NLRC3 IS A NEGATIVE REGULATOR OF DNA-INDUCED IMMUNE RESPONSE

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ABSTRACT

Lu Zhang: NLRC3 IS A NEGATIVE REGULATOR OF DNA-INDUCED IMMUNE RESPONSE
(Under the direction of Jenny P-Y. Ting)

Innate immunity provides the first line of defense and the pattern recognition receptors (PRRs) are indispensable in sensing foreign insults and self-damage. Recent research interests have been focused on elucidating cytosolic DNA recognition and downstream signaling activation. Stimulator of interferon genes (STING, also named MITA, MYPS or ERIS) is an intracellular DNA sensor that activates type I interferon production through its interaction with TANK-binding kinase 1 (TBK1). STING recognizes cytosolic dsDNA, DNA viruses, cyclic di-GMP (c-di-GMP), c-di-GMP-producing bacteria as well as other intracellular DNA sensors. Here we found that the nucleotide-binding, leucine-rich repeat containing protein, NLRC3, inhibited STING-dependent innate immune activation in response to cytosolic dsDNA, c-di-GMP and DNA viruses. Mechanistically, NLRC3 associated with both STING and TBK1, and impeded STING-TBK1 interaction and downstream type I interferon production. Using purified recombinant proteins NLRC3 was found to interact directly with STING. Furthermore, NLRC3 blocked proper trafficking of STING to perinuclear and punctated region, which is known to be essential for its activation. In animals, herpes simplex virus 1 (HSV-1)-infected Nlr3-/- mice exhibited enhanced innate immunity, reduced morbidity and viral load. This demonstrates the intersection of two key pathways of innate immune regulation, NLR and STING, to fine tune host response to intracellular DNA,
DNA virus and c-di-GMP. In addition, NLRC3 directly bound to DNA, specifically to double-stranded and single-stranded junction of DNA and DNA replication fork with 25 base pair single-stranded region shown by incubation of purified recombinant NLRC3 and DNA in a cell-free system. In an ultraviolet (UV) light-induced DNA damage model, Nlrc3-/- cells displayed elevated activation of innate immune activation, potentially triggered by damaged DNA. These findings collectively suggest a novel role of NLRC3 in STING-dependent pathways, DNA-damage-induced innate immune activation and pathways beyond immune regulation.
To my parents Qingde Zhang and Bang Liu, the best parents in the world. Without you I never would have made it to this point. Because of you, I found my interests in basic research in college, where I met my wonderful husband, Rui. Rui, thank you for all your love and support, thank you for the commitment to spend the rest of our lives together in a place we call home.
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<tbody>
<tr>
<td>AIM2</td>
<td>absent in melanoma 2</td>
</tr>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>ARD</td>
<td>Ankyrin-repeat domain</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a caspase activation and recruitment domain</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutation</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>BER</td>
<td>base-excision repair</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CBP</td>
<td>cyclic-AMP-responsive element-binding protein</td>
</tr>
<tr>
<td>CDN</td>
<td>cyclic dinucleotide</td>
</tr>
<tr>
<td>cGAS</td>
<td>cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC class II transactivator</td>
</tr>
<tr>
<td>CTD</td>
<td>C terminal domain</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IFN-regulatory factor</td>
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<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DC</td>
<td>dendritic cells</td>
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<tr>
<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>DSBs</td>
<td>double strand breaks</td>
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<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ERIS</td>
<td>endoplasmic reticulum interferon stimulator</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>HGPS</td>
<td>Hutchinson-Gilford progeria syndrome</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
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<tr>
<td>IAD</td>
<td>IRF associated domain</td>
</tr>
<tr>
<td>IFI16</td>
<td>gamma-interferon-inducible protein 16</td>
</tr>
<tr>
<td>IFN-I</td>
<td>type I interferon</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor or NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory transcription factor</td>
</tr>
<tr>
<td>ISD</td>
<td>interferon stimulating DNA</td>
</tr>
<tr>
<td>ISG</td>
<td>interferon-stimulated gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>interferon-stimulated response elements</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeats</td>
</tr>
<tr>
<td>LTβ</td>
<td>lymphotoxin β</td>
</tr>
<tr>
<td>Mab21</td>
<td>male abnormal 21</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signaling protein</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MITA</td>
<td>mediator of interferon regulatory factor 3 activation</td>
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<tr>
<td>MPYS</td>
<td>plasma membrane tetraspanner</td>
</tr>
<tr>
<td>MRN</td>
<td>MRE11-RAD50-NBS1</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
</tr>
<tr>
<td>NBS</td>
<td>Nijmegen breakage syndrome</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide-excision repair</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide-binding domain and leucine-rich repeat containing proteins</td>
</tr>
<tr>
<td>NTase</td>
<td>nucleotidyltransferase</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PGI2</td>
<td>prostaglandin I2</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RHD</td>
<td>acid rel homology domain</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
</tbody>
</table>
STING: stimulator of interferon genes
TAD: transactivation domain
TBK1: TANK-binding kinase
TLR: Toll-like receptor
TMEM173: transmembrane protein 173
TNF: tumor necrosis factor
TRAF: TNF receptor-associated factor
VACV: vaccinia virus
XP: xeroderma pigmentosum
ZBP1: Z-DNA binding protein 1
1 Overview of anti-viral innate immunity

Innate immunity

The immune system is one of the most important systems for the body. It is the defense line of the body to fight against microbial pathogen invasion, monitor and clear abnormal cells (Janeway, 1993). It also has the ability to elicit immune-tolerance and regulation. In general, immune system is composed of immune molecules, immune cells and immune organs. Upon invasion of the pathogen, the host will elicit a series of controlled responses. Local cells and tissues at the site of infection will produce anti-microbial peptides to inhibit microbe replication. Cytokines and chemokines will also be produced to recruit immune cells to the site of infection to clear the invading pathogen, repair the damaged tissue and gain immune memory against the pathogen.

Based on the specificity of pathogen recognition and the immune response, immune system can be classified to two sub-systems: innate immune system and adaptive immune system. Adaptive immunity (also known as acquired immunity or cell-mediated immunity) specifically recognizes pathogen epitopes through a large variety of antigen receptors on T cells and B cells, which will then undergo colonial expansion and activation into effector cells. Adaptive immunity features in antigen specificity and immune memory, yet it is slow to take action, usually become functional after 96 hours of infection (Janeway CA Jr, 2001).
On the contrary, innate immunity features in fast recognition and clearance of pathogen. We are living in an environment in continues contact of bacteria, virus and parasites, but staying healthy most of the time. This is primarily attributed to surveillance by the innate immune system, which is the first line of defense and recognizes and clears pathogens within hours or even minutes after infection. Innate immune defense mechanisms are composed of (1) Physical barriers (skin and mucosal surface, blood-brain barrier and placental barrier, etc.). (2) Chemical barriers (include physical pH, antimicrobial peptides and so on). (3) Phagocytosis mediated mainly by phagocytes. Phagocytes recognize invading microbial pathogens by scavenger receptors, pattern recognition receptors (PRR) and others to internalize and clear the pathogens. (4) Inflammation response: Pathogen infection can induce resident macrophages or other immune cells to secret cytokines such as tumor necrosis factor (TNF) or interleukin 1 (IL-1) which will induce the expression of adhesion molecules on endothelial cells to allow transmigration of immune cells from the blood stream. In addition, those inflammatory cytokines can also induce the expression of certain molecules such as bradykinin and prostaglandin I2 (PGI2) and cause vasodilatation, leading to increased blood flow and increased body temperature. Increased body temperature actively inhibits grow and replication of the invading pathogen and helps the elimination of pathogens. Thus, inflammation response is not only a pathological response, but also an active strategy to eliminate pathogens. (5) Bio-active molecules. Some immune cells are able to synthesize molecules to inhibit or kill microbial pathogens, such as reactive oxide species (ROS), reactive nitrogen species (RNS) and defensins. In addition, complement system is also an important branch of innate immunity. Apart from a direct killing of the
microbial pathogens, complement system can synergize with the recruitment of phagocytes and enhance phagocytosis (Janeway CA Jr, 2001).

**Pattern recognition mechanisms**

Adaptive immunity recognizes pathogens through a large variety of specific T-cell receptors (range from $10^{13}$ to $10^{15}$). The innate immune system lacks this large repertoire of specific receptors, thus the recognition of pathogens by innate immune system is less specific. Research showed that the recognition of microbial pathogens by innate immune system is achieved by Pathogen-associated Molecular Patterns (PAMPs) (Kaufmann, 2007). The molecules within the infected host that recognize these PAMPs are called pattern recognition receptors (PRRs) (Blasius and Beutler, 2010; Kawai and Akira, 2010; Medzhitov, 2007; Takeuchi and Akira, 2010). So far, four different families of PRRs have been described. Our lab studies a family of innate immune proteins called the NLRs (nucleotide-binding domain (NBD) leucine rich-repeat (LRR) containing proteins, also known as NOD-like receptors) (Davis et al., 2011; Ting et al., 2008a; Ting et al., 2008b). NLRs do not appear to directly bind pathogen ligands. Instead, many appear to modulate the function of PRRs (Davis et al., 2011; Schneider et al., 2012; Ting et al., 2008a; Ting et al., 2008b). In this dissertation, I will concentrate on a new NLR family member that modulates host responses to DNA and DNA viruses, which is a novel function for NLR proteins.
**Type I interferon signaling pathway**

Type I interferon (IFN-I) and proinflammatory cytokines are induced upon the recognition of PAMPs by PRRs. IFN-I binds to IFNα receptor (IFNαR) on cell surface and activate the downstream signaling cascade to trigger the production of a high level of IFN-I and Interferon induced gene (ISG) expression, therefore mediating cellular antiviral innate immune response. NF-κB and IRFs activation are two major events in antiviral innate immune response (Seth et al., 2006). We will introduce the function and activation mechanism of NF-κB and IRFs in this section.

**NF-κB**

In mammalian cells, NF-κB family is consist of five transcription factors: p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (Rel A), Rel B and c-Rel (Li and Verma, 2002). They are encoded by NFKB1, NFKB2, RELA, RELB and REL genes respectively (Hoffmann and Baltimore, 2006). All family members have a highly conserved 300-amino acid rel homology domain (RHD) at N-terminus, which serve as the major function domain of NF-κB. RHD domains are responsible for DNA-binding and dimerization. In addition, there is a nuclear localization sequence in RHD domain to mediate nuclear translocation of activated NF-κB (Baldwin, 1996; Ghosh et al., 1995; Ghosh et al., 1998).

NF-κB signaling pathway can be classified into two categories: canonical signaling pathway and noncanonical signaling pathway (Razani et al., 2011). Canonical NF-κB pathway can be activated by inflammatory signals, such as cytokines, PAMPs and danger-associated molecular patterns (DAMPs). When cells sense these stimuli, cell surface or cytosolic receptors are activated which in turn activate the IKK complex.
Activated IKK complex then phosphorylates IκBs, including IκBα, IκBβ and IκBε. Phosphorylated IκB recruits the E3 ubiquitin ligase, SCF/βTRCP complex and gets ubiquinated. Ubiquitinated IκB then undergoes proteosome-dependent degradation. NF-κB is then released and translocates to the nucleus to initiate the downstream gene transcription (Figure 1). P65 and c-Rel are the two major functional NF-κB proteins in the canonical pathway. They can form homodimer or heterodimer with p50, which lacks a transactivation domain (TAD). Therefore, there are four different transcription factors that can be activated when canonical NF-κB pathway is activated, these are: p65:p65, p65:p50, c-Rel:c-Rel, c-Rel:p50 (Delhase et al., 1999; Ea et al., 2006; Xu et al., 2009).

Activation of noncanonical NF-κB pathway (Figure 1) is not dependent on the activation of IKKγ complex, but on the IKKα-NIK kinase complex (Claudio et al., 2002). Previous studies showed that lymphotoxin β (LTβ), B-cell activating factor (BAFF), CD40 ligand and Receptor activator of nuclear factor kappa-B ligand (RANKL) can activate NF-κB through the non-canonical pathway (Claudio et al., 2002; Darnay et al., 1999; Garceau et al., 2000; Yin et al., 2001). Once the relative receptors are activated, TRAF2 recruits cIAP1 and cIAP2 and activates these two molecules by K63 linked ubiquitination. Activated cIAP1 and cIAP2 then associates with TRAF3 to mediate K48 linked ubiquitination of TRAF3, leading to the latter’s proteosome-dependent degradation (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Degraded TRAF3 no longer mediates ubiquitination and degradation of NIK (He et al., 2006; Liao et al., 2004). Thus accumulated NIK can phosphorylate the downstream substrate p100 and IKKα.

Phosphorylated p100 recruits the SCF/βTRCP complex and becomes ubiquitinated. Ankyrin-repeat domain (ARD) in the C-terminus of p100 will be degraded.
through 26S-proteosome. The end product of this event is p52, which can form a heterodimer with RelB or RelA and translocates to the nucleus to initiate downstream gene transcription (Xiao et al., 2004; Xiao et al., 2001).

**IRFs**

IRF proteins are a group of ubiquitously expressed transcription factors. They were firstly identified as transcription regulators of IFN-I and ISGs expression. Later, IRFs were found to be crucial in regulating innate immunity, adaptive immunity, apoptosis and tumorigenesis (Lohoff and Mak, 2005; Taniguchi et al., 2001). There are 9 members in the IRF family: IRF1, IRF2, IRF3, IRF4 (also known as RIP, LSIRF or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP) and IRF9 (also known as ISGF3γ) (Barnes et al., 2001; Hiscott et al., 1999; Mamane et al., 1999; Miyamoto et al., 1988; Nguyen et al., 1997; Taniguchi et al., 2001). IRF protein family members are characterized by conserved 120-amino-acid-long DNA-binding domain (DBD), containing 5 proline repeats on the N terminus (Darnell et al., 1994; Tanaka et al., 1993). The DBD domain on IRFs can form helix-loop-helix structure and therefore able to bind to interferon-stimulated response elements (ISRE) in the promoter region of interferon stimulated genes and induced the downstream gene transcription. Except for IRF1 and IRF2, all other IRFs members have IRF associated domain (IAD), which are responsible for mediating homodimer or heterodimer among different IRFs (Qin et al., 2005; Takahasi et al., 2003; Taniguchi et al., 2001).

Among all IRF proteins, IRF3 and IRF7 are known to play important role in inducing IFN-I expression (Figure 2). IRF3 is constitutively expressed in most cell types,
but stays in an inactive state. Upon stimulation, IRF3 gets activated following a two-step phosphorylation by upstream protein kinases. Firstly, serine and threonine in amino acid 396-405 are phosphorylated to allow IRF3 to bind to the cyclic-AMP-responsive element-binding protein (CBP). CBP then mediates phosphorylation of S385 and S386, which are important for the dimerization of IRF3 (Lin et al., 1998; Wathelet et al., 1998). IRF7 is different from IRF3 since it is only expressed in dendritic cells and B cells in the resting state. The expression of IRF7 is inducible by viral infection or IFN-I stimulation in other cell types. Similar to IRF3, IRF7 is activated by the phosphorylation of S477 and S479 (Ning et al., 2011). TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKe are two major kinases that activate IRF3 and IRF7. TBK1 and IKKe share 64% of homology in amino acid sequence. They also function similarly in phosphorylating IRF3 and IRF7 (Hacker and Karin, 2006). However, TBK1 is expressed ubiquitously in almost all cell types, whereas the expression of IKKe is limited to peripheral blood cells, T cells and bone marrow derived macrophages (BMDMs) in the resting state. Expression of IKKe is inducible in other cell types by viral infection, TNF or LPS stimulation. There are other kinases that can mediate the phosphorylation of IRF7 in addition to TBK1 and IKKe in some cell types, such as IRAK1 and IKKe in plasmacytoid cells (Bulek et al., 2011; Hemmi et al., 2004).

1.2 Cytosolic DNA recognition

The concept of PRRs recognizing PAMPs was introduced in the previous sections. It is worth mentioning that the surveillance system conducted by PRRs is compartmentalized (Gurtler and Bowie, 2013). Microbial-associated molecules found in
different compartments (including extracellular, endosomal, and cytosol) are monitored by PRRs and cause the host to mount immune responses against self or non-self insults. We will focus on the knowledge and recent progress of cytosolic DNA sensing in this section.

DNA was found to activate the host immune response over 50 years ago (Isaacs et al., 1963; Rotem et al., 1963) and the importance of elucidating DNA recognition pathways was realized for a long time (Atianand and Fitzgerald, 2013). However, it was only in the past six years that the route by which DNA is detected and the downstream signaling pathways activated by DNA have begun to be elucidated.

In healthy eukaryotic cells, DNA is strictly located in the nucleus (O'Neill, 2013). The presence of self DNA and DNA derived from microbial and viral pathogens in cytosol or endosomes would considered to be aberrant, thus the cells will mount innate immune response against DNA in aberrant locations (Ishii et al., 2006; Kerur et al., 2011; Lund et al., 2003; Stetson and Medzhitov, 2006).

Up to now, more than ten DNA receptors or sensor have been identified (Table 1). In the recent years, significant attention has been drawn to cytosolic DNA receptors and the mechanisms by which they recognize DNA and activate the downstream signaling. One of the key findings is the discovery of a transmembrane protein, STING (also known as MITA, MPYS, ERIS and TMEM173) (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008). It was firstly identified to be an important adaptor protein to activate IFN-I downstream of DNA and RNA receptors. The work was published by four different groups around the same time. Later on, more functions of STING have been revealed. The next section will introduce
the function and molecular mechanism of STING and several other important cytosolic DNA receptors.

DAI

Toll-like receptor 9 was found to be the DNA receptor that recognizes endosomal DNA and hypomethylated DNA (Krieg et al., 1995). Later on, it was found that cytosolic dsDNA could trigger innate immune response, specifically IFN-I induction, independent of TLR9. DNA-dependent activator of IFN-regulatory factor (DAI) was previously identified in tumor stromal tissue and named DLM-1 or Z-DNA binding protein 1 (ZBP1). Overexpression of DAI led to an increased in IFN-I production after B-DNA stimulation while shRNA-mediated knockdown of DAI led to decreased IFN-I and ISGs production leading to the conclusion that DAI is important for DNA recognition. In addition, the expression level of DAI was highly inducible by B-DNA or IFN-I stimulation. Mechanistically, DAI binds to B form of DNA in the cell cytosol and also binds to IRF3 and TBK1. The recognition of B-DNA by DAI enhances DNA binding to IRF3 and TBK1, both of which activate the downstream IFN-I pathway (Takaoka et al., 2007). However, Dai<sup>-/-</sup> mice showed normal IFN-I production after B-DNA stimulation. This suggests that there might be other DNA receptors that DAI are redundant to (Lippmann et al., 2008). This finding then led to the researchers to look for other cytosolic DNA receptors.

AIM2

It has been shown that NLRP3 forms inflammasome and activate caspase-1 in response to high concentrations of internalized adenovirus DNA (Muruve et al., 2008).
However, researchers also found that Nlrp3 deficient cells displayed a normal caspase-1 activity in response to transfected double stranded DNA from various source (Hornung et al., 2008; Muruve et al., 2008). This suggests that intracellular dsDNA-induced caspase-1 activation is NLRP3 independent, and other factors are responsible for sensing intracellular dsDNA and activation of caspase-1. Using both human and mouse cells, researcher found that absent in melanoma 2 (AIM2), pyrin and HIN domain-containing protein (PYHIN) 8 family member, serves as a receptor to cytosolic DNA and activate both caspase-1 and NF-κB (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010; Schroder et al., 2009). Knockdown and knockout of Aim2 inhibited caspase-1 activation after stimulation with both cytosolic dsDNA and dsDNA viruses (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Mechanistically, AIM2 binds to dsDNA through HIN200 domain and associates with the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) through pyrin domain (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Taken together, AIM2 is a cytoplasmic DNA receptor that binds to dsDNA and forms AIM2-containing inflammasome to activate caspase-1. However, AIM2 is not responsible for cytosolic dsDNA-induced IFN-I activation (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010; Schroder et al., 2009).

**RNA POL III**

RNA-sensing pathway was better studied prior to the discovery of DNA sensors. RIG-I is known as the cytoplasmic dsRNA sensor or RNA with 5’-triphosphate group (5’-ppp) (Takeuchi and Akira, 2008; Yoneyama et al., 2004). However, at that time, a
mystery in the field was that when AT-rich DNA was introduced into the cytosol, RIG-I deficient cells showed decreased IFN-I production compared to WT cells. Later on, RNA polymerase III was identified as the missing link. RNA polymerase III utilizes AT-rich dsDNA as a template to transcribe dsRNA with 5'-ppp. 5'-ppp dsRNA then serves as RIG-I ligand and activates RIG-I-dependent pathways. Researchers also tested a panel of stimuli and found that RNA polymerase III mediates herpes simplex virus type 1, Epstein Barr virus (EBV) and Legionella pneumophila induced IFN-I production (Chiu et al., 2009; Choi et al., 2009). RNA polymerase III was previously known to localize in both nucleus and cytoplasm (Jaehning and Roeder, 1977), however, its function in the cytoplasm remained unknown until it was found to detect dsDNA and transcribe cytosolic DNA into RIG-I ligand.

**IFI16**

Gamma-interferon-inducible protein 16 (IFI16) is the second member of the PYHIN family found as cytosolic DNA sensors (Jin et al., 2012; Unterholzner et al., 2010). After AIM2 was identified as a DNA sensor and shown to function in forming inflammasome and activation of caspase-1, researchers searched for additional cytoplasmic DNA sensors function in IFN-I pathway. They used a DNA motif derived from vaccinia virus (VACV), a potent inducer of IFN-I, to affinity purify DNA-binding proteins, and identified IFI16. Further experiments showed that overexpression of IFI16 enhanced cytosolic dsDNA and dsDNA virus-induced IFN-I activation (Figure 3) (Cavlar et al., 2012); knockdown of IFI16 using shRNA reduced production of IFN-I and ISGs. Mechanistically, IFI16 directly binds to dsDNA and recruits STING to the interaction
complex upon stimulation with dsDNA (Unterholzner et al., 2010). Later on, it was revealed that IFI16 forms inflammasome with ASC and caspase-1 in the nucleus and peri-nuclear region upon KSHV infection (Kerur et al., 2011). However, further studies are needed to elucidate how IFN16 distinguishes self- and foreign-DNA in the nucleus and whether KSHV-induced IFN-I pathway is also dependent on IFI16.

**DDX41**

Several members of DExD/H-box helicase superfamily have been identified to be cytosolic nucleic acid sensors in myeloid dendritic cells as well as plasmacytoid dendritic cells, including DDX1-DDX21-DHX36 complex, DHX33, DHX36 and DHX9 (Kim et al., 2010; Mitoma et al., 2013; Zhang et al., 2011a; Zhang et al., 2011c). Yong-Jun Liu’s group screened all 59 members of the DExD/H-box helicase superfamily by using small interfering RNA (siRNA). This led them to the identification of another cytocolic DNA senser, DDX41 (Figure 3) (Cavlar et al., 2012; Zhang et al., 2011b). The results showed that overexpression of DDX41 and STING enhanced IFN-b promoter activity. Conversely, reduction of DDX41 by shRNA resulted in a dampened IFN-I and proinflammatory cytokine production in response to dsDNA transfection and HSV-1 infection. Mechanistically, DDX41 associates with both dsDNA and STING in the cytosol and causing activation of the downstream signaling pathways, including IRF3 phosphorylation, TBK1 activation, p65 activation and MAPK activation. Later on, it was also found that DDX41 associates with a bacterial second messenger, c-di-GMP, and activates the STING-dependent pathways (Parvatiyar et al., 2012).
Although it is now well accepted that STING is a central adaptor protein for cytosolic DNA sensing and that all cytosolic dsDNAs activate STING and the downstream pathways in a sequence-independent manner, it is yet to be elucidated how DNA sensors activate STING. Chen’s group hypothesized that cytoplasmic dsDNA directly bound to DNA receptor, which then directly or indirectly activated the adaptor protein, STING. They carried out a well-designed *in vitro* experimental system using two cell lines, one as a screening cell line to screen for the candidate DNA receptor(s), the other as a reporter cell line to indicate the activation of STING-dependent pathways. These results showed that the potential molecule(s) induced by dsDNA stimulation and activated STING are not DNA, RNA or protein and is not heat-sensitive. Further investigation showed that the STING-activating molecule is cyclic GMP-AMP (cGAMP) and is synthesized by cGAMP synthase (cGAS), a nucleotidyltransferase (NTase) and male abnormal 21 (Mab21) domain containing protein (Figure 4) (Ablasser and Hornung, 2013). Collectively, the studies suggested that cGAS directly bound to cytosolic dsDNA, which then led to the synthesis of a second messenger, cGAMP. cGAMP then directly bound to and activated the adaptor protein STING and STING-dependent pathways (Sun et al., 2013; Wu et al., 2013). The conclusions were later on confirmed in gene knockout cells and mice (Li et al., 2013).

In addition, cGAS was found to be crucial in regulating HIV-induced IFN-I production. RNA from HIV infection would be reverse-transcribe into dsDNA, which served as a ligand for cGAS. Upon binding to dsDNA, cGAS would be activated and
convert GMP and AMP into cGAMP, which will then activate STING and induced IFN-I and proinflammatory cytokine production (Gao et al., 2013).

**STING**

STING, also known as mediator of interferon regulatory factor 3 activation (MITA) (Zhong et al., 2008), plasma membrane tetraspanner (MPYS) (Jin et al., 2008) or endoplasmic reticulum interferon stimulator (ERIS) (Sun et al., 2009) and transmembrane protein 173 (TMEM173), was first discovered by an expression cloning technique using cDNA library (Ishikawa and Barber, 2008; Zhong et al., 2008). The readout of the screening process was IFN-β promoter activity induced by the transfected expression construct in 293T cells. Studies showed that STING is located to ER, mitochondria outer membrane or mitochondria-associated ER membrane in unstimulated cells. It is also ubiquitously expressed by all human tissues tested, and more predominantly expressed in most macrophages, dendritic cells, T cells, B cells and a variety of epithelial cells, endothelial cells and fibroblasts. Functionally, STING was shown to be an adaptor protein that associates with different cytosolic DNA sensors, such as IFI16, DDX41, cGAS (Figure 2-4). Upon activation, STING recruits a downstream protein kinase, TBK1 and become phosphorylated by TBK1 and then recruits transcription factor IRF3 to the interaction complex. STING has been shown to directly bind to cytosolic dsDNA and cyclic dinucleotide (CDNs) (Burdette et al., 2011). The crystal structure of cytosolic C-terminal domain (CTD) of STING binding to c-di-
GMP has been resolved by several groups and they showed that the CTD of STING forms a homodimer and c-di-GMP binds to the pocket formed by this dimer (Huang et al., 2012; Ouyang et al., 2012; Shang et al., 2012; Shu et al., 2012). The c-di-GMP bound form of STING has a stronger association with TBK1 and resulted in an enhanced IFN-I production (Barber, 2014; Gurtler and Bowie, 2013).

1.3 NLR proteins are negative regulators of innate immunity

The nucleotide-binding domain and leucine-rich-repeat-containing (NLR) protein family is discovered by genomic mining of proteins with similar structures as MHC class II transactivator (CIITA) (Ting et al., 2008a). NLR proteins have tripartite domain architecture: N-terminal effector domain, central nucleotide binding domain and C-terminal leucine-rich-repeat domain. NLR family members have been further categorized as several different sub-families according to their specific effector domains. NLRC proteins are one sub-family that contains one or more caspase recruitment domains (CARD) (Harton et al., 2002; Inohara and Nunez, 2003; Ting et al., 2008a; Tschopp et al., 2003). Five NLR proteins belong to this sub-family, including NOD1, NOD2, NLRC3, NLRC4 and NLRC5. NLRP proteins have a pyrin domain (PYR) as effector domain. Other effector domains include transactivation domain (AD), baculovirus inhibitor of apoptosis repeat domains (BIR) and uncharacterized domain (X) (Ting et al., 2008a).

NLR proteins were also found to be evolutionarily conserved from echinoderm sea urchin to human, though some metazoan genomes do not have these genes, such as Drosophila and Caenorhabditis (Rast et al., 2006; Ting and Davis, 2005).
The functional and mechanistic characterization of NLR proteins has been a major focus of research in the past decade. NLR proteins are pivotal in host proinflammatory responses to various stimuli. The NLR family includes about 22 members (human) that have distinct functions in various signaling pathways (Davis et al., 2011; Ting et al., 2010; Ting et al., 2008a). Functionally, NLR proteins can be classified into two categories: inflammasome-forming NLRs and noninflammasome NLRs (Ting et al., 2010). Inflammasome regulates activation of caspase-1 and is a big protein complex that is formed by multi-components, including NLRs, ASC and caspase-1. Activation of caspase-1 results in the proteolytic cleavage of IL-1β and IL-18 (Martinon et al., 2002). Electron microscopy (EM) has shown that inflammasome structures share striking structural similarities to the apoptosome (Faustin et al., 2007). NLRP1, 2, 3, 6, 12, NLRC4, NOD2 are all found to form inflammasome with caspase-1, with or without the adaptor ASC to facilitate IL-1β processing when ectopically overexpressed (Agostini et al., 2004; Grenier et al., 2002; Martinon et al., 2002; Poyet et al., 2001; Wang et al., 2002).

Although inflammasome-forming NLRs are thought to be crucial in infectious disease, metabolic disorders, tumorigenesis and autoimmune disorders, studies are converging to suggest that non-inflammasome NLRs are equally important (Ting et al., 2010). Studies have shown that NLRs function in the regulation of type I interferon production, NF-κB activity, MAPK activation, host antimicrobial response and sterile inflammation. NOD1 and NOD2 represent the first NLRs that mediate non-inflammasome functions (Ting et al., 2010). Recently, there is an emerging role of
nonflammasome NLRs as negative regulators of innate immunity. We will focus on introducing the functions of several negative-regulator-NLRs in this section.

**NLRX1**

NLRX1 was the first NLR molecule shown to regulate intracellular RNA and RNA virus sensing pathway. Overexpression of NLRX1 dampened IFN-I and proinflammatory cytokine production in response to several RNA virus and RNA analogue poly(I:C). Knockdown of NLRX1 resulted in an enhanced production of IFN-I and proinflammatory cytokine (Moore et al., 2008). These findings were later confirmed by using gene-deletion cells and mice (Allen et al., 2011; Xia et al., 2011). Mechanistically, NLRX1 localized to mitochondria membrane and associated with as MAVS to block MAVS-RIG-I association, which is required for the downstream signaling. NLRX1 has also been shown to directly bind to dsRNA by crystallography (Hong et al., 2012; Xiao and Ting, 2012). Studies performed in Nlrx1−/− mice also showed that NLRX1 is crucial in regulating lung inflammation in response to Influenza A virus (IAV) infection or intranasal delivery of LPS. Nlrx1−/− mice showed an enhanced production of IFN-β and IL6 and an increased cellular infiltration in the lung (Allen et al., 2011; Xia et al., 2011). There are also studies showing that NLRX1 positively regulates production of reactive oxygen species (ROS) (Tattoli et al., 2008). It is possible that NLRX1 can function both as a negative regulator of RIG-I-MAVS signaling and as a positive regulator of induction of ROS. However, the physiological relevance of the latter has not been verified in gene-deletion animals. The literature also indicates that NLRX1 augments RNA virus-induced cellular autophagy response (Lei et al., 2012). Nlrx1−/− cells showed an
increased IFN-I and proinflammatory cytokine production and a decreased autophagosome formation, this gave the host cell an advantage to fight against viral infection. Mechanistically, NLRX1 recruits TUFM, which in turn recruits ATG5-ATG12 and forms a protein complex to regulate autophagy response.

Most recently, the function of NLRX1 has again been expanded. The literature showed that $Nlrx1^{-/-}$ mice are more susceptible to experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS) (Eitas et al., 2014). Phenotypically, $Nlrx1^{-/-}$ mice showed a worsened clinical scores and increased tissue damage in central nervous system (CNS) during EAE, accompanied by increased inflammatory and cytokine responses (Eitas et al., 2014). This again affirms that NLRX1 reduces inflammatory responses.

**NLRP6**

NLRP6 was shown to suppress bacteria infection-induced NF-κB activation and MAPK activation (Anand and Kanneganti, 2012; Anand et al., 2012). During *Listeria monocytogenes* and *Salmonella typhimurium* infection, Nlrp6 deficiency led to an elevated level of p65 activation and ERK phosphorylation. Nlrp6 deficient cells also showed faster bacteria clearance.

There are also studies showed that in colitis model and colon cancer models, *Nlrp6^{-/-}* animals showed increased inflammation and tumorigenesis (Elinav et al., 2011). In the absence of NLRP6, researchers observed increased CCL5 and reduced IL-18 production in the colon. However, there is still a question on whether NLRP6 affect inflammasome function since there was no evidence showing that IL-1β production was
affected due to deficiency of Nlrp6. Further biochemical and molecular evidence are yet
to be done.

NLRP12

NLRP12 was the first studied inhibitory NLR protein. It was found to inhibit both canonical (Williams et al., 2005) and noncanonical NF-κB pathway (Lich et al., 2007). The inhibitory function of NLRP12 in the noncanonical pathway is through its association of NIK. It has been shown that upon binding to NIK, NLRP12 can de-stabilize NIK and thus inhibit the downstream signaling. However, when looking at the activation of canonical NF-κB activation, a modest effect was observed when NLRP12 expression was knocked down with shRNA, although it was confirmed later in gene deletion cells that NLRP12 inhibits canonical NF-κB activation (Zaki et al., 2011).

The function of NLRP12 in regulating NF-κB activation led researchers to the studies of the role of NLRP12 in inflammation-associated diseases. In a colitis and colitis-associated colon cancer model, Nlrp12−/− animals showed an increased number of polyps and increased disease susceptibility (Allen et al., 2012; Zaki et al., 2011). Mechanistically, canonical and noncanonical NF-κB was hyper-activated in Nlrp12−/− mice and resulted in an elevated production of the downstream chemokines and proinflammatory cytokines, including Cxcl12, Cxcl13, TNF and IL6, which are associated with several forms of solid cancer. There were also an elevated activation of ERK and AKT signaling pathways in Nlrp12−/− mice, which are tightly associated with cell growth and tumorigenesis. Therefore, NLRP12 serves as a checkpoint protein for
canonical and noncanonical NF-κB activation, inflammation and inflammation-associated tumorigenesis.

It has also been proposed that NLRP12 forms an inflammasome under certain stimuli, such as attenuated *Yersinia* infection (Vladimer et al., 2012). It was shown that NLRP12 recognizes acylated lipid A in *in vitro* assays. In addition, *Nlrp12*−/− mice showed a dampened IL-18 production and were more susceptible to an attenuated strain of *Yersinia pestis* infection. In humans, there is an association between inflammasome activation and NLRP12 polymorphisms during periodic fever syndromes (Jeru et al., 2008). Most recently, it was shown that NLRP12 negatively regulates *Salmonella enterica* serovar Typhimurium-induced innate immune responses (Zaki et al., 2014). This effect is independent of inflammasome activation. In this study, it was found that *Nlrp12*−/− mice showed reduced bacteria burden in both the liver and spleen after salmonella infection, but increased inflammatory cytokine and antibacterial peptide production. A further investigation showed NLRP12 inhibits canonical NF-κB and ERK activation, as well as NO production.

1.4 The intersection of innate immunity and DNA damage-repair response

**Brief overview of DNA damage response**

Cells in an organism are continuously challenged with genotoxic stress, which leads to various forms of DNA damages. It has been reported that each cell in the human body faces thousands of DNA damage incidents every day (Lindahl and Barnes, 2000). The sources that cause DNA damage could be endogenous cell metabolites or exogenous insults, such as irradiation, pathogen invasion and environmental toxic...
chemicals (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). Though the majority of DNA damages are challenges to genomic integrity, there are also programmed-DNA lesions, such as developmentally-regulated genome rearrangements in lymphocytes and germ cells (Jackson and Bartek, 2009; Longhese et al., 2009; Tsai and Lieber, 2010).

DNA damage could be detrimental to cells if dysregulated, leading to gene mutation, aberration of chromosomal structures and loss of genetic information. Thus, eukaryotic cells have evolved a signal transduction network during DNA damage, named DNA damage response (DDR), in order to preserve genome integrity and avoid the adverse effects caused by DNA damage (Hoeijmakers, 2001; Tsai and Lieber, 2010).

DDR signaling pathway are tightly regulated temporally and spatially to sense DNA damage as soon as it occurs, then amplify the signal by recruiting the responsible factors and then activate the downstream effector proteins to repair damage and appropriately determine cell fate (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The signal transduction of DDR is through protein-protein interaction and cascades of protein post-translational modifications, which allow the signaling to be fast and reversibly-regulated (Polo and Jackson, 2011).

The major players in DDR signaling pathway (Figure 5) is a group of phosphatidylinositol 3-kinase-like proteins: DNA-PK, ATM and ATR (Yang et al., 2003). DNA-PK is required for NHEJ repair and can be directed to the site of DNA damage by Ku. DNA-PK is also required for V(D)J recombination which utilizes NHEJ. DNA-PK deficient mice suffer from severe combined immunodeficiency due to impaired V(D)J
recombination (Lee and Kim, 2002; Smith and Jackson, 1999). ATM senses the double strand DNA breaks (DSB), the most toxic form of DNA damage to cells. ATM directly phosphorylates the relevant transcription factors, such as the tumor suppressor p53 (Banin et al., 1998; Canman et al., 1998). There is also evidence that DNA damage can activate the transcription factor NF-κB in an ATM-dependent manner (Piret et al., 1999). However, the mechanism remains to be elucidated.

**DNA damage response-associated diseases**

The DNA-damage-and-repair response is an ancient and conserved way of protecting our genomic stability and integrity (Kastan, 2008). The consequence of DNA damage if left unrepaired is diverse (Bohr, 2002). Acute and short-term DNA-damage results in cell cycle-arrest or even cell death. Accumulating and long-term DNA damage leads to gene mutation and oncogenesis, as well as other genetic diseases (Greenberg et al., 2006; O'Driscoll, 2012).

There are various different types of DNA damage and no single repair system is capable of repairing all kinds of damages (Iyama and Wilson, 2013). Fortunately, DNA damage is counteracted by a sophisticated DNA repair system with different signaling pathways that can cope with different DNA damage specifically. DNA repair responses arose very early in evolution and are highly conserved. Accumulating studies showed that there are four main DNA damage repair pathways to cope with different type of DNA damage in mammals. They are homologous recombination (HR), non-homologous end joining (NHEJ), base-excision repair (BER) and nucleotide-excision repair (NER).
These four different pathways are partly overlapping and can cover almost all kinds of DNA damage (Maisonobe et al., 2013).

Homologous recombination and end-joining pathways are developed to deal with double strand breaks (DSBs), which is the most toxic and problematic form of DNA damage (Carr and Lambert, 2013). Homologous recombination usually takes place in S and G2 phase of DNA replication, when DNA is already replicated and has a second copy to align the breaks. On the other hand, end joining is less accurate and usually happens during G1 phase of cell cycle when the sister chromatin is not available.

BER and NER are in charge of repairing damages on a single strand of DNA (Seo and Jung, 2004). BER is responsible for repairing small chemical alteration of bases. This kind of lesions often cause miscoding and lead to gene mutation though they sometimes do not even impede transcription or replication. Thus, BER is particularly crucial for preventing gene mutagenesis (Parsons and Dianov, 2013). NER targets a wide class of helix-distorting lesions, which usually interfere with transcription and replication (Melis et al., 2013). The other interesting fact worth mentioning is that BER-targeted lesions are usually endogenously originated, though not exclusively so, whereas NER-targeted lesions are usually caused by exogenous insults.

As we mentioned above, DNA damage can be very toxic if left unattended. One of the clearest associations of DNA damage with diseases is cancer (Wallace et al., 2012).

*Cancer*
Genome instability is a fundamental feature of cancer. Cancer can be thought of as a disease of our genes and is driven by genome instability and fuelled by a variety of DNA damage and replication errors (O'Driscoll, 2012). An example that illustrates the intimate relationship between cancer and DNA damage is that most carcinogens work by causing DNA damage and generating gene mutations. In addition, inherited DNA repair defects often serve as predisposing factors for cancer, such as xeroderma pigmentosum (XP) (Niedernhofer et al., 2011; Schwertman et al., 2013), a syndrome associated with inborn defects in NER (Sepe et al., 2013). Patients with XP exhibit more than 1000-fold of incidence of sun-induced skin cancer compare to patients without the disease. Mutations in mice that target almost all of the factors in NER signaling pathway showed a pronounced cancer predisposition (de Boer and Hoeijmakers, 1999). Another prime example is the inherited mutations in BRCA1 and BRCA2, which are associated with a strong predisposition to breast cancer (Lord and Ashworth, 2013).

Oncogene activation or tumor suppressor inactivation can both lead to abnormal cell proliferation, which generates DNA-replication-associated genomic stress, thus activating DDR, more specifically, ATM/ATR-dependent pathways to induce cell death or senescence (Bartkova et al., 2005; Gorgoulis et al., 2005).

Thus, DDR is activated to create a barrier to protect against tumorigenesis and malignancy. However, defects in this DDR barrier can also lead to higher frequency of cancer incidence.

*Immune deficiency*
DDR factors are required during immune system development, specifically during V(D)J recombination to generate a big repertoire of immune cell receptors. Ataxia telangiectasia (AT) patients and Nijmegen breakage syndrome (NBS) patients have mutation or defects in ATM and NBS1, respectively (Marechal and Zou, 2013). One of the most challenging symptoms is that those patients are highly prone to sometimes-fatal infections, and this is largely due to immune deficiency. Furthermore, class-switch recombination is affected in AT patients.

**Aging**

There is an emerging interest in understanding the mechanism of aging as a means to increase longevity and mitigate aging-associated diseases. One of the theories for the cause of aging is the accumulation of DNA lesions (Schumacher et al., 2008). The links between DNA damage and aging are briefly summarized as followings. Firstly, patients with inherited defects in DDR often suffer from a premature or accelerated aging symptom, such as Werner's syndrome, which is caused by mutation in WRN gene; Hutchinson-Gilford progeria syndrome (HGPS), which has defects in Lamin-A (responsible for DSB repair signaling pathway) (Musich and Zou, 2011). Researchers also generated several mouse models with defects in DDR factors, some of the animals display a dramatic accelerated or premature ageing phenotype, including most of NER factor deficiency animals (Schwertman et al., 2013). Secondly, there is evidence showing that insulin-like growth factor 1 (IGF-1) and other growth hormone regulate longevity and that DNA damage downregulates this signaling pathway (Schumacher et al., 2008). Thirdly, cell apoptosis and senescence are also
thought to contribute to aging by exhaustion of stem cell in the process of tissue regeneration. DNA damage was thought to contribute to aging by inducing cell senescence and apoptosis, as well as causing stem cell exhaustion (Insinga et al., 2014).

**Interplay of DNA damage and innate immune responses**

Evolutionarily, DNA damage response should arose earlier than immune response given the fact that cells must protect itself from errors in replication or environmental stimulus that could break genomic integrity for basic cell survival. DNA damage responses have been studied intensively in the last 50 years, and much has been elucidated. By contrast the molecular mechanisms by which innate immune responses respond to and recognize nucleic acids has a history that is less than a decade. However, there are accumulating evidence showing the crosstalk between these two distinct and interwoven pathways. Several work has shown that factors in DNA damage response pathway play a role in innate immune signaling and vice versa. Understanding the interplay of DNA damage responses and innate immune responses potentially have translational implications and could help develop new treatment for immune disorders. Next, we will review several the factors that are involved in both pathways.

*IFI16*

IFI16 was first identified as a cytosolic dsDNA sensor that recognizes cytosolic pathogen dsDNA and triggers IFN-I production (Jin et al., 2012; Unterholzner et al.,
2010). It was then shown that IFI16 is also involved in autoimmune disorders (Costa et al., 2011). Specifically, antibodies against IFI16 were found in the sera of SLE patients. In assays performed in cell culture, IFI16 translocates to the cytoplasm from nucleus after UV-B irradiation. Although the detailed mechanisms are lacking, this finding provides evidence that innate immune sensors can be involved in cellular response against UV-B irradiation.

**DNA-PK**

On the contrary to IFI16, DNA-PK was first found to be crucial in DDR. It was one of several major players in DSB signaling pathway (Anderson, 1993; Anderson and Lees-Miller, 1992; Sirbu and Cortez, 2013). DNA-PK can be activated and recruited by Ku70 and 80, which recognize DSB (Sirbu and Cortez, 2013). Previous work also showed that DNA-PK is able to activate NF-κB through activation of MEK-ERK-IKK signaling pathway after DNA damage (Sabatel et al., 2011). Recently, DNA-PK was characterized as a cytosolic dsDNA sensor that directly binds to bacterial or viral dsDNA and activate transcription factor NF-κB and IRF3 which then can trigger IFN-I production (Ferguson et al., 2012). There are also studies showing that DNA-PK can interact with transcription factor Aire, which then upregulates the expression level of TLR1, TLR3 and TLR8 (Kong et al., 2011).

Collectively, DNA-PK is crucial in both innate immune response and DNA damage response. It will be interesting to investigate how DNA-PK copes with different upstream signals and activates the right downstream factor to guide the appropriate
cellular response to the stimuli. It is also worth looking at how different pathways downstream of DNA-PK affect each other.

**MRE11**

MRE11 was part of the MRE11-RAD50-NBS1 (MRN) complex that is responsible for DSB repair signaling pathway. MRN complex was shown to bind to DSB directly in vitro and in vivo and was thought to be important for the initiation of DSB repair process and ATM recruitment and activation (Lavin, 2007; Lee and Paull, 2007). Later on, MRE11 was shown to directly bind to cytosolic dsDNA and activate IFN-I response in a STING-dependent manner. MRE11 can also regulate STING trafficking upon binding to cytosolic dsDNA, which is crucial for downstream signaling (Kondo et al., 2013). These findings again reinforce point that there is crosstalk between DNA damage response and innate immune response.

**AIM2**

Absent in melanoma 2 (AIM2) was named based on the finding that the expression of AIM2 is extremely low in melanoma. However, there is no detailed mechanistic study to show how AIM2 expression is regulated in melanoma or the significance of the low expression of AIM2. Functional characterization of AIM2 showed that AIM2 forms inflammasome with ASC and caspase-1 in the cytosol to process pro-IL1b and pro-IL18 to mature form in response to intracellular dsDNA stimulation (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010). Unpublished data in the lab shows that *Aim2<sup>−/−</sup>* mice develop more tumor than WT mice
in a DSS/AOM animal model. Mechanistically, AIM2 associate with DNA-PK and AKT to
downregulate tumorigenesis in an inflammasome-independent manner. It will be
interesting to explore whether AIM2 affect DNA damage response due to its direct
association with DNA-PK and the fact that a lot of DNA damage response defect mouse
model display a colitis-associated colon cancer phenotype.
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<td>STING, TBK1</td>
<td>IFN-1, TNF and IL6</td>
</tr>
<tr>
<td>DDX41</td>
<td>dsDNA, c-di-GMP, c-di-AMP</td>
<td>STING, TBK1</td>
<td>IFN-1, TNF and IL6</td>
</tr>
<tr>
<td>cGAS</td>
<td>dsDNA</td>
<td>STING</td>
<td>IFN-1, TNF and IL6</td>
</tr>
<tr>
<td>STING</td>
<td>dsDNA, c-di-GMP, c-di-AMP, cGAMP</td>
<td>IFI16, DDX41, cGAS, TBK1, IRF3, MRE11, DNA-PK, STAT6</td>
<td>IFN-1, CCL2, CCL20, MCP-3, TNF and IL6</td>
</tr>
</tbody>
</table>
Figure 1. Canonical and noncanonical NF-κB signaling pathway

(Adapted from Vucic et al., 2011)
Figure 2. Nucleic acid induced IRF3/7 activation

(Adapted from www.invivogen.com)
Figure 3. IFI16 and DDX41 downstream signaling

(Adapted from Cavlar et al., 2012)
Figure 4. cGAS downstream signaling

(Adapted from Ablasser et al., 2013)
Figure 5. DNA damage response pathway

(Adapted from Bean et al., 2013)
REFERENCES


CHAPTER TWO: NLRC3, A MEMBER OF THE NLR FAMILY OF PROTEINS, IS A NEGATIVE REGULATOR OF INNATE IMMUNE SIGNALING INDUCED BY THE DNA SENSOR STING

SUMMARY

Stimulator of interferon genes (STING, also named MITA, MYPS or ERIS) is an intracellular DNA sensor that induces type I interferon through its interaction with TANK-binding kinase 1 (TBK1). Here we found that the nucleotide-binding, leucine-rich repeat containing protein, NLRC3, reduced STING-dependent innate immune activation in response to cytosolic DNA, cyclic di-GMP (c-di-GMP) and DNA viruses. NLRC3 associated with both STING and TBK1, and impeded STING-TBK1 interaction and downstream type I interferon production. Using purified recombinant proteins NLRC3 was found to interact directly with STING. Furthermore, NLRC3 prevented proper trafficking of STING to perinuclear and punctated region, known to be important for its activation. In animals, herpes simplex virus 1 (HSV-1)-infected Nlr3−/− mice exhibited enhanced innate immunity, reduced morbidity and viral load. This demonstrates the intersection of two key pathways of innate immune regulation, NLR and STING, to fine tune host response to intracellular DNA, DNA virus and c-di-GMP.

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INTRODUCTION

Nucleic acid and small nucleic acid-like molecules can act as pathogen associated molecular patterns (PAMPs) to cause type I interferon (IFN-I) and other cytokine induction (Takeuchi and Akira, 2007). Among DNA sensors, STING (also referred to as mediator of IRF3 activation (MITA), plasma membrane tetraspanner (MPYS) or endoplasmic reticulum IFN stimulator (ERIS) has emerged as central for DNA-induced IFN-I activation (Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008). Other DNA sensors such as gamma-interferon-inducible protein16 (IFI16) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) are known to activate IFN-I in a STING-dependent manner (Unterholzner et al., 2010; Zhang et al., 2011).

The nucleotide binding domain (NBD) and leucine-rich repeat (LRR) containing proteins, NLRs, are intracellular sensors that regulate inflammatory responses (Eisenbarth and Flavell, 2009; Shaw et al., 2010). Most NLRs positively influence inflammatory responses, particularly the inflammasome NLRs. However emerging studies of gene-deficient mice have revealed that several NLRs negatively affect innate immune responses (Allen et al., 2011; Allen et al., 2012; Anand et al., 2012; Cui et al., 2010; Schneider et al., 2012; Xia et al., 2011; Zaki et al., 2011). Notably, we have previously shown that a caspase activating and recruitment domain (CARD) containing NLR, NLRC3, reduces LPS-induced nuclear factor kappa B (NF-κB) activation through inhibiting the adaptor protein TNF receptor associated factor 6 (TRAF6) (Schneider et al., 2012). However, the intersection of NLRs with DNA-sensing molecules has not been described. In this report, we find that NLRC3 deficiency also leads to increased innate immune response to intracellular DNA and c-di-GMP in both hematopoietic and non-
hematopoietic cells. NLRC3 interacts with STING and the protein kinase TBK1, leading to reduced STING-TBK1 association, improper STING trafficking and decreased activation of innate immune cytokines. However this interference is separate from the previously described function of NLRC3 in impeding TRAF6 activation during LPS response. This work reveals the intersection of NLR with STING-mediated DNA sensing and unveils the multi-facet function of NLR family.
RESULTS

NLRC3 deficiency leads to elevated of DNA- and HSV-1-induced IFN-I and cytokine production

During our screening of NLR-deficient cells for new functions, we observed that IFN-I protein (Figure 1A) was higher in Nlrc3−/− bone marrow-derived macrophages (BMDM) than wildtype (WT) cells. This enhancement was observed in response to transfected poly(dA:dT) but not to extracellular poly(dA:dT), poly(I:C) or LPS (Figure 1A). Interleukin-6 (IL-6) protein was also higher in Nlrc3−/− BMDM in the presence of intracellular poly(dA:dT) but not extracellular poly(dA:dT) (Figure 1B). In addition, the effect of NLRC3 was extended to the interferon stimulatory DNA (ISD), which has been used to more specifically demonstrate cytoplasmic DNA sensing (Chiu et al., 2009; Stetson and Medzhitov, 2006). NLRC3 also negatively regulates IFN-I (Figure 1C-E) and IL-6 (Figure S1A) responses to ISD in mouse embryonic fibroblasts (MEFs). These results suggest that NLRC3 functions as a negative regulator of cytoplasmic DNA sensing. To determine its role in a more physiologic setting, Ifna4 and Ifnb response to a DNA virus, Herpes simplex virus 1 (HSV-1) was tested and found to be higher in Nlrc3−/− BMDMs (Figure 1F-G) and peritoneal macrophages (Figure 1H-I). The impact of NLRC3 is not limited to type I IFN because tumor necrosis factor (TNF) protein and transcript were similarly increased (Figure 1J-K). However, NLRC3 did not affect various responses to the Sendai RNA virus (SeV) (Figure 1K).

To assess if the suppressive role of NLRC3 on DNA-induced IFN-I and cytokine response is exhibited in non-immune cells, pairs of Nlrc3+/− and Nlrc3−/− MEFs were isolated from siblings from heterozygous matings. Ifnb and Tnf transcripts were
significantly increased in Nlrc3−/− MEFs in response to HSV-1 (Figure 1L-M), as were IFN-β and IL-6 proteins (Figure 1N-O). However, Nlrc3−/− MEFs responded normally to SeV (Figure 1O). The lack of an effect of NLRC3 on poly(I:C) or RNA virus-induced cytokine responses was more extensively analyzed. Wildtype and Nlrc3−/− cells responded similarly to Sendai virus, intracellular or extracellular poly(I:C), and vesicular stomatitis virus (VSV) under a variety of test conditions (Figure S2).

Due to concerns about variations in MEFs, we isolated a second pair of sibling-matched MEFs, and identical effects of Nlrc3 deletion on Ifna4 and Ifnb transcripts was observed, indicating that the suppressive effect of NLRC3 was not due to artificial differences in one specific pair of gene-sufficient and deficient MEFs (Figure S1B-D). Similar results were observed when IFNβ protein was measured. Consistent with increased cytokines which would be expected to reduce viral load, HSV-1 genomic DNA copy number was significantly reduced in Nlrc3−/− MEFs (Figure 1P) and BMDMs (Figure 1Q). However HSV-1-mediated cell death was not altered in Nlrc3−/− MEFs, indicating that the observed differences were not due to different cell viability (Figure S3). These data demonstrate that NLRC3 attenuates cytokine response to intracellular DNA without affecting cell viability.

**NLRC3 deficiency causes increased IFN-β and IL-6 production in response to c-di-GMP and c-di-GMP**

C-di-GMP, a small di-nucleotide monophosphate, is a second messenger of bacteria such as *Listeria monocytogenes* and *Burkholderia thailandensis*, and activates the IFN-I response via interaction with STING(Burdette et al., 2011; Jin et al., 2011; Sauer et al., 2011). Nlrc3−/− MEFs produced more IFN-β and IL-6 proteins in response to
transfected c-di-GMP (Figure 2A-B). Additionally, Nlrc3−/− MEFs produced increased IFN-I and IL-6 in response to infection with c-di-GMP producing L. monocytogenes (Figure 2C-E). Increased IFNβ was also observed in Nlrc3−/− cells infected with another c-di-GMP producing bacteria, B. thailandensis (Figure 2F). Thus Nlrc3-deficiency leads to increased innate immune response to cytoplasmic DNA, c-di-GMP, and bacteria that produce c-di-GMP.

**NLRC3 inhibits the STING-dependent pathway**

Cytoplasmic DNA and c-di-GMP induce IFN-I through the STING molecule, which led us to examine both functional and molecular interactions between NLRC3 and STING (Burdette et al., 2011; Huang et al., 2012; Ouyang et al., 2012; Shang et al., 2012; Shu et al., 2012). To investigate if NLRC3 affects the STING pathway, we examined the impact of NLRC3 on the activation of IFN-β promoter-luciferase by STING. This reporter assay was internally controlled by the co-transfection of a Renilla luciferase construct. NLRC3 inhibited IFN-β promoter activation by STING by 9.72 fold. STING operates by interaction and activation of its downstream kinase, TBK1 (Tanaka and Chen, 2012). NLRC3 dramatically reduced IFN-β promoter activation by TBK1. However NLRC3 had no direct effect on the downstream interferon regulatory transcription factor 3 (IRF3), indicating that NLRC3 likely functions at the upstream STING-TBK level (Figure 3A). As a specificity control, another NLR, NLRP11, did not reduce IFN-β promoter activation by TBK1 (Figure 3B). NLRC3 also inhibited a second promoter driven by the canonical interferon-stimulated responsive element (ISRE), which is known to be activated by STING and TBK1 (Ishikawa and Barber, 2008; Zhong et al., 2008) (Figure 3C). However NLRC3 had no effect on the activation of the
ISRE promoter by mitochondrial antiviral signaling protein (MAVS) (also referred to as interferon-beta promoter stimulator 1 (IPS-1), virus-induced signaling adapter (VISA) and CARD adaptor inducing IFN-β (CARDIF)), which is important for RNA sensing, nor did it affect promoter activation by the downstream IRF3 (Figure 3C). In addition, NLRC3 inhibited NF-κB promoter activated by STING, and reduced MAVS activation slightly but did not affect retinoic acid-inducible gene 1 (RIG-I) (Figure 3D). We also observed that NLRC3 inhibited c-di-GMP and poly(dA:dT)-induced ISRE activation (Figure 3E). These experiments indicate that the predominant effect of NLRC3 is on the STING pathway. As an additional specificity control for NLR proteins, overexpression of NLRC5, which has been reported to inhibit various innate immune pathways when tested in an overexpression system (Cui et al., 2010) did not inhibit STING or TBK1-induced ISRE activation (Figure 3F). These experiments suggest that NLRC3 down-regulates innate immunity caused by STING and TBK1.

**NLRC3 associates with STING and TBK1 and alters the STING-TBK1 interaction after stimulation**

To explore the mechanism by which NLRC3 interferes with STING and TBK1 function, we tested if NLRC3 interacts with STING and/or TBK1. Transient transfection and co-immuno-precipitation followed by immunoblot showed that HA-NLRC3 strongly associated with Flag-STING and more modestly with Flag-TBK1, but not with Flag-IRF3 (Figure 4A), suggesting it interacts with the upstream STING-TBK complex but not with the downstream IRF3. This agrees with earlier data indicating that NLRC3 affected STING and TBK1 function but not IRF3 function (Figure 3A). Immunoblot of the input protein indicates that all of the proteins are expressed in readily detectable amounts (Figure 4A, right panel). In a more physiologic approach, HA-NLRC3 also associated
with endogenous STING (Figure 4B, top lane) and TBK1 (Figure 4C) in a hemi-endogenous system, but not with IRF3 (data not shown). These experiments indicate that NLRC3 can associate with STING and TBK1.

To further investigate whether the association between NLRC3 and STING is direct, we prepared purified, recombinant full length NLRC3 and truncated STING protein (amino acid 139-379 and 139-344) and performed a protein pull-down assay. The results show NLRC3 and STING directly bind to each other in a reciprocal pull-down assay (Figure 4D-E).

Next, a domain mapping experiment was conducted with NLRC3 deletion constructs (Figure 4F). Full-length NLRC3, CARD-NBD and NBD alone strongly associated with STING, while the CARD or LRR domain alone either did not associate, or did not associate strongly, with STING (Figure 4F). The CARD domain alone did not express in high amounts, however a prolonged exposure did not reveal any interaction (not shown). The presence of LRR reduced the association of NBD with STING suggesting that the LRR is an inhibitory domain. These data indicate that the primary interaction domain in NLRC3 is the region that includes the NBD domain. A reciprocal experiment was performed to map the interaction domain in STING (Figure 4G). The first 240 residues of the N-terminus or the C-terminal 111-379 residues did not interact with NLRC3, while the C-terminal residues 81-379 interacted with NLRC3. This indicates that the STING c-terminus soluble tail and residues 81-111 are required for interaction with NLRC3. The C terminal residues 139-344 was shown to directly bind NLRC3 as demonstrated in Figure 4D-E, thus this region contains residues necessary and sufficient for association with NLRC3. However, a confounding issue with STING is
that it is membrane bound and the transmembrane domain is required for STING localization to the ER. To examine this with the truncation mutants, we performed subcellular fractionation assay and showed that truncations 41-379 and 81-379 are membrane associated while 111-379 and 221-379 lose their membrane localization, indicating that residues 81-111 contained a sequence important for membrane-localization (Figure S4A). These results indicate that only the membrane-associated form of STING interacted with NLRC3. The interaction of STING with TBK1 produced the same results in that STING truncation mutant 81-379 but not 111-379 interacted with TBK1 (Figure S4B), which is also consistent with previous findings (Zhong et al., 2008). We also mapped the domains on TBK1 that bind to NLRC3. The result shows that N-terminus of TBK-1, which contained the kinase domain, is required for NLRC3 association (Figure 4H).

Upon DNA stimulation, the association of STING with TBK1 is essential to activate downstream signals (Ishikawa and Barber, 2008; Sun et al., 2009; Tanaka and Chen, 2012; Zhong et al., 2008). Thus we tested if the presence of NLRC3 interfered with the association of STING and TBK1. To pursue this in a physiologic system that did not involve overexpressed proteins, the association of STING and TBK1 was tested in \textit{Nlrc3}^{-/-} and control BMDMs in response to HSV-1 infection. The avoidance of overexpressed protein for this analysis is because overexpressed NLRs are prone to artifacts. The results show stronger STING-TBK1 association in \textit{Nlrc3}^{-/-} cells than WT controls 2-4 hours post-infection (Figure 4I, top lane; quantitation to the right). However, the association of STING-TBK1 was not enhanced by HSV-1. Because HSV-1 encodes a complex array of immune evasion and regulatory proteins that might
obscure the outcome, we resort to ISD as a simplified system to examine responses to DNA without the confounding regulatory functions associated with HSV-1. The result shows enhanced STING-TBK1 association in WT cells after ISD stimulation, which was further potentiated in \( Nlrc3^{-/-} \) cells 2-4 hours post-stimulation (Figure 4J, top lane; quantitation to the right). However at the six hour timepoint, STING-TBK1 interaction was more pronounced in WT cells. These results indicate that NLRC3 interfered with STING-TBK1 association at the 2-4 hr timepoint.

**NLRC3 blocks STING trafficking**

STING has been shown to traffic from the ER to a perinuclear/golgi location and to endoplasmic-associated puncta after DNA stimulation (Ishikawa et al., 2009; Saitoh et al., 2009). STING is reported to colocalize with TBK1 at these puncta, which represent the proposed platform for TBK1-mediated IRF3 activation. In cells transfected with an empty vector (EV), ISD caused STING to present in a perinuclear pattern (Figure 5A panel ii) followed by a punctated appearance (Figure 5A panel iii). However the presence of NLRC3 dramatically reduced the trafficking of STING to the perinuclear region (12-fold) (Figure 5A panel v) and completely prevented STING’s movement to puncta (Figure 5A panel vi). Thus NLRC3 reduced STING trafficking after ISD stimulation.

To further pursue this finding using a biochemical approach, we examined if the absence of NLRC3 affected STING and TBK1 co-localization by fast protein liquid chromatography (FPLC). This was performed using cell lysates prepared from HSV-1 infected and un-infected WT and \( Nlrc3^{-/-} \) primary MEFs. Similar to Figure 4I-J, this
strategy did not involve any over-expressed proteins, thus providing a physiologically relevant condition to test the impact of NLRC3 on STING and TBK1. Whole cell lysates were fractionated by FPLC followed by immunoblotting of the fractions for STING and TBK1. In mock, uninfected wildtype controls (Figure 5B, top four rows, densitometry results in Figure 5C left panel, quantitation in Figure 5D), a majority of TBK1 and STING resided in different fractions and only a small portion of STING and TBK1 was detected in the same fractions. In uninfected Nlrc3−/− cells, 2.09-fold more STING and TBK1 were found in the same fractions compared to wildtype controls. Upon HSV-1 stimulation, 4.41-fold more STING and TBK1 were detected in the same fractions in Nlrc3−/− cells than controls (Figure 5B, bottom four rows, densitometry results in Figure 5C right panel, quantitation in Figure 5D). The cumulative data in this Figure are consistent with a model where NLRC3 interacts with STING and TBK1 to impede the interaction, since removal of NLRC3 by gene deletion led to more association of these two proteins. The inhibitory effect of NLRC3 on STING-TBK1 association was observed at the uninfected state, and became more pronounce upon HSV-1 infection.

**Nlrc3−/− cells exhibit elevated signal transduction after HSV-1 infection**

To examine for changes in downstream signals that are known to be activated by STING and TBK1, we examined for changes in protein phosphorylation that lie downstream of STING activation post-HSV-1 infection. Phosphorylation of TBK1, IRF3, p65 and JNK were induced 4-6 hours post-infection in wildtype controls (Figure 6A). The amount of phospho-TBK1 and phospho-IRF3 4-6 hours post-infection were higher in Nlrc3−/− than control MEFs, while the phosphorylation of JNK was enhanced throughout all of the timepoints measured in Nlrc3−/− cells. HSV-1 infection did not
increase phosphorylation of ERK or p38, and NLRC3 did not alter these signals. HSV-1 infection induced p65 nuclear translocation was also visualized by confocal microscopy and was found to be significantly augmented in Nlrc3−/− cells (Figure 6B). Our earlier data indicate that NLRC3 affected the sensing of intracellular DNA. To study if downstream signals induced by DNA are affected by NLRC3, we assessed phosphorylation induced by ISD transfected into MEFs. Intracellular ISD caused increased phosphorylation of TBK1 and p-JNK in wildtype controls, and these responses, but not p-ERK, were further augmented in Nlrc3−/− cells, supporting the model that NLRC3 regulates signaling responses caused by intracellular DNA (Figure 6C). As a specificity control, intracellular poly(I:C) was transfected into cells, and it did not cause increases in the phosphorylation of multiple key pathways in Nlrc3−/− cells relative to controls (Figure 6D). These data suggest that NLRC3 is a negative regulator of innate immune signals generated upon HSV-1 infection and ISD stimulation.

However, this function of NLRC3 is distinct from its regulation of NF-κB signaling induced by TRAF6 during an LPS response (Schneider et al., 2012), as TRAF6 was not required for HSV-1-induced IFN-α activation (Figure S5A-B). TRAF6 also did not associate with STING in co-IP assays (Figure S5C).

**NLRC3 deficiency augments host response to HSV-1 in vivo**

Next, to examine the in vivo importance of NLRC3, Nlrc3−/− and control mice were infected intravenously (i.v.) with HSV-1, and survival, weight change and morbidity were monitored (Figure 7A-B). Infected control mice exhibited significant lethargy and lack of movement (Movie S1), while infected Nlrc3−/− mice were active and mobile (Movie S2). Many control mice had to be euthanized 6-8 days post-infection when their body
temperature was <32 °C, whereas 100% of similarly infected Nlrc3−/− mice showed a more modest temperature drop ranging from 34.2 °C to 35.9 °C. Control mice also exhibited rapid weight loss after HSV-1 infection and had to be sacrificed due to a >20% weight loss. In contrast, Nlrc3−/− mice maximally lost up to 11% of body weight and recovered 100% of body weight by day 9. Sera from HSV-1-infected Nlrc3−/− mice showed increased IFNβ, TNF and IL-6 six hours post-infection when compared to controls (Figure 7C-E). HSV-1 genomic DNA copy number was significantly reduced in Nlrc3−/− mice (Figure 7F). In contrast, weight loss or serum IFNβ level in Nlrc3−/− mice was not significantly different from WT mice after infection with VSV (Figure S6). Thus NLRC3 attenuates physiologic host response to HSV-1, a DNA virus, but not VSV, a RNA virus.
DISCUSSION

This study identifies NLRC3 as a negative regulator of type I IFN and proinflammatory cytokine production triggered by cytoplasmic DNA and HSV-1. It also reduced the response caused by c-di-GMP, which provided us with the clue that linked NLRC3 to the STING pathway. Mechanistically, NLRC3 inhibits type I IFN promoter activation by STING and TBK, but not by the RIG-I-MAVS pathway. NLRC3 can directly interact with STING to reduce STING-TBK1 association, which is normally required for interferon induction. Furthermore, NLRC3 blocks ISD-induced STING trafficking to perinuclear and punctated regions, which is important for signal transduction downstream of STING (Ishikawa et al., 2009; Saitoh et al., 2009). Ablation of the Nlrc3 gene led to enhanced anti-viral cytokine production and viral clearance in culture. Most important, HSV-1-infected Nlrc3−/− mice exhibited greatly reduced morbidity, enhanced interferon and cytokine production and reduced viral load. This work demonstrates that NLR is a negative regulator of innate immunity triggered by the STING pathway.

There are multiple papers by several group that identify the negative regulatory functions of NLRs. Studies of gene deletion strains show that NLRX1 inhibits RNA virus and LPS induced cytokines in a cell-specific fashion (Allen et al., 2011; Xia et al., 2011), NLRP12 reduces canonical and non-canonical NF-κB (Allen et al., 2012; Zaki et al., 2011), NLRP6 impedes MAPK and NF-κB activation(Anand et al., 2012), and NLRC5 inhibits NF-κB and MAPK activation in some, but not all, gene deletion strains (Cui et al., 2010; Kumar et al., 2011). In addition, an in vitro study shows that NLRP4 reduces IFN production induced by nucleic acids (Cui et al., 2012). These findings indicate a broad function for NLRs in attenuating innate immune responses. However, none of the
previously studied NLRs have been linked to the STING-mediated DNA-sensing pathway.

While our previous work showed a function of NLRC3 in reducing the activation of TRAF6 in response to LPS (Schneider et al., 2012), this report shows that intracellular DNA sensing during HSV-1 infection is independent of TRAF6. Furthermore, the present report also shows that NLRC3 does not affect IFN-I induction by LPS. Thus the impact of NLRC3 on LPS-induced cytokines such as TNF and IL-6 shown in our previous work (Schneider et al., 2012) likely occurs via a different path from IFN-I production caused by intracellular DNA. However, a recent paper indicates that TRAF6 is involved in cellular response to DNA and RNA (Konno et al., 2009). This may likely explain the more robust impact of NLRC3 in some experiments that used ISD instead of HSV-1. Further investigation is needed to fully assess the contribution of each pathway in response to nucleic acids in a NLRC3-dependent fashion.

The involvement of NLRC3 in two different responses (LPS-induced proinflammatory cytokines and intracellular DNA induced IFN-I response) is in line with other NLRs, which serve multiple functions. For example, NLRP3 and NLRP1 are involved in inflammasome function, but also in pyroptosis (Eisenbarth and Flavell, 2009; Kovarova et al., 2012; Masters et al., 2012). NOD2 activates NF-κB, MAVS-induced type I IFN and autophagy (Cooney et al., 2010; Homer et al., 2010; Sabbah et al., 2009; Travassos et al., 2010). NLRP6 mediates inflammasome activation (Elinav et al., 2011), inhibits NF-κB activation (Anand et al., 2012) and promotes epithelium repair and renewal (Chen et al., 2011; Normand et al., 2011).
It is well-accepted that cytosolic DNA is immune stimulatory, and STING is the central adaptor protein for multiple intracellular DNA-sensing pathways (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008). Additionally, STING also mediates responses to RNA (Ishikawa et al., 2009; Sun et al., 2009; Zhong et al., 2008), cyclic dinucleotides (Jin et al., 2011; Sauer et al., 2011), cyclic GMP-AMP (Wu et al., 2013), bacterial (Gratz et al., 2011; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Jin et al., 2011; Manzanillo et al., 2012; Watson et al., 2012), viral (Holm et al., 2012; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Sun et al., 2009; Zhong et al., 2008), eukaryotic pathogen-derived (Sharma et al., 2011) and self DNA (Gall et al., 2012). It also intersects with other DNA sensors such as IFI16 and DDX41 (Unterholzner et al., 2010; Zhang et al., 2011). Thus it is significant that NLRC3 impacts this central DNA sensing molecule. In contrast to its intersection with STING-TBK1, we have not found a direct effect of NLRC3 on IFI16 or DXD41 (not shown). We also have not found a consistent function for NLRC3 in altering host response to intracellular poly(I:C) or the RNA viruses tested. While previous work has shown a consistent role for STING in host response to DNA virus, the results are less consistent for RNA virus. For example, IFN\(\beta\) production and IRF3 nuclear translocation status are comparable between VSV-infected WT and \(\text{Sting}^{-/-}\) MEFs and BMDMs, while \(\text{Sting}^{-/-}\) dendritic cells produced less IFNa after VSV infection (Ishikawa et al., 2009). It is possible that an investigation of IFNa in dendritic cells might reveal a function for NLRC3 in response to VSV. It is also possible that NLRC3 inhibits RNA virus in a time- and dose-dependent fashion which was missed. Finally, NLRC3 only partially shuts off STING function, hence residual function might promote anti-RNA viral response.
The main finding of this work is that NLRC3 interacts with STING biochemically and functionally. It would follow that NLRC3 should reduce signals that lie downstream of STING activation. This is supported by the observation that Nlrc3-/- cells showed increased p-IRF3 (Figure 6A) and NF-κB phosphorylation/translocation (Figures 6A-B) after HSV-1 infection. The luciferase data showed that NLRC3 did not affect IRF3 activation of an ISRE promoter, hence the impact of NLRC3 is not directly on IRF3. We further showed that NLRC3 affected NF-κB activation by STING but not RIG-I or MAVS (Figure 3D), hence NLRC3 did not indiscriminately inhibit NF-κB activation. Instead it only inhibited NF-κB activation downstream of STING activation. Together, these data lead to the conclusion that NLRC3 negatively impacts STING, which then affects downstream events such as IRF3 and NF-κB activation.

In addition to pathogen-driven responses, DNA-dependent immune response triggered by self-DNA is associated with several diseases. As an example, DNase II deficient mice were unable to digest self-DNA from apoptotic cells and mice lacking DNase II died during embryonic development partly due to anemia (Kawane et al., 2001), which was rescued when STING was also removed (Ahn et al., 2012). This suggests that the cytosolic DNA-sensing pathway is involved in the pathology evoked by DNA sensing by STING.

In summary, our findings show the attenuation of DNA and c-di-GMP sensing by NLRC3 and reveal the intersection two pivotal pathways, NLR and STING in the control of innate immune responses. This work expands the function of NLRs to the important task of regulating host response elicited by intracellular DNA and c-di-GMP.
EXPERIMENTAL PROCEDURES

Cell culture

HEK293T cells were purchased from ATCC and maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin and 100µg/ml streptomycin. Nlrc3+/+ and Nlrc3−/− MEFs were generated from 13.5-day embryos and maintained in the complete DMEM medium described above with 1mM sodium pyruvate, 4mM L-glutamine and non-essential amino acid. BMDMs were generated in the presence of L-929 conditional medium as previously described. All cells were grown in a 37°C incubator supplied with 5% CO₂.

Reagents and antibodies

Poly (dA:dT) was purchased from InvivoGen, c-di-GMP from KeraFast, cytotoxicity detection kit from Roche, HSV-1 (KOS strain) from ATCC and propagated in Vero cells, Sendai virus (Cantell strain) from Charles River and VSV (Indiana strain) from ATCC and propagated in Vero cells. TNF and IL-6 ELISA kits were from BD Biosciences, mouse anti-IFN-β antibody was from Cosmo Bio, anti-IFN-β was from R&D system, anti-phospho-TBK1, TBK1, phospho-IRF3, IRF3, phospho-p65, p65, phospho-JNK, phospho-ERK, phospho-p38 were from Cell Signaling Technology, and anti-b-actin antibody was from Santa Cruz.

Experimental animals and and in vivo virus infection

The C57BL/6 Nlrc3+/− mice have been described (Schneider et al., 2012). C57BL/6 littermates were produced and used in selected experiments. Mice were
treated in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Eight-10 weeks old mice were infected with HSV (2x10^7 pfu of viruses per mouse) or VSV (5x10^7 pfu of viruses per mouse) by intravenous injection. The temperature and weight of the mice were monitored accordingly. For cytokine studies, mice were sacrificed 6 hours post-infection and sera were collected through cardiac puncture. For HSV-1 genome copy number measurement, brains were harvested 5 days post-infection. For all experiments, wild-type and *Nlrc3*^-/-^ mice were matched for age and sex.

**Plasmids and molecular cloning**

FLAG-tagged STING (originally cloned and named as MITA) full-length and domain truncation expression plasmids have been described (Zhong et al., 2008). HA-tagged NLRC3 full-length and domain truncation expression plasmids were cloned into pcDNA3.1.

**Real time RT-PCR**

Total RNA was extracted and assayed by Real-time PCR as described using SYBR green master mix or Tagman assay *(Schneider et al., 2012)*. Primers used: mIFNβ: 5’- ATGAGTGGTGGTTGCAGGC-3’, 5’- ATGAGTGGTGGTTGCAGGC-3’; mIFN-a4: 5’- CCTGTGTGATGGAAGGACC-3’, 5’- TCACCTCCCAGGCAGTACAGA-3’; actin: 5’- agggctatgctccctcac-3’, 5’- ctctcagctgtggtgtaa-3’; mTNF: Mm00443258_m1, mIL-6: Mm00446190_m1.)
HSV-1 genomic DNA copy number measurement

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer’s protocol. HSV-1 genomic DNA copy numbers were then determined by real-time PCR using HSV-1 specific primer: 5’-TGGGACACATGCCTTCTTG-3’, 5’-ACCCTTAGTCAGACTCTGTTACTTACCC-3’.

Virus, bacteria infection and poly(dA:dT) stimulation

BMDMs or MEFs were plated in 24-well plates and grown to 80% confluence overnight. The cells were washed with PBS and infected with HSV-1 (KOS strain), SeV (Cantell strain) or VSV (Indiana strain) at the indicated multiplicity of infection (MOI) at 37 °C in serum-free DMEM for 1h. The cells were then washed with warm PBS and cultured in complete DMEM.

*L. monocytogenes* (43251) or *B. thailandis* were grown to log phase and added to the cell cultures at a MOI of 10. After 30 min, gentamicin (50 µg/ml; Life Technologies) was added to the medium. The medium was changed after 1 hour of infection. Poly(dA:dT), poly(I:C) or ISD were added or transfected using lipofectamine 2000 (Invitrogen) at 4 µg/ml to BMDMs or MEFs for the indicated time. LPS were added into cell culture at 100 ng/ml. c-di-GMP were transfected at 1 µg/ml, 2 µg/ml or 4 µg/ml.

Co-immunoprecipitation

Procedures were done following the previous publication. (Schneider et al., 2012)
Transfection and Luciferase reporter analysis

HEK293T cells were seeded in 24-well plates at the density of 1.0×10^5 per well and transfected the following day by lipofectamine 2000 following the manufacturer’s instruction. 10 ng of pRT-TK Renilla luciferase reporter plasmid and 100 ng of firefly luciferase reporter plasmids were transfected together with indicated expression plasmids. Luciferase activity was measured 24 hours post-transfection using the Dual-Glo® Luciferase Assay System.

Recombinant protein purification and in vitro pulldown

Recombinant baculovirus expressing NLRC3 carrying an N-terminal Halo-Tag™ (HALO) and C-terminal hexahistidine tag (6XHIS) was generated as described by Mo et al. (Mo et al., 2012). For assays using immobilized Halo ligand capture (HaloLink™, Promega) for protein capture, recombinant HALO-NLRC3-6XHIS was partially purified using immobilized Ni^{2+} chromatography (PrepEase® Ni-TED, USB). HaloLink™ resin or HaloLink™ resin incubated with 1 µg of HALO-NLRC3-6XHIS was added to IP buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM β-mercaptoethanol and 0.1% CHAPS and 1 µg of recombinant STING protein (Ouyang et al., 2012). After 4 hours rotating at 4°C, the HALO-resin associated proteins were isolated by centrifugation for 30 s at 13k x g, the resins were washed 3 times with IP buffer supplemented with 300 mM NaCl. The resin associated STING proteins were liberated with 1x SDS-PAGE loading buffer and analyzed using immunoblot analysis. For assays using anti-STING antibody co-immunoprecipitation tag-free NLRC3 was purified to near homogeneity using tandem immobilized Ni^{2+} chromatography and HALO-ligand affinity
chromatography followed by TEV–protease treatment to remove both the HALO and 6XHIS as previously described. Purified NLRC3 (1 µg) and purified STING proteins (1 µg) were combined in IP buffer and incubated with anti-STING antibody while rotating at 4° C overnight. The STING protein and associated NLRC3 were captured using protein A paramagnetic beads (Miltenyi) according to the manufacturers protocols and assayed by immunoblot for STING (Cell Signaling) and NLRC3 (Sigma) as described.

**Fast protein liquid chromatography (FPLC)**

*Nlrc3<sup>+/+</sup>* or *Nlrc3<sup>−/−</sup>* MEFs were harvested after indicated treatment and lysed in hypotonic buffer (25 mM Tris-HCL, pH 7.5, 2mM DTT) with a dounce homogenizer on ice. The lysate was then centrifuged at 16,000g for 10 minutes. The precipitate was lysed in CHAPS buffer (50mM Tris-HCL, pH 7.5, 150 mM NaCl, 1mM DTT, 0.5 mM EDTA, 5% glycerol and 0.1% CHAPS) with a dounce homogenizer on ice and then centrifuged at 16,000g for 10 minutes. The protein concentrations of the supernatant were determined by Bio-Rad Protein Assay. Equal amount of protein from *Nlrc3<sup>+/+</sup>* or *Nlrc3<sup>−/−</sup>* cells were subjected to size exclusion chromatography (Superose 6). The indicated fractions were subjected to western blot analysis with the indicated antibodies. Densitometry for western blot was performed using ImageJ.

**Confocal microscopy**

*Nlrc3<sup>+/+</sup>* or *Nlrc3<sup>−/−</sup>* BMDMs (4 × 10<sup>5</sup>) were seeded onto coverslips in 24-well dishes and were grown overnight before inoculation with HSV-1 at the MOI of 1 for 3 hours. Cells were fixed with 4% paraformaldehyde (PFA), followed by ice-cold methanol. Cells were stained with anti-p65 (D14E12; Cell Signaling) and Alexa Fluor 546-
conjugated anti-rabbit antibody (A-11035; Invitrogen) and then were counterstained for nucleic acids with Hoechst 33342. In experiments using overexpressed protein, HEK293T cells (2.5 × 10^5) were reverse transfected using Lipofectamine 2000 with STING-HA (100 mg) and NLRC3-FLAG (375 mg) directly onto poly-L-lysine coated coverslips. After 24 h, cells were transfected with ISD (4 mg/ml) for 4 h, followed by PFA fixation. Cells were stained with anti-HA (3724S; Cell Signaling) and anti-FLAG (F1804, Sigma) followed with AF546-conjugated anti-rabbit antibody and AF488-conjugated anti-mouse IgG1 antibody (A-11035 and A11029; Invitrogen), and then counterstained for nucleic acids with Hoechst 33342. Cells were analyzed with a Zeiss LSM 710 laser-scanning confocal microscope.

**Statistical Analysis**

Statistical analysis was carried out with Prism 5.0 for Macintosh. All data are shown as mean ± s.d. The mean values for biochemical data from each group were compared by Student's t-test. Comparisons between multiple time points were analyzed by repeated-measurements analysis of variance with Bonferroni post-tests. In all tests, P-values of less than 0.05 were considered statistically significant. *P < 0.05, **P <0.01, ***P <0.001.
ACKNOWLEDGMENTS

Supported by NIH grants CA156330, P01DK094779, and R37-AI029564, (J.P.-Y.T.); AI088255 (J.A.D.); AI107810 (B.D.); and DE 018281 and U19 AI109665 (B.D. and J.P.-Y.T.); Burroughs Wellcome Fund Career Award for Medical Scientists (J.A.D.); and MOST grants 2014CB910400 and 2013CB911103 and NSFC grants 31200559 and 31330019 (S.O. and Z.-J.L.). We thank T.W. Mak for sharing Traf6+/+, Traf6+/−, and Traf6−/− cells, A. Baldwin and L. Su for materials, E. Miao for Burkholderia thailandensis, and R. Chen for support and discussion.
Figure 1 NLRC3 attenuates DNA- and HSV-1-induced cytokines
Figure 1. NLRC3 Attenuates DNA- and HSV-1-Induced Cytokines

(A and B) WT and Nlrc3−/− BMDMs were transfected (intracellular) or incubated (extracellular) with poly(dA:dT), transfected with poly(I:C), or treated with LPS. IFN-β and IL-6 were measured 16 hr after treatment. (C–E) Nlrc3+/+ and Nlrc3−/− MEFs were transfected with ISD, and Ifna4 (C) and Ifnb (D) transcripts or IFN-β (E) measured 6 hr after transfection. HSV-1 was used at MOI 0.05, 0.1, and 1 for (F)–(J) unless specified. (F and G) BMDMs were infected with HSV-1, and Ifna4 (F) and Ifnb1 (G) transcripts were measured. (H–J) Peritoneal macrophages were infected with HSV-1, and Ifna4 (H), Ifnb1 (I), and Tnf (J) transcripts were measured. (K) Peritoneal macrophages were infected with HSV-1 (MOI 1) or SeV (80 HA unit/ml) and TNF assayed. (L and M) Primary MEFs were infected with HSV-1, and Ifnb1 (K) and Tnf (L) transcripts were measured. (N and O) Primary MEFs were infected with HSV-1, and IFN-β (N) and IL-6 (O) proteins were measured. (P and Q) Primary MEFs (P) or BMDMs (Q) were infected with HSV-1, genomic DNA was extracted, and HSV relative genome copy number was determined by real-time PCR. All cytokines were determined by ELISA and transcripts determined by real-time PCR. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test). All data are representative of at least three independent experiments.
Figure 2. NLRC3 deficiency causes increased IFN-β and IL-6 production in response to c-di-GMP and c-di-GMP.
Figure 2. NLRC3 deficiency causes increased IFN-β and IL-6 production in response to c-di-GMP and c-di-GMP

(A and B) c-di-GMP was transfected into primary MEFs, and IFN-β (A) and IL-6 (B) were measured 24 and 48 hr after transfection, respectively, by ELISA. (C–E) Primary MEFs were infected with L. monocytogenes at MOI of 10, and Ifna4 transcripts (C) or IFN-β (D) and IL-6 (E) proteins were measured 6 hr after infection. (F) BMDMs were infected with a second bacterial strain, B. thailandensis, and IFN-β was measured 6 hr after infection. *p < 0.05, **p < 0.01 (Student’s t test). All data are representative of at least three independent experiments.
Figure 3. NLRC3 suppresses STING-TBK1-mediated IFN-β, ISRE, and NF-κB reporter activation

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E

F
Figure 3. NLRC3 suppresses STING-TBK1-mediated IFN-β, ISRE, and NF-κB reporter activation

(A–D) HEK293T cells were transfected with the IFN-β (A, B), ISRE (C), or NF-κB (D) promoter reporter with the internal control Renilla luciferase reporter pLR-TK and empty vector or NLRC3 plasmid as indicated. Luciferase assays were performed 24 hr after transfection. (E) HEK293T cells were transfected with the ISRE promoter reporter with the internal control Renilla luciferase reporter pLR-TK. Stimulatory ligands were transfected 24 hr after plasmid transfection. Luciferase assays were performed 16 hr after ligand stimulation. (F) Same as (A)–(D), except cells were transfected with empty vector or NLRC5. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test). Data are representative of at least two independent experiments.
Figure 4. NLRC3 associates with STING and TBK1 to interfere with their Interaction
Figure 4. NLRC3 associates with STING and TBK1 to interfere with their Interaction

(A) HEK293T cells were transfected with indicated plasmids and coimmunoprecipitation assay was performed 24 hr after transfection. (B and C) HEK293T cells were transfected with HA-tagged NLRC3 (B) or empty vector (C). Immunoprecipitation and immunoblots in (A)–(C) were performed with indicated antibodies. (D and E) Recombinant NLRC3 (D) and STING (E) in vitro binding assay was performed. Immunoblots were performed with the antibodies indicated. (F–H) HEK293T cells were transfected with indicated plasmids; constructs are shown at the top, the corresponding blots are shown below. The smaller peptides in (G) produced by 1–379 might be degradative products. (I and J) WT and Nlrc3−/− BMDMs were infected with HSV-1 (I) or stimulated with ISD (J); immunoprecipitation and immunoblot were performed with the indicated antibodies at the indicated time points. Densitometric measurements of STING-TBK1 coprecipitation are shown to the right of each immunoblot. Data are representative of at least three independent experiments.
Figure 5. NLRC3 blocks STING trafficking
Figure 5. NLRC3 blocks STING trafficking

(A) HEK293T cells were transfected with STING with an empty vector (EV) or with NLRC3 followed by stimulation with ISD. The percentages indicate the number of cells showing STING localized to the perinuclear region (ii and v) or forming puncta (iii and vi). A minimum of 100 cells were counted per group. (B) WT and Nlrc3−/− MEFs were mock-treated or infected with HSV-1 (MOI = 1) for 4 hr. Cell lysate were subjected to FPLC, and fractions were collected and immunoblotted for STING and TBK1. The blue boxes indicate fractions that contained both TBK1 and STING. (C) Densitometric tracing of STING in WT (black line) and Nlrc3−/− cells (red) was performed with ImageJ. (D) The areas depicted in (C) were calculated by area under curve in Prism. The areas where STING-TBK1 were localized in the same fractions are depicted for uninfected WT and Nlrc3−/− MEFs and for HSV-1-infected WT and Nlrc3−/− MEFs. In each case, the value for WT cells was set as 1.0. Data are representative of two independent experiments.
Figure 6. NLRC3 deficiency enhances immune signaling

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Figure 6. NLRC3 deficiency enhances immune signaling

(A) Immunoblot of phosphorylated (p-) TBK1, IRF3, p65, JNK, ERK, and p38 in lysates of WT and Nlrc3−/− MEFs infected with HSV-1 (MOI 1) for indicated time points. Densitometric measurements are depicted to the right. (B) BMDMs isolated from WT or Nlrc3−/− mice were infected with HSV-1 (MOI 1) for 2.5 hr. Cells were fixed and stained for endogenous p65 (red) or Hoechst, which stains the nucleus (blue). The merged purple color is indicative of nuclear p65. (C) Similar to (A), except cells were transfected with ISD. (D) Similar to (A), except cells were transfected with poly(I:C). Data are representative of at least two independent experiments.
Figure 7. *Nlrc3*^−/−^ mice are more resistant to HSV-1 infection
**Figure 7.** *Nlrc3*^−/−^ mice are more resistant to HSV-1 infection

WT and *Nlrc3*^−/−^ mice were infected i.v. with HSV-1 (2 × 10^7^ pfu) and tested for (A) body weight, (B) survival rate, (C–E) serum cytokines (6 hr after infection), and (F) HSV-1 genomic DNA (harvested 5 days after infection and measured by real-time PCR analysis). *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test). Data are representative of at least three independent experiments.
Supplemental Figure 1. *Nlrc3* deficiency leads to enhanced IFN-I production in different littermate-matched MEF pairs, related to Figure 1.
Supplemental Figure 1: *Nlrc3* deficiency leads to enhanced IFN-I production in different littermate-matched MEF pairs, related to Figure 1.

Different pairs of *Nlrc3*+/+ and *Nlrc3*−/− MEFs were generated from siblings produced from independent heterozygous matings. Different MEF pairs were tested independently and yielded consistent result. (A) *Nlrc3*+/+ and *Nlrc3*−/− MEFs were seeded in 24-well plate overnight and transfected with ISD at 4 μg/ml. Cells were harvested after 6 hours after transfection. Message RNA of *Il6* was determined by real-time PCR. (B-C) A pair of *Nlrc3*+/+ and *Nlrc3*−/− MEFs other than those shown in Figure 1 were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 0.2, 1 or 5. Cells were harvested at indicated time points. *Ifna4* and *Ifnb* level were determined by real-time PCR. (D) Supernatants were harvested from the same experiment and IFN-β ELISA was performed. *P* < 0.05, **P* <0.01, ***P* <0.001 (Student’s *t*-test). Data are representative of three independent experiments.
Supplemental Figure 2. Nlrc3 deficiency does not alter SeV-induced IFN-\(\lambda\) production, related to Figure 1
Supplemental Figure 2: *Nlrc3* deficiency does not alter SeV-induced IFN-I production, related to Figure 1.

(A-B) *Nlrc3*+/+ and *Nlrc3*−/− MEFs were seeded in 24-well plate overnight and infected with SeV at HA unit of 8, 80 and 800. Cells were harvested after 6 hours of infection. Message RNA of *Ifna4* and *Ifnb* were determined by real-time PCR. (C-J) Poly(I:C) were either added (extra-) or transfected (intra-) into *Nlrc3*+/+ and *Nlrc3*−/− MEFs for indicated time point. Message RNA of *Ifna4, Ifnb, Tnf* and *Il6* were determined by real-time PCR. (K-N) *Nlrc3*+/+ and *Nlrc3*−/− MEFs were seeded in 24-well plate overnight and infected with VSV at MOI of 1, 5 and 25. Cells were harvested after 6 or 16 hours of infection. Message RNA of *Ifna4, Ifnb, Tnf* and *Il6* were determined by real-time PCR. Data are representative of at least three independent experiments.
Supplemental Figure 3. Deficiency of Nlrc3 does not alter HSV-1, L. monocytogenes or B. thailandensis-induced cell death, related to Figure 1.
Supplemental Figure 3. Deficiency of \textit{Nlrc3} does not alter HSV-1, \textit{L. monocytogenes} or \textit{B. thailandensis}-induced cell death, related to Figure 1.

(A) \textit{Nlrc3}^{+/+} and \textit{Nlrc3}^{-/-} MEFs were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 0.2, 1 or 5. Supernatant were harvested at indicated time points. Cytotoxicity was determined by Cytotoxicity Detection Kit (Roche). (B) \textit{Nlrc3}^{+/+} and \textit{Nlrc3}^{-/-} MEFs were seeded in 24-well plate overnight and infected with \textit{Listeria monocytogenes} at MOI of 10 or 50. Supernatant were harvested at 6 hours post infection. Cytotoxicity was determined by the Cytotoxicity Detection Kit. (C) \textit{Nlrc3}^{+/+} and \textit{Nlrc3}^{-/-} MEFs were seeded in 24-well plate overnight and infected \textit{Burkholderia thailandensis} at MOI of 10 or 50. Supernatant were harvested at 6 hours post infection. Cytotoxicity was determined by the Cytotoxicity Detection Kit. Data are representative of at least two independent experiments.
Supplemental Figure 4. Domain Mapping of STING-TBK1 interaction regions, related to Figure 2

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IP: anti-HA
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IB: anti-HA
Supplemental Figure 4. Domain Mapping of STING-TBK1 interaction regions, related to Figure 2

(A) HEK293T cells were transfected with STING truncation constructs. Cells were harvested 24 hours post transfection and fractionated using Subcellular Protein Fractionation Kit (Thermo) according to manufacturer's instruction. Western blot were performed using indicated antibodies. CE: cytosolic extract; ME: membrane extract; NE: nuclear extract. Constructs 41-379 and 81-379 were membrane associated, while 111-379 lost its membrane association. (B) HEK293T cells were transfected with Flag-tagged TBK1 and HA-tagged STING/MITA truncation plasmids depicted above. The N-terminal N-240 residues are not sufficient for interaction, and only membrane-associated 41-379 and 81-379 associated with TBK1. Co-immunoprecipitation assays were performed 24 hours post-transfection with indicated antibodies. The kinase domain is necessary and sufficient for interaction with NLRC3. Data are representative of at least two independent experiments.
Supplemental Figure 5. TRAF6 is not required for HSV-1-induced IFN-I response, related to Figure 3.
Supplemental Figure 5. TRAF6 is not required for HSV-1-induced IFN-I response, related to Figure 3

(A-B) Traf6+/+, Traf6+/− and Traf6−/− MEFs were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 1 or SeV at HA unit of 80. Cells were harvested after 6 hours of infection. Message RNA of Ifna4 and Ifnb were determined by real-time PCR.

(C) HEK293T cells were transfected with Flag-tagged STING/MITA and HA-tagged TBK1, HA-tagged NLRC3 and V5-tagged TRAF6. Co-immunoprecipitation assays were performed 24 hours post-transfection with indicated antibodies. *P < 0.05; ns, no significant difference (Student’s t-test). Data are representative of two independent experiments.
Supplemental Figure 6. Nlrc3 is not required for antiviral response against VSV in vivo, related to Figure 7
Supplemental Figure 6. Nlrc3 is not required for antiviral response against VSV in vivo, related to Figure 7

(A) WT and Nlrc3<sup>-/-</sup> mice were infected i.v. with VSV (5x10<sup>7</sup> pfu) and body weight was monitored. (B) WT and Nlrc3<sup>-/-</sup> mice were infected i.v. with VSV (5x10<sup>7</sup> pfu), serum were harvested 6 hours post-infection and measured for IFN-β. ns, no significant difference (Student’s t-test). Data are representative of two independent experiments.
REFERENCES


CHAPTER THREE: NLRC3 NEGATIVELY REGULATES DNA DAMAGE-INDUCED INNATE IMMUNE RESPONSE

INTRODUCTION

Innate immunity provides us the first line of defense against pathogens and self-derived danger factors. Exogenous DNA from invading pathogens can act as pathogen-associated molecular patterns, while self-derived endogenous DNA can act as damage-associated molecular patterns to trigger innate immune responses and inflammation. Studies on intracellular DNA sensors have drawn a lot of interest in recent years. Among all the DNA sensors identified, STING (also referred to as mediator of IRF3 activation (MITA), plasma membrane tetraspanner (MPYS) or endoplasmic reticulum IFN stimulator (ERIS) has emerged as a key molecule for intracellular DNA-induced IFN-I activation (Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008). Other known DNA sensors activate STING upon binding to dsDNA. There is also evidence that STING can directly bind to DNA and trigger the downstream signaling event (Abe et al., 2013). Our previous studies showed that an NLR family protein, NLRC3, negatively regulates STING-dependent intracellular DNA- and DNA virus-induced IFN-I production (Zhang et al., 2014). Nlrc3−/− mice are more resistant to HSV-1 infection and produced significantly more IFN-β systemically after HSV-1 infection. Mechanistically, NLRC3 directly binds to STING in a cell-free system and blocks STING trafficking from ER to perinuclear region and puncta-like structures after ISD stimulation. This established the first link between NLR proteins and the STING-dependent pathway.
On the other hand, DNA damage response is a response system for cells to react with genomic stress. There is emerging evidence showing that DNA damage can also trigger innate immune responses to help cope with genomic stress. Previous studies suggested that UV and DNA-damage-inducing molecules can activate NF-κB and IRF3 partially through TLR2 and TLR4 (Kim et al., 1999; Wang et al., 2011). Some forms of DNA damage also causes oxidative damage and can induce innate immune reactions or inflammation. Although there is one report to indicate that the NLRP3 inflammasome affects DNA damage response (Licandro et al., 2013), the role of noninflammasome NLR has not been investigated in DNA damage response. The studies on whether NLR proteins regulate DNA damage-induced innate immune response and inflammation are also lacking.

In this chapter, we show that NLRC3 directly binds to DNA and negatively regulates UV-induced TBK1, JNK and p53 phosphorylation. However, NLRC3 does not affect cell cycle progress at resting stage. Further investigations and mechanistic studies are needed to demonstrate the role of NLRC3 in DNA damage response or DNA damage-induced immune response.
RESULTS

NLRC3 associates with biotinylated HSV60mer

We previously found that NLRC3 acts as a negative regulator of intracellular DNA-induced IFN-I signaling pathway. This Chapter explores whether NLRC3 directly binds to dsDNA in the cytoplasm. To achieve this, we transfected NLRC3 expression construct into HEK293T cells and incubate the cell lysate with biotin-labeled HSV60mer, which is a 60-base pair dsDNA derived from HSV-1 genome that is known as a potent activator of IFN-I (Unterholzner et al., 2010). Streptavidin resin was used to precipitate the protein(s) associated with HSV60mer. Western blot data showed that NLRC3 indeed associated with biotin-labeled HSV60mer (Figure 1). As expected, the positive controls, including DDX41 (Zhang et al., 2011), IFI16 (Unterholzner et al., 2010) and STING (Abe et al., 2013), all bound DNA. However, NLRP11 (another NLR protein) failed to associate with HSV60mer. These data suggested that NLRC3 associates with dsDNA.

Recombinant NLRC3 directly binds to DNA

To further investigate whether the association between NLRC3 and dsDNA was direct, we prepared purified recombinant full-length NLRC3 from an insect cell protein purification system (Zhang et al., 2014). Coomassie blue staining and Western blot analysis showed the successful purification of full-length NLRC3 (Figure 2A). We then incubated purified NLRC3 with DNA and analyzed it under electronic microscopy. The image indicated that NLRC3 directly associated with the DNA construct we used (Figure 2B-C). Interestingly, NLRC3 specifically bound to the junction of single stranded region.
and double-stranded region of DNA. We next investigated whether recombinant NLRC3 is able to bind to replication fork, a more physiological relevant form of DNA with single-strand and double-strand junction. The result showed that NLRC3 directly bound to single-strand and double-strand junction of a replication fork with 25 bp of single strand region (Figure 2D). Collectively, these data demonstrated that NLRC3 is able to directly bind to DNA, and specifically to DNA with single-strand and double-strand junction.

**NLRC3 deficiency leads to increased UV-induced TBK1 activation**

DNA damage could trigger inflammation and immune response (Ferguson, 2010; Kidane et al., 2014), especially the damage induced by UV irradiation (Gehrke et al., 2013). Since we previously found that NLRC3 is a negative regulator of intracellular DNA-initiated innate immune response, we investigated whether NLRC3 has any effect in UV-damage induced immune activation. We treated WT and \( Nlrc3^{-/-} \) MEFs with UV-C irradiation and explored the activation status of several key molecules in both DNA-damage pathway and innate immune signaling pathway. Western blot analysis of the samples showed that there were elevated levels of p-TBK1, p-JNK and p-p53 in \( Nlrc3^{-/-} \) deficient cells compared to the littermate-controlled WT cells. Increased TBK1 phosphorylation due to the loss to NLRC3 indicates that UV irradiation can induce activation of the TBK1-dependent pathway and that NLRC3 negatively regulates this process. A modest increase in JNK phosphorylation was observed in \( Nlrc3^{-/-} \) cells, suggesting that NLRC3 might also have a negative role in JNK-dependent pathway, such as cell growth, survival or apoptosis. Furthermore, a modest increase of p53 phosphorylation at the serine 18 site (equivalent to serine 15 in human) in \( Nlrc3^{-/-} \) deficient cells suggests that p53-dependent DNA damage response might be
downregulated by NLRC3. Collectively, our data demonstrates that NLRC3 plays crucial negative regulatory role in both UV-induced DNA damage response and innate immune response.

**NLRC3 deficiency does not interfere with cell cycle progression**

Given the observation that NLRC3 might regulate DNA damage response, we next examined whether NLRC3 affected one of the fundamental effect of DNA damage response, cell cycle progression. Using PI staining of DNA content and flow cytometry assay, we did not observe any significant difference in cell cycle comparing WT and Nlrc3-deficient cells at the resting stage. It is possible that NLRC3 regulates cell cycle progression only after an insult, such as UV irradiation. It is also possible that NLRC3 is not involved in the regulation of cell cycle progression but rather in other biological or pathological processes induced by UV irradiation.
DISCUSSION AND FUTURE STUDIES

In this study, we have identified several novel features of NLRC3. First we find that NLRC3 can bind to DNA in intact cells. Additionally, recombinant full-length NLRC3 directly binds to DNA and more specifically to the single-stranded region within a double-stranded vector in a cell free system. Finally NLRC3 profoundly inhibits UV-induced activation of TBK1, and modestly reduces JNK and p53, although deficiency of NLRC3 does not affect cell cycle progression at the resting stage in MEFs. Further analysis of the impact of NLRC3 during cell cycle progression upon UV treatment and other insults is planned. Collectively, this work demonstrates that NLRC3 may play a negative regulatory role in UV-induced innate immune response and DNA damage response.

Our previous study showed that NLRC3 serves as a negative regulator of intracellular DNA-initiated innate immune response. Mechanistically, NLRC3 directly associates with STING to impede STING-TBK1 association and blocks STING trafficking. While this clearly illustrates how NLRC3 elicits the inhibitory effect in IFN-I pathway, a key question is whether NLRC3 directly or indirectly forms a complex with DNA. In this study, our results show that NLRC3 associates with HSV60mer. However, further studies are needed to elucidate the functional and mechanistic details of NLRC3-DNA-binding. Questions that need to be addressed include: first, which domain within NLRC3 is responsible for binding to DNA; second, does NLRC3 competes with other cytosolic DNA receptors for DNA-binding, thus inhibiting the signaling activation of IFN-I production; third, does NLRC3 binds to DNA directly independent of other factors. Our further experiments addressed the third question and show that purified recombinant
NLRC3 directly binds to DNA in a cell free system shown by EM. Furthermore, the result indicates that NLRC3 binds to single-strand and double-strand junction of DNA, leading us to speculate that NLRC3 might directly bind to the replication fork. This finding indicates that NLRC3 might be involved in the DNA replication process or DNA damage-induced single strand-double strand junction formation. Thus, another key question is the sub-cellular localization of NLRC3 at the resting stage as well as DNA-damaged stage or viral-infected state and whether there is a portion of NLRC3 that translocates to the nucleus after stimulation. However, we found that NLRC3 does not interfere with cell cycle progression or replication rate in MEFs by measuring DNA content at resting stage. It is possible that NLRC3 regulate cell replication after DNA damage induction instead of at resting stage. It is also possible that the failure to detect cell cycle progression difference in \textit{Nlrc3}\textsuperscript{−/−} cells is due to the fact that MEFs are not rapid replicating cells, thus NLRC3 might regulate cell cycle progression of replication in a rapid replicating cell type such as lymphocytes.

We also show for the first time that UV irradiation induces TBK1 phosphorylation and that NLRC3 negatively regulate this process. The impact of UV radiation on TBK1 has not been documented. It will be of interest to assess if NLRC3 impacts TBK1 phosphorylation during other forms of DNA damage, such as those caused by X-irradiation. One possibility is that UV irradiation causes leakage of DNA from nucleus to the cytoplasm. The free-floating DNA in the cytoplasm can then serve as a danger signal and activate intracellular DNA sensors to activate the innate immune response. Further experiments to measure the cytoplasm DNA content after UV irradiation are needed to test this hypothesis. A recent study showed that UV irradiation induces
inflammatory and innate immune responses to promote metastases in a melanoma mouse model. It was shown that UV-irradiated keratinocytes secret high mobility group box 1 (HMGB1), which initiate an influx of neutrophils. Neutrophils secret proinflammatory cytokines, such as TNF, to induce angiogenesis and facilitate the migration of melanoma cells to the blood vessel and distant organs (Bald et al., 2014).

In conclusion, our study suggests that NLRC3 has a potential regulatory role in UV irradiation induced DNA damage and repair response and innate immune response. This work has the potential to expand the function of NLRs to a highly conserved DNA damage and repair pathway. This also establishes the possible link between NLRs and DNA damage response. Since DNA damage is involved in the pathology of many diseases, including tumorigenesis, tumor cell metastases, autoimmune diseases. Our finding shed light on the potential role of NLRC3 in those disease models and may provide new drug target for new drug and therapy design.
EXPERIMENTAL PROCEDURES

Cell culture

HEK293T cells were purchased from ATCC. Littermate-controlled Nlrc3+/+ and Nlrc3−/− MEFs were generated from 13.5-day embryos. Both HEK293T and MEFs were maintained as previously described (Zhang et al., 2014).

Reagents and antibodies

Biotinylated HSV60mer were purchased from Integrated DNA Technologies (IDT), with the sequence: 5’-TAAGACACGATGCAGATAAAATCTGGTTTGTAAAATT
ATTAAAGGTACAAATTGCCCCTAGC-3’; Streptavidin agarose beads were from Thermo; anti-phospho-TBK1, TBK1, γH2AX, phospho-JNK, phospho-p53 (Ser15), phospho-AKT were from Cell Signaling Technology, and anti-β-actin antibody was from Santa Cruz.

Plasmids and molecular cloning

STING (originally cloned and named as MITA) expression plasmids have been described previously (Zhong et al., 2008). IFI16 expression construct was a gift from Dr. Andrew Bowie. DDX41 construct was a gift from Dr. Yongjun Liu. NLRC3 and NLRP11 expression plasmids were cloned into pcDNA3.1.

Transfection and DNA-binding assay

HEK293T cells were seeded in 24-well plates at the density of 1.0×10^5 per well and transfected the following day by lipofectamine 2000 following the manufacturer’s instruction. Cells were lysed in CHAPS lysis buffer (10 mM CHAPS in PBS, 1 mM NaF,
1 mM Na$_3$VO$_4$, and protease inhibitors) for 1 hr at 4°C. After centrifugation, cell lysates were incubated with 10 µl biotinylated HSV60mer conjugated agarose beads at 4°C for 3 hr on the rotater. The agarose beads were then washed five times by CHAPS wash buffer (10 mM CHAPS in PBS, 300 mM NaCl, 1 mM NaF, and 1 mM Na$_3$VO$_4$).

**Recombinant protein purification**

Recombinant baculovirus expressing NLRC3 carrying an N-terminal Halo-Tag ™ (HALO) and C-terminal hexahistidine tag (6XHIS) was kindly generated Dr. Alex Duncan and Dr. Jinyao Mo as described (Mo et al., 2012; Zhang et al., 2014). Tag-free NLRC3 was purified to near homogeneity using tandem immobilized Ni$^{2+}$ chromatography and HALO-ligand affinity chromatography followed by TEV–protease treatment to remove both the HALO and 6XHIS as previously described.

**Cell-free protein-DNA binding assay and electronic microscopy**

The visualization of DNA bound to NLRC3 was kindly performed by Dr. Jack Griffith and Dr. Oya Bermek. DNA constructs with 400bp single strand tail and replication fork with 25 bp single strand region was generated as previously described (Arat and Griffith, 2012; Compton et al., 2010; Subramanian and Griffith, 2005). 900 ng of purified full-length NLRC3 was incubated with 56ng of DNA construct in 10 mM HEPES, 50 mM NaCl for 20 min at room temperature. The product was then mounted onto charged carbon-coated copper grids and shadowed with tungsten as previously described (Compton et al., 2010).
Cell Cycle determination

Littermate-controlled WT and \( Nlrc3^{\pm} \) MEFs were harvested and fixed. DNA content was then measured through Fluorescence-activated cell sorting (FACS) according to *Current Protocols in Cell Biology*, chapter 8.4.4 (Darzynkiewicz et al., 2001). Data was analyzed in *FlowJo*.
Figure 1. NLRC3 associates with biotinylated HSV60mer
Figure 1. NLRC3 associates with biotinylated HSV60mer.

HA- or Flag-tagged expression constructs of NLRC3, DDX41, IFI16, STING and NLRP11 were transfected to 293T cells respectively. Cell were lysed in CHAPS buffer 24 hours after transfection and incubated with biotinylated HSV60mer-conjugated agarose beads and analyzed by immunoblotting with anti-HA and anti-Flag antibody.
Figure 2. Recombinant NLRC3 directly binds to DNA
Figure 2. Recombinant NLRC3 directly binds to DNA.

(A) Full-length recombinant NLRC3 was purified from Sf9 cells and the purity was illustrated by coomassie blue staining (left panel), specificity was illustrated by immunoblotting with anti-NLRC3 antibody (right panel). (B-C) Recombinant NLRC3 from (A) was incubated with 3.4kb dsDNA with 400bp single strand tail. The products were visualized under EM. Blue arrow points at free protein and red arrows point at DNA-bound protein. (D) Recombinant NLRC3 from (A) was incubated with a replication fork construct with 25bp single strand region. The products were visualized under EM. Red arrow points at DNA-bound protein.
Figure 3. NLRC3 deficiency leads to increased UV-induced TBK1 and p53 phosphorylation.
Figure 3. NLRC3 deficiency leads to increased UV-induced TBK1 and p53 phosphorylation.

Littermate-controlled WT (W) and \textit{Nlrc3}\textsuperscript{-/-} (K) MEFs were irradiated with 120 mJ/cm\textsuperscript{2} UV-C and incubated for indicated time. Cells were lysed with RIPA lysis buffer and Immunoblots were then performed with indicated antibodies.
Figure 4. NLRC3 deficiency does not interfere with cell cycle progression at the resting state.
Figure 4. NLRC3 deficiency does not interfere with cell cycle progression at the resting stage.

Littermate-controlled WT and Nlrc3<sup>-/-</sup> MEFs were fixed and stained with propidium iodide (PI). Cell cycle distribution profiles were determined by flow cytometry and the graph shows the distribution of percentage of cells at different stages of the cell cycle.
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CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

Summary of findings

Over the course of our studies, it has become clear that NLRC3 is a negative regulator of intracellular DNA-, DNA virus-, c-di-GMP- and c-di-GMP-producing bacteria-induced IFN-I production (Figure 1). Mechanistically, NLRC3 targets the central DNA-sensing molecule STING to elicit the inhibitory effect. NLRC3 directly binds to STING to impede STING-TBK1 association and block STING trafficking from ER to perinuclear region and puncta structures. More importantly, Nlrc3\(^{-/-}\) mice are more resistant to HSV-1 infection. Specifically, Nlrc3\(^{-/-}\) mice produce significantly higher amount of anti-viral cytokines systemically, which result in a reduced viral load. Furthermore, this inhibitory effect of NLRC3 on IFN-I production is not dependent on its previous reported function to dampen TRAF6-dependent signaling pathway, since we find that TRAF6 is not required for DNA-initiated IFN-I production. Our further investigation on the role NLRC3 in DNA-induced IFN-I signaling pathway shows that NLRC3 directly binds to DNA. Interestingly, purified recombinant NLRC3 specifically binds to the single-strand and double-strand junction of DNA as well as to replication fork. This finding gives rise to the hypothesis that NLRC3 might interfere with replication under normal or DNA-damaged conditions. We then find that NLRC3 negatively regulates UV irradiation-induced TBK1 activation, which has not been previously linked to UV-induced response, but potentially can affect downstream signals that have a role such as NF-κB. NLRC3 also inhibits UV irradiation-induced p53 and JNK activation,
potentially affecting cell growth or proliferation. However, NLRC3 does not affect cell cycle progression in MEFs at resting stage, although the important issue is whether NLRC3 affects cell growth or apoptosis upon genotoxic stress induced by DNA damage.

Our discoveries have also raised several interesting questions and provided various future directions to follow up.

**Further investigation on the mechanism by which NLRC3 functions**

Apart from the crucial function in cyclic dinucleotide and cytosolic DNA-induced IFN-I signaling pathway, STING has also been reported to positively regulate VSV-induced IFN-I production and antiviral innate immune response in vivo. However, NLRC3 does not regulate the VSV-sensing arm of STING function (Ishikawa and Barber, 2008; Ishikawa et al., 2009). It is not known how NLRC3 distinguishes different upstream signals and to specifically affect the DNA-sensing portion of STING function. One hypothesis is that there are other factors involved to help NLRC3 to achieve the specificity of signaling regulation. Alternatively, we have only tested two RNA viruses. Further testing of additional RNA virus is needed to understand if NLRC3 can also affect the function of STING during infection by RNA virus.

*Nlrc3*−/− mice are more resistant to HSV-1 infection and produce increased level of IFN-β systemically. Though IFN-β is one of the major contributors of antiviral innate immune response, there is no clear evidence that the increased resistance of HSV-1 in *Nlrc3*−/− mice is due to increased IFN-β production. To explore whether IFN-β is the direct cause of decreased morbidity and mortality after HSV-1 infection in *Nlrc3*−/− mice, future work should include crossing *Nlrc3*−/− to *Ifnar1*−/− mice and determine whether the
double KO mice lose the phenotype observed in \textit{Nlrc3}\textsuperscript{--/} mice. If \textit{Nlrc3/Ifnar1} double knockout mice show a normal morbidity and mortality after HSV-1 infection, the conclusion would be that increased IFN-I production serves as a direct cause of increased antiviral effect and resistance to HSV-1 infection in \textit{Nlrc3}\textsuperscript{--/} mice. If \textit{Nlrc3/Ifnar1} double knockout mice mimic the phenotype of \textit{Nlrc3}\textsuperscript{--/} mice, the conclusion would be that there are other factors contributing to increased morbidity and mortality in \textit{Nlrc3}\textsuperscript{--/} mice. In fact, IFN-I production is not the sole factor to control viral infection in vivo. As mentioned in Chapter 1 of this dissertation, it is a multi-cell type, multi-organ effort to elicit antiviral response. If \textit{Nlrc3/Ifnar1} double knockout mice do not mimic the phenotype WT mice, it is likely that other cytokines are involved as STING regulates a host of cytokine responses. Alternately, it is also possible that the impact of NLRC3 lies beyond cytokine changes. This will provide another direction of study to explore other factors that NLRC3 is affecting to cause the dramatic change in antiviral innate immune responses. For example, NLRC3 could directly interact with viral protein or nuclei acid to affect viral replication or infection efficiency.

\textbf{Exploring the role of NLRC3 in HIV-induced innate immune response}

It was recently discovered that HIV induces IFN-I activation in a STING dependent manner (Gao et al., 2013). Specifically, HIV infection activates cGAS and induces the production of cGAMP, which then directly binds to STING and activates the downstream IRF3-dependent IFN-I production. Inhibition of HIV reverse transcriptase significantly dampens HIV-induced IFN-I production, suggesting that DNA reverse transcribed by HIV RNA is the molecule that activates IFN-I. Now that NLRC3 inhibits
STING-dependent cytosolic dsDNA sensing, it is plausible that NLRC3 also regulates HIV-induced IFN-I production. This is another interesting future direction to follow up on and will provide more detailed mechanisms on the regulation of HIV-induced innate immune responses.

**Exploring the role of NLRC3 in Autophagy**

In addition to function in IFN-I signaling pathways, the role of STING has been explored extensively in the past few years. STING has been shown to function in several other biological and pathological processes apart from regulating IFN-I production. Given that NLRC3 negatively regulates STING-dependent IFN-I production, it is possible that NLRC3 can also regulate other STING-dependent pathways.

It has been reported that HSV-1 activates autophagy in myloid cells in a STING-dependent manner (Rasmussen et al., 2011). Autophagosome formation induced by HSV-1 infection does not require viral gene expression, but is dependent on viral entry and the release of genomic DNA in the cytosol. This is another way for the host to combat viral infection in addition to IFN-I production. Furthermore, some bacteria infection also induces autophagy in a STING dependent manner (Watson et al., 2012). DNA from Mycobacterium tuberculosis activates cytosolic surveillance pathway through STING-dependent pathways, which then facilitate the host cells to mark bacteria with ubiquitin. Ubiquitin-coated bacteria can then be targeted and delivered to autophagosome by ubiquitin-autophagy receptors p62 and NDP52 and the DNA-responsive kinase TBK-1. This is a major strategy for the host to clear Mycobacterium tuberculosis infection. Our studies show a profound effect of NLRC3 on TBK-1
activation, especially upon UV-radiation. Thus, it will be of great interests to explore the function of NLRC3 in virus- or bacteria- infection induced autophagosome activation. This could be another side of the host antiviral response that NLRC3 is modulating.

**Studying the role of NLRC3 in adaptive immunity**

Despite the finding that STING is recognized as a key molecule in innate immune responses, there is also evidence suggesting that STING might also modulate adaptive immunity. Studies have shown that upon activation, STING recruits STAT6 to ER, where STAT6 undergoes phosphorylation and dimerization independent of JAK, but dependent on TBK1 (Ito et al., 2011). Activated STAT6-dimer then translocates to the nucleus and initiates the transcription of a specific set of antiviral chemokines, which is different from the traditional signaling pathway downstream of IL4 and IL13. STAT6 responsive antiviral chemokines include CCL2 (also known as MCP-1), which is important for attracting monocytes, macrophages and T cells (Yadav et al., 2010); and CCL20 (also known as MIP3A), which is responsible for recruiting CCR6-expressing B cells, T cells and dendritic cells (Ito et al., 2011). Regulation of those two chemokines production by STING could potentially affect immune cell homing and adaptive immunity. In addition, an independent group has also shown that STING regulates MCP-1 and MCP-3 production after *Listeria monocytogenes* infection *in vivo* to modulate Ly6C<sup>hi</sup> monocyte migration.

Taken together, it is plausible that NLRC3 might also regulate chemokine production and cell migration after pathogen infection, thus potentially affecting adaptive immunity. On the other hand, it has been reported previously that NLRC3
negatively regulates T cell function through modulating NFAT activity (Conti et al., 2005), though it was not studies using gene deletion cells or animals. Therefore, to study the role of NLRC3 in adaptive immunity, specifically studying the role of NLRC3 in T cells would be a good start. Furthermore, given that NLRC3 regulates antiviral response, it is possible that NLRC3 affects T cell function under the context of viral infection.

**Examine the potential function of NLRC3 in autoimmune diseases**

The immune system protects us from infections and self-danger factors. However, overzealous or uncontrolled immune response can also be harmful and results in autoimmune diseases, such as Systemic Lupus Erythematosus (SLE) and Aicardi-Goutières syndrome (AGS). There are accumulating evidence that IFN-I is pathogenic in SLE patients. Several cytosolic DNA or RNA sensors have been shown to mediate the pathogenesis of SLE. It has also been shown that mutations in the human 3’ repair exonuclease 1 (Trex1) gene is associated with Aicardi-Goutières syndrome (AGS). Mechanistic studies show that TREX1 negatively regulates STING activity, loss of TREX1 results in uncontrolled STING activation and overzealous IFN-I production, which is a major cause of AGS (Gall et al., 2012). A most recent study showed that gain of function of the intracellular sensor MDA5 is associated with autoimmune disorders (Gall et al., 2012).

Thus, investigating the role of NLRC3 is another worthy future direction. Our preliminary data showed that the expression level of NLRC3 is significantly decreased in SLE patients compared to healthy controls. In addition, we also have preliminary data showing that Nlrc3-deficiency resulted in significantly elevated level of autoantibody in a
SLE mouse model. This is consistent with our hypothesis that loss of NLRC3 increased the susceptibility of host to autoimmune diseases through uncontrolled STING activation.

**Evaluate the role of NLRC3 in cancer**

Cancer is tightly associated with DNA damage and chronic inflammation. Studies have reported that DNA damage induce IRF3 and NF-κB activation (Kim et al., 1999; Wang et al., 2011). Our previous studies suggested that NLRC3 is important in innate immune response and inflammation regulation, specifically IRF3 and NF-κB activation. In addition, NLRC3 also might regulate DNA damage response or DNA damage-induced immune response. Therefore, it will be interesting to examine the role of NLRC3 in an inflammation-associated cancer model or DNA-damage-induced cancer model. Unpublished data in our lab suggest that Nlrc3−/− mice displayed an increased tumor number and size in AOM/DSS-induced colitis-associated colon cancer model. This supports our hypothesis that loss of NLRC3 results in elevated level of inflammation and increased tumorigenesis.

On the other hand, genotoxic damage is important in multiple diseases, with cancer being a major outcome. As an example, UV-irradiation is a big risk factor for melanoma development. A recent study showed that UV-induced DNA damage causes inflammation, which facilitate the metastasis of melanoma. In addition, my preliminary results showed that deficiency of NLRC3 led to increased phosphorylation of AKT (data not shown), which is usually elevated as well in tumors. Therefore, I propose that NLRC3 might play an important role in UV-induced skin inflammation and loss of NLRC3 results in elevated tumorigenesis and metastasis in melanoma.
Regulation of NLRC3 function

Now realizing NLRC3 is of great importance in so many biological and pathological processes. It would serve as a potential drug target for many diseases. Understanding how NLRC3 is regulated at the transcription, translation and post-translation levels would be crucial to uncover strategies utilized by cells to modulate the related biological and pathological processes. However the regulation of NLR proteins has not been intensively studied. We have evidence that transcripts level of NLRC3 goes down after stimulated with LPS (Schneider et al., 2012) and ISD (data not shown). However, how the process is regulated is not clear. The best studies NLR function regulation is NLRC4, which undergoes phosphorylation on Serine 533 by PKCδ is essential for NLRC4-inflammasome activation (Qu et al., 2012). Thus, future studies will be focused on how NLRs are being regulated.

Concluding remarks

This dissertation mainly focused on the central theme of understanding the roles of NLRC3 in self-DNA and DNA derived from pathogens induced host innate immune response and inflammation. The first half of the research centered on the identification of an inhibitory function for NLRC3 in STING-dependent IFN-I signaling pathway and the investigation of the molecular mechanism. The second half of the research focused on investigation of its inhibitory role in DNA-damage induced response. These studies represent the first links between NLR and STING-dependent pathways and contribute to the understanding of an intriguing interplay between DNA damage and innate immune
responses. Our findings also point to potential future directions to evaluate the roles of NLRC3 in several disease pathogenesis processes and shed light on the potential drug target.
Figure 1. NLRC3 inhibits STING-dependent DNA-sensing pathway
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