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The Role of MDA5 and TLR3 in Response to dsRNA and Viral Infection

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THE ROLE OF MDA5 AND TLR3 IN RESPONSE TO DSRNA AND VIRAL INFECTION

by

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ABSTRACT OF THE DISSERTATION

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The innate immune system consists of a number of genetically encoded receptors that detect the products of viral replication and initiate signaling cascades leading to activation of the antiviral response. During the course of infection, many viruses produce dsRNA that can be recognized by two major arms of the innate immune system: the toll-like receptors (TLR) and the Rig-I-like receptors (RLR). Among the TLRs, TLR3 binds dsRNA within the endosomal compartment and initiates signaling through its downstream adapter TRIF. Melanoma differentiation-induced gene 5 (MDA5) is a member of the RLR family that recognizes dsRNA within the cytosolic compartment and signals through the adaptor IPS-1. Although TLR3 and MDA5 initially employ distinct downstream adaptors, both are known to induce the production of cytokines and cell surface molecules involved in the antiviral response, which raises the question of whether they are redundant or functionally distinct. Using mice that are genetically deficient for MDA5, TLR3, or both MDA5 and TLR3 (double knockout, DKO), we have

demonstrated that these receptors have unique functions necessary for controlling viral infection.

Using two models of viral infection, murine norovirus (MNV) and encephalomyocarditis virus (EMCV), we demonstrate that both MDA5 and TLR3 limit viral replication. Neither MDA5-/- nor TLR3-/- animals controlled MNV and EMCV infection as well as wild type (WT) controls, but DKO mice were more susceptible to infection than either single knockout. Furthermore, we find that MDA5 and TLR3 play distinct roles in activating the natural killer (NK) cell response to the dsRNA analogue poly I:C (pIC). We demonstrate that the discrete functions of MDA5 and TLR3 are dependent on their expression in different cell types as well as their unique capacities to control production of cytokines. In addition, we show that the individual contribution of each sensor is necessary at distinct phases of the innate immune response, with TLR3 acting initially and MDA5 acting at later time points. These results illustrate how cooperation between the TLR and RLR pathways is necessary for the development of a complete antiviral response.

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LIST OF ABBREVIATIONS

MDA5	Melanoma differentiation associated protein-5
TLR	Toll-like receptor
RLR	Rig-I-like receptor
Rig-I	Retinoic acid-inducible gene-I
PRR	Pattern Recognition Receptor
PAMP	Pathogen-associated molecular pattern
IFN	Interferon
IFNAR	Interferon-α/β receptor
EMCV	Encephalomyocarditis virus
MNV	Murine norovirus
pIC	Poly (I:C)
dsRNA	double-stranded RNA
DC	dendritic cell
NK (cell)	Natural killer cell
KO	knock out
WT	wild type

Chapter 1

INTRODUCTION

Abstract

Viral pathogens have been discovered in all species from single-cell bacteria to the largest mammals. In order to protect themselves from the pathogenic effects of these invaders, organisms have been required to develop mechanisms to detect and limit viral infection. In mammals this requirement has evolved the adaptive immune system, which is able to generate highly specific antibodies and T cells that recognize specific viral proteins and peptides that either block infection or target infected cells for destruction. Initiation of the adaptive immune response, however, is a slow process that requires days to weeks for maximum effect. To provide protection during the initial hours and days of infection mammals have maintained a system of pattern-recognition receptors (PRR) first seen in lower organisms that recognize broad motifs common to viral pathogens and thus serve as the initial sensors of viral infection. These sensors initiate the adaptive antiviral response as well as trigger the innate immune pathways that protect the host during early infection while the more specific adaptive response develops. This chapter will provide an introduction to the role of small non-enveloped RNA viruses in human disease, describe the innate immune response to infection with these viruses, and detail the molecular pathways responsible for the detection of viral infection.

Small RNA viruses in disease

Members of the picornaviruses and caliciviruses families consist of small, non-enveloped viruses containing a positive-sense RNA genome[1]. Although small, viruses from these families are responsible for a surprisingly large number and range of human diseases as shown in Table 1.1. These viruses are typically transmitted in a fecal-oral manner and replicate in the alimentary tract, although several can also be spread through respiratory transmission. Several picornavirus family members can also cause systemic as well as gastrointestinal illness in humans and animals, such as encephalitits, myocarditis, hepatitis, and have been linked to type 1 diabetes[1]. Caliciviruses have been shown to be the most common cause of nonbacterial epidemic gastroenteritis worldwide[2-6] and have mostly been detected in the gastrointestinal tract.

Picornavirus and calicivirus genomes both contain a unique 5' covalent linkage to a protein called VPg, which has been linked to a role in viral RNA synthesis [7-9]. Picornaviruses contain a single long open reading frame (ORF) and viral proteins are obtained from processing of the polyprotein[1]. Caliciviruses have at least two ORFs and the exact number depends on the genus[10,11]. Picornaviruses gain entry to cells by attaching to host cell membrane receptors. A variety of receptors are employed by different viruses, including CD155 (poliovirus), VCAM-1 (EMCV), DAF (coxsackie A viruses), and Coxsackievirus-Adenovirus Receptor (coxsackie B viruses)[1]. The cellular receptors for caliciviruses are currently unknown, but human volunteer studies suggest that replication occurs in the upper intestinal tract [12,13]. The use of different receptors for entry by picornavirus and calicivirus family members likely results in the distinct viral

tropism and ability to cause different diseases, some of which will be discussed in more detail below.

Myocarditits

Myocarditis is a cardiac disease in which the myocardium becomes inflamed and injured in the absence of an ischemic event. Most cases of myocarditis have been linked to an infectious origin, with viruses being the most common cause in North America[14]. At least 20 viruses have been linked to the disease[15], but in humans, coxsackie viruses seem to be the dominant pathogen[16,17]. The incidence of myocarditis is unclear because most cases are likely to be asymptomatic and even in cases in which endometrial biopsy is available there is disagreement among pathologists about the exact diagnostic criteria[18]. However, most studies have shown that a majority of cases occur in children and young adults. The disease consists of three phases: acute, subacute, and chronic. In the acute phase there is active viral replication in the myocardium, the initial production of inflammatory cytokines, and a limited macrophage and NK cell infiltrate. The subacute phase consists of viral release, a large lymphocytic infiltrate, and a more massive cytokine response leading to viral clearance. The chronic phase typically consists of very low levels or the absence of viral particles but also fibrosis and remodeling of the myocardium leading to dilated cardiomyopathy and congestive heart failure[14].

The availability of several mouse models of myocarditis has begun to reveal some of the mechanisms of disease progression. A.BY/SnJ and SWR/J strains of mice have been shown to be susceptible to a more chronic form of myocarditis while C57BL/6 and DBA1/J strains have a more acute, rapidly clearing form of the disease when infected

with either coxsackie B virus (CVB) or EMCV[19-21]. Using these models, there are currently three main theories concerning the mechanism of myocardial damage that occurs in viral myocarditis[14]. 1. Excessive immune-mediated destruction of virally infected and surrounding myocardium by immune infiltrates. 2. Autoimmune destruction of myocardium by self-reactive cells and antibodies triggered by release of cardiac proteins or viral mimicry. 3. Direct myocardial injury by virus. There is evidence of all three forms of myocardial damage in different models[22-24]. Based on these somewhat contradictory results, it is currently unclear whether the immune system is protective or pathogenic in viral myocarditis. Recent experiments have demonstrated that anti-inflammatory cytokine IL-10 and in a human clinical trial, anti-viral cytokine IFNβ have protective effects in myocarditis and result in improved cardiac function[25,26]. However, in other clinical trials, general immunosuppression with prednisone had no significant effect on the outcome of myocarditis[27]. These studies highlight the complexity of the balance between the protective and harmful effects of the immune response to myocarditis and the potential that myocarditis is not one disease but rather a collection of diseases with differing etiologies.

Diabetes

Type 1 diabetes mellitus (T1DM) is caused by progressive destruction of the beta (β) cells in the pancreatic islets leading to hypoinsulinemia and hyperglycemia. Population based and familial studies as well as animal models have revealed that several genetic factors contribute to the risk of developing the disease, however, the precise mechanisms that lead to the initiation and development of T1DM remain unknown [28,29].

The high discordance rate (<50%) of T1DM in monozygotic twins as well the rapidly increasing incidence rates in certain geographic locations strongly suggest that environmental factors also contribute to the disease[30,31]. It has long been hypothesized that viral infection may be related to T1DM[32]. Indeed, human studies have found virus-specific antibodies in the serum and viral antigen in the pancreatic islets of recent onset T1DM patients[33-35]. There are several theories explaining how viral infection could lead to T1DM including: (i) direct virus-mediated destruction of β-cells, (ii) molecular mimicry of host proteins by viral antigens[36], (iii) release of novel antigens from β-cells upon viral infection leading to activation of auto-reactive cells[37], (iv) the production of inflammatory and immune cytokines that cause bystander activation[38], and (v) production of type I interferon (IFN) by infected cells leading to increased immune targeting of β-cells[39]. Although a number of different viruses have been linked to T1DM, the most common association in humans is with coxsackieviruses[40-42].

A variety of animal models have also provided evidence that viruses can trigger T1DM. Infection of the Biobreeding (DR-BB) rat with Kilham rat virus and susceptible mouse strains with encephalomyocarditis virus (EMCV) lead to diabetes in those normally resistant animals[43,44]. In addition, T1DM was induced in mice after infection with a CVB4 strain isolated from a human patient with diabetic ketoacidosis[40]. Conversely, there is also evidence that viruses can be protective and limit development of T1DM in susceptible individuals. Indeed, this is proposed in the hygiene hypothesis[45], originally proposed for asthma, which attempts to explain the increasing incidence of autoimmune diseases as a result of lower incidence of enterovirus infection. In support of this theory, NOD mice, which develop an autoimmune T cell mediated T1DM similar

to human disease, are protected from diabetes development if infected with EMCV of CVB before the initiation of disease[46]. Further studies have suggested that viruses which replicate quickly cause T1DM, while slowly replicating viruses are protective for the disease[47]. One potential explanation for this apparent paradox has been suggested by histological studies from human pancreatic tissues. Studies from Dotta at al have shown two distinct patterns of T1DM in human pancreatic islets[35]. Pattern A is the classic autoimmune form consisting of a T cell infiltrate similar to the disease seen in the NOD mouse. Pattern B samples do not have T cell infiltrates, instead displaying marked increases in natural killer (NK) cells and macrophages within the islets. Pattern B samples are also more likely to include viral antigen than pattern A. This suggests that T1DM may consist of multiple disease processes in which virus play distinct, even contradictory roles.

Gastroenteritis

In humans, viral gastroenteritis results in vomiting, diarrhea, fever, malaise, and abdominal pain within 24-48 hours after infection. These symptoms usually clear within 48-72 hours, but virus can persist for 3-6 weeks post-infection[48,49]. Viral gastroenteritis can occur in clusters in a variety of settings such as hospitals, nursing homes, day care centers, and cruise ships. Advances in diagnostic techniques has revealed that 90% of viral gastroenteritic outbreaks and up to 36% of sporadic gastroenteritis cases can be attributed to Norwalk virus and other human noroviruses[2-6]. There is great genetic diversity among both human and animal noroviruses. A recent comparison of norovirus sequences from around the world has suggested that a new pandemic strain emerges

every 2-4 years, indicating the importance of understanding the pathogenesis of infection[50].

Until recently the inability to culture norovirus *in vitro* as well as the lack of an animal model have limited investigation into its pathogenicity. The discovery and subsequent culture of murine norovirus-1 (MNV-1) has thus led to advances in understanding both the viral lifecycle as well as the host response to infection[51,52]. These studies have demonstrated that mice defective in the innate immune response are more susceptible to severe norovirus infection. Similarly, human studies have revealed increased susceptibility to norovirus infection in the very young, elderly, and immunocompromised individuals[50]. Recent reports have also suggested additional disease susceptibility factors. Noroviruses have been shown to bind to histo-blood group antigens of the ABO, Lewis, and secretor families, and strain-specific susceptibility to infection is dependent on blood group antigen and secretor status[53-56]. However, the wide diversity of norovirus strains suggests that individuals who are resistant to one strain, may be susceptible to another.

Innate Immune response to small RNA virus infection

During viral infection a variety of cellular responders are activated to contain and clear the infection. Upon penetration of the epithelial barriers and infection within host tissues viruses, their replication products, and infected cells are recognized by tissue phagocytic cells such as macrophages, neutrophils and dendritic cells, which produce inflammatory and antiviral cytokines that serve to activate the innate immune response. These cytokines, which will be detailed in the following sections, activate NK cells and other innate lymphocytes, induce phagocyte maturation, lead to production of acute phase antiviral products from the liver, and upregulate cell intrinsic antiviral responses. All of these effects constitute the innate antiviral response.

Cellular

NK cells

NK cells develop from the common lymphocyte progenitor, but unlike lymphocytes of the adaptive immune system, do not undergo genetic recombination of their antigen receptors genes. Instead NK cells employ a variety of invariant receptors that recognize cell surface receptors that are differentially modulated on virally infected cells[57]. NK cells express both activating and inhibitory receptors on their cell surface and the two-receptor hypothesis describes how these opposing receptors control their activation[58]. Activating receptors bind ligands that are induced upon cellular stress such as viral infection or transformation and signal for NK cytotoxicity against cells expressing the target ligand. Inhibitory receptors bind MHC class I molecules which

traditionally present peptides to CD8 T cells of the adaptive immune system and are normally expressed on healthy cells. The presence of inhibitory receptors prevents NK cytotoxicity against uninfected targets. However, during infection, many viruses downregulate MHC class I surface expression in order to evade the adaptive immune response. If this occurs, then NK cell inhibitory receptors do not engage their ligand resulting in an activating response and cytotoxicity. In addition to their cell surface receptors, NK cells are activated by type I IFN and inflammatory cytokines produced during viral infection, which result in increased baseline NK cell activation and cytotoxic ability[59]. NK cells have been demonstrated to be important for control of both CVB and EMCV infection in the context of myocarditis and diabetes[60,61].

Innate-like lymphocytes

Innate-like lymphocytes (ILL) are cells of the lymphocyte lineage, which, similar to NK cells, function in the innate immune system. There are two main types of ILLs involved in control of viral infection, intraepitheial γ:δ T cells and NK T cells. Among γ:δ T cells, there are two subsets, one which rearranges antigen receptor genes similar to α:β T cells known as lymphoid γ:δ T cells, and intraepithelial γ:δ T cells. Unlike other T cell types, intraepithelial γ:δ T cells have antigen receptors of very limited diversity[62]. These cells are typically located in the skin and mucosal surfaces and are thought to recognize a poorly defined selection of pathogen and host derived ligands directly, independent of presentation on MHC molecules[63]. NK T cells express an invariant T cell antigen receptor α chain and a limited selection of T cell β chains[64]. With this unique antigen receptor, NK T cells have been demonstrated to recognize lipid antigens presented on CD1d molecules. These cells are located within lymphatic tissues and are

thought to play a role in rapid secretion of cytokines early during infection. Intraepithelial γ:δ T cells have been demonstrated to play a role in CVB myocarditis[65], while NK T cells have been implicated in EMCV-induced diabetes in animal models[66].

Phagocytes

Macrophages, dendritic cells, and neutrophils have all been implicated in the innate response to picornavirus infection[67-70]. These cells are known to phagocytose infected cells as well as function in the production of antiviral and inflammatory cytokines. Phagocytosis of virally infected cells serves two functions. First, phagocytosis by macrophages and neutrophils results in degradation of viral particles by lysosomal enzymes as well as reactive oxygen and nitrogen species leading to clearance of infectious particles and control of infection. Second, phagocytosis and degradation allows for recognition of viral products by PRR within macrophages and neutrophils. These receptors, which will be described in detail in later sections, stimulate the activation of phagocytic cells leading to increased antigen presentation, cytokine production, and inflammatory mediators. Unlike macrophages and neutrophils, which function at the site of infection, dendritic cells (DC) gather antigen from the site of infection, then migrate to lymphatic tissues, where they function in the initiation of the adaptive immune response. As part of this response, they produce large amounts of cytokines, which besides informing the adaptive response, contribute to activation of the innate response. In particular, DCs have been implicated in activation of the NK cell response to viral infection[71].

Cytokines

Viral infection induces the production of a variety of antiviral and inflammatory cytokines from both infiltrating immune cells as well as infected cells themselves. Type I IFN is acknowledged as a critical mediator of the antiviral response necessary to limit viral infection, but inflammatory cytokines also recruit immune cells necessary for control of infection.

Interferons

The initiation of IFN production is an essential step in the antiviral response. There are three type of IFN produced during infection. Type I IFNs (IFN-α, IFN-β, IFN-ω, IFN-ε, and IFN-κ) fight viruses both directly by inhibiting viral replication in cells and indirectly by stimulating the innate and adaptive immune responses[72]. Type II IFN, also known as IFNγ, has distinct functions compared to type I and type III IFNs. This cytokine is produced by activated T cells, NK cells, and NK T cells and acts primarily on macrophages leading to their activation and increased ability to kill intracellular pathogens as well as stimulation of the adaptive immune response[73]. It has a much more limited role on virally infected cells themselves. Recent studies have led to the identification of type III IFNs (IFN-λ). These include three proteins, named IFN-l1, IFN-l2, and IFN-l3, or interleukin-29 (IL-29) (l1) and IL-28A/B (l2/3). Although genetically distinct from type I IFNs, type III IFNs have similar biological antiviral functions [74-76]. In contrast to type II IFN, type I and type III IFN can be produced by almost all nucleated cells allowing for a variety of cells to initiate an antiviral response. A major difference between type I and type III IFN, however, is that while the type I IFN receptor is ubiquitously expressed, the type III IFN receptor has a more limited distribution, suggesting some cellular specificity in the IFN response[77,78].

IFN-α and IFN-β bind to the IFNα receptor (IFNAR) in an autocrine or paracrine manner. Activation of this receptor leads to JAK/STAT signal transduction pathways [79,80] and the induction of a variety of IFN-induced genes. These genes increase the cellular resistance to viral infection and sensitize virus-infected cells to apoptosis [81]. Interestingly, several viral sensors, which will be discussed in more detail later, are among those genes induced by IFN. They in turn enable the production of IFN, creating a positive feedback loop that enhances the response. In addition type I IFNs directly activate DC and NK cells, and promote the survival and effector functions of T and B cells, providing a link between the innate response to infection and the adaptive immune response [82-85]. The importance of type I IFN in control of viral infection is evidenced by several mutations in mice and humans that affect this pathway and lead to severe sensitivity to viral infection[86-88], as well as the number of viruses that encode inhibitors of IFN pathway components[89].

Although type I IFN is critical for the control of virus infection, it has also been linked to a variety of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, systemic sclerosis, and myositis[72]. Autoimmune phenomena has also been seen in patients treated with IFN in the context of viral infection[90]. It has recently been suggested that IFN is an initiating event in T1DM. This is suggested by reports that IFN-induced gene upregulation is seen in islet samples from newly diabetic patients[35,91]. In this context, IFN could function to upregulate antigen presentation of β-cell antigens as well as activate immune cells leading to autoimmune destruction of islets[92] or perhaps lead to direct cytotoxic effects[93]. Paradoxically, stimulation with IFN-inducing agents, such as dsRNA or virus, have a

protective effect in the NOD mouse and BB rat models of T1D[46,94]. Additionally, production of IFNα and IFNβ was shown to be critical for prevention of CVB-induced diabetes in a mouse model[95]. In this system IFN reduced the permissiveness of β-cells to infection and limited NK-mediated death of these cells. These disparate results illustrate that although IFN is necessary for control of viral infection, disregulation or overproduction may also induce autoimmune pathology.

Inflammatory cytokines

In addition to IFN signaling, inflammatory cytokine and chemokines also play a role in control of viral replication. Both dendritic cells (DC) and macrophages produce TNFα, IL-6, MCP-1, and IL-12 in response to viral infection. In addition these same inflammatory cytokines are often detected in the serum of virally infected animals. Inflammatory cytokines activate the vascular endothelium as well as stimulate the recruitment of immune cells such as monocytes and neutrophils. IL-6 has been shown to be important to limit damage during CVB infection[96], while TNFα has been shown to lead to enhanced pathogenicity with the same virus[97]. These results demonstrate that although the inflammatory response is important in the clearance of viral infection, a prolonged inflammatory state can also lead to adverse reactions including necrosis of local tissue and autoimmune diseases, so careful regulation is critical.

Sensors involved in detection of viral products

The innate immune response provides protection during the early stages of viral infection. However, activation of both the cellular and cytokine responses requires the host to recognize an ongoing infection. To accomplish this, organisms take advantage of pathogen associated molecular patterns (PAMP), which are specific for microbial components typically seen in the context of infection. These PAMPs are recognized by pattern recognition receptors (PRR) of the innate immune system that rapidly signal for the initiation of the antiviral response. Two distinct groups of PRRs include the Rig-I like receptors (RLR) and the Toll-like receptors (TLR). These two pathways, along with other components, provide a means for cells to detect the presence of viral pathogens.

Rig-I like receptors

RLRs are cytoplasmic proteins that recognize viral products that have gained access to the cytosol. There are currently three known members of this family: retinoic acid-inducible gene I (Rig-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (Lgp2)[98]. Rig-I and MDA5 both contain a DExD/H box helicase domain that binds double stranded RNA (dsRNA) and two N-terminal caspase recruitment (CARD) domains involved in signaling [99-102]. Lgp2 contains the helicase domain, but lacks the CARD domains, and was originally thought to be a negative regulator [103,104]. However, recent reports indicate that it may have an activating function in response to certain viruses[105,106]. Both Rig-I and Lgp2 also contain a C-terminal repressor domain that blocks signaling in the absence of ligand binding [102]. Rig-I binds preferentially to ssRNAs that are phosphorylated at the 5' end [107,108] and

contain homopolyuridine or homopolyriboadenine motifs as well as short dsRNA [109-111]. MDA5 recognizes long dsRNAs and does not require 5' phosphorylation [110-113]. Crystallization studies have determined that the differential ligand binding capacities of Rig-I and MDA5 are dependent on structural differences in the C-terminal domain of the protein[114,115]. The different ligand preferences of the two proteins is thought to result in specificity for the recognition of individual viruses.

Both MDA5 and Rig-I signal through CARD-CARD interactions with IPS-1 (also known as MAVS, VISA, or Cardiff), which is located on the outer mitochondrial associated membrane [116-119]. Downstream of IPS-1 [120], TRAF3 activates TBK1 and IKKe, which phosphorylate IFN regulatory factor 3 (IRF-3) and IRF-7 [121,122]. Activated IRF-3 and IRF-7 translocate into the nucleus and bind IFN stimulated response elements (ISREs), inducing the expression of type I IFNs [123]. IPS-1 also interacts with FAS-associated death domain-containing protein (FADD) [124]. FADD activates caspases-8 and -10, and the activation of the caspase death effector domains activates NF-kB, leading to the production of inflammatory cytokines [125]. A schematic of RLR signaling is shown in Figure 1.1.

Toll like receptors

TLRs are transmembrane proteins that contain luminal leucine-rich repeats (LRRs) that sense pathogen associated molecular patterns and cytoplasmic Toll/IL-1 receptor homology (TIR) domains that signal through downstream adaptors [98]. There are 10 members of the TLR family in humans and 13 in mice. TLRs involved in the detection of viral nucleic acids are located on the cell surface (TLR3) or in endosomal

compartments (TLR3, 7, 8, and 9) [126]. TLR3 recognizes dsRNA, which constitutes the genome of dsRNA viruses and is also an intermediate produced during replication of single stranded (ss) RNA viruses [127]. TLR7 and 8 recognize ssRNA as well as imidazolequinilone compounds, which are known to have antiviral properties [126,128-131]. TLR9 recognizes unmethylated CpG-containing DNA, which is commonly found in the genomes of DNA viruses[132,133].

TLR3 signals through the adaptor protein TRIF [134,135]. TRIF interacts with TRAF3 and TRAF6 through TRAF-binding motifs and with RIP1 and RIP3 through RHIM motifs [136-138]. TRAF3 leads to the secretion of type I IFNs, while TRAF6 and RIP1 lead to NF-kB activation and production of inflammatory cytokines [139]. TLR7, 8, and 9 signal through the adaptor protein myeloid differentiation primary-response gene 88 (MyD88). MyD88 contains a TIR domain as well as a death domain that allows it to serve as an adaptor for TLR signaling. MyD88 associates with a signaling complex consisting of TRAF6, BTK, IRAK4, and IRAK1 [140]. Signaling through this complex activates IRF7, NF-kB, and MAP kinase pathways [141-143]. Thus, although RLRs and TLRs signal through different pathways, both appear to be able to activate the production of type I IFNs (i. e. IFN-a and IFN-b) and inflammatory cytokines.

Two additional TLR family members that signal through MyD88 have been implicated in the recognition of non-nucleic acid viral components. TLR2 is known to detect a variety of lipoproteins as well as yeast-associated zymosan, however, it has also been demonstrated to have a role in the recognition of viral envelope proteins [144]. A recent study has also described a role for TLR2 in detection and the early IFN response to

poxvirus infection[145]. Similarly, while TLR4 has traditionally been known as the sensor of LPS, it can also respond to viral-derived envelope glycoproteins [146].

1.9 Additional sensors (non-TLR, RLR)

The TLRs and RLRs have been shown to play a role primarily in RNA virus infection. Recently, the array of innate immune sensors of viral infection has been shown to include additional cytosolic proteins that are involved in the recognition of DNA viruses. A DNA binding protein, named DNA-dependent activator of IFN-regulatory factors (DAI), Z-DNA binding protein 1 (ZBP1), or DLM-1, binds cytosolic DNA inducing type I IFN and other genes involved in innate immunity [147,148]. Accordingly, RNA interference of mRNA for DAI in cells inhibits DNA-mediated antiviral responses. Furthermore, NALP3, a component of the cytosolic molecular complex termed the inflammasome, has been shown to recognize adenoviral DNA, inducing activation of caspase 1 and maturation of pro-interleukin-1β in macrophages [149]. Correspondingly, mice lacking NALP3 or its signaling adaptor, ASC, display reduced innate inflammatory responses to adenovirus particles. Even more recent studies have suggested that Aim2 also recognizes DNA virus infection[150]. Similar to NALP3, Aim2 activates the inflammasome pathway leading to IL-1β production. Cells are also known to produce type I IFN in response to cytoplasmic dsDNA[151]. The initial sensor in this pathway is currently unknown, however, STING is known to play a role in the downstream signaling pathway[152]. The discovery of these sensors has provided further insight into the innate response against DNA viruses.

Besides RLR and TLR classes of sensors, other proteins are known to detect viral products and contribute to the immune response, especially RNase L and protein kinase R (PKR). RNase L has recently been reported to be involved in the RLR response to viral nucleic acids [153]. It is proposed that 2',5'-linked oligoadenylate generated by viral infection activates RNase L to cleave self RNA into small RNA products, which are responsible for RLR signaling. However, it is not yet known how these small self-RNAs are recognized by MDA5 and RIG-I. PKR has been shown to dimerize upon binding of dsRNA. The activated PKR dimer phosphorylates eukaryotic initiation factor 2-a (eIF2-a) which results in inhibition of translation, preventing viral replication [154]. Recent results indicate that PKR is also necessary for the stabilization of type I IFN mRNA transcripts[155]. Like RLRs, RNase L and PKR are upregulated in response to type I IFN, demonstrating their important role in the pre-programmed antiviral response.

The role of RLRs and TLRs in viral infection

RLRs

Among the RLRs, ligand preferences appear to determine which virus is recognized by which sensor. The current paradigm is that RIG-I recognizes RNA containing 5'-triphosphates, while MDA5 recognizes dsRNA. Therefore it is not surprising that RIG-I has been shown to detect Influenza A and B viruses, vesicular stomatitis virus (VSV), and some Flaviviruses (Japanese Encephalitis Virus and Hepatitis C Virus) [112,156,157]. Likewise, MDA5 detects picornaviruses such as encephalomyocarditis virus (EMCV), Mengo virus and Theilers virus [112,113]. These viruses contain a 5' VPg cap instead of 5'triphosphate and make large amounts of dsRNA during replication.

However, other results do not neatly fit this paradigm. RIG-I and MDA5 play redundant roles in the recognition of West Nile Virus [158], Dengue virus, [157] paramyxovirus, and reovirus [157], most of which contain 5'triphosphates. In addition, although sendai virus has been shown to activate RIG-I, it encodes for a protein, the V protein, that is a specific inhibitor of MDA5 [159]. Furthermore, murine hepatitis virus, a member of the coronavirus family that does not contain VPg has recently been shown to be recognized by MDA5[160]. One explanation is that although RIG-I preferentially recognizes 5-triphosphates and polyuridine rich regions, it can also recognize short dsRNA, while MDA5 recognizes long dsRNA [110]. The ability of MDA5 and Rig-I to specifically detect certain viruses, while also detecting common pathogens illustrates the need for multiple sensors to recognize and control the wide variety of viral pathogens.

TLRs

Compared to the RLRs, the role of TLRs in anti-viral responses is more intricate [161,162]. TLR3 was originally shown to detect dsRNA[127]. Accordingly, TLR3 has been implicated in the detection of several RNA viruses such as EMCV [163], CVB [70], RSV [164,165], West Nile Virus [166], and Punta Toro Virus [167]. However, in another study TLR3 did not contribute to viral pathogenesis in vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV) and reovirus infections [168]. In addition, TLR3 has been implicated in recognition of DNA viruses. TLR3-deficient mice are more susceptible to MCMV infection than wild type mice[169] and have defective T cell responses to HSV[170]. Thus, TLR3 may recognize not only RNA viruses, but also DNA viruses, most likely through RNA intermediates that are generated during viral replication.

TLR7 has been shown to contribute to the detection of RSV, Sendai virus, influenza, HIV, VSV, and Coxsackie virus B3 (CVB3) [171], while TLR8 has been implicated in detection of influenza and paramyxovirus as well as HIV [129,146,172]. TLR9 plays a role in recognition of Herpes Simplex virus and cytomegalovirus infection [169,173-175]. TLR2 and 4 have been shown to play a role in the recognition of enveloped viruses. Both Herpesviruses, which contain a DNA genome, and RSV, which has a ssRNA genome, have been reported to be recognized by these sensors [176-178]. In summary, TLR7 and 8 recognize ssRNA viruses, while TLR9 recognize DNA viruses. TLR2 and 4 recognize enveloped viruses, while TLR3 plays a role in recognition of both RNA and DNA viruses. Overall, TLR viral specificities exhibit a significant overlap with those of RLRs.

Pattern recognition receptors in human disease

Recent studies have implicated the dsRNA sensors MDA5 and TLR3 in human diseases. Genome-wide analysis of single nucleotide polymorphisms (SNPs) identified a polymorphism in the gene encoding human MDA5, interferon induced helicase 1 (Ifih1), that is associated with an increased risk of T1DM[179]. This polymorphism, A946T, is located near the N-terminus of the protein in an area that has been suggested to be a regulatory region in Rig-I and LGP-2. This suggested that a genetic predisposition to T1DM may occur from an altered capacity to either detect viral infection or regulate IFN production through MDA5. More recent studies indicate that very rare polymorphisms in Ifih1, which result in inability to produce IFN in response to virus or dsRNA, are protective for T1DM[180,181]. This strengthens the link between viruses and diabetes and

suggests that IFN production by MDA5 may be a pathogenic instigator of T1DM. Meanwhile, another recent human study demonstrated that a dominant negative form of TLR3 causes susceptibility to neonatal Herpes Simplex-1 Encephalitis (HSE) [182]. This report indicates that TLR3 plays a role in protection in humans from viral infections, although whether TLR3 signaling is important for additional viruses remains to be seen.

Conclusions

PRRs such as MDA5 and TLR3 play important roles in control of viral infection and have been implicated in human diseases. Although much has been learned about the downstream signaling pathways and molecular ligands associated with these proteins, how they function *in vivo* remains poorly understood. In this study, we will describe the investigation of MDA5 and TLR3 function in several *in vivo* systems. Chapter 2 focuses on understanding how MDA5 and TLR3 control MNV-1 infection. In Chapter 3, we make use of an EMCV infection model to study the role of MDA5 and TLR3 in myocarditis and diabetes. Chapter 4 describes how MDA5 and TLR3 contribute distinct functions in NK cell activation by dsRNA. Finally, Chapter 5 will describe how this work has contributed to our knowledge of how MDA5 and TLR3 function *in vivo* as well as potential applications for future studies.

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Figure Legends

Table 1.1. Diseases Caused by Picornavirus and Calicivirus Family Members

List of common diseases caused by picornaviruses and caliciviruses.

Figure 1: Cytoplasmic and Endosomal Sensors of Viral Nucleic Acids

This figure illustrates the detection of viral products by RLR and TLR family members and the downstream signaling pathways leading to IFN and inflammatory cytokine production.

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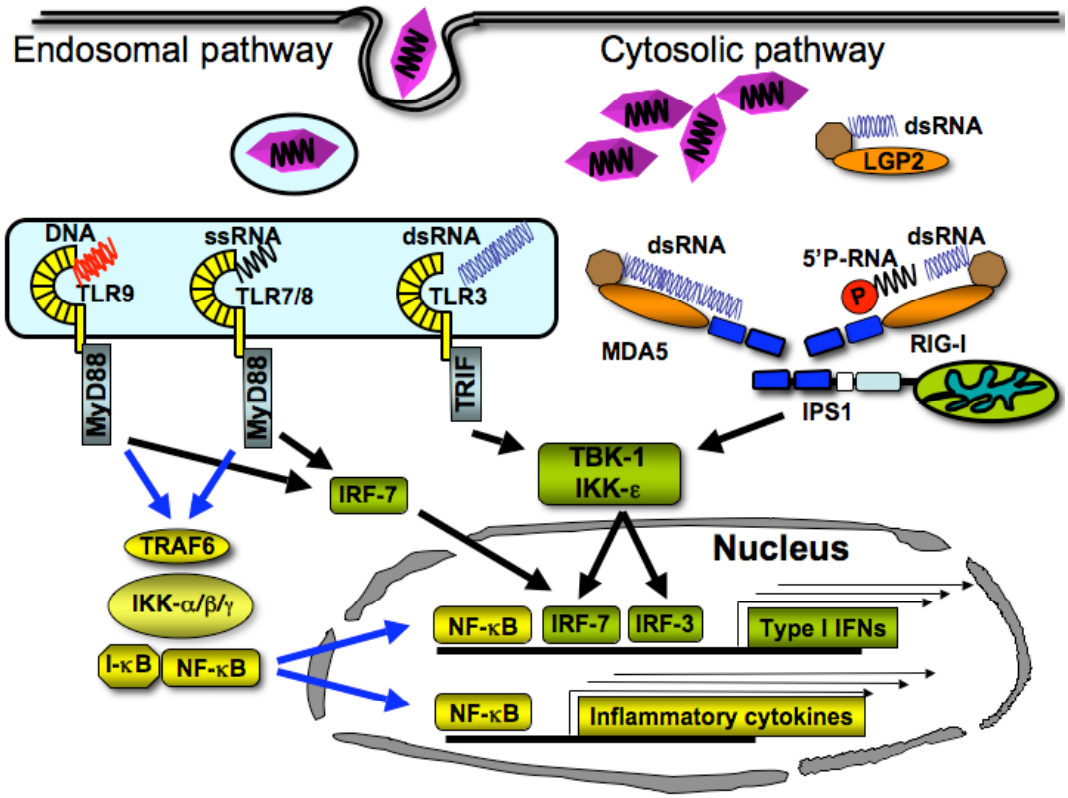
Table 1.1: Diseases Caused by Picornavirus and Calicivirus Family Members	
Virus	Disease
Picornaviruses	
Poliovirus	Poliomyelitis
Coxsackievirus A	Hand Foot and Mouth Disease, Meningitis, Conjunctivitis
Coxsackievirus B	Myocarditis, diabetes
Echovirus	Myocarditis, Meningitis
Rhinovirus	Common cold
Hepatitis A virus	Hepatitis
Foot and Mouse Disease Virus	Foot and Mouth Disease
Encephalomyocarditis Virus	Myocarditis, Encephalitis
Caliciviruses	
Norwalk virus (norovirus)	Gastroenteritis
Sapporo virus	Gastroenteritis

Figure 1.1


Endosomal pathway
Cytosolic pathway
dsRNA
LGP2
DNA
ssRNA
dsRNA
TLR9
TLR7/8
TLR3
dsRNA
5'P-RNA
dsRNA
P
MDA5
RIG-I
MyD88
MyD88
TRIF
IPS1
TBK-1
IKK-ε
IRF-7
TRAF6
IKK-α/β/γ
I-κB
NF-κB
Nucleus
NF-κB
IRF-7
IRF-3
Type I IFNs
NF-κB
Inflammatory cytokines

CHAPTER 2

MDA5 Recognition of a Murine Norovirus

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Abstract

Noroviruses are important human pathogens responsible for most cases of viral epidemic gastroenteritis worldwide. Murine norovirus-1 (MNV-1) is one of several murine noroviruses isolated from research mouse facilities and has been used as a model for human norovirus. MNV-1 infection has been shown to require innate immunity for clearance, however, the initial host protein that recognizes MNV-1 infection is unknown. Because noroviruses are RNA viruses, we investigated whether MDA5 and TLR3, cellular sensors that recognize dsRNA, are important for the host response to MNV-1. We have shown that MDA5 but not TLR3 deficient dendritic cells(DC) have a defect in cytokine response to MNV-1. In addition, MNV-1 replicates to higher levels in MDA5 -/- DCs as well as in MDA5 -/- mice *in vivo*. This is the first demonstration of an innate immune sensor for norovirus. Knowledge of the host response to MNV-1 may provide keys for prevention and treatment of the human disease.

Author Summary

Gastroenteritis is a common disease in both developed and developing countries. The two main causes of this affliction are bacteria and viruses. The primary viruses implicated in gastroenteritis are a family of viruses called noroviruses, which include Norwalk virus, notorious for several recent outbreaks on cruise ships. We are interested in how the innate immune system detects viral infection and signals the body to respond to the threat. To learn more about this we studied two classes of proteins, both of which are thought to detect signs of viral infection. We discovered that one of these proteins, Melanoma differentiation associated protein-5 (MDA-5), is responsible for detecting a mouse norovirus that is similar to the human pathogen. These findings allow us to better understand the pathogenesis of norovirus infection and may provide clues for controlling the human disease.

Introduction

Norwalk virus and other human noroviruses are common human pathogens responsible for most of the nonbacterial epidemic gastroenteritis in both developed and developing countries[Estes, 2006 #6; Widdowson, 2005 #12; Lopman, 2004 #10; Fankhauser, 1998 #8; Mead, 1999 #11]. In humans, norovirus infection results in vomiting, diarrhea, fever, malaise, and abdominal pain within 24 hours after infection. These symptoms usually clear within 48 hours, but the virus can persist for 3-6 weeks post-infection[Dolin, 2004 #9; Graham, 1994 #23]. Until recently the lack of ability to culture the virus has prevented us from investigating its pathogenicity. The discovery and subsequent culture of murine norovirus-1 (MNV-1) has led to advances in understanding of both the viral lifecycle as well as the host response to infection[Wobus, 2004 #2; Karst, 2003 #19].

Noroviruses are in the *Calicivirus* family and are nonenveloped viruses containing a single-stranded positive-sense RNA genome. This genome is covalently linked at the 5' end to the viral nonstructural protein VPg[Green, 2001 #31]. It encodes four open reading frames (ORFs)[Jiang, 1993 #20; Lambden, 1993 #21; Clarke, 2000 #22; Thackray, 2007 #1]. ORF1 encodes a polyprotein that is cleaved into at least six nonstructural proteins by the viral 3C-like protease[Blakeney, 2003 #43; Liu, 1996 #45; Liu, 1999 #46; Sosnovtsev, 2006 #44]. ORF2 encodes the major capsid protein, viral protein 1[Prasad, 1999 #47; Jiang, 1993 #20], while ORF3 encodes the small basic protein, viral protein 2[Bertolotti-Ciarlet, 2002 #48; Glass, 2000 #49]. OFR4 was recently discovered and has yet to be characterized[Thackray, 2007 #1].

The rapid clearance of MNV-1 infection indicates an important role for the innate immune system, since clearance occurs before the typical initiation of adaptive

immunity[Mumphrey, 2007 #5]. Previous work has revealed that MNV-1 infection of mice lacking either the type I and type II interferon (IFNα/β/γ) receptors or the STAT-1 molecule is lethal [Mumphrey, 2007 #5; Karst, 2003 #19]. Several proteins are known to initiate the IFN response to viruses[Takeuchi, 2007 #63], including Toll-like receptors (TLR)[Iwasaki, 2004 #42], Rig-I-like helicases (RLH)[Sumpter, 2005 #38; Pichlmair, 2007 #51], PKR[Garcia, 2007 #32], and RNase L[Malathi, 2007 #15]. However, the initial sensor responsible for recognition of noroviruses and subsequent activation of cytokine response has not been determined.

TLRs are located on the plasma membrane and in endosomal compartments. Among the TLRs, TLR 7 and 8 recognize ssRNA[Lund, 2004 #36; Heil, 2004 #41; Diebold, 2004 #52], TLR9 recognizes DNA[Bauer, 2001 #39; Hemmi, 2000 #53], while TLR3 signals in response to dsRNA[Alexopoulou, 2001 #54]. The RLHs are cytoplasmic sensors located intracellularly[Pichlmair, 2007 #51], which include Rig-I and MDA-5[Takeuchi, 2007 #24] [Fujita, 2007 #35; Yoneyama, 2004 #55] and signal through IPS-1/MAVS/Cardiff/VISA[Xu, 2005 #61; Meylan, 2005 #60; Sun, 2006 #59]}[Perry, 2005 #62]. Rig-I has recently been shown to preferentially recognize 5'-phosphorylated RNA[Hornung, 2006 #26; Pichlmair, 2006 #27], while MDA5 responds to dsRNA [Yoneyama, 2005 #37]. Recently it has been shown that the lack of Rig-I does not confer susceptibility to human norovirus *in vitro*[Guix, 2007 #7]. Because MDA5[Loo, 2008 #17; Kato, 2006 #13; Gitlin, 2006 #18; Fredericksen, 2008 #58], and possibly TLR3 [Edelmann, 2004 #56; Wang, 2004 #57] have been shown to play a role in host response to other RNA viruses we investigated if these sensors might be involved in MNV-1 recognition.

Results

MDA-5 is required for cytokine response to MNV-1 by Bone Marrow-Derived DC. Previous studies have shown a requirement for the type I IFN response for control of MNV-1 infection[Wobus, 2004 #2]. Since both MDA5 and TLR3 have been shown to be involved in type I IFN and cytokine signaling in response to infection with other viruses, we were interested to see if they may play a role in MNV-1 infection.

MNV-1 infection has a limited cell tropism- infecting only DC and macrophage lineages *in vitro*[Wobus, 2004 #2; Ward, 2006 #50]. In order to test whether the MDA5 or TLR3 sensors were important, BMDCs from Wild Type as well as TLR3 -/- and MDA5 -/- mice were cultured for 7 days and then stimulated with various MOI of MNV-1 isolates. After 24 hours supernatants from the *in vitro* infections were harvested and tested for cytokine production.

Interestingly, although WT and TLR3 DCs produced similar levels of IFNα and inflammatory cytokines in response to MNV stimulation, MDA5 deficient DCs produced significantly less IFNα, IL-6 , MCP-1 and TNFα (figure 1) and IFNβ (data not shown). This indicates a role for MDA5 in the detection of MNV-1 infection.

MNV-1 replicates more efficiently in MDA-5 deficient DCs. Because the MDA5 -/- BMDCs had a defect in cytokine response to MNV-1, we wanted to test if this deficiency had an effect on the course of viral infection. To address this issue, we infected BMDCs *in vitro* with MNV-1 and harvested samples at 6-hour time-points post-infection. The infections were done at a saturating and non-saturating MOI to test for effects on viral replication and spreading.

Viral titers were identical in WT and MDA5 KO mice up to 12 hours post-infection at a saturating MOI (figure 2a). However, starting at 18 hours pi, titers from MDA5 KO BMDCs began to increase over WT BMDCs, and leveled out to a 1-log difference at 24 and 48 hours. At a lower MOI there was no significant difference between viral titers in the WT and KO cells until the 48-hour time point (figure 2b). At both MOIs the kinetics of MNV-1 infection appears similar in WT and MDA5 -/- BMDCs; the difference mainly appears to be in the total amount of viral replication seen in the KO BMDCs. These results as well as the defect in cytokine response seen previously provide further evidence for the role of type I IFN (IFNα IFNβ) in preventing MNV-1 growth.

MDA5 limits MNV-1 replication *in vivo.* MNV-1 infection naturally occurs after fecal-oral transmission[Wobus, 2004 #2]. In order to show that MDA5 plays a role in MNV-1 detection *in vivo* we infected WT or MDA5 -/- mice with MNV-1.CW3 perorally. Organs were then harvested from infected as well as mock-infected mice on days 1, 3, and 5 post-infection and viral titers were determined for each sample.

At d3 post-infection MDA5 KO animals had a one log increase in viral titers compared to wild type animals in the mesenteric lymph nodes, spleen, and proximal intestine (figure 3). Minimal or negative titers were seen in distal intestine, and stool as well as liver and lung (data not shown) by viral plaque assay in both WT and MDA5 KO animals, indicating that MNV-1 infection remained locally contained. Consistent with lack of systemic infection, serum samples taken at d1, 3, and 5 timepoints post-infection were found to be negative for IFNα and IFNβ (data not shown). Organs harvested at d1 and d5 also contained minimal detectable levels of MNV-1 in both WT and MDA5 KO mice indicating that the infection developed and was cleared in the timeframe that has

been reported earlier[Mumphrey, 2007 #5]. Similar to our *in vitro* data, the kinetics of MNV-1 infection is similar in WT and MDA5 -/- mice *in vivo*, while there is a significant increase in maximum titers in the KO animals. This suggests that MDA5 controls the amplitude of infection.

MDA-5 recognizes replication competent viral RNA. Although MDA5 detects norovirus, it is unclear which RNA feature is essential for recognition. Rig-I recognizes viruses through 5'-phosphorylation, however, in norovirues this feature is absent because of a 5' VPg cap[Green, 2001 #31]. To test whether 5' RNA configuration is essential for MDA5 recognition we infected BMDCs with MNV and then harvested the total RNA from infected as well as mock-infected cells before peak infection to isolate potential intermediates. BMDCs from WT or MDA5 deficient mice were then stimulated with the harvested RNA, as well as RNA treated with calf intestinal phosphatase (CIP,) which removes 5' phosphates, and proteinase K (PK), which degrades proteins including VPg and prevents viral replication *in vitro*[Guix, 2007 #7].

Both WT and MDA5 -/- DCs produced limited inflammatory cytokines in response to mock-infected RNA, however, WT but not KO BMDCs produced large amounts of TNFα and IFNβ in response to RNA from MNV-infected cells (figure 4). CIP treatment of the RNA had no significant effect on cytokine production in either cell type, as predicted by the existence of VPg instead of 5' phosphorylation. However, the addition of PK to the RNA abrogated the cytokine response in WT BMDCs. This suggests that MDA5 does not recognize naked viral RNA, but either directly recognizes RNA linked to VPg or recognizes a RNA product generated during viral replication. Because MDA5 has been previously shown to recognize uncapped poly I:C[Kato, 2006

#13; Gitlin, 2006 #18], it is most likely that the PK effect reflects the requirement for viral replication and the subsequent generation of RNA species that are recognized by MDA5.

Discussion

We have provided the first description of an initial sensor of norovirus infection. MDA5 recognizes MNV-1 and stimulates antigen presenting cells to produce type I interferon as well as IL-6, MCP-1, and TNFα that function to recruit other immune cells as well as activate antiviral pathways in host cells. Deficiency of this sensor results in lack of cytokine production as well as increased MNV-1 replication in deficient cells and mice.

It is interesting to note that although MDA5 deficient cells have a defect in IFNα production, the mice do not have as severe a phenotype as the IFNαβγR or Stat1 deficient mice. These mice completely lose the ability to respond to IFN because they lack either its receptor or a critical downstream signaling molecule. As a result these mice have widespread MNV-1 dissemination that often results in lethality. The more mild phenotype seen in the MDA5 KO mice resembles that seen in the IFNαβR KO mice and likely results from the retained ability of IFNγ production. Additionally, it is also possible that other sensors of viral products or cell damage are able to respond and trigger an IFN and cytokine response. Further investigation is needed to determine if mice that are deficient in multiple nucleic acid sensors lack all ability to respond to MNV-1 and whether they therefore have a more severe phenotype. Data from our lab and others[Guix, 2007 #7] from *in vitro* experiments suggest that TLR3 and Rig-I are unlikely candidates for additional MNV-1 sensors, but TLR7 remains to be tested, as does the role of other sensors *in vivo*.

Although the putative recognition structure for Rig-I has previously been determined[Hornung, 2006 #26; Pichlmair, 2006 #27], the RNA structure recognized by MDA5 in viral infection remains unclear. We demonstrated that MDA5 recognition of

MNV RNA is abrogated by treatment with PK, which degrades VPg, preventing viral replication. This suggests that MDA5 does not recognize naked RNA, but rather a RNA intermediate that is abundantly generated during replication. Although we cannot rule out the possibility that MDA5 recognizes the VPg-RNA structure itself, this is unlikely because MDA5 is known to respond to poly I:C which has no protein cap. Learning more about which viruses are recognized by MDA5 may provide hints as to what this protein recognizes. This information could then be used to design adjuvants to manipulate the immune response for both vaccine design as well as in viral infection.

Materials and Methods

Cell Lines. RAW264.7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Hyclone), 100U penicillin/ml, 100μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine.

Bone Marrow-derived DC. Bone marrow was flushed from the femurs of mice and cultured as described previously[Barchet, 2005 #28]. Briefly, cells were cultured in RPMI (Gibco) with 10% fetal calf serum (Hyclone), Glutamax, Na Pyruvate, Non-Essential AAs, and Kanamycin for 7-8 days at 37 degrees.

Mice. MDA5 -/- mice were described previously[Gitlin, 2006 #18]. For the infection studies mice backcrossed onto a pure 129/SVJ background were used. Control WT mice were age and sex matched and were obtained from littermate controls and from Jackson Lab for 129/SVJ. TLR3 -/- mice were kindly provided by Richard Flavell [Alexopoulou, 2001 #29]. All mice were bred and housed in a pathogen free facility and regularly tested for MNV-1 antibodies.

In vitro stimulations. BMDCs were counted and plated at 200,000 cells/well in a 96 well plate. MNV-1 was added at various MOI to the cultures, or alternatively 500ng RNA was complexed with lipofectamine 2000 (invitrogen) and added according to manufactures instructions. After 20-24 hours supernatants were harvested and stored at -20 degrees until cytokine analysis. IFNα and IFNβ levels from the supernatants were measured by ELISA (PBL Biomedical Laboratory, New Brunswick, NJ), while IL-6, MCP-1, and TNFα levels were determined by cytokine bead array (BD Biosciences).

***In vivo* infections.** WT or MDA5 KO mice were infected perorally with $1X10^7$ PFU MNV1.CW3[Thackray, 2007 #1] or mock-infected with media only. Three days post-

infection the following organs were harvested and stored at -80 degrees until assayed: spleen, liver, mesenteric lymph node, lung, proximal intestine, distal intestine, stool, and serum.

MNV-1 plaque assay. Tissue samples were homogenized in 1 ml complete DMEM by bead beating with 1.0-mm zirconia/silica beads (BioSpec Products, Inc.). Tissue homogenates were diluted 1:10 in complete DMEM and tested for viral titers by using a plaque assay that has been previously described [Wobus, 2004 #2]. Briefly, $2X10^6$ RAW264.7 cells were seeded into each well of six-well plates, and infected the next day with 10-fold dilutions of tissue homogenate in duplicate. After a 1-hr infection, the inoculum was removed and wells were overlaid with 1.5% SeaPlaque agarose (Cambridge Biosciences) in complete minimal essential medium and incubated at 37C. After 48 hrs, a second overlay was added containing 1.5% SeaKem agarose (Cambridge Biosciences) and 0.01% neutral red in complete minimal essential medium. After 8 hrs, plaques were then visualized.

RNA preparation. $5*10^6$ BMDC were infected with MNV-1 at MOI 1 or mock-infected for 10 hours. Total RNA was harvested using the Genelute Total RNA Isolation Kit (Sigma). Purified RNA was incubated with either 10 units Calf Intestinal Phosphatase (New England Bioloabs) in NEB buffer 2 or with 200μg/ml proteinase K (Sigma) in 0.1M NaCl, 10mM Tris (pH 8), 1mM EDTA, 0.5% sodium dodecyl sulfate or left untreated for one hour at 37 degrees then precipitated with LiCl (Ambion), washed, and resuspended for stimulations.

Acknowledgements

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Figure Legends

Figure 2.1: MDA5 is required for cytokine response to MNV *in vitro*. Bone marrow-derived dendritic cells from wild type, TLR3 KO, or MDA5 KO mice were stimulated *in vitro* with MNV at the indicated MOI. 24 hours later, cell culture supernatants were harvested and examined for IFNα by ELISA or for IL-6, TNFα, and MCP-1 by cytokine bead array. Data shown is the average of three independent experiments. Statistical analysis was done using student's t test where * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$.

Figure 2.2: MDA5 deficiency leads to increased MNV titers *in vitro*. Bone marrow-derived dendritic cells from wild type or MDA5 KO mice were infected with MNV at MOI 5 or 0.05. Samples were taken at 6-hour time-points and stored at -80 degrees. Viral titers were determined in duplicate on RAW cells for each sample and statistical significance was determined using student's t test. Data shown is the average of four independent experiments.

Figure 2.3: MNV replicates more efficiently in MDA5 KO hosts. Wild type or MDA5 deficient mice were inoculated perorally with $1X10^7$ PFU MNV-1.CW3 or mock infected with media only. Organs were harvested 3 days post-infection and viral titers of Mesenteric Lymph Node (MLN), Spleen , Proximal Intestine (prox intest), Distal Intestine (dist intest), and Stool were determined by viral plaque assay. Statistical significance was calculated using the Mann Whitney test, and P values comparing infection between WT and MDA5 KO mice are as follows: MLN $P<0.0001$; Spl

P=0.0003; PI P=0.0002. Mock-infected animals showed no detectable MNV-1 at all time-points tested. Data shown is from at least 9 animals.

Figure 2.4: Proteinase K and RNase abrogates MDA5 recognition of viral RNA. WT or MDA5 -/- BMDCs were stimulated with RNA from MNV infected or mock infected lysates that was treated with CIP, PK or untreated. After incubation for 20 hours, supernatants were harvested and cytokines analyzed with CBA and IFNβ ELISA. Data shown is from three independent experiments and statistics were calculated by student's t test

Figure 2.1

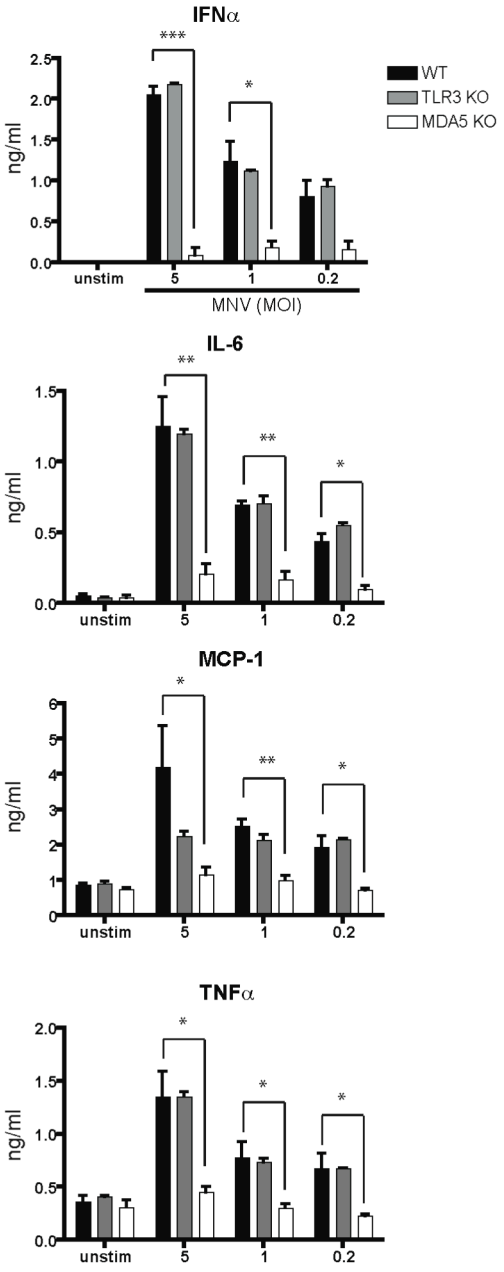

IFNα
***
*
WT
TLR3 KO
MDA5 KO
ng/ml
2.5
2.0
1.5
1.0
0.5
0.0
unstim
5
1
0.2
MNV (MOI)
IL-6
**
**
*
ng/ml
1.5
1.0
0.5
0.0
unstim
5
1
0.2
MCP-1
*
**
*
ng/ml
6
5
4
3
2
1
0
unstim
5
1
0.2
TNFα
*
*
*
ng/ml
2.0
1.5
1.0
0.5
0.0
unstim
5
1
0.2

Figure 2.2

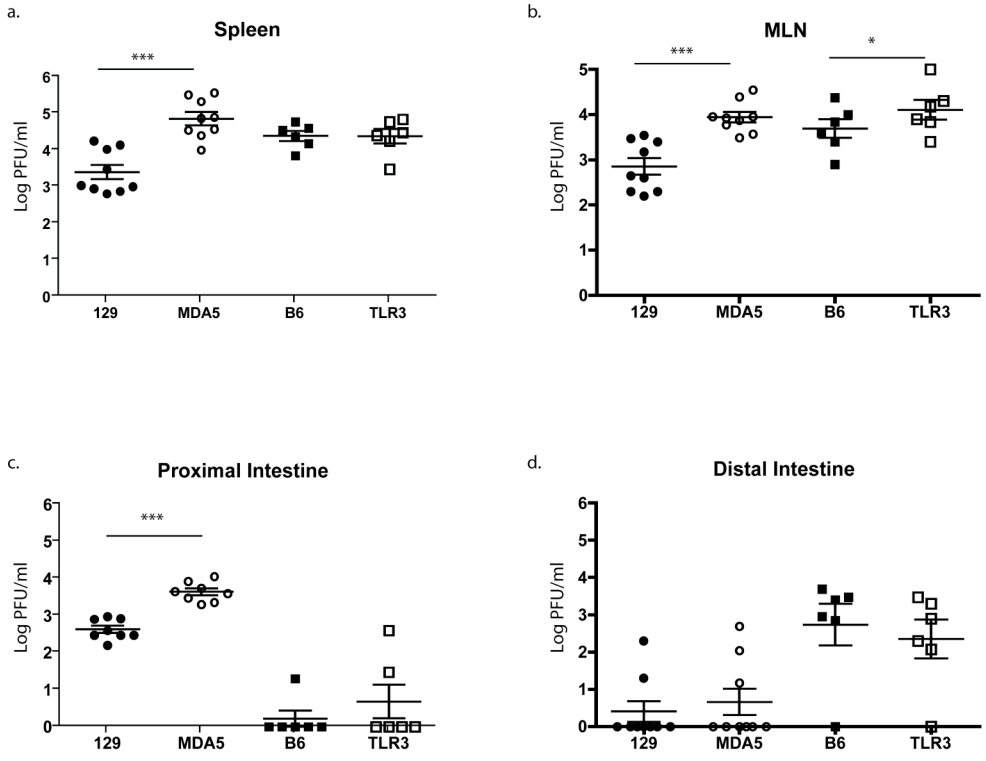

a.
Spleen
***
Log PFU/ml
0
1
2
3
4
5
6
129
MDA5
B6
TLR3
b.
MLN
***
*
Log PFU/ml
0
1
2
3
4
5
129
MDA5
B6
TLR3
c.
Proximal Intestine
***
Log PFU/ml
0
1
2
3
4
5
6
129
MDA5
B6
TLR3
d.
Distal Intestine
Log PFU/ml
0
1
2
3
4
5
6
129
MDA5
B6
TLR3

Figure 2.3

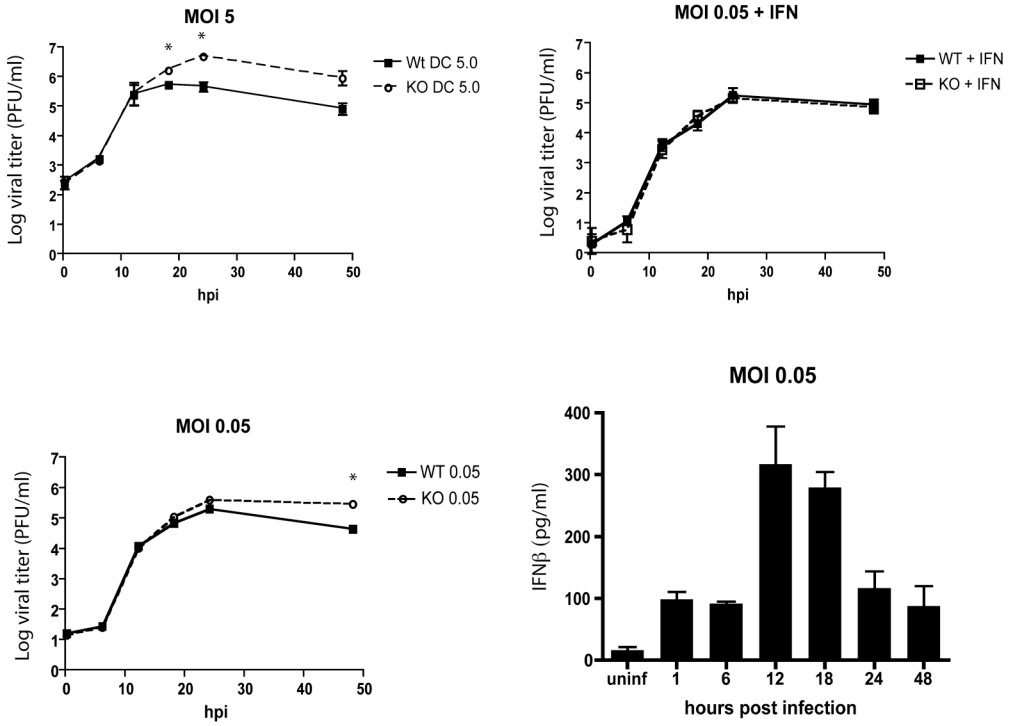
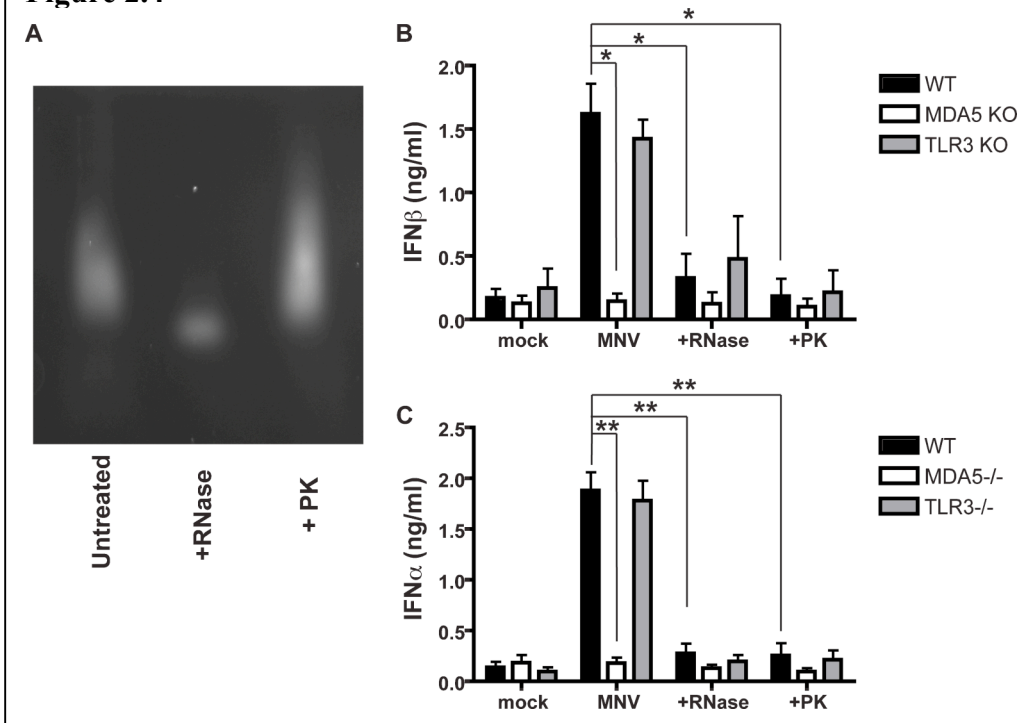

MOI 5
Log viral titer (PFU/ml)
hpi
Wt DC 5.0
KO DC 5.0
MOI 0.05 + IFN
WT + IFN
KO + IFN
MOI 0.05
WT 0.05
KO 0.05
IFNβ (pg/ml)
uninf
1
6
12
18
24
48
hours post infection

Figure 2.4


A
Untreated
+RNase
+ PK
B
IFNβ (ng/ml)
2.0
1.5
1.0
0.5
0.0
mock
MNV
+RNase
+PK
WT
MDA5 KO
TLR3 KO
C
IFNα (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
mock
MNV
+RNase
+PK
WT
MDA5-/-
TLR3-/-

Chapter 3

RNA Sensors in Myeloid Cells Prevent Virus-Induced Diabetes

This chapter has been submitted for publication as:

McCartney SA, Vermi W, Lonardi S, Rossini C, Otero K, Calderon B, Gilfillan S, Diamond MS, Unanue ER, and Colonna M. *RNA sensors prevent virus-induced diabetes in mice.*

Abstract

Viruses have long been implicated in the etiology of type I diabetes mellitus (T1DM). This association was further strengthened by the finding of genetic susceptibility to T1DM associated with polymorphisms in the human gene encoding MDA5, a dsRNA sensor involved in the recognition of viral infection. In addition to MDA5, the endosomal dsRNA sensor TLR3 has also been implicated in animal models of diabetes and viral infection. It has been suggested that these sensors may induce T1DM through the production of type I IFN in response to infection, which may lead to increased stimulation of autoimmune T cells. In this study we addressed the relative contributions of MDA5 and TLR3 to the host response to picornavirus infection and diabetes using a model of encephalomyocarditis virus strain D (EMCV-D). By analyzing EMCV-D infection in MDA5$^{-/-}$, TLR3$^{-/-}$ and MDA5$^{-/-}$TLR3$^{-/-}$ double knockout (DKO) mice we found that MDA5 and TLR3 have different impacts in the control of viral infection and tissue damage in heart and pancreas. EMCV-D infection caused primarily heart damage in MDA5$^{-/-}$ mice but severe diabetes in TLR3$^{-/-}$ mice. We further determined that the development of diabetes in the TLR3$^{-/-}$ animals was due to virus-induced β-cell damage rather than T cell-mediated autoimmunity, and resulted from the impaired capacity of hematopoietic cells, especially DC, to induce an early IFN-β response that restricts β-cell infection. These findings indicate that IFN produced by dsRNA sensors limits diabetes induced by viral infection, and provides evidence that these proteins can be protective as well as pathogenic.

Introduction

Innate immune responses to viruses depend on molecular sensors that promptly detect viral products and trigger the secretion of type I interferons, i.e. IFN-β and IFN-α (Takeuchi and Akira 2007). Two types of sensors detect double-stranded (ds) RNA generated during infection with RNA viruses: Toll-like receptor (TLR) 3 and RIG-I-like receptors (RLR). TLR3 senses the dsRNAs that reach the endosomal compartment due to the phagocytosis of virally infected cells(Kawai and Akira). RLRs include two IFN-inducible helicases, MDA5 and RIG-I, which sense the dsRNAs that are generated in the cytoplasm during viral replication. MDA5 specializes in the detection of picornaviruses, whereas RIG-I senses most of the other RNA viruses(Yoneyama and Fujita 2009). These viral specificities depend on the ability of MDA5 and RIG-I to detect RNA molecules with different lengths, structures, and 5' caps (Cui, Eisenacher et al. 2008; Kato, Takeuchi et al. 2008; Saito and Gale 2008; Takahasi, Kumeta et al. 2009).

TLR3 deliver its intracellular signal through the adaptor TRIF, activating the transcription factor IRF3, which induces IFN-β production(Yamamoto, Sato et al. 2002). RIG-I and MDA5 signal through another adaptor, IPS-1, that activates IRF3 and IRF7, inducing both IFN-β and IFN-α production(Kawai, Takahashi et al. 2005; Meylan, Curran et al. 2005; Seth, Sun et al. 2005; Xu, Wang et al. 2005). A third RLR, Lgp2, detects dsRNA but does not contain signaling domains and is thought to positively regulate MDA5 and negatively regulate RIG-I (Satoh, Kato et al.; Rothenfusser, Goutagny et al. 2005; Venkataraman, Valdes et al. 2007). Type I IFNs induce an antiviral state in uninfected cells and promote apoptosis of infected cells, limiting viral replication

and spreading(Garcia-Sastre and Biron 2006). Moreover, type I IFNs promote subsequent NK cell, T cell and B cell responses(Stetson and Medzhitov 2006), facilitating complete viral clearance.

Type I diabetes (T1DM) is an autoimmune disease caused by selective destruction of β-cells of the endocrine pancreas by autoreactive T cells. Although predisposing genetic factors, particularly MHC class II, play a predominant role in the pathogenesis of T1DM, clinical (Yoon, Austin et al. 1979; Hyoty, Hiltunen et al. 1995; Andreoletti, Hober et al. 1997; Ylipaasto, Klingel et al. 2004) and experimental (Craighead and McLane 1968; Yoon, McClintock et al. 1980; Guberski, Thomas et al. 1991)studies have suggested that viral infections may contribute to T1DM, particularly infections by RNA viruses such as Coxsackie B4 and enteroviruses. These viruses may induce T1DM by causing β-cell damage and subsequent release of autoantigens that induce and/or trigger autoreactive T cells(Horwitz, Ilic et al. 2002). Additionally, excessive type I IFN and cytokine response to viral infection can activate and attract pre-existing autoreactive T cells that have escaped thymic selection (von Herrath 2009). Consistent with a diabetogenic role of type I IFN response to viruses, genetic studies have recently showed that resistance to T1DM is highly associated with MDA5 polymorphisms that reduce response to dsRNA (Nejentsev, Walker et al. 2009; Shigemoto, Kageyama et al. 2009). Additional studies demonstrated that TLR3 activation by synthetic dsRNA precipitate disease in mouse models of T1D (Wen, Peng et al. 2004; Lang, Recher et al. 2005). Altogether, these studies suggest that RNA sensors may contribute to the incidence T1DM.

In this study we addressed the relative contributions of MDA5 and TLR3 in host response to picornavirus infection and diabetes in the model of encephalomyocarditis virus strain D (EMCV-D). EMCV-D is picornavirus family member that has preferential tropism for pancreatic β-cells, and can induce diabetes in selective mice strains such as DBA/2(Gaines, Kayes et al. 1986; Cerutis, Bruner et al. 1989). EMCV-D, like other EMCV strains, also induces myocarditis. The role of MDA5 and TLR3 in EMCV infection has been matter of debate. Initial studies indicated that MDA5 is essential for protection from EMCV infection, while TLR3 was dispensable (Gitlin, Barchet et al. 2006; Kato, Takeuchi et al. 2006); however, a subsequent study suggested that TLR3 provides protection from EMCV-induced myocarditis (Hardarson, Baker et al. 2007). By analyzing EMCV-D infection in $MDA5^{-/-}$, $TLR3^{-/-}$ and $MDA5^{-/-}TLR3^{-/-}$ double knockout (DKO) mice we found that MDA5 and TLR3 have different impacts in the control of viral infection and tissue damage in heart and pancreas. EMCV-D infection caused mainly heart damage in $MDA5^{-/-}$ mice and diabetes in $TLR3^{-/-}$ mice. Remarkably, diabetes was due to virus-induced β-cell damage rather than T cell-mediated autoimmunity, and depended on the impaired capacity of hematopoietic cells, especially DC, to induce an early IFN-β response that restricts β-cell infection. These results indicate that MDA5 and TLR3 have different impacts on anti-viral responses in distinct organs because they have different cellular distribution and mediate type I IFN responses of different magnitude and kinetics. Moreover, RNA sensors are not always diabetogenic; in fact their impact depends on the pathogenetic mechanism of diabetes. While in autoimmune diabetes engagement of RNA sensors by viruses may unveil or

trigger a latent autoimmune condition, in diabetes caused by direct viral damage of β-cells, a normal function of RNA sensors in innate immune cells is essential for protection.

Results

Both MDA5 and TLR3 contribute to survival from EMCV-D infection

To evaluate the roles of MDA5 and TLR3 in the response to EMCV-D, we infected WT, MDA5$^{-/-}$, TLR3$^{-/-}$ and DKO mice with EMCV-D. All mice were on a C57BL/6 background. Similar to previous reports, WT C57Bl/6 mice survived EMCV-D infection; however, MDA5$^{-/-}$ mice were highly susceptible and died on day 5 post-infection (PI) (Fig. 1). TLR3$^{-/-}$ mice were also more susceptible to EMCV-D than WT mice, but less susceptible than MDA5$^{-/-}$ mice, dying on average at day 20 PI with a proportion of mice surviving infection. The contribution of TLR3 to anti-EMCV-D defense was further confirmed by the more severe sensitivity of DKO mice to EMCV-D compared to MDA5$^{-/-}$ mice. DKO mice died at day 2 PI (Fig. 1), or at day 3 PI with an extremely low inoculum of 1 PFU/mouse (data not shown). These data demonstrate that both MDA5 and TLR3 contribute to the containment of EMCV-D infection in vivo although MDA5 appears to have a predominant role over TLR3.

Infection of MDA5$^{-/-}$ and TLR3$^{-/-}$ mice with EMCV-D results in different heart and pancreatic diseases

Previous studies have implicated MDA5 (Kato, Takeuchi et al. 2006) and TLR3 (Hardarson, Baker et al. 2007) in protection from EMCV-induced viral myocarditis using different strains of EMCV. To test the relative contributions of MDA5 and TLR3 to the protection of heart from EMCV-D, we measured serum troponin in WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice after infection, which reflects the extent of cardiac damage. Similar to previous findings (Kato, Takeuchi et al. 2006), MDA5$^{-/-}$ mice had highly elevated

troponin levels by day 4 (Fig. 2A). Correspondingly, the hearts of MDA5$^{-/-}$ mice showed high EMCV-D titers, marked immunoreactivity for EMCV-D antigens and histological evidence of myocarditis at this time-point (Fig. 2B and Fig. S1). TLR3$^{-/-}$ mice showed milder signs of heart infection than MDA5$^{-/-}$ mice. Troponin levels were normal with the exception of few TLR3$^{-/-}$ mice with elevated troponin at day 7. Heart viral titers were slightly higher than those of WT mice at all time points, and histopathology was modest (Fig. 2 and Fig. S1). The contribution of TLR3 to anti-EMCV-D response in the heart became more evident in the DKO mice, which showed earlier increase in troponin levels, higher levels of EMCV replication in the heart and stronger immunostaining for EMCV antigens than MDA5$^{-/-}$ mice. These results indicate that MDA5 plays a dominant role in the protection from EMCV-D-induced myocarditis, whereas TLR3 contribution is secondary.

EMCV-D is also known to infect the pancreas, particularly the β-cells of the pancreatic islets, leading to the development of diabetes in susceptible mouse strains like DBA/2 (Gaines, Kayes et al. 1986). C57Bl/6 mice are normally resistant to EMCV-induced diabetes, and accordingly WT mice maintained normal serum glucose levels after EMCV-D infection (Fig. 3A). Additionally, WT mice showed no increase of the pancreatic enzymes amylase or lipase in the serum, suggesting lack of significant damage of exocrine pancreas (Fig. 3B). EMCV-D titers in the pancreas were increased at day 1-3 PI but were cleared within a week. Deficiency of MDA5 was not as harmful for the pancreas as it was for the heart. MDA5$^{-/-}$ mice showed no detectable hyperglycemia and a slight increase in serum amylase and lipase at day 2 and 4 PI (Fig. 3 and data not shown).

Viral titers were similar to those of WT animals at day 1 PI, but increased at day 2 PI and remained elevated until day 4-5 PI, when most of the mice died (Fig. 3). Thus, MDA5 is essential for EMCV-D control at late rather than early timepoints, most likely because MDA5 expression must be induced by type I IFN. Similar to MDA5$^{-/-}$ mice, TLR3$^{-/-}$ mice showed minimal increase in amylase or lipase in the serum (Fig. 3). Surprisingly, however, TLR3$^{-/-}$ mice developed marked hyperglycemia by day 5 (Fig. 3A) and by day 7 had almost undetectable islet mass as assessed by hematoxylin-eosin staining (Fig. S2). TLR3$^{-/-}$ mice showed higher viral titers in the pancreas at days 1 and 2 PI compared to WT and MDA5$^{-/-}$ mice, but normal clearance of EMCV-D by day 7. Thus, TLR3 plays as role in the early control of EMCV-D infection, which appears to be essential for the protection of the endocrine pancreas.

Finally, examination of pancreas of DKO mice demonstrated an impressive increase of serum levels of amylase and lipase as compared to WT mice, which was paralleled by a large increase in viral titers and extensive destruction of pancreatic architecture in both the exocrine and endocrine tissue on day 2 (Fig. 3A and S2). This virus-induced pancreatitis is likely to be the major cause of the death of DKO mice at day 2 PI, given the comparatively moderate defect in the heart at this timepoint. Despite a severe EMCV infection of the islets, DKO mice did not develop detectable hyperglycemia, perhaps due to the concomitant pancreatitis that is rapidly followed by death. Altogether, these results reveal that MDA5 and TLR3 play partially redundant roles in EMCV-D control in the exocrine pancreas, such that only DKO mice develop an overt pancreatitis. However, TLR3 is essential to prevent damage of pancreatic islets, perhaps because of its unique

role in the early control of EMCV-D infection. MDA5 does control EMCV-D infection at later timepoints, but it may be too late to effectively protect endocrine pancreas. Overall, the development of three completely different disease outcomes after infection with the same dose of EMCV-D demonstrates that MDA5 and TLR3 have different impacts on anti-viral responses in distinct organs.

EMCV-D-induced β-cell damage in TLR3$^{-/-}$ mice is T cell-independent

T1D is the result of the autoimmune destruction of pancreatic islets by the autoreactive T cells. EMCV-D-induced diabetes in TLR3$^{-/-}$ mice, however, was not paralleled by an obvious T cell infiltration of pancreatic islets at any time after infection (data not shown), making the involvement of an autoimmune mechanism unlikely. At early time points, pancreatic islets of TLR3$^{-/-}$ mice showed marked staining for viral antigens compared to WT mice supporting the viral titer data (Fig. 4A). Additionally, islets of TLR3$^{-/-}$ mice showed marked apoptosis as assessed by staining for caspase 3 (Fig. 4B), and a robust infiltrate of myeloid cells that was consistent with the presence of monocytes/macrophages and/or dendritic cells recruited from either the blood or local tissue (Fig. 4C). In contrast, WT mice showed only a limited increase of myeloid cells mostly located around but not within the pancreatic islets. Altogether, the extensive viral infiltration and apoptosis in the pancreatic islets of TLR3$^{-/-}$ mice suggest that diabetes is due to failure to control local infection.

TLR3 must be present in the hematopoietic compartment to prevent EMCV-D-induced diabetes

We next asked whether TLR3 and MDA5 play their protective roles against EMCV-D infection by acting directly in the infected tissues or in the innate immune system. To test this hypothesis, we generated bone marrow chimeras between WT and MDA5$^{-/-}$ mice as well as between WT and TLR3$^{-/-}$ mice and measured survival and blood glucose after EMCV-D infection. Survival curves showed that chimeras containing MDA5$^{-/-}$ hematopoietic cells and WT stroma (MDA5->WT) were resistant to EMCV-D infection, while WT->MDA5$^{-/-}$ chimeras were highly susceptible to infection similar to MDA5$^{-/-}$ mice. Conversely, TLR3$^{-/-}$->WT chimeras were more sensitive to EMCV-D infection than WT->TLR3$^{-/-}$ chimeras, although not as susceptible as WT->MDA5$^{-/-}$ chimeras (Fig. 5A). These results demonstrate that the overall resistance to EMCV-D infection is largely dependent on MDA5 function in radio-resistant stromal tissues, while TLR3 contributes to the anti-EMCV-D defense in part, especially in hematopoietic cells. However, the analysis of blood glucose after EMCV-D infection revealed a quite different picture of MDA5 and TLR3 impact on the protection of pancreatic islets. TLR3$^{-/-}$->WT chimeras developed diabetes after EMCV-D infection, whereas WT->TLR3$^{-/-}$ chimeras as well as MDA5->WT and WT->MDA5$^{-/-}$ chimeras were protected from diabetes (Fig. 5B). Thus, protection of endocrine pancreas from EMCV-D infection and diabetes is largely dependent on TLR3 expression in the radio-sensitive hematopoietic compartment.

Early IFN-β response through TLR3-IRF3 prevents EMCV-D-induced diabetes

The selective impact of TLR3 on the control of EMCV-D-induced diabetes indicated that TLR3 has a special role in the response to the infection. We noticed that TLR3$^{-/-}$ mice exhibit higher viral titers than WT and MDA5$^{-/-}$ mice at early timepoints PI (see Fig. 3C).

In contrast, MDA5$^{-/-}$ mice showed higher viral titers than WT and TLR3$^{-/-}$ mice at late timepoints (see Figs. 2B and 3C). Thus, we hypothesized that TLR3 may prevent EMCV-D-induced diabetes through the induction of type I IFN early after infection. To test this hypothesis we measured type I IFN in the serum of infected mice using a sensitive bioassay. In the MDA5$^{-/-}$ mice type I IFN was severely reduced as compared to WT mice; the residual type I IFN detected in MDA5$^{-/-}$ mice was dependent on TLR3, since no type I IFN was observed in the serum of DKO mice (Fig. 6A). Thus, the quantitative impact of MDA5 and TLR3 on type I IFN response is different, MDA5 being responsible for most of it. MDA5 and TLR3 also contributed differently to the kinetics of type I IFN response. While type I IFN was detected as early as 15 hours after EMCV-D infection in the serum of WT and MDA5$^{-/-}$ mice, in TLR3$^{-/-}$ mice was detected only later, suggesting that TLR3 induces an early IFN response to EMCV-D that is essential to protect β-cells.

TLR3-mediated signals activate the transcription factor IRF3, which binds the IFN-β promoter inducing IFNβ production(Honda, Takaoka et al. 2006). If TLR3-mediated IFN-β production is necessary to protect from EMCV-D-induced diabetes, one would expect that mice lacking IRF3 or IFN-β would also develop diabetes after EMCV-D infection. To test this, we infected IRF3$^{-/-}$ and IFN-β$^{-/-}$ mice with EMCV-D and measured blood glucose. Both IRF3$^{-/-}$ and IFN-β$^{-/-}$ mice developed hyperglycemia after infection. However, both knockout mice were much more susceptible to EMCV-D and died earlier than TLR3$^{-/-}$ mice (Fig. 6B,C), most likely because a global defect of IRF3 and IFN-β impairs more anti-viral pathways than that triggered by TLR3 only. Since TLR3 must be expressed in the hematopoietic compartment to protect from diabetes, we

hypothesized that a defect of IRF3 and IFN-β limited to the hematopoietic compartment may be sufficient to induce diabetes. To test this, we created IRF3$^{-/-}$->WT and IFN-β$^{-/-}$->WT bone marrow chimeras, which were then infected with EMCV-D. Both chimeras developed hyperglycemia after infection, and had a mild survival defect similar to the TLR3$^{-/-}$ chimeras (Fig. 6D,E). This data strongly supports the hypothesis that early IFN-β production by hematopoietic cells through the TLR3-IRF3 pathway is critical for protection from EMCV-D-induced diabetes.

Dendritic cells are necessary for protection from EMCV-D-induced diabetes

We finally investigated which hematopoietic cell types are implicated in the TLR3-mediated control of EMCV-D-induced diabetes. We had previously observed the accumulation of myeloid cells around the islets of WT mice after EMCV-D infection (see Fig. 5). Interestingly, these cells expressed TLR3 (Fig. 7A), suggesting that they may be responsible for the early type I IFN secretion that protects β-cells from EMCV-D. We asked whether protective myeloid cells are macrophages or DC. To directly assess the involvement of macrophages in preventing virus-induced diabetes, we infected WT mice with EMCV-D after the injection of clodronate-containing liposomes, which deplete macrophages(Van Rooijen 1989). Macrophage depletion increased susceptibility to EMCV-D infection as reflected by survival, but had no effect on diabetes induction in WT mice, suggesting that macrophages were dispensable for the protection of β-cells (Fig. 7B,C). To assess the involvement of DC in preventing virus-induced diabetes, we infected with EMCV-D CD11c-diphtheria toxin receptor (CD11c-DTR) transgenic mice, which can be depleted of DC by injection of diphtheria toxin. DC-depleted mice were

not only highly susceptible to EMCV-D infection, but also developed diabetes, suggesting that DCs are critical for protection of β-cells from EMCV-D (Fig. 7D,E). Because DC activate cytotoxic lymphocytes such as NK cells and CD8 T cells through secretion of type I IFN, inflammatory cytokines and through cell-cell interaction, we asked whether cytotoxic cellular responses contribute to EMCV-D clearance and protection from diabetes. However, antibody-mediated depletion of NK cells or CD8 T cells had no effect on the development of diabetes or survival of EMCV-D infection in WT mice (data not shown). We conclude that DC-mediated protection of β-cells from EMCV-D is independent of priming and/or activation of cytotoxic lymphocytes.

Discussion

It is common knowledge that both RLR and TLR3 mediate responses to dsRNA and, possibly, RNA viruses. However, while RLR have been convincingly shown to be major players in innate responses to viruses in vivo, the literature is still contradictory as to the in vivo role of TLR3. Initial studies did not detect any role of TLR3 in anti-viral innate defense to EMCV (Kato, Takeuchi et al. 2006). However, more recent studies have revealed that TLR3 does in fact contribute to EMCV anti-viral responses (Hardarson, Baker et al. 2007). In this work we have demonstrated that both MDA5 and TLR3 provide protection from EMCV-D infection, but their contributions are different in different tissues. In the heart, MDA5 plays the major role in determining resistance, in the endocrine pancreas TLR3 is more prominent, in the exocrine pancreas MDA5 and TLR3 are both important. Moreover, our bone marrow chimera studies demonstrated that MDA5 and TLR3 act predominantly in stromal and hematopoietic compartments, respectively. Thus, the differential roles of MDA5 and TLR3 are related to the distinct cellular distribution of these sensors, as well as the tissue tropism of the viruses.

The type I IFN response to RNA viruses *in vivo* has been mainly attributed to RLRs. In this study we confirmed that MDA5 induces the bulk of type I IFN production in response to EMCV-D infection. However, using a sensitive bioassay for type I IFN, we found that TLR3-mediated type I IFN is small but rapid. Thus, MDA5 and TLR3 induce type I IFN responses with different magnitude and kinetics. The reduced magnitude of type I IFN responses in the $MDA5^{-/-}$ mice explains the major survival defect, while $TLR3^{-/-}$ mice show a comparatively mild defect. On the other hand, the delayed kinetics

of the type I IFN response in TLR3$^{-/-}$ mice is responsible for the lack of viral control in endocrine pancreas and diabetes, highlighting the importance of this early response. Another study recently demonstrated that TLR2 is critical for early IFN production in response to several DNA viruses(Barbalat, Lau et al. 2009). In that study, TLR2 expression in inflammatory monocytes was particularly critical for the response. In EMCV infection, dendritic cells were critical for early protection from diabetes. There are several known subsets of CD11c+ DCs which could play a role and it remains to be seen precisely which subset is responsible for this phenotype. Because CD11c is expressed on a variety of cells of the myeloid lineage, we also cannot completely exclude a contribution from monocytes and inflammatory macrophages. Together, these results suggest that early TLR-mediated production of IFN by myeloid cells is an important component of the anti-viral response.

Because of different cellular distributions as well as extent and timing of anti-viral responses, dsRNA sensors have different impact in distinct organs. This was seen in our study contrasting the anti-viral response seen in the heart and the pancreas between the MDA5-/- and TLR3-/- animals. This is a novel and important finding, which prompts the investigation of viral sensors in individual organs and cell types during *in vivo* infections. It is likely that anti-viral response in each organ relies on different dsRNA sensors and is highly dependent on the tropism of the virus. Moreover, dsRNA and other TLR/RLR ligands are being increasingly used as adjuvants in vaccines with the idea that stimulation of the innate immune response will lead to increased and specific adaptive immune responses. However, this work has revealed that the timing and level of expression of the

sensors for these adjuvants varies during the antiviral response. It is important that we understand the role of both the TLR and RLR systems in the immune response in different organs and cell types to best take advantage of this strategy. It may be possible to target these adjuvants to specific cells to maximize the desired immune response and minimize harmful effects of cytokine production.

Although we did not observe the development of autoimmune diabetes after EMCV-D infection, our finding that MDA5$^{-/-}$ mice were resistant to EMCV-D-induced diabetes is intriguing. Recent association studies have implicated the MDA5-encoding gene Ifih1 in susceptibility to human type I diabetes. In those studies, rare human Ifih1 alleles which were shown to be defective for IFN production were seen to be protective, resulting in failure of individuals with these alleles to develop T1D(Nejentsev, Walker et al. 2009). Similarly, our results indicate that mice lacking MDA5 appear to be protected from diabetes after EMCV-D infection, even in the presence of detectable EMCV-D in the pancreatic islets. It is possible that the diabetes phenotype in MDA5$^{-/-}$ mice is not seen as a result of the severe myocarditis the develops in these animals, however, the presence of hyperglycemia in IRF3$^{-/-}$ and IFNβ$^{-/-}$ animals, which have similar survival phenotypes and also develop myocarditis suggests that deficiency of MDA5 may be generally protective within pancreatic islets. This may be due to the detrimental effects of IFN on β-cell survival or an IFN-independent effect of MDA5 on cell survival, which has recently been reported in studies of melanoma(Besch, Poeck et al. 2009; Tormo, Checinska et al. 2009).

Despite strong clinical correlation between viruses and diabetes as well as several experimental models in which virus infection results in diabetes, the underlying mechanism of how the human disease is initiated and whether viruses are directly involved remains unclear. A recent report suggested that human diabetes can be initiated by different mechanisms(Dotta, Censini et al. 2007). This study examined pancreatic tissue of recent onset diabetic patients and reported two distinct histological patterns. Half of the patients were noted to have T cell infiltrates within the islets, similar to the autoimmune NOD mouse model. However, islets from other patients displayed coxsackievirus B4 antigen and contained NK and myeloid cell infiltrates, but no T cells. This suggests that type I diabetes may be initiated by either an autoimmune mechanism or by direct virus infection. Likewise, some studies of EMCV-D-induced diabetes have suggested that virus infection initiated an autoimmune T cell response, however, other studies have reported no T cell infiltrate in the islets. Our results indicate that EMCV-induced diabetes in TLR3-/- mice does not involve autoimmune T cells, instead diabetes results from direct viral infection of β-cells. However, this study tested mice on the C57Bl/6 background, which are resistant to autoimmunity, and it is possible that studies of EMCV on a genetic background susceptible to autoimmunity would have a different outcome. It is possible that polymorphisms in dsRNA sensors play a role in both autoimmune and virus-induced diabetes. MDA5 polymorphisms have clearly been implicated in diabetes. In addition, a recent study has implicated non-functional TLR3 alleles with human myocarditis(Gorbea, Makar et al.), suggesting that inability to control virus infection can lead to human disease. In this context, polymorphisms leading to excessive IFN production by MDA5 may lead to autoimmune diabetes while non-

functional alleles provide protection. In contrast, nonfunctional TLR3 alleles may lead to increased susceptibility to viral infection and virus-induced diabetes. Further investigation is needed to determine how these sensors function to both limit infection and trigger autoimmunity.

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Experimental Procedures

Mice and infections: MDA5-/-(Gitlin, Barchet et al. 2006), TLR3-/-(Alexopoulou, Holt et al. 2001), DKO(McCartney, Vermi et al. 2009), CD11c-DTR(Jung, Unutmaz et al. 2002), IRF3-/-(Sato, Suemori et al. 2000), and IFNβ-/- (Takaoka, Mitani et al. 2000)mice have been described previously. All mice have been backcrossed to the C57Bl/6 background. Age-matched control mice were purchased from Jackson ImmunoResearch Laboratories, Inc. All mice used in these experiments were male. EMCV-D was obtained from Dr. John Corbett and was passaged in L929 cells. Infections were done at 1000 PFU per mouse by intraperitoneal injection.

BM chimeras: Recipient mice were γ irradiated with 1000 rad. After an overnight rest, mice were reconstituted with 5 x 10^6 BM cells per mouse that had been harvested from the femurs and tibias of age-matched donors. After 6 weeks, chimeras were used for infections.

Virus titers: Organs from infected animals were frozen at -80°C immediately after harvest. Subsequently, organs were suspended in 1ml DMEM and homogenized by bead beating with 1.0mm beads (BioSpec Products, Inc). Organ homogenates were diluted 1:10 in DMEM and tested for viral titers in a plaque assay. L929 cells were seeded in 6 well plates and infected with 10-fold dilutions of tissue homogenate in duplicate. After 1 hour incubation, the inoculum was removed and wells were overlaid with complete DMEM media containing 1.5% Seaplague agarose (Cambridge Biosciences). After 48 hours, a second overlay was added containing 1.5% SeaKem agarose (Cambridge

Biosciences) and 0.01% neutral red (Sigma)in complete DMEM media. After 8 hours, plagues were then visualized.

Blood/Serum measurements: Blood was taken at various time-points after infection and centrifuged in serum separator tubes (Becton, Dickinson, and Co.) to isolate serum, which was kept at -80 until use. Serum levels of troponin (Life Diagnostics, Inc) were determined by ELISA. Blood glucose was measured by Ascencia Elite glucometer (Fisher) and care was taken to measure glucose levels at a consistent time of the day to minimize variation. Serum levels of inflammatory cytokines were measured by Bioplex assay using the mouse-23plex kit (Bio-rad) on a Luminex machine.

IFN bioassay: IFN levels in the serum were determined by bioassay (Newby, Pekosz J Virol 2007). Briefly, L929 cells were incubated for 24 hours with standards or samples, then infected for 10 hours with VSV-GFP. Cells were fixed and the percent of GFP+ cells were used to calculate IFN levels in the linear range of the standard curve. This method was found to give similar results to IFNα ELISA (PBL), but was more sensitive for low IFN values.

Liposome preparation and application: Multilamellar liposomes containing clodronic acid disodium salt (Cl_2MDP) (Sigma) in PBS or control liposomes without clodronate were prepared as described by van Rooijen(Van Rooijen 1989; Calderon, Suri et al. 2006). Briefly, 86mg of phosphatidyl choline (Sigma) and 8 mg of cholesterol (Sigma) were dissolved in 10 ml of chloroform and dried in an evaporator to form a lipid film.

The lipid was dispersed in 10 ml of PBS for control liposomes or 10 ml of Cl_2MDP solution (2.5g into 10ml DI water, adjusted to pH 7.3) for clodronate liposomes. The preparations were kept for 2 hours at room temperature, sonicated for 3 minutes, and incubated overnight at 4ºC. Liposomes were then purified and washed by centrifugation and resuspended in 4 ml of sterile PBS. A volume of 0.1 ml of liposome suspension for every 10 g of body weight was injected i.v. for 5 consecutive days starting 48 hours before EMCV infection.

Histology: Organs were harvested at indicated time-points after infection and tissue sections prepared by either formalin-fixation paraffin-embedding or frozen in OCT reagent. For EMCVpol, iba-2, activate caspase-3, synaptophysin, and H&E staining, sections were deparaffinized, rehydrated, and stained with primary antibody at 1:1000 and then secondary ab at 1:2000. For TLR3 staining, frozen sections were stained with primary antibody at 1:500 and secondary antibody at 1:2000.

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Figure Legends

Figure 3.1: Both MDA5 and TLR3 protect from EMCV-D infection.

WT, MDA5-/-, TLR3-/-, and DKO mice were infected with 1000 PFU EMCV-D i.p. and monitored for survival (n=20). WT mice survived infection, MDA5-/- mice died at average day 5, TLR3-/- died at average day 20, and DKO mice died at average day 2.

Figure 3.2: MDA5 is critical for protection in the heart.

WT, MDA5-/-, TLR3-/-, and DKO mice were infected with 1000 PFU EMCV-D i.p. Serum and heart tissue were harvested at days 2, 4, and 7 from surviving mice and were evaluated for troponin by ELISA (n>5) (A) and virus titer by plaque assay (n>6 for each time point) (B). Statistical significance was calculated by two-tailed student's t-test or Mann-Whitney test for non-Gaussian data and is indicated by *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

Figure 3.3: Both MDA5 and TLR3 control infection in the pancreas.

WT and KO mice were infected with EMCV-D as above. Serum samples were evaluated for blood glucose (n>8) (A) or amylase and lipase (n>4) (B) at the indicated times post-infection. Pancreas was harvested at the indicated times and viral titers were determined by plaque assay (C) (n>6). Statistical significance was calculated by two-tailed student's t-test and is indicated by *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

Figure 3.4: Differential roles of MDA5 and TLR3 in the pancreas.

Pancreas tissue samples from WT or knockout mice were harvested at day 2 or 4 as indicated, fixed in formalin, and paraffin embedded. Tissue sections were stained using anti-EMCVpol (A) or anti-iba-2 (B) by immunohistochemistry (n>3). Alternatively, sections were stained for anti-iba-2 (brown) and co-stained with synaptophysin (blue) (C-a); anti-EMCVpol (brown) and synaptophysis (red) (C-b); anti-EMCVpol (brown) and anti-iba-2 (red) (C-c); or active caspase-3 (brown) and synaptophysin (blue) (C-d).

Figure 3.5: MDA5 is critical in stroma, TLR3 in hematopoietic cells for control of EMCV infection.

Bone marrow chimeras were developed between WT and MDA5-/- and WT and TLR3-/- animals. Chimeras were infected with 1000PFU EMCV-D and evaluated for survival (n>10) (A) and blood glucose (n>10) (B). Statistical significance was calculated by two-tailed student's t-test and is indicated by *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

Figure 3.6: MDA5 and TLR3 control kinetically distinct IFN responses.

Serum samples from WT, MDA5-/-, TLR3-/-, and DKO mice were harvested at various time-points after EMCV-D infection and evaluated for type I IFN production by bioassay (n>5 per time point) (A). IRF3-/- and IFNb-/- mice were infected with EMCV-D and monitored for survival (n=6) (B) and blood glucose (C). IRF3-/- and IFNb-/- chimeras were infected with EMCV-D and monitored for survival (n=6) (D) and blood glucose (n=6) (E). Statistical significance was calculated by two-tailed student's t-test and is indicated by *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

Figure 3.7: CD11c+ cells required for protection from EMCV-D-diabetes.

WT mice were treated with clodronate or PBS-containing liposomes and monitored for survival (n=8) (A) and blood glucose (n=8) (B) after EMCV-D infection. CD11c-DTR mice were treated with PBS or DT then monitored for survival (n=6) (C) and blood glucose (n=6) (D) after EMCV-D infection. To visualize TLR3+ cell infiltrates in the islets, tissue sections were made from formalin-fixed paraffin-embedded WT pancreas 12 hours after EMCV infection. These sections were stained with anti-TLR3 (brown) and synaptophysin (blue) (E). Statistical significance was calculated by two-tailed student's t-test and is indicated by *= $p<0.05$.

Supplementary Figure Legends

Supplementary figure 3.1. Extensive viral replication and pathology in MDA5-/- hearts.

Heart tissue samples from WT or knockout mice were harvested at day 2, 4, or 7 as indicated, fixed in formalin, and paraffin embedded. Tissue sections were stained by H&E and evaluated for pathology (A) or stained for EMCVpol by immunohistochemistry (B).

Supplementary figure 3.2. Pathological damage of EMCV-D infection in the pancreas.

Pancreas tissue samples from WT or knockout mice were harvested at day 2, 4, or 7 as indicated, fixed in formalin, and paraffin embedded. Tissue sections were stained by H&E and evaluated for pathology

Figure 3.1

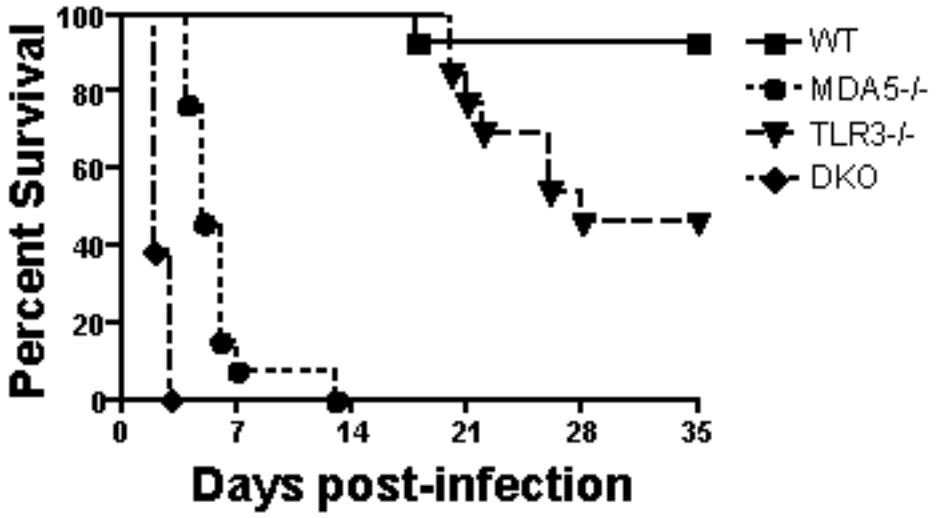

Percent Survival
100
80
60
40
20
0
0
7
14
21
28
35
Days post-infection
WT
MDA5-/-
TLR3-/-
DKO

Figure 3.2

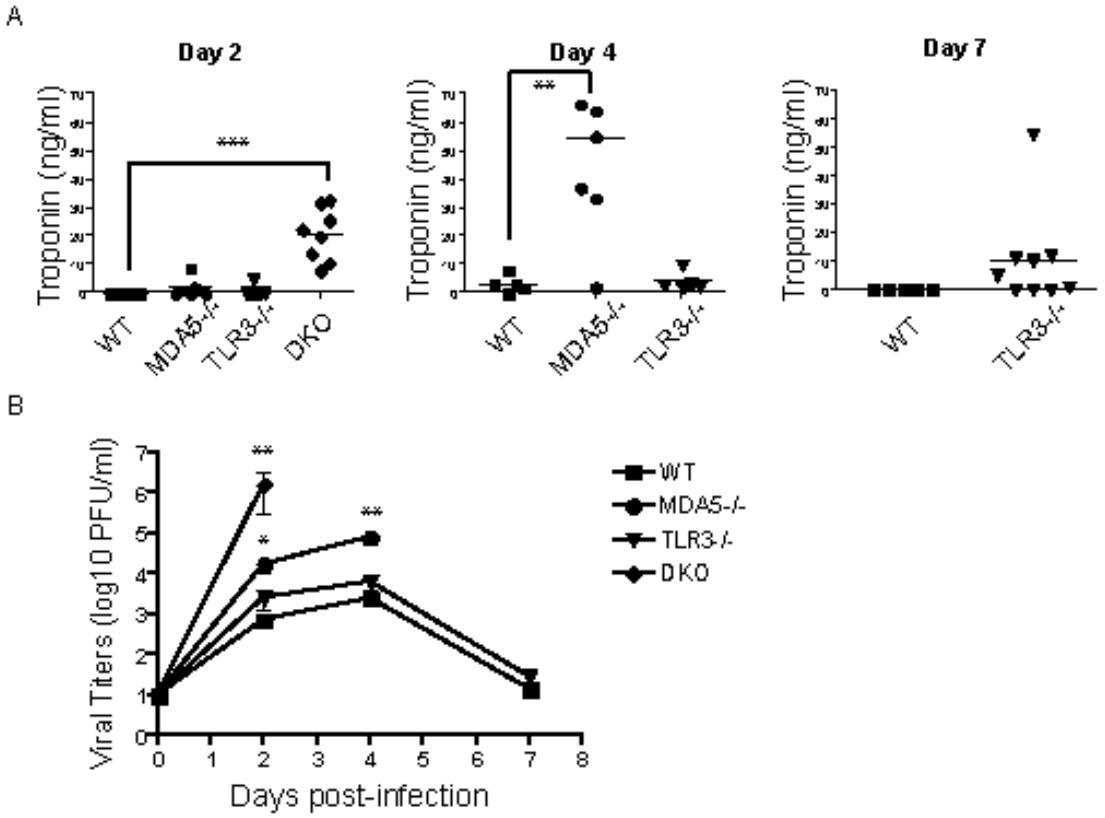

A
Day 2
Day 4
Day 7
Troponin (ng/ml)
***
**
WT
MDA5-/-
TLR3-/-
DKO
B
Viral Titers (log10 PFU/ml)
Days post-infection
WT
MDA5-/-
TLR3-/-
DKO

Figure 3.3

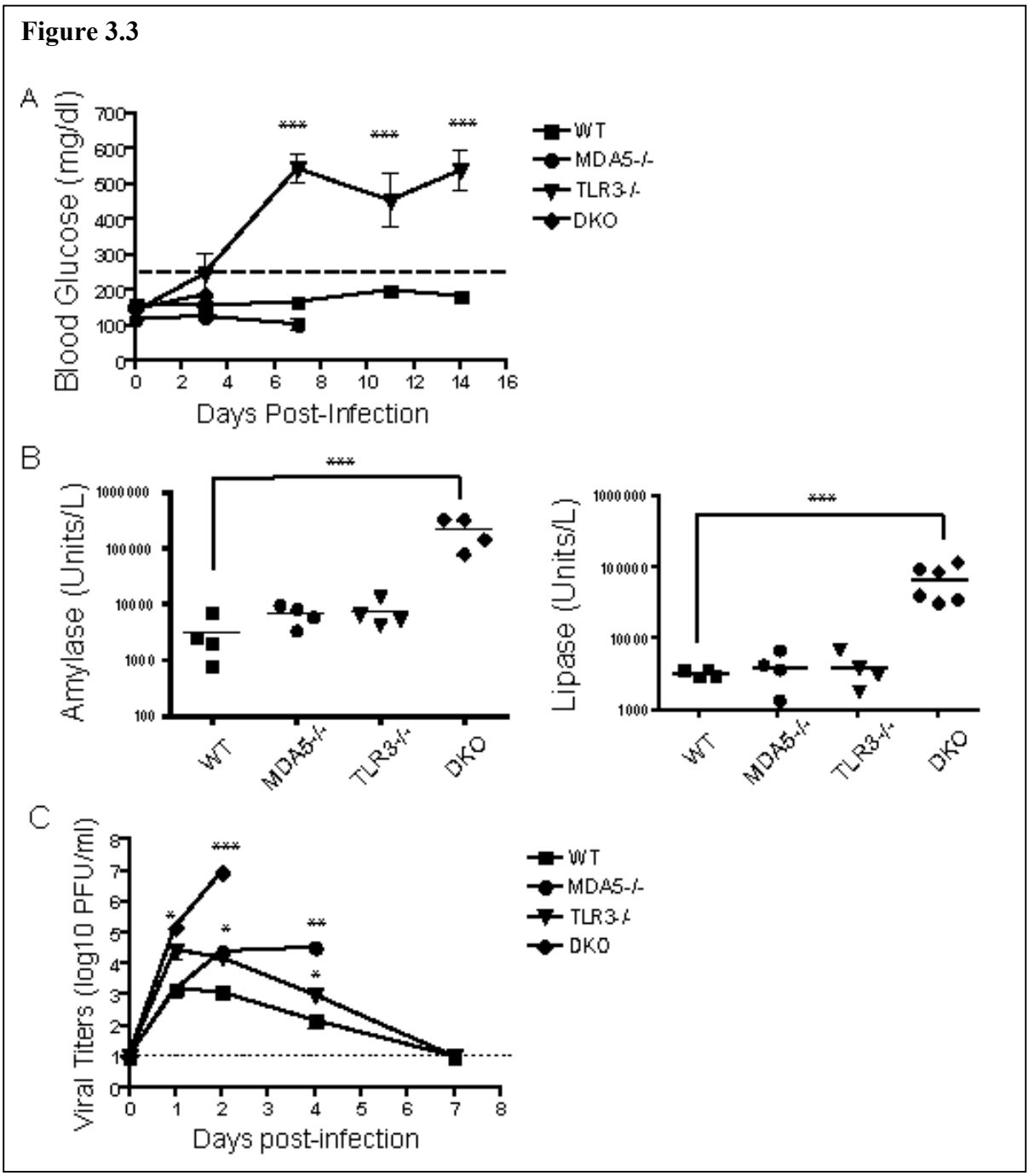

A
Blood Glucose (mg/dl)
Days Post-Infection
WT
MDA5-/-
TLR3-/-
DKO
***
B
Amylase (Units/L)
Lipase (Units/L)
WT
MDA5-/-
TLR3-/-
DKO
C
Viral Titers (log10 PFU/ml)
Days post-infection

Figure 3.4

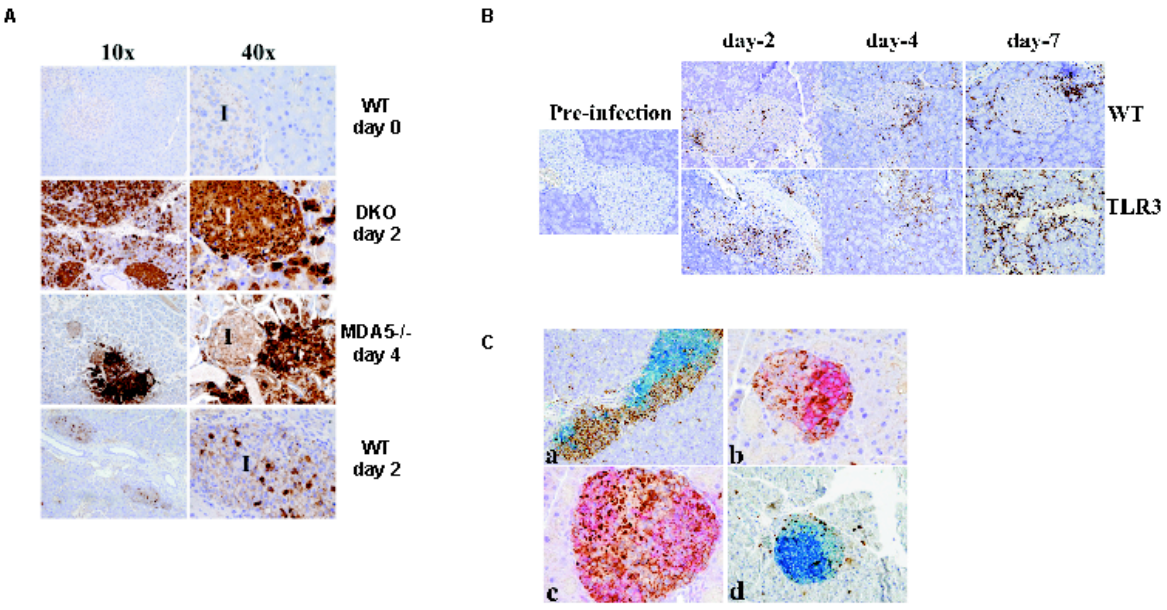

A
10x
40x
WT
day 0
DKO
day 2
MDA5-/-
day 4
WT
day 2
B
day-2
day-4
day-7
Pre-infection
WT
TLR3
C
a
b
c
d

Figure 3.5

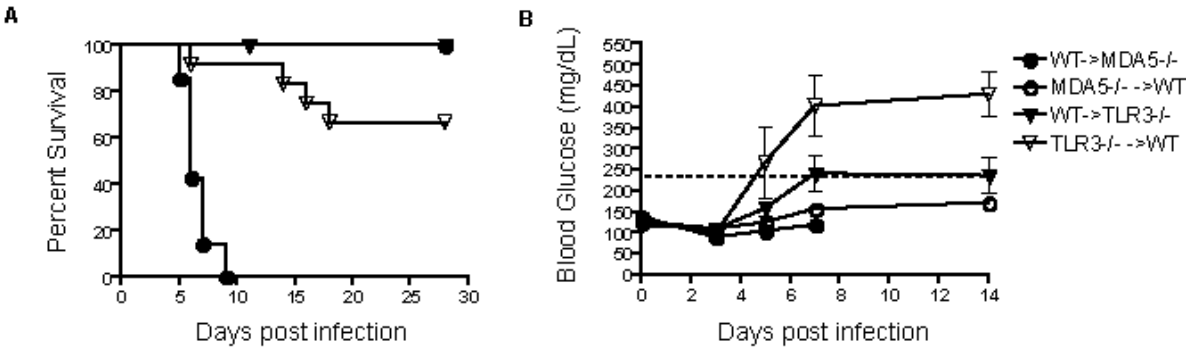

A
Percent Survival
Days post infection
B
Blood Glucose (mg/dL)
Days post infection
WT->MDA5-/-
MDA5-/- ->WT
WT->TLR3-/-
TLR3-/- ->WT

Figure 3.6

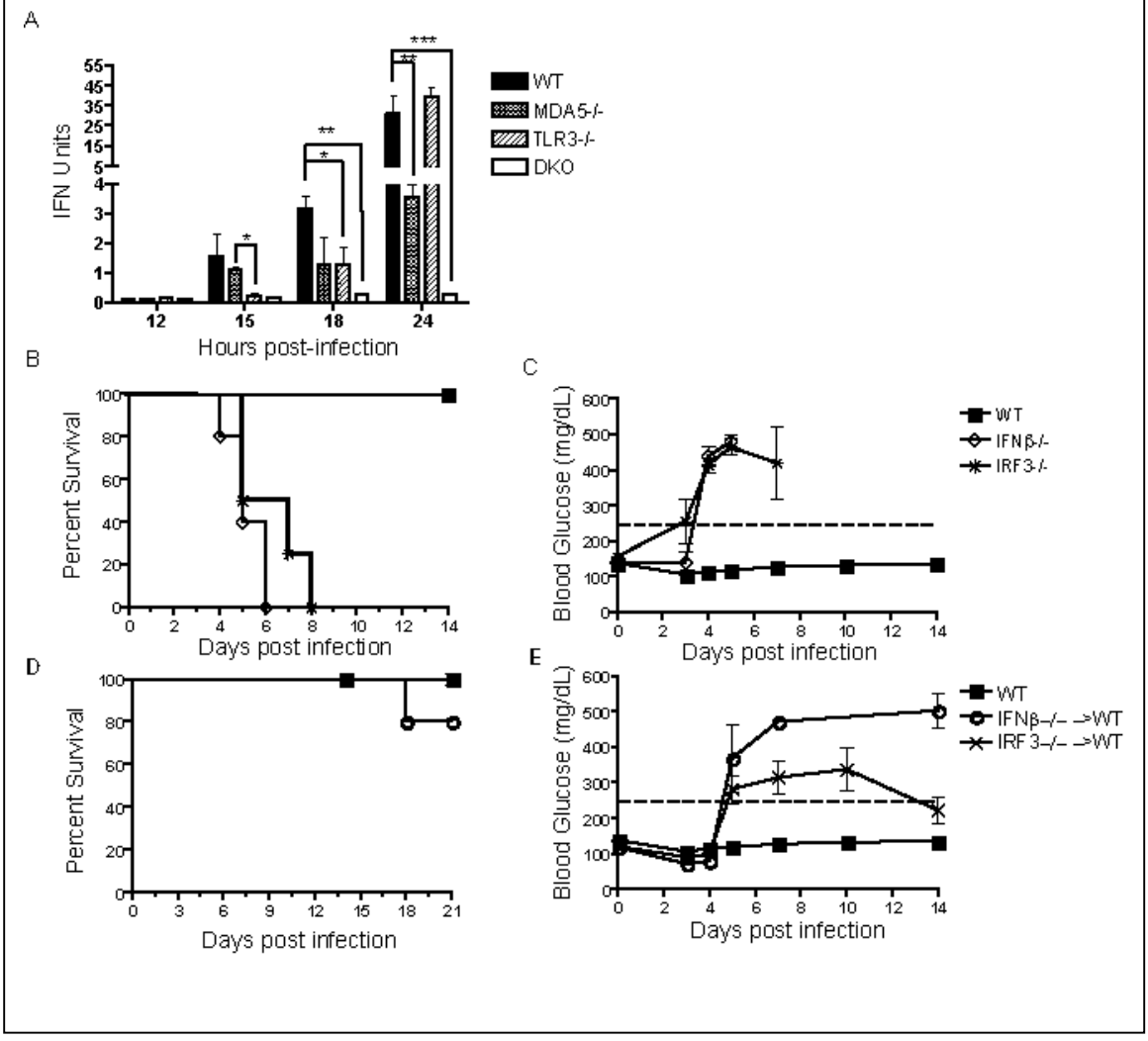
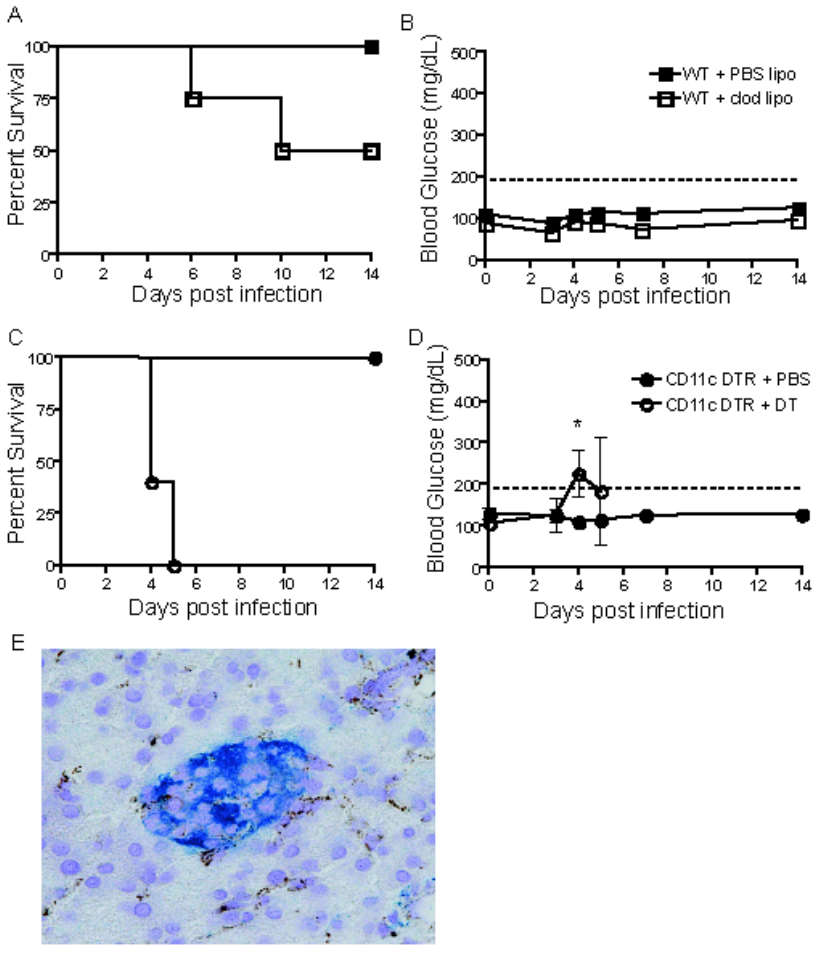
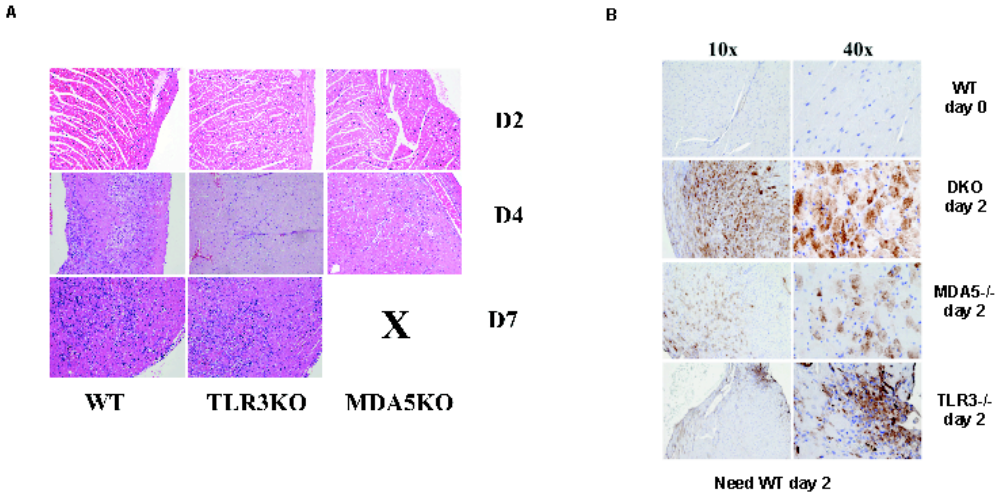

A
IFN Units
Hours post-infection
WT
MDA5-/-
TLR3-/-
DKO
B
Percent Survival
Days post infection
C
Blood Glucose (mg/dL)
Days post infection
WT
IFNβ-/-
IRF3-/-
D
Percent Survival
Days post infection
E
Blood Glucose (mg/dL)
Days post infection
WT
IFNβ-/- ->WT
IRF3-/- ->WT

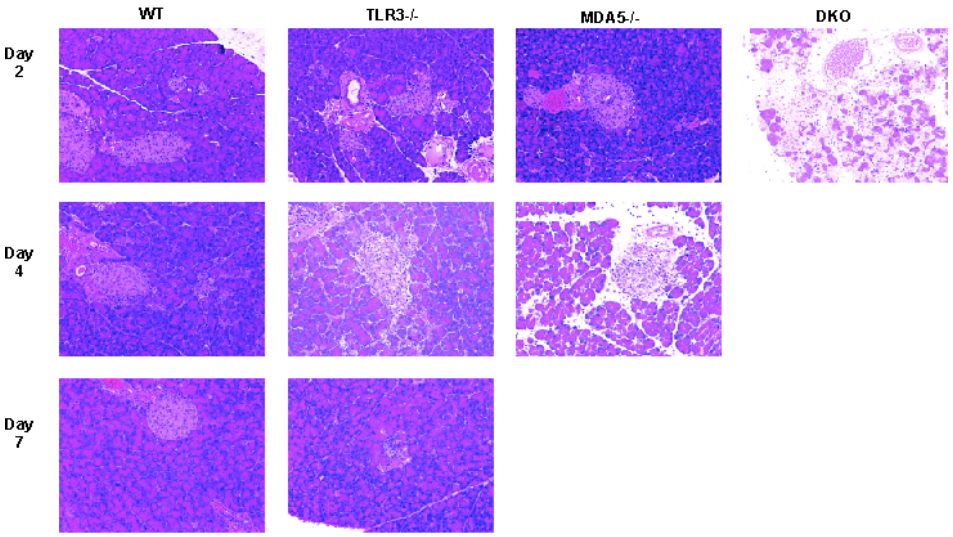
Figure 3.7


A
Percent Survival
Days post infection
B
Blood Glucose (mg/dL)
WT + PBS lipo
WT + clod lipo
Days post infection
C
Percent Survival
Days post infection
D
Blood Glucose (mg/dL)
CD11c DTR + PBS
CD11c DTR + DT
*
Days post infection
E

Supplemental Figure 3.1



Supplementary Figure 3.2



CHAPTER 4

Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells

This chapter has been published as:

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Abstract

The double stranded (ds) RNA analogue poly(I:C) is a promising adjuvant for cancer vaccines because it activates both dendritic cells (DC) and natural killer (NK) cells, concurrently promoting adaptive and innate anti-cancer responses. Poly(I:C) acts through two dsRNA sensors, Toll-like receptor 3 (TLR3) and melanoma differentiation-associated protein-5 (MDA5). Here we investigated the relative contributions of MDA5 and TLR3 to poly(I:C)-mediated NK cell activation using $MDA5^{-/-}$, $TLR3^{-/-}$ and $MDA5^{-/-}$ $TLR3^{-/-}$ mice. MDA5 was crucial for NK cell activation, whereas TLR3 had a minor impact most evident in the absence of MDA5. MDA5 and TLR3 activated NK cells indirectly through accessory cells and induced distinct stimulatory cytokines, IFN-α and IL-12 respectively. To identify the relevant accessory cells in vivo, we generated bone marrow chimeras between either wild type and $MDA5^{-/-}$ or wild type and $TLR3^{-/-}$ mice. Interestingly, multiple accessory cells were implicated, with MDA5 acting primarily in stromal cells and TLR3 predominantly in hematopoietic cells. Futhermore, poly(I:C)-mediated NK cell activation was not notably impaired in mice lacking CD8α DCs, providing further evidence that poly(I:C) acts through diverse accessory cells rather than solely through DC. These results demonstrate distinct, yet complementary roles for MDA5 and TLR3 in poly(I:C)-mediated NK cell activation.

Introduction

Microbial components play a major role in activating innate and adaptive immune responses by triggering pattern recognition receptors (Ishii et al., 2008). Poly(I:C) is an analog of viral double-stranded (ds) RNA that activates various immune cell types through two major dsRNA sensors, melanoma differentiation-associated protein-5 (MDA5) and Toll-like receptor (TLR) 3. MDA5 is a cytosolic sensor, which detects poly(I:C) that penetrates into the cytosol through yet undefined mechanisms (Ishii et al., 2008). TLR3 is located in intracellular endosomes and detects poly(I:C) that has been internalized by endocytosis (Matsumoto and Seya, 2008). Upon poly(I:C) detection, MDA5 transmits signals through the adaptor IPS1, while TLR3 signals through the adaptor TRIF (also known as TICAM1). Both these adaptors initiate downstream signaling pathways that lead to activation of a similar array of transcription factors, including IRF3, IRF7, IRF1 and NF-κB. These factors induce the expression of genes encoding type I interferons (IFN), i.e. IFN-α and IFN-β, pro-inflammatory cytokines and various molecules involved in antigen presentation (Kawai and Akira, 2008).

Poly(I:C) induces the maturation of dendritic cells (DC), boosting their ability to prime and expand antigen-specific T cell responses (Kumar et al., 2008; Longhi et al., 2009; Trumpfheller et al., 2008). Because of this DC stimulatory activity, poly(I:C) is a promising adjuvant for vaccines, particularly for cancer vaccines that must overcome both tolerance to tumor-associated self-antigens and the immunosuppressive influence of the tumor microenvironment (Steinman and Banchereau, 2007). Poly(I:C) is also extensively used to activate mouse NK cells *in vivo*. The NK cell stimulatory activity of

poly(I:C) is potentially important for anti-cancer vaccines as it may contribute to tumor eradication by inducing NK cell-mediated lysis of tumor cells. In humans, the NK cell stimulatory activity of poly(I:C) has been chiefly attributed to its ability to trigger TLR3 expressed in cultured NK cells (Hart et al., 2005; Lauzon et al., 2006; Schmidt et al., 2004; Sivori et al., 2004). Whether poly(I:C) also activates human NK cells through DC or other accessory cells has not been investigated. In mice, poly(I:C) is thought to activate NK cells primarily through DC. An initial study showed that poly(I:C) stimulates TLR3 in DC, which consequently acquire the ability to activate NK cells (Akazawa et al., 2007). A very recent study showed that poly(I:C) triggers both the TRIF and IPS-1 signaling pathways in CD8α DCs, which in turn activate NK cells in vitro (Miyake et al., 2009). These results suggest that TLR3 and MDA5 may stimulate murine NK cells indirectly through activation of DCs, particularly CD8α DCs.

In this report we investigated the relative contributions of MDA5 and TLR3 in poly(I:C)-mediated activation of NK cells using MDA5$^{-/-}$, TLR3$^{-/-}$ and MDA5/TLR3$^{-/-}$ mice. We found that MDA5 has a predominant role in NK cell activation, whereas the contribution of TLR3 is secondary and is most evident in the absence of MDA5. Both MDA5 and TLR3 activated NK cell indirectly through accessory cells, but induced different NK cell stimulatory cytokines, as MDA5 was essential for IFN-α and IFN-β whereas TLR3 was required for interleukin (IL)-12 and, in part, for IFN-β. By generating bone marrow chimeras between wild type (WT) and dsRNA sensor-deficient mice, we found that MDA5 promotes NK cell activation mainly through stromal accessory cells, whereas TLR3 acts predominantly through bone marrow-derived accessory cells. To determine

whether the hematopoietic accessory cells that activate NK cells are, indeed, CD8α DC, we examined mice deficient for the basic leucine zipper transcription factor ATF-like 3 (BATF3), which have a selective developmental defect in CD8α DC (Hildner et al., 2008). We found that poly(I:C)-mediated stimulation of NK cells was minimally affected in these mice, indicating that poly(I:C) acts through multiple accessory cells, rather than solely through CD8α DC. We conclude that MDA5 and TLR3 mediate substantially distinct and yet complementary functions during poly-I:C-mediated activation of NK cells.

Results and discussion

MDA5 has a predominant role in NK cell response to poly(I:C) *in vivo*.

To investigate the relative contributions of MDA5 and TLR3 in poly(I:C)-mediated activation of NK cells, we injected WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and MDA5/TLR3$^{-/-}$ (double knockout, DKO) mice with poly(I:C); after 24 hrs we isolated spleen NK cells and measured cytotoxicity *ex vivo*. NK cells from untreated WT and sensor-deficient mice were unable to kill targets, while NK cells from poly(I:C)-treated WT mice killed up to 50% of targets (Figure 1A). NK cells from MDA5$^{-/-}$ mice treated with poly(I:C) had a significant defect in cytotoxicity as only 20% of target cells were killed at maximum effector:target ratios. NK cells from TLR3$^{-/-}$ mice treated with poly(I:C) had a modest, but not significant cytotoxicity defect. However, NK cells from DKO mice were unable to lyse targets after poly(I:C) stimulation. These results suggest that MDA5 can largely compensate for lack of TLR3, and that TLR3 contribution to NK activation is evident only in the absence of MDA5.

Another measure of NK cell activation is CD69 upregulation. CD69 is a cell surface molecule induced by IFN-α/β that promotes lymphocyte retention in lymphoid organs (Shiow et al., 2006). Following injection of poly(I:C), CD69 upregulation was partially impaired in NK cells from MDA5$^{-/-}$ mice in comparison to those from WT mice and completely abrogated in NK cells from DKO mice (Figure 1B). There was no decrease in CD69 expression in the TLR3$^{-/-}$ mice, further implying that the effect of poly(I:C) is predominantly mediated by MDA5. To determine the contributions of MDA5 and TLR3 to NK cell production of IFN-γ in response to poly(I:C), we isolated splenocytes 3 hrs

and 4 hrs after injecting WT and dsRNA sensor-deficient mice with poly(I:C), and determined the intracellular content of IFN-γ in NK cells. NK cells isolated from both MDA5$^{-/-}$ and TLR3$^{-/-}$ mice 3 hours after poly(I:C) injection produced less IFN-γ than WT NK cells (Figure 1C). However, by 4 hours following poly(I:C) injection, TLR3$^{-/-}$ and WT NK cells generated similar amounts IFN-γ, whereas MDA5$^{-/-}$ NK cells still produced less IFN-γ than either the TLR3-/- or WT NK cells. DKO NK cells did not produce IFN-γ at any time point assessed following poly(I:C) stimulation. Altogether, these results indicate that MDA5 plays a more predominant role than TLR3 in stimulating NK cytotoxicity, CD69 upregulation and IFN-γ production.

MDA5 activates NK cells through an NK cell-extrinsic pathway

Since MDA5 is ubiquitously expressed, poly(I:C) could directly activate mouse NK cells through MDA5. However, MDA5 is also expressed in DCs, which play a crucial role in activating NK cells (Andoniou et al., 2005; Andrews et al., 2003; Ferlazzo et al., 2002; Fernandez et al., 1999; Gerosa et al., 2002; Lucas et al., 2007; Mortier et al., 2008). Thus, poly(I:C) may activate mouse NK cells through DC or other accessory cells expressing MDA5. To test whether MDA5-mediated activation of mouse NK cells occurs in an NK-intrinsic or -extrinsic manner, we co-cultured combinations of bone marrow DCs (BMDCs) and NK cells from WT or dsRNA sensor-deficient mice and measured cytotoxicity, CD69 upregulation and IFN-γ production in response to poly(I:C). Remarkably, the defect seen in NK cell activation in the MDA5$^{-/-}$ mice *in vivo* was entirely recapitulated in the co-cultures of WT NK cells with MDA5$^{-/-}$ BMDCs. MDA5$^{-/-}$ BMDCs stimulated with poly(I:C) promoted NK cytotoxicity, CD69 upregulation and

IFN-γ secretion less effectively than did poly(I:C)-activated WT BMDCs, (Figure 2A-C). Following exposure to poly(I:C), DKO BMDCs were almost entirely incapable of inducing NK activation. TLR3$^{-/-}$ BMDCs pulsed with poly(I:C) induced less IFN-γ production in NK cells than did similarly treated WT BMDCs, whereas NK cell cytotoxicity and CD69 expression were slightly augmented. No significant differences in NK cytotoxicity, CD69 expression and IFN-γ production were detected when NK cells from WT or DKO mice were co-cultured with poly(I:C)-activated WT BMDCs (Figure 2D-F). Consistent with this result, purified NK cells exhibited only modest or no increase in CD69 expression and IFNγ secretion when directly stimulated with pIC even when pretreated with IFNα and/or IL-12 to induce MDA5 and TLR3 (Supplementary Figure 1). We conclude that poly(I:C)-induced NK activation through MDA5 and TLR3 occurs extrinsic to the murine NK cell itself. Moreover, while MDA5 deficiency in BMDC severely impaired cytotoxicity and CD69 expression, TLR3 deficiency had minor impact on these functions. In fact, TLR3 deficiency caused a slight increase of cytotoxicity and CD69 expression. These *in vitro* results further corroborate the concept that MDA5 plays a predominant role in murine NK cell activation, while the contribution of TLR3 is limited but quite evident in the complete abrogation of NK activation observed in the DKO mice and cells.

MDA5 and TLR3 disparately promote the secretion of cytokines that stimulate NK cells

A variety of cytokines have been shown to activate NK cells. IFN-α/β augments NK cell lytic capacity and expression of CD69 (Gerosa et al., 2002; Gerosa et al., 2005; Swann et

al., 2007); IFN-α/β, IL-12 and IL-18 stimulate NK cell production of IFN-γ (Andoniou et al., 2005; Biron et al., 1999; Chaix et al., 2008; Ferlazzo and Munz, 2004; Nguyen et al., 2002; Trinchieri, 1995); IL-15 and IL-2 promote NK cell survival, proliferation and effector functions (Granucci et al., 2004; Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008; Waldmann and Tagaya, 1999). Since stimulation of both MDA5 and TLR3 with poly(I:C) leads to the production of IFN-α/β as well as inflammatory cytokines in DC and other cells (Kawai and Akira, 2008), we predicted that the defect in NK activation *in vivo* would be associated with a defect in cytokine production in the absence of these dsRNA sensors. We found that serum IFN-α was completely abolished in the MDA5$^{-/-}$ and DKO mice 24 hours after poly(I:C) stimulation (Figure 3A). In contrast, there was no defect in serum IFN-α in TLR3$^{-/-}$ mice compared to WT mice, consistent with previous studies (Kato et al., 2006; Kumar et al., 2008; Miyake et al., 2009). MDA5$^{-/-}$ mice had WT levels of IL-12p40 in the serum, whereas serum IL-12p40 was completely abolished in TLR3$^{-/-}$ and DKO serum 6 hours after poly(I:C) stimulation (Figure 3B). Other cytokines potentially relevant for NK cell activation, such as IFN-β, IL-18, IL-1β, IL-15 or IL-12p70 were undetectable in the serum of all mice after poly(I:C) stimulation.

Similar to our findings in the serum, we found that poly(I:C)-stimulated MDA5$^{-/-}$ and DKO BMDCs secreted less IFN-α than did WT BMDC (Figure 3C). TLR3-deficiency did not diminish but, in fact, slightly augmented the IFN-α response. MDA5$^{-/-}$ BMDC also failed to produce adequate amounts of IFN-β, although a very minor IFN-β response was detectable early after poly(I:C) stimulation (Figure 3D). The IFN-β response was partially reduced in TLR3$^{-/-}$ BMDCs and completely abolished in DKO BMDCs. Thus,

MDA5 is essential for both IFN-α and IFN-β responses to poly(I:C), whereas TLR3 is dispensable for IFN-α, although it contributes to IFN-β production. As opposed to type I IFNs, MDA5$^{-/-}$ BMDCs secreted WT levels of IL-12p40, whereas TLR3$^{-/-}$ BMDCs produced essentially no IL-12p40 (Figure 3E).

We conclude that MDA5 and TLR3 induce cytokines in response to poly(I:C) in different ways. MDA-5 is required for the IFN-α and IFN-β response to poly(I:C), but not IL-12p40. In contrast, TLR3 is required for IL-12p40 and, to a certain extent, IFN-β but is not essential for IFN-α production. Consistent with previous studies in vivo (Longhi et al., 2009), we found that poly(I:C)-induced NK cell activation in vitro was severely impaired in the presence of an antibody that blocks the receptor for type I IFN (IFNAR) (supplementary figure 2A, B). Moreover, co-cultures of NK cells and DC lacking IFNAR showed that NK cell activation requires IFN-α signaling in both NK cells and DC (supplementary figure 2C, D). Thus, type I IFNs are required for robust NK cell activation; because MDA5$^{-/-}$ mice secrete very little IFN-α and IFN-β in response to poly(I:C), the scarcity of these cytokines is probably responsible for the global defect in NK cell activation in MDA5$^{-/-}$ mice following stimulation with poly(I:C). Although IL-12 stimulates IFN-γ secretion (Trinchieri, 1995), the addition of an antibody neutralizing IL-12 to co-cultures of NK cells and DC had minimal impact on poly(I:C)-induced NK cell secretion of IFN-γ (supplementary figure 2A, B). Thus, the partial defect in IFN-γ secretion observed in TLR3$^{-/-}$ mice may be due to insufficient IFN-β, perhaps combined with the lack of IL-12 and/or other cytokines (Matikainen et al., 2001). Yet undefined TLR3-induced cell-cell interactions might also contribute to NK cell secretion of IFN-γ.

MDA5 and TLR3 function in different accessory cell populations

Our data indicate that MDA5 and TLR3 have distinct quantitative and qualitative impacts on NK cell activation by poly(I:C). One potential explanation for this is differential expression of MDA5 and TLR3 in cell types that have distinct capacities to produce cytokines. To address this possibility we investigated the expression of MDA5 and TLR3 in the spleen and liver before and after poly(I:C) stimulation by immunohistochemistry. In naïve mice, MDA5 was broadly expressed in the red pulp and the T-cell area of the spleen, and in the hepatocytes and interstitial cells of the liver (Figure 4A). In contrast, TLR3 expression was more limited, including DC of the white pulp, rare lymphoid cells in the marginal zone, red pulp macrophages as well as liver interstitial cells, likely corresponding to Kupffer cells and endothelial lining cells (Figure 4B). Poly(I:C) stimulation induced a very strong increase in MDA5 expression in both spleen and liver, with the only notable exception in the splenic B-cell area (Figure 4A). Poly (I:C) stimulation also induced broader expression of TLR3 in the spleen, particularly in the B-cell area, and in the liver, including the hepatocytes (Figure 4B). These results suggested that MDA5 and TLR3 are constitutively expressed in partially distinct cellular compartments of the spleen and liver, with MDA5 being more broadly expressed than TLR3. Administration of poly(I:C) stimulates a stronger and broader expression of both sensors, consistent with previous reports showing that type I IFNs induce MDA5 (Ishii et al., 2008) and TLR3 expression (Matsumoto and Seya, 2008); even under these conditions, however, the distribution of MDA5 and TLR3 do not entirely overlap.

To test the importance of MDA5 and TLR3 in the stromal versus hematopoietic compartments, we created radiation chimeras between WT and MDA5$^{-/-}$ as well as WT and TLR3$^{-/-}$ mice. Upon poly(I:C) stimulation, defective NK cell-mediated cytotoxicity and reduced serum levels of IFN-α were seen in MDA5$^{-/-}$ chimeras that had received WT bone marrow, while no cytotoxicity defect and minor impairment of systemic IFN-α were observed in WT chimeras that had been grafted with MDA5$^{-/-}$ bone marrow (Figure 5A, B). Conversely, a slight decrease in cytotoxicity and marked reduction of systemic IL-12p40 was evident in WT chimeras that had received TLR3$^{-/-}$ bone marrow showed, while no obvious cytotoxicity or systemic IL-12p40 defects were observed in TLR3$^{-/-}$ /WT chimeras that were grafted with WT bone marrow (Figure 5A, C). These results indicate that MDA5 activates NK cells by acting predominantly in the radio-resistant stromal cell population, while TLR3 activates NK cells mainly through radio-sensitive hematopoietic accessory cells.

Poly(I:C)-mediated NK cell activation in vivo occurs independently of CD8α DCs.

Since TLR3 is highly expressed in CD8α DC (Edwards et al., 2003) and CD8α DC specialize in the secretion of IL-12 (Maldonado-Lopez et al., 1999), it seemed plausible that the hematopoietic accessory cells involved in TLR3-induced NK cell activation were, in fact, CD8α DC. This possibility was further supported by a recent study showing that poly(IC) triggers the TRIF and IPS1 signaling pathways in CD8α DCs, inducing the secretion of IL-12 and type I IFNs that activate NK cells *in vitro* (Miyake et al., 2009). To directly test the contribution of CD8α DC to poly(I:C)-mediated NK cell activation *in*

vivo, we analyzed Batf3$^{-/-}$ mice, which selectively lack the CD8α DC population (Hildner et al., 2008). Following injection of poly(I:C), NK cells isolated from Batf3$^{-/-}$ killed RMA-S cells only slightly less efficiently than WT NK cells (Figure 6A). Upregulation of CD69, NK cell secretion of IFN-γ, serum IFN-α and serum IL-12p40 were similar in Batf3$^{-/-}$ and WT mice (Figure 6B-E). These results indicate that poly(I:C) triggers secretion of NK cell stimulatory cytokines through multiple accessory cells rather than solely through CD8α DC.

Concluding remarks

In this study we provide the first demonstration that MDA5 is essential for robust activation of murine NK cells in response to poly(I:C). While previously published studies have suggested that poly(I:C) activates NK cells primarily through TLR3, our data show that MDA5 is, in fact, more important than TLR3 for triggering all NK cell functions, including cytotoxicity, CD69 and IFN-γ production. TLR3 has a minor impact on NK cell activation and its role is most evident in DKO mice, where the lack of MDA5 and TLR3 completely abrogates the NK cell response to poly(I:C). This result also excludes any contribution of other dsRNA sensors, such as RIG-I, to poly(I:C)-mediated NK cell activation.

We demonstrated that MDA5- and TLR3-mediated NK cell activation is NK cell-extrinsic. This conclusion is supported by in vitro experiments showing that lack of both MDA5 and TLR3 in NK cells has no impact on the ability of poly(I:C) to induce NK cell activation in the presence of WT DC, whereas deficiency of MDA5 and/or TLR3 in DC impairs activation of WT NK cells. Similar results were recently obtained by Miyake et al. (Miyake et al., 2009). Moreover, the extrinsic function of MDA5 was further supported by in vivo experiments, showing that poly(I:C)-mediated NK cell activation is normal in lethally irradiated WT mice reconstituted with $MDA5^{-/-}$ bone marrow cells, which generate $MDA5^{-/-}$ NK cells. Although MDA5 is ubiquitously induced by type I IFNs and therefore may be also expressed in NK cells, NK cells most likely lack efficient mechanisms for poly(I:C) uptake, thereby preventing a direct effect of poly(I:C) on NK

cells. It remains possible that the administration of poly(I:C) with liposomal reagents that facilitate cytosolic entry of poly(I:C) may induce some direct activation of NK cells.

MDA5 and TLR3 contributed to poly(I:C)-induced NK cell activation by inducing different NK cell stimulatory cytokines. MDA5 promoted IFN-α and IFN-β secretion, whereas TLR3 was essential for IL-12p40 and, in part, for IFN-β production. Our in vitro data indicates that type I IFNs are crucial for poly(I:C)-induced NK cell activation. These data are consistent with the recent observation that NK cell secretion of IFN-γ in response to poly(I:C) is blocked by injection of an anti-IFNAR antibody in vivo (Longhi et al., 2009). The crucial role of type I IFNs in poly(I:C)-mediated NK cell activation, together with the predominant function of MDA5 in inducing IFN-α and IFN-β secretion explain the major defect in NK cell activation in $MDA5^{-/-}$ mice. Although IL-12 is a known inducer of IFN-γ (Trinchieri, 1995), blockade of IL-12 did not affect poly (I:C)-induced secretion of IFN-γ by NK cells, at least in vitro. Therefore, the transient defect in NK cell secretion of IFN-γ observed in $TLR3^{-/-}$ mice may be due to insufficient IFN-β, perhaps combined with a defect in IL-12 and/or other cytokines (Matikainen et al., 2001). While our data underscore the roles of MDA5 and TLR3 in inducing IFN-α and IL-12, MDA5 and TLR3 may also act by inducing cell surface molecules on accessory cells that activate NK cells through cell-cell interactions or local delivery of cytokines. This is the case for the α chain of the IL-15 receptor (IL-15Rα), which is induced by type I IFNs and allows accessory cells to trans-present IL-15 to NK cells (Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008). Accordingly, transcriptional analysis of WT, $MDA5^{-/-}$ and $TLR3^{-/-}$ BMDCs stimulated in vitro with poly (I:C) showed that both MDA5 and TLR3

are required for the induction of both IL-15Rα and its ligand IL-15 (supplemental figure 3). MDA5 and TLR3 may induce other cell surface molecules on accessory cells involved in NK cell activation, either indirectly through type I IFN signaling, or directly through IPS1 and TRIF and their downstream transcription factors IRF1/3/7 and NF-κB.

Our data indicates that the disparate impacts of MDA5 and TLR3 on poly(I:C)-induced secretion of IFN-α, IFN-β and IL-12p40 and NK cell activation may be related, at least in part, to the distinct cellular distribution of MDA5 and TLR3. Bone marrow chimera experiments demonstrated that MDA5 mainly acts through stromal cells. The surfeit of these cells and their general ability to produce type I IFNs can explain why MDA5 stimulation leads to a major release of IFN-α. MDA5 may have a more limited role in IL-12 secretion because, although IFN-α induces IL-12p35 (Gautier et al., 2005; Hermann et al., 1998), it also modulates IL-12 production from DC and monocytes/macrophages (Nguyen et al., 2000). In contrast, TLR3 has a more restricted distribution and acts mainly through hematopoietic cells. This may explain why TLR3 has a minor impact on systemic IFN-α and a more prominent effect on IL-12 production. Although CD8α DC express TLR3 (Edwards et al., 2003), specialize in the secretion of IL-12 (Maldonado-Lopez et al., 1999), produce type I IFNs (Longhi et al., 2009) and strongly activate NK cells in vitro in response to poly(I:C) (Miyake et al., 2009), our analysis of BATF3$^{-/-}$ mice that lack CD8α DC demonstrates that poly(I:C)-mediated NK cell activation occurs even in the absence of these cells, indicating that TLR3 acts through a variety of hematopoietic accessory cells. Immunohistochemical analysis of TLR3 expression suggests that these cells may include various DC and macrophage subsets in the white and the red pulp of

the spleen as well as marginal zone B cells. In conclusion, our studies elucidate the mechanisms by which poly(I:C) activates NK cells in vivo, demonstrating distinct, yet complementary roles for MDA5 and TLR3 in stimulating NK cell effector functions through a multiplicity of accessory cells.

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Materials and Methods

Mice, **cell lines, and antibodies.** MDA5$^{-/-}$, TLR3$^{-/-}$, and IFNAR$^{-/-}$ mice have been described previously (Alexopoulou et al., 2001; Trumpfheller et al., 2008). DKO mice were made by intercrossing MDA5$^{-/-}$ and TLR3$^{-/-}$ mice. These mice have been backcrossed to the C57Bl/6 background. Age- and sex- matched C57BL/6 control mice were purchased from the Jackson labs. Batf3$^{-/-}$ (Hildner et al., 2008) and WT (Taconic) mice were on the 129SvEv background except for those used for IFN-γ staining, which were backcrossed 6 times onto the C57BL/6 background. All mouse protocols were approved by the Washington University Animal Care Committee. RMA-S cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and Glutamax. Blocking antibodies included anti-IFNAR (MAR1-5A3), anti-IL-12 (Tosh, kindly provided by Emil R. Unanue) and anti-human IFNγR (GIR-208) as isotype control.

Bone marrow chimeras. Recipient mice were γ irradiated with 1000 rad. After an overnight rest, mice were reconstituted with 5 x 10^6 bone marrow cells per mouse that had been harvested from the femurs and tibias of age- and sex- matched donors. After 6 weeks, chimeras were used for *in vivo* pIC stimulations.

Cell preparations. Single cell suspensions were prepared from spleens and depleted of erythrocytes by ammonium chloride lysis. For NK purification, cell suspensions were incubated with anti-DX5 coated MACS beads (Miltenyi) and purified by autoMACS. Primary cells were cultured in complete media (RPMI 1640 (w/o) L-glutamine

supplemented with 10% FCS, Sodium Pyruvate, Kanamycin sulfate, Glutamax, and non-essential amino acids). BMDCs were cultured in complete media with 2% GM-CSF for 7 days and then used in assays with complete media.

NK-DC co-cultures. For NK-DC cell co-culture experiments, $1x10^5$ BMDCs were cultured with 5 $x10^4$ NK cells in the presence or absence of 25μg poly(I:C) (invivogen). In some experiments anti-IFNAR, anti-IL-12p70 or control antibody were added to the cultures before the addition of the poly(I:C). In other experiments, purified NK cells alone were stimulated with 0, 25, or 100μg poly(I:C) in the presence of 200, 1000, or 5000U IFNα (PBL); 1, 10, or 100ng/ml IL-12 (Peprotech); or 1ng/ml each IL-12 and IL-18(Peprotech). After 24 hours, supernatants were harvested for cytokine detection and NK cells were detached by washing with 1mM EDTA in PBS and analyzed by FACS.

Cytotoxicity Assays. To measure NK cytotoxicity *ex vivo*, splenocytes were prepared as described above 24 hours after injecting mice with 100μg poly(I:C) i.v. and mixed with $1x10^5$ Cr^{51}-labeled RMA-S targets in decreasing E:T ratios. To measure NK cytotoxicity in NK-DC co-cultures, Cr^{51}-labeled RMA-S targets were directly added to the NK-DC co-cultures. After 4 hours, supernatants were harvested and Cr^{51} release was measured in individual samples as well as maximum and spontaneous release samples. Specific lysis was calculated by (specific release) – (spontaneous release)/ (max release) – (spontaneous release).

FACS analysis. Splenocytes, cultured NK cells and BMDC prepared as described above were treated with Fc block (HB-197) and stained with anti-CD3, anti-NK1.1, and anti-CD69 (BD) for NK cells activation experiments or with anti-DX5 in place of NK1.1 for Batf3$^{-/-}$ mice. Samples were processed on a FACSCalibur and analyzed with CellQuest software (BD).

Ex vivo IFN-γ production. Mice were injected with 100μg poly(I:C) i.v. After 3 or 4 hours, splenocytes were prepared as described above and cultured with monensin for an additional 3 or 4 hours. After incubation, cells were stained with anti-CD3 and anti-NK1.1 or anti-DX5 (Batf3$^{-/-}$) then fixed with PFA and permeabilized with saponin buffer and stained with anti-IFN-γ. Samples were then analyzed by FACS as described above to detect percentage of IFN-γ-producing NK cells.

Cytokine measurements. Serum samples were taken at 6 and 24 hours after injecting mice with 100μg poly(I:C); supernatants of NK-DC cultures were harvested at 0, 6, 12, and 24 hours after poly(I:C) stimulation. Type I IFNs and IL-12p40 were determined by ELISA (PBL and eBioscience, respectively); IFN-γ was assessed by CBA (BD).

Statistics. Figures were plotted using Prism4 (GraphPad Software) indicating the mean and standard deviation. Statistical significance was determined primarily by student's t test. ANOVA was used to determine significance for cytotoxicity assays. Significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Immunohistochemistry. Spleen and liver sections were obtained from frozen (for anti-TLR3 staining) and formalin-fixed paraffin-embedded tissue (for anti-MDA5 staining). Primary antibodies included anti-MDA5 (rabbit polyclonal anti-mouse, AL180, Alexis, Plymouth Meeting, PA, USA); anti-TLR3 (rat anti-mouse 11F8.1B7, kindly provided by David M. Segal, Experimental Immunology Branch, NCI); anti-B220 (Caltag Laboratories) and anti-CD3 (rabbit monoclonal SP7, Thermo scientific). Anti-TLR3 and -B220 were detected, after endogenous biotin blocking, using a rabbit anti-rat IgG (Mouse Absorbed; Vector Laboratories, Burlingame, CA, USA). For anti-MDA5 and -CD3 stainings, sections were deparaffinized and subjected to antigen retrieval by incubating in a water bath at 98°C for 40 minutes. Primary antibodies were detected using Envision Rabbit (Dako, Glostrup). Reactions were revealed by DAB.

RNA preparation and RT-PCR. BMDCs were stimulated with 25μg poly(I:C) for 6 or 12 hours then RNA was harvested from the cells by RNeasy kit (invitrogen). cDNA was synthesized from RNA (Superscipt RT kit, invitrogen) and relative levels of IL-15 and IL-15Ra were determined by semi-quantitative PCR and normalized to GAPDH using the following primers: IL-15-sense 5'-GCAGAGTTGGACGAAGAC-3' ; IL-15-antisense 5'-AGCACGAGATGGATGTATT-3' ; IL-15Rα-sense 5'-TCTCCCCACAGTTCCAAAAT-3'; IL15Rα-antisense 5'-GGCACCCAGGCTCAGTAAAA-3'; GAPDH-sense 5'-GAGCCAAAAGGGTCATCATC-3'; GAPDH-antisense 5'-CCATCCACAGTCTTCTGGGT-3'.

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Figure Legends

Figure 4.1. Poly(I:C)-induced NK cell activation is primarily mediated by MDA5. WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were injected with 100μg poly(I:C) i.v. After 24 hours, splenocytes were harvested and used as effector cells in a cytotoxicity assay with labeled RMA-S targets (experiment performed using 1 mouse for each genotype in 4 independent trials) (A) or assayed for CD69 expression by FACS, gating on NK1.1^{+}CD3^{-} splenocytes (experiment performed using 2 mice for each genotype in 3 independent trials) (B). Alternatively, 3 or 4 hours after poly(I:C) injection, splenocytes were isolated, cultured with monensin for an additional 3 or 4 hours, and analyzed for intracellular content of IFNγ by FACS, gating on NK1.1^{+}CD3^{-} cells (experiment performed using 1 mouse for each genotype in 3 independent trials) (C). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Figure 4.2. MDA5 and TLR3 activate NK cells through NK cell-extrinsic mechanisms. BMDCs from WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were cultured with purified NK cells from WT mice in the presence or absence of 25μg poly(I:C). After 24 hours NK1.1^{+}CD3^{-} cells were stained for CD69 expression (A) and IFNγ was measured from culture supernatants (B) (experiments performed using BMDCs from 1 mouse for each genotype and NK cells from 3 pooled mice in 4 independent trials). Alternatively, Cr^{51}-labeled RMAS targets were added to culture and cytotoxicity was measured (C) (experiments performed using BMDCs from 1 mouse from each genotype and NK cells from 3 pooled mice in 3 independent trials). In reverse experiments, BMDCs from WT mice were cultured with purified NK cells from WT or DKO mice with or without

poly(I:C). After 24 hours CD69 expression was determined by FACS (D) (experiments performed using BMDCs from 1 mouse and NK cells from 3 pooled mice of each genotype in 4 independent trials), IFNγ was measured in supernatants (E) (experiments performed using BMDCs from 1 mouse and NK cells from 3 pooled mice of each genotype in 4 independent trials), and cytotoxicity was measured against RMAS targets (F) (experiments performed using BMDCs from 1 mouse and NK cells from 3 pooled mice of each genotype in 3 independent trials). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Figure 4.3. MDA5 and TLR3 mediate distinct cytokine responses.

WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were injected with 100μg poly(I:C) i.v. Serum was taken at 6 and 24 hours and assayed for IFN-α (experiment performed with serum from 5 mice of each genotype in 1 independent ELISA assay) (A) or IL-12p40 (experiment performed with serum from 4 mice of each genotype in 1 independent ELISA assay) (B) by ELISA. BMDCs from WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were stimulated with 25μg poly(I:C). At various time-points after stimulation, supernatants were harvested and IFN-α (C), IFN-β (D), IL-12p40 (E) were measured by ELISA (experiments performed with supernatants from BMDCs from each genotype in 4 independent experiments and evaluated by 2 independent ELISA assays). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Figure 4.4. Expression patterns of MDA5 and TLR3 in spleen and liver.

Frozen tissue sections from spleen and liver of unstimulated MDA5$^{-/-}$ and WT mice and from poly(I:C)-injected WT mice were stained with anti-MDA5 (brown) and counterstained with Hematoxylin (blue) (A). Formalin-fixed sections from spleen and liver of unstimulated TLR3$^{-/-}$ and WT mice and poly(I:C)-injected WT mice were stained with anti-TLR3 (brown) and counterstained with Hematoxylin (blue) (B) (experiments performed using organs from 2 unstimulated and 4 pIC stimulated mice for each genotype with staining done at least in duplicate for each). WP, white pulp; RP, red pulp. Magnification = 200X, Scale bar = 100 microns. Expression of TLR3 in the B cell area of the spleen was confirmed by staining with anti-B220 and anti-TLR3 (data not shown).

Figure 4.5. MDA5 and TLR3 act in different cellular compartments.

Bone marrow chimeras consisting of WT>MDA5$^{-/-}$, MDA5$^{-/-}$>WT, WT>TLR3$^{-/-}$, and TLR3$^{-/-}$>WT mice were stimulated with 100μg poly(I:C) i.v. After 24 hours splenocytes were harvested and used as effector cells in cytotoxicity assays against Cr^{51} labeled RMAS targets (experiments performed using 1 mouse for each chimera in 3 independent trials) (A). Additionally, serum was collected to measure systemic IFN-α (B) and IL-12p40 (C) (experiments performed with serum from 4 mice of each chimera in 1 independent ELISA assay). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Figure 4.6. Poly (I:C)-induced NK cell activation is independent of CD8α DCs.

WT and Batf3$^{-/-}$ mice were injected with 100μg poly(I:C) i.v. After 24 hours, splenocytes were harvested and used as effector cells in a cytotoxicity assay with labeled

RMA-S targets (experiments performed using 1 mouse for each genotype in 3 independent trials) (A) or assayed for CD69 expression by FACS, gating on $DX5^{+}CD3^{-}$ splenocytes (experiments performed using 2 mice for each genotype in 2 independent trials) (B). Alternatively, 3 hours after poly(I:C) injection, splenocytes were isolated and cultured with monensin for 3 additional hours, at which time $DX5^{+}CD3^{-}$ cells were analyzed by FACS for intracellular IFN-γ content (experiments performed using 1 mouse for each genotype in 3 independent trials) (C). Serum samples from poly(I:C) injected mice were taken at 24 hours and IFN-α (D) and IL-12p40 (E) were measured by ELISA (experiments performed with serum from 4 mice of each chimera in 1 independent ELISA assay). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Supplementary Figure Legends

Supplementary Figure 4.1. Limited effect of poly(I:C) on purified NK cells.

Purified NK cells from WT and DKO mice were cultured with increasing doses of pIC along with IFNα or IL-12 alone or in combination. As a positive control, NK cells were cultured with the combination of IL-12 and IL-18. After 24 hours, NK cells were analyzed by FACS for CD69 expression (A-C) or IFN-γ was measured from culture supernatants by CBA (D-F) (experiments performed using pooled NK cells from 5 mice for each genotype in 3 independent trials).

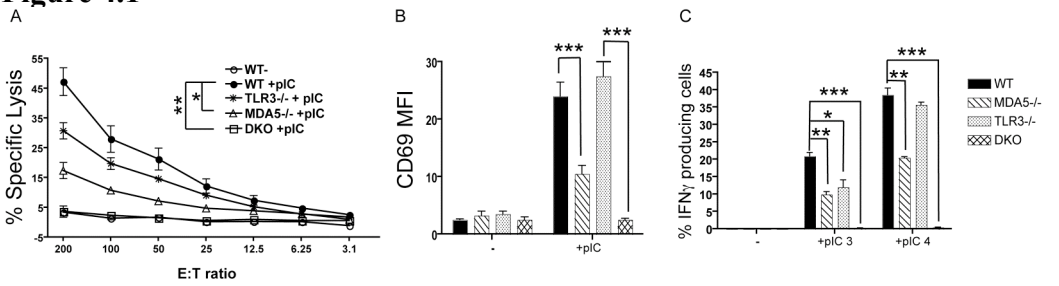
Supplementary Figure 4.2. Type I IFN is essential for poly(I:C)-induced NK cell activation through NK cell intrinsic and extrinsic mechanisms.

(A, B) WT BMDCs were cultured with purified NK cells from WT mice in the presence of poly(I:C) and anti-mouse IFNAR (MAR1), anti-mouse IL-12, or anti-human IFNGR (GIR) as control. After 24 hours, NK cells were analyzed by FACS for CD69 expression (A) or IFN-γ was measured in the co-culture supernatants (B) (experiments performed using BMDCs from 1 mouse and NK cells from 3 pooled mice in 3 independent trials). (C, D) Different combinations of WT and IFNAR$^{-/-}$ BMDCs and NK cells were co-cultured with poly(I:C). After 24 hours, CD69 expression (C) and IFN-γ production (D) were determined (experiments performed using BMDCs from 1 mouse for each genotype and NK cells from 3 pooled mice in 3 independent trials). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Supplementary Figure 4.3. MDA5 and TLR3 are required for poly (I:C)-induced expression of IL-15Rα and IL-15.

BMDCs from WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were stimulated with poly(IC) for 6 or 12 hours. RNA was harvested and levels of IL-15 and IL-15Rα were determined by semi-quantitative PCR normalized to GAPDH (experiments performed using BMDCs from 1 mouse for each genotype and in 4 independent trials). Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$.

Figure 4.1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3.1
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TLR3-/- + pIC
MDA5-/- +pIC
DKO +pIC
**
*
B
CD69 MFI
***
***
-
+pIC
C
% IFNγ producing cells
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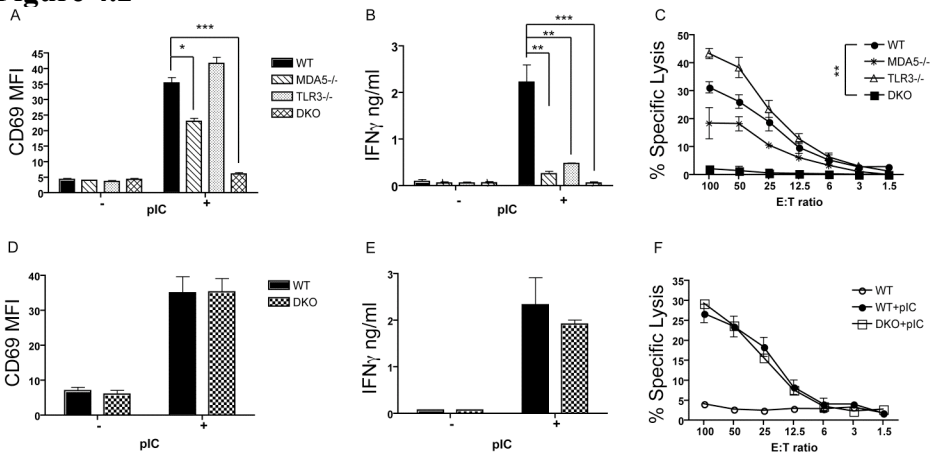
Figure 4.2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TLR3-/-
DKO
E:T ratio
D
CD69 MFI
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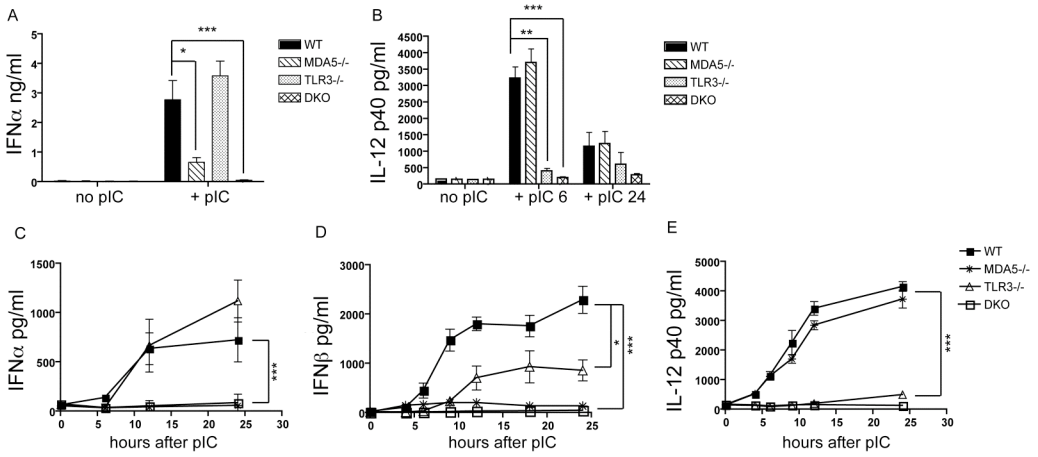
E:T ratio

Figure 4.3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E
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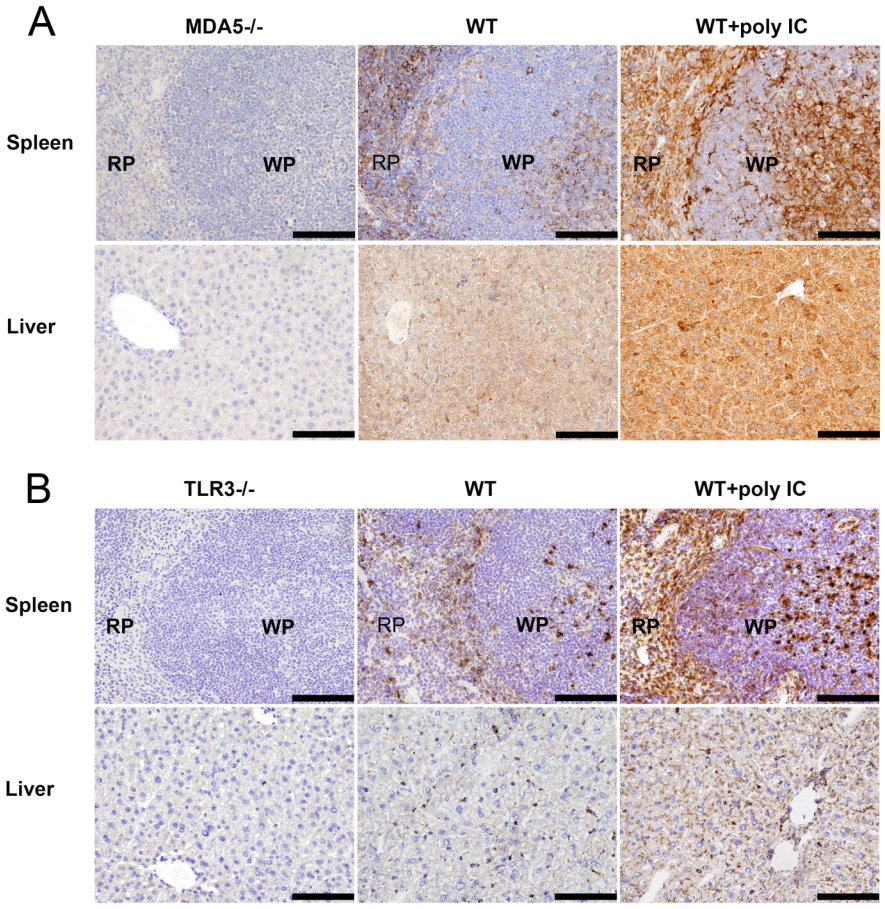
hours after pIC

Figure 4.4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Liver
RP
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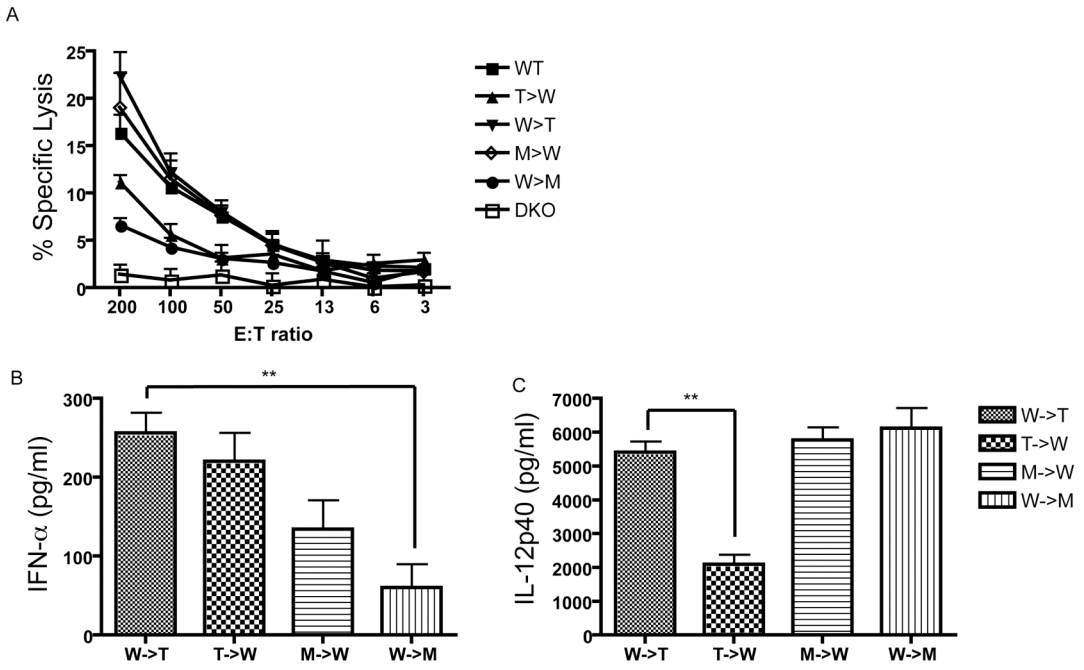
WT+poly IC
Spleen
Liver
RP
WP

Figure 4.5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W>M
DKO
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IFN-α (pg/ml)
300
200
100
0
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M->W
W->M
C
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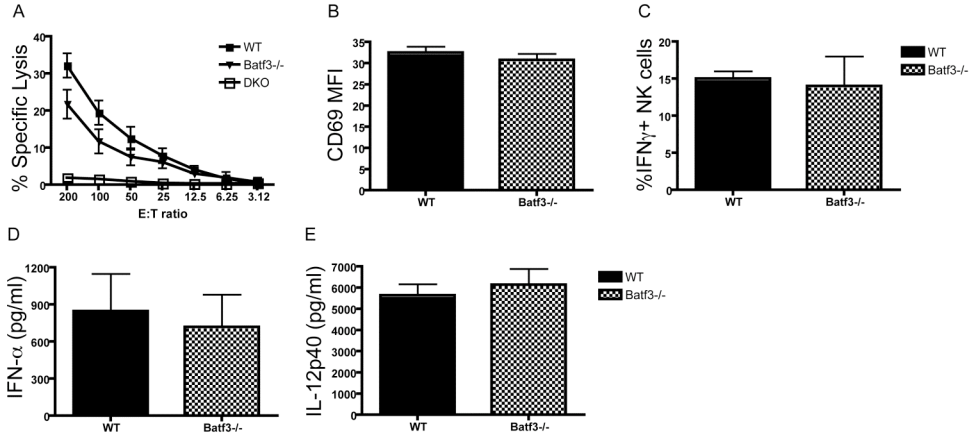
W->M

Figure 4.6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E:T ratio
B
CD69 MFI
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C
%IFNγ+ NK cells
WT
Batf3-/-
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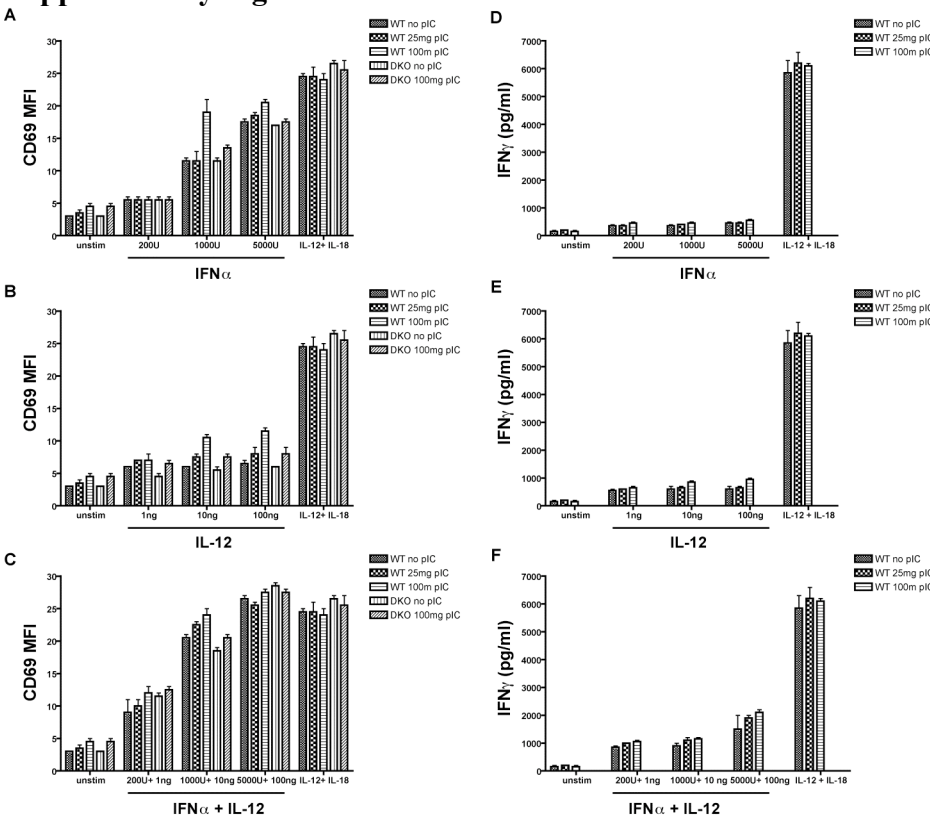
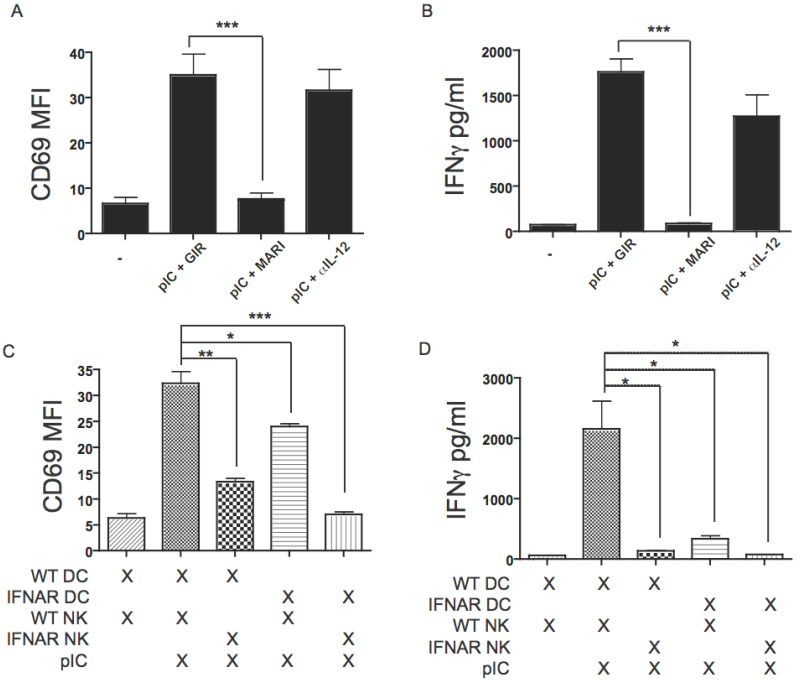
WT
Batf3-/-

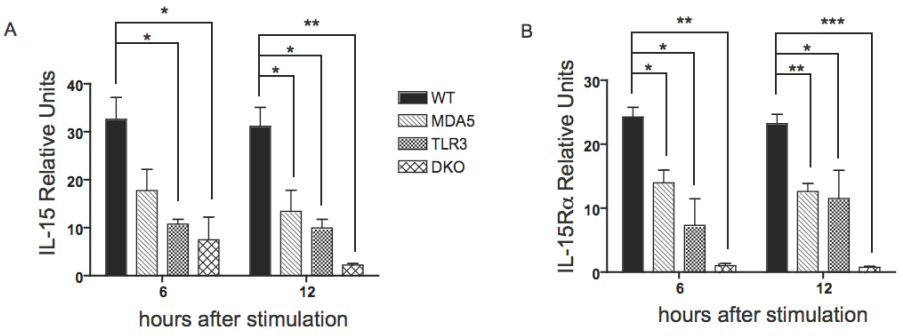
Supplementary Figure 4.1



Supplementary Figure 4.2



Supplementary Figure 4.3



Chapter 5

DISCUSSION

Abstract

Organisms use a number of proteins to detect viral infection and activate the antiviral response. The preceding chapters have provided evidence that two of these proteins, the dsRNA sensors MDA5 and TLR3, have distinct functional roles in activation of the innate immune system and control of viral infection. It is likely that the role of these sensors, and other sensors of the innate immune system, are dependent on several factors including viral tropism, cell and tissue specificity of PRRs, and downstream signaling components. The combinations of these factors that occur during a given viral infection are critical for successful control of that infection and determination of the disease pathology. This chapter addresses how the results in the previous chapters contribute to our understanding of how the RLR and TLR pathways work together to coordinate control of infection. It also discusses potential mechanisms by which these pathways may contribute to human disease and how we can apply this knowledge to develop new therapeutic targets.

Understanding the diversity between viral sensors

In this work we tested whether viral sensors of the TLR and RLR families serve redundant or non-redundant functions. It has previously been appreciated that one way by which viral sensors can be seen to have differential effects is by the recognition of different ligands and thus different viruses, such as for Rig-I and MDA5. However, several recent studies have demonstrated that both Rig-I and MDA5 function in a variety of viral infections, suggesting that different sensors may also recognize different components of the same virus, ensuring that multiple pathways are activated. Additionally, different downstream signaling components could ensure that PRRs activate the production of distinct cytokines. Finally, the differential expression of viral sensors among tissues and cells types could contribute to their distinct roles in viral infection. Results obtained in chapters 2, 3, and 4 help to highlight the importance of these factors in the antiviral response.

PRR signaling results in distinct cytokine responses

Both RLRs and TLRs are able to initiate IFN and inflammatory cytokine pathways *in vitro*. More recent studies, however, have revealed differential signaling by RLRs and TLRs in individual cell types. One study found that influenza infection in bronchial epithelial cells led to TLR3-dependent inflammatory cytokine induction and Rig-I- dependent IFN response(1). Another recent study has demonstrated that human keratinocytes contain functional TLR, RLR, and PKR signaling pathways and with the use of siRNA and small molecule inhibitors they were able to show that TLR3 provides

the main stimulus for NF-kB signaling, while RLRs are the primary initiators of IRF3 and IFN signaling in this cell type (2).

This work has also demonstrated that there is some specialization in cytokine production that is dependent on whether the signal is transduced by MDA5 or TLR3. In chapter 4, in response to pIC, MDA5 was found to be critical for robust type I IFN production, while TLR3 was necessary for IL-12 production both *in vivo* and *in vitro*, supporting previous findings. However, we also determined that there are important kinetic differences in MDA5 and TLR3 cytokine production. Both in response to pIC *in vitro* and viral infection *in vivo*, TLR3 was found to function at early time points for type I IFN production. In contrast, MDA5 was shown to be important for robust IFN production at late time points. There are several possibilities for why MDA5 and TLR3 are important for IFN production at different time points. The first is cellular location. TLR3 is located in the endosomal compartment, while MDA5 is cytoplasmic. To detect extracellular components, cells perform phagocytosis, which brings external contents into endosomal compartments. This would allow initial contact with TLR3, with subsequent internalization events from the endosomal to the cytoplasmic compartment being necessary for contact with MDA5. Second, while TLR3 is constitutively expressed on several cell types, MDA5 is an IFN-induced gene, and requires IFN production to be upregulated. Therefore, early IFN production by TLR3 may serve to upregulate MDA5, allowing for robust IFN production if the viral pathogen remains present, while preventing needless systemic IFN responses if the TLR3 response is necessary to control infection. *In vivo* another possibility for kinetic differences in MDA5 and TLR3 IFN

production is distribution in different cell types, which will be discussed in the next section.

The stimulation of different signaling pathways by TLRs and RLRs could have important implications. Type I IFN and inflammatory cytokines promote distinct immune responses. While IFN is known to activate a variety of immune cells, it also promotes intracellular antiviral responses. In contrast, inflammatory cytokines typically act to promote inflammation and the recruitment of immune cells without specific antiviral activities. If we are able to distinguish which receptor is preferentially responsible for the production of each cytokine, then we may be able to target activating ligands to the endosomal or cytoplasmic compartments to activate MDA5 or TLR3 specifically to induce a particular cytokine response. It may also be possible to take advantage of the kinetic difference in MDA5 and TLR3 signaling to initially target TLR3 in order to initiate a robust early IFN response, rather than targeting cells which have yet to express MDA5. These techniques could be a tremendous advantage to the fields of vaccine development as well as infectious diseases and tumor immunology since antiviral sensors and their ability to induce cytokines are potential targets for therapy and prevention. However, more work is needed to understand how individual and combinations of cytokines act to control immune activation if we are to take advantage of this knowledge.

Differential distribution of PRRs

The distribution of viral sensors in different cell and tissue types may be another mechanism to differentiate their actions. This has been previously described in

comparison between conventional dendritic cells (cDC) and plasmacytoid DCs (pDC). cDCs are specialized for pathogen detection and antigen presentation, while pDC specialize in the secretion of type I IFNs in response to viruses (3, 4). In humans cDCs express TLR1, 2, 3, 4, 5, 6 and 8, while pDCs preferentially express TLR7 and 9. CDCs are capable of expressing high levels of RIG-I and MDA5, while pDCs also express these cytoplasmic sensors, but, paradoxically, the sensors do not appear to function (5) unless the pDCs themselves are infected(6). Although both cell types express different sensors, they both respond to viruses and initiate an antiviral response.

Our data suggests that a similar mechanism exists between hematopoietic and stromal tissues. In the response to both dsRNA and viral infection, we found that TLR3 functions in hematopoietic cells, while MDA5 functions in stromal tissues. Expression patterns of the two sensors demonstrate that while MDA5 can be expressed on all cell types after IFN stimulation, TLR3 is expressed on a more narrow range of cells primarily, but not limited to, hematopoietic myeloid cells. It is likely that these different expression patterns *in vivo* explain the functional differences between the two sensors. The presence of TLR3 on hematopoietic cells suggests that these cells are important for initial detection of viral infection. In this context TLR3 may be necessary to recognize dsRNA from other cells that have been infected instead of by the infected cell itself. This is supported by viral replication experiments in which MDA5, but not TLR3, is necessary to limit viral replication of both MNV-1 (chapter 2) and EMCV (data not shown) in infected dendritic cells *in vitro*. Production of early IFN by TLR3 may then lead to induction of MDA5 on surrounding cells. This could lead to additional IFN production as well as IFN-independent effects of MDA5, such as apoptosis, in infected cells that are

necessary to control infection. However, this paradigm may not occur for all tissues, and it is possible that different organs could have different expression patterns of PRRs.

The differential distribution of viral sensors may also be necessary to combat a variety of different viral tropisms. Even within the picrona- and calici- virus families, that is a broad distribution of viruses that infect different tissues as reviewed in chapter 1. This likely explains the differential importance of MDA5 and TLR3 in control of their infection. Infection of MDA5/TLR3 DKO mice with EMCV results in similar susceptibility compared to mice lacking IFNAR, suggesting that these sensors are mostly sufficient to control this infection. However, infection of MDA5-/- or DKO (data not shown) mice with MNV-1 or CVB(7) results in a much less severe phenotype than IFNAR-/- animals, suggesting that additional antiviral sensors are necessary to for complete control of these infections. This could be related to the production of distinct viral nucleic acid intermediates, but also could represent infection within different cell types that are protected by different sensors. Thus, studying the expression patterns of TLR and RLR family members in different organs and in response to different infection may be necessary to understand their functional roles.

Implications for human diseases

T1DM

Viruses have long been implicated in T1DM. However, a direct causal role in initiation of the disease has never been proven. Recent studies implicating MDA5, a dsRNA sensor involved in detection of viral infection provided hope that such a mechanism may be discovered. In chapter 3 we tested the importance of MDA5 in prevention of virus-induced diabetes. Interestingly, mice lacking MDA5 did not develop diabetes after EMCV-D infection, but rather developed myocarditis. However, mice lacking TLR3 were highly susceptible to diabetes after EMCV infection, although TLR3 has not been implicated in diabetes in mice or humans. Is it possible to make sense of this paradox? Results from human studies suggest a potential explanation. Work by Dotta and others revealed two distinct histological patterns in recent onset diabetic patients, one characterized by T cell infiltrate and the other by myeloid cell infiltrate. In addition, viral antigens were detected in samples containing the myeloid infiltrate, but not the T cell infiltrate, suggesting two potential mechanisms of T1DM- autoimmune and viral. In this model MDA5 is implicated in autoimmune diabetes, while TLR3 may play a role in diabetes initiated by viral infection.

Several studies have implicated MDA5 in autoimmune diabetes. Work by the Todd group identified genetic polymorphisms that both increase and decrease susceptibility to T1DM(8, 9). The mechanism by which these polymorphisms affect T1DM susceptibility remain unclear, however, polymorphisms that result in defective MDA5 IFN production appear to mediate resistance to T1DM. This is interesting because of the known role for type I IFN in autoimmune diseases, and the myriad of

effects type I IFN has on both β cells and the immune response in the islets. In contrast, our results suggest that MDA5 does not play a role in virus-induced diabetes. This was somewhat surprising because MDA5 is known to limit viral replication. One potential explanation is that the severe myocarditis in these animals masked the diabetes phenotype. Indeed, MDA5-/- animals were seen to have increased viral titers in the pancreas as well as increased insulitis upon histological examination compared to WT mice, suggesting that in the absence of myocarditis, diabetes would have developed. This could be directly tested using mice lacking MDA5 only in the β-cells. More importantly, this study did not address the role of MDA5 in autoimmune diabetes. The animals used in this work were on the C57Bl/6 background, which do not develop spontaneous autoimmunity. To properly evaluate the role of MDA5 in autoimmune T1DM, it is necessary to have the mutation on the NOD background either by backcrossing or making the knockout in newly created embryonic stem cells derived from NOD. These mice could then be tested for development of diabetes to determine how a lack of MDA5 affects autoimmunity in these animals.

Data implicating TLR3 in autoimmune diabetes is less supportive than that of MDA5. The primary association was seen using a model of diabetes in which a mouse expressing a transgene for LCMV-GP developed autoimmune diabetes only after the administration of pIC or virus(10). The authors suggested based on these results that TLR3 was important for autoimmune diabetes, although this was never directly tested. However, the existence of MDA5 was not known at that time, and it is likely that the response to pIC was dependent on MDA5 and not TLR3 in these transgenic animals. Moreover, mice lacking TLR3, which were backcrossed to the NOD background

demonstrated no defect in the development of diabetes compared to WT NOD animals(11). In humans, genome wide association studies have also provided no evidence that human TLR3 polymorphisms are implicated in T1DM. Our data, however, reveals a role for TLR3 in virus-induced diabetes. The pancreatic infiltrates seen in TLR3-/- animals closely resemble "pattern B" infiltrates seen by Dotta(12). The question remains, how prevalent is the autoimmune versus virus-induced diabetes? Clinically, there appears to be no difference between pattern A and pattern B patients, the difference was only detected histologically. In addition, this study included only 6 patients, and it is unclear how representative this sample is to the population at large. Recent work from nPOD (network of pancreatic organ donors) has begun to look at the pancreas of diabetic patients on a more global scale. This project contains clinical as well as histological data, so it is possible that there will be clinical correlations to the histological findings that can be used to identify distinctions among T1DM patients. If this is indeed possible, it would be interesting to know whether there are different genetic risk factors between the two populations.

Myocarditis

Research into the mechanism of myocarditis has focused on whether the disease is mediated primarily by viral infection or autoimmunity. Our results with EMCV infection indicate that a virus-dependent mechanism occurs in this system. Increased viral titers in MDA5-/- animals, and to a lesser extent, TLR3-/- animals correlated with increased myocarditis as measured by histopathology and serum troponin levels. We were unable to detect T cell infiltrates in the heart in these animals, however, we cannot rule out

immunopathology mediated by innate cells in this system. It is known that the genetic background influences the response to EMCV infection in mouse models. The mice we used in this study, strain C57Bl/6, develop acute myocarditis in response to EMCV, and it would be interesting to known the effect of MDA5 and TLR3 deficiency in mouse strains which develop chronic myocarditis consistent with autoimmune etiology. It is possible that on those backgrounds MDA5 and TLR3 may contribute to autoimmunity A very recent human study has revealed that TLR3 polymorphisms are associated with viral myocarditis and dilated cardiomyopathy(13). In this study susceptibility to myocarditis correlated with TLR3 alleles demonstrating reduced downstream signaling capacity *in vitro*. This suggests that select cases of human myocarditis correlate with failure to control viral infection, similar to our findings in the mouse system, and may explain the benefit of IFNβ therapy seen in clinical trials.

Gastroenteritis

In our study, we demonstrate that both MDA5 and TLR3 were necessary for complete control of MNV infection. The absence of either sensor resulted in an increase in viral titers, while deficiency of both sensors had a more profound defect (data not shown). It is unclear whether human MDA5 and TLR3 polymorphisms result in differential susceptibility to norovirus infection, but it is likely, based on the mouse data, that inactivating mutations may lead to more severe norovirus infection. Recent findings have demonstrated the importance of both innate immune genes and environmental factors in the pathogenesis of inflammatory bowel disease. Genes involved in autophagy as well as PRRs for bacterial ligands have been implicated in the disease process.

Moreover, the presence of intestinal viruses such as MNV has been suggested to be important for the full development of the disease phenotype in genetically susceptible animals(14). In this model, genetic factors do not directly contribute to disease pathology in the absence of an environmental stimulus, but when both environmental and genetic factors are present, disease pathology results. It is possible that by regulating the level of viral infection in the gastrointestinal tract, polymorphisms in MDA5 and TLR3 could contribute to development of inflammatory bowel disease by altering the environmental components of the disease. This remains to be investigated.

Implications for immune-targeted therapeutics

Stimulation with pIC activates both the MDA5 and TLR3 pathways, leading to cytokine production and immune activation. We demonstrate that activation through the two sensors has unique properties, which may be useful for the targeting of therapeutics. Work by the Steinman group has shown that pIC is a very strong adjuvant that leads to robust activation of a T cell response in the context of a tumor vaccine(15). However, work from our lab has shown that pIC stimulation of MDA5 and TLR3 has distinct effects on the T cell response. While TLR3 is necessary for a robust primary response, MDA5 is critical for the memory T cell response(16). These results suggest that targeting pIC to the correct hematopoietic or stromal compartment may help to ensure the desired T cell response in the context of vaccination.

Cell-specific targeting of pIC may also be useful in cancer therapy. There has been much effort towards the development of cancer vaccines that would activate the immune response to attack tumor cells. To date, this strategy has been largely

unsuccessful. There is hope that the use of pIC will boost the effectiveness of this therapy. PIC has several beneficial effects on the immune response to tumors. It activates NK cells, which can kill transformed cells; it activates antigen presenting cells, priming the T cell response; and it activates T cells directly. By targeting pIC treatment directly to specific immune cell types it may be possible to enhance the anti-tumor immune response. In addition, pIC has a direct anti-tumor role in some cancers. Studies of breast and liver cancers demonstrate that tumor cells that express TLR3 are susceptible to apoptosis upon pIC treatment(17, 18). More recent evidence also implicates MDA5 in susceptibility to pIC. Many melanomas upregulate MDA5 and are thus highly susceptible to apoptosis upon delivery of pIC intracellularly. This effect was partially dependent on MDA5-mediated IFN production, but partially independent of IFN(19, 20), suggesting that MDA5 plays a direct role in apoptosis. Further studies are necessary to determine whether MDA5 is upregulated in other types of cancers and if it can be exploited. These exciting advances suggest that the ability of pIC to activate both anti-tumor immunity and direct tumor cytotoxicity make it a potentially useful therapeutic agent.

Conclusions

In this work we have shown that the dsRNA sensors MDA5 and TLR3 play distinct roles in controlling viral infection. These sensors have unique cellular and tissue expression profiles and stimulate production of distinct cytokine patterns when exposed to their ligands. The ability of these sensors to recognize viral infection and initiate production of interferon and inflammatory cytokines, both of which may play a role in the development of autoimmunity, suggests that alteration of their activity could lead to human disease. Indeed, polymorphisms in the genes encoding MDA5 and TLR3 have been associated with T1DM, myocarditis, and encephalitis, and we are beginning to understand the mechanisms by which these diseases develop. In addition to understanding the underlying disease mechanisms caused by these genes, it may be possible to use our understanding of tissue distribution and receptor-specific signaling pathways to target these pathways for human therapeutics. Knowledge of MDA5 and TLR3 function may lead to important advances in vaccines, autoimmunity, and cancer therapy. We hope that this work will lead to further discoveries in these fields.

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2. **McCartney S,** Vermi W, Gilfillan S, Diamond M, Colonna M. Timing of type I IFN production is critical for the development of virus-induced diabetes and myocarditis. Abstract for poster presentation. *NHLBI Physician-Scientist Trainee Conference.* May 10, 2010, Bethesda, MD.

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