus A/PR/8/34 (Peng et al., 2010). A unique lncRNA signature was expressed in the lungs during infection, that was dependent on type I interferon and STAT1 signaling. Although they didn't characterize specific lncRNAs identified from their screen, they were able to reveal large swaths of lncRNA signatures that are differentially expressed among the four strains of mice and correlate to their lncRNAs that are mediated through the type I interferon signaling pathway.

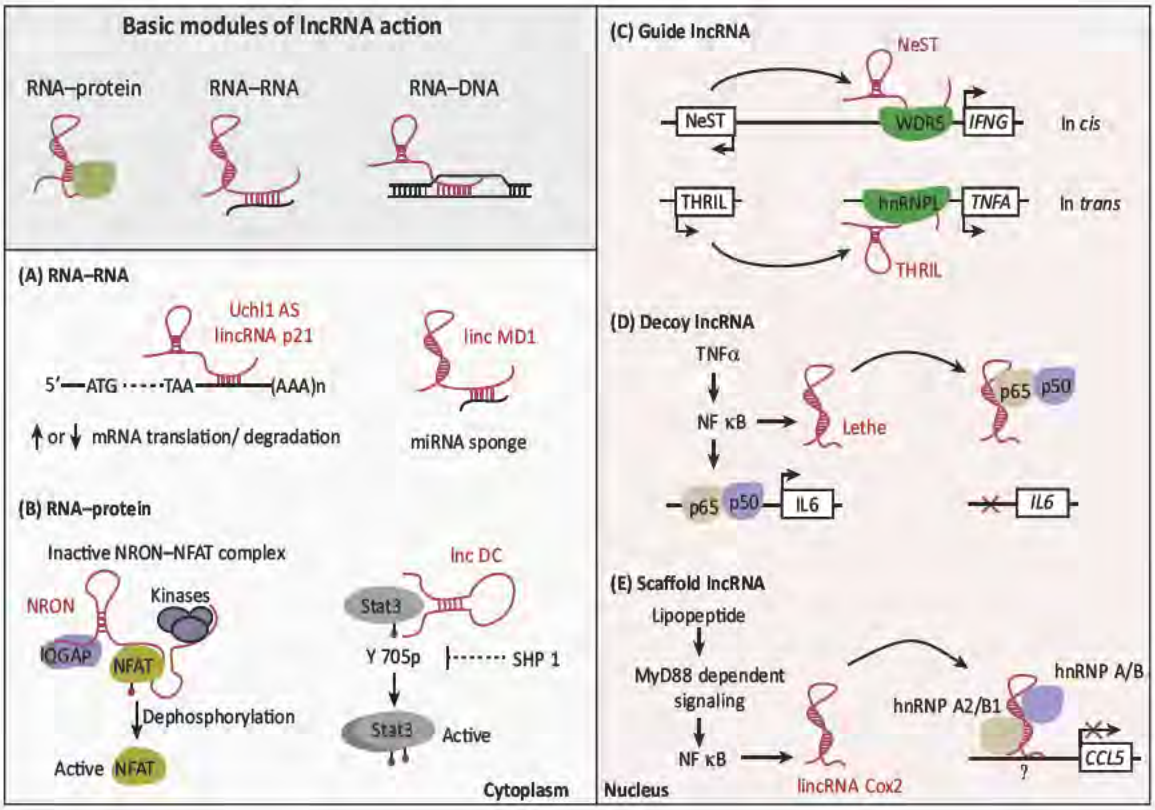


Figure 1.2. lncRNA regulation in the innate immune response. lncRNAs can mediate their functions via RNA-protein, RNA-RNA or RNA-DNA interactions (A) Examples of lncRNAs that bind to other RNAs to stabilize/degrade mRNA or act as a miR sponge in the cytoplasm; (B) NRON-NFAT and lnc-DC exert functions in the cytoplasm by RNA-protein interactions. (C) Nuclear lncRNA NeST acts in cis to promote the transcription of IFNg whereas THRIL acts in trans to mediate transcription of TNFa. (D) Lethe and lincRNA-Cox2 facilitate RNA-protein interactions to sequester p65 from binding to induce NF-kB-dependent transcription or through lincRNA-Cox2 binding of hnRNPA2/B1 to inhibit CCL5 transcription. (adapted from (Atianand and Fitzgerald, 2014))

Recently, a lncRNA microarray from human alveolar epithelial cells (A549) identified 9 lncRNAs that were differentially expressed after infection with influenza virus (Ouyang et al., 2014). The lncRNA Negative regulator of antiviral response (NRAV), had a markedly reduced expression upon infection. Ectopic expression of this lncRNA *in vivo* in mice suppressed MxA, IFITM3, and the expression of other ISGs, resulting in increased weight loss and decreased survival of infected mice. This was also seen in mice infected with Sendai Virus (SeV), Muscovy Duck Revirus, and HSV. Although located in both nuclear and cytoplasmic compartments, NRAV was shown to have no effect on ISG mRNA stability in the cytosol, and most likely regulates histone modifications at ISG gene loci in the nucleus as indicated by ChIP assays showing decreased H3K4me3, an indicator of active transcription, and increased H3K27me3, an indicator of transcriptional repression.

Objectives for Chapter 2

As described above, many groups are now unraveling the bewilderingly complex array of transcripts to dissect their functions in various processes. In Chapter 2, I dissect the role of a particular lncRNA, AS-IL1α,in its involvement in the inducible immune response. The objectives for chapter 2 are in three parts, (1) to characterize this natural antisense transcript as a *bone fide* lncRNA,, (2) to determine the role of AS-IL1α in the inducible immune response, namely explore

its protein-targets, and (3) to determine the mechanism by which AS-IL1α exerts its functions.

Now, I will describe the protein players involved in the signaling pathways that confer the innate immune response.

Pattern recognition receptors (PRRs)

The innate immune response is initiated when a specific moiety of molecular structures termed pathogen associated molecular patterns (PAMPS) (Thompson et al., 2011) that are conserved among certain classes of pathogens or molecules present themselves during conditions of injury or infection. Other molecules that may be released from stressed or dying cells and stem from either host or pathogen called danger-associated molecular patterns (DAMPS) (Land, 2015) also trigger the innate immune system. Both PAMPS and DAMPS engage germline-encoded evolutionarily conserved receptor proteins called pattern recognition receptors (PRRs). Activated PRRs initiate signaling cascades that result in gene transcription and the production of effector molecules and mediate downstream molecular signals. Several groups of PRRs have been described and are characterized into four main families: the toll-like receptors (TLRs) (Beutler, 2009), the retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) (Loo et al., 2008), C-type lectin receptors (CLRs) (Williams, 2013), and nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) (Davis et al., 2011). The effectors of these signals, typically in the form of cytokines and chemokines, coordinate the appropriate signals to drive the adaptive immune

response to produce long-term immunological memory against the original pathogen.

Triggers of inflammation include proteins, lipids, bacterial or fungal cell walls and membranes, carbohydrates, and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Modifications and specific arrangements of these molecular components are conserved among certain pathogens and are potent ligands for PRRs to initiate inflammation. The following sections will briefly review the specific type of PRRs that make up the innate immune receptor repertoire.

Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are type I transmembrane proteins that traffic between the plasma membrane and endosomal vesicles. Those located on the plasma membrane (TLR1,2,4,5,6) are usually specific for hydrophobic lipids and proteins while those found on the endosome sense nucleic acids (TLR3,7,8,9) (**Figure 1.1**). To date, 10 human TLRs (TLR1-10), and 13 (TLR1-13) murine TLRs have been identified (Gürtler and Bowie, 2013). All TLRs share a common architecture consisting of extracellular leucine-rich repeats (LRRs) and a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain common to the Interleukin (IL)-1 receptor (Dinarello, 2013). These receptors signal by dimerization, and differentially recruit the adaptor proteins, Myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (Mal aka TIRAP) (Fitzgerald et al., 2001) and/or TIR-domain-containing adaptor inducing IFN-β (TRIF) aka TIR domain-

containing adaptor molecule 1 (TICAM) or TRIF-related adaptor molecule (TRAM) aka TICAM2 (**Figure 1.3**). The adaptors initiate signal cascades culminating in the activation of differential pathways that converge onto nuclear factor of kappa B (NF-kB), mitogen-activated protein kinase (MAPK) or interferon regulator factors (IRFs). Together, these transcription factors drive expression of cytokines, chemokines, and/or type I interferons (IFNs) and also mediate downstream cellular proliferation, maturation, and survival.

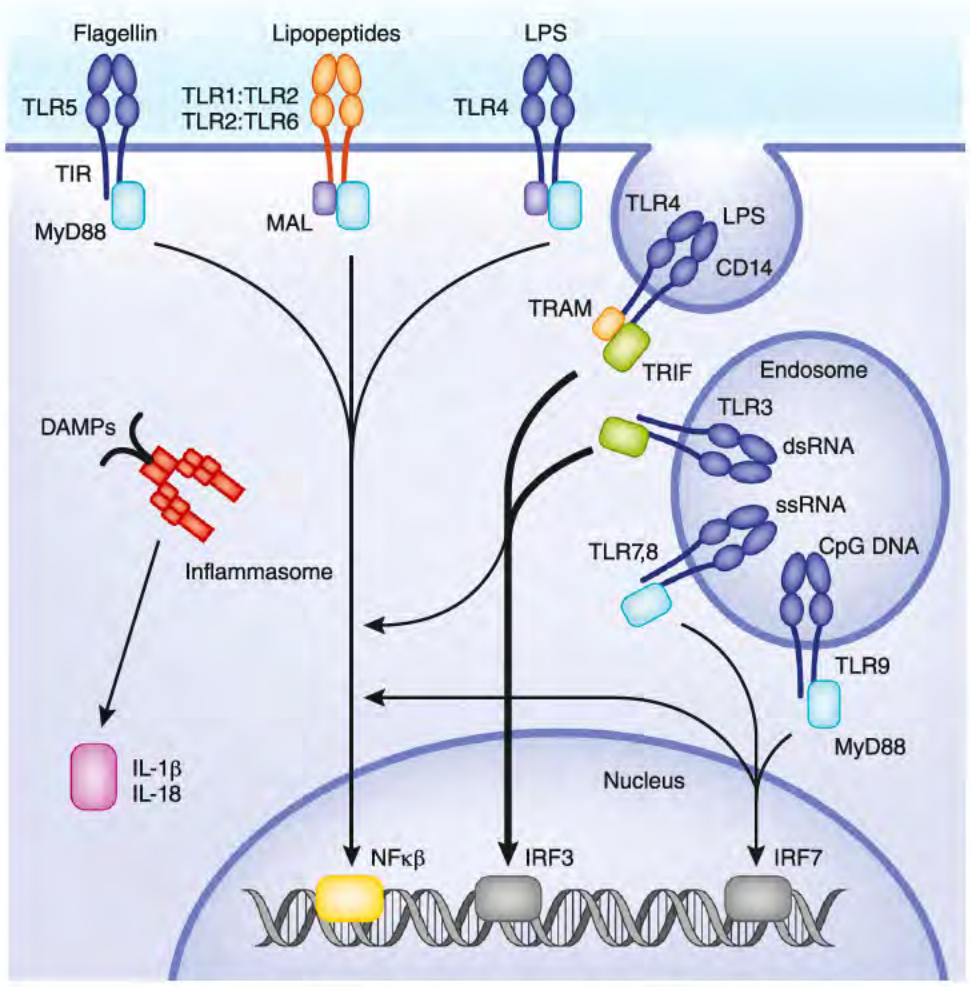


Figure 1.3. Toll-like receptor signaling.

TLRs 1-2, 4-6 are expressed on the cell surface, while TLRs 3, 7, 8, 9 are expressed in intracellular compartment called the endosome. Upon ligand binding, TLRs initiate signaling cascades involving MyD88 or TRIF that culminates in the activation of TRAF3 and TRAF6 that activate transcription factors NF-kB or IRFs to induce the production of proinflammatory cytokines like IL-6, TNF-a, IL-1a, or type I IFNs. (adapted from Hato et al., 2014)

Cytosolic nucleic acid sensors induce type I IFNs

Many viruses, bacterial pathogens, and parasites directly penetrate the cell without encountering TLRs. The interferon-stimulating properties of nucleic acids cannot solely be attributed to TLR signaling alone. For example, in TLR9 knockout cells, transfection of pathogen-derived dsDNA still induced a robust Type I IFN response (Ishii et al., 2006; Stetson and Medzhitov, 2006). This led scientists to hypothesize that another class of sensors exist that are directly responsible for Type I IFN production.

All viruses, and many bacteria and protozoa enter the cell's cytoplasmic compartment during their life cycles. During development and replication, these pathogens hijack the host's cellular machinery in order to gather nutrients or produce large numbers of progeny. The process leads to accumulation of viral nucleic acids in the cell, which are potent PAMPs (Hornung and Latz, 2010). Cytosolic nucleic acid sensors detect a variety of nucleic acids. These sensors can be divided into RNA sensors (RLRs), and DNA responders (NLRs, ALRs) (Ting et al., 2010). Cytosolic sensors can activate three distinct inflammatory pathways, the type I interferons through transcription factors IRF3 and -7, NF-kB, or MAPK pathways. Additionally, nucleic acid sensors can trigger the assembly of the inflammasome, a multiprotein complex that results in the caspase-1-dependent activation of the cytokines, IL-1β, IL-1α (to a lesser extent and elaborated later), and IL-18. Members of the NLR family of receptors were the first shown to activate the inflammasome pathway. Additionally, AIM2, a member

of the PYHIN family, has also been shown to assemble into inflammasomes (Rathinam et al., 2010).

Rig-I-like receptors (RLRs)

The RLR receptor family is composed of three DExD/H box RNA helicases: retinoic acid inducible gene-1 (RIG-I) encoded by the DDX58 gene, melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology-2 (LGP-2) (Rothenfusser et al., 2005). RIG-I and MDA-5 contain two tandem N-terminal caspase activation and recruitment domains (CARDs) followed by a DExD/H box RNA helicase domain (DEAD domain) with ATPase activity, and a C-terminal repressor domain (CTD). Although they have the same domain structure, RIG-I and MDA-5 recognize distinct RNA ligands as demonstrated by their crystal structures (Bin Wu et al., 2014; Niland et al., 2012). RIG-I recognizes small 5'ppp modified RNA and preferentially bind RNA less than 1000 nucleotides (nt) long. Its DEAD domain binds to the 5'ppp for specific recognition (Bin Wu et al., 2013; Wilusz et al., 2009). On the other hand, MDA5 binds to the RNA phosphodiester backbone and initiates filament formation upon binding. This allows it to preferentially recognize double-stranded (ds)RNA species >2000 nt in length (Bin Wu et al., 2014). Because 5'ppp modifications, and dsRNA species are not common features of mammalian RNA, but rather in viral genomes, these two receptors are strong agonists for sensing active viral infections. Additionally, recognition does not depend on sequence specificity. RIG-I has been implicated in sensing Sendai Virus (SeV), influenza A and B,

hepatitis C (HCV), vesicular stomatitis virus (VSV) and respiratory syncytial virus (RSV). MDA-5 recognizes encephalomyocarditis virus (EMCV), coxsackie B virus (CVS) and Polio. MDA-5 also detects the transfected synthetic viral RNA-mimic, polyinosinic:polycytidylic (poly(IC)). Upon binding to their cognate ligands, MDA-5 and RIG-I alike form helical filaments that allow for protein-protein interactions through their CARD domains to recruit the adaptor mitochondrial antiviral signaling protein (MAVS), aka VISA, IPS-1 or CARDIF (Bin Wu et al., 2013). MAVS then activates the IKK-related kinase (TBK1) aka IKKi, and in turn, activates the transcription factors IRF3 and -7, leading to robust transcription of type I interferons.

Unlike RIG-I and MDA5, LPG-2 lacks the two N-terminal CARD domains and was initially shown to negatively regulate RLR activity (Rothenfusser et al., 2005). Overexpression of LGP-2 decreased the capacity of SeV and NDV to induce type I IFNs. However, LGP-2 deficient mice had observable increased interferon signaling upon infection, which is contrary to its suggested function as a negative regulator (Satoh et al., 2010).

Interferon-inducible cytosolic DNA receptors

Similar to RLRs, cytosolic DNA receptors mediate innate immune responses independently of TLR signaling. Unlike RNAs that are highly modified in microbial species, DNA sensors typically recognize non-specific, modification-independent DNA backbones. It is speculated that the innate immune trigger is

due to the incorrect tropism of DNA where it should be in the nucleus rather than in the cytoplasm. Several DNA sensors have been described, and can be grouped by the signaling pathways they activate. Receptors like DAI and IFI16 induce the expression of proinflammatory cytokines and type I IFNs upon DNA ligation through signaling involving the adaptor stimulator of interferon genes (STING) aka TMEM173 and either NF-kB for proinflammatory cytokines or IRFs for type I IFNs. Helicases DHX9 or -36 interact directly with DNA and MyD88 to activate NF-kB and IRF7. These helicases activate both proinflammatory cytokines and type I IFNs through MyD88-dependent, TLR-independent mechanisms. RNA polymerase III (RNAPIII) reverse-transcribes certain types of dsDNA like AT-rich DNA to an RNA ligand that activates RIG-I to induce type I IFN as described in the RIG-I section (Hornung et al., 2009). Flightless I interacting protein-1 (LRRfip1) through β-catenin, responds to microbial DNA through its leucine-rich repeats (LRR) and activates IRF3 via CBP/p300 coactivation pathways (Bagashev et al., 2010). Lastly, cGAS has recently been identified to recognize cytosolic DNA and triggering type I IFN signaling in a STING-depedent manner through cyclic di-nucleotide second messenger signaling.

Nod-like receptors (NLRs) and the inflammasome

There are 22 human Nod-like receptors (NLRs) and 34 murine NLRs in the protein family that consists of cytoplasmic pathogen censors that contain a

central nucleotide binding and oligomerization domain (NACHT) common to all NLRs and C-terminal leucine-rich repeats (LRRs) (Inohara et al., 2005). The N-terminal portion of most NLRs harbor protein-binding CARD domain, a pyrin domain, and a baculovirus inhibitor of apoptosis protein repeat (BIR) domain. NLRs that harbor a pyrin or BIR domain in their N-terminus are not involved in the transcriptional activation of inflammatory cytokines and are components of the inflammasome that regulate caspase-1 activity. All caspases are cysteine proteases that initiate or execute cellular programs, leading to inflammation and/or cell death. They are transcribed and translated as inactive zymogens that require proteolytic cleavage for activation. NOD1 and NO2 harbor CARDs in addition to LRR and NOD domains activate NF-kB via RIP2/RICK adaptor to drive the transcription of proinflammatory cytokines. They recognize bacterial peptidoglycans, g-D-glutamyl-mesodiaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively. Many TLRs and NLRs synergistically recognize pathogens to mediate the activity of IL-1β and IL-18. IL-1β, the original pyrogen, is a pleotropic cytokine that induces fever, activates monocytes, macrophages and neutrophils and drives acute-phase protein synthesis. IL-18 activates natural killer (NK) cell activity and IFN-γ production to induce Th1 and Th17 adaptive immune responses. Many stimuli activate TLR ligands as mentioned above also activate NF-kB-dependent transcription of IL-1β and IL-18. Unlike many others, these cytokines lack leader sequences and are retained in the cell rather than secreted out via secretory vesicles. This list isn't exhaustive, and intense

investigation is underway in the NLR field (Davis et al., 2011).

IL-1 signaling pathway

IL-1 represents two proteins, IL-1α and IL-1β. Both are transcribed and translated as a precursor 33 kilodalton (pro) form, without a signal peptide. The *IL1A* gene is located adjacent to the *IL1B* gene on the long arm of chromosome 2. IL-1α is transcribed and translated into a 271 amino acid precursor. Similar to IL-1β, IL-1α lacks a signal peptide sequence and the precursor can be processed to produce a shorter mature protein, which is the carboxyl terminal part of the precursor. The differences between IL-1α and IL-1β are mainly in the cell sources that secrete them. They are also regulated differently, but post-secretion, act upon the same IL-1 receptor and downstream effects are identical. The signaling complex recruits MyD88, four IL-1R activating kinases and results in the activation of NF-kB, c-Jun N-terminal kinase (JNK) and p38-mitogen activated protein kinase (MAPK) target gene transcription. Pro-IL-1α is fully active and is usually found dispersed throughout the cytoplasm of cells (Kim et al., 2013). The reverse is true for pro- IL-1β, which is inactive until cleaved by caspase-1, which is regulated by the inflammasome. Although IL-1α can be found outside of cells in regions of high inflammation, functions for IL-1α are also intracellular. For example, following stimulation with cytokines and TLR ligands, intracellular pro-IL-1α translocates to the nucleus, where it participates in the

activation of transcription factors NF-kB and AP-1 to drive the synthesis of proiflammatory cytokines, including IL-6 and more IL-1α.

Both IL-1α and IL-1β can be secreted by a number of cells including macrophages, monocytes, neutrophils, lymphocytes, and epithelial cells. IL-1α and IL-1β both signal through the IL-1R. IL-1R is expressed on many cell types, including lymphocytes and thymocytes. The IL-1R consists of an extracellular ligand-binding domain, organized similarly to members of the Ig-superfamily, and a cytoplasmic region containing a TIR domain. Signaling through IL-1R requires many of the same adaptor proteins that are associated with TLR signaling, including MyD88, IRAK, TRAFs and lead to NF-kB activation and proinflammatory cytokine production.

As mentioned earlier, IL-1α can be active in its pro-form. Although it can be cleaved by caspase-1 into its mature form for secretion out of the cell to signal through IL-1R on neighboring cells like IL-1β, this isn't necessary for its function. Indeed, for this reason, IL-1α is classified as an alarmin (**Figure 1.4**). Alarmins are molecules that are passively released by necrotic cells, and can subsequently activate neighboring cells to trigger highly inflammatory states. Because dysregulation of inflammation lies at the heart of many inflammatory triggers, hyper expression of IL-1α in a cell could be deleterious to the local cellular environment. For this reason, IL-1α requires extra attention and requires tight regulation.

Under circumstances where normal cell turnover and apoptosis is

occurring, IL-1α translocates into the nucleus and becomes tightly bound to chromatin and does not become active. Only under necrotic conditions, does cytoplasmic IL-1α, cleaved or non-cleaved become released into the extracellular space and initiate IL-1R signaling (Cohen et al., 2010a). Additionally, IL-1α sequesters to IL-1 receptor, type II (IL-1R2), which acts as a decoy receptor for IL-1α to remain inactive until there is an inflammatory trigger (**Figure 1.4**)

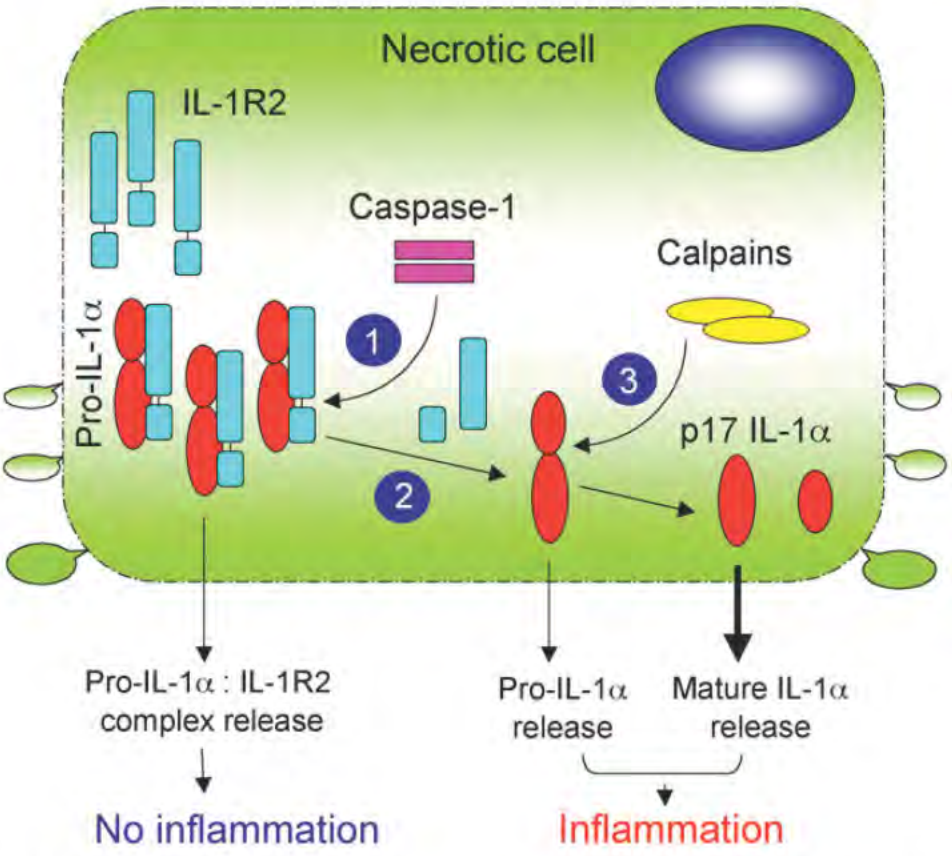


Figure 1.4. Model of IL-1α activation. IL-1α is transcribed and translated into a p33 form where it is sequestered by the cytosol through IL-1R2 binding. Under normal apoptosis pro-IL-1α stays sequestered to IL-1R2 and no inflammation occurs. (1) Active caspase-1, through inflammasome activation, can cleave IL-1R2 and liberates pro-IL-1α to be available for (2) release from necrotic cells and is fully active. It can also be cleaved by calpains, leaving a highly active p17 mature IL-1α form. Both pro- and mature forms can be active. (Adapted from (Di Paolo and Shayakhmetov, 2013)

Malaria

The *Plasmodium spp*. parasite was identified as the causative agent of malaria in 1880, and the mosquito was identified as the vector in 1897 (Cox, 2010). Despite subsequent efforts in all areas of biology, epidemiology, and public health, there is still no effective vaccine for the prevention of malaria (Arama and Troye-Blomberg, 2014). Physical barriers such as bed nets and screens, as well as chemical prevention methods like insecticides and mosquito repellents, have drastically slowed the transmission of the disease. Additional discoveries of therapeutics have dampened the overall malaria burden worldwide. However, this disease still accounts for 584,000[1] number of deaths people annually according to the World Health Organization. The risk of malaria is highly dependent on interactions between the host, parasite, mosquito vector and environment. Changes in any one of these elements may drastically impact the risk of infection. In the following sections, I will describe the *Plasmodium* species and the innate sensing of malaria parasites that contribute to the outcome of infection.

Four Plasmodium spp. cause malaria worldwide, with *Plasmodium falciparum* causing the most mortality. They are protozoan parasites of the apicomplexan phylum. Malarial disease ranges from severe, life-threatening illness, to mild febrile illness to asymptomatic infections. Severe malaria is characterized by coma, hyperparasitemia, hypoglycemia, or anemia and typically

[1] http://www.who.int/mediacentre/factsheets/fs094/en/

presents itself in young children or adults without previous exposure. As the population exposed to malaria ages, lower parasitemia and rates of disease occur. Repeated exposure to parasites results in mild febrile illnesses to asymptomatic or subclinical disease. Cerebral malaria (CM) is among the most severe complications by *P. falciparum* infection, described by diffuse encephalopathy, associated with loss of conscience and muscular tone. The degree of loss of consciousness ranges from confusion to coma. Typical CM is associated with poor prognosis, with a 20-30% mortality (Brewster 1990).

Mouse Cerebral Malaria

Rodent malaria parasites in mice is the choice model for studying the pathogenesis of CM. Four species and 113 subtypes of rodent *Plasmodium* exists (Carter and Walliker, 1976), but only *P. berghei* and *P. yoelii* have been used consistently for CM studies. *P. berghei* is the only species that is able to induce CM in mice, rats, and hamsters (Bafort et al., 1980; Mackey et al., 1980; Mercado, 1965; Rest, 1983). Among all strains of P. berghei isolated, four of them *P. berghei SP11, ANKA (PbA), NK65, and Kyberg (K173*) have been used to study CM. This is worth mentioning because the use of these different isolates have caused a lot of confusion in the field. For a long time, only PbA was reported to induce neurological signs in mice. *NK65* was only shown to induce neurological symptoms in one occasion with rats (Kamiyama et al., 1987). And low-dose injection of *K173* showed CM-associated neurological signs, but may go away with higher-dose injections (Rae et al., 2004). Most studies of CM have

been performed using multiclonal parasite lines and phenotypes differ drastically from lab to lab as the parasite passage increases. Indeed, for these reasons, there has been extensive contention and debate over the relevance of the rodent models, and it is evident that experimental models do not reproduce all the features of CM. Additionally, the mouse model lacks parasite sequestration in the brain, which is a key manifestation in human disease, and have been observed to preferentially sequester to different organs bringing the question of what the mouse model actually dies from. This, however, is debatable as a number of studies have observed parasite sequestration (Engwerda et al., 2002; Hearn et al., 2000), but it is difficult to discern if the iRBCs are located in the blood stream in the brain or actually bound to brain endothelial cells. To add even more confusion to the existing data, only some strains of mice are susceptible to CM (CBA/J, C57BL/6J, 129/Ola, etc) (Grau 1990, Mackey 1980, Amani 1998, Bagot 2002). Despite all of this, though not perfect, these animal models have been instrumental in our understanding of certain mechanisms associated with CM (de Souza et al., 2010; Longley et al., 2011).

Plasmodium life cycle

If the lack of a consensus mouse model and Plasmodium strain isn't enough, to further complicate the process, malaria parasites undergo various life stages in multiple hosts. An infected female *Anopheles* mosquito takes a blood meal, and deposits sporozoites into the dermis of the mammalian host. They enter the bloodstream and migrate to the liver where they undergo a replication

amplification stage in hepatocytes for 48 hours (mice) and up to 2 weeks (humans). Merozoites are the form that is released after rupture of infected hepatocytes and invade red blood cells (RBCs) and initiate the asexual replication cycle. (**Figure 1.5)** Each cycle of invasion and replication occurs from 24-72 hours depending on the Plasmodium species. Clinical symptoms including fever and inflammation occur during this stage.

Failure to control a malaria infection is in part, related to the complexity of the parasite and the interaction with the immune system of the host. Although there are now more efforts being made to understanding the nature and control of immunity or the pathological consequences of the host's response to *Plasmodium*, a lot remains to be learned. Key questions include what molecular contributions of Plasmodium initiates the immune response. How is that regulated? What are the mechanisms that determine the severity of disease? Because of the different location of the parasite and the different antigens expressed at the liver and blood stages, the relevant immune responses and their specificity and regulation will not be the same for the liver and blood stages of infection. A thorough understanding of the mechanisms and antigens recognized at both of these stages, and the differentiation of immunity to disease and infection will be important for the construction of an effective vaccine.

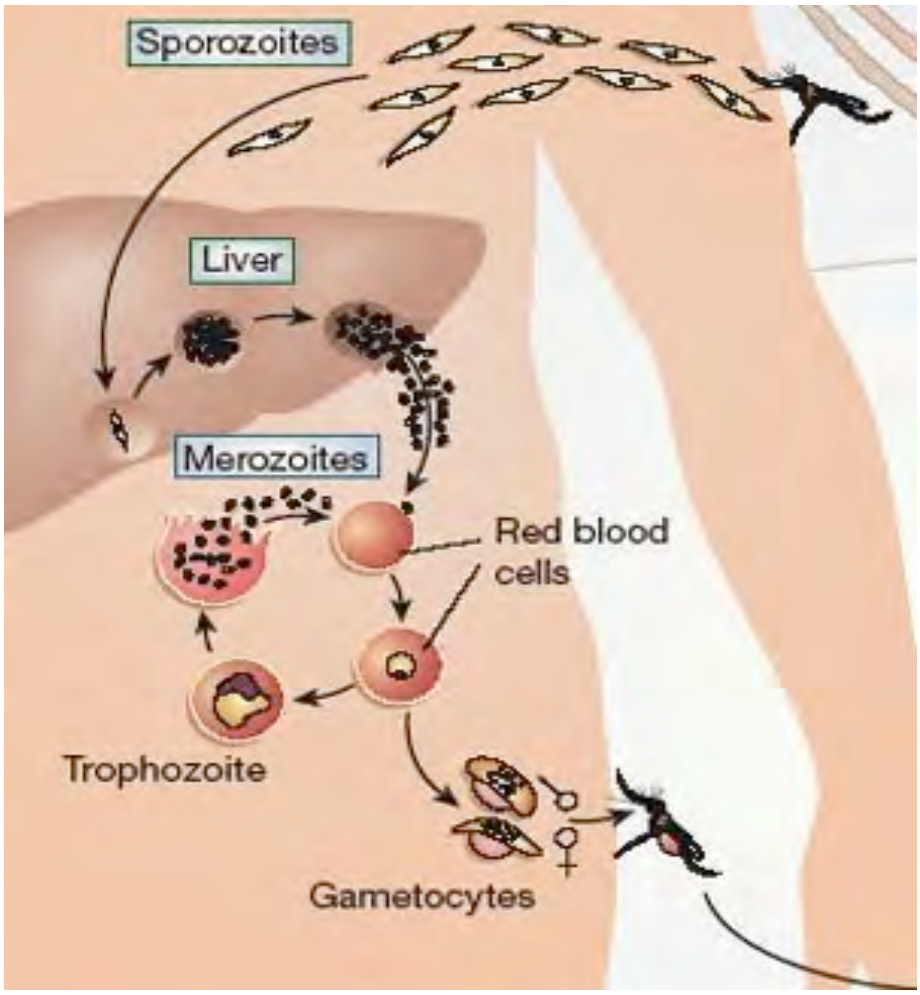


Figure 1.5. Plasmodium life cycle.

Three distinct stages of the parasite life cycle. Infection initiates upon deposition of sporozoites (spz) by infected-mosquitoes taking a blood meal from a mammalian host. The sporozoites migrate to the liver and matures divides in liver hepatocytes. The mature merozoite emerges from hepatocytes to initiate the invasion of red blood cells (RBCs) in repeated cycles for the rest of the infection until the host clears or becomes moribund to infection. The transmission cycle continues when another blood meal is taken by a mosquito and takes up Plasmodium-infected blood. (image from Menard 2005).

For each stage of infection, there are several potential targets of the protective immune response; molecules on the surface of the sporozoites, infected liver cells, blood stage merozoites, and infected red blood cells have been identified.

Innate immune contribution to illness during malaria

The “cytokine storm” observed during malaria is thought to be a major contributor to malaria-dependent mortality. Through pattern recognition receptors (PRRs), parasite molecules are detected and trigger signaling cascades that converge into a productive inflammatory response. This triggers the activation and selection of B and T cells that undergo receptor class-switching and somatic hypermutation that yields in adaptive immunity toward the original pathogen. In the case of *Plasmodium* parasites, these PRRs include the toll-like receptor (TLR) family (Gazzinelli et al., 2004). For example, in humans, polymorphisms in TLR2, 4, 9 and Mal (also known as TIRAP) have been associated to be protective in the clinical outcome of malaria (Khor et al., 2007; Mockenhaupt et al., 2006). Glycosylphospatidylinositols (GPIs) from the parasite, which anchor a range of Plasmodium molecules to cell surfaces, are considered likely candidates to induce host inflammatory responses, fever and other pathology. GPI anchors have been shown to trigger TLR2 (Zhu et al., 2005), (Nebl et al., 2005), and to a lesser extent, TLR4 (Krishnegowda et al., 2005) and were thought to be the dominant immunostimulatory PAMP. TLR1-2 is activated by GPI anchors containing three fatty acid chains, whereas TLR2-6 is activated by two fatty chained GPI anchors. Activation of these TLRs mediate MyD88 signaling which

leads to NF-kB nuclear activity and induction of TNF-α, nitric oxide (NO) and pro-IL1b transcription. However, GPI anchors but do not encompass the entire immune stimulatory activity of malaria (Wu et al., 2010). Others claim that murine cerebral malaria is independent of TLR signaling (Togbe et al., 2007).

IL-1β-mediated fever is one of the hallmarks of malaria that causes many of the symptoms associated with disease (Brown et al., 1999; Clark et al., 1994; Clark and Rockett, 1994). The inflammasome is central to IL-1β regulation. Mice-deficient in MyD88 and IL1R-IL18R signaling have decreased production of IL-12 and attenuated pathology during infection with murine *Plasmodium spp.* (Adachi et al., 2001; Franklin et al., 2009; Pichyangkul et al., 2004). Studies of cerebral malaria in mice lacking TLRs have suggested that although TLRs contribute to experimental cerebral malaria (ECM), additional signaling mechanisms also contribute to this extreme manifestation of disease (Togbe et al., 2007). Parasites consume hemoglobin as a source of nutrients during the blood-stage infection and release toxic iron-containing heme. In order to detoxify the heme molecules, the parasite converts the heme into an inert crystal called hemozoin (Hz). This byproduct has been shown to activate TLR9 (Coban et al., 2007) and was later discovered that DNA-bound to Hz was the actual trigger for the TLR9-dependent inflammation (Parroche et al., 2007).

Polymorphisms in the type I IFN receptor (*IFNAR1*) (Aucan et al., 2003) are linked to increased survival from malaria. A number of reports have revealed

the ability of *Plasmodium* to induce type I IFNs (Aucan et al., 2003) (Pichyangkul et al., 2004; Vigário et al., 2007). Type I IFNs are typically induced in response to nucleic acids. Although plasmacytoid dendritic cells (pDCs) are a major source during virus infections and may regulate the IFN response to schizonts (Pichyangkul et al., 2004) many other cell types can also produce these cytokines. Several additional nucleic acid sensors have also been implicated in IFN gene regulation, however their role in malaria has not been thoroughly explored. In this study we investigated the role of TLR-independent DNA sensors in type I IFN production during malaria in response to both liver-stage and blood-stage infections.

Objectives for Chapter 3

In Chapter 3, the objectives are to define the PAMP and innate immune response to liver-stage infections by PbA in mice. We found that RNA serves as a PAMP by activating MDA5-MAVS, and mediating Type I IFN production and subsequent interferon-stimulatory gene (ISG) production. These proteins play a role in dampening the initial parasite load. Next, we look at the blood-stage infection by PbA iRBCs. We demonstrate that in this situation, type I IFNs contribute to cerebral malaria through a STING-dependent pathway. We also define a new A/T-rich DNA motif that activates this response. Furthermore, Hz has been shown to activate TLR9, but we show that it also triggers inflammasome activation to mediate robust IL-1β production. Each part of the study reinforces the notion that there are multiple PAMPS that contribute to the

inflammatory response during malaria infection. Each of these PAMPS exhibits themselves in different forms dependent on the life-stage of the parasite.

Preface to Chapter 2:

Components of this chapter has been submitted to the following publications:

Jennie Chan, Maninjay Atianand, Zhaozhao Jiang, Susan Carpenter, Dan Aiello, Roland Elling, Katherine A. Fitzgerald*, and Daniel R. Caffrey*. 2015. *Cutting Edge*: *A Natural Antisense Transcript, AS-IL1α, controls inducible transcription of the pro-inflammatory cytokine IL-1α*. Journal of Immunology. In revision.

* equal contribution.

CHAPTER 2: A Natural Antisense Transcript controls inducible transcription of the proinflammatory cytokine IL-1α.

Abstract

Natural antisense transcripts (NATs) are a class of long noncoding RNAs (lncRNAs) that are complementary to other protein-coding genes. Although thousands of NATs are encoded by mammalian genomes, their functions in innate immunity are unknown. Here, we identify and characterize a novel NAT, AS-IL1α that is partially complementary to IL-1α. Similar to IL-1α, AS-IL1α is expressed at low levels in resting macrophages and is induced following infection with Listeria monocytogenes or stimulation with TLR ligands (Pam3CSK4, LPS, PolyI:C). Inducible expression of IL-1α mRNA and protein were significantly reduced in macrophages expressing shRNA that target AS-IL1α. AS-IL1α does not alter the stability of IL-1α mRNA, as expected of many natural antisense transcripts. Instead, AS-IL1α was required for the recruitment of RNA Polymerase II (RNAPII) to the IL-1α promoter to initiate transcription in the nucleus. In summary, our studies identify AS-IL1α as important regulator of IL-1α transcription during the inflammatory response.

Introduction

lncRNAs are differentially regulated in dendritic cells after lipopolysaccharide (LPS) (Guttman et al., 2009) and macrophages after Pam_3CSK_4 stimulation (Carpenter et al., 2013). There is a strong correlation between the immune protein coding genes that were strongly induced and neighboring lncRNAs suggesting that these genes may be co-regulated and may represent a new component of the inducible immune response. These observations, along with other lncRNAs that have been implicated in regulating the immune system, either by cell activation processes or regulating the effector cytokines themselves, indicate that lncRNAs are regulated and may be important in mitigating various immune processes and functions. Through our own RNA-sequencing data, we have implicated a type of lncRNA called a natural antisense transcript (NAT) that regulates the transcription of the pro-inflammatory cytokine, IL-1α.

Natural antisense transcripts (NATs) are one class of lncRNAs that are defined as being complementary to one or more protein-coding genes (Werner et al., 2009). Some estimates indicate that ~50-70% of lncRNAs are NATs, making them a substantial proportion of the noncoding genome (Derrien et al., 2012; Khorkova et al., 2014; Werner et al., 2009). NATs have been shown to regulate the expression of their sense gene pair or of related genes in both activating and repressive contexts (Liu et al., 2014; Matsui et al., 2008; Vigetti et al., 2014). Similar to the broader class of lncRNAs, it is thought that NATs function in a cell-

type specific manner. However, the functions of NATs in innate immunity are relatively unexplored.

Macrophages constitute the first line of defense against microbial pathogens and participate in triggering inflammation by exogenous and endogenous stimuli. Production of pro-inflammatory cytokines is among the earliest of the host's cellular responses to infection. Of particular relevance to this study are the pro-inflammatory cytokines, IL-1α and IL-1β. These cytokines are induced rapidly and amplify inflammation via IL-1 receptors expressed on adjacent cells (Chen et al., 2007). The significant potential of these cytokines to cause tissue damage highlight the need for rigorous control of their production and activity. IL-1β requires the activation of the inflammasome to convert it to the mature biologically active cytokine. Although IL-1α may be cleaved into its mature protein, it can also be activated in its full-length form and is therefore considered an endogenous alarmin, which are molecules that upon release from damaged or dying cells, will trigger the immune system (Kim et al., 2013). IL-1α appears to be primarily regulated during transcription and splicing as it is translated as an active protein (Dinarello et al., 2012).

This chapter contains three parts: (1) to characterize the molecular and cellular structure of AS-IL1α, (2) to determine the role of AS-IL1α on other genes upon perturbation of its own expression in order to determine its targets, and (3) to elucidate some mechanistic insights to how AS-IL1α may exhibit its functions.

Materials and methods

In vivo infections. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at UMASS Medical School. Mouse strains C57Bl/6 were maintained in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee (IACUC). *Listeria monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (NTNU, Trondheim, Norway) and infected as described (Severa et al., 2014) for 24 hours before harvesting the spleen.

RNA-sequencing. Single cell suspensions from spleens were used to make total RNA. 4 µg of total RNA was used to generate libraries for RNA-sequencing (Illumina unstranded mRNA kits). Samples were sized, quantified and validated on a Bioanalyzer tp ensure RNA quality. Libraries were sequenced on a High-Seq System (Illumina 2000, San Diego, CA) as paired-end 50 reads. Sequence reads were aligned to the mouse genome (assembly NCBI m37/mm9) using TopHat (Trapnell et al., 2009). Gene level read counts based on the resulting alignments were calculated using HTSeq (http://www-huber.embl.de/users/anders/HTSeq/) and the Ensembl65 gtf transcript annotation file. The DESeq R package (Anders and Huber, 2010) was used to normalize gene counts, calculate fold change in gene expression, estimate p-values and adjusted p-values for change in gene expression values, and to perform a variance stabilized transformation on read counts. The Circos program

(Krzywinski et al., 2009) was used to visualize genome-wide gene expression changes. Log2 fold change values were computed by subtracting the log2 variance stabilized counts of the unstimulated sample from the log2 variance stabilized counts of the Listeria monocytogenes-stimulated splenocytes All protein coding genes that were annotated with particular GO IDs (GO:0006955, GO:0045088, GO:0009607, GO:0009615, GO:0006952, GO:0006954, GO:0032020, GO:0051707, GO:0051707, GO:0035455, GO:0035457, GO:0006950, GO:0001866, GO:0009611) were classified as immune genes and colored red.

Accession numbers. All raw data from RNA-sequencing are available for download from NCBI Gene Expression Omnibus under the accession number (in submission).

Cell culture. Murine bone marrow derived macrophages (mBMDMs) were derived by flushing progenitor cells out using PBS, pelleted, depleted of RBCs using RBC lysis buffer (Sigma). BMDMs were harvested from bone marrow extracted from mouse femurs and cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin, 100 ug/mL streptomycin and ciprofloxin, and 25% L929 conditioned supernatant (as the source of M-CSF) and maintained at 37*C in a humidified atmosphere of 5% CO2, in 75 or 175 cm2 culture flasks. Or Human Embryonic Kidney (HEK) 293T Cells were cultured in DMEM supplemented with 10% FCS,

100 U/ml penicillin, 100 ug/ml streptomycin and ciprofloxin. Either cell type was seeded at $1x10^5$/ml in a total volume of 15 mL (75 cm2 flask) or 35 ml (175 cm2) flask, and passaged when confluent as follows: cell monolayers were washed with medium incubated with 1 mL trypsin/EDTA (75 cm2 flask) or 2 ml (175 cm2) flask at 37*C until detachment was evident and 9 ml medium was added to terminate trypsinization. Cells were then centrifuged 400g, 5 minutes, 4*C and the pellet re-suspended in 10 ml medium. Cell number and viability was determined by mixing a 10 ul aliquot of re-suspended cells with 90 ul 0.4% trypan blue solution, and applying 10 ul of this mixture to a hemocytometer. The dye is excluded from viable cells, and under bright field microscopy membrane-damanged cells stain blue-violet, whereas undamaged cells appear translucent.

Cell stimulations. Cells were infected with L. Monocytogenes or stimulated with lipopolysaccharide (LPS) (100 ng/mL), Pam_3CSK_4 (100 nM), Poly(I:C) (25 µg/mL) and ISD (3 uM) oligonucleotides (Unterholzner et al., 2010) as described in (Severa et al., 2014). After 2 hours incubation, total RNA was harvested from all conditions (RNeasy, Qiagen).

RNA isolation and qRT-PCR. RNA was isolated from cells prepared as described above for BMDMs and maintained in culture as described. Stimulated cells were harvested into RLT buffer containing 2-bME for subsequent processing with the RNease Mini kit (Qiagen). Each RNA sample was adjusted to contain the same

quantity of RNA using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using iScript RT cDNA kit (BioRad) and qRT-PCR was performed using SYBR Green PCR Master Mix (BioRad) with the primers listed below in Table 2.1.

Table 2.1. qRT-PCR primers and shRNA targeting sequences

	shRNA target sequences
shRNA-GFP-For	5'CCGGAAGCAAGCTGACCCTGAAGTTCATCTCG AGTACTTGAAGTCCCAGTCGAACGTTTTTTTG3'
shRNA-GFP-Rev	5'AATTCAAAAAGCAAGCTGACCCTGAAGTTCATT CTCGAGTACTTGAAGTCCCAGTCGAACGTT3'
shRNA#1-For	5'CCGGAAGAAGGTATTTTTACCCATCCTCGAGGA TGGGTAAAAATACCTTCTTTTTTTG3'
shRNA#1-Rev	5'AATTCAAAAAAAGAAGGTATTTTTACCCATCCTC GAGGATGGGTAAAAATACCTTCTT 3'
shRNA#2-For	5'CCGGAACTGGCTAGGGAGGACAAAACTCGAGT TTTGTCCTCCCTAGCCAGTTTTTTTG-3'
shRNA#2-Rev	5'AATTCAAAAAAACTGGCTAGGGAGGACAAAACT CGAGTTTTGTCCTCCCTAGCCAGTT-3'
shRNA#3-For	5'CCGGAACTGGCTAGGGAGGACAAAACTCGAGT TTTGTCCTCCCTAGCCAGTTTTTTTG-3'
shRNA #3-Rev	5'AATTCAAAAAAACTGGCTAGGGAGGACAAAACT CGAGTTTTGTCCTCCCTAGCCAGTT-3'
	qRT-PCR primer sequences
AS-IL1α-For	5'- AGGCTTGGGATTCACTTGAC-3'
AS-IL1α-Rev	5'-TCTCTCTGGGCTTCAGTTCC-3'
IL-1α mature-For	5'TCTCAGATTCACAACTGTTCGTG-3'
IL-1α mature-Rev	5'AGAAAATGAGGTCGGTCTCACTA-3'
Gapdh-For	5'-CAAGGTCATCCATGACAACTTTG-3'
Gapdh-Rev	5'-GTCCACCACCCTGTTGCTGTAG-3'
18s rRNA-For	5'-CGCGGTTCTATTTTGTTGGT-3'
18s rRNA-Rev	5'-AGTCGGCATCGTTTATGGTC-3'
7SK – For	5'-AGAACCTCCAAACAAGCTCTCAAGG-3'
7SK – Rev	5'-AGAAAGGCAGACTGCCACATGCAG-3'
IL1α intron 5-exon 6 – For	5'-CACACACACACACACATCTGC-3'

IL1α intron 5-exon 6-Rev	5’-GGGCTGGTCTTCTCCTTGAG-3’
IL1α intron 1 – For	5’-CGCTCTTCCCGTTTTGTAAG-3’
IL1α intron 1 – Rev	5’-GTGGCCATGTGTGTGTCACT-3’
IL1α intron 2 – For	5’-TCCTCCTCCTCCTCCTTCTC-3’
IL1α intron 2 – Rev	5’-GAACCTGATGGCCTCTCTCA-3’
AS-IL1α-specific RT primer	
	ChIP primers
(TSS+22)IL1α-For	5’-AGTCAACTCATTGGCGCTTG-3’
(TSS+22)IL1α-Rev	5’-AGAGGACAGTCAAGGAGCAAAC-3’
(TSS+227)IL1α-For	5’-CAAGATGGCCAAAGTTCCTGAC-3’
(TSS+227)IL1α-Rev	5’-TGGAGTCAAAGGAACCTTGAGC-3’
(TSS+87)IP10-For	5’-CCGTCATTTTCTGCCTCATC-3’
(TSS+87)IP10-Rev	5’-CTGCAAGCTGAAGGGATTTC-3’
(TSS+73)Gapdh-For	5’-TAGGACTGGATAAGCAGGGC-3’
(TSS+73)Gapdh-Rev	5’-GAACAGGGAGGAGCAGAGAG-3’

Polysome profiling. Macrophages were seeded at 5 million cells in a 100 mm dish. After 16 hours of culture cells were pre-treated with cycloheximide (100 ug/ml) for 10 minutes at 37ºC or with harringtonine (2 ug/ml) for 25 minutes followed by cycloheximide (100 ug/ml) for 10 minutes. Cells were washed twice in ice-cold PBS+Cycloheximide (100 ug/ml) and scraped in 1ml of PBS+Cycloheximide (100 ug/ml). Cells were then pelleted at 500g for 5 minutes at 4ºC and lysed in 1ml of lysis buffer (10mM Tris-HCl pH. 7.5; 5mM MgCl2; 100mM KCl; 1% Triton X-100; 2mM DTT; 100 ug/ml Cycloheximide and 1X Protease-Inhibitor Cocktail EDTA-free (Roche). After incubation at 4ºC for 10 minutes, lysate was cleared at 1300.g for 10 minutes at 4ºC, the supernatant recovered and absorbance at 260nm measured. 10 A260 units complemented or not with 35 mM of EDTA were loaded on top of a 10 to 50% (Weight/Volume)

sucrose gradient (20mM HEPES-KOH pH. 7.4; 5mM MgCl2; 100mM KCl; 2mM DTT; 100 µg.ml^{-1} of Cycloheximide) and centrifuged in a SW-40ti rotor at 35,000 rpm for 2h40min at 4ºC. After centrifugation, samples were collected from the top of the gradient into 15 fractions while absorbance at 254nm was measured. Collected fractions were complemented with 0.4 femto moles of a firefly coding RNA (Spike-In), SDS (1% final), proteinase K (200 ug/ml) and incubated at 42ºC for 45 minutes. After proteinase K treatment, RNA was extracted using one volume of Phenol (pH 4.5):Chloroform:Iso-amyl alcohol (25:24:1). The recovered aqueous phase was supplemented with 20 µg of glycogen, 300 mM sodium acetate pH 5.2, and 10 mM $MgCl_2$. RNA was precipitated with 3 volumes of 100% ethanol at −20°C overnight. After a wash with 70% ethanol, RNA was re-suspended in 50 µl of water (Carpenter et al., 2013).

Generation of wildtype and AS-IL1α knockdown shRNA-mediated macrophage cell lines. shRNA sequences were designed as described (Hornung et al., 2008) in Figure 2.$$ and ordered from IDT through UMass Molecular Biology Core Labs. Forward and reverse strands were denatured and annealed by mixing 1:1 of each, diluting with nuclease-free water, and placing on a 95*C heatblock. After 10 minutes, heatblock was turned off to allow slow cooling to room temperature to allow the oligonucleotides to anneal. These oligonucleotides were ligated into a pLKO.1 TRC cloning vector (Moffat et al., 2006). (Sigmal-Aldrich) (see **Table 2.1** for sequences). Generation of the plasmid was determined by running a 1%

agarose gel and the 7 kb fragment was extracted using Qiagen Gel Extraction kit. Competent e.coli DH5α cells with plasmid DNA as follows: 50 ul competent cells were incubated on ice with 1 ug plasmid DNA for 1 hour, followed by heat shocking of cells for 2 minutes at 42*C. Cells were cooled on ice for 1 minute before being transferred to a 1 mL warm luria broth without antibiotic selection. Cells were allowed to recover for 2 hours at 37*C. Transformed cells were plated onto L-agar containing ampicillin and left for 16 hours at 37*C. This was performed on 4 shRNAs and 10 colonies were chosen from each plate. The selected single colonies were selected and grown overnight at 37*C in ampicillin-containing Luria broth. Isolation of plasmid DNA from *e. coli* DH5a was performed using 1 ml of the broth and run on a Qiagen miniprep column according to manufacturer's instructions (Qiagen). Sequencing of plasmid DNA was performed by GeneWiz for positive incorporation of shRNA oligonucleotides into pLKO.1 vector. Lentiviral particles were produced in 2x106 HEK 293T cells transfected with 4 ug shRNA, along with 1 ug pMD2 and 3 ug pSPAX using GeneJuice (Novagen) for 48 hours. Viral supernatant was collected and passed through a 0.45 um filter and added to immortalized BMDMs. Puromycin (3 ug/ml) was added to the culture media for selection of shRNA-incorporated cells. Knockdown efficiency was assessed by one-step qRT-PCR (BioRad) with the primers listed in **Table 2.1**.

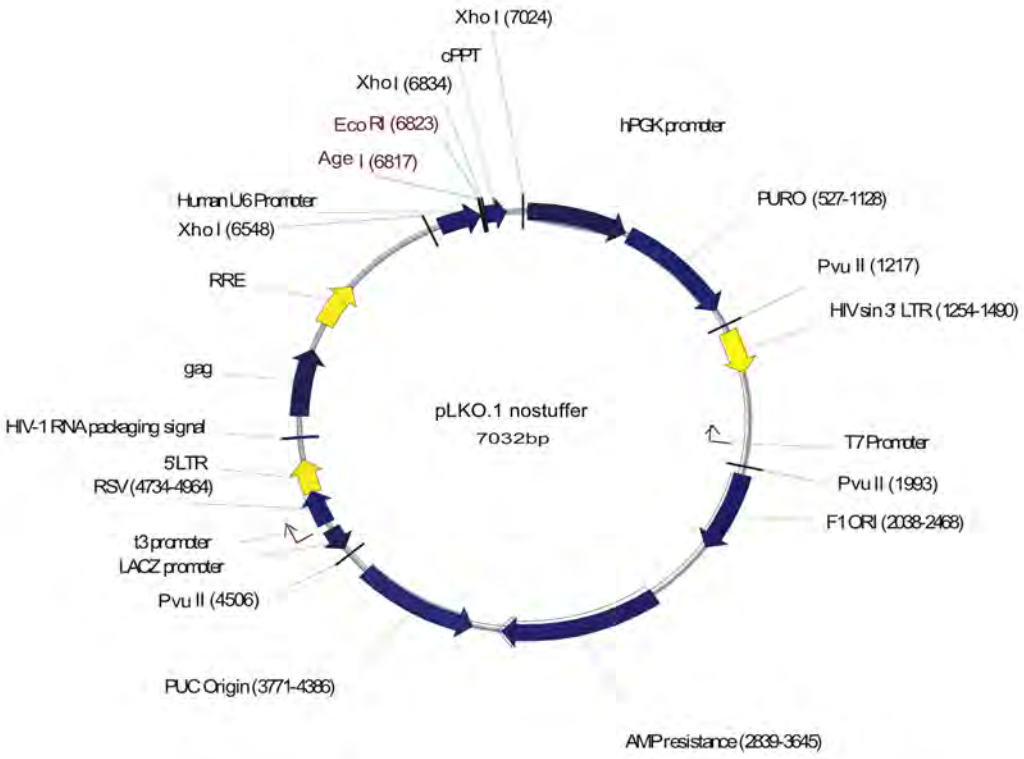


Figure 2.1. Lentiviral vector pLKO.1 map. Vector map of the lentiviral construct used to insert shRNAs targeting GFP (control) or AS-IL1α (3 lines) and transduced into immortalized macrophages. shRNA double-stranded oligonucleotides were cloned into the vector using Age I and Eco RI restriction sites. Ampicillin was used to positively select for the E.coli DH5α transformed plasmids. Puromycin was added to the culture media to positively select for transduced cells. *Vector map was downloaded from **www.addgene.com**

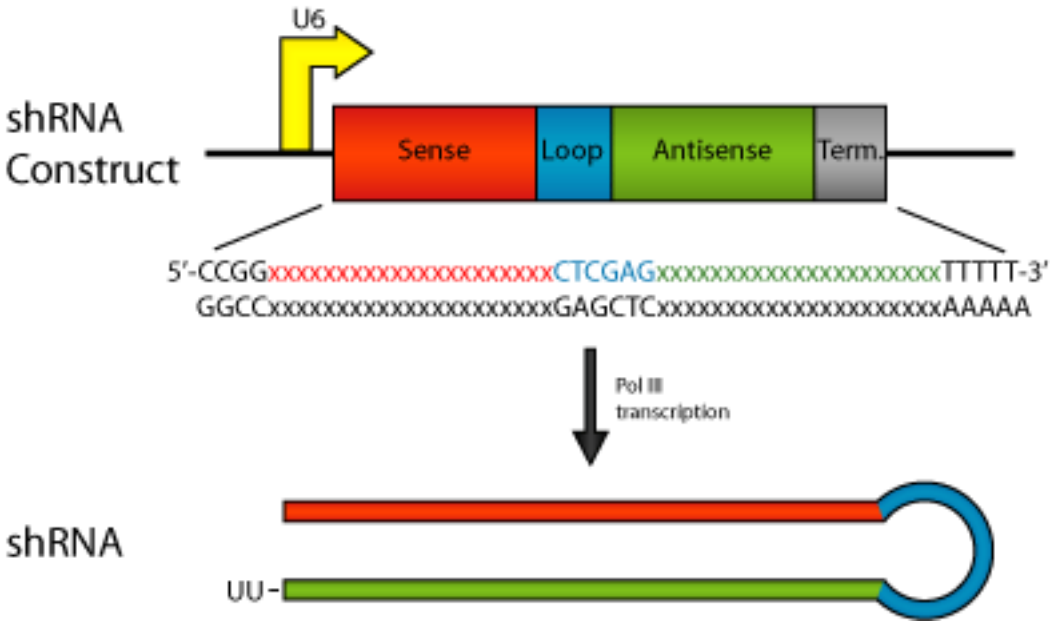


Figure 2.2. Design of shRNA sequences to insert into pLKO.1 cloning vector. A non-targeting sequence against GFP (control line) and three discrete sequences specifically targeting AS-IL1a were generated using the above formula. The two oligonucleotides were purchased from IDT through UMass Molecular Core Facility and annealed in lab before cloning into the pLKO.1 vector.

Western blot. Immortalized macrophages (imacs) were plated at a density of 1x106 cells/ml in 1 mI in a 12-well plate. They were stimulated with PBS or 100 ng of LPS/ml for 6 hours. Cells were washed with 1X PBS, scraped and centrifuged at 400g, 5 minutes 4*C. PBS was removed and lysis buffer containing the following recipe was used to resuspend the cells at 100 ul for 10 minutes on ice: Lysed cells were then centrifuged at 14000g, 10 minutes 4*C and supernatant containing protein was quantified by Bradford assay. They were then diluted to equal protein concentrations, mixed with 4x sample buffer, boiled for 5 minutes and loaded onto a 5% stacking gel and 10% resolving SDS-PAGE gel. Immunoblotting was performed using mouse IL-1α/IL-1F1 antibody (R&D Systems AB-400NA) and anti-goat IgG) (BioRad 172-1011).

Nanostring Analysis. Cells were treated with either PBS or LPS (100 ng/ml) for 6 hours and purified using an RNeasy Mini Kit (Qiagen). Total RNA was hybridized to nCounter CodeSets, which were constructed for detecting selected mouse-specific genes and levels of RNA measured using the nCounter Digital Analyzer as described (Carpenter et al., 2013; Dixit et al., 2010). To account for technical variation in lane hybridization, samples were first normalized to positive and negative internal controls provided by Nanostring that are independent of our samples. We determined the normalization factor be generating the geometric mean and normalized to reference housekeeping genes to account for sample variability. The final counts serve as the number of total counts of each gene.

Values are displayed as a heatmap generated by log2-transformation and row clustering using http://anto.umassmed.edu/~adam/heatmap/enterInformation.php

Cell fractionation. Cytosolic and nuclear fractions were prepared as described (Smale et al., 2012). The cell lysis buffer contained 0.15% NP-40, and the sucrose cushion did not contain detergent. Cells treated with PBS or LPS (100 ng/ml) for 6 hours were lysed with 0.15% NP-40. After fractionation, cytoplasmic and nucleoplasmic RNA was purified using Qiagen RNAse columns. GAPDH and 7SK were used for cytoplasmic and nuclear controls respectively. Primers are listed in the **Table 2.1**.

RNA stability experiments. α-amanitin (50 ug/ml) (Sigma-Aldrich A2263) or actinomycin D (5 ug/ml) (Sigma-Aldrich A1410) was treated onto WT or shRNA-AS-IL1α cells for 6, 12, or 24 hours after a 6 hour LPS stimulation (100 ng/ml). RNA was measured the same way. RNA was harvested and cDNA was made prior to qRT-PCR. IL-1α mRNA and 18S rRNA degradation was determined using the primers listed in **Table 2.1**.

Chromatin Immunoprecipitation (ChIP). Cells were stimulated with LPS for 6 hours, fixed in 1% formaldehyde, lysed, and sonicated to shear the chromatin. A sample of the sheared chromatin was removed from the larger sample and subjected to deproteination, quantified via Nanodrop, and run on a 2% agarose

gel to determine the size of the sheared chromatin. 5 ug of chromatin was pre-cleared with Dynabeads Protein G (10009D) from Novex/Life Technologies and subjected to immunoprecipitation with antibodies specific to RNAPII (Active Motif 102660), H3K9-acetylation (Abcam AB4441), or IgG isotype control (Abcam AB37415) overnight. Immunoprecipitated fragments were washed and deproteinated to obtain purified DNA that was used for PCR amplification at the IL-1α, IP-10, or gapdh promoter that are shown in **Table 2.1**.

Mice: $Ptpn^{spin}$ mutant or cage-matched wildtype or heterozygous control mice footpads or femurs were a gift from Thirumala-Devi Kanneganti, PhD (St. Jude) aged 4-6 weeks. Footpads were extracted for RNA, reverse transcribed and performed qRT-PCR for AS-IL1α, IL-1α and GAPDH. Bone marrow was extracted from femurs and differentiated into BMDMs as described above before plating and stimulating with LPS (100 ng/ml) for 6 hours, harvesting RNA, making cDNA, and qRT-PCR on the same genes.

Results

Identification of AS-IL1α, a natural antisense non-coding RNA in the IL-1α locus.

To assess lncRNA expression during an active infection, we infected C57Bl/6 mice with *Listeria monocytogenes (Lm)* and performed RNA-

sequencing. We chose *Lm* since it activates multiple innate pathways and drives a robust acute inflammatory response both *in vivo* and *in vitro*. Spleens from infected and non-infected mice were harvested 24 hours after infection and total RNA was prepared for RNA-sequencing. Consistent with previous reports (Leber et al., 2008), *Listeria monocytogenes* induced expression of a wide range of protein-coding immune genes (**Figure 2.3a, inner track**). We also detected many lncRNAs that were differentially expressed following *Listeria* infection (**Figure 2.3a, outer track**).

Strikingly, one of these lncRNAs (hereafter referred to as AS-IL1α) was induced 14-fold and was encoded by the opposite strand of the IL-1α locus on chromosome 2. Similar to what we had seen *in vivo* and in our RNA-seq data, AS-IL1α levels also increased in macrophages infected with *Listeria monocytogenes (*MOI 5 and 10) (**Figure 2.3b**).

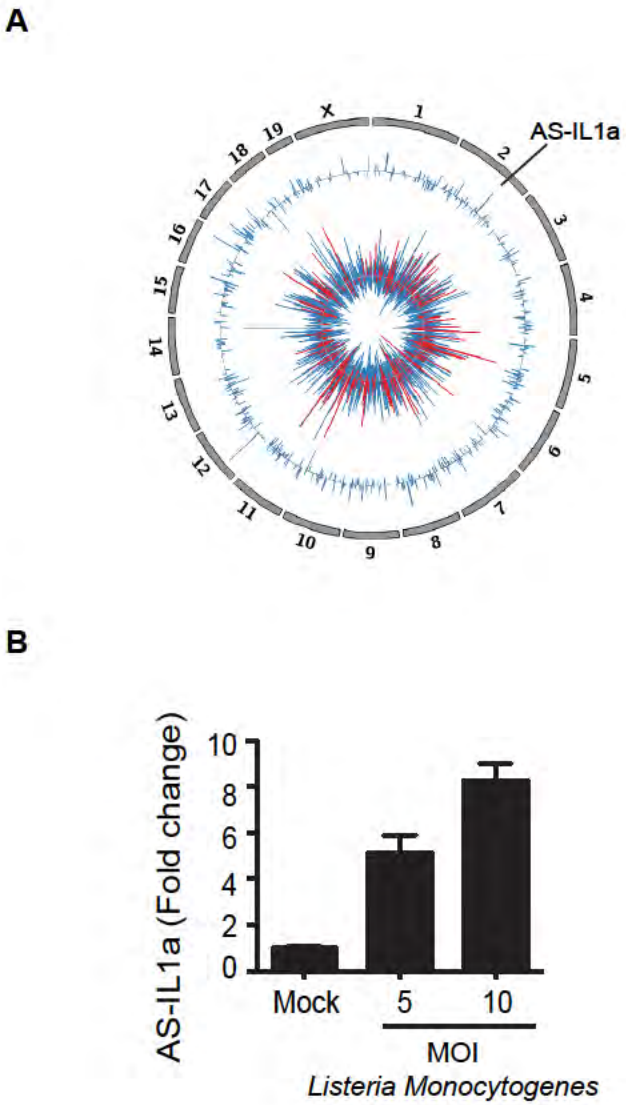


Figure 2.3. Identification of AS-IL1a.

(A) Circos plot showing differentially expressed genes (RNA-seq) in splenocytes harvested from *L. Monocytogenes* infected C57Bl/6 mice. Chromosomes are indicated on the outer tracks. The middle track shows log2 fold-change values for all lncRNAs and AS-IL1α (colored red). The inner track shows log2 fold-change values for protein-coding genes, with immune genes colored in red. (B) Macrophages infected with *L. Monocytogenes* for 3 hours at MOI 5 and MOI 10.

We used PCR to confirm the orientation and gene structure of AS-IL1α. We amplified the AS-IL1α region identified by our qRT-PCR primers, and then designed new primers to "walk" further into the gene in order to identify the full-length mature transcript in murine macrophages. The schematic drawn in **Figure 2.4** is the maximally amplified region as we have achieved today. It differs from the annotated sequence on ENSEMBL 9.0 in that we've never been able to amplify the annotated exon 1. To the best of our sequencing data, we modeled the sequence architecture as shown in **Figure 2.4**. For the sequences mapped, see **Appendix I**.

In order to function as a lncRNA, we must demonstrate that this gene does not encode for a protein (Mattick and Rinn, 2015). To assess the protein-coding potential of AS-IL1α, we performed polysome profiling which maps the portion of the transcript that actually get translated (Ingolia et al., 2012). Macrophages were treated with cycloheximide, which traps ribosomes along their RNA strands and these RNA were detected in the heavier fractions of a sucrose density gradient. To compare, cells were also treated with EDTA post-cycloheximide treatment, which disrupts all RNA-protein interactions and these RNA were detected in the lighter fractions of a gradient. A third set of cells were pre-treated with harringtonine prior to cycloheximide, which inhibits translation and polysome formation by causing ribosomes to accumulate at their initiation sites. Thus, pre-treatment with harringtonine causes these RNA species to shift to lighter fractions in a sucrose gradient. As expected, harringtonine prevented

polysome formation in both GAPDH and *IL-1*α mRNA (**Figure 2.5)**, and both mRNAs shifted to lighter fractions in the sucrose gradient. In contrast, AS-IL1α was largely unaffected by harringtonine treatment, indicating that it is unlikely to associate with ribosomes. Of note, however, is the heavy sedimentation of AS-IL1α in cycloheximide alone and cycloheximide and harringtonine treatment samples. These high sedimentation fractions are disruptable by EDTA. Most likely, there are large protein complexes co-aggregating with this transcript. Collectively, these studies indicate that AS-IL1α is unlikely to encode a protein product.

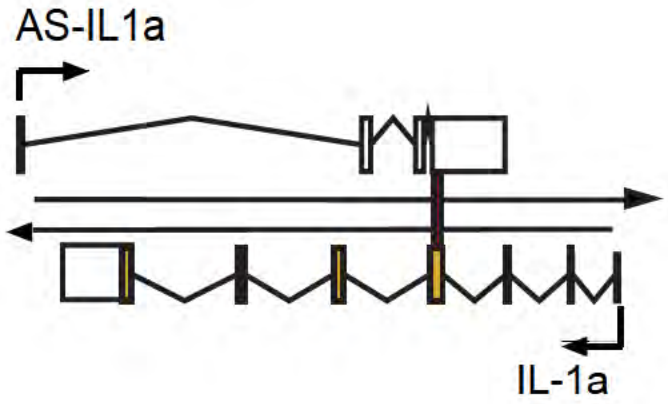


Figure 2.4. AS-IL1α schematic in murine macrophages.

AS-IL1α chromosomal localization runs antiparallel as an antisense transcript and overlaps with IL-1α protein coding gene on murine chromosome 2 in regions indicated in red. Translated exons are in yellow boxes. Non-translated regions are in white boxes

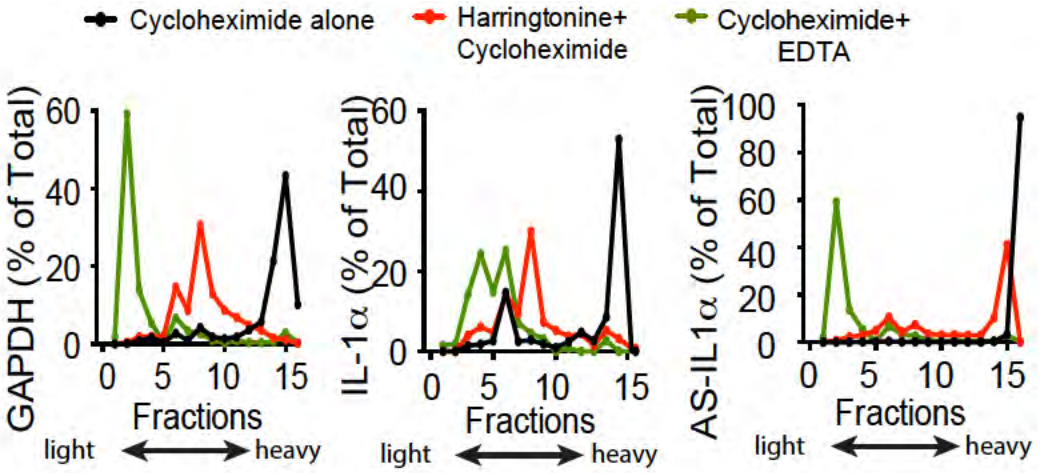


Figure 2.5. Polysome profiling of GAPDH, IL-1α and AS-IL1α transcripts.

Immortalized macrophages were stimulated with LPS and either treated with cycloheximide alone, harringtonine prior to cycloheximide treatment, or cycloheximide with EDTA to determine translational potential of AS-IL1α.

AS-IL1α is inducible in macrophages exposed to TLR ligands via NF-κB.

In order to further characterize AS-IL1α, we examined its expression in bone marrow derived macrophages (BMDMs) stimulated with ligands of TLRs and other innate receptors. We found that AS-IL1α was up-regulated after stimulation with LPS (TLR4), Pam_3CSK_4 (TLR1/2) and PolyI:C (TLR3) (**Figure 2.6a**). In contrast, Interferon stimulatory DNA (ISD), which activates the intracellular cGAS DNA sensing pathway, failed to upregulate AS-IL1α. We next wanted to understand the signaling pathway responsible for the inducible expression of AS-IL1α. We focused on LPS signaling and compared inducible levels of AS-IL1α in WT and MyD88-/-/TRIF-/- DKO cells. In the absence of MyD88/TRIF, LPS failed to upregulate AS-IL1α (**Figure 2.6b**). We also treated macrophages with Bay11-7085, an inhibitor of NF-κB, which prevented the LPS inducible expression of AS-IL1α (**Figure 2.6c**). Collectively, these data indicate that AS-IL1α is induced by multiple TLRs via MyD88/TRIF signaling and NF-κB.

Knocking down AS-IL1α inhibits inducible IL-1α expression

To determine whether AS-IL1α contributes to inflammatory gene expression in macrophages, we next generated macrophage cell lines in which AS-IL1α expression was specifically silenced by shRNA. We made three independent shRNAs that targeted AS-IL1α exons that did not overlap with those of IL-1α. Using the cloning vector, pLKO.1 (**Figure 2.1**), we transduced 293T

cells with three different shRNAs and a control non-targeting green fluorescent protein (GFP) shRNA (**Figure 2.2**). After 48 hours, the supernatants containing lentivrirus were added onto wildtype-immortalized macrophages. We confirmed that AS-IL1α was significantly silenced in LPS-stimulated cell lines that expressed these shRNA (**Figure 2.7a**). In shRNA-GFP lines, LPS strongly induced IL-1α mRNA levels whereas LPS only induced abrogated levels of IL-1α mRNA in cells expressing shRNA-AS-IL1α (**Figure 2.7b**). Consistent with the low levels of mRNA, LPS failed to induce the high levels of IL-1α protein normally detected by immunoblotting (**Figure 2.7c**). These results indicate that AS-IL1α is required for the inducible expression of IL-1α and that this NAT regulates its protein-coding partner in a positive manner.

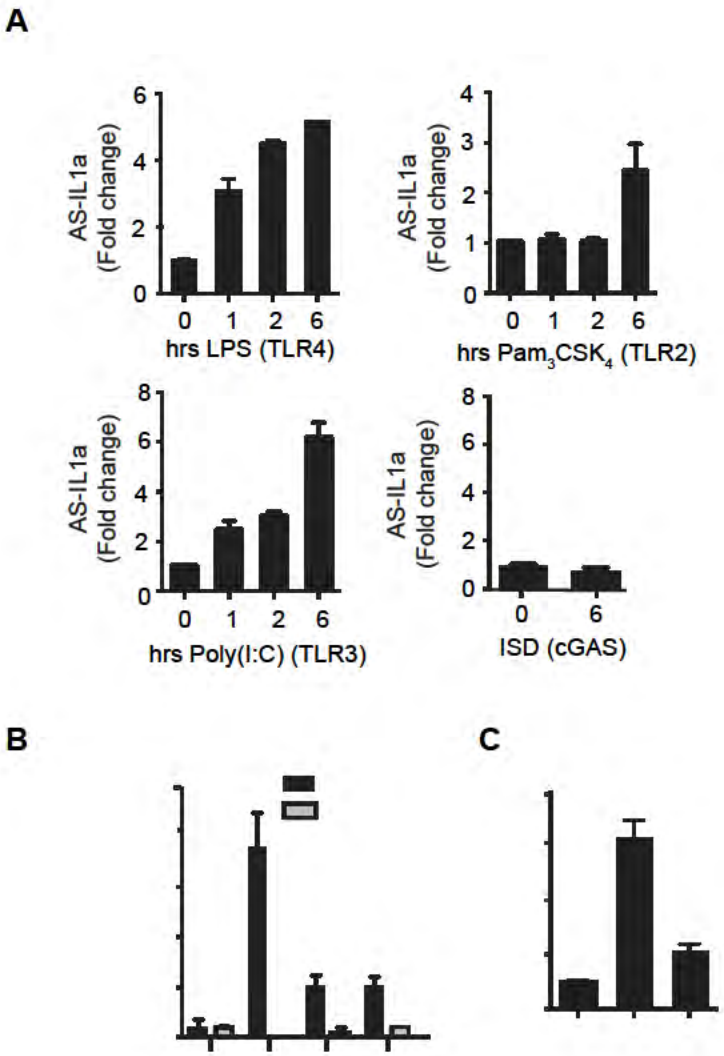


Figure 2.6. Inducibility of AS-IL1α by TLR ligands.

(A) LPS, Pam3CSK4, Poly(I:C) or ISD were stimulated on immortalized macrophages for 0, 1, 2, or 6 hours and AS-IL1α expression was measured by qRT-PCR. (B) WT and MyD88/Trif double knockout macrophages were not-treated (NT), LPS, Pam_3CSK_4 or Poly(I:C) treated for 6 hours. AS-IL1a expression was measured by qRT-PCR. (C) An NF-κB inhibitor, Bay11-7082, was treated on cells 1 hour prior to LPS stimulation (6 hours), and AS-IL1a expression was measured by qRT-PCR.

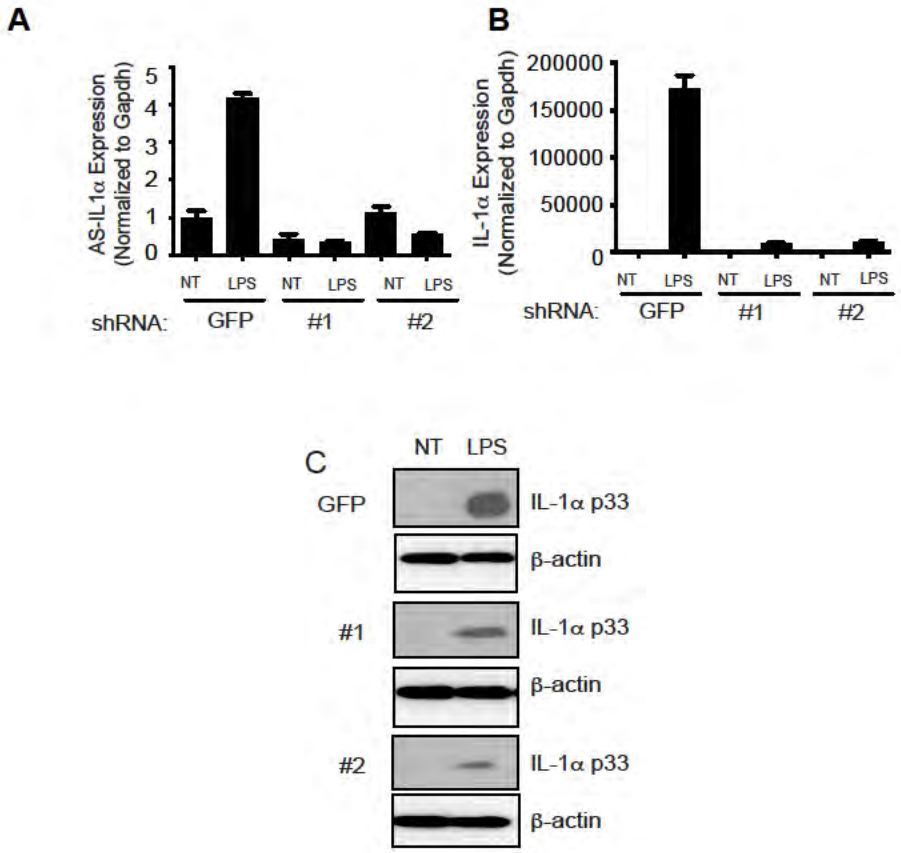


Figure 2.7. AS-IL1α regulates IL-1α expression.

(**A**) shRNA 1 and shRNA 2 target AS-IL1α in regions that do not overlap with IL-1α. (**B**) AS-IL1α RNA levels (qRT-PCR) were reduced in both shRNA 1 and shRNA 2 cell lines. shRNA GFP is a control that targets the GFP gene. (**C**) IL-1α mRNA levels (qRT-PCR) were reduced in shRNA 1 and shRNA 2 cell lines. (**D**) IL-1α p33 protein expression (Western blot) was decreased in shRNA 1 and shRNA 2 cell lines stimulated with LPS. b-actin was used as a loading control.

We next used a non-enzymatic RNA profiling technology that employs bar-coded fluorescent probes to simultaneously analyze mRNA expression levels of differentially regulated genes following LPS stimulation in control and AS-IL1α silenced macrophages (6 hours) (nCounter, Nanostring). Consistent with the qRT-PCR analysis, IL-1α was the most significantly affected gene in the shRNA lines (**Figure 2.8** and **Table 2.2** for adjusted values). We also found that IL-1β levels were decreased in the shRNA cell lines, albeit to a lesser extent. A heat map depicting immune gene expression in control shRNA-GFP and AS-IL1α shRNA cells is shown. Whether or not AS-IL1α regulates just the cis-gene IL-1α, or somehow regulating the entire IL-1 locus needs to be further determined. Additionally, whether other genes affected on the Nanostring are secondary effects of abrogated IL1α-IL1 receptor signaling is unclear.

In order to determine whether IL-1α also induces the transcription of AS-IL1α, we measured expression of AS-IL1α in macrophages from mice deficient in IL-1α. In these cells, the inducible expression of IL-1α was absent as expected. LPS treatment however, led to increased levels of AS-IL1α. These observations indicate that transcription of AS-IL1α is regulated independently of IL-1α (**Figure 2.9**).

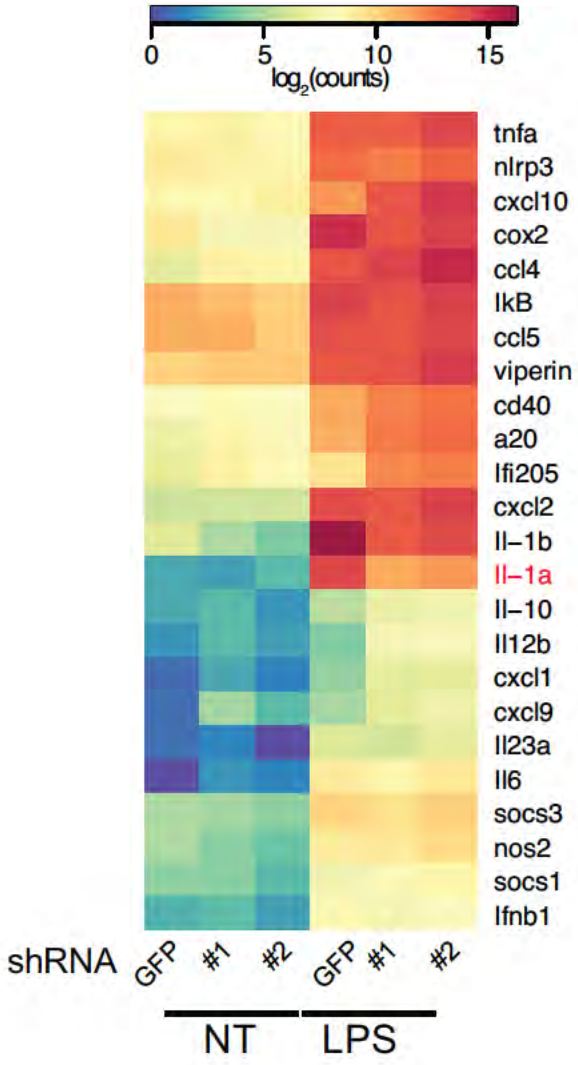


Figure 2.8. AS-IL1α targets IL-1α expression.

Mouse macrophages knocked down for GFP (control) or two independent shRNA lines targeting AS-IL1α (#1 and #1) were treated with PBS/not-treated (NT) or LPS (100 ng/ml) for 6 hours. RNA was extracted and subjected to nCounter Nanostring analysis. Gene expression profiles are displayed as a heat map (log2 transformed) with hierarchical clustering.

Table 2.2. Nanostring Adjusted mRNA counts

Treatment	NT			LPS		
shRNA:	GFP	AS-IL1α-#1	AS-IL1α-#2	GFP	AS-IL1α-#1	AS-IL1α-#2
Genes						
Il6	1	4	3	613	417	648
Il21	1	3	2	5	5	10
tlr11	1	6	5	9	12	15
nlrp12	1	1	7	3	6	12
tlr5	1	4	2	4	8	9
cxcl9	2	26	8	28	90	157
Il23a	2	3	1	78	63	103
cxcl1	2	6	3	20	107	98
Ifna4	2	6	4	9	13	15
arg2	3	15	10	16	26	38
prdm1	3	17	14	14	60	85
Ifng	3	7	2	14	4	7
Il13	4	4	3	12	9	22
Il12b	4	8	5	15	202	225
arg1	4	4	3	21	10	8
Il-4	5	7	7	13	8	15
tgfb2	5	4	8	20	17	21
Il-10	6	8	4	40	131	179
IL-1α	6	5	8	20635	2789	3892
mmp2	6	6	7	19	17	26
Il12a	7	6	4	9	5	5
nlrp6	7	8	10	30	11	12
Ifnb1	7	9	5	361	188	288
nlrc4	17	138	101	20	116	168
socs1	19	17	8	182	343	451
Il-33	20	3	4	132	6	10
socs3	30	26	19	1110	820	1200
nos2	32	18	11	600	656	933
ccr1	33	36	33	46	12	18
ccr2	35	8	8	66	6	10
cd86	38	306	247	93	141	292
IrakM	41	111	90	180	445	812
cxcl2	58	60	60	16652	13182	22428
tlr9	59	282	197	77	49	82

rybp	69	350	220	142	189	281
Il15	71	144	98	403	733	830
tlr1	72	69	35	364	42	62
IL-1β	83	30	14	77402	12299	16876
Ifi205	94	511	342	724	4958	5900
ccl4	98	484	421	13466	20942	36025
tlr3	107	263	260	189	217	407
cd18	116	214	142	151	97	141
eya4	117	621	370	47	89	135
a20	133	425	310	2434	6056	8580
yaf2	141	239	194	255	101	196
Irf1	164	414	310	916	2263	3353
cd40	244	350	280	2843	5688	7324
cd80	249	103	74	3030	314	508
tlr8	249	890	626	185	260	360
nlrc5	294	339	255	540	336	537
pstpip1	336	455	330	366	112	226
md2	379	603	442	484	208	354
tlr4	384	996	721	217	132	183
cxcl10	398	348	528	3888	12952	26125
tnfa	448	493	342	12162	11604	20561
stat3	471	1412	1146	1172	1082	1662
nfkb2	484	588	435	4369	2854	4126
duba	533	1011	638	964	692	1145
Il-18	580	775	533	1205	442	737
myd88	595	1208	931	1258	1228	2066
nlrp3	605	494	413	8239	6198	9918
Irf2	616	595	429	694	299	540
cox2	667	212	193	31295	14225	22562
oas2	675	811	619	1185	567	882
aim2	710	758	593	1523	418	798
zbp1	748	15	12	1524	5	22
Icam1	838	1867	1436	4634	6859	9755
dusp6	854	1083	809	2277	421	802
tlr2	936	582	478	2793	1867	2853
tlr7	952	840	668	991	545	870
nfkb1	988	942	741	7469	2730	4159
casp1	1010	3931	2639	1668	2516	3807
mnda	1066	4591	3242	2044	4274	6555

trex1	1071	781	692	3022	2660	4320

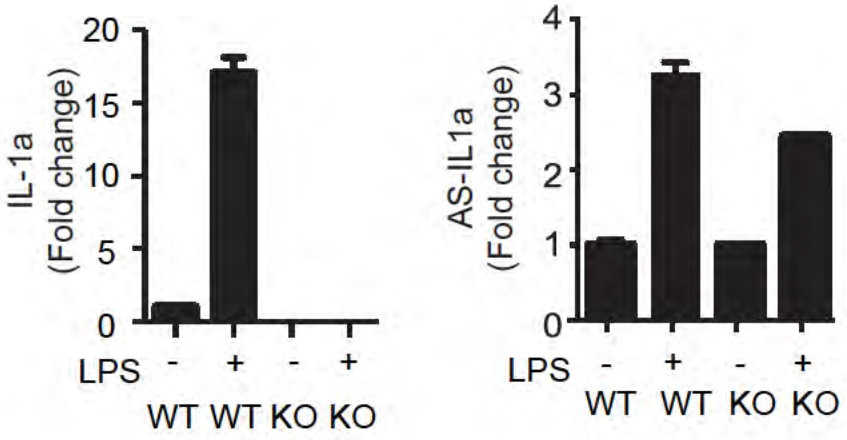


Figure 2.9. IL-1α and AS-IL1α are independently regulated.

Wildtype and IL-1α knockout macrophages were stimulated with LPS for 6 hours. AS-IL1α expression was induced even in the absence of IL-1α, indicating that AS-IL1α regulates IL-1α expression, but doesn't act reciprocally

Although AS-IL1α was predominantly located in the nucleus, we wanted to rule out the possibility that AS-IL1α controlled the stability of IL-1α. For example, AS-IL1α could function similarly to Bace1-AS, which stabilizes its cognate gene by binding to its mRNA and preventing its degradation (Faghihi et al., 2008). In order to determine if AS-IL1α changes the stability of IL-1α mRNA, we compared IL-1α mRNA levels in control shRNA-GFP expressing macrophages and the shRNA-AS-IL1α line before and after treatment with α-amanitin, a mushroom toxin, which binds to RNA polymerase II (RNAPII) and prevents new transcription. We stimulated the shRNA-GFP macrophages as well as AS-IL1α knockdown macrophages with LPS for 6 hours and then treated cells with α-amanitin at the time points indicated before harvesting the RNA (**Figure 2.10a**). We did not detect any change in the degradation rate of IL-1α mRNA in the presence or absence of AS-IL1α. We confirmed these results in two additional shRNA lines and using actinomycin D, another inhibitor that blocks transcription by intercalating into DNA (**Figure 2.10b**). As a control, we also measured levels of 18S ribosomal RNA, an RNA polymerase I transcript that is not affected by α-amanitin treatment. These results indicate that AS-IL1α does not alter the stability of IL-1α mRNA.

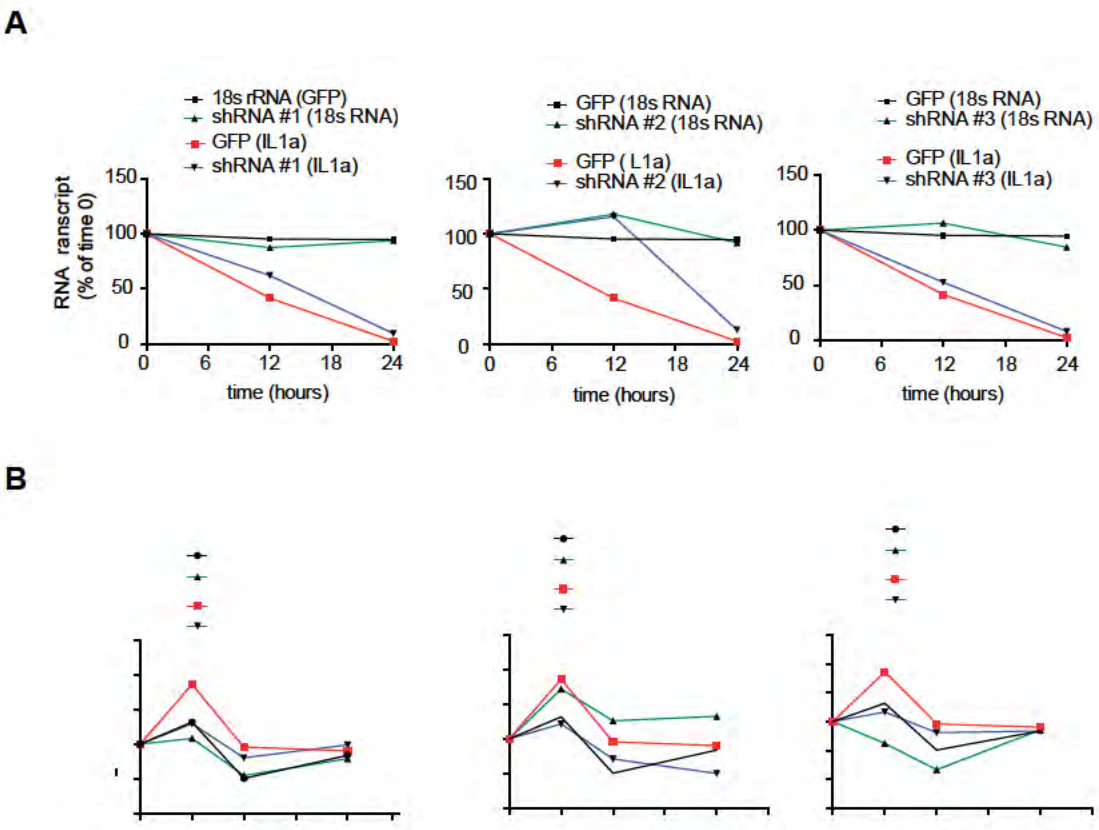


Figure 2.10. AS-IL1α does not alter RNA stability of IL-1α.

(A) a-amanitin, an RNAPII transcription inhibitor, was added to imacs in shRNA-GFP or shRNA-AS-IL-1α (cell lines #1,2, or 3) after 6 hours LPS stimulation. RNA was harvested at 12 and 24 hours after α-amanitin addition. qRT-PCR was used to measure transcript levels of 18s rRNA (an RNAPI transcript and unaffected by α-amanitin) or IL-1α to determine RNA degradation. Everything is graphed as a percentage over time zero (when α-amanitin was added). (B) Actinomycin-d, which intercalates DNA and prevents *de novo* transcription, was added to the same cell lines as in (A) after 6 hours LPS stimulation. RNA was harvested at 1 and 2 hours after actinomycin-d addition.

AS-IL1α is localized to the nucleus and enhances IL-1α expression at the transcriptional level

Long non-coding RNAs can be found in the nucleus, cytoplasm or in both compartments (Cheng et al., 2005; Louro et al., 2009; Wu et al., 2008). To gain a better understanding of how AS-IL1α might regulate IL-1α gene expression, we first examined its localization in macrophages by performing subcellular fractionation of nuclear and cytosolic compartments and analyzing RNA levels after LPS stimulation by qRT-PCR. We also measured levels of GAPDH, IL-1α as well as the nuclear RNA, 7SK. As expected, the mature IL-1α and GAPDH transcripts were localized to the cytosol, while 7SK RNA was confined to the nucleus. The induced levels of AS-IL1α were primarily nuclear (**Figure 2.11**).

Since AS-IL1α was localized to the nucleus, we hypothesized that AS-IL1α regulated the transcription of IL-1α. We designed qRT-PCR primers that spanned exon-exon boundaries to measure mature IL-1α transcripts, and compared those expression levels using primers that measured intron-containing sequences of the IL-1α pre-mRNA (see **Table 2.1** and **Appendix I** for sequences and amplicon regions). This analysis revealed that the pre-mRNA levels of IL-1α were also decreased in the three AS-IL1α shRNA lines (**Figure 2.12a**). To confirm the specificity of our assay, we made reverse transcription primers that targeted AS-IL1α only and compared it to our normal method of reverse transcription using poly-d(T) and random hexamer primers. The trends were

comparable indicating the abrogated AS-IL1α expression levels were specific to AS-IL1α and not promiscuous priming (**Figure 2.12b**).

We next performed chromatin immunoprecipitation (ChIP-PCR) assays to measure RNAPII occupancy at the region surrounding the transcription start site (TSS) of the IL-1α locus (+22bp). In order to shear the chromatin to the appropriate size after lysing and fixing the cells with formaldehyde, we took a small aliquot of the sonicated chromatin samples, and ran it on an agarose gel. Fragment sizes greater than 500 base pairs would indicate an incomplete fragmentation. Once we determined our samples were sheared properly, we were able to immunoprecipitate the samples with either RNA polymerase II (RNAPII) or Histone 3 Lysine 9 (H3K9)-acetylation (Ac), which is an indicator of active transcription. As expected, LPS treatment in the shRNA-GFP control cells resulted in robust RNAPII binding at this region. This was greatly reduced in macrophages expressing AS-IL1α shRNA (**Figure 2.13a**). We observed similar results when we measured the epigenetic mark H3K9-Ac, an indicator of active transcription at this same locus **(Figure 2.13b**). The effect of AS-IL1α shRNA was specific to the IL-1α locus, as recruitment of RNAPII to GAPDH or the IP-10 locus was unaffected in the knockdown line. These results indicate that AS-IL1α is required to facilitate RNA polymerase II dependent recruitment to the IL-1α locus to allow transcription to proceed in LPS-stimulated cells.

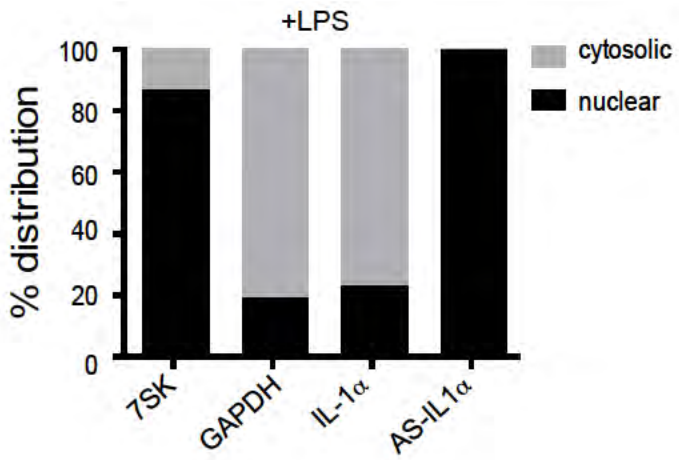


Figure 2.11. AS-IL1α is localized to the nucleus. (A) Localization of AS-IL1α in LPS-induced iBMDMs. Cytosolic and nuclear fractions were separated via a sucrose gradient and qRT-PCR was performed on fractionated RNA. Gapdh (cytosolic), IL-1α (cytosolic) and 7SK (nuclear) were also measured as controls.

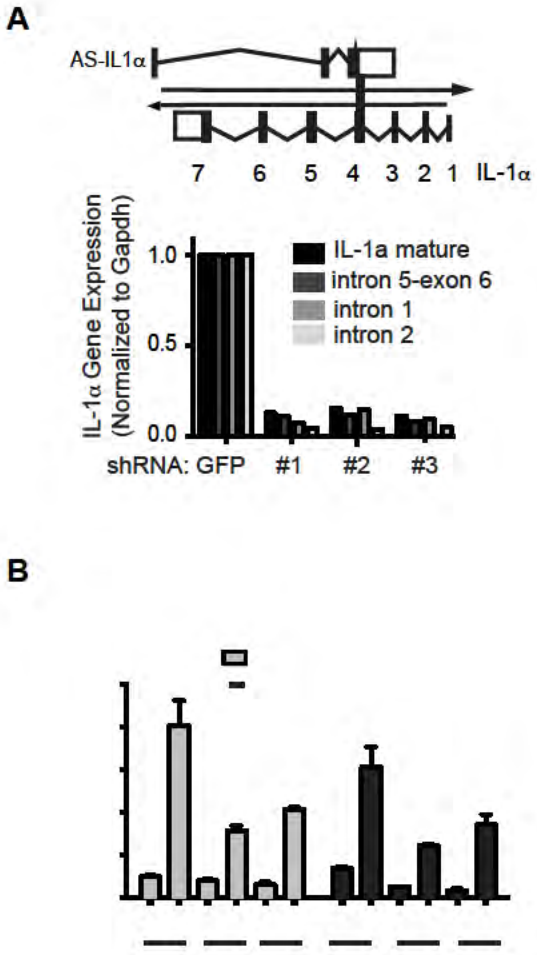


Figure 2.12. IL-1a pre-mRNA transcript is attenuated in AS-IL1a knockdown cells. (A) qRT-PCR was performed on shRNA-GFP and the three AS-IL1a KD cell lines. Primers made against the IL-1α mature mRNA (exon-exon junction), and three primers that measured IL-1α pre-spliced mRNA. Everything was shown as a percentage of the expression of shRNA-GFP. A schematic of IL-1α depicting the primers targeting regions of IL-1α mRNA and pre-mRNA is shown below. (B) Reverse transcription comparing poly-d(T) and random hexamer priming to an AS-IL1α specific primer to demonstrate specificity of amplified region prior to qRT-PCR.

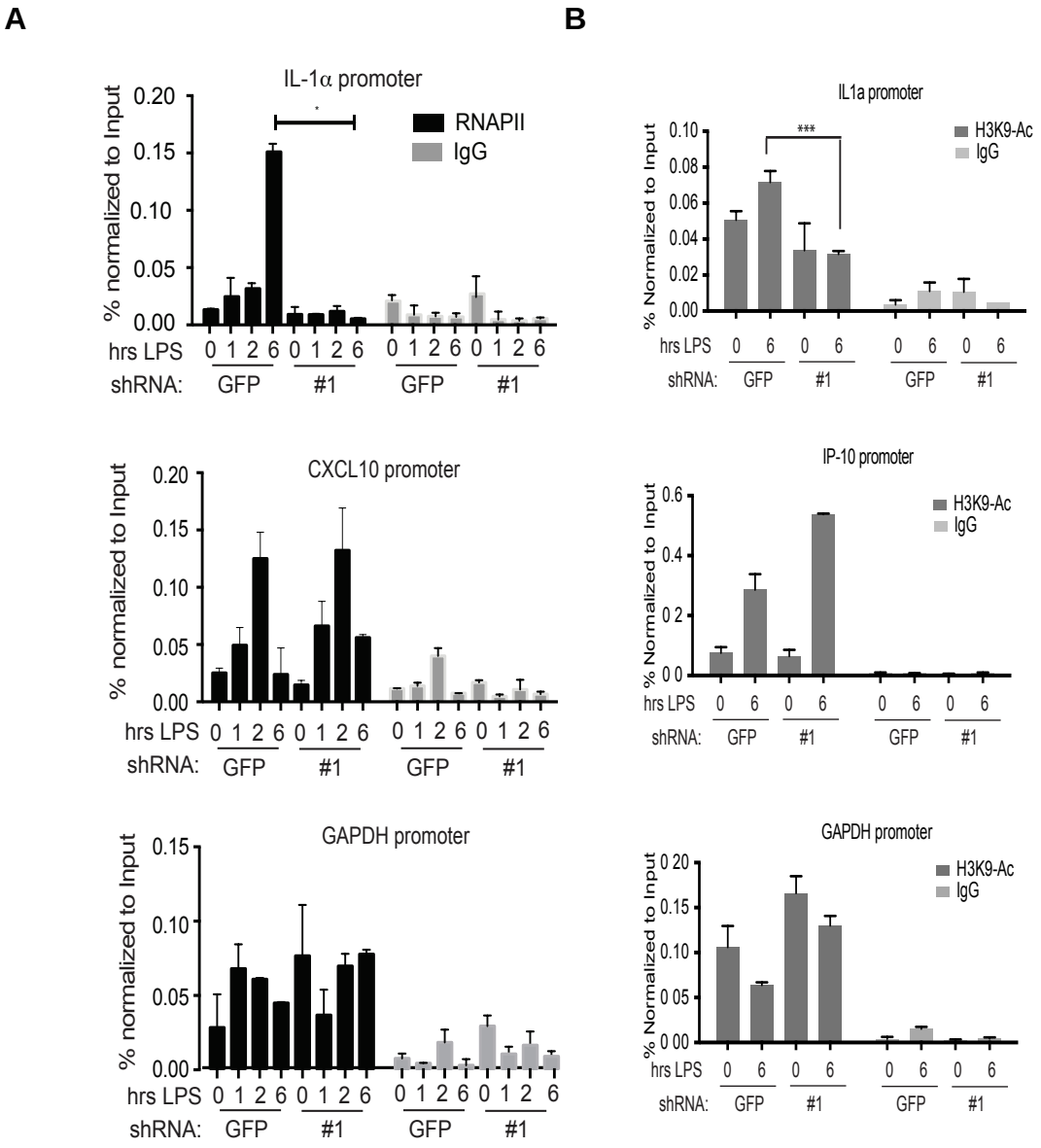


Figure 2.13. Decreased RNAPII recruitment on AS-IL1α KD lines by Chromatin immunoprecipitation (ChIP). (A) Antibodies against RNA polymerase II (RNAPII) or IgG isotype control were used. Regions of the IL-1α gene, near the transcription start site (+22), or downstream of the gene (+227) were measured for RNAPII binding RNAPII recruitment did not decrease at the CXCL10 (IP10) or Gapdh promoter. (B) ChIP of H3K9 Acetylation. ChIP comparing shRNA-GFP cells and shRNA-AS-IL1a was performed. Antibodies against H3K9 acetylation or IgG isotype controls were used. Regions of the IL-1α gene, near the transcription start site (+22) or Gapdh, and CXCL10.

Our observations thus far have been made in cell lines using shRNA technology. Additional means of creating a knockout or knockdown of AS-IL1α would have been beneficial to our study to mitigate the possibility that we were observing shRNA off-target effects. As a true NAT, the majority of this transcript overlaps within the antiparallel transcript of the protein-coding IL-1α. Deletion of AS-IL1α by CRISPR technology or TALENs, which mitigate double-stranded breaks, could very well delete both genes. Instead, we tried to use locked nucleic acid (LNA) technology, which would target only AS-IL1α -containing DNA strand. In order to determine whether or not this technology was feasible to us, we worked with Anastasia Khorkova's group, which frequently uses LNAs to delete a control gene that they frequently knockdown, the Huntington's gene (HTT) in Chinese Hamster Ovary (CHO) cells. We attempted to delete HTT in immortalized mouse macrophages and were unable to abrogate HTT expression more than 20% of the basal expression, therefore, did not consider LNAs as a sufficient means of gene knockdown in our system.

Additionally, because IL-1α has been implicated in various inflammatory diseases and is studied in various mouse models contexts, we sought to determine if AS-IL1α might be correlated in the dysregulation of IL-1α expression itself in these models. Thoureau Kanneganti's lab group utilizes a mouse with a mutation in the protein Src homology region 2 domain-containing phosphatase-1 (SHP1) called protein tysosine phosphatase N-terminal ($PTPN^{spin}$) mice (Lukens et al., 2013) that exhibits a spontaneous arthritis-like inflammatory disease in the

footpads. SHP-1 is highly expressed in hematopoetic cells and a key negative regulator of signaling (Xu et al., 2000). Mutations in this gene lead to hyperproliferation of hematopoetic cells and eventually develop an IL-1α-dependent autoinflammatory phenotype. When these mice were crossed to IL-1α knockout mice, disease was abrogated, indicating the critical activity of IL-1α in mediating disease. We wanted to know whether or not AS-IL1α was also exhibiting an increased expression in these mice. Comparing footpads of $PTPN^{spin}$ mice to littermate controls, we measured IL-1α and AS-IL1α expression. In the mice that exhibited the inflammatory phenotype (swollen footpads), there was an increased expression both IL-1α and AS-IL1α compared to WT, non-inflamed mice (**Figure 2.14a**). Additionally, when we took bone marrow from these mice and differentiated the progenitor cells into BMDMs, and stimulated them with LPS, we were able to induce much more robust levels of IL-1α and AS-IL1α (**Figure 2.14b**) indicating that these cells were much more susceptible to inflammation. Although this data is only correlative, it suggests that *in vivo*, when high levels of AS-IL1α were present, IL-1α expression was also significantly increased.

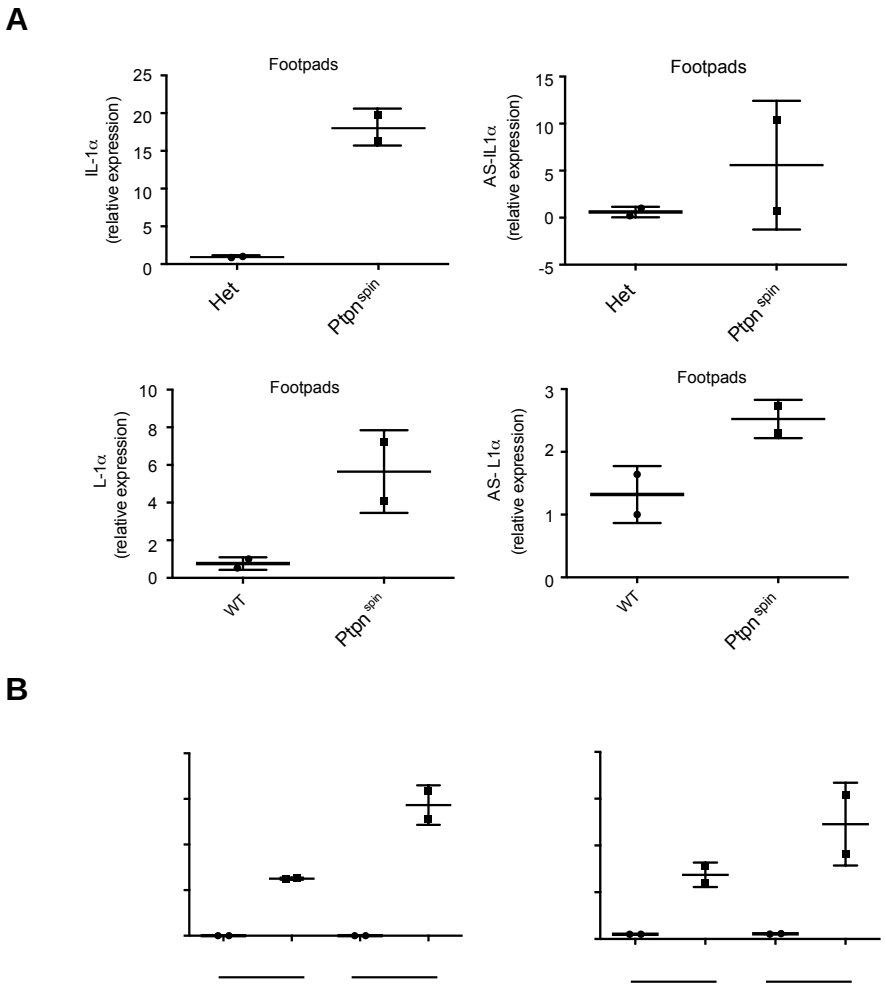


Figure 2.14. PTPN^{spin} mice have elevated IL-1α and AS-IL1α expression. (A) Footpads were harvested from Ptpn^{spin} mice and Heterozygous (Het) or wildtype (WT) littermate controls. IL-1α (left column) or AS-IL1α (right column) were measured by qRT-PCR. First experiment represented (top row) and second experiment is represented (bottom row). Two footpads from two different mice were represented per genotype. (B) BMDMs were harvested from femurs of Ptpn^{spin} mice or Het littermate controls. Each datapoint represents femurs that were differentiated into BMDMs from a different mouse. Cells were plated at 1x10^6 cells/well and stimulated with PBS or LPS (100 ng/ml) for 6 hours before harvesting for RNA and measured for IL-1α or AS-IL1α by qRT-PCR.

Discussion

Impaired transcription and release of IL-1α contributes to many inflammatory and autoimmune conditions, including familial Mediterranean fever (FMF), type 2 diabetes and certain types of cancer (Chen et al., 2007; Dinarello et al., 2012; Rider et al., 2013). These diseases are frequently treated with an IL-1R1 receptor antagonist, Anakinra. A monoclonal anti-IL1α antibody, MABp1, is also currently in Phase III clinical trials for the treatment of type 2 diabetes, advanced cancers, and acne vulgaris with some success reported thus far (MD et al., 2014). Although generally recognized as safe and effective these therapies some limitations, primary due to short half lives of these biologics *in vivo*. A better understanding of the mechanisms regulating IL-1 transcription and function could lead to improved therapeutics for a myriad of inflammatory diseases.

Large-scale sequencing projects have identified thousands of lncRNAs in the genome (Guttman et al., 2009) and lncRNAs are emerging as important regulators of gene expression in diverse biological contexts including immunity. LPS has been shown to induce widespread changes in lncRNA expression in immune cells (Guttman et al., 2009). It has also been shown that these lncRNAs in turn can promote or repress inflammatory gene expression in immune cells (Carpenter et al., 2013; Rapicavoli et al., 2013). Here, we identified, AS-IL1α, a natural antisense lncRNA that is transcribed from the opposite strand of the IL-1α locus in macrophages exposed to microbial products. Although AS-IL1α is expressed at lower levels than IL-1α, their expression dynamics are similar.

Using loss of function approaches, we uncovered an important role for this lncRNA in controlling IL-1α gene transcription. The presence of AS-IL1α therefore adds another regulatory checkpoint to ensure that production of IL-1α mRNA is carefully regulated. Indeed, disruption of AS-IL1α function could limit IL-1α transcription and potentially alleviate the damaging effects of excessive IL-1α levels during infection and autoimmune disease.

Preface to Chapter 3

Most of the data and subject content of this chapter has been extracted from the following manuscripts:

Kalantari P, DeOliveira RB*, **Chan J*,** Corbett Y, Rathinam V, Pleogh H, Gazzinelli RT, Golenbock DT, Fitzgerald KA. Dual engagement of the NLRP3 and AIM2 inflammasomes by Plasmodial-derived hemozoin and DNA during malaria. Cell Reports. 2014 Jan 16; 6(1): 196-210.

Liehl P, Zuzarte-Luis V, **Chan J**, Zillinger T, Baptista F, Carapau D, Carret C, Lassnig C, Muller M, Kalinke U, Magarida Vigario A, Golenbock DT, Strobl B, Prudencio M, Fitzgerald KA, Barchet W, Mota MM. Host-cell sensors for *Plasmodium* activate innate immunity against liver-stage infection. Nature Medicine. 2013 Dec 22; 20(1): 47-53.

Sharma S, DeOliveira RB, Kalantari P, Parroche P, Goutagny N, Jiang Z, **Chan J**, Bartholomeu DC, Lau F, Hall JP, Barber GN, Gazzinelli RT, Fitzgerald KA, Golenbock DT. Innate Immune Recognition of an AT-Rich Stem-Loop DNA Motif in the *Plasmodium falciparum* Genome. Immunity. 2011 Aug 26; 35(2): 194-207.

* equal contribution

CHAPTER 3: Characterizing the Innate immune response to *Plasmodium* infections.

Abstract

Malaria is a disease that can affect over 1/3 the world's population, with the majority occurring in developing countries. Cerebral malaria is a serious neurological manifestation of malaria disease associated with high levels of inflammatory cytokines in circulation that lead to a disruption in the integrity of the blood-brain barrier (BBB) (van der Heyde et al., 2006). The molecular mechanisms underlying the inflammatory response during malaria infection are still poorly understood. Inflammatory cytokines and type I IFNs induced when innate immune sensors recognize components of the malaria parasite can contribute to clearance of the parasite and in some circumstances, these same effectors can lead to the development of a more severe form of disease called cerebral malaria. First, we investigated the role of these inflammatory pathways during the liver stage of infection, which is typically asymptomatic. When mice are infected with liver-tropic PbA sporozoites (spz), a type I IFN response ensues. This host response is responsible for up-regulating interferon stimulated genes (ISGs) and limiting the parasite load in the liver. The expression of ISGs is abrogated in IFNAR-/- mice. In contrast to the blood-stage disease, type I IFNs are protective to the host during this stage of disease. This protective phenotype

is dependent on IRF3/7 and the adaptor MAVS suggesting that parasitic RNA is recognized by host cells.

Once liver-stage infection ends, systemic blood-stage Infection of C57BL/6 mice with *Plasmodium berghei ANKA* (PbA) leads to experimental cerebral malaria (ECM). In this model, animals succumb seizures 6-12 days post-infection. Concomitant with our findings that patients with febrile malaria present with an IFN signature, we have found that mice lacking the type I IFN receptor, IFNAR-/-, and transcription factors IRF3-/-IRF7-/- are protected from ECM-mediated death, implicating an important role of type I IFNs in exacerbating the ECM phenotype and suggesting that the nucleic-sensing pathway is activated. Our lab has identified several *Plasmodium* derived PAMPs that are involved in driving the innate immune responses. Firstly, we demonstrated that an A-T-rich motif, prevalent in the *Plasmodium* genome, is sensed by innate immune system. We next showed that pro-inflammatory responses during blood-stage infections are exacerbated by the *Plasmodium* heme metabolite, hemozoin (Hz), which triggers the inflammasome, leads to elevated IL-1β secretion and increased neutrophil recruitment. Collectively, these findings reveal a complex role for type I IFNs whereby, an early and efficacious induction of type I IFN during *Plasmodium* infections may limit parasitic loads during liver stage infection but subsequent waves of continuous type I IFNs contribute to pathology, ECM and death. This study supports the notion that malaria is a multifarious disease

and alterations in any number of variables can lead to drastically different disease outcomes.

Introduction

3.3 billion people worldwide live in malaria-endemic areas and are at risk of being infected and the latest estimate from the World Health Organization estimates that 584,000 people annually succumb to mortality by this disease (2014). People experiencing malaria typically exhibit high fever and paraoxysm. Repeated cycles of schizont rupture are thought to coincide with episodic fevers, chills, headaches and profuse sweating (Maegraith and Fletcher 1972). Fever is associated with pathophysiology and thought to contribute to morbidity, especially in children and *Plasmodium*-naïve adults. Driven by cytokines such as IL-1β and TNF-α, the clinical manifestations associated with these cytokines is also thought to be responsible for the up-regulation of adhesion molecules that result in the sequestration of infected RBCs in capillary beds into various organs (Hunt et al., 2010). While it is clear that Plasmodium infections are associated with significant immune system activation, the initial components of these immune responses are ill understood. An appropriate immune response is important for the clearance of the parasite as well as an efficacious vaccine, and thus requires a robust initiation of the innate immune system for the appropriate acquired immune response. Attaining a better understanding of the interactions between *Plasmodium* species and the innate immune system may lead to improved management of its disease-associated symptoms, and may also lead to better vaccine targets. Thus far, much of the focus on studying malaria has centered on the adaptive immune system. However, since disease

manifestations that are associated with pathophysiology are mediated by innate pro-inflammatory cytokines, our goal is to understand the early triggers of this inflammation.

Despite a substantial body of evidence that supports the role of Toll-like receptors (TLRs) in the innate immune response to parasitic diseases, and mounting evidence that this is also true for malaria, it is unclear whether or not TLRs are the primary and sole means for innate immune recognition of *Plasmodium* species. In this study, we investigate a composite innate immune response to different stages of the *Plasmodium* parasites' infection of the mammalian host. First, during its incubation phase as a sporozoite infecting hepatocytes and second, during its clinically symptomatic phase as a blood-infecting parasite. To fully elucidate the innate immune contribution at these two stages, we utilize mouse models of infection using the murine-tropic Plasmodium berghei ANKA (PbA) strain. Isolated schizonts from PbA infected mice we in a liver-stage infection as well as a blood-stage infection (bypassing the liver-stage by i.v. injections).

This project contains three parts: (1) to demonstrate that the type I interferon (IFN) response is initiated upon detection of PbA sporozoite infection in murine hepatocytes and dampens parasite load, (2) but that same type I IFN response can be deleterious to the hose during blood stage infections and contribute to mortality in a cerebral malaria mouse model, and (3) that the

inflammatory response during the blood stage infection contributes to pathology and death in the host, which is mediated by cellular PAMPs.

Materials and Methods

***Plasmodium* liver stage infection** was obtained by intravenous injection (into different mouse strains and lines) of a designated amount of sporozoites of the following species, strains and lines: *GFP*-expressing *P. berghei* ANKA wt and p36p-, *P. yoelii* 17XNL, *P. berghei* NK65 wt and *uis3*- sporozoites, all obtained by dissection of *Anopheles stephensi* infected mosquitoes bred at the IMM and NYU insectariums. *Ifnar1* flox/flox mice were used to generate LysM-Cre-*Ifnar1*flox/flox and Alb-Cre-*Ifnar1*flox/flox mice. Mavs-/- mice were obtained originally from Z.J. Chen (South Western Medical School, Dallas, TX), *Mda5*-/- mice from M. Colonna (Washington University, St. Louis, MI), *Tlr3*-/-, *Tlr4*-/-, *Trif*-/- and *Myd88*-/- from S. Akira (Osaka University, Japan) and used to generate *Trif*-/-/*Myd88*-/- mice. *Irf9*-/- *Irf7*-/-and *Irf3*-/- mice were kindly provided by T. Taniguchi (University of Tokyo, Japan) and used to generate *Irf7*-/-/*Irf3*-/- mice. IMM Animal Care Committee approved all protocols. Activation of type I IFN response was obtained by peritoneal injection of PolyI:C (Invitrogen). Parasite liver load was measured by qRT-PCR of *Plasmodium* 18S rRNA at different time points after infection. Activation of type-I IFN response was established by genomic-wide transcriptional profile, Western blot or qRT-PCR analysis by measuring the expression of several ISGs in *Plasmodium*-sporozoite versus mock-infected

(injected with salivary gland (Sg) extracts of non-infected mosquitoes) mouse livers. Data are expressed as mean±s.d. Statistically significant differences between two different groups were analyzed using the Mann Whitney test. $p<0.05$ were considered statistically significant. All statistic tests were performed using Graph Prism 5.0 or 6.0 Software.

RNA extraction. For mouse liver RNA extraction, whole livers were homogenized in 3 ml denaturing solution (4M guanidine thiocyanate; 25 mM sodium citrate pH 7, 0.5% *N*-lauroylsarcosine and, 0.7% β mercaptoethanol in DEPC-treated water). RNA was extracted using RNeasy Mini kit (Qiagen). Complementary DNA was synthesized using Transcriptor First Strand cDNA Synthesis kit (Roche). Gene expression analysis was performed using kits from Applied Biosystems. For analysis, the expression levels of all target genes were normalized against hypoxanthine guanine phosphoribosyltransferase (*Hprt*) housekeeping gene (ΔCt). Gene expression values were then calculated based on the ΔΔCt method, using the mean of control group as calibrator to which all other samples were compared. Primer pairs used to detect target gene transcripts are listed below.

Hepatocyte and nonparenchymal cell isolation. Mouse primary hepatocytes were isolated using a modified two-step perfusion protocol followed by a Percoll purification step. Briefly, mice were killed by CO_2 inhalation and immediately processed for cannulation of the portal vein using a 26-gage needle. Upon

successful cannulation, the inferior vena cava (IVC) was cut to allow fluid to drain. Liver perfusion medium (LPM, Gibco) was perfused at 8–9 ml/min for 10 min followed by liver digestion medium (LDM, Gibco) also at a rate of 8–9 mL/min for 10 min. Intermittent clamping of the IVC (3-s clamp every 30 s) was performed during LDM perfusion to improve tissue digestion. After digestion, the liver was excised and the cells were liberated by tearing and shaking of the liver with forceps. The cell suspension was then sequentially filtered through a 100-µm and a 70-µm cell strainer and spun at 50*g* for 3 min. The cell pellet was resuspended in Williams's Medium E (Gibco) with 10% of fetal bovine serum (FBS, Gibco) and carefully overlaid on a 60% Percoll solution (1:1). The cell suspension was fractionated by centrifugation at 750*g* for 20 min, without break, at 20 °C. Viable hepatocytes deposited in the pellet were washed with Williams's Medium E with 10% FBS, spun at 50*g* for 3 min and resuspended in complete Williams's Medium E (supplemented with 4% FBS and 1% penicillin/streptomycin, Gibco). Viability and yield were assessed by trypan blue staining. To obtain mouse primary nonparenchymal cells, perfused livers were squeezed in 1X PBS solution containing DNase (2 U/ml), filtered through a 70-µm filter followed by 8-min centrifugation at 1,300 r.p.m. The pellet was resuspended in 10 ml of 35% Percoll and centrifuged at 2,600 r.p.m. for 20 min without break at 20 °C. The pellet representing the nonparenchymal cells was washed in 1× PBS containing 2% serum before resuspension into lysis/binding buffer. qRT-PCR analysis of the relative expression of macrophage (*Cd68*),

neutrophil (*Ncf2*) and hepatocyte (*ApoAI*) markers was performed to confirm purity of the obtained populations.

Plasmodium blood-stage infection. The *P. berghei ANKA* (Pba) strain (gift of A. Luster, Harvard, Boston, MA) was maintained by passage in C57BL/6 mice. Mice were inoculated i.p. with 1×10^5 iRBCs and neurological symptoms and death were recorded daily. Cerebral malaria was diagnosed by clinical signs including ataxia, paralysis, deviation of the head, convulsions, and coma, followed by death. Moribund animals were scored as dead, and euthanized. For *ex vivo* infections with PbA iRBCs, BALB/c mice were injected with PbA at 1×10^5 iRBCs and parasitemia was monitored daily. Blood was collected by cardiac puncture at 65% parasitemia.

RNA extraction and real time PCR. Human PBMC, mouse BMDM, HEK293 or mouse splenocytes ($3–10\times10^6$ cells/condition), were stimulated for 6 to 17h using Hemozoin (100µM) or poly(dA-dT) (5µg/ml), CpG motifs (5µM), AT-rich ODN (3µM), or *Pf* gDNA (5µg) all of which were transfected with lipofectamine 2000 (Invitrogen). RNA was extracted with RNeasy kit (Qiagen, CA), cDNA was synthesized, and quantitative RT-PCR analysis was performed with primers as described (Charrel-Dennis et al., 2008; Goutagny et al., 2010). Gene expression data is presented as a ratio of gene copy number per 100 copies of b-actin ± SD.

***P. falciparum* culture.** Erythrocytic stages of *Pf* 3D7 isolates were cultured and

natural hemozoin and DNA from *Pf* were purified as described (Parroche et al., 2007). For iRBC preparation, malaria cultures were synchronized with sorbitol for 3 cycles. *Pf* culture stage and parasitemia was assessed daily by field staining. Where indicated, *Pf* enriched cultures at 8% parasitemia and trophozoite or schizont stages were purified as described (Baratin et al., 2005; Cooper et al., 2005; Wu et al., 2010). The culture was then adjusted to a parasitemia of 8% and used to stimulate PBMCs at 40% hematocrit.

ELISA. Cell culture supernatants were assayed for hIFN-α by ELISA (R&D or Bender MedSystems), according to the manufacturer's instructions. The mouse TNFα and IL-6 kits were from e-Biosciences, while the murine IFNβ kit is as described (Roberts et al., 2007).

Table 3.1. Primers/Oligonucleotide sequences.

Gene name	Forward Primer	Reverse Primer
Mus musculus		
AT5	GGGTAAATTTTTACTATGGG	
AT6	ATATATATTTTTACCATAAT	
Hprt	CATTATGCCGAGGATTTGGA	AATCCAGCAGGTCAGCAAAG
Ifit1	CCTTTACAGCAACCATGGGAGA	GCAGCTTCCATGTGAAGTGAC
Ifi44	TCGATTCCATGAAACCAATCAC	CAAATGCAGAATGCCATGTTTT
Usp18	CGTGCTTGAGAGGGTCA	GGTCGGGAGTCCACAACTTC

	TTTG	
Ifit3	CTGAACTGCTCAGCCCACAC	TGGACA T ACTTCCTTCCCTGA
Irf7	CTTCAGCACTTTCTTCCGAGA	TGT AGTGTGGTGACCCTTGC
Ifnb	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA
Plasmodium		
18S PbA	AAGCATTAAATAAAGCGAATACATCCTTAC	GGAGATTGGTTTTGACGTTTATGTG

Results

Liver-stage sporozoites induce a robust Type I IFN response

Clinical malaria begins when fully differentiated schizonts rupture from the liver hepatocytes and release the merozoites that commence to invade erythrocytes (Prudêncio and Mota, 2007). As such, for many decades, it was assumed that the liver-stage infection was immunologically silent (Ejigiri and Sinnis, 2009). However, we now know that as parasites develop and replicate inside hepatocytes, liver-stage specific antigens develop. Indeed, the last few vaccines developed were protein-based vaccines that targed the circumsporozoite (CS) protein, which is a major sporozoite surface antigen (Arama and Troye-Blomberg, 2014; Cohen et al., 2010b). The liver-stage, therefore, plays a critical role in the life cycle of Plasmodium development. At this key stage, when parasite load is much lower than when it reaches the blood stage, and the disease is not yet systemic, it can become a great opportunity to

fight infection. Thus, understanding the immunological events that occur during the liver stage can facilitate our progress towards discovering better therapies and vaccines.

Most studies that have examined innate immune pathways implicate proinflammatory cytokines in the pathogenesis of disease. We have also seen the role of type I IFNs as a way to inhibit cerebral malaria by reducing parasite burden in mice if administered prior to infection (Vigário et al., 2007). In order to study liver-stage infection, we harvested Anopheles mosquito salivary glands (sg) infected with *Plasmodium berghei ANKA (PbA*) and infected WT mice intravenously (IV) through the retroorbital vein. The sporozoites migrate to the liver and divide in the hepatocytes for 48 hours post-infection. We performed a transcriptomic microarray on murine liver homogenates comparing wildtype (WT) mice versus mock infection (*Anopheles* mosquito salivary glands alone) (**Figure 3.1a**). Genes that were up-regulated more than two fold (89 genes) were surprisingly all ISGs linking the Type I IFN pathway to liver-stage infections (**Figure 3.1b**). This ISG signature was abrogated in the absence of IFNAR. We also determined the parasite load by extracting total RNA from WT and IFNAR-/- livers and performed qRT-PCR on Plasmodium-specific 18S rRNA and found that in the absence of Type I IFN receptor, the parasite load increased substantially (**Figure 3.1c**). This indicates that Type I IFN signaling and ISGs are directly implicated in dampening parasite load. Among the most highly induced genes were the ISGs, Interferon-induced protein with tetrapeptide repeats 1

(IFIT1), interferon inducible 44 (IFI44), ubiquitin-specific protease 18 (USP18) also known as Ubl carboxyl-terminal hydrolase 18 (UBP18) which has been shown to cleave ISG15, IFIT3, and interferon regulatory factor 7 (IRF7). We therefore, designed qRT-PCR primers for these genes to confirm their induction during sporozoite infection. This data confirmed the findings in the microarray that showed these genes with abrogated expression in IFNAR-/- mice **(Figure 3.2**). This information contradicts the previously held notion that during the liver-stage of infection, *Plasmodium* is immunogenically silent.

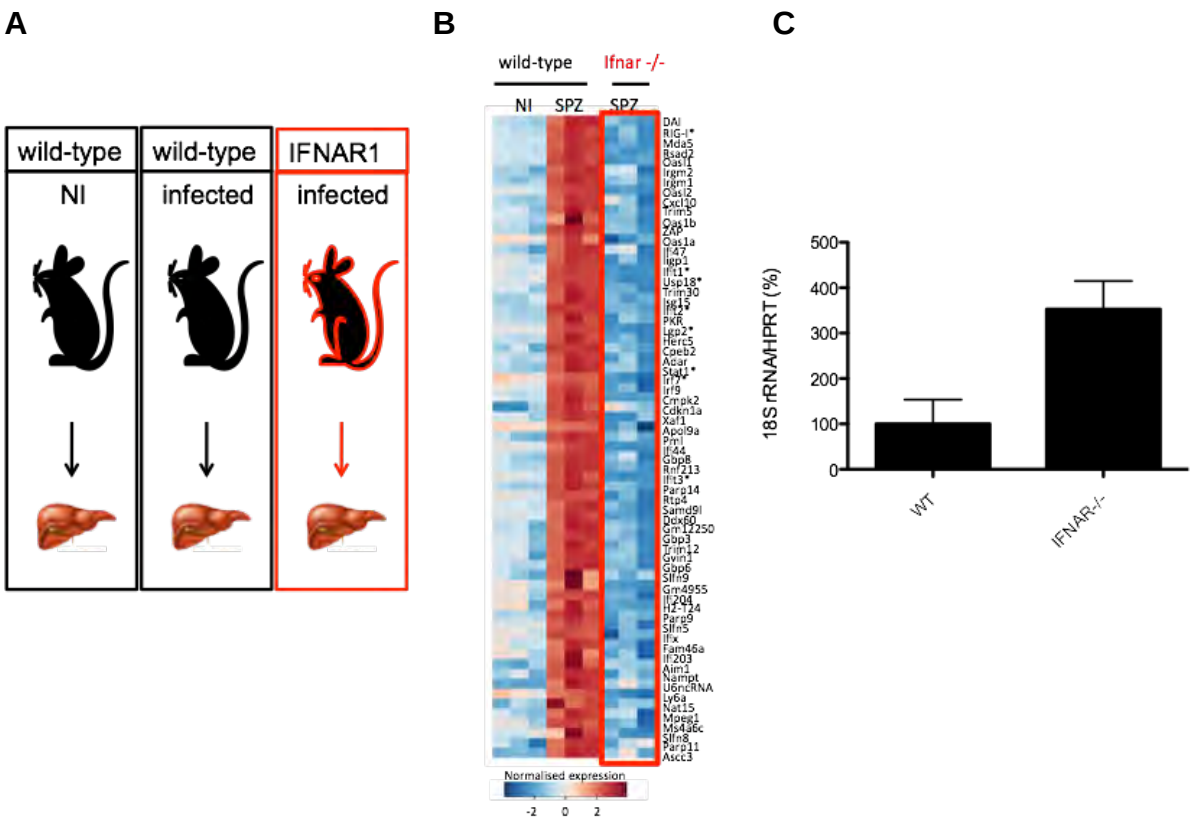


Figure 3.1. *Plasmodium* sporozoite infection induces a robust Type I IFN signature. (**A**) Schematic for wildtype or IFNAR-/- mice infected with non-infected (NI) mosquito salivary glands alone or *Plasmdium berghei*-infected salivary glands. (**B**) Heatmap of microarray data of noninfected or infected sporozoites. The Type I IFN signature disappears in IFNAR-/- mice. (**C**) Plasmodium sporozoite parasite load is measured using primers targeting the Plasmodium18S rRNA and the housekeeping gene, HPRT. In IFNAR-/- mice, the parasite load is significantly increased.

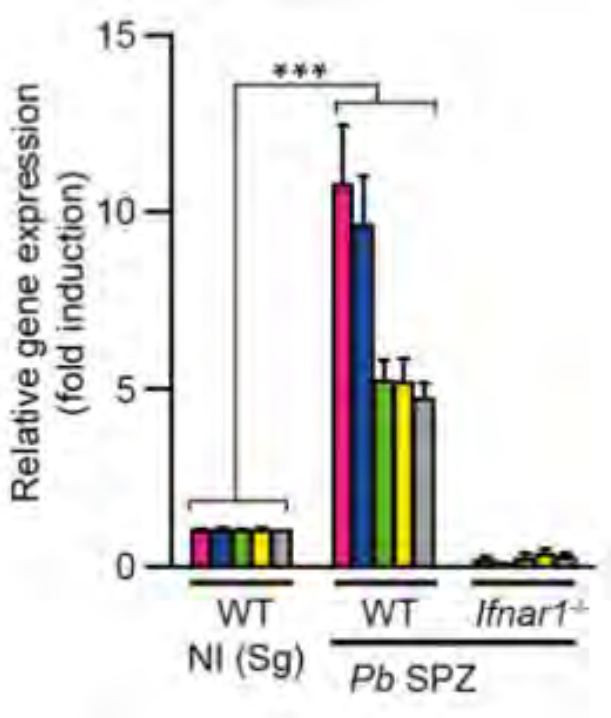


Figure 3.2. Sporozoites induce ISGs in an IFNAR-dependent manner. Wildtype (WT) or IFNAR-/- mice were injected with salivary gland homogenates from non-infected mosquitoes (NI (Sg)) or Plasmodium berghei sporozoite-infected mosquitoes (Pb SPZ) for 42 hours. IFIT1 (pink), IFI44 (blue), USP18 (green), IFIT3 (yellow) and IRF7 (grey) all were highly upregulated during the infection. This response was abrogated in IFNAR-/- mice.

We then tested whether or not this response required an active infection. We repeated the same infection protocol in WT mice as described above using increasing numbers of live sporozoites that were just harvested from mosquitoes, or heat-inactivated (dead sporozoites), irradiated sporozoites (alive but attenuated) or p36p mutant sporozoites (Douradinha et al., 2007) (genetically attenuated due to the lack of the microneme protein) (**Figure 3.3**). In the absence of a replication sufficient parasite, the ISGs IFIT1 and USP18 were severely dampened, suggesting that an active replication is required for mounting a type I IFN response. This could either be due to a potential PAMP that is only expressed by live parasites or perhaps due to insufficient parasite numbers that aren't high enough to activate the Type I IFN response.

Next, we wanted to determine what other components in the Type I IFN pathway were required for the sporozoite-induced IFN response. We infectious various knockout mice deficient for the signaling components involved in the signaling pathway to type I IFN response and found that the ISG induction was largely independent of adaptors Myd88 and Trif, as well as TLR3 and TLR4 (**Figure 3.4**), but dependent on transcription factor IRF3, and to a lesser degree IRF7 (**Figure 3.5a**). Looking upstream, we observed that the ISG response However, the adaptor MAVS-/- (IPS1) mice were completely abrogated in ISG induction (**Figure 3.5b**). This was a surprise because it meant that the likely Type I IFN trigger was an RNA species, which has never been implicated during *Plasmodium* infections. More surprisingly, the intracellular RNA receptor, MDA5

was implicated (**Figure 3.5c**). This signifies that during Plasmodium liver-stage infections, RNA can act as a major pattern associated molecular pattern (PAMP).

Because Type I IFN signaling is intact in most cells, we wanted to determine whether or not the ISG induction was due to direct hepatocyte infection or due to recruitment of liver leukocytes to the site of infection. Using wildtype C57Bl/6 mice, we separated hepatocytes from the liver using a collagenase protocol to discrupt the liver matrix as well as sort for leukocytes. We were able to determine that although leukocytes contributed slightly to the production of ISGs in response to Plasmodium sporozoites at 48 hours post-infection, just before the parasites egress from the liver-stage to the blood-stage of infection, the more prolific ISG producers were the hepatocytes (**Figure 3.6**).

During the incubation phase of infection when Plasmodium resides in hepatocytes, low levels of detection of the parasite RNA trigger a type I IFN response that can dampen the initial parasite load. This signaling is mediated by MAVS and MDA5, but it is unclear whether or not the PAMP is host or malarial-derived. In all, this part of the study reveals that after a mosquito takes a blood meal from its mammalian host, Plasmodium sporozoites get deposited into the bloodstream where it eventually settles in the liver hepatocytes. There it grows and divides for (48 hours in mice) up to 1-2 weeks in humans. It is the initial detection of RNA as a PAMP that triggers the type I IFN response, leading to the production of ISGs to dampen the liver parasite load (Schematic in **Figure 3.7**).

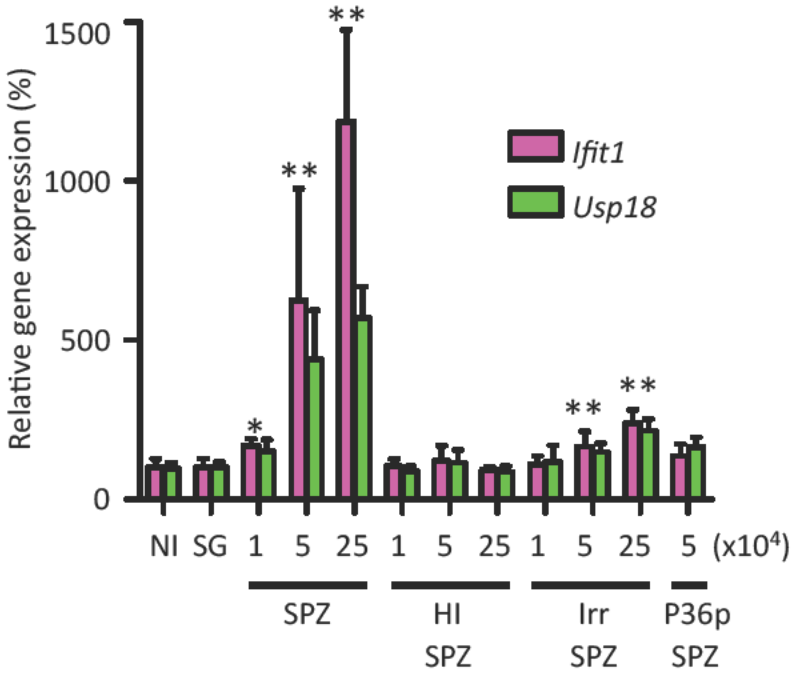


Figure 3.3. An active infection is required for ISG induction. Non-infected (NI), mosquito salivary gland alone (SG), or increasing doses of live sporozoites (SPZ), heat-inactivated (HI) spz, irradiated (Irr) spz, or genetically attenuated microneme protein p36p-/- sporozoites are infected into wildtype mice. 42 hours post-infection, livers were harvested and RNA was extracted. qRT-PCR was performed and two ISGs, IFIT1 and USP18 were measured. All expression is represented as a percentage over NI (100%).

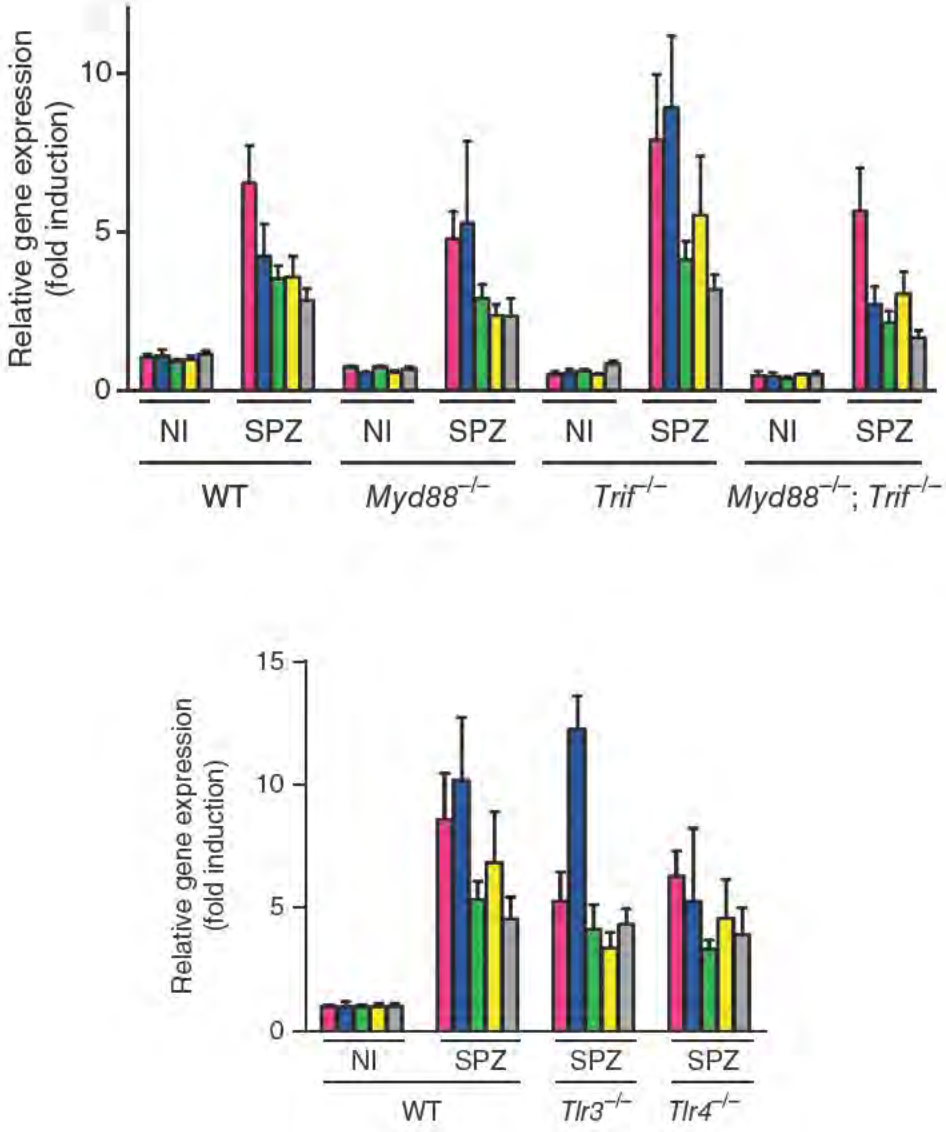
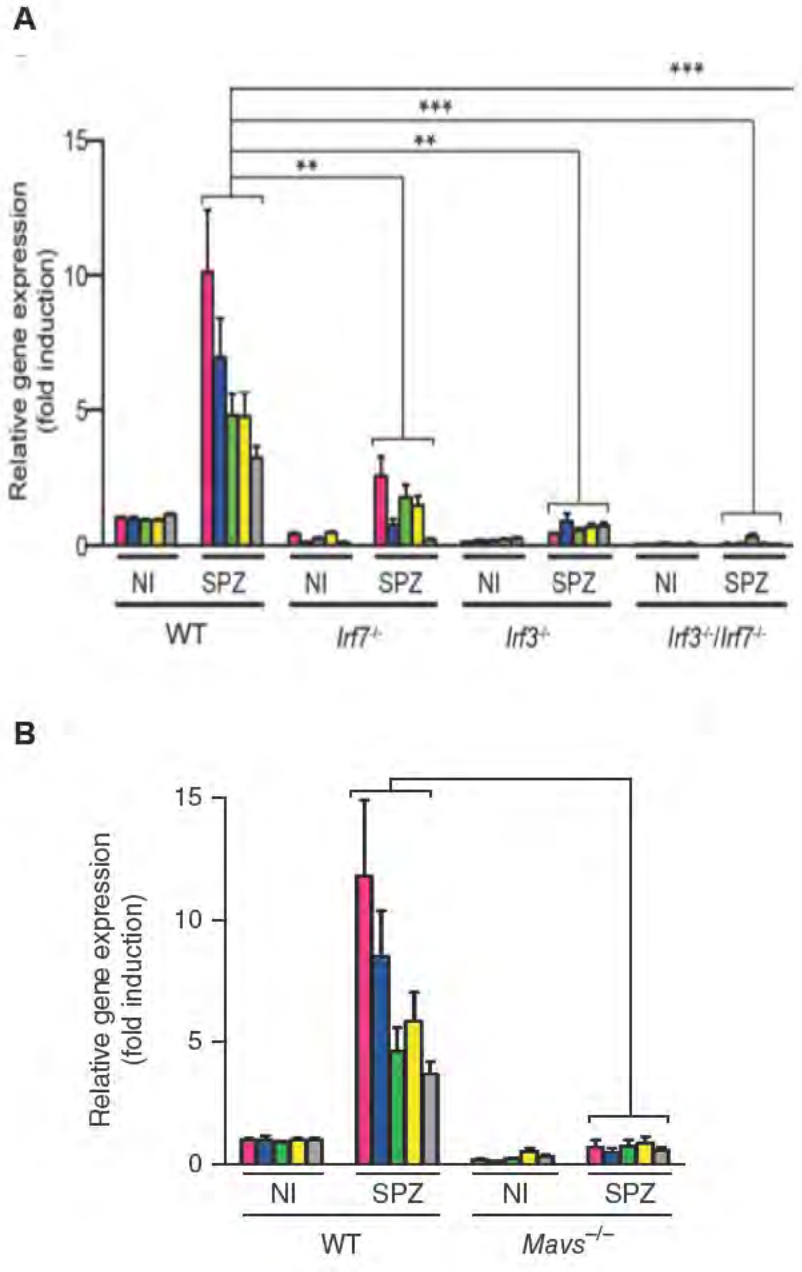


Figure 3.4. Signaling molecules not involved in the production of ISGs in the liver. Wildtype (WT) or various knockout mice as indicated were injected with salivary gland homogenates containing $5x10^4$ *Plasmodium berghei ANKA* sporozoites for 42 hours intravenously IFIT1 (pink), IFI44 (blue), USP18 (green), IFIT3 (yellow) and IRF7 (grey) are shown.


A
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Relative gene expression (fold induction)
15
10
5
0
NI
SPZ
NI
SPZ
NI
SPZ
NI
SPZ
WT
Irf7-/-
Irf3-/-
Irf3-/-Irf7-/-
B
Relative gene expression (fold induction)
15
10
5
0
NI
SPZ
NI
SPZ
WT
Mavs-/-

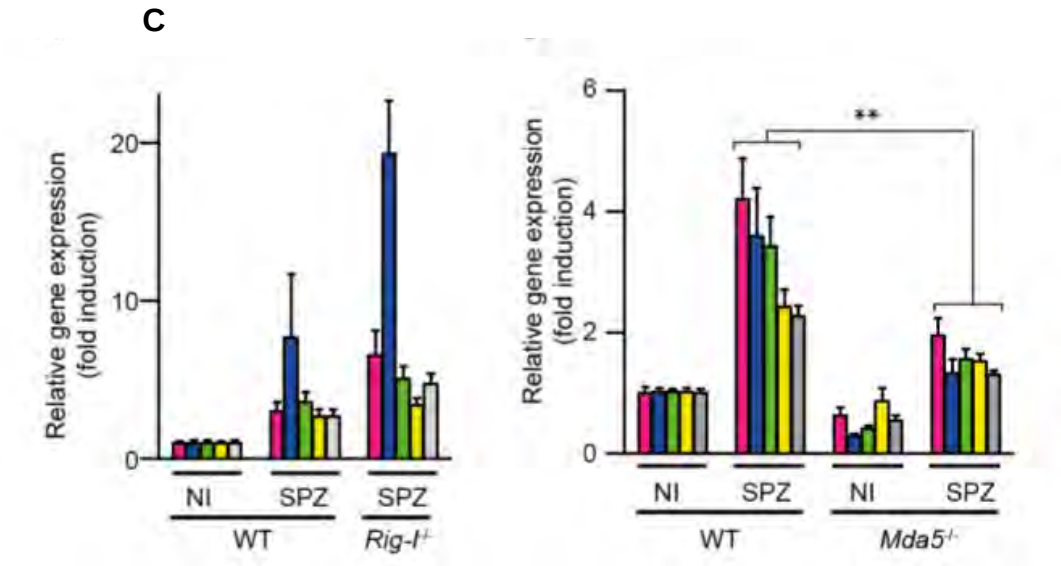

Figure 3.5. Signaling molecules involved in the production of ISGs in the liver. Wildtype (WT) or various knockout mice as indicated were injected with salivary gland homogenates containing $5x10^4$ *Plasmodium berghei ANKA* sporozoites for 42 hours intravenously. IFIT1 (pink), IFI44 (blue), USP18 (green), IFIT3 (yellow) and IRF7 (grey) are shown.

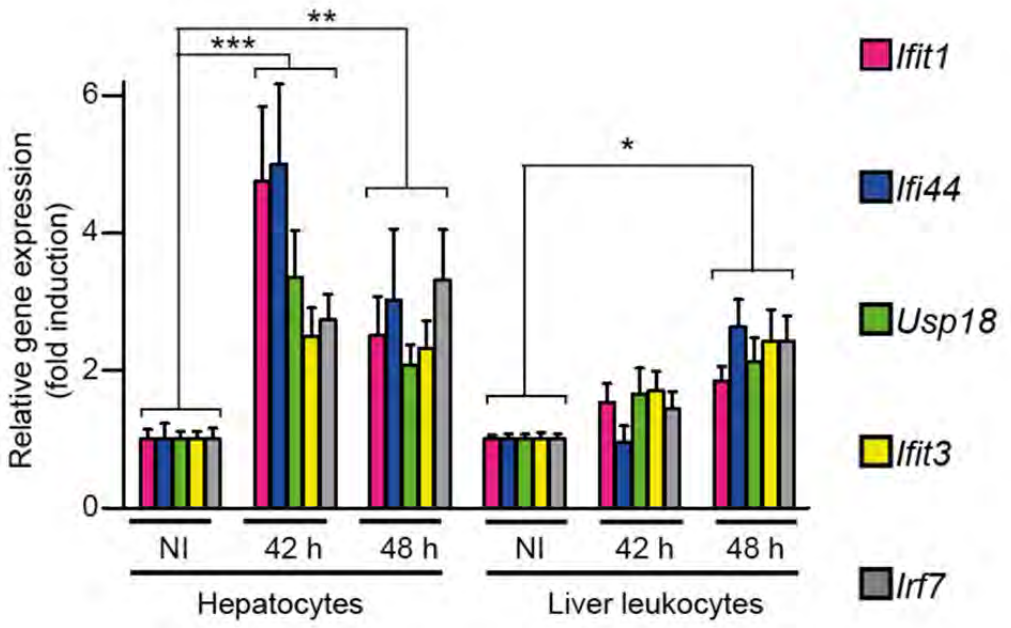

Figure 3.6. **Hepatocytes are the better producers of ISGs during liver-stage infection**. Hepatocytes and liver leukocytes were separated from parenchymal cells as described after 42 or 48 hours post infection with PbA spz. ISGs were measured by qRT-PCR.

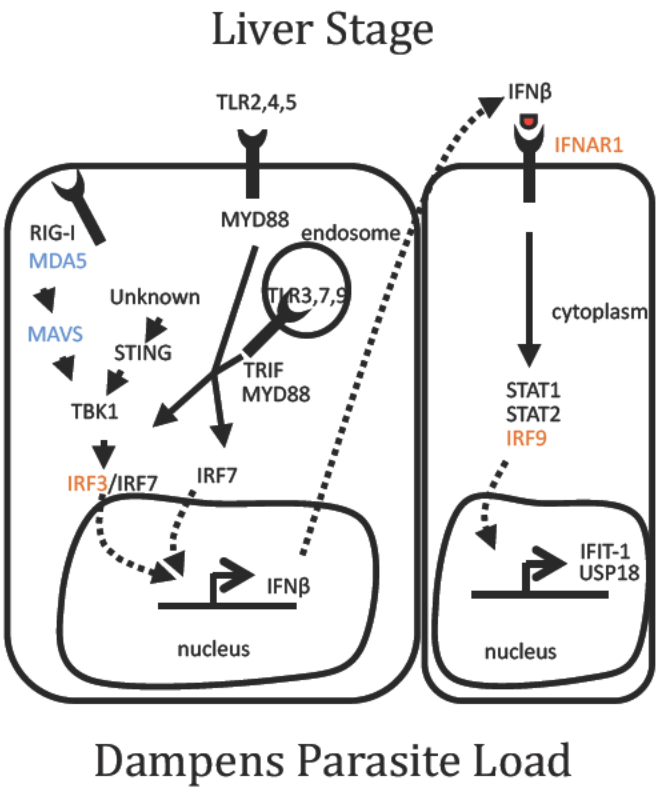

Figure 3.7. Schematic of liver-stage Plasmodium sensing in hepatocytes. Plasmodium infection of hepatocytes induces a Type I IFN response, activating MDA5 and the adaptor MAVS which leads to activation of transcription factors IRF3/7 and transcription of IFN-β which signals through IFNAR to induce ISG production (IFIT1, USP18, IFI44).

When this response isn't sufficient to eliminate infection, the sporozoites egress from the liver to initiate the blood-stage of infection, where symptoms of disease manifest itself. When merozoites infect RBCs, macrophages in the periphery phagocytose the iRBCs and can trigger an inflammatory response that activates both the type I IFN and IL-1 pathways. These pathways are responsible for the physical symptoms of disease. When unchecked, excessive cytokine production leads to vascular permeability, which includes an opening to the blood brain barrier. Pathology in the brain leads to the highly fatal manifestation of malaria called cerebral malaria and often leads to death in both mice and humans.

Type I IFNs contribute to experimental cerebral malaria during blood stage-infections

Prior to my arrival, the laboratory performed global gene expression profiling of human peripheral blood mononuclear cells (PBMCs) harvested from 14 febrile Plasmodium falciparum (Pf)-infected patients before and after treatment with anti-malarial drugs. Analyses revealed that these patients elicited a robust Type I interferon signature (data not shown). We wanted to know whether or not the same Type I IFN response could abrogate severity of disease utilizing a mouse model for cerebral malaria, one of the clinically most fatal outcomes of complicated malaria.

We infected wildtype and IFNAR-/- mice 6-10 weeks of age with *Plasmodium berghei ANKA (PbA)—i*nfected blood (iRBCs) intraperitoneally (IP) and monitored their survival and bled them to determine parasitemia over 20-25 days post-infection. This model recapitulates human disease in various aspects including neurological symptoms that include ataxia, convulsions, paralysis, coma and followed by death (Bagot et al., 2002). Wildtype C57Bl/6 (WT) mice succumb to death by cerebral malaria between 6-12 days post-infection after exhibiting neurological symptoms (**Figure 3.8a left panel**). These mice all died had exhibited cerebral complications prior to death, and similar to human disease, did not exhibit exacerbated parasitemia levels at the time of death nor anemia (**Figure 3.8a right panel**), indicating that the cause of death was not due to extreme parasite burden or excessive erythrocyte lysis. Surprisingly, IFNAR-/- mice did not succumb to cerebral malaria, indicating that the presence of Type I IFN signaling is actually detrimental to disease outcome.

We performed the same infection protocols on IRF3/7 double knockout (dKO) mice, and these survival curves phenocopied the IFNAR-/- mice data and were protected from experimental cerebral malaria (ECM) as well (**Figure 3.8b**). In this scenario, type I IFNs are pathological for blood-stage infection, which is contrary to the data from liver-stage infections where type I IFN is protective. One of the features of cerebral malaria is the association between the pathological “cytokine storm” and its association with the broken BBB that is observed in complicated cerebral malaria patients (Pino et al., 2005). On the other hand, type

I interferons have not previously been shown to be associated with contributing to malaria severity. None of these cohorts of mice had increased parasitemia upon death, suggesting that the abrogation of those specific genes did not cause increased parasite burden that was not controlled, but rather, an inflammation-depdendent phenotype that was no longer active that became protective for the mice.

Plasmodium AT-DNA are immunostimulatory and trigger type I IFN production

Because type I interferons are typically triggered by nucleic acid sensing pathways that signal through TBK1, STING, IRF3/7 and IFNAR, we hypothesized that nucleic acid sensing was involved in the innate immune response to *Plasmodium* and wanted to investigate the role of a TLR-independent DNA sensor involved in the type I IFN response. To date, Plasmodium species are the most AT-rich genome sequenced to date (up to 80% A/T) (Gardner et al., 2002). We have identified over 6000 A-T nucleotide rich motifs, ATTTTTAC (AT5 ODN or AT5 PDE or A6E) in the *Plasmodium falciparum (Pf)* genome. We made oligonucleotides from these regions of the Pf genome and transfected macrophages with poly (dA-dT) or the oligonucleotides and found that they were strong inducers of IFN–β as measured by ELISA (**Figure 3.9a**). We also repeated the same experiment using Plasmodium-infected RBCs (iRBCs) harvested from an infected C57Bl/6 mouse. As expected, uninfected RBCs were

non-immunostimulatory. However, the infected RBCs were highly stimulatory for IFN-β as measured by ELISA and that response was dependent on the transcription factors, IRF3/IRF7 and the intracellular DNA adaptor TMEM173, also known as STING (**Figure 3.9b,c**). Together, these data suggests that the high A-T content in the Plasmodium genome may specifically be triggering the Type I IFN response during blood-stage malaria that contributes to the manifestation of ECM-mediated mortality.

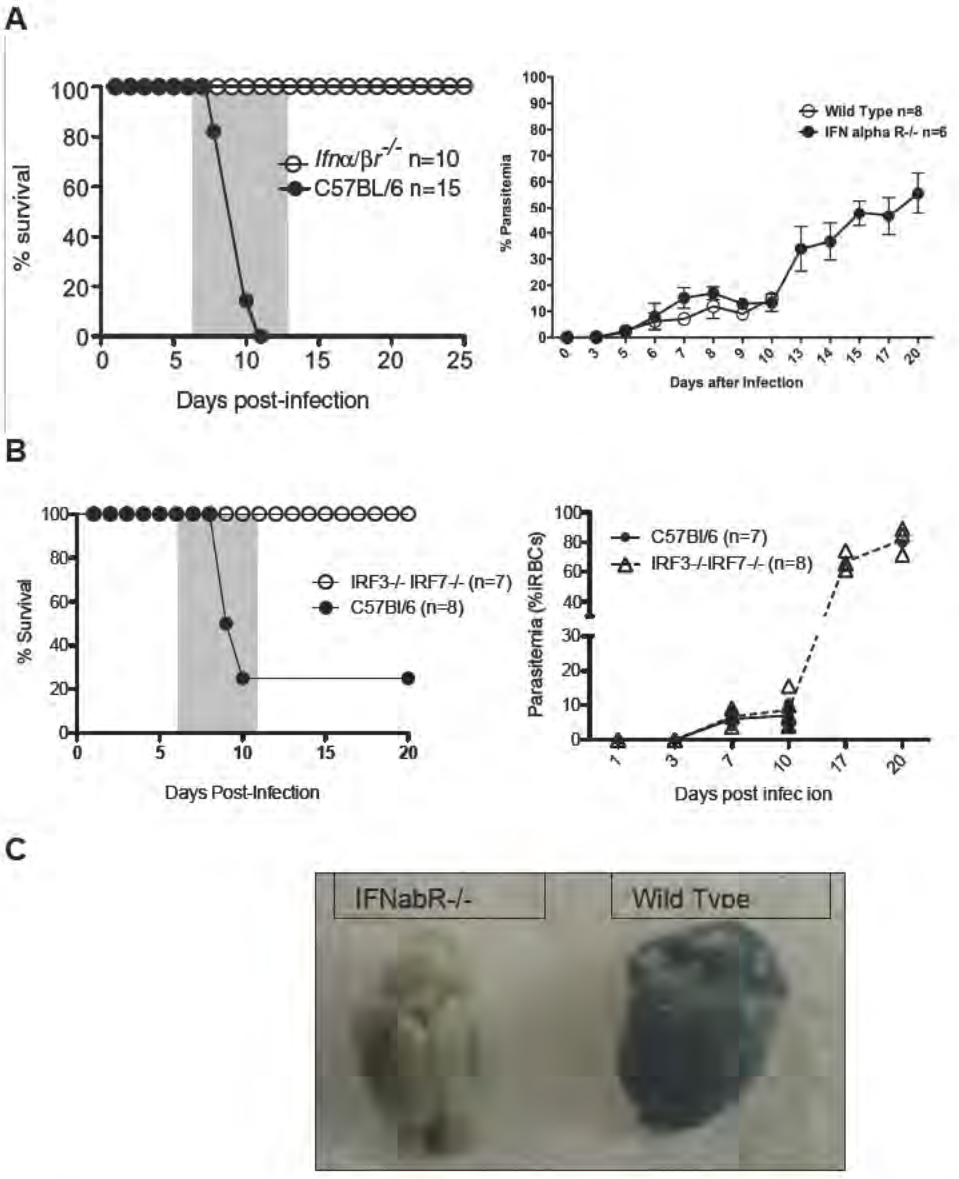

Figure 3.8. Type I IFNs contribute to blood-stage induced mortality during malaria infections. 1x10^5 iRBCs were infected into wildtype (C57Bl/6) and (A) IFNAR-/- or (B) IRF3-/-IRF7-/- mice. Tail vein bleedings were performed on mice every other day and giemsa stained to determine parasitemia levels. Mice were also monitored daily for survival and/or cerebral malaria symptoms (C) In mice about to succumb to death, 800 ul of 1% (w/v) Giemsa dye/PBS was injected into the peritoneal cavity of mice and allowed to circulate for 1 hour. Mice were then sacrificed by CO_2 asphyxiation and perfused with 10 ml PBS through the heart. Brains were harvested and blue staining is an indicator of blood-brain barrier and vascular permeability, a marker of ECM.

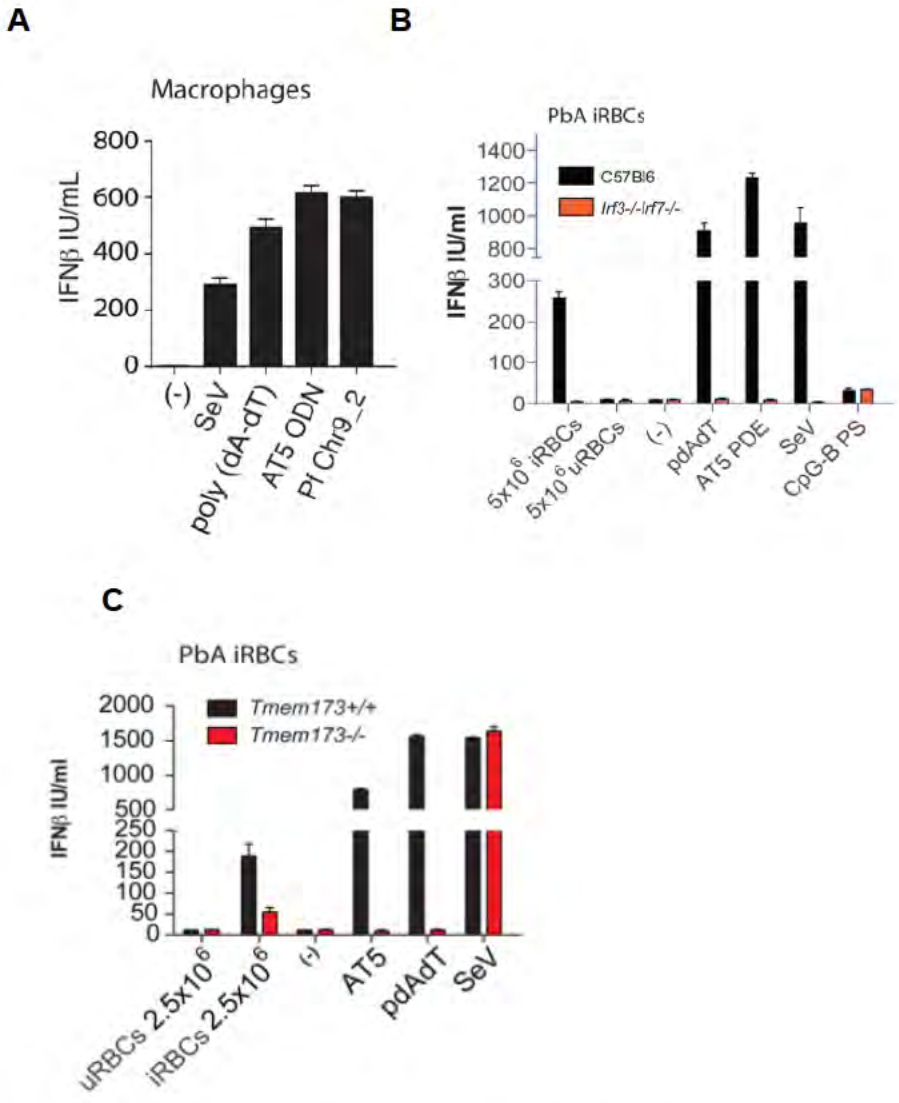

Figure 3.9. AT-DNA induces IFN-b in a IRF3/7 and STING-dependent manner. (A) immortalized macrophages were transfected with poly(dA-dT), AT5 ODN or Pf Chr9 AT-rich motifs using Lipofectamine 2000 or Sendai Virus (SeV) for 6 hours and IFN-b was measured by ELISA. (B) WT C57Bl/6 or IRF3-/-IRF7-/- or (C) TMEM173-/- (STING-/-) macrophages were transfected with AT-rich oligonucleotides using Lipofectamine 2000, SeV, or CpG-B and compared to Plasmodium infected RBCs (iRBCs) as indicated. (This figure was taken from (Sharma et al., 2011))

iRBCs, through hemozoin, activate the inflammasome to trigger IL-1β production

Because the AT-DNA activated the intracellular DNA-sensor pathway, we also wanted to look at whether or not or Plasmodium DNA would activate the inflammasome and trigger IL-1β activity. We harvested bone marrow from AIM2-/- mice and their WT littermate controls (AIM2+/+) and differentiated them into BMDMs for 7 days using L929 secreting M-CSF supernatants. As expected, Plasmodium DNA was a potent inducer of the IL-1β pathway as well. However, when we used the same AT-rich oligonucleotides that induced a potent IFN-β response, we found that they didn't depend on AIM2, as the knockout cells were just as responsive as the AIM2+/+ cells (**Figure 3.10a,b**). When transfection total genomic DNA from various Plasmodium clones, however, the IL-1β response was clearly AIM2-dependent. This tells us that although DNA is a potent trigger for the inflammasome activity, the AT-rich DNA motifs are specifically involves in activating the Type I IFN response.

Nucleic acids have been shown to be potent triggers for the innate immune response, it isn't surprising that *Plasmodial*-derived DNA will also trigger the innate signaling pathways. We show here, that AT-DNA activates the STING-IRF3/7 axis to induce type I Interferons, and although total genomic DNA triggers IL-1β via AIM2 inflammasome, the AT-DNA in particular, doesn't activate the pyrogen, IL-1β. During blood-stage infection, iRBCs are a major source of "malaria toxin". iRBCs contain hemozoin, which we believe acts as a carrier for

the DNA (Parroche et al., 2007). We tested increasing concentrations of iRBCs to determine which inflammasome was responsible for IL-1β production. We found that this response is completely NLRP3-mediated, and to a lesser degree, requires AIM2 inflammasomes as well (**Figure 3.11**). Because NLRP3 is activated by crystals like hemozoin, it is probable that iRBCs phagocytosed by macrophages, contain DNA-carrying hemozoin, which upon phagosomal destabilization, can trigger a robust IL-1β response.

We show that parasitic nucleic acids also serve as PAMPs in triggering a robust innate immune response. RNA during the liver-stage, and DNA in the blood-stage (**Figure 3.12**), perhaps carried by hemozoin, can trigger a robust type I IFN response and also induce the IL-1 pathway. If these pathways are too potent, severe complications including a highly fatal neurological development, cerebral malaria, can occur. The host immune response to parasites plays a significant role in developing ECM. As immunity to malaria is extraordinarily complex, it is often difficult to isolate the variables that contribute to disease. We have aided in identifying the molecular triggers to the immunity against malaria.

Here, we find evidence to support the existence of multiple nucleic acid sensing pathways to be active in the detection of malaria: one through the previously shown TLR9 activity through CpG motifs, a second one via interaction of malarial AT-rich motifs through an unknown cytosolic DNA receptor. Both of these pathways trigger inflammatory cytokines as well as type I IFN production.

Upon schizont rupture, hemozoin crystals containing malarial DNA activate both NLRP3 and AIM2 inflammasomes.

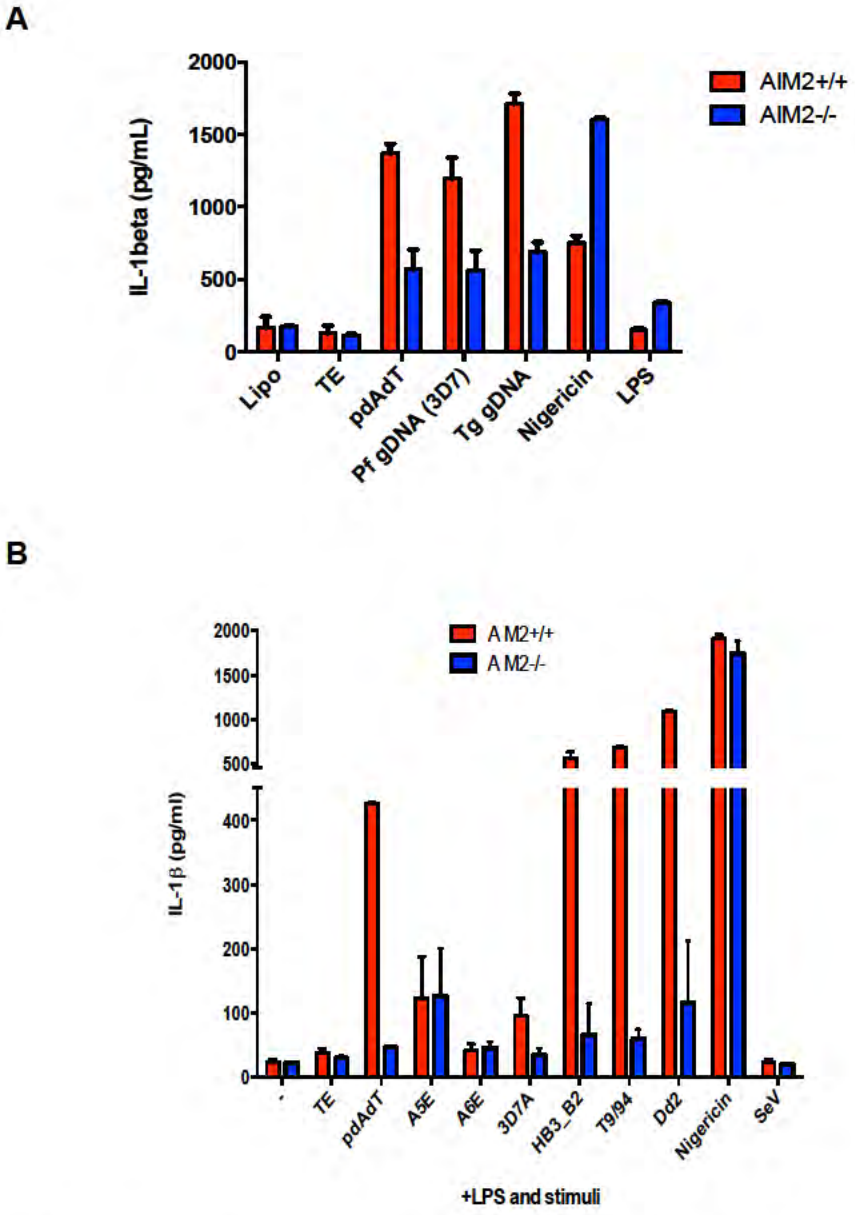

Figure 3.10. **Malaria DNA activates IL-1β via AIM2 inflammasome**. (A) Pf genomic DNA was transfected using lipofectamine 2000 compared to poly (dA-dT) or Nigericin, which depends on NLRP3, in AIM2+/+ or AIM2-/- BMDMs. (B) Pf DNA from different isolates (3D7A, H3B-B2, T9/94, Dd2) were transfected into AIM2+/+ or AIM2-/- BMDMs compared to Plasmodium-derived AT-rich DNA oligonucleotides A5E, A6E.

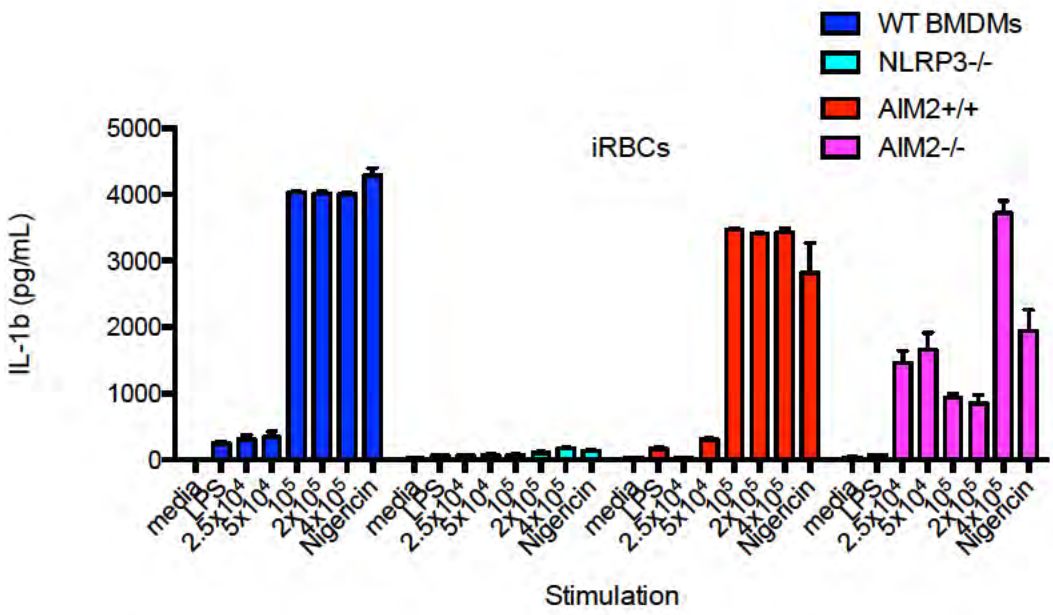

Figure 3.11. iRBCs induce IL-1β through NLRP3 and Aim2. Increasing concentrations of iRBCs were used to stimulate BMDMs from WT, NLRP3, or AIM2-/- cells.

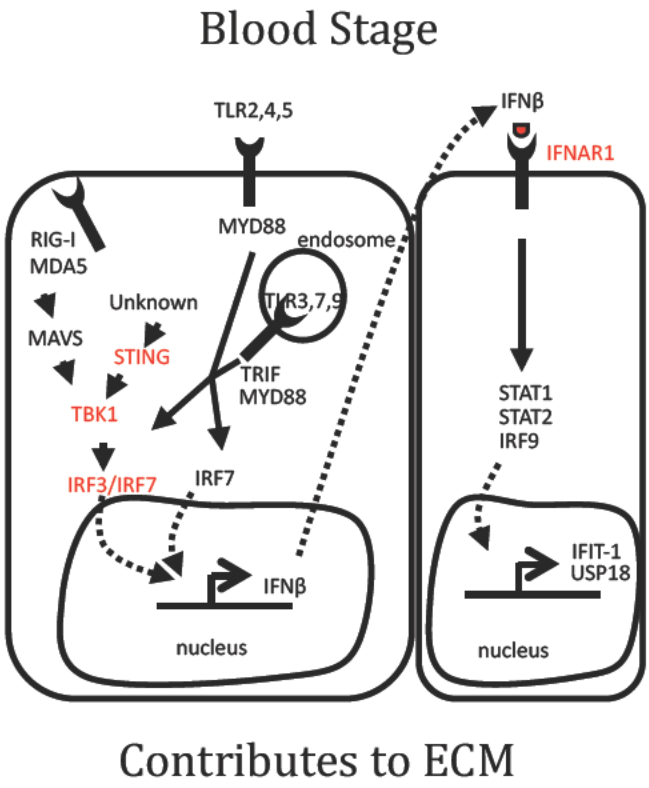

Figure 3.12. Innate immune responses during PbA blood-stage infections. Through an unknown nucleic acid receptor, A-T-rich DNA from PbA activates STING, TBK1 and triggers the activation of transcription factors IRF3/7 to induce type I IFNs and amplifies signaling through IFNAR. This process encourages cerebral malaria and causes mice to become moribund.

Discussion

The basic objective of this project was to understand how the immune system can participate or interfere with the malaria infection process. Although drastic efforts to develop an effective vaccine has yielded incomplete results, epidemiological and experimental studies have demonstrated that protective immunity can be induced against malaria (Langhorne et al., 2008). Natural immunity to malaria can be acquired with time after repeated infections, and it is thought that antibodies are made to target merozoites. Experimentally, sterile immunity has been achieved in humans against the pre-erythrocytic stage only after immunization with radiation-attenuated sporozoites (Kumar et al., 2006), and against blood stage parasites only after repeated immunization with live blood stage paraistes under drug prophylaxis. To answer these questions, we utilized a mouse model of infection to show, for the first time, that although the liver-stage infection is asymptomatic, it is not immunologically silent like previously thought. Type I IFNs are induced in infected hepatocytes. It may be below the level of detection, however, because a natural infection only occurs with a few parasites deposited into the skin and subsequently sequestered into hepatocytes to initiate infection. That signal is picked up only in an extreme dose of infection (we started with 50,000 sporozoites in the liver). However, the conclusions are clear that an innate immune response can be induced, and that response can alleviate parasite burden. Moreso, at each stage of disease, different triggers of the immune response, RNA during the liver-stage, and DNA

during the blood-stage, converges to produce type I IFNs. Triggering the immune response at the appropriate time is critical for the appropriate outcome of disease. Ultimately, further experimentation will be required to determine the exact nucleic acid triggers of the type I IFN response during both disease stages, as well as implicating the signaling molecules upstream of the transcription factors IRF3/7 during blood-stage disease to confirm the importance of the molecules STING and TBK1 during infection.

Chapter 4: Discussion, perspectives, and implications

The innate immune system is the body's first line of defense against pathogens. This response is governed by PRRs that recognize PAMPS and DAMPS and activate downstream signaling molecules. Effectors like proinflammatory cytokines, chemokines and type I IFNs mediate this response. Indeed, small changes in an organism's ability to regulate the inflammatory response can lead to deleterious effects for the organism to combat disease or to re-establish homeostasis after infection. This thesis research has focused on two independent projects: (1) we identified AS-IL1α, a TLR-inducible lncRNA that is a component of the inflammatory response, which regulates the transcription of a highly inflammatory cytokine, IL-1α and (2) we explored the role of Plasmodium PAMPS in both liver (RNA) and blood-stage (DNA+hemozoin) during malaria infection that may contribute to the pathogenesis of disease.

Notes on antisense transcription

It is well known that antisense transcription exists and a multitude of genes are transcribed from opposite chromosomal strands. It has also been widely appreciated that when the genome produces transcripts from both strands, roles can be ascribed involving gene regulation that mediates RNA interference or even gene silencing on the chromatin level. Some sense/antisense partners share promoters and as such, re-defines the notion of what a transcriptional unit is. Current studies estimate over 2481 pairs of

overlapping sense/antisense transcripts have been annotated via the Functional Annotation of Mouse (FANTOM) consortium and Riken group (Werner et al., 2009). 20.1% of these were bidirectionally transcribed loci, while 14.8% contained overlapping exons. It should be noted that these parameters are based on the latest algorithms, and much of the defined mechanisms are based on speculations from known examples. It needs to be emphasized that the only way to truly define these classes of noncoding elements is through individual interrogation, much like the protein-coding research. It is unclear why in some instances, an antisense transcript will behave similarly to RNAi, where complimentary base-pairing would lead to its expected degradation, where as in other cases like AS-IL1α, the lncRNA would function to promote more transcription. It was a surprise to us that this lncRNA behaved this way, as we expected AS-IL1α to behave like a typical antisense transcript. For decades, researchers have been exploiting antisense oligonucleotides (ASOs) to knockdown sequences of interest, including mRNAs. Indeed, there is an entire industry dedicated to manufacturing these ASOs as a method to silence genes. When a much larger antisense RNA becomes transcribed (>300 nt), perhaps, tertiary structures and folding of the transcript must dictate its functions. In the context of AS-IL1α, from the polysome profiling experiments, we were able to demonstrate the unlikelihood of this transcript to be translated. However, this experiment also revealed that AS-IL1α has a dense sedimentation, indicating that it must belong to a high molecular weight protein complex. It remains to be seen

how exactly this lncRNA functions at the IL-1α promoter. We don't know if it binds to the chromatin directly, or if it is mediated through protein:chromatin interactions. The model we have now shows that AS-IL1α gets transcribed upon TLR-stimulation mediated through NF-kB. At the same time, IL-1α is getting transcribed, too because TLR-signaling also induces IL-1α transcription (**Figure 4.1**). But the presence of AS-IL1α then drives much more rampant IL-1α transcription, which can be seen at 6 hours post-TLR stimulation and this is corroborated by the ChIP results because much more RNAPII gets recruited in the presence of AS-IL1α than in the knockdown cells.

AS-IL1α mechanism

From these studies, we observe the role of AS-IL1α in promoting the transcription of its antisense gene, IL-1α. Gathered from the polysome profiling data, and extrapolating from examples of other lncRNA antisense transcript mechanisms of action, the reigning model is that TLR signaling induces the transcription of both AS-IL1α and IL-1α. I could not confirm that AS-IL1α transcription kinetics occur faster than IL-1a transcription, but would be expected if AS-IL1α is necessary for IL-1α transcription. Unfortunately, using the typical laboratory qRT-PCR assays do not detect that level of sensitivity required to discriminate the subtle differences in induction kinetics at very early time points. In this model, even one copy of AS-IL1α RNA would be sufficient to begin driving

more transcription of its protein-coding gene. Performing a global run on assay followed by RNA-sequencing (GRO-seq) (García-Martínez et al., 2004) would interrogate the sequences that initiate transcription in WT or knockdown cells and can reveal early kinetic differences in transcription as well as the transcription of target genes that are altered. Nuclear-localized lncRNAs like AS-IL1α typically have been shown to modulate epigenetic processes to alter gene expression. In the case of AS-IL1α, I observe decreases in histone modifications and RNAPII recruitment to the IL-1α promoter in cells with abrogated AS-IL1α expression. Either AS-IL1α acts as a guide to recruit key transcription factor complex proteins to bind at the promoter and initiate transcription in *cis* at the promoter of its protein-coding partner, or may behave as a multiprotein-complex scaffold for the actual assembly of the transcription factor protein complex. It will be difficult to distinguish the subtle but important differences in mechanism. In the guide scenario, AS-IL1α is necessary to physically bring the auxillary proteins to the promoter itself, whereas in the scaffold model, AS-IL1α would be necessary for maintaining the protein complex, but the physical recruitment to the promoter itself, would be mediated by the proteins and not the RNA itself. In order to determine the order of assembly, several key experiments would need to be performed. First, the cloned lncRNA would need to be *in vitro* transcribed and biotinylated. Immunoprecipitation followed by mass spectrometry would be performed to identify key protein binding partners. Subsequent genetic manipulation via knockdown or knockout of the proteins themselves in the

presence or absence of the lncRNA would sanction the interrogation of which components are necessary and sufficient for observing the histone modifications and RNAPII recruitment that precedes transcription. RNA immunoprecipitation (RIP) studies, would also reveal whether or not the lncRNA binds directly to the chromatin (which supports the guide model) rather than the scaffold model. From the nanostring experiments, IL-1α was implicated as the most highly abrogated gene in the absence of AS-IL1α, other gene expression changes were still observed. What remains to be seen is whether or not the amelioration of AS-IL1α really only affects IL-1α gene expression and subsequent observations of gene changes are a result of dampened IL-1α signaling or if other genes are directly affected due to decreased AS-IL1α activity. Performing the same nanostring experiment on IL-1α or IL-1 receptor knockout cells to examine other gene expression changes would resolve these confounding data.

Lastly, overexpression studies would nicely complement the current study utilizing knockdown approaches. Overexpressing the lncRNA in a vector with an inducible promoter could drive AS-IL1α expression and subsequently observe all and any other genes that could be induced in the presence of the lncRNA. This would answer the question of whether or not promoter-specific localization of the lncRNA is sufficient to drive the transcription of those target genes, or if the AS-IL1α transcript itself is only mediating the transcription of IL-1α. Inconclusive data from such experiments may not indicate that the lncRNA does not regulate IL-1α, but more thorough and targeted overexpression systems would need to be

utilized in order to direct the lncRNA overexpression to the desired IL-1α promoter region. Novel techniques exploiting the CRISPR-Cas9 system allows for the attachment of an RNA to the CRISPR guide RNA to the target chromatin location without cleavage (John Rinn lab, data unpublished). In the future, this technique could be extremely useful in teasing out the specific expression localization necessary for observing *cis*-effects. Currently, probing cis effects are difficult and require the production of flox-knockout animals that can mediate allele-specific gene disruption (Dimitrova et al., 2014).

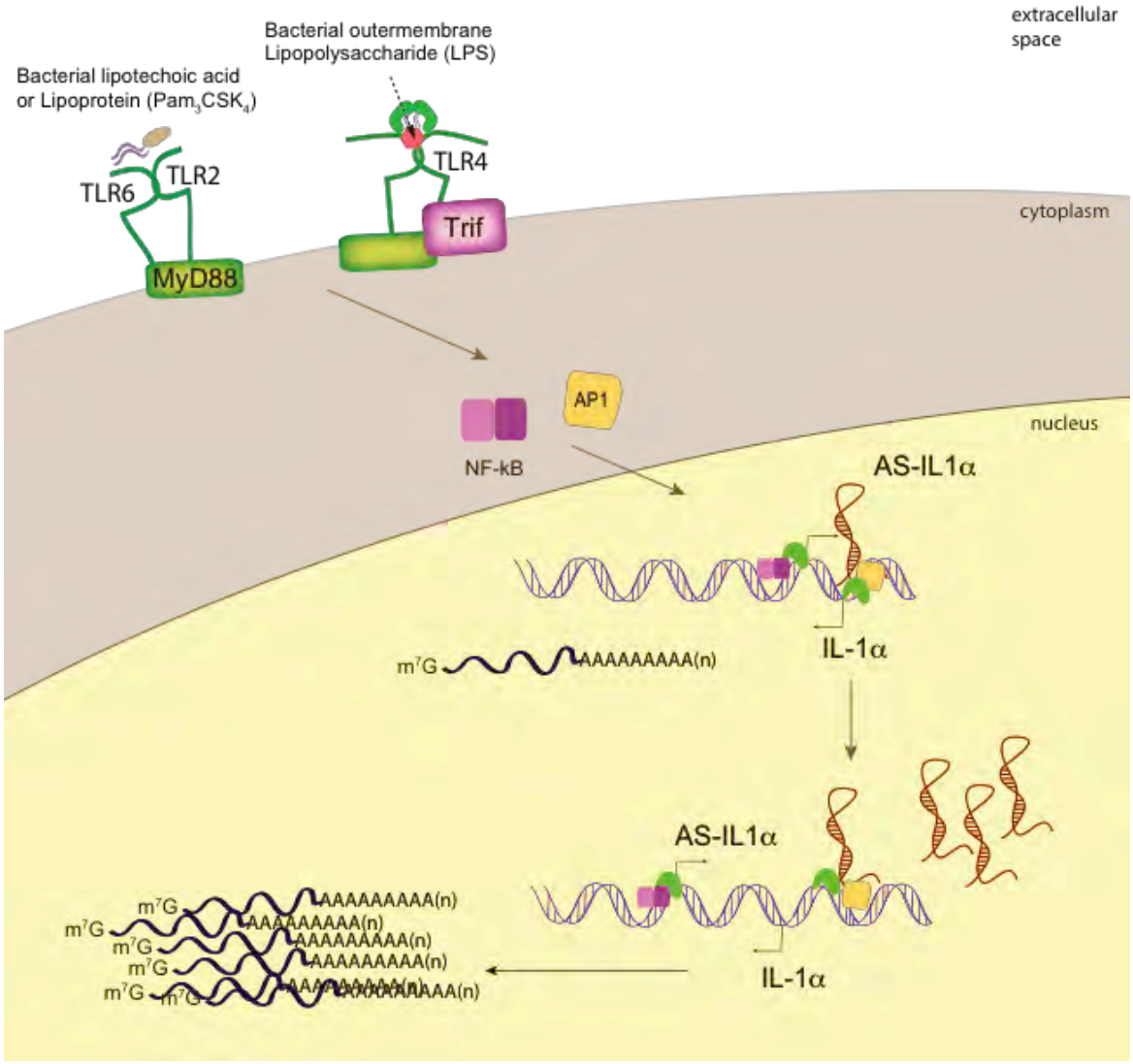

Figure 4.1. Model of AS-IL1α on IL-1α transcription. Transcription of IL-1α and AS-IL-1α occurs after TLR signaling and activation of the transcription factors NF-kB and AP-1, but not via intracellular nucleic acid receptors. Once AS-IL1α is transcribed, it leads to more RNAPII recruitment (green) to transcribe more IL-1α mRNA .

AS-IL1α orthologs

Identifying putative AS-IL1α orthologs would strengthen the case for the biological importance of this lncRNA in regulating IL-1α mRNA expression. The implications for such an ortholog would be 1) that there was a selective advantage for organisms across multiple species to maintain this gene, revealing the necessity of added regulation on the cytokine, IL-1α, and 2) that sequence specificity in part, mediates its function. Utilizing nucleotide Basic Local Alignment Search Tool (BLASTN) and both the predicted nucleotide sequence obtained from ENSEMBL 7.0 and confirmed cDNA sequencing data (**Figure 4.2**), a syntenic region at the IL-1α locus was identified in the human genome. More specifically, regions of sequence conservation at the IL-1α gene, but beyond the IL-1α gene borders are observed. It is unclear, however, if these regions of conservation represent a potential un-annotated gene that might be orthologous to AS-IL1α and experimental confirmation of inducibility and cloning would need to be performed to validate functional gene orthologs. Additional searches for sequence conservation was performed using BLASTN to query the mature AS-IL1α sequence and compared against sequenced model organisms in the NCBI database, ranging from *e. coli* to *Homo sapiens.* Initial results using this method indicated that based on sequence alone, AS-IL1α may be present in rodents, including hamsters and rats as well. If these *in silico* interrogations are correct, then perhaps this lncRNA arose separately in the rodent evolutionary lineage, or other organisms also express this lncRNA, but have not maintained sequence

conservation due to the lack of selective pressure on the importance of the actual nucleotide sequence itself. One can argue that these searches are preliminary at best, albeit superficial, but are essential first steps in gene discovery.

Implications on IL-1α

Although the IL-1α molecule has long been recognized, implicating its disctinct role in various diseases has been limited as both IL-1α and IL-1β activate the same IL-1 receptor and elicits the same downstream signaling events. One critical difference is that unlike IL-1β, IL-1α is active even in its uncleaved form and doesn't require inflammasome activation in order to mediate its functions. That means, once translation is completed, IL-1α is fully capable of activating the immune response. Of note, IL-1α, not IL-1β is thought to be responsible for early neutrophil recruitment (Ozaki et al., 1987). *In vivo*, this is probably due to IL-1α bypassing the requirement for cleavage. Hence, in situations where sterile inflammation or high levels of necrosis is observed, IL-1α propogates inflammation and induces a feed-forward signaling loop that promotes more inflammation and eventually pathology. In inflammatory bowel disease (IBD) models, it should be noted that IL-1α propogates colitis, whereas IL-1β exascerbates it (Bersudsky et al., 2014). Only treatment with anti-IL-1α antibodies was able to ameliorate disease, whereas anti-IL1β nor IL-1R blocades were sufficient to abrogate disease.

In the context of this study, IL-1α transcription was investigated in macrophages as a component of the TLR-inducible immune response. However, it should be noted that IL-1α is constitutively expressed in non-hematopoetic cells, namely epithelial cells like keratinocytes. It should be interesting to explore the necessity of this lncRNA in those cell types, whether it is expressed at all, or if it is basally transcribed at low levels.

Another unique feature of IL-1α is its nuclear localization sequence present in the N-terminal half of the precursor deemed the propiece. The IL-1α propiece translocates to the nucleus and participates in the regulation of transcription. Therefore, IL-1α, like IL-1 family members IL-33 and IL-37, is a dual-function cytokine, binding to chromatin as well as to its cell surface receptor. Some cancer cells can express membrane-bound IL-1α, which can increase immunogenicity of tumor cells and serve in anti-tumor immune surveillance and tumor regression. However, in the tumor microenvironment, the precursor to IL-1α released from dying tumor cells is inflammatory and, similar to IL-1β, increases tumor invasiveness and angiogenesis.

Future Directions and lncRNAs in innate immunity

lncRNA research is a relatively new field in biology. Without a doubt, there is one lncRNA (Xist) that has huge implications in vital life if perturbed. But currently, the data is limited on how much these lncRNAs affect normal physiological functions *in vivo* or whether the aberrant expression would lead to

disease outcomes. In order to fully appreciate lncRNAs, and in our case, AS-IL1α, it would be necessary to generate a knockout mouse and determine if in the absence of this lncRNA, the alterations in IL-1α expression would abrogate or exacerbate various diseases within an entire organism.

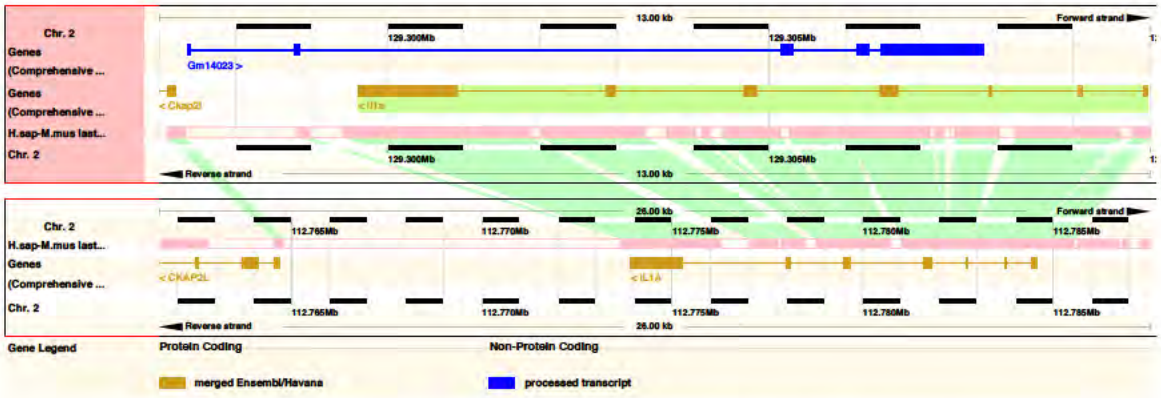

Figure 4.2. Syntenic alignment of human and murine genomic regions at the AS-IL1α and IL-1α locus. Utilizing Nucleotide Basic Local Alignment Search Tool (nBLAST), and ENSEMBL 7.0, the IL-1α genomic locus was compared between human and murine nucleotide sequences. Regions of nucleotide consensus is shown in highlighted green. Although AS-IL1α was only annotated in the murine track (indicated as GM14023 in blue), and no such processed transcript was identified from the human data, there are large regions of sequence homology between the two species.

The global view of lncRNA research

With the results from the Encyclopedia of the DNA element (ENCODE) project and revealed new layers of complexicity previously unappreciated in the eukaryotic transcriptomes, more biologists are beginning to take interest in this novel class of regulatory RNAs. At the Keystone research symposia in 2013, there were two general groups delving into lncRNA research. The first group consists of the obstinate molecular biologists analyzing the genome on a global scale, mobilizing new technology to look at these nucleic acids in novel ways to find common pathways, or new themes and patterns to group these RNAs. The other group, passionate about their disease system, be it cancer, or development, or inflammation, had specific questions and utilized RNA-sequencing technology to discover potential gene candidates that might be perturbed in their systems, with the end goal of new discoveries for therapy or basic biology in specific signaling pathways. It is quite a fascinating time to witness and partake in research with this group of people. It will be interesting to see how the research evolves.

Innate immunity to malaria

In many ways, *Plasmodium* infections are the “Goldilocks” of disease responses. As we have seen in Chapter 3, excessive or subpar immune activation at the wrong place or wrong time could result in deleterious outcomes. During the initial stage of infection in liver hepatocytes, robust activation of the cytosolic RLRs

could wield a type I IFN signaling cascade that is sufficient to severely dampen parasite load (**Figure 4.3**). The problem is, unlike in our disease model where we inject 50,000 sporozoites into the mouse, a real mosquito-dependent infection would only release a few sporozoites. So although the innate immune system can be induced, the question is whether or not in an actual infection, the infectious agent initiates at a level below detection. We haven't tested whether or not this initial response in the liver stage actually affects the final disease outcome in the ECM mouse model. Here, we isolated the parasite in separate stages and infected the mice at each stage of its life cycle rather than letting it progress from sporozoite stage to end-stage disease. Through collaborative discussion, it was said that in some instances, using the usual 50,000 sporozoite infection will still lead to ECM if disease was allowed to progress to blood stage, but that result is inconsistent.

It has been noted that the cytokine storm patients exhibit during malaria infection strongly coincides with disease severity and outcomes. We see that in the blood-stage model of disease where the absence of IFNAR and its upstream signaling molecules is actually protective of disease. This excessive inflammatory response is driven by AT-DNA and hemozoin coated with DNA, as well as other previously identified malarial toxins like GPI-anchors. (**Figure 4.3**) Being able to dissect the molecular interactions between host and pathogens will be crucial to our ability to develop new therapeutics. For the first time, we were able to show that type I IFNs are important for mediating disease severity in malaria and an

immunostimulatory AT-rich DNA motif that activates an unidentified nucleic acid sensor. It remains to be seen if cGAS is that receptor or if there is another.

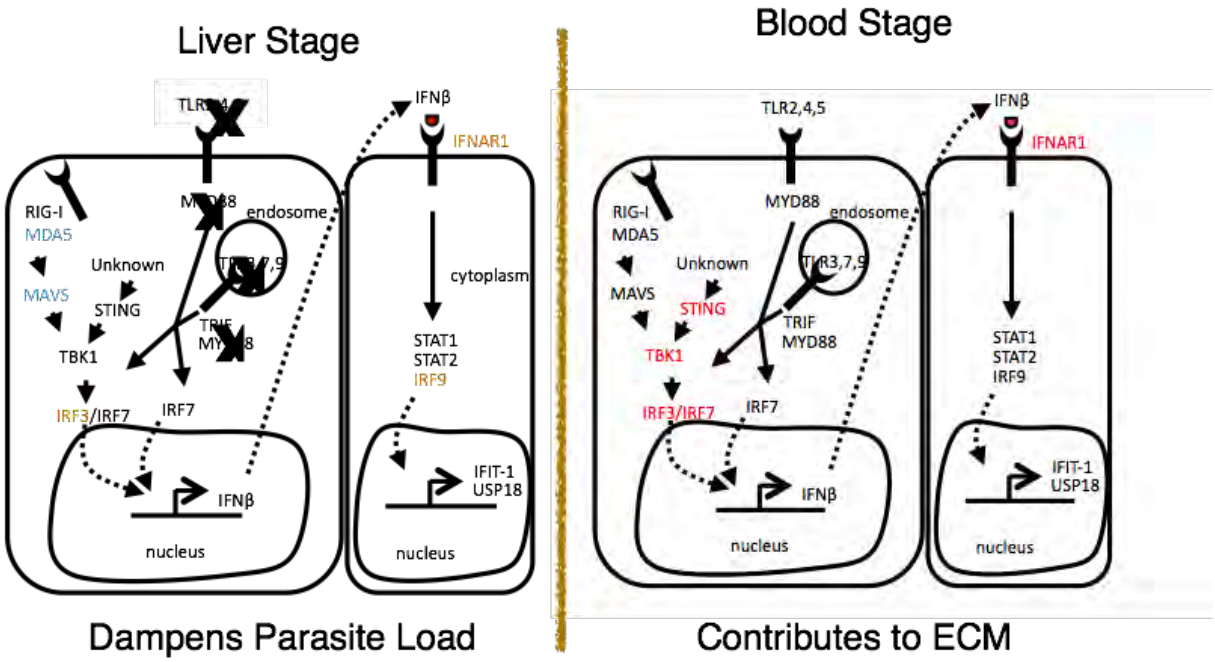

Figure 4.3. Schematic for innate immune detection during liver and blood stage malaria infection. Upon infection by sporozoites into liver hepatocytes, presumably RNA (from either host or parasite) activates the RLR, MDA5 and the adaptor MAVs leading to activation of the transcription factor IRF3, and to a lesser degree, IRF7 and transcription of type I IFNs. This leads to activation of IFNAR1 for the production of ISGS like IFIT1 and USP18. Detection of *Plasmodium* spz leads to dampened parasite load. However, any spz that matures into blood-infecting merozoites, will be phagocytosed by circulating macrophages as an iRBC and triggers a cytosolic nucleic acid sensor leading to activation of STING/TBK1, IRF3/7 and IFNAR. This signaling cascade during systemic infection contributes to increased inflammation and ultimately the manifestations of ECM that lead to death. This signaling is triggered in part, by AT-rich DNA and hemozoin.

The convergence of genetics, parasite, kinetics of immune responses

It is hypothesized that malaria transmission began roughly 6,000 years ago based on DNA sequencing from parasites of infected mosquitoes traced over the African continent (Su, 2014). Speculations are based on the emergence of parasite population, but contradict other estimates of 100,000-400,000 years. Regardless, the conclusion is that *Plasmodium* has been a strong driver of human disease and people that inherited genes that were anti-*Plasmodia*l became a strong selective advantage in areas where Plasmodium was endemic. For example, we know that humans that carry sickle-cell are much more prevalent in *Plasmodium* endemic regions (Aidoo et al., 2002) and those people confer protection against malaria. Other studies have linked genetics that confer protection to malaria are also more disposed to autoimmunity (Daniel-Ribeiro and Zanini, 2000). In these cases, perhaps the inflammatory environment is protective, similar to having a prophylactic treatment prior to infection. But there have been reports that autoimmune patients with SLE make antibodies that can cross-react to *Plasmodium* antigens (Zanini et al., 2009). It could be interesting to see if other genes linked to autoimmunity would be involved in mitigating *Plasmodium* infections. However, this parasite is complicated – with 14 chromosomes, and multiple life stages, it's very likely that this pathogen has and will continue to evolve with us. It is important to understand how and what the inflammatory triggers of disease are, in order to develop new therapeutics and potentially find the vaccine cure, but in the immediate future, the best therapies

are within the public health sector, utilizing bednets and public education outreach for improved diagnostics and upgrading infrastructure.

Appendix I: AS-IL1α sequences

***Annotated cDNA sequence from ENSEMBL*:**

Predicted Gene Gm14023: ENSMUSG00000085498

Novel processed transcript

ACTTCCGCAGCGGTGCCTCTGAGGTGGGAGCTGGTCACAGGAAAGCAGCT
TCTAACCTGCTGTATTGTCTCTAAAACCACTACTTGGTTTTGGTGGTACATTA
TATGGGTCTCATGATGGAGCCCTGGCAGGCTTGGGATTCACTTGACCGACA
AGAATAGACTTGGAATCACAAAGATTCTCCTGCATCTACTTCCCACCGGGAA
GCAAGTTCCCAAGTGACAAGGAAGACTACCAGTATTCCCAGTCTCTAATTCA
GATGAGTATATGCTAAGTATGGGGAACTGAAGCCCAGAGAGATTAAGGAAC
ATGACTAAGGCGGTGGCAGCTAGCAGAGGTCATGAGCCAATGAACCACGG
CTGCTTTCTCTCCAACAGAAGACCTTTACATGACCTTTGCAGTATGGCCAAG
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GTCTCACTGAAACTCAGCCGTCTCTTCTTCAGAATCTTCCCGTTGCTTGACG
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CCTGCAGGCTTAGTGAGAGTGGTCCCCATTGTGCTTCCTCCCTCCCTCCCT
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CTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCCCACTGCATTCC
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GTATTGTTAGGTCACCTAGATATGATTTAAAGTCATTTGAGAATGCATATATG
CTTGGGACTTAAACATCTGATTCTAAGCTCCCATGGATGCCTTTAGTATGGC
TGTTCTTCTGGAAGTAGCTTTTAAAGCTAGTATTGCAGATACACAAAATGACA
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ACCTCAGTTCTGTGGGCTATCTGGTTATCTCTAGCCTCTAGTCTCTTAGGAG
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GTTATGAAAGAGCCAATGAACATGAAGAATGCCATTTAACTACTAGTATGAA
GAACCAAGTGATCTCATTTATAAAGAGTAAATGTATTTATATCTCTAGTGAGG

GTGAGACATAAGTATGTACTACTCAGTTCTCATTGTGCTTTTCTGGTAGGTG
AACTAGTGTGG

Exon breakdown from ENSEMBL database:

ENSMUST00000144178 ENSMUSE00000837831 exon1:
ACTTCCGCAGCGGTGCCTCTGAGGTGGGAGCTG

>ENSMUST00000144178 ENSMUSE00000735388 exon2:
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GGTTTTGG TGGTACATTATATG

>ENSMUST00000144178 ENSMUSE00000742797 exon3:
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GTTCCCAAGTGACAAGGAAGACTACCAGTATTCCCAGTCTCTAATTCAGATG
AGTATATGCTAAG

>ENSMUST00000144178 ENSMUSE00000761765 exon4:
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CAGCTAGCAG
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CATGACCT TTGCAGTATGGCCAAGAAAGGAGTCCTTGA

ENSMUST00000144178 ENSMUSE00000744940 exon5:
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AGCCGTCTCTTCTTCAGAATCTTCCCGTTGCTTGACGTTGCTGATACTGTCA
CCCGGCTCTCCTTGAAGGTGAAGTTGGACATCTTTGACGTTTCAGAGGTTCT
CAGAGATACAAACTGTCTGTGCAAGTCTCATGAAGTGAGCCATAGCTTGCAT
CATAGAAGGATTTCTGTAAGGAGGAAAAGTGGAACCTGAGAAGTTATCTATG
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CTAGATATGATTTAAAGTCATTTGAGAATGCATATATGCTTGGGACTTAAACA
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GCTTTTAAAGCTAGTATTGCAGATACACAAAATGACATAACTTGGTGAGTGT
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GGTGGGCTGAAGCAACTGGCTAGGGAGGACAAAATTAACATTTTGAAGAAA
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GAACATGAAGAATGCCATTTAACTACTAGTATGAAGAACCAAGTGATCTCAT
TTATAAAGAGTAAATGTATTTATATCTCTAGTGAGGGTGAGACATAAGTATGT
ACTACTCAGT TCTCATTGTGCTTTTCTGGTAGGTGAACTAGTGTGG

Note: Only Exon 2 through 5 was confirmed through primer walking experiments, using designed primers, PCR, running amplicons on 1% agarose gel, followed by transformation using DH5α competent bacteria and sequencing positively selected clones through Genewiz.

qRT-PCR primers are highlighted in orange.

Regions of AS-IL1α (nt 498-647) comoplimentary to IL-1α indicated in red.

Appendix 2: Generating an LPS-stimulated human dendritic cell transcriptome

Preface to Appendix 2

* Library preparation was done in collaboration with Zhaozhao Jiang

Introduction

RNA-sequencing allows the annotation of all expressed transcripts, which includes determining a gene's splice junctions, quantifying expression levels of individual transcripts, idenfitying novel or rare transcripts, as well as give clues about alternative splicing of each gene. In **Figure A2.1**, I address the 5 steps involved in a typical RNA-sequencing (RNA-seq) experiment. In the first step, we must consider the question we want to address and the purpose of the RNA-seq library. In our case, we are interested in studying the role of long noncoding RNAs in the inflammatory immune response. We wanted to investigate the inducible immune system upon challenge by a potent inflammatory stimulus like lipopolysaccharide (LPS). Other labs have successfully deposited transcriptomes from murine CD11c+ dendritic cells in response to LPS (Guttman et al., 2009) or murine macrophages stimulated with Lipid A over zero to 24 hours in specific subcellular fractions (Smale et al., 2012). Each one of these data sets has produced an explosion of data that give specific insights into what genes are part of which inducible wave of transcription and how these genes may regulate or be regulated during an active inflammatory response.

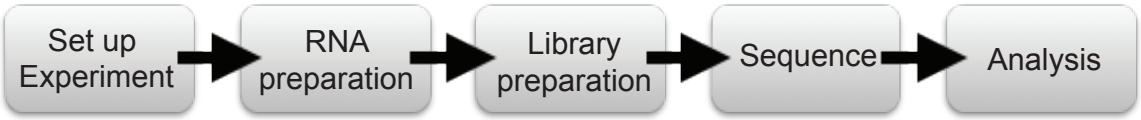


Figure A2.1. The workflow to produce an RNA-seq library.

One of the major questions our lab has been interested in is how long noncoding RNAs (lncRNAs) are involved in these pathways. However, it is problematic to identify lncRNAs in murine cells that are absolutely involved in the human inflammatory response due to the non-sequence conservation of lncRNAs from species to species. One reason is that many lncRNAs contain repeat elements that are species specific. Another is the reigning hypothesis that because regulatory RNAs exert much of their function via secondary or tertiary structures that are difficult to identify, their primary sequence is less likely to undergo evolutionary pressure for conservation. Unlike proteins where single nucleotide changes can result in deleterious translational effects leading to truncated or null proteins, single nucleotide mutations in RNAs are unlikely to be as detrimental to the functional transcript. Ultimately, in order to study lncRNAs in human health, we wanted to directly use human immune cells, but to do it in a manner that would allow us to directly compare and contrast with the existing murine datasets. In steps two through five of the workflow, building an adequate RNA-seq library requires validation and quality control for RNA quality proper selection of RNA species in order to minimize the noise associated with the technological limitations that still exists with RNA-sequencing data. In this appendix, I will go

over our workflow and explain what we ultimately did in order to guide a potential RNA-seq performer in the future.

Materials and Methods

Dendritic cell culture. About 60 ml whole blood was harvested from three human healthy donors (NY biologics). In LeucoSep tubes (Greiner Bio One), 15 ml Lymphoprep solution (Stemcell Technologies) was added and centrifuged at 2000 RPM, 30 seconds, room temperature (RT). The whole blood was diluted 1:3 in phosphate buffered saline (PBS). The more dilute the blood is, the easier it will be to remove the buffy coat where the peripheral blood mononuclear cells (PBMCs) are. The diluted blood is added to the leukosep tubes and centrifuged at 2000 RPM, 25 minutes, RT, without brakes. Remove the buffy coat and centrifuge 2000 RPM, 5 minutes, 4*C, with brakes. RBCs were lysed using RBC lysis buffer (Sigma R7757), resuspended and incubated 5 minutes on ice and centrifuged again as the previous settings. CD14+ monocytes were enriched from PBMCs by positive selecting using CD14 MicroBeads (Miltenyi Biotec 130-050-201) and MACS LS separation columns (Miltenyi Biotec 130-042-401), with MACs pre-separation filter (Miltenyi Biotec 130-041-407). Cells were plated using RPMI medium containing containing 25 mM HEPES, 5 ml NEAA (100X), 5 ml GlutaMax (100X), 1 mM Na-Pyuvate Penicillin-streptomycin (Invitrogen), 5% human serum (Omega HS-20 Heat inactivated at 55*C for 30 minutes and filtered with 0.22 uM) and 1:25 human GM-CSF + 1:50 human IL-4 for 10 days.

Cytokines. hGM-CSF or hIL-4 was produced in HEK 293 cells transduced with a lentiviral vector pAIP encoding either cytokine as described in (Reinhard et al., 2014).

Flow cytometry. Cells were plated at $1x10^6$ cells per antibody stain in a v-bottom 96-well plate and centrifuged at 400g, 5 minutes, 4*C. Media was removed and cells were resuspended in FACS buffer (PBS+2%FCS). We used the following antibodies: phycoerythrin (PE) anti-CD11c, allophycocyanin (APC) anti-DC-Sign, and fluorescence minus one (FMOs) for gating. Cells were acquired on a LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Gene expression analysis. RNA isolated from human CD14+ monocytes or DCs treated with LPS (100 ng/ml) for the indicated time points were harvested into RLP buffer containing 2-mercaptoethanol for subsequent processing with the RNease Mini kit (Qiagen). Each RNA sample was adjusted to contain the same quantity by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA was then reverse transcribed (Biorad) into cDNA for subsequent qRT-PCR analysis, or 100 ng was used to be hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies) per the manufacturer's protocol. The gene expression data first were normalized to an internal positive control set, then to an internal negative control set, and then to seven

housekeeping genes. All values were log10-transformed, and a heatmap was generated via http://anto.umassmed.edu/~adam/heatmap/enterInformation.php. qRT-PCR from above was performed using SyBr Green PCR Master mix (BioRad) with the following primer pairs: HPRT, forward, 5'-ATCAGACTGAAGAGCTATTGTAATGA-3', reverse, 5'-TGGCTTATATCCAACACTTCGTG-3'; TNF-a, forward, 5'-CCTCTCTCTAATCAGCCCTCTG-3', reverse, 5'-GAGGACCTGGGAGTAGATGAG-3'; IL-6, forward, 5'-TCTCCACAAGCGCCTTCG-3', reverse, 5'-CTCAGGGCTGAGATGCCG-3'.

RNA quality. 2-3 ul of RNA was submitted to the UMass Molecular Biology Core lab that operates an Agilent lab-on-chip system. Cloning was performed on the end point cDNA library samples. pGEMT cloning vector was used (Promega).

RNA-sequencing library. Illumina TruSeq stranded total RNA with RiboZero kit was purchased (Illumina RS-122-2201)

Results

In order to look at the inducible immune response in dendritic cells, we took 3 human healthy donor blood, harvested their PBMCs, and differentiated those cells into CD14+ dendritic cells (DCs). Once these cells were differentiated, as confirmed by flow cytometry (**Figure A2.2**), around 95% were found to be

positive for the DC markers, CD11c and CD-Sign as compared to CD14+ monocytes harvested together.

We then plated the cells and stimulated them with the Gram-negative bacteria cell wall TLR4 agonist, lipopolysaccharide (LPS). Over a time course, 0, 0.5, 1, 2, 4, 6 and 24 hours, we harvested the cells for RNA, reverse transcribed into cDNA and performed qRT-PCR on the DCs from the three different donors. Surprisingly, the three donors had quite distinct kinetics and absolute expression levels for the cytokines, TNF-α and IL-6. Interestingly, these discrepancies reveal potential genetic differences that account for the donor cells' ability to respond to LPS (**Figure A2.3**). We decided to use all three donors' as biological triplicate PBMCs to perform an RNA-seq and compare their different transcriptomes. Before we did that, we performed a Nanostring analysis (**Figure A2.4** and **Table A2.1**) on these donors' RNA and saw that although their gene expression levels differed, the kinetics of the genes expressed more or less corroborated what was in the literature in regards to early and late response genes (Smale et al., 2012).

In order to build an RNA-seq cDNA library, we must start with high quality RNA. In order to determine that our samples had intact RNA, we ran all of our samples through a Bioanalyzer (UMass Core facility), which can indicate the RNA integrity of the samples and whether or not there is contaminating DNA (**Figure A2.5a and b**) and only the 18S and 28S rRNA peaks should be visible. A typical RNA integrity number (RIN) of 8 or higher should be useds as a general

guideline for good quality RNA. Once the libraries were made, the samples are subjected to a Bioanalyzer analysis again to determine the quality of RNA at the end of the preparation. The libraries should have fragment sizes about 270-350 nucleotides long (**Figure A2.6**) compared to the average RNA fragment size prior to preparing the library at about 1500-2500 nucleotides long (**Figure A2.5**).

Discussion

Once the transcriptomes of these three donors' dendritic cells are profiled, we can utilize this data set to ask many questions regarding the transcriptional dynamics involved in LPS signaling in human cells. Comparative genomics would be a useful analysis to determine if murine lncRNAs are conserved across species in humans. We can also compare the three different donors' genome and differential expression of various genes that may contribute to their overall differences in proinflammatory cytokine expression levels. There are many avenues in which we could utilize these libraries and will generally be a good tool for the scientific community to have access to.

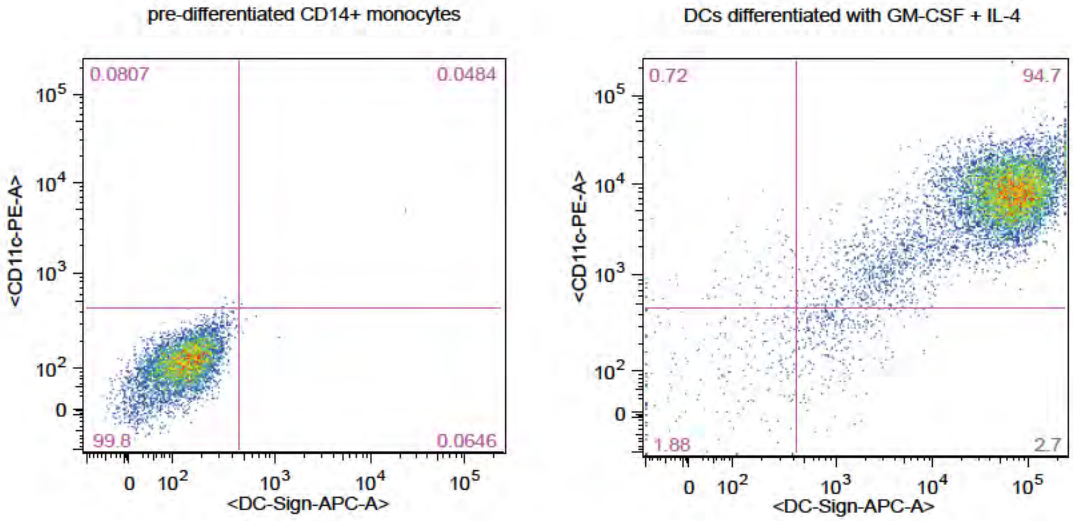


Figure A2.2. Flow cytometry scatter plot of pre-differentiated and differentiated DCs. Cells showing here have been gated for live, singlet populations and are comparing the pre-differentiated CD14+ monocytes, which are CD11c-, DC-Sign- compared to 94.7% of the differentiated CD11c+ DC-Sign+

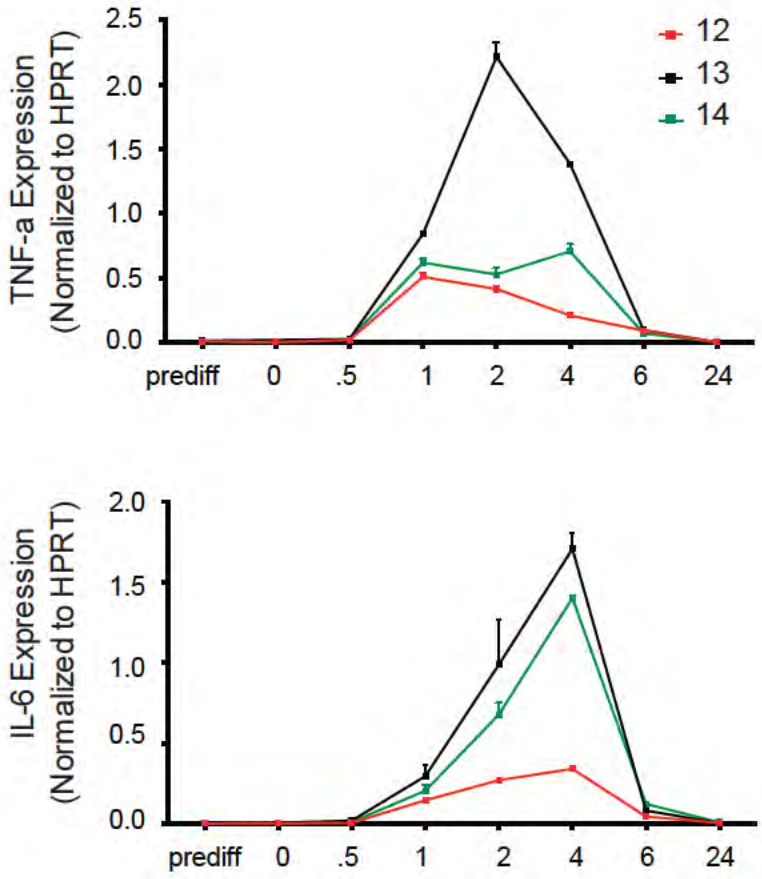


Figure A2.3. Kinetics of TNFα and IL-6 in three human donor DCs. Although the kinetics of expression for the three different human donors' DCs were similar, the absolute level of expression between the donors are drastically different. These differences may be due to the donor's genome differences or perhaps epigenetic changes may be at play.

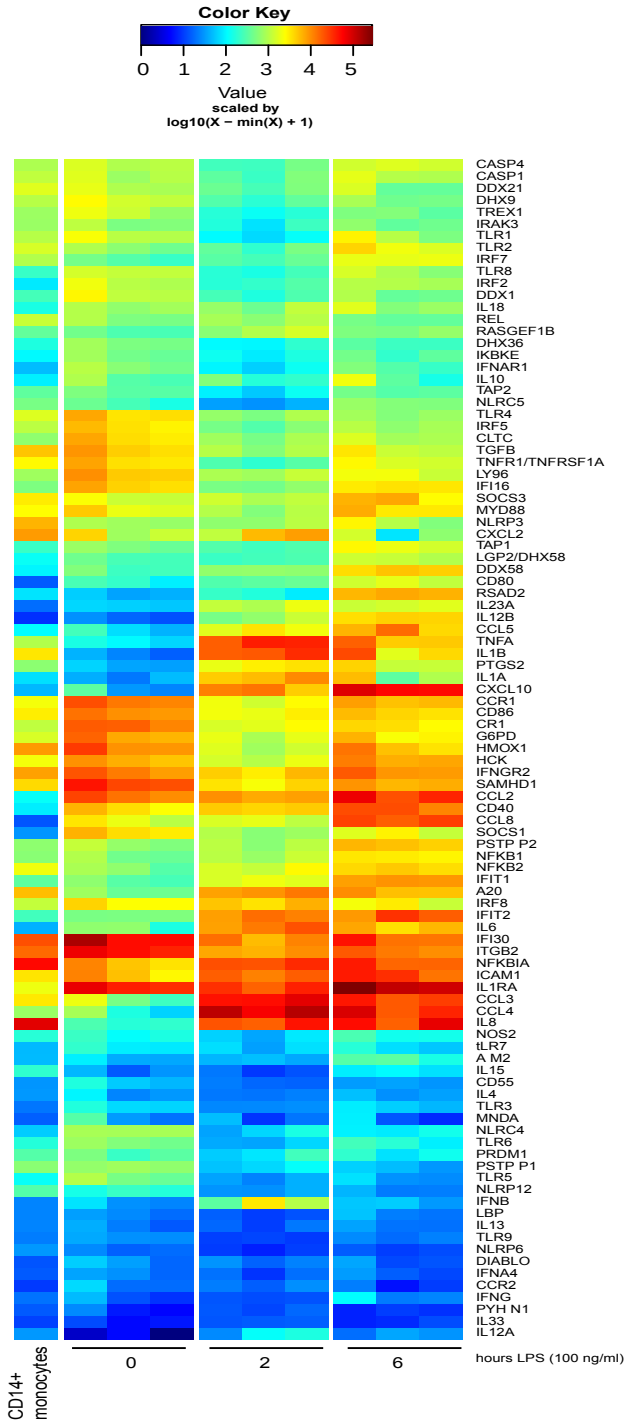


Figure A2.4. Gene expression changes from dendritic cells from 3 donors stimulated with LPS at various time points.

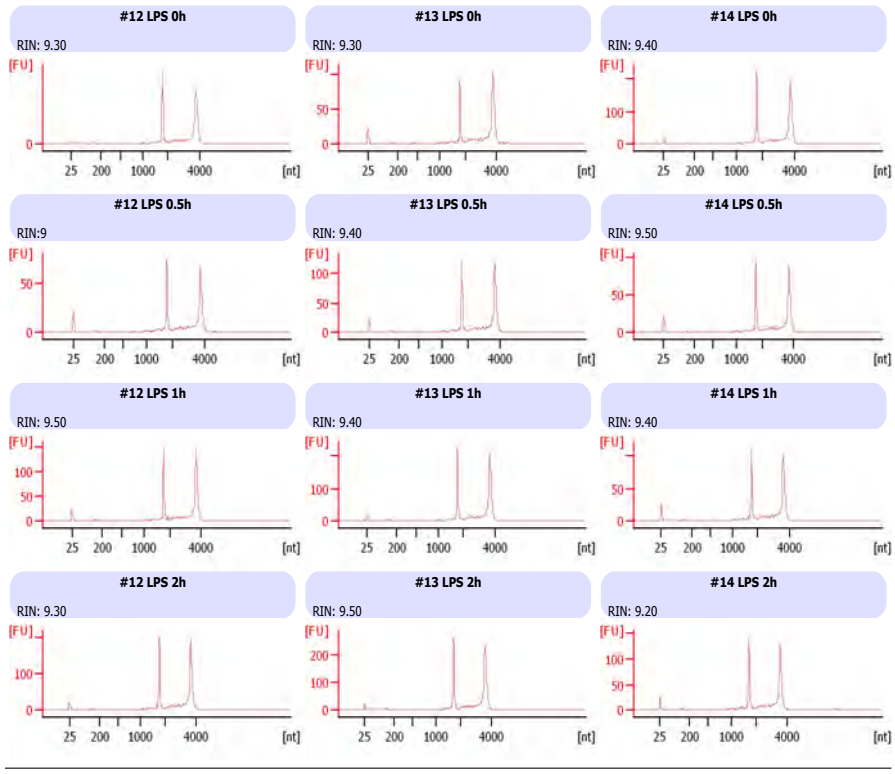
	monocytes #12	LPS 0h #12	LPS 0h #13	LPS 0h #14	LPS 2h #12	LPS 2h #13	LPS 2h #14	LPS 6h #12	LPS 6h #13	LPS6 h #14
A20	5559	778	385	410	7927	9314	13178	10142	5398	5303
AIM2	38	82	29	28	35	41	28	308	332	139
CASP1	1170	1749	742	1049	341	214	546	1861	1026	947
CASP4	931	1645	964	1099	239	229	473	1428	1698	1430
CCL2	108	25369	14337	10182	9812	7156	8070	71054	21659	36839
CCL3	3258	2030	473	225	47514	50233	80413	44160	19169	27327
CCL4	727	858	148	55	130857	62937	141676	92089	19264	37670
CCL5	99	242	64	33	1735	3481	2174	6401	15559	4020
CCL8	3	3394	2010	1077	1599	1872	1116	25654	18012	27077
CCR1	2246	22141	13163	11166	2154	1412	2625	8216	5273	5918
CCR2	-1	60	8	8	12	5	18	13	-5	0
CD40	80	6270	3835	2491	4767	4162	4775	22742	22696	11169
CD55	20	152	48	36	12	7	6	23	26	20
CD80	4	259	201	82	289	336	392	1404	1870	1173
CD86	3076	14258	9824	8769	2200	1968	3092	5770	4212	3517
CLTC	640	7488	3818	3108	787	473	917	1659	815	915
CR1	1156	18725	17796	11254	1558	1781	2627	4099	3753	2627
CXCL10	36	337	21	15	11757	13879	4671	85620	56261	52864
CXCL2	8502	4265	778	1327	1168	5857	8361	1483	71	636
DDX1	247	2792	1163	1080	249	151	282	984	369	418
DDX21	1749	1897	955	881	406	253	530	1615	365	371
DDX58	89	545	222	242	670	678	642	3814	5239	4545
DHX36	149	820	503	474	96	91	187	328	221	220
DHX9	1048	2627	1452	1194	296	175	359	885	427	459
DIABLO	3	50	24	7	20	6	14	27	1	3
G6PD	1578	17075	7250	6215	1930	892	1889	6359	2348	2873
HCK	2230	9686	6768	5112	1502	1026	2012	12439	6757	7495
HMOX1	8627	28746	9492	9086	1713	788	1413	13866	5322	3551
ICAM1	3450	11724	5411	2719	18016	11785	18394	42370	32676	13830
IFI16	511	7677	4382	3649	487	450	663	3191	3596	3362
IFI30	21877	162581	50425	51408	14842	5680	11505	47024	14189	13661
IFIT1	346	633	316	255	1585	2075	1758	8078	9458	8946
IFIT2	239	482	497	521	8255	14911	11753	8699	31614	18289
IFNA4	4	26	19	7	9	-1	9	23	5	1
IFNAR1	39	1069	590	434	89	52	117	546	302	207
IFNB	14	70	19	15	336	3272	1024	45	50	22

IFNG	9	38	3	-1	4	2	3	99	12	15
IFNGR2	8009	20615	12771	8304	4588	3062	6101	19164	9027	8551
IKBKE	94	810	448	384	120	78	147	448	193	351
IL10	81	963	309	262	552	164	193	2146	343	136
IL12A	21	-8	-5	-9	16	107	147	9	27	20
IL12B	-2	19	7	3	471	667	1321	3720	4463	4284
IL13	14	31	12	4	7	0	11	26	9	9
IL15	188	36	4	20	11	-1	3	81	97	67
IL18	142	1019	657	822	698	407	1194	1757	551	729
IL1A	66	31	11	39	4738	5710	10502	5678	338	968
IL1B	3361	33	15	5	18202	19468	33714	23507	1790	3993
IL1RA	2045	74598	40455	32690	32072	17259	39051	280798	121598	102958
IL23A	8	58	52	45	1205	938	2068	1301	1474	1772
IL33	0	2	-5	-4	4	5	5	-3	-2	2
IL4	21	89	15	21	11	10	13	41	18	25
IL6	32	638	657	146	7978	12828	21201	7555	3628	6055
IL8	81621	276	167	190	20654	17667	46266	50558	17530	76645
IRAK3	725	1137	499	549	157	68	225	661	357	428
IRF2	76	2144	1064	937	168	199	306	1037	1017	855
IRF5	1114	5840	3646	2896	465	298	597	1061	633	862
IRF7	992	456	303	210	322	249	390	1934	1873	2036
IRF8	1216	4350	2488	2558	5066	3513	6601	2242	3157	1277
ITGB2	15745	70187	50550	38083	7405	6395	10051	19529	12835	9982
LBP	14	24	16	8	5	0	3	44	12	10
LGP2/DHX58	112	424	258	245	215	242	274	1371	1277	974
LY96	776	9941	4811	4572	918	786	1274	2186	2206	1292
MNDA	5	308	23	9	40	-1	10	82	4	-2
MYD88	2547	4906	1977	1643	1010	546	1068	6961	3243	3279
NFKB1	597	989	440	394	1081	709	1326	3365	3170	2837
NFKB2	2170	895	647	306	1585	1224	2680	4024	4905	3780
NFKBIA	53306	11329	5089	3534	21833	20909	34854	42420	16415	16845
NLRC4	49	875	851	874	26	58	142	85	77	129
NLRC5	481	405	251	139	24	19	33	723	557	527
NLRP12	302	135	211	163	20	19	32	35	12	13
NLRP3	6319	953	783	698	642	647	1087	2813	1037	537
NLRP6	21	16	5	8	0	-3	0	5	0	2
NOS2	169	215	104	146	53	27	76	266	125	131
PRDM1	304	507	210	329	49	73	237	190	68	123

PSTPIP 1	605	676	783	651	43	59	106	53	40	21
PSTPIP 2	632	1242	683	531	1107	593	1102	6149	5333	4445
PTGS2	615	55	27	24	1952	3028	3578	4300	1243	1279
PYHIN1	4	16	-4	-5	4	1	7	-3	0	-2
RASGE F1B	365	441	279	261	597	1003	1550	516	482	680
REL	1295	1016	515	477	859	605	1053	465	361	365
RSAD2	70	50	25	32	205	157	79	6078	7356	6460
SAMHD 1	3973	45812	24473	21811	3476	2329	4304	9256	6069	6885
SOCS1	20	6427	3817	3145	1009	586	758	1719	2913	1238
SOCS3	3163	2431	1328	1260	1414	912	1178	6375	7421	2594
TAP1	211	732	523	390	297	235	284	2759	2004	1457
TAP2	353	516	320	318	82	47	118	452	305	340
TGFB	5103	9172	4595	3696	1041	556	1016	3392	1306	1121
TLR1	1036	2360	1078	1004	97	61	108	2916	1053	503
TLR2	1567	953	608	418	358	188	491	4277	2146	1636
TLR3	10	155	61	56	16	16	18	81	51	36
TLR4	1683	7378	3465	3746	683	496	962	814	553	733
TLR5	111	1019	478	384	26	14	32	68	19	17
TLR6	162	756	542	436	29	26	58	240	174	81
TLR7	37	194	81	76	65	24	66	166	65	45
TLR8	207	1504	1229	1196	171	141	246	1560	935	576
TLR9	12	29	19	17	0	1	-1	17	8	13
TNFA	994	143	96	57	18179	41007	38343	17588	4691	4904
TNFR1	2671	7667	3687	3275	282	183	314	2705	1599	1443
TREX1	744	2091	1334	663	167	115	168	519	549	330

Table A2.1. Nanostring analysis of human innate immune genes on three donors' dendritic cells.

A



B

C

Sample Name	Sample Comment	Status	Result Label
#12 LPS 0h		✓	RIN: 9.30
#13 LPS 0h		✓	RIN: 9.30
#14 LPS 0h		✓	RIN: 9.40
#12 LPS 0.5h		✓	RIN:9
#13 LPS 0.5h		✓	RIN: 9.40
#14 LPS 0.5h		✓	RIN: 9.50
#12 LPS 1h		✓	RIN: 9.50
#13 LPS 1h		✓	RIN: 9.40
#14 LPS 1h		✓	RIN: 9.40
#12 LPS 2h		✓	RIN: 9.30
#13 LPS 2h		✓	RIN: 9.50
#14 LPS 2h		✓	RIN: 9.20
Ladder		✓	All Other Samples

Figure A2.5. **RNA integrity Analysis by Bioanalyzer**.(A) Traces indicate average RNA length around 1500-2500 nucleotides and shouldn't be too fragmented. (B) Only the 18S and 28S rRNA bands are visible and (C) RIN 8+ should be used for successful library prerparation.

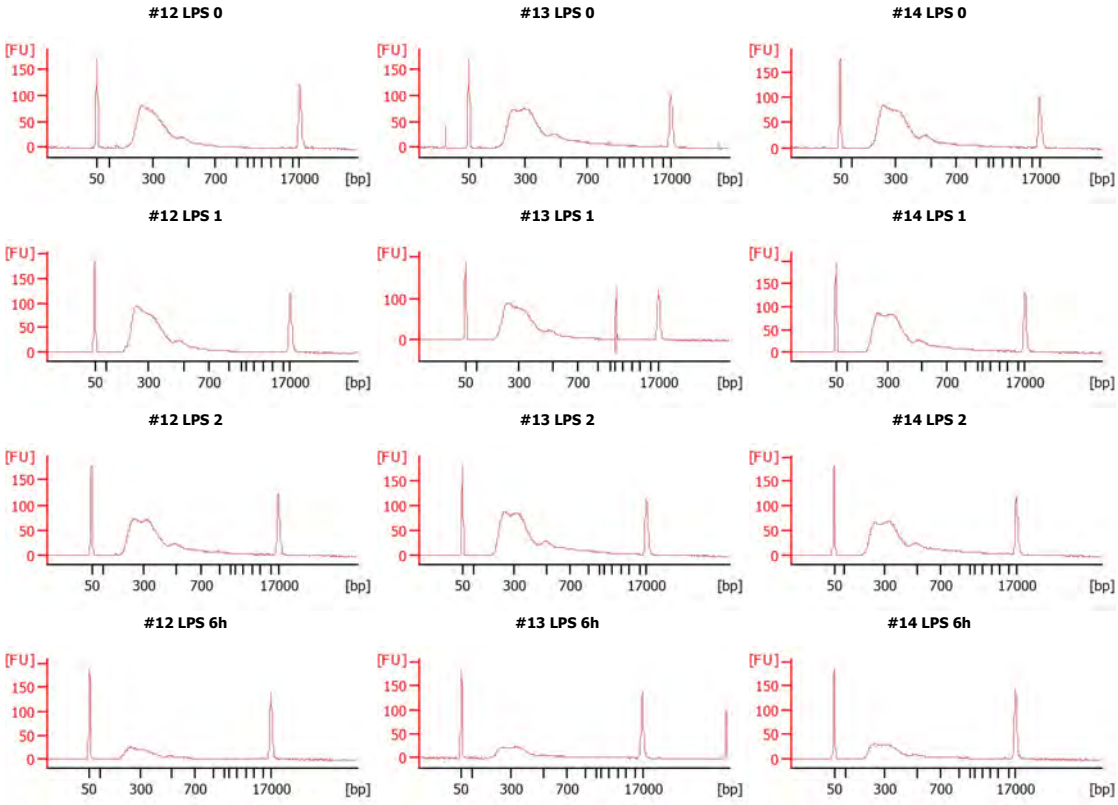


Figure A2.6. **Confirmation of correct fragment size after library preparation**. After cDNA library is prepared, the average transcript length should be approximately 270-300 basepairs. These bioanalyzer traces confirm size selection was properly performed.

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