Characterization of NLRC3 and its Mechanism of Action in Regulating T cell Function and Activation.

by

Jun Yu (Jerry) Zhou

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Immunology
University of Toronto

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Abstract

NLRC3 is a newly discovered Nod-like receptor with immune regulatory functions. Strikingly, NLRC3 is highly expressed in lymphocytes, with the highest expression in T cells. Unpublished observations from our laboratory showed that NLRC3-deficient mice cleared *Citrobacter rodentium* infection more quickly than wild-type mice, which correlated with robust CD4+ T cell responses within the lamina propria. The focus of my thesis is to determine the mechanism and function of NLRC3 within T cells using *in vitro* approaches. NLRC3 was downregulated upon T cell receptor stimulation, which was also mimicked by intracellular calcium release and mTOR inhibition. Subcellular fractionation revealed NLRC3 localization in both the cytoplasmic and nuclear fractions in Jurkat T cells. Finally, co-immunoprecipitation verified the interaction between NLRC3 and IFRD1, a target identified by yeast two-hybrid screens and implicated in gene transcriptional regulation. Altogether, these findings provide new insight into the role of NLRC3 in regulating T cell responses.
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Sincerely,
Jun Yu (Jerry) Zhou
2014
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Introduction

The mammalian innate and adaptive immune systems

The immune system serves as a protective mechanism against infection and colonization of the host by foreign pathogens. Over time, the mammalian immune system has evolved into two distinct branches, namely, the innate and adaptive immune systems. The main purpose of the innate system is to survey the body for the presence of pathogens and to initiate a response to contain and control the infection. To achieve this, the innate immune system relies on the expression of pattern recognition receptors (PRRs), present on a number of cell types, to detect pathogens and limit infections. Upon detecting a pathogen through PRRs, cells of the innate immune system rapidly activate an inflammatory response by secreting pro-inflammatory cytokines, such as IL-1b, and IL-6 (Medzhitov, Preston-Hurlburt et al. 1997). These inflammatory cytokines serve to activate neighboring cells and prevent further spread of the pathogens.

While the innate immune system is essential to prevent the pathogen from spreading, it also stimulates the activation of the adaptive immune system via cytokines and protein receptors. The adaptive immune system is essential for pathogen clearance and protection from future infection by the same pathogen via immunological memory. However, the trade-off for these highly effective mechanisms is delayed activation which can take up to two weeks after initial exposure. There are two major types of lymphocytes that participate in the execution of the adaptive immune response, B cells and T cells. B cells possess a highly specific immunoglobulin surface receptor for antigen recognition. Upon binding to a specific antigen, activated B cells, driven by B cell receptor signaling, induce the up-regulation of the key transcriptional regulator, NF-κB and NFAT, as well as other transcription factors involved in B cell function, proliferation and differentiation (Antony, Petro et al. 2003). Further activation leads to proliferation and differentiation of naïve B-cells into plasma cells, which secrete various subfamilies of soluble immunoglobulins into the blood. These immunoglobulins bind to the pathogen or its
toxins, inactivating their function to prevent any damage to the host (Brandtzaeg 2003). In addition to plasma cells, a subset of the activated B cells differentiate into memory B cells, which are rapidly activated in a subsequent re-infection. This is accompanied by a unique mechanism, known as affinity maturation, which continuously enhances the affinity of the B-cell immunoglobulin receptor toward the targeted antigen (Schatz, Oettinger et al. 1992).

T cells also possess a highly specific receptor for antigen recognition. Similar to B cells, activated T cells also up regulate the key transcriptional regulator, NF-κB, NFAT and AP-1, as well as other transcriptional factors, such as T cell specific T-bet, to proliferate and differentiate (Smith-Garvin, Koretzky et al. 2009). Activated T cells can differentiate into multiple subtypes of T cells: helper T cell, cytotoxic T cell, and regulatory T cell. Helper T cells, like the name suggests, enhance the effectiveness of the immune response via secretion of cytokines, such as TNF-α and IFNγ (Munoz-Fernandez, Fernandez et al. 1992). Cytotoxic T cells recognize pathogen-infected cells to induce apoptosis, preventing further pathogen production and spread (Barry and Bleackley 2002). Regulatory T cells help to regulate the immune system to prevent over-activation and autoimmunity via repressive signals, such as CTLA-4 and TGF-β (Vignali, Collison et al. 2008). Interestingly, T cells with innate-like properties have also been described; NK T cell possess a T cell receptor that functions to recognize self and foreign lipids (Borg, Wun et al. 2007), like a pattern recognition receptor, but it also expresses many surface markers associated with NK cells (Godfrey, MacDonald et al. 2004).

Both the innate and adaptive immune systems are essential to protect us from pathogenic infection. However, the two systems cannot function independently because they rely on each other to generate the most appropriate immune response. Therefore, the innate and adaptive immune systems have a very interactive and cross-regulating relationship.
**Pattern Recognition Receptors**

To detect the presence of a foreign organism, the innate immune system utilizes a class of protein receptors known as PRRs. In general, most PRRs recognize conserved molecules that originate from a foreign organism, and these molecules are known as pathogen-associated molecular pattern or PAMPs. Many PAMPs are essential components of the microbes and include cell wall products like lipopolysaccharide (LPS), peptidoglycan, as well as viral single or double stranded nucleic acid (Kumar, Kawai et al. 2011). By detecting a core component of the microbe, PRRs enable the innate immune system to accurately detect any intrusion by foreign organisms (Medzhitov, Preston-Hurlburt et al. 1997). Alternatively, some PRRs recognize danger signals produced by the cell during stress, and these signals are known as danger-associated molecular pattern or DAMPs (Martinon, Petrilli et al. 2006). In contrast to PAMPs, DAMPs are derived from host cellular material, such as intracellular proteins and metabolites, which are only released after cellular injury due to a pathogenic infection (la Sala, Ferrari et al. 2003, Panayi, Corrigall et al. 2004). Together, the recognition of PAMPs and DAMPs by multiple PRRs enables the cell to sense the presence of a broad range of pathogens (Table 1), and to initiate the most appropriate immune response to trigger pathogen clearance. Within the PRRs, multiple families of PRRs exist, which specialize in recognizing certain types of pathogens. The major families of PRRs will be discussed below.

Table 1. Pattern Recognition Receptor localization, ligand and functions.

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Extracellular sensors: The Toll-like receptors

Toll-like receptors were the first major family of PRRs to be discovered in animals. The importance of TLR genes was first discovered through sequence comparison of Toll protein, a Drosophila homolog, and mammalian IL-1 receptor cytoplasmic domain (Gay and Keith 1991). Toll is an essential factor in Drosophila melanogaster innate immunity against fungal and Gram-positive bacterial infection as an inducer of anti-microbial peptides (Lemaitre, Nicolas et al. 1996). 13 different TLRs are found in mammals, each specializing in the recognition of a specific type of ligand (Table 1.1). The TLRs are Type-I transmembrane receptors with an N-terminal leucine rich repeats domain and C-terminal Toll/Interleukin-1 receptor (TIR) homology domain (Sasai and Yamamoto 2013; Takeuchi and Akira 2010), which are responsible for ligand recognition and recruitment of adaptor proteins, respectively. The TLR family can be separated into two major categories based on the localization of each TLR within a cell. The two categories of TLRs are: 1) plasma membrane-associated TLRs and 2) endolysosome-associated TLRs. The localization of each TLR is associated with its ability to recognize certain types of ligands, which are based on the pathogen’s behavior and localization.

The plasma membrane associated TLRs, including TLR1, 2, 4, 5, 6, 11, have their ligand-binding domain oriented towards the extracellular environment. This specific orientation enables these TLRs to detect extracellular pathogens (O’Neill, Golenbock et al. 2013). TLR3, 7, 8, 9, 10, 12 and 13 form the second category of TLRs that are localized in the endolysosome. The endolysosome is the product of fusion between the lysosome and endocytosed vesicles. The endolysosome contains extracellular products that are cleaved by lysosome proteases. This unique localization provides the ability for the endolysosomal-associated TLRs to recognize invasive pathogens like viruses and intracellular bacteria (O’Neill, Golenbock et al. 2013). The ligands for endolysosome-
associated TLRs are generally nucleic acids, which are a core component the genetic material in all organisms. Upon recognition of their ligands, the endolysosome-associated TLRs triggers, the activation of proinflammatory pathways including type I interferons (IFN).

The signaling pathways induced by TLRs vary among the TLR family members and depend on the subsequent recruitment of adaptor proteins. To bind their ligands and activate signal transduction, TLRs form either homodimers or heterodimers with other TLRs. Successful binding of the ligand leads to the activation of the cytoplasmic TIR domain to recruit adaptor proteins. There are five TIR domain-containing adaptor proteins: Myeloid differentiation primary response gene 88, (MyD88), TIR-domain-containing adaptor-inducing interferon-β (TRIF), Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP/MAL), TRIF related adaptor molecule (TRAM) and Sterile-alpha and Armadillo motif containing protein (SARM) (Takeuchi and Akira 2010).

TLR signaling can be also separated into two signaling pathways, one dependent on the MyD88 adaptor and the second mediated by TRIF. MyD88 is the common adaptor protein for most TLRs with the exception of TLR3. Upon activation, recruitment of MyD88 to the TIR domain of the TLR enables MyD88 to interact with interleukin-1 receptor-associated kinase 4 (IRAK-4) (Suzuki, Suzuki et al. 2002). IRAK-4 is a serine/threonine kinase that activates interleukin-1 receptor-associated kinase 1 (IRAK-1) and interleukin-1 receptor-associated kinase 2 (IRAK-2) through phosphorylation, to form a multi-protein complex (Li, Strelow et al. 2002, Kawagoe, Sato et al. 2008). The IRAK complex then dissociates from MyD88 and interacts with Tumor necrosis factor (TNF) receptor associated factors 6 (TRAF6), an E3 ubiquitin ligase (Cao, Xiong et al. 1996). TRAF6 self ubiquitinates to induce the phosphorylation of IκB and MAP kinases, which then leads to the activation of the key transcriptional factors NF-κB and AP-1, respectively. Finally, NF-κB and AP-1 translocate into the nucleus to induce the expression of proinflammatory cytokines and chemokines, initiating an inflammatory response against the pathogen. Interestingly, TLR7 and TLR9 can utilize MyD88 in plasmacytoid dendritic cells to activate the type I IFN pathway, which is important for viral restriction (Beignon, McKenna et al.
In this case, MyD88 also activates interferon regulatory factor 7 (IRF7), leading to up-regulation of type I IFNs.

The second TLR pathway relies on the adaptor protein TRIF. Similar to MyD88, TRIF is recruited to TIR containing TLRs to interact with TRAF3 and TRAF6 via another adaptor protein TIRAP/MAL. TRAF3 is important for the activation of Tank binding kinase (TBK) and IκB kinase (IKK) (Hacker, Redecke et al. 2006), which subsequently phosphorylate IRF3 and IRF7 leading to production of type I IFNs. TRIF may also interact with Receptor interacting protein kinase 1 (RIP1) and Tumor necrosis factor receptor type 1- associated DEATH domain protein (TRADD), which leads to the activation of FAS-mediated apoptosis pathway (Meylan, Burns et al. 2004). This also results in NF-κB up regulation through caspase-8 and caspase-10 activation.

**Intracellular nucleic acid sensors: The RIG-I-like Receptors.**

Another major family of PRRs are the RIG-I-like receptors, or RLR. The RLR family is composed of three members, RIG-I, MDA5, and LGP2 (Takeuchi and Akira 2009). In contrast to TLRs, which are membrane bound and face the extracellular space, RLRs are localized to the cytoplasm. The localization of RLRs allows them to be key sensors of viruses, by recognizing viral ssRNA and dsRNA as PAMPs. RLRs have a conserved protein structure composed of two N-terminal CARD domains, a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain. Since RLRs are responsible for recognition of RNA viruses, their activation up-regulates the type I IFN and NF-κB pathway through the adaptor protein, mitochondrial antiviral signaling protein, MAVS (also known as IPS-1) (Kawai, Takahashi et al. 2005).

Upon detecting RNA, RIG-I and MDA5 bind MAVS to activate signaling through TRAF3 in a similar fashion as the TLR/TRIF-dependent pathway. Similarly to TLR signaling, activation of TRAF3 leads to NF-κB up-regulation via TRADD, and type I IFN up-regulation by IRF3 and IRF7 (Zeng, Sun et al. 2010). RIG-I recognize short RNA, while MDA5 recognizes long RNA (Kato, Takeuchi et al. 2006). However, RLRs do not bind to host RNA due to a difference in length and eukaryotic modification, including capping of mRNA, at
the N-terminus (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006). In contrast, LGP2 may also bind to both ssRNA and dsRNA, but it has been described as a regulator of RIG-I and MDA5 function instead of as an immune response activator. LGP2 was originally shown as a negative regulator in in vitro studies, but LGP2 deficient mice had increased cytokine production mediated by RIG-I and MDA5 (Rothenfusser, Goutagny et al. 2005, Saito, Owen et al. 2008, Satoh, Kato et al. 2010). More recently, LGP2 has been shown to be an important inducer of CD8+ T-cell survival and fitness during peripheral T-cell responses against viral infection (Suthar, Ramos et al. 2012). In this study, the ablation of LGP2 gene in mice revealed LGP2 is dispensable for the activation of the innate immune response, but essential to promote viral specific CD8+ T-cells survival (Suthar, Ramos et al. 2012). Therefore, the function of LGP2 highlights its role as a regulator of the adaptive immune system.

**Intracellular bacterial sensors: Nod-like receptors**

Nod like receptors, or NLRs, are another family of intracellular PRRs that possess diverse functions (Figure 1). Currently, 23 NLRs have been identified in humans and 34 identified in mice (Zhao and Shao 2012). The structure of the NLR family consists of a conserved tripartite domain. Similar to TLRs, the C-terminal domain contains leucine rich repeats, required for binding to ligand. The center of the protein is a NACHT domain responsible for the oligomerization of NLR proteins as well as regulation through ATP or GTP binding. The N-terminal domain varies depending on the NLR protein, but generally, it is either a CARD domain, Pyrin domain or a domain whose function is not yet identified (termed X).

NLRs can be also separated into multiple categories. The first category is canonical NLRs that function as PRRs, such as NOD1 and NOD2. NOD1 and NOD2 are intracellular sensors of the bacterial cell wall product, peptidoglycan. NOD1 binds to D-glutamyl-meso-diaminopimelic acid, or iE-DAP, which is generally found in Gram-negative bacteria (Chamaillard, Hashimoto et al. 2003, Girardin, Boneca et al. 2003). In contrast, NOD2 binds to muramyl dipeptide (MDP), which can be found in both Gram-positive and Gram-
negative bacteria (Girardin, Boneca et al. 2003, Inohara, Ogura et al. 2003).

Upon binding to peptidoglycan, NOD1 and NOD2 recruit the adaptor protein Rip2, leading to the activation of MAP kinases and NF-κB pathways, with subsequent secretion of proinflammatory cytokines.

In addition, NOD1 and NOD2 have also been reported to recruit the cellular autophagy machinery against invading pathogens. NOD1 and NOD2 activation of antibacterial autophagy is via a Rip2-independent pathway. Instead, NOD1 and NOD2 interact with an autophagy protein, ATG16L1, to recruit the host autophagy machinery and form a double membrane autophagosome around intruding bacteria (Travassos, Carneiro et al.)

Figure 1. General overview of different types of NLRs. a) NOD1 and NOD2 are intracellular sensors of peptidoglycan, which activate NF-κB through adaptor Rip2. b) Inflammasome associated NLRs form a multi-protein inflammasome complex with adaptor ASC, which facilitates the caspase 1-dependent processing of proinflammatory cytokines, including IL-1β, into the active form. c) Non-canonical NLRs do not sense PAMPs or DAMPs, but they have the ability to regulate the immune response. NLRC5 and CIITA are transcriptional regulators of MHCI/II genes. NLRX1 is uniquely localized on the inner surface of mitochondria to regulate mitochondria associated pathways.
In this case, NOD1 and NOD2 function as targeting molecules to recruit the autophagy machinery towards the plasma membrane in order to engulf invading pathogens. Interestingly, our laboratory has recently discovered that ATG16L1 also acts as a negative regulator of NOD1 and NOD2 signaling pathway by inhibiting Rip2 activation (Sorbara, Ellison et al. 2013). Therefore, NOD1 and NOD2 are responsible for the detection of intracellular bacteria and activating multiple pathogen controlling mechanisms, such as inflammatory cytokine production and autophagy.

Another category of NLRs are the inflammasome-associated NLRs. The inflammasome is a protein complex that triggers the formation of proinflammatory cytokines during infection as well as in times of cellular damage or stress. The inflammasome activates caspase-1, leading to the cleavage and activation of proinflammatory cytokines such as IL-1β (Martinon, Burns et al. 2002). In addition, the inflammasome also has the ability to activate a special form of cell death known as pyroptosis. To date, six NLRs: NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4, have been identified to be part of different inflammasome complexes (Latz, Xiao et al. 2013). The majority of these NLRs maintain the conserved tripartite structure, with the exception of NLRP1. The NLRP proteins have an N-terminal pyrin domain to recruit the adaptor protein, ASC, with the exception of NLRC4 and NLRP1, which contain an N- and C-terminal CARD, respectively (Mariathasan, Newton et al. 2004, Faustin, Lartigue et al. 2007). Nonetheless, NLRC4 and NLRP1 may still recruit the adaptor ASC, but ASC recruitment is not essential to activate caspase-1 in NLRC4 and NLRP1, although without ASC activation, slower kinetics was observed (Mariathasan, Newton et al. 2004, Faustin, Lartigue et al. 2007). The ASC adaptor protein consists of two domains, a pyrin domain and a CARD domain. Generally, ASC interacts with pyrin domain-containing NLRs while the CARD domain is utilized to bring pro-caspase 1 into close proximity to form the inflammasome (Martinon, Burns et al. 2002). The close localization of the inflammasome and pro-caspase-1 lead to the cleavage of pro-caspase-1 into its active form. Activated caspase-1 then cleaves pro-inflammatory molecules, including pro-IL-1β, from the pro-form into their active form (Martinon, Burns et al. 2002). The inflammasome may be triggered by a
variety of ligands, but specific ligand recognition relies on which NLR is involved to form the inflammasome. The ability to incorporate a selection of NLRs enables the inflammasome to survey a broad range of PAMPs and DAMPs, sensing infection, cell damage and stress, and activate the most appropriate proinflammatory response.

**Non-canonical NLRs: NLRX1, CIITA, and NLRC5**

In addition to the canonical NLRs and inflammasome-associated NLRs, there exist a number of non-canonical NLRs acting as transcriptional regulators of immune-related genes. One such NLR is the class II transactivator or CIITA, which is a transcriptional coactivator that regulates Major Histocompatibility Complex (MHC) Class II genes, and a few MHC Class I genes. CIITA has the ability to induce *de novo* transcription of the MHC class II gene, as well as to reinforce MHC class I gene expression (Devaiah and Singer 2013). CIITA elicits its function by translocating into the nucleus and forming an enhanceosome, consisting of CIITA with other transcriptional factors, which binds to the upstream regulatory element of MHC class I/class II genes (Masternak, Muhlethaler-Mottet et al. 2000). The CIITA protein maintains the conserved tripartite domain structure of NLRs (Figure 2a). The N-terminal consist of multiple regulatory domains, including an activation domain (AD), proline/serine/threonine (PST) domain and a GTP binding domain (GBD). The AD domain is required for the binding to general transcriptional factors (Fontes, Jiang et al. 1997; Sisk, Gourley et al. 2000). The function of the PST domain has not been discovered, but nonetheless is essential for CIITA function (Chin, Li et al. 1997). Finally, the GBD is required to interact with DNA binding transactivators (Chin, Li et al. 1997). The C-terminal LRR does not bind to a ligand like NOD1/NOD2, but has been shown to be important for the localization of CIITA to the nucleus (Hake, Masternak et al. 2000; Linhoff, Harton et al. 2001).

In addition, CIITA possess three nuclear localization signals (NLS), which are also required for nuclear translocation (Masternak, Muhlethaler-Mottet et al. 2000; Raval, Weissman et al. 2003). Two of the NLSs are found in the N-terminal domains and one is located in the C-terminal domain (Raval, Weissman et al. 2003). To control MHC class II
gene transcription, CIITA utilizes four upstream cis-acting elements, W/S, X1, X2 and Y boxes, at a region in the locus known as the SXY module (Figure 2b). CIITA interacts with trans-activating DNA binding factors at the cis-acting elements to regulate class II gene expression. The binding of trans-acting DNA binding factors leads to the formation of the enhanceosome in which CIITA is anchored at the site of activation (Masternak, Muhlethaler-Mottet et al. 2000). The enhanceosome enables CIITA to recruit histone modification proteins, leading to activation or suppression of the MHC class II gene and other MHC related genes, including HLA-DO, HLA-DM, Invariant chain li and some MHC I molecules as well (Wright and Ting 2006; Zika and Ting 2005).

NLRC5 is another recently discovered MHCI transcriptional activator. NLRC5 also maintains the tripartite domain of NLR (Figure 2a) and it is phylogenetically closest to CIITA (Benko, Magalhaes et al. 2010, Meissner, Li et al. 2010). NLRC5 contains a C-terminal LRRs and NACHT domain, but the N-terminus of NLRC5 is a CARD domain, albeit shaped atypically and termed an atypical CARD domain. Similar to CIITA, NLRC5 possess a single NLS within the N-terminal, and is also capable of translocating into the nucleus (Meissner, Li et al. 2010). Interestingly, NLRC5 expression is found to be highest in leukocytes, including CD4+/8+ T cell, CD19+ B cell, NK and NKT cells (Benko, Magalhaes et al. 2010, Neerincx, Lautz et al. 2010). In a steady state, NLRC5 is localized in both the nucleus and cytoplasm. NLRC5 translocates into the nucleus via importin-α while the export into the cytoplasm relies on exportin-1, also known as CRM1 (Benko, Magalhaes et al. 2010). As an MHCI transactivator, NLRC5 is a major inducer of the expression of both classical MHC class I gene, and non-classical MHC class I genes. MHC class I accessory genes and other related genes, such as TAP1, are also inducible by NLRC5 (Meissner, Li et al. 2010). In contrast to CIITA, NLRC5 has not been observed to influence the expression of MHC class II associated loci (Robbins, Truax et al. 2012). To induce the transcription of MHCI genes, NLRC5 is hypothesized to co-operate with trans-acting DNA-binding proteins to form an enhanceosome like complex on the SXY module at the MHC class I promoter in a similar fashion as CIITA (Figure 2b).
Figure 2 Non-canonical NLRs: NLRC3, NLRC5 and CIITA. a) Domain structures of NLRC3, NLRC5 and CIITA. b) Transcriptional regulation of MHC Class I and Class II locus by NLRC5 and CIITA at the promoter, respectively.
Ultimately, the NLRC5 enhanceosome can activate MHC class I gene expression via the recruitment of histone-modifying enzymes (Meissner, Liu et al. 2012; Robbins, Truax et al. 2012).

In addition to being a transcriptional activator, NLRC5 has also been reported as a negative regulator of TLR4 and RIG-I signaling pathway through interaction with IKKα, IKKβ and RIG-I (Cui, Zhu et al. 2010). However, conflicting findings have been reported to suggest NLRC5 deficiency does not affect the immune response during viral and bacterial infection (Kumar, Pandey et al. 2011). Therefore, NLRC5 is another transcriptional regulator within the NLR family that specifically regulates MHC class I gene expression.

NLRX1 is a recently identified NLR with a unique N-terminal mitochondria localization sequence. Aside from the localization signal, NLRX1 maintains the conserved NACHT domain and C-terminal LRRs. Recently, research has highlighted the function of NLRX1 as a potential regulator of innate immunity. Initially, NLRX1 had been thought to localize to the outer mitochondria membrane, acting as a negative regulator of RLR anti-viral response by interacting with the RLR pathway adaptor protein MAVS (Allen, Moore et al. 2011). However, the correct localization of NLRX1 was later confirmed by our group and another laboratory to be within the mitochondria matrix, where it can interact with UQCRC2, a mitochondria matrix protein of the respiratory chain complex III (Rebsamen, Vazquez et al. 2011, Soares, Tattoli et al. 2013). At the same time, NLRX1 deficiency did not alter anti-viral responses in BMDMs and murine embryonic fibroblasts (MEFs) from our laboratory (Soares, Tattoli et al. 2013). Alternatively, NLRX1 was shown to induce the release of reactive oxygen species in vitro (Abdul-Sater, Said-Sadier et al. 2010). Finally, other studies have shown that NLRX1 promotes autophagy during viral infection by interacting with TUFM (Lei, Wen et al. 2012, Lei, Wen et al. 2013).

The function of NLRX1 in inflammatory regulation and mitochondrial related pathways was further investigated more recently. One group showed that NLRX1 protected mice from experimental autoimmune encephalomyelitis (EAE), NLRX1 deficient mice were associated with over-reactive resident microglial cells secreting higher levels
of IL-6 and CCL2 (Eitas, Chou et al. 2014). Another group demonstrated that NLRX1 acts as a positive regulator of antiviral responses by preventing mitochondrial damage during influenza A infection (Jaworska, Coulombe et al. 2014). However, our laboratory have recently published a new paper focusing on NLRX1 as a mediator of extrinsic and intrinsic apoptosis (Soares, Tattoli et al. 2014). We observed that NLRX1 was down-regulated by glucose deprivation and SV40 transformation. SV40 transformed MEFs deficient with NLRX1 had reduced basal ROS production and exhibited increasing sensitivity to extrinsic apoptosis induced by TNF/cycloheximide (Soares, Tattoli et al. 2014). In contrast, these MEFs were resistant to intrinsic apoptosis triggered by glycolysis inhibition and endoplasmic reticulum (ER) stress (Soares, Tattoli et al. 2014). Together, these observations suggest NLRX1 is utilized by a cell to regulate its susceptibility between extrinsic and intrinsic apoptosis pathways based on nutrient availability. In addition, in vivo azoxymethane (AOM)/dextran sodium sulphate (DSS) treatment in mice, an animal model for colorectal cancer, demonstrated NLRX1 plays a protective role against inflammation-associated carcinogenesis, by reducing inflammation and number of tumors (Soares, Tattoli et al. 2014). Based on these reports, NLRX1 is a unique NLR that is involved cellular metabolism, apoptosis regulation and other mitochondrial related pathways, rather than acting as a conventional PRR.

**NLRC3 structure and function.**

Nod like receptor family CARD domain containing 3, or NLRC3, is another member of the NLR family that has been recently investigated. NLRC3 possesses the conserved structure of NLRs including a C-terminal LRR, a NACHT domain and an N-terminal domain (Figure 2a). Despite the term NLRC3, this protein does not contain a CARD-domain and the function of the N-terminal domain is currently unknown. The structure of NLRC3 protein is not determined, but in silico analysis using I-TASSER predicts NLRC3 protein to fold back its C-terminal toward the N-terminal (Figure 3). Based on its genetic sequence, the NLRC3 is most closely related to NLRX1, followed by CIITA and NLRC5 from phylogenetic studies (Benko, Magalhaes et al. 2010, Meissner, Li et al. 2010).
The function of NLRC3 is currently unclear, but a limited number of recently conducted studies provide insight into the potential function of this NLR. The first study on NLRC3 revealed that the most abundant expression of NLRC3 mRNA in both mice and human cells was found in lymphoid-progenitor derived populations, specifically CD4+ T cell, CD8+ T cell and NK cells (Conti, Davis et al. 2005). Interestingly, stimulation of the T cell receptor (TCR) pathway using either anti-CD3/CD28 or PMA/ionomycin triggers the down regulation of NLRC3 mRNA in CD4+ T cells (Conti, Davis et al. 2005). In addition, when NLRC3 is overexpressed in Jurkat T cell, the expression of IL-2 mRNA is inhibited (Conti, Davis et al. 2005). Together, these results suggested NLRC3 is potentially a negative regulator of T cell function.

Recent discoveries, on the other hand, have suggested NLRC3 as a potential negative regulator of inflammation in macrophages, via modification of the TLR receptor signaling and anti-viral response. In vitro analysis revealed that NLRC3 has an inhibitory effect on MyD88 and TRAF6 signaling, leading to lower NF-κB activation by delaying IkBα phosphorylation (Schneider, Zimmermann et al. 2012). As a result, inflammatory cytokine
production is enhanced transiently in NLRC3-deficient macrophages upon LPS challenge. In addition, NLRC3 was found to interact with TRAF6 \textit{in vitro} and to regulate the degradation of TRAF6 (Schneider, Zimmermann et al. 2012). NLRC3 was shown to regulate the degradation of TRAF6 by decreasing the total ubiquitination and K63-linked ubiquitination on TRAF6 (Schneider, Zimmermann et al. 2012). Lower ubiquitination is hypothesized to lead to increased proteosomal degradation of TRAF6 and decreased activation of TRAF6. Further evidence supporting the role of NLRC3 in TLR related pathways was provided using \textit{in vivo} LPS septic endotoxic models. Peritoneal macrophage isolated from NLRC3-deficient mice had higher expression and levels of proinflammatory cytokines following LPS challenge (Schneider, Zimmermann et al. 2012). NLRC3 deficient mice did not survive endotoxic shock and had higher serum levels of IL-6 (Schneider, Zimmermann et al. 2012). Together, these results suggest that NLRC3 is potentially a critical negative regulator of LPS induced inflammation.

In addition to regulating inflammatory cytokine production, NLRC3 was also found to be a negative regulator of the viral DNA sensor STING (Zhang, Mo et al. 2014). STING is a cytoplasmic DNA sensor that induces the production of type I IFN upon infection by intracellular pathogens (Keating, Baran et al. 2011). In addition to being a strong activator of anti-viral responses, STING has also been shown to be stimulated by intracellular bacteria, such as \textit{L. monocytogenes}, to activate type I IFN response (Woodward, Iavarone et al. 2010). The function of NLRC3 in the STING pathway was demonstrated \textit{in vitro}, which revealed that NLRC3-deficient BMDMs and MEFs have an enhanced STING response upon stimulation with STING ligands, including intracellular DNA, interferon stimulating DNA, Herpes simplex virus 1 (HSV-1) and c-di-GMP (Zhang, Mo et al. 2014). Overexpression of NLRC3 with STING using IFN-β-promoter luciferase and NF-κB luciferase reporters showed reduced activation (Zhang, Mo et al. 2014), suggesting that NLRC3 directly inhibits the STING and TBK1 activation potential. The mechanism of action was shown via co-immunoprecipitation; NLRC3 interferes with the STING-TBK1 interaction and inhibits the trafficking of STING to the perinuclear region following interferon stimulating DNA stimulation (Zhang, Mo et al. 2014). Finally, \textit{in vivo} HSV-1
infection showed that NLRC3 deficient mice had increased type I IFN and pro-inflammatory cytokine productions at 6 hours post infection (Zhang, Mo et al. 2014). The elevated immune response led to a reduction in HSV-1 viral DNA copy and protection from death (Zhang, Mo et al. 2014).

Altogether, NLRC3 is hypothesized to function as a non-canonical regulator of the immune response. However, NLRC3 as a potential negative regulator in T-cell needs to be evaluated, especially since NLRC3 mRNA expression is vastly different between myeloid cells and lymphocytes (Conti, Davis et al. 2005). In addition, studies from our laboratory have demonstrated that transfection and expression of NLRs has an inherent ability to inhibit luciferase assays, which are widely used in the previous studies to demonstrate the ability of NLRC3 to inhibit NF-κB and IFN signaling (Ling, Soares et al. 2012). The interest of our group is to determine the function and mechanism of action of NLRC3 in T cells. Preliminary work from our laboratory has shown that NLRC3-deficient mice infected with the enteric pathogen, *C. rodentium*, clear the infection more quickly than wild-type mice due to a robust T cell response in the gut lamina propria early during infection. These observations support the notion that NLRC3 functions within T cells during infection.
Hypothesis and Aims

My project focused on characterizing the properties of NLRC3 and aimed to decipher its mechanism of action using *in vitro* and biochemical techniques. My hypothesis is that during resting conditions within T cells, NLRC3 interacts with its protein partners and may regulate their function. Upon stimulation of the T cell receptor or in stress conditions, NLRC3 expression is down-regulated, releasing these protein partners that may then have alternative functions to regulate T-cell function and differentiation.

My aims in this work were to:

1) Characterize the properties of NLRC3 protein and mechanism of action. This includes investigating the expression of NLRC3 protein in T cells and T cells associated lymphoid tissue as well as determining the localization of NLRC3 protein, especially in organelles such as the nucleus and mitochondria, where other non-canonical NLRs resides. The kinetics of NLRC3 protein expression was also evaluated to compare with the rapid down regulation of NLRC3 mRNA following TCR stimulation.

2) Verify Y2H identified NLRC3 interacting protein partner through co-immunoprecipitation. My goal was to initially analyze Y2H results for common patterns, such as protein localization or multiple components of specific protein complex. Highly interesting targets were then cloned into plasmid and co-expressed with NLRC3 for co-immunoprecipitaiton. Co-immunprecipitation was used to verify whether NLRC3 interacts with the Y2H identified target, which will further suggests the mechanism of action by NLRC3.

NLRC3 is a newly discovered NLR that has been suggested to possess a negative regulatory role in regulating T cells and macrophages. By characterizing NLRC3 and
identifying its potential interacting partner, I hope to decipher mechanisms tNLRC3 utilizes to regulate T cells. This would provide a new insight into the function of NLRC3, and potentially other NLRs, in regulating T cell function and differentiation.
Material and Methods

Mice

C57BL/6 and NLRC3 deficient colony were bred and maintained under specific pathogen-free condition at the CCBR, University of Toronto, Canada. NLRC3 deficient mice were provided by Dr. Philip Rosenstiel (University of Kiel, generated by genOway) and backcrossed for 9 generations to C57BL/6 (Charles River). *In vivo* experiments were conducted with littermates from the same cross. NLRC3 deficient mice were generated by excising exons 2 and 3 of NLRC3 gene in embryonic stem cells (Figure 4a). Expression of NLRC3 in wild type and NLRC3 deficient mice were confirmed by genomic PCR amplification (Figure 4b). Heterozygous x Heterozygous breeding pairs were setup to generate WT and NLRC3 deficient littermates for experiments.

Figure 4  
a) Scheme for generating constitutive NLRC3 knock-out mice. Exon 2 and exon 3 of NLRC3 gene were excised by homologous recombination.  
b) PCR verification of NLRC3 mice genotype. Conducted by Vinicius Motta, Post Doc.
cDNA cloning of hIFRD1-FLAG and AHCYL1-FLAG.

The coding sequence of the IFRD1 gene and AHCYL1 gene were amplified using conventional PCR from IFRD1 plasmid (Origene) and AHCYL1 plasmid (kindly provided by Dr. Yun, Department of Medicine, Emory University), respectively. Cloning primer sequence: IFRD1 Forward 5’-AAT AGG TAC CAT GCC GAA GAA CAA GAA GCG-3’ and IFRD1 Reverse 5’-AAT AGG TAC CAT GCC GAA GAA CAA GAA GCG -3’; AHCYL1 Forward 5’- AGT GAC CGG TAT GCA GGA GTT CAC CAA ATT-3’ and AHCYL1 Reverse 5’- AGT GAC CGG TAT GCA GGA GTT CAC CAA ATT-3’. PCR amplified product was removed from agarose gel and purified using gel extraction kit (Fermentus). PCR products were digested using KpnI and XbaI followed by ligation to pcDNA3-FLAG vector.

In vitro expansion of primary T cells

Splenocytes were harvested from the spleen of the mouse using mechanical disruption. Splenocytes were incubated with red blood cell lysis buffer (Sigma) and resuspended in complete media (RPMI-1680 medium containing 10% FCS, penicillin/streptomycin (Gibco), L-glutamine (Gibco), 50uM β-mercaptoethanol (Sigma-Aldrich), Sodium Pyruvate (Gibco), non-essential amino acids (Gibco), 20mM HEPES (Gibco) ). IL-2 and Con-A were added to the 10mL culture flask and cells were cultured for 24 hours before centrifugation to remove complete media containing Con-A. Fresh media containing only IL-2 were added to maintain culture for 3 additional days before expanded cells were subjected to stimulation.

In vitro cell stimulation

T cell receptor simulation was conducted by culturing T cells in plate-bound anti-CD3 (2ug/mL) with soluble anti-CD28 (0.5ug/mL) or phorbol-12-myristate-13-acetate (50 ng/mL), ionomycin (Sigma) (1 μg/mL) in complete medium. Amino acid stress were induced by culturing Jurkat cells with Krebs-Ringer bicarbonate (KRB) buffer (118.5 mM NaCl, 4.74 mM KCl, 1.18 mM KH2PO4, 23.4 mM NaHCO3, 5 mM glucose, 2.5 mM CaCl2, 1.18 mM MgSO4, adjusted to pH 7.6 by
titration with 1 N NaOH) or stimulating with rapamycin (25 µg/mL) for times as indicated. Lysates from stimulated cells were collected for western blot analysis using RIPA buffer.

**Bone marrow derived macrophage (BMDM) differentiation**

Bone marrows cells were harvested from the femur and tibia of NLRC3 WT and KO mice using standard techniques and immediately resuspended in A1 medium (2mM L-glutamine, 50µM beta-mercaptoethanol, 10% FCS) upon harvest. 2x10^6 bone marrow cells were cultured in 10mL of A2 medium (A1 medium 30% and L929 conditioned medium) for 6 days in humidified incubator with 5% CO2 at 37°C, with the addition of 10mL A2 medium on day 3. On day 6, BMDMs were completed differentiated, and utilized for stimulation.

**Cytokine ELISA**

BMDM were plated in 24-well plates at a density of 3.25x10^5 cells/well for 2 hours prior to stimulation. To activate the BMDMs, wells were incubated with 20 units of murine IFNγ overnight prior to LPS stimulation. BMDM were stimulated with or without LPS (InvivoGen) with concentration and time as indicated in figure, and culture supernatants were collected and store at -80°C until utilized for ELISA. The concentrations of IL-6, TNF-α, and KC in supernatant were determined using ELISA (DuoSet from R&D system) according to the manufacturer’s recommendation.

**Subcellular Fractionation of Jurkat cells**

Jurkat cells were fractionated into the cytosolic and nuclear fraction using previously described method (Benko S et al, 2010). Briefly, Jurkat cells were resuspended in buffer A (10mM pH 7.9 HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT and protease inhibitor cocktail) and left on ice. After 15 minutes, 10% Nonidet P-40 was added to the suspension prior to vortex and centrifugation. The supernatant was collected as the cytosolic fraction while the nuclear pellet was resuspended in buffer C (20mM pH7.9 HEPES, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and protease inhibitor cocktail)
and placed on a rotating platform at 4°C. After 15 minutes, nuclear suspension was centrifuged and the supernatant was collected as the nuclear fraction. Fractions were boiled for 10 minutes with laemmli buffer and store at -80°C.

*Western Blot*

Cell lysates were prepared using RIPA buffer and run on SDS-PAGE gel electrophoresis. Proteins were transferred onto polyvinylindene fluoride membrane using semi-dry transfer apparatus and blocked with blocking solution (5% skim-milk with 0.1% Tween 20 containing TBS/T) for 1 hour. Membranes were incubated with primary antibody overnight at 4°C, followed by secondary antibody incubation for 1 hour at room temperature. The following antibodies were used: anti-tubulin (Sigma, T9026; 1:10000), anti-pERK & ERK (Cell Signalling, 9926 & 9910 1:1000), anti-Golgin 97 (Abcam, ab84340; 1:1000), anti-LAMP2 (Santa Cruz, sc-5571; 1:1000), anti-p84 (Abcam, ab131268; 1:1000), anti-NLRC3 (generated by Cedarlane via immunization) to detect endogenous NLRC3.

*Yeast Two Hybrid Screen*

Yeast Two Hybrid experiments using human NLRC3 protein were conducted by Hybrigenics (France). The human NLRC3 protein was expressed as two separate fragments by expressing each specific section of the cDNA sequence via restriction cloning into plasmid: 1) N-terminal fragment amino acid 1-136 and 2) C-terminal fragment amino acid 558-1065. Both fragments were independently screened in a T cell specific cDNA library. For each interaction, a Predicted Biological Score (PBS) is calculated based on a variety of parameters to assess the reliability of the interaction. PBS parameter includes total number of hits, sequence identity/orientation and reading frame compared to reference sequence. Based on the PBS score, each interaction is ranked from A, high confidence, to F, proven experimental artifacts. Only interaction from rank A to D was considered for further analysis with NLRC3.
Co-immunoprecipitation

Co-immunoprecipitations were conducted as previously described. Briefly, HEK293 cells were transfected with 2µg of murine NLRC3 (mNLRC3)-HA pcDNA3.1 and/or 2µg of human IFRD1 (hIRFD1)-FLAG/hAHCYL1-FLAG pcDNA3.1. Transfected cells were cultured overnight at 37°C before lysis with IP lysis buffer (50mM Tris-HCl pH7.5, 10mM MgCl₂, 10mM NaCl, 0.5% NP-40, and 10% glycerol). Cell lysates were incubated with 3µg anti-HA or 3µg anti-FLAG antibody for 2 hour prior to incubation with 30mg of Protein G beads (Sigma) for 1 hour at 4 degree. Beads were washed twice with IP lysis buffer for 30mins before boiled for 10 minutes with laemmli buffer and store at -80°C.

Statistics

Two-tailed Student’s t-tests or one-way ANOVA tests were used in Graphpad (Prism) where appropriate, and p values < 0.05 were considered significant. All experiments were repeated as indicated in figure legends.
Results

Kinetics of NLRC3 degradation following stimulation of T cells with anti-CD3/CD28.

Previously, it was reported that NLRC3 mRNA in T cells was rapidly downregulated following anti-CD3/CD28 or PMA/ionomycin stimulation, but the expression of NLRC3 protein was never evaluated in this context. The expression of NLRC3 protein in immune tissue lysates and sorted lymphocyte populations was first investigated with a rabbit anti-NLRC3 antibody that we commissioned Cedarlane to generate by immunization. Western blot analysis revealed that NLRC3 protein, detected at the predicted molecular weight of approximately 110kDa, was expressed in all harvested immune tissues, including spleen, mesenteric lymph node and thymus, as well as sorted T cell and NK-cell populations (Figure 5a). In agreement with the initially reported NLRC3 tissue array analysis (Conti, Davis et al. 2005), the highest NLRC3 protein expression was detected in the sorted T cell populations.

NLRC3 deficient mice were obtained from our collaborator, Dr. Philip Rosenstiel, and back-crossed 9 times to the C57BL/6 background. Using these mice, primary T cells were expanded ex vivo using Con-A/IL-2 and evaluated for expression of NLRC3. To ensure proper expansion, the culture was analyzed for both B/T cell population before and after 5 days of culture using flow cytometry with anti-CD19 and anti-TCRβ, respectively. Prior to the addition of Con-A/IL-2, the spleen consisted of mostly B-cells with a lesser amount of T cell. After stimulation, up to ~95% of the culture was T cells and total number of T cells were greatly increased (Figure 6a and Figure 6b). NLRC3 protein was detected only in the lysate from wild type expanded T cells but not NLRC3-deficient ex vivo expanded T cells (Figure 5b). To further investigate the relationship between NLRC3 mRNA and protein expression, magnetically sorted primary CD4+ T cells from wild type mice were stimulated with anti-CD3/CD28 for specific times before analyzing protein expression. Interestingly, NLRC3 protein remained detectable after 3 hours of stimulation and protein
Figure 5. Characterizing the expression of NLRC3 protein and its behavior following T cell receptor stimulation. a) The expression of NLRC3 protein in various lymphoid tissues and flow sorted lymphocyte populations from a wild type C57BL/6 mice. Lymphocyte populations were sorted using anti-CD3, anti-CD19 and anti-NK1.1 for T cells, B cells and NK cells, respectively. b) The expression of NLRC3 protein in ex vivo expanded T-cells derived from a wild type or NLRC3 deficient mice. C) qPCR analysis of NLRC3 mRNA after stimulation with anti-CD3/CD28 for time as indicated. Conducted by Vinicius Motta, Post Doc. d) Kinetic analysis of NLRC3 expression in magnetically sorted primary T-cells or ex vivo expanded wild type T cells stimulated with anti-CD3/CD28 for time as indicated. Experiments were repeated at least 3 times independently.
down-regulation was observed after 6 hours to 24 hours of stimulation (Figure 5d). Similarly, NLRC3 protein remained detectable up to 6 hours post-stimulation in ex vivo expanded T cells while the protein expression was absent after 24 hours of stimulation (Figure 5e). NLRC3 mRNA expression in primary T cells was rapidly down-regulated following anti-CD3/CD28 stimulation with a similar kinetics as previous reported (Conti, Davis et al. 2005). Indeed, by 3 hours post-stimulation, NLRC3 mRNA expression decreased by approximately 80%, at the same time point where NLRC3 protein expression
remained detectable in both primary and expanded T cell (Figure 5c). The difference between mRNA and protein down-regulation is likely caused by a slow protein turn-over. However, the turn-over rate of NLRC3 protein needs to be evaluated by utilizing cycloheximide to inhibit translation of newly synthesized protein, followed by using western blot analysis to examine NLRC3 expression over a period to determine its half-life.

**The absence of NLRC3 does not affect MAP kinase signaling pathway following anti-CD3/CD28 stimulation in expanded T cells.**

In addition to the rapid down-regulation of NLRC3 mRNA after TCR stimulation, NLRC3 overexpression in Jurkat T cells has also been reported to inhibit IL-2 mRNA expression, which suggested NLRC3 as a potential negative regulator of T cell function. To decipher the potential mechanism behind IL-2 mRNA inhibition, *ex vivo* expanded T cells were generated from wild type and NLRC3 deficient mice to investigate the relationship between MAP kinase signaling, IL-2 production and NLRC3. Due to excess ability of the expanded T cell and we had previously observed that positive selection of naïve T cell using MAC sorting can induce MAP kinase signaling, expanded were chosen for preliminary MAP kinases experiments. Expanded T cells were maintained in complete media with only IL-2 for at least 3 days before stimulated in fresh media with anti-CD3/CD28 for up to 60 minutes. Cell lysates were prepared at specific time points to look at early events. Short kinetic stimulation is critical for MAP kinase since phosphorylation occurs rapidly, usually after minutes of stimulation. If using the same long kinetics as to evaluate the down regulation of NLRC3, MAP kinase phosphorylation might be missed, and any difference in the kinetics or decrease of phosphorylation would be masked. Using western blot to detect phosphorylated MAP kinases, no difference was observed in both the kinetic or levels of ERK/JNK phosphorylation between wild type and NLRC3 deficient T cells (Figure 7a). Interestingly, p38 was highly phosphorylated in unstimulated cells, and p38 remained phosphorylated throughout the entire stimulation.
Figure 7. *ex vivo* expanded T-cell with NLRC3 deficiency exhibit normal MAP kinases signaling, CamKII phosphorylation and IL-2 production following *in vitro* stimulation. Kinetics of MAP kinases phosphorylation between expanded wild type and NLRC3 deficient mice T cell following: a) anti-CD3/CD28 or b) anti-CD3 only stimulation for time as indicated. c) Kinetics of CamKII phosphorylation between ex vivo wild type and NLRC3 deficient mice T-cell following anti-CD3/CD28 stimulation. d) ELISA analysis of IL-2 production between wild type and NLRC3 deficient CD4+ T cell stimulated with different concentration of anti-CD3 with or without anti-CD28. Conducted by, Vinicius Motta, Post Doc. Experiments were repeated at least 2 times independently.
Since NLRC3 deficiency did not affect the phosphorylation of MAP kinases following TCR stimulation, we continued to examine the effect of NLRC3 deficiency in the absence of TCR co-stimulation by stimulating expanded T cells with anti-CD3 alone. Similar to anti-CD3/CD28 co-stimulation, wild type and NLRC3 deficient expanded T cells did not display any differences in the kinetics or level of phosphorylation of ERK and JNK (Figure 7b). Alternatively, NLRC3 gene might regulate IL-2 mRNA via a different downstream target, such as NFAT, which is activated by calcium signaling pathway. Therefore, the effect of NLRC3 deficiency on intracellular calcium release was investigated by analyzing the protein CamKII phosphorylation after anti-CD3/CD28 stimulation. In contrast to MAP Kinase phosphorylation, CamKII phosphorylation was only induced after 15 minutes of stimulation with anti-CD3/CD28, but there was no difference in the kinetic or level of phosphorylation of CamKII between wild type and NLRC3 deficient expanded T cells (Figure 7c). Therefore, NLRC3 does not regulate MAP kinase and calcium induced signaling pathways in expanded T cells, but these observations needs to be further validated in wild type and NLRC3 deficient naïve T cells.

Nonetheless, to verify the ability of NLRC3 gene to regulate IL-2 production via an uncharacterized pathway, IL-2 cytokine ELISAs were conduct by a postdoctoral fellow, Vincius Motta, to analyze IL-2 production in absence of the NLRC3 gene. In agreement with the MAP kinases and CamKII observations, no difference was observed in IL-2 production upon stimulating wild type and NLRC3 deficient cells with anti-CD3/CD28 or anti-CD3 alone (Figure 7d). The absence of anti-CD28 equally reduced the IL-2 production in both wild type and NLRC3 deficient cells. Together, these observation indicated that NLRC3 does not inhibit IL-2 production of pre-activated T cell following re-stimulation.

**NLRC3 is not expressed in BMDM and it does not regulate proinflammatory cytokine production via LPS stimulation of TLR4.**

Recently, NLRC3 was reported as a negative regulator of both TLR4-driven proinflammatory cytokine production and of the DNA sensor STING activation in BMDM and peritoneal macrophages (Schneider, Zimmermann et al. 2012; Zhang, Mo et al. 2014). NLRC3 was shown to directly interact with TRAF6 to regulate K63-linked ubiquitination of
TRAF6. NLRC3 deficiency resulted in an increase in ubiquitination of K63, which induced rapid loss of IκBα and prolonged phosphorylation of the p65 subunit of NF-κB. On the other hand, NLRC3 was shown to interfere with STING-TBK1 interaction and transport of STING to the perinuclear region follow interferon pathway stimulation. Since STING activates type I IFN pathway through interaction with TBK1 to phosphorylate IRF3 and STAT6, disrupting STING-TBK1 complex formation reduces the ability of the complex to phosphorylate IRF3 and STAT6, which are key factors driving type I interferon production. However, earlier tissue array analysis indicated that NLRC3 mRNA expression is highest in lymphocytes but very low in myeloid cells (Conti, Davis et al. 2005). In addition, the expression of NLRC3 protein in myeloid cells has not been investigated. Therefore, the expression of NLRC3 protein in both BMDM and T cell was compared between wild type and NLRC3 deficient mice.

Using the same anti-NLRC3 antibody as above, NLRC3 protein was only detected in wild type expanded T cell lysate, included as a positive control (Figure 8a). NLRC3 protein expression was not detectable in BMDM lysates derived from both wild type and NLRC3 deficient mice. To control for equal concentration of total protein between lysates, both tubulin and β-actin were included as protein loading controls. Interestingly, the tubulin band had lower expression within BMDM lysates independent of mice genotype, but β-actin control did not reveal any difference in total protein concentration between BMDM and expanded T cells. Furthermore, a previous post-doctoral fellow, Vinicius Motta, was unable to detect full-length NLRC3 mRNA in BMDMs using qPCR analysis with primers targeting exon 2 and 3 of NLRC3 gene (Figure 8b). NLRC3 mRNA was detectable in all previously described lymphocytes, including NK cell, CD4+ T cell and CD8+ T cell; BMDM, MEF and MODE-K cells did not express detectable levels of full length NLRC3 mRNA (Figure 8b). Therefore, NLRC3 protein is expressed in T cells but is not detectable in BMDM by using our antibody.

Despite the fact that we could not detect full-length NLRC3 in BMDMs, an alternatively spliced isoform of the NLRC3 might exist and influence the function of BMDMs. Indeed, the online protein database, Uniprot, identified few potential
Figure 8. NLRC3 was not detectable in BMDMs, and NLRC3 deficient BMDMs did not produce higher levels of proinflammatory cytokines compared to wild-type mice. a) NLRC3 protein expression in ex vivo expanded T cells and BMDM derived from wild type or NLRC3 deficient mice. b) ELISA analysis of dose dependent proinflammatory cytokine production in BMDMs treated with LPS. BMDMs were stimulated by 10ng/ml or 250ng/ml of LPS for 24 hours. C) Kinetics of proinflammatory cytokine production in BMDM treated with LPS. BMDMs were stimulated by 100ng/ml of LPS for different time points. All BMDM prepared from wild type and NLRC3 deficient mice were pretreated with or without IFN-γ overnight. All ELISA has n = 2, with two independent repeats per mouse.
alternatively spliced isoforms in both human and mice NLRC3, but the expression of these isoforms has not been confirmed. Therefore, to investigate the existence of a potentially functional NLRC3 isoform, as well as the ability of NLRC3 to regulate proinflammatory cytokine production, wild type and NLRC3 deficient BMDMs were stimulated with multiple concentrations of LPS (10ng - 250ug) up to 24 hours. In addition, BMDM were primed with IFNγ to mimic a pre-stimulated and activated macrophages. Utilizing ELISA to detect IL-6, TNFα and KC in culture supernatants, there was no differences in the production of all three proinflammatory cytokines by wild type and NLRC3 deficient BMDM following both high and low doses of LPS stimulation (Figure 8c). Interestingly, IFNγ priming was required to enhance the production of IL-6 and TNFα production, while KC production was independent of IFNγ priming. In addition, and in contrast to the previously reported acute cytokine production by NLRC3-deficient peritoneal macrophage at 6 hours of stimulation, proinflammatory cytokine production was at a maximum only after 24 hour stimulation in BMDMs prepared from our group. At each of the indicated time points, there were also no differences between WT and NLRC3 deficient BMDMs, except a small decrease in IL-6 production in NLRC3 deficient BMDM at 24 hours post stimulation (Figure 8d). Similarly, the production of KC was not affected by the IFNγ priming. Together, the results indicated that NLRC3 is not detectable in BMDMs and that deficiency in NLRC3 expression in BMDMs does not limit the production of proinflammatory cytokines post LPS stimulation.

**Downregulation of NLRC3 is induced by intracellular calcium influx and mTOR inhibition.**

Unpublished previous findings from our laboratory showed that that NLRC3 mRNA down-regulation can be triggered by treatment with ionomycin or thapsigargin alone, both of which are inducers of intracellular calcium release (Figure 9). Therefore, intracellular calcium as a trigger of NLRC3 protein down-regulation was investigated in Jurkat cells. Similar to primary T cells, Jurkat cells exhibited similar down-regulation of NLRC3 upon stimulation. Indeed, using western blot analysis, NLRC3 was detectable in
non-stimulated Jurkat T cells, and NLRC3 protein down-regulation was observed after 6-8 hours post PMA/ionomycin stimulation, which is at a similar kinetic to that observed in previous experiments (Figure 10a). To investigate the activation of the diacylglycerol (DAG) and inositol triphosphate (IP$_3$) signaling pathways downstream of TCR signaling via phospholipase C, Jurkat cells were treated with either PMA or ionomycin alone to target PKCθ or intracellular calcium influx pathway, respectively. Surprisingly, PMA alone was not sufficient to induce the down regulation of NLRC3, even after 24 hours of stimulation (Figure 10b). In contrast, ionomycin treated Jurkat T cells showed down-regulation of NLRC3 after 24 hour of stimulation (Figure 10b). To further validate that NLRC3 down-regulation was induced by intracellular calcium influx, Jurkat T cells were treated with the drug thapsigargin, which prevents the removal of intracellular calcium into endoplasmic reticulum (ER). Stimulation of Jurkat T cells with thapsigargin resulted in the down-regulation of NLRC3 protein by 6 hour of stimulation, in contrast to the down-regulation
Figure 10. NLRC3 protein down regulation may be triggered by intracellular calcium influx and mTOR inhibition. Jurkat T cells were treated with a) PMA/Ionomycin or b) PMA or Ionomycin alone to stimulate TCR signaling or downstream DAG/IP₃ signaling pathways. c) Jurkat T cells were stimulated with thapsigargin to mimic intracellular calcium release. d) Jurkat T cells were maintained in (KRB) buffer only deficient in amino acids or stimulated with rapamycin (25ug/mL) to induce amino acid starvation or mTOR inhibition, respectively. Cellular lysates were treated for time as indicated before collected for western blot analysis. All experiments were repeated at least 2 times independently.
induced after 24 hours of ionomycin alone treatment (Figure 10c). The faster kinetic is likely due to the high dosage of thapsigargin in combination with its ability to influence intracellular calcium level and cellular stress. Since thapsigargin can be also utilized to induce ER stress, we continued to investigate stress conditions as a trigger of NLRC3 downregulation. Jurkat T cells were placed in an amino acid starvation buffer (KRB), or treated with rapamycin, which inhibits mTOR directly, in order to evaluate NLRC3 downregulation in response to stress pathway activations. Culturing Jurkat T cells in amino acid deprived (KRB) buffer led to a slight down-regulation of NLRC3, but NLRC3 was still detectable after 24 hours of culture (Figure 10d). In contrast, rapamycin-treated Jurkat cells displayed downregulated NLRC3 expression after 24 hours of stimulation (Figure 10d). Therefore, NLRC3 protein down-regulation is inducible by intracellular calcium release and the inhibition of mTOR. Amino acid starvation may also be a relatively inefficient trigger of NLRC3 expression down-regulation.

**NLRC3 sub-cellular localization in Jurkat T cells.**

Initially, an *in silico* analysis of NLRC3 amino acid sequence was conducted to predict NLRC3 protein localization. Complete NLRC3 amino acid sequence analysis using PSORTII identified a four amino acid nuclear localization pattern at position 65 even though the predicted localization of full length NLRC3 was 52.2% cytoplasmic and 21.7% nuclear or mitochondrial (Table 2). To further determine the importance of the four amino acid sequences, the first 150 amino acids were re-analyzed using PSORTII. This shorter fragment was predicted to be 47.8% nuclear, 30.4% mitochondrial and 17.4% cytoplasmic (Table 2). Therefore, the N-terminal fragment likely has the ability to translocate into the nucleus, but further validation this ability will be conducted in the future by *in vitro* expression of N-terminal fragment and point mutation of the NLS.
Table 2. *in silico* prediction of NLRC3 localization using PSORTII.

<table>
<thead>
<tr>
<th></th>
<th>Full amino acid sequence</th>
<th>First 150 amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NLS 4 amino acid pattern:</strong></td>
<td>RHRK at position 65</td>
<td>RHRK at position 65</td>
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<tr>
<td><strong>NLS 7 amino acid pattern:</strong></td>
<td>None</td>
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<tr>
<td><strong>Content of Basic residues</strong></td>
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<td>13.20%</td>
</tr>
<tr>
<td><strong>NLS score</strong></td>
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<td>-0.29</td>
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<tr>
<td><strong>Localization prediction</strong></td>
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<td>Nuclear</td>
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<tr>
<td><strong>% Cytoplasmic</strong></td>
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<tr>
<td><strong>% Nuclear</strong></td>
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</tr>
<tr>
<td><strong>% Mitochondrial</strong></td>
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<td>30.4</td>
</tr>
<tr>
<td><strong>% Other organelles</strong></td>
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</tr>
</tbody>
</table>

As an additional validation of the *in silico* predictions, subcellular fractionation was conducted using Jurkat T cell to separate cellular lysates into a cytoplasmic fraction and a nuclear fraction. In concordance with the previous report, NLRC3 protein was expressed in the cytoplasmic fraction (Figure 11a). Interestingly, NLRC3 protein was also detected in the nuclear fraction, which was controlled using the nuclear matrix protein marker, p84 (Figure 11a). Therefore, NLRC3 protein was expressed in both the nucleus and cytoplasm of Jurkat T cells. Since TCR signaling and intracellular calcium release can induce the downregulation of NLRC3, we also investigated whether NLRC3 could translocate between the cytoplasm and nucleus under stimulation. Jurkat T cell were stimulated with PMA/ionomycin or thapsigargin for indicated time before being subjected to subcellular fractionation. NLRC3 was continuously expressed in the cytoplasm and nucleus for up to four hours post stimulation, and translocation of NLRC3 into the nucleus or vice versa was not observed throughout the entire experiment (Figure 11b). Therefore, from these preliminary findings, NLRC3 appears to be expressed in both the nucleus and cytoplasm, but stimulation of the TCR or intracellular calcium influx does not induce a translocation of the protein to these different compartments.
Yeast-2 hybrid screen for NLRC3 interactors

To identify potential NLRC3 interacting protein partners, two Y2H screens were independently conducted in a T cell specific library using an N-terminal or C-terminal
NLRC3 fragment. Analyzing the Y2H screen results, a large list of high-confidence potential interacting partners was identified (Table 3 and Table 4). From the N-terminal fragment screen, KNPB1, RANBP5, TNPO3, which are three components of the nuclear import complex, were identified, indirectly validating our sub-cellular fractionation experiments discussed above. In addition, a few of other nuclear proteins, including IFRD1, were reported with a high confidence score. Interestingly, IFRD1 was identified in both the N-terminal and C-terminal fragment screens. Some of these potential interacting partners were screened in subsequent assays discussed below. Finally, another interesting target is adenosylhomocysteinase-Like 1(AHCYL1), also termed inositol triphosphate receptor binding protein (IRBIT). As previously mentioned, IP₃ binding to IP₃ receptor triggers the influx of intracellular calcium. At the same time, AHCYL1 is activated to modulate of IP₃ receptor activity. AHCYL1 possesses the ability to bind to the IP₃ receptor at the same site as IP₃ and therefore functions as a competitive inhibitor. Binding of AHCYL1 to the IP₃ receptor enables the cell to regulate its sensitivity to IP₃. Since NLRC3 downregulation is also triggered by intracellular calcium influx, it is possible that AHCYL1 may interact with NLRC3 when IP₃ is not present, while intracellular calcium influx could release AHCYL1 from NLRC3 and enables it to bind to IP₃ receptor, a hypothesis that remains to be tested.

Table 3. High confident score targets from NLRC3 N-terminal fragment Y2H screen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Localization</th>
<th>Function</th>
<th># of Hits</th>
<th>Y2H Score</th>
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<tbody>
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<td>Cell cycle regulation</td>
<td>2</td>
<td>C</td>
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<tr>
<td>EFTUD2</td>
<td>Nucleus</td>
<td>mRNA splicing</td>
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<td>Ubiquitin Ligase</td>
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<td>C</td>
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<tr>
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<td>Cyto/Nuc</td>
<td>Transcription regulator</td>
<td>3</td>
<td>B</td>
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<tr>
<td>Name</td>
<td>Localization</td>
<td>Function</td>
<td># of Hits</td>
<td>Y2H Score</td>
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<td>------------</td>
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<tr>
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<td>Cytoplasm</td>
<td>Amino Acid Synthetase</td>
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</tr>
<tr>
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<td>STAG2</td>
<td>Nucleus</td>
<td>Cell division</td>
<td>3</td>
<td>B</td>
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</tbody>
</table>

Table 4. High confident score targets from NLRC3 C-terminal fragment Y2H screen.
NLRC3 interacts with IFRD1 in HEK293 cells.

The T cell-specific Y2H screens identified many potential NLRC3 interacting partners, but verification of these interactions in a cell is required before determining its potential significance. The first interesting target that was verified is IFRD1. IFRD1 was identified with high confidence scores from both of the N-terminal and C-terminal independent screens (Table 3 and Table 4; Figure 12). Its expression in cells of MLN during the course of amino acid starvation was also verified by the post-doctoral fellow, Vinicius Motta, and IFRD1 is continuously expressed (Figure 13). To verify this interaction, hIFRD1 cDNA was PCR amplified with the restriction site KpnI on the N-terminus and XhoI on the C-terminus. Using restriction digest, the gel purified hIFRD1 PCR product was inserted and ligated into pcDNA3.1-FLAG plasmid. Subsequently, the hIFRD1-FLAG plasmid was transformed into E.coli for antibiotic selection and sequencing. Successfully cloned hIFRD1-FLAG plasmid was used in all subsequent experiments for verification of interaction via co-immunoprecipitation. A similar approach was also utilized to clone hAHCYL1 cDNA into pcDNA3.1-FLAG to create hAHCYL1-FLAG plasmid. For co-immunoprecipitation, we decided to overexpress mNLRC3 instead of hNLRC3. mNLRC3 is up to 78% similar to hNLRC3 and the majority of the sequence differences lie within the N-terminal region. The hNLRC3 tagged with GFP did not properly express NLRC3 in cytoplasm and instead, hNLRC3-GFP was aggregated in the golgi, which is a common artifact of GFP-tagged proteins. Therefore, we relied on mNLRC3, which we have already cloned into pcDNA3.1-HA vector and evaluated its expression. Nonetheless, hNLRC3 will be cloned and ligated into a pcDNA3.1 tagged vector to re-verify the Y2H interactions.

Co-immunoprecipitation experiments were then conducted following over-expression of mNLRC3-HA and hIFRD1-FLAG in HEK293T cells. HEK293T cells were transfected with mNLRC3-HA alone, hIFRD1-FLAG alone or both mNLRC3-HA/hIFRD1-FLAG. Using anti-FLAG antibody for immunoprecipitation, NLRC3-HA protein, after immunoprecipitation, was only detected in mNLRC3-HA/hIFRD1-FLAG co-transfected HEK293T cells (Figure 14a). Successful immunoprecipitation of hIFRD1 protein was verified by detectable hIFRD1-FLAG expression in lysates from both hIFRD1-FLAG alone and
Figure 12. Y2H identified regions of interaction in IFRD1 protein.
mNLRC3-HA/hIFRD1-FLAG co-transfected HEK293T cells after immunoprecipitation. Together, these data showed that NLRC3 interacts with IFRD1 in vitro. In contrast, when immunoprecipitation was conducted in the reverse order, using anti-HA antibody, hIFRD1-FLAG was not detected in mNLRC3-HA/hIFRD1-FLAG co-transfected lysate after immunoprecipitating mNLRC3-HA (Figure 14b). It is possible that immunoprecipitation with anti-HA blocks blocked the c-terminus, which is required for IFRD1 to interact. Since IFRD1 interacts with both the n-terminus and c-terminus of NLRC3, anti-HA antibody binding could block a c-terminal epitope and potentially prevented the interaction. Thus, we speculate that since IFRD1 utilizes its central region to interact with NLRC3, such that immunoprecipitation with anti-FLAG antibody at the C-terminal of IFRD1 does not prevent the interaction.

![IFRD1 mRNA expression](image)

**Figure 13.** IFRD1 mRNA expression in isolated MLN cells from wild type or NLRC3 deficient mice following treatment with rapamycin (ug/mL) or amino acid starvation (KRB) buffer for time as indicated. Conducted by Vinicius Motta, Post Doc.

The second target of interest was AHCYL1. We wanted to verify the interaction between AHCYL1 and NLRC3 to highlight the potential link between intracellular calcium levels and NLRC3. Using a similar setup as above, HEK293T cells were transfected with mNLRC3-HA alone, hAHCYL1-FLAG alone or both mNLRC3-HA/hAHCYL1-FLAG. First,
immunoprecipitation using anti-FLAG antibody was not successful since hAHCYL1 expression was only detectable in pre-lysates of HEK cells overexpressing hAHCYL1-FLAG and mNLRC-HA was not detected after immunoprecipitation (Figure 14c). In contrast, anti-HA successfully immunoprecipitated mNLRC3-HA but subsequent verification of the interaction using anti-FLAG antibody did not reveal any interacting hAHCYL1-FLAG protein (Figure 14d). Therefore, under these conditions and in HEK293T cells, NLRC3 does not interact with AHCYL1 but further optimization of the immunoprepitation experiment may be required to confirm the absence of the interaction between AHCYL1 and NLRC3.
Figure 14. NLRC3 interacted with Y2H identified protein target, IFRD1, but not AHCYL1 in HEK293T cells. a) anti-FLAG or b) anti-HA was used to immunoprecipitate hIFRD1-FLAG or mNLRC3-HA, respectively, prior to western blot analysis. c) anti-FLAG or d) anti-HA was used to immunoprecipitate hAHCYL1-FLAG or mNLRC3-HA, respectively, prior to western blot analysis. All experiments were repeated at least 2 times independently.
Discussion

While most members of the NLR family function as intracellular sensors of danger signals or pathogen infection, a few members, namely NLRX1, CIITA and NLRC5, possess the ability to regulate the adaptive immune response by modulating apoptosis (NLRX1) or acting as transcriptional regulators (Masternak, Muhlethaler-Mottet et al. 2000; Meissner, Li et al. 2010; Soares, Tattoli et al. 2014). My goal in this project was to study NLRC3, which belongs to the non-canonical NLR sub-family based on its phylogenetic similarity to these other NLRs (Benko, Magalhaes et al. 2010, Meissner, Li et al. 2010). We postulated that NLRC3 is perhaps more of a regulatory protein in T cells than a conventional PRR.

Studies to date have highlighted macrophage-specific NLRC3 as a potential negative regulator of inflammation via interaction with the TLR4-TRAF6 axis in the LPS stimulated signaling pathway and STING in the DNA sensing pathway (Schneider, Zimmermann et al. 2012; Zhang, Mo et al. 2014). However, the initial report on NLRC3 demonstrated high expression of this gene in lymphocytes, especially T cells, as well as its rapid mRNA down-regulation following stimulation of the TCR pathway (Conti, Davis et al. 2005). Based on these findings, NLRC3 was suggested to be a negative regulatory factor, whose function is to repress activation of its target genes. Following cell activation and down-regulation of NLRC3, the influence of these repressed genes on cell function could be revealed. In this context, a normal cell in resting conditions expresses NLRC3 but following stimulation, NLRC3 mRNA and protein are down-regulated to release NLRC3 regulated pathways from repression. The mechanism of NLRC3 as a negative regulator is currently undetermined, but the high level of NLRC3 expression in lymphocytes, especially in T cells (Conti, Davis et al. 2005), supports a potential role for this protein in regulating adaptive immune responses. In addition, NLRC3 might be an important regulator of memory T cells, which remain in a resting state but are rapidly activated upon stimulation with a specific antigen. Therefore, my interest was to decipher the function of NLRC3 and its potential mechanism of regulation in T cells.
My first goal was to characterize basic properties of NLRC3 in detail by generating an antibody against NLRC3, since none were available commercially. A short C-terminal peptide from human NLRC3 was used by Cedarlane in immunizations to generate rabbit anti-NLRC3 antibody. Generating an antibody targeting this unique region had the benefit of specifically detecting NLRC3 but not other NLRs. In addition, it may detect NLRC3 isoforms potentially expressed in our NLRC3 gene knockout in mice, which are targeted within the central NACHT domain of NLRC3. First, the ability of the generated anti-NLRC3 to recognize NLRC3 was investigated in various immune tissues, including sites containing large number of T cells, and other lymphocytes populations. The antibody was able to detect a band at ~110kDa, which is similar to the predicted size of NLRC3 protein. Successful detection was further confirmed when the same ~110kDa band was observed in the sorted T cell population. Interestingly, NLRC3 protein was also detected in the sorted NK cell population, but this is not surprising since NK cells were another lymphocyte population that was identified to highly express NLRC3 mRNA in the previous microarray analysis (Conti, Davis et al. 2005). However, sorting of the NK cells relied on NK1.1 antibody and NK1.1 is also known to be expressed by NKT cells (Sykes 1990; Arase, Arase et al. 1993). Therefore, whether NLRC3 protein is expressed by NK cell or NKT cells populations remains unknown, but it would be an interesting aspect to investigate with regards to lymphocytes possessing genes associated with both the innate and adaptive immune system.

The generated anti-NLRC3 antibody was effective in recognizing NLRC3 protein, but large amount of cellular lysates were required. To address this issue, we decided to use concanavalin-A/IL-2 to expand T cell ex vivo from the spleen. Concanavalin-A (con-A) is a lectin-binding molecule that can trigger T cell activation without antigen binding by aggregating components of the TCR (Quintans, Yokoyama et al. 1989). The ability of the antibody to recognize NLRC3 in ex vivo expanded T cell was re-assessed using these T cell derived from wild type and NLRC3-deficient splenocytes. Again, an NLRC3 protein band was observed only in the wild type expanded T cells, indicating that the antibody properly detected NLRC3 protein and NLRC3 deficient mice did not express NLRC3. In addition, it
also indicated that the expression of NLRC3 recovers at a certain time point despite the fact that the TCR was stimulated in these cells. Finally, the stable expression of NLRC3 in these expanded T cells allows them to be used as an alternative model to characterize the function of NLRC3.

The down-regulation of NLRC3 mRNA post TCR stimulation was one of the key observations suggesting NLRC3 as a potential negative regulator (Conti, Davis et al. 2005). We examined the relationship between NLRC3 mRNA down-regulation and NLRC3 protein expression in primary and ex vivo expanded T cells. It was found that while mRNA for NLRC3 disappeared by 3 hours post anti-CD3/CD28 treatment, protein expression was completely down-regulated by 24 hours post stimulation. This pattern of delayed down-regulation is likely due to the fact that NLRC3 mRNA was inhibited by TCR stimulation, which led to the complete degradation of the protein due to its normal half-life within the cell, rather than an activation-induced degradation process. To investigate this possibility, further experiments will examine NLRC3 down-regulation in the presence of cycloheximide, which inhibits translation and block synthesis of new proteins. Such an experiment will reveal the half-life kinetics of NLRC3, which will assist in determining if the timing is similar to what we see upon TCR stimulation.

The delayed kinetics of protein down-regulation was also observed in the expanded T cells. Interestingly, these cells are not naïve since they had been activated by con-A, but they have returned to a resting state after the removal of con-A from solution and NLRC3 protein expression was restored. Together, these observations strengthen the suggestion that NLRC3 is a negative regulator of T cell function and further encourages the study of NLRC3 in different T cell populations, for example naive vs. memory T cells.

The inhibition of IL-2 mRNA production in NLRC3-over-expressing Jurkat cells, which was reported in the first publication of this protein, is another major observation suggesting NLRC3 as a negative regulator (Conti, Davis et al. 2005). Since IL-2 is an important cytokine for T cell proliferation, and differentiation (Morgan, Ruscetti et al. 1976; Sakaguchi et al. 1995; Thornton, Donovan et al. 2004), the potential ability of NLRC3
to inhibit IL-2 is strong evidence supporting NLRC3 as a major regulator of T cell function and differentiation. However, in my experiments, NLRC3 deficiency did not enhance IL-2 cytokine production. It is possible that the ability for NLRC3 to inhibit IL-2 is only activated under condition in which NLRC3 is highly expressed. In this scenario, basal expression of NLRC3 is not sufficient to induce the inhibition of IL-2 and removal of NLRC3 protein has no effect. However, an enhanced NLRC3 expression under yet unidentified circumstances might regulate IL-2 production. Another possibility is that the reported IL-2 inhibition is a side-effect generated by overexpression of the plasmid rather than NLRC3 itself. Our group has previously demonstrated that overexpression of NLRC3 can lead to nonspecific post transcriptional inhibition of reporter genes (Ling, Soares et al. 2012). These possibilities remain to be determined and need further clarification.

The regulation of NLRC3 expression is tightly linked with TCR signaling. Our interest was to determine whether NLRC3 deficiency will compromise signaling downstream of TCR activation. To examine this idea, we investigated MAP kinase signaling as phosphorylation of these kinases is critically required for the activation of various genes and production of cytokines, including IL-2 (Dong, Davis et al. 2002). ERK, JNK and p38 are three MAP kinases that are commonly investigated in relation to TCR signaling. We analyzed the phosphorylation of these proteins to determine whether NLRC3 regulates the activation of these MAP kinases. No difference in kinetic or level of phosphorylation was observed in any of the three MAP kinases when we compared wild-type to NLRC3-deficient T cells. However, it is possible that MAP kinases are already activated prior to stimulation, likely by IL-2 in the complete media since con-A has already been removed from the media and cells were maintained in media with only IL-2 (Perkins, Marvel et al. 1993; Crawley, Rawlinson et al. 1997). This pre-activation could have masked the ability of NLRC3 to regulate MAP kinases because IL-2 potently induced MAP kinases phosphorylation. Therefore, NLRC3 might regulate MAP kinase phosphorylation, but only an early stage of MAP kinase activation or when MAP kinases are not activated by a strong stimulation. Nonetheless, these studies need to be repeated in primary isolated naïve T
cells instead of expanded T cells, to confirm that NLRC3 does not regulate proximal activation of T cells through the TCR.

The downstream signaling pathway after TCR activation can be separated into two pathways activated by either DAG or IP$_3$. DAG pathway leads to PKC$\theta$ activation, while IP$_3$ induces intracellular calcium influx from the ER and extracellular environment via binding to IP$_3$ receptor (Streb, Irvine et al. 1983; Nishizuka 1992). Intracellular calcium is a vital second messenger that modulates T cell metabolism and proliferation. Calcium signaling regulates T cell metabolism via the AMPK gene (Tamas, Hawley et al. 2006). In addition, intracellular calcium is essential for T cell activation by up regulating key transcriptional factors such as NFAT, NF-kB and AP-1 (Feske, Giltnane et al. 2001). We tested each of these pathways independently using Jurkat T cells. First, Jurkat T cell exhibited the same down-regulation of NLRC3 protein when treated with PMA/ionomycin. This is critical because PMA/ionomycin can simulate the last stage of TCR signaling and bypasses multiple components of TCR signaling pathway. Successful down-regulation of NLRC3 upon stimulation confirmed Jurkat T cells as a valid in vitro model. Next, we evaluated whether DAG or IP$_3$ pathway activation is sufficient to trigger down-regulation of NLRC3 protein. Interestingly, NLRC3 protein was down-regulated when Jurkat T cell were only stimulated with ionomycin. Ionomycin is a calcium ionophore that triggers the release of intracellular calcium influx, and thereby mimicking IP$_3$ activation (Liu and Hermann 1978). At the same time, NLRC3 down-regulation by intracellular calcium influx was also observed using thapsigargin, a drug which inhibits the ER calcium pump from transporting the calcium back into ER store (Thastrup, Cullen et al. 1990). This supports the idea that intracellular calcium influx downstream of TCR signaling triggers NLRC3 down-regulation, and suggests a relationship between NLRC3 and calcium related pathways.

NLRC3 was also not required for CamKII, a protein regulated by intracellular calcium (Yamauchi and Fujisawa 1981), activation downstream of TCR stimulation. Therefore, based on ionomycin as a trigger of NLRC3 down-regulation, we examined a potential role of NLRC3 in T cell by regulating CamKII phosphorylation. In expanded T cells, calcium signaling pathway was not regulated by NLRC3 since the kinetics and level of
phosphorylation of CamKII was the same between wild type and NLRC3 deficient T cells. The phosphorylation of CamKII followed normal kinetics in which it peaked after 15 minutes of stimulation regardless of NLRC3 deficiency. However, the relationship between NLRC3 and calcium signaling might become more apparent in naïve T cells, where the initial activation of the TCR and non-continuous exposure IL-2 forces T cells to rely on more specific signaling pathways to modulate metabolism and activation.

Thapsigargin has also been reported as a trigger of ER stress (Wong, Brostrom et al. 1993). This encouraged us to investigate cellular stresses as triggers of NLRC3 down-regulation. We first focused on amino acid starvation. Indeed, it was shown that levels of amino acids inside the cell affect the differentiation of T cells (Sundrud, Koralov et al. 2009). Therefore, amino acid starvation and mTOR inhibition was induced via KRB buffer and rapamycin, respectively, to investigate the effect of nutrient stress on NLRC3 expression. KRB buffer only induced a modest NLRC3 down-regulation, compared to rapamycin. This difference is likely attributed to the different targets of each treatment. KRB buffer activates the amino acid starvation response affecting both GCN2 expression and mTOR inhibition (Tattoli, Sorbara et al. 2012). Amino acid starvation likely triggers a balance between increasing GCN2 activation and mTOR inhibition. In contrast, rapamycin specifically targets mTOR and does not affect GCN2 expression. In addition, it is possible that despite incubating T cells in KRB buffer, a limited amount of amino acid is still available in the cytoplasm of the cells. Therefore, amino acid starvation pathway is not immediately activated and thus NLRC3 downregulation is delayed. Together, these results further emphasize a role of NLRC3 in T cell metabolism. It would be interesting to investigate if other nutrient stresses, such as glucose deprivation, also induce the downregulation of NLRC3. If NLRC3 downregulation is found to be trigged by different types of nutrient stress, this would support the idea that NLRC3 can regulate T cell function as a consequence of changes in cellular metabolism.

A number of recent studies have shifted to investigate the role of NLRC3 in macrophages. In these reports, NLRC3 was suggested to function as a negative regulator of inflammation through interaction with TRAF6 within the TLR4 signaling pathway and
through STING in the intracellular DNA sensing pathway (Schneider, Zimmermann et al. 2012; Zhang, Mo et al. 2014). The observation that NLRC3 has a function in macrophages was surprising since NLRC3 expression was shown to be extremely low in myeloid cells (Conti, Davis et al. 2005). We therefore determined NLRC3 protein expression in BMDMs, which had not been shown in these studies; levels of NLRC3 were only determined by qPCR in these published works. In our hands, we were unable detect NLRC3 protein and mRNA expression in BMDMs. Assuming that NLRC3 expression in macrophages was possibly beyond the level of detection with our anti-NLRC3 antibody, we investigated whether NLRC3 deficiency could impact LPS signaling in BMDMs. We investigated inflammatory cytokines production in wild-type and NLRC3-deficient BMDMs following LPS stimulation. We stimulated BMDM with various dosages of LPS, different kinetics, and in the presence or absence of IFNγ, which pre-activates macrophages. In contrast to the reported findings, we did not detect any increase in proinflammatory cytokine production in NLRC3 deficient BMDMs under any conditions. The only visible trend was a slight decrease in inflammatory cytokine expression in NLRC3 deficient BMDMs, which is contrary to the reported findings (Schneider, Zimmermann et al. 2012). It is possible that the absence of inhibition by NLRC3 is attributed to the inherent differences between peritoneal macrophages, which were used in the published report (Schneider, Zimmermann et al. 2012), and BMDMs used in our studies. Alternatively, the use of non-littermate controls by the group that published these papers might have also influenced their findings (Schneider, Zimmermann et al. 2012). In contrast, all our experiments were performed on BMDMs derived from littermate controls to minimize effects of the gut microbiota. Future studies will investigate whether these different variables are possible causes for these discrepancies.

From the two independent yeast two hybrid screens, we identified a number of targets with high confident scores. To first analyze the Y2H results, we searched protein databases and PubMed for the function of each potential target, then compared these functions to look for common patterns within the list of targets. A surprising finding was the discovery of multiple components of the nuclear import machinery that were found
to interact with the N-terminal fragment of NLRC3. The three targets include KPNB1 (importin-β), RanBP5 (importin-5) and TNPO3 (transportin-3). Although, each of these potential targets belong to the nuclear import machinery, they do not form a single complex. KPNB1 is a critical component of the nuclear import complex that is essential to transport protein into the nucleus by binding the nuclear pore complex (Moroianu, Hijikata et al. 1995). RanBP5 also participates in the nuclear import machinery and it is highly homologous to KPNB1 (Deane, Schafer et al. 1997). Both KPNB1 and RanBP5 have been shown to interact with RanGTP and nuclear pore complex (Jakel and Gorlich 1998). In contrast, TNPO3 is a nuclear import receptor for serine/arginine rich proteins, which are typically factors facilitating mRNA splicing factors (Lai, Lin et al. 2001). Together, the identification these proteins provides potential new insight into the function of NLRC3. Indeed, NLRC3 could interact with these nuclear import factors to regulate the translocation of different proteins involved in cell cycle regulation, mitosis and transcription/translation. By regulating the localization of these protein, NLRC3 may indirectly influence the differentiation and activation of T cells. Alternatively, these hits might simply be explained by the fact that NLRC3 can be translocated between the cytosol and the nucleus, and interact with these nuclear pore complex proteins transiently during translocation.

In addition, we identified multiple nuclear-localized proteins that are involved in histone modification, mitosis and translation (Table 3 and Table 4). Together, these patterns underscore a potential role for NLRC3 function in the nucleus. And indeed, both in silico analysis and our cell fractionation analyses supported the localization of NLRC3 in the nucleus. PSORTII, a program that calculates probabilities of subcellular localization of proteins, predicted a potential nuclear localization for NLRC3 and identified a four amino acid nuclear localization sequence. Currently, there are three types of nuclear localization signals known to be found in different proteins. Two types are classical NLS found in SV40 large T antigen that possess either 3/4 basic amino acid residue pattern or 7 amino acid residue pattern (Kalderon, Richardson et al. 1984). Another type is the bipartite NLS found in Xenopus nucleoplasmin and it is composed of 2 regions of basic residues separated by
a 10 residue spacer (Lanford and Butel 1984). The NLRC3 NLS follows the classic rule of 3 basic amino acid residues with a histidine in between. However, the related NLR, CIITA, have multiple NLS signals (Masternak, Muhlethaler-Mottet et al. 2000; Raval, Weissman et al. 2003); it is unsure whether the four pattern NLS found in NLRC3 would be sufficient for NLRC3 to translocate into the nucleus in T cells.

Therefore, to investigate the localization of NLRC3, we conducted subcellular fractionation using Jurkat T cells. We were able to detect NLRC3 protein in both the cytoplasmic and the nuclear fraction of Jurkat T cells. Potential contamination and incomplete fractionation was addressed using a variety of protein markers as controls. Indeed, the cytoplasmic fraction contains organelles such as Golgi and lysosome, but the nuclear fraction only contained nuclear materials. Despite the fact that previous studies identified NLRC3 as a cytoplasmic protein, these conclusions were based on over-expression experiments that could have prevented the proper localization of NLRC3 into the nucleus (Conti, Davis et al. 2005). Moreover, the cytoplasmic localization of over-expressed NLRC3 was observed in fibroblasts which do not express NLRC3 (Conti, Davis et al. 2005). It is possible that NLRC3 expression in cells other than T cells, could lead to mis-localization due to the fact that T cell specific proteins could be required for its proper localization in the nucleus and these factors are not expressed by that particular cell type. Further validation of NLRC3 sub-cellular localization could be performed using immunofluorescence microscopy of isolated T cells.

Shuttling between the cytoplasm and nucleus is a property of many transcriptional regulators. However, we did not observe a change in localization of NLRC3 following stimulation with thapsigargin or PMA/ionomycin. Based on this observation, NLRC3 is present in the cytoplasm and nucleus throughout the entire activation of TCR signaling. The absence of visible translocation could also be due to a balance between import and export into and out of the nucleus. Therefore, further analysis is required using nuclear export inhibition drug, such as leptomycin B, which was previously used to identify the nuclear localization of NLRC5 (Benko, Magalhaes et al. 2010).
The Y2H screens identified potential partners that NLRC3 may interact with to elicit its specific function. If the protein-protein interaction proves to be true, then it will assist in identifying the mechanism or pathway that NLRC3 protein operates to regulate T cell function and differentiation. Therefore, we employed co-immunoprecipitation to verify the interaction between NLRC3 and its potential interacting targets. The first target to be verified was IFRD1. IFRD1 is reported to be expressed in muscle and epithelial cells, as a regulator of regenerative pathways (Micheli, Leonardi et al. 2005, Dieplinger, Schiefermeier et al. 2007, Micheli, Leonardi et al. 2011). IFRD1 is also expressed in T cells due to the fact that we used a T cell specific library for the Y2H screen. The mechanism through which IFRD1 regulates transcription relies on its ability to interact with histone modifying proteins. In one case, IFRD1 was reported to inhibit NF-κB activation through interaction with HDAC3 in myoblasts (Micheli, Leonardi et al. 2011). IFRD1 was also reported to interact with MEF2C to prevent MEF2C binding to HDAC4 and induce inhibition of skeletal myogenesis (Micheli, Leonardi et al. 2005). Finally, IFRD1 was found to interact with the mammalian SIN3 complex, which consists of histone deacetylases and other proteins, which act to repress specific genes in epithelial cells (Vietor, Vadivelu et al. 2002). It is possible that NLRC3 might contribute to histone modifications in T cells through the ability to interact with IFRD1, thereby potentially regulating genes involved in T cell differentiation and function.

IFRD1 was identified in both the C-terminal and N-terminal NLRC3 Y2H screen, utilizing an overlapping region to interact with both fragments. This supports the I-TASSER in silico prediction of NLRC3 protein structure in which the N-terminus is in close proximity of the C-terminus. Therefore, the overlapping region of IFRD1 may interact with both the C-terminal and N-terminal of NLRC3. The NLRC3 IFRD1 protein-protein interaction was verified in HEK293T cells overexpression with both mNLRC3-HA and hIFRD1-FLAG. With a 79% similarity in amino acid sequence between mNLRC3 and hNLRC3, mNLRC3-HA interacted with hIFRD1-FLAG, which suggests that hNLRC3 and mNLRC3 are likely functional homologues. The double bands that are observed when mNLRC3 was overexpressed is possibly an experimental artifact due to the overexpression system.
Overexpressed mNLRC3 could be processed, modified or expressed utilizing an alternative downstream start codon that is in frame. However, the exact cause that resulted in the expression of two mNLRC3 proteins with different molecular weights is currently unclear. The interaction between mNLRC3-HA and hIFRD1-FLAG was verifiable by utilizing anti-FLAG for immunoprecipitation and anti-HA for western blot, but not vice versa. This issue is likely attributed to the anti-HA antibody being incompatible with immunoprecipitation in the interaction between mNLRC3-FLAG and hIFRD1-FLAG. It is possible that immunoprecipitation with anti-HA blocks the site that is required for IFRD1 to interact. Nonetheless, further analysis will be conducted by overexpressing hNLRC3 instead of mNLRC3.

The second potential target that was verified is AHCYL1. AHCYL1 is an interesting target since AHCYL1 competitively inhibits IP$_3$R after IP$_3$ triggers the release of calcium into the cytoplasm (Ando, Mizutani et al. 2003, Ando, Mizutani et al. 2006). To date, the majority of reports focus on the function of AHCYL1 as a regulator of ion exchange transporters and ion channels, but the function of AHCYL1 in T cells is unclear. Nonetheless, since calcium signaling triggers NLRC3 down-regulation, the interaction between AHCYL1 and NLRC3 is worth investigating. Unfortunately, we were not able to verify the interaction between mNLRC3-HA and hAHCYL1-FLAG utilizing the same setup as above for verifying IFRD1 interaction. Utilizing anti-HA antibody for immunoprecipitation, AHCYL1-FLAG failed to interact with mNLRC3-HA. Again, the anti-HA antibody might be incompatible with immunoprecipitation in this interaction, but it is also possible that AHCYL1 does not interact with mNLRC3 in HEK293 cell because of a specific factor from T cells required for the interaction to occur. Moreover, experiments utilizing an anti-FLAG antibody to immunoprecipitate did not reveal a clear observation. Immunoprecipitation was not efficient since hAHCYL1-FLAG was not concentrated in contrast to hIFRD1-FLAG. This could be due to a non-optimal lysis environment or improper expression of AHCYL1-FLAG in HEK293 cells prior to immunoprecipitation. Nonetheless, the Y2H identified an interaction between NLRC3 and AHCYL1 with high confidence, but a non T-cell might not possess T cell-specific factors that are required for
the interaction to occur. Therefore, verifying the interaction between NLRC3 and AHCYL1 might also require further optimization or utilizing Jurkat cell as an alternative.

All together, the characterization process have revealed several interesting properties of NLRC3. NLRC3 mRNA rapidly decreases following stimulation, but NLRC3 protein down-regulation occurs at a delayed kinetic. NLRC3 does not regulate proximal TCR signaling and calcium dependent pathway following TCR stimulation. However, NLRC3 down-regulation can be triggered by intracellular calcium influx, induced after IP3 pathway activation, and mTOR inhibition. Finally, NLRC3 was found not only in the cytoplasm, but also in the nucleus, contrary to previous reported observations (Conti, Davis et al. 2005). On the other hand, verification of Y2H candidates through co-immunoprecipitation have confirmed the interaction between NLRC3 and IFRD1. Based on these observation, a model could proposed for NLRC3 (Figure 15a and Figure 15b): During resting condition, NLRC3 interacts with IFRD1, to regulate the function of IFRD1 and possibly, epigenetic modification. However, the activation of the TCR or amino acid starvation responses leads to intracellular calcium influx or mTOR inhibition, respectively. This triggers the down regulation of NLRC3 mRNA followed by protein. In absence of NLRC3, IFRD1 could activate its alternative function, which was prevented by NLRC3 interaction, and to induce a changes in T cell function. Therefore NLRC3 might contribute to histone modifications in T cells through the ability to interact with IFRD1, thereby potentially regulating genes involved in T cell differentiation and function.
Figure 15. Proposed model for NLRC3: a) During resting condition, NLRC3 can interact with IFRD1 and regulate the function of IFRD1. b) After TCR activation or in stress conditions, NLRC3 is downregulated, which allows IFRD1 to activate its alternative function that was tightly regulated when NLRC3 was present.
Future Directions

At this stage of the project, a major focus in the future will be attempting to verify each of the interesting Y2H targets through co-immunoprecipitation, especially with human NLRC3. The identified targets have opened up numerous avenues for us to explore with respect to the function of NLRC3. These include cellular metabolism, regulation of gene expression, cell cycle regulation and cell division. Understanding how NLRC3 is implicated in these mechanisms will give us new insight into the regulation of immunity.

In addition, continued work will be conducted to further investigate the interaction between NLRC3 and IFRD1 in detail. Since IFRD1 has been shown to interact with multiple histone modification proteins, such as HDAC3 and HDAC1 (Vietor, Vadivelu et al. 2002; Micheli, Leonardi et al. 2011), we hypothesize that NLRC3 utilizes this interaction to regulate T cell gene expression at the epigenetic level. This may include regulating the differentiation of naïve T cells into other T cell subtypes; indeed, recent preliminary data suggest a potential link between NLRC3 and differentiation of T cells into regulatory T cells. Therefore, initial experiments will investigate histone modification status of various histone proteins between wild type and NLRC3 deficient T cell at a global level. Global histone analysis can be performed by comparing the methylation and acetylation of specific histone proteins between wild type and NLRC3-deficient T cell using western blot. Wild type and NLRC3 deficient naïve T cell can also be stimulated through the TCR to evaluate the kinetic of histone modification following activation. Follow-up experiments will examine histone modification at specific loci, such as FOXP3, which is the transcriptional regulator of T regulatory cell differentiation (Hori, Nomura et al. 2003), as well as at the promoter of proinflammatory cytokine genes, like IL-6 and IL-1. Since the regulation of transcription factors frequently depends on histone modification and previous findings showing elevated proinflammatory cytokines in the absence of NLRC3, these loci may be regulated by NLRC3 indirectly due to its ability to interact with IFRD1, which has the potential to induce epigenetic changes.
In vivo experiments involving specific T cell subtypes or lymphocytes would be another interesting perspective to investigate NLRC3 function. For example, a good experimental model to study effector and memory T responses during acute and persistent viral infection is the lymphocytic choriomeningitis virus (LCMV) infection model. The Armstrong variant of LCMV is used to investigate acute antiviral responses by CD8+ T cells while the chronic clone 13 variant is used to investigate the dependence on CD4+ T helper cell response (Wherry, Blattman et al. 2003). The flexibility of the two LCMV variants enables us to investigate both the functional difference of NLRC3 between CD4+ T cell and CD8+ T cell. It's possible that NLRC3 regulates different targets between CD4+ and CD8+ T cells since their role in the immune system is very different or NLRC3 is dispensable in one of the T cell subtypes but not the other. In addition, we can investigate whether NLRC3 is required to protect against acute or chronic viral infection. LCMV infected mice would also possess LCMV specific memory T cell. The function of NLRC3 in memory T cells could be investigated with mice that recovered from LCMV infection. In addition, we have generated NLRC3 conditional knockout mice using LCK-CRE crossed with NLRC3-floxed mice. This specifically removes NLRC3 in LCK expressing lymphocytes, which are T cells. NLRC3 conditional knockout mice could address whether NLRC3 is only involved in regulating T cell function, or could other immune cell types, such as macrophages, express NLRC3 and utilizes it to also regulate an adaptive response.

NK cells are another cell type that highly expresses NLRC3 mRNA, but whether it is NK cell, NKT cell or both that expresses NLRC3 remains to be defined. This can be easily examined by separating NK cells and NK T cells from NK1.1 expressing cells, follow by qPCR analysis of NLRC3 mRNA in these different populations. The potential of NLRC3 expression in NK T cell is interesting to investigate since NK T cell possess both properties of NK cell and T cell (Sykes 1990; Arase, Arase et al. 1993), and therefore, could possess a combination of cellular machinery from both NK cell and T cell components. In addition, NK T cells are a major producers of cytokines such as IFNγ (Matsuda, Gapin et al. 2003; Stetson, Mohrs et al. 2003). Since NLRC3 has been suggested as a negative regulator of
inflammation in macrophages, NLRC3 could also be a potential regulator of NK T cell cytokine production.

NLRC3 is a newly identified NLR that is highly expressed in T lymphocytes. NLRC3, from both observations made in our laboratory and by other groups, has been shown to be related to T cell function and differentiation. The exact mechanism of action is still unclear but preliminary results have suggested it acts as a negative regulator of T cell function. Up to date, NLRC3 is the only NLR that has been found to be specifically expressed in T cells and few other lymphocyte populations. This clearly supports NLRC3 as an important player in T cell biology. Since we could not verify expression and the ability for NLRC3 to negatively regulate proinflammatory cytokine production in macrophages, future progress will continue to evaluate the function of NLRC3 in T cells, but with much greater detail, especially utilizing various in vivo animal models, such as c. rodentium infection, and in vitro biochemical techniques. In addition, NLRC3 conditional knockout mice have been recently generated by our lab, which will be used to investigate, specifically in T cells, the outcome of NLRC3 deficiency. Lastly, study of NLRC3 will contribute greatly towards furthering our understanding of how the innate and adaptive immune systems interact to regulate the immune response.
References


Ando, H., et al. (2003). "IRBIT, a novel inositol 1,4,5-trisphosphate (IP3) receptor-binding protein, is released from the IP3 receptor upon IP3 binding to the receptor." J Biol Chem 278(12): 10602-10612.


Zhang, L., et al. (2014). "NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING." Immunity 40(3): 329-341.