ROLE OF NF-κB-ASSOCIATED FACTORS
IN T CELL DEVELOPMENT AND FUNCTION

A Dissertation in
Cell and Molecular Biology

by
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ABSTRACT

The immune system is a defensive mechanism that protects an organism from disease by identifying and neutralizing invading pathogens. The proper functioning of the mammalian immune system requires participation and cooperation of different immune cell types, with the ability to distinguish self from non-self. Despite its protective functions, the immune system can also be a double-edged sword, as aberrant activation of the immune system can cause chronic inflammation and autoimmune diseases. Therefore, it is important to understand the mechanism by which immune responses are generated and regulated. Various intracellular signal transduction pathways and transcription factors are involved in the activation and regulation of immune responses.

NF-κB is a key transcription factor in the immune system that regulates the development, function, homeostasis, and survival of various immune cells. NF-κB activation typically occurs after a stimulatory signal received at the cell surface is relayed to NF-κB through complex intracellular signaling pathways. Therefore, the outcome of NF-κB activation is critically determined by the function of molecules involved in the NF-κB signaling pathways. This thesis addresses the role of NF-κB-associated molecules in the development, differentiation, and function of T cells. Our studies show that CYLD, a negative regulator of NF-κB activation, is critical for development of natural killer T (NKT) cells. In addition, Carma1, a key molecule involved in the T cell receptor-mediated NF-κB activation, is required for the development of suppressive regulatory T cells (Tregs). Lastly, p105, a NF-κB precursor molecule with NF-κB
inhibitor function, plays an important role in the regulation of T cell homeostasis and prevention of chronic inflammation. The major findings are summarized below.

1. **CYLD regulates IL-7 signaling and ICOS expression in the development of natural killer T cells.** Natural killer T (NKT) cells modulate immune responses against pathogens and tumors. Utilizing CYLD-deficient mice, we show that CYLD, a deubiquitylase that negatively regulates NFκB activation, plays a critical role in NKT cell development. Unlike most of the known regulators of NKT cell development, CYLD is dispensable for NKT cell maturation but is required for the survival of immature NKT cells. The survival defect of immature NKT cells was associated with decreased expression of ICOS, a costimulatory molecule required for the survival and homeostasis of NKT cells, and signaling attenuation of an NKT-survival cytokine, IL-7. CYLD positively regulate ICOS expression and IL-7 signaling by preventing hyper-activation of NF-κB in immature NKT cells. Thus, CYLD regulates NKT cell development through a mechanism involving modulation of IL-7 signaling and ICOS expression.

2. **Carma1 regulates development of regulatory T cells in the thymus.** Regulatory T cells (Tregs) play a critical role in the preservation of self-tolerance and prevention of autoimmunity. We show that Carma1, a signaling molecule involved in the T cell receptor (TCR)-mediated NF-κB activation, is essential for development of Tregs in the thymus. Carma1 deficiency leads to attenuation of IL-2 signaling, a cytokine that is critical for the development of Tregs in the thymus. This IL-2 signaling defect in Carma1-knockout mice is associated with low expression of IL-2 receptor β subunit (IL-
2Rβ). Thus, Carma1 regulates thymic Treg development through modulation of IL-2 signaling via the IL-2Rβ.

3. **NF-kappa B1 p105 regulates T cell homeostasis and prevents chronic inflammation.** NF-κB transcription factors are regulated by a family of inhibitors called IκBs. NF-κB1 precursor, p105, contains IκB-like domain in their C-terminal and can function as NF-κB inhibitors. In this study, we show that p105 plays an important role in the regulation of T cell homeostasis and prevention of chronic inflammation. Mice that express mature NF-κB1, p50, in the absence of p105, spontaneously develop T cell-mediated intestinal inflammation. Consistent with the inflammatory phenotype, p105-deficient mice have increased frequency of memory/effector T cells in the peripheral lymphoid organs. Additionally, p105 deficiency renders CD4⁺ T cells more resistant to Treg-mediated suppression, while promoting differentiation of inflammatory Th17 CD4⁺ helper cells. Therefore, p105 plays critical roles in the regulation of T cell homeostasis and differentiation and the control of chronic inflammation.
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<tr>
<td>α-GalCer</td>
<td>α-galactosylceramide</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>APECED</td>
<td>autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
</tr>
<tr>
<td>CTLA</td>
<td>cytotoxic T lymphocyte antigen</td>
</tr>
<tr>
<td>CYLD</td>
<td>cylindromatosis</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>double-negative</td>
</tr>
<tr>
<td>DP</td>
<td>double-positive</td>
</tr>
<tr>
<td>DUB</td>
<td>deubiquitylating enzyme</td>
</tr>
<tr>
<td>E1</td>
<td>ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalitis</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoresis mobility shift assays</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible co-stimulator</td>
</tr>
<tr>
<td>ICS</td>
<td>intracellular cytokine staining</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
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<tr>
<td>IPEX</td>
<td>Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked</td>
</tr>
<tr>
<td>JAMM</td>
<td>JAB1/MPN/Mov34 metalloenzyme</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MJD</td>
<td>Machado-Josephin domain</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>NKT</td>
<td>natural killer T</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>RHD</td>
<td>Rel homology domain</td>
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<tr>
<td>RIP1</td>
<td>receptor-interacting protein 1</td>
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<tr>
<td>SP</td>
<td>single-positive</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>TAK1</td>
<td>TGF activated kinase 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>transcriptional activation domain</td>
</tr>
<tr>
<td>TECs</td>
<td>thymic epithelial cells</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
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<tr>
<td>Tregs</td>
<td>regulatory T cells</td>
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<tr>
<td>UCH</td>
<td>ubiquitin C-terminal hydrolase</td>
</tr>
<tr>
<td>USP/UBP</td>
<td>ubiquitin-specific protease</td>
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1.1 Immune System

The immune system is a defensive mechanism within an organism that provides protection against invading pathogens from the environment. Without a properly functioning immune system, humans would succumb to diseases caused by various pathogens such as bacteria, viruses, parasites and fungi. Through evolution, vertebrates have developed a sophisticated multi-layered immune system comprised of many types of cells, tissues and organs. In the vertebrate immune system, there are two separate but collaborative components: innate immunity that provides rapid but short-term protection and adaptive immunity that provides slower but long-term protection.

1.1.1 Innate Immunity

Innate immunity is a set of broad defensive mechanisms that provide an immediate but non-specific response. Anatomical barriers such as skin and mucous membranes of respiratory and gastrointestinal tracts provide the physical barriers to prevent entry by pathogens. Physiological barriers, such as low pH level in the stomach, ensure that only few of the ingested pathogens can survive. When pathogens invade the host tissue, subsequent tissue damage triggers an inflammatory response leading to vasodilatation, increased capillary permeability, and recruitment of various cells of the immune system. Recruitment of phagocytes such as monocytes, neutrophils, tissue macrophages and dendritic cells, is an important innate defense mechanism, which precedes ingestion of extracellular pathogens by phagocytosis. A limited number of germline-encoded receptors, known as pattern recognition receptors (PRR), allow phagocytes to recognize the pathogens. PRRs bind to pathogen-
associated molecular patterns (PAMPs) expressed on pathogens, which can consist of cell-wall structures, proteins, and nucleic acid materials. The activation of the complement system, consisting of diverse serum proteins, contributes to the innate immune response by facilitating phagocytosis through opsonization, direct lysis of pathogens, and propagation of inflammatory responses. However, some pathogens, such as intracellular bacteria and viruses, can evade these defense mechanisms by hiding inside the host cell. To combat these pathogens, the innate immune system employs the services of natural killer (NK) cells.

NK cells are unique members of the innate immune system that share common early progenitor cells with T cells of the adaptive immune system. They contain large numbers of cytotoxic granules containing perforin and granzymes, which can be released upon activation to eliminate pathogen infected cells. The activity of NK cells is regulated by a balance of signals from inhibitory and activating receptors on the cell surface. The importance of NK cell function is demonstrated by various immune deficiency syndromes that feature NK cells defects, which share severe herpes virus infection as common clinical sequelae. In addition to their direct lytic function, NK cells also express molecules such as CD40L and OX40L, which can provide co-stimulatory signals for T and B cells of the adaptive immunity. Therefore, NK cells serve as excellent examples of functional integration between innate immunity and adaptive immunity.

1.1.2 Adaptive Immunity

The adaptive immune system has developed in vertebrates to provide a stronger immune response when innate immune response alone may be insufficient. The distinguishing features of adaptive immunity are its ability to mount antigen-specific
responses and provide immunological memory. The adaptive immune response is mediated by B and T lymphocytes. B cells and T cells recognize pathogen-derived antigens through their cell surface receptors, B-cell receptor (BCR) and T-cell receptor (TCR), respectively, which consist of an invariant domain and a highly variable domain that confers antigen specificity. Antigen specificity is generated through somatic hyper-mutation and recombination of antigen receptor gene segments, which allow generation of vast numbers of antigen receptors from a limited number of receptor genes.

The major function of B cells is to produce immunoglobulins (Igs) (antibodies) that circulate in blood and neutralize pathogens, in a process known as humoral immunity. B cells produce five isotypes of antibodies: IgA, IgD, IgE, IgG, and IgM, each of which has unique functional property. After cognate antigen recognition by BCR, a membrane-bound antibody, B cells are activated to expand and differentiate into antibody-secreting plasma cells. Although most of the plasma cells are short-lived, some plasma cells survive to become long-lived antigen-specific memory B cells. Antibodies produced by B cells circulate in blood and bind antigens, facilitating phagocytosis and complement activation.

In contrast to B cells, which can directly recognize antigens through the BCR, T cells recognize processed antigenic peptides in the context of class I and class II major histocompatibility complex (MHC) molecules. Cells of the innate immune system, dendritic cells (DCs) in particular, play an essential role in the T cell activation by serving as antigen presenting cells (APCs). After phagocytosis of pathogen, DCs degrade the pathogen and present pathogen-specific antigens in complex with MHC. Exogenous antigens are usually presented in complex with MHC class II by professional APCs, whereas endogenous antigens are presented in complex with MHC class I, which is expressed by all host cells. However,
some APCs can present exogenous antigens in complex with MHC class I through a process called cross-presentation \(^9\). Importantly, each MHC class type stimulates a distinct subset of T cell population.

T cells are divided into two major functional groups, which can be identified by the expression of different TCR coreceptors: CD4\(^+\) T cells and CD8\(^+\) T cells. In addition to serving as identifying markers, CD4 and CD8 TCR coreceptors enhance T cell stimulation by enhancing TCR-MHC/peptide binding \(^{10,11}\). CD8\(^+\) T cells are activated by TCR stimulation by peptide-MHC class I complex in the presence of CD28 costimulation. Upon activation, CD8\(^+\) T cells undergo clonal expansion and gain cytotoxic effector function. When cytotoxic CD8\(^+\) T cells encounter infected or dysfunctional host cells, they release substances, such as perforin and granzymes that cause target cell apoptosis. CD4\(^+\) T cells, on the other hand, express TCR that recognizes peptide-MHC class II complex. Activated CD4\(^+\) T cells, for the most part, do not have direct cytotoxic function, but they manage overall immune response through secretion of various cytokines, which facilitate the function of other immune cells. However, CD4\(^+\) T cells make up a heterogeneous group of effector cells with distinct functions.

1.1.3  **CD4\(^+\) T helper cell differentiation**

The ability of CD4\(^+\) T cells to have a broad impact on the immune response depends on their capacity to differentiate into a variety of effector subsets. When naïve CD4\(^+\) T cells are stimulated, they can differentiate into T helper (Th)1, Th2, Th17, or regulatory T cells (Tregs). The differentiation program is primarily driven by the cytokines present in the cellular microenvironment, which activate lineage-specific transcription factors that mediate
the development and function of each subset. Each subset has specific functional characteristics, which often oppose the functions of other subsets. Therefore, a balance between the CD4+ T cell subsets is important, since skewing of T helper activity towards any particular subset can lead to development of disease.

Th1 cells are characterized by their production of signature cytokine IFN$\gamma$, which boosts cellular immune response by activating cytotoxic CD8+ T cells and macrophages. The development of Th1 cells is facilitated primarily by cytokines IL-12 and IFN$\gamma$, leading to activation of signal transducer and activator of transcription (STAT) 4 and STAT1, respectively. The STAT signaling pathway activates the transcription factor, T-bet, which controls the expression of IFN$\gamma$. Th2 cells are characterized by production IL-4, IL-5, and IL-13 cytokines. Th2 cytokines boosts humoral response by inducing B cell proliferation, antibody production and antibody isotype switching. GATA3 transcription factor induction by IL-4-dependent STAT6 signaling facilitates Th2 differentiation and cytokine production. Interestingly, Th1 and Th2 subsets each have the capacity to inhibit the development of the other subset. For instance, the expression of T-bet transcription factor in a fully differentiated Th2 cells can inhibit Th2 cytokine production. T-bet was shown to physically bind GATA3 and inhibited GATA-3 mediated Th2 cytokine transcription. On the other hand, GATA3 has been shown to inhibit Th1 differentiation through downregulation of STAT4, which leads to a reduction in T-bet expression. Overall, the regulation of Th1/Th2 responses is critical since aberrant Th1 responses play critical roles in autoimmune diseases, such as diabetes and arthritis, whereas aberrant Th2 cells can cause allergy and asthma.
More recently, a new subset of T helper cells, termed Th17 cells based on their production of the pro-inflammatory cytokine, IL-17, has been identified. Th17 cells initially garnered great interest because multiple studies showed that they were the critical mediators of experimental autoimmune encephalitis (EAE), which had long been considered to be a Th1-mediated disease. Since then, Th17 cells have been implicated in a variety of diseases including, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and asthma. IL-17 mediates pro-inflammatory responses by inducing production of various cytokines and chemokines from innate immune cells such as macrophages and neutrophils, as well as non-immune cells such as endothelial cells and epithelial cells.

The initial studies also showed that IL-23, a newly discovered cytokine that shares a common subunit with IL-12, drove Th17 cell function. However, de novo generation of Th17 cells does not require IL-23, but depends on the combination of the immunosuppressive cytokine, transforming growth factor (TGF)β, and the pro-inflammatory cytokine IL-6. The IL-6 is critically important, since in the presence of TGFβ alone, CD4+ T cells differentiate into suppressive regulatory T cells (Tregs). TGFβ and IL-6, in combination, strongly induce expression of an orphan nuclear receptor, RORγt, which has been shown to be required for Th17 differentiation. Subsequently, a related transcription factor, RORα, was shown to also facilitate Th17 differentiation. STAT3, a mediator of the IL-6 signal, induces IL-17 production indirectly by driving RORγt expression and also directly by inducing IL-17 transcription at the IL-17 promoter.

The mechanisms that inhibit Th17 activities are of great interest, given the involvement of Th17 cells in a variety of inflammatory diseases. In this regard, reciprocal inhibition between different T helper cells also affects Th17 differentiation. For instance,
Th17 differentiation can be inhibited by IFNγ and IL-4, the prototypical Th1 and Th2 cytokines, respectively. Conversely, even prior to identification of Th17 population, IL-6 had been shown to inhibit Th1 differentiation. In addition, IL-27 has been shown to be a negative regulator of Th17 responses in studies where IL-27 receptor-deficient mice developed more severe inflammation in the EAE model. IL-27 also has been implicated in the generation of regulatory T cells, immunosuppressive cells that are closely related to Th17 cells in developmental lineage.

1.1.4 Immune regulation by regulatory T cells

The presence of regulatory T cells (Tregs) that can suppress immune responses was first suggested nearly 40 years ago. However, little progress had been made for 25 years in identifying such a population, until Sakaguchi and colleagues defined the suppressor cells as a subset of CD4+ T cells that co-express CD25 (IL-2Rα). They showed that CD4+CD25− T cells caused widespread inflammation when transferred into immunodeficient mice, which was prevented by co-transfer of CD4+CD25+ T cells. The next great leap in the understanding of Tregs occurred with the discovery of Foxp3 transcription factor. Foxp3 was originally discovered to be mutated in IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in humans and Scurfy strain of mice, both of which are characterized by development of severe autoimmunity. Subsequent studies showed that Foxp3 expression is limited to Tregs and is required for Treg development. Furthermore, sustained expression Foxp3 is necessary for Tregs to retain their suppressive functions. Therefore, Foxp3 plays a critical role in the development and maintenance of Tregs.
Since modulation of Treg function presents potential opportunities of clinical intervention in a variety of immunologic disorders, it is important to understand the mechanism by which Tregs wield their suppressive function. However, to date, the complete mechanism of Treg function remains elusive, with various experimental approaches, both in vitro and in vivo, yielding different, and at times conflicting, results. Nevertheless, suppressive actions of Tregs can be roughly grouped into two parts: their ability to directly suppress effector T cells and their ability to suppress APC function.

There are several potential means by which Tregs directly regulate effector T cells. Many studies have shown that Tregs can directly inhibit IL-2 production in effector T cells. Without IL-2, effector T cells cannot proliferate or survive. Another potential mechanism of Treg function is their ability to lyse target cells. Both human and mouse Tregs have been shown to express granzymes upon activation and kill responder cells. Secretion of suppressive cytokines such as IL-10 and TGFβ, could also mediate Treg function. However, the role of TGFβ and IL-10 remains controversial, as in vitro studies negate their importance, whereas in vivo studies seem to indicate their role in Treg-mediated suppression. Recently, a new cytokine, IL-35, has been shown to be produced by Tregs and required for their maximal suppressive activity.

The other major mechanism of Treg-mediated suppression is their ability to regulate the ability of APCs to activate effector T cells. Tregs constitutively express cytotoxic T lymphocyte antigen 4 (CTLA-4), which is a potent negative regulator for T cells responses, as indicated in CTLA-4-deficient mice that develop severe multi-organ inflammation. In vitro studies have indicated that CTLA-4 on Tregs suppress expression of co-stimulatory molecules CD80/86 on APCs, preventing them from providing costimulatory signal to
effector T cells. However, since effector T cells also express CTLA-4 following activation, they could attenuate their own activation, independent of Tregs. Therefore, multi-organ inflammation seen in CTLA-4-deficient mice could be due to lack to auto-inhibitory feedback loop. However, the importance of CTLA-4 for Treg function was recently demonstrated in a study where Treg-specific CTLA-4 deficiency was sufficient for causing systemic autoimmunity.

Tregs are essential for maintenance of self-tolerance and regulating immune responses. It is likely that the function of Tregs depends on a combination of suppressive mechanisms.

1.1.5 Immune regulation by natural killer T cells

Natural killer T (NKT) cells are a unique T cell subset with broad capacity to regulate the immune response. NKT cells are defined as T cells that co-express NK cell receptors, such as NK1.1, along with semi-invariant TCR. In mice, the vast majority of NKT cells, termed type I NKT cells, expresses TCR that consists of invariant Vα14-Jα18 TCRα-chain and either Vβ2, Vβ7, or Vβ8 TCRβ-chain. In humans, NKT cells express Vα24-Jα18/Vβ11 invariant TCRs. There are also non-Vα14 NKT cells, termed type II NKT cells, but much less is known about them due to difficulty in identifying such populations. NKT cells are reactive to substances that are presented in complex with CD1d, a MHC class I-like molecule.

The first NKT stimulant was identified during the process of developing anti-tumor agents. Researchers at Kirin Pharmaceuticals showed that a glycolipid, α-galactosylceramide (α-GalCer) from a marine sponge, had anti-metastatic effect in a mouse tumor model. A
subsequent study showed that the α-GalCer/CD1d complex activates NKT cells, and suggested a role for NKT cells in the prevention of tumor metastasis\textsuperscript{70}. The discovery of α-GalCer not only facilitated the study of NKT function, but also led to the generation of tetramers which allowed specific identification of NKT cells\textsuperscript{71}. Because α-GalCer was extracted from a marine sponge, its physiological relevance in mediating in vivo NKT cell function was in doubt. However, recent studies have identified physiologically relevant ligands that stimulate NKT cells. For example, lipopolysaccharide (LPS)-related lipids in the cell wall of Sphingomonas, a Gram-negative, LPS-negative bacteria, strongly stimulated NKT cells\textsuperscript{72, 73}, which indicated that NKT cells have a role in controlling bacterial infection. In addition, endogenous self-ligand, glycosphingolipid, iGb3, has been found to activate both mouse and human NKT cells\textsuperscript{74}. iGb3 was suggested to be an important physiological ligand that facilitates NKT cell development, because mice that could not generate iGb3 in the lysosome had severe reduction in NKT cells\textsuperscript{74}. However, subsequent studies have indicated that generalized lysosomal lipid storage defect, but not iGb3 specific defect, leads to NKT cell deficiency\textsuperscript{75, 76}. Additional experiments are necessary to determine the endogenous ligands that regulate NKT cell development and function.

Administration of NKT ligands leads to the activation of NKT cells that is characterized by a rapid burst of IL-4 (Th2), which is followed by a more sustained production of IFNγ (Th1)\textsuperscript{70, 77}. The mechanism that determines the production of Th1 versus Th2 cytokine is unclear, but a number of studies have suggested that the decision may be ligand-dependent\textsuperscript{78, 79}. In addition to cytokine production, activated NKT cells also can reciprocally activate DCs. Activated NKT cells upregulate CD40L on the cell surface and bind CD40 on DCs, which leads to up-regulation of CD80, CD86, and IL-12 production by
DCs\textsuperscript{80,81} (Fig. 1-1). In addition, NKT cells also induce MHC expression on DCs \textsuperscript{82,83}.

Therefore, NKT cells can facilitate a strong activation of adaptive immune responses directly via cytokine production and indirectly via stimulating DCs.

![Fig. 1-1 Reciprocal activation between NKT cells and DCs](image-url)
In addition to their suggested role in tumor immunity and infection control, as discussed previously, NKT cells have been implicated in diseases such as diabetes, systemic lupus erythematosus, asthma, atherosclerosis, and primary biliary cirrhosis. However, many of the reported findings depend on the use of artificial experimental methods, including the use of α-GalCer agonist, and remain to be verified in a more physiological setting. Therefore, a better mechanistic understanding of NKT cell function is necessary to address their role in different diseases.

1.2 T cell development

1.2.1 Conventional T cell development

As described in the previous section, T cells play a critical role in the adaptive immune response. T cells develop in the thymus from hematopoietic stem cells that originate from the bone marrow. Thymocyte development progresses through ordered stages that are defined by expression of surface molecules, such as CD4, CD8, CD25 and CD44. The major thymic subsets are: CD4⁻CD8⁻ double-negative (DN) cells, CD4⁺CD8⁺ double positive (DP) cells and CD8⁺ or CD4⁺ single-positive (SP) cells. The DN cells can be further sub-divided into CD44⁻CD25⁻ (DN1), CD44⁻CD25⁺ (DN2), CD44⁺CD25⁺ (DN3), and CD44⁺CD25⁻ (DN4) stages. The developmental progression of thymocytes is critically dependent on interactions between thymocytes and non-hematopoietic thymic epithelial cells (TECs).

There are two major checkpoints during thymocyte development that ensure the generation of functional T cells. The first checkpoint, termed β-selection, occurs around DN3 stage, where only the thymocytes that express functional TCRβ are allowed to progress to DP
stage. TCRβ forms the pre-TCR complex with invariant pre-TCRα and CD3. Signaling through the pre-TCR induces proliferation, expression of CD4 and CD8, expression TCRα and suppression of pre-TCRα expression, resulting in progression to the DP stage. On the other hand, the cells that do not express functional TCRβ die by apoptosis.

At the DP stage, thymocytes are subjected to the second checkpoint, consisting of positive and negative selection. During positive selection, DP thymocytes that express randomly rearranged αβ TCRs are presented with self-antigens in the context of MHC on the cortical TECs. Only the cells that can bind self-antigen-MHC complex with sufficient affinity are allowed to survive, whereas thymocytes with low affinity receptors undergo death by neglect. Because antigen is presented in the context of MHC, positive selection also determines the thymocyte fate, where DPs that are selected on MHC class I develop into CD8\(^+\) cells and DPs selected on MHC class II develop into CD4\(^+\) cells. During negative selection, thymocytes that have a strong affinity for self-antigens are eliminated. Therefore, negative selection is an important mechanism that provides central tolerance and prevents autoimmunity. The importance of proper negative selection is highlighted in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). APECED is caused by genetic defect in Aire, which facilitates negative selection by regulating expression of self-antigens in the medullary TECs and thymic DCs. Without negative selection, autoreactive T cells are allowed to escape cell death and cause pathology in the periphery. Thymocytes that survive positive and negative selections eventually develop into MHC-restricted and self-tolerant conventional CD4\(^+\) or CD8\(^+\) SP αβ T cells and exit the thymus.
1.2.2 Natural killer T cell development

Several early reports indicated the importance of the thymus in NKT cell development. NKT cells are absent in athymic (nude) mice \(^{94}\), and neonatal thymectomy prevents development of NKT cells \(^{95}\). It is generally accepted that NKT cell precursors diverge from conventional T cells at the DP stage with expression of invariant TCR\(\alpha\) chain (V\(\alpha\)14-J\(\alpha\)18 in mice, V\(\alpha\)24-J\(\alpha\)18 in humans) \(^{96-98}\) (Fig. 1-2). Invariant TCR\(\alpha\) chain is paired with a limited selection of TCR\(\beta\) chains to make up the TCR (V\(\beta\)8.2, V\(\beta\)7, V\(\beta\)2 in mice, and V\(\beta\)11 in humans) \(^{67}\). Unlike the invariant TCR\(\alpha\) chain, there is no evidence of canonical TCR\(\beta\) usage and the complementarity-determining region (CDR), which determines antigen specificity, of the TCR\(\beta\) chain exhibits sequence diversity \(^{99}\). The plasticity of the TCR\(\beta\) chain most likely affords NKT cells the ability to recognize a range of glycolipid antigens.

Unlike conventional T cells that require antigen presentation by TECs, the positive selection of NKT cells is driven by antigen presentation by MHC class I-like molecule, CD1d, on cortical DP thymocytes \(^{100,101}\) (Fig. 1-2). The importance of the CD1d molecule is clearly demonstrated by NKT cell deficiency in CD1d\(^{-/-}\) mice \(^{102}\). Endogenous glycosphingolipid, iGb3, was suggested to be the critical antigen that drives positive selection of NKT cells \(^{74}\). However, more recent studies have suggested that iGb3 may not be the dominant antigen involved in NKT cell selection \(^{103,104}\). Further studies are necessary to determine the nature of selecting antigen for NKT cells.

There is also evidence for negative selection of highly self-reactive NKT cell precursors. The presence of \(\alpha\)-GalCer, a strong agonist, abrogates NKT cell development both \emph{in vivo}, and \emph{in vitro} fetal thymic organ culture systems \(^{105,106}\). In addition,
overexpression of CD1d molecule also prevents NKT cell development. However, further experiments are necessary to determine the endogenous strong agonist that mediates negative selection. It also remains to be determined at what stage the negative selection occurs.

Following selection, NKT cell precursors progress through an ordered developmental pathway, which can be tracked by expression of cell surface receptors (Fig. 1-2). DP NKT precursors first down-regulate CD8 expression to become CD4 SP cells. Based largely on intrathymic cell transfer studies, it has been shown that immature CD44⁺NK1.1⁻ cells develop into CD44⁺NK1.1⁻ intermediate stage cells. The vast majority of intermediate stage cells exit the thymus and complete their maturation in the periphery, which is marked by expression of NK1.1. However, some CD44⁺NK1.1⁻ cells complete maturation in the thymus to become mature CD44⁺NK1.1⁺ NKTs. Consistent with their developing status, the two immature NK1.1⁻ subsets undergo massive cell proliferation and cell death, while there is little turnover of NK1.1⁺ mature cells. Through the maturation process, some NKT cells lose CD4 expression to become DN NKT cells. Even though there are studies showing some functional difference between CD4⁺ NKT cells and DN NKT cells, little is known about the mechanism that regulates the divergence of NKT cell subsets.

During development, the proliferation and survival of immature NKT cells depend on support of various cytokines. For example, IL-7 plays a critical role in the survival and homeostasis of immature NKT cells. IL-7-deficient mice have significantly reduced numbers of NKT cells in both the thymus and the periphery, although NKT maturation is intact. IL-7 promotes the proliferation and survival of NK1.1⁻ immature NKT subsets. A related cytokine, IL-15, also influences development and survival of NKT cells and
IL-15 deficient mice do not have mature NK1.1⁺ NKT cells. However, the downstream cytokine signaling mechanisms remain poorly defined in NKT cells.

The survival and homeostasis of NKT cells also requires the expression of inducible co-stimulator (ICOS) molecule. ICOS is a costimulatory molecule that is typically expressed on activated T cells. ICOS provides costimulation to T cells upon engagement by its ligand, ICOS ligand (ICOSL), and regulates the expansion and effector functions of CD4⁺ T cells. Interestingly, NKT cells constitutively express ICOS, with peak expression occurring at the CD44⁺NK1.1⁻ immature stage. ICOS deficiency leads to defects in survival, homeostasis, and function of NKT cells. However, the factors that regulate expression of ICOS on NKT cells are currently not known.
Fig. 1-2 NKT cell development
1.2.3 Regulatory T cell development

Unlike other T cell subsets that are thymus-dependent for development, Foxp3+ Tregs can be generated in thymus-dependent and -independent manners. Foxp3 induction in the thymus generates ‘natural’ Tregs (nTregs), whereas Foxp3 induction in the periphery leads to conversion of naïve CD4+ T cells into ‘inducible’ Tregs (iTregs). Even though two pathways each generate Foxp3-expressing Tregs, the developmental requirements for each pathway are distinct.

The importance of the thymus in Treg development was suggested by early work on neonatal thymectomized mice. The removal of the thymus between day 2 and day 4 produced T cell-mediated inflammation, suggesting that cells from the thymus can mediate immune tolerance. Following identification of Tregs as CD4+CD25+ T cells by Sakaguchi and colleagues, CD4+CD25+ T cells were shown to originate in the thymus and induce CD25 expression at the CD4+ SP stage. The accumulated evidence suggests that TCR signaling and cytokine stimulation primarily determine Treg differentiation at the CD4+ SP stage.

In a TCR transgenic mouse model, Tregs were shown to develop only in the presence of cognate antigen for the TCR. Furthermore, Treg development required higher affinity antigen-TCR interaction compared to conventional T cell development. Direct sequence analysis of TCR repertoire used by Tregs versus conventional T cells revealed that TCR sequences of Tregs were diverse and showed little overlap with conventional T cells, suggesting that the development of the two populations depends on different types of self-antigens. These results suggest that Treg selection requires TCRs with affinities for self-antigens between conventional T cell positive selection and negative selection of auto-reactive T cells. TCR signaling in the presence of proper costimulation leads to induction of Foxp3.
Downstream of TCR signaling, several transcription factors have been identified to play a role in Treg development. TCR signaling activates NFAT and AP-1 transcription factors, which bind to the Foxp3 promoter and induce Foxp3 transcription. In addition, the deletion of various members in the TCR-dependent NF-κB signaling pathway, including PKCθ, Bcl-10, IKKβ and Carma1, has been linked to severe impairment in Treg development. However, it is currently unknown how NF-κBs or members of the NF-κB pathway regulate Treg development and/or Foxp3 induction.

In addition to the TCR signal, thymic Treg development requires a second signal provided primarily by IL-2, and to a lesser degree, two other common gamma-chain (γc) cytokines, IL-7 and IL-15. IL-2 signaling is mediated through IL-2 receptor (IL-2R), which consists of α, β, γc subunits. Deficiency of either IL-2 or CD25 (IL-2Rα) chain leads to about a 50% reduction in the numbers of thymic Foxp3+ cells, whereas IL-15 or IL-7 deficiency alone does not affect generation of Foxp3+ cells. In contrast, mice lacking γc or mice with combined deficiency of IL-2, IL-7, and IL-15 completely lack thymic and peripheral Foxp3+ Tregs, suggesting some functional redundancy between IL-2 and IL-7/IL-15 cytokines. However, the mechanism by which IL-2 and its downstream transcription factor, STAT5, mediates Treg development is still unclear.

Peripheral development of Tregs from conventional naïve CD4+ T cells requires TGF-β signaling in addition to TCR signaling. TGF-β cooperates with the TCR signal to induce Foxp3 expression in naïve CD4+ T cells. IL-2 is also required for TGF-β-mediated Foxp3 induction. IL-2 also promotes Treg differentiation by inhibiting differentiation of CD4+ T cells into Th17 cells, which can occur in the presence of IL-6 in addition to TGF-β.
The two distinct Treg developmental pathways raise interesting questions about purpose of their existence. First, it is possible that the two subsets are functionally redundant. In fact, it is currently difficult to distinguish between the two subsets based on phenotypic markers. However, it is also possible that two Treg populations with different functional characteristics may be needed for proper immune regulation. Since nTregs are generated from thymocytes with a stronger affinity for self-antigens and iTregs are generated from naïve conventional T cells from the periphery, it has been suggested that the primary function of nTregs is to guard against autoimmunity, whereas iTregs regulates immune responses against foreign antigens. Further characterization of the two Treg subsets is needed to address this important question.

1.3 NF-κB Signaling Pathway

In order for the cells of the immune system to carry out their functions, they require efficient mechanisms to respond to various stimuli from their surrounding environment. Stimulatory signals, which are typically induced via engagement of cell surface receptors, are then transmitted inside the cell via a complex network of signal transduction pathways, which eventually leads to activation of transcription factors and induction of gene expression. NF-κB was initially discovered as a transcription factor that binds to the enhancer element of the kappa light chain gene (the κB site) in B cells. Since then, NF-κB has been demonstrated to be a critical regulator of both innate and adaptive immune responses. NF-κB dysfunction has been associated with common pathological conditions, such as cancer,
atherosclerosis, and diabetes. Due to the critical functions of NF-κB, the mechanisms of NF-κB activation and regulation have been intensely studied since its initial discovery.

1.3.1 NF-κB Family Members and IκB inhibitors

There are five members in the mammalian NF-κB family: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) (Fig. 1-3). NF-κB members are ubiquitously expressed and they share evolutionarily conserved structural domains. All five members have the N-terminal Rel homology domain (RHD), which facilitates protein dimerization and DNA binding. RelA (p65), RelB, and c-Rel are produced as mature proteins and they contain the C-terminal transcriptional activation domain (TD) (Fig. 1-3). On the other hand, NF-κB1 (p50) and NF-κB2 (p52) do not have TD and are produced as p105 and p100 precursor proteins, respectively (Fig. 1-3). While the processing of p105 is constitutive and co-translational, p100 processing is a tightly regulated process in response to stimulation. RelA and c-Rel predominantly form heterodimers with p50, while RelB predominantly interacts with p52. Since p50 and 52 do not contain TAD, they rely on other factors to drive transcription. Otherwise, p50 and p52 homodimers may even suppress transcription.

Prior to activation, NF-κB dimers are sequestered in the cytoplasm by a family of NF-κB inhibitors called IκBs. There are nine IκB family members: IκBα, IκBβ, IκBγ, IκBε, IκBζ, IκBNS, Bcl-3, and NF-κB precursors p105 and p100. The characteristic feature of IκBs is the presence of multiple ankyrin repeats, which allow them to interact with NF-κB members and mask nuclear localization signals (NLS) located within the RHD domain (Fig. 1-3). p105 and p100 NF-κB precursors also contain C-terminal ankyrin repeats, allowing
them to function as IκBs\textsuperscript{155,156} (Fig. 1-3). Proteasome-mediated processing of the p105 and p100 precursors removes the C-terminal portion, generating mature p50 and p52, respectively\textsuperscript{153}.

Different IκB family members have distinct functions, depending on their NF-κB binding targets. For example, IκBα, the prototypical IκB member, primarily sequesters RelA/p50 and c-Rel/p50 heterodimers. Upon stimulation, IκBα is degraded, allowing functional NFκB dimers to translocate to the nucleus, in a process termed canonical NF-κB activation\textsuperscript{157}. On the other hand, p100 precursor protein specifically interacts with RelB. In response to more limited variety of stimuli, including lymphotoxin, B cell activating factor (BAFF), CD40 ligand, p100 is processed to p52 and functional RelB/p52 dimer is generated in a process termed, non-canonical NF-κB activation\textsuperscript{151-153}. 
Fig. 1-3 NF-κB Family Members
1.3.2 Canonical NF-κB activation

The activation of the canonical NF-κB pathway can be initiated by various cell surface receptors, including TCR, BCR, tumor necrosis factor receptor (TNFR), and cytokine receptors, via distinct intracellular signaling pathways (Fig. 1-4). However, activation of IκB kinase (IKK) complex is a central event in canonical NF-κB activation, regardless of the origins of the initial stimuli. IKK is a 700-900 kDa complex that is composed of three major subunits: two catalytic subunits, IKKα(IKK1) and IKKβ(IKK2) and a regulatory subunit IKKγ(NEMO). Of the two catalytic subunits, IKKβ is the critical subunit in the canonical NF-κB pathway, as IKKβ-deficiency causes severe NF-κB defects. Although IKKα retains some IKK activity in the absence of IKKβ, demonstrating minimal functional redundancy, deletion of IKKα has little effect on canonical NF-κB activation. In addition to IKKβ, IKKγ is an essential non-catalytic subunit in canonical NF-κB signaling. In the absence of IKKγ, the IKK complex cannot be activated.

The active IKK complex phosphorylates IκBα on Ser32 and Ser36, leading to its K48-linked polyubiquitylation at Lys19 by E3 ligase, SCF-βTRCP. The ubiquitylated IκBα is degraded via the 26S proteasome, thereby exposing the NLS sequence on functional NF-κB heterodimers and inducing nuclear translocation (Fig. 1-4).
Fig. 1-4 Canonical NF-κB Activation
1.3.3 Role of NF-κB in T cell development

Since conventional T cell development in the thymus is largely determined by the strength of signal from the TCR, understanding the mechanism of subsequent signaling and transcriptional events is important for a further understanding of T cell development. However, in this regard, the role of NF-κB in conventional T cell development has been incompletely defined. In mice with deficiencies in individual NF-κB members, conventional T cell development remains relatively unaffected. However, inhibition of NF-κB in mice via expression of dominant negative IκBα, leads to a loss of CD8 SP thymocytes and CD8+ T cells in the periphery. Consistently, T cell-specific deletion of IKKβ or IKKγ has also been shown to decrease CD8+ T cells, which suggests the involvement of canonical NF-κB pathway. In addition, a recent study has shown that activation of NF-κB is only found in CD8 SP population and plays an important role in positive selection of CD8 SP thymocytes. Therefore, NF-κB activation seems to have a pro-survival function during CD8+ T cell development. However, it is unclear what role NF-κB plays in the development of CD4+ T cells and whether differential NF-κB activation influences CD4/CD8 lineage decisions.

The NF-κB pathway is also involved in the development of non-conventional T cells, such as NKT cells. Mice, in which canonical NF-κB is inhibited by dominant negative IκBα, demonstrates severe loss of NKT cells and a block in transition from immature NK1.1- to mature NK1.1+ NKT cells. Similar defects are seen in mice with a canonical NF-κB defect caused by disruption of a key signaling molecule PKCθ or IKKβ. Furthermore, canonical NF-κB was required in NKT cell-intrinsic manner. Defects in NKT cell development is also seen in RelB-deficient mice. However, RelB expression was required in thymic stromal cells to support NKT cell development. These studies indicate that distinct
members of NF-κB are required in both hematopoietic and non-hematopoietic compartments for proper development of NKT cells.

The NF-κB pathway has also been implicated in the development of Tregs. Deficiency of several molecules in TCR-induced NF-κB pathway has been associated with a decrease in thymic Tregs. In addition, defects in specific NF-κB members, such as in c-Rel/p50 double-knockout mice, also lead to Treg deficiency. However, the exact mechanism by which TCR-mediated NF-κB activation regulates Treg development remains to be investigated.

1.4 Regulation of NF-κB and T cell function by ubiquitylation and deubiquitylation

1.4.1 Ubiquitylation

Ubiquitin is a highly conserved and ubiquitously expressed 76-amino acid peptide which was initially discovered as a lymphocyte differentiation factor. A seminal report by Hershko and colleagues showed ubiquitylation, a process of tagging ubiquitin to a protein substrate, targets proteins for degradation. Since then, detailed mechanisms and critical players involved in ubiquitylation have been identified. The conjugation of ubiquitin to target protein occurs in a stepwise manner and requires the participation of three critical enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). In the first step, E1 activates ubiquitin through formation of a high-energy thiol ester bond. In the second step, activated ubiquitin is transferred to an E2, and in the final step,
ubiquitin is transferred to a lysine residue of target protein by an E3. At each step, the number of enzyme involved increases. A single E1 mediates all ubiquitylation in eukaryotes, whereas there are about 11 members in the E2 family in yeast and at least 25 mammalian members. There is even greater enzymatic diversity for E3s, which demonstrate substrate specificity.

The manner in which ubiquitin is conjugated to target protein yields different outcomes. A target protein could be tagged with a single ubiquitin molecule at one or more lysine residues in a process known as monoubiquitylation. Monoubiquitylation plays important functions in a variety of cellular process, such as receptor-mediated endocytosis, gene transcription, and retrovirus budding from plasma membrane. Since the ubiquitin molecule contains several lysine residues, ubiquitin itself can also serve as a target for other ubiquitin molecules, which results in polyubiquitylation. Of the seven lysine residues on ubiquitin, lysine 48(K48) and lysine 63(K63) are most commonly involved in polyubiquitylation. K48 polyubiquitylation is a well-described process in which the ubiquitin chain, with minimum of four residues, tags proteins for degradation by the 26S proteasome. More recently, the importance of K63 polyubiquitylation has been highlighted in various cellular processes. K63 polyubiquitylation does not target proteins for degradation, but is involved in processes such as DNA repair, translation, and cell signaling. Both K48 polyubiquitylation and K63 polyubiquitylation are critically involved in the TCR-mediated NF-κB signaling pathway.

1.4.2 Regulation of TCR induced NF-κB activation by polyubiquitylation
Stimulation of TCR initiates a signaling cascade that ultimately results in activation of serine/threonine kinase, PKCθ. PKCθ activation facilitates formation of the CBM complex composed of Carma1, BCL10 and MALT at the lipid raft. MALT1-Bcl10 then interacts with TRAF6, a K63 type E3 ligase, which leads to oligomerization and activation of its E3 ligase activity (Fig. 1-5). Through the interaction with E2 enzyme, Ubc13/UEV1A, active TRAF6 then polyubiquitylates IKKγ, leading to IKK complex activation (Fig. 1-5). TRAF6 activation also leads to its auto-ubiquitylation, allowing it to activate TAK1-TAB2 complex, which in turn can activate IKK complex (Fig. 1-5). Interestingly, the CBM complex can also directly polyubiquitylate IKKγ (albeit at a different lysine residue) through its interaction with Ubc13/UEV1A, independent of TRAF6 (Fig. 1-5). Activated IKK complex phosphorylates of IκBα, leading to its K48-polyubiquitylation mediated by E3 ligase, SCF-βTRCP (Fig. 1-4). K48 polyubiquitin chain facilitates binding to 26S proteasome, which leads to IκBα degradation and NF-κB activation.
Fig. 1-5 Regulation of TCR induced NF-κB activation by polyubiquitylation
1.4.3 Deubiquitylation

Deubiquitylating enzymes (DUBs) are proteases that can cleave ubiquitin molecules from target proteins or pro-ubiquitin molecules. DUBs have the important function of reversing and regulating ubiquitylation, which is involved variety of cellular processes as mentioned in the preceding section. In this manner, the function of DUBs is analogous to phosphatases, which can reverse phosphorylation mediated by kinases. Just as E3 ligase has substrate specificity during ubiquitylation, DUBs also show substrate specificity. DUBs contain different protein-binding domains and also demonstrate preferences for different (e.g. K48 vs. K63) ubiquitin chains, which in concert, confers upon them specificity.

There are about 100 putative DUBs encoded in the human genome, but the mechanism of DUB function and their targets have remained largely unknown until recently. DUBs are classified into five families based on sequence homology: ubiquitin C-terminal hydrolase (UCH), Machado-Josephin domain (MJD), the JAB1/MPN/Mov34 metalloenzyme (JAMM), the ubiquitin-specific protease (USP/UBP), and the ovarian tumor domain (OTU). UCH family members have been shown to process short ubiquitylated substrates and regulate cellular ubiquitin level by converting pro-ubiquitin to active ubiquitin. So far, not much is known about MJD and JAMM family members. The USPs, with 53 members in human genome, make up the biggest DUB family, and are characterized by the presence of two conserved sequence motifs — the cysteine and histidine boxes — in their catalytic domain. OTUs, which share conserved OTU domain, make up the second largest group with 24 members in the human genome. The following section focuses on CYLD, a USP family protein, which has been one of the few well-characterized DUBs thus far.
1.4.4 CYLD, deubiquitylase with tumor suppressor function

CYLD was first identified as a tumor suppressor, which is deficient in familial cylindromatosis, which predisposes patients to develop rare benign tumor of skin appendages, termed cylindromas. Familial cylindromatosis is inherited in an autosomal dominant manner and thought to arise from the loss of heterozygocity in the apocrine and eccrine cells of the skin. Cylindromas are typically located in head and neck region and can cause significant morbidity due to their location, size and complications such as ulceration and infections. In subsequent studies, mutations of CYLD gene, located on chromosome 16q12-q13 in humans, have been found in diseases such as Brooke-Spiegler Syndrome and Multiple Familial Trichoepithelioma, which all cause skin tumors.

The CYLD gene contains 20 exons, which encode a 120kDa protein composed of 956 amino-acids. CYLD has four major functional domains: three N-terminal CAP-GLY domains which facilitate cytoskeletal associations, two proline-rich domains which mediate protein-protein interactions, four Cys-X-X-Cys motifs that represent finger-like metal binding domains and a C-terminal DUB catalytic domain. Intact DUB domain is absolutely required for DUB activity of CYLD, and the clinical significance of the CYLD’s DUB activity is highlighted by the fact that most of the mutations found in familial cylindromatosis are located in the C-terminal DUB region.

The biological function of CYLD was initially reported in vitro. In yeast-two hybrid and transfection experiments, CYLD was demonstrated to bind to TRAF2 and IKKγ/NEMO, the regulatory subunit of the IKK complex. CYLD inhibited ubiquitylation of overexpressed TRAF2, TRAF6 and IKKγ and inhibited NF-κB activation downstream of TNFR and Toll-like receptor (TLR) 4. Subsequent in vivo studies on CYLD revealed
that CYLD interacts with wide-ranging target proteins including tyrosine kinase LCK\(^{212}\), the NF-κB co-activator protein BCL-3\(^{213}\), TGF-activated kinase 1 (TAK1)\(^{214}\) and receptor-interacting protein 1 (RIP1)\(^{215}\). A shared feature of CYLD binding proteins is their conjugation to K63 type ubiquitin chains\(^{216}\). While both structural and biochemical studies indicate that CYLD has DUB activity specific for K63 ubiquitin chains\(^{211, 213, 217}\), there is also some evidence that CYLD may act on K48 type ubiquitin chains as well\(^{210, 212, 218}\). The detailed mechanism of CYLD DUB activity remains to be investigated. However, what is clearly evident thus far is the fact that through these different partners, CYLD regulates diverse biological processes such as, lymphocyte development\(^{212, 219}\), lymphocyte activation\(^{214, 219}\), inflammation and tumorigenesis\(^{213, 214, 220}\), cell migration\(^{221}\), spermatogenesis\(^{215}\), and osteoclastogenesis\(^{222}\).

### 1.4.5 Functional role of CYLD in T cell development and activation

The first report of \textit{in vivo} CYLD function demonstrated that CYLD plays a critical role in thymocyte development\(^{212}\). Thymocyte development sequentially progresses through double-negative (DN), double-positive (DP) and single-positive (SP) stages that are defined by expression of CD4 and CD8 T cell co-receptors\(^{91}\). CYLD knockout mice had relatively normal total thymocyte numbers and produced normal numbers of DN and DP stage thymocytes. However, the numbers of CD4 SP and CD8 SP, as well as the peripheral T cell numbers were drastically decreased\(^{212}\). CYLD regulates DP-SP transition by positively regulating LCK function, a key proximal-TCR signaling molecule required for thymocyte development\(^{223}\). CYLD was dispensable for LCK activation but was required for active LCK to bind and activate its target, ZAP70\(^{212}\). Ubiquitylation of LCK by an E3 ligase, Cbl
(Casitas B-lineage lymphoma), had been reported to inhibit LCK function and ZAP70 activation \(^{224}\). At least \textit{in vitro}, CYLD could reverse CBL-mediated ubiquitylation of LCK \(^{212}\). It is possible that loss of CYLD leads to hyper-ubiquitylation of LCK, resulting in decreased activation of ZAP70 and attenuated DP-SP thymocyte transition.

Despite the defect in T cell development, remaining conventional T cells in CYLD knockout mice were hyper responsive to stimulation \(^{214,220}\). The CYLD knockout mice also developed spontaneous or chemically-induced intestinal inflammation \(^{214,220}\). Hyperactive T cells seemed to be important in disease pathogenesis, since CYLD knockout T cells were sufficient to replicate the pathology in RAG1-knockout recipients \(^{214}\). Consistent with their hyperactive phenotype, CYLD knockout T cells had constitutive activation of NF-κB, which was associated with constitutively active upstream kinase, TAK1 \(^{214}\). TAK1 had been identified as an ubiquitin-dependent kinase \(^{225}\), in which polyubiquitylation was shown to mediate autoactivation of TAK1 (Fig. 1-5) \(^{226}\). Interestingly, CYLD was shown to bind and deubiquitylate TAK1 and suppress TAK1 activity under overexpression conditions \(^{214}\). Therefore, CYLD seems to play a critical role in preventing T cell hyperactivation by modulating TAK1 activity.
CHAPTER II

DEUBIQUITINASE CYLD REGULATES IL-7 SIGNALING
AND ICOS EXPRESSION IN THE DEVELOPMENT OF
NATURAL KILLER T CELLS
ABSTRACT

Natural killer T (NKT) cells modulate immune responses against pathogens and tumors. We show here that CYLD, a tumor suppressor with deubiquitylase function, plays a pivotal role in NKT cell development. Unlike other known NKT regulators, CYLD is dispensable for intrathymic NKT cell maturation but is obligatory for the survival of immature NKT cells. Interestingly, CYLD deficiency impairs the expression of ICOS, a costimulatory molecule required for the survival and homeostasis of NKT cells, and this molecular defect is coupled with signaling attenuation of an NKT-survival cytokine, IL-7. CYLD exerts these important functions by preventing hyper-activation of NF-κB in developing NKT cells. Thus, CYLD regulates NKT cell development through an unusual mechanism involving modulation of IL-7 signaling and ICOS expression.
INTRODUCTION

Natural killer T (NKT) cells are a subset of innate immune cells that expresses a semi-invariant T-cell receptor (TCR) recognizing glycolipid antigens presented by the non-polymorphic MHC class I-related protein CD1d \(^{227}\). In particular, the type I NKT cells, or invariant NKT cells (hereafter called NKT cells), express a TCR repertoire that consists of V\(\alpha\)14-J\(\alpha\)18 (in mice) or V\(\alpha\)24-J\(\alpha\)18 (in human). A well-characterized antigen of NKT cells is \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer), a synthetic glycolipid originally derived from a marine sponge \(^{228}\). However, more recent studies have identified NKT-specific glycolipid antigens derived from microbes and self-tissues \(^{227}\). In response to antigen stimulation, NKT cells rapidly produce cytokines, predominantly IL-4 and IFN-\(\gamma\), thereby modulating the nature and magnitude of immune responses \(^{227, 229}\). Strong evidence suggests that NKT cells also play a role in anti-tumor immunity as well as immunological tolerance \(^{230, 231}\).

The development of NKT cells occurs in the thymus and originates from rare CD4\(^+\)CD8\(^+\) double-positive (DP) thymocytes with a rearranged semi-invariant TCR. The positive selection of NKT cells is unique in that it is mediated by the DP thymocytes through recognition of self-glycolipids presented by the CD1d molecule \(^{232}\). After positive selection, immature NKT cells proceed through a three-stage maturation process, which can be defined based on their surface expression of CD44 and NK1.1 markers \(^{113}\). The stage 1 NKT cells (CD44\(^-\)NK1.1\(^-\)) first develop into transitional or stage 2 NKT cells (CD44\(^-\)NK1.1\(^+\)), which then gain expression of NK1.1 and become mature (stage 3) NKT cells (CD44\(^+\)NK1.1\(^+\)). This last step of NKT cell maturation can occur either in the periphery or within the thymus. A large set of signaling molecules are known to regulate the maturation process of NKT cells.
One such molecule is NF-κB, which is required for the progression of NKT cells from NK1.1− to NK1.1+ stages\textsuperscript{177-179}.

A hallmark of the NK1.1− immature NKT cells is their active proliferation and turnover, as opposed to the largely non-dividing nature of the mature CD44\textsuperscript{+}NK1.1+ population\textsuperscript{234}. The expansion and survival of immature NKT cells form an important step in NKT cell development, although the underlying signaling mechanism is poorly understood. Nevertheless, the cytokine IL-7 has a pivotal role in mediating the survival and homeostasis of developing NKT cells\textsuperscript{112,113}. IL-7 knockout mice have a drastically reduced number of NKT cells in both the thymus and the periphery, despite their competence in NKT maturation\textsuperscript{112}. IL-7 promotes the proliferation and survival of stage 1 and stage 2 immature NKT cells\textsuperscript{114,115}. Another cytokine, IL-15, appears to have a role in the homeostasis of the CD44\textsuperscript{+}NK1.1+ mature NKT population\textsuperscript{112}.

Recent studies demonstrate that NKT cells constitutively express high levels of ICOS\textsuperscript{119-121}, a costimulatory molecule that is typically expressed in activated T cells\textsuperscript{117}. Like CD28, ICOS provides a costimulatory signal to T cells upon engagement by its ligand, ICOS ligand (ICOSL), and regulates the expansion and effector functions of CD4 T cells. The constitutive expression of ICOS in NKT cells is crucial for their survival, homeostasis, and function\textsuperscript{119-121}. However, despite the critical role of ICOS in NKT regulation, how the homeostatic expression of ICOS in NKT cells is regulated is largely unknown.

An emerging signaling mechanism that regulates the development and activation of immune cells is protein ubiquitylation, a reversible process controlled by ubiquitylating enzymes and deubiquitylases (DUBs), respectively\textsuperscript{235}. In particular, lysine 63 (K63)-linked polyubiquitin chains, which are often conjugated on signaling adaptors, function as a platform
mediating the recruitment and activation of protein kinases\textsuperscript{236, 237}. The best-known ubiquitin-dependent signaling event is activation of NF-κB, a family of transcription factors that regulates both innate and adaptive immune responses\textsuperscript{153, 238}. The activity of NF-κB is primarily regulated through its subcellular localization. NF-κB proteins are normally retained in the cytoplasmic compartment due to their association with a family of inhibitory proteins, predominantly IκBα\textsuperscript{153}. NF-κB activation by many immune receptor signals involves ubiquitin-dependent activation of IκB kinase (IKK), which phosphorylates IκBα and triggers IκBα proteolysis, leading to nuclear translocation of the activated NF-κB complexes. Recent studies have identified a DUB, CYLD, which specifically deconjugates K63-linked ubiquitin chains and negatively regulates the activation of NF-κB\textsuperscript{239}. However, since the NF-κB-regulatory function of CYLD is cell type- and stimulus-specific, how CYLD regulates specific biological processes remains poorly understood.

Since NF-κB is required for NKT cell maturation\textsuperscript{177-179}, we carried out studies to examine whether CYLD regulates the activation of NF-κB in developing NKT cells and whether CYLD deficiency causes over production of NKT cells. To our surprise, we found that the CYLD knockout (KO) mice have drastically reduced NKT cell numbers in both the thymus and the periphery. However, this defect is not due to the blockade of thymic NKT cell maturation but rather because of massive apoptosis of immature NKT cells. The CYLD KO NKT cells have impaired expression of ICOS and attenuated IL-7 signaling, and these coupled molecular defects are attributed to the aberrant activation of NF-κB. These results suggest that although NF-κB is required for NKT cell maturation, deregulated NF-κB activation, as a result of CYLD deficiency, interferes with NKT cell production due to impaired survival and homeostasis of developing NKT cells. Thus, CYLD regulates NKT
cell development via a novel mechanism that ensures effective IL-7R signaling, ICOS expression, and homeostasis of immature NKT cells.
RESULTS

Impaired NKT cell response in CYLD KO mice

Upon stimulation by antigens, NKT cells rapidly produce IL-4 and IFN-γ\textsuperscript{240}. To investigate the role of CYLD in regulating NKT cell function, we examined α-GalCer-stimulated cytokine production in CYLD KO and wildtype (WT) mice. As expected, injection of WT mice with α-GalCer induced rapid and transient production of IL-4 and more delayed and persistent production of IFN-γ (Fig. 2-1A). Although individual variation was seen among the different mice, the general pattern of cytokine induction was consistent. Remarkably, the α-GalCer-induced production of both IL-4 and IFN-γ was severely attenuated in the CYLD KO mice (Fig. 2-1A). Similar results were obtained with a modified version of α-GalCer, OCH\textsuperscript{241} (Supplementary Fig. 2-1). Thus, CYLD has an essential role in NKT cell response.

CYLD KO mice have a drastic reduction in NKT cell numbers

The impaired NKT response in CYLD KO mice could be due to either a defect of the CYLD KO NKT cells in responding to antigen stimulation or the reduction in NKT cell numbers. To examine the former possibility, we performed intracellular cytokine staining (ICS) assays to examine the production of IL-4 and IFN-γ by splenic NKT cells derived from untreated or OCH-injected mice. Under untreated conditions, the CYLD KO mice displayed a low, but significant, proportion of NKT cells spontaneously producing IL-4 and IFN-γ (Supplementary Fig. 2-2A and B, top panels). However, compared to the WT NKT cells, the
CYLD KO NKT cells were hypo-responsive to OCH stimulation, yielding a lower frequency of cytokine-producing cells (Supplementary Fig. 2-2A and B, lower panels).

To further investigate how CYLD regulates NKT response in vivo, we examined the frequency of NKT cells in both the thymus and the peripheral organs. The thymus of adult CYLD KO mice had a significant reduction in the frequency of NKT cells, as detected based on their expression of T-cell receptor β (TCRβ) and binding to antigen-loaded CD1d tetramer (Fig. 2-1B). Similar or more striking NKT defect was detected in the spleen and liver (Fig. 2-1B). The absolute NKT cell number was also drastically reduced in the different organs of CYLD KO mice, with the most striking result detected in the spleen (Fig. 2-1C). The loss of NKT cells in CYLD KO mice occurred in both the CD4⁺ and CD4⁻CD8⁻ DN NKT populations, since the ratio of these two NKT sub-populations was comparable between the CYLD KO and WT mice (Fig. 2-1D). Thus, the CYLD deficiency causes severe loss of both CD4⁺ and DN NKT cells.

A hallmark of NKT cell TCR repertoire is the involvement of an invariant alpha chain (Vα14-Jα18 in mice) and a restricted number of Vβ gene families. Transgenic mice expressing the rearranged Vα14-Jα18 have been frequently used for the study of NKT cell development and function, since these animals produce a substantially high numbers of NKT cells. To examine whether CYLD is also required for NKT cell production in the Vα14-Jα18 transgenic (Va14Tg) mice, we crossed the CYLD KO mice with Vα14Tg mice to generate CYLD KO-Vα14Tg and WT-Vα14Tg littermates. As expected, a substantially increased frequency of NKT cells was detected in the thymus and spleen of the Vα14Tg mice (Fig. 2-1E, left panels). Importantly, the production of transgenic NKT cells was also dependent on CYLD, since the CYLD KO-Vα14Tg mice had a greatly reduced frequency of
NKT cells (Fig. 2-1E, right panels). This finding suggests that the defect of CYLD KO mice in NKT cell development resides in a stage following the TCRα chain rearrangement.

**CYLD KO mice have a cell intrinsic defect in NKT development**

The positive selection of NKT cells is unique in that it requires antigen presentation by cortical DP thymocytes, instead of thymic stromal cells. However, the thymic epithelial cells are nevertheless required for the development of NKT cells. To determine whether the NKT defect of CYLD KO mice was intrinsic to the NKT cell lineage or due to abnormal function of antigen-presenting DP thymocytes or thymic environment, we performed mixed bone marrow adoptive transfer studies. For these studies, WT mice were crossed with SJL mice for one generation to obtain the CD45.1 congenital marker. Bone marrow cells from WT mice (CD45.1+) were mixed with those from CYLD KO mice (CD45.1–) in 1:1 ratio and adoptively transferred into lethally irradiated WT or CYLD KO recipient mice. Since the WT bone marrow cells produce WT antigen-presenting cells (DP thymocytes and dendritic cells), impaired NKT cell development within the WT recipients (also possess normal stromal cells) would suggest a cell intrinsic defect.

The WT bone marrow cells showed a similar potency for NKT cell generation in the WT and KO recipient mice, since comparable frequencies of NKT cells were detected in the thymus (Fig. 2-2A, upper panels) and spleen (Fig. 2-2B, upper panels) of these chimeras. This result suggested that the CYLD deficiency did not affect the overall environment for NKT cell development. Consistent with this finding, the CYLD KO bone marrow cells displayed cell intrinsic defect in NKT cell development, since a substantially lower frequency
of CYLD KO NKT cells (CD45.1−) was generated in both the WT and CYLD KO recipients. These results establish a cell intrinsic function of CYLD in the development of NKT cells.

**CYLD is dispensable for NKT cell maturation**

A major post-selection step of NKT cell development is thymic maturation, the block of which is frequently seen in mutant mice with NKT deficiencies. To further understand the mechanism by which CYLD regulates NKT development, we examined the maturation of thymic NKT cells in control and CYLD KO mice based on their surface expression of CD44 and NK1.1 markers. In adult WT mice, the majority of thymic NKT cells were mature, stage 3, cells (CD44+ NK1.1+) (Fig. 2-3A). In contrast to many other known NKT-regulatory factors, CYLD was dispensable for NKT cell maturation. Despite the reduced numbers of NKT cells in the CYLD KO thymus (Fig. 2-1C), there was no obvious defect in the progression of NKT cells from stage 1 (CD44− NK1.1−) to stage 2 (CD44− NK1.1+) and stage 3 (CD44+ NK1.1+). In fact, the frequency of the stage 3 mature NKT cells was even increased in the CYLD KO thymus. The spleen of CYLD KO mice also contained increased percentage of CD44+ NK1.1+ NKT cells and decreased percentage of CD44− NK1.1− NKT cells (Fig. 2-3A, lower panels). Parallel studies using the Vα14Tg mice revealed that loss of CYLD also did not inhibit, but rather appeared to enhance, the maturation of the transgenic NKT cells (Fig. 2-3B).

To further confirm that the CYLD deficiency does not block the process of NKT maturation, we examined the thymic NKT populations in young mice (1 and 2 weeks old). Notably, at young ages, the CYLD KO mice only displayed a moderate reduction in the frequency of thymic NKT cells (Fig. 2-3C). Moreover, as seen with the adult mice, the young
CYLD KO mice displayed increased frequency of mature NKT cell population compared to their WT controls (Fig. 2-3D, top right panel). Thus, the NKT cell deficiency in CYLD KO mice was not due to the block of NKT maturation.

**Loss of CYLD causes a hyper-activation phenotype of developing NKT cells**

Thymic NKT cells are characterized by their activated phenotype, displaying constitutive CD69\(^{243}\). It has been unclear whether the activation of these developing NKT cells is subject to negative regulation. The elevated maturation phenotype of CYLD KO NKT cells prompted us to examine whether CYLD controls the activation level of developing NKT cells. In WT mice, a small proportion of thymic NKT cells exhibited a CD69\(^{\text{hi}}\) phenotype, which was not detected in the splenic NKT cells (Fig. 2-3E). Interestingly, both the thymic and splenic NKT cells derived from CYLD KO mice expressed considerably higher levels of CD69 than the WT NKT cells (Fig. 2-3E). In WT NKT cells, the high level CD69 expression was mainly detected in the CD44\(^{+}\)NK1.1\(^{+}\) mature NKT stage (Fig. 2-3F, left panels). In sharp contrast, all three maturation stages of NKT cells of the CYLD KO mice displayed high levels of CD69 (Fig. 2-3F, left panels). This hyper-activation phenotype was also detected in the CYLD KO-V\(\alpha\)14Tg NKT cells (Fig. 2-3F, right panels). These results suggest that the CYLD deficiency causes a hyper-activation phenotype of the developing NKT cells.

**CYLD is critical for the survival of developing NKT cells**

The finding that CYLD KO mice had no defect in NKT cell maturation but nevertheless displayed a severe NKT cell deficiency suggests the possibility that CYLD might regulate the survival and homeostasis of developing NKT cells. We thus examined the effect
of CYLD deficiency on the survival of thymic NKT cells. Whereas the WT NKT cells contained a small proportion of apoptotic cells (annexinV-positive), it is remarkable that the majority of CYLD KO NKT cells were undergoing apoptosis (Fig. 2-4A, left panel). This result explained the drastic loss of NKT cells in CYLD KO mice. A similar result was obtained with the CYLD KO-Vα14Tg mice, although these transgenic mice had a relatively larger population of healthy (annexinV\textsuperscript{low}) NKT cells than the regular CYLD KO mice (Fig. 2-4A, right panel).

We next examined the apoptosis of different stages of the developing NKT cells. Consistent with a prior study\textsuperscript{234}, the stage 1 (CD44\textsuperscript{–}NK1.1\textsuperscript{–}) and stage 2 (CD44\textsuperscript{+}NK1.1\textsuperscript{–}) immature NKT cells were undergoing rapid turnover displaying a high proportion of apoptotic cells, whereas only a small proportion of apoptotic cells was detected in the CD44\textsuperscript{+}NK1.1\textsuperscript{+} mature NKT population (Fig. 2-4B, upper panels). Furthermore, the CYLD deficiency greatly enhanced the level of apoptosis in the stage 1 and stage 2 NKT cells (Fig. 2-4B, lower panels). Compared to their WT counterpart, the CYLD KO mature NKT cells also displayed increased levels of apoptosis, but the overall level was considerably lower than that of the immature NKT cells (Fig. 2-4B). This result was consistent with the drastic loss of immature NKT cells within the thymus of CYLD KO mice (Fig. 2-3A and B). Thus, CYLD has a critical role in mediating the survival of immature NKT cells.

Since the CYLD KO NKT cells displayed a hyper-activated phenotype (Fig. 2-3E and F), we examined the expression of Fas, a death receptor thought to mediate activation-induced apoptosis of peripheral NKT cells\textsuperscript{114}. The level of Fas was moderately increased in the stage 2 and stage 3 CYLD KO NKT cells; however, the loss of CYLD did not alter the expression of Fas in the stage 1 NKT cells (Fig. 2-4C) despite the massive apoptosis of this
subpopulation in CYLD KO mice (Fig. 2-4B). We also analyzed the expression of Fas ligand (FasL) expression but did not detect appreciable differences between the WT and CYLD KO NKT cells (Supplementary Fig. 2-3). These results suggest the involvement of other factors that contribute to the massive apoptosis of the CYLD KO NKT cells.

**CYLD regulates IL-7R expression and signaling**

The phenotype of the CYLD KO mice in NKT cell development is reminiscent of that of the IL-7 knockout mice, which have reduced numbers of NKT cells without affecting NKT maturation. Since IL-7 is critical for the survival and homeostasis of thymic NKT cells, we examined whether CYLD deficiency affected IL-7-mediated NKT cell survival. As expected, in vitro incubation of thymocytes caused spontaneous apoptosis of both WT and CYLD knockout NKT cells (Fig. 2-5A, shaded curve), suggesting the requirement of homeostatic factors for NKT survival. Moreover, the apoptosis of WT NKT cells was almost completely protected by IL-7 (Fig. 2-5A, top panel). Interestingly, however, IL-7 was largely ineffective in protecting the CYLD KO NKT cells from undergoing apoptosis (Fig. 2-5A, lower panel). As an alternative way to examine the effect of IL-7 on the homeostasis of NKT cells, we analyzed the frequency of NKT cells after being cultured in the absence or presence of IL-7. Consistent with its strong survival effect, IL-7 substantially increased the frequency of NKT cells in the WT thymocyte culture (Fig. 2-5B, left panels). In sharp contrast, IL-7 only had a weak effect on the frequency of CYLD KO NKT cells. These findings emphasize an important role for CYLD in mediating the survival response of NKT cells to IL-7.

To address the mechanism by which CYLD regulates IL-7-mediated NKT cell survival, we examined the effect of CYLD deficiency on the expression of IL-7R. IL-7R is
composed of a specific subunit, IL-7Rα, and the common γc subunit shared with several other cytokine receptors. The CYLD deficiency did not appreciably affect the expression of γc (Supplementary Fig. 2-4). On the other hand, the CYLD KO NKT cells had substantially lower levels of IL-7Rα, as demonstrated using NKT cells derived from both regular CYLD KO (Fig. 2-5C, left panel) and CYLD KO-Vα14Tg mice (Fig. 2-5C, right panel). The reduced IL-7Rα expression was seen in all stages of the developing CYLD KO NKT cells (Supplementary Fig. 2-4) and occurred at the mRNA level (Fig. 2-5D), suggesting the requirement of CYLD in IL-7Rα gene expression. Parallel analysis of IL-2Rβ, a subunit of both IL-2R and IL-15R, did not reveal significant difference between the WT and CYLD-knockout NKT cells (Supplementary Fig. 2-4).

We next examined the role of CYLD in regulating IL-7R signaling by detecting IL-7-stimulated STAT5 tyrosine phosphorylation (Tyr-701), a primary mechanism of STAT5 activation. IL-7 stimulated the phosphorylation of STAT5 in all three maturation stages of the thymic NKT cells, as demonstrated by the increased flow cytometric intensity of phospho-STAT5 (P-STAT5) staining (Fig. 2-5E). Consistent with their reduced expression of IL-7Rα and survival response to IL-7, the CYLD KO NKT cells were severely attenuated in IL-7-stimulated STAT5 phosphorylation. Together, these findings demonstrate an important function of CYLD in regulating IL-7R expression and signaling.

**Homeostatic expression of ICOS is dependent on CYLD and coupled with IL-7 signaling**

In addition to IL-7, the costimulatory molecule ICOS plays a critical role in the survival and homeostasis of NKT cells. Notably, a recent Systems Genetics study identified ICOS as a gene, whose expression is tightly associated with that of IL-7Rα in
thymocytes. Because of the critical role of ICOS in NKT regulation, we examined the expression of this costimulatory molecule in the WT and CYLD KO NKT cells. Remarkably, the level of ICOS was drastically reduced in both the thymic and peripheral NKT cells of CYLD KO mice (Fig. 2-6A). This molecular defect was detected in all three maturation stages of the CYLD KO NKT cells (Fig. 2-6B). Moreover, the role of CYLD in regulating ICOS expression was not limited to the NKT populations, since the CYLD KO CD4 SP thymocytes and Treg cells also displayed reduced levels of ICOS expression (Fig. 2-6C). Similarly, the CYLD deficiency also impaired the ICOS expression in peripheral naïve and memory CD4+ T cells (Fig. 2-6D). CYLD did not seem to regulate the stability of ICOS protein, since incubation of the CYLD KO thymocytes with a proteasome inhibitor, MG132, did not cause an increase in the level of ICOS (data not shown). On the other hand, the CYLD deficiency greatly reduced the expression level of ICOS mRNA (Fig. 2-6E), indicating a role for CYLD in regulating ICOS gene expression.

In addition to the homeostatic expression of ICOS in NKT cells and other T-cell subsets, ICOS expression can be induced by TCR ligation in conventional αβ T cells. To assess the molecular mechanism by which CYLD regulates ICOS expression, we examined whether CYLD is also required for TCR-stimulated expression of ICOS. Splenic T cells and thymocytes were stimulated with anti-CD3/anti-CD28 for different times and subjected to flow cytometry to detect ICOS expression. As expected, TCR ligation enhanced the expression of ICOS in WT T cells (data not shown) and thymocytes (Supplementary Fig. 2-5). However, in contrast to the homeostatic expression of ICOS, the inducible expression of ICOS was not significantly affected by the CYLD deficiency (Supplementary Fig. 2-5 and
data not shown). Thus, CYLD has a specific role in mediating the homeostatic expression of ICOS.

To date, how the homeostatic expression of ICOS is regulated is largely unknown. Since IL-7 signaling plays an important role in NKT homeostasis and is regulated by CYLD, we tested the possible involvement of this cytokine in the TCR-independent expression of ICOS. Interestingly, incubation of WT NKT cells in vitro with IL-7 resulted in marked increase in the expression level of ICOS in all three stages of the developing NKT cells (Fig. 2-6F). Furthermore, consistent with their attenuated IL-7 signaling, the CYLD KO NKT cells were hyporesponsive to IL-7-mediated ICOS induction. These results indicate a role for IL-7 in mediating the homeostatic expression of ICOS and partially explain how CYLD regulates both IL-7 signaling and ICOS expression. However, it is important to note that the CYLD KO NKT cells expressed a substantially lower level of ICOS compared to WT NKT cells even when they were cultured without IL-7 (Fig. 2-6F). It is thus likely that the attenuated IL-7 signaling may only partially contribute to the impaired ICOS expression in CYLD KO NKT cells.

**ICOS/ICOSL interaction is critical for thymic NKT homeostasis**

Recent gene targeting studies reveals an important role for ICOS/ICOSL interaction in mediating the survival and homeostasis of NKT cells in both the periphery \(^{120,121}\) and thymus \(^{121}\). To examine whether somatic disruption of the ICOS/ICOSL interaction affects the homeostasis of thymic NKT cells, we injected a recombinant ICOS-Fc fusion protein into WT mice (i.v.) \(^{248}\). Remarkably, a single injection of ICOS-Fc led to marked reduction in the frequency of thymic NKT cells (Fig. 2-6G). This effect was specific, since it was not detected
in mice injected with a control Fc protein (Fig. 2-6G). These results support the previous finding that ICOS is crucial for NKT cell homeostasis and further emphasize the significance of CYLD-mediated ICOS expression in NKT development.

**Loss of CYLD causes hyper-activation of NF-κB in NKT cells, which is responsible for impaired ICOS expression and attenuated IL-7 response**

TCR-stimulated ICOS gene expression involves the transcription factors T-bet and GATA-3, although the transcription factors regulating homeostatic expression of ICOS have not been defined. Since T-bet and GATA-3 are also required for NKT cell development, we examined whether the CYLD deficiency reduced the expression of T-bet or GATA-3. Interestingly, the CYLD KO NKT cells did not have T-bet defect but even displayed hyper-expression of T-bet mRNA (Supplementary Fig. 2-6A). However, a seemingly higher level of T-bet expression was due to the presence of a higher frequency of mature NKT cells in CYLD KO mice, since comparable levels of T-bet mRNA were detected in the fractionated NKT populations of WT and KO mice (Supplementary Fig. 2-6A). Flow cytometry also revealed a similar level of T-bet protein in the CYLD KO and WT NKT cells (Supplementary Fig. 2-6B). We also did not detect a significant effect of CYLD deficiency on the expression of GATA-3 in NKT cells (data not shown).

Recent work implicated CYLD as a negative regulator of NF-κB signaling, although this function is cell type-specific. We thus examined whether CYLD controls the activation of NF-κB in NKT cells and whether deregulated NF-κB activation contributes to the impaired ICOS expression and IL-7-mediated NKT cell survival. WT NKT cells displayed a low basal level of NF-κB activity, as detected by electrophoresis mobility shift
assays (EMSA) (Fig. 2-7A). The NF-κB activity was strikingly higher in the CYLD KO NKT cells (Fig. 2-7A). We also examined the NF-κB activation at different maturation stages of NKT and detected aberrant NF-κB activation in all three stages of the CYLD KO NKT cells (Fig. 2-7B and C). Furthermore, the critical role of CYLD in negatively regulating NF-κB activation was also detected in total thymocytes (Fig. 2-7E). Thus, CYLD has a crucial role in maintaining the normal level of NF-κB in developing NKT cells and possibly other thymic T-cell populations.

The deregulated activation of NF-κB in CYLD KO NKT cells was consistent with the hyper-expression of the activation marker CD69 (Fig. 2-3E and F). Furthermore, since NF-κB is required for NKT cell maturation, the deregulated NF-κB activation may explain the accelerated NKT maturation in CYLD KO mice (Fig. 2-3D). To examine whether the deregulated NF-κB activation also contributed to the attenuated expression of ICOS and impaired IL-7R signaling, we employed a genetic system involving NF-κB inhibition by an IκBα super-repressor (IκBαSR) transgene. We crossed the CYLD KO mice with a T lineage-specific IκBαSR transgenic mouse. The aberrant activation of NF-κB in thymocytes was completely blocked in the CYLD KO-IκBαSR mice (Fig. 2-7E), suggesting the involvement of canonical NF-κB signaling. IκBαSR also blocked the basal NF-κB activity in WT-IκBαSR thymocytes (Fig. 2-7E). Importantly, inhibition of NF-κB completely restored the expression of ICOS in the CYLD KO NKT cells (Fig. 2-7F) as well as in CD4 SP thymocytes and Treg cells (data not shown). Expression of IκBαSR in the WT mice also led to a weak increase in the ICOS expression. These results, for the first time, demonstrate that NF-κB activation negatively regulates the homeostatic expression of ICOS in NKT cells. Moreover, we found that the defect of CYLD KO NKT cells in responding to the survival signal of IL-7
was also rescued by the IκBαSR (Fig. 2-7G). Thus, although NF-κB is essential for the progression of immature NKT cells to mature NKT cells\textsuperscript{177-179}, the magnitude of NF-κB activation must be tightly regulated, since deregulated NF-κB activation impairs critical molecular events (ICOS expression and IL-7R response) involved in NKT cell survival and homeostasis.
DISCUSSION

NKT cell development requires a specific set of signaling factors, most of which mediate intrathymic maturation of NKT cells. Data presented in this paper provide the first example of how negative regulation of NKT cell signaling contributes to NKT cell development. In contrast to the known NKT regulators, CYLD is dispensable for NKT cell maturation. In fact, the CYLD KO mice contain a substantially higher frequency of NK1.1+ mature NKT cells. This phenotype is associated with a hyper-activation phenotype, particularly in the immature NKT populations. However, although loss of CYLD appears to accelerate the process of NKT cell maturation, the CYLD KO mice display a severe reduction in the number of NKT cells in both the thymus and the periphery. This deficiency is due to the massive apoptosis of immature NKT cells. Thus, in contrast to its pro-apoptotic function implicated in other cell types, particularly tumor cells, CYLD has a potent anti-apoptotic role in immature NKT cells, which is crucial for NKT cell development.

The hyper-activated phenotype of CYLD KO NKT cells indicated the involvement of an activation-induced mechanism in their apoptosis induction. However, although CYLD KO NKT cells express higher levels of Fas, this is detected mainly in the stage 2 (CD44+NK1.1−) and mature (CD44−NK1.1+) NKT populations. On the other hand, the stage 1 (CD44−NK1.1−) CYLD KO NKT cells do not display a significant increase in Fas expression, despite their strikingly high level of apoptosis. Moreover, the CYLD deficiency does not alter the expression level of FasL. Thus, although Fas/FasL interaction may contribute to thymic NKT apoptosis, the massive NKT cell death in CYLD KO mice clearly involves additional mechanisms. Indeed, the survival defect of CYLD KO NKT cells was associated with
attenuated signaling of IL-7, a cytokine that is crucial for the survival and homeostasis of immature NKT cells.\textsuperscript{112,113} CYLD regulates IL-7 signaling at least partially through modulating the expression of IL-7R\(\alpha\), since the level of IL-7R\(\alpha\) protein and mRNA was reduced in CYLD KO NKT cells. Consistently, the CYLD KO NKT cells are hyporesponsive to IL-7-stimulated STAT5 phosphorylation. When cultured \textit{in vitro}, the apoptosis of WT thymic NKT cells was efficiently blocked by IL-7; in sharp contrast, IL-7 was largely ineffective in protecting the CYLD KO NKT cells from undergoing apoptosis. These findings suggest that modulation of IL-7 signaling may form an important part of the mechanism by which CYLD regulates the survival and development of NKT cells.

The critical role of CYLD in NKT cell development is also linked to the regulation of ICOS, a costimulatory molecule that is constitutively expressed in NKT cells.\textsuperscript{119,120} Gene targeting studies reveal an important function of ICOS in mediating the survival and homeostasis of NKT cells in both the periphery and the thymus.\textsuperscript{120,121} Consistently, we showed that disruption of the ICOS-ICOSL interaction by injecting mice with ICOS-Fc drastically reduces the number of thymic NKT cells. To date, little is known how the homeostatic expression of ICOS is regulated. Our current study demonstrated a pivotal role for CYLD in regulating this molecular event. The expression of both ICOS protein and ICOS mRNA is reduced to near background level in the CYLD KO NKT cells. This novel function of CYLD is not limited to NKT cells, since the CYLD KO thymocytes and peripheral T cells also display reduced ICOS expression. Our finding that CYLD regulates expression of both ICOS and IL-7R\(\alpha\) is consistent with a recent Systems Genetics study suggesting tight association between these two genes in thymocytes.\textsuperscript{246} We further found that IL-7 is a cytokine that stimulates the expression of ICOS. This latter finding indicates that the
impaired ICOS expression in CYLD KO NKT cells is at least partially attributed to the attenuated IL-7 signaling. On the other hand, since the defect of CYLD KO NKT cells in ICOS expression is so severe, the possibility for the involvement of additional mechanisms cannot be excluded.

The current study revealed a crucial role for CYLD in preventing aberrant activation of NF-κB in developing NKT cells. Loss of CYLD causes hyper-activation of NF-κB in all three maturation stages of the thymic NKT cells. Notably, deregulated NF-κB activation is often associated with enhanced cell survival and activation. However, although the CYLD KO NKT cells do display a hyper-activated phenotype, these mutant cells do not have enhanced survival ability but rather undergo active apoptosis. Our data suggest that this unusual function of NF-κB involves suppression of IL-7 signaling and ICOS expression. When the CYLD KO mice were crossed with IκBαSR transgenic mice, the constitutive NF-κB activity is inhibited. Concurrently, the ICOS expression is completely restored in the NKT cells derived from the CYLD KO-IκBαSR mice. Inhibition of NF-κB by IκBαSR also restores the ability of CYLD KO NKT cells to respond to IL-7. Thus, although NF-κB is required for NKT cell maturation, deregulated NF-κB activation interferes with ICOS expression and IL-7 signaling and, thereby, causes detrimental effect on the survival and development of NKT cells.

We have previously shown that CYLD regulates TCR-proximal signaling and ERK activation in DP thymocytes and is important for the transition of DP thymocytes to SP thymocytes. However, this signaling function of CYLD appears to be specific for DP thymocytes, since TCR-proximal signaling and ERK activation are normal in CYLD KO peripheral T cells. Similarly, the function of CYLD in NKT cells unlikely involves
regulation of TCR signaling or ERK activation, since loss of CYLD does not block the maturation of NKT cells but rather causes a hyper-activation phenotype of the developing NKT cells. In summary, our current study establishes CYLD as a critical regulator of NKT cell development and uncovers a novel mechanism that regulates the survival and homeostasis of immature NKT cells.
MATERIALS AND METHODS

Mice

Generation and genotyping of CYLD WT (CYLD\(^{+/+}\)) and CYLD KO (CYLD\(^{-/-}\)) mice have been described previously\(^\text{253, 254}\). V\(\alpha\)14 transgenic (Tg) mice expressing the rearranged V\(\alpha\)14-J\(\alpha\)281 TCR \(\alpha\) chain have been described\(^\text{242}\). CYLD KO female mice were mated with V\(\alpha\)14 Tg male to generate CYLD\(^{+/+}\)-V\(\alpha\)14 Tg mice, which were subsequently intercrossed to generate CYLD\(^{+/+}\)-V\(\alpha\)14 Tg and CYLD\(^{-/-}\)-V\(\alpha\)14 Tg (also called WT-Va14Tg and KO-Va14Tg) mice. I\(\kappa\)B\(\alpha\)SR transgenic mice express a degradation-resistant form of I\(\kappa\)B\(\alpha\) (I\(\kappa\)B\(\alpha\) super-repressor or I\(\kappa\)B\(\alpha\)SR) under the control of a T-cell specific proximal lck promoter\(^\text{252}\) and were obtained from Jackson Laboratory. CYLD KO female mice were crossed to I\(\kappa\)B\(\alpha\)SR male mice to generate CYLD\(^{+/+}\)-I\(\kappa\)B\(\alpha\)SR, which were then intercrossed to produce CYLD\(^{+/+}\)-I\(\kappa\)B\(\alpha\)SR and CYLD\(^{-/-}\)-I\(\kappa\)B\(\alpha\)SR (also called WT-I\(\kappa\)B\(\alpha\)SR and KO-I\(\kappa\)B\(\alpha\)SR) mice. B6.SJL (CD45.1\(^\text{+}\)) mice were obtained from Jackson Laboratory and then crossed to CYLD\(^{+/+}\) (CD45.2) mice to produce CYLD\(^{+/+}\) (CD45.1\(^+\)CD45.2\(^+\)) mice. All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center and Pennsylvania State University College of Medicine.

Antibodies and reagents

Functional grade anti-mCD3\(\varepsilon\) (145-2C11) and anti-mCD28 (37.51) antibodies were purchased from eBioscience. Fluorescence-labeled anti-mCD4 (L3T4), anti-mCD25 (PC61.5), anti-mCD62L (MEL-14), anti-CD44 (IM7), anti-mCD69 (H1.2F3), anti-mNK1.1
(PK136), anti-mTCRβ (H57-597), anti-mIL-2Rβ (TM-b1), anti-mICOS (7E.17G9), anti-mIL-7Rα (A7R34), anti-mCD45.1 (A20), anti-T-bet (eBio4B10), anti-mIL-4 (11B11), anti-mIFNγ (XMG1.2), anti-mFoxP3 (FJK-16s), streptavidin, along with biotinylated anti-mICOS (7E.17G9) and anti-Rat IgG2b isotype control antibodies were purchased from eBioscience. Biotinylated anti-mCD132 and fluorescence-labeled anti-mFas (Jo2), anti-mFasL (MFL3), anti-GATA3 (L50-823), anti-pSTAT5(pY694) (clone 47) were from BD Biosciences. ICOS-Fc and hu-Fc control antibodies were provided by Amgen. Anti-CYLD antibody has been described\(^{255}\). Anti-tubulin (TU-02) was from Santa Cruz. α-GalCer was purchased from Alexis Biochemicals. PBS-57 loaded CD1d tetramers, conjugated to APC or PE, and α-GalCer analog, OCH, were provided by the NIH Tetramer Core Facility. IL-7 and IL-15 were purchased from Peprotech. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma, and monensin was from eBioscience.

**Flow Cytometry and Cell Sorting**

Thymocytes and splenocytes were prepared by gentle homogenization over nylon mesh. Mononuclear cells were isolated after RBC lysis (Sigma) and debris filtration over nylon mesh. To isolate intrahepatic lymphocytes, harvested livers were processed through a 200 micron metal screen (Bellco) and treated with 20U/ml collagenase IV (Sigma) for 30 min at 37°C. Following digestion, liver slurry was subjected to centrifugation (20 min, 2800 rpm) over 35%/75% Percoll (GE Healthcare). Intrahepatic lymphocytes were isolated from the Percoll interphase. Following isolation, cells were stained with appropriate fluorescence-labeled antibodies in the presence of Fc-block to prevent non-specific binding. When biotinylated antibodies were used, cells were washed in PBS prior to staining with
fluorescence-labeled streptavidin. Annexin V (BD Biosciences) staining and Foxp3 (eBiosciences) staining were performed according to manufacturers’ recommendations. Data was acquired on FACSCanto or LSRII (BD Biosciences) and analyzed using FlowJo (Treestar). When necessary, thymic NKT cells were enriched by depleting CD8⁺ cells using either CD8 MicroBeads (Miltenyi) or Easysep CD8 positive selection kit (Stemcell). In some experiments, complete depletion of CD8⁺ cells were ensured by subsequent depletion of any remaining CD8⁺ cells by flow cytometry. For purification of pure total NKT or NKT subset populations, thymocytes were first depleted of CD8⁺ cells as described above and stained with CD1d-tetramer, anti-TCRβ, anti-CD44 and anti-NK1.1 antibodies and indicated populations were isolated by flow cytometry. Typical purity of isolation was greater than 98%.

**In vivo NKT cell stimulation**

CYLD⁺/⁺ and CYLD⁻/⁻ mice received 100 µg/kg body weight of α-GalCer or OCH in 200 µl of PBS via the tail vein. For serum cytokine determination, mice were serially bled at indicated time points via the tail vein. The blood was allowed to clot, and the serum was separated from the clot by centrifugation. Sandwich ELISA was performed to determine IL-4 and IFNγ concentrations using capture and biotinylated detection antibodies according to manufacturer’s recommendations (eBioscience). For intracellular cytokine staining, splenocytes were isolated 1hr post α-GalCer/OCH injection and incubated *in vitro* for 2 hours in presence of 2µM monensin. After staining with anti-TCRβ and CD1d-tetramer, cells were washed and fixed in Cytofix/Cytoperm buffer (BD Biosciences). Cells were then washed and incubated with anti-IL-4 and anti-IFNγ in Perm/Wash buffer (BD Biosciences).
Bone marrow chimera

B6.SJL and CYLD\(^{+/+}\) mice were crossed for one generation to generate CD45.1\(^+\) CYLD\(^{+/+}\) mice (CD45.1\(^+\)CD45.2\(^+\)). CYLD\(^{+/+}\) (CD45.1\(^+\)CD45.2\(^+\)) and CYLD\(^{+/−}\) (CD45.1\(^+\)CD45.2\(^−\)) recipient mice were lethally irradiated with 1000 rads in a cesium source. 12 hours post irradiation, CYLD\(^{+/+}\) and CYLD\(^{+/−}\) recipient mice received the mixture containing equal proportions of CYLD\(^{+/+}\) (CD45.1\(^+\)CD45.2\(^+\)) and CYLD\(^{+/−}\) (CD45.1\(^+\)CD45.2\(^−\)) T cell–depleted bone marrow (5x10\(^6\) cells/recipient). Anti-CD90 magnetic beads (Miltenyi) were used for T cell depletion. Thymic and splenic reconstitution was analyzed 6 weeks after injection based on CD45.1 expression (positive in CYLD\(^{+/+}\) cells and negative in CYLD\(^{+/−}\) cells).

IL-7 Stimulation

Total thymocytes or NKT-enriched thymocytes were stimulated in vitro with IL-7 (1-50 ng/ml) for indicated periods prior to subsequent analysis. To determine the level of IL-7 signaling, the amount of phosphorylated STAT5 (p-STAT5) measured by flow cytometry. Briefly, cells were incubated with IL-7 (50 ng/ml) for 30 min at 37°C. Cells were washed in cold-PBS and fixed in 4% paraformaldehyde for 10 min at 37°C. Cells were permeabilized in ice-cold 90% methanol for 30 min on ice. Following two washes with incubation buffer (PBS+0.5% BSA), cells were stained with p-STAT5 antibody for 1 hour at room temperature. Cells were washed twice with incubation buffer and data was acquired by flow cytometry.

ICOS-Fc Administration

CYLD\(^{+/+}\) mice received 100 \(\mu\)g of ICOS-Fc or hu-Fc control in 200 \(\mu\)l of PBS via the tail vein. 48 hrs post injection, thymocytes were isolated for analysis.
**Real-time quantitative RT-PCR**

Total RNA was isolated from purified NKT cells using TRI reagent (Molecular Research Center, Inc.) and subjected to cDNA synthesis using RNase H-reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed in triplicates, using iCycler Sequence Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad). The expression of individual genes was calculated by a standard curve method and normalized to the expression of GAPDH. The gene-specific primer sets were: mIL-7Rα, 5’-GCAGAGCGGACCACACTACA-3’ (forward) and 5’-ATTTTTGCAAAGTTAATTCT-3’ (reverse); mlCOS, 5’-TACTTCTGCAGCCTGTCCAT-3’ (forward) and 5’-CAGCAGAGCTGGGATTACATA-3’ (reverse); mT-bet, 5’-GCCAGGGAACCCTATATG-3’ (forward) and 5’-GACGATCATCTGGGTACATTTGT-3’ (reverse); mGAPDH, 5’-CCGGAATTCAACAGCAACTCCACTC-3’ (forward) and 5’-CGCGATCCAGGGTCTTTACTCTCTTG-3’ (reverse).

**Western blotting and electrophoresis mobility shift assay (EMSA)**

Whole-cell lysates were prepared from thymocytes and subjected to IB as previously described. Nuclear extracts were prepared and subjected to EMSA using a 32P-radiolabeled κB oligonucleotide probe (CAA CGG CAG GGG AAT TCC CCT CTC TT) or a control probe bound by the constitutive transcription factor NF-Y (AAG AGA TTA ACC AAT CAC GTA CGG TCT)
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FIGURE AND LEGENDS

Fig. 2-1. CYLD<sup>−/−</sup> mice have a defect in NKT cell development. (A) Age- and sex-matched CYLD knockout (KO) and WT (WT) control mice (3 mice/group) were injected with α-GalCer (αGC; 100mg/kg body weight). At the indicated times, blood was drawn and ELISA was performed to determine the concentration of IL-4 and IFN-γ in the sera. The values of individual mice were plotted (note, the mutant mouse curves are overlapping). (B) Cells were prepared from the indicated organs of CYLD KO and WT mice and subjected to flow cytometry to determine the frequency of NKT cells based on their binding to PBS57-loaded CD1d tetramer and expression of TCRβ. (C) Absolute NKT cell numbers in individual thymi, spleens, and livers of WT and CYLD KO mice. Data are presented as mean ± s.d. of 3 mice. (D) CD1d tetramer-positive NKT cells from WT and CYLD KO thymuses were analyzed based on expression of TCRβ and CD4. (E) Flow cytometric analysis of NKT cell frequency in the thymocytes and splenocytes of WT or CYLD KO mice crossed with Vα14Tg mice. Representative results four independent experiments are shown.
Fig. 2-1
**Fig. 2-2. CYLD has a cell intrinsic role in regulating NKT cell development.** CYLD WT mice were crossed with SJL mice for one generation to obtain the CD45.1 congenic marker. Bone marrow cells derived from the WT/SJL (WT) and CYLD KO (KO) mice were mixed (1:1 ratio) and adoptively transferred into γ-irradiated WT or CYLD KO recipient mice. After 6 weeks, flow cytometry was performed to determine the frequency of WT (CD45.1⁺) and KO (CD45.1⁻) NKT cells in the thymus and spleen of the recipient mice. The representative results are shown from experiment with two WT and two CYLD KO recipient mice.
**Fig. 2-3. CYLD deficiency does not block NKT cell maturation and causes a hyper-activated phenotype of NKT cells.** (A) Thymocytes (Thy) and splenocytes (Spl) were isolated from adult WT and CYLD KO mice (6 wk) and subjected to flow cytometry. Gated NKT cells (TCRβ⁺ and CD1d tetramer⁺) were analyzed for expression of CD44 and NK1.1. (B) Thymic and splenic NKT cells from WT-Vα14Tg and CYLD KO-Vα14Tg mice were analyzed as in A. (C) The frequency of thymic NKT cells from young (1 and 2 weeks) WT and CYLD KO mice were analyzed by flow cytometry. (D) Thymic NKT cells from young (1 and 2 weeks) WT and CYLD KO mice were analyzed as in A. (E) Gated thymic NKT cells were analyzed for CD69 expression. (F) CD69 expression was determined on gated subpopulations of NKT cells from WT and CYLD KO mice (left panels) or WT-Vα14Tg and CYLD KO-Vα14Tg mice (right panels).
**Fig. 2-4. Enhanced apoptosis in CYLD⁺ NKT cells.** (A) Thymocytes derived from WT and CYLD KO mice (left panel) or WT-Vα14Tg and CYLD KO-Vα14Tg mice (right panel) were subjected to flow cytometry. Gated NKT cells were analyzed for apoptosis based on AnnexinV staining. (B) Apoptosis analysis of the indicated stages of thymic NKT cells from WT or CYLD KO mice. (C) Fas expression on the indicated stages of thymic NKT cells of WT-Vα14Tg and CYLD KO-Vα14Tg mice.
Fig. 2-4
Fig. 2-5. Regulation of IL-7Rα expression and IL-7 signaling by CYLD. (A) Thymocytes derived from WT and CYLD KO mice were enriched for NKT cells by depleting CD8⁺ cells by magnetic beads. The cells were incubated in vitro either without (NT) or with IL-7 (50 ng/ml) for 48 hr, and apoptosis of NKT cells was analyzed based on AnnexinV staining. (B) NKT-enriched thymocytes were incubated as in A. After 48 hr, the frequency of NKT cells was determined by flow cytometry. (C) IL-7Rα expression on gated thymic NKT cells from WT and CYLD KO mice (left panel) or WT-Vα14Tg and CYLD KO-Vα14Tg mice (right panel). (D) Real-time PCR was performed to determine the relative level of IL-7Rα mRNA in thymic total NKT cells from WT and CYLD KO mice (left panel) and thymic NKT cell subsets from WT-Vα14Tg (WT) and CYLD KO-Vα14Tg (KO) mice (right panel). Data are presented as fold relative to KO sample (left panel) or KO sample of NK1.1⁺ cell sample (right panel). (E) NKT-enriched thymocytes derived from WT and CYLD KO mice were stimulated with IL-7 (50ng/ml) for 30 min at 37°C and subjected to intracellular staining of tyrosine-phosphorylated STAT5 (P-STAT5) and NKT surface markers. STAT5 phosphorylation was analyzed in the gated populations of NKT cells. A dotted line was used to mark the peak intensity of STAT5 phosphorylation in WT cells.
**Fig. 2-6. Impaired ICOS expression in CYLD<sup>−/−</sup> NKT cells and other T-cell subsets.** (A) ICOS expression was analyzed by flow cytometry on gated NKT cells from the thymus (Thy) and spleen (Spl) of WT or CYLD KO mice. The staining with isotype control Ig was included to determine the background staining level. (B) ICOS expression was analyzed on the gated populations of NKT cells derived from WT and CYLD KO mice (upper panels) or WT-Vα14Tg and CYLD KO-Vα14Tg mice (lower panels). (C) ICOS expression was analyzed on gated CD4<sup>+</sup> single positive (CD4 SP) thymocytes or thymic Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>). (D) ICOS expression was analyzed on gated naïve (CD44<sup>lo</sup>CD62L<sup>hi</sup>) and memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) CD4<sup>+</sup> T cells from spleen of WT and CYLD KO mice. (E) Total NKT cells were purified, by cell sorting, from the thymocytes of WT and CYLD KO mice and subjected to real-time PCR assays. The ICOS mRNA level is presented as fold relative to the KO sample. (F) Thymocytes derived from WT-Vα14Tg or CYLD KO-Vα14Tg were incubated without (NT) or with IL-7 (50ng/ml) for 48 hrs. The ICOS expression on the different populations of NKT cells was analyzed by flow cytometry. (G) WT mice were injected iv with either control Ig or ICOS-Fc (100 mg). After 48 hrs, the frequency of thymic NKT cells was analyzed by flow cytometry.
Fig. 2-6
Fig. 2-7. Hyper-activation of NF-κB is responsible for impaired ICOS expression and IL-7 response in CYLD<sup>−/−</sup> NKT cells. (A) Total thymic NKT cells of WT-β<sup>α</sup>14Tg and CYLD KO-β<sup>α</sup>14Tg mice were purified by flow cytometric cell sorting and immediately subjected to nuclear extract preparation. EMSAs were performed using 32P-radiolabelled probes for NF-κB (upper) and a constitutive transcription factor, NF-Y (lower). (B) The NKT cells from A were further separated into the indicated populations by flow cytometric cell sorting and subjected to EMSA as in A. (C) The bands in B were quantified and presented as ratio between NF-kB and NF-Y. (D) CYLD KO mice were crossed with IκBαSR transgenic mice to produce the indicated genotypes of littermates. Total cell lysates were prepared with thymocytes derived from the indicated mice and subjected to IB to detect the expression of CYLD and IκBα as well as the loading control tubulin. The larger size of the transgenic IκBα in the IκBαSR mice was probably due to its fusion with a FLAG tag<sup>252</sup>. (E) EMSA was performed using nuclear extracts of thymocytes derived from the indicated mice (age-matched). (F) Thymocytes derived from the indicated mice (age-matched) were depleted of CD<sup>8</sup><sup>+</sup> cells and subjected to flow cytometry to determine the expression level of ICOS on gated NKT (TCR<sup>β</sup><sup>+</sup>CD1dTetramer<sup>+</sup>) cells. Data are presented as a flow cytometry profile (upper) and a mean fluorescence index (MFI) bar graph (lower). (G) NKT cell enriched thymocytes from indicated mice were cultured for 48 hr either in the absence (NT) or presence of IL-7 (50ng/ml). NKT cell apoptosis was determined based on annexinV staining on the gated NKT cells.
Supplementary Fig. 2-1. CYLD knockout mice have impaired induction of IL-4 and IFN-γ by OCH. Age-matched CYLD knockout (KO) and wildtype (WT) mice received OCH (2μg). At the indicated times, sera were withdrawn from the individual mice and subjected to ELISA to detect the concentration of IL-4 and IFN-γ.
Supplementary Fig. 2-2. CYLD-deficient NKT cells are hyporesponsive to OCH stimulation. Age- and sex-matched WT and CYLD KO mice were injected (i.v.) with either PBS (NT) or OCH (2 µg). After 1 hr, splenocytes were subjected to ICS to detect the production of IL-4 (A) and IFN-γ (B) by NKT cells (gated on TCRβ⁺ cells).
Supplementary Fig. 2-3. CYLD deficiency does not significantly alter the expression of Fas ligand (FasL). Thymocytes and splenocytes of WT or CYLD KO mice were subjected to flow cytometry assays to detect FasL-expressing populations of NKT cells (gated on TCRβ⁺CD1dTetramer⁺ cells).
Supplementary Fig. 2-4. Surface expression of IL-7Rα, IL-2Rβ, and common γc on thymic NKT populations of WT-Vα14Tg and CYLD KO-Vα14Tg mice. Thymocytes derived from WT-Vα14Tg (WT) or CYLD KO-Vα14Tg (KO) mice and subjected to flow cytometry to detect IL-7Rα, IL-2Rβ, and common γc on the indicated NKT populations. NKT cells were gated based on their TCRβ⁺CD1dTetramer⁺ staining as well as the population-specific CD44/NK1.1 markers.
Supplementary Fig. 2-5. CYLD is not required for TCR-stimulated ICOS expression.

Thymocytes from wildtype (WT) or CYLD-knockout (KO) mice were incubated for the indicated times with medium (NT) or plate-bound anti-CD3 (5µg/ml) and soluble anti-CD28 (5µg/ml). The surface expression of ICOS was detected by flow cytometry.
Supplementary Fig. 2-6. Normal T-bet expression in CYLD KO NKT cells. (A) RNA was isolated from sorted total NKT cells or NKT cell subpopulations derived from thymi of WT-Vα14Tg or CYLD KO-Vα14Tg mice. Real-time PCR was performed to determine the relative T-bet mRNA level (presented as fold relative to KO samples). (B) Flow cytometry to analyze the expression level of T-bet in gated NKT cells.
CHAPTER III

ROLE OF CARMA1 IN DEVELOPMENT OF REGULATORY T CELLS THROUGH REGULATION OF IL-2 SIGNALING
ABSTRACT

Regulatory T cells (Tregs) play a critical role in the preservation of self-tolerance and prevention of autoimmunity. Despite their importance, the process of Treg development has been incompletely understood. In this study, we show that Carma1, a previously well-characterized adaptor protein downstream of the T cell receptor (TCR), is essential for the development of Tregs in the thymus. Interestingly, Carma1 deficiency impairs the response of Treg precursor to IL-2, a cytokine that is critical for the development of thymic Tregs. This IL-2 signaling defect in Carma1 knockout mice is associated with a lower expression of IL-2Rβ at the protein level. Thus, Carma1 regulates Treg development through modulation of IL-2 signaling via the IL-2Rβ.
INTRODUCTION

Regulatory T cells (Tregs) play critical functions in maintaining self-tolerance and preventing autoimmunity. There are two major types of Tregs: thymic-driven naturally occurring Tregs (nTregs) and Tregs that develop from naïve CD4 T cells in the periphery (iTregs). In this chapter, Tregs refer to the iTregs. Tregs express cell surface markers such as CD4 and CD25 along with transcription factor, Foxp3. Prior to discovery of Foxp3 as a Treg marker, Tregs could not be precisely identified because Treg markers such as CD25, are expressed by other T cell subsets, including activated CD4+ T cells. Expression of Foxp3 not only defines the Treg population, but it is also critical for Treg development and function. Defective Foxp3 gene has been identified in Scurfy strain of mice and has been shown to cause IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in humans. Both mice and humans with Foxp3 mutations develop an autoimmune phenotype with marked hyperactivation of T cells and hypersecretion of cytokines. Despite significant research efforts directed at understanding the process of thymic Treg development, the exact mechanism has not been fully elucidated.

The T cell receptor (TCR) signal is one of the key requirements for Treg development. The importance of a TCR signal in Treg development has been demonstrated in experiments involving TCR transgenic mice. These experiments showed that Tregs developed only in the presence of cognate antigen (Ag) for the transgenic TCR. Furthermore, during thymocyte development, the strength and duration of TCR signaling has been suggested to determine the cell-fate decisions. Weak or strong TCR signals arising from TCR interaction with self-Ag of variable affinities, lead to positive selection or negative selection of
conventional T cells, respectively. TCR signal of intermediate strength has been shown to facilitate Treg development. In addition to a TCR signal, coordinated costimulation by CD28 has critical role in Treg development, as mice lacking CD28 or its ligands, CD80/CD86, exhibit significant deficiency in Tregs.

In addition to a TCR signal, common-gamma chain (γc) cytokines, namely IL-2, IL-7, and IL-15, provides the second critical signal for thymic Treg development. Of these, IL-2 is the most important, as IL-2 or CD25 (IL-2Rα) deficiency leads to ~50% reduction in thymic Tregs, whereas IL-7 or IL-15 deficiencies do not affect Treg development. However, IL-7 and IL-15 clearly contribute to Treg development because when IL-7 and IL-15 signals are disrupted in addition to IL-2 signal, it leads to a complete depletion of Tregs in the thymus. It is likely that in the absence of IL-2 signaling, IL-7 and IL-15 can partially compensate to provide the necessary signals for developing Tregs. STAT5, a signaling molecule downstream of γc-receptors, also has a non-redundant role in Treg development since its disruption leads to a complete depletion of Tregs.

Though TCR signaling is clearly required for Treg development, it is less clear how TCR downstream signaling pathways are involved. TCR engagement leads to the activation of protein kinase C (PKC)-θ. Activated PKC-θ phosphorylates Carma1, which then recruits Bcl10 and MALT1 molecules to form the Carma1-Bcl10-MALT1 (CBM) adapter complex. Activation of the CBM complex leads to induction of IKK complex, eventually leading to NF-κB activation. Deficiency of several molecules in this signaling pathway has been associated with a decrease in thymic Tregs. However, the exact mechanism by which TCR-mediated NF-κB activation regulates Treg development remains to be investigated.
In this study, we demonstrate that Carma1-deficient mice develop skin inflammation with age, which was associated with cell-intrinsic defect in thymic Treg development. Carma1-deficient thymic precursors failed to induce Foxp3 expression in response to IL-2. We show that Carma1-deficient thymocytes were defective in IL-2 and IL-15 signaling, which was associated with defect in IL-2Rβ protein expression.
RESULTS

Carma1⁻/⁻ mice develop skin inflammation

During the course of our studies with Carma1⁻/⁻ mice, we noticed an interesting phenotype as the mice aged. Starting from approximately 10 weeks of age, Carma1⁻/⁻ mice spontaneously exhibited signs of dermatitis, especially around the nostril and eyes (Figure 3-1A). There were loss of hair and skin thickening in the affected area. Carma1⁻/⁻ mice also had enlarged spleens and lymph nodes (Figure 3-1B). This previously unreported phenotype in Carma1⁻/⁻ mice was quite prevalent, as a significant majority of Carma1⁻/⁻ mice were affected by 24 weeks (Figure 3-1C), and almost all of the Carma1⁻/⁻ mice were afflicted as they aged further. Upon a subsequent search of literature, we found that one group had reported a similar finding using a slightly different approach. They showed that mice with a chemically induced point mutation in Carma1 exhibited similar signs of dermatitis. Therefore, Carma1 seems to play an important role in preventing development of autoimmune phenotype in mice.

Carma1⁻/⁻ mice have significantly reduced Treg population

The development of skin inflammation, such as in atopic dermatitis, is often associated with pathologic T cell function, typically featuring T cell hyper-activation. Therefore, we examined whether skin inflammation in our Carma1⁻/⁻ mice was due to dysfunctional T cell compartment. Consistent with previous studies, Carma1⁻/⁻ mice had relatively normal conventional CD4⁺ and CD8⁺ T cell development and peripheral T cell numbers. In addition, due to the essential role of Carma1 in the TCR signaling pathway,
Carma1−/− T cells were hypo-responsive to TCR stimulation (results not shown). Therefore, it is less likely that hyperactive effector T cells caused skin inflammation in our Carma1−/− mice. However, previous studies did not address the status of Tregs in Carma1−/− mice, which play an important role in immune regulation. Since our Carma1−/− mice developed an inflammatory phenotype with age, we examined whether Carma1−/− have Treg deficiency. Interestingly, Carma1−/− mice were severely depleted of Foxp3+ Treg cells in the spleen and lymph nodes (Figure 3-2B and 3-2C). Since the majority of Tregs develop in the thymus, we examined the status of Tregs in Carma1−/− thymus. Remarkably, the percentage of Tregs in the thymus was significantly diminished in Carma1−/− mice compared to wildtype controls (Figure 3-2A). Thus, Carma1 is required for normal development of Tregs in mice.

**Carma1 has cell intrinsic function in Treg development**

Similar to conventional CD4+ T cells, the positive selection of Tregs in the thymus is driven by thymic epithelial cells (TECs)269. Although it is possible that Carma1 deficiency may affect the function of TECs, given its predominant expression in lymphocytes270,271 and its established function in T cells, it is much more likely that Carma1 deficiency affects the function of thymocytes. To determine whether the Treg defect in the Carma1−/− mice was intrinsic to the Treg precursors, we performed a mixed bone marrow chimera experiment. We first crossed Carma1+/+ mice (CD45.2+) with SJL mice (CD45.1+) to obtain the congenic marker. We obtained T cell-depleted bone marrow from Carma1+/+ (CD45.1+CD45.2+) and Carma1−/− (CD45.1−CD45.2+) mice and mixed them at 1:1 ratio. Bone marrow mixture was adoptively transferred into lethally irradiated recipient Carma1−/− (CD45.1−CD45.2+) mice. Impaired development of Carma1−/− Tregs in the wildtype recipient mice would suggest a cell-
intrinsic defect. In the thymus, both Carma1\(^{+/+}\) and Carma1\(^{-/-}\) donor cells generated the conventional CD4 SP and CD8 SP thymic subsets at the expected ratios (Figure 3-2D). However, whereas Carma1\(^{+/+}\) donor cells were able to generate CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) Tregs, Carma1\(^{-/-}\) donor cells displayed a cell-intrinsic defect in the development of Tregs. (Figure 3-2E, upper panels) And as expected, the percentage of Carma1\(^{-/-}\) derived Tregs was severely diminished in the periphery, compared to the wildtype (Figure 3-2E, lower panels). These results suggest that Carma1 has a cell-intrinsic function in the development of Tregs.

**Carma1\(^{-/-}\) mice demonstrate a defective IL-2 response**

Recently, a two-step model of thymic Treg development has been proposed.\(^{272, 273}\) In the first step, TCR signaling induces CD4\(^{+}\)CD8\(^{-}\) Treg precursors to express CD25 (IL-2R\(\alpha\)), which allows them to become sensitive to IL-2 stimulation. In the second step, IL-2 stimulation induces Foxp3 expression in CD4\(^{+}\)CD8\(^{-}\)CD25\(^{+}\) intermediate stage cells in a TCR-independent manner, leading to the generation of CD4\(^{+}\)CD8\(^{-}\)CD25\(^{+}\)Foxp3\(^{+}\)Tregs.\(^{273}\) As a critical regulator of TCR-induced NF-\(\kappa\)B activation, Carma1 may influence this potential developmental program at each step. Since CD25 (IL-2R\(\alpha\)) is a downstream target of NF-\(\kappa\)B,\(^{274}\) the NF-\(\kappa\)B defect caused by Carma1 deficiency could prevent CD4\(^{+}\)CD8\(^{-}\)Treg precursor from expressing CD25 and progressing to the intermediate stage. However, the CD4\(^{+}\)CD8\(^{-}\)CD25\(^{+}\)Foxp3\(^{-}\) intermediate stage population was only slightly diminished in Carma1\(^{-/-}\) thymus compared to wildtype (Figure 3-2A). In contrast, the percentage of CD4\(^{+}\)CD8\(^{-}\)CD25\(^{+}\)Foxp3\(^{-}\) population in the spleen, which most likely represents activated CD4 T cells, was significantly diminished in Carma1\(^{-/-}\) mice (Figure 3-2B). Since IL-2 is also a downstream target of NF-\(\kappa\)B,\(^{275}\) the Treg defect in Carma1\(^{-/-}\) mice could be due to failure of
Foxp3 induction by IL-2. Indeed, Carma1−/− thymocytes express a lower level of IL-2 transcripts (Figure 3-3A). However, our bone marrow chimera study, in which Carma1−/− Treg development was defective in the presence of wildtype thymocytes, suggests that lower IL-2 production could not be the major cause for Treg defect (Figure 3-2E).

Our data thus far suggests that regulation of Treg development by Carma1 may involve additional mechanisms other than NF-κB. To support this theory, we examined Treg development in IκBα-Super-Repressor (SR) mice. Due to overexpression of dominant negative form of IκBα, NF-κB activation is severely attenuated in these mice (Figure 3-3B, right panel, compare lanes 6 and 8), which is similar to what is typically seen in Carma1−/− mice (Figure 3-3B, left panel, compare lane 2 and 4). However, in contrast to Carma1−/− mice, Treg development was relatively normal in IκBα-SR mice (Figure 3-3C). These results suggest that Carma1 may regulate Treg development through a NF-κB independent mechanism.

Since Carma1−/− mice have near total loss of thymic Tregs but a smaller decrease in CD4+CD8−CD25+Foxp3− intermediate stage cells, we examined the IL-2 responsiveness in CD4+CD8−CD25+Foxp3− intermediate stage cells. We first crossed our Carma1−/− mice to Foxp3-GFP reporter mice to facilitate the purification of thymic Treg precursor populations. CD4+CD8−CD25+GFP+ and CD4+CD8−CD25+GFP− Treg precursor populations from Carma1+/+ and Carma1−/− thymi were purified by flow cytometry. Purified Treg precursors were incubated in vitro in the presence of IL-2 without further TCR stimulation. After 5 days of culture, IL-2 alone induced Foxp3 expression in a significant percentage of wildtype CD4+CD8−CD25+GFP+ intermediate precursor cells (Figure 3-4A, left panels). There was no Foxp3 induction in CD4+CD8−CD25+GFP− precursors, indicating the necessity of CD25
expression in IL-2 responsiveness (Figure 3-4B). Interestingly, IL-2-mediated Foxp3 expression was significantly diminished in Carma1<sup>−/−</sup> CD4<sup>+</sup>CD8<sup>−</sup>CD25<sup>+</sup>GFP<sup>−</sup> intermediate stage cells (Figure 3-4A, right panels). Thus, Carma1 regulates IL-2 responsiveness in thymic Treg precursors.

**Carma1<sup>−/−</sup> mice have an IL-2 signaling defect**

Since Carma1<sup>−/−</sup> Treg precursors showed a diminished response to IL-2, we examined the IL-2 signaling pathway in Carma1<sup>−/−</sup> thymocytes. Total thymocytes were isolated from Carma1<sup>+/+</sup> and Carma1<sup>−/−</sup> mice and stimulated with the indicated dose of IL-2 for 20 minutes. IL-2 stimulation led to rapid phosphorylation of the downstream signaling molecule, STAT5, in the wildtype thymocytes (Figure 3-4C, lanes1-3). However, IL-2 signaling was significantly diminished in the Carma1<sup>−/−</sup> thymocytes (Figure 3-4C, lanes 4-6). Consistent with intact Treg development, IL-2 signaling in IκBα-SR mice was normal (Figure 3-4C, lanes 7-12). IL-2 signaling was also defective in peripheral Carma1<sup>−/−</sup> T cells (Figure 3-4D). Furthermore, IL-15 signaling was attenuated in Carma1<sup>−/−</sup> thymocytes (Figure 3-4E). To assess the IL-2 signaling pathway in different thymic Treg precursors, thymocytes from Carma1-Foxp3-GFP mice were stimulated with IL-2 for 30 minutes and subjected to phospho-flow cytometry. In Carma1<sup>+/+</sup> CD4 SP thymocytes (Figure 3-5A, row1), both CD25<sup>+</sup>GFP<sup>−</sup> intermediate stage precursor cells and CD25<sup>+</sup>GFP<sup>+</sup> Tregs rapidly phosphorylated STAT5 upon IL-2 stimulation (Figure 3-5A, rows 3-4). However, in Carma1<sup>−/−</sup> thymocytes, IL-2 signaling was significantly attenuated in both populations (Figure 3-5B, rows 3-4). Thus, Carma1 positively regulates IL-2 signaling in thymic Tregs and Treg precursor cells.
**Carma1**<sup>−/−</sup> expressed reduced level of IL-2Rβ

Since Carma1<sup>−/−</sup> thymocytes were attenuated in both IL-2 and IL-15 signaling, we examined whether Carma1 regulates the expression of IL-2Rβ (CD122), a shared subunit between IL-2 and IL-15 receptors. Interestingly, both the Carma1<sup>−/−</sup> Treg intermediate population (CD25<sup>+</sup>GFP<sup>−</sup>) and Carma1<sup>−/−</sup> Foxp3<sup>+</sup> Treg population expressed a diminished level of IL-2Rβ in the thymus (Figure 3-6A). However, the transcription of IL-2Rβ was relatively normal in both GFP<sup>−</sup>(Foxp3<sup>−</sup>) and GFP<sup>+</sup>(Foxp3<sup>+</sup>) Carma1<sup>−/−</sup> thymic populations (Figure 3-6B). These results indicate that Carma1 regulates IL-2 signaling by modulating the expression of IL-2Rβ at the protein level.
DISCUSSION

Thymic Treg development requires a set of signaling events, most notably the engagement of the TCR and stimulation by IL-2 \(^{257}\). In this chapter, we provide clear evidence that Carma1, a critical signaling molecule downstream of the TCR, is required for thymic Treg development. Upon TCR stimulation, Carma1 recruits Bcl-10 and MALT1 to form the CBM complex, which eventually leads to NF-κB activation. The deletion of a single member of the CBM complex leads to defective NF-κB activation, which shows that the three members are functionally non-redundant \(^{195}\). The importance of this signaling pathway in Treg development has been demonstrated by examining the role of various players in the pathway \(^{130, 133, 134}\). Notably, Bcl-10, a member of the CBM complex, has been shown to be required for normal Treg development \(^{134}\). The requirement for other CBM members in Treg development has been unknown until very recently \(^{129, 131, 132}\).

NF-κB regulates the production of IL-2 and expression of IL-2Rα \(^{274, 276}\), which have important functions in Treg development. Previous reports \(^{266, 268}\), as well as our own data, clearly demonstrate that Carma1 \(^{-/-}\) thymocytes produce less IL-2. However, the result of our mixed bone marrow chimera study indicate that Carma1 \(^{-/-}\) Tregs fail to develop even in the presence of Carma1 \(^{+/+}\) cells that produce normal amounts of IL-2. Furthermore, despite a near complete absence of Foxp3\(^{+}\) Tregs, Carma1 \(^{-/-}\) mice express a relatively normal level of CD25 (IL-2Rα) on CD4\(^{+}\)CD8\(^{-}\) thymocytes. This is in contrast to Treg defect caused by Bcl-10, where a decrease in Treg population was relatively proportional to decrease in CD25 expression \(^{134}\). In addition, the Treg defect seen in our Carma1 \(^{-/-}\) mice is much more severe than the reported defect caused by deficiency of IL-2 or IL-2Rα (CD25) \(^{135}\).
Despite the reported importance of TCR-induced NF-κB activation in Treg development, Treg development was relatively normal in our IκBα-SR mice, which have a near complete inhibition of inducible NFκB activity. The discrepancy between our results and the literature could be due to the fact that inhibition of NF-κB occurred at different stages in the TCR-induced NF-κB signaling cascade. Many of the NF-κB pathway molecules implicated in Treg development, such as PKCθ, Bcl-10 and IKK complex, are all upstream of IκBα. It is possible that these upstream signaling molecules activate other signaling pathways in addition to NF-κB, which may contribute to Treg development. For example, PKCθ is a known activator of AP-1 transcription factor^{277}, which has been implicated in Treg development^{128}. Since Carma1 is also an upstream signaling molecule with the ability to activate other pathways such as c-Jun-N-terminal kinase (JNK) pathway^{265}, it is possible that Carma1 may affect Treg development independent of the NF-κB pathway.

During our search for additional mechanisms by which Carma1 regulate Treg development, we found that Carma1−/− thymocytes were defective in the IL-2 response. When the CD4+CD8−CD25+Foxp3− thymic Treg intermediate population was stimulated with IL-2, a significant percentage of Carma1+/+ cells became Foxp3+ Tregs, whereas the response in Carma1−/− cells was defective. We further showed that Carma1−/− cells have defects in the IL-2 and IL-15 signaling pathway, which was associated with diminished expression of a shared receptor subunit, IL-2Rβ, on the cell surface. In support of our data, a recent paper demonstrated the importance of IL-2Rβ-mediated STAT5 signaling in Treg development^{278}. One potential explanation for a more severe Treg defect seen in our Carma1−/− mice as compared to IL-2−/− or IL-2Rα−/− mice is that there is some degree of functional redundancy between IL-2 and IL-15, as suggested previously^{136}.
It is possible that Carma1 may regulate IL-2Rβ expression in either a NF-κB-dependent or -independent manner. Interestingly, a recent study has suggested a NF-κB-independent function for a CBM complex member, Bcl-10. A specific point mutation on Bcl-10 was shown to cause a defect in TCR-induced actin polymerization, without affecting NF-κB activation\textsuperscript{279}. From this report, it is worth noting that Carma1 did not affect TCR-induced actin polymerization. However, a separate study has shown that disruption of tubulin polymerization, but not actin polymerization, negatively affects IL-2Rβ expression on the cell surface\textsuperscript{280}. Currently, it is not known whether any of the CBM complex members regulates tubulin polymerization. Based on our data, it is interesting to speculate whether Carma1 can regulate protein expression of IL-2Rβ independent of NF-κB through tubulin polymerization. Specific post-translational modifications, such as phosphorylation and ubiquitylation, may modulate NF-κB-dependent and -independent functions of Carma1, as is the case for Bcl-10\textsuperscript{279}.

Treg deficiency in Carma1\textsuperscript{-/-} mice was associated with a skin inflammatory condition, which increased in prevalence with age. Future studies will determine whether the Treg deficiency is directly responsible for the disease phenotype. Even though Carma1\textsuperscript{-/-} conventional T cells are hypo-responsive to stimulation \textit{ex vivo}, they may still be able to cause disease \textit{in vivo} without the presence of suppressive Tregs. In summary, our study indicates a critical role for Carma1 in Treg development and suggests a novel mechanism by which Carma1 may regulate IL-2 signaling in Treg precursor cells.
MATERIALS AND METHODS

Mice

Carma1 mice were graciously provided by Dr. Josef Penninger. Foxp3-GFP reporter mice were from the Jackson Laboratory. Carma1-/- mice were mated to Foxp3-GFP mice to generate Carma1 +/- Foxp3-GFP mice, which were then intercrossed to generate Carma1 +/- Foxp3-GFP and Carma1 +/- Foxp3-GFP mice. B6.SJL (CD45.1+) mice were obtained from Jackson Laboratory and then crossed to Carma1 +/- (CD45.2+) mice to produce Carma1 +/- (CD45.1+CD45.2+) mice. IκBαSR transgenic mice express a degradation-resistant form of IκBα (IκBαsuper-repressor or IκBαSR) under the control of a T-cell specific proximal lck promoter and were obtained from Jackson Laboratory. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Antibodies and Reagents

Fluorescently-labeled anti-mCD4 (L3T4), anti-mCD8 (53-6.7), anti-mCD25 (PC61.5), anti-mIL-2Rβ/CD122 (TM-b1), anti-mCD45.1 (A20), anti-mFoxp3 (FJK-16s), were purchased from eBioscience. Fluorescently-labeled anti-pSTAT5(pY694) (clone 47) was from BD Biosciences. Anti-pSTAT5(pY694) and anti-STAT5 for western blotting were generously provided by Matt Morrison at Cell Signaling Technology. Anti-tubulin (TU-02) was from Santa Cruz. Human IL-2 was provided by NCI Biological Resources Branch. IL-7 and IL-15 were purchased from Peprotech.
**Bone marrow chimera**

B6.SJL and Carma1\(^{+/+}\) mice were intercrossed for one generation to generate CD45.1\(^{+}\) Carma1\(^{+/+}\) mice (CD45.1\(^{+}\)CD45.2\(^{+}\)). Carma1\(^{+/+}\) (CD45.1\(^{+}\)CD45.2\(^{+}\)) recipient mice were lethally irradiated. 12 hrs post irradiation, a mixture containing equal proportions of Carma1\(^{+/+}\) (CD45.1\(^{+}\)CD45.2\(^{+}\)) and Carma1\(^{-/-}\) (CD45.1\(^{-}\)CD45.2\(^{+}\)) T cell–depleted bone marrow (5x10\(^{6}\) cells/recipient) were injected intravenously into the recipient mice. Anti-CD90 magnetic beads (Miltenyi) were used for mature T cell depletion. Thymic and splenic reconstitution was analyzed 6 weeks after injection based on CD45.1 expression (positive in Carma1\(^{+/+}\) cells and negative in Carma1\(^{-/-}\) cells).

**Flow cytometry and cell sorting**

Cells from thymus, spleen and lymph nodes were prepared via gentle homogenization of organs over nylon mesh (Small Parts). Mononuclear cells were isolated after RBC lysis (Sigma) and debris filtration over nylon mesh. Flow cytometry data was acquired on LSRII (BD Bioscience) and analyzed using FlowJo (TreeStar). To purify splenic T cells, anti-CD90 magnetic beads (Miltenyi) were used. For isolating purified thymic Treg precursor populations, CD4SP thymocytes were first enriched by depleting CD8\(^{+}\) cells using CD8 MicroBeads (Miltenyi). Tregs (CD4\(^{-}\)CD8\(^{-}\)CD25\(^{+}\)GFP\(^{+}\)) and thymic Treg precursors (CD4\(^{-}\)CD8\(^{-}\)CD25\(^{+}\)GFP\(^{-}\) and CD4\(^{-}\)CD8\(^{-}\)CD25\(^{-}\)GFP\(^{-}\)) were isolated by flow cytometry sorting using FACSaria (BD Bioscience). The purity of isolated population was verified to be >98%.

**Cytokine Stimulation**
For Foxp3 induction, purified thymic Treg precursors were incubated for 5 days in the presence of hIL-2 (50U/ml). Foxp3 staining was performed according to the manufacturer’s recommendations (eBioscience). To examine cytokine signaling, cells were stimulated with indicated amounts of hIL-2/mIL-7/mIL-15 for 20-30min at 37°C. For western blotting, cells were washed in cold PBS and rapidly lysed to obtain whole cell lysates. For flow cytometry, cells were washed in cold-PBS and fixed in 4% paraformaldehyde for 10min at 37°C. Cells were spun down and permealized in ice-cold 90% methanol for 30min on ice. Following two washes with incubation buffer (PBS+0.5%BSA), cells were stained with p-STAT5 antibody and other appropriate cell surface antibodies for 1hr at room temperature. Cells were washed twice with incubation buffer and data was acquired by flow cytometry.

**Real-time quantitative RT-PCR**

Total RNA was isolated using TRI reagent (Molecular Research Center, Inc.) and subjected to cDNA synthesis using RNase H-reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed in triplicates, using iCycler Sequence Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad). The expression of individual genes was calculated by a standard curve method and normalized to the expression of GAPDH. The gene-specific primer sets were: mIL-2 5’-CCTGAGCAGGATGGAGAATTA-3’ (forward) and 5’-TCCAGAACATGCCGCAGAG-3’ (reverse); and mIL-2Rβ 5’-GACAGTTCTCAAGTGCCACA-3’ (forward) and 5’-GAGCCATTTCTGAAGGTCTC-3’ (reverse).

**Western blotting**
Whole-cell lysates were prepared from thymocytes and splenocytes and subjected to IB as previously described\textsuperscript{256}.
ACKNOWLEDGEMENTS

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FIGURES AND LEGENDS

Fig. 3-1. Carma1<sup>−/−</sup> mice develop skin inflammation. (A) Representative photograph of 16 week-old Carma1<sup>+/+</sup> (+/+) and Carma1<sup>−/−</sup> (-/-) mice are shown. Carma1<sup>−/−</sup> mice develop skin inflammation around nostril and eyes (arrows). (B) Mice from (A) were sacrificed and their organs were harvested. Carma1<sup>−/−</sup> (-/-) mice have enlarged lymph nodes and spleen compared to Carma1<sup>+/+</sup> (+/+) mice. (C) The frequency of mice that develop skin inflammation by the age of 5-6 months was tabulated.
Fig. 3-1
**Fig. 3-2 Carma1−/− mice have cell-intrinsic defect in Treg development.** The percentages of Carma1+/+ (left panels) and Carma1−/− (right panels) Foxp3+ Treg were determined in thymus (A), spleen (B) and lymph node (C). The numbers indicate the percentages out of gated CD4+CD8− T cells. (D) 6 weeks following mixed bone marrow transfer, thymocyte development was examined. Carma1+/+ (+/+ ) (left panel) and Carma1−/− (−/−) (right panel) donor cells were distinguished based on expression of CD45.1 congenic marker. The numbers indicate the percent of gated total Carma1+/+ (CD45.1+) or Carma1−/− (CD45.1−) population.
Fig. 3-2
**Fig. 3-3** NF-κB activation is dispensable for Treg development. (A) Real-time PCR was performed to determine the relative level of IL-2 expression in total thymocytes from Carma1+/+ and Carma1–/– mice. (B) Total thymocytes from Carma1+/+, Carma1–/–, IκBα-SR (IκBαΔN), and control mice were subjected to nuclear extract preparation. EMSAs were performed using 32P-radiolabelled probes for NF-κB (upper panels) and a constitutive transcription factor, NF-Y (lower panels). (C) Flow cytometry was performed on thymocytes and splenocytes from control mice and IκBα-SR (IκBαΔN) mice. The numbers indicate percent of gated CD4+CD8– population.
Fig. 3-3
**Fig. 3-4 Carma1−/− mice have IL-2 signaling defect.** Thymic Treg precursor populations CD4⁺CD8⁻CD25⁺Foxp3⁻ (A) and CD4⁺CD8⁻CD25⁻Foxp3⁻ (B) were sorted by flow cytometry and cultured *in vitro* in the presence of IL-2. After 5 days, the percentages of Foxp3⁺ Tregs were determined in each culture conditions. (C) Total thymocytes from Carma1⁺/⁺ and Carma1⁻/⁻ mice were stimulated with IL-2 for 20 minutes and immediately lysed to determine the level of p-STAT5 by immunoblotting. (D) Total T cells from Carma1⁺/⁺ and Carma1⁻/⁻ spleens were stimulated with IL-2 for 20 minutes and immediately lysed to determine the level of p-STAT5 by immunoblotting. (E) Total thymocytes from Carma1⁺/⁺ and Carma1⁻/⁻ mice were stimulated with IL-15 for 20 minutes and immediately lysed to determine the level of p-STAT5 by immunoblotting.
Fig. 3-4
Carma1–/– thymic Treg precursors have IL-2 signaling defect. Thymocytes from Carma1+/+ (A) and Carma1−/− (B) were isolated and stimulated with IL-2 for 30 minutes. After stimulation, p-STAT5 level was determined in Foxp3+ Tregs and thymic Treg precursor populations.
Fig. 3-5
Fig. 3-6 Carma1−/− mice express a reduced level of IL-2Rβ. (A) The expression of IL-2Rβ was determined on Foxp3+ Tregs and Foxp3− Treg precursors from Carma1+/+ (left panels) and Carma1−/− (right panels) mice. (B) Real-time PCR was performed to determine the relative level of IL-2Rβ expression in both GFP−(Foxp3−) and GFP+(Foxp3+) Carma1−/− thymic populations.
Fig. 3-6
CHAPTER IV

NF-κB1 p105 REGULATES T CELL HOMEOSTASIS AND PREVENTS CHRONIC INFLAMMATION
ABSTRACT

Transcription factor NF-κB is regulated by a family of inhibitors, IκBs, as well as the NF-κB1 and NF-κB2 precursor proteins, p105 and p100. Although the different NF-κB inhibitors can all inhibit NF-κB in vitro, their physiological functions are incompletely understood. In this study, we demonstrate that p105 plays an important role in the regulation of T cell homeostasis and prevention of chronic inflammation. Mice lacking p105, but expressing the mature NF-κB1 p50, spontaneously develop intestinal inflammation with features of human inflammatory bowel disease. This inflammatory disorder occurs under specific pathogen-free conditions and critically involves T cells. Consistently, the p105-deficient mice have reduced frequency of naive T cells and increased frequency of memory/effector T cells in the peripheral lymphoid organs. Although p105 is dispensable for the production of immunosuppressive regulatory T cells, p105 deficiency renders CD4 T cells more resistant to Treg-mediated inhibition. We further show that the loss of p105 results in hyperproduction of Th17 subset of inflammatory T cells. Together, these findings suggest a critical role for NF-κB1 p105 in the regulation of T cell homeostasis and differentiation and the control of chronic inflammation.
INTRODUCTION

The NF-κB family of transcription factors has diverse biological functions, including regulation of immune and inflammatory responses. In mammalian cells, the NF-κB family is composed of five members, NF-κB1, NF-κB2, RelA, RelB, and c-Rel, which function as various hetero- and homo-dimers. NF-κB1 and NF-κB2 are produced as precursor proteins, p105 and p100, which undergo proteolytic processing to generate the mature NF-κB1 p50 and NF-κB2 p52, respectively. The mature NF-κB proteins are normally sequestered in the cytoplasmic environment through physical association with a family of inhibitory proteins, IκBs. In response to various stimuli, NF-κB dimers translocate to the nucleus as a result of IκB degradation. The presence of different IκB members, with distinct properties in signal response, creates a level of complexity in NF-κB regulation.

In addition to the typical IκBs, NF-κB inhibitors also include the NF-κB1 and NF-κB2 precursor proteins, p105 and p100, respectively. The C-terminal portion of these precursor proteins share structural homology with IκBs, characterized by the presence of ankyrin repeats. Like IκBs, p105 and p100 bind to NF-κB members and prevent the nuclear translocation of NF-κB. Thus, in addition to producing the mature NF-κB1 (p50) and NF-κB2 (p52) proteins, p105 and p100 have important roles in regulating the biological function of NF-κB. Recent studies demonstrate that p100 regulates a so-called noncanonical NF-κB signaling pathway that mediates activation of the RelB/p52 NF-κB complex and specific adaptive immune functions, including B cell maturation and lymphoid organogenesis. However, the physiological function of p105 remains obscure. Unlike the signal-regulated processing of p100, the processing of p105 occurs constitutively and co-translationally,
yielding high steady levels of both p105 and p50 in most cell types. In response to various
stimuli, p105 undergoes degradation without generating p50, which may contribute to the
activation of p105-sequestered NF-κB members. At least in macrophages, p105 also has a
role in regulating MAPK signaling downstream of TLRs. p105 controls the stability and
activity of Tpl2, a MAPK kinase kinase-mediating activation of the MEK1 and its
downstream target ERK. Thus, loss of p105 causes dramatic reduction in the steady level
of Tpl2, resulting in attenuated activation of MEK/ERK by the TLR signals.

The in vivo function of p105 in NF-κB regulation has been investigated using a
knockin approach, which involved the insertion of a stop codon in the processing site of p105,
allowing the direct production of p50 without generating p105. In various cell types, these
p105-deficient (p105–/–) animals have aberrant activation of NF-κB, particularly the p50-
containing complexes. Probably due to the repressor function of p50 homodimers, the p105-
deficient immune cells display hypoactivation of proinflammatory cytokines. Surprisingly,
however, the p105–/– animals display inflammatory and autoimmune phenotypes characterized
by immune cell infiltration into the lung and liver. To date, the mechanism by which p105
regulates autoimmunity and inflammation has not been defined.

Autoimmunity and chronic inflammation often result from aberrant T cell responses,
which in turn can be caused by impaired production or function of regulatory T cells (Tregs).
Tregs suppress the activation and differentiation of naïve T cells as well as the effector
functions of differentiated T cells. Defect in Treg function breaks peripheral tolerance,
causing spontaneous activation of naïve T cells and multiorgan inflammation. Autoimmunity
and inflammation can also result from imbalanced differentiation of naïve CD4 T cells to
effector T cells, including Th1, Th2, and Th17 cells. In particular, Th17 cells are
involved in various autoimmune and inflammatory diseases\textsuperscript{143}, such as experimental autoimmune encephalitis\textsuperscript{23,293}, rheumatoid arthritis\textsuperscript{294-296}, and inflammatory bowel disease (IBD)\textsuperscript{297-300}. These inflammatory T cells produce IL-17 family of cytokines, which mediate inflammation by recruiting innate immune cells and inducing proinflammatory cytokines\textsuperscript{23,292,301,302}. Thus, characterization of the factors that regulate T cell responses and differentiation is important for understanding the molecular mechanisms underlying autoimmune and inflammatory diseases.

In this study, we show that the IκB-like protein p105 plays a critical role in regulating T cell homeostasis and differentiation and preventing T cell mediated inflammation. In addition to the previously identified autoimmune symptoms, the p105-deficient mice spontaneously develop intestinal inflammation with characteristics of human IBD. By crossing p105\textsuperscript{\textminus/\textminus} mice to the Rag1\textsuperscript{\textminus/\textminus} background and performing T cell adoptive transfer, we show that T cells play an important role in mediating the autoimmune and inflammatory disorders of the p105\textsuperscript{\textminus/\textminus} mice. Interestingly, loss of p105 causes reduction in the naïve T cell population and concurrent increase in the memory/effector T cell population, with a particular expansion of Th17 cells. Although p105 is dispensable for Treg development, the p105-deficient CD4 T cells are less sensitive to Treg-mediated suppression. We further show that p105 deficiency in macrophages promotes TLR-mediated induction of IL-6, a key cytokine that stimulates the differentiation of naïve CD4 T cells to Th17 cells\textsuperscript{143}. These findings establish p105 as a critical factor that maintains naïve T cell phenotype and prevents abnormal induction of inflammatory T cells.
RESULTS

Genetic deficiency of p105 causes colonic inflammation

To investigate the in vivo function of p105 in regulating inflammation, we examined the histological phenotypes of various organs isolated from the p105-deficient (p105<sup>−/−</sup>) mice. Even when housed under stringent pathogen-free conditions, the p105<sup>−/−</sup> mice displayed leukocyte infiltration into the lung and liver (data not shown). More strikingly, these mutant animals had intestinal inflammation with prominent features of IBD (Fig. 4-1). The colons of p105<sup>−/−</sup> mice were often devoid of solid feces and evidently shorter and more rigid (Fig. 4-1A), typical macroscopic features of IBD and experimental colitis<sup>303, 304</sup>. Large leukocyte follicles (colonic patches) were frequently detected in the colons of p105<sup>−/−</sup> mice at different ages but were not found in the control colons (Fig. 4-1B). Other histological features of the p105<sup>−/−</sup> colons included crypt damages, sporadic leukocyte infiltrations, and thickening of mucosal layer (Fig. 4-1B). Microscopic analyses of multiple histology slides also revealed higher inflammation scores in both the proximal and distal colons of the p105<sup>−/−</sup> mice (Fig. 4-1C). Consistent with the histology results, the colonic tissue of p105<sup>−/−</sup> mice expressed various proinflammatory genes, including IL-1β, TNF-α, IL-6, and IL-12 (Fig. 4-1D).

T cells are involved in the inflammation of p105<sup>−/−</sup> mice

Chronic inflammation is often mediated by aberrant responses of T cells<sup>305</sup>. To examine the role of T cells in mediating the inflammatory disorders of p105<sup>−/−</sup> mice, we crossed the p105<sup>−/−</sup> mice with Rag1<sup>−/−</sup> mice to produce p105<sup>−/−</sup>Rag1<sup>−/−</sup> mice. In contrast to the prominent colonic inflammation of p105<sup>−/−</sup> mice, no obvious colonic inflammation was
detected in the lymphocyte-free p105\(--/\)Rag1\(--/\) mice (Fig. 4-2A). Similarly, the inflammatory phenotype in the lung was also lost after the p105\(--/\) mice had been crossed to the Rag1\(--/\) background (Fig. 4-2A). Thus, lymphocytes play an important role in mediating the chronic inflammations in p105\(--/\) mice. To investigate whether p105\(--/\) T cells were sufficient for inducing inflammation in recipient mice, we adoptively transferred T cells derived from p105\(--/\) or WT mice into Rag1\(--/\) mice. Interestingly, transfer of p105\(--/\) T cells into Rag1\(--/\) mice was sufficient to cause colonic inflammation within 6 wks, as demonstrated by both colonic patch formation (Fig. 4-2B) and expression of proinflammatory cytokines (Fig. 4-2C). In contrast, colonic patches were rarely found in Rag1\(--/\) mice that had received WT T cells (Fig. 4-2B), and these control mice also did not show aberrant expression of proinflammatory cytokines in the colonic tissue (Fig. 4-2C). Thus, T cells play an important role in the development of colitis in p105\(--/\) mice.

**p105 deficiency causes abnormal activation and homeostasis of T cells**

Because of the involvement of T cells in the inflammatory phenotype of p105\(--/\) mice, we examined the effect of p105 deficiency on the activation of T cells. Compared with WT CD4 naive T cells, the p105\(--/\) CD4 naive T cells displayed a low, but significant, increase in the proliferation potential upon stimulation with anti-CD3 and anti-CD28 (Fig. 4-3A). This phenotype was in sharp contrast to the hypo-proliferation of the nfkB1\(--/\) T cells that lack both p105 and p50. Parallel DNA binding assays revealed that loss of p105 caused a marked activation of NF-κB in CD4 T cells, and this phenotype was dependent on p50 because the NF-κB activation was attenuated in nfkB1\(--/\) T cells (Fig. 4-3B). To assess the role of p105 in regulating the homeostasis of T cells in vivo, we analyzed the naive and memory T cell
populations based on their surface markers. In WT mice, the majority of T cells in the spleen and lymph nodes displayed a naive phenotype, characterized by the expression of low levels of CD44 and high levels of CD62 ligand (CD62L) (Fig. 4-3C). Remarkably, the CD44\textsuperscript{low}CD62L\textsuperscript{high} population of naive T cells was substantially reduced in the p105\textsuperscript{−/−} mice, with a concurrent increase in the population of CD4 T cells displaying memory or effector markers (CD44\textsuperscript{high}CD62L\textsuperscript{low}) (Fig. 4-3C). This abnormality in T cell homeostasis was not seen in nfkb1\textsuperscript{−/−} mice lacking the mature NF-κB1 protein p50 (Fig. 4-3C), thus suggesting that the IkB-like molecule p105 has a critical role in maintaining the naive T cell homeostasis.

**Loss of p105 does not inhibit Treg development but renders naive T cells more resistant to Treg-mediated suppression**

Spontaneous activation of naive T cells and development of autoimmune and inflammatory disorders are often caused by defect in the production of Tregs, because these cells suppress the activation of CD4 T cells by self-Ags\textsuperscript{257,289}. The fact that p105\textsuperscript{−/−} naive T cells exhibit memory/effector phenotype in vivo prompted us to examine whether the loss of p105 blocked the production of Tregs. As expected, WT mice had a population of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} Tregs in the thymus, spleen, and mLN (Fig. 4-4A, left panels). Surprisingly, despite their inflammatory phenotype, the p105\textsuperscript{−/−} mice produced even a higher frequency of Tregs in both the thymus and peripheral lymphoid organs (Fig. 4-4A, right panels). This phenotype was not seen in the nfkb1\textsuperscript{−/−} mice, lacking both p105 and p50, suggesting the involvement of deregulated activation of p50-containing NF-κB complexes (Fig. 4-4A, central panels). To examine whether p105 is required for the effector function of Tregs, we activated effector CD4 T cells in vitro in the presence of either WT or p105-
deficient Tregs. Both the WT and p105−/− Tregs potently inhibited the proliferation of the effector CD4 T cells (Fig. 4-4B). Thus, p105 is dispensable for both the production and effector function of Tregs. We next examined whether p105 regulates the sensitivity of CD4 effector T cells to Treg-mediated inhibition. Effector CD4 T cells derived from WT or p105−/− mice were activated in the presence or absence of WT Tregs and subjected to proliferation assays. The WT effector CD4 T cells were efficiently suppressed by Tregs even at a high effector:Treg ratio (12:1, Fig. 4-4C). In sharp contrast, the p105-deficient effector CD4 T cells were remarkably resistant to Treg-mediated inhibition (Fig. 4-4C). Even at low effector:Treg ratios, the proliferation of these mutant cells was only partially inhibited. Together, these results suggest that although p105 is not required for the production and function of Treg cells, it regulates the responsiveness of effector T cells to Treg-mediated inhibition. These findings provide insight into the spontaneous activation of T cells in the p105−/− mice.

**Loss of p105 results in aberrant production of Th17 inflammatory T cells**

Inflammatory T cells play an important role in the induction of chronic inflammation, including IBD. We thus examined whether the p105 deficiency affected the production of Th1 and Th17 cells, both being implicated in IBD pathogenesis. T cells isolated from the spleen of WT, nfkβ1−/−, or p105−/− mice were either untreated or stimulated briefly with mitogens to induce the production of cytokines by the effector T cells. ICS analysis of the WT CD4+ T cells revealed a low percentage (0.18%) of Th17 cells and a higher percentage of Th1 cells (7.13%), based on their production of IL-17 and IFN-γ, respectively (Fig. 4-5A, lower left panel). Interestingly, a >10 times higher frequency of Th17 cells was detected in
the p105\(-/-\) mice (Fig. 4-5A, lower right panel). The p105\(-/-\) mice also displayed a moderate increase in the frequency of Th1 cells (Fig. 4-5A, lower right panel). In contrast, the overproduction of Th17 cells was not seen in the nfkb1\(-/-\) mice (Fig. 4-5A, lower central panel). In fact, these mutant mice produced reduced frequency of Th17 cells.

Because p105\(-/-\) mice had a higher number of memory T cells, we analyzed the frequency of Th1 and Th17 cells within sorted CD4\(^+\) memory T cell population (CD4\(^+\)CD25\(-\)CD62L\(\text{low}\)CD44\(\text{high}\)). This analysis further demonstrated the aberrant production of Th17 T cells by the p105\(-/-\) mice (Fig. 4-5B). However, the frequency of Th1 cells within the memory T cell population was not enhanced in the p105\(-/-\) mice (Fig. 4-5B). Thus, the p105 deficiency causes overproduction of inflammatory T cells, particularly the Th17 cells.

Th17 cells are known to infiltrate into the colonic mucosa and mediate inflammation\(^{32}\). To assess the presence of Th17 cells in the inflamed colons of p105\(-/-\) mice, we analyzed the expression of Th17 marker genes, including those encoding IL-17A, IL-17F, IL-21, IL-22, IL-23R, and the orphan nuclear hormone receptor ROR\(\gamma\)t. Real-time PCR analyses detected a low level of expression of these genes in the colonic tissue of WT mice (Fig. 4-5C). In sharp contrast, the expression level of all of these Th17 marker genes was strikingly elevated in the colons of p105\(-/-\) mice (Fig. 4-5C). Furthermore, the aberrant expression of Th17 marker genes was dependent on p50, because it was not detected in the nfkb1\(-/-\) colons (Fig. 4-5C). These results indicate that the p105 deficiency causes enhanced frequency of Th17 cells both in the lymphoid organs and at the site of inflammation.

**p105 has no T cell intrinsic role in Th17 differentiation but controls TLR-mediated induction of IL-6 in macrophages**
The hyper production of Th17 cells in p105$^{-/-}$ mice could be due to enhanced response of naive CD4 T cells to Th17 differentiation signals or deregulated cytokine production by innate immune cells. To examine whether p105 had a T cell intrinsic role in the regulation of Th17 differentiation, we analyzed the in vitro differentiation ability of naive CD4$^{+}$ T cells derived from WT, nfkb1$^{-/-}$, or p105$^{-/-}$ mice. As expected, WT CD4$^{+}$ T cells differentiated into Th17 cells when stimulated under Th17 differentiation conditions (with rIL-6 and TGF-β and blocking Abs for IFN-γ and IL-4) (Fig. 4-6A). However, the Th17 differentiation efficiency was only slightly enhanced in the p105$^{-/-}$ T cells, thus suggesting that p105 might not play an important T cell intrinsic role in regulating Th17 differentiation. Consistently, the loss of p50 in nfkb1$^{-/-}$ T cells only moderately affected the Th17 differentiation (Fig. 4-6A).

The results described above indicate the involvement of an indirect mechanism by which p105 regulates Th17 cell differentiation. In this regard, a striking finding in our gene expression analysis was the high level expression of IL-6 in the colons of p105$^{-/-}$ mice (Fig. 4-1D). Because IL-6 is a key proinflammatory cytokine that stimulates Th17 cell differentiation, we examined the effect of p105 deficiency on IL-6 gene induction in macrophages. In WT macrophages, IL-6 gene expression was stimulated by the TLR ligands LPS and CpG and weakly induced by the CD40 agonistic Ab (anti-CD40) (Fig. 4-6B). Interestingly, the IL-6 gene induction was dramatically promoted in the p105-deficient macrophages (Fig. 4-6B). This effect was not seen in p105$^{-/-}$ bone marrow-derived dendritic cells (data not shown), suggesting cell-type specificity. Because p105 has an important role in regulating Tpl2/ERK signaling in macrophages$^{285,286}$, we performed parallel studies using the nfkb1$^{-/-}$ macrophages, because these cells had a similar Tpl2/ERK signaling defect due to the lack of p105. In contrast to the p105$^{-/-}$ cells, the nfkb1$^{-/-}$ cells did not show hyper induction
of IL-6 (Fig. 4-6B). Thus, the aberrant induction of IL-6 gene in p105−/+ macrophages is unlikely due to the defect inTpl2/ERK signaling but rather the deregulated activation of p50-containing NF-κB complexes.
DISCUSSION

NF-κB1 is co-translationally produced as two proteins, p105 and p50, both being abundantly expressed in various cell types. Strong evidence suggests that p105 functions as an IκB-like molecule that inhibits the nuclear translocation of NF-κB members. Like IκBs, p105 undergoes phosphorylation and degradation in response to immune stimuli, which is thought to mediate nuclear translocation of NF-κB. However, it has been unclear what physiological functions p105 plays, particularly in the regulation of immune responses.

Although extensive studies have been performed with nfκb1−/− mice, these animals are not particularly useful for the study of p105 function because they lack not only p105 but also the mature NF-κB1 p50. The p105−/− mice, in contrast, serve as a powerful model for the study of p105 function because they are specifically deficient in p105. It was previously shown that the p105−/− mice show autoimmune symptoms, characterized by immune cell infiltration into the liver and lung, although the underlying mechanism was not resolved. In the present study, we demonstrate that p105 has an important role in regulating the homeostasis of naive CD4 T cells. Loss of p105 causes a dramatic reduction in the naive CD4 T cell population with concurrent increase in the population of CD4 T cells with memory or effector markers. We have obtained strong evidence that T cells play a critical role in mediating the autoimmune phenotype of p105−/− mice. Interestingly, in addition to lung and liver infiltrations, the p105−/− mice spontaneously develop intestinal inflammation with features of human IBD. Because the animals were housed in the specific pathogen-free facility, such an inflammatory disorder must be autoimmune in nature, probably due to the deregulated T cell responses. Indeed, the intestinal inflammation, as well as the liver and lung infiltration, was prevented when the
p105<sup>−/−</sup> mice were bred to the Rag1<sup>−/−</sup> background lacking lymphocytes. Furthermore, adoptive transfer of p105<sup>−/−</sup> T cells was sufficient for inducing the autoimmune and inflammatory phenotypes.

Peripheral T cells are maintained predominantly in their naive state, which is important for normal function of the adaptive immune system<sup>307</sup>. The loss of naive T cells can be caused by various conditions, such as defect in thymocyte development, naive T cell apoptosis, and activation and differentiation of naive T cells by low-affinity self-Ags<sup>308</sup>. The development of thymocytes does not require p105, although the peripheral T cell number is moderately reduced in the p105<sup>−/−</sup> mice (data not shown and Ref. 288). Because the reduction in naive T cells in p105<sup>−/−</sup> mice is associated with an increase in memory/effector T cells, it is likely that loss of p105 causes abnormal activation of the naive T cells. The p105<sup>−/−</sup> naive CD4 T cells show a moderate increase in the in vitro response to anti-CD3 stimulation. More significantly, these cells are resistant to Treg-mediated inhibition. Although how p105 regulates this novel function remains to be further investigated, deregulated activation of p50-containing NF-κB complexes appears to be responsible for the resistance of CD4 T cells to Tregs, because the nfkb1<sup>−/−</sup> CD4 effector T cells (lacking both p105 and p50) are sensitive to Tregs (data not shown). In line with these findings, a previous study, using fibroblasts, suggests that NF-κB inhibits the signaling induced by TGF-β<sup>309</sup>, which is known as a major effector molecule of Tregs<sup>310</sup>. Future studies will examine whether p105 regulates TGF-β-mediated T cell suppression.

Our data suggest that the intestinal inflammation of p105<sup>−/−</sup> mice is also associated with increased frequency of Th17 cells. In peripheral lymphoid organs, the p105<sup>−/−</sup> mice contained more than 10-fold Th17 cells compared with their WT controls. The percentage of
Th17 cells within the memory/effector population of CD4 T cells is also dramatically increased in the p105^{−/−} mice. Given the important role of Th17 cells in mediating autoimmunity and inflammation^{297,298,300}, the increase in this subset of CD4 effector T cells may contribute to the development of colitis and other autoimmune disorders in the p105^{−/−} mice. Consistent with this idea, our real-time RT-PCR analyses revealed high levels of expression of Th17-specific genes in the colons of the p105^{−/−} mice. The hyperproduction of Th17 cells in p105^{−/−} mice involves activation of p50-containing NF-κB complexes, because this phenotype is not seen in the nfb1^{−/−} mice lacking both p105 and p50. In fact, the nfb1^{−/−} mice have reduced numbers of Th17 cells, a finding that is consistent with a previous report that the nfb1^{−/−} mice are refractory to the induction of experimental autoimmune encephalitis and rheumatoid arthritis^{311,312}.

The roles of p105 in regulating T cell homeostasis and Th17 differentiation may involve different mechanisms. p105 has a T cell intrinsic role in regulating the response of CD4 T cells to Tregs. In contrast, regulation of Th17 cell differentiation appears to involve an indirect mechanism, because the loss of p105 in T cells has little effect on their commitment to the Th17 lineage. More significantly, p105 negatively regulates the induction of IL-6 gene by TLR and CD40 signals in innate immune cells. Hyper expression of IL-6 is readily detected in both in vitro activated p105^{−/−} macrophages and colonic tissues derived from p105^{−/−} mice. Biochemical and genetic evidence suggests that IL-6 is a critical cytokine that stimulates Th17 cell differentiation^{143}. Notably, IL-6 not only participates in the induction of Th17 cell differentiation from naive CD4 T cells but also reprograms Tregs to become Th17 cells^{313}. Because the p105^{−/−} mice have increased frequency of Tregs, it suggests the
intriguing possibility that the aberrantly expressed IL-6 in p105\(^{−/−}\) mice may promote Th17 production from both naive T cells and Tregs. Future studies will examine this possibility.

In macrophages, p105 is required for TLR-stimulated Tpl2/ERK signaling in addition to the regulation of NF-κB. Because p105-deficient macrophages have a defect in ERK activation, the functional phenotypes of the p105\(^{−/−}\) mice may be contributed by deregulated activation of NF-κB or attenuated activation of ERK. The former possibility is strongly suggested by our parallel studies using the nfkb1\(^{−/−}\) mice, which lack both p105 and the mature NF-κB1 protein p50. Like the p105\(^{−/−}\) mice, the nfkb1\(^{−/−}\) mice have ERK signaling defect due to the lack of p105\(^{286}\). However, the loss of p50 in nfkb1\(^{−/−}\) mice is sufficient to correct the phenotypes of the p105\(^{−/−}\) mice in T cell homeostasis and autoimmune/inflammatory disorders. The nfkb1\(^{−/−}\) macrophages also do not display the aberrant induction of IL-6.

Taken together, these results suggest that p105 has a critical role in controlling the activation of p50-containing NF-κB complexes, the deregulation of which may contribute to abnormal T cell response and differentiation as well as development of autoimmune and inflammatory disorders.
MATERIALS AND METHODS

Mice

The nfκb1 knockout mice (in C57BL6/129 genetic background), which are deficient in both p105 and p50, were purchased from The Jackson Laboratory. The p105/− mice (in C57BL6/129 genetic background), lacking p105 expression and competent in p50 expression, were provided by Bristol-Myers Squibb. Rag1/− mice (in C57BL6 genetic background) were purchased from Taconic Farms. All mice were housed in specific pathogen-free cages and monitored periodically for the lack of specific pathogens. Animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine and the University of Texas MD Anderson Cancer Center.

Abs and reagents

Functional grade anti-mCD3ε (145–2C11), anti-mCD28 (37.51), and anti-mCD40, as well as the blocking Abs for mIFN-γ (XMG1.2) and mIL-4 (11B11), were from eBioscience. Fluorescence-labeled anti-mCD4 (L3T4), anti-mCD25 (PC61.5), anti-mCD62L (MEL-14), anti-CD44 (IM7), anti-mIL-17A (eBio17B7), and anti-mIFN-γ (XMG1.2) were also purchased from eBioscience. The recombinant mIL-6 and hTGF-β were purchased from PeproTech. PMA and ionomycin were from Sigma-Aldrich, and monensin was from eBioscience.

Histology
Colons were removed from sacrificed mice and flushed with PBS. Distal and proximal halves of the colons were opened longitudinally, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for H&E staining. Slides were analyzed blindly and scored for the degree of inflammation (0–40 scale) as described. Liver and lung sections were prepared similarly, and pictures were taken from typical sections.

**Flow cytometry and cell sorting**

Splenic and lymph node cell suspensions were prepared by gentle homogenization using a tissue homogenizer. Mononuclear cells were isolated by centrifugation over lymphocyte separation medium (Mediatech) and subjected to flow cytometry analyses as previously described. For isolating CD4 T cell subsets, CD4+ T cells were first enriched using CD4 microbeads (Miltenyi Biotec), and the effector (CD4+CD25-), Treg (CD4+CD25+), naive (CD4+CD25-CD44lowCD62Lhigh), and memory (CD4+CD25-CD44highCD62Llow) CD4 T cells were isolated by flow cytometry sorting using MoFlo (DakoCytomation) or FACSARia (BD Bioscience). Purity of isolated population was verified to be >98%.

**Intracellular cytokine staining (ICS)**

T cells isolated from spleen and mesentery lymph nodes (mLN) or from in vitro cultures were stimulated for 5 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of monensin (2 µM). After the stimulation period, cells were fixed in 2% paraformaldehyde and permeabilized in 0.5% saponin before staining for relevant cytokines. The stained cells were analyzed by flow cytometry.
In vitro T cell activation and differentiation

Purified naive CD4\(^+\) T cells were stimulated in replicate wells of 96-well plates (1 x 10\(^5\) cells/well) with plate-bound anti-CD3 and anti-CD28. After the indicated times of stimulation, the cells were labeled for 6 h with \(^{3}\text{H}\)thymidine for proliferation assays based on thymidine incorporation. For in vitro Th17 cell differentiation, naive CD4 T cells were stimulated with anti-CD3 and anti-CD28 in the presence of anti-IFN-\(\gamma\) (10 \(\mu\)g/ml), anti-IL-4 (10 \(\mu\)g/ml), IL-6 (20 ng/ml), and TGF-\(\beta\) (5 ng/ml). After the indicated times, the cells were subjected to ICS and real-time PCR analyses.

Treg assays

Five x 10\(^4\)/well effector CD4 T cells were stimulated with soluble anti-CD3 (1 \(\mu\)g/ml) and 1 x 10\(^5\)/well irradiated (3000 RAD) APCs (splenocytes) either in the presence or absence of Tregs. The proliferation of the effector T cells was analyzed as described above.

Preparation and stimulation of bone marrow-derived macrophages

Bone marrow-derived macrophages cells were prepared from the indicated age-matched mice using a procedure described previously\(^{316}\). Before stimulation, the cells were seeded onto 100-mm petri dishes and starved for 12–16 h in DMEM supplemented with 0.5% FBS. The cells were then stimulated with the indicated inducers and collected for total RNA preparation.

Real-time quantitative RT-PCR
Total RNA was isolated from colonic tissues, T cells, or macrophages using TRI reagent (Molecular Research Center) and subjected to cDNA synthesis using RNase H-reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using iCycler Sequence Detection System (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). The expression of individual genes was calculated by a standard curve method and normalized to the expression of actin. The gene-specific primer sets (all for murine genes) were: IL-17a, 5'-AGCGATGGGTGGATGCTCATGTTAG-3' and 5'-AGCTTTCCCCTCCGCATTTGACACACACGACCACAACGACACAG-3'; IL-17f, 5'-CCCATGGGATTACAAACAATCACCTCCAC-3' and 5'-CAGGGGCTCCACCGAGACAT-3'; IL-21, 5'-ATCTTGAACCTTCTATCAGCTCCAC-3' and 5'-GCATTAGCTATGTGCTCTGTTTC-3'; IL-22, 5'-TCC GAG GAG TCA TTA A-3' and 5'-AGA ACG TCT TCC AGG GTG CTA AA-3'; IL-23R, 5'-CCAAGAAGACCATTCCCAG-3' and 5'-TCAGTGCTACAATTTCAAGAGAACA-3'; RORγt, 5'-CAAGTCTATCTGGGATCCACTAC-3' and 5'-TGCAGGAGTAGGCACATTACA-3'; IL-1b, 5'-GCTCTCCACCTCAATGGACAG-3' and 5'-GCTCTCCACCTCAATGGACAG-3'; IL-6, 5'-CAGGGGCTCCACCGAGACAT-3' and 5'-TCCACGATTCTCCACCGAGACATCA-3'; IL-12 p35, 5'-ACTAGAGAGACTTTCTTCCACAACAAGAG-3' and 5'-GCACAGGGTCATCATCACAAGAC-3'; TNF-α, 5'-CATCTTCTCATAAATTCTGAGTGACAA-3' and 5'-CATCTTCTCATAAATTCTGAGTGACAA-3'; actin, 5'-CGTGAAAAAGATGACCCAGATCA-3' and 5'-CAGGGGCTCCACCGAGACATC-3'.

T cell adoptive transfer
Lymphocytes were isolated from mesenteric lymph nodes of wild-type (WT) and p105−/− mice (6–8 wk old) and the total T cell populations were isolated by Thy-1.2 microbeads (Miltenyi Biotec). Seven x 10⁶ Thy1.2⁺ T cells were injected into 4–6 wk old RAG1−/− recipients via the tail-vein. At 6 wk post adoptive transfer, colons were collected from sacrificed recipient mice and subjected to histology and real-time PCR analyses.

Electrophoresis mobility shift assay (EMSA)

EMSA was performed as previously described 317. In brief, nuclear extracts were prepared and subjected to EMSA using a ³²P-radiolabeled κB oligonucleotide probe (CAA CGG CAG GGG AAT TCC CCT CTC CTT) or a control probe bound by the constitutive transcription factor NF-Y (AAG AGA TTA ACC AAT CAC GTA CGG TCT).
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**FIGURES AND LEGENDS**

**Fig. 4-1. Spontaneous development of colonic inflammation in p105<sup>−/−</sup> mice.**  

*A*, Picture to compare the colons of WT and p105<sup>−/−</sup> mice, showing the loss of fecal material and reduced length of the p105<sup>−/−</sup> colon. Data are representative of multiple mice.  

*B*, H&E staining of tissue sections of the distal portion of the colon from 8-wk-old control WT and p105<sup>−/−</sup> mice. An inflammatory cell follicle in the colonic mucosa (colonic patch) is indicated by an arrowhead. Original magnification, x20 and x40.  

*C*, Histological scores of mucosal inflammation in WT and p105<sup>−/−</sup> mice. Data were from three WT and three p105<sup>−/−</sup> mice (8 wk of age), each with two colon sections. Mean scores are shown in each group along with SD.  

*D*, Real-time PCR showing the constitutive expression of several proinflammatory genes in the colon of p105<sup>−/−</sup> mice but not the WT mice. Data are presented as mean ± SD of three replicates.
Fig. 4-1
Fig 4-2. T cells are involved in the development of autoimmune and inflammatory disorders in p105<sup>−/−</sup> mice.  

A, Colons and lungs from p105<sup>−/−</sup> mice on Rag1<sup>+/+</sup> (p105<sup>−/−</sup>/Rag1<sup>+/+</sup>) or Rag1 knockout (p105<sup>−/−</sup>/Rag1<sup>−/−</sup>) background were subjected to histology analyses. Colonic patches (upper) and lung infiltrations (lower) are indicated by arrowheads, which were detected in p105<sup>−/−</sup>/Rag1<sup>+/+</sup> mice but not in p105<sup>−/−</sup>/Rag1KO mice. Original magnification, x20.

B, Induction of inflammatory cell infiltration by adoptively transferred p105<sup>−/−</sup> T cells. Rag1<sup>−/−</sup> mice (4–6 wk old) were i.v. injected with T cells isolated from the mesenteric lymph nodes of WT or p105<sup>−/−</sup> mice. After 6 wk, the recipient mice were sacrificed for histology analyses of lymphocyte infiltration in the colon. An arrowhead indicates a colonic patch detected in a recipient of p105<sup>−/−</sup> T cells. Data are representative of three mice per group. Original magnification, x20.

C, RNA samples were isolated from colons of the adoptively transferred mice described in B and subjected to real-time PCR analyses to detect the relative mRNA expression of the indicated genes.
Fig. 4-2
Fig. 4-3. Activation and homeostasis of p105-deficient CD4 T cells. A, WT, nfkb1−/−, and p105−/− naive CD4 T cells were either not treated (NT) or stimulated in triplicate wells with the indicated amounts of plate-bound anti-CD3 plus soluble anti-CD28. After 48 h, cell proliferation was measured by thymidine incorporation. Data are presented as mean ± SD of three replicates. B, CD4 T cells were either not treated (NT) or stimulated for 24 h with anti-CD3 and anti-CD28. Nuclear extracts were subjected to EMSA using a 32P-radiolabeled kB probe (upper panel) or a probe that binds the constitutive nuclear protein NF Y (NF-Y). C, Mesenteric lymph node cells and splenocytes of the indicated mice were subjected to flow cytometry analyses to measure the percentage of naive (CD44highCD62Llow) and memory/effector (CD44lowCD62Lhigh) CD4 T cells within the CD4 T cell population.
Fig. 4-3
Fig. 4-4. The p105 deficiency does not inhibit the development and effector function of Tregs but renders CD4 effector T cells insensitive to Treg-mediated suppression. A, Thymocytes, splenocytes, and mLN cells were subjected to flow cytometry, and the frequency of CD4⁺CD25⁺FoxP⁺ Tregs were presented as percentage of total CD4 T cells. B, WT effector CD4 T cells were activated in the presence of the indicated ratios of either WT or p105⁻/⁻ Tregs, and the proliferation of effector T cells was analyzed based on [³H]thymidine incorporation. The level of Treg suppression was calculated as percentage of suppression based on the proliferation of effector T cells in the absence of Tregs. C, WT and p105⁻/⁻ naive CD4 T cells were stimulated either in the absence or presence of the indicated ratios of WT Tregs, and the proliferation of the effector CD4 T cells was analyzed based on [³H]thymidine incorporation. Suppression was calculated as described in B.
Fig. 4-4
**Fig. 4-5. Loss of p105 causes aberrant production of Th17 cells.**

_A_, Th17 frequency in total T cells. T cells were isolated from the spleen of WT or p105<sup>−/−</sup> mice and either left nontreated (NT) or stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 5 h. The IL-17-producing Th17 cells and the IFN-γ-producing Th1 cells were quantified by intracellular cytokine staining and flow cytometry. Numbers represent percentage of CD4<sup>+</sup> gated cells. _B_, Th17 frequency in sorted memory CD4<sup>+</sup> T cells (CD62L<sup>low</sup>, CD44<sup>high</sup>, CD25<sup>−</sup>, CD4<sup>+</sup>), determined as in _A_. _C_, Expression of Th17 marker genes in the colons of p105<sup>−/−</sup> mice. Real-time PCR was performed to analyze the relative RNA concentration of the indicated genes in the colons of WT and p105<sup>−/−</sup> mice.
Fig. 4-6. p105 deficiency has no effect on Th17 differentiation from naive CD4 T cells in vitro but causes hyperproduction of IL-6 in macrophages. A, In vitro differentiation of Th17 cells. Purified native CD4$^+$ T cells from WT, nfkbl$^{-/-}$, and p105$^{-/-}$ mice were stimulated for 4 days with anti-CD3 and anti-CD28 either in the absence (no cytokine) or presence of IL-6 plus TGF-β. Th17 and Th1 cells were analyzed by ICS and flow cytometry. B, Macrophages were prepared from the bone marrow cells of the indicated genotypes of mice. The cells were either not treated (NT) or stimulated for 4 h with LPS (1 µg/ml), CpG (1 µM), or an agonistic Ab against murine CD40 (10 µg/ml). Real time RT-PCR was performed to measure the relative levels of IL-6 induction.
Fig. 4-6
CHAPTER V

OVERVIEW AND DISCUSSION
5.1 Overview of major findings

The primary goal of my thesis research is to understand the physiological role of NF-κB family members and NF-κB signaling in the regulation of T cell development and function. To address these questions, I have employed a variety of knockout mouse models that have perturbations in NF-κB signaling pathway. The major findings are summarized below.

1. CYLD, a negative regulator of NF-κB, is critically required for NKT cell development. The loss of CYLD leads to a severe defect in the number of NKT cells, despite normal NKT cell maturation. Immature CYLD KO NKT cells undergo massive cell death, which is associated with attenuated IL-7 signaling and ICOS expression, two factors that have been shown to be critical in NKT cell survival and homeostasis. Interestingly, attenuated IL-7 signaling and ICOS expression in CYLD KO NKT cells are associated with hyper-activation of NF-κB. Inhibition of NF-κB in CYLD KO NKT cells restores ICOS expression and IL-7 signaling.

2. Carma1 is necessary for the development of thymic Foxp3+ Tregs. We show that mice lacking Carma1, a well-known mediator of TCR-induced NF-κB activation, develop a skin inflammatory condition with age and have a severe defect in development of thymic Tregs. Carma1 deficiency caused attenuation of IL-2 signaling, a cytokine that is critical for the development of Tregs in the thymus. IL-2 signaling defect in Carma1 KO mice is associated with lower expression of IL-2Rβ on thymocytes.
3. In mice that express a dominant negative form of IκBα, thymic Treg development is relatively normal, despite near complete inhibition of inducible NF-κB activity. This result suggested that NF-κB activity may not be absolutely required for thymic Treg development.

4. p105, a NF-κB1 precursor molecule, is required for CD4+ T cell homeostasis. Mice that lack IκB-like p105 molecule develop T-cell mediated spontaneous intestinal inflammation. Consistently, the CD4+ T cell population from p105−/− mice contains a higher percentage of activated/memory cells and that is more resistant to suppression by Tregs in vitro. In addition, the loss of p105 promotes the differentiation of inflammatory Th17 cells, which is at least partially driven by hyper-production of IL-6 from p105 deficient macrophages.
5.2 Discussion and Future Directions

5.2.1 Potential mechanisms of CYLD function in NKT cell development

Previous data from our laboratory has shown that CYLD has a critical function in the conventional T cell development. In addition, the data presented in Chapter 2 clearly demonstrate that CYLD is critically required for NKT cell development in the thymus. However, the exact mechanism by which CYLD regulates NKT cell development remains to be determined. Since CYLD deficiency results in constitutive activation of NF-κB in thymocytes, it is reasonable to attribute the NKT cell development defect in CYLD-/- mice to aberrant NF-κB activation. However, it is also possible that CYLD has a NF-κB-independent function. In order to address this question, we crossed the CYLD mice with IκBα-SR mice to examine the effect of CYLD deficiency in the absence of aberrant NF-κB activation.

Unfortunately, because NF-κB is required for NKT cell survival and maturation, inhibition of NF-κB did not rescue the NKT cell defect in CYLD-/- mice. However, inhibition of NF-κB did rescue the development of CD4 SP thymocytes in CYLD-/- mice (preliminary data not shown). To directly address whether aberrant NF-κB activation is primarily responsible for NKT cell defect, it would be interesting to study the development of NKT cells in IKKβ-transgenic mice. IKKβ-transgenic mice share several phenotypic features with CYLD-/- mice, including constitutive activation of NF-κB and defects in conventional T cell development. Similar NKT cell defects in IKKβ-transgenic mice will support the notion that aberrant NF-κB activation is primarily responsible for NKT cell defect in CYLD-/- mice.
5.2.2 Clinical implications of NKT cell defects and dysfunction

The current literature suggests that, due to their immuno-modulatory functions, NKT cells contribute to pathogenesis of numerous diseases such as cancer, infections and autoimmune diseases. While the role of NKT cells in various experimental animal models has been established, there is still no clear consensus about the significance of NKT cell defects in humans. Although many correlative studies performed in humans are consistent with animal studies, there are often conflicting reports and the studies showing direct causal relationship between NKT cells and human diseases are rare. The difficulties in replicating the animal results in humans are many folds. First, there are some important differences in the NKT cell subset heterogeneity between mouse and human. For example, in addition to having CD4⁺CD8⁻ and CD4⁺CD8⁻ NKT cells, which are also present in mouse, humans also have CD4⁺CD8⁺ NKT cells, which are not present in mouse. Different NKT cell subsets have been shown to have distinct cytokine profiles. Therefore, inconsistent results between studies may be due to presence or absence of particular subsets and the definitions used to classify different NKT cell subsets. Second, there are important differences in the frequency of NKT cells between mouse and human. Whereas NKT cells constitute up to 0.5% of lymphocytes in blood and up to 35% of liver lymphocytes in mice, the frequencies of NKT cells in human vary considerably, ranging anywhere from 0.001-0.01% in peripheral blood mononuclear cells and up to 1% in the liver. The combination of limited volume of study sample and the low frequency of NKT cells in the blood is a limiting factor in characterizing human NKT cells. Further characterization of individual NKT cell subsets and the use of multi-parameter flow cytometry are critical in delineating the relationship between NKT cells and human disease.
The ultimate goal of the NKT cell field would be to clearly define the role of NKT cells in human diseases and subsequently develop NKT cell-based therapies. Currently, however, NKT cells are rarely considered for therapeutic purpose and NKT cell-based clinical trials are few in number. In addition, when immuno-modulatory therapies are used to treat various diseases, its affect on NKT cells are rarely considered. For example, numerous animal studies have shown the importance of NKT cells for tolerance in organ transplantation models. However, in organ transplantation setting, significant effort are made towards maintaining immunosuppression with agents, such as cyclosporine and FK506, to prevent rejection. Both cyclosporine and FK506 are calcineurin inhibitors and known inhibitors of NF-κB signaling pathway. However, based on previous data and the results discussed in this thesis, the modulation of NF-κB activation is critical for development and function of NKT cells. Therefore, while these powerful immunosuppressive agents are necessary to prevent graft rejection through the inhibition of conventional T cell activation, they may have unintended adverse impact on the NKT cell population, which maybe counterproductive in maintaining immune tolerance. Therefore, better mechanistic understanding of NKT cell function in transplantation and other disease settings are necessary to optimize immuno-modulatory therapies in the future.

5.2.3 Effect of aberrant NF-κB activation on IL-7Rα and ICOS expression

The NKT cell deficiency in CYLD-/- mice was associated with massive cell death at the NK1.1- immature stages. Consistently, CYLD-/- NKT cells had defects in ICOS mRNA expression and IL-7 signaling, both of which facilitate survival and homeostasis of NKT cells. In addition, the IL-7 signaling defect was associated with decreased IL-7Rα mRNA
expression on CYLD-/- NKT cells. While NF-κB inhibition could not rescue NKT cell numbers in CYLD-/- mice, as previously mentioned, it did reverse ICOS and IL-7Rα mRNA expression defects in CYLD-/- NKT cells. This suggests that constitutive NF-κB hyperactivation negatively regulates ICOS and IL-7Rα transcription. Interestingly, ICOS 5’promoter region contains a NF-κB binding site. It has been shown that prototypical canonical NF-κB heterodimer, RelA/p50, as well as p50 homodimers can bind the ICOS promoter, but c-Rel and p52 cannot. Binding of RelB was not assessed. Significantly, a polymorphism at the NF-κB binding site, which prevents NF-κB binding, leads to increased expression of ICOS. These results indicate that NF-κB can bind the ICOS promoter region and inhibit their transcription. Furthermore, a binding site for NF-κB was also identified in the conserved noncoding sequence region upstream from IL-7Rα promoter. Although, the functional relevance of this binding site is unclear, it is possible that NF-κB binding may inhibit IL-7Rα transcription. Future studies are necessary to examine the occupancy status of these NF-κB binding sites and attempt to identify individual NF-κB members that are involved in this process.

5.2.4 Effect of aberrant NF-κB activation on SLAM-SAP signaling pathway

Recently, signaling lymphocytic activation molecule (SLAM) family receptors and SLAM-associated protein (SAP) family of adaptors have been shown to be involved in the development of NKT cells. Homotypic interaction between SLAM receptors leads to recruitment and activation of SAP-FynT signaling cascade, ultimately resulting in inhibition of Ras-MAPK activation that is induced by a variety of stimuli. In addition, SLAM-SAP signaling cascade is linked to NF-κB pathway through PKCθ and Bcl10. It is currently
unknown, how SLAM-derived NF-κB signal and TCR-derived NF-κB signal are integrated and how they influence the development of NKT cells. Furthermore, it needs to be determined whether aberrant NF-κB activation influences SLAM-SAP signaling. Interestingly, SLAM receptor has been identified as a target of MARCH9 transmembrane ubiquitin ligase. Ubiquitylation of SLAM may influence its surface expression level and it is possible that CYLD may regulate SLAM expression via deubiquitylation.

5.2.5 Potential mechanisms of Carma1 function in Treg development

The data presented in Chapter 3 clearly demonstrate that Carma1 is essential for thymic Treg development. We identified an IL-2 signaling defect in Carma1-/− CD4+CD25+Foxp3- Treg precursors, which was associated with attenuated Foxp3 induction upon in vitro stimulation. However, it is certainly possible that CD4+CD25+Foxp3- cells are a group of heterogeneous cells, of which only some are Treg precursors. Therefore, IL-2 signaling defect seen in the Carma1-/− mice may simply be due to diminished frequency of Treg precursors, which could not be detected with the cell-surface staining strategy used in our study. In the future, we will employ additional Treg-associated markers, such as CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR), to better define the Treg precursor population.

Given the fact that Treg development occurs relatively normally in IκBα-SR mice with similar defects in NF-κB activation, it is possible that Carma1 may regulate Treg development via a NF-κB-independent mechanism. In addition to NF-κB, T cell stimulation also activates activator protein-1 (AP-1) transcription factor and Jun N-terminal kinase (JNK). Carma1 has been found to be required for JNK2 activation and accumulation of c-Jun (a
subunit of AP-1 dimer) following T cell stimulation. Moreover, under normal conditions, JNK2 binds c-Jun and mediates its degradation. Upon activation of JNK2, c-Jun is released and allowed to accumulate in the cell. In the absence of Carma1, JNK2 cannot be activated, which then leads to constitutive degradation of c-Jun. Interestingly, AP-1 binding sites have been identified in human Foxp3 promoter region, have been shown to positively regulate Foxp3 expression in vitro. Therefore, Carma1 may regulate Foxp3 expression through AP-1 transcription factors, independent of NF-κB.

5.2.6 Role of NF-κB in TGFβ-mediated T cell suppression

Deregulated activation of p50-containing NF-κB complexes, in the absence of p105, resulted in T cell hyperactivation and intestinal inflammation. While p105/-/- mice had functional Tregs in vitro, p105/-/- effector T cells showed resistance to suppression by Tregs in vitro. One potential mechanism that may be involved is impaired signaling of TGF-β, one of the Treg effector molecules. TGF-β initiates signaling through serine-threonine kinases consisting of type I and type II TGF-β receptors. Upon TGF-β binding, type I TGF-β receptor is activated by constitutively active type II TGF-β receptor, which in turn leads to activation of Smad2 and Smad3 molecules. Once activated, Smad2 and Smad3 translocate to the nucleus and participate in transactivation of target genes. On the other hand, Smad7, is a TGF-β-inducible antagonist of TGF-β signaling, which participates in autoinhibitory feedback loop. It has been shown that activation of NF-κB can also induce Smad7 expression. Future studies will examine TGF-β signaling and Smad7 expression level in p105/-/- effector T cells.
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