2012-07-10

RNA-Sensing Pattern Recognition Receptors and Their Effects on T-Cell Immune Responses: A Dissertation

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RNA-SENSING PATTERN RECOGNITION RECEPTORS AND THEIR EFFECTS ON T-CELL IMMUNE RESPONSES

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JULY 10, 2012

IMMUNOLOGY AND VIROLOGY

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Program in Immunology and Virology
July 10, 2012

ACKNOWLEDGEMENTS

I would like to thank the people who made it possible for me to attend graduate school. Thank you to my mentor, Dr. Dan Libraty for having me as his graduate student. This would not be possible without your tutelage and support. Thank you to my QE, TRAC and DEC committee members. Dr. Kate Fitzgerald and Dr. Evelyn Kurt-Jones have been members in all my committees. Thank you for your continuous guidance. To Dr. Eva Szomolanyi-Tsuda and Dr. Lyn Schmidt, I really appreciate all the helpful suggestions. I am very grateful to Dr. Anuja Mathew who taught me how to handle mice. She also guided me in my thesis writing. I would like to thank Dr. Jennifer Wang for the valuable techniques and discussions. Thank you to the Rock, Fitzgerald, Kurt-Jones and Finberg laboratories for the knockout mice. I also would like to thank the Finberg and Fitzgerald lab members for welcoming me in their lab meetings.

I would like to thank my CIDVR family and my fellow graduate students. Thank you to my labmates, Kris, Lei and Chinmay. Thanks for all the unforgettable moments and the memories.

To my Boston Filipino family, thank you for being my family here in MA.

To Gen, thank you for encouraging me to apply at UMass.

To my family, thank you for everything.

To my twin sister, thank you!

ABSTRACT

Virus infection is sensed by the innate immune system through germline encoded pattern recognition receptors (PRRs). Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) serve as PRRs that recognize different viral components. Microbial nucleic acids such as Ribonucleic acid (RNA) are important virus-derived pathogen-associated molecular patterns (PAMPs) to be recognized by PRRs. Virus recognition may occur at multiple stages of the viral life cycle. Replication intermediates such as single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) are detected by the RNA-sensing PRRs that initiate innate and adaptive immune responses. Triggering of the innate immune system is a critical event that can shape the adaptive immune response to virus infection. Better vaccination strategies that lead to improved T-cell and antibody responses are needed for protection against pathogens. We sought to delineate the RNA-sensing PRR pathways that are activated during infection with an RNA virus, the signaling mediators involved and the influence on subsequent virus-specific adaptive immune responses.

To analyze the role of RNA-sensing PRRs in T-cell immune responses *in vitro*, we performed direct co-stimulation experiments on CD4+ T-cells of high purity. We utilized synthetic RNA-like immune response modifiers (IRMs) R-848 (MyD88-dependent) and poly I:C (MyD88-independent) as RNA PAMPs to

determine the direct effects of RNA-sensing PRR activation on CD4+ T-cells. RNA PAMPs can act directly on CD4+ T-cells and modulate their function and phenotype. Maximal direct co-stimulatory effects were observed in CD4+ T-cells cultured with poly I:C compared to R-848. The cytoplasmic dsRNA-dependent protein kinase R (PKR) was also involved in poly I:C-mediated signaling in CD4+ T-cells.

We found differences in the RNA-sensing PRRs activated by R-848 between mouse and human CD4+ T-cells. We observed minimal direct co-stimulatory effects by R-848 in mouse CD4+ T-cells. In contrast, augmentation of Th1 responses by R-848 was observed in human CD4+ T-cells. TLR8 activation in human CD4+ T-cells may explain the observed differences.

We next explored the signaling pathways activated by RNA PAMPs in conventional dendritic cells (cDCs) and CD4+ T-cells that drive Th1 CD4 T-cell responses in isolated cDC/CD4 T-cell interactions. Allogeneic cDCs and CD4+ T-cells of high purity were cultured together with R-848 and poly I:C in MHC congenic mixed leukocyte reactions (MLRs). R-848 and poly I:C stimulation of type I IFN production and signaling was essential but not sufficient for driving CD4+ Th1 responses. The early production of IL-1α and IL-1β was equally critical.

To analyze the role of RNA-sensing PRRs in T-cell immune responses *in vivo*, we utilized a mouse model of heterosubtypic influenza A virus (IAV) infections. Using MyD88$^{-/-}$, TLR7$^{-/-}$ and IL-1-deficient mice, we explored the role

of MyD88-signaling in the generation of heterosubtypic memory CD4+ T-cell, CD8+ T-cell and antibody responses. We found that MyD88 signaling played an important role in anti-IAV spleen and lung CD4+ T-cell, spleen CD8+ T-cell and Th1 antibody immune responses. Anti-IAV lung heterosubtypic CD8+ T-cell responses were not dependent on MyD88 signaling.

Our *in vitro* and *in vivo* results show the pivotal role of RNA-sensing PRR pathway activation in T-cell immune responses. Understanding the complexity of the PRR pathways involved during viral infections and defining the subsequent immune response would have important implications for the generation of more effective vaccine strategies.

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ABBREVIATIONS

1^o	primary
2^o	secondary
Ab	antibody
APC	allophycocyanin
APC	antigen presenting cell
ASC	apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain
B6	C57BL/6J
B6.H2d	MHC congenic *H2*^{d} mice, B6.C-H2d/bByJ
cDC	conventional dendritic cell
CFSE	Carboxyfluorescein succinimidyl ester
DAMPs	damage-associated molecular patterns
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
H#, HA	hemagglutinin
HK/X31	Influenza A virus A/HK/X31
ICS	intracellular cytokine staining

IFN	interferon
Ig	immunoglobulin
IAV	Influenza A virus
IFNAR	type I IFN receptor
IL	interleukin
IL-1R	interleukin-1 receptor
i.n.	intranasally
IPS-1	Interferon-β promoter stimulator 1
IRM	immune response modifier
LDA	Live/Dead Aqua
LRR	leucine-rich-repeats
M	matrix
MACS	magnetic-activated cell sorting
MDA5	melanoma differentiation associated protein 5
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MMTV	mouse mammary tumor virus
MyD88	Myeloid differentiation primary response gene 88
N#, NA	neuraminidase
NACHT	neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E
NIH	National Institutes of Health
NLR	NOD-like receptor

NLRP3	NACHT-LRR-PYD-containing protein 3
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NP	nucleoprotein
Poly I:C	polyinosinic:polycytidylic acid
PA	polymerase subunit of the polymerase complex heterotrimer
PAMPs	pathogen-associated molecular patterns
pDC	plasmacytoid DC
PFU	plaque forming units
PKR	protein kinase R/protein kinase dsRNA-dependent
PR/8	Influenza A virus A/Puerto Rico/8/34
PRR	pattern recognition receptor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PYD	pyrin domain
qRT-PCR	quantitative RT-PCR
R-848	resiquimod
rFlt3L	recombinant fms-related tyrosine kinase 3 ligand
RIG-I	retinoic-acid-inducible protein I
RNA	ribonucleic acid
rpm	revolutions per minute

RPMI	Rosewell Park Memorial Institute cell culture medium
RSV	respiratory syncytial virus
RT-PCR	real time, reverse transcription polymerase chain reaction
SEM	standard error of the mean
sIL-1Ra	soluble IL-1 receptor antagonist
SSC	side scatter
Th1	T-helper type 1
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumor necrosis factor-α
TRIF	TIR-domain-containing adapter-inducing interferon-β

PREFACE

Parts of this thesis have appeared in separate publications:

Chapter IV:
Madera R, Wang J, Libraty D (2011). The Combination of Early and Rapid Type I IFN, IL-1α, and IL-1β Production Are Essential Mediators of RNA-Like Adjuvant Driven CD4+ Th1 Responses. *PLoS ONE* **6**(12): e29412.

Chapter V:
Madera R, Libraty D (2012). The Role of MyD88 Signaling in Heterosubtypic Influenza A Virus Infections. *Submitted for publication.*

Other work performed during thesis studies that is not presented in this dissertation has appeared in a separate publication:

Wang J, Zhang L, Madera R, Woda M, Libraty D (2012) Plasmacytoid Dendritic Cell Interferon-alpha Production to R-848 Stimulation is Decreased in Male Infants. *BMC Immunology.* **13**(1):35.

CHAPTER I:

INTRODUCTION

Over the past decade, there has been rapid advancement in our knowledge about the contributions of the innate immune system to the recognition of infection with RNA viruses. This initial host response is mediated by pattern recognition receptors (PRRs). These receptors are encoded by genes in the germline DNA and respond with the immediate activation of effectors. They do not require the gene rearrangements essential to recognition by the adaptive immune response (66). Sensing by PRRs of conserved molecular structures of pathogens, referred to as pathogen-associated molecular patterns (PAMPs), is the first crucial step in the development of virus-specific adaptive immune responses. To delineate the complexity of RNA-sensing PRR pathways that are activated during infection with an RNA virus, the signaling mediators involved and the subsequent adaptive immune responses is the focus of this dissertation.

A. Pattern Recognition Receptors and Pathogen-Associated Molecular Patterns

The recognition of evolutionarily conserved microbial structures, now known as PAMPs, was first proposed by Janeway in 1989 (64, 65). He proposed that the basis of pathogen non-self recognition lies in the ability of the host to recognize conserved microbial products or components that are unique to

pathogens and are not produced by the host. This recognition, mediated by PRRs, allows for the innate immune system to discriminate between “infectious non-self” and “non-infectious self” (110). PRRs possess certain common characteristics: (i) PRRs recognize conserved molecular patterns that are essential to the virus or bacteria and are therefore difficult for the microbe to alter; (ii) PRRs are expressed constitutively in the host and detect the pathogen regardless of their life cycle; and (iii) PRRs are germline encoded, nonclonal and independent of immunologic memory (reviewed in (3)). Today, several structurally and functionally distinct classes of PRRs are known to recognize various PAMPs and induce various host defense pathways (reviewed in (83, 84)) (Table 1.1). The three major classes of PRRs are the Toll-like receptors (TLRs) that detect PAMPs either at the cell surface or in the lumen of intracellular vesicles; and the cytosolic PRRs retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) for the detection of intracellular PAMPs (82, 83, 113).

i. Toll-like receptors (TLRs)

TLRs are the most extensively studied class of PRRs. Discovered in the mid-1990’s to be structurally related to the Drosophila Toll (111, 135), TLRs are type I transmembrane proteins composed of three major domains – the ectodomain with leucine-rich repeats (LRRs) that mediate recognition of their respective PAMPs, the transmembrane domain and the intracellular domain that

is homologous to the interleukin-1 receptor's (IL-1R) Toll/IL-1 receptor homology (TIR) domain, which is required for downstream signal transduction (Figure 1.1) (reviewed in (82)). To date, there are more than 12 members of the mammalian TLR family. 10 TLRs have been identified to be functional in humans and 12 are functional in mice with TLR1-TLR9 being conserved in both species (82, 83). TLR10 in mice is non-functional due to a retrovirus insertion (45) while TLR11, TLR12 and TLR13 are believed to have been lost in the human genome (82).

TLRs are largely divided into two sub-populations with regard to their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5 and TLR6 that are localized on the cell surface and largely recognize microbial membrane components such as lipid, lipoproteins and proteins; the other group is composed of TLR3, TLR7, TLR8 and TLR9, which are expressed within intracellular vesicles and recognize microbial nucleic acids (Table 1.1) (reviewed in (84)). TLR4 is an exception. It is expressed on the cell surface, initially transmits signals for early phase activation and is then endocytosed and delivered to intracellular vesicles for the sequential induction of late-phase signaling pathways (8, 56, 72). TLR11, a relative of surface-expressed TLR5, was recently shown to be also expressed in intracellular compartments (128) but is known to recognize the protozoan profilin-like protein (173). TLR13 is localized intracellularly; although the cognate PAMP is yet to be identified, it has been implicated to have a role in the recognition of vesicular stomatitis virus (146).

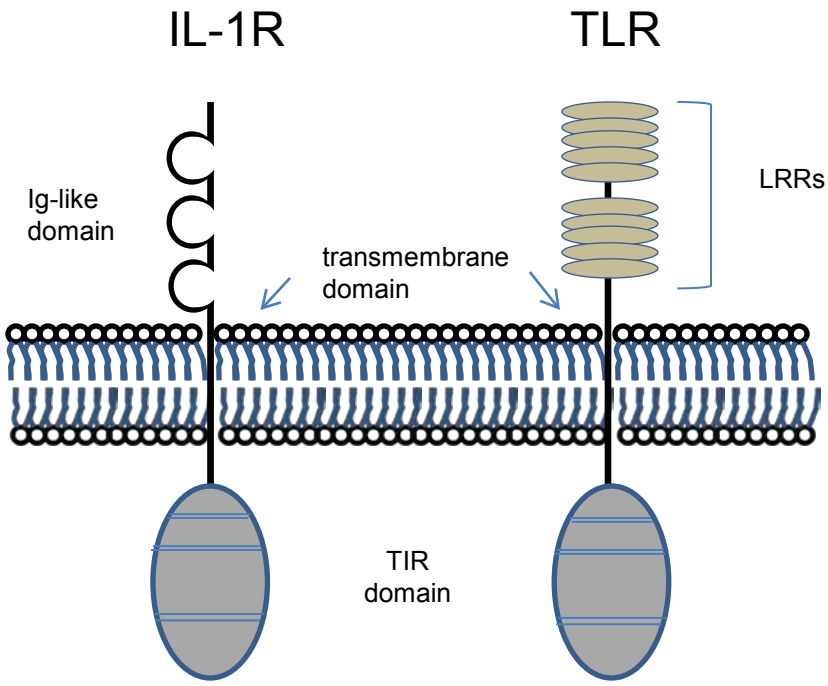


Figure 1.1. Toll-like receptors. The mammalian homolog of the Drosophila Toll receptor. Toll-like receptors (TLRs) are type I transmembrane proteins with three major domains. The N-terminal pathogen-associated molecular pattern (PAMP)-binding ectodomain that contains multiple leucine-rich repeats (LRRs), the transmembrane domain and the intracellular domain of the interluekin-1 receptor (IL-1R) called Toll/IL-1R homology (TIR) domain which mediates signaling events upon receptor activation.

Table 1.1. PRRs and PAMPs

Species	PAMPs/Activators	PRRs involved in recognition
Bacteria, Mycobacteria	LPS	TLR4
	Lipoproteins, lipoteichoic acid, peptidoglycan, lipoarabinomannan	TLR2/1, TLR2/6 NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5 IPAF, NAIP5
	DNA	TLR9 AIM2
	RNA	TLR7 NALP3
Viruses	DNA	TLR9 AIM2, DAI, IFI16, DDX41
	RNA	TLR3, TLR7, TLR8 RIG-I, MDA5, NALP3
	Structural protein	TLR2, TLR4
Fungus	Zymosan, β-glucan	TLR2, TLR6 Dectin-1, NALP3
	Mannan	TLR2, TLR4
	DNA	TLR9
	RNA	TLR7
Parasites	tGPI-mucin (Trypanosoma)	TLR2
	Glycoinositolphospholipids (Trypanosoma)	TLR4
	DNA	TLR9
	Hemozoin (Plasmodium)	TLR9
	Profilin-like molecule	TLR11

** Adapted from Kawaii and Akira, 2011*

There are four known signaling adaptors in TLR signaling: MyD88, MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1) and TRIF-related adaptor molecule (TRAM, also known as TICAM2) (reviewed in ((82, 121)). MyD88 is used by all TLRs except TLR3 which signals through the adaptor molecule TRIF. Thus, TLR signaling can be largely classified as either MyD88-dependent pathways or MyD88-independent /TRIF-dependent pathways. TLR4 is the only TLR that uses all four adaptors and thus activates both the MyD88-dependent and MyD88-independent pathways (Figure 1.2).

ii. Retinoic acid-inducible gene-I (RIG-I)-like receptors

RLRs are a family of DExD/H box RNA helicases that function as cytoplasmic sensors of viral RNA PAMPs. The RLRs include RIG-I, melanoma differentiation-associated gene (MDA-5) and laboratory of genetics and physiology 2 (LGP2) (reviewed in (99, 141)). RLRs are interferon-inducible proteins that are expressed at low concentrations in the resting cell and are greatly increased upon stimulation or activation (75, 174). Key structural domains include: caspase activation and recruitment domain (CARD), ATPase containing DEAD box helicase (DEAD helicase) and C-terminal domain (CTD) (Figure 1.3). The CTD of RIG-I and MDA-5 also encodes a repressor domain (RD). LGP2 lacks the N-terminal CARD domains and is therefore unable to signal via the adaptor interferon-β promoter stimulator 1 (IPS-1). Currently, LGP2 is known to

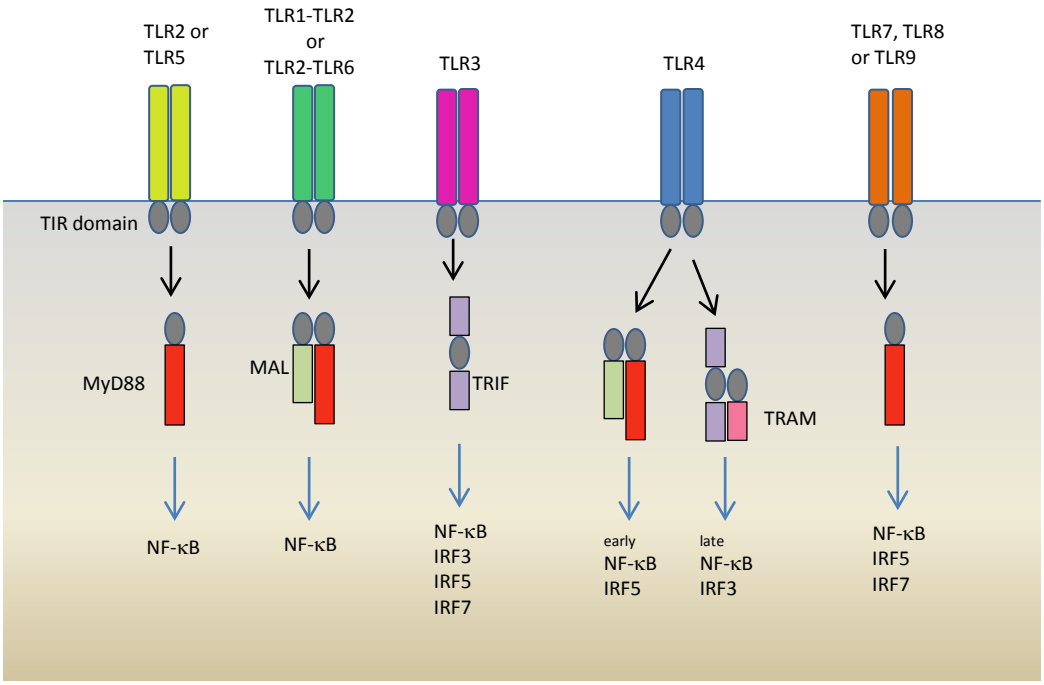


Figure 1.2. Adaptor molecules of TLR signaling. MyD88, MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1) and TRIF-related adaptor molecule (TRAM, also known as TICAM2) are the four known signaling adaptors of TLRs. Recognition by TLRs of its cognate ligand induces homo- or heterodimer formation of TLRs and the activation of signaling cascades leading to the activation of key transcription factors -- interferon regulatory factors (IRFs) and nuclear factor κB (NF-κB).

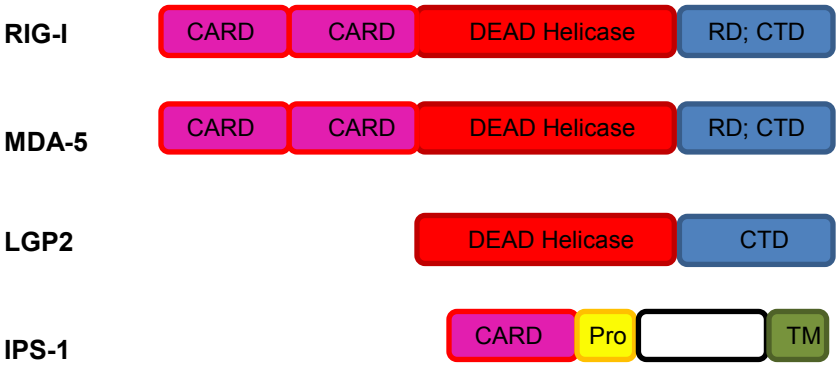


Figure 1.3. Simple representation of RLRs and their adaptor IPS-1. Retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) -- RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 consist of caspase activation and recruitment domain (CARD), ATPase containing DEAD box helicase (DEAD helicase) and C-terminal domain (CTD). LGP2 consists only of DEAD helicase, C-terminal domain (CTD) and no repressor domain (RD). RLRs signal downstream via the adaptor, interferon (IFN) β-promoter stimulator 1 (IPS-1) that consists of CARD, a proline-rich region (Pro), and a transmembrane domain (TM) on its C terminus.

function as a regulator of RIG-I and MDA-5 signaling in an inhibitory or synergistic way, respectively (129, 137, 138, 166, 174).

Although very similar in structure, the exact molecular signatures recognized by RIG-I and MDA5 are still not fully understood (6). RIG-I preferentially recognizes RNA sequences with 5' triphosphorylated (5'ppp) ends, which serve in part to distinguish a non-self RNA PAMP (52). RIG-I can also bind to ssRNA and shows a preference for shorter RNA fragments compared to MDA-5 which preferentially recognizes long dsRNAs (greater than 1 kb in length) such as the high-molecular-weight polyinosinic:polycytidylic acid (poly I:C) fragments (80).

Both RIG-I and MDA-5 interact downstream with the adaptor IPS-1 through CARD repeats. IPS-1 is thought to be not directly involved in the signaling process but serves to orchestrate the molecular interactions which subsequently lead to the production of proinflammatory cytokines and type I IFN (Figure 1.4) (69).

iii. Nucleotide-binding oligomerization domain (NOD)-like receptors

NLRs comprise a large number of family member proteins that is characterized by the presence of conserved NOD motif (reviewed in (77)). The domain structure of NLR protein resembles the pro-apoptotic APAF1 and a subset of plant disease-resistance (R) genes (161). NLRs are defined by three characteristics: an N-terminal effector domain, a central nucleotide binding

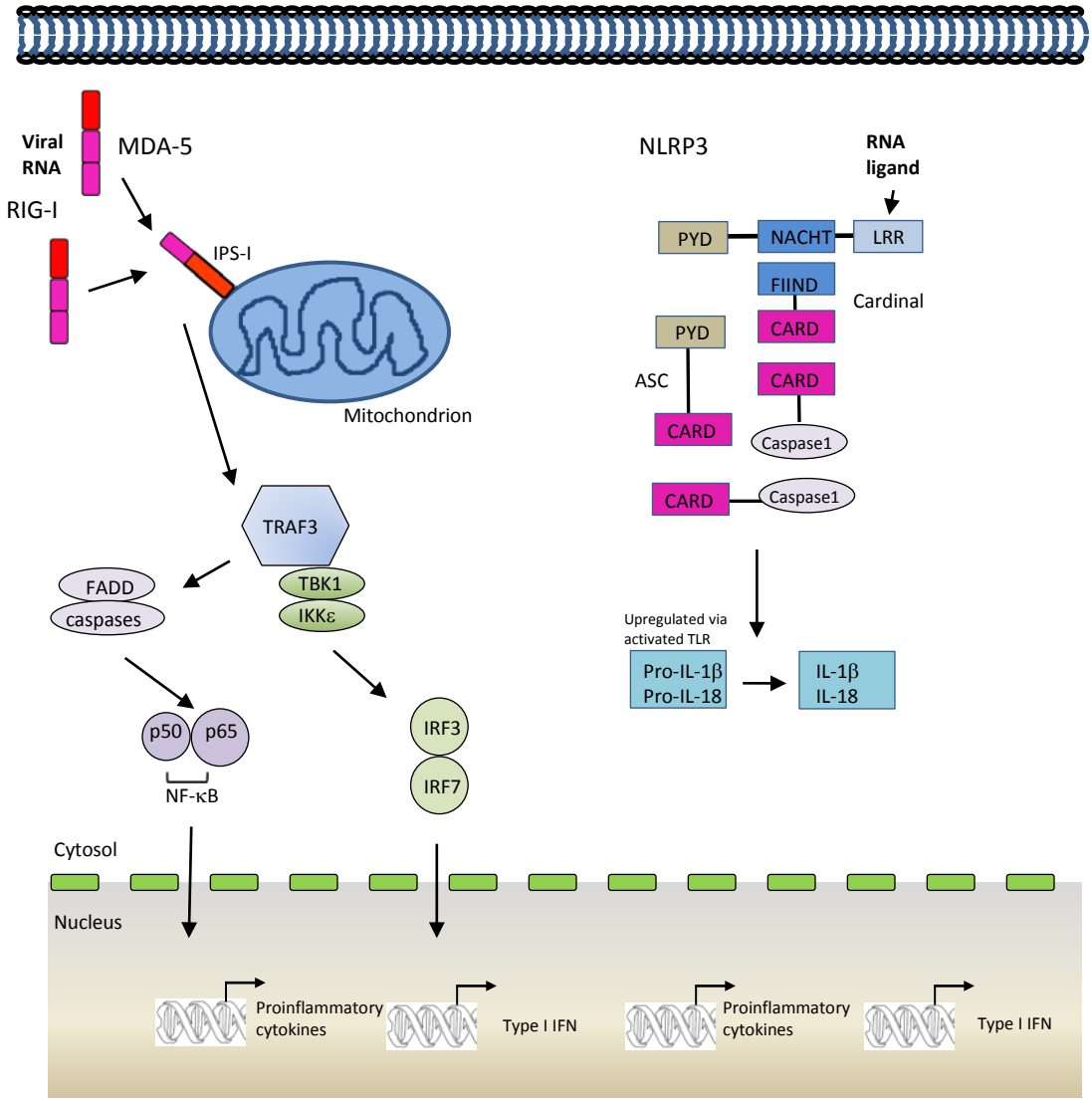


Figure 1.4. RIG-I, MDA-5 and NLRP3 signaling. The double-stranded RNA (dsRNA) receptors retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are cytosolic receptors expressed in most cells. RIG-I and MDA5 signal through a common adaptor molecule, interferon (IFN) β-promoter stimulator 1 (IPS-I), which is mitochondria associated. Downstream signaling of IPS-I leads to activation of transcription factors interferon-regulatory factor 7 (IRF7), IRF3 and nuclear factor κB (NF-κB) that induces the production of proinflammatory cytokines and type I interferons (IFNs). Inactive NACHT-LRR-Pyrin (PYD)-containing protein 3 (NLPR3) oligomerizes upon stimulation of an unknown mechanism. NLRP3 forms an inflammasome complex with proteins, apoptosis-associated speck-like protein (ASC), Cardinal and pro-caspase 1, leading to the activation of caspase-1, which mediates the processing of pro-IL-1β and pro-IL-18 (induced by TLR signaling) to mature IL-1β and IL-18, respectively. FIIND, domain with function to find; FADD, fas-associated death domain; NACHT, neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E and TP-1; LRR, leucine-rich repeat; CARD, caspase activation and recruitment domains.

domain (NBD) and C-terminal repeats. N-terminal effector domains consist of either a PYD, CARD, baculovirus inhibitor of apoptosis repeat (BIR) domains or a transactivation domain. The NACHT-LRR-PYD-containing protein 3 (NLRP3, also known as NALP3, cryopyrin) is one of the best characterized NLRs. Upon activation, NLRP3 interacts with apoptosis-associated speck-like protein containing a CARD (ASC) that is essential for the binding and recruitment of pro-caspase 1 to form an inflammasome complex. This activates caspase 1 that mediates the cleaving of pro-IL-1β and pro-IL-18 into active IL-1β and IL-18, respectively (Figure 1.4) (69). It is not yet known if NLRP3 directly detects nucleic acids or if NLRP3 is indirectly activated following nucleic acid detection by an unknown sensor that may interact with NLRP3. However, based on the broad range of NLRP3 activators, it is likely that NLRP3 may respond to a less specific stimulus such as cellular stress or alterations of host metabolites downstream of pathogen infection (171).

B. RNA-sensing PRRs and RNA PAMPs

Nucleic acid motifs are the main virus-derived PAMPs recognized by the PRRs of the innate immune system (Table 1.1). The focus of this dissertation is on RNA-sensing PRRs and the viral PAMPs that are detected during RNA virus infection. This includes TLR3, TLR7, TLR8, RIG-I, MDA-5 and NLRP3. Viral ssRNA is sensed by TLR7 and TLR8 in the endosomes and by RIG-I in the cytosol of many cell types. Viral dsRNA is recognized by RIG-I and MDA5 in the

cytosol, by TLR3 in the endosome of innate immune cells and by NLR, NLRP3. (Figures 1.4 and 1.5) (reviewed in (69)).

C. dsRNA recognition: The Pre-TLR/PRR Era

Recognition of extracellular or cytoplasmic dsRNA is thought to occur primarily via two different classes of PRRs – through TLRs (TLR3) and RLRs (RIG-I and MDA-5), respectively. However, there is a third classic molecule, recently classified in reviews as also a PRR for cytoplasmic dsRNA -- protein kinase R (PKR) (65, 113). PKR is a 68-kDa cytoplasmic dsRNA-dependent serine/threonine kinase. Upon binding of dsRNA, PKR is activated and undergoes dimerization and autophosphorylation. PKR phosphorylates its physiological substrate, eukaryotic initiation factor 2α (eIF-2α), resulting in the block of translation of both viral and cellular RNAs (reviewed in (33)). Previous studies demonstrated that PKR is required for IFN production in bone marrow-derived DCs, mouse embryonic fibroblasts and in the recognition of particular viruses (25, 35). PKR has also been implicated to enhance the induction of IFN-β mediated by cytoplasmic RNA PRRs (109). Though PKR is firmly established to play a central role in IFN-dependent antiviral actions, it is likely that PKR, as a PRR, plays a much broader function in the initiation and mounting of adaptive immune responses.

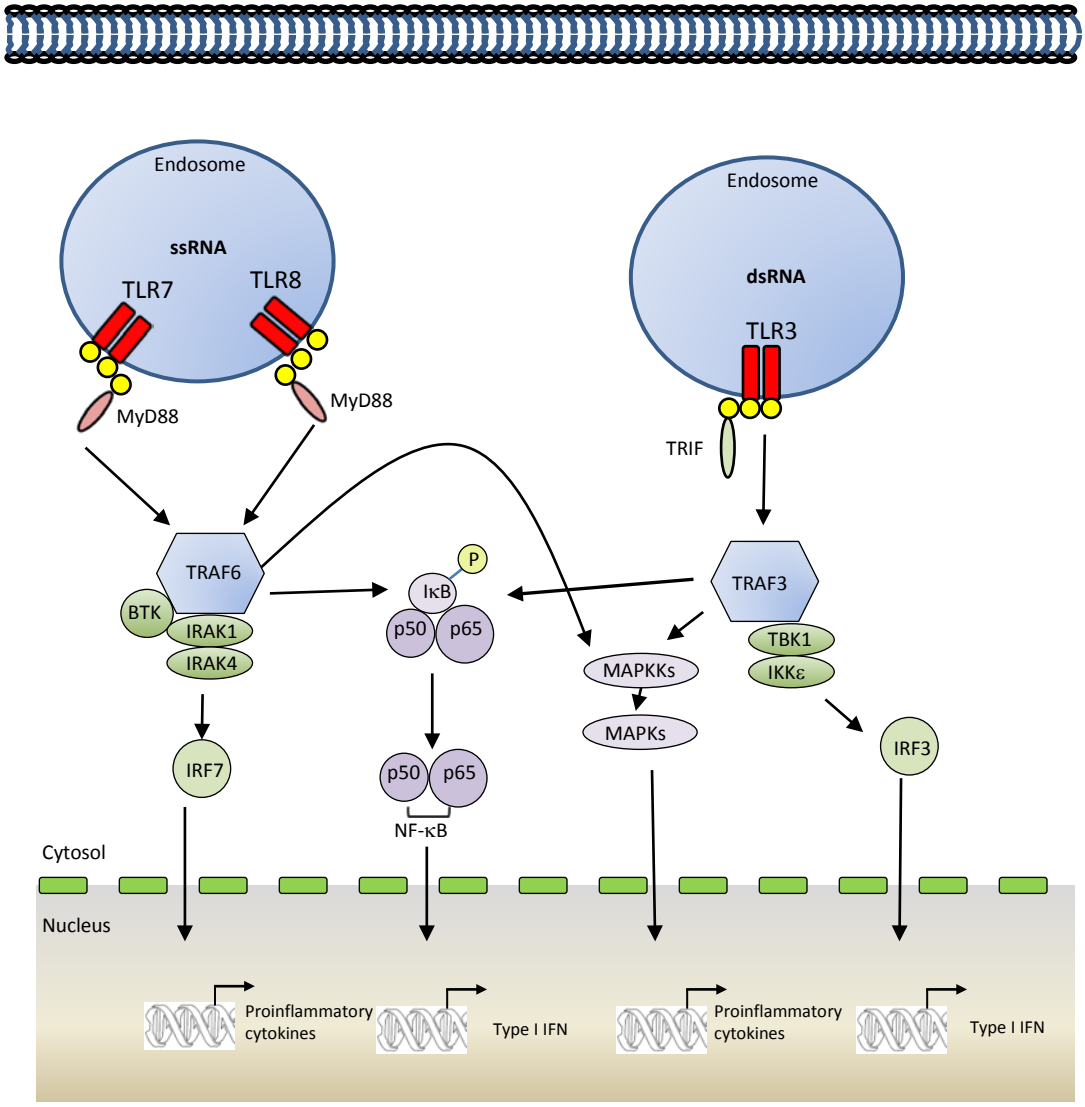


Figure 1.5. TLR7/TLR8 and TLR3 signaling. Endosomal Toll-like receptor 7 (TLR7), TLR8 and TLR3 recognize RNA ligands. TLR7 and TLR8 recognize single-stranded RNA (ssRNA) while double-stranded RNA (dsRNA) is recognized by TLR3. TLR7 and TLR8 signal through the adaptor protein, MyD88, which leads to the downstream activation of transcription factors interferon-regulatory factor 7 (IRF7) and nuclear factor κB (NF-κB). By contrast, TLR3 signals through the adaptor TRIF for the activation of IRF3 and NF-κB. TLR7, TLR8 and TLR3 signaling also activate the mitogen-activated protein kinases (MAPKs) pathway. Overall, these pathways potently induces the production of proinflammatory cytokines and type I interferons (IFNs). TRAF, tumor-necrosis factor (TNF)-receptor-associated factor; BTK, Bruton's tyrosine kinase; IRAK, interleukin-1 receptor-associated kinase; IκB, inhibitor of NF-κB; TBK1, TANK-binding kinase 1; IKKε, inhibitor of NF-κB kinase ε.

D. Expression of RNA-sensing PRRs on different cell types

PRRs have evolved to efficiently recognize PAMPs and this includes specific expression of PRRs in specific cell types (reviewed in (3, 82, 83)). RIG-I and MDA-5 are expressed in most cell types and are upregulated upon stimulation or activation. Most antigen presenting cells (APCs) express TLR3, TLR7 and TLR8. TLR3 is detected in conventional DCs, macrophages and also in non-immune cells such as fibroblasts and epithelial cells. TLR7 is highly expressed in plasmacytoid DCs (pDCs), a DC subset that can secrete vast amounts of type I IFN in response to viral infection. TLR8 is also expressed in various tissues with the highest expression in monocytes. CD4+ and CD8+ T-cells also express these RNA-sensing TLRs (53, 175). NLRP3 is expressed in T-cells (92). The expression of these different RNA-sensing PRRs in different cell types suggests sophisticated mechanisms for detecting RNA viruses. Previous studies have mainly focused on RNA-sensing PRR activation in APCs. Delineating the role of PRR activation within specific cell types, such as in T-cells, would further advance our current knowledge in the contribution of RNA-sensing PRRs in innate and adaptive immune responses.

E. RNA PAMPs as adjuvants

Adjuvants are important in eliciting robust protective immune responses from vaccines but many of their underlying mechanisms are yet to be fully elucidated (51). Vaccine adjuvants mainly target professional antigen-presenting

cells (APCs) such as dendritic cells and activate innate immunity through pattern recognition receptor (PRR) pathways (51, 108). For protection against most viruses and intracellular pathogens, adjuvants that stimulate CD4+ T helper type 1 (Th1) responses are desirable (67). CD4+ T-cell help is known to be required for optimizing B-cell and CD8+ T-cell responses, and can also provide protection through direct cytotoxic effector functions (116, 178). Unfortunately, potent CD4+ T-cell adjuvant activity in humans has often been associated with unacceptable toxicity (*e.g.* complete Freund's adjuvant (68)). Therefore, one of the major challenges in adjuvant research has been to gain CD4+ Th1 stimulatory activity while minimizing potential toxicity.

RNA-like immune response modifiers (IRMs) can skew acquired immune responses towards a Th1 phenotype while suppressing Th2 responses (98, 149, 163, 165). Among these RNA-like IRMs, resiquimod (R-848) and poly I:C are being evaluated as T-cell adjuvants for vaccine development (98, 130, 132, 176). R-848 is a synthetic imidazoquinoline-like molecule that triggers cellular responses via the endosomal TLR7 and TLR8 and MyD88-dependent signaling (24, 48). Poly I:C is a synthetic analog of viral dsRNA that activates MyD88-independent immune responses through TLR3/TRIF and the MDA5/IPS-1) signaling pathways (Figures 1.4 and 1.5) (106, 168).

These RNA-sensing PRRs and signaling pathways are present in APCs and CD4+ T-cells (71, 83). RNA-like IRM activation of MyD88-dependent and MyD88-independent signaling pathways can induce a broad range of cell-specific

responses, including NF-ƙB activation, type I interferon (IFN) and pro-inflammatory cytokine production, and co-stimulatory molecule upregulation (83, 149, 163). The ability of RNA-like adjuvants to stimulate CD4+ Th1 responses likely depends on a combination of key signaling pathways in APCs and CD4+ T-cells. A better understanding of the critical signaling pathways by which RNA-like IRMs stimulate CD4+ Th1 responses will help in the establishment of effective strategies in the generation of rationally designed vaccine adjuvants.

F. Influenza A virus recognition by RNA-sensing PRRs

Influenza A virus (IAV) is a negative-sense single-stranded segmented RNA virus of the orthomyxovirus family. IAV can be recognized predominantly by RNA-sensing PRRs TLR3,TLR7, RIG-I and NLRP3 (Figure 1.6) (reviewed in (57)). RIG-I recognizes the 5'ppp of IAV genomic RNA in IAV-infected fibroblasts (127). TLR7 recognizes the IAV genomic RNA in the endosome of pDCs and helped identify ssRNA as a ligand for TLR7 (24). TLR3 has also been involved in the immune responses of lung epithelial cells to IAV (42). The NLRP3 inflammasome can also be activated during IAV infection (78, 124, 158). The NLRP3 inflammasome detects IAV infection by sensing disturbances in intracellular ionic concentrations induced by the IAV M2 protein (59). Further investigation is needed to elucidate the role of RNA-sensing PRRs in mediating host innate and adaptive immune responses to IAV.

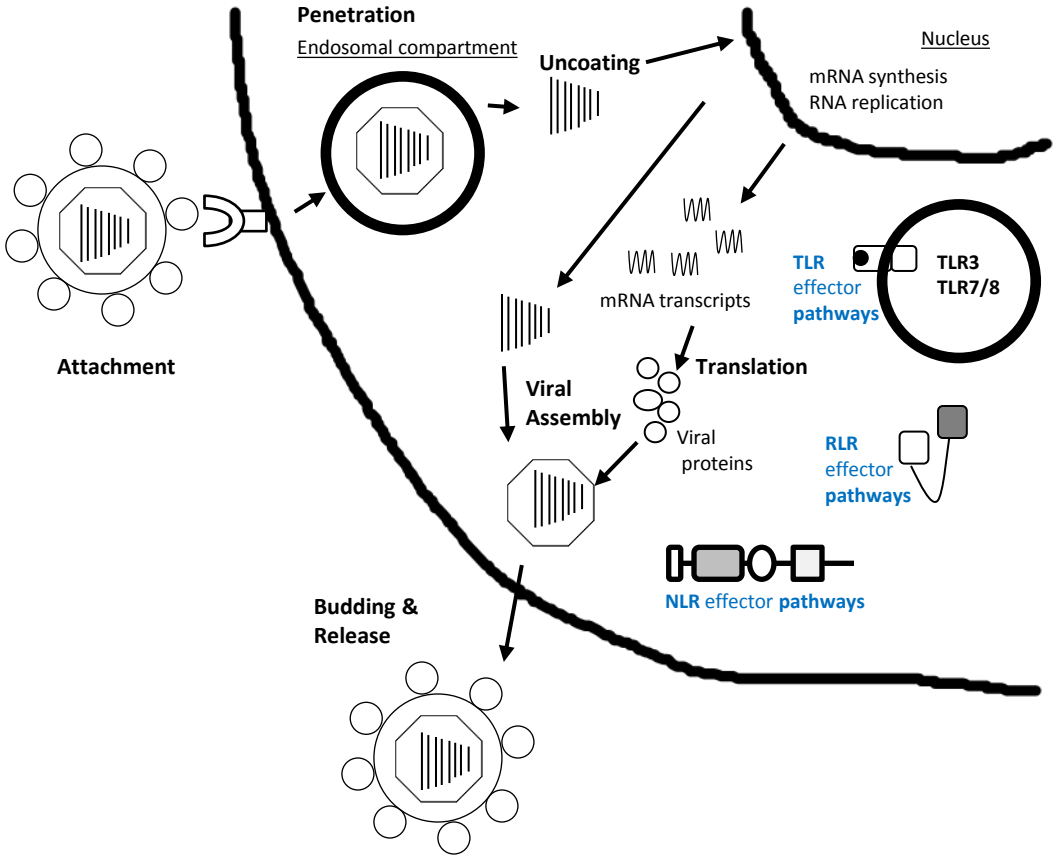


Figure 1.6. Recognition of Influenza A virus (IAV) by RNA-sensing PRRs. The TLR, RLR and NLR pathways are able to target multiple steps in the viral life cycle. The viral genome and replicative intermediates of IAV are recognized intracellularly, either in the endosome or cytosol by RNA-sensing PRRs including by TLR3, TLR7/8, RIG-I and NLRP3.

G. Adaptive Immune Response to Influenza A virus

Both cellular (CD8+ and CD4+ T-cells) and humoral immunity contribute significantly to the clearance of influenza viruses and protection against IAV infection.

i. Role of CD8+ T-cells

CD8+ T-cells play a major role in the control of primary and secondary IAV infections. Three to four days after intranasal infection, activation and the expansion of naïve CD8+ T-cells occur in the draining mediastinal lymph nodes. Five to seven days after infection, the influenza-specific CD8+ T-cells migrate to the lungs and infected airway epithelium where they exert effector functions, lysing infected target cells and producing antiviral cytokines. Influenza-specific CD8+ T-cells recognize multiple viral epitopes on target cells and APCs. In C57BL/6J (B6) ($H2D^b$) mice, the dominant CD8+ T-cell epitopes identified are on the nucleoprotein $(NP)_{366-374}$ (162) and polymerase $(PA)_{224-233}$ (10). A small proportion of influenza-specific CD8+ T-cells recognize other viral proteins (reviewed in (151)). Viral clearance is observed 10 days after primary IAV infection which coincides with the peak of CD8+ T-cell responses to $NP_{366-374}$ and $PA_{224-233}$ (86, 150). IAV-specific CD8+ T-cell responses to a subsequent infection are detected around 2 days faster than the primary response with more efficient clearance of IAV (reviewed in (91)).

ii. Role of CD4+ T-cells

The elimination of the IAV from the respiratory tract is mediated by CD8+ T-cells and B-cells, however, optimal influenza-specific Ab production by B cells is dependent on CD4+ T-cell help (155). Though IAV-specific Abs can be generated without CD4+ T-cells, Ab production is more effective and vigorous following interactions between B cells and CD4+ T-cells (96, 139). For CD8+ T-cells, the priming of naïve CD8+ T-cells is dependent on CD4+ T helper cell activity (12) and is necessary for the maintenance of protective CD8+ memory T-cell populations (73).

Memory CD4+ T-cells also enhance the production of innate inflammatory cytokines and chemokines in the lung during influenza infection that leads to early control of IAV (154). Recently, a study on infected human subjects found that the number of pre-existing influenza-specific CD4+ T-cells were inversely related to the severity of illness after challenge and suggest that activation of long-lived cross-protective CD4+ T-cells should be one of the goals of influenza vaccine development (87, 172)

iii. Role of humoral immunity

Antibodies play a substantial role in terminating influenza virus infections. Protective antibodies are directed against hemagglutinin (HA) and neuraminidase (NA) which are found on the external surface of the influenza virus. The importance of humoral responses has been clearly demonstrated in severe combined immunodeficiency (SCID) mice that can be protected by passive

administration of HA-specific antibodies (123). The main antibody isotypes in the influenza-specific humoral response are IgA, IgM and IgG. Mucosal or secretory IgA affords local protection from infection of airway epithelial cells. Complement-mediated neutralization of influenza can be mediated by IgM Ab while serum IgG predominantly transudates into the respiratory tract and contributes to long-lived protection (117) (reviewed in (91)).

H. Heterosubtypic Immunity to IAV

Heterosubtypic immunity to IAV was first demonstrated in 1965 (144). Heterosubtypic immunity is defined as immunity generated by a given IAV subtype or its antigens that protects against challenge with other IAV subtypes (e.g. immunity to H1N1 protecting against infection with H3N2) (reviewed in (38)). Using two IAV subtypes with different HA and NA proteins allows examination of primary IAV-specific responses and facilitates the detailed dissection of secondary T-cell responses without the difficulties associated with cross-neutralizing antibodies (151). Heterosubtypic immunity induced does not prevent infection like the neutralizing antibodies against the viral gylcoproteins HA and NA (14). However, heterosubtypic immunity does confer a certain degree of protection, reduces mortality otherwise caused by IAV infection and has been shown to be long lasting (38). Understanding the mechanism of hetrosubtypic immunity has been the focus of numerous studies. It has been demonstrated in mice that virus-specific CD4+ T-cells, CD8+ T-cells, virus-specific antibodies and

B-cells contribute to heterosubtypic immunity (38). CD8+ T-cells predominantly recognize cross-reactive epitopes found in conserved proteins such as the NP and matrix (M) protein of IAV. CD8+ T-cells are therefore able to recognize and kill virus-infected cells based on MHC class I restricted presentation of conserved IAV epitopes (159).

The role of CD4+ T-cells in heterosubtypic immunity to IAV has been studied less extensively than CD8+ T-cells and remains controversial. CD4+ T-cells are needed for antibody class switching and B cell somatic hypermutation and are therefore important in the development of effective anti-IAV antibody responses(159).

Though other effector immune cells could potentially contribute to heterosubtypic immunity to IAV (i.e. NKT cells and $\gamma\delta$ T-cells), it is evident that T cells can mediate protective immunity. Elucidation and a better understanding of the mechanisms involved in the heterosubtypic immunity to IAV would contribute towards the development of vaccines that confer protection against all IAV subtypes.

I. Thesis Objectives

The overall goal of this thesis is to delineate the role of RNA-sensing pattern recognition receptors in the shaping and modulation of T-cell immune responses to RNA viruses. **We hypothesize that the activation of the RNA-sensing pattern recognition receptor pathways in T-cells and in antigen**

presenting cells are crucial in the development of virus-specific T-cell immune responses.

This thesis is presented in three parts:

Chapter III: Direct co-stimulatory effects of RNA PAMPs on conventional CD4+ T-cells

Aim: Delineate the signaling pathways activated by RNA PAMPs directly on conventional CD4 T-cells

Hypothesis: RNA PAMPs can act directly on CD4 T-cells and modulate their function and phenotype

Chapter IV: Critical RNA-sensing PRR pathways are activated by RNA PAMPs/adjuvants that drive Th1 CD4+ T-cell responses in cDC/CD4+ T-cell interactions

Aim: Delineate the signaling pathways activated by RNA PAMPs that drive Th1 CD4+ T-cell responses in cDC/CD4+ T-cell interactions

Hypothesis: RNA PAMP activation of TLR7→MyD88 signaling in cDC augments alloreactive CD4+ Th1 responses through type I IFN and early IL-1α & IL-1β production and signaling.

Chapter V: Contributions of RNA-sensing PRR pathways on Th1 responses to IAV infection

Aim: Delineate the contributions of RNA-sensing PRR pathways on Th1 responses to an RNA virus

Hypothesis: MyD88-signaling is essential in the RNA-sensing pathways activated in the development of heterosubtypic immunity to Influenza A.

CHAPTER II:

MATERIALS AND METHODS

A. Mice

C57BL/6J (B6), BALB/cJ, B6.C-H2d/bByJ (B6.H2d), F1 and F2 of C57BL/6J x 129S1/SvlmJ mice were purchased from The Jackson Laboratory (Maine, USA). MyD88$^{-/-}$, TLR7$^{-/-}$, TLR3$^{-/-}$, IL-1R$^{-/-}$, IL-18R$^{-/-}$, MAVS$^{-/-}$, MDA5$^{-/-}$, NLRP3$^{-/-}$, ASC$^{-/-}$, TRIF$^{-/-}$, MAVS/TRIF$^{-/-}$ and type I IFN receptor$^{-/-}$ (IFNAR$^{-/-}$) mice were provided by Drs. R. Finberg, E. Kurt-Jones and K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$ and IL-1$\alpha\beta^{-/-}$ mice were provided by Drs. K. Rock and H. Kono (University of Massachusetts Medical School). The genetically modified knockout mice were backcrossed eight or more generations onto the B6 background and were then intercrossed to obtain the knockout genotypes. For mice not fully backcrossed onto the B6 background, F1 or F2 of C57BL/6J x 129S1/SvlmJ were used as wild type controls. The knockout genotypes were confirmed by PCR genotyping. All animal procedures were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Massachusetts Medical School Animal Care and Use Committee (Protocol A-1884).

B. Reagents /Antibodies

Resiquimod (R-848) was purchased from 3M Pharmaceuticals (St. Paul, MN), polyinosinic:polycytidylic acid (poly I:C) was purchased from Alexis Biochemicals (San Diego, CA). For the anti-CD3 stimulation experiments: poly (dT), CL075 and 2-aminopurine were purchased from Invivogen (San Diego, CA); RNA-dependent protein kinase inhibitor (PKR inhibitor) and RNA-dependent protein kinase inhibitor negative control were purchased from Calbiochem (San Diego, CA).

Antibodies used in all experiments were purchased from BD Biosciences (San Jose, CA) and eBiosciences (San Diego, CA). The antibodies/markers used in experiments are listed in Table 2.1. For experiments where anti-CD3 and anti-CD28 were used, functional grade purified anti-mouse CD3ε (clone 1 45-2C11) and functional grade purified anti-mouse CD28 (clone 37.51) were purchased from eBiosciences.

For the add-in and blocking MLR experiments, the following reagents were used: universal Type I IFN (PBL Biomedical Laboratories, Piscataway, NJ), mouse rIL-1α, rIL-1β and soluble IL-1 receptor antagonist (sIL-1Ra) (R & D Systems, Minneapolis, MN), Anakinra^{TM} (sIL-1R antagonist) (Amgen, Thousand Oaks, CA), anti-mouse IFN α/β receptor IgG_1 (anti-IFNAR) (Leinco, St. Louis, MO), and purified mouse IgG_1 isotype control (BD Biosciences).

Table 2.1. List of antibodies used.

Isolation of mouse CD4+ T-cells	Isolation of human CD4+ T-cells	cDC sorting for MLR	MLR ICS	IAV ICS	Tetramer analysis
CD3-Pacific Blue/V450*	CD4 - FITC	CD11c - FITC	CD3-Pacific Blue/V450	CD3-Pacific Blue/V450	CD3 - PerCPCy5.5
CD4 - FITC	CD8 - PerCPCy5.5	CD11b - PE	CD4 - PerCPCy5.5	CD4 - PerCPCy5.5	CD8 - Alexa 647
CD8 - PerCPCy5.5	TCR$\gamma\delta$ - PE	CD45R - PerCP	TCRβ - PE	CD8 - PE	CD44 - FITC
TCR$\gamma\delta$ - PE	CD56 - APC		DX5 - PECy7	DX5 - PECy7	CD8 tetramer - PE
DX5 - APC			IFN-γ - APC	IFN-γ - APC	
			TNFα - Alexa 700	TNFα - Alexa 700	
			IL-2 - FITC	IL-2 - FITC	
			LDA	LDA	

** For anti-CD3 stimulation experiments, cells were not surface stained with anti-CD3 antibodies.*

C. Isolation of CD4+ T-cells

Murine conventional CD4+ T-cells were isolated from splenocytes by magnetic bead enrichment for CD4+ T-cells (MACS, Miltenyi Biotec, Auburn, CA) followed by flow cytometry sorting for the $CD3^+CD4^+CD8^-DX5^-TCR\beta^+$ lymphocytes. Analysis of the CD4+ T-cells after FACS consistently demonstrated ≥99% purity. The sorted CD4+ T-cells were at least 80% $CD62L^{hi}$ indicating mostly naïve CD4+ T-cells.

Human CD4+ T-cells were isolated from blood samples of healthy adult volunteers. PBMCs were purified by Histopaque ® (Sigma, St. Loius, MO) density gradient centrifugation. Blood was carefully layered onto Histopaque® and centrifuged for 30 min at 1,400 rpm with no brake. The interface with the PBMCs was collected and washed before magnetic bead enrichment for CD4+ T-cells (MACS). The cells were then surface stained with antibodies for flow cytometry sorting ($CD4^+CD8^-TCR\gamma\delta^-CD56^-$).

D. Anti-CD3 stimulation of CD4+ T-cells

Purified murine and human CD4+ T-cells (2×10^5 cells/ well) were cultured in anti-CD3 coated 96-well flat-bottom plates. Plates were coated with 0 to 8 μg/ml concentrations of anti-CD3 in PBS overnight. The murine TLR7 agonist, R-848, and the TLR3 and MDA5 agonist, poly I:C, were added at final concentrations of 20 μM and 100 μg/ml, respectively. These agonist concentrations produced maximal T-cell responses in preliminary experiments.

Anti-CD28 (1.5 μg/ml) was also added as positive control. After 3 days, cell-free culture supernatants were collected for IFN-γ ELISA.

In some of the anti-CD3 stimulation experiments the following were also added: 3μM and 6μM poly (dT) (TLR7/modulator), 50ng/ml and 100ng/ml CL075 (TLR8/7 IRM), 2mM 2-aminopurine (PKR inhibitor), 10μM PKR inhibitor and 10μM PKR inhibitor negative control. The concentrations of these reagents were based on concentrations reported in literature.

E. Enzyme-linked immunosorbent assay

Cell culture supernatants were collected and analyzed for human IFN-γ, mouse IFN-γ, IL-1α and IL-1β by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BD Biosciences, San Jose, CA). The ELISA plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD), and the reactions were stopped with 2N sulfuric acid. Optical densities were read at 450nm using a Spectra MAX microplate reader and analyzed with Softmax® Pro 5 Software (Molecular Devices, Sunnyvale, CA).

F. CFSE staining

FACS-sorted CD4+ T-cells were washed and resuspended at 10^7cells/ml in PBS with 2% FBS and 1mM EDTA. CFSE was added at

1μM final concentration and incubated for 15 min at 30°C. The staining reaction was stopped by washing twice with RPMI medium with 10% FBS.

G. Generation and isolation of conventional DCs

Bone-marrow derived conventional dendritic cells (cDCs) were prepared as described previously (167). In brief, bone marrow cells were cultured in RPMI supplemented with 10% FBS, 50 μM 2-mercaptoethanol and 20 ng/ml recombinant *fms*-related tyrosine kinase 3 ligand (rFlt3L,R&D Systems) for 7 days. Bone-marrow derived dendritic cells were scraped off the culture dish, washed with PBS and then stained with fluorochrome-conjugated antibodies for fluorescence-activated cell sorting (FACS). cDCs ($CD11c^{+}CD11b^{+}CD45R^{-/lo}$) were isolated by sorting on a BD FACSAria flow cytometer (BD Biosciences).

H. Mixed Leukocyte Reactions

FACS-sorted cDCs (2×10^4 cells) and CD4+ T cells (2×10^5 cells) were co-cultured for six days in allogeneic and MHC congenic mixed leukocyte reactions (MLRs). Initial experiments were performed using cells from wild-type B6 ($H2^b$) and BALB/c ($H2^d$) mice to induce an allogeneic response. We subsequently switched to using MHC congenic $H2^d$ mice on the B6 background, B6.C-H2d/bByJ (B6.H2d) in order to exclude B6 and BALB/c strain differences (97). No apparent differences between the allogeneic and MHC congenic systems were observed. The murine TLR7 agonist, R-848, and the TLR3 and

MDA5 agonist, poly I:C, were added to MLRs at final concentrations of 20 μM and 100 μg/ml, respectively. These agonist concentrations produced maximal T-cell responses in preliminary experiments. After six days, cell-free culture supernatants were collected for ELISA and cells were collected for ICS analysis.

I. Addition of type I IFN and IL-1 and blockade of type I IFN and IL-1α/β signaling

With concentrations based on previously published literature, universal type I IFN (200U/ml) (39) and rIL-1α and rIL-1β (125 pg/ml each) (119) were added in some MHC congenic MLRs. To block endogenous type I IFN and IL-1 signaling, anti-mouse IFN α/β receptor IgG_1 (5 μg/ml) (148) and Anakinra™ (10 μg/ml) (79) or recombinant mouse sIL-1Ra (1 μg/ml) (169) were added to R-848 stimulated MHC congenic MLRs. Purified mouse IgG_1 isotype antibody (5 μg/ml) was used as a control. The concentrations used in the addition and blocking experiments were based on concentrations reported in literature. After six days, cell-free culture supernatants were collected for ELISA and cells were collected for ICS.

J. Intracellular cytokine staining

i. ICS for MLRs

IFN-γ and TNF-α-secreting CD4+ T-cells were quantified by intracellular cytokine staining (ICS) assay. After 6 days in culture, co-cultured cDCs and

CD4+ T-cells in MLRs were collected, washed in RPMI media with 10% FBS and then cultured for 5 to 6 hours without restimulation in the presence of Brefeldin A (BD Biosciences, San Jose, CA). Cells were stained with surface antibodies, and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) before intracellular staining with antibodies and fixation. CD4+ T-cells were analyzed using a BD FACSAria cytometer. LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) was used to exclude nonviable cells from analysis. IFN-γ or TNFα secreting CD4+ and CD8+ T-cells were identified as $LDA^{-}/CD3^{+}/CD4^{+}$ or $CD8^{+}$/ IFN-γ^{+} or TNFα^{+} cells (Figure 2.1). Data was analyzed using FlowJo software (Treestar, Ashland, OR).

ii. ICS for influenza A virus-specific CD4+ and CD8+ T-cells

The spleen & lung cell suspensions were washed with media, and then stimulated for 6 hours with immunodominant influenza A virus (IAV) internal protein CD4+ or CD8+ T-cell peptides (CD4+ T-cell epitope -- nucleoprotein $(NP)_{311\text{-}325}$, AnaSpec, Inc., San Diego, CA) (CD8+ T-cell epitopes -- $NP_{366\text{-}374}$ and polymerase $(PA)_{224\text{-}233}$, AnaSpec, Inc.) (9, 19, 162). The peptide stimulations (10μg/ml each) were done in the presence of 1μl Brefeldin A (BD Biosciences). This was followed by staining as described in ICS for MLRs.

K. Luminex analysis

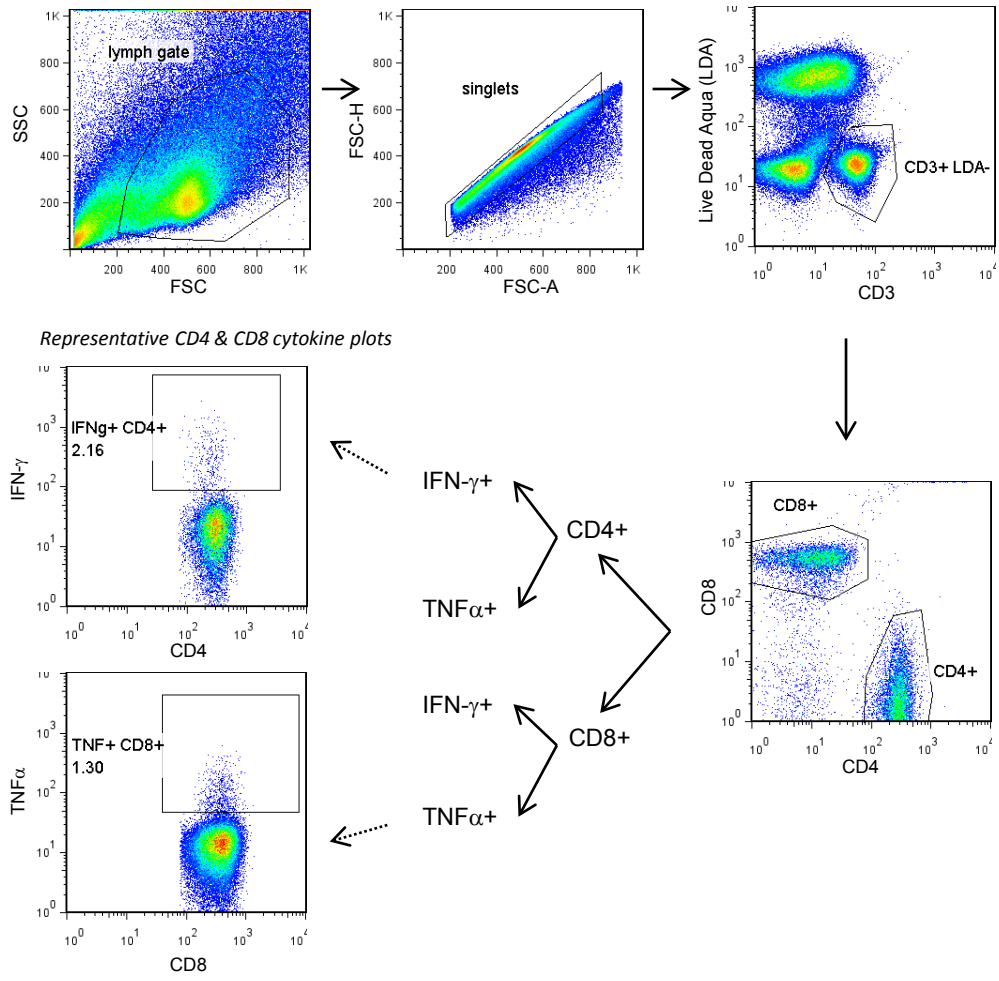


Figure 2.1. Intracellular cytokine staining for influenza A virus (IAV)-specific T-cells. Interferon-γ (IFN-γ) or tumor necrosis factor-α (TNFα)-secreting CD4+ and CD8+ T-cells were gated as live/dead aqua $(LDA)^-/CD3^+/CD4^+$ or $CD8^+$ lymphocytes. Spleen and lung cell suspensions were stimulated for 6 hours with MHC class I or II-restricted IAV peptides, as described in previous figures, in the presence of Brefeldin A. One representative example is shown.

Cell culture supernatants collected from mouse and human anti-CD3 stimulation experiments were sent for Luminex® multiplex cytokine analyses to the Luminex Core of the Baylor Institute for Immunology Research.

L. Quantitative RT-PCR

IL-1α, IL-1β and IFN-β mRNA expression were determined using RNeasy Plus Mini Kit for RNA extraction (Qiagen, Valencia, CA) and TaqMan RNA-to-C_T 1-step kit for RT-PCR (Applied Biosystems, Carlsbad, CA). All primers and probes used were TaqMan Pre-Developed Assay Reagents for Gene Expression (Applied Biosystems, Carlsbad, CA; IL-1α - Mm00439620_m1, IL-1β - Mm01336189_m1 and IFN-β - Mm00439546_s1). Levels of gene expression were normalized to β-actin for each sample. Fold increase in gene expression was determined using the $\Delta\Delta C_T$ method. Samples were run in triplicates and the results are presented as linear-fold changes in gene expression.

M. IL-1β cleavage assay

cDCs (>90% purity) were cultured in serum-free medium. To promote maturation and release of IL-1β, adenosine-5'-triphosphate (ATP, 5mM, Sigma, St. Louis, MO) was added one hour prior to harvesting supernatants. As a positive control, cDCs were primed with lipopolysaccharide (200 ng/ml) for 3 hours prior to stimulation with Nigericin (10μM, Sigma, St. Louis, MO). Cell

culture supernatants were collected after six hours and stored at -20^{o}C until used in Western blot assay. Proteins were precipitated from supernatants by adding an equal volume of methanol and 0.25 volume of chloroform, mixed, and centrifuged at 16,100x*g* at room temperature for 10 min. The upper phase was discarded without disrupting the intermediate phase before adding another equal volume of methanol. The resulting mixture was mixed and then centrifuged at 16,100x*g* for 5 minutes. The liquid phase was decanted and the protein pellet was air dried for 3 to 5 min. Dried pellets were resuspended in Laemmli's SDS sample buffer, heated at 100^{o}C for 5 minutes, and then loaded into 4-20% precast polyacrylamide gels (Bio-RAD, Hercules, CA). Proteins were transferred to Hybond-P PVDF membranes (GE Healthcare, Pittsburgh, PA) and a goat anti-mouse IL-1β antibody (R&D Systems, Minneapolis, MN) was used to detect IL-1β.

N. Influenza A Viruses

Purified influenza A viruses (IAV) influenza A/Puerto Rico/8/34 H1N1 (PR/8) and influenza A/HK/X31 H3N2 (HK/X31) stocks were purchased from Charles River (Wilmington, MA). The HK/X31 virus is a laboratory reassortant that contains the same internal proteins as PR/8 (88).

O. Heterosubtypic IAV infection model

Primary IAV infections were carried out by intranasal infection of naïve mice with 10 plaque forming units (pfu) of the PR/8 virus (a sublethal dose). After 21 days, the mice were challenged with 10^4 pfu of the HK/X31 virus by intranasal infection (a sublethal dose). 7 days later, the mice were sacrificed and T-cell and Ab responses to immunodominant internal PR/8 proteins were examined. For homotypic IAV infections, primary IAV infections were carried out by intranasal infection of naïve mice with 10 pfu PR/8 virus and challenged 21 days later with $2x10^5$ pfu PR/8.

P. Spleen and lung sample preparation for ICS analysis

IFN-γ and TNFα-secreting CD4+ and CD8+ T-cells in mouse spleens and lungs were identified by ICS. Spleens were collected and homogenized by passing through 40 μm nylon strainers and lysing RBCs with ammonium chloride in Tris-HCl buffer (Sigma, St. Louis, MO). Lungs were treated with liberase enzyme solution (0.14 U/ml, Roche, Indianapolis, IN) and DNAse I (2,000 U/ml, New England BioLabs Inc., Ipswich, MA) for 45 min at 37^{o}C prior to homogenization. The spleen & lung cell suspensions were washed with media, stimulated with 10μg/ml CD4+ or CD8+ T-cell peptides (CD4+ T-cell epitope -- $NP_{311-325}$; CD8+ T-cell epitopes -- $NP_{366-374}$ and $PA_{224-233}$) and then stained for ICS.

Q. MHC class I tetramer staining

MHC Class I peptide tetramers (PR/8 IAV nucleoprotein $(NP)_{366-374}/D^b$) were generated by the NIH Tetramer Facility (Atlanta, GA). Tetramer staining was performed for 30 min, on ice, followed by staining for CD8+ T-cells. LDA was used to exclude nonviable cells from analysis.

R. IAV NP protein-specific IgG2c, IgG1 and total IgG serum ELISA

Sera from IAV infected mice were obtained 21 days after primary infection and 7 days after secondary infection. Anti-NP specific antibodies were determined by ELISA. Briefly, 1 μg/ml of purified recombinant IAV PR/8 NP (Imgenex, San Diego, CA) was used as antigen on 96-well flat-bottomed microtiter plates (Thermo Scientific, Rochester, NY) coated overnight at 4°C. Serum samples were serially diluted (4-fold, starting dilution 1:1,000) in 2% fetal bovine serum in PBS (Figure 2.2). Diluted sera were added to plates and incubated for 2 hours at room temperature. Then, horseradish peroxidase (HRP)-conjugated anti-mouse total IgG, IgG2c and IgG1 (dilution 1:2,000) were used as detecting antibodies (Southern Biotech, Birmingham, AL). The ELISA plates were developed using TMB, and the reactions were stopped with 2N sulfuric acid. Relative antibody concentrations were determined using optical spectrophotometer readings at 450nm using a Spectra MAX microplate reader and analyzed with Softmax® Pro 5 Software (Molecular Devices, Sunnyvale,

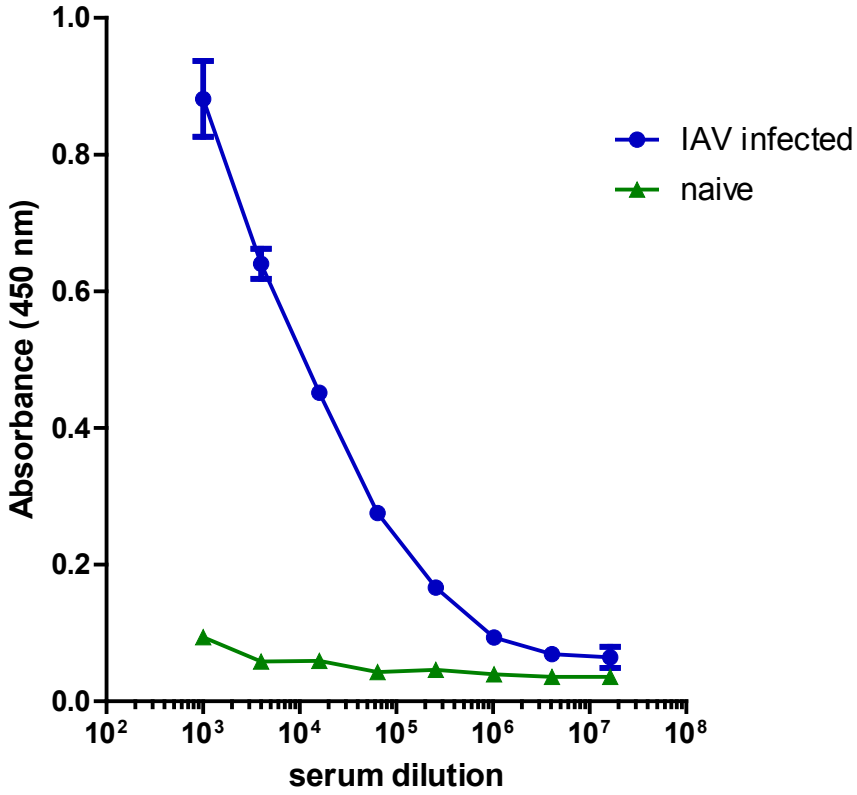


Figure 2.2. Serial dilution of naïve and IAV immune sera for NP-specific ELISA. Representative sample curve of serially diluted naïve and immune sera. Samples were diluted 4-fold (1:4,000 to 1: 16,384,000) and detected for total IgG, IgG2c and IgG1. Titers for anti-NP antibodies were defined as the reciprocal of the serum dilution that gave 50% binding in the linear range of the assay.

CA). The titers for anti-NP total IgG, IgG2c and IgG1 antibodies were defined as the reciprocal of the serum dilution that gave 50% binding in the linear range of the assay.

S. Statistical Analysis

Statistical analysis was done using GraphPad Prism Software version 5.04 (GraphPad, San Diego, CA). Comparisons between two normally distributed variables were performed using the unpaired Student's t-test. Comparisons between two non-normally distributed variables were performed using the nonparametric Mann Whitney U test. A difference was considered significant if the p-value was < 0.05

CHAPTER III

DIRECT CO-STIMULATORY EFFECTS OF RNA PAMPS ON CONVENTIONAL CD4+ T-CELLS

A. Introduction

PRRs are expressed in various cell types and at greatly varying expression levels. In addition to DC and monocytes, TLRs are expressed on B-cells, NK cells, and T-cells (53, 71). Recent studies suggested that different TLRs are expressed on different T-cell subsets (17, 53, 175). The expression of TLRs on T-cells could allow TLR ligands to directly influence T-cell function. Several studies have examined the direct influence of TLR signaling on T-cells (17, 53, 61, 134). PAMPs can directly promote CD4+ T-cell activation, survival, proliferation, cytokine production and affect memory CD4+ T-cell phenotypes (17, 34, 147). In this chapter, we focused on RNA PAMPs and their direct co-stimulatory effects on conventional CD4+ T-cells. CD4+ T-cells were isolated from mouse spleens and human PBMCs. The CD4+ T-cells were enriched by MACS and then purified by flow cytomtery in order to eliminate the potential effects of bystander cells (e.g. natural killer cells, $\gamma\delta$ T-cells). We used gene-deficient mice to delineate the RNA-sensing PRR pathways that are activated on CD4+ T-cells. FACS-sorted CD4+ T-cells were cultured on anti-CD3 coated plates in the presence of RNA-like IRMs R-848 and poly I:C for 3 days. We assessed IFN-γ levels in cell culture supernatants by ELISA and Th1 and Th2

cytokines by Luminex ® as measures of the direct co-stimulatory effects of the RNA-like IRMs.

B. RNA PAMPs can act directly on CD4+ T-cells and modulate their functions and phenotype

In initial experiments, different concentrations of anti-CD3 (0 to 8 μg/ml) were used to stimulate mouse CD4+ T- cells. The co-stimulatory effects of the TLR7-MyD88-dependent IRM R-848 and the MyD88-independent IRM poly I:C were assessed. We observed an increase in IFN-γ levels with poly I:C and minimal direct co-stimulatory effect with R-848 (Figure 3.1.) Succeeding experiments were done using 6 μg/ml and 8 μg/ml anti-CD3 where maximal direct co-stimulatory effects were observed.

Higher concentrations of Th1 cytokines (IFN-γ, TNFα and IL-2) and Th2 cytokines (IL-4, IL-13 and IL-10) were observed with poly I:C-stimulated mouse CD4+ T-cells compared to R-848-stimulated mouse CD4+ T-cells using Luminex® analyses (Figure 3.2.). CD4+ T-cells were also stained with CFSE to determine whether CD4+ T-cells proliferated during the 3-day culture. Proliferation was observed in poly I:C-stimulated mouse CD4+ T-cells while proliferation observed in R-848-stimulated CD4+ T-cells was comparable to unstimulated mouse CD4+ T-cells (Figure 3.3).

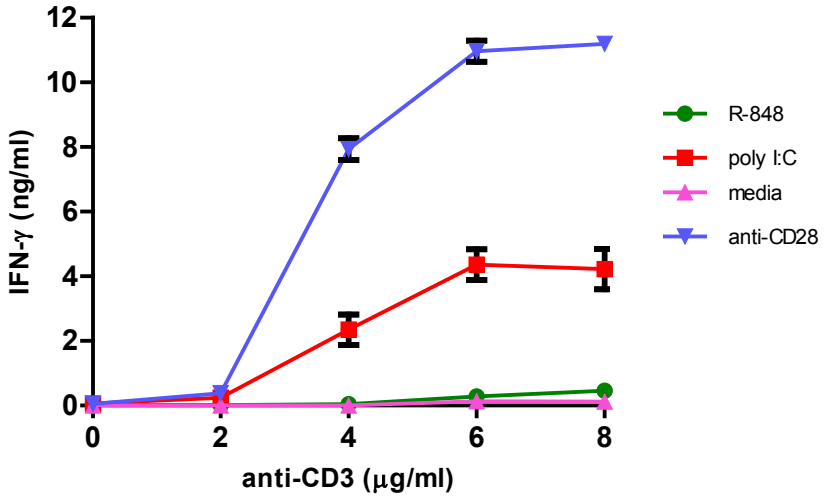


Figure 3.1. R-848 treatment has minimal direct co-stimulatory effect compared to poly I:C in mouse CD4+ T-cells. FACS-sorted mouse CD4+ T-cells were cultured for 3 days in anti-CD3 coated plates in the presence of R-848 or poly I:C. Anti-CD28 was added as positive control. IFN-γ production in anti-CD3 stimulated (0 to 8μg/ml) CD4+ T-cells in the presence of R-848 and poly I:C. One representative experiment of at least three independent experiments is shown.

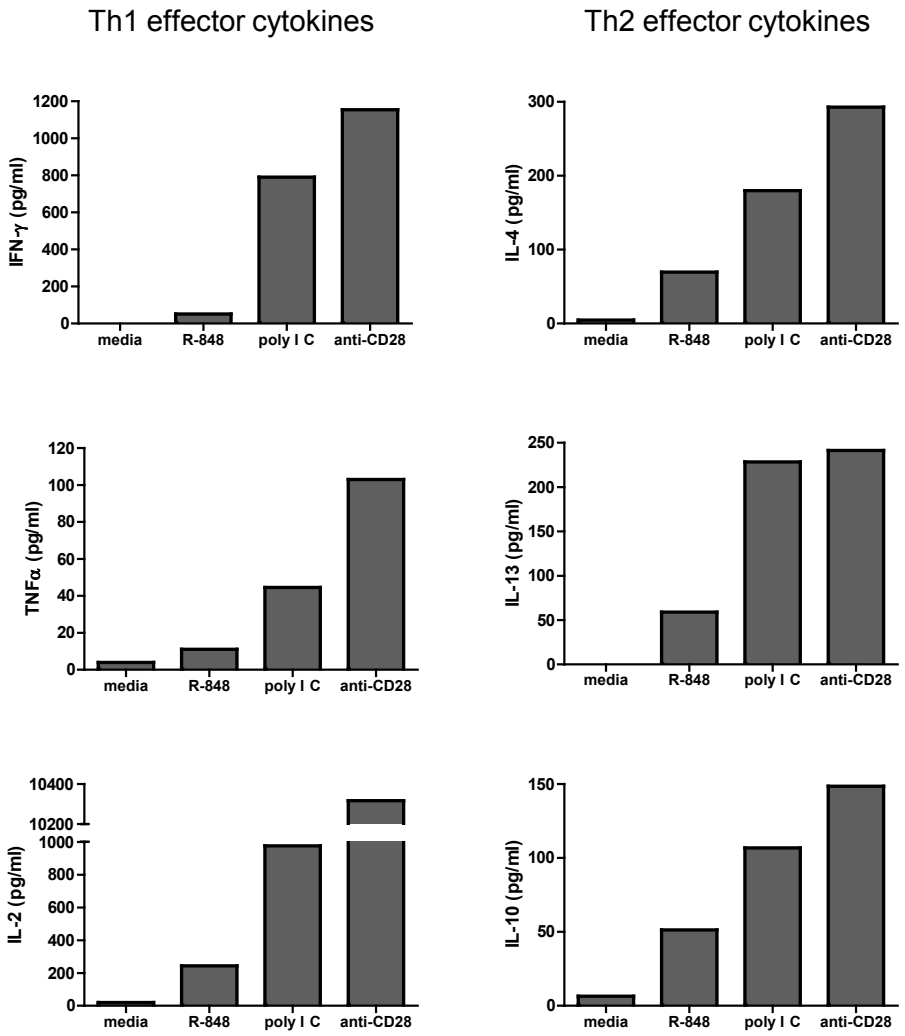


Figure 3.2. Luminex analysis of effector cytokines in anti-CD3 stimulated mouse CD4+ T-cells in response to R-848 or poly I:C. Cell culture supernatants were collected 3 days post stimulation of mouse CD4+ T-cells with anti-CD3 (8 μg/ml) Supernatants were analyzed by Luminex®. Replicates were pooled into one sample for analysis. One representative experiment of at least two independent experiments is shown.

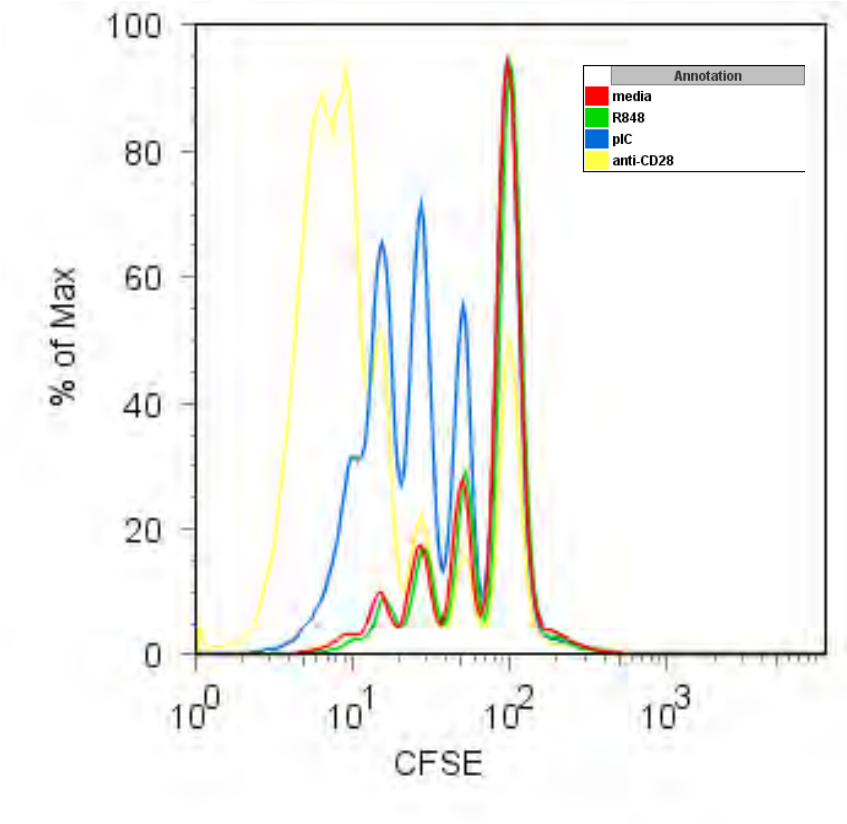


Figure 3.3. Poly I:C treatment induces CD4+ T-cells to proliferate. FACS-sorted mouse CD4+ T-cells were stained with CFSE and cultured for 3 days in 6 μg/ml anti-CD3 coated plates in the presence of R-848 (20 μM) or poly I:C (100 μg/ml). Anti-CD28 (1.5 μg/ml) was added as positive control (n=1). Cell proliferation was assayed by flow cytometry as shown by the shifting of CFSE peaks to the left.

C. Differences on RNA PAMPs direct co-stimulation in mouse and human CD4+ T-cells

PRRs, in particular TLRs, are differentially expressed in mouse and human CD4+ T-cells (71). Recognition is also species-specific (48). To investigate whether there are differences in the direct responsiveness of human and mouse CD4+ T-cells to RNA-like IRMs (R-848 and poly I:C), CD4+ T-cells were isolated from human PBMCs. Similar to mouse CD4+ T-cells, human CD4+ T-cells were initially cultured at different anti-CD3 concentrations. In contrast to anti-CD3 stimulated mouse CD4+ T-cells, we detected IFN-γ production by human CD4+ T-cells in response to R-848 at anti-CD3 stimulation concentrations as low as 2 μg/ml (Figure 3.4). At 6 μg/ml, higher IFN-γ production was observed on R-848 stimulated human CD4+ T-cells compared to poly I:C stimulated human CD4+ T-cells (Figure 3.4). On the other hand, comparable Th1 and Th2 effector cytokines levels were detected in R-848 and poly I:C stimulated human CD4+ T-cells, though predominantly slightly higher for poly I:C at 8 μg/ml anti-CD3 concentration (Figure 3.5).

The imidazoquinoline-like molecule R-848 also activates TLR8 signaling in addition to TLR7 (48). Previous studies on TLR8 signaling suggested TLR8 to be non-functional in mice (70) but it was later demonstrated to be functional with the addition of poly (dT) in combination with TLR8 IRM in transfected HEK293 cells with murine TLR8 and in primary mouse cells (37). To investigate whether TLR8 activation would explain the observed differences between mouse and human

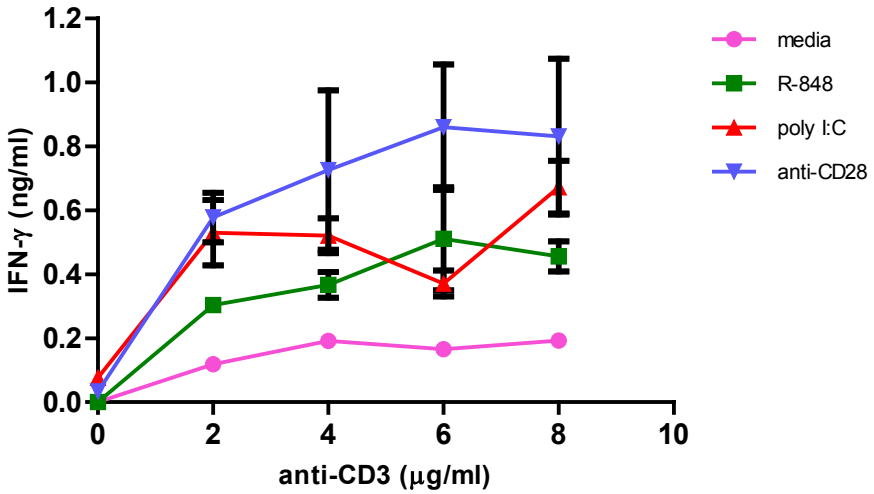


Figure 3.4. R-848 and poly I:C have comparable direct co-stimulatory effects in anti-CD3 stimulated human CD4+ T-cells. FACS-sorted human CD4+ T-cells were cultured for 3 days in anti-CD3 coated plates in the presence of R-848 or poly I:C. Anti-CD28 was also added as positive control. IFN-γ production of anti-CD3 stimulated (0 to 8μg/ml) CD4+ T-cells in the presence of R-848 and poly I:C. One representative experiment of three independent experiments is shown.

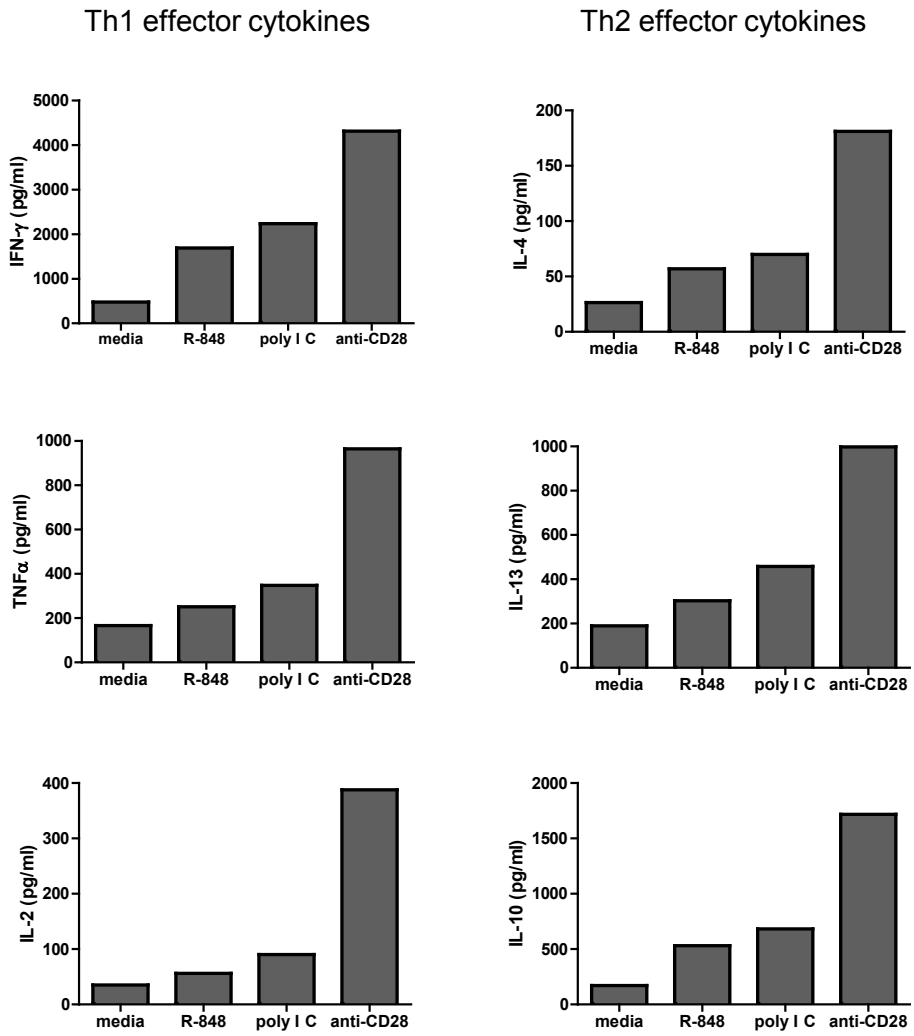


Figure 3.5. Luminex analysis of effector cytokines in anti-CD3 stimulated human CD4+ T-cells. Cell culture supernatants were collected at day 3 post stimulation of human CD4+ T-cells with anti-CD3 (8 μg/ml) + R-848 (20 μM), poly I:C (100 μg/ml) or anti CD28 (1.5 μg/ml). Supernatants were analyzed by Luminex® system for multiple cytokines. Replicates were pooled into one sample for analysis.

CD4+ T-cell responses to R-848 and poly I:C, we performed two preliminary TLR8 stimulation experiments. These experiments were done only once and we did not pursue these experiments. First, mouse CD4+ T-cells were cultured with the combination of R-848 and poly (dT) (3μM and 6μM) based on previous literature that functional murine TLR8 response needs poly (dT) enhancement (37). A slight, not statistically significant increase in IFN-γ production was observed in R-848/poly (dT)-stimulated mouse CD4+ T-cells (Figure 3.6a). We also explored the direct co-stimulation of TLR8 in human CD4+ T-cells in which we utilized CL075, a thiazoloquinolone derivative that stimulates TLR8 in human PBMCs (36). IFN-γ was produced by anti-CD3 stimulated human CD4+ T-cells with the addition of 50 ng/ml and 100 ng/ml of CL075 (Figure 3.6b). However, IFN-γ production was also observed in unstimulated human CD4+ T-cells (media only). These two preliminary experiments did not directly show R-848-mediated TLR8 signaling as the difference between mouse and human CD4+ T-cells. More definitive experiments such as TLR8 signaling blockade in human CD4+ T-cells and the use of TLR8-deficient mice would address and investigate TLR8 direct co-stimulation on CD4+ T-cells.

D. Delineating poly I:C signaling in mouse CD4+ T-cells

With the robust response that we observed in poly I:C direct co-stimulation of CD4+ T-cells, we focused on poly I:C signaling on CD4+ T-cells. MyD88-independent poly I:C signals though TLR3/TRIF and the MDA5/IPS-1 signaling

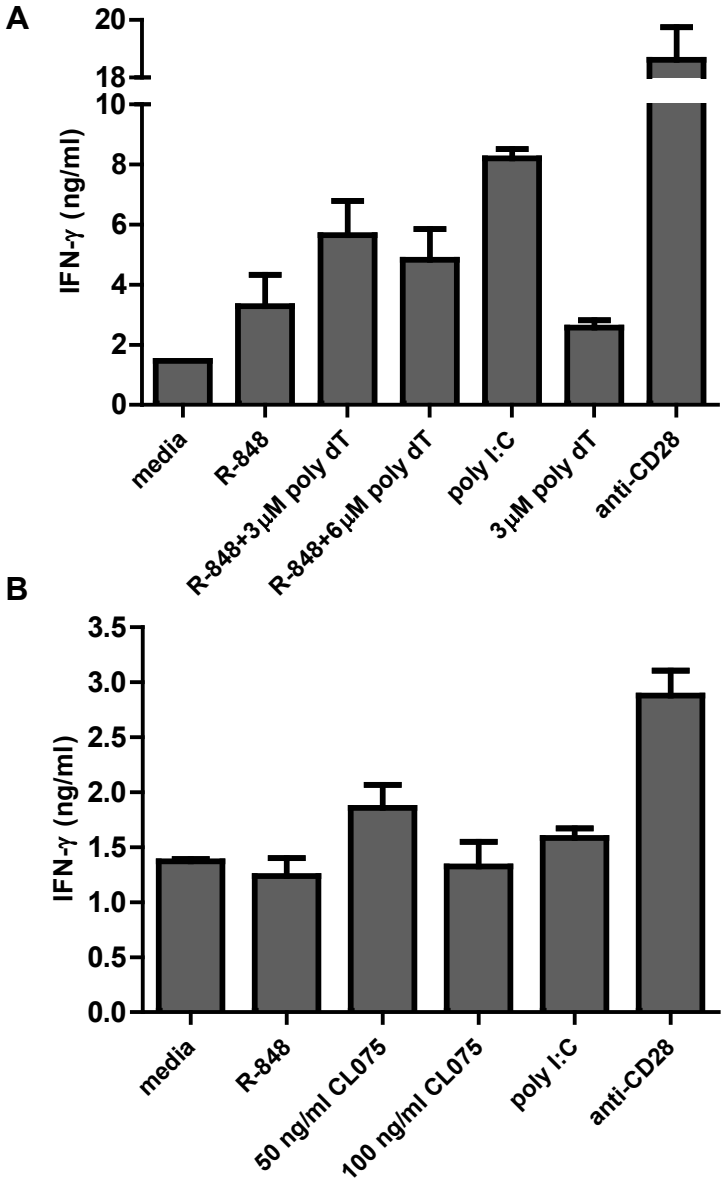


Figure 3.6. Direct co-stimulatory activity by stimulation of TLR8 in mouse and human CD4+ T-cells. FACS-sorted mouse and human CD4+ T-cells were cultured for 3 days in anti-CD3 coated plates (8μg/ml) in the presence of R-848, poly I:C, poly(dT) (previously shown to activate murine TLR8 in combination with TLR8 IRMs) and CL075 (TLR8/7 IRM that stimulates TLR8 in human PBMCs). Anti-CD28 was added as positive control. (A) Mouse CD4+ T-cells (B) human CD4+ T-cells. Bars represent mean ± SEM (n=1).

pathways (106, 168). Poly I:C also activates the NOD-like receptor (NLR) NLRP3 that oligomerizes upon activation and recruits ASC and procaspase-1 to form an inflammasome complex that activates caspase 1 (69). To delineate the RNA-sensing PRR pathways activated by poly I:C in anti-CD3 stimulated CD4+ T-cells, we utilized mice deficient in TLR3, TRIF, IPS-1, MDA-5, NLRP3, ASC, TRIF/MDA-5 and MyD88. Some of the knockout mice used were not fully backcrossed to the B6 background. F1 and F2 mice of C57BL/6J x 129S1/SvlmJ mice were used as wild type controls.

Unexpectedly, poly I:C-induced IFN-γ production by mouse CD4+ T-cells was not abrogated in TLR3 deficient mice though a partial decrease was observed in IPS-1 deficient mice in one out of two experiments performed (Figure 3.7a). Statistical analysis showed no significant differences between the TLR3 and IPS-1 deficient mice. TRIF$^{-/-}$, MDA5$^{-/-}$ and TRIF$^{-/-}$/MDA5$^{-/-}$ double-deficient CD4+ T-cells also gave similar results (Figure 3.7b&c). Poly I:C-induced IFN-γ production by mouse CD4+ T-cells was also not abrogated in NLRP3$^{-/-}$ and ASC$^{-/-}$ mice (Figure 3.7d). On the other hand, poly I:C signaling in CD4+ T-cells was not affected in MyD88$^{-/-}$ CD4+T-cells, as expected (Figure 3.7d). Taken together, our data suggest that poly I:C signals through MyD88-independent signaling pathways other than TLR3/TRIF and RLR pathways.

The RNA-activated protein kinase (PKR) also recognizes poly I:C (109). To investigate PKR involvement in poly I:C signaling in CD4+ T-cells, mouse CD4+ T-cells were cultured in the presence of PKR inhibitors, 2-aminopurine and

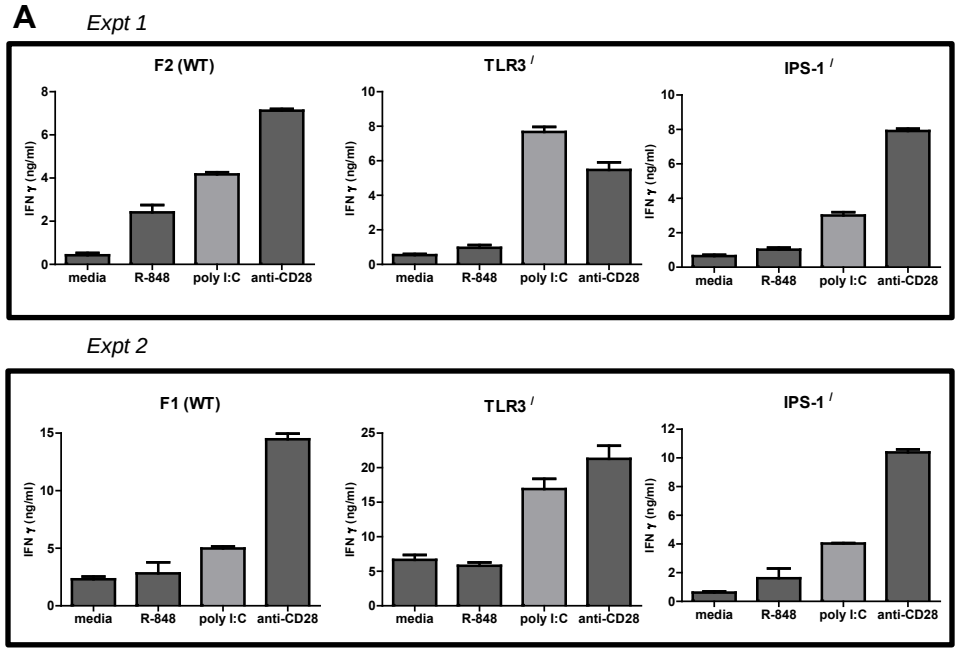


Figure 3.7. Signaling pathways induced following poly I:C treatment. FACS-sorted mouse CD4+ T-cells from mice deficient in specific pattern recognition receptors and signaling mediators were stimulated with anti-CD3 (6μg/ml) to delineate poly I:C signaling in CD4+ T cells. FACS-sorted mouse CD4+ T-cells were cultured for 3 days in the presence of R-848, poly I:C and anti-CD28. Cell culture supernatants were measured for the presence of IFN-γ by ELISA. Some of the knockout mice used were not yet fully backcrossed to B6 background. F1 and F2 (C57BL/6J x 129S1/SvlmJ) were used as wild type controls. (A) Anti-CD3 stimulation of F1, TLR3$^{-/-}$ and IPS-1$^{-/-}$ CD4+ T-cells (n=2, differences not significant)…

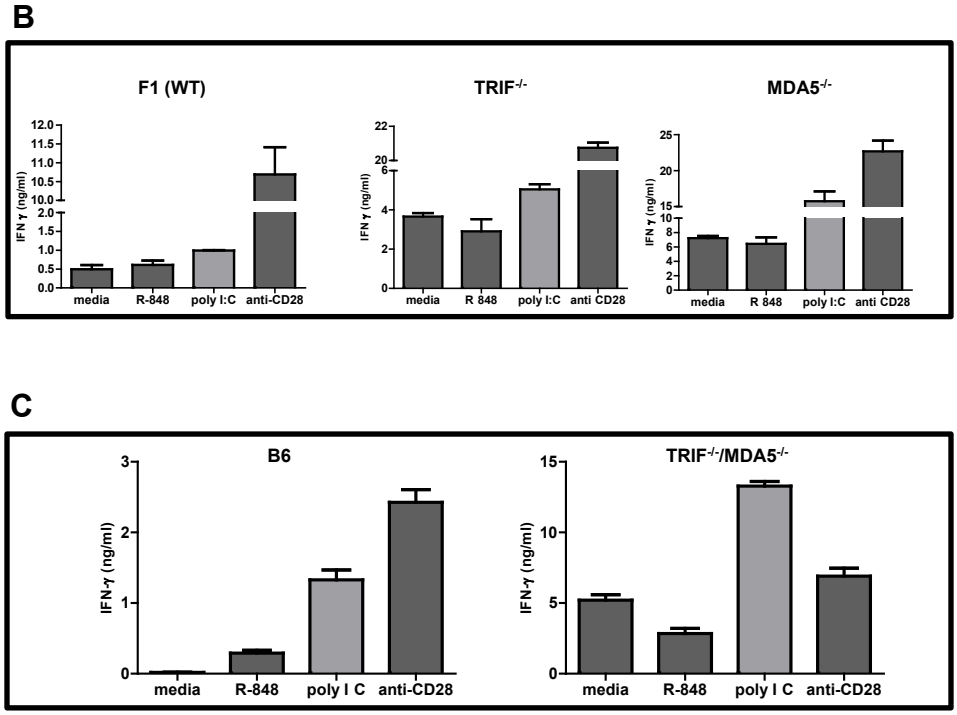


Figure 3.7. Signaling pathways induced following poly I:C treatment. *(continued)* …(B) F1, TRIF$^{-/-}$ and MDA5$^{-/-}$ (n=1); (C) B6 and TRIF$^{-/-}$/MDA5$^{-/-}$ (n=1) double knockout and…

D

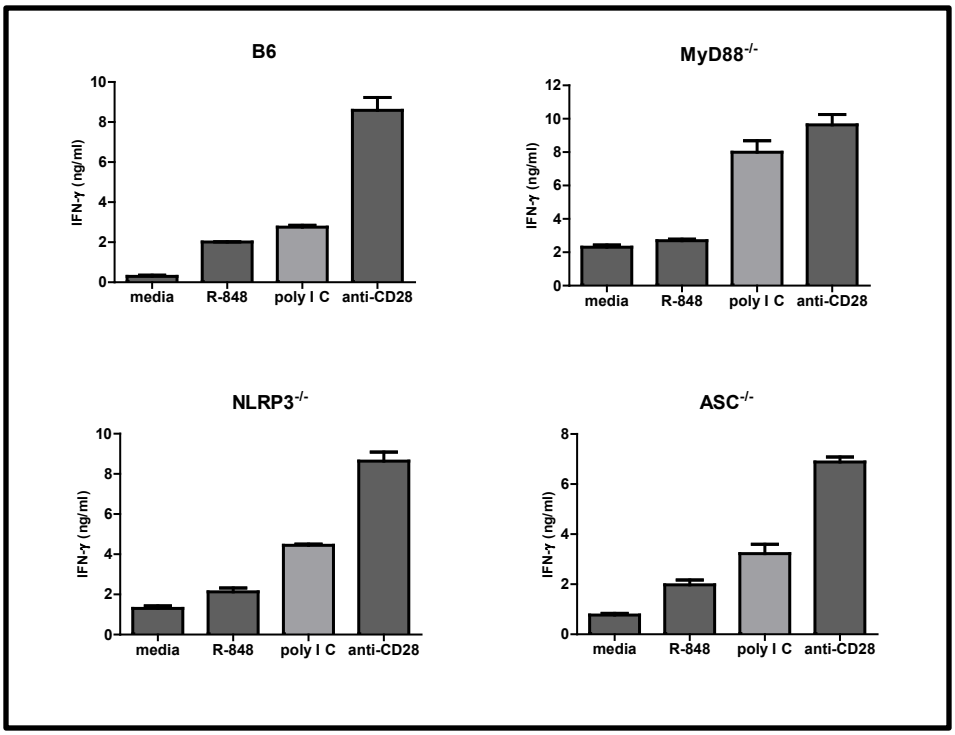


Figure 3.7. Signaling pathways induced following poly I:C treatment. *(continued)*
…(D) B6, MyD88$^{-/-}$, NALP3$^{-/-}$ and ASC$^{-/-}$ (n=1). Bars represent mean ± SEM.

RNA-dependent protein kinase inhibitor. The presence of these inhibitors abrogated IFN-γ production induced by poly I:C in mouse CD4+ T-cells. However, the inhibitors also abrogated the anti-CD28 positive control stimulation. The PKR inhibitors may be general serine/threonine kinase inhibitors or that these inhibitors induced cell death. More definitive experiments must be performed such as the use of PKR-deficient mice to firmly establish PKR involvement in poly I:C induced IFN-γ production by mouse CD4+ T-cells and viability assays such as MTT or LDH assays to address cell death.

E. Chapter Discussion

Sensing of RNA PAMPs by RNA-sensing PRRs is essential to recognize RNA viruses. In this chapter, we focused on the RNA-sensing PRRs expressed in CD4+ T-cells and their role in modulating CD4+ responses.

We focused on isolating ultrapure CD4+ T-cells to eliminate the secretion of cytokines by other cell types. In initial experiments, we utilized magnetically enriched (MACS) CD4+ T-cells and observed high IFN-γ production by R-848 stimulated CD4+ T-cells. However, minimal IFN-γ production was observed with R-848 when CD4+ T-cells were sorted by FACS (>99.5% purity). The difference observed between MACS-enriched and FACS-sorted T-cells suggests that IFN-γ may have been produced by other non-CD4+ T-cells such as NK cells and γδ T-cells. Poly I:C was previously shown to have direct co-stimulatory effect on human γδ T cells (170). Hence, CD4+ T-cells of high purity are required in the

anti-CD3 stimulation experiments to ensure that the IFN-γ measured is produced by CD4+ T-cells.

RNA-like IRMs were previously shown to exert extrinsic activity on T-cells by activating and maturing APCs (2, 4, 94). Induction of cytokine production and proliferation in ultrapure CD4+ T-cells in our hands (where APCs were eliminated) suggest that there are differential intrinsic effects of RNA PAMPs and RNA-sensing PRR recognition in CD4+ T-cells. The minimal responses observed in the R-848-stimulated CD4+ T cell only (APC-free) system suggests the importance of R-848 signaling and RNA-sensing PRR recognition in APCs and non-CD4+ T-cells. On the other hand, the direct poly I:C signaling and RNA-PRR recognition in CD4+ T-cells may be sufficient to drive, not only Th1, but Th2 responses.

The observed higher R-848 induced Th1 responses in human CD4+ T-cells compared to R-848 stimulated mouse CD4+ T-cells suggest that R-848 activates TLR8 in human CD4+ T-cells. Although TLR7 and TLR8 are close phylogenetic relatives, mouse TLR8 was previously thought to be nonfunctional (37). The activation of murine TLR8 by RNA-like IRM requires poly (dT). The combination of R-848 and poly(dT) in anti-CD3 stimulation experiments slightly enhanced the IFN-γ production by mouse CD4+ T-cells. The observed increase suggests TLR8 signaling or the enhancement of TLR8 signaling by poly (dT), however, an increase in IFN-γ production in the presence of poly(dT) alone was also observed. This experiment was only done once and therefore more

definitive experiments should be performed. The use of other IRMs such as 3M-001 (TLR7-selective) and 3M-002 (TLR8-selective) in anti-CD3 stimulated mouse CD4+ T-cells will allow us to delineate the contributions of each of these TLRs. These IRMs were used to demonstrate TLR8 to be functional in primary mouse PBMCs (37). The use of TLR8-deficient mice would provide more definitive results in the direct TLR8 co-stimulation of R-848 in CD4+ T-cells.

Though we observed minimal direct co-stimulation effect with R-848, the multiple cytokine analyses with Luminex® showed the increase of Th1 and Th2 cytokine production with both poly I:C and R-848. The up regulation of Th1 responses for direct lysis and Th2 responses for stimulating antibodies are desirable for viral vaccines. With our results we showed MyD88-dependent (R-848) and MyD88-independent (poly I:C) pathways can be directly stimulated in CD4+ T-cells to induce Th1 or Th2 responses. These results show the potential of R-848 or poly I:C as adjuvants in vaccines.

The robust response that we observed in poly I:C stimulated CD4+ T-cells made it a more likely candidate than R-848 for delineating the RNA-sensing PRRs pathways activated intrinsically in CD4+ T-cells. Since poly I:C is recognized by TLR3, MDA5 and NLRP3, we utilized mice deficient in these RNA-sensing PRRs and their signaling mediators. Interestingly, IFN-γ production by CD4+ T-cells was not abrogated in these gene-deficient CD4+ T-cells. These results led us to explore the involvement of PKR. Work from as early as the 1980's indicates that PKR is activated by cytoplasmic dsRNA. In some reviews,

PKR is described as a PRR for dsRNA (66, 113). Researchers demonstrated that PKR was required for IFN production in bone marrow-derived DCs (25) and mouse embryonic fibroblasts (35) following poly I:C stimulation. PKR has also been shown to contribute to West Nile virus like particle-induced interferon production and could serve as a PRR for recognition of WNV infection (35).

We observed complete abrogation of IFN-γ production by CD4+ T-cells with the use of PKR inhibitors, however, more definitive experiments should be performed, such as the use of PKR-deficient mice or the knockdown of PKR in CD4+ T-cells, to further address PKR involvement on the poly I:C direct co-stimulation of CD4+ T-cells.

We observed distinguishable differences between R-848 and poly I:C mediated co-stimulation of CD4+ T-cells but to fully discern the differences, more experiments should be performed. These include: the activation of transcription factors (IRFs, NF-κB, MAPKs), stimulation at different time points, determining cell death and viability after stimulation and to look at the cytotoxic activity of the RNA-like IRM treated CD4+ T-cells (by determining production of proteases involved in cell killing such as granzyme B). To further delineate poly I:C co-stimulation signaling, treatment with Con A (an inhibitor of the V-type ATPase would abolish acidification of endosomes and thereby, poly I:C binding to TLR3) and Lyo-Vec-complexed poly I:C (which is recognized by MDA5 but not by TLR3) can be used to differentiate between TLR3 and MDA5 signaling in CD4+ T-cells (112).

F. Chapter Summary

This chapter focused on the RNA-sensing PRRs expressed in mouse and human CD4+ T-cells. In these series of experiments, we have shown RNA-like IRMs to directly act on mouse and human CD4+ T-cells and enhance IFN-γ production. With the use of ultrapure CD4+ T-cells (without the confounding effects of non-CD4+ T-cell cytokine-producing lymphocytes) and mice deficient in specific PRRs and signaling mediators, we show variability in the responses elicited by RNA PAMPs and the direct co-stimulatory effects of RNA-like IRMs in mouse and human CD4+ T-cells. The results presented in this chapter suggest that recognition of RNA PAMPs and activation of the PRR pathways directly on CD4+ T-cells (intrinsic effect) induced cytokine production and proliferation in CD4+ T-cells.

CHAPTER IV

CRITICAL RNA-SENSING PRR PATHWAYS ARE ACTIVATED BY RNA PAMPS/ADJUVANTS THAT DRIVE Th1 CD4+ T-CELL RESPONSES IN cDC/CD4+ T-CELL INTERACTIONS

A. Introduction

In this chapter, we set out to delineate the essential signaling pathways by which the RNA-like IRMs, R-848 and poly I:C, augment CD4+ Th1 responses. Highly purified conventional dendritic cells (cDCs) and conventional CD4+ T-cells were co-cultured in mixed leukocyte reactions (MLRs) in order to evaluate specific RNA-like adjuvant effects on these central mediators of primary immune responses. We found that R-848 was a more effective CD4+ Th1 adjuvant than poly I:C in isolated cDC/CD4+ T-cell interactions. Type I IFN production and Type I IFN receptor signaling in cDCs were necessary but not sufficient for the CD4+ T-cell adjuvant activity of R-848. Early and rapid IL-1α production and IL-1β secretion from cDCs were also necessary for CD4+ Th1 adjuvant properties of R-848. Moreover, addition and blocking of these essential cytokines affect RNA-like IRM stimulated CD4+ Th1 responses. Our results provide important insights into the key signaling pathways responsible for RNA-like IRM CD4+ T-cell activation, and will help in the rational design of improved vaccine adjuvants.

B. R-848>poly I:C augmented alloreactive CD4+ Th1 responses in a cDC/CD4+ T-cell MLR

We utilized a MHC congenic MLR to investigate the essential signaling pathways by which RNA-like IRMs stimulate CD4+ Th1 responses. Highly purified murine cDCs and highly purified MHC congenic conventional CD4+ T-cells were co-cultured for six days in the presence or absence of RNA-like IRMs. The cDCs and CD4+ T-cells were sorted by flow cytometry to high purity in order to eliminate the potential effects of bystander cells (*e.g.* natural killer cells, γδ T-cells). Alloreactive CD4+ T-cell cytokine production was measured in cell culture supernatants and by ICS. At maximal doses, the murine TLR7 agonist R-848 was a strong stimulator of alloreactive IFN-γ-producing CD4+ T-cells, whereas, the TLR3 and MDA5 agonist poly I:C was less potent in this system (Figures 4.1a&b). Nearly all R-848 or poly I:C stimulated IFN-γ+ CD4+ T-cells also produced TNF-α (Figure 4.1b). We used IFN-γ expression and production as a measure of RNA-like IRM augmentation of CD4+ Th1 responses. Our data indicate that RNA-like IRM activation of MyD88-dependent signaling pathways (R-848) stimulated CD4+ Th1 responses better than MyD88-independent signaling pathways (poly I:C) in isolated cDC/CD4+ T-cell interactions.

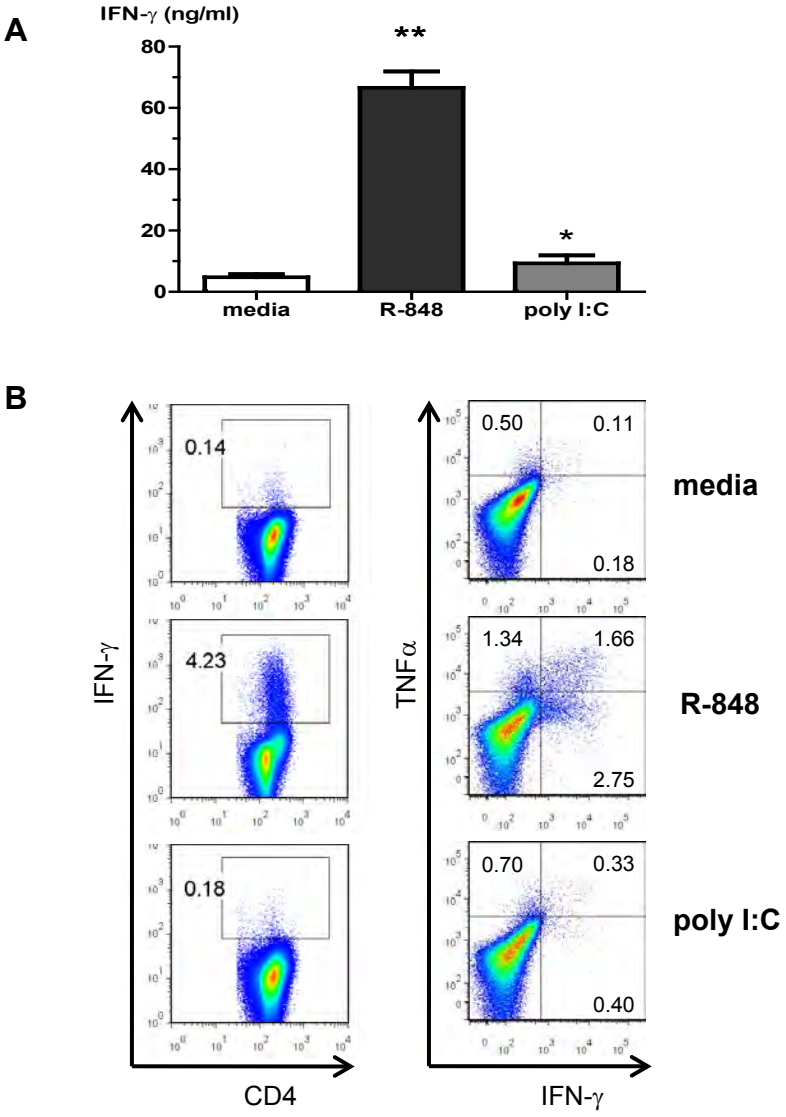


Figure 4.1. R-848 > poly I:C induces IFN-γ+ and TNF-α+ alloreactive CD4+ T-cells in a congenic MLR. Highly purified bone marrow-derived conventional dendritic cells (cDCs)(CD11c$^+$CD11b$^+$CD45R$^{-/lo}$) and conventional CD4+ T-cells (CD3$^+$ CD4$^+$CD8$^-$ TCRb$^+$DX5$^-$) were co-cultured for 6 d in the presence or absence of RNA-like IRMs. (A) Cell culture supernatant IFN-γ levels measured by ELISA. Values are mean ± SEM (n=3), **R-848 p<0.0001 compared to media control; *poly I:C p=0.0343 compared to media control (representative of at least three independent experiments shown). (B) ICS staining for IFN-γ and TNF-α producing conventional CD4+ T-cells. Values shown are the % of CD3$^+$CD4$^+$ T-cells producing IFN-γ (left column) and % IFN-γ$^+$TNF-α$^-$, IFN-γ$^-$TNF-α$^+$, or IFN-γ$^+$TNF-α$^+$ (right column) (representative of three independent experiments shown, left column and right column were from different experiments).

C. Type I IFN signaling in cDCs was necessary to stimulate CD4+ Th1 responses in a cDC/CD4+ T-cell MLR

RNA-like IRMs and RNA-sensing PRRs induce Type I IFN production and signaling in cDCs (98, 130). Type I IFN can drive DC maturation and activation (140). IFN-γ responses were abolished when cDCs from IFNAR$^{-/-}$ mice were used in MLRs compared to cDCs from wild-type B6 mice (Figures 4.2 a&b). The small increase in alloreactive CD4+ T-cell IFN-γ responses induced by poly I:C was also abrogated when cDCs from IFNAR$^{-/-}$ mice were used. Type I IFN signaling in cDCs was essential for the ability of R-848 and poly I:C to augment alloreactive CD4+ T-cell IFN-γ production in isolated cDC/CD4+ T-cell interactions. However, R-848 induced much lower IFN-β mRNA levels in cDCs compared to poly I:C (Figure 4.2c), and yet stimulated much higher CD4+ T-cell IFN-γ responses compared to poly I:C. This suggested that Type I IFN production and signaling in cDCs was essential but not sufficient for optimal RNA-like IRM stimulation of CD4+ Th1 responses.

D. R-848 stimulation of CD4+ Th1 responses was dependent on MyD88-mediated signaling in cDC and conventional CD4+ T-cells

R-848 activates murine TLR7/MyD88-dependent signaling (49). R-848 has been reported to have direct effects on cDCs and CD4+ T-cells (17, 71, 134). We therefore examined the role of MyD88-dependent signaling pathways in cDCs and CD4+ T-cells during R-848 stimulation of alloreactive CD4+ Th1 responses.

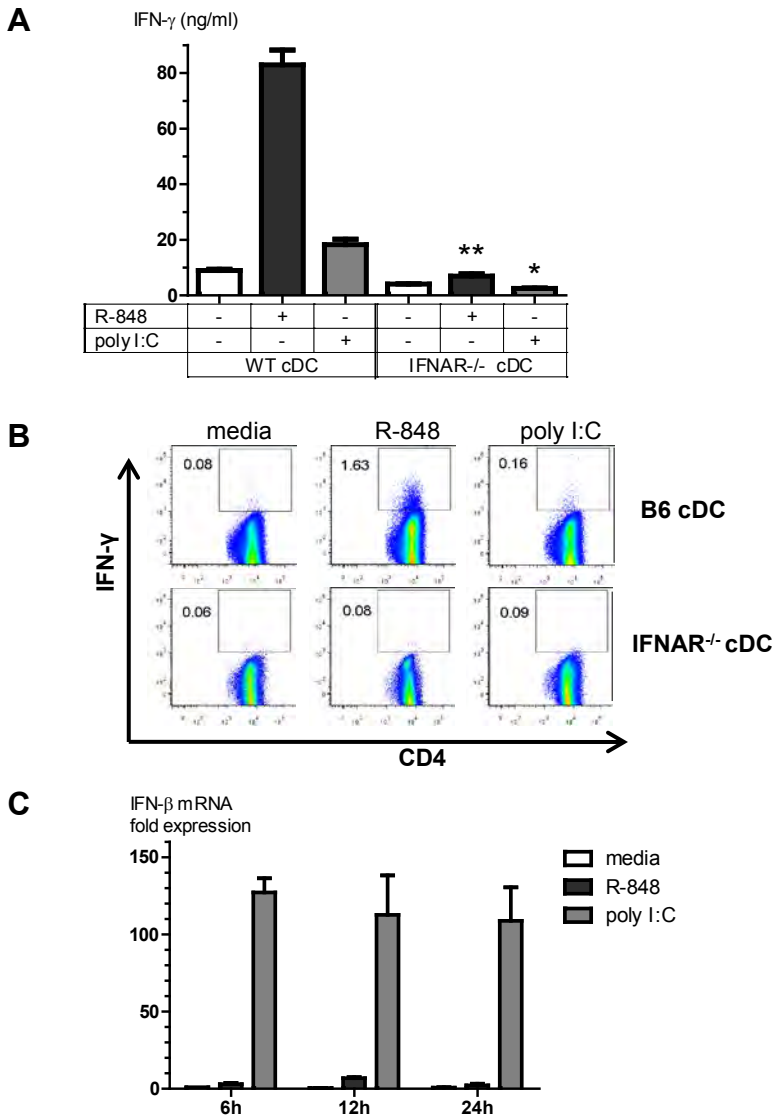


Figure 4.2. Type I IFN is essential for RNA-like IRMs to stimulate CD4+ Th1 responses in a cDC/CD4+ T-cell MLR. (A) IFN-γ levels in cell culture supernatants measured by ELISA in MHC congenic MLRs utilizing B6 or IFNAR$^{-/-}$ cDCs and B6.H2d CD4+ T-cells. Values are mean ± SEM (n=3). ** p=0.0008 compared to R-848 stimulated B6 cDC MLR, * p=0.0037 compared to poly I:C stimulated B6 cDC MLR. (B) ICS staining for IFN-γ producing conventional CD3^{+}CD4^{+} T-cells in MHC congenic MLRs utilizing B6 or IFNAR$^{-/-}$ cDCs and B6.H2d CD4+ T-cells (representative of three independent experiments shown). Values shown are the % of IFN-γ^{+} conventional CD3^{+}CD4^{+} T-cells. (C) cDCs were stimulated with R-848, poly I:C, or media (unstimulated control), and cellular RNA was collected at the indicated time points. IFN-β mRNA levels were measured by qRT-PCR, as described in the Methods section. Bars represent fold-change in IFN-β mRNA relative to unstimulated control at the 6 h time point (mean ± SEM, n=3).

Not surprisingly, IFN-γ production was entirely abrogated when MyD88$^{-/-}$ or TLR7$^{-/-}$ cDCs and B6.H2d CD4+ T-cells were used in MHC congenic MLRs (Figure 4.3a). IFN-γ production was also decreased when B6.H2d cDCs and MyD88$^{-/-}$ conventional CD4+ T-cells were used in MLRs (Figure 4.3b). This MyD88-dependence in conventional CD4+ T-cells was not mediated through TLR7 or IL-18 receptor signaling (Figure 4.3b and Figure 4.4 a&b). The data indicate that R-848 activation of TLR7/MyD88-dependent signaling in cDCs was critical for augmenting alloreactive CD4+ Th1 responses, and that TLR7-independent/MyD88-dependent signaling in CD4+ T-cells was also essential.

E. cDC IL-1α and IL-1β production and IL-1R-mediated signaling in cDCs and conventional CD4+ T-cells, were essential for R-848 stimulation of CD4+ Th1 responses

We next explored the role of IL-1R/MyD88 signaling in R-848 stimulation of CD4+ Th1 responses. When IL-1R$^{-/-}$ cDCs and B6.H2d CD4+ T-cells were used in the MHC congenic MLRs, IFN-γ production was 45 ± 9% lower compared to responses when wild-type cDCs were used (mean±SEM, *n*=3, p=0.03) (Figures 4.5 a&b). Similarly, when B6.H2d cDCs and IL-1R$^{-/-}$ CD4+ T-cells were used in MHC congenic MLRs, IFN-γ production was 55 ± 5% lower compared to responses when wild-type CD4+ T-cells were used (mean±SEM, *n*=3, p=0.01)(Figures 4.5 c&d). The observed effect of eliminating IL-1R-mediated signaling on R-848 stimulation of CD4+ Th1 responses in both cDC and CD4+ T-

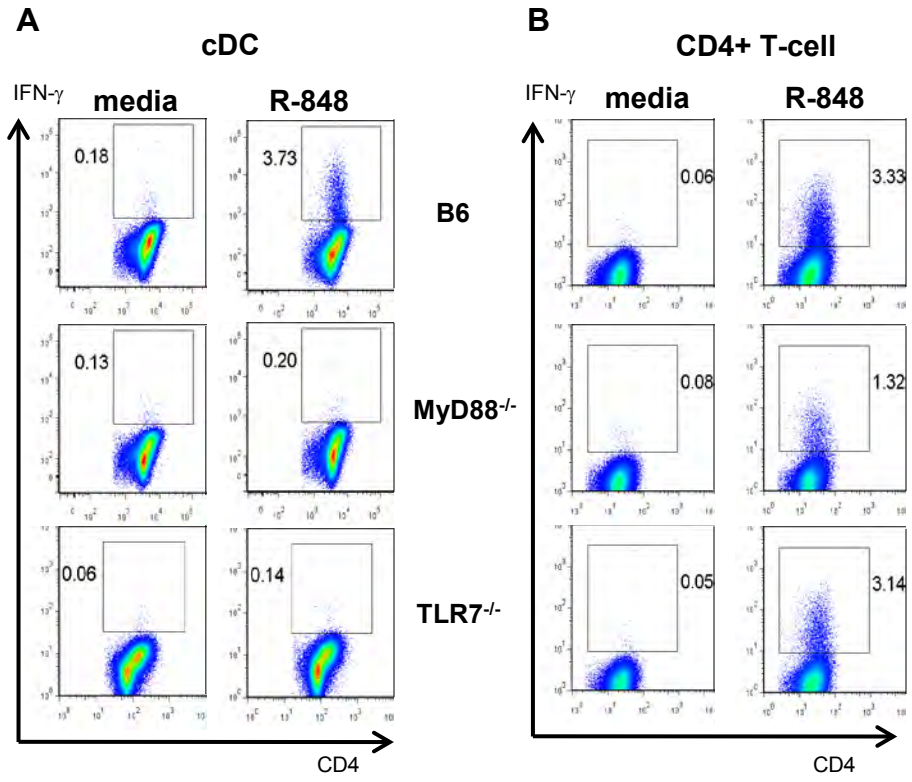


Figure 4.3. TLR7 signaling in cDCs, and TLR7-independent/MyD88-mediated signaling in CD4+ T-cells, are essential for R-848 induced CD4+ Th1 responses. (A) ICS staining for IFN-γ producing CD4+ T-cells in MHC congenic MLRs utilizing B6, MyD88$^{-/-}$, or TLR7$^{-/-}$ cDCs and B6.H2d CD4+ T-cells (representative of two independent experiment shown) (B) ICS staining for IFN-γ producing CD4+ T-cells in MHC congenic MLRs utilizing B6.H2d cDCs and B6, MyD88$^{-/-}$, or TLR7$^{-/-}$ CD4+ T-cells (representative of two independent experiments shown). Values shown are the frequencies of IFN-γ^{+} conventional CD3^{+}CD4^{+} T-cells.

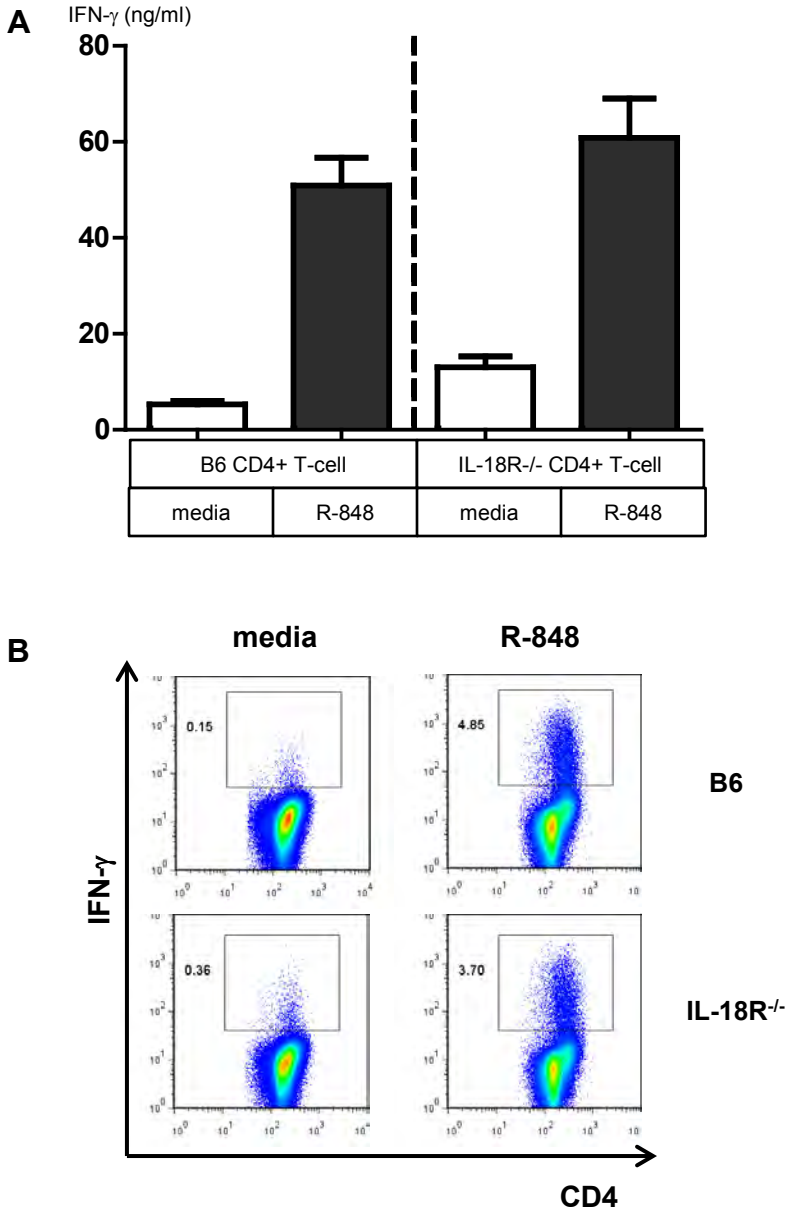


Figure 4.4. MyD88-dependence in conventional CD4+ T-cells was not mediated through IL-18 receptor signaling. (A) Cell culture supernatant IFN-γ levels (day 6) in MHC congenic MLRs utilizing B6.H2d cDCs and B6 or IL-18R$^{-/-}$ CD4+ T-cells. Values are mean ± SEM. (B) ICS staining for IFN-γ producing CD4+ T-cells in MHC congenic MLRs utilizing B6.H2d cDCs and B6 or IL-18R$^{-/-}$ CD4+ T-cells. Values shown are the frequencies of IFN-γ^{+} CD3^{+}CD4^{+} T-cells (n=3).

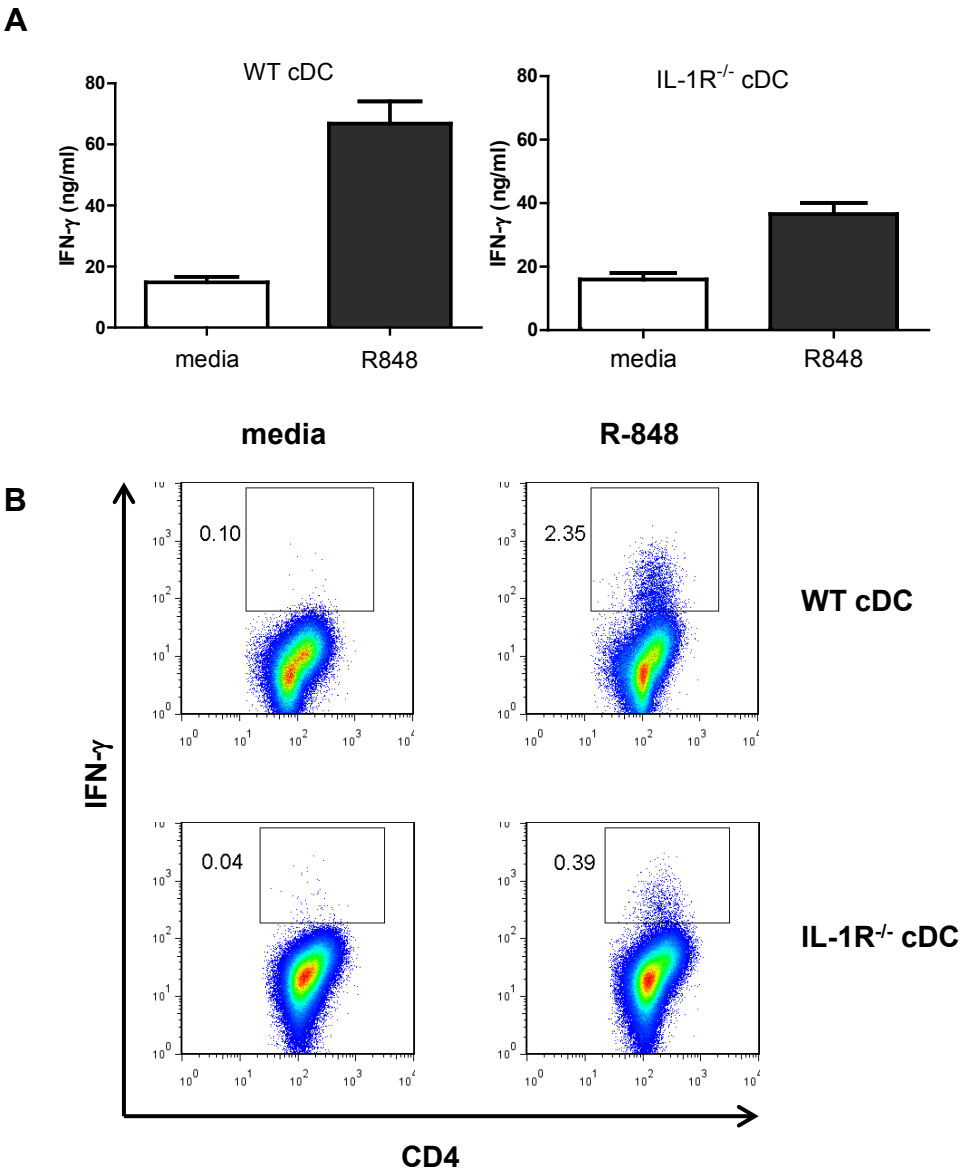


Figure 4.5. IL-1R-mediated signaling in cDCs and conventional CD4+ T-cells are essential for R-848 stimulation of CD4+ Th1 responses. (A) IFN-γ levels in cell culture supernatants in MHC congenic MLRs utilizing B6 or IL-1R$^{-/-}$ cDCs and B6.H2d CD4+ T-cells. Values are mean ± SEM (n=3). (B) ICS staining for IFN-γ producing conventional CD3^{+}CD4^{+} T-cells in MHC congenic MLRs utilizing B6 or IL-1R$^{-/-}$ cDCs (allogeneic MLR, representative of two experiments, n=3). Values shown are the % of IFN-γ^{+} conventional CD3^{+}CD4^{+} T-cells…

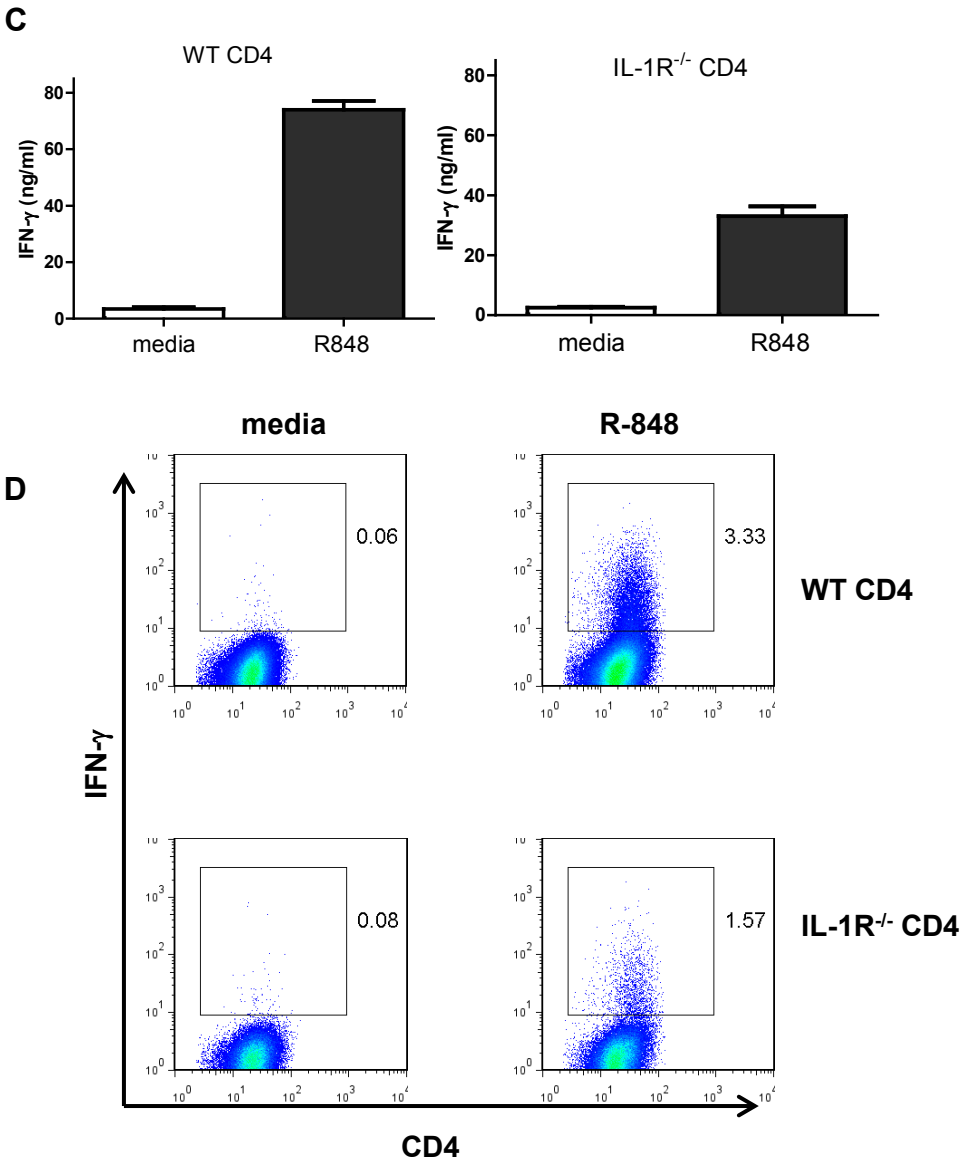


Figure 4.5. IL-1R-mediated signaling in cDCs and conventional CD4+ T-cells are essential for R-848 stimulation of CD4+ Th1 responses. *(continued)…* (C) IFN-γ levels in cell culture supernatants in MHC congenic MLRs utilizing B6.H2d cDCs and B6 or IL-1R$^{-/-}$ CD4+ T-cells. Values are mean ± SEM (n=3). (D) ICS staining for IFN-γ producing conventional CD3^{+}CD4^{+} T-cells in MHC congenic MLRs utilizing B6.H2d cDCs and B6 or IL-1R$^{-/-}$ CD4+ T-cells (congenic MLR, only one experiment done, n=3). Values shown are the % of IFN-γ^{+} conventional CD3^{+}CD4^{+} T-cells.

cells prompted us to examine IL-1α and IL-1β production by cDCs. R-848-induced alloreactive CD4+ T-cell IFN-γ production was decreased when IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, or IL-1$\alpha\beta^{-/-}$ cDCs and B6.H2d CD4+ T-cells were used in the MLRs (Figures 4.6 a&b). IL-1α and IL-1β had largely non-redundant roles in driving the alloreactive CD4+ Th1 responses to R-848 in this system. Collectively, our data suggest that the Type I IFN, IL-1α, and IL-1β are all necessary for the optimal stimulation of CD4+ Th1 responses by RNA-like IRMs.

F. R-848 rapidly increases cDC production of pro-IL-1α and pro-IL-1β mRNA and protein

We next explored the differences in the kinetics of IL-1α and IL-1β production by R-848- and poly I:C-stimulated cDCs. R-848 stimulation of cDCs produced a rapid upregulation of pro-IL-1α and pro-IL-1β mRNA (Figures 4.7 a&b), whereas, poly I:C stimulation of cDCs produced a slower and more gradual increase in pro-IL-1α and pro-IL-1β mRNA expression (Figures 4.7 c&d). Biologically active IL-1α (precursor and mature IL-1α) is primarily intracellular and membrane-associated, while biologically active IL-1β (mature IL-1β) is primarily secreted (27). Comparable levels of IL-1α protein (precursor and mature forms) were measured in the cell lysates from R-848 and poly I:C-stimulated cDCs at early time points (Figure 4.8). R-848 and extracellular ATP stimulation of cDCs rapidly induced higher levels of secreted mature IL-1β

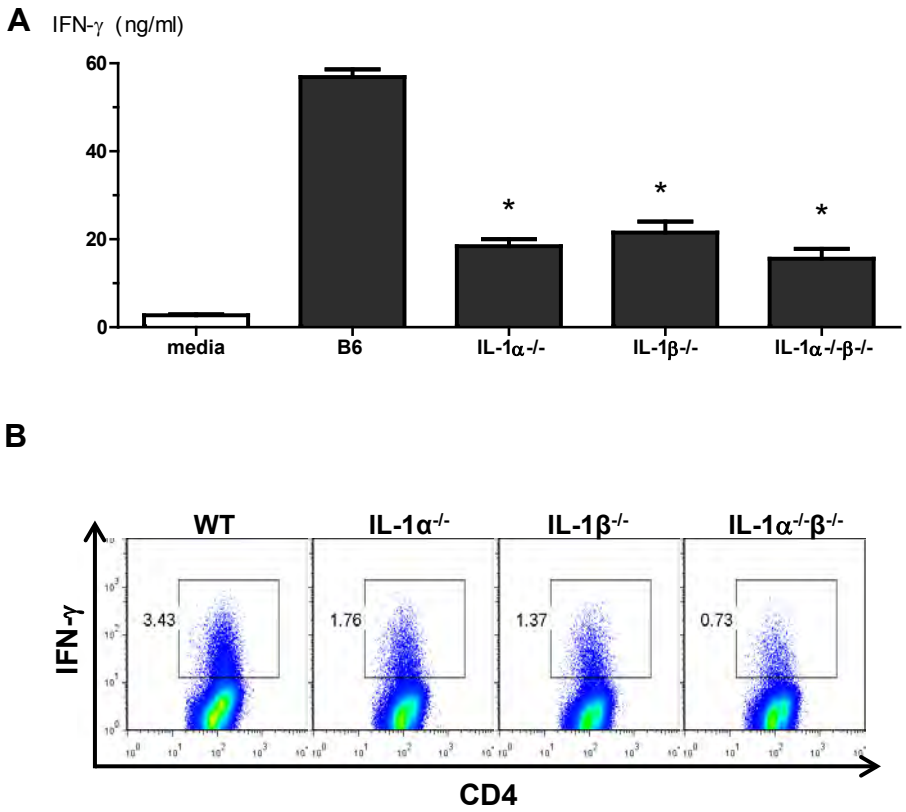


Figure 4.6. IL- 1α and IL-1β are essential for R-848 stimulation of CD4+ Th1 responses. (A) IFN-γ levels in cell culture supernatants (day 6) in MHC congenic MLRs utilizing B6, IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$ or IL-1$\alpha^{-/-}\beta^{-/-}$ cDCs and B6.H2d CD4+ T-cells. Values are mean ± SEM (n=6). *p<0.05 compared to R-848 stimulated B6 cDC MLR. (B) ICS staining for IFN-γ producing CD4+ T-cells in MHC congenic MLRs utilizing B6, IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$ or IL-1 $\alpha^{-/-}\beta^{-/-}$ cDCs and B6.H2d CD4+ T-cells (representative of two independent experiments shown). Values shown are the % of IFN-γ^{+} conventional $CD3^{+}CD4^{+}$ T-cells.

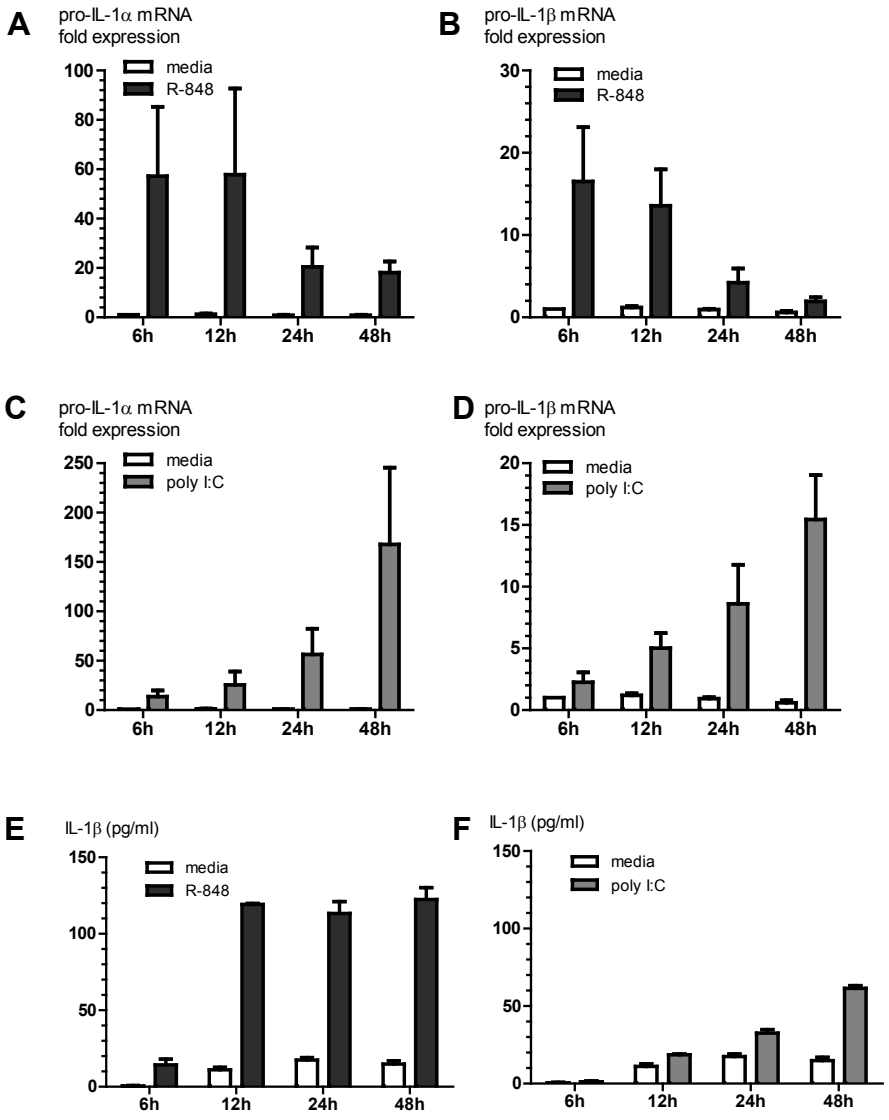


Figure 4.7. R-848 induces a rapid increase in pro-IL- 1α and pro-IL-1β mRNA expression and protein production in cDCs. cDCs were stimulated with R-848, poly I:C, or media (unstimulated control), and cellular RNA and cell culture supernatants were collected at the indicated time points. Pro-IL- 1α (A&B) and pro-IL-1β (C&D) mRNA levels were measured by qRT-PCR, as described in the Methods section. Bars represent fold-change in pro-IL- 1α or pro-IL-1β mRNA relative to unstimulated control at the 6 h time point (mean ± SEM, n=3). (E&F) IL-1β protein levels (precursor and mature forms) were measured in cell culture supernatants by ELISA (mean ± SEM, n=3).

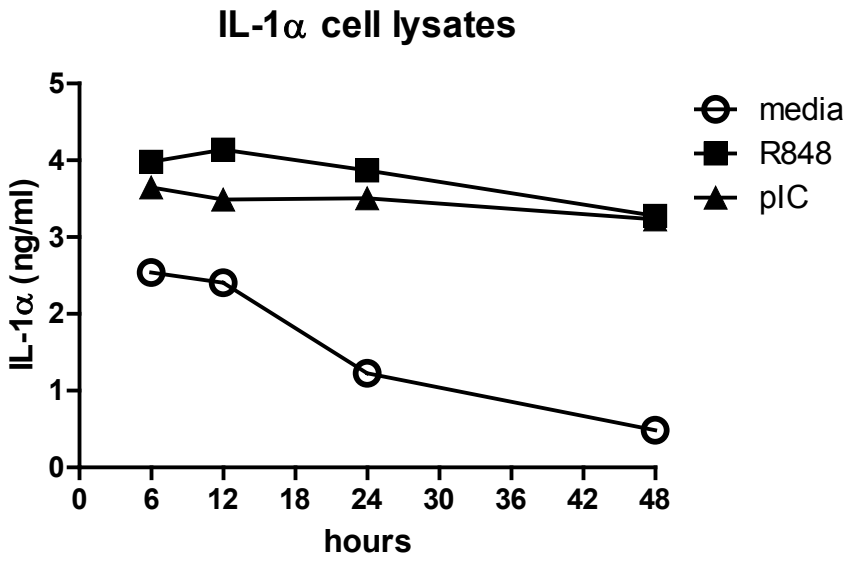


Figure 4.8. Comparable levels of IL-1α protein (precursor and mature forms) were measured in R-848 and poly I:C-stimulated cDCs. cDCs were stimulated with R-848, poly I:C, or media (unstimulated control), and the cell lysates were collected at the indicated time points. Pro-IL- 1α protein levels were measured by ELISA (representative of one out of two independent experiments shown).

protein in cell-free culture supernatants compared to poly I:C and extracellular ATP (Figures 4.7 e&f and Figure 4.9).

To further show the essential roles of type I IFN, IL-1α and IL-1β in driving CD4+ Th1 responses, we added recombinant type I IFN, IL-1α and IL-1β to the MLR to determine whether these cytokines could increase IFN-γ levels in CD4+ T-cells. We found that type I IFN (200 U/ml), rIL-1α (125 pg/ml) and rIL-1β (125 pg/ml) were sufficient to induce a three-fold increase in IFN-γ production. IFN-γ levels following treatment with recombinant cytokines were comparable to responses when poly I:C was used in MLR (Figure 4.10).

In MHC congenic MLRs, poly I:C treatment induced rapid and sustained Type I IFN production (Figure 4.2c). Pro-IL-1α and pro-IL-1β were induced by poly I:C at later time points compared to R-848 (Figure 4.7). To further examine the early roles of IL-1α and IL-1β, we added mouse rIL-1α and rIL-1β at the same time as poly I:C in MHC congenic MLRs. Addition of rIL-1α and rIL-1β increased poly I:C induced cytokine-producing (IFN-γ, TNF-α and IL-2) CD4+ T-cells (Figure 4.11). The data indicate that early IL-1α and IL-1β production and signaling are essential for the robust RNA-like IRM stimulation of CD4+ Th1 responses.

G. Inhibition of IL-1 and Type I IFN signaling abrogated R-848 stimulation of CD4+ Th1 responses

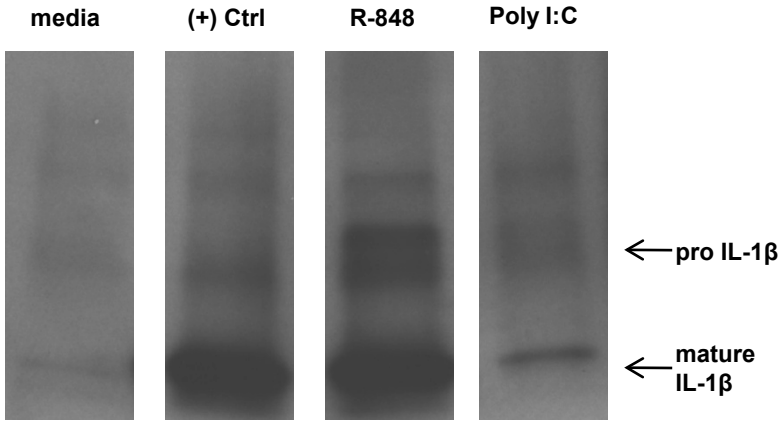


Fig. 4.9. R-848 and extracellular ATP stimulates early mature IL-1β secretion from cDCs. Non-primed DCs were stimulated for 6 h with R-848 or poly I:C with extracellular ATP (5 mM). As a positive control, cDCs were primed with lipopolysaccharide (200 ng/ml) for 3 hours prior to stimulation with nigericin (10 μM). Mature IL-1β protein was detected by Western blot in cell culture supernatants. Data shown is representative of three independent experiments.

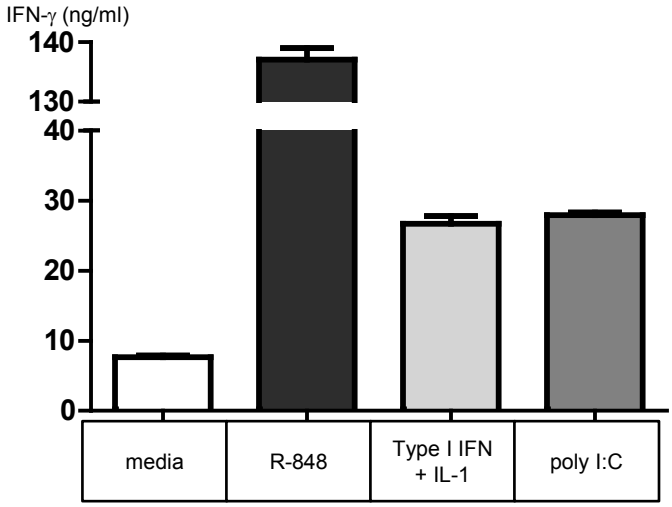


Figure 4.10. Addition of type I IFN, rIL-1α and rIL-1β increased IFN-γ production in CD4+ T-cells. IFN-γ levels (day 6) in cell culture supernatants in MHC congenic MLRs. 200 U/ml type I IFN and 125 pg/ml of rIL-1α and rIL-1β were added to MHC congenic cDC/CD4+ MLRs. Values shown are mean IFN-γ levels (ng/ml) ± SEM (n=2).

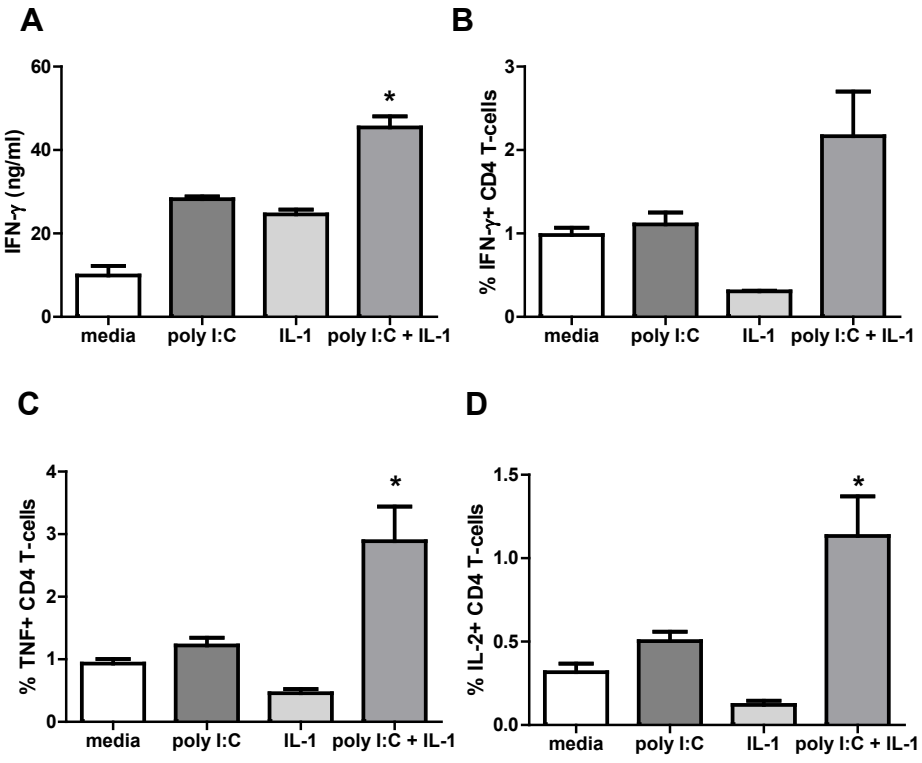
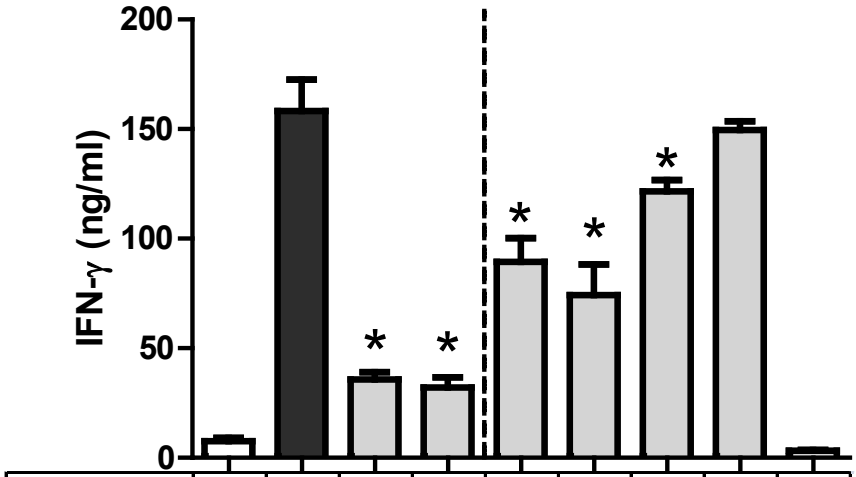


Figure 4.11. The addition of rIL- 1α and rIL-1β increased poly I:C-induced CD4+ Th1 responses in a cDC/CD4+ T-cell MLR. (A) IFN-γ levels in cell culture supernatants in poly I:C stimulated MHC congenic MLR with mouse rIL- 1α and rIL-1β. ICS staining for IFN-γ (B), TNF-α (C) and IL-2 (D) producing conventional CD4+ T-cells. Values are mean ± SEM (n=3) (representative of two independent experiments shown). *p<0.05 compared to media control.

To further assess the role of IL-1 in R-848-induced CD4+ Th1 responses, a natural inhibitor of IL-1 signaling, sIL-1Ra, and its synthetic form, Anakinra^{TM}, were added to R-848 stimulated MHC congenic MLRs. To inhibit Type I IFN signaling, antibodies against the IFN α/β receptor were also added. Inhibition of both IL-1 and Type I IFN signaling diminished the robust R-848 induced CD4+ Th1 responses (Figure 4.12). Inhibition of either IL-1 or Type I IFN signaling also reduced R-848 induced CD4+ Th1 responses but to a lesser degree (Figure 4.12). Taken together, Type I IFN production and signaling by itself was not sufficient to drive RNA-like IRM stimulated CD4+ Th1 responses. The combined actions of Type I IFN, IL-1α, and IL-1β were essential for the optimal activation of alloreactive CD4+ Th1 responses in cDC/conventional CD4+ T-cell interactions.

H. Chapter Discussion

There is a growing need for novel vaccine adjuvants with potent T-cell immune stimulatory effects. In this chapter, we investigated the critical cellular signaling pathways used by RNA-like IRMs (R-848 and poly I:C) to stimulate CD4+ Th1 responses during alloreactive cDC/CD4+ T-cell interactions. RNA-like IRM stimulation of Type I IFN production and signaling was essential but not sufficient for driving CD4+ Th1 responses. The rapid and early production of IL-1α and IL-1β was equally critical for the optimal activation of Th1 CD4+ T-cells. In cDCs, R-848 activation of TLR7/MyD88-dependent signaling led to a rapid upregulation of pro-IL-1α and pro-IL-1β production compared to poly I:C



R-848	-	+	+	+	+	+	+	+	-
Anakinra	-	-	+	-	+	-	-	-	-
sIL-1Ra	-	-	-	+	-	+	-	-	-
anti-IFNAR	-	-	+	+	-	-	+	-	-
IgG1 isotype	-	-	-	-	-	-	-	+	+

Figure 4.12. Inhibition of Type I IFN, IL- 1α and IL-1β signaling decreases R-848-induced CD4+ Th1 responses. Type I IFN and IL-1 signaling was blocked with anti-mouse IFN α/β receptor, Anakinra™ and sIL-1Ra. IFN-γ levels (day 6) in cell culture supernatants were measured in MHC congenic MLRs with treated and mock treated cells. Blocking of either Type I IFN or IL-1α/β signaling decreased IFN-γ levels in cell culture supernatants. Values are mean ± SEM (n=6). *p<0.05 compared to R-848 stimulated MLR (black bar).

activation of MyD88-independent signaling pathways. R-848 stimulated the early production and secretion of mature IL-1β from cDCs and augmented alloreactive CD4+ Th1 responses to a greater degree than poly I:C. Our data suggests that the CD4+ T-cell adjuvant activity of RNA-like IRMs is mediated by a critical combination of rapid Type I IFN, IL-1α and mature IL-1β production.

The RNA-like IRMs, R-848 and poly I:C, are well-known inducers of Type I IFN in many cell types and have been shown to be potent CD4+ T-cell adjuvants (2, 130, 132, 165). Longhi *et al.* (98) primed and boosted mice with a dendritic cell targeted HIV gag protein vaccine and TLR agonists. They found poly I:C to be the most effective inducer of Type I IFN and the superior adjuvant to elicit CD4+ T cell immunity. Antibody blocking or deletion of Type I IFN receptor markedly reduced DC maturation, T-cell proliferation, and the development of adaptive Th1 immunity in response to the HIV gag protein. Ichinohe et al. (60) also investigated the mucosal adjuvant effect of poly I:C by intranasal co-administration with inactivated influenza virus HA vaccines and observed cross-protection against heterologous infection. The authors observed upregulated expression of Type I IFN, and Th1/Th2 cytokines following the administration of HA vaccine with poly I:C. Vasilakos *et al.* (165) demonstrated that mice immunized with chicken ovalbumin and R-848 induced high levels of Type I IFN, and neutralizing antibodies to Type I IFN inhibited ovalbumin-specific CD4+ Th1 responses. Similarly, in isolated cDC/CD4+ T-cell interactions, we found that Type I IFN production and signaling was necessary for the activation of Th1

alloreactive CD4+ T-cells by RNA-like IRMs. However, poly I:C was a more potent inducer of Type I IFN in highly purified cDCs compared to R-848, yet it was less potent at stimulating alloreactive CD4+ Th1 responses. This suggested that Type I IFN production and signaling in cDCs was essential but not sufficient for RNA-like IRMs to augment CD4+ Th1 responses in isolated alloreactive cDC/CD4+ T-cell interactions.

We found that the ability of R-848 to augment alloreactive CD4+ Th1 responses in a cDC/CD4+ T-cell MLR was also dependent on the early and rapid production of functional IL-1α and IL-1β from cDCs. MyD88-dependent IL-1R mediated signaling in both cDCs and CD4+ T-cells was important in mediating the CD4+ Th1 stimulatory effect of R-848. IL-1 stimulates cDC maturation and activation (43, 101) and can act directly on CD4+ T-cells to enhance their differentiation and cytokine production (11). T-cells can also produce IL-1 and appears to serve an autocrine role in T-cell activation (156, 164).

The Th1 adjuvant effects of IL-1 have been previously reported in *in vivo* studies. Ben-Sasson et al. (11), used recipient mice that were immunized with pigeon cytochrome C peptide together with different proinflammatory cytokines (administered through a miniosmotic pump). Only IL-1 augmented primary immune responses among a series of cytokines tested, including TNFα, IL-1, IL-6, IL-18 and IL-33. IL-1α and IL-1β displayed similar potency (11). The timing and the concentration of IL-1 are critical for an optimal effect. Staruch et al. (152) have shown that the optimal adjuvant effect of IL-1 was 2 hours after the priming

dose of bovine serum albumin (BSA) protein antigen. These authors have also observed dose-dependent enhancement of CD4+ T-cell responses to BSA. Similarly, in our MHC congenic MLR system, we found that the early addition of IL-1 was necessary for increased adjuvant effects of poly I:C.

The observed comparable decreases in the frequencies of IFN-γ producing CD4+ T cells when utilizing IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$ and IL-1$\alpha\beta^{-/-}$ cDCs in the MLRs suggested that IL-1α and IL-1β have largely non-redundant roles in stimulating alloreactive CD4+ Th1 responses. Though IL-1α and IL-1β both bind and activate IL-1R (26), differences in their localization might explain their apparent non-redundant effects (27, 29, 103). Biologically active IL-1α (precursor and mature forms) is predominantly cell- and membrane-associated (93), and likely acts in an autocrine or even intracrine manner in antigen-presenting cells (27). The pro-IL-1α is processed by calpain (7, 102) and heat shock, calcium ionophores and ATP have all been shown to stimulate the release of mature IL-1α *in vitro* (102). In contrast, pro-IL-1β is biologically inactive and has different posttranslational processing requirements than pro-IL-1α. Caspase-1-containing inflammasomes (*e.g.* NLRP3 inflammasome) cleave pro-IL-1β into the biologically active mature form which is secreted into the extracellular environment in a non-classical manner (29, 104). Thus, mature IL-1β likely acts predominantly in a paracrine or endocrine manner, activating IL-1R signaling in effector cells such as CD4+ T-cells. The preferential cell association of IL-1α, in

contrast to the existence of IL-1β primarily as a soluble protein; difference in processing and release may all be important in explaining the non-redundant effects for these two types of IL-1.

Other studies conducted on IL-1α and IL-1β-deficient mice have also shown non-redundant roles for these cytokines in immune responses and disease pathogenesis. IL-1β, but not IL-1α, was required for antigen-specific T-cell activation in delayed-type hypersensitivity responses (120) and in T-cell dependent antibody production against sheep red blood cells (55, 118). IL-1α, but not IL-1β, modulated the antiviral and immunoregulatory activities of IFN-γ (55), and was necessary for cell- and tissue-injury induced sterile inflammatory responses (18). Further studies are needed to discriminate the potential non-redundant roles of IL-1α and IL-1β in the RNA-like adjuvant augmentation of CD4+ Th1 responses.

IL-1α is secreted to a much lesser extent than IL-1β. It is not commonly detected in body fluids except during severe inflammation in which, pro-IL-1α is possibly released from necrotizing cells (18, 102, 115). It is therefore possible that RNA-like IRMs trigger cell death that consequently releases IL-1. We performed some cell death assays (MTT assay, data not shown) but we did not see any significant differences. In our ICS analyses, we used LIVE/DEAD cell stain kit to exclude nonviable cells. No notable increase in dead lymphocytes was observed in the presence of RNA-like IRMs (data not shown). Hence, our

observations suggest that the concentrations of RNA-like IRMs that we used in MLRs did not cause cell death.

In cDCs, R-848 induced less Type I IFN but early and more robust IL-1α and IL-1β production than poly I:C. R-848 was also a more potent CD4+ T-cell adjuvant compared to poly I:C in the cDC/CD4+ T-cell MHC congenic MLR system. However, poly I:C has also been shown to be a potent CD4+ T-cell adjuvant *in vivo* (60, 98, 163). When administered as an adjuvant in mice, the main source of poly I:C-induced Type I IFN has been reported to be non-hematopoietic cells (98). In addition, poly I:C has been shown to induce mRNA expression (105) and protein production of IL-1 both *in vitro* (5, 131) and *in vivo* (5, 30, 133). In our isolated cDC/CD4+ T-cell MHC congenic MLR, poly I:C stimulation did not induce sufficient Type I IFN and IL-1. The increase in CD4+ Th1 responses with exogenous rIL-1 in poly I:C stimulated MHC congenic MLRs supports this hypothesis. Furthermore, increased Type I IFN production has been shown to inhibit IL-1 production (40). In the said paper, the investigators found that type I IFN strongly suppressed IL-1 production through two distinct mechanisms – STAT1 dependent inhibition of NLRP3 and NLRP1 inflammasome activity and the enhanced production of IL-10 of bone marrow-derive macrophages that decreased the levels of pro-1L-1α and pro-IL-1β. The delayed IL-1 production observed in poly I:C stimulated MHC congenic MLRs may be an effect of the robust poly I:C-induced type I IFN in the isolated cDC/CD4+ T-cell MLR system that consequently inhibited IL-1 production.

The essential roles of these cytokines were also evident in the abrogation of CD4+ Th1 responses upon Type I IFN and IL-1 signaling blockade in R-848 stimulated MHC congenic MLRs. The observed differences in the cDC production of type I IFN, IL-1α and IL-1β between R-848 and poly I:C in MHC congenic MLRs indicate that there are complex signaling cascades for the initiation of CD4+ Th1 responses. Complete elucidation and a better understanding of these mechanisms would offer further possibilities for the use of these RNA-like IRMs as adjuvants.

We observed that the ability of R-848 to augment alloreactive CD4+ Th1 responses was also dependent on MyD88-mediated signaling in the CD4+ T-cells but not fully MyD88-dependent as observed in cDCs. This suggests that MyD88-dependent signaling is important in R-848 stimulated cDC cytokine production and is also intrinsically essential in CD4+ T-cells. IL-1R mediated signaling could only explain part of this profound CD4+ T-cell MyD88 signaling dependence. The isolated removal of CD4+ T-cell TLR7 or IL-18 receptor signaling did not affect R-848 induced alloreactive CD4+ Th1 responses. It is possible that there is overlap and a partial redundancy of the signaling effects in CD4+ T-cells among IL-1R, IL-18R, and TLR7/MyD88 dependent pathways. It is also be possible that other MyD88-dependent signaling pathways in CD4+ T-cells, yet to be identified, are involved in priming and activation (177).

In summary, RNA-like IRMs are effective CD4+ Th1 adjuvants and their ability to induce Type I IFN is critical for their T-cell stimulatory activity. In MLRs utilizing

two specific cell types, cDCs and conventional CD4+ T-cells, we have shown that early and rapid IL-1α and mature IL-1β production are equally critical with Type I IFN production. Upon RNA-like IRM stimulation, Type I IFN, IL-1α and IL-1β mainly produced by cDCs drive the activation of CD4+ Th1 responses. R-848-mediated CD4+ Th1 responses are mainly TLR7/MyD88-dependent in cDCs. MyD88 signaling is also important in CD4+ T-cells but not mediated by TLR7 signaling. Together, these results show that the CD4+ T-cell adjuvant activity of RNA-like IRMs involves a complex interplay of critical PRR and innate immune signaling pathways in different cells.

I. Chapter Summary

There is a growing need for novel vaccine adjuvants that can provide safe and potent T-helper type 1 (Th1) activity. RNA-like immune response modifiers (IRMs) are candidate T-cell adjuvants that skew acquired immune responses towards a Th1 phenotype. In this chapter, we set out to delineate the essential signaling pathways by which the RNA-like IRMs, resiquimod (R-848) and polyinosinic:polycytidylic acid (poly I:C), augment CD4+ T-helper 1 (Th1) responses. Highly purified murine conventional dendritic cells (cDCs) and conventional CD4+ T-cells were co-cultured in allogeneic and MHC congenic mixed leukocyte reactions. The activation of CD4+ Th1 cells was examined utilizing cells from mice deficient in specific RNA-sensing pattern recognition receptors and signaling mediators. R-848 and poly I:C stimulation of Type I

interferon production and signaling in cDCs was essential but not sufficient for driving CD4+ Th1 responses. The early and rapid production of IL-1α and IL-1β was equally critical for the optimal activation of Th1 CD4+ T-cells. R-848 activation of Toll-like receptor 7/MyD88-dependent signaling in cDCs led to a rapid upregulation of pro-IL-1α and pro-IL-1β production compared to poly I:C activation of MyD88-independent signaling pathways. The *in vitro* data show that CD4+ T-cell adjuvant activity of RNA-like IRMs is mediated by a critical combination of early and rapid Type I interferon, IL-1α and IL-1β production. These results provide important insights into the key signaling pathways responsible for RNA-like IRM CD4+ Th1 activation. A better understanding of the critical signaling pathways by which RNA-like IRMs stimulate CD4+ Th1 responses is relevant to the rational design of improved vaccine adjuvants.

CHAPTER V:

CONTRIBUTIONS OF RNA-SENSING PRR PATHWAYS ON Th1 RESPONSES TO INFLUENZA A VIRUS INFECTION

A. Introduction

To explore further the contributions of RNA-sensing PRRs in the modulation of immune responses, we utilized an influenza A virus (IAV) mouse infection model and mice deficient in specific RNA-sensing PRRs and signaling mediators.

RNA-sensing PRRs, mediate the initial recognition of IAV and shape the adaptive immune response (69). This includes the MyD88 signaling dependent TLR7 (24) and the MyD88 signaling independent receptors TLR3 (42), RIG-I (81) and NLRP3 (78). MyD88 signaling has been found to be essential for the optimal protection against various pathogens including homologous challenge with IAV (145). However, the role of MyD88 signaling in heterosubtypic IAV infections is not well characterized.

In this chapter, we utilized a murine model of heterosubtypic IAV infections (9, 22) in which we examined the role of MyD88 signaling in heterosubtypic adaptive immune responses (T-cell and Ab responses) to the internal proteins of IAV.

B. Heterosubtypic immunity to influenza A viruses

Mice were infected intranasally with a sublethal dose of A/Puerto Rico/8/34 (PR/8) (H1N1). Mice were then challenged intranasally 21 days later with a sublethal dose of A/HK/X31 (HK/X31) (H3N2), a strain that contains all the internal proteins of PR/8 (6 out of 8 of the total genome) (88). We examined memory T-cell and Ab responses to influenza A PR/8 internal proteins 7 days after the second IAV infection (Figure 5.1). This allowed us to examine heterosubtypic immune responses to IAVs without the confounding effects of anti-hemagglutinin protective Abs.

C. A MyD88-mediated signaling pathway is required for the induction of heterosubtypic CD4+ T-cell immune responses to IAV.

In a murine homologous IAV challenge model, splenic CD4+ T-cell immune responses have been previously reported to be dependent on TLR7/MyD88 signaling (90). We infected B6 mice with10 pfu PR/8 and later challenged these mice with 10^4 pfu HK/31. We found that anti-IAV $NP_{311-325}$ CD4+ T-cell IFN-γ and TNFα production in the lung and spleen were dependent on MyD88 signaling (Figures 5.2 & 5.3). Some of these $NP_{311-325}$ specific CD4+ T-cells produced both cytokines (Figure 5.4). Anti-IAV $NP_{311-325}$ CD4+ Th1 cytokine production in the lung and spleen was MyD88 dependent, but the frequencies of CD3+CD4+ T-cells at these two sites were not (Figure 5.2c & 5.3c). We further found that lung anti-IAV $NP_{311-325}$ CD4+ Th1 cytokine production was partially dependent (but not statistically significant) on TLR7

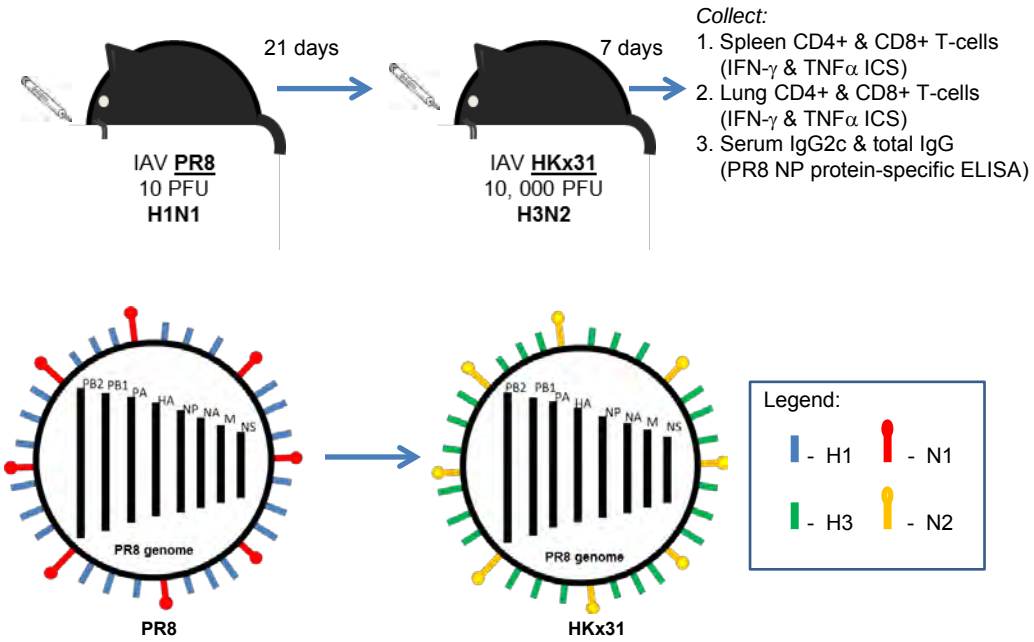


Figure 5.1. Heterosubtypic immunity to influenza A virus (IAV) internal proteins. B6 mice were infected intranasally (i.n.) with 10 plaque forming units (pfu) of PR/8 and challenged 21 days later with 10,000 pfu of HK/X31. 7 days after HK/X31 infection, spleen and lungs were collected for intracellular cytokine staining (ICS) and the serum was used to measure NP-specific IgG levels by ELISA. PR/8 and HK/X31 IAVs have different hemagglutinin (HA) and neuraminidase (NA) surface proteins and are not recognized by any cross-reactive neutralizing antibodies. These IAV strains share 6 internal genes derived from PR/8 – NP, the polymerase complex heterotrimer (PB2, PB1 and PA), matrix protein (M) and nonstructural protein (NS).

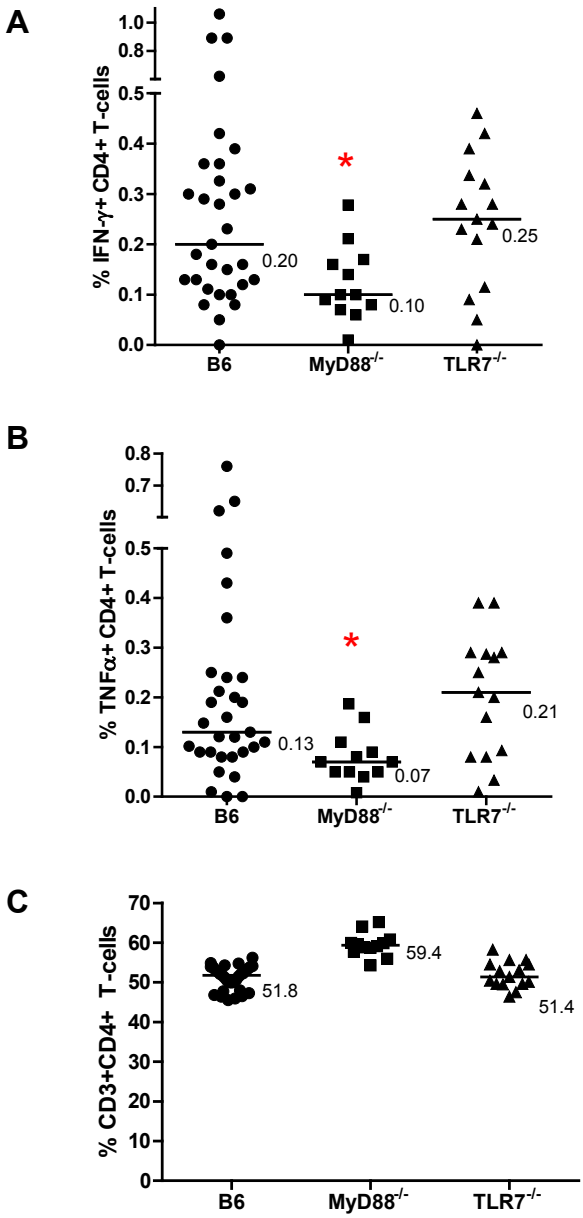


Figure 5.2. IFN-γ or TNFα production by IAV-specific CD4+ T-cells in the spleen. Splenocytes from mice infected with PR/8 and HK/X31 IAVs, as described in Figure 5.1, were stimulated for 6 hrs with nucleoprotein $(NP)_{311\text{-}325}$ peptide, a MHC Class II restricted PR/8 IAV epitope. $NP_{311\text{-}325}$-specific cytokine-producing CD4+ T-cells were identified as $CD3^+CD4^+CD8^-IFN\text{-}\gamma^+$ (A) or $TNF\alpha^+$ T cells (B). $NP_{311\text{-}325}$-specific cytokine-producing CD4+ T-cells were analyzed in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice. The frequencies of spleen CD3+CD4+ T-cells in these mouse strains were also determined (C). Values shown (A & B) are the % of IFN-γ^+ or $TNF\alpha^+$ $NP_{311\text{-}325}$-specific splenic CD4+ T-cells minus unstimulated background. Bars are median values. *$p<0.05$ compared to $NP_{311\text{-}325}$-specific B6 splenic CD4+ T-cells. (B6 n=31; MyD88$^{-/-}$ n=12; TLR7$^{-/-}$ n=15).

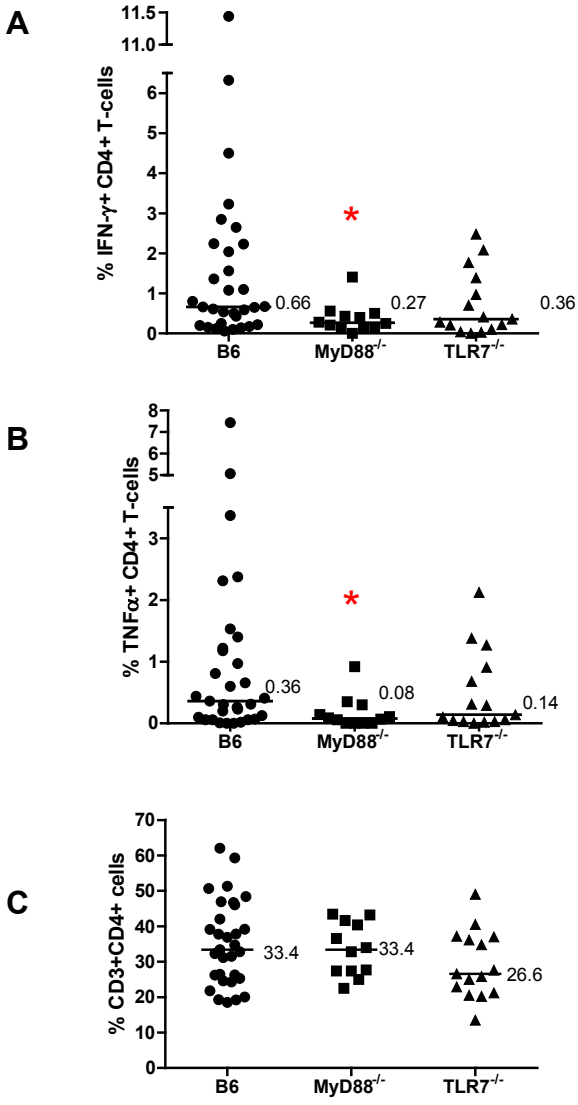


Figure 5.3. IFN-γ or TNFα production by IAV-specific CD4+ T-cells in the lung. Lungs from mice infected with PR/8 and HK/X31 IAVs, were homogenized and cell suspensions were stimulated for 6 hrs with nucleoprotein $(NP)_{311-325}$ peptide. The frequency of $NP_{311-325}$-specific IFN-γ^+ (A) or TNFα^+ (B) producing CD4+ T-cells and the frequencies of CD3+CD4+ T-cells in the lung (C) were determined in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice. Values shown (A & B) are the frequency of IFN-γ^+ or TNFα^+ $NP_{311-325}$-specific CD4+ T-cells minus the frequency of cells obtained when stimulated with media. Bars are median values. *$p<0.05$ compared to $NP_{311-325}$-specific B6 lung CD4+ T-cells. (B6 n=31; MyD88$^{-/-}$ n=12; TLR7$^{-/-}$ n=15)

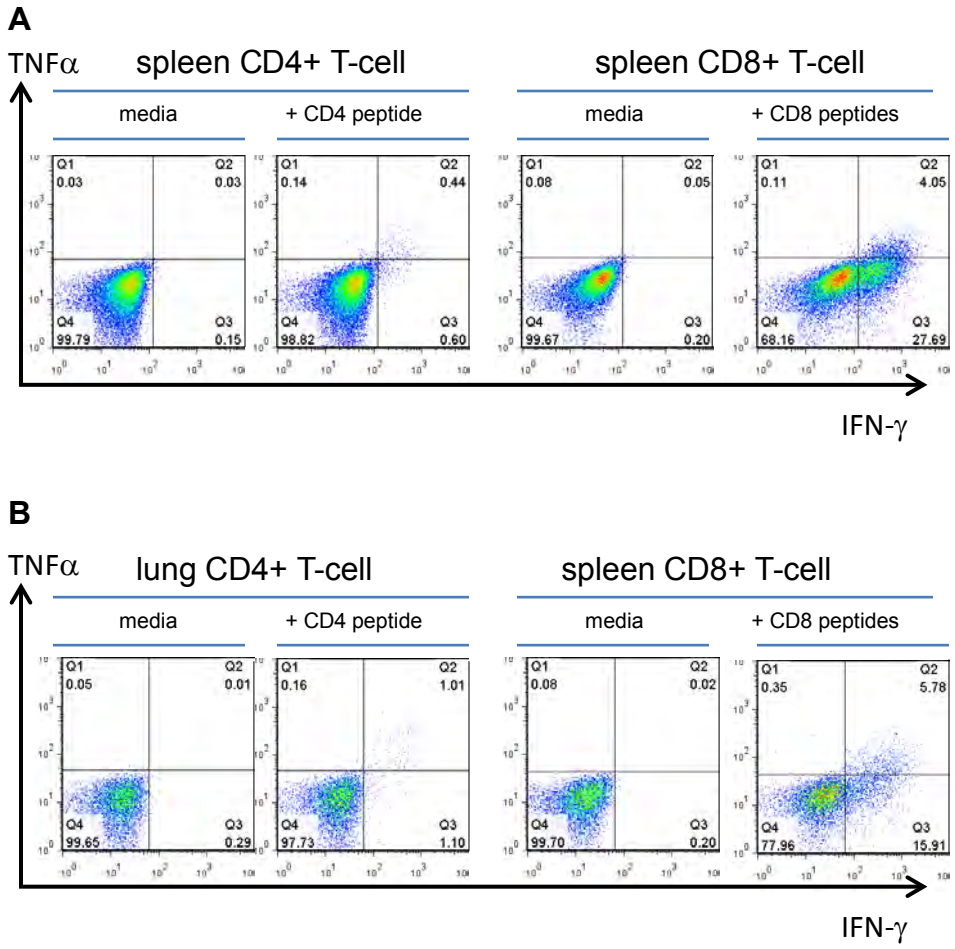


Figure 5. 4. IAV-specific CD4+ & CD8+ T-cells in the spleen and lungs produce both IFN-γ and TNFα. Mice were infected with 10 PFU PR8 followed by infection, 21 days later, with 10,000 PFU HK/X31. Seven days after the second infection, lung and spleen cells were stimulated for 5 to 6 hrs with $NP_{311-325}$ or $NP_{366-374}$ and $PA_{224-233}$ peptide mix. The frequency of IAV-specific IFN-γ and TNFα producing CD4+ and CD8+ T-cells in spleen (A) and in the lungs (B) were determined. Representative plots are shown above.

signaling, but anti-IAV $NP_{311-325}$ CD4+ Th1 cytokine production in the spleen was TLR7 independent (Figures 5.2 & 5.3). The MyD88 dependent IL-1α/β signaling has been reported to influence antibody responses and CD4+ T-cells (142). To further investigate the MyD88 dependence on CD4+ T-cells, we infected IL-1-deficient mice with IAV. Anti-IAV $NP_{311-325}$ CD4+ Th1 cytokine production in the lung and spleen was also unchanged in IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, IL-1$\alpha\beta^{-/-}$, or IL-1R$^{-/-}$ mice compared to production by CD4+ T-cells in wild type mice (Figure 5.5).

D. Anti-IAV NP Th1 antibody responses are partially dependent on TLR7 and MyD88 signaling in a heterosubtypic infection model.

In C57BL/6 mice, CD4+ Th1 cells play a role in antibody isotype switching to IgG2c (47). In a murine homologous IAV challenge model, anti-IAV HA IgG2a/c levels were reported to be TLR7 and MyD88 dependent (90). In our murine heterosubtypic IAV challenge model, we also found that IgG2c directed against the IAV NP protein was MyD88 dependent. We observed a decrease (but not statistically significant) in TLR7$^{-/-}$ mice antibody responses. The partial TLR7 and MyD88 dependence for anti-NP IgG2c Ab was seen only after the secondary heterosubtypic IAV infection (Figure 5.6). Th2 dependent IgG_1 levels directed against IAV NP protein were also dependent on MyD88 signaling after the secondary heterosubtypic IAV infection (Figure 5.6).

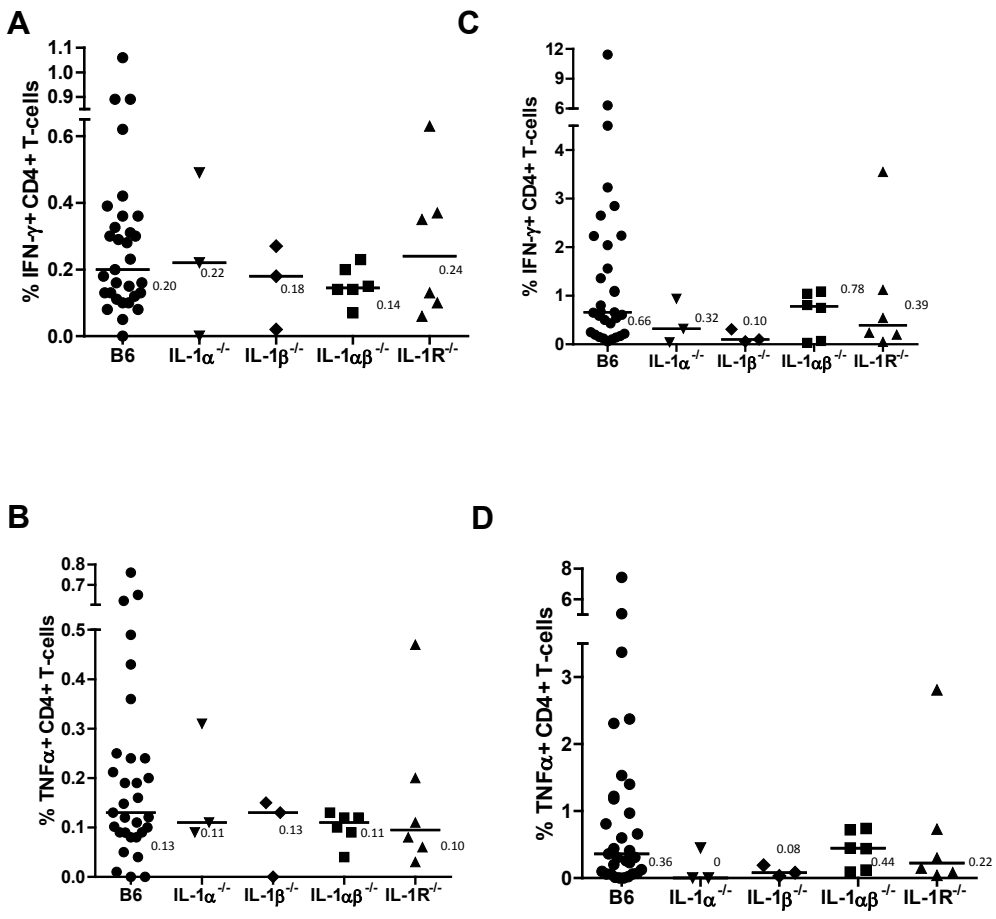


Figure 5.5. IFN-γ or TNFα production by IAV-specific CD4+ T-cells in IL-1-deficient mice. Frequencies of IFN-γ or TNFα-secreting nucleoprotein $(NP)_{311-325}$-specific CD4+ T-cells were assessed in IAV infected B6 and IL-1-deficient mice (IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1αβ$^{-/-}$, or IL-1R$^{-/-}$) in the spleen (A&B) and lungs (C&D). Mice were infected with PR/8 and HK/X31 IAVs, as described in Figure 5.1. Values shown are the frequency of IFN-γ^{+} or TNFα^{+} $NP_{311-325}$-specific CD4+ T-cells minus the frequency of cells obtained when stimulated with media. Bars are median values. (B6 n=31, IL-1α$^{-/-}$ n=3, IL-1β$^{-/-}$ n=3, IL-1αβ$^{-/-}$ n=6, or IL-1R$^{-/-}$ n=6)

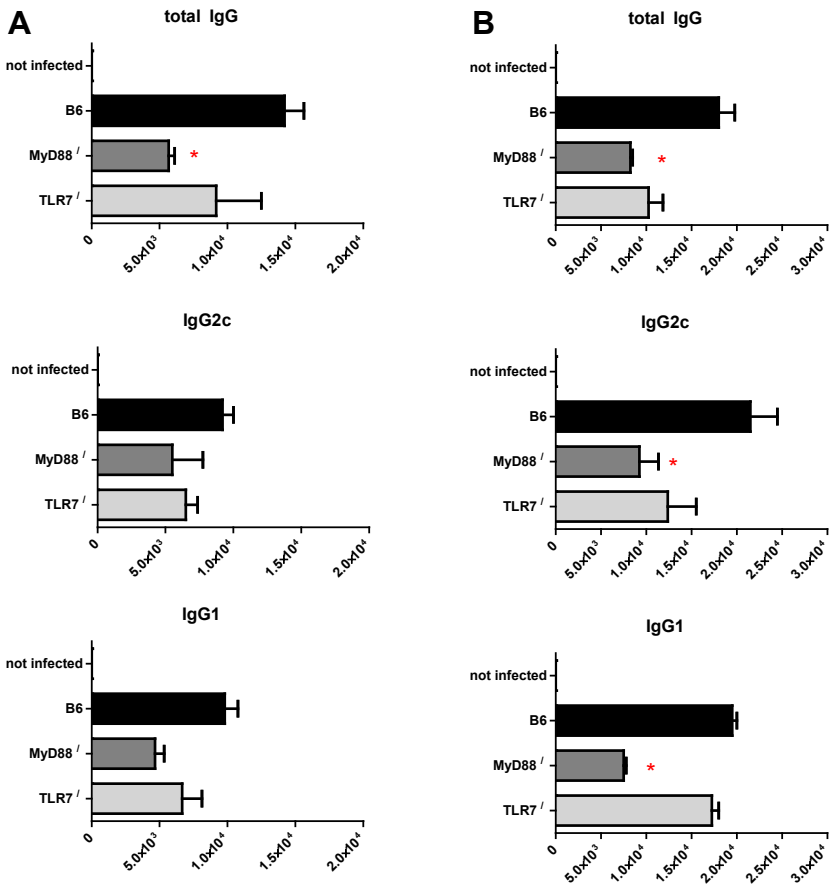


Figure 5.6. Antibody titers of nucleoprotein (NP)-specific IgG2c, IgG1 and total IgG in the sera of immunized mice. B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice were infected with PR/8 and HK/X31 IAVs, as described in Figure 5.1. Mouse sera were collected 20 days after primary PR/8 IAV infection (A) and 7 days after secondary heterosubtypic HK/X31 IAV infection (B). *p<0.05 compared to B6 sera. (in A: B6 n=5; MyD88$^{-/-}$ n=3; TLR7$^{-/-}$ n=3; in B: B6 n=4; MyD88$^{-/-}$ n=4; TLR7$^{-/-}$ n=4)

E. A MyD88-mediated signaling pathway is required for the induction of some heterosubtypic CD8+ T-cell immune responses to IAV.

We examined CD8+ T-cell immune responses to peptides $NP_{366-374}$ or $PA_{224-233}$, two MHC Class I-restricted immunodominant epitopes in C57BL/6 mice (10, 21). Anti-NP or PA CD8+ T-cell type 1 cytokine production in the spleen was dependent on MyD88 signaling, but largely independent of TLR7 signaling (Figures 5.7a&b). There were no differences in anti-NP or PA lung CD8+ T-cell cytokine production (IFN-γ or TNFα) and cell frequencies between wild type, $MyD88^{-/-}$, or $TLR7^{-/-}$ mice (Figures 5.8a-c). There was also no difference in anti-NP or PA spleen CD8+ T-cell type 1 cytokine production between wild type and IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, IL-1$\alpha\beta^{-/-}$, or IL-1$R^{-/-}$ mice (Figure 5.9). We found that the frequency of CD3+CD8+ T-cells in the spleen was MyD88 dependent unlike the frequencies of CD3+CD4+ T-cells in the spleen which were MyD88 independent (Figures 5.2c & 5.7c). This finding was confirmed by MHC Class I tetramer staining of $NP_{366-374}$ CD8+ T-cells in the spleen (Figure 5.10).

F. Similarities and diferences between homotypic (1^o PR/8 and 2^o PR/8) and heterosubtypic (1^o PR/8 and 2^o HK/X31) IAV infection

Similar to a previous study (90), we also performed homotypic IAV rechallenge experiments and compared their results with our heterosubtypic IAV challenge model. We observed that production of IFN-γ and TNFα by CD4+ (Figure 5.11) and CD8+ (Figure 5.12) T-cells was dependent on MyD88 signaling

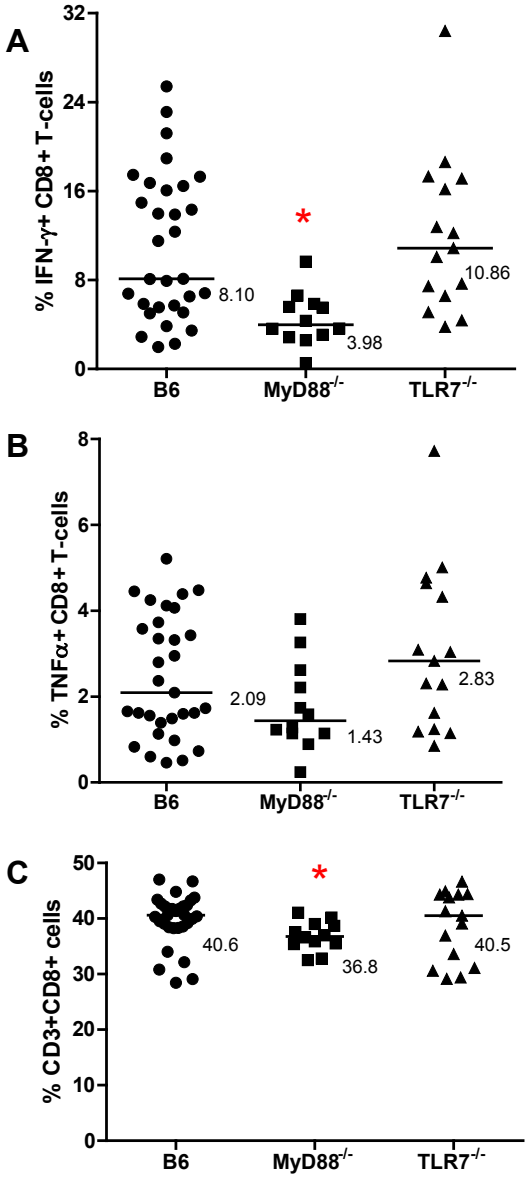


Figure 5.7. IFN-γ or TNFα production by IAV-specific CD8+ T-cells in the spleen. Splenocytes from mice infected with PR/8 and HK/X31 IAVs, were stimulated for 6 hrs with a mixture of nucleoprotein $(NP)_{366-374}$ and polymerase $(PA)_{224-233}$ peptides. $NP_{366-374}$ or $PA_{224-233}$-specific cytokine-producing CD8+ T-cells were identified as $CD3^+CD8^+CD4^-$ IFN-γ^+ (A) or TNFα^+ (B). $NP_{366-374}$ or $PA_{224-233}$-specific cytokine-producing CD8+ T-cells were analyzed in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice. The frequencies of spleen CD3+CD8+ T-cells in these mouse strains were also determined (C). Values shown (A & B) are the frequencies of IFN-γ^+ or TNFα^+ $NP_{366-374}$ or $PA_{224-233}$-specific splenic CD8+ T-cells minus frequencies of cells obtained with media stimulation. Bars are median values. *$p<0.05$ compared to $NP_{366-374}$ or $PA_{224-233}$-specific B6 splenic CD8+ T-cells. (B6 n=31; MyD88$^{-/-}$ n=12; TLR7$^{-/-}$ n=15)

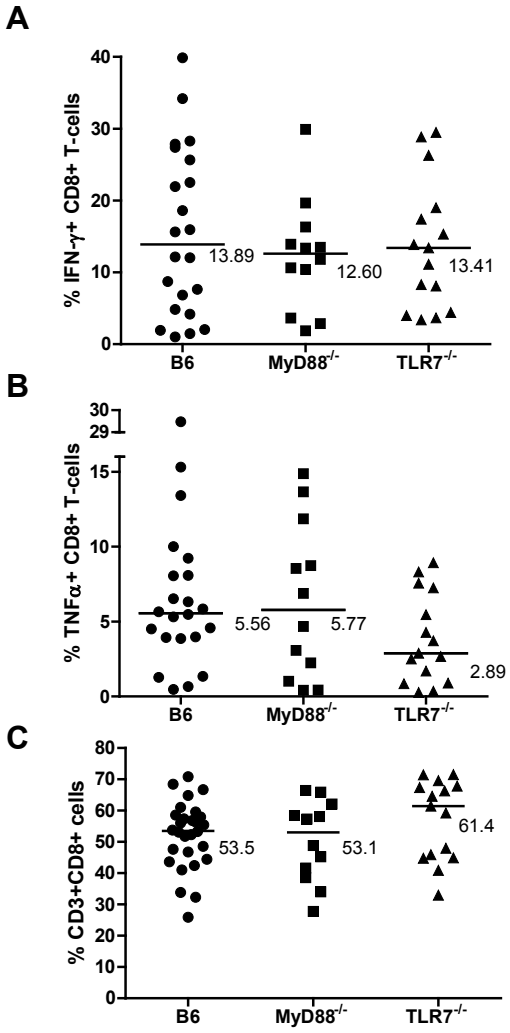


Figure 5.8. IFN-γ or TNFα production by IAV-specific CD8+ T-cells in the lung. Lungs from mice infected with PR/8 and HK/X31 IAVs, were homogenized and stimulated for 6 hrs with a mixture of nucleoprotein $(NP)_{366-374}$ and polymerase $(PA)_{224-233}$ peptides. MHC Class I restricted PR/8 IAV epitopes. $NP_{366-374}$ or $PA_{224-233}$-specific cytokine-producing CD8+ T-cells were identified as $CD3^+CD8^+CD4^-IFN\text{-}\gamma^+$ (A) or $TNF\alpha^+$ (B). The frequencies of lung CD3+CD8+ T-cells (C) were also determined in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice. Values shown (A & B) are the frequencies of IFN-γ^+ or $TNF\alpha^+$ $NP_{366-374}$ or $PA_{224-233}$-specific CD8+ T-cells minus frequencies of cells obtained with media stimulation. Bars are median values. *$p<0.05$ compared to $NP_{366-374}$ or $PA_{224-233}$-specific B6 lung CD8+ T-cells. (B6 n=22; MyD88 n=12; TLR7 n=15)

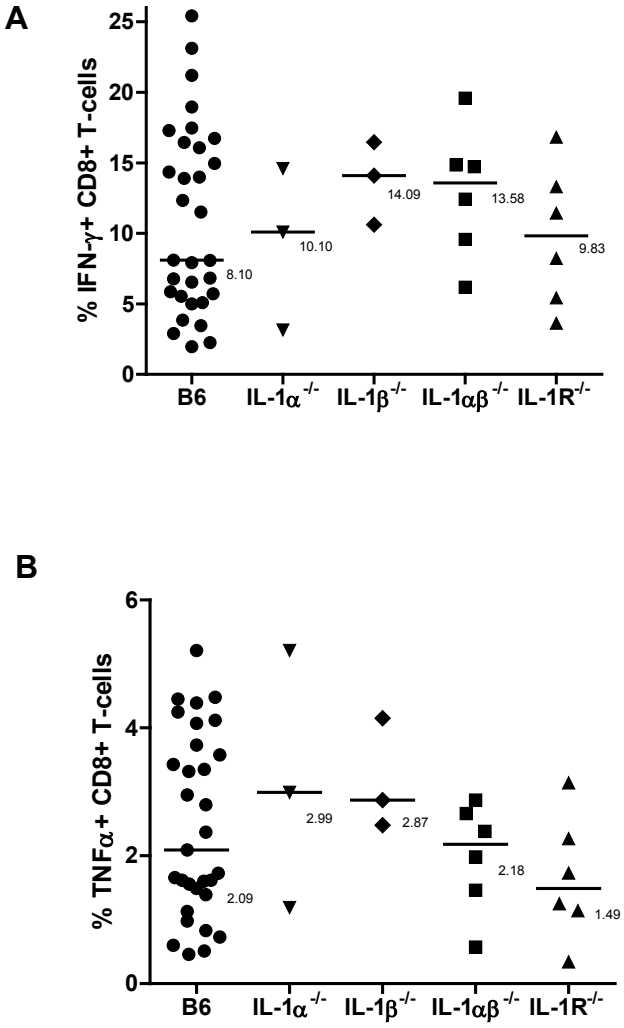


Figure 5.9. IFN-γ or TNFα production by IAV-specific CD8+ T-cells in IL-1-deficient mice. IFN-γ or TNFα-secreting nucleoprotein $(NP)_{366-374}$ or polymerase $(PA)_{224-233}$ -specific CD8+ T-cells in heterosubtypic IAV infected IL-1-deficient mice (IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, IL-1$\alpha\beta^{-/-}$, or IL-1$R^{-/-}$) in the spleen (A&B). Mice were infected with PR/8 and HK/X31 IAVs, as described in Figure 5.1. Values shown are the frequencies of IFN-γ^+ or TNFα^+ $NP_{366-374}$ or $PA_{224-233}$-specific CD8+ T-cells minus frequencies of cells obtained with media stimulation. Bars are median values.(B6 n=31, IL-1$\alpha^{-/-}$ n=3, IL-1$\beta^{-/-}$ n=3, IL-1$\alpha\beta^{-/-}$ n=6, or IL-1$R^{-/-}$ n=6)

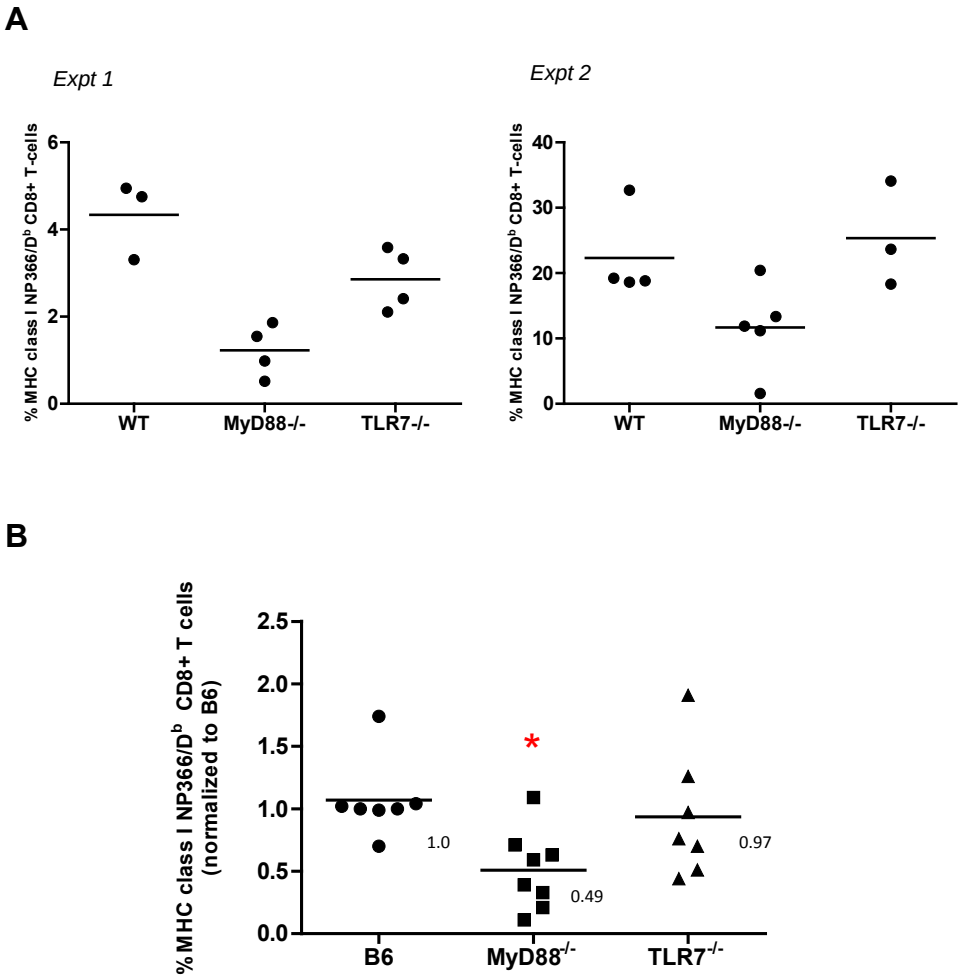


Figure 5.10. MHC class I influenza A virus (IAV)-specific CD8+ T-cell tetramer staining. MHC Class I peptide tetramers (PR/8 IAV nucleoprotein $(NP)_{366\text{-}374}/D^b$) were generated by the NIH Tetramer Facility (Atlanta, GA). Tetramer staining was performed for 30 min, on ice, followed by staining for CD8+ T-cells. Live/Dead Aqua (LDA) was used to exclude nonviable cells from analysis. At least 200,000 events were collected for analysis. Data was analyzed using FlowJo software (Treestar, Ashland, OR). The % of MHC class I NP366/Db CD8+ T-cells in two experiments were shown (A). For statistical analysis, the relative frequencies of MHC class I tetramer positive CD8+ T-cells in the spleen normalized to B6 mice (using one B6 IAV-infected mice as basis per experiment) following heterosubtypic IAV infection were determined (B). (B6 n=7; MyD88 n=9; TLR7 n=7)

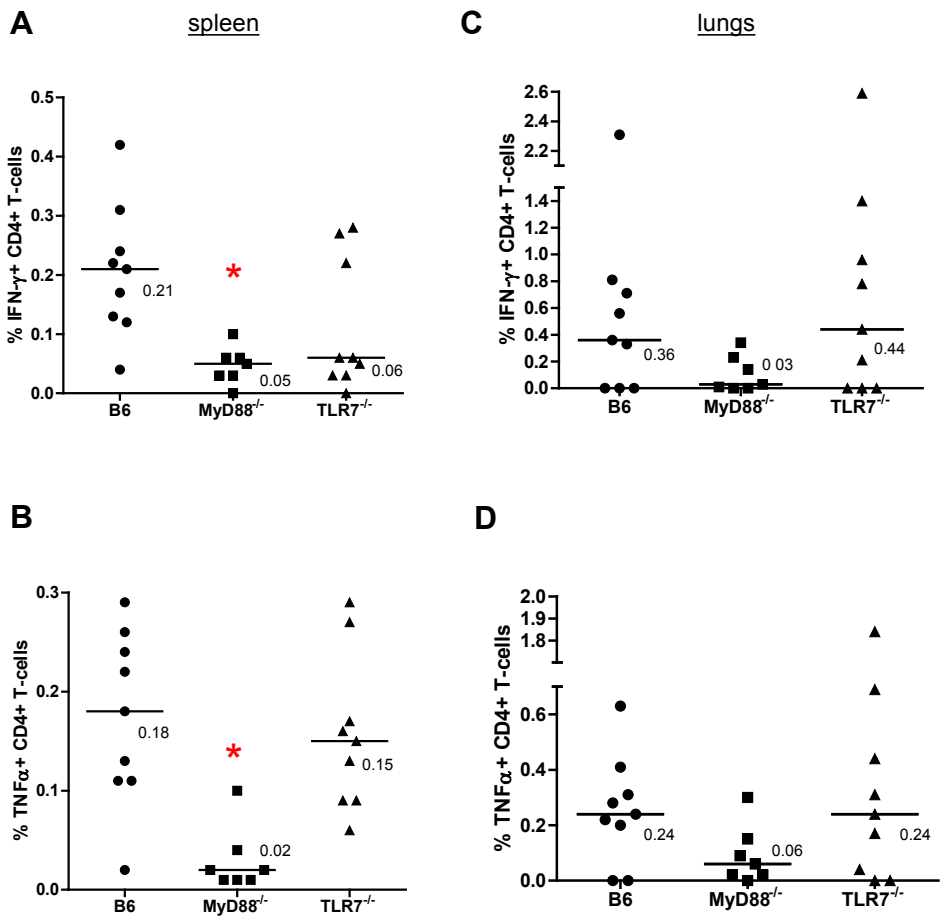


Figure 5.11. IFN-γ and TNFα production by CD4+ T-cells in the spleen and lung in mice infected sequentially with PR8 IAV (PR8+PR8). Splenic and lung CD4+ T-cells from mice infected primarily with 10 PFU PR8 followed by infection, 21 days later, with 2 x 10^5 PFU PR8. Seven days after second PR8 infection, lung and spleen cells were stimulated for 5 to 6 hrs with $NP_{311-325}$ peptide . The frequencies of IAV-specific IFN-γ and TNFα producing CD4+ T-cells in spleen (A&B) and in the lungs (C&D) in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ infected mice were determined. Values shown are the frequencies of IFN-γ$^+$ or TNFα$^+$ CD4+ T-cells when stimulated with $NP_{311-325}$ minus frequencies obtained when CD4+ T-cells were stimulated with media. Bars represent median values. *p<0.05 compared to $NP_{311-325}$ stimulated B6 spleen or lung CD4+ T-cells. (B6 n=9; MyD88$^{-/-}$ n= 7; TLR7$^{-/-}$ n=9).

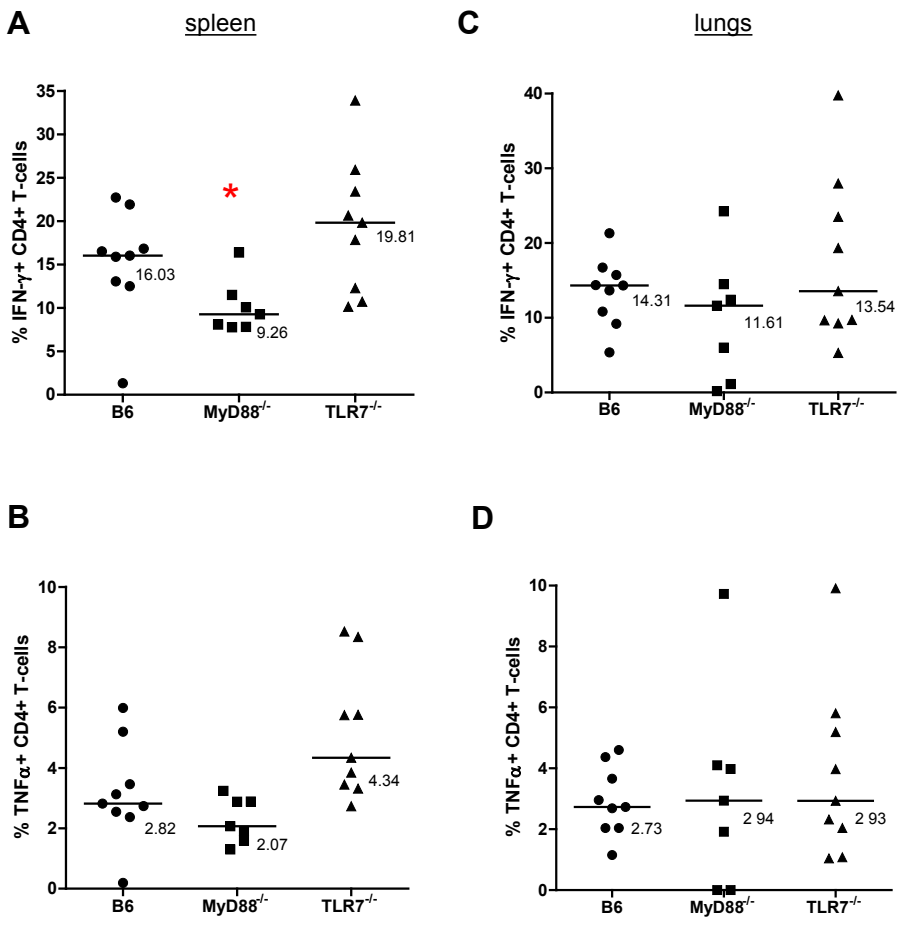


Figure 5.12. IFN-γ and TNFα production by spleen and lung CD8+ T-cells infected sequentially with PR8 IAV (PR8+PR8). Splenic and lung CD8+ T-cells from mice infected primarily with 10 PFU PR8 followed by infection, 21 days later, with 2 x 10^5 PFU PR8. Seven days after the second PR8 infection, lung and spleen cells were stimulated for 5 to 6 hrs with a $NP_{366-374}$ and $PA_{224-233}$ peptide mixture. The frequencies of IAV-specific IFN-γ and TNFα producing CD8+ T-cells in spleen (A&B) and in the lungs (C&D) in B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ infected mice were determined. Values shown are the frequencies of IFN-γ^+ or TNFα^+ $NP_{311-325}$ stimulated CD8+ T-cells minus frequencies of cells obtained with media stimulation. Bars represent median values. *$p<0.05$ compared to $NP_{366-374}$ and $PA_{224-233}$ peptide mix stimulated B6 spleen or lung CD8+ T-cells. (B6 n=9; MyD88$^{-/-}$ n= 7; TLR7$^{-/-}$ n=9).

as has been previously reported (90). In contrast, frequencies of MHC class I IAV-specific CD8+ tetramer staining cells in homotypic IAV infection were not dependent on MyD88 signaling (Figure 5.10 and 5.13).

G. Chapter Discussion

The murine model used in this study provided an experimental system for analyzing heterosubtypic cell-mediated immunity. We found that MyD88 signaling, but not TLR7 signaling, was essential in heterosubtypic CD4+ and CD8+ T-cell responses with the exception of anti-influenza CD8+ T-cells in the lung where responses were MyD88 independent

The initial induction of influenza specific immune responses is mediated by PRR signaling pathways. PRR signaling pathways can be broadly categorized into MyD88 dependent and MyD88 independent pathways (57). We examined immune responses elicited during serial sublethal heterosubtypic IAV infections in a murine model. This model most closely reflects serial natural IAV infections in humans. Our findings point to an important role for MyD88 dependent signaling in anti-IAV heterosubtypic memory T-cell immune responses. MyD88 dependent signaling was important for Th1 cytokine production by anti-IAV heterosubtypic memory CD4+ T-cells while no notable differences in the frequencies were observed in MyD88-deficient IAV infected mice. MyD88 dependent signaling was also important for NP-specific serum IgG2c and IgG1 antibody responses.

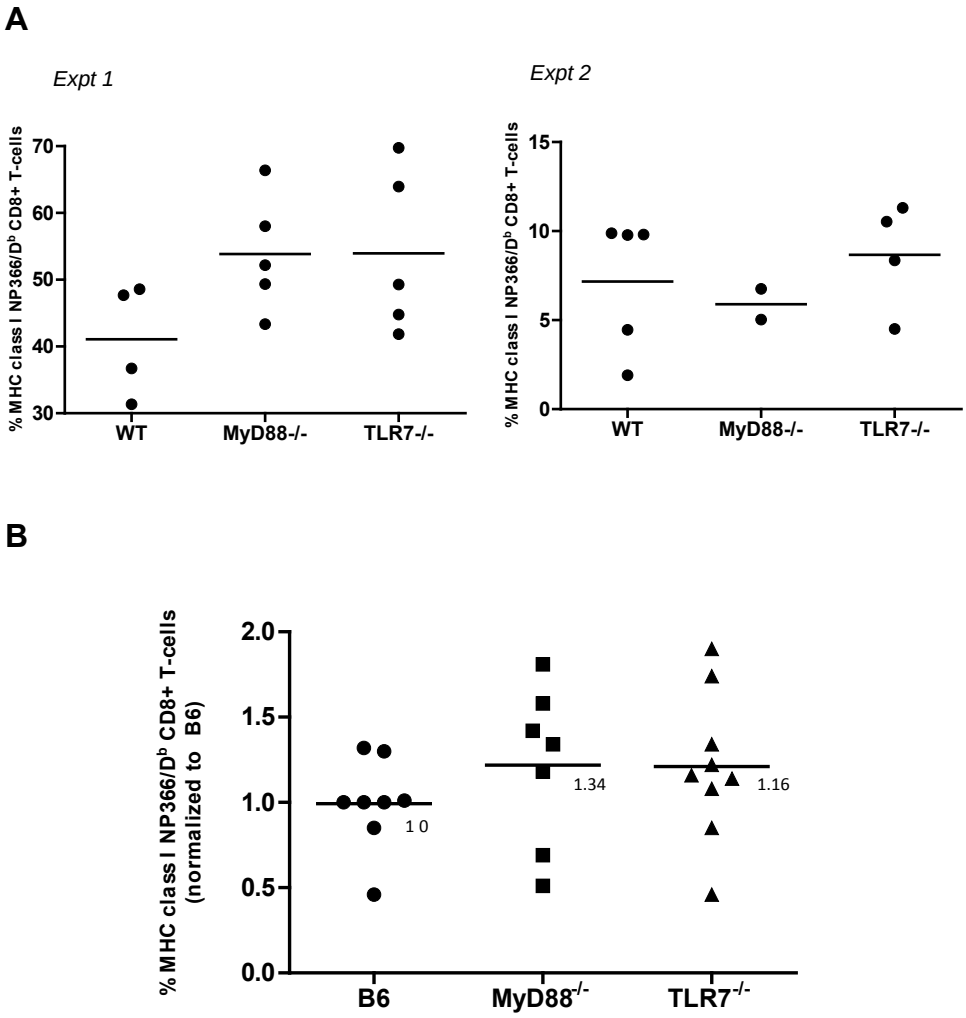


Figure 5.13. MHC class I IAV-specific CD8+ T-cell tetramer staining (homotypic, PR8+PR8). B6, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice were infected with 10 PFU PR8 followed by infection, 21 days later, with 2 x 10^5 PFU PR8. Spleens were collected 7 days after homologous PR8 challenge and stained with MHC Class I peptide tetramers $NP_{366-374}/D^b$. The % of MHC class I NP366/Db CD8+ T-cells in two experiments were shown (A). For statistical analysis, the relative frequencies of MHC class I tetramer positive CD8+ T-cells in the spleen normalized to B6 mice (using one B6 IAV-infected mice as basis per experiment) following homotypic IAV infection were determined (B). (B6 n=8; MyD88 n=7; TLR7 n=9)

For anti-IAV heterosubtypic memory lung and spleen CD4+ T-cells, MyD88 signaling was not required to achieve the cell frequencies but was needed for Th1 cytokine production. In our murine model, there was minimal proliferation of anti-IAV heterosubtypic memory CD4+ T-cells by *in vivo* EdU incorporation (data not shown). This implies that the majority of anti-IAV $NP_{311-325}$ memory lung and spleen CD4+ T-cells were recruited to the sites, likely from regional lymph nodes. A similar finding has been reported for anti-IAV heterosubtypic memory CD8+ T-cells in a murine model (62, 89).

MyD88 signaling is essential in the regulation of murine CD4+ T-cell responses in several models (31, 34, 177). In the LCMV infection model, MyD88 deficient mice failed to develop LCMV-specific CD4+ T-cells that can produce Th1 cytokines and the reconstitution of MyD88 expression in CD4+ T-cells rescued the LCMV-specific response (177). In the murine model of inflammatory bowel disease (31), $MyD88^{-/-}$ CD4+ T-cells showed decreased proliferation and defective T-cell function both *in vitro* and *in vivo*. In an OVA-expressing recombinant Salmonella vaccine (63) that elicits Th1-biased cell-mediated and serum Ab responses in B6 mice, $MyD88^{-/-}$ mice exhibited greatly reduced Th1-dependent Ab responses. The CD4+ T-cells from vaccinated MyD88 deficient mice also failed to produce IFN-γ (63).

There are several ways in which MyD88 signaling could be involved in anti-IAV heterosubtypic memory CD4+ Th1 cytokine responses (87, 172). In addition to cytokine production and providing T-helper function to CD8+ T-cells,

IAV infection can induce cytolytic CD4+ effectors that reside in the lung (15, 16). Memory CD4+ T-cells can also direct enhanced protection from IAV infection through mobilization of immune effectors in the lung, independent of their helper functions (157). The MyD88-dependent IL-1 and TLR7 signaling pathways were reported to be important in eliciting IAV immune responses (20, 85). TLR7-deficient mice have a Th2 bias in response to IAV and reduced levels of IAV-specific Th1 responses (47, 90). Administration of IL-1 as mucosal adjuvant can confer significant protection against lethal IAV infection (85) and increased mortality during IAV virus infection was observed in the absence of IL-1R1 (142). However, our heterosubtypic IAV data showed that dependence on TLR7 or IL-1 signaling exclusively was minimal suggesting a much broader role of MyD88 signaling in eliciting IAV-specific immune responses other than TLR7 and IL-1.

Virus-induced activation of T-cells involves the dynamic interaction of APCs and T-cells through virus antigen presentation by APCs to T-cells, upregulation of co-stimulatory molecules and the production of cytokines that influence the activation and the generation of effector and memory-T cells. These crucial events and other immune cells (such as B-cells and pDCs) were previously reported in literature to be affected by MyD88 signaling. MyD88 signaling is required for the DC activation/production of early inflammatory cytokines and the production of Th1 cytokines by T-cells against IAV infection (145). MyD88 signaling pathways in B-cells are essential for effective generation of long term humoral immunity (generation of long-lived antibody secreting

plasma cells) against virus infections and after influenza virus-like particle (VLP) vaccination (41, 76). MyD88 signaling is also critical for regulating anti-IAV B-cell Ab isotype switching (47). MyD88-dependent signaling is also important in plasmacytoid DC (pDC)-mediated protection in the lungs against IAV infection (74).

MyD88 might also play an intrinsic role in Th1 cytokine production within CD4+ T-cells. Such a role has been reported in a LCMV murine infection model in which MyD88 signaling within the CD4+ T-cells is essential for normal CD4+ T-cell function (177). In a Toxoplasma gondii murine resistance model (95), T-cell expression of MyD88 is also necessary for the Th1 response and the prolonged resistance to Toxoplasma gondii.

The involvement of MyD88 (mostly via TLR7) has been examined in several independent IAV studies (47, 90, 100, 145) with a range of results that are difficult to generalize, except that all report that CD8+ T-cell responses were not affected by the absence of MyD88 signaling. For anti-IAV heterosubtypic memory CD8+ T-cells, we also observed the pulmonary Th1 cytokine response and cell frequency to be MyD88 independent. This finding was consistent with other reports that anti-IAV memory CD8+ T-cells in the lung were largely recruited from regional draining lymph nodes (62, 89).

The heterosubtypic anti-NP IgG2c antibody response was dependent on MyD88 signaling but not through TLR7. This is in contrast to previously reported TLR7/MyD88 dependence in anti-hemagglutinin Th1 antibody responses in a

homologous IAV challenge model (90). In addition, we observed reduced anti-NP total IgG and IgG1 antibody responses. This is in contrast to previously reported striking Th2 bias observed in IAV-infected TLR7$^{-/-}$ and MyD88$^{-/-}$ mice (142). Differences in experimental conditions (such as virus strain or stock, infection volume, route of immunization/infection, homotypic vs heterosubtypic challenge) may explain these contrasting results. The MyD88-dependence observed in IAV-specific antibody (total IgG, IgG2c and IgG1) responses suggests an important role not only in T-cells but other immune cells such as B-cells and pDCs (1, 41, 47, 74, 76). The antibody response to IAV infection is largely dependent on CD4+ T-cell help for B cells (155). CD4+ T-cells provide cognate signals and secrete factors that drive B-cell activation and regulate Ab isotype switching. The MyD88-dependence observed in heterosubtypic anti-NP antibody response may be a consequence of MyD88$^{-/-}$ CD4+ T-cell functional defect.

Further studies that would better address the importance of the MyD88 pathway in heterosubtypic IAV infections include adoptive transfer experiments to address whether MyD88 signaling is involved in the activation of APCs (Is the impaired CD4+ T-cell response in MyD88KO mice due to the failure of the APC system?) or if MyD88 is intrinsically involved in CD4+ T-cell functional maturation in response to heterosubtypic IAV infections. The latter could be addressed by the adoptive transfer of naïve MyD88$^{-/-}$ CD4+ T-cells into T-cell deficient mice, infect heterosubtypically with IAV, restimulate CD4+ T-cells with IAV-specific peptides and determine IFN-γ and TNFα production in response to IAV.

Reconstitution of MyD88 expression in MyD88$^{-/-}$ CD4+ T-cells by MyD88-expressing lentivirus, as previously done in murine LCMV model (177), could also show the importance of intrinsic MyD88 signaling in CD4+ T-cells.

In IAV-specific CD8+ T-cells, we observed an interesting difference in MyD88 dependence between CD8+ T-cells in the spleen and in the lung. Upon restimulation, IFN-γ and TNFα production by MyD88$^{-/-}$ CD8+ T-cells in spleen was impaired in contrast to MyD88$^{-/-}$ CD8+ T-cells in the lung. This difference was not addressed in this study but possible explanations include *(i)* antigen persistence and the control of local T-cell memory by respiratory DCs (89) and the MyD88$^{-/-}$ effect reduction of T-cell activation in the spleen (145); *(ii)* MyD88-dependent proliferation and intracellular expression of antiviral cytokines by CD8+T-cells may be needed in the spleen but not in the lung (due to the preferential localization of memory IAV T cells to the draining lymph nodes (89)); *(iii)* compromised IAV-specific CD8+ T-cell effector recall/memory as a result of defective CD4+ T-cell function in MyD88$^{-/-}$ CD4+ mice (9, 12, 47, 96); *(iv)* MyD88 may be crucial for the optimal local recruitment of immune cells to the site of infection (46); and *(v)* non-MyD88 dependent signaling pathways such as inflammasome activation may play a role at the site of IAV infection (lung) that can compensate or redundant for MyD88 in inducing protective anti-IAV immunity (57, 58).

In conclusion, MyD88 dependent signaling, not all of it through TLR7, played important roles in T-cell and antibody memory immune responses in

heterosubtypic IAV infections. Homosubtypic (PR8+PR8) IAV infections further supported the importance of MyD88 signaling. Though not directly addressed, differences observed between heterosubtypic and homotypic IAV infections may be attributed to the protective neutralizing Abs generated during homosubtypic infections.

H. Chapter Summary

We used a mouse model of heterosubtypic influenza A virus infections to determine the role of MyD88 signaling in CD4+ T-cell, CD8+ T-cell, and antibody responses. We found that MyD88 signaling played an important role in anti-IAV CD4+ T-cell responses in the lung and spleen and in CD8+ T-cell responses in the spleen following heterosubtypic and homotypic challenge with IAV. Following heterosubtypic IAV challenge, MyD88 dependent signaling was important for T-helper 1 cytokine production of memory CD4+ T-cells. TLR7 dependent signaling played a role only in anti-IAV heterosubtypic memory lung CD4+ Th1 responses. Our results have important implications for the generation of effective universal influenza vaccines.

CHAPTER VI:

FINAL SUMMARY AND IMPLICATIONS

The immune response to viral infections is determined by a complex interplay of interactions between the pathogen and the host. Viruses not only need the host for replication, but the virus itself is assembled using the machinery of the host cell and the virion can contain material derived from the host cell. Discrimination of 'self' from the viral 'non-self' is mediated by PRRs. RNA replicative intermediates such as ssRNA and dsRNA generated during the virus life cycle are detected by RNA-sensing PRRs.

In this thesis, we focused on delineating the effects of key RNA-sensing PRR pathways in the innate immune response on eliciting adaptive immune T cell responses. With the use of RNA-like IRMs R-848 and poly I:C, we first looked at direct co-stimulation by RNA PAMPs and the role of RNA-sensing signaling pathways in CD4+ T-cells. In preliminary experiments using MACS-enriched (not FACS sorted) CD4+ T-cells, we observed R-848 augmentation of IFN-γ production by CD4+ T-cells. However, R-848 augmentation was not observed in FACS-sorted CD4+ T-cells. This suggested production of cytokines by non-CD4+ T-cells in response to R-848. The use of CD4+ T-cells of high purity (>99.5% purity) eliminated the potential of bystander cells (e.g. NKT cells and $\gamma\delta$ T-cells) to secrete cytokiness. Using FACS-sorted CD4+ T-cells from

specific knockout mice, we explored the RNA-sensing PRR signaling involved or activated by RNA-like IRMs in CD4+ T-cells.

We found that different RNA-like IRMs can directly co-stimulate CD4+ T-cells . The RNA-like IRM poly I:C augmented CD4+ T-cell responses better than R-848. The differences that we observed between R-848 and poly I:C are indicative of the complex interactions between the numerous PRR signaling pathways activated during infection by a RNA virus. The generation of virus-specific immune responses involves the interplay of several PRRs and the delineation of all of these signaling events is yet to be achieved.

On the other hand, we observed the robust proliferation and production of effector cytokines by CD4+ T-cells in response to poly I:C. In addition to direct TLR3 and RLR signaling in CD4+ T-cells, T-cells express NLRP3 and thereby may be activated by poly I:C (92). We have also shown that direct co-stimulation of CD4+ T cells by poly I:C involves the activation of PKR signaling. Though more definite experiments such as the use of PKR-deficient mice could further address this, it is suggested from the anti-CD3 stimulation experiments with TLR3$^{-/-}$, TRIF$^{-/-}$, IPS-1$^{-/-}$, MDA-5$^{-/-}$, NLRP3$^{-/-}$, ASC$^{-/-}$, TRIF$^{-/-}$/MDA-5$^{-/-}$ and even MyD88$^{-/-}$ CD4+ T-cells that PKR or other signaling pathways were activated and involved in the poly I:C direct co-stimulation of CD4+ T-cells.

We also noted a difference in the ability of R848 to induce cytokine production between human and mouse CD4+ T cells. Human CD4+ T-cells were easily stimulated to secrete IFN-γ with R-848 while there was minimal secretion

of IFN-γ by mouse CD4+ T cells. We explored the possibility that TLR8 signaling in human CD4+ T-cells may account for these differences since TLR8 signaling in the mouse requires poly(dT) signaling to function (37). Addition of poly(dT) to R-848 slightly increased IFN-γ production by mouse CD4+ T-cells. Anti-CD3 stimulation of human CD4+ T-cells with the CL075 (TLR8/7 IRM previously shown to stimulate human PBMCs) resulted in the slight augmentation of IFN-γ production. Further definitive experiments should be performed to address TLR8 direct co-stimulation in mouse and human CD4+ T-cells.

We focused mainly on RNA-sensing PRR pathways on CD4+ T-cells since CD4+ T-cells play a pivotal role in virus clearance, primarily through the augmentation of the B cell and CD8+ T-cell responses (28). We next explored RNA-sensing PRR activation in either CD4+ T-cell or in cDCs in isolated cDC/CD4+ T-cell interactions (MLRs). In contrast to anti-CD3 stimulation experiments, we observed augmentation of Th1 cytokine production in MHC congenic MLRs by R-848 > poly I:C. This difference between R-848-mediated CD4+ Th1 responses between CD4+ T-cells alone and isolated cDC/CD4+ T-cells interactions suggests a pivotal role for APCs in triggering cytokine responses in CD4+ T-cells.

RNA-like IRMs and RNA-sensing PRRs induce Type I IFN production and signaling in cDCs (98, 130). With the use of IFNAR$^{-/-}$ cDC in MHC congenic MLRs, we observed abrogated CD4+ Th1 responses in both R-848 and poly I:C stimulated MLRs. However, we observed much lower IFN-β mRNA expression in

R-848 stimulated cDCs than poly I:C stimulated cDCs. This suggested type I IFN to be essential but not sufficient for the optimal stimulation of CD4+ Th1 responses by RNA-like IRMs.

R-848-mediated TLR7/MyD88 signaling has been reported to have direct effects on cDC and CD4+ T-cells(17, 71, 134). Using MyD88$^{-/-}$ and TLR7$^{-/-}$ mice to look into R-848 signaling in either cDC or CD4+ T-cells in isolated cDC/CD4+ interactions, we have shown that TLR7/MyD88 signaling in cDCs and TLR7-independent but MyD88-mediated signaling in CD4+ T-cells are essential for R-848 induced CD4+ Th1 responses.

The MyD88-dependence observed in both cDC and CD4+ T-cells led us to explore the role of IL-1R/MyD88 signaling in R-848 stimulation of CD4+ Th1 responses. The observed partial abrogation in R-848 MHC MLRs in the absence of IL-1R signaling in either cDC or CD4+ T-cells led us to discover the role of early and rapid IL-1α and IL-1β production in RNA-like IRM driven CD4+ Th1 responses. Taken together, the data indicated that combined actions of type I IFN and the early IL-1α and IL-1β production and signaling are essential for the robust RNA-like IRM stimulation of CD4+ Th1 responses. The essential roles of these cytokines were evident in the experiments involving addition and inhibition of IL-1 and type I IFN.

To explore the contributions of RNA-sensing PRRs in the modulation of immune responses *in vivo*, we utilized a murine model of heterosubtypic IAV infections, in which, mice were infected and challenged with sublethal doses of

IAV PR/8 and HK/X31, respectively. In a mouse homosubtypic IAV challenge model, splenic CD4+ T-cell immune responses have been previously reported to be dependent on TLR7/MyD88 signaling (90). In our model, we found that MyD88 signaling played an important role in anti-IAV heterosubtypic recall lung and spleen CD4+ T-cell and spleen CD4+ T-cell immune responses. However, these MyD88-dependent immune responses were not solely dependent on TLR7 signaling. TLR7 signaling played a role only in anti-IAV heterosubtypic lung CD4+ T-cell responses. For anti-IAV heterosubtypic memory lung CD8+ T-cells, the Th1 cytokine response was MyD88-independent.

Our results showing differential responses in CD4+ T-cells and CD8+ T-cells in the spleen and lung, indicate the complexities of innate recognition and PRR signaling. In this thesis, we did not address the roles for RLR signaling and NLRP3 inflammasome activation during heterosubtypic IAV infection *in vivo*. Several studies have reported that IAV is recognized by RLRs but the delineation of the RNA-sensing pathways and their cooperative interactions with other signaling pathways remains incomplete (reviewed in (141)). Several recent studies have established a role for the NLRP3 inflammasome during IAV infection but contradictory results have been obtained (reviewed in (77)). Elucidation and a clearer understanding of these effector pathways would be needed to develop improved vaccine and adjuvant strategies against seasonal and pandemic influenza.

A. Vaccine design

Since T-cells can mediate cross-protective (heterosubtypic) immunity against influenza viruses by recognition of conserved viral proteins, targeting of long-lived protective T-cells should be one of the goals of future vaccine development. Recent studies in human and mouse influenza models have identified CD4+ T-cells to have more prominent protective roles other than promoting antibody responses. In a human influenza infection model using volunteers seronegative to the challenge influenza virus, investigators found pre-existing influenza-specific CD4+ T-cells, rather than CD8+ T-cells to correlate with disease protection (172). Their results suggested that CD4+ T-cells might exert direct cytotoxic activity against virus-infected cells. In mice, lethal PR/8 infection induced a population of IFN-γ expressing cytotoxic CD4+ T-cells (16). These findings support the concept that perforin mediated cytotoxicity may play a role in protection conferred by CD4+ T-cells. In this thesis, we have shown RNA-like IRMs to influence CD4+ Th1 responses. Although we have not studied whether RNA-like IRMs induced cytolytic activity in CD4+ T-cells, these observations strengthen the concept that CD4+ T-cells may be targeted to induce effective and long-lasting protection against influenza.

With the recent progress in PRR pathways and vaccine immunology, it is becoming apparent that the triggering of the innate immune mechanism is the initial event that crucially determines the outcome of the adaptive immune response (23). In vaccination, vaccines are thought to contain two types of

immune triggers: PAMPs and damage-associated molecular patterns (DAMPs) (114) that can stimulate the immune system through PRR activation in APCs such DCs and indirectly by PRR activation in bystander cells (Figure 6.1). Depending on the cytokine milieu generated, CD4+ T-cells develop into various Th subtypes that activate cognate B-cells, induce antibody production and isotype switching. CD4+ T-cell help is also essential in the generation of effector CD8+ T-cells and long lasting memory CD8+ T-cells. In this thesis, we looked at an isolated cDC-CD4+ T-cell interaction that provides a snapshot on how PRRs can activate directly or indirectly cDCs and CD4+ T-cells (Figure 6.1).

Another novel approach is to counteract viral subversion of innate immune responses by targeting an alternative PRR pathway to help elicit an adaptive immune response. Viruses possess different immune evasion strategies. Influenza NS1 is the most well-characterized of the proteins that subvert RIG-I mediated type I IFN responses at multiple steps and interestingly, no direct viral mechanism against TLR signaling has been described (6, 143). It is therefore possible to stimulate TLR signaling to counteract NS1 inhibition of RIG-I signaling.

Currently PRRs (TLRs) are being explored as immune adjuvants (reviewed in (153)). In Chapter IV, we have explored the role of RNA-like IRMs as adjuvants. Our results provide important insights into the key signaling pathways responsible for RNA-like IRM CD4+ Th1 activation. TLR7 (e.g. imiquimod) and TLR7/8 (resiquimod) agonists were shown to contribute to the

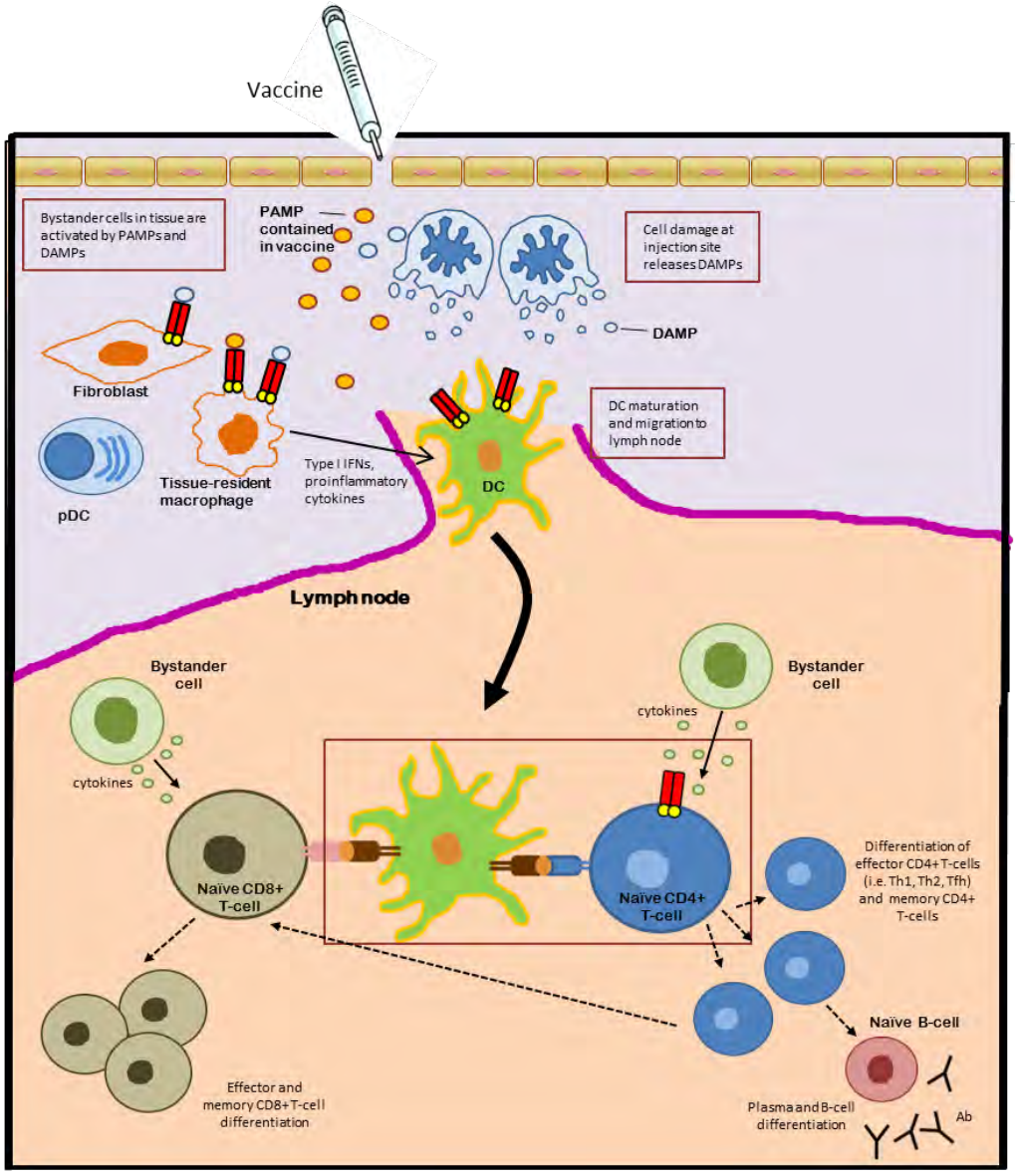


Figure 6.1. Induction of adaptive immune responses to vaccines through PRR-mediated DC activation. Vaccines may contain pathogen-associated molecular patterns (PAMPs) or may induce the local release of damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs are detected directly by pattern-recognition receptors (PRRs) expressed by dendritic cells (DCs), leading to DC activation, maturation and migration to the lymph nodes. Alternatively, PRR-mediated recognition of PAMPs and DAMPs by bystander cells may induce the release of tissue-derived factors, such as cytokines, that may cooperate in the activation and orientation of the DC response. In the lymph nodes, the activated DCs may present antigens to T cells, provide them with co-stimulatory signals and stimulate their differentiation by providing a favorable cytokine milieu. Some cytokines — such as interleukin 4 (IL 4) and type I interferons (IFNs) — may be provided by bystander cells. Depending on the cytokine milieu, CD4+ T cells may differentiate into various T helper (TH) cell subtypes that help in the activation of cognate B cells, antibody production and isotype switching. Depending on the balance between activating cytokines (and most often with the help of TH1 cell-derived IL 2), activated CD8+ T cells differentiate into effector and memory CD8+ T cells. In this thesis, we looked at isolated DC and CD4+ T-cell interactions and showed that PRRs can be activated by PAMPs in both DC and CD4+ T-cells. (Adapted from Desmet and Ishii, Nature Reviews Immunology July 2012 Vol. 12 p. 479-491)

immunogenicity of a variety of vaccine adjuvants (107). In addition to stimulating T cells to proliferate and produce effector cytokines, memory T cells also respond to TLR-mediated activation (17). TLR7/8 stimulation promotes maturation of DCs, stimulates B-cells to secrete antibodies and cytokines (13) and trigger NK cells to produce IFN-γ (44). With a better understanding of the RNA-sensing PRR pathways, incorporation of RNA PAMPs or RNA-like IRMs in vaccine formulations as means to improve vaccine efficacy could be developed in the future.

B. The therapeutic applications of manipulation of PRR signaling pathways

Our current understanding of the RNA-sensing PRRs pathways has led to numerous applications with promising potential. One therapeutic application is the possible use of PRRs in the management of chronic viral infections (160). In chronic viral hepatitis, ANA275 or isatoribine (a TLR7 agonist) has been used in clinical trials (54). Manipulation of the NS3/4a-inhibited dsRNA RIG-I/TRIF/IRF3 signaling in Hepatitis C virus (HCV) infections is also in progress. The use of several drugs (SCH6 (Schering-Plough), BILN 2061(Boehringer-Engelheim), VX-950 (Vertex/Mitsubishi) and SCH 503034(Schering-Plough)) that restore HCV-inhibited cellular interferon pathways has been observed to confer antiviral immune response (160).

In this thesis, we have shown RNA-like IRMs to induce Th1 responses and this could be useful for targeting tumors. TLR7 and TLR8 activation can

reverse the suppressive function of regulatory T-cells (122). When combined with the ability of TLR7 and TLR8 to activate DCs into producing multiple cytokines, this results in a strong anti-tumor response (50). New TLR7 agonists that target γδ T-cells are also being studied for treating carcinoma (136). The double stranded RNA mimic IPH-3102 is being developed for the treatment of breast cancer and as vaccine adjuvant (125). Hence, the activation of RNA-sensing PRRs is a promising approach in anti-tumor/anti-cancer strategies.

TLR7 and TLR8 agonists have also been studied as treatment for allergies and asthma. Since the primary feature of an allergic response is usually a Th2 response, this is counterbalanced by the induction of TLR7/TLR8-induced Th1 response. This has been a focus for novel therapeutics in these disease areas (32, 50).

In addition to development of PRR/TLR agonists, significant research has also been done on antagonists. These compounds could be used to treat sepsis, systemic lupus erythematosus (SLE) and rheumatoid arthritis (50). For example, the quinazoline derivative CDP-52364 inhibits TLR7, TLR8 and TLR9 signaling that also inhibits disease progression of SLE and other autoimmune diseases in animal models (50, 126). Delineating the critical PRR pathways and having a clearer understanding of the interplay of different PRR signaling pathways would contribute towards more effective approaches in antagonizing PRR signaling in certain diseases.

Details of PRR signaling pathways continue to be uncovered and this offers new opportunities in targeting innate immune signaling. The ability to specifically target key processes such as development of long lasting memory T-cells, might prevent uncontrolled infection and treatment of multiple diseases.

CHAPTER VII:

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