UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cardif (MAVS) regulates the maturation of Natural Killer Cells and $CD4^+$ T Helper Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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Chair

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DEDICATION

This dissertation is dedicated to my mom, family, and friends who have continually supported me throughout my life in all of my endeavors.

By the grace of God, I am what I am.

EPIGRAPH

If any of you lacks wisdom, let him ask God, who gives generously to all without reproach, and it will be given him. But let him ask in faith, with no doubting, for the one who doubts is like the wave of the sea that is driven and tossed by the wind.

James 1:5-6

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LIST OF ABBREVIATIONS AND SYMBOLS

Cardif	CARD adaptor inducing interferon-β
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic Cell
IFNγ	Interferon Gamma
IPS-1	IFN-β promoter stimulator-1
KLRG1	Killer cell lectin-like receptor subfamily G member 1
MAVS	Mitochondrial antiviral signaling
MCMV	Mouse cytomegalovirus
MDA5	melanoma differentiation associated factor 5
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
NLRP3	NOD-like receptor family, pyrin domain containing 3
RIG-I	Retinoic acid inducible gene-1
STAT	Signal Transducers and Activators of Transcription
T_{eff}	T effector cell
T_{reg}	T regulatory cell
VISA	Virus-induced signaling adaptor

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ABSTRACT OF THE DISSERTATION

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Professor Jeffrey Esko, Chair

Cardif, also known as IPS-1, VISA and, MAVS, is an intracellular adaptor protein that functions downstream of the RIG-I family of pattern recognition receptors. Cardif is required for the production of type I-IFNs and other inflammatory cytokines after RIG-I like receptors recognize intracellular

antigenic RNA. Studies have recently shown that Cardif may have other roles in the immune system in addition to its role in viral immunity. In this study, we find that the absence of Cardif alters normal natural killer cell development and maturation. Cardif$^{-/-}$ mice have a 35% loss of mature CD27^{-}CD11b^{+} NK cells in the periphery. Additionally, Cardif$^{-/-}$ NK cells have altered surface marker expression, lower cytotoxicity, decreased intracellular STAT1 levels, increased apoptosis and decreased proliferation compared to wild-type NK cells. Mixed chimeric mice revealed that the defective maturation and increased apoptotic rate of peripheral Cardif-/- NK cells is cell-intrinsic. Surprisingly, Cardif$^{-/-}$ mice showed enhanced control of mouse cytomegalovirus (MCMV, a DNA b-herpesvirus), commensurate with increased activation and IFNγ production by these immature NK cell subsets. In addition, Cardif$^{-/-}$ mice have an increased T_{eff}:T_{reg} ratio and increased production of IFNγ by CD8^{+} T cells. These results indicate that the skewed differentiation and altered STAT expression of Cardif$^{-/-}$ NK cells can result in their hyper-responsiveness in some settings, and support recent findings that Cardif-dependent signaling can regulate aspects of immune cell development and/or function distinct from its well characterized role in mediating cell-intrinsic defense to RNA viruses.

Chapter 1

Introduction to Cardif

Introduction

Pathogens that invade the body have specific differences in the structures of their macromolecules that make them identifiable to the immune system. These differences in macromolecular structures are known as pathogen associated molecular patterns (PAMPs) and are recognized by pattern recognition receptors (PRRs) found within host immune and non-immune cells (1, 2). Retinoic acid inducible gene-1 (RIG-I)-like receptors (RLRs) are a subset of PRRs that are DExD/H box RNA helicases and recognize viral RNA found within the cytoplasm of cells (3). There are three members of the RLR family, however only two members of the RLR family, RIG-I and MDA5 (melanoma differentiation associated factor 5), have caspase recruitment and activation domains (CARDs) that allow for downstream signaling after activation. These RLRs induce the production of type-I IFNs and NFκB regulated genes after engaging viral RNA (4). The adaptor protein that interacts with RIG-I and MDA5 and allows downstream signaling was discovered by four different groups and is thus known by four names: Cardif (CARD adaptor inducing interferon-β), MAVS (mitochondrial antiviral signaling), IPS-1 (IFN-β promoter stimulator-1), and VISA (virus-induced signaling adaptor) (5–8). We will refer to this protein as Cardif.

Cardif is a ubiquitously expressed protein that is located on the outer membranes of both mitochondria and peroxisomes of immune and non-

immune cells (4, 9, 10). The mitochondrial localization of Cardif is essential to its function in viral signaling. Cardif has 3 main domains: a transmembrane domain, a linker region, and a CARD domain (11). The transmembrane domain connects Cardif to the membranes of mitochondria and peroxisomes, while the linker region of Cardif provides a platform for downstream signaling molecules. The CARD domain is essential for the interaction of RIG-I and MDA5 with Cardif.

Signaling Pathway

RIG-I and MDA5 initiate signaling through CARD-CARD interactions with Cardif. RIG-I binds 5' di- and tri-phosphate, short (<1 kbp), double stranded RNA (dsRNA) (12). RNA from sendai virus, rabies virus, influenza virus, ebola virus, and hepatitis C virus are all detected by RIG-I. MDA5 binds long dsRNA (>1 kbp) that is produced by picornaviruses such as polio (13, 14). Some viruses such as Dengue and West Nile virus are detected by both RIG-I and MDA5 (12).Once RIG-I or MDA5 binds to viral RNA they undergo a conformational change that reveals their CARD domain. Both RIG-I and MDA5 are known to form oligomers with K63-polyubiquitin (11, 15, 16). The K63-polyubiquitination of RLRs is vital to their activation and association with Cardif (1). The CARD domains of the oligomerized RIG-I or MDA5 then interact with the CARD domain of Cardif. Once a Cardif molecule has been engaged by

RIG-I or MDA5, it aggregates with other Cardif molecules forming a polymer on the mitochondria. This prion-like manner of signal propagation via Cardif produces a robust antiviral response to relatively small amounts of viral RNA. The Cardif polymer also forms a scaffold for downstream signaling (17, 18). Cardif interacts with cytoplasmic adaptor molecules TRAF3, TRAF2, and TRAF6 to activate transcription factors NFκB, IRF3, and IRF7 (Fig. 1.1). These transcription factors induce the expression of NFκB target genes, as well as type I IFN to initiate an antiviral state (4, 13).

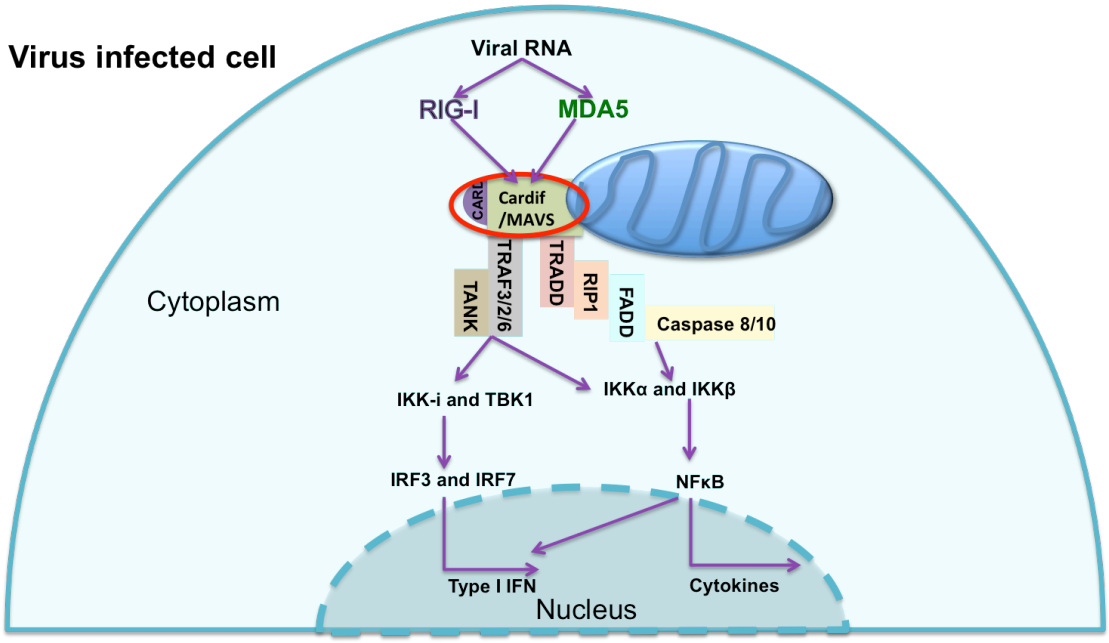

Figure 1.1. Model of the Cardif Pathway. Cardif, located on the mitochondria is activated by RIG-I or MDA5 after they are engaged by and RNA ligand. Cardif then serves as a scaffold for downstream signaling resulting in the production of type I IFN and transcription of NFκB target genes.

Roles outside of viral defense

The RIG-I/MDA-5-Cardif pathway is important for antiviral immunity in response to a number of viruses. This pathway has also been identified as an inducer of apoptosis.

Cardif is vital for signaling in response to viral pathogenic nucleic acids sensed by MDA5 and RIG-1. However, there have been reports that suggest that both RIG-I and Cardif may play roles in immune regulation that are separate from their roles in viral defense (19–23). Wang et al. report that RIG-$I^{-/-}$ mice develop colitis and are more susceptible to dextran sulfate-induced colitis (20). Xu et al. report that $Cardif^{-/-}$ B cells have a cell-intrinsic defect in CD23 and TLR7 expression (21). Additionally, $Cardif^{-/-}$ mice develop more severe disease in the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (24). Cardif is also required for the optimal activation of the NLRP3 inflammasome (22). RIG-I activity under the control of IRF1 has been implicated in the progression of atherosclerosis. Wang et al. propose that 25-hydroxycholesterol induces IL-8 production in macrophages by inducing IRF1 and subsequent RIG-I expression and activation (23). In addition, macrophages found in atherosclerotic plaques express high levels of RIG-I (25).

Just as Cardif has been implicated in controlling autoimmunity in mouse models of autoimmunity, Cardif has been implicated in human autoimmunity

as well. Pothlichet et al. described a loss-of-function MAVS (Cardif) variant in humans that is associated with SLE. Patients with this variant were characterized by low type I IFN levels and a lack of autoantibodies specific for RNA-binding protein (26). Similarly, Molineros et al. described mutations of *IFIH1* (MDA5) that are associated with an increased risk of SLE with downregulation of type I IFN signaling (27). These reports as well as others, suggest that Cardif is active even in the absence of pathogenic viral RNA.

Non-viral activators of RIG-I/MDA5-Cardif pathway

Several publications have reported endogenous agonists of RIG-I and MDA5 that lead to Cardif activation such as the small self-RNAs produced by RNase-L, IRE1α, and endogenous retroviral elements in the human genome (28–33). Dupuis-Maurin et al. discovered that overexpression of the transcription factor Sp1 (specificity protein 1) activates the Rig-I-Cardif pathway by stimulating the OAS-RNase-L pathway which produces small self-RNAs (34). Tonic Rig-I/MDA-5-Cardif signaling activity stimulated by endogenous agonists to RIG-I and MDA5 can lead to low-level production of IFN-β as well as NF-κB activation. Cardif may also be involved in other signaling cascades rather than the well-known RIG-I/MDA-5-Cardif axis. Subramanian et al. recently reported the activity of Cardif in the NLRP3 inflammasome (22).

Summary and Key Questions

Cardif is established as important adaptor protein for antiviral signaling. However, the role of Cardif outside of viral infection is largely unknown. The mechanism for Cardif activity outside of viral infection is unknown as well. In this dissertation we explore the role of Cardif in the development and maturation of natural killer cells and T cells.

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Chapter 2

Introduction to Natural Killer (NK) Cells and T cells

Natural Killer Cells

Natural killer (NK) cells are cytotoxic innate lymphocytes that are best known for their ability to lyse stressed cells such as, virally infected cells and tumor cells (1, 2). They are also involved in immune homeostasis, and inflammatory responses due to their production of various cytokines and interaction with dendritic cells and macrophages. The importance of NK cells is apparent in patients that have an NK cell deficiency. Rare disorders that result in NK cell deficiencies render patients particularly susceptible to herpes viruses, often resulting in death, and malignant tumor growth (2–4).

Development and Maturation

Natural killer cells develop in the fetal liver before birth and the bone marrow after birth (5). The stages of NK cell development have been heavily characterized in mice, however development in humans is not as well characterized. The following NK stages have been characterized in mice. NK cells develop from the common lymphoid progenitor (CLP) in bone marrow and proceed to progress through a series of developmental stages before becoming mature NK cells. The first stage is the pre-NKP (pre-NK precursor) or pre-pro NKP stage (6, 7). Pre-NKP cells are $Lin^{-}CD27^{+}CD244^{+}CD122^{-}IL7R\alpha^{+}Flk2^{-}$. NKP cells, which are $Lin^{-}CD27^{+}CD244^{+}CD122^{+}Flk2^{-}$ are the next

stage of NK cell development. The sequential acquisition of NK cell markers NK1.1 and NKp46, identify immature NK (iNK) cells. Mature NK cells (mNK) express CD49b.

There are addition levels of maturation within mNK cells that are characterized by the expression of CD27 and CD11b. The maturation level of mNK cells can be divided into four stages of successive progression: $CD27^-CD11b^-$→ $CD27^+CD11b^-$→ $CD27^+CD11b^+$→ $CD27^-CD11b^+$ (Fig. 1) (8, 9). $CD27^-CD11b^-$ NK cells constitute a minute percentage of NK cells in most organs. NK cells progress quickly though this stage onto $CD27^+CD11b^-$ NK cells. These CD27 single-positive NK cells are highly proliferative however, they are less cytotoxic and they secrete less IFNγ upon stimulation. The expression of CD11b marks another level of maturity within mNK cells. $CD27^+CD11b^+$ NK cells are fully functional and highly proliferative. $CD27^-CD11b^+$ NK cells are terminally differentiated and considered to be the last stage of NK cell maturation. However, they are less proliferative and require more stimulation to induce killing and IFNγ secretion than $CD27^+CD11b^+$ NK cells (8, 10). $CD27^-CD11b^+$ NK cells also acquire the expression of KLRG1 which marks the last stage of NK cell development (11).

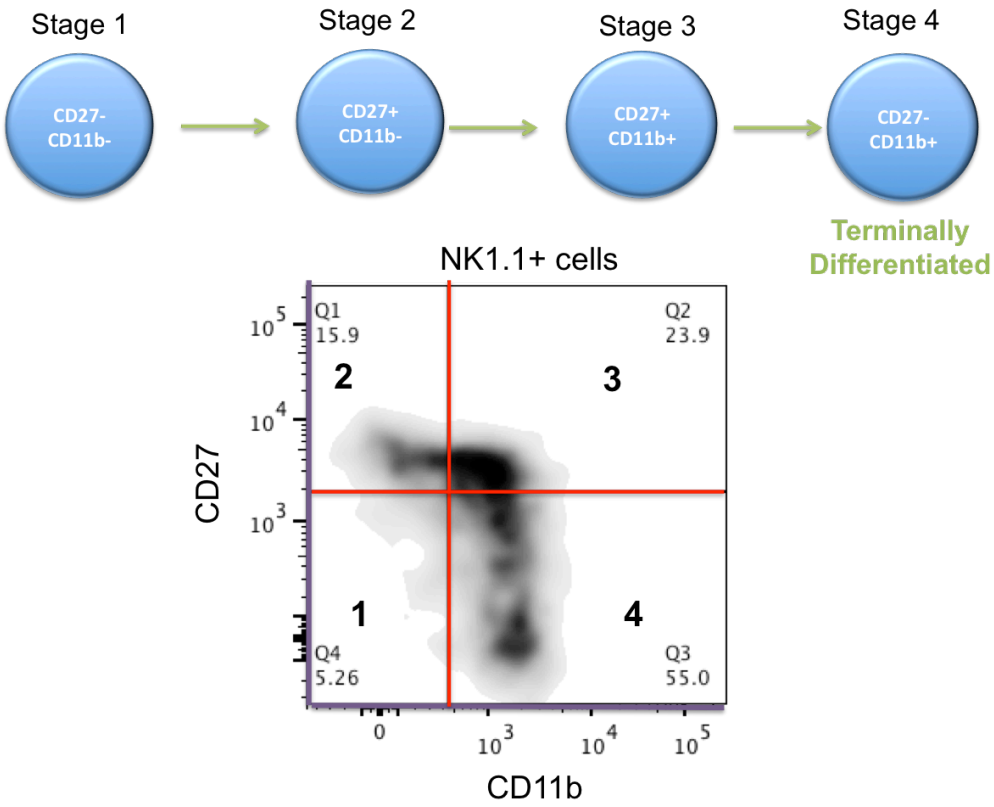

Figure 2.1. The four stages of NK cell maturation. In mice, NK cells mature in a sequential manner through four additional stages after the expression of NK1.1. These four stages are characterized by CD27 and CD11b expression. The last stage of NK cell maturation is a termianally differentiated NK cell that is CD27-CD11b+.

NK cell egress from the bone marrow is coordinated by the expression of $S1P_5$ and CXCR4 on NK cells(10, 12, 13). $S1P_5$ is a sphingosine-phosphate (S1P) G-protein-coupled receptor that binds the lysophospholipid, S1P (12). The expression of $S1P_5$ promotes egress from bone marrow and lymph nodes to the blood, spleen, and liver(1, 13, 14). $S1P_5$ is acquired with maturation of NK cells. $CD27^+CD11b^-$ NK cells express the lowest levels of $S1P_5$ while $CD27^-CD11b^+$ NK cells express the highest amounts (10). As the amount of $S1P_5$ increases egress from bone marrow increases. This relationship between $S1P_5$ and NK maturity accounts for the low percentage of $CD27^-$ $CD11b^+$ NK cells in the bone marrow and lymph nodes. The chemokine receptor CXCR4 has an inverse relationship with NK cell maturation and bone marrow egress. CXCR4 expression decreases as NK cells mature and its expression promotes bone marrow localization (14). CXCR4 desensitization, which uncouples the receptor from G-proteins is particularly important for the egress of $CD27^+CD11b^-$ and $CD27^+CD11b^+$ NK cells from the bone marrow(15). Desensitization and decreased expression of CXCR4 as well as, the increase of $S1P_5$ surface expression are both required for NK cell egress from the bone marrow (14, 15). CX_3CR1 is expressed on terminally differentiated NK cells that also express the inhibitory receptor KLRG1. CX_3CR1expression also promotes egress from the bone marrow and lymph nodes (16).

The expressions of the aforementioned chemotactic receptors play an important role in NK cell tissue distribution. NK cells are able to egress from the bone marrow at all stages of NK cell maturation. However, as mentioned previously, the probability of egress is increased as NK cells mature. Terminally differentiated, $CD27^{-}CD11b^{+}$ NK cells have the highest probability of egress from the bone marrow. Consequently, the predominant NK cells in the bone marrow and lymph node are $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells. While the predominant NK cells in the spleen, liver, blood, and lung are the terminally differentiated $CD27^{-}CD11b^{+}$ NK cells(8, 15). Once in the periphery, $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells may continually mature to the final $CD27^{-}CD11b^{+}KLRG1^{+}$ NK cell stage (9, 11).

Cytokine Production and Degranulation

NK cells kill target cells via the release of perforin and granzymes contained in lytic granules that form pores and induce apoptosis in target cells (17–19). NK cells also release a variety of inflammatory cytokines such as IFNγ and TNFα, among other cytokines. NK cell cytokine release, the polarization of lytic granules towards the target cell, and degranulation depend on signals from activating and inhibitory receptors that interact with target cells (1, 17).

Activating and Inhibitory Receptors/Licensing

The combined stimulation of activating receptors and inhibitory receptors shape the response of NK cells. There are a variety of activating receptors that recognize stress markers on the surface of target cells. In addition, NK cells express the Fc receptor CD16, which binds to the Fc portion of antibodies and allows NK cells to kill antibody-coated target cells (17). The signals for activation must overcome the signals for inhibition in order for NK cells respond to a stimulus. However, NK cells that have not interacted with MHC-1 via inhibitory receptors, will not respond to ligands for activating receptors. This is believed to be due to NK cell "licensing"(17, 18, 20). It is theorized that NK cells are "licensed" by interacting with inhibitory receptors via MHC-I. Without this interaction, NK cells are hyporesponsive to activation stimuli, and they are unable to detect MHC-I loss or reduction on transformed cells (2). In addition, NK cell licensing also protects against NK-mediated autoimmunity (20). However, unlicensed donor NK cells offer the greatest protection against leukemia relapse in human leukemia patients receiving bone marrow transplants (20). Cytokines in an inflammatory environment can also induce responsiveness in NK cells that have not been licensed (17, 21, 22). Indeed, unlicensed NK cells perform better than licensed NK cells in MCMV infection (22–24).

Roles During Infection, viruses, and cancer

Though NK cells are lymphocytes, they had been classified as innate immune cells due to their ability to respond quickly to infection, and their lack of clonal expansion upon recognition of an antigen and long-lived immune memory cells. However, studies of NK cells and MCMV infection found that NK cells that recognize the MCMV antigen m157 via the activating NK receptor Ly49H, expand in manner similar to T- and B-cell clonal expansion, and they remain as long-lived memory cells in the periphery after infection (18, 25–28). Upon MCMV infection, mouse Ly49H+ NK cells expand and their numbers grow to 3-10 times greater than their numbers prior to infection (18). Similarly, in humans NKG2C+ NK cells proliferate in response to HCMV infection (18).

During infection, NK cells can be specifically activated as in the case of MCMV infection and Ly49H+ NK cells, or they can be nonspecifically activated by cytokines such as, IL-12 and type I IFNs produced by dendritic cells (1). This activation leads to NK cell proliferation, killing, and cytokine production. The IFNγ and TNFα produced by NK cells in turn also induces dendritic cell maturation (29). NK cells also aid in the control of immunopathology by killing activated macrophages (1).

NK cells are active in tumor regulation as well as viral infections. NK cells lyse tumor cells that lack MHC-I or express ligands for NK activating

receptors *in vivo* (1). In addition NK cells protect against the spontaneous development of tumors in experimental models of spontaneous tumor development (30, 31).

Role of Dendritic Cells and IL-15 in NK Cell Development and Function

NK cells function *in vivo* is largely shaped through interaction with dendritic cells. Dendritic cells (DCs) promote NK cell activation via cytokine production and the formation of an immunological synapse with NK cells. This cell-cell contact can activate NK cells by engaging receptors such as CD27 and NKG2D on the surface of NK cells (29). DCs also produce IL-12, IL-18, type I IFNs, and IL-15, which are all known to impact NK cell function (29).

The cytokine IL-15 in particular, is an exceptionally important cytokine to the development, survival, proliferation, and function of NK cells (32–34). IL-15 is expressed by a variety of cells; DCs in particular are known for their expression of IL-15 (14, 32). Unlike most cytokines, IL-15 isn't primarily secreted. Instead, it forms a complex with IL-15rα within the cell and is then presented on the surface of the producing cell (34, 35). This is called transpresentation. The receptor IL-15Rβ/γc on the recipient cell, which also recognizes IL-2, then engages the IL-15/IL-5rα complex on the presenting cell. NK cells are dependent upon IL-15 for development as early as the NK cell precursor stage when they begin expressing CD122 (IL-15Rβ). This early

reliance on IL-15 is obvious in experimental models that have low or absent IL-15 that result in a marked NK cell deficiency (33, 34). Less than optimal amount of IL-15 also effects NK cell maturation and function in a dose-dependent manner. Very low amounts of IL-15 result in low total numbers of NK cells that are mostly immature and low functioning (36). While medium amounts of IL-15 result in normal total numbers of NK cells that have somewhat mature and express lower amounts of IFNγ upon stimulation (32, 36). DCs also prime NK cells for their effector functions in inflammatory conditions by transpresenting IL-15 (37).

T Cells

Like NK cells, T cells are also lymphocytes that develop from the common lymphoid progenitor (CLP) cell (38, 39). T cells however, develop in the thymus where they undergo a rigorous selection process before they are released to the periphery (40). There are a variety of T cells however, for the purposes of this introduction we will focus on $CD4^+$ T cells, $CD8^+$ T cells.

T Cell Development

Lymphoid progenitors migrate to the thymus from the bone marrow early after birth (41). While in the thymus, thymocytes undergo a selection process that selects for T cells that are reactive but not self-reactive (Fig. 2.2). The first step is the production of double-positive (DP) thymocytes from double-negative thymocytes. DP thymocytes express both CD4 and CD8 while DN thymocytes express neither receptor (41). DP thymocytes then undergo a process of selection in the cortex of the thymus. During this process, cortical thymic epithelial cells (cTECs) present self-antigens to DP thymocytes (39, 42). The reaction of these DP thymocytes to the presented self-antigen results in 3 different reactions based upon the affinity of the pre-TCR towards the presented antigens: death by neglect caused by no affinity to cTEC presented antigens, positive selection caused by intermediate affinity to cTEC presented antigens, and negative selection caused by high affinity to cTEC presented

antigens (39, 40). After this process, positively selected DP thymocytes develop into single positive (SP) $CD4^+$ or $CD8^+$ thymocytes and relocate to the medulla of the thymus (39, 41). While in the medulla, SP thymocytes interact with various other antigen-presenting cells that present a plethora of self-antigens. During this process, which takes 3-4 days, SP thymocytes face death due to negative selection. If SP thymocytes are not negatively selected during this time, they are able to exit the thymus and move on to the periphery. While in the periphery, T cells require consistent contact with self-antigen presented to the TCR by either MHC-I for $CD8^+$ T cells, or MHC-II for $CD4^+$ T cells for survival (43).

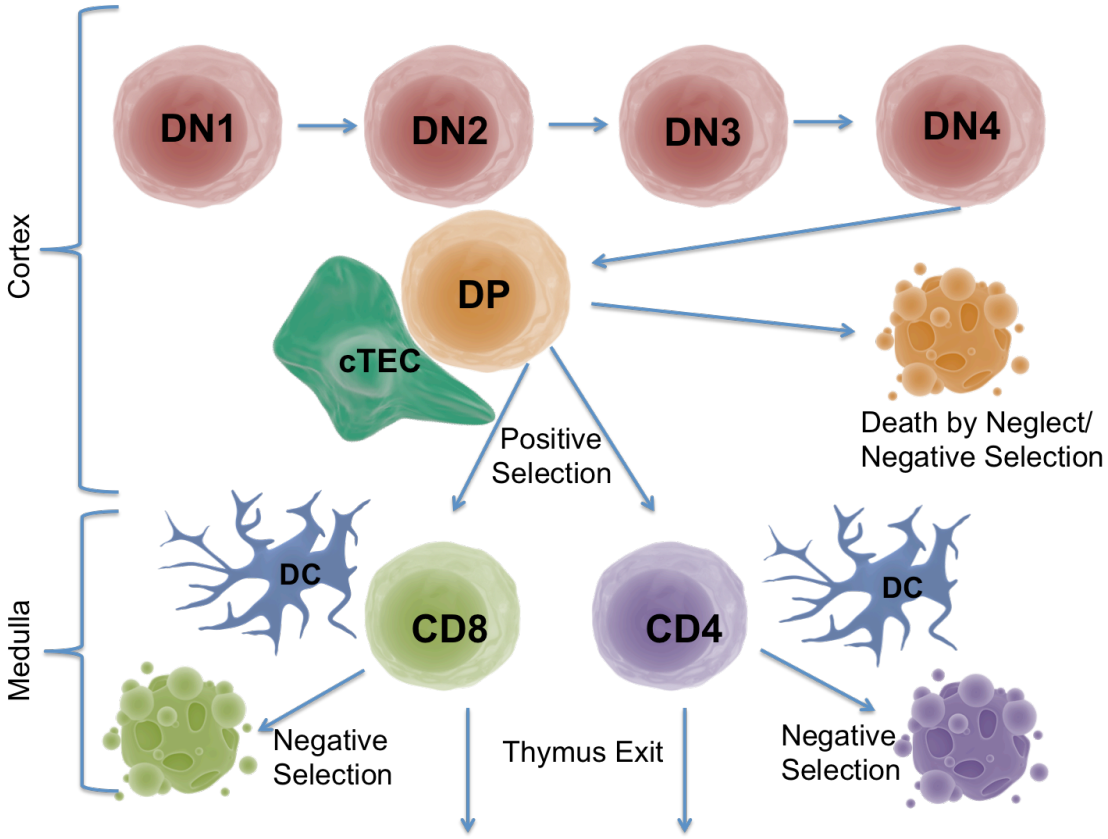

Figure 2.2. Thymocyte Development. Double negative thymocytes progress through 4 stages before developing into double positive thymocytes. Double positive thymocytes interact with cortical epithelial cells and either die (due to neglect or negative selection) or become single positive CD4+ or CD8+ cells. These cells are able to exit the thymus if they do not undergo apoptosis as a result of negative selection.

CD4$^+$ T cells

CD4$^+$ T cells, also known as T-helper cells, are lymphocytes that guide immune responses and are vital to adaptive immunity (44). The absence of CD4$^+$ T cells leaves humans and mice highly susceptible to infections that would otherwise be easily controlled by the immune system. This phenomenon is seen in AIDS patients that have little to no CD4$^+$ T cells due to infection by HIV (44). CD4$^+$ T cells start as naïve cells that have not encountered antigen. Once they encounter antigen, naïve CD4$^+$ T cells then differentiate into one of their specialized subsets: Th1, Th2, Treg (T regulatory cells), Th17, Th22, and Th9 cells (45). These subsets are largely determined by their cytokine production and the expression of a specific transcription factor. The cytokine production profiles are as follows: Th1 cells produce IFNγ and TNF; Th2 cells produce IL-4, IL-5, and IL-13; Tregs produce IL-10 and TGFβ; Th17 cells produce IL-17, IL-22, IL-21, and IL-25; Th22 cells produce Il-22; Th9 cells produce IL-9 (45–47). The cytokines present during the activation of a naïve CD4$^+$ T cell determine their differentiation into a specific subset (45–50). The subsets of CD4$^+$ T cells are known to be plastic; different environments can cause them to change into a different subset (44, 51–53, 50, 54). All of the subsets are effector or inflammatory subsets except Tregs (47). Tregs are regulatory cells that control inflammation. Tregs develop in the thymus or they are induced when naïve CD4$^+$ T cells encounter an antigen and

are in the presence of TGFβ (40, 55, 56). The absence or reduction of Treg numbers leads to chronic inflammation and autoimmunity. The maintenance of Treg numbers, and the ratio of Tregs to T effector cells are crucial for balanced immune responses (56).

$CD8^+$ T cells

$CD8^+$ T cells, like NK cells are cytotoxic cells (18). However, unlike NK cells, their ability to kill target cells is restricted to their TCR engagement and recognition of a specific antigen presented by MHC-I. Once a $CD8^+$ T cell recognizes its antigen, it becomes activated and undergoes clonal expansion, resulting in the production of a multitude of $CD8^+$ T cells that recognize the same antigen. $CD8^+$ T cells also produce memory cells that persist long after an infection has been resolved. These memory cells respond quickly when challenged with the same antigen. Like NK cells, activated $CD8^+$ T cells produce large amounts of IFNγ upon activation, and primarily kill target cells with perforin and granzymes (18, 57).

Similar cytokines also maintain and activate $CD8^+$ T cells and NK cells. $CD8^+$ T cells, like NK cells, are activated by IL-12 and type I IFNs during infection. Furthermore, IL-15 is important for $CD8^+$ T cell maintenance, especially for memory $CD8^+$ T cells (18, 34, 43). IL-$15^{-/-}$ and IL-15r$\alpha^{-/-}$ mice have 50% fewer $CD8^+$ T cells compared to wild type mice (34).

Summary and Key Questions

The two main outcomes of Cardif signaling, type I IFN production and nuclear factor-κB (NF-κB) signaling affect both NK cells and T cells, directly, and indirectly. Type I IFNs are known to increase the expression of IL-15 in DCs (58). As previously mentioned, IL-15 is important for both NK cell and CD8+ T cell homeostasis (33, 34). In addition, type I IFNs directly act on NK cells and T cells to maintain STAT levels which prime cells for activation upon infection (59–61). NF-κB is the family name of a group of transcription factors that regulate various genes involved in a number of physiological processes (62). The NF-κB pathway has been identified as a regulator of activation, differentiation, development, and survival of T cells (62). NF-κB has also been implicated in directing the differentiation of $CD4^+$ T cell subsets (62). NF-κB is important for thymic Treg development and Treg function (62, 63). In addition, NF-κB activation is required for NK cell cytotoxicity and perforin expression (19, 64).

In this dissertation, we investigate the effect that Cardif has on NK and T cell development, maturation, and function. We have identified possible molecular mechanisms for Cardif's effect on NK and T cells. In addition, we have found links between our data and clinical data of Cardif polymorphisms in patients suffering from autoimmune disease.

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Chapter 3

Cardif Regulates the Maturation of Natural Killer cells

Introduction

Pattern recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) (1, 2). Retinoic acid inducible gene-1 (RIG-I)-like receptors (RLRs) are a subset of PRRs that recognize intracellular viral nucleic acids and induce the production of type-I IFNs and NFκB regulated genes (3). Two members of the RLR family, RIG-I and MDA5, have caspase recruitment and activation domains (CARDs) that allow for downstream signaling after activation. The adaptor protein that interacts with RIG-I and MDA5 and allows downstream signaling was discovered by four different groups and is thus known by four names: Cardif (CARD adaptor inducing interferon-β), MAVS (mitochondrial antiviral signaling), IPS-1 (IFN-β promoter stimulator-1), and VISA (virus-induced signaling adaptor) (4–7). We will refer to this protein as Cardif.

RIG-I and MDA5 initiate signaling through CARD-CARD interactions with Cardif, which is a ubiquitously expressed protein that is located on the outer mitochondrial membrane of both immune and non-immune cells (3, 8). The mitochondrial localization of Cardif is essential to its signaling function. Once Cardif has been engaged by RIG-I or MDA5, it aggregates with other Cardif molecules. This aggregation is essential to propagation of downstream signals (9). Cardif interacts with cytoplasmic adaptor molecules TRAF3, TRAF2, and

TRAF6 to activate transcription factors NFκB, IRF3, and IRF7 to induce expression of type I IFN genes and IFN-induced genes (3, 10).

Cardif is vital for signaling in response to viral pathogenic nucleic acids sensed by MDA5 and RIG-1. However, there have been reports that suggest that both RIG-I and Cardif may play roles in immune regulation that are separate from their roles in viral defense (11–15). Wang et al. report that RIG-$I^{-/-}$ mice develop colitis and are more susceptible to dextran sulfate-induced colitis (12). Xu et al. report that $Cardif^{-/-}$ B cells have a cell-intrinsic defect in CD23 and TLR7 expression (13). Additionally, $Cardif^{-/-}$ mice develop more severe disease in the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (16). Cardif is also required for the optimal activation of the NLRP3 inflammasome (14). RIG-I activity under the control of IRF1 has been implicated in the progression of atherosclerosis. Wang et al. propose that 25-hydroxycholesterol induces IL-8 production in macrophages by inducing IRF1 and subsequent RIG-I expression and activation (15). These reports as well as others, suggest that Cardif is active even in the absence of pathogenic viral RNA.

Natural killer (NK) cells are innate cytotoxic lymphocytes that target virally infected, stressed or cancerous cells (17). NK cells primarily develop in the bone marrow although some peripheral organs such as the liver can house and develop NK cells (18–20). Mature NK (mNK) cells are the primary NK

cells found in peripheral organs such as the spleen, liver, and lymph nodes where they undergo additional maturation (21). CD49b acquisition is the earliest stage of NK maturity. The acquisition of CD11b, CD43, and KLRG1 occur after CD49b, and identify more advanced stages of NK maturation. Surface markers CD27 and CD11b can be used to further delineate stages of maturation within immature NK and $CD49b^+$ NK (mNK) cells (22–24). Maturation using these markers is divided into four stages that progress in the following order: $CD27^-CD11b^-$ (Stage 1) → $CD27^+CD11b^-$ (Stage 2) → $CD27^+CD11b^+$ (Stage 3) → $CD27^-CD11b^+$ (Stage 4). Very few $CD27^-CD11b^-$ NK cells are found in the peripheral organs and the majority of $CD27^-CD11b^-$ NK cells do not express CD49b. $CD27^+CD11b^-$ NK cells are highly proliferative and have the ability to degranulate but are not as cytotoxic as $CD27^+CD11b^+$ or $CD27^-CD11b^+$ NK cells. $CD27^+CD11b^+$ NK cells are highly responsive to cytokine and DC stimulation in vitro compared to $CD27^-CD11b^+$ NK cells (22). $CD27^-CD11b^+$ NK cells are terminally differentiated, long-lived, and express the inhibitory NK receptor KLRG1 on their surface (25). $CD27^+CD11b^+$ NK cells are most prevalent in bone marrow and lymph nodes while $CD27^-CD11b^+$ NK cells are the predominant stage found in spleen, liver, blood, and lung.

NK cell numbers, maturation, and function are diminished in the absence of type I IFN signaling. $IFNAR^{-/-}$ mice that lack the ability to respond to type I IFN have fewer NK cells and are unable to control the growth of tumor

cells that are normally susceptible to NK killing (26–29). IFNAR-deficient NK cells are also unable to kill some NK target cells *in vitro*. It has been suggested that consistent and low levels of type I IFN are required to maintain NK cell numbers and functionality *in vivo* as well (30). Constitutive type I IFN signaling maintains STAT1 levels in NK cells. High STAT1 levels are required to maintain NK cytotoxicity while STAT4 leads to IFNγ production (26, 31, 32). It has been suggested that many of the effects of type I IFN deficiency in NK cells can be attributed to the reduction of intracellular STAT1 levels.

Considering the above studies and observations, we sought to understand how the absence of Cardif affects the development, maturation, and function of NK cells. In this study, we found that Cardif expression is required for maintaining optimal NK cell numbers in the periphery and for full NK cell maturation. As such, we found a marked reduction in the number of $CD49b^{+}$ and $CD27^{-}CD11b^{+}$ NK cells with a concomitant increase in $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells in the periphery of mice lacking Cardif. These NK cells had decreased cytotoxicity although they produced comparable amounts of IFNγ. Mixed bone marrow chimeras revealed that the maturation of peripheral $Cardif^{-/-}$ NK cells is cell-intrinsic. Similar to NK cells from $IFNAR^{-/-}$ mice, $Cardif^{-/-}$ NK cells have decreased proliferation and decreased STAT1 activation. Our results suggest that Cardif is intrinsically important for NK cell subset maintenance and function.

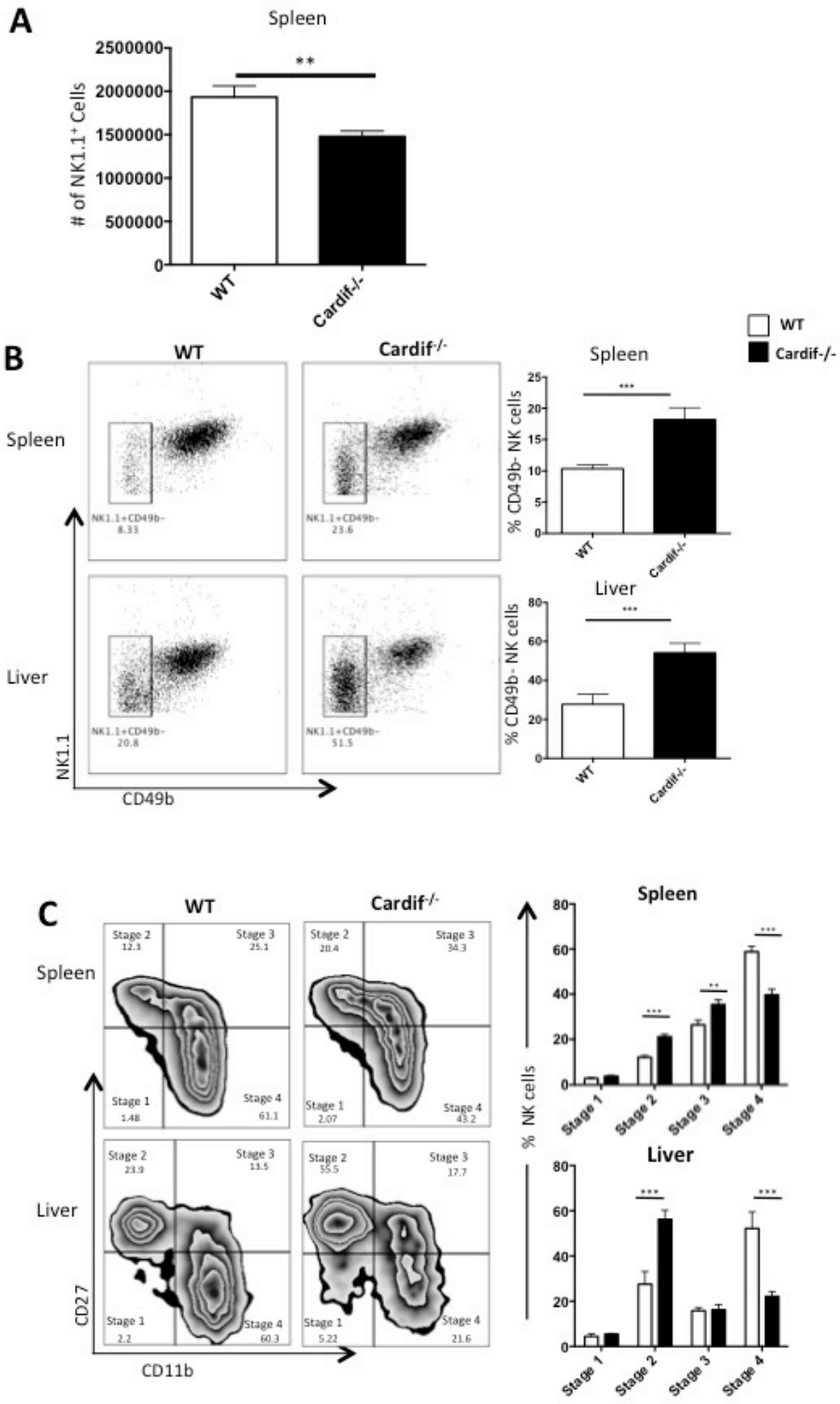
Results

NK maturation is impaired in Cardif$^{-/-}$ mice

Cardif is reportedly active in the absence of a viral infection and is a potent inducer of type I IFN (3, 13, 16). Studies have shown that type I IFN regulates NK cell maturation and function (27, 30). With this information, we hypothesized that Cardif may influence NK cell maturation and function. To test this hypothesis, we characterized NK cells in the bone marrow and in the periphery of wild-type (WT) and Cardif$^{-/-}$ mice. We found that, compared to WT mice, Cardif$^{-/-}$ mice have fewer numbers of CD19^{-}CD3^{-}NK1.1^{+} NK cells in spleen but not in bone marrow (Fig. 3.1A).

The acquisition of CD49b marks the developmental step from immature NK (iNK) to mature NK (mNK) cells (21, 36). We found that the frequency of CD49b^{-} (immature) NK cells in Cardif$^{-/-}$ spleen and liver is significantly higher than in WT NK cells (Fig. 3.1B). This 2-fold increase of CD49b^{-} NK cells in the spleens and livers of Cardif$^{-/-}$ mice suggests a delay in mNK development and maintenance in the periphery.

Figure 3.1. Cardif is required for optimal NK cell numbers in the periphery and for the terminal differentiation of NK cells. (A) The absolute number of $NK1.1^{+}CD3^{-}CD19^{-}$ cells was determined in the spleen. (B) Representative plots (*left and middle panels*) and bar graphs (*right panels*) show the percentage of $CD49b^{-}$ NK cells ($NK1.1^{+}CD3^{-}CD19^{-}$) in the spleen (*upper panels*) and liver (*lower panels*). (C) Representative plots and bar graphs plots (*left and middle panels*) and bar graphs (*right panels*) show the distribution of mNK ($NK1.1^{+}CD3^{-}CD19^{-}$ $CD49b^{+}$) cells based on CD27 and CD11b expression in the spleen (*upper panels*) and liver (*lower panels*). Stages 1-4 are as follows: stage 1($CD27^{-}CD11b^{-}$), stage 2 ($CD27^{+}CD11b^{-}$), stage 3 ($CD27^{+}CD11b^{-}$), and stage 4 ($CD27^{-}CD11b^{+}$). Experiments were repeated at least three times. For all, n=3-5 mice/group/experiment. Data are means ± SEM. $^{**}p<0.01$, $^{***}p<0.001$.


A
Spleen
# of NK1.1+ Cells
2500000
2000000
1500000
1000000
500000
0
**
WT
Cardif-/-
B
WT
Cardif-/-
Spleen
Liver
NK1.1+CD49b-
8.33
NK1.1+CD49b-
23.6
NK1.1+CD49b-
20.8
NK1.1+CD49b-
51.5
NK1.1
CD49b
Spleen
% CD49b- NK cells
***
Liver
% CD49b- NK cells
***
WT
Cardif-/-
C
WT
Cardif-/-
Spleen
Liver
Stage 1
Stage 2
Stage 3
Stage 4
12.3
25.1
1.48
61.1
20.4
34.3
2.07
43.2
23.9
13.5
2.2
60.3
55.5
17.7
5.22
21.6
CD27
CD11b
Spleen
% NK cells
Liver
% NK cells
***
**
***
***
***
Stage 1
Stage 2
Stage 3
Stage 4

In addition to a reduction in mature NK cells ($NK1.1^{+}CD3^{-}CD19^{-}CD49b^{+}$), we observed a significant reduction in terminally differentiated $CD27^{-}CD11b^{+}$ mNK cells in the spleen and liver of $Cardif^{-/-}$ mice (Fig. 3.1C). This reduction of $CD27^{-}CD11b^{+}$ mNK cells is accompanied by a concomitant increase in frequencies of $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ immature NK cells. $Cardif^{-/-}$ mice have a 35-40% loss of terminally differentiated mature $CD27^{-}CD11b^{+}$ NK cells with a concomitant increase in less mature ($CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$) NK cells in the spleen. Additionally, there is a >50% decrease in terminally differentiated $CD27^{-}CD11b^{+}$ NK cells and a 50% increase in $CD27^{+}CD11b^{+}$ NK cells in liver of $Cardif^{-/-}$ mice (Fig. 3.1C). NK cells in the blood and lymph nodes of $Cardif^{-/-}$ mice displayed similar alterations in NK maturation (data not shown). However, we found no differences in NK maturation in the bone marrow (Fig. 3.2). Taken together, these data suggest that Cardif is important for NK maturation but does not impair NK development in bone marrow.

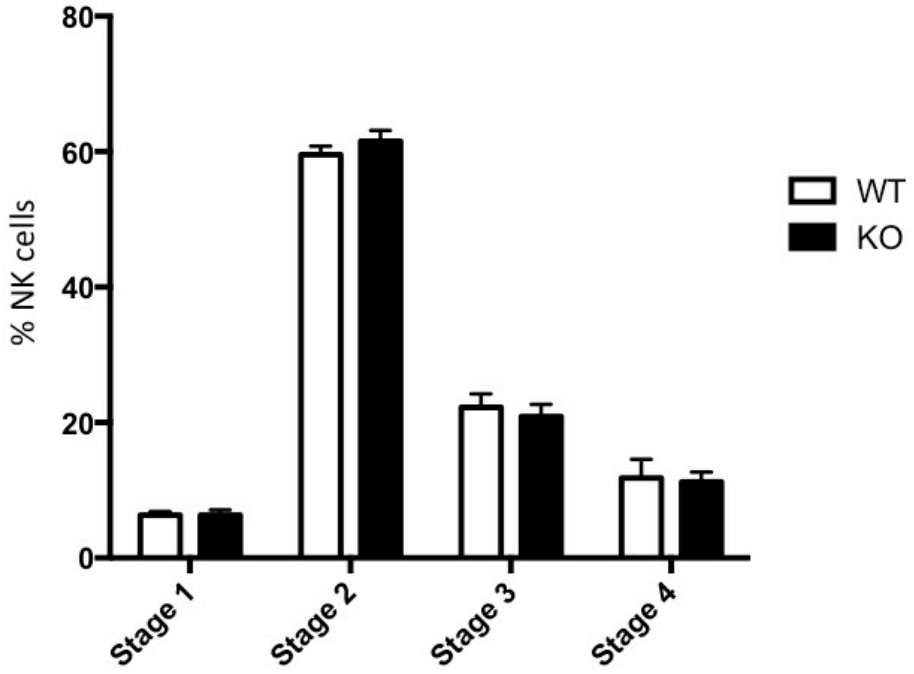
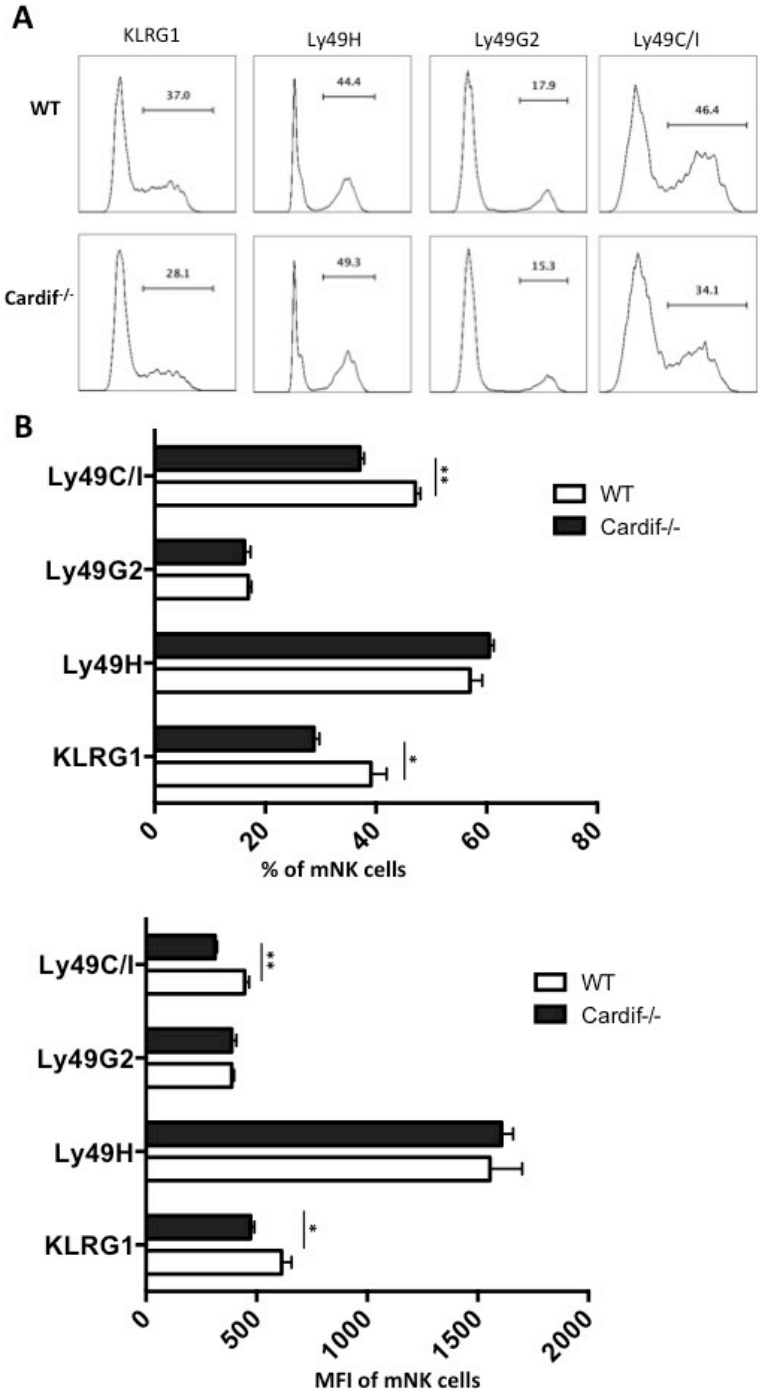


Figure 3.2. Bone marrow resident Cardif$^{-/-}$ NK cells are similar to WT. Bar graph shows the percentage of NK (NK1.1^{+}CD3^{-}CD19^{-}) cells that express CD27 and CD11b in the bone marow. Stages 1-4 are as follows: stage 1(CD27^{-}CD11b^{-}), stage 2 (CD27^{+}CD11b^{-}), stage 3 (CD27^{+}CD11b^{-}), and stage 4 (CD27^{-}CD11b^{+}). Experiments were repeated at least twice with at least 3 mice/group/experiment. Data are means ± SEM.

Inhibitory receptor expression is reduced in Cardif$^{-/-}$ mice

Next, we wanted to examine homeostatic NK function in the absence of Cardif signaling. Ly49 receptor expression on NK cells is required for NK cell "licensing", the process that allows NK cells to become active cytolytic cells following their encounter with self MHC-1 expressing cells (37–39). The expression of the inhibitory receptors such as Ly49C/I and KLRG1 on NK cells is associated with NK cell maturity (23, 25). We investigated the expression of KLRG1 and select Ly49 receptors in Cardif$^{-/-}$ mice. We found that significantly fewer Cardif$^{-/-}$ mNK ($NK1.1^{+}CD3^{-}CD19^{-}CD49b^{+}$) cells express KLRG1 (Fig. 3.3A, B) (21). Moreover, there is a decrease in the surface expression of KLRG1 on Cardif$^{-/-}$ mNK cells (Fig. 3.3B). We found that WT and Cardif$^{-/-}$ mNK cells express similar amounts of the activating Ly49H receptor and the inhibitory Ly49G2 receptor (Fig. 3.3A, 3.3B). However, the number of Ly49C/I^{+} mNK cells is markedly decreased in Cardif$^{-/-}$ mice. Likewise, the surface expression of Ly49C/I on Cardif$^{-/-}$ mNK cells is significantly lower than WT mNK cells. The decrease in the frequency and cellular expression of KLRG1 and Ly49C/I which are associated with NK cell activation and maturation, suggest that Cardif is involved in late mNK cell maturation (18, 40).

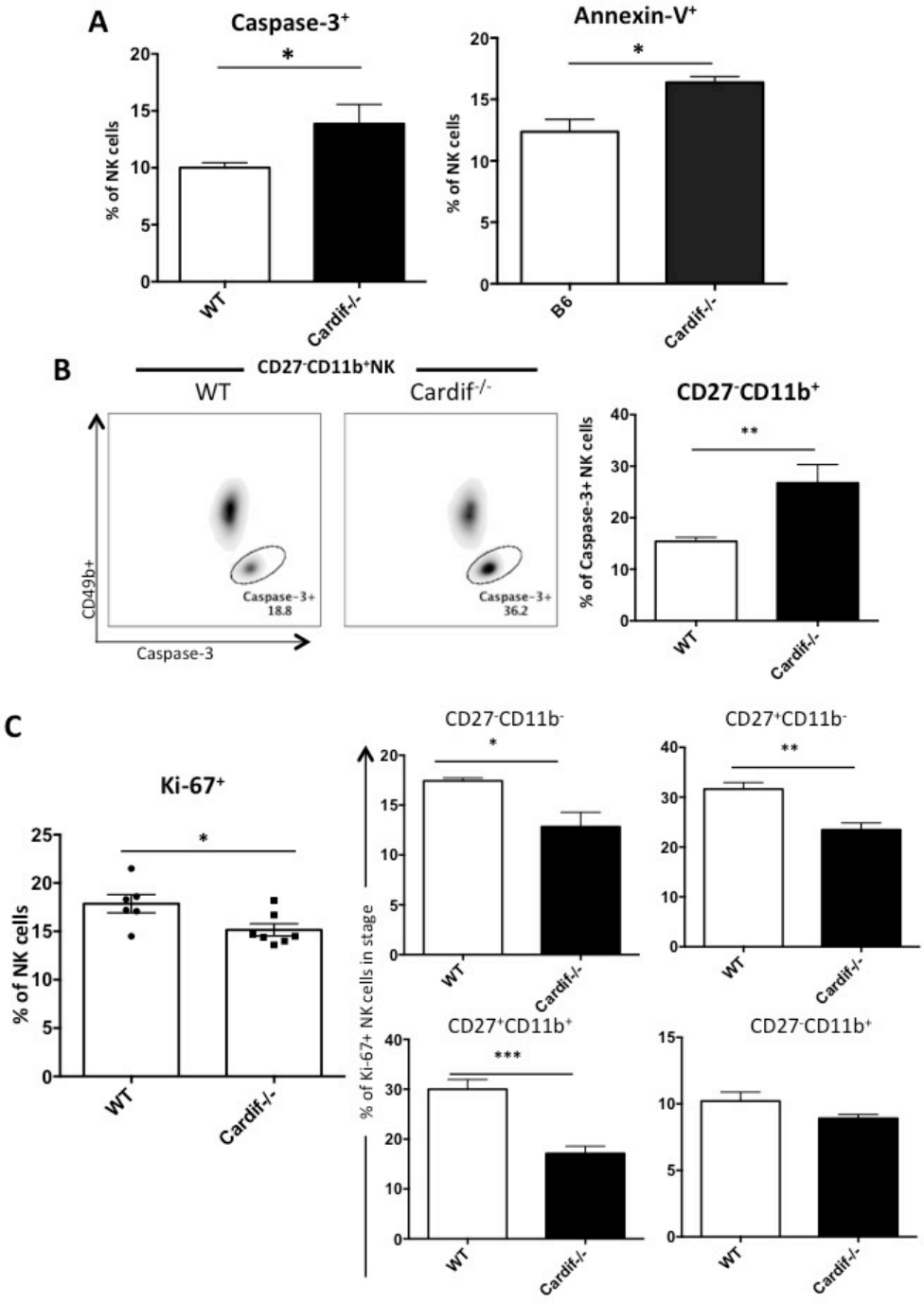
Figure 3.3. Cardif$^{-/-}$ NK cells are less mature. (A) Representative histograms show the percentages of KLRG1^{+} cells, and cells expressing select activating and inhibitory Ly49 receptors in WT and Cardif$^{-/-}$ mNK populations (NK1.1^{+}CD3^{-}CD19^{-} CD49b^{+}). (B) Bar graphs depict the percentage (*left panel*) and amount of expression (*right panel*) of NK maturation markers and Ly49 receptors. Amount of expression was determined by flow cytometric analysis of mean fluorescence intensity (MFI). Experiments were repeated twice. For all, n=3 mice/group/experiment. Data are means ± SEM. *p< 0.05, **p<0.01.


A
KLRG1
Ly49H
Ly49G2
Ly49C/I
WT
Cardif-/-
37.0
44.4
17.9
46.4
28.1
49.3
15.3
34.1
B
Ly49C/I
Ly49G2
Ly49H
KLRG1
WT
Cardif-/-
**
*
0
20
40
60
80
% of mNK cells
Ly49C/I
Ly49G2
Ly49H
KLRG1
WT
Cardif-/-
**
*
0
500
1000
1500
2000
MFI of mNK cells

Cardif$^{-/-}$ NK cells are more apoptotic and less proliferative

In order to identify mechanistically why there are fewer mature NK cells in Cardif$^{-/-}$ mice, we investigated the survival and proliferation of NK cells in Cardif$^{-/-}$ mice. We found that more Cardif$^{-/-}$ NK cells were caspase-3^{+}, suggesting that they were more apoptotic (Fig. 3.4A). We confirmed the increase in apoptosis using Annexin V, as we found more Annexin-V^{+} NK cells in Cardif-/- mice, (Fig. 3.4A). The greatest increase in apoptotic mNK cells was observed in CD27^{-}CD11b^{+} NK cells, where 2-fold more CD27^{-}CD11b^{+} Cardif$^{-/-}$ NK cells were apoptotic as noted by caspase-3 expression (Fig. 3.4B). This increase of caspase-3^{+} CD27^{-}CD11b^{+} NK cells in Cardif$^{-/-}$ mice accounts for the increase in frequencies of total caspase-3^{+} NK cells. These data suggest that Cardif-/- NK cells, particularly, CD27^{-}CD11b^{+} NK cells, are more apoptotic in the absence of Cardif signaling. We used Ki-67 to identify proliferating NK cells in Cardif$^{-/-}$ and WT mice. We found a ~15% decrease in proliferation of total splenic Cardif$^{-/-}$ NK cells compared to WT NK cells (Fig. 3.4C). However, upon further analysis, we found that CD27^{+}CD11b^{-} and CD27^{+}CD11b^{+} Cardif$^{-/-}$ NK cells have a ~25% and ~30% reduction in proliferation, respectively. The decrease in NK proliferation is most pronounced in CD27^{+}CD11b^{-} and CD27^{+}CD11b^{+} NK cells, the stages that are known to mature into CD27^{-} CD11b^{+} mNK cells (22, 23). Thus, in the absence of Cardif these immature NK cells proliferate less and are prone to apoptosis.

Figure 3.4. Cardif$^{-/-}$ NK cells are more apoptotic and less proliferative. (A) Bar graphs show the percentage of Caspase-3^{+} (*upper panel*) and Annexin-V^{+} (*lower panels*) NK cells (NK1.1^{+}CD3^{-}CD19^{-}) from WT and Cardif$^{-/-}$ mice. (B) Representative plots and bar graph of the percentage of WT and Cardif$^{-/-}$ caspase-3^{+} CD27^{-}CD11b^{+} NK cells. (C) Bar graphs depict the percentage of Ki-67^{+} total NK cells (*left panel*), as well as stage 1-4 NK cells (*middle and right panels*). Experiments were repeated twice. For all, n=4-5 mice/group/experiment. Data are means ± SEM. *$p<$ 0.05, **$p<$0.01, ***$p<$0.001.


A
Caspase-3+
Annexin-V+
% of NK cells
WT
Cardif-/-
B6
B
CD27-CD11b+NK
WT
Cardif-/-
CD49b+
Caspase-3
Caspase-3+ 18.8
Caspase-3+ 36.2
CD27-CD11b+
% of Caspase-3+ NK cells
C
Ki-67+
CD27-CD11b-
CD27+CD11b-
CD27+CD11b+
CD27-CD11b+
% of Ki-67+ NK cells in stage

Cardif deficiency alters NK cell function

Since we found that NK cells in Cardif-/- mice are less mature and fewer are Ly49C/I^{+}, we hypothesized that Cardif-deficient NK cells may not function as well as WT NK cells. Fernandez, et al., has previously shown that unlicensed Ly49C/I^{-} NK cells are less cytotoxic than Ly49C/I^{+} NK cells (41). We stimulated WT and NK Cardif$^{-/-}$ NK cells with either IL-12 and IL-18, or PMA (phorbol 12-myristate 13-acetate) and ionomycin *ex vivo*. We found that granzyme B and CD107a, a marker of degranulation, were significantly reduced in Cardif$^{-/-}$ NK cells compared to WT NK cells, suggesting that Cardif$^{-/-}$ NK cells are likely impaired in their ability to kill target cells (Fig. 3.5). The production of IFNγ however was comparable between NK cells from both WT and Cardif$^{-/-}$ mice. Next, we tested the ability of Cardif$^{-/-}$ NK cells to directly kill target cells in vitro. YAC-1 cells served as target cells in the assay. Cardif$^{-/-}$ NK cells had ~20-25% less cytotoxic activity than WT NK cells when cultured with target B cells (Fig. 3.6). These data indicate that Cardif plays an important role in manipulating the functions of NK cells.

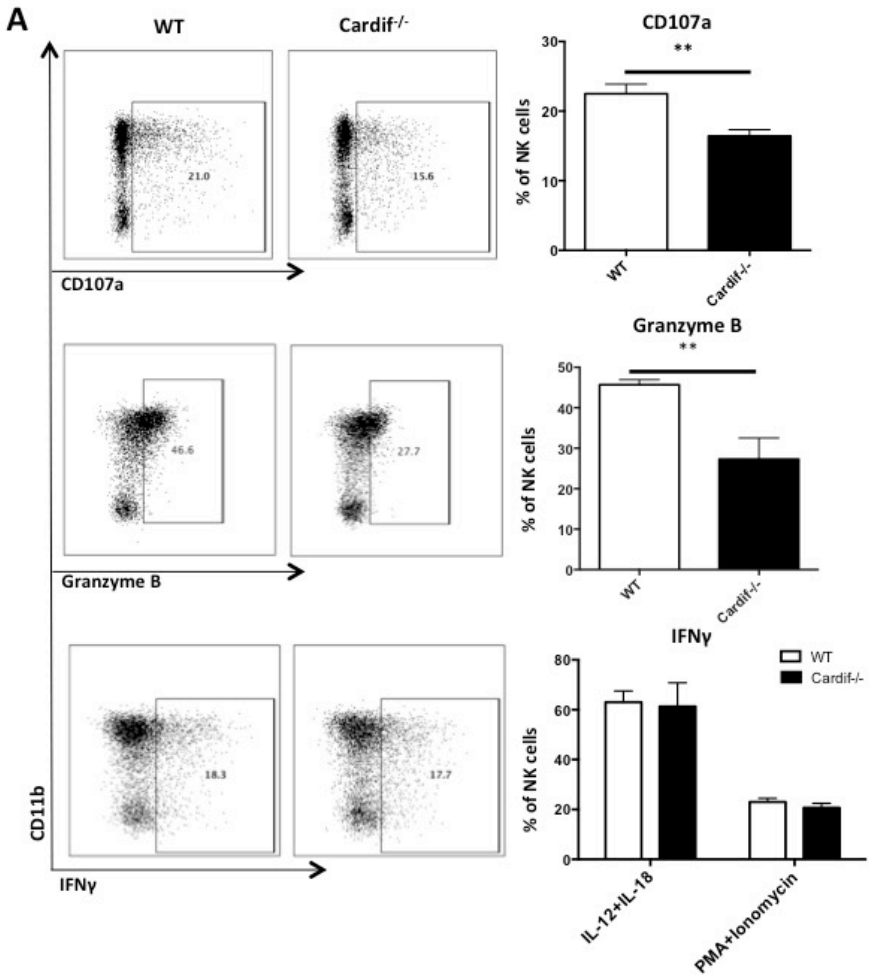


Figure 3.5. Cardif$^{-/-}$ NK cells are less functional than WT mNK cells. (A) Representative plots and bar graphs show the percentage of CD107a^{+} (*upper panels*), granzyme B^{+} (*middle panels*), and IFNγ^{+} (*lower panels*) mNK (NK1.1^{+}CD3^{-}CD19^{-} CD49b^{+}) cells in WT and Cardif$^{-/-}$ samples. Experiments were repeated at least twice. For all, n=3-5 mice/group/experiment. Data are means ± SEM. *p< 0.05, **p<0.01, ***p<0.001.

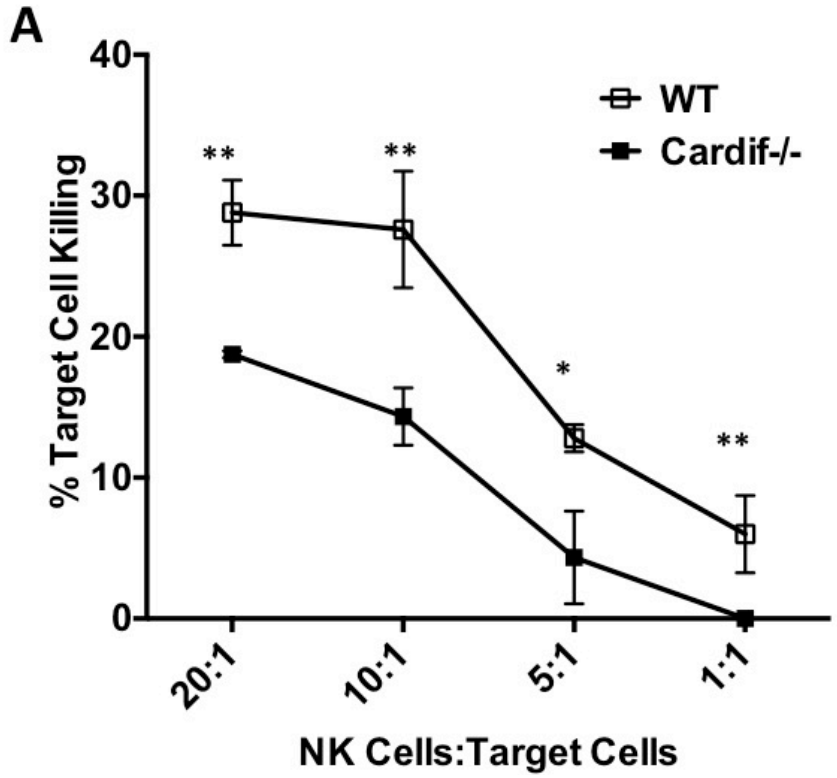
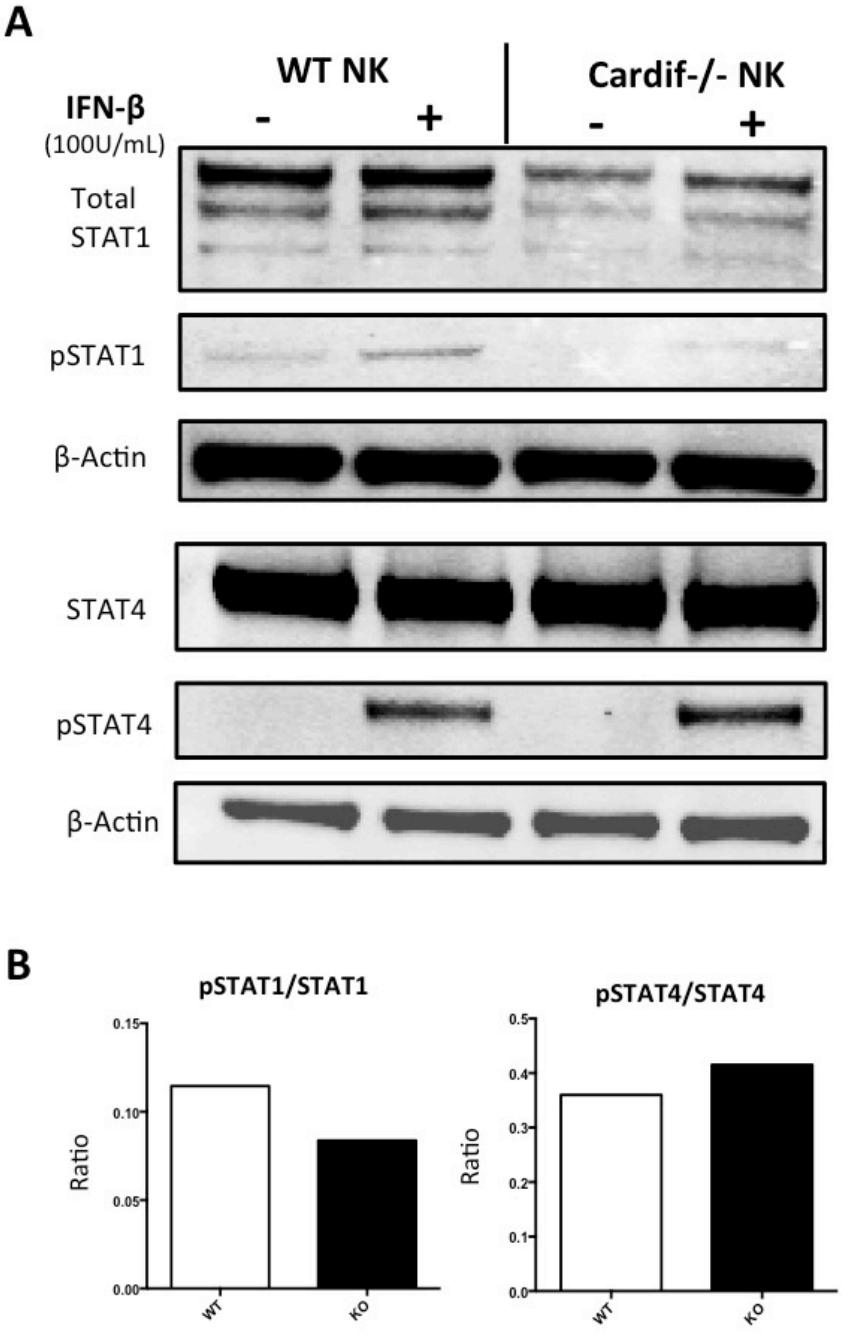


Figure 3.6. Cardif$^{-/-}$ NK cells are less cytotoxic than WT mNK cells. (A) Representative line graph depicting the cytotoxic activity of WT and Cardif$^{-/-}$ NK cells against target YAC-1 cells at various ratios. Ratios are NK cells : Target cells. Experiments were repeated at least twice. For all, n=3-5 mice/group/experiment. Data are means ± SEM. *$p<$ 0.05, **$p<$0.01, ***$p<$0.001.

STAT1 protein is reduced in Cardif$^{-/-}$ NK cells

Reports have shown that the maintenance of STAT1 levels in NK cells is important for NK cell cytotoxicity upon activation, while the levels of STAT4 are important for IFNγ production (31). In earlier experiments, we found that Cardif$^{-/-}$ NK cells are less cytotoxic than WT NK cells while IFNγ production was the same (Fig. 3.5 and Fig. 3.6). With these observations, we decided to measure STAT1 and STAT4 phosphorylation in NK cells and found that there were dramatic reductions in both the phosphorylation and the levels of STAT1 protein in Cardif$^{-/-}$ NK cells (Fig. 3.7A). STAT4 protein and phosphorylation seemed similar between Cardif$^{-/-}$ and WT NK cells but we found that the pSTAT4/STAT4 ratio was higher in Cardif$^{-/-}$ NK cells. Based on a known role for STAT1 in regulating NK cytolytic functions, these data indicate that the reduced cytolytic function of Cardif$^{-/-}$ NK cells is most likely due to the low intracellular amount of STAT1 and phosphorylated STAT1. The effects of an increase in the pSTAT4/STAT4 ratio in Cardif$^{-/-}$ NK cells was most likely masked due to the already high amounts of STAT4 present in WT NK cells.

Figure 3.7. Cardif$^{-/-}$ mNK cells have decreased levels of STAT1 and phosphorylated STAT1. (A) Western blot shows the amount of total STAT1 (*top panel*), pSTAT1 (*2nd panel*), STAT4 (*4th pan*el), and pSTAT4 (*bottom panel*) protein in WT (*le*ft *panels*) and Cardif$^{-/-}$ (*right panels*) mNK cells. Cells were treated with PBS or 100U/ml of IFN-β. Afterwards, 30ug of protein from cell lysates were loaded per well. β-Actin was used to normalize protein data. (B) pSTAT1/STAT1 and pSTAT4/STAT4 ratios derived western blot quantification. Experiments were repeated twice. For all, n=4 mice/group/experiment. Data are means ± SEM. *$p<$ 0.05, **$p<$0.01, ***$p<$0.001.

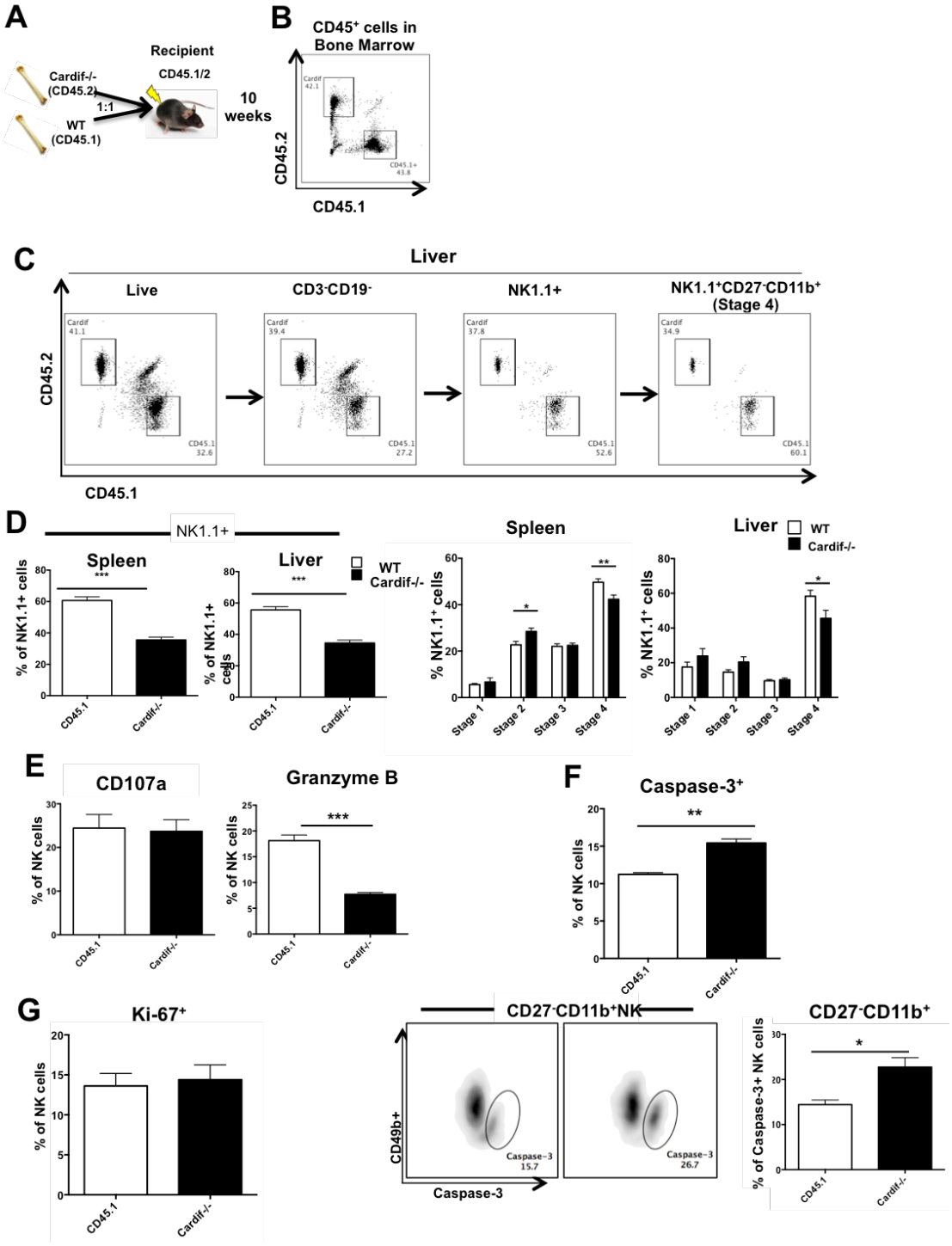

A
WT NK
Cardif-/- NK
IFN-β
(100U/mL)
-
+
-
+
Total
STAT1
pSTAT1
β-Actin
STAT4
pSTAT4
β-Actin
B
pSTAT1/STAT1
Ratio
0.15
0.10
0.05
0.00
WT
KO
pSTAT4/STAT4
Ratio
0.5
0.4
0.3
0.2
0.1
0.0
WT
KO

Impaired NK cell phenotype in Cardif$^{-/-}$ mice is cell-intrinsic

To determine whether the impact of Cardif on NK cell maturation is cell-intrinsic, we used a mixed chimera approach. WT CD45.1^{+} bone marrow and CD45.2^{+}Cardif$^{-/-}$ bone marrow were mixed 1:1 and transplanted into irradiated CD45.1^{+}/CD45.2^{+} recipient mice (Fig. 3.8A). After 10 weeks of reconstitution we assessed the phenotype of NK cells in various organs. We found that CD45.1^{+} and Cardif$^{-/-}$ cells contributed equally to the total number of CD45^{+} cells in the reconstituted bone marrow (Fig. 3.8B). However, there were slight, but consistently lower percentages of Cardif$^{-/-}$ origin NK (CD45.1^{-} CD45.2^{+}NK1.1^{+}CD3^{-}CD19^{-}) cells in the bone marrow of chimeric mice compared to WT CD45.1 origin NK cells (data not shown). We found that this NK cell specific phenotype was more pronounced in the periphery (Fig. 3.8C). We found that Cardif$^{-/-}$ NK cells reconstituted only ~40% while CD45.1^{+} NK cells reconstituted ~60% of total NK cells in the spleen and liver (Fig. 3.8D). We also observed a slight decrease of the CD27^{-}CD11b^{+} subset within Cardif$^{-/-}$ NK cells in the spleen and liver, similar to what we observed in the global knockout mice (Fig. 3.8E and 3.1C). The decrease in Cardif$^{-/-}$ CD27^{-}CD11b^{+} NK cells was accompanied by an increase in CD27^{+}CD11b^{-} Cardif$^{-/-}$ NK cells in both the spleen and liver of the mixed bone marrow chimeric mice (Fig. 3.8E).

We also investigated the function of Cardif$^{-/-}$ NK cells in the chimeric mice. We found that although Cardif$^{-/-}$ NK cells and WT NK cells displayed a similar amount of degranulation, granzyme B production was lower in Cardif$^{-/-}$ NK cells (Fig. 3.8F). In addition, we found that more Cardif$^{-/-}$ NK cells are caspase-3^{+} compared to WT NK cells in chimeric mice (Fig. 3.8G). Similar to global knockout mice, CD27^{-}CD11b^{+} Cardif$^{-/-}$ NK cells in chimeric mice have the greatest increase in apoptosis (Fig. 3.8G). We examined proliferation of WT and Cardif$^{-/-}$ NK cells in chimeric mice using Ki-67 and found that both WT and Cardif$^{-/-}$ NK cells proliferated at the same rate (Fig. 3.8H).

Figure 3.8. Cardif$^{-/-}$ NK cells show maturation defect in 1:1 bone marrow chimeric mice. (A) Schematic diagram of method used to create 1:1 mixed bone marrow chimeric mice. Cardif$^{-/-}$ (CD45.2) and CD45.1 bone marrow were intravenously injected into CD45.1/.2 recipient mice in a 1:1 ratio. Mice were harvested10 weeks after injection. (B) Representative plot depicting equal amounts of WT and Cardif$^{-/-}$ CD45^{+} cells in the bone marrow. (C) Representative plots that show the ratios of CD45.1 and Cardif$^{-/-}$ cells in the liver in specific populations: live cells→CD3^{-}CD19^{-}→ NK1.1^{+}CD3^{-}CD19^{-}→ NK1.1^{+}CD3^{-}CD19^{-}→ NK1.1^{+}CD3^{-}CD19^{-}CD27^{-}CD11b^{+} (*upper panels*). (D) Representative bar graphs show the contribution of cells from CD45.1 and Cardif$^{-/-}$ origins to total NK1.1^{+}CD3^{-}CD19^{-} cells in spleen and liver. (E) Representative bar graphs depict the distribution of WT (CD45.1) and Cardif$^{-/-}$ NK cells based on the 4 maturation stages, denoted by CD27 and CD11b in the spleen and liver of 1:1 mixed bone marrow chimeric mice. (F) Bar graphs depict the production of CD107a (*left panel)* and Granzyme B (*right panel*) by NK cells. (G) Bar graphs depict the percentage of Caspase-3^{+} NK cells. Representative plots and bar graph depict the percentage of Caspase-3^{+} CD27^{-}CD11b^{+} NK cells (*middle and right panels*). (H) Bar graphs depict the percentage of Ki-67^{+} NK cells. Experiments were performed twice (A-E) or once (F-H). For all, n=6 mice/group/experiment. Data are means ± SEM. **p*< 0.05, ***p*<0.01, ****p*<0.001.


A
Recipient
CD45.1/2
Cardif-/-
(CD45.2)
1:1
WT
(CD45.1)
10
weeks
B
CD45+ cells in
Bone Marrow
CD45.2
CD45.1
C
Liver
Live
CD3-CD19-
NK1.1+
NK1.1+CD27-CD11b+
(Stage 4)
CD45.2
CD45.1
D
NK1.1+
Spleen
Liver
WT
Cardif-/-
% of NK1.1+ cells
% of NK1.1+ cells
% NK1.1+ cells
% NK1.1+ cells
CD45.1
Cardif-/-
Stage 1
Stage 2
Stage 3
Stage 4
E
CD107a
Granzyme B
% of NK cells
% of NK cells
F
Caspase-3+
% of NK cells
G
Ki-67+
% of NK cells
CD27-CD11b+NK
CD49b+
Caspase-3
Caspase-3
15.7
Caspase-3
26.7
CD27-CD11b+
% of Caspase-3+ NK cells

Signaling via Cardif leads to the production of Type I IFN. We, along with other groups, have found that similar to Cardif$^{-/-}$ mice, IFNAR1$^{-/-}$ mice have fewer terminally differentiated NK cells and abrogated functionality in the absence of type I IFN signaling (27, 28, 42, 43) (Fig. 3.9). Gough et al., have theorized that low homeostatic levels of type I IFN are required in the absence of infection to sustain levels of STAT proteins in the cytoplasm of various immune cells and consequently maintain normal cell function upon activation (30). Thus, we hypothesized that IFN-β signaling was likely impaired in response to Cardif deletion since Cardif$^{-/-}$ NK cells have low intracellular levels of STAT1 (Fig. 3.7).

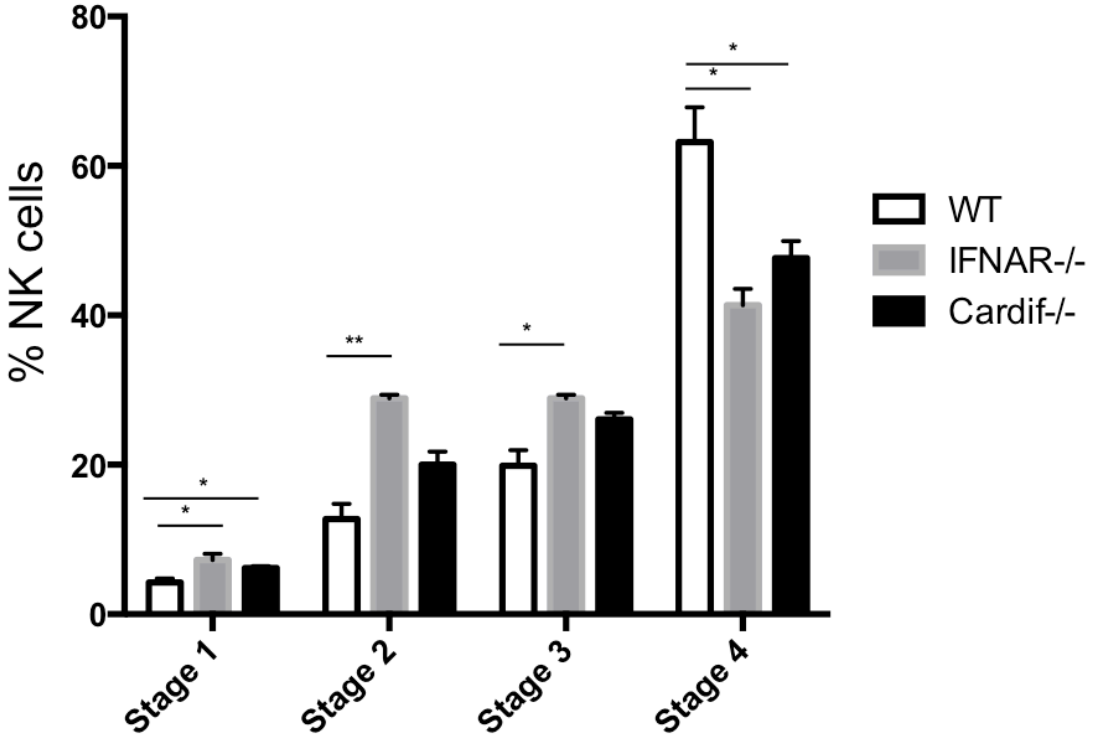


Figure 3.9. Impaired Cardif-/- NK cell maturation is similar to IFNAR-/- NK cell maturation impairment. Bar graphs show the percentage of mNK ($NK1.1^{+}CD3^{-}CD19^{-}$ $CD49b^{+}$) cells that express CD27 and CD11b in the spleen. Stages 1-4 are as follows: stage 1($CD27^{-}CD11b^{-}$), stage 2 ($CD27^{+}CD11b^{-}$), stage 3 ($CD27^{+}CD11b^{-}$), and stage 4 ($CD27^{-}CD11b^{+}$). Experiments were repeated at least twice with at least 3 mice/group/experiment. Data are means ± SEM. *$p<$ 0.05, **$p<$0.01, ***$p<$0.001.

In an attempt to rescue NK maturation, we treated WT and Cardif$^{-/-}$ NK cells with IFN-β *in vitro*. Similar to Brady et al., we noticed drastically reduced levels of CD11b expression on NK cells in culture (44). This made it difficult to confidently identify the maturation stages of NK cells. We did however, measure total expression of CD11b and KLRG1 in the cultured NK cells. Both CD11b and KLRG1 expression significantly increased with IFN-β treatment in WT and Cardif$^{-/-}$ NK cells (Fig. 3.10). Still, CD11b and KLRG1 levels were not restored to those of WT with IFN-β stimulation.

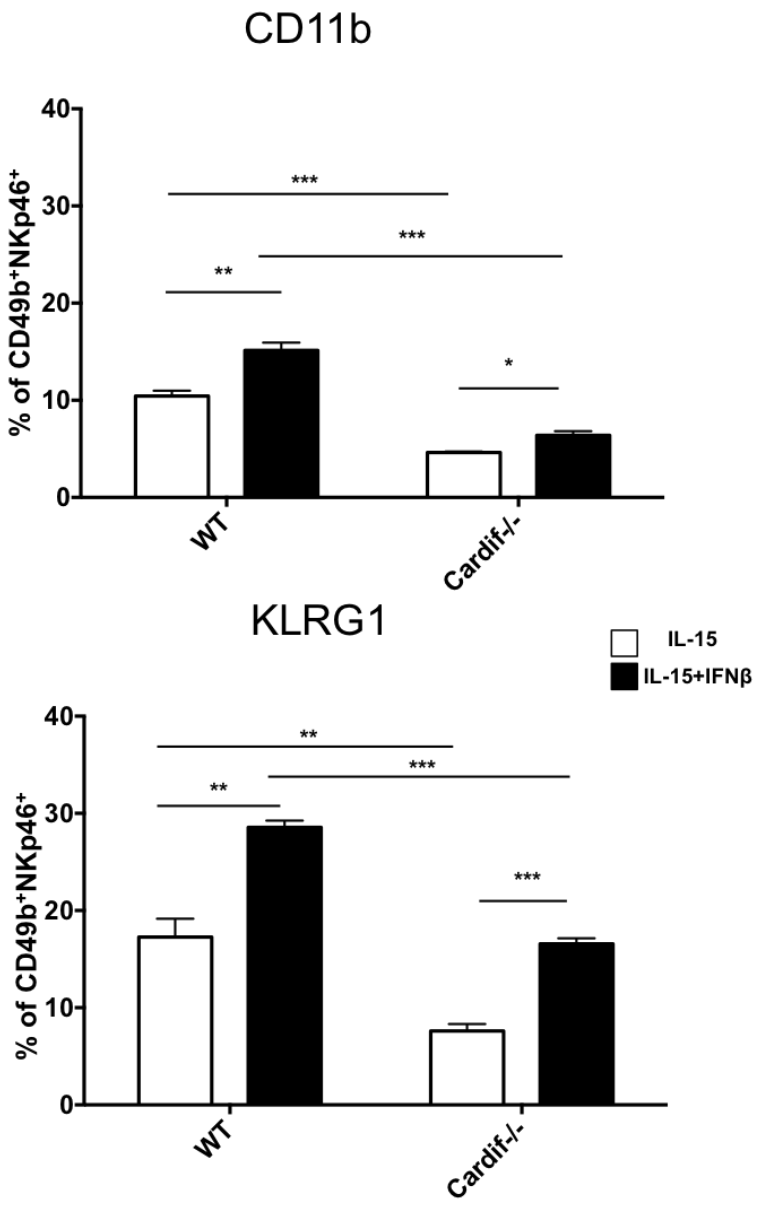


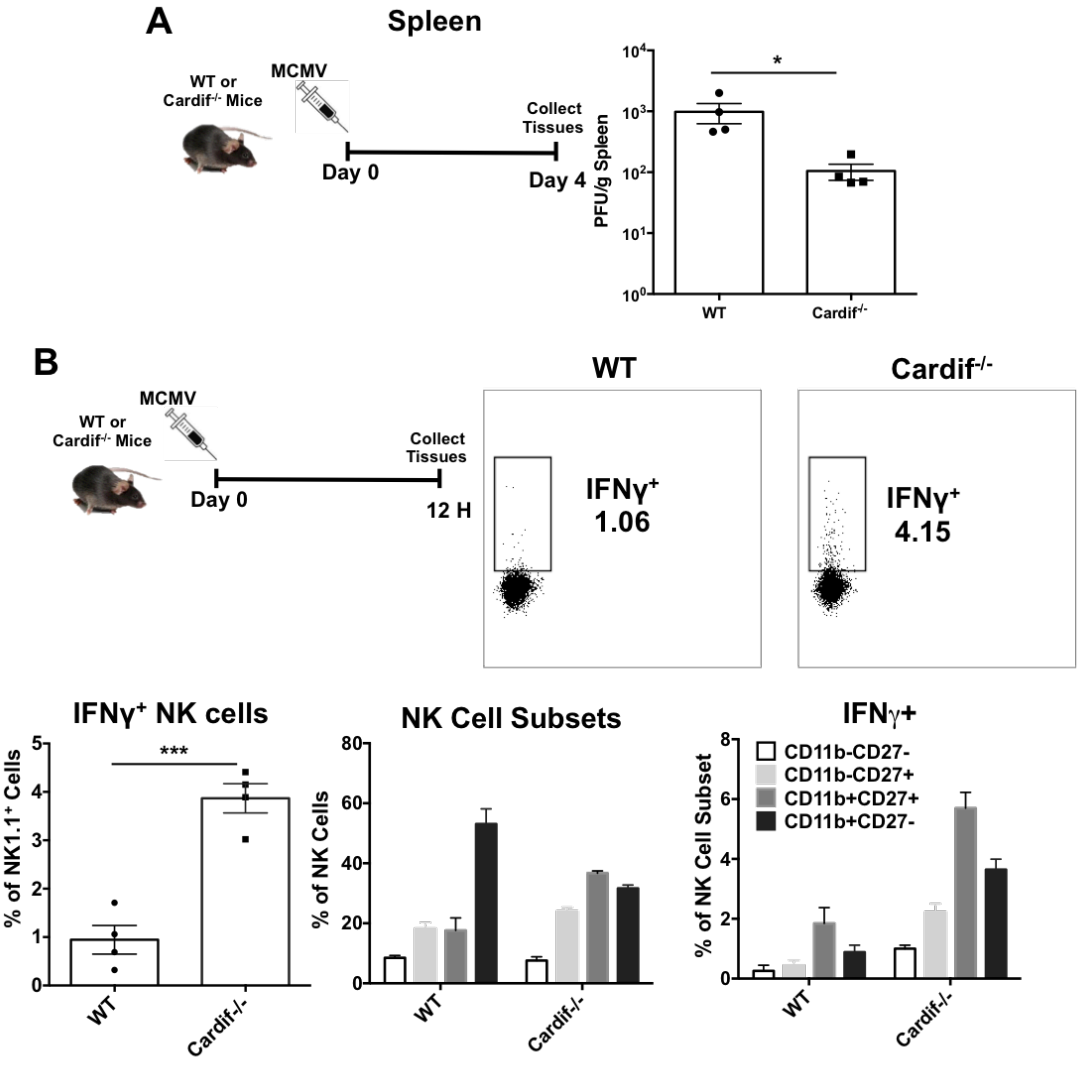
Figure 3.10. Exogenous IFN-β is unable to completely rescue the impaired maturation phenotype of Cardif$^{-/-}$ NK cells. (A) Bar graphs depicting the amount of NK cells (CD49b^{+}NKp46^{+}) expressing the maturation markers CD11b and after a 5-day *in* vitro treatment with IL-15 or IL-15+IFN-β. For all, n=3 mice/group/experiment. Data are means ± SEM. *$p<$ 0.05, **p<0.01, ***p<0.001.

Cardif$^{-/-}$ mice have increased control of MCMV infection

Many groups have clearly shown that NK cells are required for the control of MCMV (murine cytomegalovirus) in mice (45). NK cell function is often tested in vivo by examining the ability of infected mice to control MCMV. We decided to test Cardif$^{-/-}$ NK cell function in vivo by infecting Cardif$^{-/-}$ mice with MCMV and assessing MCMV replication. We found that at 4 days post-infection (dpi) Cardif$^{-/-}$ mice possess 10-fold lower amounts of MCMV within the spleen (Fig. 3.11A). Verma et al. described a burst of IFNγ that is produced by NK cells at 12h post infection in response to Type I IFN, that contributes to the early control of MCMV infection (46). This early control results in a baseline of viral replication that remains relatively constant through days 4 and 5 dpi. Mack et al. have also described an early production of IFNγ by NK cells that is STAT4 and Type I IFN dependent during LCMV infection (47). We hypothesized that the altered STAT4:STAT1 ratio present in Cardif$^{-/-}$ NK cells actually resulted in an increase in IFNγ production at 12h after viral infection. This increase in IFNγ production at 12h likely resulted in increased viral control at 4 dpi. Indeed, we found that 12h post infection, Cardif$^{-/-}$ NK cells produced ~80% more IFNγ than WT NK cells (Fig. 3.11B). All Cardif$^{-/-}$ NK cell subsets produced more IFNγ^{+} than their WT counterparts; however, the highest percentage of IFNγ^{+} cells was found in the less mature CD27^{+}CD11b^{+} NK cell subset rather than the mature CD27^{-}CD11b^{+} NK cells. The

$CD27^+CD11b^+$ NK subset had the highest percentage of $IFN\gamma^+$ cells in infected WT and $Cardif^{-/-}$ spleens, but $Cardif^{-/-}$ spleens had nearly twice as many $CD27^+CD11b^+$ NK cells compared to WT spleens (Fig. 3.11B). These data suggest that the increased prevalence of the $CD27^+CD11b^+$ NK cell subset as well as the augmented STAT4:STAT1 ratio contributed to the amplified production of IFNγ by $Cardif^{-/-}$ NK cells in response to MCMV infection in the spleen.

Figure 3.11. Cardif$^{-/-}$ mice have greater control of MCMV infection. (A) Schematic diagram of experimental procedures used for MCMV infection experiment (*left panel*). Bar graph portraying the amount of PFU/g of MCMV in the spleens of mice 4 d.p.i. (*right panel*). (B) Schematic diagram of experimental procedures used for MCMV infection experiment (*upper left panel*). Representative plots portraying IFNγ production by NK cells (*upper right panel*). Representative bar graph portraying the amount of PFU/g of MCMV in the spleens of mice 12h post infection (*lower left panel*). Bar graph portraying the percentage of NK cell subsets in the spleens of mice 12h post infection (*lower middle panel*). Bar graph portraying the percentage of IFNγ^{+} cells within NK cell subsets in the spleens of mice 12h post infection (*lower right panel*). For all, n ≥ 3 mice/group. Experiments were performed once (A) or twice (B). Data are means ± SEM. **p*< 0.05, ***p*<0.01, ****p*<0.001.


A
Spleen
WT or Cardif-/- Mice
MCMV
Day 0
Collect Tissues
Day 4
PFU/g Spleen
*
WT
Cardif-/-
B
MCMV
WT or Cardif-/- Mice
Day 0
Collect Tissues
12 H
WT
Cardif-/-
IFNγ+ 1.06
IFNγ+ 4.15
IFNγ+ NK cells
% of NK1.1+ Cells
***
NK Cell Subsets
% of NK Cells
IFNγ+
% of NK Cell Subset
CD11b-CD27-
CD11b-CD27+
CD11b+CD27+
CD11b+CD27-

Summary

We have discovered a novel intrinsic role for Cardif in the maturation and function of NK cells. It is widely accepted that Cardif is vital for antiviral signaling in response to RIG-I and MDA-5 activation; however, several studies indicate that Cardif and other components in this pathway have relatively unexplored non-viral functions (13–16). Cardif has been shown to reduce the severity of EAE in mice, activate the NLRP3 inflammasome, and regulate TLR7 expression in B cells. There are currently no reports however, describing the requirement for, or involvement of, Cardif in NK cell development. In this report, we describe a novel and cell-intrinsic role for Cardif in the homeostatic maturation and function of NK cells.

We discovered that Cardif mice have fewer splenic NK cells when compared to WT mice, and Cardif$^{-/-}$ NK cells are ~ 40-45% more likely to lack CD49b, a marker of mature NK cells. Splenic and liver Cardif$^{-/-}$ NK cells populations also have ~30% and ~60% fewer terminally differentiated NK cells, respectively. Markers associated with NK maturation, Ly49C/I and KLRG1 are also lower in Cardif$^{-/-}$ NK cells. Functionally, we found that Cardif$^{-/-}$ NK cells had lower cytotoxic activity, as Cardif$^{-/-}$ NK cells did not degranulate nor produce as much granzyme B, or kill target cells as well as WT NK cells. However, we observed no changes in IFNγ production in Cardif-deficient NK cells. We did not measure production of other cytokines by Cardif NK cells in

the current study. Since, STAT1 has been shown to regulate the cytotoxicity functions of NK cells while STAT4 governs IFNγ production by NK cells, we measured total STAT protein and protein phosphorylation. STAT1 protein levels and phosphorylation were considerably reduced in Cardif$^{-/-}$ NK cells. In contrast, pSTAT4/STAT4 protein levels were higher in Cardif$^{-/-}$ NK cells. These data suggest that the diminished cytotoxicity of Cardif$^{-/-}$ NK cells is due to the reduced intracellular levels of STAT1. Though there is an increase of pSTAT4/STAT4 in Cardif$^{-/-}$ NK cells, this increase may not have translated into increased levels of IFNγ because the levels of STAT4 are already high in WT NK cells. A slight increase in STAT4 in NK cells may not lead to increased production of IFNγ upon *in vitro* stimulation but the altered STAT1/STAT4 ratio could have greater consequences upon infection *in vivo.*

Cardif likely plays a role in stabilizing NK cells and preventing their apoptosis. A recent report indicated that Cardif associates with caspase-8 to induce apoptosis via caspase-3 in response to viral infection (48). Our data shows that Cardif deficiency increases apoptosis in NK cells, particularly in terminally differentiated NK cells. Moreover, our data suggests that the presence of Cardif decreases NK cell proliferation, predominantly in CD27^{+}CD11b^{-} and CD27^{+}CD11b^{+} NK cells. Cardif's apparent role in apoptosis and proliferation in NK cells explains much of the immature phenotype seen in NK cells from Cardif$^{-/-}$ mice (Fig. 3.1). The lack of proliferation in CD27^{+}CD11b^{-}

and $CD27^{+}CD11b^{+}$ NK cells suggests a lack of differentiation into terminally differentiated stage 4 NK cells, likely as a result of increased apoptosis. Together our data suggest that $Cardif^{-/-}$ mice have fewer total NK cells with a lower percentage of $CD27^{-}CD11b^{+}$ NK cells due to an increase of $CD27^{+}CD11b^{+}$NK cell death and a decrease in proliferation of $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells. NF-κB is a major downstream target of Cardif signaling along with Type-I IFN production (5, 6). NF-κB is known to be involved in cell survival in multiple cell types, including B and T cells (49). Yet, little is known about NF-κB activity in NK cells. A few reports suggest that changes in NF-kB signaling regulate NK cell proliferation, Ly49 expression, and increased apoptosis (49–51). NF-κB signaling is tightly regulated in NK cells and changes in NF-κB activity are likely to perturb NK cell survival. We did not measure NF-kB signaling in NK cells in the current study, but we surmise that the absence of Cardif decreases NF-κB signaling, thus impacting NK cell survival.

Our mixed bone marrow chimera experiments revealed that Cardif is intrinsically required for optimal terminal differentiation of NK cells. Moreover, Cardif is intrinsically required for optimal fitness and survival of NK cells in peripheral organs as $Cardif^{-/-}$ NK cell numbers were lower than WT NK cell numbers in the periphery of the mixed chimeric mice and $CD27^{-}CD11b^{+}$ terminally differentiated NK cells were nearly twice as likely to be derived from

WT mice rather than Cardif$^{-/-}$ mice in the periphery of mixed chimeric mice. We also found that increased apoptosis and decreased granzyme B levels in Cardif$^{-/-}$ NK cells are cell intrinsic. The decreased proliferation and increased degranulation that was observed in Cardif$^{-/-}$ mice however is not cell intrinsic.

These data suggest that Cardif affects NK maturation and function via cell-extrinsic and cell-intrinsic factors. We initially thought that the cell-extrinsic factor acting upon Cardif$^{-/-}$ NK cells was IFN-β; however, we found that treatment with IFN-β *in vitro*, though somewhat effective, is not able to completely rescue the maturation phenotype that is present in Cardif$^{-/-}$ NK cells. Type I IFN is known to indirectly affect the differentiation of NK cells by upregulating the amount of IL-15 and IL-15Rα present on dendritic cells (52, 53). IL-15 transpresentation by dendritic cells has been shown to have a dose-dependent effect on the development, maturation, and proliferation of NK cells and NKT cells (54–56). IL-15 transpresentation is required for the expression of NK1.1 on NKT cells in the thymus. We have found that fewer NKT cells in the thymus express NK1.1 in young Cardif$^{-/-}$ mice, which leads us to believe that there may be a perturbation in Type-I IFN production in Cardif$^{-/-}$ mice that leads to lower amounts of IL-15 transpresentation (data not shown). We also found that Cardif$^{-/-}$ dendritic cells express lower amounts of IL-15/IL-15Rα complexes when stimulated with TLR9 agonists (data not shown). The involvement of IL-15 transpresentation in the NK phenotype of Cardif$^{-/-}$ mice is

also supported by our data that informs us that Cardif$^{-/-}$ NK proliferation and cytotoxicity, two NK functions that are mediated by IL-15 transpresentation, is normal in a wild type environment. Thus it seems that Cardif regulates the maturation and function of NK cells by direct and indirect mechanisms.

We hypothesized that impaired IFN-β signaling and expression would be responsible for the NK cell phenotype present in Cardif mice, since Cardif signaling often leads to type I IFN production. We found however, that though exogenous addition of IFN-β was effective at increasing CD11b and KLRG1 levels, it was unable to completely rescue the immature phenotype of Cardif$^{-/-}$ NK cells. Further studies to delineate the role of type-I IFNs in the terminal differentiation of NK cells in the absence of Cardif and other RIG-I molecules will be of interest, particularly in the context of viral immunity and autoimmune diseases.

We used the MCMV infection model to test the function of Cardif$^{-/-}$ NK cells in the context of an NK-mediated disease. Surprisingly, we found that Cardif$^{-/-}$ mice controlled MCMV replication in the spleen better than WT mice 4 days post infection. This increased viral control was characterized by increased percentages of IFNγ^{+} NK cells in the spleen 12h post infection. It has been reported that this early production of IFNγ by NK cells, occurs in response to Type I IFN produced by marginal zone stromal cells in the spleen (46). Data from Mack et al. shows that very early after LCMV infection, a short

burst of IFNγ is produced by NK cells via the Type I IFN pathway rather than through IL-12 pathways (32). Also, Doring et al., has shown that Cardif$^{-/-}$ pDCs, mDCs, and macrophages produce IFN-α in response to MCMV infection at a rate similar to their WT counterparts (57). This suggests that the production of type I-IFN is not altered in Cardif$^{-/-}$ mice. Instead, it seems that Cardif$^{-/-}$ NK cells are more prone to produce IFNγ in response to Type I IFN stimulation. Hayakawa et al. found that CD27^{+}CD11b^{+} NK cells produced more IFNγ than CD27^{-}CD11b^{+} NK cells in response to cytokines and DC cross talk (22). We also found that CD27^{+}CD11b^{+} NK cells were more prone to become IFNγ^{+} in response to MCMV infection. The prevalence and contribution of CD27^{+}CD11b^{+} NK cells can partially account for the increased production of IFNγ by Cardif$^{-/-}$ NK cells. Not only did the immaturity of Cardif$^{-/-}$ NK cells result in increased IFNγ, the prevalence of unlicensed, Ly49C/I^{-} NK cells in Cardif$^{-/-}$ mice could also have also contributed. It has been reported that unlicensed NK cells are most active in the response to MCMV infection (58, 59). These unlicensed Ly49C/I^{-} NK cells do not function as well as licensed NK cells in the absence of viral infection, which explains the reduced cytotoxicity of Cardif$^{-/-}$ NK cells *in vitro* (38). However upon viral infection, these unlicensed NK cells benefit from the lack of inhibition and are more readily activated. Also, the altered STAT1/STAT4 ratio in Cardif$^{-/-}$ NK cells could prolong the production of IFNγ, since it would take longer for levels of STAT1 to raise high enough to

effectively block the pathway to STAT4 downstream of the Type I IFN receptor. The early control of MCMV infection by NK cells sets a “baseline” of MCMV production, that is not appreciably surpassed throughout the course of MCMV infection (46). This “baseline” of viral production gives insight into the lower levels of MCMV in Cardif$^{-/-}$ mice 4 days post infection. Taken together, these data suggest that Cardif$^{-/-}$ NK cells are more active than WT NK cells in response to viral infection. This conclusion is supported by findings of autoimmunity in humans.

Materials and Methods

Mice

Cardif$^{-/-}$ mice were a kind gift from the Shresta laboratory at La Jolla Institute for Allergy and Immunology. Cardif$^{-/-}$ mice were generated as described in Michallet, et al., and are on a C57BL/6 background (33). C57BL/6 mice purchased from The Jackson Laboratory (000664) or wild-type littermates were used as controls in experiments. Male and female mice were used at 6-12 weeks of age. All experiments adhered to the guidelines outlined by the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were euthanized by CO_2 inhalation.

Flow cytometry

Spleen and liver were harvested and pushed through a 40-µm strainer. Liver was perfused with PBS prior to collection and placed in RPMI supplemented with 10µm HEPES and 10% FBS. In addition, lymphocytes were separated from hepatocytes via density centrifugation. Red blood cells were lysed with RBC lysis buffer according to the manufacturer's protocol (BioLegend).

Cells were resuspended in FACS buffer (1%BSA and 0.1% sodium azide in PBS) and 1-4 x 10^6 cells were incubated in 100µl with anti-CD16/CD32 Ab (2.4G2) for 30 min on ice to block FCγRII/III receptor binding. Samples were then incubated with a mixture of fluorochrome-conjugated antibodies (BioLegend, eBiosciences, BD Biosciences) for 30 min on ice in the dark. LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) was used to determine cell viability. Intracellular staining of antibodies was performed after cells were fixed and permeabilized using 0.55% paraformaldehyde and Permeabilization Buffer (BD Biosciences). Samples were analyzed for cellular fluorescence on an LSR II (BD Biosciences) and data were analyzed with FlowJo software (Treestar). A complete flow cytometry gating strategy for peripheral NK cells is shown in figure 3.12.

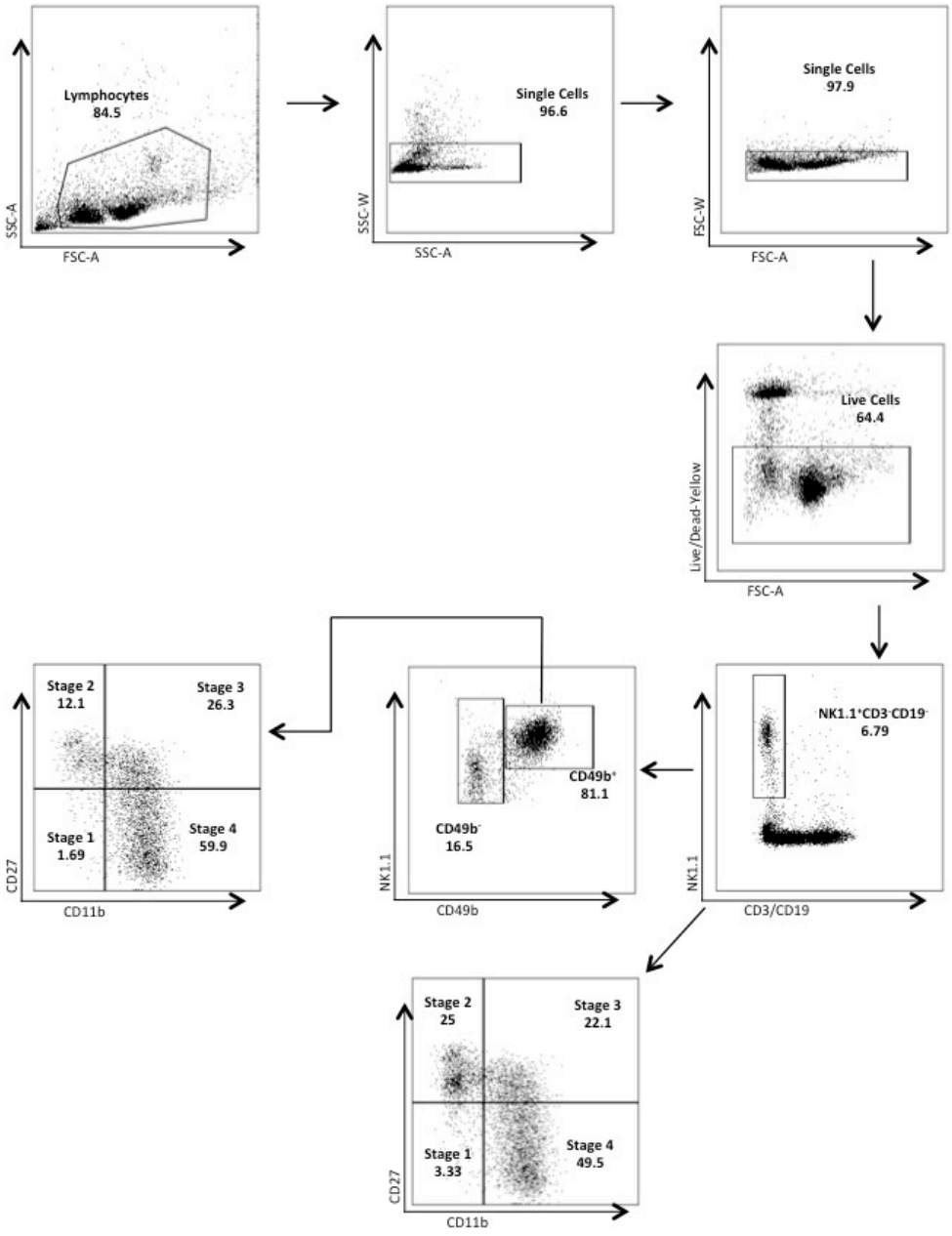


Figure 3.12. Gating strategy used to identify NK cells, mNK cells, and NK cell maturation stages. Briefly, lymphocytes were selected and then single and dead cells were excluded. NK cells were identified by the expression of NK1.1 and the absence of CD3 and CD19. NK cells were then either immediately divided into four stages based upon the expression of CD27 and CD11b or they were divided into these stages after CD49b positive NK cells were selected. The above plots are representative.

Ex vivo stimulation

Spleen was harvested and pushed through a 40-µm strainer. Red blood cells were lysed with RBC lysis buffer according to the manufacturer's protocol (BioLegend). Splenocytes were cultured in 96-well plates for 4-5 hours in the presence of either PMA (50ng/mL) and ionomycin (1µg/mL), or IL-12 (20ng/mL) and IL-18 (20ng/mL). When measuring degranulation, FITC-conjugated CD107a antibody (0.05ug/mL) was added to the stimulation media. After stimulation, splenocytes were washed with PBS and then prepared for flow cytometry analysis as described above.

In vitro culture

NK cells were enriched using EasySep™ Mouse NK Cell Enrichment kit (Stem Cell Technologies). Spleens from 3 or more mice were pooled together. Purity of NK cells was ~80-85% as reported by supplier. Murine cytokines were used in the following concentrations: IL-15 (50ng/mL) (PeproTech), IL-18 (50ng/mL) (PeproTech), IFN-β (10U/mL) (Millipore). There were triplicates of each condition. Cells were stimulated with PMA (50ng/mL) and ionomycin (1µg/mL) for 4 hours. After 4 hours, cells were harvested and processed for FACS analysis.

For type I IFN rescue experiments, NK cells were cultured in the presence of IL-15 only or IL-15 with IFN-β for 5 days at a concentration of $5e^6$

cells/ml in a 96 well round bottom plate. IL-15 was vital for the survival of NK cells in culture. After 5 days of cytokine treatment, NK cells were stimulated with PMA and ionomycin at the aforementioned concentrations and analyzed by flow cytometry.

Cytotoxicity Assay

Enriched NK cells were cultured at various ratios together with calcein labeled-YAC-1 cells for 4 hours. YAC-1 cells were labeled with 0.5uM calcein. Specific lysis was determined by measuring percent specific release of calcein using the following formula: Percent specific release = (experimental release – spontaneous release)/ (maximum release – spontaneous release) × 100.

Bone Marrow transplantation

Bone marrow transplantation studies were performed as previously described (34). CD54.1/.2 mice were irradiated with two doses of 550 rads each, 4 hours apart. Bone marrow cells were isolated from CD45.1 and Cardif$^{-/-}$ mice and processed under sterile conditions. A single-cell suspension in PBS was obtained with a 1:1 ratio of CD45.1 and Cardif$^{-/-}$ bone marrow cells. Approximately 5e^{6} cells were retro-orbitally injected into recipient CD54.1/.2 mice in a volume of 200µl. Mice received autoclaved water treated with antibiotics (trimethoprim-sulfamethoxazole), one week before and continued

until one week after injection. Mice were euthanized for experiments 8-10 weeks after injection.

Apoptosis and proliferation analysis

Annexin V (Invitrogen) and propidium iodide (Invitrogen) were used to identify apoptotic and dead cells respectively. Cells were stained according to the protocol provided by the manufacturer. FITC conjugated Caspase-3 (BD Biosciences) was also used to identify apoptotic cells. Cells were stained for intracellular caspase-3 according to the manufacturer's protocol. For proliferation, Ki-67 (eBioscience) was used to identify proliferating cells ex vivo. Splenocytes were stained for intracellular Ki-67 using the Foxp3/Transcription factor staining buffer set (eBioscience), and cells were stained according to the protocol provided by the manufacturer.

Western blot analysis

NK cells were isolated using EasySep™ Mouse CD49b positive selection kit (Stemcell technologies). Spleens from 7 or more mice were pooled together. Purity of NK cells was ~90% as reported by supplier. Isolated NK cells were then divided and either left untreated or treated with 100U/mL IFN-β (Millipore) for 15 min at 37°C. Cells were then lysed for protein collection using RIPA Buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA,

1 mM EGTA, 1 mM $NaVO_4$, 1 mM NaF, 0.5% NP40, 0.1% Brij35, 0.1% deoxycholic acid). Total was quantified using BCA Protein Assay Reagent (Thermo Scientific). Afterwards, 30µg of each protein sample was loaded into SDS-PAGE. The following antibodies were used at specified concentrations for immunoblots: STAT1 (1:1000; Cell Signaling #9172), pSTAT1 (Tyr701) (1:1000; Cell Signaling #9171), STAT4 (1:1000; Cell Signaling #2653), pSTAT4 (1:1000; Cell Signaling #5267), and β-Actin (1:2000; Cell Signaling #9774). Western blots were quantified using ImageJ software (NIH).

MCMV Infection

MCMV was prepared as described in Verma et al., 2013 (35). Briefly, salivary gland extract stocks of the MCMV K181 strain (referred to as MCMV throughout) were prepared in BALB/c mice as described previously. Cardif$^{-/-}$ and WT mice were infected intra-peritoneally (i.p.) with 1 x 10^5 PFU of the salivary gland-derived K181 strain of MCMV. The numbers of MCMV PFU in organs were measured by plaque assay in NIH 3T3 cells with a sensitivity of 3 to 10 PFU/g of organ.

Statistical analysis

All data are presented with mean ± SEM as determined by Prism software (GraphPad). Unpaired *t*-tests were used to compare WT and Cardif$^{-/-}$ samples. *P* values of 0.05 or less were considered significant for all experiments.

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Chapter 4

Cardif is Required for the Production of IFNγ by $CD4^+$ and $CD8^+$ T cells

Introduction

$CD4^+$ T cell subsets

T helper (CD4+) lymphocytes are immune cells important in mediating the adaptive immune response. There are several subsets of CD4+ T cells, each with a distinct phenotype. These CD4+ T cell subsets include TH1, TH2, TH17, and Tregs (1–3). TH1, TH2, and TH17 cells are effector or inflammatory subsets of $CD4^+$ T cells that are equipped to fight specific types of immune challenges. T regulatory cells however, are known for their regulation of immune responses and anti-inflammatory nature. Tregs are $CD4^+CD25^{hi}$ T cells that express the transcription factor Foxp3 (4, 5). Tregs can develop in the periphery or within the thymus (6–9). They are indispensible in the regulation of autoimmunity. Mutated FOXP3 causes IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome) in humans (10, 11). When cultured *in vitro* with other T cells, Tregs can suppress proliferation. Tregs also suppress inflammatory responses and autoimmunity *in vivo*. Interestingly however, the different $CD4^+$ T cell subsets are plastic, meaning that in certain conditions one type of $CD4^+$ T cell can change into another (1, 2, 12–15). TH17 and Tregs are particularly linked, since they both require TGF-β for their differentiation, and their driving transcription factors, RORγt and FoxP3 respectively, are both induced during their generation (2). The conditions that predispose tolerant and anti-

inflammatory Tregs to change into inflammatory effector $CD4^+$ T cells is an active area of research that seeks to better understand autoimmunity and loss of tolerance.

$CD8^+$ T cells

$CD8^+$ T cells, like $CD4^+$ T cells, are also important mediators of the adaptive immune system. Each $CD8^+$ T cell recognizes a specific antigen. Upon recognition, the $CD8^+$ that recognizes the antigen undergoes clonal expansion to produce a multitude of clones that recognize the same antigen(16). $CD8^+$ T cells mediate immune responses by recognizing and killing virally infected cells and releasing inflammatory cytokines such as IFNγ that prime other immune cells for robust responses.

Cardif and T cells

RIG-I, MDA5, and Cardif are all expressed in $CD4^+$ and $CD8^+$ T cells. However, there is very little research on the roles of RIG-I, MDA5, and Cardif in $CD4^+$ and $CD8^+$ T cells. Anz et al., found that RIG-I and MDA5 are expressed at higher levels than both TLR3 and TLR7 in Teff and Treg cells (17). MDA5 expression in Treg cells is vital for the inhibition of Treg function upon infection with EMCV, a viral agonist for MDA5.

Type I IFN is known to affect the development of T cells and Cardif leads to the production of type I IFN. Cardif has also been shown to affect immune homeostasis even in the absence of infection (18–21). With this information we hypothesized that Cardif could possibly affect the development and function of T cells. We sought out to determine the effect that Cardif has on the development and function of T cells.

Results

Cardif$^{-/-}$ mice have fewer CD4+ and CD8+ cells in the Thymus

Cardif is present in all lymphoid cells and is reportedly active in the absence of viral infection. Cardif potently induces type I IFN and NFκB target genes during viral infections. Both Type I IFN and NFκB are known to regulate T cell activity. The cytokines produced as a result of Cardif activity could regulate the differentiation of various CD4^{+} T cell subsets as well as the activity of CD8+ T cells. We analyzed various lymphoid organs of Cardif$^{-/-}$ mice to test if the absence of Cardif would affect T cell differentiation and development.

We found that in the thymus there was an alteration in the distribution of CD4+ and CD8+ cells in the T cell compartment (Fig. 4.1). We observed that the percentage of CD8+CD4- and CD4+CD8- cells were lower while the percentage of CD4+CD8+ cells was higher in Cardif$^{-/-}$ mice. This finding suggests that Cardif is involved in the development of CD4+ and CD8+ single-positive cells from CD4+CD8+ double positive cells in the thymus.

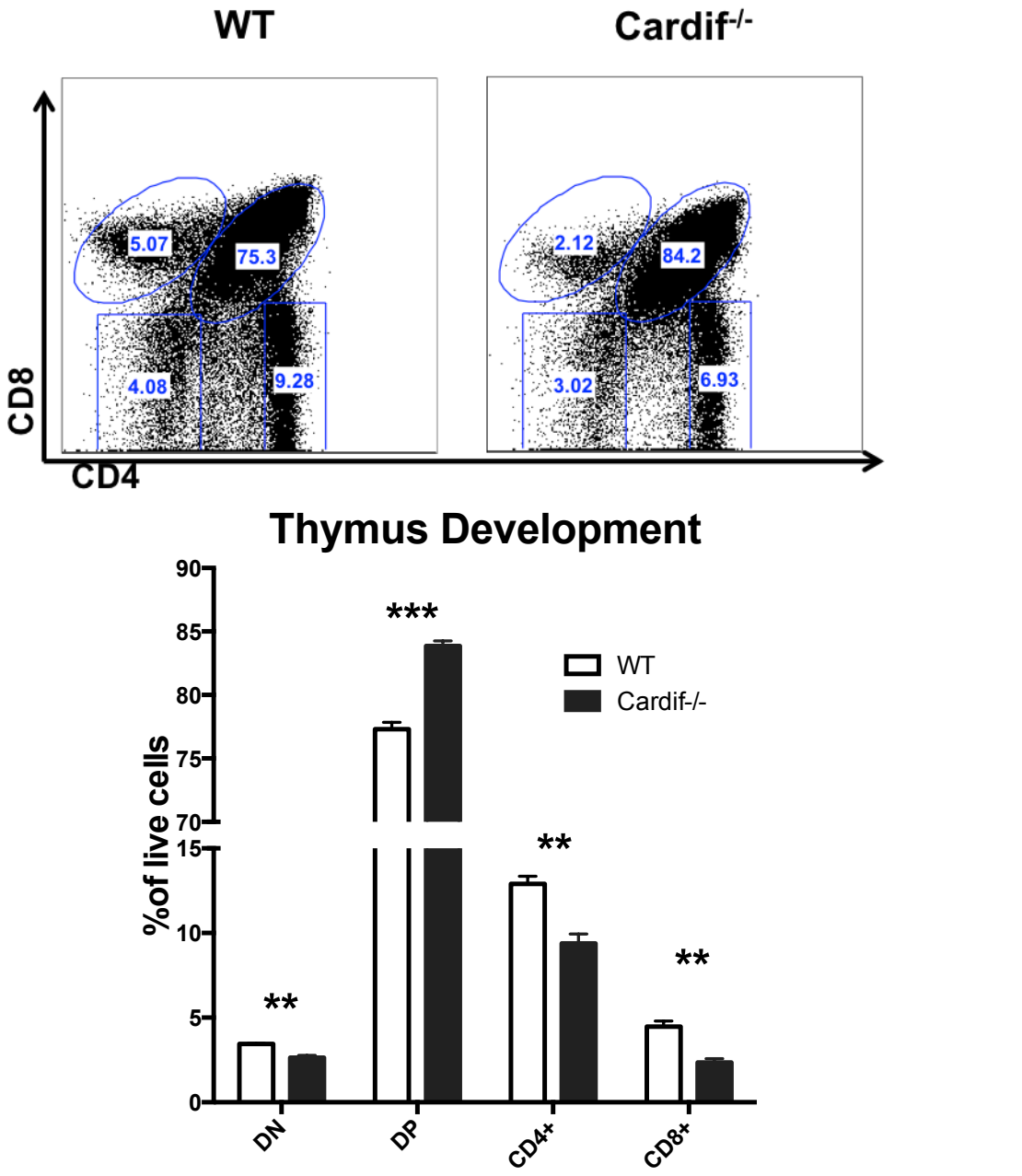

Figure 4.1. There are fewer CD4+ and CD8+ cells in the thymus of Cardif$^{-/-}$ mice. The frequencies of CD4+CD8-, CD4-CD8+, CD4+, CD8+, and CD4-CD8- cells in the thymus of wild type and Cardif$^{-/-}$ mice were calculated via flow cytometry. Experiment was performed once with at least 3 mice/group/experiment. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Cardif$^{-/-}$ mice have more TH1 and TH17 cells

We found no difference in the total numbers or frequency of CD4^{+} or CD8^{+} cells in the spleen or lymph nodes of Cardif$^{-/-}$ mice (Fig. 4.2). We did find however, that there was a ~50% increase in the frequency of TH1 cells in the spleen. TH2, TH17 and Treg cell percentages were roughly the same in the spleens of Cardif$^{-/-}$ mice. In the lymph nodes of Cardif$^{-/-}$ mice, we found that there was a trend of increased frequencies of TH1 cells (Fig. 4.3). TH2, Treg, and TH17 cell frequencies were the same in the lymph nodes of Cardif$^{-/-}$ and WT mice. From these data we concluded that Cardif is involved in the ability of CD4+ T cells to differentiate into IFNγ producing TH1 cells.

Teff:Treg percentage is greater in Cardif$^{-/-}$ mice

The ratio of T effector cells to Treg cells (T_{eff}:T_{reg}) in lymphoid organs is known to be a better indicator of the effectiveness or activity of T_{reg} cells than the frequency of T_{reg} cells alone. We calculated the ratio of T_{eff}:T_{reg} in the spleen and lymph nodes of Cardif$^{-/-}$ mice and found that there was a ~40% increase in the T_{eff}:T_{reg} ratio in the spleen compared to WT mice (Fig. 4.4). There was also an increase in the T_{eff}:T_{reg} ratio in the lymph nodes of Cardif$^{-/-}$ mice however the increase was not statistically significant. The increased T_{eff}:T_{reg} ratio in Cardif$^{-/-}$ mice may predispose these mice to chronic

inflammation due to the lack of an effective amount of Treg cells to regulate T effector cell activity.

Fewer Cardif$^{-/-}$ naive T cells differentiate into Tregs

Naïve CD4^{+} T cells can differentiate into iTregs (induced Tregs) in the presence of high amounts of TGF-β. We decided to investigate the ability of naïve Cardif$^{-/-}$ CD4^{+} T cells to differentiate into Tregs *in vivo*. We found that naïve Cardif$^{-/-}$ CD4^{+} T cells do not differentiate into Tregs as readily as their wild type counterparts (Fig. 4.5). In addition the Tregs produced from naïve Cardif$^{-/-}$ CD4^{+} T cells express lower amount of FOXP3. This data suggests that the imbalance in T_{eff}:T_{reg} cells in Cardif$^{-/-}$ mice is partially due to the decreased tendency of naïve CD4^{+} T cells to differentiate into Tregs.

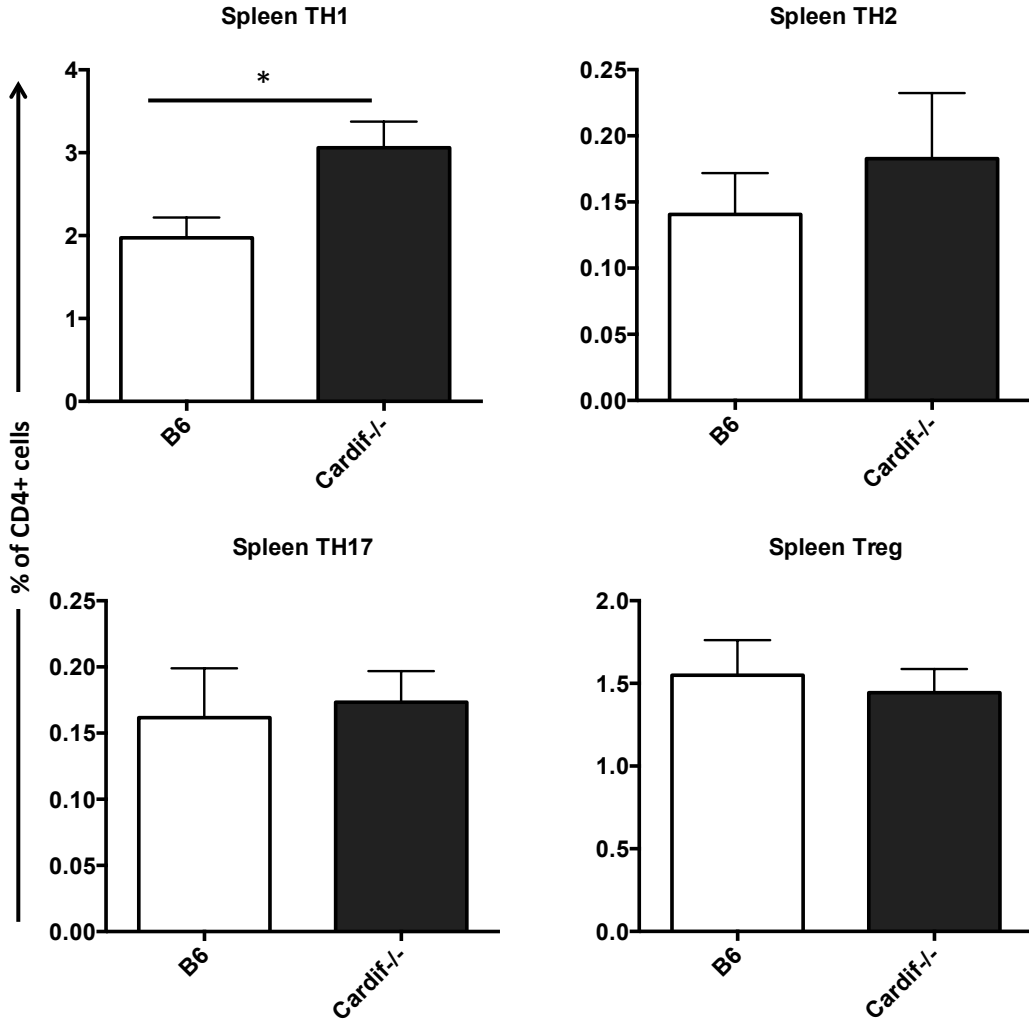

Figure 4.2. There are more IFNγ producing TH1 cells in the spleen of Cardif$^{-/-}$ mice. The frequencies of TH1, TH2, TH17, and Treg cells in spleens of wild type and Cardif$^{-/-}$ mice were calculated via flow cytometry. TH1 cells are CD4+ and IFNγ+; TH2 cells are CD4+ and IL-4+; TH17 cells are CD4+and IL-17+; Treg cells are CD4+, CD25+, and FoxP3+. Experiments were performed 4 times with at least 3 mice/group/experiment. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

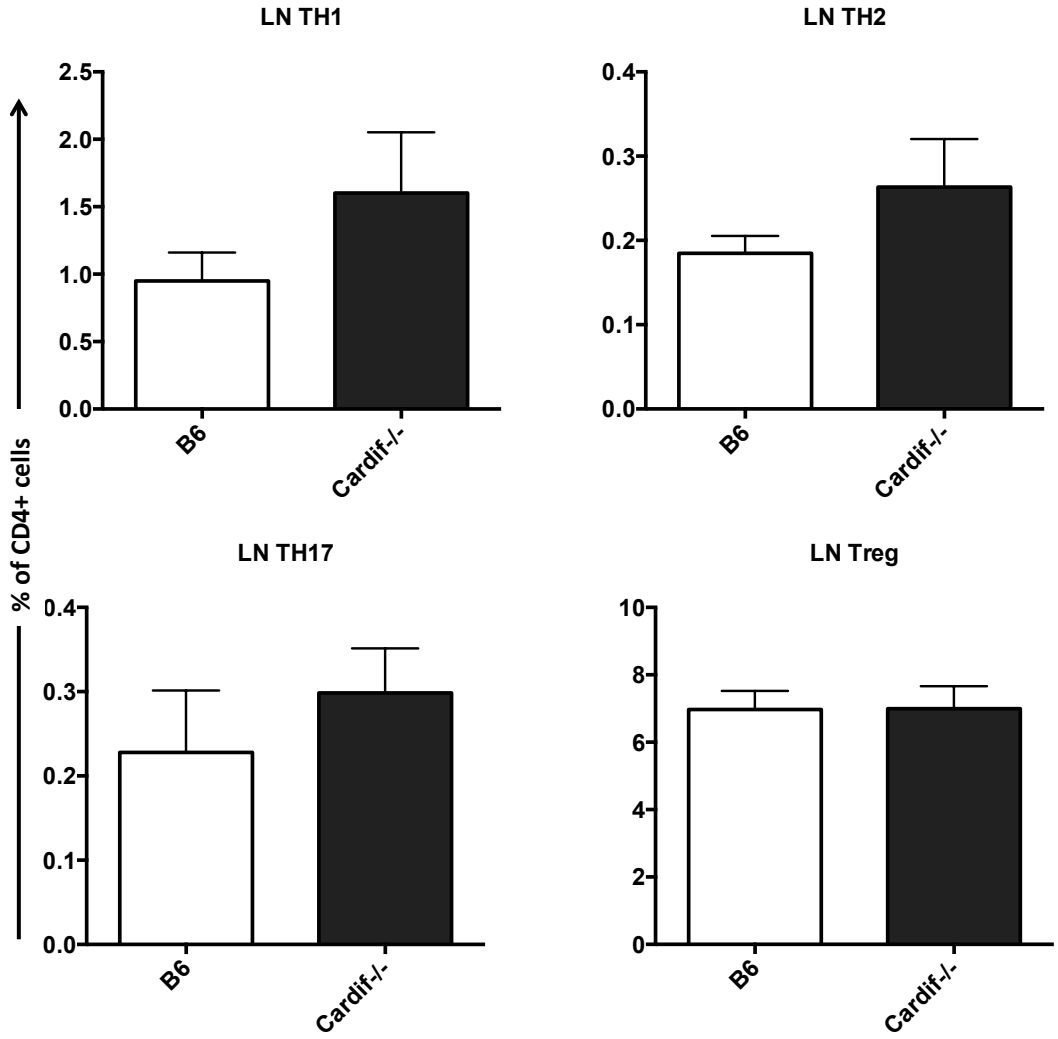

Figure 4.3. There are more TH1 and TH2 cells in the lymph nodes of Cardif$^{-/-}$ mice. The frequencies of TH1, TH2, TH17, and Treg cells in spleens of wild type and Cardif$^{-/-}$ mice were calculated via flow cytometry. TH1 cells are CD4+ and IFNγ+; TH2 cells are CD4+ and IL-4+; TH17 cells are CD4+and IL-17+; Treg cells are CD4+, CD25+, and FoxP3+. Experiments were performed 4 times with at least 3 mice/group/experiment. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

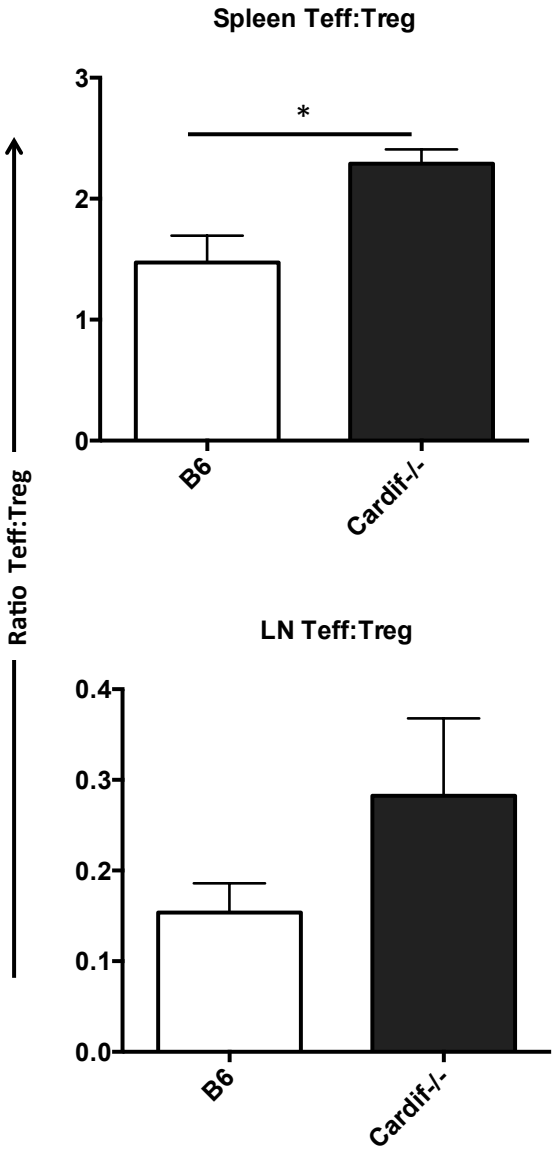

Figure 4.4. Cardif$^{-/-}$ mice have a higher T effector to Treg ratio than wild type mice. The ratio of Teff:Treg in the spleens and lymph nodes of wild type and Cardif$^{-/-}$ mice were calculated via flow cytometry. Experiments were performed 3 times with at least 3 mice/group/experiment. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

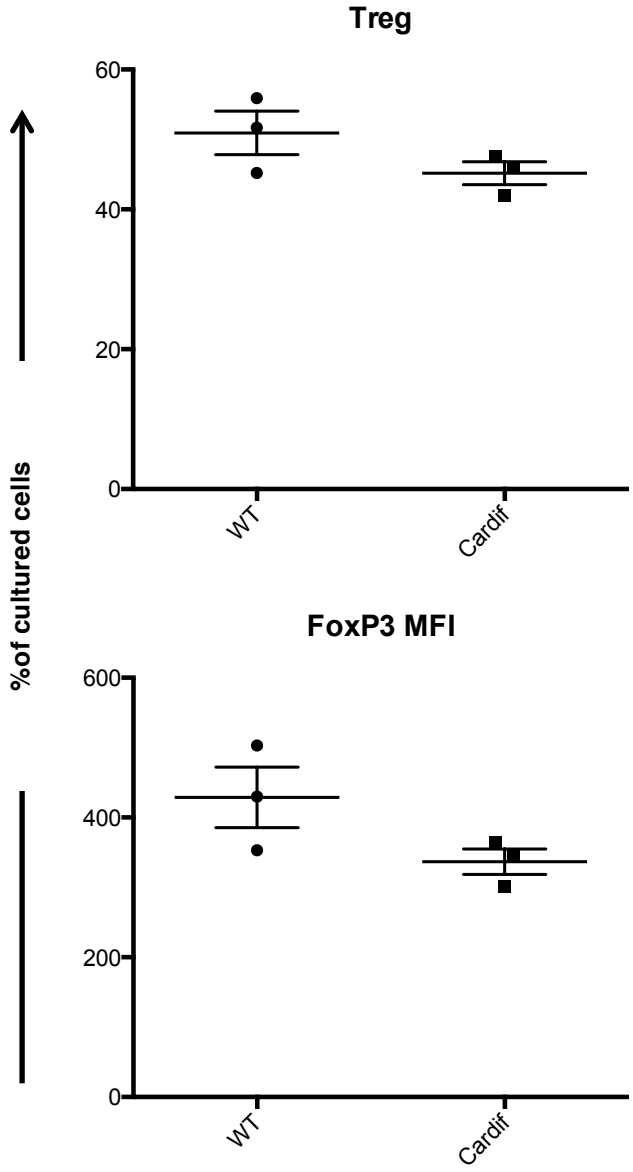

Figure 4.5. Cardif$^{-/-}$ naïve CD4^{+} T cells do not differentiate into Tregs *in vitro* as readily as their WT counterparts. Naïve CD4^{+} T cells from WT and Cardif$^{-/-}$ mice were cultured for 4 days. The percentage of Tregs was determined by FACS analysis. Experiments were performed with 3 mice/group. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

CD8 T cells produce more IFNγ

The production of IFNγ by $CD8^+$ T cells is an indicator of their functional capacity. Although there was not a change in the frequency of $CD8^+$ T cells in the periphery, we decided to test if there was a change in the function of $Cardif^{-/-}$ $CD8^+$ T cells. We found that there is a ~75% increase in the frequency of IFNγ^+ $CD8^+$ T cells in the spleens of $Cardif^{-/-}$ mice. Similarly, there was a ~50% increase in IFNγ^+ $CD8^+$ T cells in the lymph nodes of $Cardif^{-/-}$ mice (Fig. 4.6).

The increase in IFNγ production by CD8 T cells as well as CD4 T cells in the absence of Cardif suggests that Cardif is involved in regulating the production of IFNγ by T cells in WT mice.

Changes in T cells due to Cardif are cell-intrinsic

We decided to investigate whether the effects of $Cardif^{-/-}$ that we observed in T cells were cell-intrinsic. In order to test this we adoptively transferred naïve $CD4^+$ T cells from WT or $Cardif^{-/-}$ mice into $Rag^{-/-}$ mice. $Rag^{-/-}$ mice either received naïve $CD4^+$ T cells from WT or $Cardif^{-/-}$ mice. $Rag^{-/-}$ mice lack T cells and B cells, so all T cells harvested after the adoptive transfer would have come from the donor naïve $CD4^+$ T cells. After 10 days, we harvested spleens from the $Rag^{-/-}$ mice and analyzed them for $CD4^+$ T cell subsets via FACS analysis. We found that the adoptively transferred $Cardif^{-/-}$

$CD4^+$ T cells maintained their phenotype in the $Rag^{-/-}$ mice (Fig. 4.7). $Cardif^{-/-}$ $CD4^+$ T cells had significantly higher percentages of IFNγ-producing TH1 cells and there was a trend of higher percentages of TH2 and TH17 cells. There was also a trend of a slight reduction in the percentage of Tregs in $Cardif^{-/-}$ $CD4^+$ T cells compared to WT.

To further characterize the cell-intrinsic nature of Cardif on T cells, we used a mixed-bone marrow chimera approach. We sought to determine how WT and $Cardif^{-/-}$ T cells develop and differentiate in the same environment. In order to answer this question we used irradiated CD45.1/CD45.2 heterozygous mice as recipients and we transferred bone marrow from $CD45.2^+$ $Cardif^{-/-}$ and $CD45.1^+$ WT mice at a 1:1 ratio. We found that similar to the data from the thymus in the global knockout mice, there were lower percentages of $CD8^+$ T cells originating from $Cardif^{-/-}$ bone marrow (Fig. 4.8). We also noticed a significant increase of $Cardif^{-/-}$ TH17 cells in the spleen of the chimeric mice and a slight but significant decrease in $Cardif^{-/-}$ Tregs in the spleen (Fig. 4.9). However, unlike the global knockout and adoptive transfer experiments, we did not see an increase in $Cardif^{-/-}$ TH1 cells in the spleen or lymph node. Most surprisingly, we noticed that the previous phenotype of increased IFNγ production by $Cardif^{-/-}$ $CD8^+$ T cells is completely reversed in the bone marrow chimera (Fig. 4.10). We found that $Cardif^{-/-}$ $CD8^+$ T cells

produced significantly less IFNγ than WT $CD8^+$ T cells when in a wild type environment.

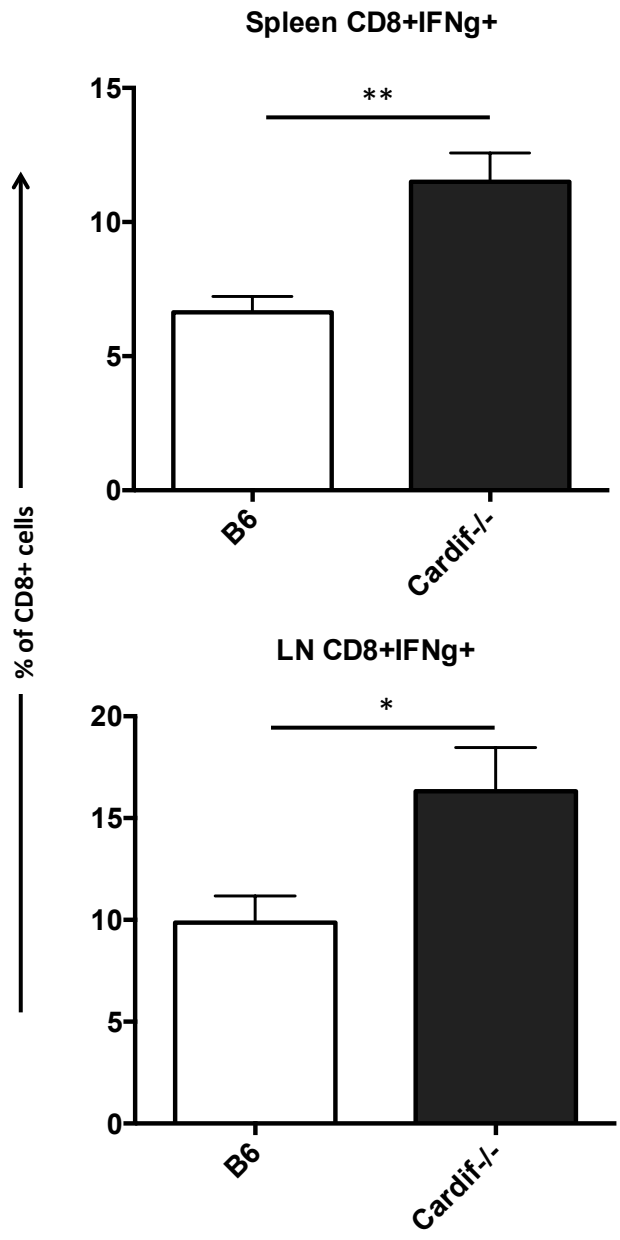

Figure 4.6. CD8+ cells from Cardif$^{-/-}$ mice produce more IFNγ than wild type CD8+ cells. The frequencies of IFNγ producing CD8+ cells in spleens and lymph nodes of wild type and Cardif$^{-/-}$ mice were calculated via flow cytometry. Experiments were performed 3 times with at least 3 mice/group/experiment. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

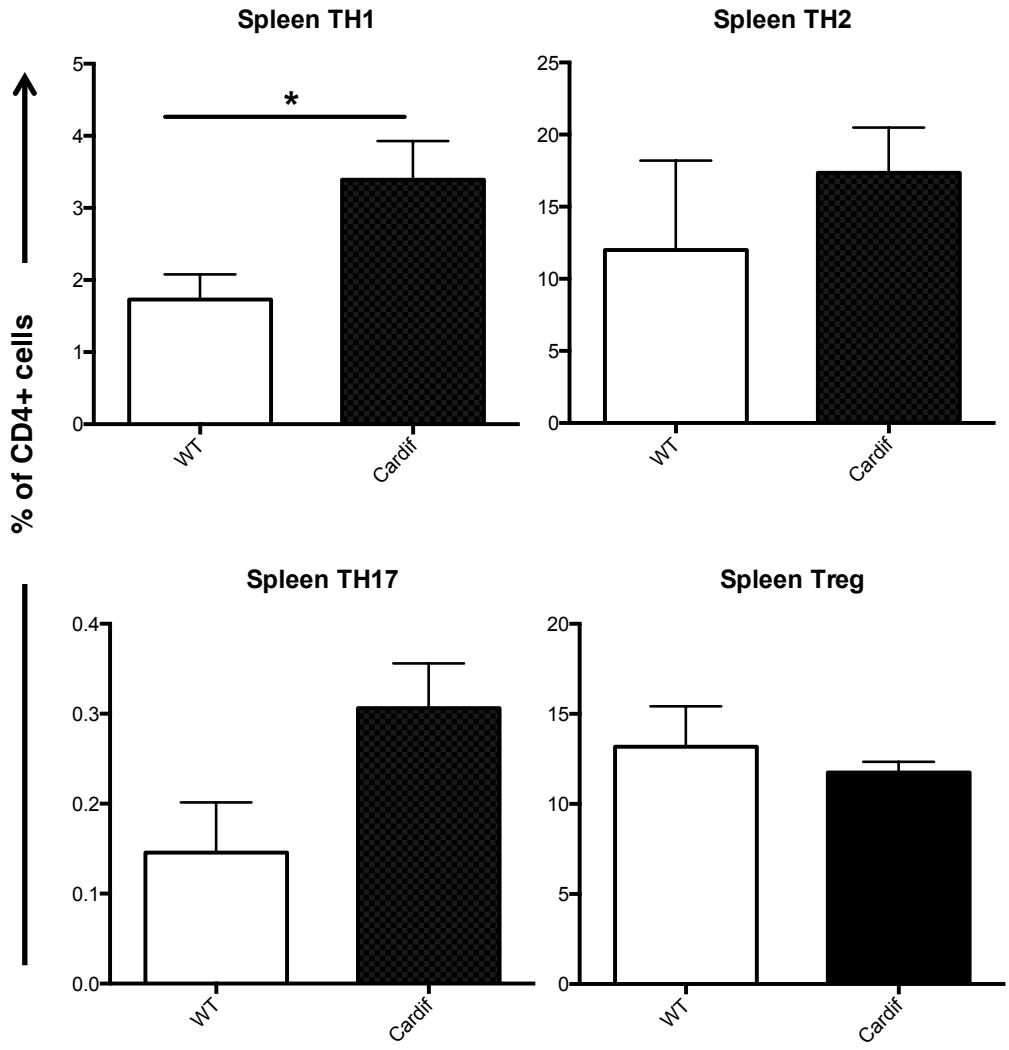

Figure 4.7. Cardif$^{-/-}$ CD4^{+} T cells produce more TH1 cells than WT in a similar environment. The frequencies of CD4+ T cell subsets in spleens of RAG$^{-/-}$ mice after 10-day adoptive transfer of WT or Cardif$^{-/-}$ naïve CD4^{+} cells was calculated via flow cytometry. Experiments had 4 mice/group. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

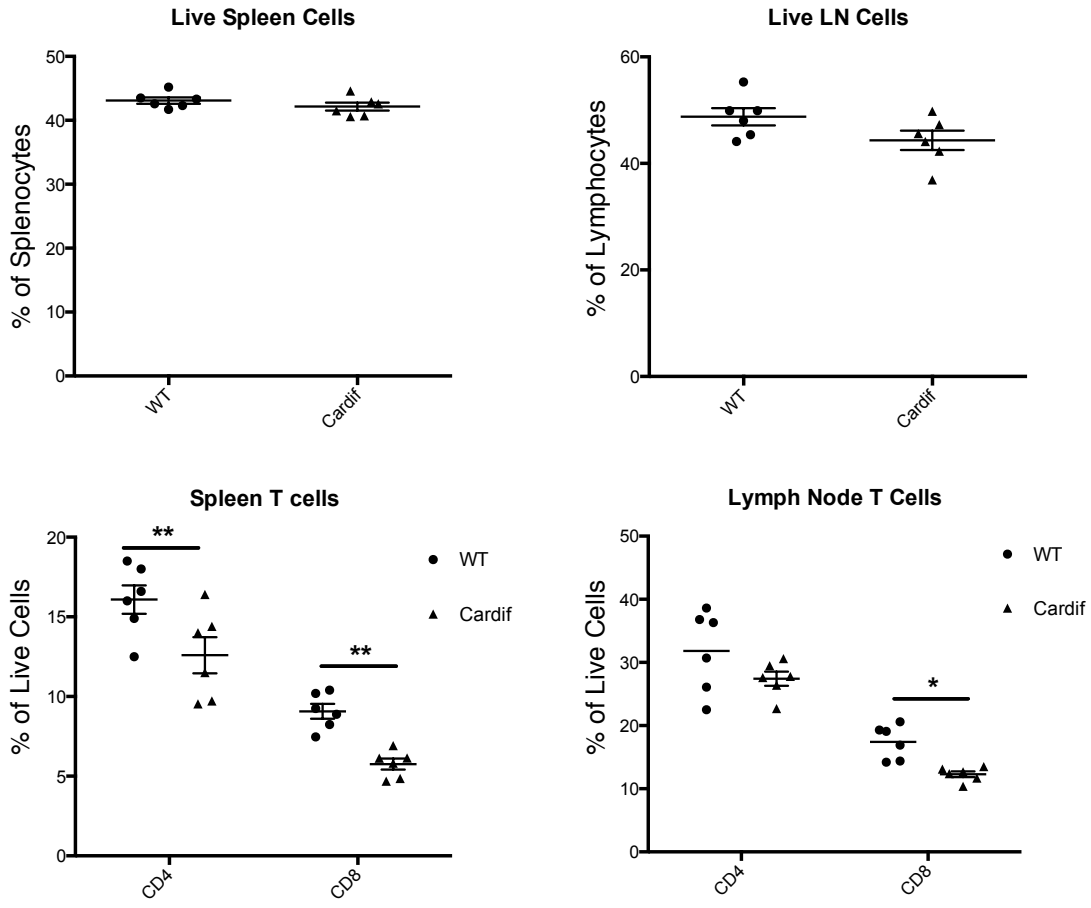

Figure 4.8. Cardif$^{-/-}$ CD8^{+} T cells are outcompeted in a WT environment. The frequencies of CD4^{+} and CD8^{+} T cells from WT and Cardif$^{-/-}$ donors in the spleens and lymph nodes of recipient mice mice after a 6-week bone marrow transplant was calculated via flow cytometry. Experiments had 7 mice/group. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

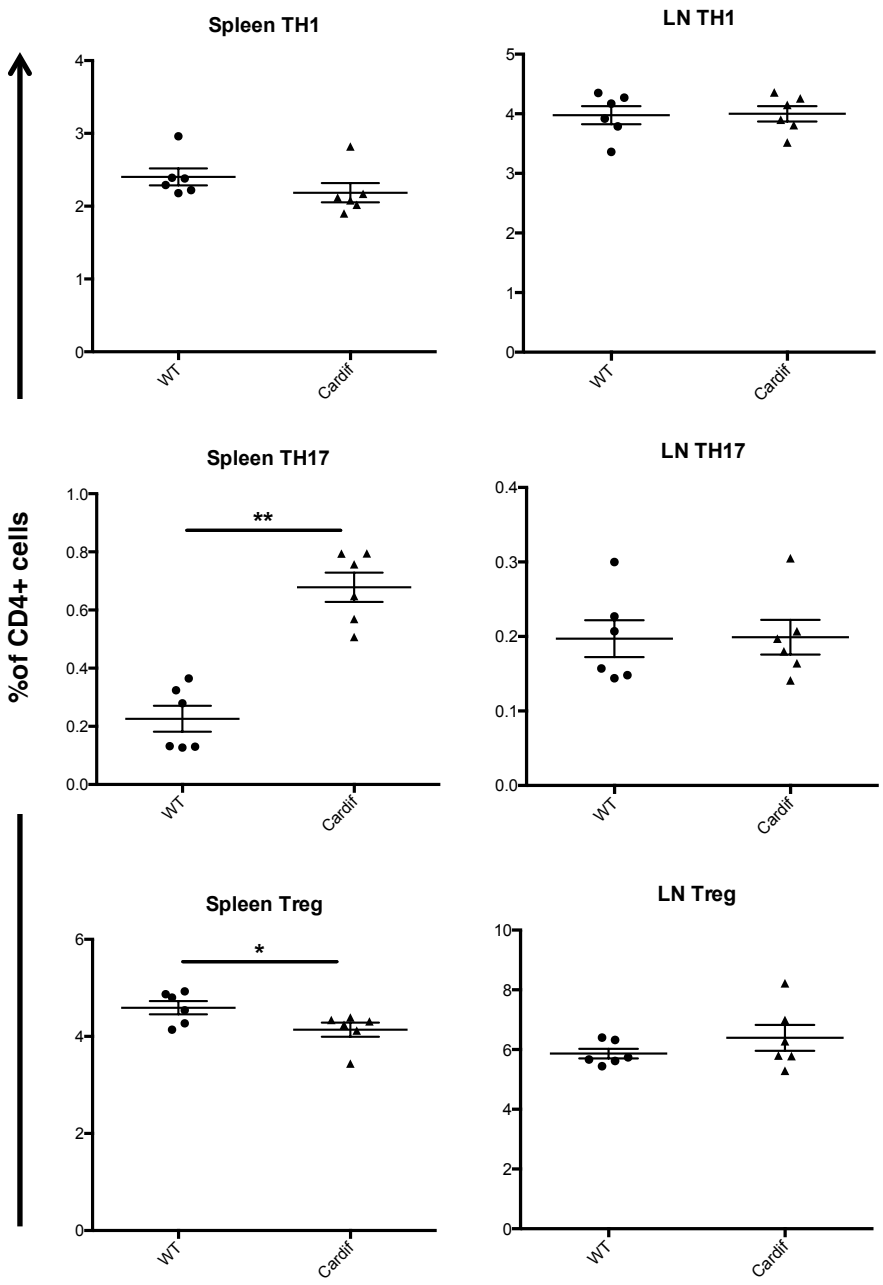

Figure 4.9. Cardif$^{-/-}$ CD4^{+} T cells produce more TH17 cells in the spleens of mixed bone marrow chimeric mice. The frequencies of CD4^{+} T cell subsets from WT and Cardif$^{-/-}$ donors in the spleens and lymph nodes of recipient mice after a 6-week bone marrow transplant was calculated via flow cytometry. Experiments had 7 mice/group. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

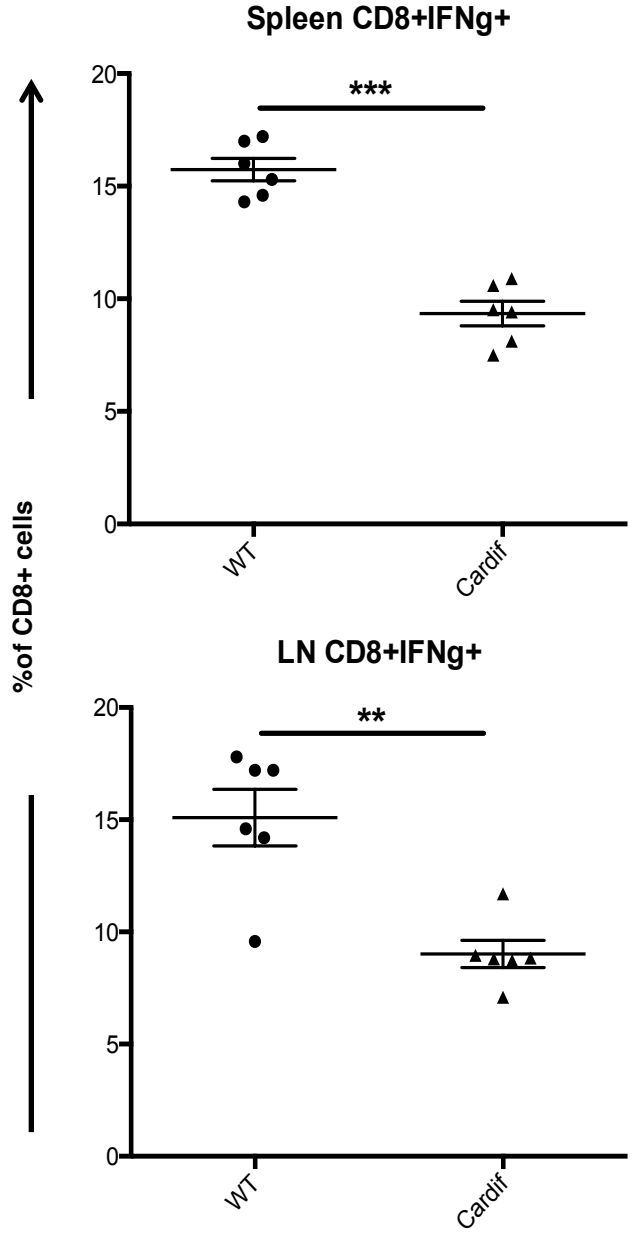

Figure 4.10. Cardif-/- CD8+ T cells produce fewer IFNγ+ cells in the spleens and lymph nodes of mixed bone marrow chimeric mice. The frequencies of CD8+IFNγ+ cells from WT and Cardif-/- donors in the spleens and lymph nodes of recipient mice after a 6-week bone marrow transplant was calculated via flow cytometry. Experiments had 7 mice/group. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Summary

We have found that Cardif$^{-/-}$ mice have altered T cell numbers and functionality. There are fewer CD4+ and CD8^{+} T cells in the thymus of Cardif$^{-/-}$ mice while there are greater numbers of double positive thymocytes. In addition, Cardif$^{-/-}$ mice have an increased T_{eff}:T_{reg} ratio in the periphery and naïve Cardif$^{-/-}$ CD4^{+} T cells do not differentiate into Tregs as readily as WT CD4^{+} T cells. Furthermore, CD8^{+} T cells from Cardif$^{-/-}$ mice produce more IFNγ when stimulated *ex vivo.*

We used adoptive transfers and mixed bone marrow chimeras to characterize the mechanism of Cardif's effects on T cells. We observed greater amounts of TH1 cells in the spleens of Rag$^{-/-}$ mice that received Cardif$^{-/-}$ naïve cells rather than WT naïve cells. Low percentages of Cardif$^{-/-}$ CD4^{+} and CD8^{+} T cells in the periphery of mixed bone marrow chimeric mice revealed that CD4^{+} and CD8^{+} T cells from Cardif$^{-/-}$ mice were not as fit as their WT counterparts while in a wild type environment. Unexpectedly, we found that fewer Cardif$^{-/-}$ CD8^{+} T cells from mixed bone marrow chimeric mice produced IFNγ when stimulated compared to CD8^{+} T cells of WT origin. This signifies

that Cardif extrinsically regulates the production of IFNγ by $CD8^+$ T cells. $Cardif^{-/-}$ CD8+ T cells may require the environment that is present in $Cardif^{-/-}$ mice in order to produce high amounts of IFNγ. Like NK cells, it is possible that STAT4 levels are increased in CD8+ T cells resulting in increased IFNγ production upon stimulation (22). However, in a wild-type environment, $Cardif^{-/-}$ CD8+ T cells do not receive the signals required to maintain high levels of STAT4. Yet, it seems that Cardif regulates $CD4^+$ T cell differentiation by both extrinsic and intrinsic methods, as the increased T_{eff} differentiation of $Cardif^{-/-}$ CD4+ T cells persisted in wild-type environments.

These data reveal a novel role for Cardif in the regulation of $CD4^+$ T cell subset regulation and $CD8^+$ T cell function and development. Cardif regulates these processes via cell-intrinsic and cell-extrinsic mechanisms, which could involve but is not limited to altered antigen-presenting cell (APC) maturation and homeostatic type I IFN production. Future studies should seek to identify the effect of Cardif on APCs and their interactions with T cells.

Materials and Methods

Mice

Cardif$^{-/-}$ mice were a kind gift from the Shresta laboratory at La Jolla Institute for Allergy and Immunology. Cardif$^{-/-}$ mice were generated as described in Michallet, et al., and are on a C57BL/6 background (23). C57BL/6 mice purchased from The Jackson Laboratory (000664) or wild-type littermates were used as controls in experiments. Male and female mice were used at 6-12 weeks of age. All experiments adhered to the guidelines outlined by the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were euthanized by CO_2 inhalation.

Flow cytometry

Spleen and lymph nodes were harvested and pushed through a 40-µm strainer. Red blood cells were lysed with RBC lysis buffer according to the manufacturer's protocol (BioLegend).

Cells were resuspended in FACS buffer (1%BSA and 0.1% sodium azide in PBS) or PBS and 1-4 x 10^6 cells were incubated with a mixture of fluorochrome-conjugated antibodies (BioLegend, eBiosciences, BD Biosciences) for 30 min on ice in the dark. LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) was used to determine cell viability. Intracellular staining of

antibodies was performed after cells were fixed and permeabilized using 0.55% paraformaldehyde and Permeabilization Buffer (BD Biosciences). Samples were analyzed for cellular fluorescence on an LSR II (BD Biosciences) and data were analyzed with FlowJo software (Treestar).

Ex vivo stimulation

Spleen was harvested and pushed through a 40-µm strainer. Red blood cells were lysed with RBC lysis buffer according to the manufacturer's protocol (BioLegend). Splenocytes (5 million/mL) were cultured in 96-well plates for 4-5 hours in the presence of PMA (50ng/mL) and ionomycin (1µg/mL). After stimulation, splenocytes were washed with PBS and then prepared for flow cytometry analysis as described above.

In vitro Treg differentiation

Naïve CD4+ T cells from WT and Cardif$^{-/-}$ were enriched using the CD4^{+}CD62L^{+} T Cell Isolation kit (Miltenyi Biotec). Spleens from 3 or more mice were pooled together. 96-well plates were coated with 1µg/mL anti-CD3. Murine cytokines were used in the following concentrations: anti-CD28 (1µg/mL) and TGF-β (5ng/mL) (Millipore). After 3-4 days, cells were harvested and processed for FACS analysis.

Adoptive Transfer

For homeostatic proliferation experiments, naïve CD4+ T cells from WT and Cardif$^{-/-}$ were enriched using the CD4^{+}CD62L^{+} T Cell Isolation kit (Miltenyi Biotec). Each mouse received 2x10^{6} naïve cells from WT or Cardif$^{-/-}$ origin. Cells were transplanted via retro-orbital injection into RAG-/- mice. After 10 days, the spleens and lymph nodes were harvested, processed, stained, and analyzed via flow cytometry.

Bone Marrow transplantation

Bone marrow transplantation studies were performed as previously described (24). CD54.1/.2 mice were irradiated with two doses of 550 rads each, 4 hours apart. Bone marrow cells were isolated from CD45.1 and Cardif$^{-/-}$ mice and processed under sterile conditions. A single-cell suspension in PBS was obtained with a 1:1 ratio of CD45.1 and Cardif$^{-/-}$ bone marrow cells. Approximately 5e^{6} cells were retro-orbitally injected into recipient CD54.1/.2 mice in a volume of 200µl. Mice received autoclaved water treated with antibiotics (trimethoprim-sulfamethoxazole), one week before and continued until one week after injection. Mice were euthanized for experiments 6 weeks after injection.

Statistical analysis

All data are presented with mean ± SEM as determined by Prism software (GraphPad). Unpaired *t*-tests were used to compare WT and Cardif$^{-/-}$

samples. *P* values of 0.05 or less were considered significant for all experiments.

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Chapter 5

Discussion

Cardif is a mitochondrial-localized adaptor protein that activates downstream signaling following its engagement with viral-RNA bound RLRs (RIG-I like receptors). Multiple groups have recently characterized new roles for Cardif besides the aforementioned pathway. We have discovered that Cardif$^{-/-}$ mice have reduced numbers of mature NK cells and increased numbers of immature and unlicensed NK cells. These NK cells do not function as well as their WT counterparts *in vitro* when responding to NK cell targets or anti-NK1.1 activation. In addition, Cardif$^{-/-}$ mice have low numbers of licensed NK cells as well as an altered STAT1:STAT4 ratio in NK cells, both of which inhibit their propensity to engage in cytotoxic responses. However, in the context of MCMV Cardif$^{-/-}$ NK cells produce more IFNγ due to their altered STAT1:STAT4 ratio and they are also more inclined to activate in response to MCMV infected cells due to the lack of licensed NK cells. Thus the very qualities that make Cardif$^{-/-}$ NK cells less fit in the absence of viral infection, are the same qualities that make them more active in response to MCMV, and possibly perpetrators in autoimmune disease.

Cardif$^{-/-}$ mice also have reduced numbers of CD8^{+} T cells in the thymus. These Cardif$^{-/-}$ CD8^{+} T cells also produce IFNγ more readily than WT CD8^{+} T cells when stimulated *ex vivo.* In addition, Cardif$^{-/-}$ mice have a higher T_{eff}:T_{reg} ratio and naïve Cardif$^{-/-}$ CD4^{+} T cells do not differentiate into Tregs as readily as naïve WT CD4^{+} T cells. Adoptive transfer and bone marrow chimera

studies revealed that Cardif has a cell-intrinsic and cell-extrinsic effect on T cells.

In total, our studies identify a pivotal role for Cardif, in the absence of viral signaling, in the maintenance of NK and T cell homeostasis. However, there are still remaining questions regarding the exact mechanisms for how Cardif regulates immune in the absence of viral infection.

Cardif and basal Type I IFN production

It is possible that the effect of Cardif on NK and T cells is partially due to a decrease in basal type I IFN signaling. Cardif signaling leads to type I IFN production, and basal type I IFN is known to be important for NK and T cells. Type I IFN can affect these cells specifically however, in the case of NK cells and $CD8^+$ T cells, IL-15 has a more profound impact (1). IL-15 is an interferon stimulated gene (ISG) and the amount of IL-15/IL-15rα complexes present on the surface of accessory cells such as DCs and macrophages, depends on type I IFN signaling (1, 2) (Fig.1). IL-15 has a dose-dependent affect on NK cells which is directly proportional to NK cell fitness (3). Low levels of IL-15 presentation result in impaired NK cell development and numbers. Mid-level IL-15 presentation however results in normal NK cell development but impaired maturation and function. We hypothesize that in $Cardif^{-/-}$ mice there is a slight reduction in type I IFN production, which then leads to mid-level

presentation of IL-15 by accessory cells and consequently, impaired NK cell maturation. Type I IFN may also have direct effects on NK cells that alter their maturation and function in Cardif$^{-/-}$ mice (4–6).

Recent studies that found that basal systemic IFNβ production is regulated by commensal bacteria support our hypothesis (7–12). Multiple studies found that commensal bacteria provide tonic immune stimulation that leads to type I IFN production which calibrates the activation threshold of the innate immune system in response to viral infection (9, 10, 12). NK cell priming is compromised in germ-free mice, which in turn hinders NK cell mediated antiviral immunity (10). In addition, optimal CD8^{+} T cell clonal expansion requires commensal bacteria (12).The Cardif pathway has been specifically implicated in monitoring commensal bacteria (8, 11). Together these studies suggest that the Cardif pathway is integral to the basal production of type I IFNs in response to commensal bacteria, which primes CD8^{+} T cells and APCs; APCs in turn prime and activate NK cells.

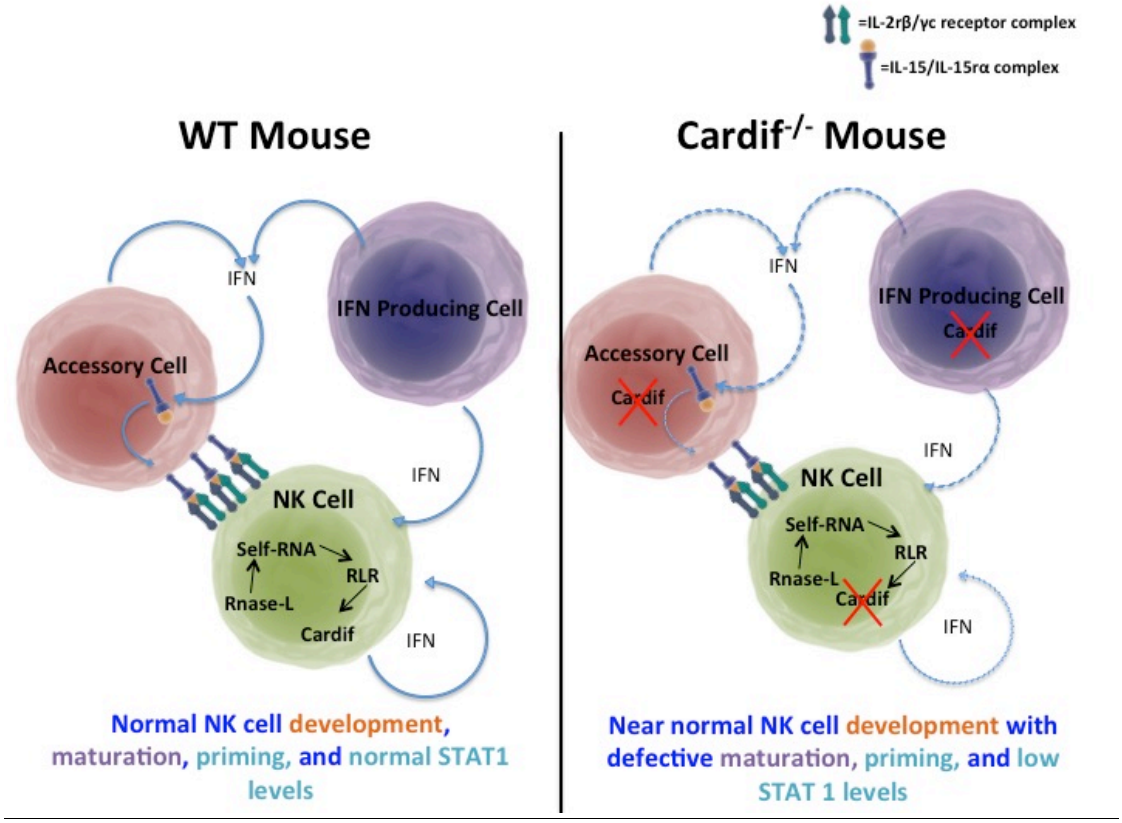

Figure 5.1. The affect of Cardif on NK cells and accessory cells causes defective NK maturation and function. Cardif expression in all cells leads to homeostatic type I IFN production. Type I IFN maintains optimal presentation of IL-15/IL-15rα complexes on the surface of accessory cells. Cardif may be activated by self-RNA ligands that activate RLRs.

Cardif and SLE

Increased Cardif NK cell and T cell activation in viral infections due to a lack of regulation could possibly lead to autoimmunity or autoinflammatory disease upon challenge. Indeed, Cardif has recently been associated with incidence of systemic lupus erythematosus (SLE) (13). Pothlichet et al. described a loss-of-function MAVS (Cardif) variant in humans that is associated with SLE in African-Americans (14). Low type I IFN levels, and a lack of autoantibodies specific for RNA-binding protein characterized patients with this variant. Similarly, Molineros et al. described a risk allele of *IFIH1* (MDA5) in African Americans that is associated with an increased risk of SLE with downregulation of type I IFN signaling (15).

Interestingly, SLE patients have reduced absolute numbers and a reduction in the cytolytic activity of NK cells, similar to the phenotype observed in Cardif$^{-/-}$ mice (16–20). SLE patients also have a reduction in Treg numbers (21). Furthermore, just as in Cardif$^{-/-}$ mice, the NK cells of SLE patients have an increase in the number of immature NK cells and a concomitant decrease of mature cytotoxic NK cells (16, 18, 20, 22). NK cells of SLE patients also have increased IFNγ expression (18, 19). Future studies should investigate Cardif signaling in NK cells in the context of SLE to further delineate the effect of Cardif on NK cells and SLE pathogenesis.

Basal activators of Cardif pathway

Our data suggests that Cardif regulates NK and T cell maturation and function in the absence of viral infection, and is consequently active without the presence of a virus. Several publications suggest that this is the case; however there isn't a clear mechanism for this action of Cardif (14, 23–25). There may be tonic signaling through the Rig-I/MDA-5-Cardif axis that is triggered by endogenous agonists. There have been reports of endogenous agonists of Rig-I such as the small self-RNAs produced by RNase-L, IRE1α, and endogenous retroviral elements in the human genome (26–31). Recently, Dupuis-Maurin et al. discovered that overexpression of the transcription factor Sp1 (specificity protein 1) activates the Rig-I-Cardif pathway in the absence of a pathogen by stimulating the OAS-RNase-L pathway to produce small self-RNAs (32). Tonic Rig-I/MDA-5-Cardif signaling activity stimulated by Sp1 or other transcription factors may lead to low-level production of IFN-β as well as NF-κB activation. It is also possible that Cardif is involved in other signaling cascades rather than the RIG-I/MDA-5-Cardif axis. Subramanian et al. recently reported the activity of Cardif in the NLRP3 inflammasome (33). Future studies are needed to identify exact mechanisms for Cardif's role in NK cell maturation.

Future Studies

The role of Cardif in the homeostasis of the immune system is an exciting new area of research in immunology. Future studies should focus on: 1) the mechanism for Cardif's activation outside of viral infection, 2) the role of Cardif in accessory cell presentation of IL-15, and 3) the direct products of constitutive Cardif activation and signaling. Cardif is active outside of viral infection. Studies that focus on the mechanism for Cardif activation during homeostasis could further delineate the cause of the NK cell phenotype in Cardif$^{-/-}$ mice and also reveal previously unknown immune homeostatic processes. Cardif is ubiquitously expressed and has roles in the function of cell types besides NK cells. It is possible that Cardif activity could regulate type I IFN production, which in turn regulates IL-15 transpresentation on DCs. IL-15 transpresentation is known to regulate NK cell proliferation, development, and maturation. Cardif activation is known to lead to the production of type I IFN as well as NF-κB target genes, however it is unknown of these products are involved in immune regulation in response to Cardif activation. Studies that would determine the products of Cardif activation in the absence of infection, would uncover the mechanism used by Cardif to regulate immune cells. These studies would not only help define the mechanism for the NK and T cell phenotype in Cardif$^{-/-}$ mice, they would also uncover new participants and mechanisms involved in immune homeostasis.

In sum, we have found that Cardif, a protein widely known for its role in antiviral signaling, is not only active in the absence of viral infection, it plays an integral role in NK cell and T cell homeostasis. Future studies will seek to identify sources of basal Cardif activation and the effect of Cardif on APCs.

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