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The Molecular Mechanism of Host Responses to Viral Infection

By
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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

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Professor Laurent Coscoy
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Professor Hei Sook Sul

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The Molecular Mechanism of Host Responses to Viral Infection

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By

Suruchi Nandu Schock
Abstract

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University of California, Berkeley

Professor Astar Winoto, Chair

All living organisms, including humans, are constantly under attack by various pathogens such as viruses. Activation of appropriate host innate immune pathways like interferon and/or cell death is often critical for efficient viral clearance. Using biochemical and immunological methods, I have studied these two aspects of the antiviral response. I initially examined the role and regulation of the virus-induced host protein Tripartite Motif Containing Protein 21 (TRIM21) and the adaptor molecule Fas-Associated Death Domain (FADD) in the context of RNA virus infection. I found that TRIM21 functions in concert with FADD to negatively regulate ubiquitination of the transcription factor IRF7, thereby functioning in a negative feedback loop for the viral-induced interferon response. I have also identified the presence of a novel complex consisting of FADD, TRIM21 and RIP1 where TRIM21 and RIP1 regulate each other’s ubiquitination status. FADD and RIP1 have been recently implicated in an alternative form of programmed cell death: necroptosis, whose physiological function is not completely clear but it has been suggested to serve as a backup host pathway to fight viral infection. To investigate this possibility, I have screened seven viruses for their ability to induce necroptosis. I found two of them, Sendai virus and MHV68, are capable of inducing necroptosis, particularly in conditions when apoptosis is blocked. I found that MHV68 activates the cytoplasmic sensor molecule STING, leading to production of tumor necrosis factor (TNF) and subsequently causing necroptotic death. In contrast, Sendai virus induced death occurs independently of TNF or the adaptor STING. Instead, Sendai virus-mediated necroptosis requires the RNA sensor RIG-I in conjunction with the deubiquitin protein CYLD and several Sendai virus proteins, leading to de-ubiquitination of RIP1 and formation of RIP1/3 necrosome to promote necroptosis. These data are consistent with the notion that necroptosis may be an additional antiviral mechanism that hosts can employ when apoptosis is blocked. Necrotic cells can then release inflammatory contents which may help alert the immune system. These findings offer insight into the complex host-pathogen relationship, and with continued study may help guide the judicious development of antiviral drugs and vaccines.
To my mom,

Who inspired my curiosity and encouraged and supported all my passions
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Chapter 1: General Introduction

“An inefficient virus kills its host. A clever virus stays with it.”
Dr. James Lovelock

The Innate Immune Antiviral Response

General Overview

The relationship between host cells and viruses can be thought of as an intricate game of chess in which each player makes a move that the other must acknowledge and subsequently respond to in a quick and efficient manner. The host employs a number of antiviral strategies in the hopes of winning this match; preventing the spread of and eventually eradicating the virus. The virus counters each of these moves with the hope of evading the host’s antiviral attempts and elongating the match into a stalemate, thereby allowing the virus to maintain its parasitic existence for as long as possible.

To yield a successful infection, a virus must recognize, bind, and garner entry into its target cells. In addition to nonspecific interactions with heparan sulfate or other carbohydrates found on cells, receptors on viral envelopes mediate specific attachment to complementary receptors found on susceptible cells. Once attached, viral entry occurs through membrane fusion, endocytosis, or injection of genetic material into the cell [1]. These entry processes utilize viral proteins as well as naturally occurring cellular pathways such as receptor mediated endocytosis for their completion. Often, viral particles must traffic to appropriate sites within the cell to carry out replication. Once there, transcription, translation, and replication of the viral genome yield the production of fully assembled viral particles. Release of these virions through membrane budding or cell lysis facilitate the infection of subsequent cells and promote viral spread.

Left unchecked, viral infection can cause detrimental immunopathology of the organism or even worse, death. Activation of the immune response is critical in limiting this spread and for efficient viral clearance. This includes the initial triggering of innate immune mechanisms from primary infected cells, such as production of antiviral cytokines (e.g. Interferonα/β). These cytokines are responsible for the up-regulation of antiviral proteins, enhancement of antigen processing pathways and MHC expression, and the overall maintenance of an “antiviral” state. Moreover, the production of chemokines and pro-inflammatory cytokines allow for the recruitment and activation of immune cells such as natural killer (NK) cells, macrophages and dendritic cells to the site of infection. These cells use a variety of mechanisms including cytotoxicity, phagocytosis, antigen presentation, and secretion of pro-inflammatory cytokines to exert direct and indirect antiviral effects during an infection. The trafficking of dendritic cells to draining lymph nodes instigates the activation of the adaptive immune response. T cells are activated and antiviral antibodies are eventually produced. While it is clear that innate immune mechanisms are vital for initial restriction of viral growth, the coordinated efforts of the innate and adaptive arms of the immune system offer superior defense.
Intrinsic Innate Immune Recognition of Viral Infection

Immune cells possess the ability to recognize and respond to viral infection through the production of pro-inflammatory cytokines, and chemokines. This is accomplished through germline-encoded receptors which recognize conserved viral traits (e.g. DNA/ RNA). The best studied of these receptors are the Toll-like receptors (TLRs). TLRs are transmembrane proteins found on the plasma membrane (TLRs 1, 2, 4, 5, and 6) or on endosomal membranes (TLRs 3, 7, 8, and 9) of cells. Each member is activated by a unique pathogen-associated molecular pattern (PAMP), with most endosomal members reacting to viral nucleic acids. Studies show that TLR3 responds to double stranded RNA, both as a common intermediate of viral replication as well as the synthetic analog poly(I:C) [2, 3]. This receptor is important in antiviral immunity, as patients with a dominant negative mutation for TLR3 exhibit severe herpes simplex virus 1 (HSV-1) infection resulting in encephalitis [4]. TLR7 is another endosomal-resident receptor which responds to long ssRNA molecules. TLR7 is abundant in plasmacytoid dendritic cells and along with TLR9, responsible for production of large amounts of IFNα upon viral infection [5].

Once engaged, TLRs activate signaling pathways through the use of two main adaptors: MyD88 and/or TRIF. All TLRs, with the exception of TLR3, are able to bind MyD88 and begin signaling cascades which result in the activation of nuclear factor kappa b (NF-κB), mitogen activated protein kinase (MAPK), and interferon regulatory factors (IRF) proteins. Alternatively, TLR3 (and TLR4) use TRIF to directly bind to TRAF6 and TBK1 for activation of NF-κB and IRF3/7, respectively. These transcription factors then go on to promote expression of antiviral proteins, pro-inflammatory cytokines and chemokines.

In addition to membrane bound TLRs, which are important in sensing virus from extracellular compartments, cells contain intracellular cytoplasmic receptors. These receptors are responsible for detecting virally derived nucleic acids generated during infection and replication of the virus in the cytoplasm. The best studied of these receptors are the RIG-I-like Receptors (RLRs). This DExD/H-box subfamily of helicases include: retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 contain two N-terminal CARD domains important in downstream signaling through adaptor molecule MAVS (IPS-1/VISA). This domain is followed by an RNA helicase domain, which is present in the case of RIG-I, and is dependent on ATP binding for its function. Finally, RIG-I contains a C-terminal repressor domain responsible for holding it in an auto-inhibited conformation until ligand binding. The third receptor of this family, LGP2, contains a helicase domain and lacks the CARD domain. While the importance of RIG-I and MDA5 in antiviral immunity to various viruses is well established, the generation of LGP2 deficient mice indicate contrasting regulatory roles for LGP2 depending on the infecting virus [6].

Analysis of RIG-I and MDA5 deficient animals reveal that each receptor is responsible for responding to unique RNA viruses. RIG-I is required for efficient recognition of Sendai virus, Newcastle disease virus, Influenza A and B, and vesicular stomatitis virus. In contrast, MDA5 has been reported to be necessary for recognition of encephalomyocarditis virus (EMCV), Theiler’s virus, and mengo virus [7, 8]. Until recently, the specific ligands for these receptors remained unclear. It is now generally accepted that the ligand of RIG-I is a short, blunt end, double-stranded RNA (dsRNA) molecule. This RNA can also contain a 5’ triphosphate [9, 10]. Moreover, studies show that the molecular species recognized by MDA5 is a long dsRNA molecule [11]. These are distinguished from cellular RNAs, which are capped and primarily single-stranded. Upon ligand binding, RIG-I and MDA-5 undergo conformational changes and begin a signal transduction pathway utilizing the mitochondrial-associated protein.
MAVS. This signaling cascade results in activation of NF-κB, MAPK, and IRFs. It was recently reported that many of the proteins recruited downstream of RIG-I/MAVS share similarity with those recruited downstream of the death receptor TNFR [12], suggesting an interesting mode of regulation as well as cross-talk between these disparate cellular responses. In addition to TLRs and RLRs, recent advances have identified a slew of new cytoplasmic sensors. These include RNA-sensing DDX and DHX proteins, protein kinase R (PKR), and members of the Interferon-induced protein with tetratricopeptide repeats (IFITs) family. These proteins have been reported to bind viral RNA or its synthetic analog poly(I:C), and/or stimulate IFN responses [13]. Stimulator of interferon genes (STING) is a recently acknowledged ER-localized sensor identified for its ability to induce interferon-β production upon overexpression [14]. It has been reported to become active in response to RNA viruses, dsDNA and cyclic-dinucleotides. In the case of RNA viruses, STING has been suggested to function through an interaction with RIG-I. In fact, murine embryonic fibroblasts lacking STING show reduced IFN-β production upon infection with the single-stranded RNA virus, Sendai virus [15]. RNA sensing is a critically important host immune advantage as both RNA and DNA viruses are capable of activating RNA sensors. Both families of virus undergo an RNA stage at some point during replication and based on localization, secondary structures, and differential modifications, allow cells to distinguish between RNAs of cellular or viral origin. In some instances, AT-rich dsDNA can even activate RIG-I through production of 5’ triphosphate containing RNA molecules by host protein RNA Polymerase III [16]. Although much of the work on pathogen recognition has focused on the identification and characterization of RNA sensors or TLRs, several studies suggest the presence of cytoplasmic DNA receptors responsible for inducing IFN responses [17, 18]. Accordingly, a number of putative cytoplasmic DNA sensors have been recently reported.

One of the first putative cytoplasmic DNA sensors to be discovered was DNA-dependent activator of IFN-regulatory factors (DAI). DAI is able to bind DNA and loss of it in L929 cells results in diminished interferon activation. In addition, DAI was shown to promote interferon production by interacting with IRF3 and TBK1 [19]. Although these data as well as a few other studies suggest an important role for DAI in DNA sensing, DAI deficient animals lack a significant defect in response to DNA vaccination [20]. Thus, the prevailing notion is that redundancy within the DNA sensors must be what accounts for the discrepancies seen between in vitro and in vivo data. In addition, another family of intracellular receptors has recently been suggested to play a role in antiviral immunity: the Nod-like receptors (NLRs). The primary function of NLRs is their ability to assemble into large multimeric protein complexes, termed inflammasomes. These complexes lead to caspase-1-dependent processing of cytokines IL-1β and IL-18 as well as the non-classical cell death response of pyroptosis. Although inflammasome activation has been studied largely in response to intracellular bacterial infection, several lines of evidence support the notion that inflammasomes may form in response to viral infection [21]. Infection of multiple RNA and DNA viruses, including Sendai virus, Influenza, varicella-zoster, and Kaposi’s sarcoma-associated herpesvirus (KSHV), result in inflammasome assembly, caspase-1 activation, or IL-1β/IL-18 secretion [22-24]. Two additional DNA sensors, Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) and IFI16 have been implicated in sensing of viral DNA; however further characterization is needed to solidify their role in this setting [25, 26].
**Virus-induced Interferon Production**

Regardless of which pattern recognition receptor is utilized for detection of a virus, cells are able to trigger appropriate signaling pathways resulting in global gene-expression changes. The major consequence of these changes is the production and secretion of chemokines and pro-inflammatory cytokines (e.g., TNFα, IL-6, IFN). As discussed briefly above, the production of these molecules assist with the recruitment and activation of immune effector cells. The major cytokine expressed early during viral infection is interferon (IFN).

Named for its ability to “interfere” with viral replication, three classes of IFN have been discovered, each classified by the receptor it signals through [27]. The most recently discovered Type III IFNs include three IFNλ subtypes which signal through a receptor complex of IL10R2 and IFNLR1. Although relatively novel, these IFNs have been reported to regulate antiviral responses similarly to their Type I and Type II counterparts [28, 29]. IFNγ comprises the only member of the Type II class of IFN. It is produced primarily by activated T cells and NK cells and signals through an IFNγ receptor complex. Though many of the cellular responses modulated by IFNγ overlap with those of Type I IFNs, IFNγ is critically important in skewing the immune response towards a Th1 character (innate-immunity driven phenotype). Type I IFNs are the largest class of IFN and are comprised of multiple IFNα members, and a single IFNβ, IFNκ, IFNε, IFNo, IFNτ, and IFNδ. All Type I IFNs signal through the interferon-alpha receptor (IFNAR).

Rapid production of Type I IFN is one of the earliest hallmarks of viral infection. As previously discussed, recognition of viral features by the cell’s pattern recognition receptors activate NF-κB and IRF3 transcription factors. These factors translocate to the nuclease and mediate expression of IFNβ, the first Type I IFN produced. Secreted IFNβ then binds to its cognate receptor (IFNAR) on the cell surface. This occurs on both the IFNβ producing cell as well as on neighboring cells. It is the ability of uninfected neighboring cells to respond to IFNβ that allows them to activate an “antiviral state”; upregulating antiviral proteins, MHC proteins, and preparing for potential viral infection. After the binding of IFNβ to its receptor, the downstream activation of the Jak/STAT pathway results in the expression of a multitude of antiviral genes. One such gene is the transcription factor, IRF7. In a positive feedback loop for Type I IFN, IRF7 augments the expression of IFNα/β which then signals to further increase expression of IFN stimulatory genes. Other notable antiviral genes upregulated upon IFNα/β production include PKR, 2’-5’ oligoadenylate synthetase (OAS) and Mx proteins. The functions of these genes range from protein synthesis inhibition and viral RNA degradation to cell death induction [30, 31].

**Regulation of Type I Interferon**

The importance of Type I IFNs, particularly IFNα/β, in antiviral immunity is well established. Type I IFN is indispensable for control of viral infection as evidenced by Type I IFN Receptor (IFNAR−/−) knockout mice. These mice, which are unresponsive to IFNα/β, succumb quickly to viral infection, despite having an intact adaptive immune response [32]. While the induction of cytokines is critical for resistance against viral infection, unregulated expression can yield adverse effects. Aberrant activation of IRFs or production of Type I IFN and pro-inflammatory cytokines have been implicated in the development of autoimmune diseases such as systemic lupus erythematosus [33]. Thus, strict regulation over the induction and duration of
these responses is vital in mounting an effective immune response while maintaining cell homeostasis.

Several studies have found activation of the Ras/Raf/Mek pathway to negatively regulate IFN-induced gene expression [34-36]. Cells permit viral replication even in the presence of IFN due to Ras/Mek-mediated reduction in STAT2 levels [35]. Additionally, IRF3 can be targeted by host proteins Pin1, MafB, and TRIM21 for negative regulation [37, 38]. Moreover, our lab has shown that IRF7 is able to be targeted by a member of the Tripartite-Motif Containing (TRIM) Family of proteins, TRIM21. In concert with Fas-associated death domain (FADD), TRIM21 is able to target IRF7 for ubiquitin-mediated degradation post-infection, thus effectively downregulating IFN production [39].

In addition to host regulation of anti-viral pathways, viruses also manipulate these pathways for their own benefit. For example, poxviruses encode soluble versions of IFN receptors which are able to neutralize IFN from acting on functional cell surface receptors [40]. Sendai virus C proteins are also able to counteract Jak/STAT signaling and prevent the initiation of an antiviral state [41, 42]. Alternatively, viruses encode proteins to target IFN-induced genes like PKR [43]. Programmed cell death, or apoptosis, is another common mechanism to restrict viral growth. Accordingly, a number of viruses have been shown to encode proteins known as viral FLIPs. These proteins contain a death effector domain (DED), which allow them to bind other DED-containing proteins like FADD or caspase-8, thereby disrupting apoptotic signaling [44, 45]. As we continue to uncover new viral proteins we will likely find novel ways in which viruses are able to manipulate host pathways.

Cellular Death Pathways

Apoptosis

Apoptosis describes a regulated form of cellular suicide characterized by membrane blebbing, nuclear condensation, and caspase activation [46]. The intracellular components of the cell are maintained within a membrane to be phagocytosed and cleared without aberrant activation of the immune system. Defects in apoptosis cause altered organismal development, the survival of autoreactive lymphocytes, and inefficient restriction of pathogens. Two pathways exist to mediate apoptosis: the intrinsic and extrinsic. The intrinsic pathway involves the transduction of signals through the mitochondria leading to the release of cytochrome C and subsequent activation of caspasers. The Bcl2 family of proteins play a role in this intrinsic pathway [47]. In contrast, the extrinsic pathway of apoptosis is initiated through a diverse group of receptors belonging to the Tumor Necrosis Factor Receptor (TNFR) superfamily.

The prototypic example of a death receptor is FasL/Fas system. Upon ligand binding, a death-inducing-signaling complex (DISC) assembles. Homotypic interaction between the death domains of Fas and adaptor molecule FADD allow for its recruitment. The death effector domains of FADD and caspase-8 mediate their interaction, thus completing DISC formation. This then allows for the downstream activation of effector caspases and cellular demise [48]. While the primary function of FasL/Fas is to signal apoptosis, the TNF/TNFR system is able to promote both cell survival and cell death. After binding of TNF to its receptor, the formation of a receptor-associated “complex I” ensues. This includes adaptor molecule TRADD as well as RIP1, cIAPs and TRAFs. cIAP mediated ubiquitination of RIP1 enables the recruitment of
downstream kinases and effector molecules to activate NF-κB and MAPKs [49]. Signaling through this complex promotes cell survival. Internalization of the receptor leads to the formation of cytoplasmic “complex II.” This complex now forms the familiar DISC consisting of deubiquitinated RIP1 and the recruitment of FADD and caspase-8 [50]. Signaling through this complex yields apoptotic death (Figure 1.1). Association of the NF-κB driven protein, cFLIP, with complex II allows apoptosis to be inhibited during normal basal states. However, when NF-κB activity is turned off, synthesis of cFLIP is stopped and complex II can become active, giving way to apoptosis [51].

In addition to apoptosis, other forms of cellular death have been described. For instance, autophagy has been described as a degradative process for the recycling of dysfunctional organelles or other cellular components by lysosomes. It is mediated by Atg proteins and occurs through formation of vesicles known as autophagosomes [52]. Pyroptosis, in contrast to apoptosis, is an inflammatory type of cell death. Triggered by microbial infection or various pathological stimuli, this caspase-1-dependent death response causes rupture of the cell and release of pro-inflammatory contents [53]. Finally, another form of inflammatory death is that of necrosis or oncosis. Considered an unregulated and passive form of death, it occurs in response to cellular stresses or cell injury. This causes organelle swelling and permeabilization of the membrane. The subsequent leakage of intracellular contents acts as pro-inflammatory agents, thus recruiting immune cells and initiating an inflammatory response.

**Necroptosis**

Recently, a non-classical form of programmed cell death has been defined and termed necroptosis, or programmed necrosis. Although morphologically similar to necrosis, necroptosis is a regulated form of death initiated through death receptor ligation, DNA damage, or infection. Often considered a “backup” mechanism, this form of death prevails when apoptosis has been inhibited. Thus, many of these studies involve disrupting apoptotic signaling or inhibiting caspase-8 using the small molecule inhibitor zVAD.fmk [54]. Necroptosis involves the kinase activity of RIP1 and its family member RIP3. Treatment with small molecule Necrostatin-1, an inhibitor or RIP1 kinase activity, rescues RIP1 dependent necroptosis. Execution of this form of death causes the production of mitochondrial reactive oxygen species (ROS) and permeabilization of mitochondrial, lysosomal and plasma membranes. Hence, in contrast to apoptosis, programmed necrosis is considered an inflammatory type of death. Although earlier observations of TNF treatment or caspase inhibition inducing necrotic morphology were made, it was not until 2003 that Chan et. al introduced the term “programmed necrosis” to describe the non-apoptotic form of death requiring RIP1 and occurring downstream of multiple death receptors (e.g. TNFR-1, Fas, TRAILR) [55, 56].

Over the last decade a wealth of knowledge has been gained regarding the inducers of necroptosis, the proteins involved, and its role in various disease states. The most extensively studied trigger of necroptosis is the TNF pathway. Ligation of TNF to its receptor can result in either NFκB-mediated cell survival signals or caspase-dependent apoptosis (as described previously). However, in cases of caspase-8 depletion, or inhibition by either pharmacological drugs or viral proteins, recruitment of RIP3 leads to formation of a third complex known as “complex IIb” (or the necrosome) [57]. RIP1 and RIP3 contain an N-terminal kinase domain and a C-terminal RIP homotypic interaction motif (RHIM). Interaction between the two is mediated through their RHIM domains. The mutual phosphorylation of RIP1 and RIP3 via their kinase domains is essential for executing necroptosis (Figure 1.1) [58]. As a recently described
phenomenon, the downstream signaling events leading to execution of necroptosis are only now beginning to be uncovered. Recently, it was discovered that RIP3 dependent phosphorylation of the mitochondrial protein, mixed lineage kinase domain-like protein (MLKL), and in certain cells, the further recruitment of PGAM5 and Drp1, are required to begin the execution phase of this death [59, 60].

**Regulation of Necroptosis**

Many proteins are involved in mediating the transition between cell survival, apoptosis and necroptosis. Here we describe several proteins and the capacities in which they regulate necroptosis. FADD is an adapter molecule, critical for signaling downstream of all death receptors. Loss of FADD renders embryonic lethality of mice. This lethality is rescued upon additional loss of RIP1 indicating that during development FADD holds aberrant necroptotic cell death at bay [61]. In response to TCR stimulation, FADD deficient T cells are unable to clonally expand, instead undergoing premature necroptosis [62]. Additionally, dendritic cell specific deletion of FADD results in necroptotic loss of these cells and systemic inflammation triggered by the microbiota [63]. Thus, FADD appears to antagonize necroptosis in most cases studied. Curiously, FADD deficient MEFs are resistant to necroptosis, suggesting that FADD may in fact play alternate roles dependent on cell type [64]. Similarly to FADD, caspase-8 deficiency yields embryonic lethality of mice, which is rescued upon RIP3 loss [65]. Additionally, caspase-8 loss in intestinal epithelial cells yields higher susceptibility to colitis development which could be rescued with RIP3 deletion [66]. The negative regulation of necroptosis appears to be due to caspase-8 cleavage of RIP1 and CYLD proteins [55, 67]. CIAP and CYLD proteins play opposing roles in regards to RIP1; cIAPs promote ubiquitination while CYLD removes ubiquitin chains. The ubiquitin status of RIP1 modulates whether it is involved in cell survival, apoptosis or necroptosis promotion, with the latter two occurring upon deubiquitination. Thus, CYLD has become an important promoter of necroptosis [68, 69]. Alternatively, depletion of cIAP proteins through addition of IAP antagonists greatly sensitizes cells to TNF-induced necroptosis [69]. Thus, it is clear based on the above data and accumulating evidence that many of the same proteins involved in cell survival and apoptosis play the additional role of regulating necroptosis. As additional triggers of apoptosis and necroptosis are identified, we will likely begin to uncover new proteins and pathways required for maintaining the balance between survival and these distinct forms of death.

**Emerging Triggers of Necroptosis**

Necroptosis has been largely characterized downstream of the TNFR, however numerous other triggers are emerging. In addition to the TNF receptor, the death receptors Fas and TRAILR can also initiate necroptosis [70, 71]. In response to DNA damaging agents, Tenev et. al. reported the assembly of a large 2MDa size complex referred to as the “rioptosome.” This complex can also stimulate caspase-independent necrosis [72]. Recently, activation of TLR3 and TLR4 by poly(I:C) and LPS, respectively, has been shown to trigger necrotic death of macrophages. Both of these TLRs recruit Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF), which can directly bind RIP1 or RIP3 via their RHIM domains [73]. As previously mentioned, T cell receptor stimulation can yield necroptosis in the absence of caspase-8 or FADD [62, 74].
Although mostly studied in contrived situations, cell death is a physiologically relevant mechanism to contain pathogen spread. Thus the study of necroptosis induction upon pathogen infection has seen a recent rise. Vaccinia virus (VV) encodes B13R, an inhibitor of caspases. This caspase inhibitor is required for VV pathogenesis, however it also results in sensitization of cells to TNF-induced necrosis. In agreement with necroptosis acting as a host protective antiviral response, RIP3\textsuperscript{\textminus/\textminus} mice succumb to VV infection due to higher viral titers than their wildtype counterparts [58]. Coincidentally, the herpesvirus MCMV, has been found to encode for a necroptosis inhibitor protein, which when mutated results in attenuated infection [75]. Interestingly, unlike VV necroptosis, which promotes RIP1-RIP3 dependent necroptosis, infection with the mutant MCMV results in necroptosis through activation of the DNA sensor DAI. DAI does so through homotypic interaction with RIP3 via their RHIM domains [76]. Even reovirus and influenza virus have been reported to promote necroptosis under the right conditions [77, 78]. However, whether necroptosis is always a benefit for the host is unclear. In certain cases it appears that this form of death may actually enhance detrimental pathology without affecting viral load or spread. Thus, accumulating evidence from viral studies suggest that necroptosis may play an important role in innate immune responses, but it remains to be determined what the viral and pathological outcomes for the host would be. Other than viral infection specifically, interferon cytokines have also been implicated in necroptosis induction. Robinson et. al report that infection of macrophages with the bacterial pathogen Salmonella Typhimurium causes Type I IFN dependent necroptosis [79]. Thapa et. al, report that IFN-induced necroptosis is due to the upregulation of PKR and its interaction with RIP1 [31]. Given the diversity of signals able to elicit necroptosis it will become interesting to find at what point these different signals converge to induce necroptotic signaling and whether the execution mechanisms are the same regardless of initiating upstream signal.
Figure 1.1. Cell survival and cell death pathways originating from TNFRI.

The ligation of TNF to its receptor mediates the assembly of Complex I which consists of the receptor, TRAF2/5, TRADD, cIAPs, and RIP1. cIAPs facilitate the K63 linked ubiquitination of RIP1. Signaling through this complex results in NF-κB and MAPK activation and promotes cell survival. Internalization of Complex I and CYLD-mediated deubiquitination of RIP1 allows for recruitment of FADD and caspase-8 and formation of the death-inducing signaling complex (DISC). This complex results in apoptotic signaling. In cases of FADD or caspase-8 deletion or inhibition (through zVAD.fmk), RIP3 is recruited. Auto and trans-phosphorylation of RIP1 and RIP3 forms the necrosome (Complex IIb) inducing necroptosis. Necrostatin-1 (Nec-1) inhibits RIP1 kinase activity thereby preventing necroptosis signaling from occurring. Figure adapted from Han et. al. [80].
Chapter 2: The Mutual Regulation of Ubiquitination by RIP1 and TRIM21

Introduction

Infection with viruses leads to the activation of numerous host antiviral pathways which are critical for efficient viral clearance. One such response is the production of Type I interferon (IFNα and IFNβ). Type I IFN is indispensable for viral control as Type I IFN Receptor (IFNAR−/−) knockout mice rapidly succumb to viral infection despite having an intact adaptive immune system [32]. Viral activation of the ssRNA sensor RIG-I activates signaling complexes responsible for the phosphorylation and activation of interferon regulatory factors (IRFs). After phosphorylation and dimerization, these transcription factors translocate to the nucleus to mediate Type I IFN production. Although the induction of cytokines is critical for resistance against viral infection, aberrant activation of IRFs or production of Type I IFN has been implicated in the development of autoimmune diseases such as systemic lupus erythematosus [33]. Thus, strict regulation over the induction and duration of the IFN response is vital in mounting an effective immune response and maintaining homeostasis.

Fas-associated death domain (FADD) is a multifaceted adaptor molecule primarily involved in the transduction of apoptotic signals from members of the TNFR family of death receptors. However, it has recently been implicated in both the induction and termination of antiviral IFN responses. Activation of RIG-I leads to recruitment of FADD and RIP1 via adaptor molecule TRADD, which is crucial in activation of IFN responses [12]. Loss of FADD or RIP1 yields cells sensitive to viral infection due to defects in dsRNA-activated gene expression [81]. Additionally, overexpression of RIP1 can mediate activation IRF7 [82]. Thus FADD and RIP1 appear to contribute to the induction phase of IFN production. In contrast, we recently reported the identification of a novel interaction between FADD and Tripartite Motif Containing Protein 21 (TRIM21) which is responsible for negatively regulating IFNα after viral infection [39].

TRIM21 (aka Ro52) is a member of the tripartite motif family of proteins (TRIM). This family consists of over 70 members that are defined by the presence of three characteristic domains: RING finger, B-box, and Coiled-coil. TRIM21 is expressed in many tissues with the highest expression found in immune cells. Its expression can be further upregulated upon IFN treatment or viral infection [83, 84]. Like many of its family members TRIM21 is a functional E3 ubiquitin ligase. It is this ubiquitin ligase activity that underlies its ability to target transcription factor IRF7 for ubiquitin-mediated degradation, thereby downregulating the IFNα response. In addition to IRF7, TRIM21 has also been reported to target cell cycle protein p27, IRF3, IRF8 and itself for ubiquitination [85-88]. Although TRIM21 is capable of targeting multiple proteins, it remains unclear how TRIM21’s ligase function is activated as well as how it recruits appropriate target molecules.

E3 ubiquitin ligases function primarily by catalyzing the addition of ubiquitin molecules to a target protein. Based on the length and linkage variation of these ubiquitin chains, it promotes the signaling or degradation of these proteins. In addition to target proteins, many ubiquitin ligases also promote self-ubiquitination. It has recently been appreciated that this auto-ubiquitination may be functionally significant. It has been reported that auto-ubiquitination of Mdm2 enhances its ability polyubiquitinate p53, while BCA2 auto-ubiquitination affects its own protein stability [89, 90]. Thus, we postulated that TRIM21 activation may also be regulated through its auto-ubiquitination status.
Here we report that FADD enhances K63 linked auto-ubiquitination of TRIM21 while RIP1 counteracts this enhancement. We find that FADD and TRIM21 can be found in complex with RIP1 or IRF7, but that these complexes are distinct from each other. Thus we hypothesized that FADD enhanced auto-ubiquitination of TRIM21 may activate TRIM21 or mediate the recruitment of IRF7. Conversely we believed that RIP1 could negatively regulate TRIM21 function. However, we find TRIM21 auto-ubiquitination to play a nonessential role in its ligase activity or recruitment of IRF7. Surprisingly however, we observe that RIP1 not only affects TRIM21 ubiquitination, but conversely TRIM21 downregulates RIP1 ubiquitination. Although this deubiquitination does not affect the ability of cells to undergo necroptosis, we propose that TRIM21 may regulate RIP1’s ability to activate NF-κB or undergo apoptosis.
Materials and Methods

Cells and reagents – 293T and L929 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine, and Na pyruvate. The following antibodies were used for Western Blot and Immunoprecipitations: anti-Ro52 and anti-FADD M-19 (Santa Cruz), anti-FLAG M2 (Sigma), anti-RIP1 and anti-hFADD (BD Biosciences), anti-HA (Abcam), anti-GAPDH (Cell Signaling), anti-IRF7 (Invitrogen), anti-TRIM21 (Proteintech). zVAD.fmk was purchased from Enzo Life Sciences. Recombinant murine TNFα was purchased from eBioscience. Sendai virus strain Cantell was obtained from Charles River Laboratories.

Constructs – The expression plasmid pCDNA3-HA-TRIM21 was a gift from Dr. Gemana Meroni. TRIM21 was cloned in pCI and pCI-FLAG-STR. The MSCV2.2-IRF7-FLAG construct was a gift from Dr. Gregory Barton. IRF7 was cloned into pCI and pCI-HA vectors. RIP1 cDNA was obtained from Open Biosystems and cloned into pCI. pCS2-Ub-His, pCS2-Ub K48R-His, and pCS2-Ub-K63R constructs were a gift from Dr. Michael Rape.

Transfections – 293T cells were seeded into 6 well plates at 2x10^5 cells/ml in Antibiotic-free media. The following day, transfections of cells were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol.

Cell lysis and Co-immunoprecipitations – Cells were lysed on ice with 1% Nonidet P-40, 50mM Tris-Cl at pH7.6, 150mM NaCl, 1mM EDTA, and 10% glycerol and supplemented with protease and phosphatase inhibitors (1mM Na3V04 and NaF, 1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 5μg/ml pepstatin, 10μg/ml aprotinin, and leupeptin). Immunoprecipitations were performed by incubating cell lysates with the indicated antibody for 2 hours followed by Protein A-immobilized or Protein G-immobilized beads (Pierce) or ANTI-FLAG M2 Affinity Gel (Sigma) addition and further incubated (1 hour to overnight) at 4° C. Immunoprecipitates were washed with buffer, resolved by SDS-PAGE and analyzed by Western blotting. For experiments with Sendai virus, 24 hours post-transfection, cells were infected with 50 HA U/ml and incubated for 18 hours before lysis as described above. Alternatively, cell lysates were incubated with RIP1 antibody or IgG2a control antibody (UCSF Monoclonal Antibody Core) for 2 hours before addition of Protein A beads for an additional 1 hour. All samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 4% BSA-TBST and incubated with indicated primary and secondary antibodies before visualization by Super Signal West Pico Chemiluminescent Substrate (ThermoScientific).

Luciferase Assay- 293T cells were transfected with MSCV-Flag-IRF7, IFNα4-luciferase, and renilla constructs. They were also transfected with pCI, hTRIM21, Flag-FADD, and/or RIP1. 24 hours post-transfection, cells were infected with 50HA U/ml of SeV. 18 hours post-infection, cells were harvested and luciferase assay performed using Dual-Luciferase Reporter System (Promega) as per the manufacturer’s instructions.

Determination of Cell Death
L929 cells were settled into a 96well plate and transfected with 50ng/well of empty vector control or FLAG-TRIM21 using Lipofectamine 2000 as per the manufacturer’s protocol.
24 hours, cells were pretreated with zVAD.fmk at 10uM for 1 hour and then treated with TNFα at 10ng/ml or infected with Sendai virus at 10 HA U/ml for an additional 18 hours. Cell survival was monitored using CellTiter-Glo Luminescent Cell Viability Assay (Promega) as per the manufacturer’s protocol.
Results

FADD, TRIM21 and RIP1 interact together

The interaction between the adaptor molecule FADD and the Receptor-interacting serine/threonine protein kinase1 (RIP1) is well documented, particularly for the induction of cell death. However, our lab recently identified a novel non-apoptotic function for FADD which is mediated through its interaction with the E3 ubiquitin ligase, TRIM21 [39]. Although we investigated the function of FADD and TRIM21, our initial pull-down of FADD also brought down RIP1 (data not shown). However, the potential significance of this previously unidentified interaction was ignored as we initially focused on the novel interaction of FADD and TRIM21. Though we elucidated a role for FADD and TRIM21 in targeting IRF7 for ubiquitin-mediated degradation, the nature of what regulates TRIM21 activation remained unclear. Therefore I began by re-examining the significance of TRIM21’s potential interaction with FADD and RIP1 and to determine whether RIP1 may function in regulating TRIM21.

First, to determine whether FADD could indeed interact with the E3 ubiquitin Ligase TRIM21, as well as RIP1, 293T cells were transfected with constructs for FLAG-FADD, TRIM21 or RIP1. FADD was immunoprecipitated using anti-FLAG antibodies and associating proteins were determined by Western Blot (Figure 2.1A). FADD was able to interact not only with RIP1 but also TRIM21 (Figure 2.1A lane 10). As the interaction between FADD/RIP1 and FADD/TRIM21 has been previously reported, we sought to confirm that the interaction between these three proteins was within one complex. Thus, 293T cells were transfected with RIP1 and TRIM21 and lysates subsequently immunoprecipitated using anti-RIP1 or an isotype control antibody (Figure 2.1B). TRIM21 was able to be co-immunoprecipitated with RIP1 but not the control antibody. Additionally, 293T cells were transfected with RIP1, FADD, Flag-Strep-TRIM21, and ubiquitin. This time, anti-FLAG antibodies immunoprecipitated TRIM21 and were able to show that FADD and RIP1 interacted (Figure 2.1C lanes 13 and 14). In fact, it appears that FADD interaction is enhanced with the addition of ubiquitin. These results demonstrate that FADD, TRIM21 and RIP1 form a novel complex within cells even in the absence of additional stimuli.

The interaction between FADD/RIP1/TRIM21 is distinct from that of FADD/TRIM21/IRF7

We have previously reported that FADD and TRIM21 are able to interact with IRF7 and target it for proteosomal degradation [39]. Therefore we next investigated whether RIP1 may also be involved in IRF7 regulation. The reported role of RIP1 in antiviral responses occurs mainly downstream of TLRs and aids the induction of NF-κB activation [91]. However, RIP1 deficient cells have a defect in IFN production and it has been proposed that latent infection membrane protein 1 (LMP1) from Epstein-Barr virus mediates RIP1 dependent IRF7 activation [82]. Thus, RIP1 appears to be important in the induction of IFN and may affect the ability of TRIM21 and FADD to target IRF7 for degradation. To determine whether FADD/TRIM21/RIP1/IRF7 can interact together, 293T cells were transfected with all four constructs and anti-FLAG antibody was used to immunoprecipitate FADD (Figure 2.2A). Complex formation was observed in unstimulated cells as well as Sendai virus stimulated cells as interaction between FADD/TRIM21/IRF7 is enhanced upon viral infection. RIP1 and TRIM21
were able to interact with FADD while IRF7 did not. This was true whether cells were infected with Sendai virus or not (Figure 2.2A right blot, lanes 5 and 11). This indicates that FADD and TRIM21 have a preference for binding RIP1 over IRF7. Conversely, when anti-HA antibodies were used to immunoprecipitate IRF7, TRIM21 and FADD associated while RIP1 did not (Figure 2.2B lane 10). Furthermore, upon titrating increasing amounts of IRF7 and immunoprecipitating FADD, a change in complex formation could be seen (Figure 2.2C). As RIP1 was lost from the complex, IRF7 was incorporated. It appears that RIP1 and IRF7 compete for interaction with FADD and TRIM21. Thus, FADD and TRIM21 can be found in two distinct pools within cells: one containing IRF7 and another containing RIP1.

**RIP1 affects TRIM21 auto-ubiquitination but not its ability to downregulate the IFNα response**

In trying to understand what the functional significance of this newfound complex was, I discovered that RIP1 is able to inhibit FADD-induced TRIM21 auto-ubiquitination (Figure 2.3A compare lanes 9 and 11). TRIM21 is an E3 ubiquitin ligase which is capable of ubiquitinating target proteins. It has been noted that TRIM21 auto-ubiquitinates itself and that this is enhanced in the presence of FADD (Figure 2.3A lanes 3 and 9). However, the function of this auto-ubiquitination has yet to be elucidated. We hypothesized that this may function in regulating TRIM21’s activity or its ability to recruit target proteins. To discount the idea that TRIM21’s auto-ubiquitination does not merely regulate its degradation, we investigated the ubiquitin linkage type of its ubiquitin chains. Ubiquitin molecules contain seven lysines which can be utilized to build polyubiquitin chains. In particular, it has been found that ubiquitin chains linked through lysine at position 48 primarily signal a protein for ubiquitin-mediated degradation. In contrast, chains built with lysine at position 63 can initiate cell signaling and act as scaffolds to recruit relevant proteins. Thus, to determine whether the auto-ubiquitination mediated by FADD on TRIM21 was degradative or not, we transfected cells with ubiquitin mutants which replaced either lysine 48 (K48R) or lysine 63 (K63R) with an arginine. Thus these mutants were no longer able to mediate elongation of chains linked through their mutated lysine. In fact, we found that ubiquitination of TRIM21 largely consisted of K63 linked chains and not K48. This was evidenced by decreased TRIM21-ubiquitination upon addition of the K63R mutant, which was not seen with the K48R mutant (Figure 2.3B compare lanes 4, 5, and 6). This is in agreement with the fact that TRIM21 protein levels remained unchanged upon addition of FADD. Thus, auto-ubiquitination does not regulate the turnover of TRIM21 and may in fact regulate some other function of TRIM21.

Given that FADD enhances TRIM21 ubiquitination and both proteins act synergistically to reduce IFNα production, we reasoned that K63-linked auto-ubiquitination on TRIM21 could act as a scaffold to recruit IRF7 into the active complex. As RIP1 expression was seen to diminish TRIM21 auto-ubiquitination, RIP1 may negatively regulate TRIM21 activity through modulation of its ubiquitination status. Thus, FADD and RIP1 may play opposing roles in the activation of TRIM21. To investigate RIP1’s role, 293T cells were transfected with an IFNα4 luciferase reporter construct as well as expression vectors for RIP1, TRIM21, and FADD-FLAG (Figure 2.4). In addition, each of these samples was transfected with IRF7, as 293T cells require exogenous expression of this protein for activation of the IFNα4 reporter. These cells were subsequently infected with Sendai virus at 50HA U/ml to induce activation of the reporter construct. As expected, the addition of TRIM21 and FADD inhibited IFNα4 activation [39].
However, the additional expression of RIP1 did not rescue this decrease indicating that RIP1 was not involved in keeping TRIM21 in an inactive state. In addition, we investigated the requirement of auto-ubiquitination for recruitment of IRF7. Two TRIM21 mutants were generated. The first (CysTRIM21) consisted of mutation of two conserved cysteine residues (C31A, C36A) found in the RING domain. This mutant had abolished catalytic activity and lost the ability to auto-ubiquitinate itself (data not shown). The second mutant (LysTRIM21) consisted of mutation of two lysines in the B30.2 domain to arginine (K341R, K351R). This mutant lacked the majority of the auto-ubiquitination seen but still maintained a higher molecular weight band likely corresponding to a mono-ubiquitinated form of TRIM21 (data not shown).

To determine whether the auto-ubiquitination of TRIM21 is required for IRF7 interaction, we performed a co-immunoprecipitation study utilizing the CysTRIM21 mutant, as this had lost all auto-ubiquitination. In agreement with our luciferase data, we found that IRF7 was indeed able to interact with CysTRIM21 as well as wildtype TRIM21, indicating that the auto-ubiquitination of TRIM21 is not required for interaction (data not shown). Altogether, these results indicate that loss of TRIM21 auto-ubiquitination, whether through RIP1 expression or mutation, is not required to target IRF7 or regulate the IFNα response.

TRIM21’s potential regulation of RIP1

While our prior experiments indicate that RIP1 is not involved in IRF7 regulation through TRIM21, we became interested in understanding what the function of this novel FADD/RIP1/TRIM21 complex may be. Again, because TRIM21 is an E3 ubiquitin ligase, we were interested in whether it may affect the ubiquitination status of RIP1. RIP1 is ubiquitinated in response to TNFα stimulation as well as during viral infection. Its polyubiquitination is mediated primarily by E3 ubiquitin ligases cIAP1 and cIAP2; however there is also evidence to suggest TRAF2 and A20 can do so. The proteins CYLD, A20 and USP7 are responsible for the deubiquitination of RIP1 and help determine which of RIP1’s diverse cellular responses it will participate in.

To determine if TRIM21 functions in RIP1 ubiquitination, 293T cells were transfected with FADD, TRIM21 and RIP1. Cellular lysates were immunoblotted for RIP1 and its ubiquitination-smear monitored (Figure 2.5A). On its own, RIP1 can be found to be heavily ubiquitinated (Figure 2.5A lane 6). In this form, RIP1 primarily activates NF-κB and promotes cell-survival [92]. As expected, when RIP1 is expressed in conjunction with FADD it is found in its deubiquitinated form (Figure 2.5A lanes 4 and 5). When in complex with FADD, RIP1 promotes apoptosis or necroptosis [68, 93]. It is interesting to note that the expression of FADD alone is sufficient to activate the relevant RIP1 deubiquitinase protein in 293T cells (Figure 2.5A lane 5). Unexpectedly, the expression of TRIM21 (a functioning ubiquitin ligase) decreases RIP1 ubiquitination, although not to the same level as FADD expression (Figure 2.5A lane 7).

Given that TRIM21, FADD and RIP1 can be found in the same complex and that TRIM21 leads to diminished RIP1 ubiquitination, we investigated the possibility that TRIM21 plays an active role in promoting RIP1 functions which require its deubiquitinated form. Two such responses include apoptosis and necroptosis. Our lab had previously investigated a role for TRIM21 in Fas-induced apoptosis and not found an involvement in this. Hence, I began by investigating a role for TRIM21 in the recently identified, caspase-independent, RIP1 kinase-dependent death of necroptosis. To do this we utilized the necroptosis-prone fibrosarcoma cell line, L929 cells. This murine cell line is sensitive to TNFα and zVAD.fmk-induced necroptosis.
[94]. Thus, L929 cells overexpressing empty vector control or TRIM21 were pretreated with zVAD.fmk for an hour followed by treatment of TNFα or infection with Sendai virus (10HA U/ml). Cell survival was monitored using CellTiter-Glo (Figure 2.5B). We found no significant difference in the ability of these cells to undergo programmed necrosis whether they expressed TRIM21 or not. In addition to L929 cells, the role of TRIM21 in necroptosis induction was studied in other cellular models including Etoposide-induced necroptosis of MDA-MB231 or Hs578t cells. No role for TRIM21 was found in the induction of necroptosis in these systems either (data not shown). Thus, it appears that although capable of regulating RIP1 ubiquitination, TRIM21 does not affect the ability of cells to undergo necroptosis; however it is likely involved in other RIP1-dependent processes.
Figure 2.1. FADD forms a complex with TRIM21 and RIP1.
A, 293T cells were transfected as indicated. Anti-FLAG antibody was used to
immunoprecipitate FADD and associating proteins were detected by indicated antibodies
by Western Blot. Whole cell lysates (WCL) were blotted to control for protein expression.
B, 293T cells were transfected with RIP1 and hTRIM21 constructs. RIP1 antibody or control
(Ctl) were used for immunoprecipitation and indicated antibodies used for Western Blot.
C, 293T cells were transfected with the indicated constructs and anti-FLAG antibody used
to immunoprecipitate Flag-Strep-TRIM21. Interacting proteins were detected by Western Blot.
Figure 2.2. FADD, TRIM21 and RIP1 form a distinct complex from FADD, TRIM21 and IRF7.

A, 293T cells were transfected with indicated constructs and left uninfected or infected with Sendai virus at 50HA U/ml for 18 hours. FLAG antibody was used to immunoprecipitate FADD and interacting proteins determined by Western Blot (right). WCL samples (left). B, 293T cells were transfected with indicated constructs and HA antibody was used for immunoprecipitation of IRF7. Interacting proteins were determined by Western Blot with indicated antibodies. C, 293T cells were transfected with indicated constructs. HA-IRF7 was titrated up in concentration starting with lane 3. FLAG antibody immunoprecipitated FADD and proteins found in the complex were determined by Western Blot.
Figure 2.3. RIP1 decreases FADD-mediated, K63 linked auto-ubiquitination of TRIM21.
A. 293T cells were transfected with the indicated constructs and TRIM21 ubiquitination was detected by Western Blot using TRIM21 antibody. B, Which ubiquitin linkage was utilized for TRIM21 auto-ubiquitination was determined by transfecting 293T cells with a FADD-FLAG, hTRIM21, and Ubiquitin constructs. WT: wildtype, K48R: has mutation at lysine 48 to arginine, K63R: has mutation at lysine 63 to arginine.
Figure 2.4. RIP1 does not affect the ability of FADD and TRIM21 to negatively regulate IFNα production.

293T cells were transfected with an IFNα-luciferase reporter construct and IRF7 in every sample. Samples were also transfected with empty vector (pCI), RIP1, TRIM21, and/or FADD. 24 hours post-transfection, cells were infected with 50HA U/ml of Sendai Virus for 18 hours and dual-luciferase reporter assays were performed (firefly and renilla).
Figure 2.5. TRIM21 decreases ubiquitination of RIP1 but does not affect the ability to undergo necroptosis.

A, 293T cells were transfected with FADD-FLAG, hTRIM21, or RIP1, and RIP1 ubiquitination was detected in whole cell lysates by Western Blot. B, L929 cells were transfected with 50 ng of pCI or FLAG-TRIM21. 24 hours post-transfection, cells were pretreated with zVAD (10uM) for 1 hour, followed by TNFα (10ng/ml) and Sendai Virus (SeV 10HA U/ml) stimulations. After 18 hours cell survival was monitored using Cell Titer-Glo Assay and death was normalized to the pCI Mock treated sample.
Discussion

The ability of one protein to be involved in various distinct cellular processes is a common theme in biology. For example, FADD is a multifunctional adaptor molecule with functions ranging from apoptosis and cell cycle regulation to mediating the downregulation of interferon post-viral infection. RIP1 is another example of a multifunctional protein involved in promoting both cell survival as well as cell death. Understanding a protein’s ability to reconcile its role in these often antithetical responses is of utmost importance, particularly as their dysregulation can lead to disease states. Post-translational modifications represent one mechanism by which a protein may regulate its activity or its ability to interact with appropriate cellular partners, thereby modulating which response it is involved in.

We began by investigating the role of RIP1 in TRIM21 regulation and identified a novel complex consisting of FADD, RIP1 and TRIM21. This complex was found to be distinct from the IFNα-downregulating complex of FADD, TRIM21 and IRF7 that has been previously reported [39]. We find TRIM21 catalyzes K63 linked auto-ubiquitination on itself which is enhanced upon interaction with FADD. RIP1 expression decreases these chains, suggestive of a potential regulation mechanism. Auto-ubiquitination of E3 ligases is common and in some cases has been reported to provide a mechanism of regulation. In the case of RING-finger E3 ubiquitin ligase BCA2, auto-ubiquitination was found to regulate its own protein stability [90]. In contrast, the K63 linked auto-ubiquitination of TRAF6 is responsible for the recruitment and assembly of Tak1/TAB1/TAB2 signaling complex; however the absolute requirement for this auto-ubiquitination has recently been questioned [95, 96]. Moreover, K6 linked auto-ubiquitination of BRCA1 has been shown to recruit UBXN1 which is then responsible for inhibiting BRCA1’s enzymatic activity [97]. Thus, auto-ubiquitination serves as a diverse way to regulate E3 ubiquitin ligases. Consequently, we hypothesized that akin to TRAF6 or BRCA1, RIP1’s ability to inhibit TRIM21 ubiquitination may regulate its activity and affect its ability to target IRF7. However, we found auto-ubiquitination to be dispensible for interaction between TRIM21, FADD and IRF7 and expression of RIP1 did not affect TRIM21’s function in the IFNα response. This suggests that TRIM21 recruits target proteins in an ubiquitin-independent manner. In fact, during the course of this study, a group reported that tyrosine phosphorylation of TRIM21 is required for its interaction with IRF3 [98]. However, we did not find conclusive evidence that phosphorylation was required for IRF7 recruitment (data not shown), thus it is possible that TRIM21 utilizes different tactics for the recruitment of different substrates. Additionally, the finding that RIP1 affects TRIM21’s ubiquitination status, yet does not affect its ability to target IRF7, does not preclude the notion that auto-ubiquitination of TRIM21 may regulate a yet untested or unknown function of TRIM21.

In addition to studying the regulation of TRIM21, we investigated the significance of the novel FADD, RIP1, TRIM21 interaction. We discovered that TRIM21 is able to negatively regulate RIP1 ubiquitination, however the mechanism by which it does so is still unclear. TRIM21 is able to catalyze ubiquitination of target proteins to mediate their degradation, as is the case for IRF7; however RIP1 protein levels appear unchanged upon TRIM21 expression, suggesting that regulation is occurring at the level of deubiquitination. As TRIM21 is not a reported deubiquitinase, it may affect the ubiquitination status of RIP1 in an indirect manner. TRIM21 may promote the activation of a known RIP1 deubiquitinating enzyme such as CYLD or A20 [99, 100]. Alternatively, TRIM21 may be causing the degradation of a RIP1 specific
ubiquitin ligase like cIAP1/2 or TRAF2 [49, 100-102]. Further studies are required to resolve this TRIM21-dependent mechanism.

RIP1 is a dynamic protein capable of acting in range of cellular responses including cell survival, apoptosis and necroptosis; and it has recently been appreciated that the status of RIP1’s ubiquitination is a major factor which mediates these varied functions. Although the expression of TRIM21 did not affect the ability of cells to undergo necroptosis, it is possible that TRIM21 affects RIP1 mediated activation of NF-κB or apoptosis. Two groups have generated TRIM21-deficient mice. One group found that their mice manifested signs of systemic autoimmunity due to dysregulation of the IL23-Th17 pathway [103]. The other group found no significant abnormalities of their mice, but observed that TRIM21-deficient MEFs produced increased pro-inflammatory cytokines upon TLR stimulation [84]. These studies show that TRIM21 is clearly responsible for the negative regulation of multiple cytokine pathways, however no mechanism was ever implicated. We suggest that one mechanism by which TRIM21 may accomplish this regulation is through modulation of RIP1’s ubiquitination status. Further study is required to solidify this claim, however if found to be true, it would suggest a novel mechanism of RIP1 regulation and explain why TRIM21-deficient mice exhibit dysregulated production of cytokines. Additionally, we found that FADD expression results in complete deubiquitination of RIP1. This finding itself is not unexpected as it is well documented that when participating in FADD-mediated apoptosis RIP1 is no longer modified. However, how FADD stimulates the deubiquitination of RIP1 is not well-documented and may prove to be insightful in understanding the dynamics of cell survival versus cell death induction.

Finally, the unregulated production of cytokines is associated with many pathological states. For example, the autoimmune diseases, Systemic lupus erythematosus (SLE) and Sjögren’s Syndrome (SS) present with upregulated levels of IL-6, IL12/IL23p40, TNFα and type I IFN cytokines [104]. TRIM21 was first described as an autoantigen in both of these diseases and polymorphisms of this gene have been linked to their development [105]. Thus, further detailed studies understanding the mechanism by which TRIM21 regulates cytokine production may prove to be insightful in developing novel treatment strategies to target these and other diseases.
Chapter 3: An RNA and DNA virus: different mechanisms, same outcome.

Introduction

Cells have many tools in their arsenal to counteract viral infection. One such tool is the ability to undergo programmed cell death, specifically apoptosis. Apoptosis is a caspase-dependent form of “suicide” which involves condensation of chromatin, membrane blebbing and formation of apoptotic bodies. Rapid, phagocytic consumption of these apoptotic bodies circumvent an inflammatory immune response [106]. The antiviral IFN cytokines promote apoptosis of virally infected cells through upregulation of double-stranded RNA protein kinase, PKR or sensitization towards death receptor mediated death [107, 108]. Alternatively, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells induce apoptosis of virally infected cells through targeted secretion of pore-forming perforin/granzyme molecules. Granzymes bypass upstream signaling requirements, activate caspases and relieve inhibitors of apoptosis [109]. In addition to granzymes, CTLs activate death receptor-mediated apoptosis of infected cells. Binding of Fas/FasL or TNFR/TNF cause clustering of death receptors and their intracellular death domains. Recruitment of adaptor molecules Fas-associated death domain (FADD) or Tumor necrosis factor receptor type 1-associated death domain (TRADD) initiate signaling cascades convergent on caspase activation and resulting in apoptosis.

By and large, apoptosis remains an important antiviral mechanism; eliminating a virus’ niche to limit pathogen spread and allowing clearance of dead/dying infected cells. In certain cases, viruses utilize the formation of apoptotic bodies and subsequent phagocytosis as a means of dissemination without eliciting a host response [110]. However, the detriment of apoptosis on most viruses can be recognized by the fact that many viruses encode proteins which inhibit various points of apoptotic signaling. Several viruses encode proteins which inhibit death receptor mediated FADD or caspase-8 functions (viral-FLIPs), disrupt IFN production or signaling, encode homologs of anti-apoptotic Bcl-2 or antagonize PKR functions [111, 112].

Recently the identification of a novel form of programmed cell death, “necroptosis,” has been reported to occur upon inhibition of apoptosis. Necroptosis is initiated upon death receptor ligation, particularly, TNFR. Signaling is dependent on the deubiquitination of RIP1 and its recruitment to family member, RIP3, thus forming the “necrosome.” Auto and trans-phosphorylation of these proteins is essential for induction of downstream signaling events which include phosphorylation of mitochondrial protein MLKL [59]. Inhibition of RIP1 kinase activity, through use of small molecule, Necrostatin-1 or deletion of RIP3 prevents necroptosis [113]. Initially thought of as a backup mode of death, the significance of necroptosis in various facets of biology is beginning to be elucidated. For example, the lethality of FADD and caspase-8 knockout mice can be rescued by additional loss of RIP1 or RIP3, respectively [61, 65]. Programmed necrosis has also been implicated in maintenance of T cell homeostasis and induction of pathological states such as ischemia reperfusion [62, 114].

Given that viruses encode apoptotic inhibitors, and disruption of apoptotic signaling is important in triggering programmed necrosis, it is unsurprising that necroptosis has recently surfaced as a potential antiviral mechanism. In fact, RIP3 deficient mice are more susceptible to vaccinia virus infection than wild-type mice [58]. In addition, the herpesvirus MCMV has been reported to encode an inhibitor of necroptosis [76, 115]. Moreover, reovirus and influenza virus have also been suggested to elicit programmed necrosis under particular conditions [77, 78]. The identification of viruses as physiologic inducers of necroptosis has emerged, however the
prevalence of this phenomenon and molecular mechanisms leading to this form of cellular
demise remain to be fully characterized. Therefore we sought to perform a screen of multiple
DNA and RNA viruses for their ability to elicit necroptosis. Here we report the identification of
two additional viruses, MHV68 and Sendai virus as potent inducers of necroptosis.
Characterization of these responses allows us to identify a novel recognition pathway for
MHV68 as well a previously uncharacterized pathway for Sendai virus-dependent necroptosis
induction.
Materials and Methods

Cells and Reagents - L929 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine, and Na pyruvate. LA4 cells (ATCC) were maintained in Ham’s F12K media supplemented with 15% FBS, penicillin and streptomycin, and L-glutamine. Anti-TNF neutralizing antibody (clone MP6-XT22) and anti-rat control were purchased from R&D Systems. Jak1 inhibitor was purchased from Calbiochem. zVAD.fmk was purchased from Enzo Life Sciences. Necrostatin-1 was Sigma-Aldrich. Compound3 was a generous gift from Dr. Patrick Harran [116]. CellTiter-Glo was used to determine cell survival (Promega). Type I IFN and IFNγ were purchased from PBL InterferonSource and PeproTech Inc. respectively. TLR ligands: poly(I:C) (InvivoGen), bacterial lipopeptide-BLP and pTCpG (Integrated DNA Technologies Inc.) and LPS (Sigma-Aldrich) were used. The following antibodies were used: anti-RIP1 (BD Biosciences), anti-RIP3 (ProSci Inc.), anti-Ubiquitin (Santa Cruz Biotechnology).

Viruses - Sendai virus (Cantell Strain), MHV68, WSN, MCMV, HSV-1 (Strain 17), and LCMV were purchased from ATCC. MHV68 was propagated in 3T3 cells. KSHV was a generous gift from Dr. Britt Glaunsinger. The following Sendai virus (Z strain) and mutants (WT, d2Y, and V[-]) were generously provided by Dr. Takashi Irie and as described [117]. UV irradiation of virus was done by placing virus into sterile tissue culture plate ~8 inches from UV light in tissue culture hood for 30-45 minutes.

Virus induced Necroptosis and Cell Death Determination – L929 cells were seeded in 96 well plates. The following day cells were pretreated with zVAD and/or Necrostatin-1 for 1 hour before addition of virus. Following 16-18 hours of infection, cell survival was monitored by the ATP assay, CellTiter-Glo (Promega), per manufacturer’s protocol. Mock samples were set to 100% survival and all other samples were expressed relative to mock.

TLR or IFN stimulation of L929 cells- L929 cells were settled into 96 well plates. The following day, zVAD was added to cells for 1 hour before addition of TLR ligands. LPS was titrated from 0.1ng to 5ng per well and BLP from 1ng to 100ng. Poly(I:C) and pTCpG were transfected into cells with Lipofectamine 2000 as per manufacturer’s protocol. Poly(I:C) was titrated from 1μg to 10μg per well and pTCpG was titrated from 0.1μM to 10μM. Cell death was assayed between 7-18 hours post treatment. For Interferon experiments, L929 cells in 96 well plates were treated with zVAD and 100-1000U/ml of Universal Type I IFN (PBL Interferon Source) or 100-1000ng/ml of IFNγ (PeproTech Inc.). Cell survival was monitored after 16-18 hours post treatment. For the Jak1 inhibitor experiment, L929 cells were treated with zVAD, followed by Jak1 inhibitor (5μM) and then infected with viruses as indicated. Cell survival was monitored 16-18 hours post infection.

ELISA-To determine TNFα secretion, cells were treated as explained in figure legend. Samples of virally infected supernatants were then used to determine TNFα by ELISA (eBioscience).

TNFα Neutralization Assay- L929 cells were treated with anti-TNF or anti-rat control antibodies at 2μg/ml prior to addition of zVAD and infection with Sendai virus or MHV68 with
indicated concentrations. 16-18 hours post-infection cell survival was monitored by CellTiter-Glo ATP Assay.

**shRNA and siRNA knockdown** - LMP-RIG-I and LMP-control shRNA retroviral constructs were a gift from Dr. Russell Vance. Sequences are as described [118]. RIG-I and control retroviruses were produced in Pheonix cells and L929 cells were infected. A stably expressing cell line was generated by puromycin (4ug/ml) selection. PTY-STING and PTY-control shRNA lentiviral constructs were a gift from Dr. Zhijian Cheng [119]. 293T cells were used to produce lentivirus. L929 cells were infected and a stably expressing cell line was generated with puromycin selection. To knockdown CYLD expression, L929 cells were transfected with 75nM of control or CYLD siRNA (Dharmafect) using Dharmafect1 Transfection Reagent. After 48 hours, cells were split into 96 well plates and the following day were treated with indicated reagents or harvested for Western Blot analysis.

**NP protein qRT-PCR** – To determine if Sendai virus mutants were able to establish an efficient infection and cause IFNβ production, L929 cells were infected with Sendai virus (Cantell Strain) at 1 or 5 HA U/ml or Z strain: WT, V(-), d2Y mutants at MOI 200. After 16-18 hours of incubation, RNA was harvested by Trizol (as per manufacturer’s protocol) and cDNA prepared. qRT-PCR was carried out for structural protein NP and normalized to γ-Actin. Expression was plotted on log scale relative to mock infected samples.

**Immunoprecipitations** – To observe RIP1 ubiquitination, L929 cells were seeded into 15cm dishes. Cells were then treated with zVAD (20μM) or TNFα (100ng/ml) followed by Sendai virus infection (10HA U/ml) for the indicated times. TNFα treated cells were harvested after 5 hours. Cells were harvested in SDS lysis buffer (1%SDS, 50mM Tris-Cl at pH7.4, 5mM EDTA, 10mM DTT, 15U/ml DNaseI) and supplemented with protease and phosphatase inhibitors (1mM Na3V04 and NaF, 1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 5μg/ml pepstatin, 10μg/ml aprotinin, and leupeptin). Anti-RIP1 (2ug) antibody and Protein A-immobilized beads (Pierce) were added to 1mg of cell lysate and incubated overnight at 4°C. Ubiquitination was determined by SDS-PAGE and Western Blot. To determine phosphorylation of RIP3, L929 cells were seeded into 15cm dishes. Cells were treated as indicated and previously discussed. Cells were harvested in NP40 lysis buffer [1% Nonidet P-40, 50mM Tris-Cl at pH7.6, 150mM NaCl, 1mM EDTA, and 10% glycerol and supplemented with protease and phosphatase inhibitors (1mM Na3V04 and NaF, 1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 5μg/ml pepstatin, 10μg/ml aprotinin, and leupeptin)]. Anti-RIP3 (2ug) antibody and Protein A-immobilized beads were added to 1mg of lysate and incubated overnight at 4 °C. To detect RIP3, SDS-PAGE and Western Blotting were carried out. Anti-Ig, light chain specific (Jackson Immuno) secondary antibody was used to minimize masking of phosphorylation by heavy chain.
Results

Sendai virus and MHV68 cause necroptosis of L929 cells

To investigate whether inducing necroptosis is a common cell death mechanism of various viruses, a screen of RNA and DNA viruses was carried out in L929 cells. L929 cells are a murine fibrosarcoma cell line which readily undergo necroptosis in response to zVAD.fmk or TNFα treatment. Addition of the pan-caspase inhibitor, zVAD, causes necroptosis of these cells due to inhibition of caspase-8 and autocrine production of TNFα [94]. Much of what has been learned about necroptosis is due to the study of this phenomenon in this sensitive cell line [57, 120]. Thus, for study of virus-induced necroptosis, L929 were treated with zVAD at 10μM one hour prior to infection with either DNA viruses: MCMV, MHV68, HSV-1 or RNA viruses: SeV, and LCMV. Cell survival was determined 16-18 hours post-infection by ATP assay. Treatment with zVAD alone causes only about 50% cell death at the concentration used, however additional infection with SeV or MHV68 dramatically enhanced cell death, while other viruses did not (Figure 3.1A). Influenza virus (WSN), a related single-stranded RNA virus, and KSHV, the human homolog of MHV68, were then examined for their ability to induce death. Neither of these related viruses induced death as dramatically as SeV or MHV68 (Figure 3.1B). An alternate strain of influenza, PR8 was also tested and was incapable of inducing cell death (data not shown). Addition of the RIP1 kinase inhibitor Necrostatin-1 was able to rescue SeV and MHV68 induced death (Figure 3.1C) [113]. Thus, SeV and MHV68 and not related viruses specifically induce RIP1 kinase-dependent necroptosis of L929 cells.

To further characterize the virus induced phenomenon, we investigated the kinetics of cell death upon zVAD and SeV treatment. Necroptosis occurred dramatically after 10 hours of treatment with zVAD and SeV (Figure 3.2A) and occurred in a dose-dependent manner with increasing titers of virus inducing increasing levels of cell death (Figure 3.2B). Similar observations were made with MHV68 (data not shown). Additionally, L929 cells were treated with zVAD, infected with live virus or ultraviolet-irradiated (UV) virus and monitored for cell survival. UV-inactivated viruses were able to rescue necroptosis implicating viral replication or expression of a particular viral protein(s) in inducing necroptosis.

Activation of TLRs and IFN signaling do not yield necroptosis of L929 cells

Activation of TLR3 or TLR4 and zVAD treatment in macrophages has been reported to induce necroptosis, as does treatment of FADD-deficient dendritic cells with LPS [63, 73]. Thus, we investigated whether L929 cells may be undergoing necroptosis by virally-activated TLRs. L929 cells were treated with zVAD and subsequently treated with ligands for TLR2, TLR3, TLR4, and TLR9 (BLP, poly(I:C), LPS, pTCpG respectively). As TLR3 and TLR9 are endosomally located receptors, poly(I:C) and pTCpG ligands were transfected into L929 cells. We found that zVAD treatment and TLR stimulation did not cause necroptosis of L929 cells, with the exception of poly(I:C) which did cause some amount of death, but not as dramatically as viral infection (Figure 3.3A). Recently, Type I and Type II IFN have been suggested to mediate necroptosis of FADD deficient MEF cells [31]. Type I IFN is also implicated in necroptosis of macrophages after infection with pathogen, Salmonella enterica serovar Typhimurium [79]. Viral infection of L929 cells with SeV does cause upregulation of IFNβ (data not shown), thus we treated L929 cells with zVAD as well as Type I or Type II IFN to determine whether that may
account for necroptosis induction (Figure 3.3B). Alternatively, L929 cells were treated with an inhibitor of Jak1 kinase, an important signaling molecule downstream of the interferon receptor (Figure 3.3C). Neither addition of IFN nor inhibition of its signaling suggested a role for interferon-induced necroptosis.

**TNFα production is dispensable for Sendai virus induced necroptosis but is responsible for MHV68 induced death.**

As mentioned previously, L929 cells are extremely sensitive to TNF induced necroptosis. Accordingly, we hypothesized that TNFα production may be responsible for the virally induced necroptosis observed. To determine how much TNFα is produced by virally infected cells, L929 cells were treated with zVAD and/or infected with SeV and MHV68 at different doses. After 16-18 hours of incubation, supernatants were assayed for TNFα by ELISA. We found that SeV was a potent inducer of TNFα, with 10-60pg/ml produced upon infection with 10 or 100HA U/ml, respectively. In contrast, infection of MHV68 at an MOI of 2 or 5 caused production of only 2-10pg/ml of TNFα. Addition of zVAD did not significantly alter levels of TNFα by either virus (Figure 3.4A). To examine whether TNFα was responsible for the necroptosis seen, L929 cells were treated with anti-control or anti-TNF neutralizing antibodies prior to addition of zVAD and virus. As a control, 10ng/ml of TNFα was added. This is 100 fold more TNFα than what is produced upon infection of SeV at its highest dose. As expected, we found that addition of the anti-TNF neutralizing antibody significantly rescued TNFα induced death. In addition, we were able to rescue zVAD and MHV68 induced death. Surprisingly, addition of the anti-TNF neutralizing antibody was not able to significantly rescue SeV death at either the low or high infection (Figure 3.4B). Moreover, we were able to rescue death of zVAD and TNF with the anti-TNF neutralizing antibody, to address the possibility that zVAD and TNF synergize to cause necroptosis (data not shown). Altogether, this data suggests that zVAD and MHV68-induced necroptosis is due primarily to the production of TNFα after infection, while SeV-induced necroptosis occurs through an alternative mechanism.

**Infection of lung cells with Sendai virus causes TNF independent necroptosis.**

SeV and MHV68 are very different mouse-specific viruses. SeV is a small single-stranded RNA virus, while MHV68 is a much larger double-stranded DNA virus. Although so different, the natural route of infection for both of these viruses is intranasally. MHV68 undergoes productive infection of lung epithelia and eventually forms a latent infection in splenic B cells [121]. The primary site of SeV infection is also the lung and severe infections cause lethal pneumonia of the mice [122]. Therefore, we hypothesized that lung cells may be a noteworthy pool of cells to examine for their ability to undergo virus induced necroptosis. The lung adenoma cell line, LA4 cells, were thus treated with zVAD, TNF, Nec-1 and infected with SeV or MHV68 (Figure 3.5A). Treatment of LA4 cells with TNF induced significant cell death. This was found to be apoptotic death as zVAD treatment rescued the majority of it. However, these cells could also undergo RIP1 dependent necroptosis as it took the addition of zVAD and Nec-1 to fully rescue TNF stimulated death. Interestingly, we found that infection with SeV alone caused very little cell death, but was significantly enhanced upon addition of zVAD. This could be rescued with additional Nec-1 treatment. MHV68 however did not cause death of these cells in the presence or absence of zVAD (Figure 3.5A right panel). ELISA was used to
determine TNF production by these cells as previously described. We found that infection with SeV leads to very little TNF production from LA4 cells, particularly in comparison to that produced by L929 cells (Figure 3.5B). In agreement with this, addition of anti-TNF neutralizing antibodies was incapable of rescuing much death after zVAD and SeV infection, although they completely rescued TNF stimulated death (Figure 3.5C). Finally, treatment of LA4 cells with zVAD and UV-irradiated SeV rescued cell death. Infection of LA4 cells with other viruses was also unable to induce necroptosis of these cells (Figure 3.5D). Thus, we find that the more physiological target of Sendai virus, the lung epithelial LA4 cell line is also able to undergo TNF-independent, RIP1 kinase-dependent necroptosis in response to zVAD and virus infection.

Sendai virus necroptosis is initiated through RIG-I signaling and MHV68 through STING.

As we have established that SeV necroptosis is independent of TNFα, we sought to explore which signaling pathways are necessary for the cell death observed. SeV is well established as a potent activator of the cytoplasmic RNA sensor RIG-I [123, 124]. Recently, the molecule Stimulator of IFN Genes (STING) has received attention as an activator of RIG-I signaling and as a downstream component of a cytoplasmic DNA sensor [125]. In fact, STING deficient MEFs were shown to have defective IFNβ production upon SeV infection [15]. Thus to determine whether necroptosis induction may be initiated through activation of these pathways, L929 cells stably expressing RIG-I shRNA or STING-shRNA were generated. L929 cells express very low levels of RIG-I at basal states, and upregulate protein expression upon viral infection. Thus knockdown of RIG-I was confirmed by Western blot of SeV infected cells. Knockdown of RIG-I was able to rescue zVAD and SeV infected cell death potently, while MHV68 infection yielded very mild rescue (Figure 3.6A). Interestingly, knockdown of STING was unable to rescue SeV induced necroptosis, but significantly rescued MHV68 induced death (Figure 3.6). The above data suggest that SeV initiates necroptosis through activation of RIG-I and that this occurs independently of STING. MHV68 on the other hand appears to be able to signal through a previously unidentified STING-dependent pathway.

RIP1 ubiquitination status regulates Sendai virus induced necroptosis.

Necroptosis induction has been largely studied downstream of the TNF receptor. RIP1 and its ubiquitination status have been recently appreciated as a major player in the regulation of necroptosis. When leading to NF-κB activation, RIP1 is heavily ubiquitinated. However, upon deubiquitination, RIP1 becomes associated with apoptotic or necroptotic signaling machinery. Curiously, many of the same death inducing proteins have been found to assemble into signaling complexes downstream of RIG-I activation [12]. Therefore to determine whether RIP1 ubiquitination status is important in SeV induced necroptosis we knocked down expression of the RIP1 deubiquitinase, CYLD. Knockdown was confirmed by Western blot. Downregulation of CYLD expression was able to rescue zVAD and SeV infected death of L929 cells (Figure 3.7A). To confirm that RIP1 ubiquitination is important for necroptosis, we also used the small molecule, Compound 3. Addition of this Smac mimetic compound is known to downregulate expression of cIAP1 and cIAP2, which are the E3 ubiquitin ligases that ubiquitinate RIP1. Thus Compound 3 would reduce the requirement of CYLD and sensitize the cells to necroptosis. Similar to CYLD knockdown, treatment of L929 cells with Compound 3 and infection of SeV was able to cause necroptosis although not as potently as with zVAD (Figure 3.7B). Together,
these data suggest that modulation of RIP1 ubiquitination is critically important in SeV stimulated necroptosis.

To determine whether treatment of zVAD and SeV infected L929 cells actually exhibit altered RIP1 ubiquitination in unmanipulated cells, L929 cells were treated with zVAD, SeV, and/or TNF for various time points. It was recently reported that CYLD regulated RIP1 is found within an NP40 insoluble compartment in necroptosing cells, and thus we also looked at this compartment [68]. RIP1 was immunoprecipitated from these lysates and ubiquitination was monitored by Western Blot (Figure 3.8A). We found that zVAD treatment alone causes the heavy ubiquitination of RIP1 6 hours post-infection and that this is further enhanced by 10 hours post-infection. Cells infected with Sendai virus alone do not appear to ubiquitinate RIP1. Cells treated with zVAD and infected with Sendai virus have moderate ubiquitination of RIP1 at 6 hours which is lost by 10 hours post-infection. Thus, it appears that zVAD and SeV infection enhances the kinetics of RIP1 deubiquitination compared to zVAD alone and thus may allow for rapid and effective necroptosis.

RIP1 and RIP3 are found to be phosphorylated during necroptosis signaling, hence phosphorylated RIP3 is often used to monitor initiation of necroptosis [68]. Thus, we immunoprecipitated RIP3 from L929 cells treated with zVAD and SeV and monitored phosphorylation by Western blot. In support of the notion that zVAD and SeV enhance the kinetics of necroptosis, we found RIP3 phosphorylation to occur as early as 2 hours after zVAD and SeV infection, which was not seen until 10 hours in zVAD alone treated cells (Figure 3.8B). We also observe that RIP3 phosphorylation is lost by 10 hrs post zVAD and SeV treatment. One explanation for this is that the cells which had phosphorylated RIP3 earlier have begun dying at this timepoint. Therefore those cells which have survived are those that have not phosphorylated their RIP3. Interestingly, we find that RIP3 can also be ubiquitinated, a modification previously unreported. It appears that RIP3 is not ubiquitinated in cells treated with zVAD and infected with SeV. However, further studies are required to determine what the significance of this ubiquitination may be.

Sendai virus accessory proteins Y1 and Y2 may promote zVAD enhanced necroptosis.

Sendai virus is a small nonsegmented, negative, single-standed RNA virus. It consists of 6 structural genes: NP, P, M, F, HN and L [126]. The P gene of SeV is unique in its ability to produce up to 8 different accessory proteins via overlapping reading frames or mRNA editing (Figure 3.8A). Collectively referred to as “C proteins,” C, C, Y1, and Y2 are a set of nested genes initiated at different start codons but which share the same termination site. The C proteins have been the most extensively studied of the SeV accessory proteins and reported to antagonize IFN action, downregulate viral RNA synthesis, and inhibit apoptosis [41, 127, 128].

We have shown that UV-irradiated SeV is incapable of initiating necroptosis of L929 or LA4 cells. Furthermore, infection of cells with other RNA viruses (ie. LCMV, WSN) was unable to cause necroptosis. Therefore we hypothesized that a viral protein(s) specific to SeV may be responsible for the dramatic cell death seen upon zVAD addition and viral infection. To try and narrow down which SeV genes may be responsible, we obtained SeV mutants from Dr. Takashi Irie and Dr. Atsushi Kato. These viruses were of the Z strain of SeV and included a wildtype virus, d2Y and V(-) mutants. The d2Y virus lacks both the Y1 and Y2 accessory proteins, while the V(-) mutant lacks just the V accessory protein [129]. The growth kinetics and viral protein expression of the d2Y mutant is reported to be comparable to that of WT while that of the V(-)
A mutant is comparable or even slightly enhanced [129, 130]. Accordingly, we found expression of the SeV structural protein NP to be comparable between the WT and d2Y virus, and slightly enhanced in V(-) virus upon infection of L929 cells (Figure 3.9B). Curiously, we found that viruses provided by Irie and Kato yielded higher expression of SeV proteins than our lab WT virus. These differences may be due to variation found between strains of SeV. Moreover, we found that all viruses were able to induce IFNβ production by L929 cells and that replication of viral genomic RNA was equivalent between Z strain viruses (data not shown).

To determine whether the Y1, Y2, or V proteins are important in induction of necroptosis, L929 cells were treated with zVAD and subsequently infected with the lab strain of SeV or the WT and mutant viruses of the Z strain (Figure 3.9C). We were able to see that the WT Z strain virus was capable of inducing death similarly to the lab strain. Interestingly, the V(-) mutant exhibited slightly enhanced cell death, while the d2Y mutant partially rescued the death. This data suggests that the Y1 and/or Y2 proteins of SeV may be important in promoting necroptosis. In support of the idea that expression of a viral protein(s) is necessary, we found that addition of the translation inhibitor cycloheximide is able to fully rescue zVAD and SeV induced death (Figure 3.9D). Given that the d2Y mutant was unable to fully rescue necroptosis, we propose that the combination of multiple proteins may be required to fully sensitize cells towards necroptosis.
Figure 3.1. Sendai virus and MHV68 cause RIP1-dependent necroptosis of L929 cells.
A, L929 cells were treated with zVAD (10μM) and infected with MCMV, MHV68, HSV-1, LCMV (at MOI 5) or SeV (at 50HA U/ml) as indicated. Cell survival was monitored by CellTiter-Glo. B, L929 cells were treated with zVAD (10μM) and infected with KSHV, WSN (MOI 5), SeV (10HA U/ml). Cell survival was monitored by CellTiter-Glo. C, L929 cells were treated with zVAD (10μM), Necrostatin-1 (20μM) and infected with SeV (100HA U/ml), MHV68, or MCMV (MOI 5). Cell survival was monitored by CellTiter-Glo.
Figure 3.2. Characterization of viral necroptosis response.
A, FS-FADD-L929 cells were treated with zVAD (10μM) and infected with SeV (10HA U/ml) for the indicated times and cell survival monitored. B, L929 cells were treated with zVAD (10μM) and infected with the indicated amounts of SeV. 16-18 hours post-infection, cell survival was monitored. C, L929 cells were treated with zVAD (10μM) and infected with live or UV-irradiated Sev(10HA U/ml) or MHV68 (MOI 2). Cell survival was monitored 18 hours post-infection.
Figure 3.3. Viral necroptosis is independent of TLR and IFN signaling.
A, L929 cells were treated with zVAD (20μM) and subsequently treated with increasing doses of indicated TLR ligands. Cell survival was determined by CellTiter-Glo. B, L929 cells were treated with zvAD (20μM) and subsequently treated with increasing amounts of Type I IFN or IFNγ. After 16-18 hours cell death was determined. C, L929 cells were with zVAD (10μM.) followed by Jak1 inhibitor (5μM) before addition of SeV (10HA U/ml) or MHV68 (MOI 2). Cell Survival monitored by CellTiter-Glo.
Figure 3.4. Sendai virus induced necroptosis is TNF-independent. MHV68 induced necroptosis is TNF-dependent.
A, L929 cells were treated with zVAD and infected with SeV or MHV68 at the indicated titers (10 or 100HA U/ml)(MOI 2 or 5). After 18 hours, secreted TNF was determined by ELISA. B, L929 cells were treated with TNF neutralizing or control antibodies prior to addition of zVAD (20μM) and infection with Sendai virus or MHV68 at indicated titers. Rescue from cell death was monitored by CellTiter-Glo.
Figure 3.5. Sendai virus infection of lung epithelial cells causes TNF-independent necroptosis.

A, LA-4 cells were treated with zVAD (20μM), TNF (100ng/ml), or Nec-1 (20μM) and infected with Sendai virus (100HA U/ml) or MHV68 (MOI 10). Cell survival was monitored 24 hours post-infection. B, TNF levels in supernatants of Sendai virus infected LA-4 cells were detected by ELISA. C, LA-4 cells were treated with anti-control or anti-TNF neutralizing antibodies and treated with zVAD, TNF and infected with Sendai virus (100HA U/ml) before cell survival was assayed. D, LA-4 cells were treated with zVAD (20μM) and infected with live SeV (100HA U/ml), HSV-1, LCMV, MCMV (MOI5) or UV-inactivated SeV. Cell survival was monitored.
Figure 3.6. Sendai virus induces necroptosis through RIG-I while MHV68 signals through STING.
A, L929 cells stably expressing mouse RIG-I shRNA or control shRNA were treated with zVAD (10μM) and infected with SeV (10HA U/ml) or MHV68 (MOI 5). Cell survival was monitored after 16-18 hours post-infection with CellTiterGlo. Knockdown was confirmed by Western blot of L929 cells infected with SeV to upregulate RIG-I protein expression. B, L929 cells stably expressing mouse STING shRNA or control shRNA were treated with zVAD (20μM) and infected with SeV (10HA U/ml) or MHV68 (MOI 10). Knockdown was confirmed by Western blot.
Figure 3.7. CYLD is necessary for Sendai virus induced necroptosis while loss of cIAP proteins sensitizes towards Sendai virus-induced death.

A, CYLD expression was knocked down upon transfection of CYLD or control siRNA. L929 cells were then stimulated with TNF (1ng/ml), zVAD (10μM), and infected with SeV (5HA U/ml). Cell survival was determined by CellTiter-Glo. Knockdown was monitored by Western blot. B, L929 cells were treated Compound3 (100nM) and zVAD (20μM) and then infected with SeV (10 or 100HA U/ml). Cell survival was compared by CellTiter-Glo.
Figure 3.8. zVAD and Sendai virus infection enhance kinetics of RIP1 deubiquitination and RIP3 phosphorylation.
A, L929 cells were treated with zVAD (20μM) and infected with SeV (10HA U/ml) or TNF (100ng/ml) and lysed in SDS lysis buffer. RIP1 was immunoprecipitated and ubiquitination detected by Western blot. B, L929 cells were treated as in A, but lysed in NP40 lysis buffer. RIP3 was immunoprecipitated and phosphorylated RIP3 (*) and ubiquitination was detected by Western blot.
Figure 3.9. Sendai virus d2Y mutant partially rescues necroptosis of L929 cells.
A, Schematic of six structural Sendai virus proteins (NP, P, M, F, HN, L) and accessory proteins (V, W, X, C’, C, Y1, Y2). B, Relative expression of SeV NP protein in L929 cells infected with SeV (1 or 5HA U/ml) and WT, d2Y, V(-) mutants (MOI 100). qPCR values are normalized to Actin and plotted relative to Mock cells (log10 scale) C, L929 cells treated with zVAD (20μM) and infected with SeV (1HA U/ml) or WT, d2Y, V(-) mutants (MOI 100) Cell survival was monitored. D, To determine whether de novo protein synthesis was required for Sendai virus induced death, L929 cells were treated with cycloheximide (CHX 100μg/ml) before addition of zVAD (10μM) and infected with SeV (10HA U/ml).
Figure 3.10. Model of Sendai virus and MHV68-induced necroptosis.
We find that MHV68 induces necroptosis of L929 cells through recognition by STING. Activation of STING likely causes production of TNFα through an unknown mechanism. TNFα in conjunction with zVAD.fmk secretion causes necroptosis. Sendai virus signals through RIG-I and yields assembly of a downstream signaling complex. Addition of zVAD.fmk in conjunction with a viral protein(s) transitions RIP1 away from NF-κB signaling and into the necrosome with RIP3. This transition is also dependent on CYLD function. This causes necroptosis.
Discussion

Activation of pathogen recognition receptors mediates the stimulation of multiple antiviral responses. For example, recognition of viral RNA by RIG-I leads to activation of NF-κB and IRF transcription factors. These molecules promote the expression of pro-inflammatory and interferon cytokines. While pro-inflammatory cytokines help recruit appropriate immune effector cells, Type I IFNs are critical for the upregulation of antiviral genes as well as sensitization of cells to apoptosis [131]. Here we propose that in addition to the aforementioned functions, the RIG-I pathway appears capable of initiating an alternative form of cell death, necroptosis; and that this phenomenon may occur through modulation of proteins similar to those reportedly involved in TNF-mediated necroptosis. Additionally, we describe a novel mechanism of MHV68 recognition and signaling which is capable of inducing TNF production and thereby necroptosis of L929 cells.

Necroptosis or programmed necrosis has been described to occur upon death receptor ligation, DNA damage, and more recently pathogen infection [132]. Although examples exist for viruses which provoke necroptosis, the prevalence of this phenomenon has not been clearly studied. Thus, we sought to perform a screen of viruses for their ability to induce necroptosis. We found that the DNA virus, MHV68, and the RNA virus, Sendai virus, were capable of causing RIP1 kinase-dependent programmed necrosis upon addition of the pan-caspase inhibitor zVAD. Although activation of certain TLRs and IFN signaling have recently been implicated in necroptosis, we found no role for either of these mechanisms in our system [31, 79]. Instead, we report that MHV68 is able to induce necroptosis through adaptor molecule, STING and cause production of TNF which is responsible for the death seen.

STING involvement in MHV68 recognition has not previously been reported. In fact, recognition of this virus has been described to occur primarily through TLR2 and TLR9 [133, 134]. A recent study however, has shown that deficiency of the cytosolic DNA sensor, cGAS, renders mice susceptible to MHV68 replication, while infection of cGAS deficient bone marrow-derived macrophages yields impaired IFNβ expression [135]. Furthermore, the second messenger, cGAMP, which is produced by cGAS is able to be sensed by STING and promote IFN production [136]. We suggest the existence of a novel STING-mediated MHV68 recognition pathway. We believe that zVAD and MHV68 infection (perhaps through activation of cGAS) stimulate a STING-dependent pathway for TNF-induced necroptosis (Figure 3.10). As STING has been primarily studied for its ability to enhance IFN production, further study would be required to understand the molecular mechanism linking STING activation to TNF production, though such a link has recently been proposed in dendritic cells and macrophages [137]. It is also possible that an alternative DNA sensor, like IFI16, may be responsible for sensing MHV68 in these cells.

We also report that treatment of L929 cells with zVAD and infection with Sendai virus induces necroptosis through a different mechanism than that of MHV68 (Figure 3.10). Based on our data, we propose a model that requires the culmination of three events for Sendai virus-induced necroptosis to occur. This includes activation of the RIG-I pathway, a defect or inhibition of caspase-8, and the presence of a viral protein(s) to push the system towards necroptosis. Clearly, not all viruses that signal through RIG-I are able to promote necroptosis, as evidenced by a lack of cell death upon influenza virus or LCMV infection. An alternate explanation for lack of death with these viruses, could be that they encode an inhibitor of necroptosis, similar to the one expressed by MCMV [75]. However, UV-inactivation of these
viruses was still unable to cause necroptotic death (data not shown). We find that the d2Y mutant of Sendai virus is able to moderately rescue necroptosis of L929 cells, suggesting that the Y1 and/or Y2 proteins may be in part responsible for manipulating this intrinsic pathway. Loss of Y1 and Y2 only partially rescued necroptosis suggesting the importance of other potential protein(s). Given that zVAD and SeV treated cells require the deubiquitinase CYLD, and that the kinetics of RIP1 deubiquitination and RIP3 phosphorylation appear to be enhanced, we hypothesize that the viral proteins involved in necroptosis function to regulate RIP1 ubiquitination, in either a direct or indirect manner. Further studies will be required before concluding which proteins are responsible for this.

We were surprised to find that Sendai virus induced necroptosis was independent of TNF production even though infected cells produced ample amounts of TNF. Interestingly, even apoptosis of Sendai virus infected cells has been reported to be TNF independent [138], perhaps suggesting the presence of a TNF-antagonizing protein. In fact, knockout of the C proteins of SeV, (of which Y1 and Y2 are included) induce apoptosis of infected cells, although whether this is due to loss of TNF-inhibition has not been analyzed [128].

The activation of pattern recognition receptors such as TLRs and DAI have begun to be implicated in necroptosis induction, thus revealing the importance of this phenomenon in pathogen infection [73, 76]. However the role of RIG-I signaling in necroptosis induction has not yet been revealed. Interestingly, the signaling platform assembled upon RIG-I activation has been reported to share many of the same proteins as that found downstream of the TNFR [12]. Upon activation, RIG-I associates with the mitochondrial adaptor molecule MAVS (IPS-1/VISA/Cardif) [139-142]. This then leads to assembly of a number of proteins including TRADD, RIP1 and FADD; proteins also shown to be involved in TNFR necroptotic pathway [80]. The ubiquitination of RIP1 by cIAP or TRAF proteins mediates the recruitment of NEMO/IKKγ; activation of this pathway yields NF-κB or IRF activation [143]. The deubiquitinase CYLD has been shown to negatively regulate RIG-I signaling by targeting RIG-I directly [144]. In addition, caspase-8 has been suggested to cleave RIP1 as a way of downregulating RIG-I signaling, although we have yet to observe this cleavage [145]. Many of the same proteins, complexes, and functions are important in the TNFR pathway. Ligation of the TNFR causes assembly of a similar complex consisting of RIP1, cIAP, TRAF, and NEMO/IKKγ. Ubiquitination of RIP1 again, facilitates NF-κB activation. Upon deubiquitination by CYLD protein, RIP1 is able to interact with either FADD and caspase-8 or RIP3 to promote apoptosis or necroptosis respectively [80]. Additionally, caspase-8 has been shown to inhibit necrototic signaling by cleavage of RIP1 or CYLD [67]. Given the similarities between RIG-I and the TNF-necroptosis pathways, it is therefore conceivable that activation of RIG-I by SeV promotes the assembly of these proteins, and that in cases of caspase-8 loss or inhibition, particular viral proteins may promote the transition of these molecules away from RIG-I signaling and into the necrototpic pathway.

Sendai virus and MHV68 are both pathogens of the respiratory system. Interestingly, the other known necroptosis-inducing viruses, Vaccinia virus, Reovirus, and MCMV are all also capable of initiating respiratory infections [146-148]. Thus, we hypothesized that lung epithelial cells may be an important physiological cell type in which to study virus induced necroptosis. Indeed, we found that zVAD treatment and infection of lung epithelial cells with Sendai virus, but not MHV68 caused necroptosis of these cells. Whether necroptosis of infected lung cells occurs in vivo remains to be determined. We also investigated the ability of a number of other cell types to undergo necroptosis in response to Sendai virus infection, however found that most
did not (data not shown). Thus, the susceptibility of cells to virus induced necroptosis may be cell type specific.

It is important to note that these experiments were done in the presence of the caspase inhibitor zVAD. As necroptotic cell death is unmasked upon inhibition of apoptosis, zVAD addition is an attractive way to mimic this state. However, we can imagine that virus induced necroptosis may occur physiologically in cells which express low levels of caspase-8 or other negative regulators of necroptosis. A number of lung cancers for example, have been reported to lose expression of caspase-8 upon transformation [149]. Alternatively, many viruses including VV, KSHV, and cowpox virus encode inhibitors of apoptosis as a mechanism of immune evasion. Apoptosis of infected cells is an important innate immune mechanism for limiting viral propagation. The viral-FLIP family encode for a protein that specifically counteracts apoptosis signaling through inhibition of caspase-8 or FADD [150]. Thus, viruses utilize this protein to maintain their replicative niche. However in some cases, inhibition of apoptosis may be futile, as it can open the infected cell up to undergoing necroptotic death as opposed to apoptotic death. In fact, we see that expression of MC159, a vFLIP from molluscum contagiosum virus, in L929 cells causes necroptosis similarly to zVAD treatment (data not shown).

Although our understanding of virus induced necroptosis is far from complete, it appears that this mechanism may be more prevalent than previously thought. It is also interesting to note that different viruses appear to trigger necroptosis through very different pathways. Here we identify two additional pathways, RIG-I and STING, which when given the right conditions, are capable of inducing necroptosis. Although potentially important as an antiviral mechanism, we must remember that necroptotic death is inflammatory and may therefore elicit unwanted pathology. In addition, some viruses may usurp this pathway as a mechanism for viral dissemination. Thus, as we begin to identify more pathways and viral triggers of necroptosis, it will become increasingly important to determine whether this has negative or positive consequences on viral infection and host pathology.
Concluding Remarks

Host-pathogen interactions are a compelling area of study. Pathogens continually evolve alongside their hosts and thus studying the dynamic tug-of-war which exists between these organisms allows us to make conscientious decisions regarding therapeutics. Multiple antiviral mechanisms exist, however two critical components of this overall response is the production of interferon cytokines and cell death of infected cells. My thesis work examines both of these phenomena. We find that FADD mediates non-degradative, K63 linked auto-ubiquitination of TRIM21. However, this modification does not play a clear role in activation of the ligase or its ability to downregulate IFNα through IRF7 degradation. Interestingly, identification of a new complex of proteins, RIP1, FADD, and TRIM21 may be important in negatively regulating the ubiquitination status of RIP1. RIP1 is a complex component of pro-survival and cell death signaling. Initial characterization along with descriptions reported in the literature support the notion that TRIM21’s regulation of pro-inflammatory cytokine production may in fact be attributed to this novel interaction and function with RIP1. Further studies will be required to confirm and dissect the molecular mechanism by which TRIM21 may accomplish this. TRIM21 has been implicated in playing a role not only during viral infection but also in multiple autoimmune diseases. Thus, it will be interesting to put this function into the context of viral infection or other pathological states.

Traditionally, cell death, particularly apoptosis has been shown to limit viral spread and dissemination. However with many viruses encoding for apoptotic inhibitors and the recent identification of caspase-independent death, necroptosis, it has raised the question of what contribution necroptotic death plays during antiviral responses. In this thesis, I report the identification of two additional viruses which are capable of inducing necroptotic cell death through two completely different mechanisms. We find that MHV68 is able to induce TNF-mediated necroptosis in a STING-dependent manner. Sendai virus induced necroptosis appears to be more complicated. We propose a model in which RIG-I activation in conjunction with caspase-8 inhibition and particular viral proteins, mediate the transition of RIP1 from an NF-κB activating complex to promotion of necroptosis. We will continue to dissect the molecular mechanism of this response and try to identify the viral components required.

It has been suggested thus far that necroptosis, like apoptosis, mainly plays an antiviral role, limiting viral replication and spread. However data are beginning to emerge suggesting that in some cases this may not necessarily be the case. In fact necroptosis may be responsible for the detrimental pathology seen during certain viral infections. As more viruses are discovered to elicit this type of death, it will become increasingly important to understand the contribution of this mechanism towards viral load and dissemination versus host pathology. Indeed, we have infected wildtype and RIP3−/− mice with MHV68 and found no difference in viral loads (data not shown). However, the pathology elicited in each of these mice would need to be further characterized. We are also in the midst of doing a similar study with Sendai virus (data not shown). Significant gains have been made in the field of virus-induced necroptosis, however a number of questions remain to be answered. Given that new triggers and pathways leading to necroptosis continue to be discovered, what is the point of convergence between these? Necroptosis results in the release of damage-associated molecular patterns (DAMPs) and inflammation. How do these DAMPs lead to inflammation? Perhaps some DAMPs are more important than others. Particularly in the case of viruses, is release of DAMPs beneficial or detrimental to the host? Are virus-specific T cell responses altered when infected cells die by
necroptotic death? Does this allow the exposure of previously hidden antigens which shape the T cell response? It is clear that we are far from understanding the intricacies of necroptosis and its importance in host antiviral responses however it will be exciting to see what progress is made in the coming years and whether it may change the way we treat certain infections and diseases.
References


Appendix

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**Figure A.1. Cloning of TRIM family proteins.**
TRIM family proteins were obtained and cloned into various vectors with epitope tags. Indicated restriction enzymes (RE) were used for cloning into indicated vector and can be used to pop insert out.
**Figure A.2. Cloned Constructs.**

Various proteins were cloned into indicated vectors using the indicated restriction enzymes (RE). Sendai virus proteins were cloned into pCI and MSCV-PIG retroviral vector and tagged with a FLAG tag. Original muSOX protein was obtained from Britt Glaunsinger lab. Original MC159, K13, and E8 were from Francis Chan lab. Original Ubiquitin and mutants were obtained from Michael Rape lab.